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Functional diversity of dissimilatory nitrate reducers in estuarine sediments

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Gent, 23 November 2015
Helen Decleyre

List of acronyms

A

AA	amino acid
anammox	anaerobic ammonium oxidation
Asp	aspartic acid
ATP	adenosine triphosphate

B

(p)BLAST	(protein) basic local alignment tool
bp	base pair
BSA	bovin serum albumin

C

c	cytochrome
cAMP	cyclic adenosine monophosphate
cbaA	nitric oxide reductase Cu_ANor gene
cEPS	colloidal exopolysaccharides
chl *a*	chlorophyll *a*
cNirK	copper-dependent nitrite reductase
cnor/cNor	nitric oxide reductase gene / enzyme accepting electrons from heme *c*
Cys	cysteine
Cu_ANor	nitric oxide reductase accepting electron from cytochrome *c*
CuNir	copper-dependent nitrite reductase
CO_2	carbon dioxide

D

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNRA	dissimilatory nitrate/nitrite reduction to ammonium

DOC	dissolved organic carbon

E

EPS	exopolysaccharides

G

GC	gas chromatograph
GTR+G	general time reversible model with gamma distributed rates

H

HBM	high microphytobenthos biomass
HGT	horizontal gene transfer
His	histidine
HMM	hidden markov model

I

IDT	integrated DNA technologies
IMG	integrated microbial genome database

L

LBM	low microphytobenthos biomass
Lipo	lipoprotein

M

MALDI-TOF	matrix-assisted laser desorption/ionization time of flight
MB	marine broth
Met	methionine
MID	multiplex identifier
ML	maximum likelihood
MPB	microphytobenthos
MUSCLE	multiple sequence comparison by log expectation

N

NADH	nicotinamide adenine dinucleotide

nar /NarG	membrane-bound nitrate reductase gene / enzyme
nap/NapA	periplasmic nitrate reductase gene / enzyme
NCBI	National center for biotechnology information
NH_4^+	ammonium
nirk/NirK	periplasmic copper-dependent nitrite reductase gene / enzyme
nirS/NirS	periplasmic cytochrome cd_1-dependent nitrite reductase gene / enzyme
nirB/NirB	cytoplasmic NADH-dependent nitrite reductase gene / enzyme
nor	nitric oxide reductase gene
nosZ/N_2OR	periplasmic nitrous oxide reductase gene / enzyme
nrfA/NrfA	periplasmic cytochrome *c* nitrite reductase gene / enzyme
NSW	natural seawater
NO	nitric oxide
NO_3^-	nitrate
NO_2^-	nitrite
N_2	dinitrogen gas
N_2H_4	hydrazine
N_2O	nitrous oxide

O

OD	optical density
OTU	operational taxonomic unit

P

(q)PCR	(quantitative) polymerase chain reaction
Permanova	permutational analysis of variance
pmf	proton motive force

Q

qnor/qNor	nitric oxide reductase gene / enzyme accepting electrons from quinols

R

RAxML	randomized axelerated maximum likelihood

rRNA	ribosomal ribonucleic acid

T

Tat	twin-arginine
Tg	teragrams
TOM	total organic matter

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Chapter 1

Introduction

1.1 Nitrogen cycling in marine ecosystems

Marine ecosystems represent interconnected waters comprising gradients from estuaries where fluvial freshwater and saline seawater mingle, through the relatively shallow coastal ocean on the continental shelves, to the deep open ocean. Marine water depth and salinity increase as distances from the shore increase and these waters are very dynamic and tightly connected through a network of surface and deep-water currents. Coastal shallow waters can be well mixed throughout the water column, and linkage between sediments and shallow waters is frequently observed. The water columns of deeper, saline waters are stratified by sharp temperature, salinity and density transitions. The environments of open ocean, continental shelf and estuaries differ substantially in the quantity and source of their inorganic and nutrient inputs. Marine and coastal ecosystems provide a wide range of valuable ecosystem services to humans including supply of food, flood prevention, coastal protection, culture, recreation, etc. and also support services like habitat provision, nutrient cycling and primary productivity (Barbier et al., 2011).

The productivity, diversity and dynamics of marine ecosystems are limited by the supply of biologically available nitrogen. To be able to keep up with the growing global populations since the previous century, human activities involved in food and energy production such as animal husbandry, increased production and use of agricultural fertilizers (the Haber–Bosch process), and fossil fuel combustion have substantially altered the global nitrogen cycle (Galloway et al., 2004;Howarth, 2008;Voss, 2011;Steffen et al., 2015). In the 1990s, fixed nitrogen resulting from anthropogenic activities amounted to 156 teragrams (Tg) N yr^{-1}, which is more than globally supplied by natural biological nitrogen fixation on land (107 Tg N yr^{-1}) (Galloway et al., 2004) and almost equal to nitrogen fixed in the ocean (177 Tg N yr^{-1}) (Grosskopf et al., 2012). Anthropogenically-derived nitrogen contributes worldwide between 48-66 Tg N yr^{-1} to estuaries and coastal areas through terrestrial and fluvial run off (Boyer et al., 2006;Voss, 2011). The negative consequences of these nitrogen additions are substantial and manifold and often lead to a cascade of effects, *e.g.* nitrogen oxide emitted can first cause photochemical smog and subsequently, after atmospheric oxidation to nitric acid and deposition on the ground, can lead to ecosystem acidification and eutrophication[1] (Galloway et al., 2003). Coastal eutrophication, a major problem in marine ecosystems, in turn has many negative effects such as biodiversity loss in both benthic and planktonic communities (Oviatt and Gold, 2005;Voss, 2011), promotion of harmful algal blooms (Anderson et al., 2002), excessive oxygen demands resulting in hypoxic/anoxic zones which negatively affects benthic metabolism and nitrogen mineralization (Karlson et al., 2007;Diaz and Rosenberg, 2008;Voss, 2011), decreased water quality (Paerl et al., 2006), decreased water transparency resulting in coral reef degradation and seagrass destruction (Smith and Schindler, 2009) and so on.

It is generally supposed that the increased anthropogenic nitrogen concentrations in rivers have no or

[1] Eutrophication : excessive plant and algal growth resulting from increased nutrient loading

very limited impact on the open ocean. Before reaching the sea, nutrients delivered through rivers have to cross estuarine zones which are often biogeochemically very active systems that act as filters for fluvial nutrients through extensive (bacterial) nitrogen processing (Billen, 2011). Estuarine systems where substantial nitrogen processing is occurring, are furthermore often characterized by high concentrations of nitrous oxide (N_2O), acting as a source for this greenhouse gas with a global warming potential that is 310x higher compared to the generally known greenhouse gas carbon dioxide (CO_2). They were shown to emit higher concentrations of nitrous oxide relative to open waters (Bange, 2006), and contribute approximately 30% to the global nitrous oxide budget (Voss et al., 2013). Sedimentary nitrification – the aerobic oxidation of ammonium to nitrite and subsequently nitrate – and to a lesser extent denitrification – the sequential anaerobic reduction of nitrate/nitrite to dinitrogen gas – were proposed to be the main estuarine sources of nitrous oxide (Bange, 2006).

1.1.1 Estuarine nitrogen cycling

Estuaries are semi-enclosed water bodies, with free connection to the sea, where saline seawater is diluted by fluvial freshwater inflow resulting in the presence of a broad salinity gradient. These ecosystems come in different sizes and shapes ranging from shallow coastal lagoons to deep glacial fjords, tectonic depressions and drowned river valleys. Within and around estuaries, many different habitat types are found, including shallow open waters, freshwater and salt marshes, intertidal mud and sand flats, oyster reefs and seagrass beds. Human pressure on estuarine environments is extensive, as they often represent important economic regions with high population densities and, consequently, perturbation of the natural functioning of these systems is very high.

Estuarine filtering capacities are facilitated by their water circulation patterns which involves a downslope seaward flow of low density fluvial water at the surface and a bottom seawater inflow (Voss, 2011). This two-layer circulation pattern plays a crucial role in nitrogen transformations in estuaries by trapping particles and nutrients. Large sedimentary particles are carried downwards to the bottom of the estuary when the flow velocity decreases at the mouth of the estuary. The landward moving of the seawater at the bottom in turn carries these settled particles back up into the estuary. Furthermore, when the particles transported by rivers enter more saline waters, the increase in ionic strength will result in aggregation and subsequent formation of larger and thus faster settling particles, *i.e.* this process is called flocculation. Upper reaches of estuaries are thus characterized by high levels of suspended particles that are known as the turbidity maximum. Wetland systems at the border of the estuary such as intertidal flats further retain particles. The net result is that nitrogen and organic matter remain in estuarine systems. Circulation patterns are highly variable and are influenced by the shape and size of the estuary and, consequently, the filtering effect for riverine nutrients varies between different types of estuaries.

Sources of nitrogen in estuaries are fluvial nitrate and ammonium transport (global amount of 66 Tg N yr^{-1}) (Seitzinger et al., 2005), nitrate in groundwater (4 Tg N yr^{-1}) (Voss, 2011), atmospheric nitrogen

deposition of NO_x and NH_4^+ (8.4 Tg N yr^{-1}) (Galloway et al., 2004) and biological dinitrogen fixation (15 Tg N yr^{-1}) (Capone, 1988). The latter process involves conversion of dinitrogen gas (N_2) into ammonium (NH_4^+) (Figure 1, step 1). Dinitrogen fixation results in the addition of extra nitrogen to the system as it was previously unavailable to plants and animals with exception of a small group of microorganisms, *i.e.* diazotrophs, making it a scarce nutrient in many ecosystems, often limiting primary production. Bioavailable ammonium from the sources described above can subsequently be (i) assimilated into organic material which ultimately can be re-mineralized to ammonium (Figure 1, steps 2 and 3) or (ii) it can be oxidized to nitrate in an aerobic, two-step process called nitrification. During nitrification, ammonia will first be converted to nitrite by ammonia-oxidizing bacteria or archaea while, secondly, nitrite is converted to nitrate, the most oxidized form of nitrogen, by nitrite-oxidizing bacteria (Figure 1, steps 4 and 5). Nitrous oxide (N_2O) is a by-product of this process under oxygen limiting conditions. Nitrate, nitrite and ammonium can be lost from estuarine systems through burial in coastal sediments (global amount of 4 Tg N yr^{-1}) (Middelburg et al., 1996) and/or gaseous emission in the form of dinitrogen gas (8 Tg N yr^{-1} by denitrification) (Seitzinger et al., 2006) or nitrous oxide (0.31 Tg N yr^{-1}) (Bange, 2006). For a long time, it was thought that denitrification – the respiratory anaerobic reduction of nitrate/nitrite to dinitrogen gas (Figure 1, step 6) – was the sole, predominant process involved in fixed nitrogen reduction in marine environments (Burgin and Hamilton, 2007). However, alternative nitrogen reducing pathways such as dissimilatory nitrate/nitrite reduction to ammonium (DNRA) – anaerobic reduction of nitrate to nitrite and subsequently ammonium (Figure 1, step 7) – and anaerobic ammonium oxidation (anammox) – combination of nitrite with ammonium to produce dinitrogen gas (Figure 1, step 8) – can compete with denitrification for nitrate and subsequently might contribute significantly to the reduction of nitrogen of anthropogenic origin. The ability of anammox bacteria to perform DNRA under ammonium limited conditions (Kartal et al., 2007a) and convert formate/acetate to carbon dioxide (CO_2) while reducing nitrate/nitrite (Guven et al., 2005) allows them to directly compete for both nitrate and nitrite. The major difference between these three processes is that bioavailable nitrogen is permanently removed by denitrification and anammox, while DNRA results in the retention of nitrogen in the environment thereby enhancing eutrophication. Denitrification and DNRA mostly depend on the oxidation of organic matter during nitrate reduction (see § 1.2.1 and § 1.2.2). None of these three processes strictly depend on the presence of nitrate, and therefore nitrite is considered the key branching point of the nitrogen cycle. Nitrite can be consumed in different ways indicating that the so-called nitrogen cycle is not so much a cycle as it is a network of transformations.

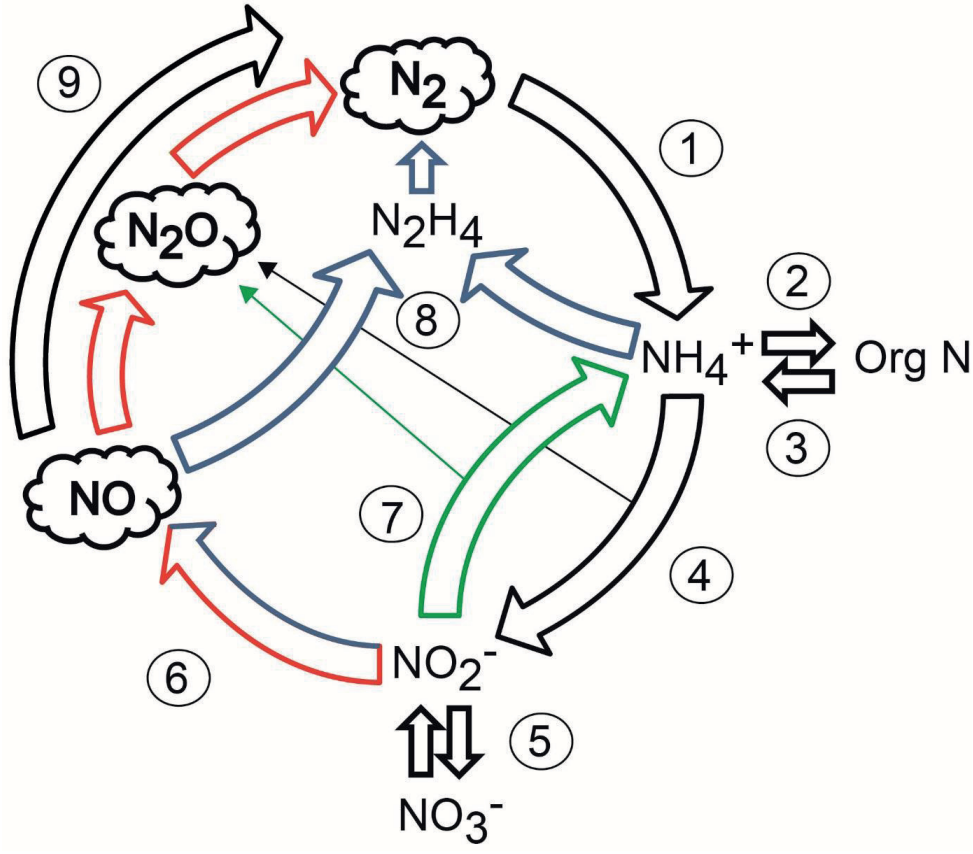

Figure 1 The microbial nitrogen cycle. Microbial processes involved in dissimilatory nitrate reduction are indicated in green, red and blue. Numbers indicated on the figure represent the different processes of the nitrogen cycle. 1: Nitrogen fixation, 2: Nitrogen assimilation, 3: Remineralisation, 4: Ammonia oxidation, 5: Nitrite oxidation, 6: Denitrification, 7: Dissimilatory nitrate reduction to ammonium (DNRA), 8: Anaerobic ammonium oxidation (anammox), 9: Nitrite-dependent anaerobic methance oxidation.

1.1.2 Complexity of highly dynamic estuarine intertidal sediments

Estuarine intertidal mudflats are highly dynamic due to their tidal regime, non-vegetated, soft sediment habitats located between the high- and low-water level (Dyer et al., 2000). They form extremely productive systems and may provide up to 50% of the primary production of estuaries, especially due to the formation of microphytobenthos biofilms (agglomerates of prokaryotes, diatoms and protozoa) at their surface (Underwood and Kromkamp, 1999;Miles and Sundback, 2000). Sediments in such an environment are vertically stratified as a result of available electron donors, acceptors and respiration processes (Middelburg and Levin, 2009). The vertical sequence of electron acceptors follows the order of decreasing free energy yield. Therefore, estuarine mudflat sediments are typically characterized by a thin oxic surface layer (limited to the upper 2-3 mm), an anoxic but oxidized zone in which nitrate, manganese and iron are the main electron acceptors, and a sulfidic zone, in which sulfate reduction is the predominant process. This vertical distribution of electron acceptors can, however, be disrupted or modified by activities of diverse benthic organisms such as macrofauna, meiofauna, diatoms and prokaryotes. Bioturbation (*i.e.*, particle reworking and burrow ventilation, (Kristensen et al., 2012)) by macrofaunal organisms[2] can significantly extend the oxic zone at the surface of the sediment and alter the physical structure and chemical composition of the sediment, resulting in formation of unique niches for sediment microorganisms to inhabit. Bacterial abundance and activity have been shown to be ten-fold higher in the macrofaunal burrow walls compared to the surrounding sediment (Bird et al., 2000;Papaspyrou et al., 2005). Meiofauna[3] abundances are generally higher in the upper mm of muddy sediments and their bioturbating activities were found to not deepen oxygen penetration within the sediment significantly (Bonaglia et al., 2014). However, these benthic organisms seem to contribute significantly to microscale transport of solutes (like oxygen, ammonium and nitrate), thereby stimulating microbial mineralization (Rysgaard et al., 2000). Activity of benthic diatoms further complicate matters. The often patchy distribution of these organisms and the temporal variations in light intensity regulate the benthic photosynthesis and consequently the bentic O_2 dynamics. Vertical migration in and out of the sediment prevents diatoms, the most dominant members of microphytobenthos in mudflats, from being grazed by organisms arriving with the water during inundation and, during day time, allows them to position themselves at sediment depth with optimum light conditions. Excretion of photosynthesates by diatoms was furthermore shown to stimulate heterotrophic bacterial activity (Wenzhofer and Glud, 2004). In contrast, the production of cytotoxins by diatoms can also inhibit bacterial growth (Ianora and Miralto, 2010). Moreover, different benthic organisms interact through grazing activities of higher trophic levels on lower trophic levels. The highly dynamic nature of these intertidal environments and the complex interaction between different trophic levels (Figure 2) make it challenging to examine these

[2] Macrofauna : benthic invertebrates > 1mm (*e.g.* bivalves, gastropods and crustaceans)
[3] Meiofauna : benthic invertebrates (*e.g.* nematodes and copepods) that pass through a 1 mm sieve but are retained on a 32µm sieve

systems as a whole. Controlled field and laboratory experiments often result in reduced complexity which make it difficult to extrapolate observations made at specific spatial and temporal scales to the ecosystem level.

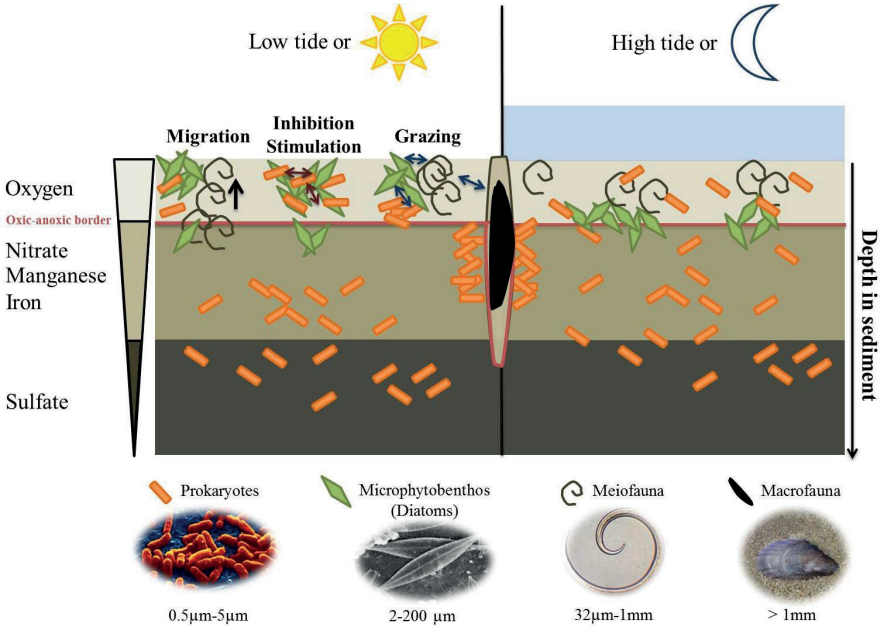

Figure 2 Schematic illustration representing the dynamic nature of muddy sediments and the complex interaction of their different inhabitants. More information can be found in the text.

Because of their dynamic environment and their interactions, examining how different benthic organisms operate in their natural habitat is not always that straightforward. This is especially the case for the smaller (µm) benthic inhabitants (prokaryotes, diatoms and meiofauna) of such sediments. The microenvironment experienced by these organisms varies according to their size and mobility (Stocker, 2012), however, currently it is not possible to interrogate these small scales *in situ* due to the absence of appropriate sampling methods. The introduction of microsensors allowed studying spatio-temporal variability of some environmental parameters at an appropriate µm-scale, nevertheless this information cannot directly be linked to microbial community analyses. Relatively large sample volumes (compared to individual cell sizes) and destructive sampling techniques resulting in disruption of spatial variation of microbial communities are generally the starting point for these analyses (Koester et al., 2008) making it impossible to examine such communities on an adequate scale.

In conclusion, the dynamic nature of intertidal sediments and the diversity of trophic levels pose particular challenges for the study of the nitrogen cycle, even more because some of the eukaryotic communities have been shown to directly and indirectly impact nitrate reducing processes (see 1.3.4).

1.2 Dissimilatory nitrate reducing processes

1.2.1 Denitrification

Bacterial denitrification is a facultative, respiratory process that encompasses the stepwise dissimilatory reduction of nitrate or nitrite over nitric and nitrous oxide to dinitrogen gas with concomitant energy conservation under low oxygen or anoxic conditions (Figure 3). The trait of denitrification is phylogenetically widespread (Zumft, 1997) and is found among Bacteria, Archaea and Eukaryotes (Risgaard-Petersen et al., 2006;Shoun et al., 2012). This thesis focused on dissimilatory nitrate reducing processes in bacteria and therefore Archaeal and fungal denitrification will not be further addressed. Denitrification is a modular process and comprises a series of sequentially expressed metalloproteins that are located in the periplasm or the cytoplasm, known as nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) reductases that are each encoded by specific genes (Figure 3). The defining step of denitrification is the respiration of nitrite to nitric oxide which results in the conversion of fixed, non-gaseous and biologically preferred form of nitrogen to a gaseous form that is released into the atmosphere (Zumft, 1997). Two types of denitrification can be distinguished based on carbon source used for cell biomass production: heterotrophic denitrifiers use organic substrates as carbon source and electron donor, while autotrophic denitrifiers use a variety of reduced compounds (*e.g.* S^0, S^{2-}, $S_2O_3^{2-}$, SO_3^{2-}, HS^-, H_2, Fe^{2+}) as electron donor and inorganic carbon dioxide as carbon source. Similarly to aerobic respiration, energy conservation occurs through the generation of a proton motive force across the bacterial membrane, which is exploited for ATP (adenosine triphosphate) synthesis.

Pre-genomic evidence already suggested the existence of partial denitrification, *i.e.* lack of certain genes and enzymes involved in denitrification (Denariaz et al., 1989;Toffanin et al., 1996), and recently it was shown to be widespread based on whole genome sequence analyses (Graf et al., 2014). Such truncated pathways make the identification of a bacterium as a true denitrifier difficult. Therefore, in this work, a bacterium is considered a denitrifier if anaerobic growth coincided with at least 80% of nitrate or nitrite was converted to gas (Mahne and Tiedje, 1995) and, adhering to the original description of denitrification (Zumft, 1997), when its genome contains a nitrite reductase.

Under oxic conditions, denitrifying bacteria are able to respire oxygen which results in more energy making oxygen the preferred electron acceptor over nitrate. Vice versa, there are no denitrifiers known in which denitrification is the only means of ATP production, indicating that denitrification is never a constitutive physiological trait (Tiedje, 1988). From a bioenergetic viewpoint, denitrification in the presence of high oxygen concentrations makes no sense which agrees with the numerous regulatory systems described in well-studied denitrifiers like *Paracoccus denitrificans* and *Peusdomonas stutzeri* shutting down denitrification once oxygen is detected. Furthermore, some enzymes involved in

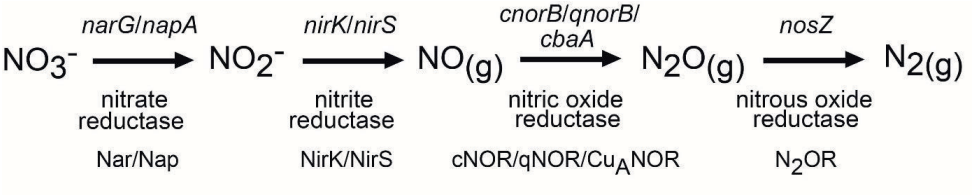

Figure 3 Schematic representation of the modularity of denitrification with all different genes, enzymes and intermediates involved. Although not strictly unique to denitrification, nitrate reduction to nitrite and nitrous oxide reductase are also indicated. Nitrite-dependent anaerobic methane oxididation (methanotrophic denitrification), which does not involve production of nitrous oxide, is not included. Gaseous nature of intermediates are indicated with (g). Abbreviations: Nar, membrane-bound nitrate reductase encoded by *narG*: Nap, periplasmic nitrate reductase encoded by *napA*: NirK, periplasmic copper-dependent nitrite reductase encoded by *nirK*; NirS, cytochrome cd_1-dependent nitrite reductase encoded by *nirS*; cNor, membrane-bound nitric oxide reductase accepting electrons from cytochrome *c* and encoded by *cnorB*; qNor, membrane-bound nitric oxide reductase accepting electrons from quinol and encoded by *qnorB*; Cu_ANor, membrane-bound nitric oxide unique in gram-positive bacteria and encoded by *cba*; N_2OR, periplasmic nitrous oxide reductase encoded by *nosZ*.

denitrification, *i.e.* membrane-bound nitrate reductase NarG and nitrous oxide reductase NosZ, were shown to be inhibited by the presence of oxygen (Patureau et al., 2000;Moir and Wood, 2001). Nevertheless, aerobic denitrification exists and was shown to occur in marine environments (Gao et al., 2010) although often incomplete resulting in nitrous oxide emission. This type of denitrification might provide an advantage in environments of alternating oxic/anoxic conditions where it is simply impossible to rebuild the denitrifying respiratory chain each time oxygen comes or goes (Patureau et al., 2000). Microorganisms capable of both aerobic and anaerobic denitrification would have the best chances of survival in these habitats (Gao et al., 2010).

1.2.1.1 Enzymes and genes involved in bacterial denitrification

Nitrate reductase. Nitrate reductase catalyses the two-electron reduction of nitrate to nitrite (Figure 3). Two distinct classes of nitrate reductases exist: the membrane-bound nitrate reductase Nar encoded by the *nar* gene cluster *narGHI* (Gonzalez et al., 2006) and the periplasmic nitrate reductase Nap encoded by the *nap* gene cluster. In *Bacillus azotoformans*, however, the periplasmic Nap nitrate reductase has been observed to be membrane-bound (Suharti and de Vries, 2005;Heylen and Keltjens, 2012). Similar observations were made for other periplasmic enzymes (see further) involved in denitrification in gram-positive bacteria, probably resulting from the very limited periplasmic space present in these bacteria. Nar is a trimeric enzyme complex with NarG and NarH forming a complex at the cytoplasmic side of the membrane attached by the membrane anchor NarI. NarG contains a [4Fe-4S] cluster and the active site, *i.e.* the molybdenum *bis* molybdopterin guanine dinucleotide (Mo-bis-MGD) cofactor. Electrons are transferred from NarI which receives electrons from the quinone pool in the membrane to NarH and subsequently to the active site in NarG (Kraft et al., 2011). For each pair of

electrons, two protons are translocated across the membrane and therefore this enzyme complex contributes to proton motive force (pmf) generation and subsequent ATP production (Chen and Strous, 2013). The periplasmic Nap consists of two subunits, NapA which contains Mo-bis-MGD and a [4Fe--4S] cluster, and NapB which is a cytochrome *c* (Kraft et al., 2011). Electrons are transferred from the membrane-associated quinol pool to the periplasmic NapAB complex by a membrane-bound NapC or NapGH (Shapleigh, 2013). In general, Nap-dependent quinol oxidation appears to be not involved in pmf generation (Simon and Klotz, 2013).
Nar enzymes are exclusively expressed under anaerobic conditions, whereas Nap was found to be also expressed under aerobic conditions. Furthermore, many denitrifiers contain both types of nitrate reductases. Based on these observations, both types of nitrate reductase might perform distinct functions within the cell: energy conservation with Nar and redox homeostasis (Ellington et al., 2006), aerobic denitrification (Kraft et al., 2011) and transition from oxic to anoxic conditions (Zumft, 1997) with Nap. Nitrate reduction is generally considered not to be the starting point of the denitrification pathway as nitrate reducing bacteria are not per se denitrifying bacteria and nitrate reduction is also involved in DNRA (see § 1.2.2).

Nitrite reductase. The key step in denitrification is the reduction of nitrite to nitric oxide as it results in the formation of the first gaseous product (Figure 3). Two isofunctional, evolutionary unrelated, periplasmic enzymes catalyse the one-electron reduction of nitrite to nitric oxide in gram-negative bacteria: (i) a copper-containing enzyme NirK (or cNirK/CuNIR) encoded by the *nirK* gene and (ii) a cytochrome cd_1 nitrite reductase, NirS (or cd_1NIR) encoded by the *nirS* gene (Zumft, 1997). Typical electron donors for both enzymes are periplasmic electron-transferring proteins that include (i) various forms of small copper-containing proteins named azurin, pseudoazurin or cupredoxin and (ii) numerous monoheme and diheme cytochromes *c* like cytochromes c_{550}, c_{551}, c_{552} or c_{553} (Zumft, 1997;Murphy et al., 2002;Pearson et al., 2003;Nojiri et al., 2009). Nitrite reductase from *B. azotoformans* accepts electrons from menaquinol via a membrane bound cytochrome c (Suharti and de Vries, 2005).

NirK enzymes are homotrimeric with each monomer typically containing two copper centres, *i.e.* T1Cu and T2Cu (Nojiri et al., 2009). The copper in T1Cu is bound by Cys, Met and two His residues and was shown to be involved in electron transfer to the active site, while T2Cu copper is ligated by three His residues and a water molecule, and was shown to be the site of nitrite binding (Libby and Averill, 1992;Abraham et al., 1993). Two other residues of T2Cu, *i.e.* Asp and His, play an essential role in nitrite reductase functioning as they form a hydrogen bond network that functions as proton donor to nitrite (Boulanger et al., 2000). The catalytic mechanisms proceeds in an irreversible, ordered way where nitrite binds to T2Cu, thereby replacing the water ligand, before electrons are transferred from the T1Cu to T2Cu. Nitrite protonation and electron transfer from T2Cu allow the N-O bond cleavage and release of a water molecule. Reduction of nitrite by NirK mainly results in nitric oxide,

however, small amounts (3–6%) of nitrous oxide have been observed if nitric oxide accumulated (Rinaldo and Cutruzzola, 2007).

NirS enzymes consist of two monomers of ~60kDa subunits, with each monomer comprising two domains. The smaller domain forms an α-helical domain around the covalently bound heme *c*, while the larger domain contains an eight bladed β-propeller in which the central channel accommodates the catalytic site heme d_1 ligated to a conserved His residue (Baker et al., 1997). Heme *c* will be reduced by external electron donors and is involved in intramolecular electron transfer. The mechanism of nitrite reduction by NirS involves binding of nitrite to heme d_1 which is reduced to nitric oxide with electrons transported from heme *c* via c-type cytochromes. Why this enzyme uses this unusual heme d_1 is uncertain, however it has been suggested the heme d_1 helps promoting a catalytic site with a low affinity for nitric oxide and higher affinity for nitrite (Rinaldo et al., 2011). Nitric oxide has a high affinity for reduced metal centers, so ensuring product release is critical for efficient Nir function.

Some organisms contain more than one *nir* gene copy (Etchebehere & Tiedje 2005) and, recently, both types of *nir* were found to be not mutually exclusive (Graf et al. 2014), although functionality of the two different nitrite reductases present in the same genome has not yet been demonstrated. Neither NirK nor NirS contribute to proton motive force generation directly due to their periplasmic location (Chen and Strous, 2013). Expression of NirS requires the presence of at least three or four genes involved in the maturation and assemblage of the haem d_1 in the reaction center (Philippot, 2002;Van Spanning, 2011). In contrast, NirK requires only *nirK* gene expression although co-transcription of the *nirV* gene has been observed (Jain and Shapleigh, 2001). Although NirV has been proposed to be involved in proper insertion of the copper reaction center, it has also been observed in combination with NirS and even within non-denitrifying bacteria suggesting a broader role of this enzyme.

Nitric oxide reductase. The product of nitrite reduction, nitric oxide (NO), is a very reactive radical and its accumulation has to be prevented. The two-electron reduction of nitric oxide to nitrous oxide (N_2O) is catalysed by nitric oxide reductase, an integral membrane protein with the active site faced towards the periplasm (Figure 3). Respiratory nitric oxide reductases belong to the heme-copper oxidase (HCO) super family and can be divided in three groups: (i) cNor, also known as short-chain Nor, is encoded by the *cnorB* gene, accepts electrons from either cytochrome *c*/cupredoxins and consists of two subunits, NorB and NorC, (ii) qNor, or long chain Nor, is encoded by the *qnorB* gene, accepts electrons of ubiquibol/menaquinol and has a similar primary structure as cNor, but consists of a single subunit with a 300 bp N-terminal extension of NorB, and (iii) Cu_ANor, encoded by the gene *cbaA*, is only detected in gram-positive bacteria, accepts electrons from cytochrome c_{551} but, in contrast to what was originally assumed, lacks menaquinol activity (Al-Attar and de Vries, 2015) and is composed of two subunits, a small Cu_A-type subunit and a large subunit NorB. NorC is a *c*-type cytochrome proposed to accept electrons from the respiratory chain and then transfer them to a *b*-type heme in NorB. This electron is next passed on to the dinuclear heme b_3::Fe_B active site which is where

nitric oxide is bound and reduced to nitrous oxide. Indications that cNor contributes to pmf generation are absent as channels previously shown to serve pmf generation in oxygen reducing HCOs are not present in these enzymes (Simon and Klotz, 2013). In contrast, qNor enzyme were found to contain a water channel from the cytoplasm that might serve in proton donation (Matsumoto et al., 2012) while Cu_ANor effectively contributes to pmf generation (Al-Attar and de Vries, 2015). cNor enzymes were found to be unique to denitrifying bacteria, whereas qNor is present in both denitrifying and non-denitrifying bacteria (Hendriks et al., 2000), with especially gram-positive bacteria preferring qNor (Heylen and Keltjens, 2012).

Nitrous oxide reductase. The multi-copper enzyme nitrous oxide reductase N_2OR encoded by the *nosZ* gene, is the last of the three enzymes required for complete denitrification and reduces nitrous oxide to dinitrogen gas (N_2) (Figure 3). It is a periplasmic enzyme in gram-negative bacteria while it appears to be associated with the membrane by a lipid anchor in gram-positive bacteria. Nitrous oxide reductase is unique in the fact that it is the only known enzyme able to remove the ozone depleting greenhouse gas nitrous oxide and organisms containing this enzyme therefor act as sink for nitrous oxide. Two phylogenetical variants of NosZ have been described, each with their distinct regulatory and functional components: (i) typical NosZ commonly found in *Alpha*-, *Beta*-, and *Gammaproteobacteria* performing complete denitrification, although typical NosZ is also found in non-denitrifying bacteria (Zumft, 1997;Zumft and Heinz, 2007), and (ii) atypical NosZ found in taxonomically diverse bacteria comprising a more diverse nitrogen metabolism, *e.g.* performing DNRA or not denitrifying at all (Sanford et al., 2012). Epsilonproteobacterial NosZ are characterized by an additional C-terminally fused monoheme cytochrome *c* domain (Simon et al., 2004). N_2OR is a homodimer with two copper centers per monomer, Cu_A and Cu_Z (Moura and Moura, 2001). Cu_A, the electron entry site in each monomer, is a binuclear copper center. The catalytic Cu_Z sites are tetranuclear copper sites, ligated by seven His residues, and a bridging sulfur atom (Brown et al., 2000). In the functional homodimer, the Cu_A electron input site of one monomer is in close contact with the catalytic Cu_Z center of the other monomer. N_2OR receives electrons from the cytochrome *c*/cupredoxin pool in an electron neutral process and hence does not result in the generation of ATP (Simon and Klotz, 2013). Differences in the degree of conservation in two out of the seven conserved His residues involved in binding of the catalytic center CuZ suggest structural and possibly mechanistic differences between typical and atypical NosZ proteins (Sanford et al., 2012). Similarly to Nir-type denitrifiers, niche differentiation between typical and atypical NosZ-type denitrifiers has been suggested (Jones et al., 2014).

1.2.1.2 Bioenergetics of denitrification

From a theoretical, thermodynamic perspective, nitrate and oxygen are almost equally good electron acceptors when comparing the overall redox potentials of aerobic respiration (ΔG_0'=-2.870kJ per mol glucose) and denitrification (ΔG_0'=-2.670kJ per mol glucose). However, from a bioenergetic

perspective, much more energy is conserved during aerobic respiration making oxygen a much better electron acceptor. The core respiratory machinery for both forms of respiration is the same, but each pathway adds its own modules, *i.e.* a terminal oxidase in aerobic respiration and nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase in denitrification. During aerobic respiration, up to ten protons are translocated per electron pair, resulting in the conservation of approximately 50% of the energy in the form of a proton motive force that subsequently can be used for ATP generation. In contrast, canonical denitrification translocates at most six protons per electron pair when NADH is used as electron donor. Overall, only 30% of the energy is conserved in the form of a proton motive force (Chen and Strous, 2013). This can be explained by the fact that so far only the Nar (Bertero et al., 2003) and Cu_ANor (Al-Attar and de Vries, 2015) modules were experimentally shown to be involved in proton translocation, although qNor (Matsumoto et al., 2012) enzymes may also be involved in pmf generation and ATP production.

1.2.2 Dissimilatory nitrate/nitrite reduction to ammonium (DNRA)

Dissimilatory nitrate/nitrite reduction to ammonium (DNRA) or respiratory ammonification is a two-step anaerobic process involving the two-electron reduction of nitrate to nitrite followed by the six-electron reduction of nitrite to ammonium (Einsle et al., 1999) (Figure 4). It contrasts with denitrification as it results in the retention of nitrogen in the ecosystem, *i.e.* as ammonium, and there are indications that small amounts of nitrous oxide are released as a by-product of DNRA although the mechanism behind this is not understood (Smith, 1982;Cruz-Garcia et al., 2007). DNRA has often been seen as a short circuit of the biological nitrogen cycle as it bypasses both denitrification and dinitrogen fixation. Energy conservation can occur in two different ways: (i) respiratory DNRA, where electrons are transported from non-fermentable substrates (*e.g.* H_2, sulfide or formate) to nitrite generating a proton motive force used for ATP production (Simon, 2002), while (ii) during fermentative DNRA, nitrite acts as an electron-sink allowing re-oxidation of NADH with the generation of one extra ATP by substrate level phosphorylation for each acetate produced (Polcyn and Podeszwa, 2009). The potential to perform DNRA has been found, similarly to denitrification, to be phylogenetically widespread and can be found in members of the *Bacteroidetes* (Mohan et al., 2004), *Proteobacteria* (*Gamma*, *Delta* and *Epsilon*) (Smith et al., 2007), *Actinobacteria*, *Firmicutes*, *Acidobacteria*, *Chloroflexi* and *Planctomycetes* (Welsh et al., 2014). In contrast, only very few cultured DNRA representatives are currently available (Cole et al., 1974;Bonin, 1996;Hoffmann et al., 1998;Mania et al., 2014;Yoon et al., 2015b) in addition to the model organisms *Escherichia coli*, *Wollinella succinogenes*, *Bacillus subtilis* (Cole, 1996;Nakano and Zuber, 1998;Simon, 2002), and the more recent *Bacillus vireti* (Mania et al., 2014) and *Shewanella loihica* (Yoon et al., 2015a;Yoon et al., 2015b). Similarly to denitrification, defining a DNRA bacterium is not that straightforward as one of the two enzymes involved, *i.e.* NirB (see § 1.2.2.1), can also function in assimilatory processes.

Therefore, in this work, a bacterium is considered a DNRA bacterium if the sum of nitrate reduction products (nitrite and nitrous oxide) was less than 70% of the consumed nitrate (Bonin, 1996) with concomitant ammonium production or when its genome contains a *nrfA* and/or *nirB* gene.

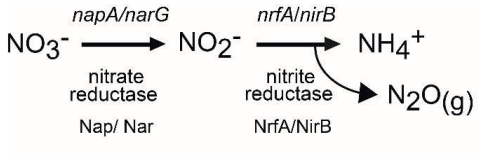

Figure 4 Schematic representation of the DNRA pathway with all different genes, enzymes and intermediates involved. Although Nap is considered to be the predominant nitrate reductase involved, some organisms do contain Nar. Abbreviations: Nar, membrane bound nitrate reductase encoded by *narG*: Nap, periplasmic nitrate reductase encoded by *napA*: NrfA, periplasmic cytochrome *c* nitrite reductase encoded by *nrfA*; NirB, NADH-dependent nitrite reductase encoded by *nirB*.

Comparison of free energy change between denitrification (ΔG_0'= -2.670 kJ/mol glucose, Reaction (1)) and DNRA (ΔG_0'= -1.870 kJ/mol glucose, Reaction (2)) points to a higher amount of free energy for denitrification compared to DNRA (Strohm et al., 2007) indicating that denitrification would be favoured over DNRA under low C:N conditions (electron donor limiting conditions).

(1) $5\ C_6H_{12}O_6 + 24\ NO_3^- + 24\ H^+ \rightarrow 30\ CO_2 + 12\ N_2 + 42\ H_2O$ ($\Delta G°$'= -2,670 kJ per mol glucose)

(2) $C_6H_{12}O_6 + 3\ NO_3^- + 6H^+ \rightarrow 6\ CO_2 + 3\ NH_4^+ + 3\ H_20$ ($\Delta G°$'= -1,870 kJ per mol glucose)

However, under high C:N conditions (electron acceptor limiting conditions), DNRA has the advantage that eight electrons are transferred per mole nitrate compared to five electrons in denitrification, and therefore the energy yield per mol nitrate is higher for DNRA compared to denitrification (Reaction (3) and (4)) (Tiedje, 1982;Strohm et al., 2007). Comparison of the growth yield of the denitrifying bacterium *Paracoccus denitrificans* and the DNRA bacterium *Wollinella succinogenes*, obtained after performing standardized growth experiments, furthermore indicated that DNRA results in twice as much cell mass per mole of nitrate compared to denitrification (Strohm et al., 2007). Inefficient energy conservation during denitrification is the main explanation for this observation (see § 1.2.1.1). From a biokinetic point of view, the dominance of DNRA over denitrification at high C:N conditions can be explained by the higher nitrate affinity (low Ks value) of such organisms.

(3) $5\ C_6H_{12}O_6 + 24\ NO_3^- + 24\ H^+ \rightarrow 30\ CO_2 + 12\ N_2 + 42\ H_2O$ ($\Delta G°$'= -556 kJ per nitrate)

(4) $C_6H_{12}O_6 + 3\ NO_3^- + 6H^+ \rightarrow 6\ CO_2 + 3\ NH_4^+ + 3\ H_20$ ($\Delta G°$'= -623 kJ per nitrate)

1.2.2.1 Enzymes and genes involved in DNRA

The reduction of nitrate to nitrite is assumed to be mostly catalyzed by the periplasmic nitrate reductase Nap, although some organisms contain the membrane-bound nitrate reductase, Nar (Simon,

2002;Kraft et al., 2011). The presence of the Nap pathway enables periplasmic ammonification without the need to transport nitrate or nitrite across the membrane. Reduction of nitrite to ammonium can be catalyzed by the cytoplasmic NADH-dependent nitrite reductase NirB or its to two-subunit variant NirBD (Mohan and Cole, 2007) and/or the periplasmic pentaheme cytochrome *c* nitrite reductase NrfA or the membrane-bound menaquinol-reactive complex NrfHA (Figure 4) (Einsle et al., 1999;Simon, 2002). Variation in the presence and gene expression of the corresponding genes *nrfA* and *nirB* has been observed depending on organism and growth condition. *E. coli* and *B. vireti* were shown to contain and express both enzymes (Cole, 1996;Mania et al., 2014), while *W. succinogenes* (Simon, 2002) and *B. subtilis* (Nakano and Zuber, 1998) contain only NrfA and NirB respectively. Complementary and differential expression of NapA/NrfA and NarG/NirB was observed with NapA/NrfA expression being optimal under nitrate limiting conditions, while NarG/NirB expression was optimal in intermediate to high nitrate conditions (Wang et al., 1999;Wang and Gunsalus, 2000). This observation indicates a role of NrfA in providing adequate membrane potential needed for ATP synthesis in concert with periplasmic NapA nitrate reductase which conserves energy less efficiently compared to NarG (Richardson, 2000). In contrast, NarG and NirB are expressed in nitrate sufficient conditions with NarG activity resulting in proton motive force generation needed for ATP formation (Simon and Klotz, 2013) and NirB involved in detoxification of the accumulated nitrite by converting it to ammonium. This differential and complementary expression in response to their environmental signals allows the organisms to (i) compete more effectively for substrates in nitrate limiting conditions and (ii) energy can be conserved by preventing the synthesis of unneeded respiratory enzymes.

1.2.2.2 Occurrence of denitrification and DNRA within a single organism

Until recently, it was thought that denitrification and DNRA are incompatible within a single organism (Tiedje, 1988). Genome sequencing however indicated that bacteria harboring both the pathway for denitrification and DNRA are not rare in the environment (Heylen and Keltjens, 2012;Sanford et al., 2012;Yoon et al., 2013). The marine bacterium *Shewanella loihicia* PV-4 was shown to possess two non-identical *nrfA* genes and the complete set of denitrification genes (*nir*, *nor*, *nos*). This strain appeared valuable in examining ecophysiological features of denitrification and DNRA. The effect of numerous environmental parameters on the occurrence of either denitrification or DNRA has been examined using continuous (chemostat) cultures (Yoon et al., 2015a;Yoon et al., 2015b). At high C:N ratio, ammonium was the main product of nitrate reduction, while at low C:N denitrification was the dominant pathway. This observation agrees with the hypothesis that the ratio C:N is an important factor in partitioning of nitrate reduction between DNRA and denitrification (Tiedje, 1982). In addition, high pH, high temperature and high nitrite-to-nitrate ratios also favored DNRA over denitrification. Although pure culture studies have limited predictive power for environmental

processes, they might at least give indications on potential controls and explain some previous field observations (see § 1.3).

1.2.3 Anaerobic oxidation to ammonium (anammox)

Anammox bacteria were discovered more than 20 years ago in a waste water treatment plant (Mulder et al., 1995;vandeGraaf et al., 1997;Strous et al., 1999). These chemolithoautotrophic bacteria are able to gain energy by the formation of nitrogen gas from nitrite and ammonium under anoxic conditions (Jetten et al., 2005), with hydrazine and nitric oxide as two intermediates (Figure 1) (Kartal et al., 2011). Similarly to denitrification, this process results in the loss of nitrogen from the system. Anammox bacteria were found to form a monophyletic order within the Planctomycetales and in total five different genera have been described so far: *Candidatus* Brocadia (Strous et al., 1999;Kartal et al., 2008), *Candidatus* Kuenenia (Schmid et al., 2000;Strous et al., 2006), *Candidatus* Scalindua (Kuypers et al., 2003;Schmid et al., 2003;van de Vossenberg et al., 2008), *Candidatus* Jettenia (Quan et al., 2008) and *Candidatus* Anammoxoglobus (Kartal et al., 2007b). Anammox bacteria are metabolically diverse, as they can use other inorganic as well as organic compounds as alternative electron donors and acceptors (Strous et al., 2006;van de Vossenberg et al., 2008). Furthermore, a shortage of ammonium can be surmounted via DNRA by the anammox bacterium using formate as electron donor (Kartal et al., 2007a). These organisms are typically slow-growing with a doubling time between 7 and 14 days, although shorter doubling times are also observed (Lotti et al., 2014), and so far only strongly enriched bioreactor cultures (up to 98% pure) are available. Another intrinsic feature of anammox bacteria is the presence of the anammoxosome, a highly curved organelle that makes up 30% of the cell volume (Jetten et al., 2005). This organelle is involved in energy production and the unique ladderane lipids of its membrane might play an important role in energy conservation by preventing passive proton diffusion across the membrane (van Niftrik and Jetten, 2012). It took more than two decades to figure out the exact pathway involved in converting nitrite and ammonium to dinitrogen gas, and the discovery was greatly enhanced with the availability of the *Ca*. Kuenenia stuttgartiensis genome. The anammox process consists of three consecutive steps (Kartal et al., 2011): (i) the one electron reduction of nitrite to nitric oxide mainly catalysed by nitrite reductase NirS, (ii) the three electron condensation of ammonium and nitric oxide to form hydrazine (N_2H_4) catalysed by hydrazine synthase, an enzyme uniquely found in anammox bacteria and, (iii) the oxidation of hydrazine to dinitrogen gas catalysed by hydrazine dehydrogenase. Step 1 was found to be catalysed by NirK in anammox strain KSU-1 and *Ca*. Jettenia asiatica (Hira et al., 2012;Hu et al., 2012) and *Ca*. Brocadia fulgida was found to contain no previously described *nir*-type reductase (Gori et al., 2011). Anammox bacteria are chemolithoautotrophs that fix carbon through the acetyl coenzyme A pathway (Strous et al., 2006) and conserve energy through the generation of a proton motive force for ATP synthesis by

membrane-bound ATP synthase. This thesis focused on denitrification and DNRA in estuarine sediments and therefore energetics and enzymology of anammox was not addressed in detail.

1.3 Importance of anammox, denitrification and DNRA in marine environments

1.3.1 How to study nitrogen reduction in the environment

Molecular community analysis of nitrate/nitrite reducing bacteria in the environment depends on primers targeting different functional marker genes rather than 16S rRNA genes due to the broad taxonomic distribution of these organisms (Philippot, 2002;Jones et al., 2008;Welsh et al., 2014). Anammox bacteria are an exception as they form a taxonomically coherent group within the *Planctomycetales*. Nevertheless, the use of rRNA genes to study diversity has as main disadvantages that it is generally not related to the physiology of the organism and primer specificity related issues limit diversity captured (Schmid et al., 2005). Functional gene markers like (i) *nirK*, *nirS*, and *nosZ* for denitrification (Thröback et al., 2004), (ii) *nrfA* for DNRA (Mohan et al., 2004;Takeuchi, 2006;Smith et al., 2007;Welsh et al., 2014) and (iii) the single copy and unique anammox gene hydrazine synthase *hzs* (Harhangi et al., 2012), have often been used to examine these communities in marine environments using amplicon-based sequencing approaches and quantitative PCR (Santoro et al., 2006;Takeuchi, 2006;Smith et al., 2007;Magalhaes et al., 2008;Mosier and Francis, 2010;Hou et al., 2013;Russ et al., 2013). Ladderane lipids, exclusively present in anammox bacteria, were found to be a valid complementary tool to PCR-based screening for anammox in the environment (Kuypers et al., 2003;Russ et al., 2013).

Presence or high abundance of functional genes does not necessarily imply importance of a particular process in that environment. Therefore, the effective and relative contribution of nitrate reducing processes can be assessed using (i) isotopic labelling experiments with ^{15}N-nitrite, ^{15}N-nitrate or ^{15}N-ammonium, (ii) the acetylene inhibition technique or (iii) determination of gene transcript abundances (mRNA). The advantage of labelling experiments is that, based on the recovery of the label in the produced nitrogen compound, different processes can be distinguished at once: denitrification converts ^{15}N-nitrate or ^{15}N-nitrite to $^{15}N^{15}N$ while anammox results in $^{14}N^{15}N$ (in the presence of unlabelled ammonium). The contribution of DNRA can be recognised by the conversion of ^{15}N-nitrate to ^{15}N-ammonium. Anammox bacteria can, however, through DNRA under ammonium limiting conditions also produce of ^{15}N-ammonium, which is subsequently combined with ^{15}N-nitrite to produce dinitrogen gas, thereby mimicking denitrification and resulting in the underestimation of the anammox process (Kartal et al., 2007a). DNRA rates, in contrast, can also be underestimated through incorporation of ^{15}N-ammonium into the bacterial cell. The acetylene (C_2H_2) inhibition technique provides a simple alternative way for measuring denitrification rates in diverse environments. This method can however not distinguish between denitrification and anammox, nor

can it provide information on DNRA activity. The main disadvantage of gene transcript determination is its dependence on primers (see further).

1.3.2 Dominance of anammox in oxygen minimum zones and anoxic basins

The geographic distribution of anammox bacteria in the environment resulted in the hypothesis that *Ca*. Scalindua predominates in marine environments whereas *Ca*. Brocadia, *Ca*. Kuenenia, and *Ca*. Anammoxoglobus mainly occur in freshwater habitats, indicating that salinity is an important environmental driver (Schmid et al., 2007). The anammox process was found to contribute mainly to fixed nitrogen removal in oxygen minimum zones (OMZ) in oceans (Kuypers et al., 2005;Lam et al., 2009) and marine anoxic basins (Dalsgaard et al., 2005;Jensen et al., 2008). Activity estimates suggested that up to 20-65% of all fixed nitrogen was removed by anammox in these environments (Dalsgaard et al., 2003;Dalsgaard et al., 2005;Lam et al., 2009;Lam and Kuypers, 2011). In contrast, the anammox process was found to be of minor importance (less than 20%) in estuarine sediments (Trimmer et al., 2003;Dale et al., 2009;Dong et al., 2011;Hou et al., 2013), probably due to the combination of (i) high variability of estuarine environments, (ii) anammox being outcompeted in the presence of high carbon concentration by denitrification or DNRA (Risgaard-Petersen et al., 2004;van Niftrik and Jetten, 2012) and (iii) increased process rates with increasing depth indicating minor importance of anammox in shallow estuarine sediments (Thamdrup, 2012).

1.3.3 Nitrate partioning to DNRA and denitrification

In the past, denitrification has often been considered the dominant nitrate reducing pathway in marine sediments, while DNRA was considered less important or was ignored. However, some recent ^{15}N-studies reported on the predominance of DNRA over denitrification in different marine and estuarine environments (Song et al., 2014), although this was not always the case (Giblin et al., 2013) and sometimes appeared to be dependent on the season (Smith et al., 2015), indicating that DNRA can be an important pathway in benthic sediment nitrogen cycling. The ratio of carbon to nitrate has been suggested to be an important determinant in the fate of nitrate, *i.e*. low C:N ratio favouring denitrification and high C:N supporting DNRA (Tiedje, 1982). Although, this has been observed in lab experiments (Streminska et al., 2012;Kraft et al., 2014), such a clear trend is not always observed in marine environments (Porubsky et al., 2008;Drake et al., 2009;Koop-Jakobsen and Giblin, 2010). High carbon concentrations moreover stimulate sulfate reduction producing sulfide, which is known to stimulate DNRA but also autotrophic denitrification (Burgin and Hamilton, 2007;Moraes et al., 2012).

Predominance of DNRA over denitrification has been reported at higher temperatures, *i.e*. during summer and early autumn (Smith et al., 2015) and in many tropical estuaries (Dong et al., 2011), while DNRA was less important in cold sediments (Crowe et al., 2012). Giblin and colleagues (2010) concluded, based on a 12-year survey of the Parker river estuary, that large variation in seasonal and

inter-annual variability in denitrification and DNRA was primarily driven by salinity and not temperature, with high salinity favouring DNRA. This observation contrasts with the recently observed decrease in transcript numbers of the *nrfA* gene from the Colne estuary head to mouth additionally supported by a decrease in DNRA process rates (Smith et al., 2015). Congruence between *nrfA* gene abundances and DNRA rates was also observed in the New River estuary and it was suggested that *nrfA* gene abundances may have the potential to assess DNRA activity in sediments (Song et al., 2014). Conversely, the marine strain *S. loihica* PV-4 was shown to contain two differentially expressed *nrfA* genes, *i.e.* one highly expressed under high C:N ratios indicating its involvement in DNRA while the second copy was continuously expressed, although orders of a magnitude lower, under both high and low C:N (Yoon et al., 2015b). This demonstrates that the use of both *nrfA* gene abundances and transcript abundances as a proxy for DNRA activity should be cautiously interpreted.

The effect of pH on nitrate removal in marine environments is unknown, however recent batch culture experiments with the marine bacterium *S. loihica* PV-4 indicated that DNRA is favoured at elevated pH (≥ 7.5) while denitrification was favoured under more acidic conditions in batch and chemostat cultures (Yoon et al., 2015b).

The nitrite-to-nitrate ratio has been suggested to be another potential environmental driver, although incongruence between different studies is observed, *i.e.* dominance of DNRA at high nitrite conditions (Yoon et al., 2015a) versus dominance of denitrification at high nitrite conditions (Kraft et al., 2014). The latter study might be more relevant as a sediment community was used instead of a single organism. This study furthermore demonstrated that pH, presence of sulfide and temperature could be ruled out as important drivers. Microbial generation times related to specific nitrite reductase features were found to be essential, *i.e.* at short generation times, a bottle neck in electron supply to NrfA results in dominance of denitrification over DNRA (Kraft et al., 2014). This parameter is however characteristic to chemostat-based experiments, where it is controlled by the dilution rate applied.

Nir and *nrf* community composition and diversity, potentially important regulators of marine nitrate reduction, were shown to be variable along a salinity gradient (Takeuchi, 2006;Francis et al., 2013;Song et al., 2014). Other additional environmental drivers like [chlorophyll *a*], [oxygen], [nitrate], [nitrite] and [ammonium] were previously reported to alter Nir-type denitrifier community composition and showed differential responses between both Nir-type denitrifiers to environmental gradients (Abell et al., 2010;Jones and Hallin, 2010). In molecular surveys, NirS-type denitrifiers are often found to dominate over NirK-type denitrifiers in the marine environment. This observation should however be taken with care as (i) only very few studies quantify both types of denitrifiers in environmental surveys and (ii) *nirK* gene sequence divergence is larger than *nirS* gene sequence divergence as reflected in the limited applicability of current *nirK* primers (Penton et al., 2013).

NirK-type denitrifiers were suggested to contribute more to nitrous oxide emission compared to NirS-type denitrifiers based on positive correlations between NirK abundance and nitrous oxide emissions (Clark et al., 2012) and the negative association between the ratio nitrous oxide/dinitrogen gas and NirS-type denitrifiers (Cuhel et al., 2010). The recently discovered atypical NosZ reductases were also detected in non-denitrifying nitrous oxide reducers, potentially acting as an important nitrous oxide sink, and were completely missed using currently available *nosZ* primers (Sanford et al., 2012). Re-evaluation of the link between NosZ community structure or abundance and potential nitrous oxide flux is necessary to increase the accuracy of predictive gas flux models.

1.3.4 The role of intertidal benthic organisms in nitrogen cycling

Benthic Bacteria and Archaea involved in intertidal mudflat nitrogen cycling share their habitat with a diverse set of invertebrate organisms. Benthic macrofauna species can affect benthic bacterial and archaeal nitrogen cycling through their bioturbation activities (*i.e.*, particle reworking and burrow ventilation, (Kristensen et al., 2012)). Their burrow walls act as extensions of the sediment oxic-anoxic interface thereby stimulating coupled bacterial nitrification-denitrification, although this stimulation process appears to be regulated by macrofaunal density (Braeckman et al., 2010;Laverock et al., 2011;Kristensen et al., 2012). Nitrification– the aerobic oxidation of ammonia to nitrate over nitrite – forms an important source of nitrate within the sediment that can diffuse from the oxic to the anoxic zone where it becomes available to organisms capable of anaerobic nitrate reduction, hence the term coupled nitrification-denitrification. Similarly to denitrification, nitrification can be influenced by the presence of benthic organsisms through the alteration of the oxic sediment environment. The presence of bioturbating polychaetes or bivalves was also shown to negatively impact denitrification through stimulation of bacterial sulfate reduction but, in contrast, stimulated DNRA (Bonaglia et al., 2013;Bonaglia et al., 2014). Finally, macrofaunal grazing on Bacteria, Archaea and microalgae might also liberate intracellular pools of fixed nitrogen and thereby fuel nitrogen cycling.

The general role of meiofauna in benthic nitrogen cycling is much less understood compared to macrofauna, notwithstanding that they dominate in terms of biomass, abundance and diversity, and many interactions between meiofauna and prokaryotes have already been described such as organic matter remineralization (Nascimento et al., 2012) and degradation of pollutants (Naslund et al., 2010). Nematode bioturbation has been shown to stimulate coupled bacterial nitrification-denitrification at the oxic-anoxic interface of marine sediments, although this stimulating effect was counteracted by the presence of macrofaunal bivalves (Bonaglia et al., 2014). In another study, copepods were shown to enhance DNRA while reducing denitrification rates (Stock et al., 2014). Similar to macrofauna, grazing by meiofauna might also result in the remineralization of intracellular fixed nitrogen pools from Bacteria, Archaea and benthic microalgae. Recently, it has been shown that the meiofaunal group foraminifera are capable of complete denitrification (Risgaard-Petersen et al., 2006;Hogslund et al., 2008), with the ability to denitrify attributed to the foraminifera itself and not to endobiotic bacteria.

These organisms can move towards the sediment surface where nitrate concentrations are highest and can reach outside the diffusive boundary layer which provides them a competitive advantage compared to nitrate reducing bacteria (Thamdrup, 2012).
Direct involvement of Eukaryotes in the dissimilatory parts of the nitrogen cycle has also been shown. Microphytobenthos, consisting predominantly of diatoms in muddy environments, have been shown to (i) negatively influence bacterial denitrification (Risgaard-Petersen, 2003;Sundback et al., 2006), with these microalgae outcompeting bacteria for nitrogen, (ii) enhance bacterial denitrification (An and Joye, 2001;Tobias et al., 2003;Porubsky et al., 2009) or (iii) be neutral (Stock et al., 2014). Benthic diatoms can store nitrate intracellularly exceeding ambient levels by several orders of magnitude, and use DNRA as dark survival strategy, releasing ammonium to the environment (Kamp et al., 2011;Stief et al., 2013). Diverse fungi were shown to reduce nitrate to nitrite (Shoun and Tanimoto, 1991;Tsuruta et al., 1998), while some subsequently reduce nitrite to either nitrous oxide via denitrification or to ammonium through DRNA. Complete denitrification in fungi has never been observed. Fermentative DNRA was shown to occur only under anoxic conditions, while respiratory denitrification occurred under oxygen limiting conditions (Zhou et al., 2001;Zhou et al., 2002). Influences of the estuarine fauna on the anammox process remain unclear as no studies have focused on this process. A schematic overview of all currently known influences – both biotic and abiotic – on denitrification, DNRA and anammox in marine environments is represented in Figure 5.

Originally, it was believed that prokaryotes were the sole organisms capable of nitrate respiration. Recent evidence indicates otherwise, however, Prokaryotes and, more specifically, Bacteria remain the major players in nitrate reducing pathways like denitrification and DNRA. Nevertheless, it is important to realize that other processes such as nitrite-dependent anaerobic methane oxidation (Ettwig et al., 2010) or the recently discovered reverse-hydroxylamine:ubiquinone reductase module (reverse-HURM) pathway, a nitrate/nitrite ammonification pathway that provides the organisms with ammonium for biomass synthesis (Hanson et al., 2013), might also contribute to nitrogen removal or retention in marine environment.

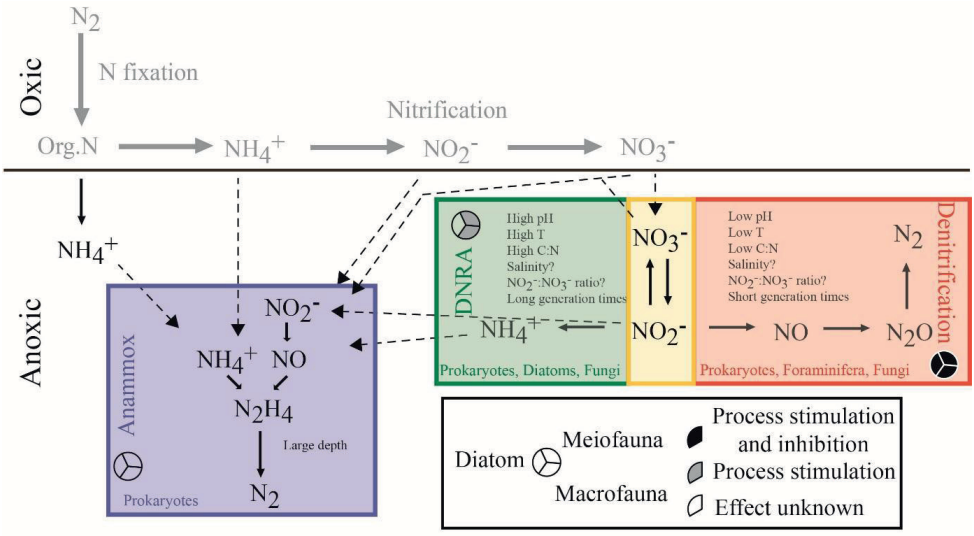

Figure 5 Schematic illustration of nitrogen cycling in estuarine sediments, with specific focus on dissimilatory nitrate reducing processes, *i.e.* denitrification (red), DNRA (green) and anammox (blue). Different organisms (Prokaryotes, Diatoms, Foraminifera and Fungi) known to perform each process are included in the respective process colour. Effects of macrofauna, meiofauna and diatoms previously described in literature are indicated using small pie charts. Their contributions are presented in a simplified way as only individual effects of each trophic level are shown on a single process. Parameters involved in determining the dominance of a certain process are also given and in case of ambiguity a question mark was added. Solid black arrows represent nitrogen conversion through the nitrogen cycle. Striped arrows indicate diffusion of nitrogen cycling intermediates that can subsequently be used in other processes. Full terms of abbreviations included are DNRA: dissimilatory nitrate reduction to ammonium, Org.N: organic nitrogen, T: temperature, C: carbon, N: nitrogen.

1.4 Conceptual framework of the thesis

1.4.1 Current research status

While estuaries are important conduits canalizing fertilizer run-off from land to sea, they also play an important role in removing this anthropogenic nitrogen via benthic nitrogen reduction before water enters the open ocean. Denitrification, sometimes coupled to nitrification, was identified in the past as the dominant nitrogen removing pathway in these systems. Numerous studies on the importance of estuarine denitrification and the influence of the diverse estuarine fauna on this process have been performed for many years. Nevertheless, knowledge on the diversity and distribution of bacterial players involved, remains limited. In addition, environmental surveys largely depend on PCR-based approaches using functional markers like *nirK* and *nirS* as a proxy to examine presence, abundances and activities of denitrifying communities. Very few attempts have been made to target both markers simultaneously, either due to unsuccessful amplification of *nirK* or the assumption that *nirS* denitrifiers are more important *in situ* because of their numerical dominance. Restricted applicability of current *nirK* primers has been known for many years and a few attempts have been made to improve coverage, but these attempts failed repeatedly (Thröback et al., 2004;Green et al., 2010). Why these sequences are so diverse, what makes them so different compared to cytochrome cd_1-dependent nitrite reductase (*nirS)* sequences and what this means biologically, remain major questions to be answered.

Over the last years, evidence on the importance of DNRA in marine ecosystems has increased, further complicating the understanding of nitrogen cycling in these environments. A major question concerns the variation in the relative importance of these processes in marine systems and the environmental parameters involved. From the viewpoint of eutrophication this is essential as denitrification is the main process resulting in effective loss of nitrogen. Current knowledge on DNRA is based mostly on a few model organisms like *E. coli* and *W. succinogenes*, while only limited cultured marine representatives are currently available. Isolating DNRA bacteria relevant in marine/estuarine environments will certainly assist the understanding of the ecophysiology of key species involved *in situ*.

1.4.2 Introduction to the study area

The Paulina Polder intertidal mudflat, located along the polyhaline part of the Westerschelde estuary, The Netherlands, was used as study area in this thesis. The Westerschelde estuary is a well-mixed, turbid, macrotidal estuary situated in NW Europe, near the border between The Netherlands and Belgium (Figure 6). The catchment area is urban with a total population of more than 10 million people and densities varying from 100 to more than 2000 inhabitants km². The tidal regime is semidiurnal with mean neap and spring tides of 2.7 and 4.5 m respectively (Regnier et al., 1998). The

yearly average fluvial freshwater discharge amounts 105 m^3 s^{-1} (Baeyens et al., 1998) and salinity ranges from approximately 12 PSU at the estuary head to 32 PSU at the mouth. The residence time of the water in the estuary is rather high, ranging from 10–15 days in the most seaward region to 50–70 days in the upstream regions (Soetaert and Herman, 1995). The Westerschelde estuary is a highly heterotrophic system were annual gross bacterial production exceeds net primary production (Goosen et al., 1999). This eutrophied estuary has a nitrogen load of 5×10^9 mol N yr^{-1} (Soetaert and Herman, 1995) with nitrate being the predominant form of reactive nitrogen (Soetaert et al., 2006) and denitrification the main nitrate removing process (Dahnke et al., 2012;Van Colen et al., 2012).

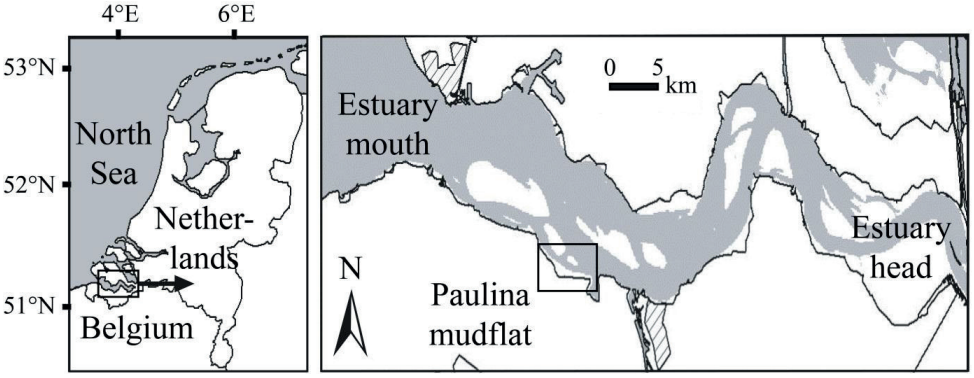

Figure 6 Geographical location of the Paulina polder tidal mudflat (Westerschelde estuary, SW Netherlands). Estuary head and mouth are indicated.

1.4.3 Thesis outline

The main objectives of this thesis were (i) to obtain an insight into total bacterial and denitrifier community diversity, abundance and composition in estuarine sediments, and assess the effect of microphytobenthos presence on these community features, (ii) to evaluate classification and diversity of NirK sequences and the variability of the dissimilatory nitrogen cycling gene inventory in NirK-type denitrifiers, (iii) to assess the relative importance of distinct nitrate removing pathways within estuarine sediments and (iv) to provide new cultured marine representatives of nitrogen removing bacteria for future studies to unravel ecophysiological features of these groups of bacteria in marine environments.

The description of the experimental work performed is organized into three chapters (2-4) which are published papers or submitted manuscripts, and at the end of each chapter a reflection and discussion

section is included with hindsight reflections, updated information and/or some (experimental) aspects that could not be addressed in detail in the manuscript:

In **Chapter II**, we report on the effect of microphytobenthos (MPB) biomass on total bacterial and denitrifier community diversity and abundances in estuarine sediments. MPB have been shown to intervene in the nitrogen cycle, however if and how they shape denitrifying communities *in situ* is unknown. In addition to nitrogen, other types of algal-bacterial coupling have been reported to influence the total bacterial community, nevertheless the effects of increased MPB biomass remains ambiguous. We assessed total bacterial and denitrifying guild community structure and diversity using amplicon-based pyrosequencing and their abundances through qPCR. In contrast to many other studies, denitrifying guild communities were evaluated using both *nirk* and *nirS* genes as functional markers.

In **Chapter III**, we examined the nowadays generally accepted underestimation of denitrifying organisms in amplicon-based environmental surveys. *NirK*-type denitrifiers were choosen as a case study as this gene is often excluded in such surveys as a result of unsuccesfull amplification of the *nirK* gene. An in-detail study of taxonomically diverse, full-length copper-dependent nitrite reductases, NirK, extracted from publically available genomes, was performed with re-assessment of previous described NirK phylogenetic clades. The potential of NirK-type denitrifiers to partition nitrate between denitrification and DNRA was also evaluated.

In **Chapter IV** we surveyed the occurrence and the relative importance of three nitrate removing processes, *i.e*. denitrification, DNRA and nitrate reduction to nitrite, in estuarine sediments using the acetylene inhibition technique. In the past, it was generally assumed that denitrification formed the main nitrate removing pathway within such sediments, however more recent reports indicated that DNRA can contribute equally or even more to nitrate removal compared to denitrification. Additionally, we were specifically interested in the identity of DNRA bacteria present within these sediments as not much is known about these organisms in marine environments. Therefore, an isolation campaign targeting marine nitrate reducing bacteria was performed.

Chapter 2

A doubling of microphytobenthos biomass coincides with a tenfold increase in denitrifiers and total bacterial abundances in intertidal sediments of a temperate estuary

Redrafted from:

Decleyre H., Heylen K., Sabbe K., Tytgat B., Deforce D., Van Nieuwerbrugh F., Van Colen C. and Willems A. (2015). A doubling of microphytobenthos biomass coincides with a tenfold increase in denitrifiers and total bacterial abundances in intertidal sediments of a temperate estuary. PLoS ONE 10(5): e0126583. doi:10.1371/journal.pone.0126583.

Author's contributions:

HD, KH and AW designed the experiments. HD performed the experiments. HD and KH analyzed the data. CVC, KS, DD, FVN and BT contributed analysis tools. HD, KH, AW, CVC and KS wrote the paper.

Summary

Surface sediments are important systems for the removal of anthropogenically derived inorganic nitrogen in estuaries. They are often characterized by the presence of a microphytobenthos (MPB) biofilm, which can impact bacterial communities in underlying sediments for example by secretion of extracellular polymeric substances (EPS) and competition for nutrients (including nitrogen). Pyrosequencing and qPCR was performed on two intertidal surface sediments of the Westerschelde estuary characterized by a two-fold difference in MPB biomass but no difference in MPB composition. Doubling of MPB biomass was accompanied by a disproportionately (ten-fold) increase in total bacterial abundances while, unexpectedly, no difference in general community structure was observed, despite significantly lower bacterial richness and distinct community membership, mostly for non-abundant taxa. Denitrifier abundances corresponded likewise while community structure, both for *nirS* and *nirK* denitrifiers, remained unchanged, suggesting that competition with diatoms for nitrate is negligible at concentrations in the investigated sediments (appr. 1 mg/l NO_3^-). This study indicates that MPB biomass increase has a general, significantly positive effect on total bacterial and denitrifier abundances, with stimulation or inhibition of specific bacterial groups that however do not result in a re-structured community.

2.1 Introduction

The rate of terrestrial nitrogen input has more than doubled in the past century, mostly through fossil fuel combustion and increased use of agricultural fertilizers (Vitousek et al., 1997;Canfield et al., 2010). When it is not removed by biotic uptake or dissimilatory nitrate reduction in streams and rivers, excessive, anthropogenically-derived nitrogen ends up in estuaries and coastal areas (Turner and Rabalais, 2003), where it is implicated in eutrophication (Paerl et al., 2006) that can generate an excessive biochemical oxygen demand resulting in hypoxic zones (Diaz and Rosenberg, 2008), and can promote harmful algal blooms (Anderson et al., 2002). Estuarine surface sediments, lying at the interface between the oxidized water column or atmosphere and the deeper reduced sediment, can serve as important removal sites for inorganic nitrogen (Seitzinger, 1988). Benthic dissimilatory nitrate reduction includes three primarily anoxic processes with varying importance (Smith et al., 2007;Dong et al., 2009;Dong et al., 2011;Giblin et al., 2013): nitrate can be retained in the system as biologically available ammonium via dissimilatory nitrate/nitrite reduction to ammonium (DNRA) or lost by reduction to a gaseous product via anaerobic ammonium oxidation (anammox) or denitrification, *i.e*. the respiratory reduction of nitrate to either the potent greenhouse gas nitrous oxide or the harmless dinitrogen gas. Denitrification is most likely to dominate in temperate coastal areas (Giblin et al., 2013). It is proposed to be favoured over DNRA with decreasing salinity (Gardner and McCarthy, 2009;Giblin et al., 2013) and lower temperatures (Dong et al., 2011;Giblin et al., 2013), and outcompetes anammox in highly variable, eutrophic estuaries (Trimmer et al., 2003;Rich et al., 2008;Dale et al., 2009).

Denitrification is performed by a wide variety of phylogenetically unrelated microorganisms. Therefore, denitrifying communities are commonly characterized using the two genes encoding cytochrome cd_1 and copper-containing nitrite reductases (*nirS* and *nirK* respectively) as a proxy (Philippot and Hallin, 2005). These key enzymes convert fixed nitrogen to a gaseous form, as such distinguishing dissimilatory nitrate-reducing bacteria that produce nitrite as end-product from true denitrifiers. The two forms are functionally equivalent but structurally different and respond to different environmental drivers, most of which are currently still unknown (Zumft, 1997;Jones and Hallin, 2010). Recently, elaborate genome analyses showed that both *nir* genes are not mutually exclusive in a single organism (Graf et al., 2014). However, the functionality of these two nitrite reductases in one organism remains to be demonstrated. *NirS* denitrifiers are more widespread, whereas *nirK* denitrifiers comprise more diverse taxa (Zumft, 1997). Despite the importance of denitrification in estuarine systems, little is known about the diversity and distribution of the two denitrifying guilds in these ecosystems and almost no attempts are made to target both, either due to unsuccessful amplification of *nirK* or the assumption that *nirS* denitrifiers are more important *in situ* because of their numerical dominance (Francis et al., 2013).

In addition to denitrifying microorganisms, biofilm-producing microphytobenthos (MPB) present in the uppermost mm of sediments can also influence benthic nitrate reduction. MPB metabolism decouples nitrification-denitrification through (i) competition for ammonium and nitrate between MPB and bacteria (Hochard et al., 2010) and (ii) pH increase via CO_2 removal from the pore water (Bartoli et al., 2012). MPB inorganic nitrogen assimilation can even exceed N consumption via denitrification by one to two orders of magnitude (Sundback and Miles, 2000;Hochard et al., 2010), depending on the *in situ* nitrate concentrations. Diatoms, which often dominate primary production in estuarine intertidal sediments, are also known to store nitrate intracellularly up to a few 100 mM (Lomas and Glibert, 2000;Kamp et al., 2011;Stief et al., 2013), and even use DNRA as dark survival strategy, releasing ammonium to the environment. Thus, MPB intervene in nitrogen cycling but if and how they shape the denitrifier communities *in situ* is not known.

Besides for inorganic nitrogen, other types of algal-bacterial coupling exist in these complex estuarine ecosystem (Haynes et al., 2007). Photosynthetically fixed carbon by MPB is transferred to heterotrophic bacteria within hours, resulting in a quick use of labile biofilm DOC and hydrolysed EPS (Middelburg et al., 2000;Risgaard-Petersen, 2003;Bellinger et al., 2009;McKew et al., 2013;Taylor et al., 2013), while MPB can also produce cytotoxins that can inhibit bacterial growth (Ianora and Miralto, 2010). These algal-bacterial interactions are species-specific, both for diatoms and bacteria (Hanlon et al., 2006;Bellinger et al., 2009;Amin et al., 2012). However, the effects of increased MPB biomass on bacteria in underlying sediments, including higher bacterial enzymatic activity (Hanlon et al., 2006) and EPS production (Yallop et al., 2000) but without or with only a small increase in total bacterial cell numbers (Yallop et al., 2000;Gurung et al., 2001;Belzile et al., 2008;Lubarsky et al., 2010;Orvain et al., 2014), remains ambiguous.

Based on the current knowledge, we hypothesized that higher MPB biomass (i) does not affect total bacterial abundances, (ii) negatively impacts denitrifier abundances, (iii) results in different total bacterial and denitrifier community structure, and (iv) generates differential responses of *nirK* and *nirS* denitrifier communities. To investigate these hypotheses, we sampled estuarine sediments at the Paulina polder tidal flat (Westerschelde estuary, SW Netherlands), characterized by the presence of MPB biofilms stabilizing sediment surfaces (Lubarsky et al., 2010;Stal, 2010). The eutrophied Westerschelde estuary has a nitrogen load of 5×10^9 mol N yr^{-1} (Soetaert and Herman, 1995) with nitrate being the predominant form of reactive nitrogen (Soetaert et al., 2006) and denitrification as the main nitrate removing process (Dahnke et al., 2012;Van Colen et al., 2012). Sediment samples solely differed in MPB biomass but not MPB composition. Abundance and diversity of the total bacterial community as well as both *nirS* and *nirK* denitrifying guilds were assessed using qPCR and 454 pyrosequencing. Abundant and non-abundant fractions of all three bacterial communities (*i.e.* all bacteria, *nirK*, *nirS*) were examined separately to also assess influences of the low-abundant fraction of the bacterial community on diversity parameters.

2.2 Materials and Methods

Sampling and analytical procedures

Samples were collected in October 2011 at the Paulina polder mudflat (51° 21' 24" N, 3° 42' 51" E) in collaboration with NIOZ, which provided the necessary permit for field sampling, issued by the 'Provincie Zeeland, The Netherlands; Directie Ruimte, Milieu en Water'. A plexiglas corer (inner Ø 3.2 cm) was used to collect triplicate samples of bacterial communities in two muddy sediments. To assess the sole effect of MPB on total bacterial and denitrifier abundance and diversity, two adjacent (± 6 m), physico-chemically similar sediments with visually different MPB biofilm development (high (HBM) or low (LBM) biomass of MPB) were sampled (MPB biofilms are visible as a brown film on the sediment; Figure 1). In both LBM and HBM sediments, the three replicates were taken as close together as technical constraints would allow, *i.e.* within a 10 x 10 cm square (Figure 1). The sediment cores were sealed and kept at 4 °C until further processing. In the lab, the upper cm of the sediment was sampled and stored in sterile falcon tubes at -20 °C until DNA extraction. At each location three further cores (each in triplicate) were taken for nutrient analyses (inner Ø 6.2 cm), determination of total organic matter (TOM) and grain size (inner Ø 3.2 cm) and determination of extracellular polymeric substances (EPS) and chlorophyll *a* (inner Ø 3.2 cm). These additional cores were taken adjacent to the cores for bacterial community structure analysis. The upper one cm of these cores was immediately frozen at -80 °C (for pigment analysis) or -20 °C (for all other parameters) until further analysis. The samples were analysed for $NO_3^-/NO_2^-/NH_4^+/Si/PO_4^-$ pore water concentrations (SAN^{plus} segmented flow analyzer, SKALAR), the total amount of organic matter (loss of mass after incineration at 500 °C for 2 hours) and grain size distribution using laser diffraction (Malvern Mastersizer 2000). To assess MPB biomass in the sediment (Jeffrey et al., 1997), chl *a* concentration was measured by HPLC analysis after pigment extraction using 10 ml 90% acetone - 10% milliQ water solution (Wright and Jeffrey, 1997). Colloidal extracellular polymeric substances (cEPS) were determined by spectrophotometry using the phenol-sulfuric acid assay (Dubois et al., 1956). Furthermore, an additional single core was taken from each sediment type (inner Ø 6.2 cm, n=1) to measure profiles of dissolved oxygen concentrations (vertical increments of 0.2 mm, Figure S1) and pH (increments of 1mm) in the laboratory in triplicate using Unisense microsensors (25 µm and 500 µm tip size for oxygen and pH respectively) and was used to determine the mm depth of the oxic-anoxic border and the geometric mean of the pH. Mean difference analysis using a t-test confirmed that both HBM and LBM sediments indeed only differed in parameters which could be related to MPB activity (*i.e.* chl *a*, EPS, phosphate, silicate), but not in other physical or chemical parameters (Table 1).

DNA extraction

DNA was extracted in triplicate from each HBM and LBM biological replicate (only top 0-1cm) separately to account for both technical and biological variation using a slight modification of Boon et

al. (2000). In brief, one gram of sediment, 750 µl 1x TE buffer (1 mM EDTA, 10 mM Tris-HCL, pH8) and 0.5 g of glass beads (Ø 0.1 mm) were added to a 2 ml Safe lock tube (Eppendorf). The mixture was vigorously shaken three times for 90 s using a bead beater (Mixer Mill MM200, Retsch) at a frequency of 30 Hz. Then, 150 µl of lyzosyme (6 mg /150 µl 1x TE buffer) was added and gently mixed for 5 minutes at room temperature. Subsequently, 40 µl of 20% SDS was added, and samples were again slowly mixed for 5 min at room temperature, with subsequent addition of 250 µl 8M ammonium acetate. The supernatant was collected after centrifugation at 7 000 rpm for 15 min at 4 °C. A chloroform-isoamylalcohol (24:1) purification was performed, followed by centrifugation at 7 000 rpm for 15 min at 4 °C. The aqueous phase was transferred to a new Safe lock tube, and 0.8 volume of isopropanol was added. The precipitation was performed for 1 h at -20 °C. Finally, the pellet was obtained by centrifugation at 12 000 rpm for 25 min, washed in 70% ethanol (5 min at 12 000 rpm) and resolved in 50 µl of 1x TE buffer. DNA yields were determined using a Nanodrop 2000 spectrophotometer (Thermo, Scientific) and the quality was checked by gel electrophoresis. Finally, DNA triplicates of a single biological replicate were mixed together for further analysis.

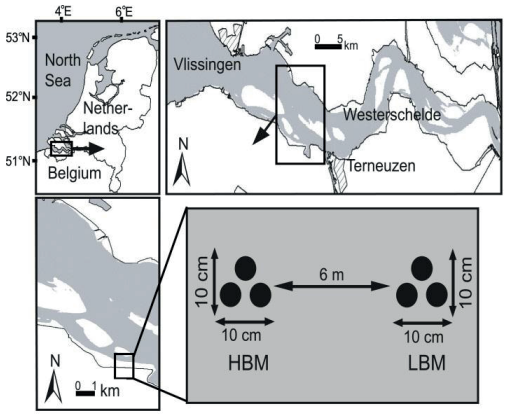

Figure 1 Geographical location of the Paulina tidal flat (Westerschelde estuary, SW Netherlands) and sampling design. For both estuarine sediments types (HBM and LBM) triplicate samples were taken as close as technical constraints allowed. Additional cores for measuring physico-chemical parameters were taken in immediate vicinity of the sample cores (not shown on figure).

Barcoded amplicon sequencing of *nirK*, *nirS* and 16S rRNA genes

Amplicon libraries were prepared using a two-step PCR procedure as recommended by Berry et al. (2011) for the 16S rRNA, *nirK* and *nirS* genes. Six different multiplex identifiers (MIDs) were used to identify the different replicates per sediment type (Table S1). Targeting the V3-V1 region of the 16S

rRNA, 27 cycles of amplification were performed with the F19-38 (CTGGCTCAGGAYGAACGCTG (Koort et al., 2005)) / 518R (ATTACCGCGGCTGCTG (Yu and Morrison, 2004)) primer set. Sequencing starting from the V3 region was selected as it was previously shown to result in good taxonomic assignment (Huse et al., 2008;Mizrahi-Man O, 2013). Furthermore, the V1-V3 region is often targeted in sequencing projects and therefore well represented in publically available databases. In a second step, 2 µl of the PCR products of the first reaction was amplified in a 5 cycle PCR with the barcoded PCR primers including the 10 basepair-MID, a sequencing key and the sequencing primer (Table S1). Each PCR reaction was performed in a 25 µl volume using 1.25 U FastStart High Fidelity Enzyme Blend (Roche), 0.1 µM primers, 0.2 mM dNTP mix and 0.1 mg bovine serum albumin (BSA, only used on the first round of PCR) and milliQ water. All reactions were performed in triplicate to take into account technical variation and were pooled before determination of PCR product quantity and quality. The thermal program consisted of an initial denaturation step of 95 °C for 3 min, a cycling program of 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 120 s. Before sequencing, PCR amplicons were purified using the MinElute purification kit (Qiagen) and quantified with a fluorescent stain-based kit (Quant-iT Pico Green, Invitrogen). The quality of the PCR product was assessed on a Bioanalyzer 2100 using a high sensitivity DNA chip (Aligent). Amplicon libraries for the *nirK* and *nirS* genes using the primer pairs F1aCu (ATCATGGTSCTGCCGCG) - R3Cu (GCCTCGATCAGRTTGTGGTT) (Hallin and Lindgren, 1999) and Cd1aF (GTSAACGTSAAGGARACSGG) - R3cd (GARTTCGGRTGSGTCTTGA) (Michotey et al., 2000;Throbäck et al., 2004) were prepared in a similar way as described above except that the number of cycles in the first PCR round was increased to 35 cycles. All amplicons were sequenced unidirectionally, starting from the forward primer for *nirK* and *nirS* and the reverse primer for 16S rRNA gene. Sequencing was performed on a GS FLX Titanium at the NXTGNT sequencing facility of Ghent University.

Sequence analysis

All sequence data were screened and de-multiplexed using default setting of MOTHUR v.1.30.1 (Schloss et al., 2009). Sequences containing homopolymers of more than 8 nucleotides, mismatches to the barcode (>1) and primers (>2) and sequences shorter than 200 bp were discarded. Subsequently, a MOTHUR-implemented version of pyronoise was used to further denoise the data. Chimera sequences were removed using the Uchime algorithm (Edgar et al., 2011) and, in case of the 16S rRNA gene, potential chloroplast and mitochondrial sequences were also removed. The 16S rRNA gene sequences were aligned using the SILVA reference alignment (release 102) and binned into operational taxonomic units (OTUs) at a 97% gene sequence identity threshold. Sequences were classified using a MOTHUR formatted version of the RDP training set (v.9).

Prior to alignment, the *nirK*/*nirS* gene sequence data were checked for the presence of specific functional/conserved regions and screened using the HMM FRAME algorithm (Zhang and Sun, 2011) included in the FunFrame pipeline (Weisman et al., 2013) to detect and correct frameshift errors. The obtained HMM alignment scores were used for further quality filtering. The cytochrome d_1 HMM from Pfam (accession PF02239.10) was used for the *nirS* gene sequence dataset and sequences with a HMM score ≥ 107 were retained. The *nirK* primers targeted a region that overlaps with two domains, plastocyanin-like 1 and plastocyanin-like 2, of the *nirK* gene and a HMM was designed covering the primer target region based on sequences obtained from the Fungene database using the HMMER3 program (Hmmer.org). *NirK* gene sequences with a HMM score ≥ 48 were retained and for both genes pairwise alignment was performed with the remaining sequences using MOTHUR v.1.30.1 (Schloss et al., 2009). Cut-offs for binning *nirS* and *nirK* gene sequences into OTUs were determined experimentally with the focus on only retaining functional diversity, *i.e*. binning identical amino acid sequences. A range of threshold distances (5-20%) was tested for both the *nirK* and *nirS* gene sequences using MOTHUR v.1.30.1 (Schloss et al., 2009). Subsequently, all OTU representative gene sequences per threshold distance were translated *in silico* and pairwise distance matrices of amino acid sequences of all OTU representatives were determined using MEGA 5.10 (Kumar et al., 2008). At a cut-off of 82% gene sequence identity, all pairwise distances of *in silico* translated AA sequences of all *nirK* OTU representatives were > 0, indicating that the sequences of all OTU representatives had a unique AA sequence (~functional diversity). In case of the *nirS* gene, a similarly obtained cut-off of 80% sequence identity was used for OTU binning. Maximum likelihood phylogenies for both genes were calculated using RAxML 7.4.2 (Stamatakis, 2006;Ott et al., 2010). Protein BLAST searches with the OTU representatives were performed to determine the closest relatives using three different NCBI databases: a non-redundant protein database with/without uncultured/environmental sequences and the whole genome database. If possible, depending on alignable length and e-value, the first five hits were used for further analysis. Node confidence was determined using 1 000 bootstrap replicates.

Quantification of *nirK*, *nirS*, and 16S rRNA genes

Quantitative real-time PCR (qPCR) of *nirK*, *nirS* and 16S rRNA genes was carried out using a Lightcycler 480 II (Roche, Applied Science). Standard curves were prepared from serial dilutions of linearized plasmid with the *nirK* gene from *Alcaligenes faecalis* LMG 1229^T, *nirS* gene from *Paracoccus* sp. R-24615 and 16S rRNA gene from *Flavobacterium swingsii* LMG 25510, containing between 10^9 to 10^1 target gene copies calculated directly from the concentration of the extracted plasmid. DNA concentrations were determined using the Nanodrop 2000 spectrophotometer (Thermo, Scientific). The qPCR assays were carried out in a 20 µl reaction volume composed of SensiMix™ SYBR No-ROX (Bioline GmbH, Luckenwalde, Germany), 0.4 µM of each primer, 2.5 µl of template DNA (10 ng/µl), 0.1 mg BSA (not used in the *nirS* assay) and sterilized milliQ water. The same primers as for pyrosequencing were used and the thermal protocol can be found in Table S2. All

reactions per sample were performed in triplicate. Agarose gel electrophoresis, melting curve analysis, cloning and sequencing of the obtained amplicons indicated that the amplification was specific. PCR inhibition was determined by spiking sediment DNA with a known amount of standard DNA and corrected for according to Zaprasis et al. (2010).

Statistical analysis

Chao1 estimations, Inverse Simpson diversity indices, rarefaction curves, Venn diagrams, community membership (Jaccard dissimilarity index) and structure (Bray Curtis dissimilarity index) were calculated using MOTHUR v.1.30.1 (Schloss et al., 2009). Mean differences between HBM and LBM sediment samples were tested using a t-test. Further, correlations between physico-chemical parameters and abundance data from the six collected samples were analysed using product moment correlations (Statistica 5.0, Statsoft 1984-1995). Homogeneities of variances were checked using the Levene's test ($p > 0.05$). For PO_4^{3-}, non-parametric Mann-Whitney U tests and Spearman's rank correlations were used since the necessary assumptions for heterogeneity of variances and normal distributions were not met. Permutational analyses of variance (Permanova) were conducted using the Permanova add-on software for Primer v6 (Anderson et al., 2008).

Nucleotide sequence accession numbers

Complete amplicon libraries of 16S rRNA, *nirK* and *nirS* gene sequences derived from barcoded amplicon sequencing were deposited in the Genbank SRA database under study accession number SRP035903.

2.3 Results

Sediments sampling and physico-chemical analysis

Two adjacent (± 6 m) sediments with visually different MPB biofilm development were sampled in triplicate. MPB communities in all HBM and LBM replicates were dominated mainly by *Navicula* spp. (*Navicula arenaria* var. *rostellata*, *N. phyllepta* and *N. gregaria*) and contained to a lesser degree also *Gyrosigma fasciola*, *Amphora copulata* and an unknown *Nitzschia* species. No significant differences could be observed between the HBM and LBM sediments in TOM, % mud content and pore water concentrations of NO_3^-, NO_2^- and NH_4^+ (Table 1). So, the only detected differences between HBM and LBM sediments were specifically related to the presence and activity of MPB: chlorophyll *a*, a proxy for MPB biomass, and EPS were significantly higher in the HBM sediments, while PO_4^{3-} and Si were significantly lower ($p < 0.05$, Table 1).

Table 1 Physico-chemical parameters of both estuarine sediments (HBM and LBM) (n=3).

Parameter	HBM	LBM
pH	6.69 ± 0.26	7.07 ± 0.19
oxic-anoxic interface (mm depth)	5.25 ± 0.99	6.51 ± 0.64
TOM (%)[a]	5.73 ± 0.67	4.65 ± 0.42
Chl *a* (µg/g dw)[*]	28.61 ± 1.61	13.26 ± 3.48
EPS (mg/mg dry sediment)[b,*]	0.0004 ± 0.00015	0.0002 ± 0.00003
% mud [c]	51.26 ± 9.92	45.94 ± 5.08
NH_4^+ (µg/l)[d]	3 632.58 ± 434.83	4 445.93 ± 2870.13
NO_3^- (µg/l)[d]	1 376.85 ± 281.89	761.33 ± 351.88
NO_2^- (µg/l)[d]	10.17 ± 2.31	16.24 ± 4.51
PO_4^{3-} (µg/l)[d,*]	1 058.94 ± 66.53	1 918.8 ± 1276.08
Si (µg/l)[d,*]	2 125.45 ± 622.9	4 356.73 ± 206.36

Significant differences (*) between both sediments (t-test, $p \leq 0.05$) were detected using a t-test ($p \leq 0.05$); for PO_4^{3-}, a non-parametric test (Mann-Whitney U) was performed. Reliability of significance testing was checked using the Levene's p-value (> 0.05). Millimeter depth till oxic-anoxic border and pH were excluded from statistical testing as no biological replicates were taken for these parameters.
[a] TOM, total organic matter, [b] Extracellular polymeric substances, [c] Percentage mud (particle size < 63µm) determined using the Wenthworth grain size chart, [d] Pore water concentrations.

Total bacterial community diversity, estimated richness and structure

The rarefaction curves (Figure S2) and the ratio observed:estimated 16S rRNA OTU richness for samples HBM1, HBM3, LBM1 and LBM2 (0.72-0.84, Table 2) indicated that the current sampling effort was almost sufficient to capture total bacterial diversity, but showed clear differences between HBM and LBM samples (0.72-0.76 vs. 0.84-0.85; Table 2). The 16S rRNA gene rarefaction curves of a replicate per sample type (HBM2 and LBM3) flattened very quickly in comparison with the other

Table 2 Overview of 16S rRNA (n=2), *nirK* (n=3) and *nirS* (n=3) gene sequences derived from HBM and LBM estuarine sediments.

					Richness		Diversity
Target	**Sample**	**# sequences**	**Library coverage[a] (%)**	**# OTUs observed[b]**	**# OTUs estimated[c]**	**Observed/estimated ratio**	**Inverse Simpson [d]**
16S rRNA	**HBM1**	31 415	99.57	779 (A:14-NA:765)	1021 (927-1172)	0.76	26.70 (26.07-27.36)
	HBM3	37 298	99.5	821 (A:13-NA:808)	1138 (1008-1347)	0.72	34.83 (34.06-35.63)
	LBM1	42 512	98.86	1235 (A:16-NA:1219)	1468 (1385-1578)	0.84	24.71 (24.21-25.23)
	LBM2	38 721	99.02	1232 (A:15-NA:1217)	1454 (1379-1554)	0.85	33.76 (33.04-34.52)
nirK	**HBM1**	13 510	99.88	59 (A: 11-NA:48)	63 (57-89)	0.94	4.30 (4.20-4.42)
	HBM2	13 602	99.9	52 (A:7-NA:45)	61 (57-80)	0.85	4.67 (4.55-4.79)
	HBM3	9 028	99.84	59 (A:13-NA:46)	65 (56-98)	0.91	3.41 (3.32-3.51)
	LBM1	11 701	99.87	56 (A:5-NA:51)	68 (59-101)	0.87	4.05 (3.93-4.18)
	LBM2	9 779	99.82	54 (A:8-NA:46)	68 (59-98)	0.82	2.11 (2.06-2.16)
	LBM3	10 802	99.89	59 (A:10-NA:49)	57 (54-75)	0.95	3.50 (3.42-3.59)
nirS	**HBM1**	6 365	99.64	58 (A:7-NA:51)	71 (59-108)	0.82	3.56 (3.48-3.65)
	HBM2	16 427	99.76	61 (A:8-NA:53)	59 (50-91)	1.03	3.44 (3.38-3.50)
	HBM3	4 948	99.71	47 (A:7-NA:40)	60 (51-93)	0.78	1.98 (1.92-2.04)
	LBM1	6 948	99.69	57 (A:8-NA:49)	65 (56-97)	0.88	3.27 (3.20-3.34)
	LBM2	6 684	99.68	57 (A:7-NA:50)	66 (56-97)	0.86	2.31 (2.25-2.38)
	LBM3	17 548	99.68	72 (A:7-NA:65)	70 (59-103)	1.03	3.62 (3.56-3.68)

[a] Good's coverage estimates sampling completeness and calculates the probability that a randomly selected amplicon sequence from a sample has already been sequenced; [b] A, abundant OTUs (> 1% relative abundance); NA, non-abundant (< 1% relative abundance); [c] Chao1 richness with upper and lower 95% confidence intervals; [d] Inverse Simpson diversity index with upper and lower 95% confidence intervals.

replicates (Figure S2). As no such discrepancies were observed when using the same DNA material for *nir* gene sequencing and qPCR (see below), we suspect that the limited numbers of OTUs observed for HBM2 and LBM3 resulted from inconsistent emulsion PCR and therefore excluded both samples from further analyses. After removal of HBM2 and LBM3 sequences from the data set, a total number of 149 946 sequences were binned into 2 482 OTUs across all samples at a cut-off of 0.03. In total, 2.69% of all sequences were determined as chimera or chloroplast and removed from the data set. Both the observed and estimated OTU richness in LBM samples were significantly ($p < 0.05$) higher than in HBM samples, with the majority of OTUs being non-abundant (i.e. relative abundance < 1%; Table 2). Singletons (832 OTUs) and doubletons (485 OTUs) together accounted for approximately half of the total number of OTUs. Given the higher OTU richness in the LBM sediments, % coverage was also significantly lower than in the HBM sediments ($p < 0.05$, Table 2). Despite this higher richness in LBM samples, the inverse Simpson diversity index did not significantly differ between the HBM and LBM sediments (Table 2). Replicates of each sediment type shared only about half of their total number of OTUs (Figure 2A-B); this variation among replicates could be attributed to a high dissimilarity in non-abundant OTUs. Both sample types showed clear differences in community members, although not significant and again mostly among non-abundant OTUs (Table 3), with 78.5% of HBM OTUs and 85.8% of LBM OTUs being unique (Figure 2C). Nevertheless, community structures (Bray-Curtis dissimilarity index, which takes into account community membership as well as OTU relative abundances) did not show significant differences at any level (total community, abundant and non-abundant fraction) between both sample types (Table 3).

Table 3 Dissimilarity in community membership (Jaccard) and structure (Bray-Curtis) between both estuarine sediment types (HBM and LBM) based on 16S rRNA, *nirK* and *nirS* genes.

		Jaccard	Bray-Curtis
All OTUs	**16S rRNA**	0.732897	0.299214
	nirK	0.153846	0.185174
	nirS	0.255556	0.065105
Abundant OTUs	**16S rRNA**	0.357143	0.260035
	nirK	0.411765	0.181191
	nirS	0.222222	0.0589
Non-abundant OTUs	**16S rRNA**	0.735594	0.402036
	nirK	0.213333	0.430228
	nirS	0.277108	0.48913

Three different levels were assessed: total community (all), abundant and non-abundant fraction. An OTU was defined as abundant when its relative abundance was larger than 1%. Permanova analyses were performed to determine significant differences in community structure and membership.

Taxonomic diversity in HBM and LBM samples

The estuarine sediments harboured bacteria belonging to a broad range of known phyla, with approximately one third of the OTUs remaining unclassified (30.58% or 759 OTUs, Table 4). The majority of known OTUs belonged to the *Proteobacteria* (representing one third of all OTUs), *Bacteroidetes*, *Planctomycetes*, *Actinobacteria*, *Acidobacteria* and *Verrucomicrobia*. Candidate phyla OD1, OP11, SR1, TM7 and WS 3 were represented by only a few OTUs (Table 4). Twenty-one OTUs were found to have a relative abundance of more than 1% in at least one of the replicates of both sediment types. These abundant OTUs belonged to *Alpha-* (1 OTU), *Beta-* (1 OTU), *Gamma-* (6 OTUs), *Delta-* (4 OTUs) and unclassified *Proteobacteria* (1 OTU), *Actinobacteria* (1 OTU), *Firmicutes* (1 OTU), *Cyanobacteria* (1 OTU), *Bacteroidetes* (2 OTUs), *Planctomycetes* (1 OTU) and two OTUs with no taxonomic identification (Figure 2D). The phyla *Armatimonadetes*, *Chlorobi*, *Spirochaetes* and *Tenericutes* appeared to be unique to LBM samples, although they were only represented by one to five OTUs. A significant difference in the number of OTUs between HBM and LBM samples was found for five phyla, namely for *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* (more specifically *Alpha-*, *Gamma-*, *Delta-* and unclassified), *Spirochaetes*, and *Verrucomicrobia*, as well as the unclassified fraction ($p < 0.05$) (Table 4). Eighteen bacterial taxa (families or genera within *Alpha-*, *Gammaproteobacteria or Bacteroidetes)* were detected that were previously described to harbour diatom-associated bacteria (Amin et al., 2012) (Figure 3).

Denitrifying guild diversity, estimated richness and structure

In total, 68 422 quality filtered *nirK* gene sequences, including 4 693 unique sequences, were assigned to 78 OTUs, with six singleton OTUs and two doubletons. Comparable numbers of OTUs were found in HBM and LBM samples (Table 2). Rarefaction curves (Figure S3A) and the ratio observed:estimated OTU richness (0.82-0.94, Table 2) indicated that the sequencing effort was almost sufficient to cover the whole *nirK* diversity detectable with the applied primer set in both sediment types. HBM and LBM samples had similar richness and diversity values (Table 2), with both containing nine abundant OTUs and being dominated by OTUs 1 and 2 (Figure S4A). Although three biological replicates were taken from each sediment type, differences in relative abundances of abundant OTUs between replicates within sample types were as large as between sample types (Figure S4A), while non-abundant OTUs were very similar.

Looking at the *in silico* translated amino acid (AA) sequences of *nirK*, the majority of all OTU representatives clustered together with *Alphaproteobacteria* – spread over four clusters - and *Betaproteobacteria* (Figure S5). One distinct cluster (supported by a high bootstrap value of 100%) did not contain known cultivated representatives. Sequences derived from *Bacteriodetes* and *Firmicutes* were included but proved unrelated to our OTU representatives. Most OTU representatives

clustered together with sequences obtained from marine environments, with the exception of two OTUs (OTU 11 and 66) closely related to sequences from soil.

In total, 58 920 quality filtered *nirS* gene sequences, with 5 391 unique sequences, were assigned to 90 OTUs, with twelve singleton OTUs and eighteen doubletons. Comparable numbers of OTUs were found in HBM and LBM samples (Table 2). As for *nirK*, rarefaction curves (Figure S3B) and the ratio observed:estimated OTU richness (0.78-1.03, Table 2) indicated that the current sequencing effort was nearly sufficient to completely catalogue the *nirS* diversity detectable with the applied primer set in both sample types. HBM and LBM did not significantly differ in *nirS* gene richness and diversity. OTUs 1 - 3 dominated in both sediments, and nine OTUs were abundant (Figure S4B). In contrast to *nirK*, replicates of a single sediment type differed less in relative abundances of abundant OTUs (Figure S4).

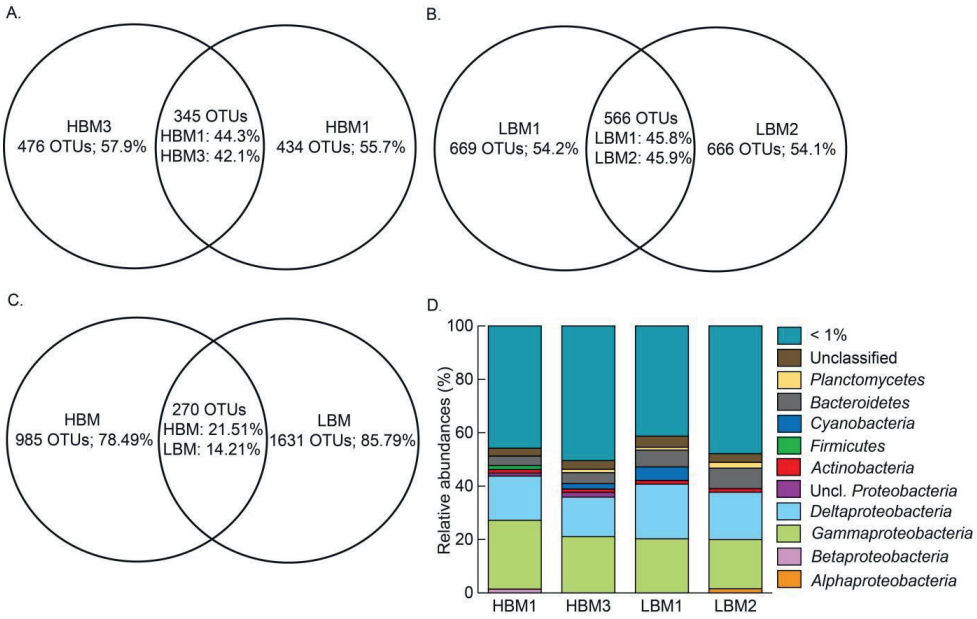

Figure 2 Distribution of 16S rRNA gene OTUs. A-C, Venn diagrams representing the number of observed OTUs for the 16S rRNA gene. Comparisons are shown between (A) HBM replicates, (B) LBM replicates, (C) HBM (n=2) and LBM (n=2) samples. The number and percentage of unique and shared OTUs are given. D, The relative abundance of abundant 16S rRNA derived OTUs, grouped per phylum, from HBM (n=2) and LBM (n=2) sediment samples. Sequences were assigned to OTUs using sequence dissimilarity treshold of 3%. All OTUs with a relative abundance below 1% were grouped. Uncl. stands for unclassified.

Table 4 Detection and distribution of bacterial phyla (or subdivisions of *Proteobacteria*).

Phylum		Total	HBM	LBM
		% (# OTUs)	% (# OTUs)	% (# OTUs)
Acidobacteria		3.63 (90)	3.59 (45)	4.10 (78)
Actinobacteria		4.67 (116)	6.06 (76)*	4.73 (90)*
Armatimonadetes		0.04 (1)	0 (0)	0.05 (1)
Bacteroidetes		9.31 (231)	8.45 (106)*	9.21 (175)*
Chlamydiae		0.16 (4)	0.16 (2)	0.16 (3)
Chlorobi		0.08 (2)	0 (0)	0.11 (2)
Chloroflexi		1.69 (42)	1.83 (23)	1.68 (32)
Cyanobacteria		0.73 (18)	0.80 (10)	0.58 (11)
Deinococcus-Thermus		0.04 (1)	0.08 (1)	0 (0)
Firmicutes		2.98 (74)	3.19 (40)	2.74 (52)
Fusobacteria		0.08 (2)	0.08 (1)	0.11 (2)
OD1		1.49 (37)	1.91 (24)	1.10 (21)
OP11		0.32 (8)	0.32 (4)	0.26 (5)
Planctomycetes		6.21 (154)	4.06 (51)	7.21 (137)
Proteobacteria	*Alphaproteobacteria*	5.04 (125)	5.82 (73)*	4.94 (94)*
	Betaproteobacteria	1.09 (27)	1.59 (20)	0.95 (18)
	Gammaproteobacteria	14.67 (364)	15.62 (196)*	14.52 (276)*
	Deltaproteobacteria	6.93 (172)	6.69 (84)*	7.63 (145)*
	Zetaproteobacteria	0.04 (1)	0.08 (1)	0 (0)
	Epsilonproteobacteria	0.08 (2)	0.08 (1)	0.11 (2)
	Unclassified	2.54 (63)	2.23 (28)*	2.47 (47)*
Spirochaetes		0.20 (5)	0 (0)*	0.26 (5)*
SR1		0.16 (4)	0.08 (1)	0.16 (3)
Synergistetes		0.12 (3)	0.16 (2)	0.05 (1)
Tenericutes		0.04 (1)	0 (0)	0.05 (1)
TM7		1.09 (27)	1.59 (20)	1.00 (19)
Verrucomicrobia		5.48 (136)	6.14 (77)*	5.89 (112)*
WS3		0.52 (13)	0.64 (8)	0.53 (10)
Unclassified		30.58 (759)	28.76 (361)*	29.41 (559)*

Percentages OTU per phylum are given for both sediment types (Total) and for each sediment type separately (HBM and LBM), exact numbers are given between brackets. Phyla containing previously described diatom-associated bacteria are underlined. Significant differences (*) in the number of OTUs between HBM and LBM samples were detected using a t-test ($p \leq 0.05$). Reliability of significance testing was checked using the Levene's p-value (>0.05).

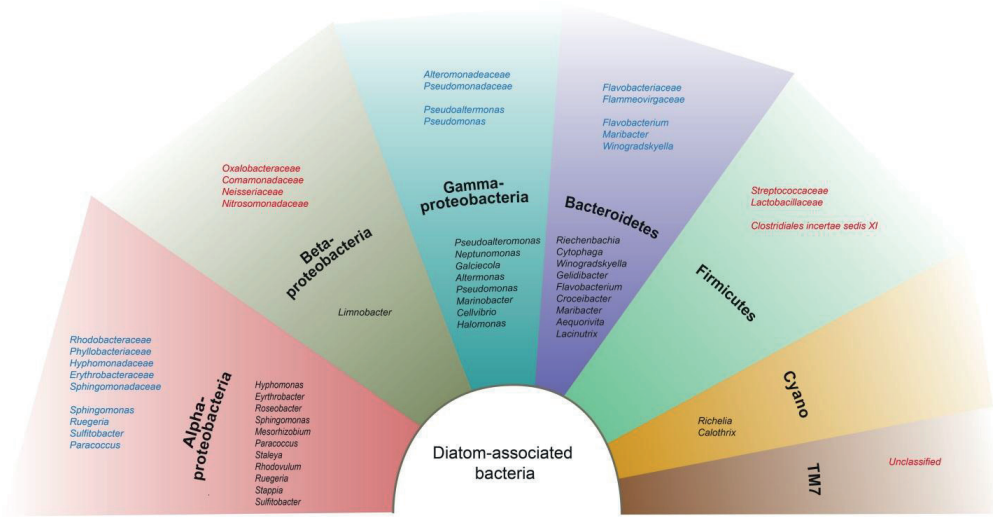

Figure 3 Overview of diatom-associated bacteria found in different phyla. The inner tier represents diatom-associated bacterial taxa reported by Amin et al. (35). The outer tier depicts diatom-associated bacterial taxa found in our study, either previously reported (blue) or representing potentially new diatom- bacteria associations (red). The highest taxonomic identification of these taxa is shown. Diatom-bacteria associations were identified based on the difference in relative abundances of specific taxa (i.e. number of sequences per taxon) between HBM and LBM sediment samples.

Looking at the *in silico* translated amino acid (AA) sequences of *nirS,* most OTU representatives clustered together with *Alpha-, Beta* and also *Gammaproteobacteria* (Figure S6). Multiple clusters without cultured representatives were found, albeit not always supported by high bootstrap values. Most of the OTU representatives clustered together with sequences obtained from marine environments; eight OTUs resembled sequences derived from soil environments or activated sludge.

NirK and *nirS* community membership and structure were very similar between HBM and LBM samples (Table 3), with only a few unique OTUs for each sediment type (Figure 4A-B). Similar trends were found when considering the abundant and non-abundant community levels separately (Table 3). However, it is interesting to note that OTUs 12 and 13 were exclusively present in the *nirK* abundant HBM community fraction (Figure S4).

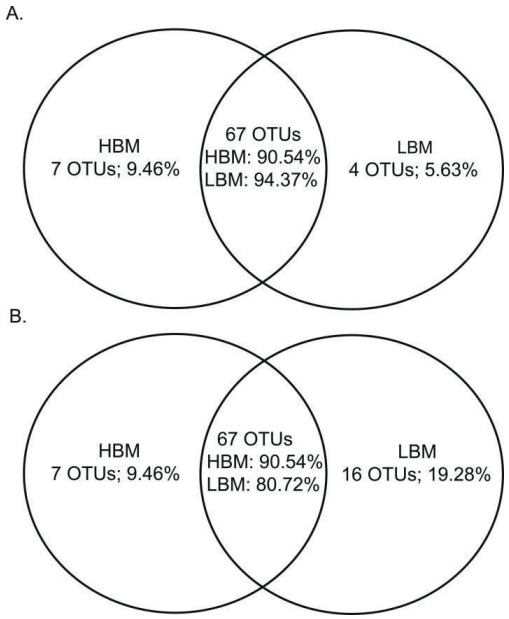

Figure 4 Venn diagrams representing number of observed OTUs for the nirK (A) and nirS (B) genes. Comparison is shown between HBM and LBM samples (n=3) for both genes. The number and percentage of unique and shared OTUs are given.

Quantification of the denitrifying guild abundance relative to total bacterial abundance

The abundances of both *nirK* and *nirS* denitrifiers and the total bacterial communities of HBM and LBM samples were determined via qPCR (Table 5). The PCR efficiency of the *nirS* assay (62.9%) was lower than for the other genes, probably due to the exclusion of BSA in this assay because of negative effects on the melting curves. Inhibition factors per gene type were experimentally determined and gene copy numbers were corrected accordingly. The level of inhibition for both *nir* genes was similar, but much lower than for the 16S rRNA gene. Total bacterial abundances ranged from 8.25×10^5 to 7.98×10^7 copies/g sediment. Final copy numbers of *nirK* and *nirS* ranged from 1.48×10^3 to 6.09×10^4 copies/g sediment and 3.66×10^4 to 3.35×10^5 copies/g sediment respectively (Table 5).

Significant differences in the abundance of 16S rRNA, *nirK* and *nirS* genes were detected between HBM and LBM samples ($p < 0.05$), with the abundances of all three genes being a log unit higher in HBM samples. *NirS* genes were also consistently approximately a log unit more abundant than *nirK* genes (Table 5). For all three genes, a strong positive correlation was found with chl *a* and EPS whereas a significantly negative correlation was found for Si. Both are in agreement with the higher abundances of the all three genes in HBM sediments ($p < 0.05$, Table S3). Furthermore, a positive

correlation was found between 16S rRNA abundances and concentration NO_3^- and *nirK* abundances and % TOM ($p < 0.05$, Table S3).

Table 5 Absolute and relative quantification of 16S rRNA, *nirK* and *nirS* genes in both estuarine sediment types (HBM and LBM).

Sample	Absolute quantification			Relative quantification	
	16S rRNA[a] gene copies / g sediment	*nirK*[b] gene copies / g sediment	*nirS*[c] gene copies / g sediment	ratio *nirK*/16S rRNA	ratio *nirS*/16S rRNA
HBM1	$(6.68 \pm 0.85) \times 10^7$	$(3.39 \pm 0.38) \times 10^4$	$(2.93 \pm 0.16) \times 10^5$	0.0005	0.0044
HBM2	$(3.75 \pm 0.13) \times 10^7$	$(5.40 \pm 0.72) \times 10^4$	$(2.53 \pm 0.18) \times 10^5$	0.0014	0.0068
HBM3	$(7.98 \pm 0.48) \times 10^7$	$(6.09 \pm 0.70) \times 10^4$	$(3.35 \pm 0.26) \times 10^5$	0.0008	0.0042
LBM1	$(5.56 \pm 0.64) \times 10^6$	$(1.48 \pm 0.15) \times 10^3$	$(3.66 \pm 0.60) \times 10^4$	0.0003	0.0066
LBM2	$(5.19 \pm 0.40) \times 10^6$	$(8.61 \pm 0.36) \times 10^3$	$(6.00 \pm 0.55) \times 10^4$	0.0017	0.0116
LBM3	$(8.25 \pm 0.24) \times 10^5$	$(2.72 \pm 0.37) \times 10^3$	$(6.23 \pm 0.14) \times 10^4$	0.0033	0.0755

Analyses was performed in triplicate for each biological replicate (n=3). Gene copy numbers given were corrected for PCR inhibition.

[a] Detection limit, 10^1 copies; PCR efficiency, 90 %; standard curve R^2, 0.99; inhibition factor range: 0.07-0.10; [b] Detection limit, 10^1 copies; PCR efficiency, 87 %; standard curve R^2, 0.99; inhibition factor range: 0.36-0.62; [c] Detection limit, 10^1 copies; PCR efficiency, 62.9%, standard curve R^2, 0.99; inhibition factor range: 0.31-0.55

2.4 Discussion

In intertidal sediments of the Westerschelde estuary, a two-fold increase in biomass (*i.e.* chl *a*) of otherwise compositionally identical MPB communities coincided with a disproportionate (ten-fold) increase in both total bacterial and denitrifying community abundances in underlying sediments (Table 5). This contrasts with previous studies that reported correlations between MPB biomass and bacterial abundances with a more moderate quantitative influence (two to three-fold), but, unlike here, these studies did not exclude variation of other environmental factors non-related to MPB (e.g. salinity, temperature, seasonality) (Gurung et al., 2001;Belzile et al., 2008;Orvain et al., 2014). Unexpectedly, this two-fold increase in MPB biomass also corresponded with a ten-fold increase in denitrifier abundance. Potential competition with diatoms for nitrate appeared negligible at the NO_3^- concentration in the investigated sediments (appr. 1 mg/l, *i.e.* 10x higher than in sediments with comparable MPB biomass with competition (Sundback and Miles, 2000;Hochard et al., 2010)). Thus, microphytobenthos was no specific determinant of denitrifying guilds but rather influenced the bacterial population more generally. This agrees with the facultative nature of the denitrification capacity, and the assumption that the population distribution of denitrifiers is determined by their general ability to compete for natural carbon substrates under aerobic conditions (Tiedje, 1988). Unfortunately, our results could not ascertain whether the MPB directly affected the bacterial abundance or whether (identical) non-measured variables influenced both MPB and bacteria simultaneously. Estuarine MPB biofilms can be extremely variable in time (seasonality) and space (from cm to km), with complex interactions of biotic and abiotic parameters responsible for their patchiness (Jesus et al., 2005). Tidal height, sediment type and hydrodynamism (Jesus et al., 2009;Fonseca et al., 2013), and intraspecies competition (Seuront and Spilmont, 2002;Brito et al., 2009) are unlikely causes as both samples, from the same sediment type, were taken at the same tidal height and both biofilms contained the same MPB species. However, grazing of MPB, alone or with bacteria, by higher organisms such as grazing benthic deposit feeding invertebrates like harpacticoid copepods (BuffanDubau et al., 1996), (bacterivorous) nematodes (Moens and Vincx, 1997;Moens et al., 1999), polychaetes and bivalves (Van Colen et al., 2014) is a plausible factor.

Besides with chl *a*, total bacterial abundances correlated with other parameters related to MPB activity, namely EPS, produced by both diatoms and bacteria (Lubarsky et al., 2010) and a potential nutrient source for bacteria and diatoms (Stal, 2010;McKew et al., 2013;Taylor et al., 2013), and Si, a major limiting nutrient for diatoms and hence an important factor controlling primary production (Martin-Jezequel et al., 2000). Taxon-specific effects of diatom-derived EPS (*i.e.* a shift to algal organic matter degrading taxa) could explain the significant decreases in number of OTUs in *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, *Spirochaetes*, and *Verrucomicrobia* (Table 4) in HBM compared to LBM as well as the big, albeit not significant, difference in community membership, specifically for non-abundant taxa (Table 3). The same effects could also be the result of potential bactericidal effects

of MPB, not measured in this study (e.g. by polyunsaturated aldehydes (PUAs) (Ianora and Miralto, 2010)). In addition to a shift in number of OTUs, diatom-derived EPS (and PUA) might also explain the increase in total bacterial abundances in HBM samples, as specialist EPS-degrading bacteria are specifically favoured by these additional nutrient sources (Haynes et al., 2007;Ribalet et al., 2008;Taylor et al., 2013). Using the relative abundances of specific taxa (*i.e*. number of sequences per taxon) as a proxy, our data also suggest that growth of other than the typical diatom-associated bacteria (Amin et al., 2012) appears to have been facilitated (Figure 3). Specifically the phyla *Betaproteobacteria* (*Oxalobacteraceae*, *Comamonadaceae*, *Neisseriaceae, Nitrosomonadaceae)*, *Firmicutes (Streptococcaceae, Lactobacillaceae*, *Clostridales incertae sedis XI)*, and the candidate division TM7 demonstrated a more than 30% increase in their sequence abundances in HBM compared to LBM sediment (Table S4). *Bacteroidetes* and *Gammaproteobacteria*, the two phyla that are already known to contain taxa able to assimilate diatom-derived EPS (Haynes et al., 2007) as well as to thrive in the presence of PUAs (Ribalet et al., 2008), were dominant in both sediment types (if unclassified phyla are ignored), but specific known diatom-associated bacterial genera (Amin et al., 2012) within both phyla (*Pseudoalteromonas*, *Maribacter* and *Winogradskyella)* were only represented by few sequences (<40 sequences) in HBM samples. The only exception was *Pseudomonas* (Amin et al., 2012) well represented (appr. 5000 sequences) and with an increase of over 30% in its relative abundance in HBM sediments. However, considering the relative increase in sequence abundance, *Pseudomonas* and the above mentioned taxa could not have been solely responsible for the observed tenfold increase in total bacterial abundance. To our surprise, despite all these pronounced differences between both sample types, the Bray-Curtis index for community structure remained unaltered due to the dominant influence of relative abundances of abundant OTUs.

Temporal and spatial heterogeneity can be considerable at the micro- and mesoscale in natural environments, and especially in marine sediments (Lozupone and Knight, 2007), stressing the importance of replication. Biological replicates are often pooled before analyses, resulting in loss of spatial and experimental variability (Prosser, 2010). In our study, replicate environmental samples were not pooled and a remarkable degree of variability between replicates of a single sediment type was observed, sharing less than 50% of their OTUs (Figure 2A-B). These differences probably result from the patchiness of the sediments that were sampled and/or incomplete sequencing, indicated by the 16S rRNA gene rarefaction curves, resulting in a snapshot of a subset of especially non-abundant taxa. Unfortunately, due to technical issues only two replicates per sampling site in the 16S rRNA gene sequences analysis were analysed, resulting in a considerable loss of information. Next to patchiness, this variability might also result from stochastic processes as explained by the neutral community assembly theory (Nemergut et al., 2013). Despite containing bacteria belonging to different OTUs, calculated richness and diversity indices were similar for different replicates in both sample types (Table 2), which is in agreement with Bowen et al. (2012) who reported similar estimates

of richness and diversity when comparing individual and homogenized replicate sediment samples of a salt marsh. Only 14-21% of OTUs were shared between both sediment types, again with the non-abundant fraction being responsible for most of the differences in community members (Figure 2, Table 3). Abundant and non-abundant fractions of bacterial communities clearly have a distinct influence on community composition analysis with the non-abundant fraction as major determining factor for phylogenetic diversity (Sogin et al., 2006). The potential role of these non-abundant taxa remains unclear although it has been suggested that these non-abundant taxa are disproportionally active in comparison to more abundant taxa (Campbell et al., 2011;Nemergut et al., 2013) or can serve as a microbial seed bank (Jones and Lennon, 2010;Lennon and Jones, 2011).

The two-fold increase in MPB biomass was accompanied by a ten-fold increase of denitrifier abundances, while community structure remained unchanged. *NirK* and *nirS* gene abundances correlated well with chl *a*, Si and EPS concentration. The only available comparable study also showed a positive correlation of MPB biomass with *nirK* but not with *nirS* (Abell et al., 2010), albeit at much lower concentration of chl *a* (ng instead of µg/g dry weight) than here. Interestingly, *nirK* and not *nirS* abundances correlated with levels of organic matter, despite this feature not being significantly different among HBM and LBM samples. This observation confirms differential responses to environmental variables by both *nir* communities (Smith and Ogram, 2008;Jones and Hallin, 2010), and organic matter as important driver of *nirK* denitrifier abundances (Kandeler et al., 2006;Barta et al., 2010). Other differential drivers (temperature, salinity and concentration of O_2, NO_3^-, NO_2^-, NH_4^+) of denitrifier guilds composition (Liu et al., 2003;Taroncher-Oldenburg et al., 2003;Jayakumar et al., 2004;Hannig et al., 2006;Abell et al., 2010;Jones and Hallin, 2010) could not be confirmed here because of absence of correlation or the spatial proximity of the sediment types. In congruence with the few other reports on denitrifier marker genes in estuarine sediments (Smith et al., 2007;Abell et al., 2010;Mosier and Francis, 2010), *nirS* genes were one order of magnitude more abundant than *nirK* genes, regardless of sample type. Nevertheless, establishing the *in situ* importance of either *nir* populations in this system requires further studies acquiring gene transcript data and activity measurements (Jones and Lennon, 2010;Campbell et al., 2011;Bodelier et al., 2013;Hunt et al., 2013).

Our *nir* sequences had a close match with sequences from estuarine and marine sediments (Nogales et al., 2002;Santoro et al., 2006;Dang et al., 2009;Mosier and Francis, 2010;Sokoll et al., 2012;Francis et al., 2013) as well as from soil and activated sludge (Hallin et al., 2006;Hashimoto et al., 2009), suggesting that the Westerschelde estuary has both tidal and terrestrial influences. They were affiliated with sequences derived from *Alpha-*, *Beta-* and *Gammaproteobacteria* which is similar to previous studies using the same primer sets (Palmer et al., 2012;Depkat-Jakob et al., 2013). However, we need to keep in mind the limitation of the PCR-based approach to evaluate denitrifier diversity and abundance. It is widely recognized that available primer sets only target part of the denitrifier

population because of high sequence divergence in these molecular markers (Green et al., 2010;Palmer et al., 2012;Penton et al., 2013). With a rough calculation of the potentially undetected functional diversity for *nirK* based on data from the present study we want to put this relevant but often ignored issue into context. All retrieved *nirK* sequences (assigned to approximately 60 OTUs, see Table 2) were related to those from *Alpha-* and *Betaproteobacteria*, with both taxa together responsible for a little over 6% of the total bacterial community diversity (based on number of 16S rRNA OTUs). Let's assume the following: (i) all *Proteobacterial nirK* OTUs were detected (although this seems highly unlikely considering the reported difference in amplification success among denitrifiers, even within the same genera (Heylen et al., 2006a;Heylen et al., 2007) and reports of *Delta-*, *Gamma-* and *Epsilonproteobacteria* harbouring *nirK* which were not picked up in this study), (ii) other phyla harbouring *nirK* denitrifiers are restricted to *Firmicutes* and *Bacteroidetes*, accounting for over 12% of the community diversity (whole genome studies confirm that denitrifiers from both phyla almost exclusively contain *nirK* but also demonstrate that other phyla do as well), and (iii) the ratio denitrifiers/non-denitrifiers is stable over all phylogenetic lineages, then the undetected *nirK* denitrifier diversity is actually double of what we could detect (120 OTU undetected vs 60 detected). So at best, our study considered one third of this specific denitrifier guild when addressing our research questions. Currently, only shotgun metagenomics can completely uncover *in situ* functional diversity and overcome the limitations of PCR-based sequencing which is essential for further identification of the actual key denitrifiers. Bioinformatic approaches to resolve the bottleneck of detecting target genes in short-read metagenomic libraries are currently under development (Orellana et al., 2014) making it a valid alternative for future phylogenetic studies.

In conclusion, our study indicated that a disproportional tenfold increase in bacterial cell numbers in the sediments coincided with a doubling in biomass of MPB. A similar correlation between MPB biomass and both *nirK* and *nirS* denitrifiers is evidence for the lack of competition for nitrate between denitrifiers and MPB and suggests that MPB are a general determinant of bacterial populations. No causal relation between MPB biomass and bacterial abundance could be inferred as no plausible environmental variable accounting for the doubling in MPB biomass could be deduced from this dataset. Surprisingly, although bacterial abundance did increase tenfold, no significant differences in total bacterial community structure between both sediment types could be detected.

2.5 Acknowledgement

We thank Dirk Van Gansbeke and Bart Beuselinck for performing the physico-chemical analyses. We also thank NIOZ for facilitating the sampling campaign.

2.6 Supplementary information

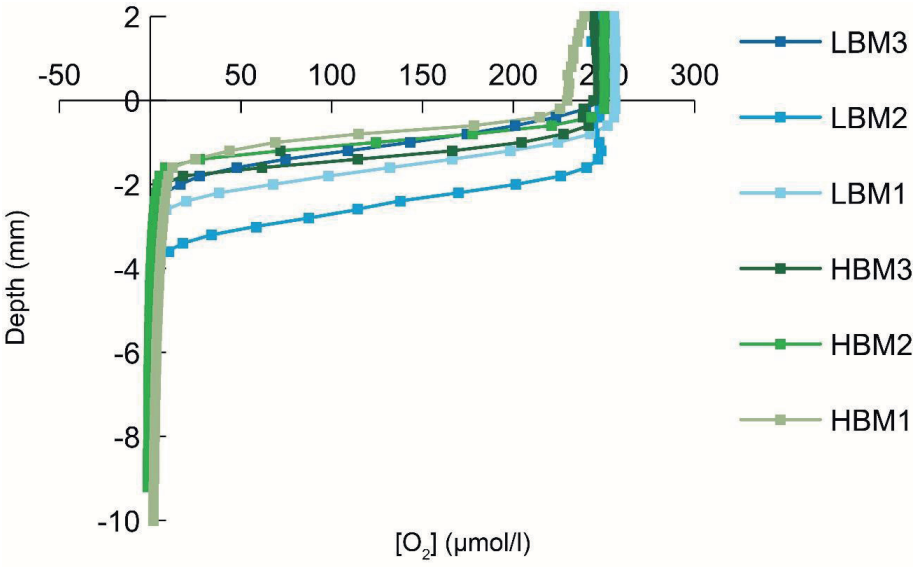

Figure S 1 Oxygen profiles showing oxygen penetration depth and depth of oxic-anoxic border in HBM and LBM sediment samples (n=3).

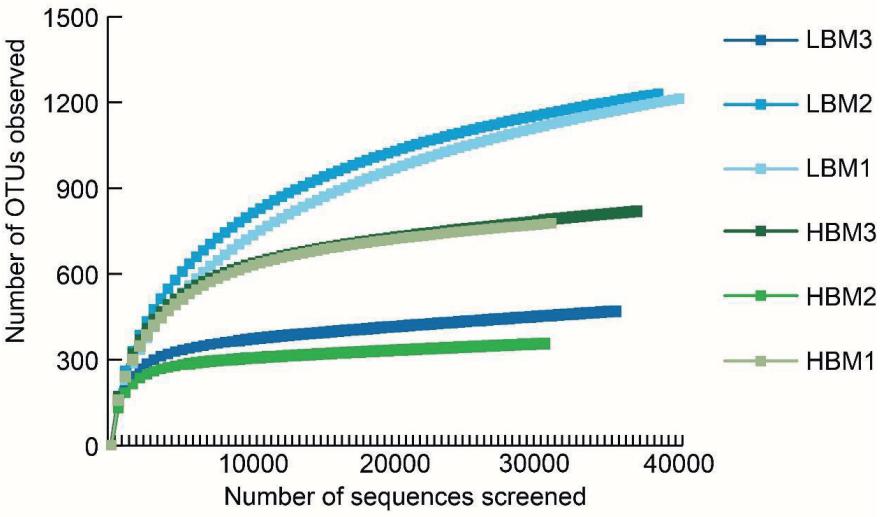

Figure S 2 Rarefaction curves of the 16S rRNA gene sequences plotting the number of OTUs observed at 3% sequences divergence as function of the number of sequences screened in each replicate.

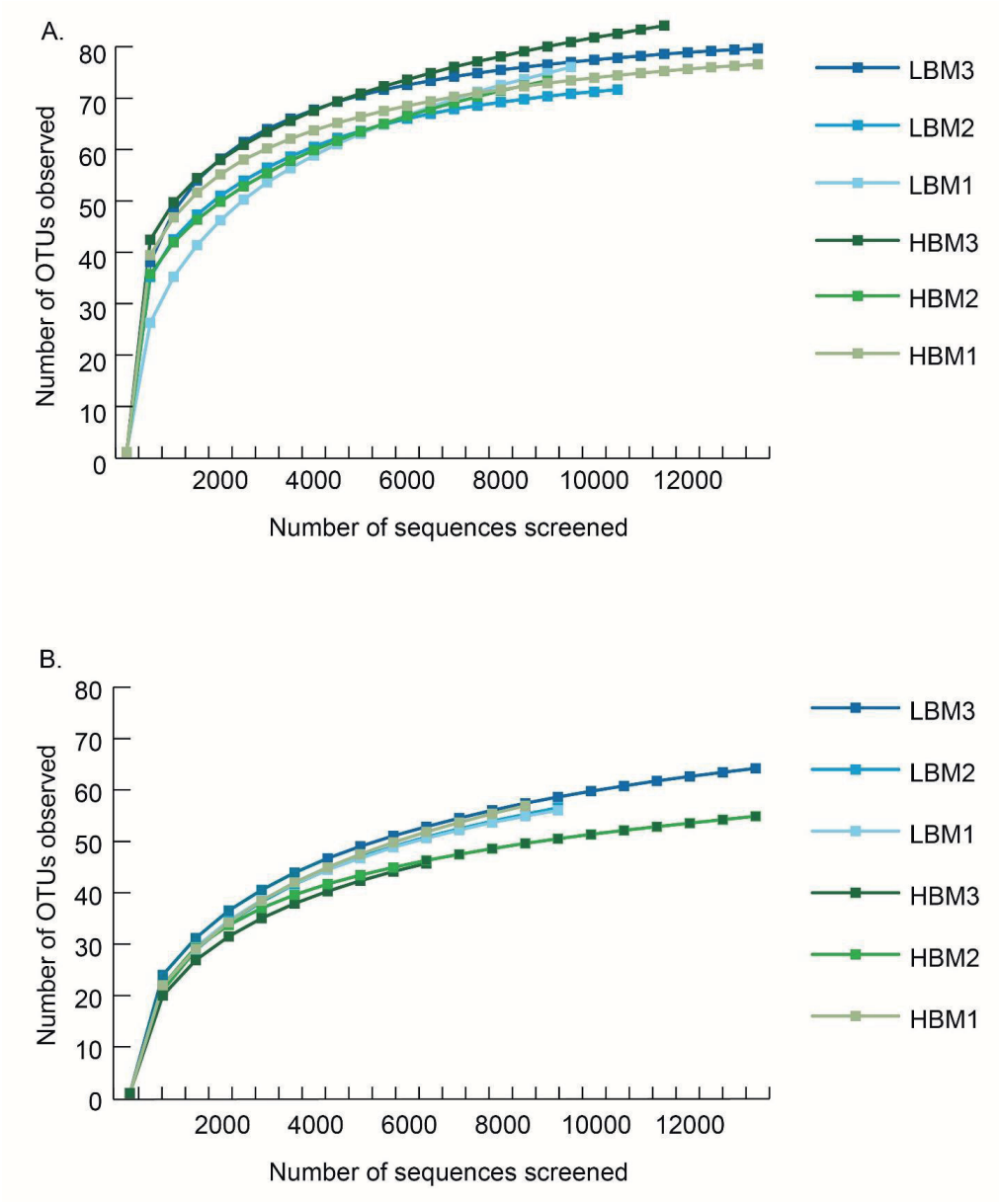

Figure S 3 Rarefaction curves for *nirK* (A) and *nirS* (B) plotting the number of OTUs observed at 18 and 20 % sequence divergence respectively as function of the number of sequences screened in each replicate.

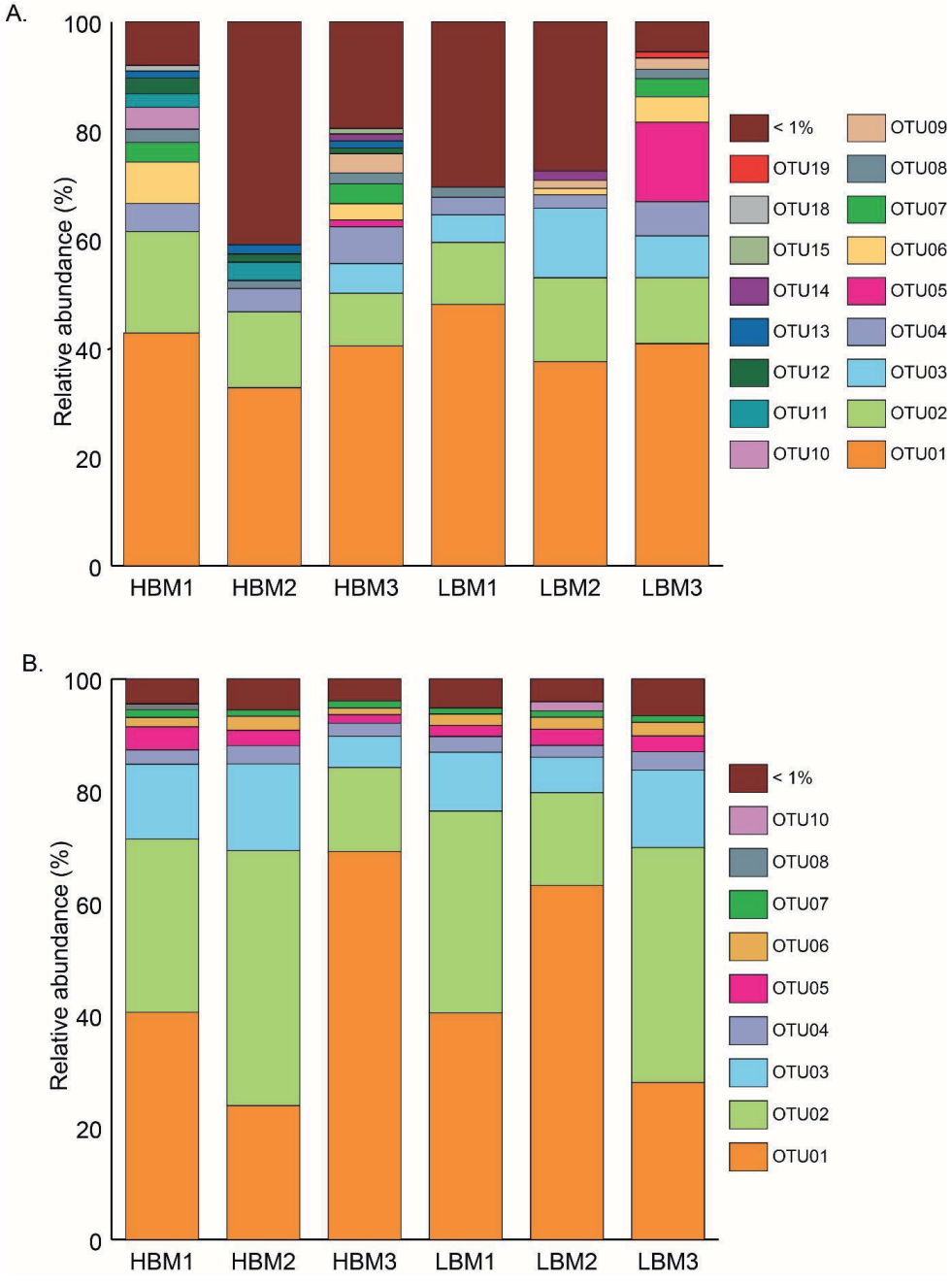

Figure S 4 Relative abundances of (A) *nirK* and (B) *nirS* derived OTUs from HBM (n=3) and LBM (n=3) sediment samples. Sequences were assigned to OTUs using sequence dissimilarity tresholds of 18% and 20% respectively. All OTUs with a relative abundance below 1% were grouped.

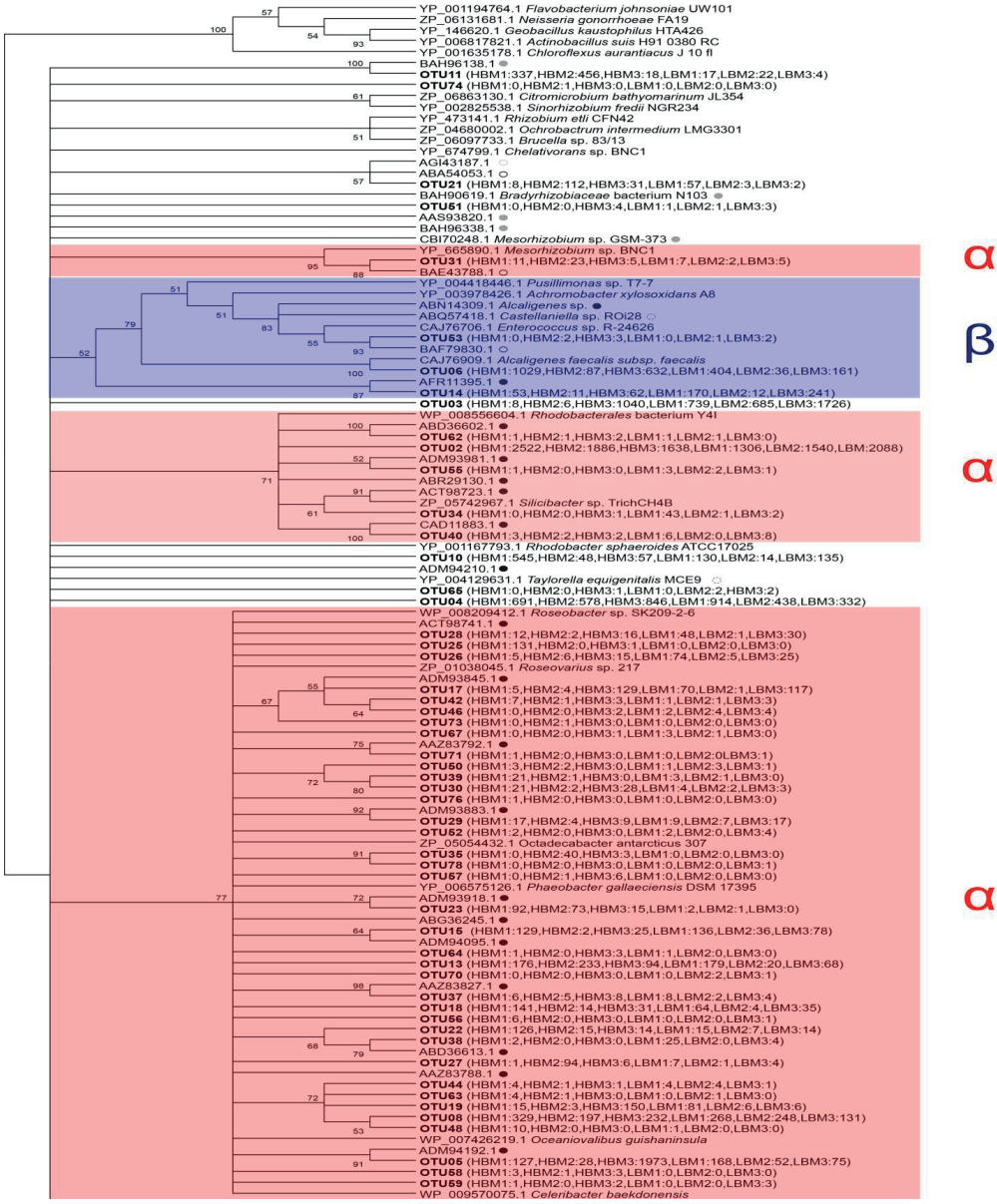

Figure S 5 continues on the next page

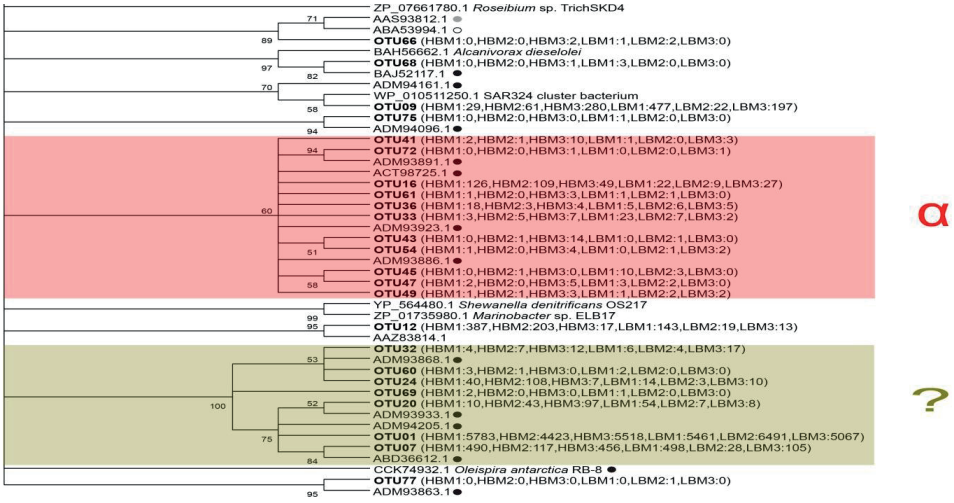

Figure S 5 Maximum likelihood tree of representative *nirK* sequences retrieved from HBM and LBM sediment samples. The tree is based on *in silico* translated amino-acid sequences. One representative per OTU is shown. Environmental sequences were included from marine sediments (black dot), soil (grey dot), urethral sample (discontinuous black circle), activated sludge (black circle) and MFC cathode (grey circle). Sequences only represented by an accession number are unknown environmental sequences. Values in parentheses represent the number of sequences that are present from that OTU in a particular replicate. Red colored boxes indicate sequences affiliated to *Alphaproteobacterial nirK* sequences and the blue box represent sequences affiliated to *Betaproteobacterial nirK* sequences. The percentages of replicates trees (n=1000) in which the OTUs clustered together, are shown next to the branches. Bootstrap values below 50% are not shown.

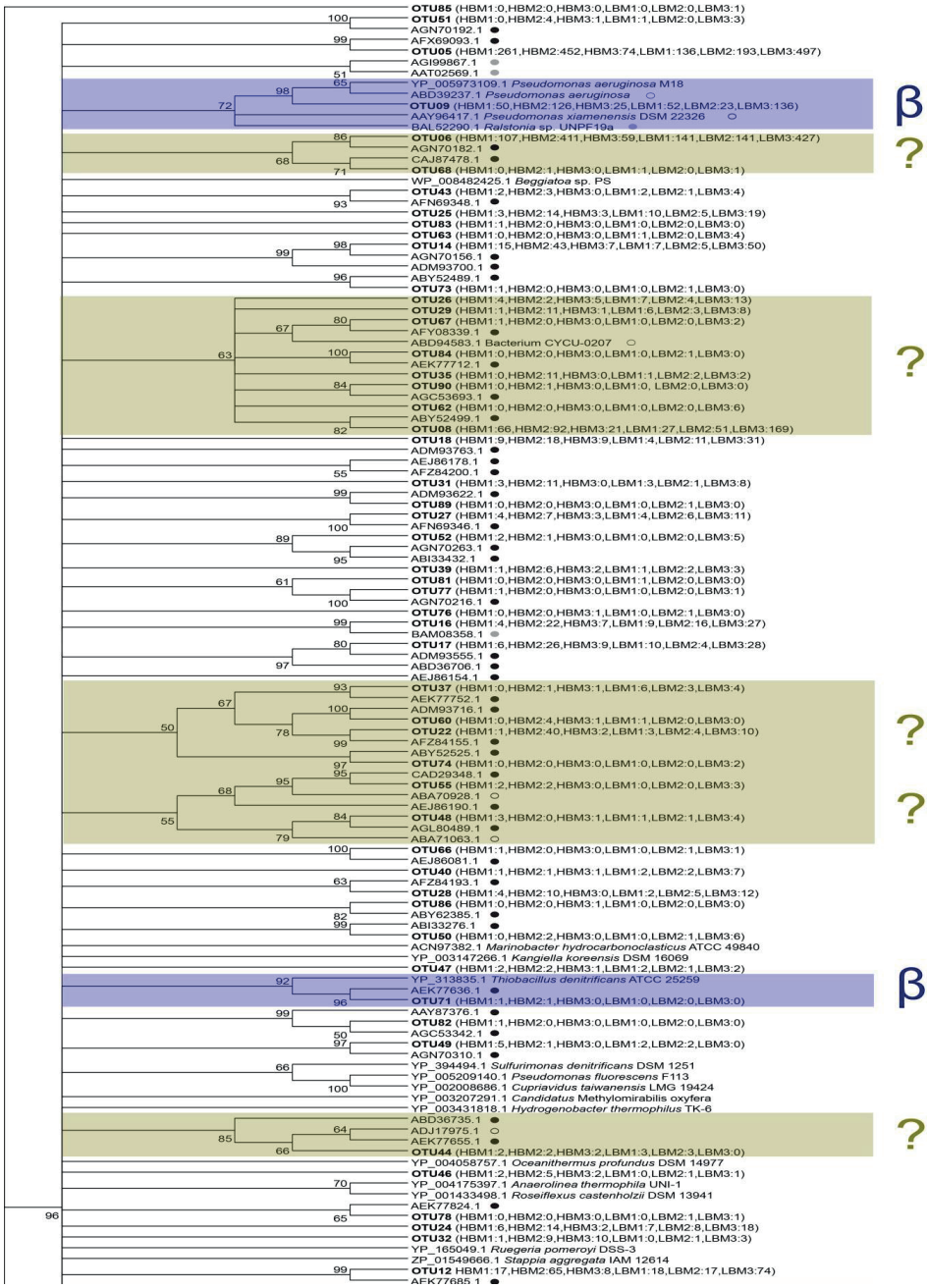

Figure S 6 continues on the next page

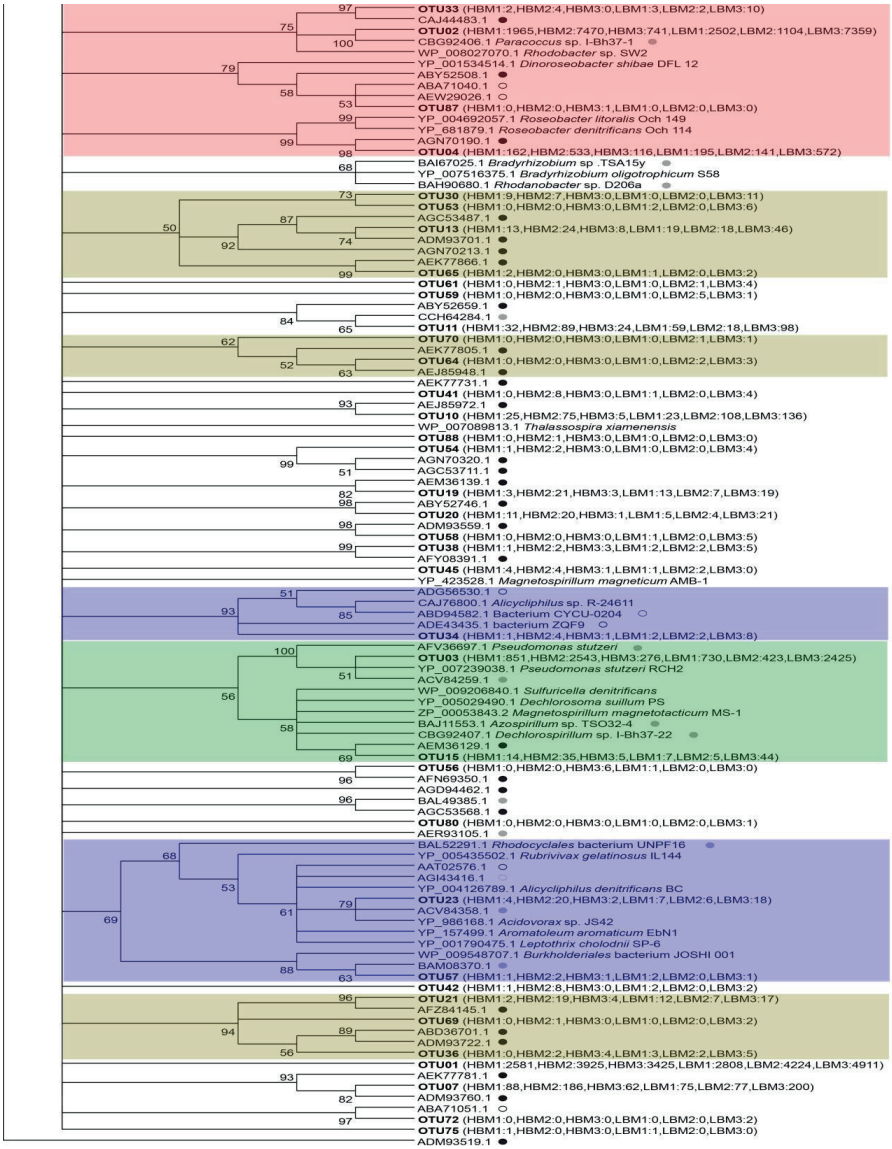

Figure S 6 Maximum likelihood tree of representative *nirS* sequences retrieved from HBM and LBM sediment samples. The tree is based on *in silico* translated amino-acid sequences. One representative per OTU is shown. Environmental sequences were included from marine sediments (black dot), soil (grey dot), activated sludge (black circle), MFC cathode (grey circle) and a membrane reactor (striped dot). Sequences only represented by an accession number are unknown environmental sequences. Values in parentheses represent the number of sequences that are present from that OTU in a particular replicate. Red colored boxes indicate sequences affiliated to *Alphaproteobacterial nirS* sequences, blue boxes represent sequences affiliated to *Betaproteobacterial nirS* sequences, and the green box represents sequences derived from *Gammaproteobacteria nirS* sequences. The percentages of replicates trees (n=1000) in which the OTUs clustered together, are shown next to the branches. Bootstrap values below 50% are not shown.

Table S 1 Complete primer sequences used for 454 pyrosequencing consisting of the adaptor sequence capable of binding to the Lib-L DNA capture beads used for unidirectional sequencing, a key, a particular multiplex identifier (MID) and the primer**.**

Gene	MID	Adaptor sequence	Key	MID	Primer (5'-3')
16S	MID1	CCATCTCATCCCTGCGTGTCTCCGA	TCA	ACGAGTGCG	CAGGTTCRTCCTGAGCCAG
	MID2	CCATCTCATCCCTGCGTGTCTCCGA	TCA	ACGCTCGAC	CAGGTTCRTCCTGAGCCAG
	MID3	CCATCTCATCCCTGCGTGTCTCCGA	TCA	AGACGCACT	CAGGTTCRTCCTGAGCCAG
	MID4	CCATCTCATCCCTGCGTGTCTCCGA	TCA	AGCACTGTA	CAGGTTCRTCCTGAGCCAG
	MID5	CCATCTCATCCCTGCGTGTCTCCGA	TCA	ATCAGACAC	CAGGTTCRTCCTGAGCCAG
	MID6	CCATCTCATCCCTGCGTGTCTCCGA	TCA	ATATCGCGA	CAGGTTCRTCCTGAGCCAG
nirK	MID1	CCATCTCATCCCTGCGTGTCTCCGA	TCA	ACGAGTGCG	ATCATGGTSCTGCCGCG
	MID2	CCATCTCATCCCTGCGTGTCTCCGA	TCA	ACGCTCGAC	ATCATGGTSCTGCCGCG
	MID3	CCATCTCATCCCTGCGTGTCTCCGA	TCA	AGACGCACT	ATCATGGTSCTGCCGCG
	MID4	CCATCTCATCCCTGCGTGTCTCCGA	TCA	AGCACTGTA	ATCATGGTSCTGCCGCG
	MID5	CCATCTCATCCCTGCGTGTCTCCGA	TCA	ATCAGACAC	ATCATGGTSCTGCCGCG
	MID1	CCATCTCATCCCTGCGTGTCTCCGA	TCA	CATAGTAGT	ATCATGGTSCTGCCGCG
nirS	MID7	CCATCTCATCCCTGCGTGTCTCCGA	TCA	CGTGTCTCTA	GTRAACGTSAAGGARACSG
	MID8	CCATCTCATCCCTGCGTGTCTCCGA	TCA	CTCGCGTGTC	GTRAACGTSAAGGARACSG
	MID9	CCATCTCATCCCTGCGTGTCTCCGA	TCA	TAGTATCAG	GTRAACGTSAAGGARACSG
	MID1	CCATCTCATCCCTGCGTGTCTCCGA	TCA	TCTCTATGCG	GTRAACGTSAAGGARACSG
	MID1	CCATCTCATCCCTGCGTGTCTCCGA	TCA	TGATACGTCT	GTRAACGTSAAGGARACSG
	MID1	CCATCTCATCCCTGCGTGTCTCCGA	TCA	TACTGAGCT	GTRAACGTSAAGGARACSG

Table S 2 Temperature-time profiles for primers used in qPCR of 16S rRNA, *nirK* and *nirS* genes.

Primer set	Temperature (°C) /time (s)		
	Aric/t-518R	F1aCu-R3Cu	Cd1aF-R3cd
Initial denaturation	95/600	95/600	95/600
Denaturation	95/10	95/10	95/10
Annealing	60/15	63/15	58/15
Elongation	72/20	72/20	72/30
Number of cycle	45	45	45

Table S 3 Correlation matrix of the 16S rRNA, *nirK* and *nirS* gene abundances with environmental parameters. Product moment correlation coefficients are given, significant correlations are indicated in bold. Millimeter depth till oxic-anoxic border and pH were excluded from correlation analyses as no biological replicates were taken for these parameters.

	16S rRNA abundance	*nirK* gene abundance	*nirS* gene abundance	% mud	NO_3^--N	NO_2^--N	NH_4^+-N	PO_4^{3-}	%TOM	Si	chl *a*	EPS
16S rRNA abundance	1											
nirK gene abundance	**0.86**	1										
nirS gene abundance	**0.97**	**0.94**	1									
% mud	0.25	0.15	0.28	1								
NO_3^--N	**0.83**	0.65	0.77	0.56	1							
NO_2^--N	-0.58	-0.67	-0.7	-0.52	-0.69	1						
NH_4^+-N	-0.24	-0.37	-0.39	0.09	-0.69	0.7	1					
PO_4^{3-}	-0.54	-0.65	-0.67	-0.02	-0.11	**0.83**	**0.94**	1				
%TOM	0.72	**0.87**	0.76	-0.05	0.38	-0.24	-0.01	-0.28	1			
Si	**-0.98**	**-0.88**	**-0.99**	-0.24	-0.77	0.65	0.38	0.65	-0.71	1		
chl *a*	**0.84**	**0.9**	**0.9**	0.46	0.67	-0.59	-0.13	-0.42	**0.84**	**-0.85**	1	
EPS	**0.95**	**0.91**	**0.9**	-0.42	0.7	-0.39	-0.15	-0.45	**0.86**	**-0.9**	0.79	1

Table S 4 The number of OTUs and sequences of specific taxa found to increase when MPB biomass doubled. The number of OTUs and sequences in HBM and LMB samples are given.

Taxonomy		Total # OTUs	HBM # seqs	LBM # seqs
Betaproteobacteria				
	Oxalobacteraceae	3	479	213
	Comamonadaceae	8	96	41
	Neisseriaceae	5	183	14
	Nitrosomonadaceae	1	68	22
Firmicutes				
	Streptococcaceae	7	758	174
	Lactobacillaceae	3	40	4
	Clostridiales_Incertae_Sedis_XI	10	158	20
TM7		27	405	266

Reflection & discussion

The aim of this research was to assess the effect of microphytobenthos (MPB) on total bacterial and denitrifier abundances and community structure. Anthropogenic related activities have altered estuarine nitrogen cycling substantially due to increased fluvial nitrogen effluxes, however, little is known on the organisms involved in nitrogen removing pathways that counteract negative effects of this increased nitrogen loading. As discussed in the introduction (§ 1.1.2), MPB also influence nitrogen cycling in estuarine sediments, and were therefore included in this study. We found that a doubling of MPB biomass resulted in a disproportional increase of both total bacterial and *nirK*/*nirS* denitrifying guild abundance. Despite the observed increase in abundances, no effect in total bacterial and denitrifier community structures could be observed.

Our study is unique in that both community structure and abundances of marine denitrifying organisms were assessed and that both functionally equivalent *nirK* and *nirS* genes were used as a proxy for denitrification. Nevertheless, it is important to recognize that we did not address two other potential important nitrate reducing processes, *i.e.* anammox and DNRA. Several attempts to obtain positive amplification results with anammox specific 16S rRNA (Hu et al., 2010;Humbert et al., 2010), *nirS* (Lam et al., 2009) or *hzsA* (Harhangi et al., 2012) primers failed repeatedly with variable possible reasons like exclusion of anammox bacteria during DNA extraction or the presence of anammox bacteria under the PCR detection limit. At the time this study was performed, two different primer sets targeting the *nrfA* gene were available (Mohan et al., 2004;Takeuchi, 2006), but due to nonspecific amplification or unavoidable positive blanks, the *nrfA* gene could not be included in this study. Recently, *nrfA* primers were improved (Welsh et al., 2014) and successfully applied in quantitative and community composition analyses of environmental samples (Song et al., 2014), making future evaluation of DNRA versus denitrifying communities in our ecosystem possible. It is very likely that DNRA communities will be influenced in a similar way as denitrifying communities, since both depend on the same sources of electron donor. In contrast, chemolithoautotrophic anammox bacteria are much less likely to be affected by MPB, assuming the effect of MPB on bacterial abundances results from EPS production. However, anammox bacteria can perform DNRA under ammonium limiting conditions (Kartal et al., 2007a;Lam et al., 2009), indicating that they might nevertheless be influenced under such conditions.

The main focus of this research was to evaluate the influence of one single parameter, *i.e.* MPB differing in biomass but not composition, on total and denitrifying communities. Therefore we limited the number of sampling sites to two as including more samples would have introduced more variables and logistical demands would have been too high. As good scientific practice requires, we concentrated on true replication of each sampling site by analyzing biological and technical replicates to take into account the notorious heterogeneity and variability of this dynamic environment (Prosser,

2010). Nevertheless, it would be useful to perform a follow-up study to confirm and increase the robustness of our observations (see below). We attempted to evaluate the observed trend over a broader range of chl *a* concentrations in a follow-up study, but this was inconclusive as we could not obtain chl *a* concentrations as high as in the first experiment. In addition, physico-chemical parameters were analyzed from additional samples taken in very close proximity of the biological samples, and thus not from the same core as used for molecular analyses. This implies that (i) parameters used for correlation analyses do not precisely reflect real conditions within our biological samples and (ii) that there is no one-to-one relationship between biological samples and physico-chemical parameters. Although this might have potential effects on our study outcome, this approach derives from the technical impossibility to determine all parameters on a single biological sample due to the requirement of large amounts of sediment sample for physico-chemical analysis. In addition, the bulk sampling techniques as well as analysis tools currently applied, only provide information on a scale irrelevant for an individual bacterium, and do not allow interrogation of the local conditions experienced by bacteria (Stocker, 2012), indicating that we nevertheless would use average estimates for local conditions.

Next generation sequencing (NGS) is currently the most widely used approach in microbial ecology as it circumvents the large discrepancy between cultivated organisms and bacterial cell numbers observed in the environment, the so-called "Great plate count anomaly". Despite the current easiness to perform extensive sequencing experiments, one should keep in mind that a robust experimental design is needed to take into account the impact of technical and bioinformatics issues on the outcome of sequencing surveys. Evaluating community diversity and structure using amplicon-based pyrosequencing has two major drawbacks: (i) it depends on PCR amplification, and therefore the use of primers, and (ii) it results in rather short sequences that limit the information obtained on the gene of interest. Although primers are often used in environmental surveys, their coverage remains a continuing challenge. Both *nir* and 16S rRNA primer coverage have been shown to be limited (Ghyselinck et al., 2013;Penton et al., 2013), and the target region of 16S rRNA gene furthermore greatly influences the amount of phylogenetic diversity obtained as pyrosequencing produces short reads. Whole genome shotgun sequencing would have avoided such primer limitations, however, this approach also has some drawbacks. As we were interested in specific genes, a gene-based metagenomic approach, which involves the analysis of individual genes that have been isolated from the metagenome, would have been applicable. An important issue concerning such an approach is that in a first step, the gene (s) of interest need to be extracted from the metagenome. Two main issues are encountered: (1) previous research indicated that a Blast approach was superior to a HMM based approach due to absence of highly conserved amino acid residues in many reads, indicating that a comprehensive and well-curated set of protein or gene reference sequences is a key requirement for robust assessment of the best cut off and parameters to effectively retrieve reads encoding the gene of

interest (Orellana et al., 2014); (2) The need to determine a stringent cut off to reduce the rates of false positives and false negatives when using similarity searches to detect individual genes within the metagenome. Cut off values are probably gene specific, so the determination of these values require some further attention before starting the analysis as this will majorly influence the obtained diversity. So although shotgun sequencing is a primer independent approach, the subsequent data analysis still strongly depends on accuracy of databases. Another issue is that reads covering different parts of the same gene cannot directly be compared through sequence alignment. Therefore an alternative way for phylogenetic analysis, determination of gene diversity and/or community structure of the sample will need to be applied. A genome centric approach, *i.e.* obtaining complete genomes through the assembly of individual genes, might present a solution for this problem, however, in very complex system like marine sediments this will not be straightforward. Continuing improvements in both the technique (longer read lengths) and data-analysis (the way of extracting the gene of interest, determining appropriate cut-offs) will probably make this application in future research more appealing. Nevertheless, even without these issues, quantification of the genes of interest would still have required real-time PCR analysis as sequencing methods are only semi-quantitative. Alternatively, total bacterial abundances can also be examined using microscopy or flow cytometer counts. In the light of the rather small copy number of 16S rRNA genes observed in this study, *i.e.* one to two orders of a magnitude lower than normally observed in marine sediments (Haynes et al., 2007;Amaral-Zettler et al., 2010;Li et al., 2012), PCR independent verification of bacterial cell numbers would have been valuable.

In addition to PCR and primer related issues, amplicon-based sequencing studies are susceptible to limitations and biases inherent to molecular analysis such as those associated with DNA extraction, stability, presence of contaminants, inhibitors and sequencing errors. In this study, the MOTHUR software was used to process sequences for two main reasons: (i) it is a step-by-step analysis tool where parameters can still be adjusted if necessary, which contrasts with black box tools such as the Fungene pipeline (Fish et al., 2013) and the rdpipeline (Cole et al., 2014), and (ii) a standard sequence processing procedure for the 16S rRNA gene was available as a starting point. Bioinformatic cleaning up of sequences, *i.e.* PCR (chimera removal) and sequencing errors, majorly influence how accurately community diversity and structure can be assessed in downstream analyses. Inclusion of a mock community would have been useful as an internal control for optimizing sequence processing parameters depending on the gene of interest, *i.e.* 16S rRNA, *nirK* or *nirS* gene (Schloss et al., 2011). To simplify downstream analysis, sequences were binned at a particular cut off into operational taxonomic units (OTUs), an approach standardly performed at 97% for 16S rRNA gene sequences. However for protein coding genes, binning was less evident. OTU assignment at species-level threshold distances has been applied for binning *nir* sequences (Palmer et al., 2012;Palmer and Horn, 2012;Penton et al., 2013). Nevertheless, incongruence between *nir* and 16S rRNA gene phylogenies

makes such an approach invalid (Heylen et al., 2006a;Jones et al., 2008). Frameshift mutations result in inflated and false diversity of protein coding genes, and have often been either mistakenly ignored during DNA based functional gene analyses or erroneously discarded as sequences containing unexpected stop codons. Here, functional assessment of *nir* genes was performed (i) using the HMM-FRAME step of the FunFrame pipeline to correct sequencing errors resulting in stop codons (Weisman et al., 2013), although some remaining erroneous sequences still needed to be removed, and (ii) by binning of sequences at a level representing functional diversity.

Interesting research topics that deserve further attention in future research involve:

- obtaining further insights on the effect of different MPB biomasses by including more samples covering a wider gradient of MPB biomass, *e.g.* do bacterial abundances keep increasing with increasing biomass or is there a turning point where no or a negative effect on bacterial abundances is observed. Additionally, the effect of MPB biomass on DNRA and anammox bacteria abundances is currently unknown. It would also be interesting to evaluate if an effect on dissimilatory nitrogen reducing gene abundances also translates into increased activities (mRNA level). Finally, evaluation of the MPB effect on a broader time and spatial (other mudflats) scale would provide additional insights on MPB effects on total and functional bacterial community structure and abundances. However, one should carefully interpret results due to potentially variable physico-chemical conditions.
- evaluating whether the MPB biomass itself affects bacterial abundances directly or indirectly, through potential grazing effects of higher organisms on MPB alone or on both MPB and the bacterial community. Proxies for such interactions can be investigated using microcosm experiments. Additionally, in case of a direct MPB effect, other factors need to be unraveled: do all diatom species affect the bacterial community, or is it the presence of certain diatom species that result in the effect on bacterial abundances? Secondly, how do these organisms affect the bacterial community, through EPS production, cytotoxin production, other ways?
- examining in more detail the bacterial taxon-specific effect of MPB biomass: (a) is a similar trend observed in a repeated sampling campaign, and (b) what specific genera within the *Firmicutes*, *Betaproteobacteria* and candidate division TM7 are affected. A molecular approach using phylum and genus specific primers should provide the first insights through real-time PCR.
- evaluating, improving or redesigning current available *nir* primers (see chapter 3).

Chapter 3

Highly diverse *nirK* genes comprise two major clades that harbour ammonium-producing denitrifiers

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Author's contributions:

HD and KH designed the experiments. HD and BT performed the experiments. HD and KH analyzed the data. HD, KH and AW wrote the paper.

Summary

Background

Copper dependent nitrite reductase, NirK, catalyses the key step in denitrification, *i.e.* nitrite reduction to nitric oxide. Distinct structural NirK classes and phylogenetic clades of NirK-type denitrifiers have previously been observed based on a limited set of NirK sequences, however, their environmental distribution or ecological strategies are currently unknown. In addition, environmental *nirK*-type denitrifiers are currently underestimated in PCR-dependent surveys due to primer coverage limitations that can be attributed to their broad taxonomic diversity and enormous *nirK* sequence divergence. Therefore, we revisited reported analyses on partial NirK sequences using a taxonomically diverse, full-length NirK sequence dataset.

Results

Division of NirK sequences into two phylogenetically distinct clades was observed, with Clade I mainly comprising *Alphaproteobacteria* (plus some *Gamma*- and *Betaproteobacteria*) and Clade II harbouring more diverse taxonomic groups like *Archaea*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, *Nitrospirae*, *Firmicutes*, *Actinobacteria*, *Planctomycetes* and *Proteobacteria* (mainly *Beta* and *Gamma*). Failure of currently available primer sets to target diverse NirK-type denitrifiers in environmental surveys could be attributed to mismatches over the whole length of the primer binding regions including the 3' site, with Clade II sequences containing higher sequence divergence than Clade I sequences. Simultaneous presence of both the denitrification and DNRA pathway could be observed in 67% of all NirK-type denitrifiers.

Conclusion

The previously reported division of NirK into two distinct phylogenetic clades was confirmed using a taxonomically diverse set of full-length NirK sequences. Enormous sequence divergence of *NirK* gene sequences, probably due to variable *nirK* evolutionary trajectories, will remain an issue for covering diverse NirK-type denitrifiers in amplicon-based environmental surveys. The potential of a single organism to partition nitrate to either denitrification or dissimilatory nitrate reduction to ammonium appeared to be more widespread than originally anticipated as more than half of all NirK-type denitrifiers were shown to contain both pathways in their genome.

3.1 Introduction

Canonical denitrification is the conversion of fixed nitrogen to a gaseous end product with concomitant energy conservation (Mahne and Tiedje, 1995;Zumft, 1997). It is a facultative respiratory pathway in which nitrate, nitrite, nitric oxide and nitrous oxide are sequentially reduced to dinitrogen gas, each step catalysed by one or more metallo-enzymes (Zumft, 1997). This important process has been intensely studied, both experimentally and *in situ*, as it is part of the global nitrogen cycle and contributes to the loss of fixed nitrogen from the environment as well as the emission or mitigation of nitrous oxide, *i.e.* a greenhouse gas that has a 310x greater global warming potential than carbon dioxide and is involved in ozone destruction (Ravishankara et al., 2009). Although denitrification has long been considered as a modular process (Zumft, 1997), with certain denitrifiers lacking either nitrate reductase and/or nitrous oxide reductase, whole genome sequences revealed the existence of extremely truncated versions of the pathway with only one enzyme or discontinuous chains of two or more enzymes (Shapleigh, 2013;Graf et al., 2014). This raises the semantic though relevant question of when an organism should be considered as denitrifier. We propose that, adhering to the original description of the process, a denitrifier *sensu stricto* should be capable of acquiring energy from at least the one-electron reduction of nitrite to the gaseous nitric oxide, meaning it should at least contain a nitrite reductase.

This key enzyme in the denitrification process exists in two evolutionary unrelated variants, *i.e.* copper containing nitrite reductase NirK and cytochrome cd_1 nitrite reductase NirS encoded by the *nirK* and *nirS* gene respectively (Zumft, 1997;Shapleigh, 2006). Some organisms contain more than one *nirK* or *nirS* gene copy (Etchebehere and Tiedje, 2005) and, recently, both types of Nir were found to not be mutually exclusive (Graf et al., 2014), although functionality of the two different nitrite reductase types within one organisms still needs confirmation. NirS-type denitrifiers are often assumed to be predominant in the environment, while NirK-type denitrifiers cover more diverse taxa (Zumft, 1997). It should, however, be noted that (i) very few studies have attempted to quantify both NirK and NirS-type denitrifiers in environmental surveys, with most only including *nirS* (Bowen et al., 2013;Francis et al., 2013;Fan et al., 2015), and (ii) *nirK* gene sequence divergence exceeds that of *nirS* genes resulting in constrained applicability of the currently available *nirK* primers, leading to an underestimation of NirK-type denitrifiers (Penton et al., 2013). Both types of denitrifiers are assumed to respond differentially to environmental gradients with NirS-type denitrifying communities being shown to correlate with nitrate concentration (Smith and Ogram, 2008) and pH (Enwall et al., 2010), NirK-type denitrifying communities were reported to exhibit a greater habitat selectivity compared to NirS-type denitrifying communities (Thröback et al., 2004;Smith and Ogram, 2008) and were found to correlate with Cu content (Enwall et al., 2010) and TOM (Decleyre et al., 2015). Co-occurrence patterns of *nirS*, *nor* and *nos* genes suggested shared regulatory mechanisms that may constrain loss of *nor* and *nos* in *nirS*-type denitrifiers (Graf et al., 2014). In contrast, no such genomic linkage patterns were observed for NirK indicating that NirK-type denitrifiers are more likely to perform incomplete

denitrification. Taken together with previously reported positive correlations between NirK abundance and nitrous oxide emissions (Clark et al., 2012) and the negative relationship between the ratio nitrous oxide/dinitrogen gas and NirS-type denitrifiers (Cuhel et al., 2010), NirK-type denitrifiers might contribute significantly more to nitrous oxide emission than their NirS counterparts.

The large divergence in NirK sequences from taxonomically distant as well as closely related NirK-type denitrifiers has not yet been well-characterized despite the need for accurate assessment of abundances and community structure of these potential nitrous oxide emitters. While there are conserved catalytic domains shared by all NirK proteins, substantial variation exists in the primary structure between different members of the NirK protein family. Copper-containing nitrite reductase NirK (also sometimes designated as cNirK/CuNIR) is a periplasmic, homotrimeric enzyme with each monomer typically containing two copper centres, T1Cu ligated by four amino acid residues (two His, Cys and Met) and T2Cu ligated by three His and a water molecule (Nojiri et al., 2009). The enzyme receives one electron at the T1Cu site from an electron donor and catalyses the one-electron reduction of nitrite to nitric oxide at the T2Cu site. Moreover, an essential hydrogen bond network including Asp and His around T2Cu functions as proton donor to the substrate (Boulanger et al., 2000). Two different classes of NirK-type nitrite reductases have been designated previously based on their structure. (i) Soluble periplasmic Class I NirK found in some *Alphaproteobacteria* can be divided into two subclasses based on their optical absorption spectrum and sequence identity: green Nir types (described for *Achromobacter cycloclastes* and *Alcaligenes faecalis* S6) and blue Nir types (described for *Alcaligenes xylosoxidans*) (Ellis et al., 2007). (ii) Outer-membrane bound Class II NirK was described for *Neisseria gonorrhoeae* (Boulanger and Murphy, 2002). More divergent NirK sequences from some Gram-positive bacteria and from the ammonium oxidizing bacterium *Nitrosomonas europaea* were placed in a separate class (Philippot, 2002). Furthermore, the NirK of *Hyphomicrobium denitrificans* with an additional cupredoxin domain at the N-terminus (Ellis et al., 2007;Nojiri et al., 2007) and those of *Burkholderia*, *Ralstonia* and *Bdellovibrio* with a C-terminus extension containing a class I *c*-type heme domain (Ellis et al., 2007), do not fit the current classification. In addition to structural differences, phylogeny has been used to delineate two NirK clades supported by distinct amino acid motifs, *i.e.* TRPHL and SSFHV/I/P, around the active site His residue (Jones et al., 2008). Clade I was found to harbour Class I NirK sequences, while Clade II NirK contained more taxonomically diverse NirK-type denitrifiers including some belonging to Class II NirK.

It is clear that an unambiguous NirK classification is lacking. Furthermore, currently available primers only target Class I *nirK* sequences (Jones et al., 2008;Penton et al., 2013). So, although NirK-type denitrifiers are potential nitrous oxide emitters, the predominance of various NirK classes or clades in the environment cannot be unequivocally and systematically evaluated. This is unfortunate as it could unveil different ecological strategies or environmental distributions among denitrifiers harbouring distinct clades or classes of NirK, as was recently demonstrated for the two distinct clades of nitrous

oxide reductase NosZ (Sanford et al., 2012;Jones et al., 2013;Jones et al., 2014). Therefore, we revisited and extended the NirK analyses of Jones and colleagues (Jones et al., 2008), by performing detailed sequence and phylogenetic analyses on NirK sequences from fifteen different phyla in light of the structurally different NirK classes described so far. The previously proposed grouping of NirK in two distinct phylogenetic clades was confirmed and further underpinned by Clade II specific indels. The potential to partition nitrate between denitrification and dissimilatory nitrate reduction to ammonium appeared common to denitrifiers from both clades. Furthermore, evolutionary trajectories underpinning the extremely high NirK sequence divergence and consequences for *nirK* primer coverage were considered.

3.2 Results and discussion

Proposed NirK classification in two clades based on phylogenetic and sequence analysis

Copper dependent nitrite reductase NirK encoded by the *nirK* gene catalyses the key step of the denitrification pathway, *i.e.* the reduction of nitrite to the gas nitric oxide. In environmental surveys, this gene has been used as a proxy for denitrification despite previously reported limitations of conventional *nirK* primers (Green et al., 2010;Penton et al., 2013) resulting in the underestimation of both the presence and abundance of NirK-type denitrifiers in different environments. Broad taxonomic diversity of NirK-type denitrifiers as well as the impressive divergence among NirK sequences both account for the limited coverage of current primers. A NirK dataset composed of 267 full-length NirK sequences, extracted from whole genomes, from fifteen different Bacterial and Archaeal phyla was used for phylogenetic and sequence analysis. Prior to use of the dataset, all NirK sequences were verified for the presence of core Cu-binding sites (His59, His64, His99, Cys100, His110, Met115 and His298) and the active site residues Asp62 and His237 required for nitrite reducing activity (Figure 1) (Boulanger et al., 2000;Kataoka et al., 2000). Comparison of NirK sequences with other copper oxidases lacking Nir activity (laccases and ascorbate oxidases) indicated that the residues Asp62, His64, Cys100, His110, Met115 and His237 were not conserved. Based on this observation, we were convinced that all NirK sequences included in this study were indeed nitrite reductases. NirK sequence lengths varied substantially from 304 to 995 aa, with the majority of NirK sequences ranging between 300 and 560 aa, and some Actinobacterial sequences around 700-990 aa.

Phylogenetic analyses resulted in the distinction of two NirK clades (Figure 2): (i) Clade I mainly comprised of *Alphaproteobacteria* (plus some *Gamma*- and *Betaproteobacteria*) and (ii) Clade II harboured more diverse taxonomic groups like *Archaea*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes*, *Nitrospirae*, *Firmicutes*, *Actinobacteria*, *Planctomycetes* and *Proteobacteria* (mainly *Beta* and *Gamma*). Using an expanded dataset of over 250 full-length NirK sequences, we thus confirm the previously described existence (based on 147 partial *nirK* sequences) of two distinct NirK clades (Jones et al., 2008), and propose the systematic use of the designated NirK Clades I and II in further environmental surveys. Clade I corresponded to the previously described Class I NirK nitrite reductases (Ellis et al., 2007;Jones et al., 2008), while NirK sequences previously defined as belonging to Class II or unclassified, all clustered together in Clade II. Interestingly, this grouping into two clades is underpinned by small indels specific to Clade II (Figure 3), specifically two deletion regions (7aa) which coincided with the linker and tower loop previously observed in *Bacillus* and *Neisseria* species (Figure 2, Figure 3) (Boulanger and Murphy, 2002;Nojiri et al., 2007;Fukuda et al., 2011). The extra loop regions unique to NirK from *Bacillaceae* (Figure 1, Figure 3) (Fukuda et al., 2011) were observed in all *Bacillus* sp. with exception of the three *Paenibacillus* species. *Actinobacteria* also contained additional insertions although the size, location and presence/absence of these insertions were variable (Figure 3 and S1). We also found that the TRPHL and SSFHV/I/P motifs around the

active site His237 (Figure 2, Figure S1) thought to be unique to Clade I and II respectively (Jones et al., 2008) could not be used as good distinguishing features, as the corresponding regions within each clade were much more diverse than previously described. Approximately 61% of all Clade I NirK contained a TRPHL motif, with other previously undescribed motifs being SRPHL (32%), SYPHL (3%), SRIHL (1%) and TR/YPHI (3%). More than half of all Clade II NirK (61%) contained a SSFHV/I/P motif while 39% comprised other and more diverse motifs such as CH/TFHV (4%), LSFHV/I (5%), SNFHV/I/P (5%), SSFHL (5%), with especially Actinobacterial and Archaeal NirK containing previously undescribed motifs (Figure S1).

Figure 1 Trimmed multiple sequence alignment of NirK from *Rhodobacter sphaeroides* ATCC 17025 (R. spha), *Alcaligenes faecalis* S-6 (A.faec), G*eobacillus. kaustophilus* HTA426 (G.kaus), *Bacillus azotoformans* LMG 9581T (B.azot), *Jonesia denitrificans* DSM 20603 (J.deni), *Propionibacter acnes* C1 (P.acne), *Flavobacteria johnsoniae* UW101 (F.john), *Halomonas denitrificans* ATCC 35960 (H.deni), *Burkholderia pseudomallei* 668 (B.pseu) and *Azospirillum* sp. B506 (A.sp). Sequences belonging to Clade I NirK are underlined. Amino acid numbering was based on whole NirK gene of *Rhodobacter sphaeroides* ATCC 17025. The linker, tower and extra loop specific for *Bacillus* sp. are given in purple, red and orange respectively. Copper binding motives T1Cu and T2Cu are indicated in green and blue respectively, active site residues Asp and His required for nitrite reducing activity in yellow, conserved regions are indicated by *, deletions specific to Clade II NirK sequences are indicated by ‡ and • and the *Bacillus* specific insertion by †.

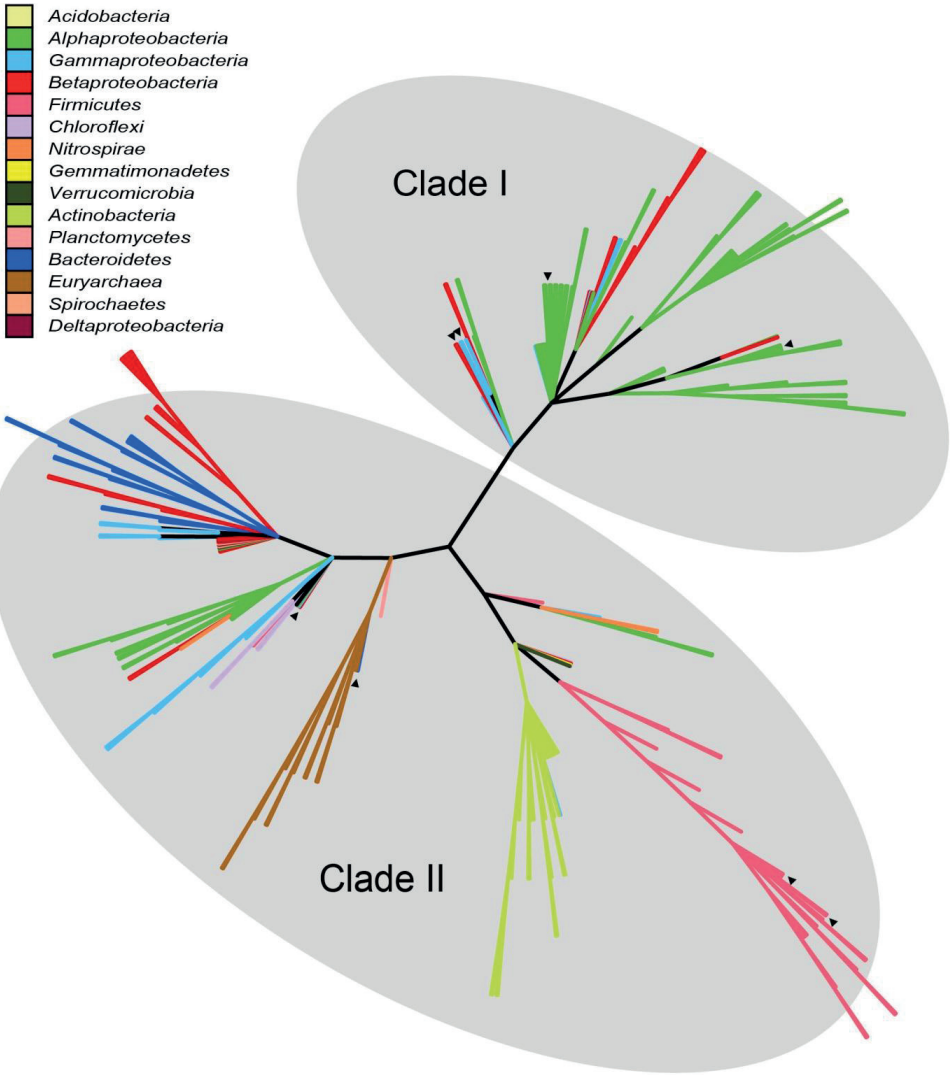

Figure 2 Unrooted bifurcating neighbour joining tree of full-length NirK sequences obtained from whole genomes. NirK Clade I encompassed *Alphaproteobacteria* and some *Gamma-* and *Betaproteobacteria*, while NirK Clade II comprised *Firmicutes*, *Actinobacteria*, *Euryarchaea*, *Chloroflexi*, *Bacteroidetes*, *Nitrospirae*, *Gemmatimonadetes*, *Planctomyces*, *Verrucomicrobia* and *Proteobacteria*. The same bifurcating tree in cladogram format with an estimation of reliability based on bootstrap analysis can be found in supplementary information (Figure S1). Physiologically characterized denitrifying strains are indicated by ▼: *Bacillus azotoformans* LMG 9581, *Bacillus bataviensis* LMG 21833 (Verbaendert et al., 2011), *Haloferax denitrificans* ATCC 35960 (Tomlinson et al., 1986), *Hyphomicrobium denitrificans* ATCC 51888 (Martineau et al., 2015), *Shewanella loihica* PV-4 (Yoon et al., 2013), *Shewanella denitrificans* OS217 (Brettar et al., 2002), *Rhodobacter sphaeroides* ATCC 17025 (Bergaust et al., 2014), *Alcaligenes faecalis* S-6 (Kakutani et al., 1981).

As could be expected from their interaction to form a hydrogen bound network involved in proton supply for substrate reduction (Boulanger and Murphy, 2002), substantial variation in AA motifs around the other essential T2Cu centre residue, Asp 62 (Figure 2), were also observed (Figure S1). Distinction between NirK Clade I and II based on signal peptide, as previously observed for the two clades of NosZ (Sanford et al., 2012), was not found.

Strikingly, the overall mean NirK similarity within the dataset was extremely low (only 10%), with especially Clade II being defined by low sequences similarity (16% compared to 66% in Clade I). This even higher than previously appreciated level of NirK sequence divergence and above mentioned substantial sequence size variation may be partly attributed to the high incidence of N- and C-terminus extensions in approximately 30% of all NirK sequences. C-terminus extensions comprising a class I *c*-type heme domain $CX_2CHX_{50}M$ previously only reported in NirKs from *Burkholderia*, *Ralstonia* and *Bdellovibrio* (Ellis et al., 2007), were observed in 39 other NirK sequences derived from *Spirochaetes*, *Verrucomicrobia*, *Alpha*-, *Beta*-, *Delta*- and *Gammaproteobacteria* (Figure 3 and Figure S1). Recent NirK analysis of the commensal bacterium *Neisseria weaveri* revealed high sequence similarity between the C-terminus *c*-type heme and other Neisserial NirK cytochrome electron carriers, *i.e*. Ccop and c_5 cytochromes (Aas et al., 2015), suggesting it might function as an alternative electron transport route to NirK providing an adaptive advantage in nitrite limiting environments. In contrast, these C-terminus extensions are not found in pathogenic *Neisseria* sp., further underlining their potential adaptive benefit linked to distinct lifestyles. The approximately 900 aa long NirK of *Propionibacterium acnes* was previously found to contain a N-terminus extension comprising a 400 aa predicted transmembrane domain of unknown function and an additional cupredoxin domain, *i.e*. CX_4HX_4M (Figure 3). All Actinobacterial NirK included in this study, were observed to contain the cupredoxin domain, however, not all contained a transmembrane domain (Figure S1), resulting in substantial sequence length variation within the *Actinobacteria* (900 aa vs. 500 aa). The cupredoxin containing N-terminus extensions were also previously reported in non-actinobacterial *Hyphomicrobium denitrificans* ATCC 51888, *Herpetosiphon aurantiacus* DSM 785 and *Nitrosospira multiformis* ATCC 25196 (Ellis et al., 2007), and were additionally observed here in six other NirK sequences belonging to *Nitrospirae*, *Alpha*- and *Betaproteobacteria* (Figure S1). The function of the N-terminus cupredoxin domain remains unknown as it located too far away from the catalytic core for effective electron transfer (Nojiri et al., 2007) and, moreover, higher catalytic activity was observed in N-terminus T1Cu mutant compared to the wild type (Yamaguchi et al., 2003). Nevertheless, its conservation and wide occurrence does suggest some adaptive benefits despite its presence being not required for NirK functioning. NirK sequences were not found to contain both N- or C-terminus extensions, which corroborates previous observations (Ellis et al., 2007).

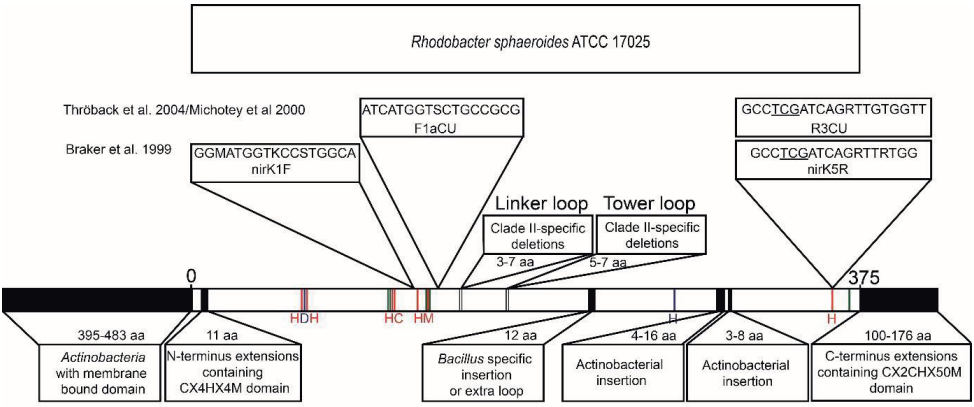

Figure 3 Schematic illustration of sequence length variation, primer target site of two frequently used primer sets F1aCu-R3Cu (Hallin and Lindgren, 1999) and nirK1F-nirK5R (Braker et al., 1998), and different types of indels and extensions observed in 267 NirK sequences. The 375 aa long sequence of *Rhodobacter sphaeroides* ATCC 17025 (pdb accession number 1ZV2) was used as reference. Size and, if possible, information on taxon or clade defined by these indels or extensions are represented. Copper binding sites, essential active site residues and conserved regions are indicated in red, blue and green respectively. The copper binding His included in the reverse primer target sites is underlined. Abbreviations of copper binding and active site residues are presented due to limited space, with H: His, C: Cys, D:Asp and M:Met.

High sequence divergence causes *nirK* primer coverage issues

Although shotgun sequencing is becoming increasingly affordable for environmental monitoring of ecosystem functions, amplification-based gene sequencing and quantification is still widely performed. Primer coverage is a well-known but continuing problem for successful amplification and sequencing of any gene from environmental or mixed samples and especially for the very diverse *nirK* gene. The most frequently used primers F1aCu-R3Cu (Hallin and Lindgren, 1999) and NirK1F-NirK5R (Braker et al., 1998) bind to supposedly conserved copper binding regions outside the Clade II indels and/or variable Asp or His motifs described higher and shown in Figure 3, yet still they only detect Clade I sequences (Jones et al., 2008;Green et al., 2010;Penton et al., 2013). Large sequence divergence seemed to exist throughout the whole *nirK* gene, even at supposedly conservative sites used for primer design (Figure 4 and S2). We directly related failure of current primers as broad range tools to mismatches over the whole primer binding regions including the 3' site. As expected from sequence variation, this was more explicit for Clade II than Clade I sequences (Figure 4 and S2).

As the sequence divergence within Clade II was very high and modification of available primers to increase coverage is not straightforward (Penton et al., 2013), we attempted to design novel *Bacteroidetes* specific *nirK* primers using the current dataset. *Bacteroidetes* NirK sequence divergence

averaged around 28%, making this taxon a better test case for primer design compared to *Actinobacteria* and *Archaea* (sequence divergence of 42% and 37% respectively). Although *Firmicutes* sequence divergence were found to be the lowest (22%), the *Bacteroidetes* groups was chosen as a test group because recently *Geobacillus* specific *nirK* primers (Verbaendert et al., 2014) were published. The novel primers (Table S1) scored significantly better for successful amplification in pure cultures than the traditional ones but unfortunately did not render amplicons from various environmental samples (Table S2). Although it cannot be excluded that *nirK*-containing *Bacteroidetes* were only present under the detection limit in these samples, it seems more likely that, despite their high degeneracy, the novel primers were too specific to capture all diversity present (Table S1). A similar observation was made for primers targeting denitrifying *Geobacillus* ((Verbaendert et al., 2014), unpublished data). Wei and colleagues, however, recently succeeded in designing new *nirK* primers targeting more diverse Clade I and numerous subgroups within Clade II *nirK* sequences (Wei et al., 2015), though no *Firmicutes nirK* were included and the dataset used for primer design was limited to 97 *nirK* sequences. *In silico* evaluation of these new primers on the *Bacteroidetes* dataset indicated that the reverse primer did not find a match for all *Bacteroidetes* sequences within the alignment. It is clear that primer coverage, albeit essential for proper experimental design of environmental surveys, remains an issue difficult to resolve.

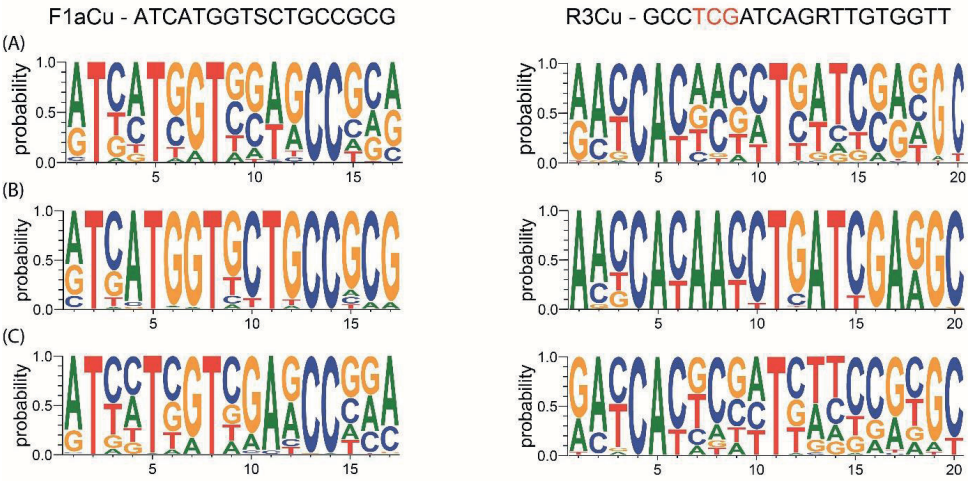

Figure 4 Sequence logo diagrams (5'-3') depicting the degree of *nirK* sequence variability of F1aCu-R3Cu primer binding regions (Hallin and Lindgren, 1999) in (a) Clade I+II *nirK*, (b) Clade I *nirK*, and (c) Clade II *nirK*. The primer sequence is given at the top and copper binding His included in the reverse primers is indicated in red. Diagrams for the primers nirK1F-nirK5R (Braker et al., 1998) can be found in Figure S2.

Evolutionary processes resulting in high sequence divergence

Phylogenetic analyses of NirK sequences (Figure 2) showed clustering of NirK-type denitrifiers according to their taxonomic origin for *Alphaproteobacteria* in Clade I, and *Actinobacteria*, *Firmicutes* and *Archaea* within Clade II. Nevertheless, high NirK sequence divergence between closely related NirK-type denitrifiers is common and generally known (Heylen et al., 2006a;Jones et al., 2008;Shapleigh, 2013), with Beta- and Gammaproteobacterial clusters also found in Clade I and *Verrucomicrobia*, *Spirochaetes*, *Chloroflexi*, *Beta-*, *Delta-* and *Gammaproteobacteria* sequences spread throughout the entire Clade II (Figure 2). Confirming previously reported incongruences between phylogenies of 16S rRNA gene sequence and denitrification genes (Philippot, 2002;Jones et al., 2008), our analyses revealed some more striking examples. Firmicute *Sulfobacillus acidophilus* TPY did not cluster together with other Firmicutes NirK sequences and was even more found to share only 5% sequence similarity with the other Firmicute NirK sequences (Figure S1). Also, the NirK from Gammaproteobacterial *Cellvibrio gilvus* ATCC 13127 grouped within the *Actinobacteria* cluster while that of the Bacteroidete *Rhodothermus marinus* SG0.5JP17-172 was found in the cluster of the *Archaea*. Jones and colleagues (Jones et al., 2008) already elaborately discussed that the evolutionary process responsible for these incongruences might not be as simple as horizontal gene transfer. They proposed that gene duplication and subsequent sequence divergence of the different copies and/or gene loss as well as lineage sorting could be primarily responsible. In that light, it is interesting to note that approximately 5% of all NirK-type denitrifiers in our dataset were found to contain more than one *nirK* gene copy in their genome. Although our observations are biased because of considerable differences in number of genomes available per phylum (Table 1), *Alphaproteobacteria* were mostly found to contain multiple copies, but also some representatives of *Betaproteobacteria*, *Firmicutes*, *Nitrospirae* and even *Archaea*. The majority of these taxa harboured two copies, with exception of *Afipia* sp. 1NLS2 which was previously described to contain three (Shapleigh, 2013). As was found for *Afipia*, the Betaproteobacterium *Pusillimonas* sp. T7-7 and the Alphaproteobacterium *Ochrobactrum anthropi* ATCC 49188 each harbored two NirK sequences belonging to different clades. All other taxa contained NirK copies belonging to either Clade I or II, with various degrees of divergence (Figure S1). Clearly, multiple *nirK* copies may provide adaptive advantages to the denitrifiers in changing environments, provided that both genes are expressed as functional nitrite reductases under different physicochemical conditions (Etchebehere and Tiedje, 2005). Differential loss and unequal evolutionary pressures on both copies could explain the observed incongruent organisms and denitrification gene phylogenies.

Compared with NirK, the overall sequence divergence of NirS appears to be lower, with no described NirS classes based on structural diversity, phylogenetic groups or amino acid motifs. The different evolutionary trajectories of both nitrite reductases are potentially related to the differential spread of the encoding genes over prokaryotic life, with NirS being limited to mostly *Proteobacteria* (Jones et

al., 2008;Wei et al., 2015). However, an additional important feature to consider is the difference in operon size between both *nir* types. NirS maturation requires the expression of a multiple gene operon consisting of at least three or four *nir* genes (Philippot, 2002), while NirK maturation does not require other genes, although sometimes it is accompanied by a second gene encoding the protein NirV (Jain and Shapleigh, 2001). NirV was detected in approximately 61% of the genomes included in our dataset, mainly in *Proteobacteria* (*Alpha* and *Beta*) and *Bacteroidetes*, confirming that the presence of NirV is not mandatory for NirK maturation (Philippot, 2002). The firmicute *Bacillus azotoformans* LMG 9581 (Verbaendert et al., 2011) and the archaeon *Haloferax denitrificans* ATCC 35960 (Tomlinson et al., 1986) have both been physiologically shown to perform denitrification, however, no NirV could be detected. Alternatively, it is also plausible that NirV was missed in these organisms due to the limited number and diversity of reference sequences available (only *Alphaproteobacteria*). The evolutionary pressure on *nirS* is thus much higher than in *nirK,* as mutations are less likely to be retained by natural selection because of incompatibility of the expressed enzyme with accessory proteins. Differential evolutionary pressures on both *nir* genes might not only explain differential mutation rates but also the higher probability for mobility and gene duplication of *nirK*. Furthermore, compared to *nirK*, occurrence of the *nirS* gene is more often linked to other denitrification genes (Graf et al., 2014). Indeed, NirK can be involved in other nitrogen processes such as anaerobic ammonium oxidation (Anammox) (Hira et al., 2012) and nitrifier denitrification[4] (Lawton et al., 2013), as well as have a function other than anaerobic respiration of nitrogen oxides (Basaglia et al., 2007). So, distinct taxonomic breadth, operon structure and metabolic versatility of *nirS*-and *nirK*-type denitrifiers could contribute to distinct evolutionary trajectories resulting in the higher level of sequence divergence for NirK.

DNRA is common in both Clade I and II NirK-type denitrifiers

Denitrification and dissimilatory nitrate reduction to ammonium (DNRA), a process also contributing to nitrous oxide emission, were long believed to be performed by distinct microbial populations. However, genome analysis of *Shewanella loihica* (Yoon et al., 2013), *Bacillus azotoformans* (Heylen and Keltjens, 2012) and other bacteria (Sanford et al., 2012) have recently revealed the gene inventory for both nitrate reducing processes to be present in one organism. Functional capacity to carry out both nitrate reducing processes and their determining environmental drivers, mainly carbon-to-nitrogen ratio and nitrite concentrations, have since been demonstrated for *S. loihica* Interestingly, all organisms containing both processes were NirK-type denitrifiers. In our dataset, approximately 67% of NirK-type denitrifiers were found to harbour both pathways in their genome (Table 1), with 25.9% belonging to Clade I and 40.8% to Clade II. Unequal distribution over different phyla was observed

[4] Nitrifier denitrification is the pathway of nitrification in which ammonia (NH_3) is oxidized to nitrite (NO_2^-) followed by the reduction of NO_2^- to nitric oxide (NO), nitrous oxide (N_2O) and molecular nitrogen (N_2). The transformations are carried out solely by autotrophic nitrifiers.

Table 1 Modularity of denitrification and DNRA observed in the 249 NirK containing genomes covering fifteen different phyla. Percentage complete denitrification (Nir+Nor+Nos), incomplete denitrification (-Nor, -Nos or -Nor/-Nos), DNRA (Nar+NrfA, Nap+NrfA, NrfA, Nar+NirB, Nap+NirB or only NirB), and combined occurrence of both processes (NirK+NrfA and/or NirB) are represented for each phylum separately and over all 249 NirK genomes.

	No.of genomes	**No. of *nirK* copies per genome**	**% complete denitrification**	**% incomplete denitrification**			**% DNRA**						**% denitrification and DNRA**
		(% of phylum)	***nir+nor+nos***	***-nor***	***-nos***	***-nor/-nos***	***nap+nrfA***	***nar+nrfA***	***nrfA***	***nap+nirB***	***nar+nirB***	***nirB***	***nirK+nrfA*and/or *nirB***
Acidobacteria	1	1 (100)	100	0	0	0	0	0	0	0	0	0	0
Actinobacteria	28	1 (100)	0	0	50	50	0	7.1	17.9	3.6	39.3	0	64.3
Alphaproteobacteria	82	1 (92.7); 2 (6.1) or 3 (1.2)	41.5	1.2	39.0	18.3	15.7	0	0	65.9	11	6.1	78.0
Bacteroidetes	21	1 (100)	42.9	19.0	28.6	9.5	9.5	0	42.9	9.5	0	9.5	66.7
Betaproteobacteria	39	1 (92.3) or 2 (7.7)	61.5	0	30.8	7.7	2.6	2.6	0	61.5	61.5	5.1	74.4
Verrucomicrobia	2	1 (100)	0	50	0	50	100	0	0	50	0	0	100
Chloroflexi	5	1 (100)	0	20	0	80	0	0	0	0	0	40	40
Deltaproteobacteria	2	1 (100)	0	0	50	50	0	0	50	0	0	50	50
Euryarcheaota	13	1 (84.6) or 2 (15.4)	46.2	0	38.5	15.3	0	0	0	0	0	7.7	7.7
Firmicutes	27	1 (96.3) or 2 (3.7)	40.8	7.4	25.9	25.9	11.1	11.1	0	37	37	3.7	55.6
Gammaproteobacteria	23	1 (100)	19.0	0	33.3	47.7	30.4	4.3	0	47.8	17.4	0	73.9
Gemmatimonadetes	1	1 (100)	0	100	0	0	0	0	0	0	0	0	0
Nitrospirae	2	1 (50) or 2 (50)	0	0	50	50	0	0	0	0	0	0	0
Planctomycetes	1	1 (100)	0	0	0	100	100	100	0	100	100	0	100
Spirochaetae	2	1 (100)	50.0	0	0	50.0	0	0	0	50	0	50	100
Total	**249**	**5.3**	**36.0**	**4.0**	**34.8**	**25.2**	**12.1**	**3.2**	**6.1**	**42.2**	**23.7**	**6.0**	**66.7**

for *nrfA* and *nirB*, the genetic markers for respiratory and fermentative DNRA respectively and the genes coding for the periplasmic pentaheme cytochrome *c* and the cytoplasmic NADH-dependent nitrite reductases respectively (Table 1). However, inadequate representation of certain phyla due to a lower number of genomes may have obscured a clear picture of their occurrence. The metabolic versatility rendered from the ability to use both processes is a clear competitive advantage in variable environments, especially those with changing carbon and/or nitrogen loads (Tiedje, 1988;Strohm et al., 2007;Yoon et al., 2015b). However, inadequate representation of certain phyla due to a lower number of genomes may have obscured a clear picture of their occurrence. The metabolic versatility rendered from the ability to use both processes is a clear competitive advantage in variable environments, especially those with changing carbon and/or nitrogen loads (Tiedje, 1988;Strohm et al., 2007;Yoon et al., 2015b). This also nicely fits with the similar preference of Nap and NrfA for low nitrate concentrations (Tiedje, 1988;Richardson, 2000). The differential preference of Nar and NrfA for high and low nitrate concentrations respectively also agrees with this negative co-occurrence pattern (Tiedje, 1988;Richardson, 2000). The inventory of denitrification genes in all 249 NirK containing genomes varied enormously (Table 1) and recently reported observations such as the simultaneous presence of NirK and NirS, the high occurrence of only Nir and Nor in *Actinobacteria* and the observation of highly truncated versions of the denitrification pathway (Graf et al., 2014) were confirmed here. Our analyses therefore suggest that NirK denitrifiers are not only more likely to contribute more to nitrous oxide emissions due to the higher occurrence of truncated denitrification (Graf et al., 2014) but also because of their ability to carry out DNRA.

In conclusion, this study confirms the existence of the two phylogenetic distinct clades of NirK based on a taxonomically diverse set of full-length NirK sequences, with Clade I harbouring Class I NirK and, Clade II containing a more diverse set of structurally different NirK. We propose a systematic usage of Clade I and Clade II designation in NirK sequence analyses of environmental surveys to ascertain potential ecophysiological differences between NirK-type denitrifiers from each clade. In amplicon-based surveys, enormous *nirK* sequence divergence, due to variable *nirK* evolutionary trajectories, will remain the major cause of limited primer coverage. The simultaneous presence of both the denitrification and DNRA pathway appears to be more widespread than originally anticipated, as more than half of all NirK-type denitrifiers were shown to contain both pathways in their genome.

3.3 Experimental procedures

Acquisition, processing and analysis of NirK sequences for database building

In total, a set of 267 NirK sequences, representing 249 microbial genome sequences across 15 phyla, were downloaded from the microbial genome (complete and DRAFT) database of GenBank. A two-step search was performed to acquire full-length NirK sequences as many genes are often misannotated or annotated in different ways. First, protein BLASTs were performed using NirK sequences with known crystal structure (*Alcaligenes faecalis* (Tocheva et al., 2004), *Rhodobacter sphaeroides* (Jacobson et al., 2005), *Neisseria gonorrhoeae* (Boulanger and Murphy, 2002), *Achromobacter xyloxidans* (Ellis et al., 2001), *Geobacillus kaustophilus* (Fukuda et al., 2011)), with additionally the NirK sequence of *Bacillus azotoformans* LMG 9581^T (Heylen and Keltjens, 2012) to increase matches with NirK sequences of Gram-positive bacteria. Then, search terms such as nitrite reductase copper, NirK and copper containing nitrite reductase were used to find additional NirK sequences via the gene search module of the integrated microbial genome (IMG) database (Markowitz et al., 2014) with following settings: Filters-Gene product Name (inexact), Sequencing status-All finished, Permanent Draft and Draft, Domain-Bacteria.

Sequence and phylogenetic analyses

All NirK sequences were aligned using the MUSCLE algorithm (Edgar, 2004) and the alignment was manually refined in Mega 6.06 (Tamura et al., 2013). Sequences were checked for the presence of the copper-binding sites of T1Cu and T2Cu. Then, the redundancy of the dataset was reduced by eliminating different strains of the same species with identical NirK sequences. Manual inspection of the NirK alignment was performed to check for N- and/or C-terminus extensions and to identify differences in insertions, deletions and conserved regions between the different taxonomic groups included. The alignment was trimmed to cover the maximum region shared by all NirK sequences before neighbour joining phylogenetic analysis in Mega 6.06 (Tamura et al., 2013), resulting in the exclusion of N- and C-terminus extension from further analysis. The number of differences method was used to compute evolutionary distances and node confidence was determined using 1000 replicates.

Signal peptide prediction was done as described by Emanuelsson et al. (2007): SignalP 4.1 was used for prediction of secretory signal proteins (Petersen et al., 2011), TatP 1.0 for twin-arginine translocation signal proteins (Bendtsen et al., 2005b), LipoP 1.0 for lipoprotein signal proteins (Rahman et al., 2008), and SecretomeP 2.0 for non-classical protein secretion (Bendtsen et al., 2005a). When both Tat and Sec signal peptides were predicted for a single sequence, the one with the highest D-value was retained. Prediction servers did not cover archaeal sequences.

Co-occurrence patterns of NirK, NrfA, Nar and Nap

To query NirK-encoding genomes to detect the presence of either (in)complete denitrification and/or DNRA, a diverse set of homologous amino acid sequences of functionally characterized NapA, NarG, NirS, NrfA, Nor and NosZ (Table S3) was used to build a pBlast database in CLC genomics Wb 7.5. NirB sequences were directly downloaded from the RAST server (Aziz et al., 2008) and checked for the presence of essential metal binding motifs using *Escherichia coli* K12 (P08201) and *Bacillus subtilis* 162 (P42435) as reference sequences. A NirV database was built to assess the occurrence of NirV in taxonomically diverse NirK-type denitrifiers. An Expect value of 0.00001, Existence 11-Extensions 1 as Gap cost and BLOSUM62 as matrix were used as pBlast settings. Co-occurrence and Pearson correlation analyses were performed in default settings using the co-occur and Hmisc package in R 3.1.2 (R core team, 2013;Veech, 2013).

3.4 Acknowledgement

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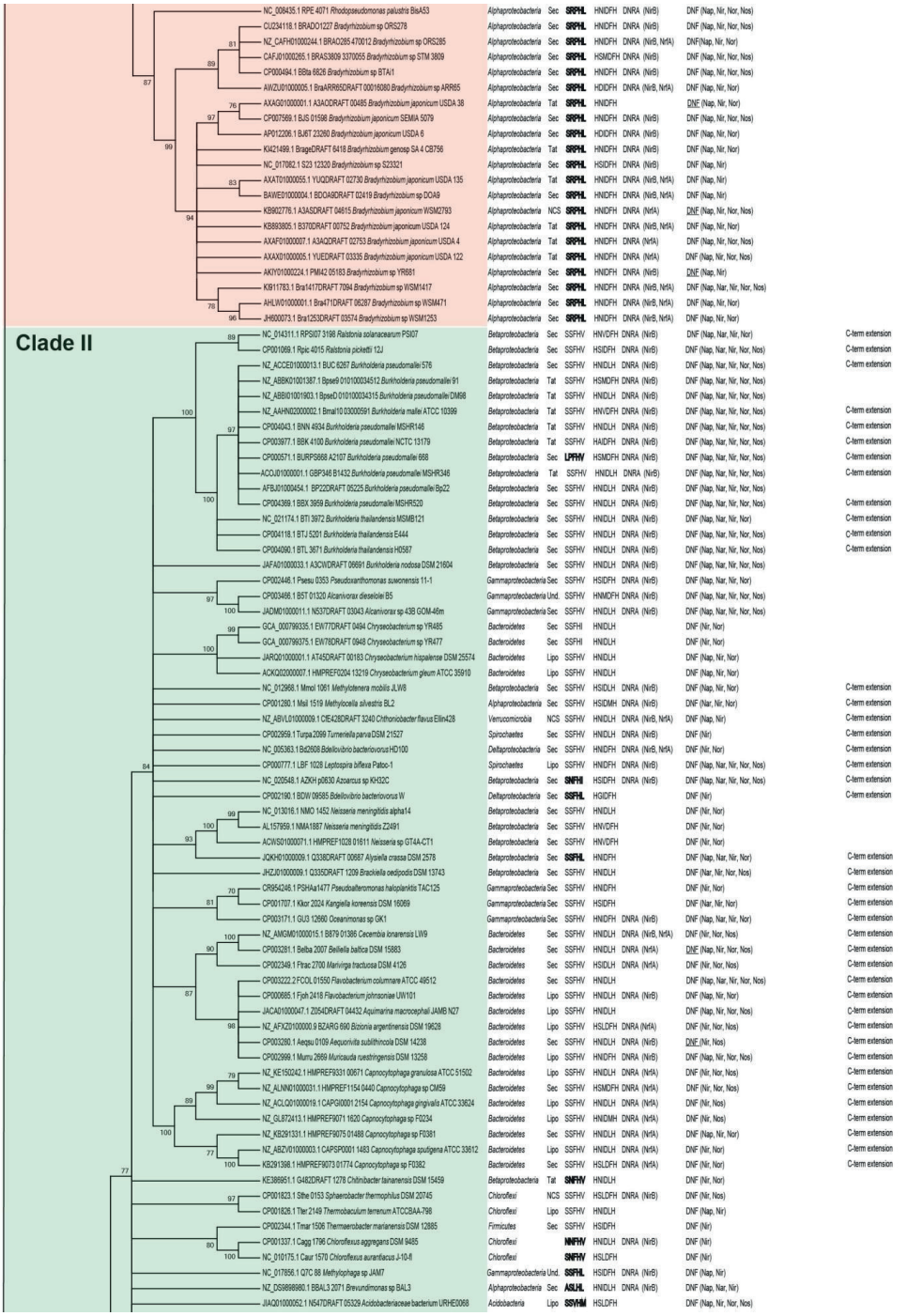
3.5 Supplementary information

Figure S 1 continues on the next page



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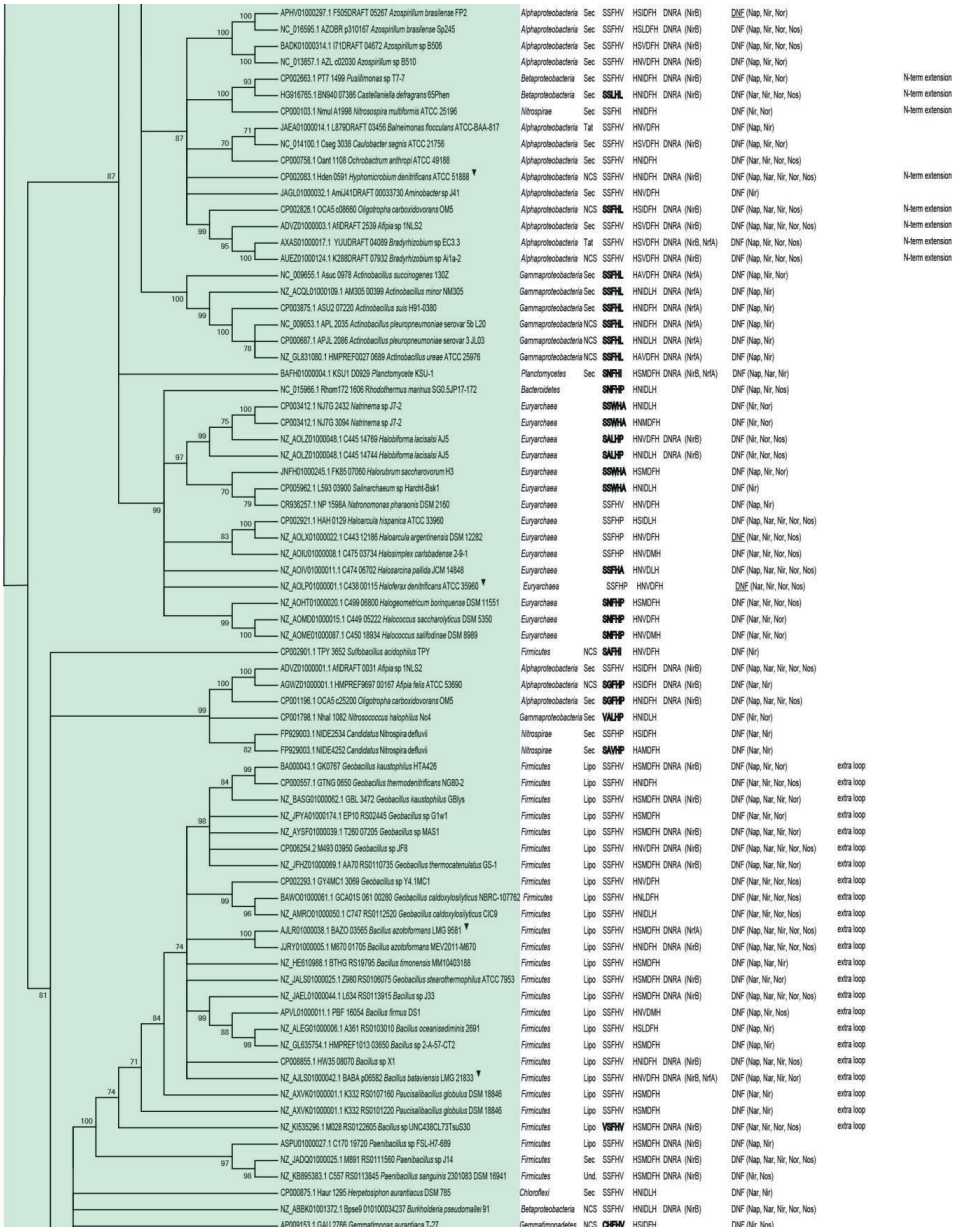

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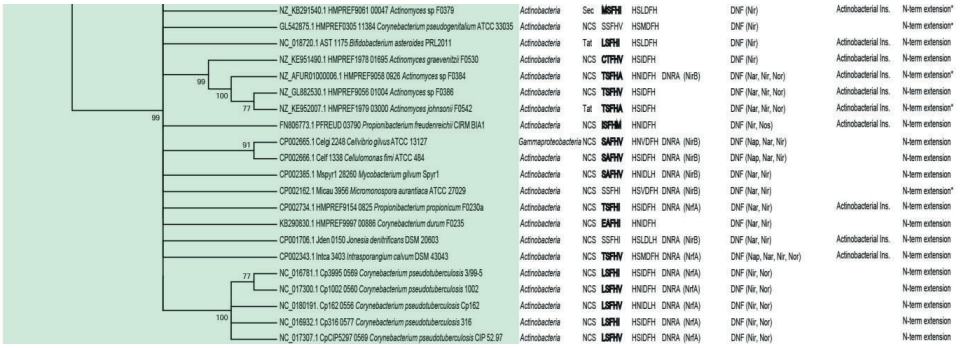

Figure S 1 Bifurcating neighbour joining tree of 267 NirK sequences representing 249 genomes. Bootstrap values < 70% after performing 1000 replications are not shown. NirK Clade I encompassed *Alphaproteobacteria* and some *Gamma*- and *Betaproteobacteria*, while NirK Clade II comprised of *Firmicutes*, *Actinobacteria*, *Euryarchaea*, *Chloroflexi*, *Bacteroidetes*, *Nitrospirae*, *Gemmatimonadetes*, *Planctomyces*, *Verrucomicrobia* and *Proteobacteria*. Additional information is added to the cladogram in the following order: taxonomic position, signal peptide prediction, observed amino acid motif around the essential T2Cu His and Asp amino acids (previously undescribed His motifs are in bold), the presence of DNRA (based on presence of NrfA and/or NirB), the presence of denitrification (DNF indicates presence of NirS), characteristic insertions or deletions and N- or C-terminus extensions. Specifically for *Actinobacteria*, an * was added if no corresponding transmembrane domain was present in the N-terminus extension. Full terms of abbreviations included are DNRA: dissimilatory nitrate/nitrite reduction to ammonium, DNF: denitrification, NCS: non-classical secretion, Del.: deletion, Ins.: insertion, Und.: undefined, Tat: twin-arginine signal peptide, Sec: secretory signal peptide, Lipo: lipoprotein signal. Physiologically characterized denitrifying strains are indicated by ▼: *Bacillus azotoformans* LMG 9581, *Bacillus bataviensis* LMG 21833 (Verbaendert et al., 2011), *Haloferax denitrificans* ATCC 35960 (Tomlinson et al., 1986), *Hyphomicrobium denitrificans* ATCC 51888 (Martineau et al., 2015), *Shewanella loihica* PV-4 (Yoon et al., 2013), *Shewanella denitrificans* OS217 (Brettar et al., 2002), *Rhodobacter sphaeroides* ATCC 17025 (Bergaust et al., 2014), *Alcaligenes faecalis* S-6 (Kakutani et al., 1981).

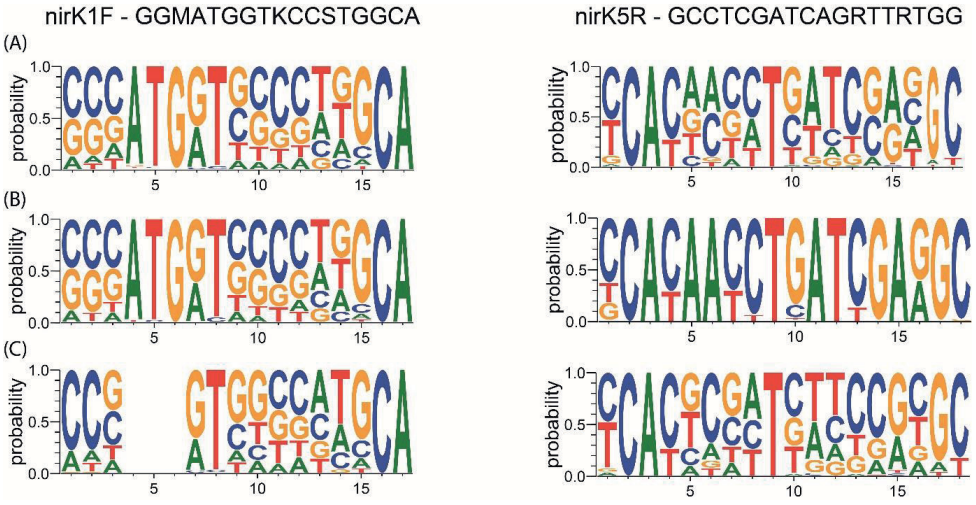

Figure S 2 Sequence logo diagrams (5'-3') depicting the degree of *nirK* sequence variability of nirK1F-nirK5R primer binding regions (Braker et al., 1998) in (a) Clade I+II *nirK*, (b) Clade I *nirK*, and (c) Clade II *nirK*. The primer sequence is given at the top and copper binding His included in the reverse primers is indicated in red. An indel in the forward primer binding region of Clade II is present.

Table S 1 Overview of sequences used for Nap, Nar, NirS, Nor, Nos and NirV database building in CLC genomics Wb 7.5 to screen NirK genomes for the presence of these nitrogen cycling genes. Unique identifiers were included for each sequence and refer to the accession number of the protein database (pdb), UniProtKB/SwissProt or Genbank. NirB sequences were automatically obtained from the RAST server, and are therefore not included in this table. Multiple copies of certain genes of *Bacillus azotoformans* and *Bacillus bataviensis* were included.

Gene	Overview sequences included		
	Species	Strain number	Unique identifier
NapA	*Escherichia coli*	K-12	CQR81706.1
	Cupriavidus necator	ATCC 17699	WP_012624252.1
	Rhodobacter sphaeroides	ATCC 17023	1OGY_A
	Desulfovibrio desulfuricans	ATCC 27774	3ML1_A
	Bacillus azotoformans	LMG 9581	WP_003332434.1
NarG	*Escherichia coli*	K-12	3IR7_A
	*Bacillus azotoformans*NarG1	LMG 9581	EKN67265.1
	*Bacillus azotoformans*NarG2	LMG 9581	EKN66862.1
	Bacillus Bataviensis	LMG 21833	EKN65800.1
NirS	*Paracoccus pantotrophus*	LMD 92.63	WP_024843009.1
	Pseudomonas aeruginosa	PAO1	WP_003111532.1
	Paracoccus denitrificans	PD1222	WP_011748767.1
	Pseudomonas stutzeri	ATCC 14405	P24040.1
	Ca. Methylomirabilis oxyfera		CBE69462.1
NrfA	*Dusulfovibrio vulgaris*	DP4	2J7A_A
	Desulfovibrio desulfuricans	ATCC 27774	1OAH_A
	Bacillus selenitireducens	MLS10	WP_013172241.1
	Bacillus azotoformans	LMG 9581	EKN68642.1
	Bacillus batavensis	LMG 21833	EKN65993.1
Nor	*Anaerophaga* sp. qNor	HS1	WP_010526654.1
	Flavobacterium johnsoniae qNor	UW101	WP_012024479.1
	Neisseria gonorrhoea qNor		WP_003689179.1
	Bacillus azotoformans qNor1	LMG 9581	EKN71191.1
	Bacillus azotoformans qNor2	LMG 9581	EKN67270.1
	Staphyllococcus aureus qNor	P72360	WP_011748763.1
	Pseudomonas aeruginosa cNor	PAO1	WP_003113237.1
	Geobacillus stearothermophilus qNor		3AYF_A
	Bacillus sp. qNor1	1NLA3E	WP_015593973.1
	Bacillus sp. qNor2	1NLA3E	WP_015594703.1
	Bacillus bataviensis qNor1	LMG 21833	EKN66169.1
	Bacillus bataviensis qNor2	LMG 21833	AJLS01000055.1
NosZ	*Paracoccus denitrificans*	DSM 413	1FWX_A
	Geobacillus thermodenitrificans	NG80-2	WP_011887499.1
	Pseudomona stutzeri	ATCC 14405	WP_003279971.1
	Achromobacter cycloclastes		2IWF_A
	Desulfotomaculum ruminis	DSM 2154	WP_013841561.1
	Desulfitobacterium hafniense	T51	WP_011458974.1
	Bacillus azotoformans NosZ1	LMG 9581	EKN71177.1
	Bacillus azotoformans NosZ2	LMG 9581	EKN68157.1
	Bacillus azotoformans NosZ3	LMG 9581	EKN63216.1
NirV	*Rhodobacter sphaeroides*	ATCC 17025	ABP70489.1
	Rhodobacter sphaeroides	KD131	WP_041669266.1
	Rhodobacter sp.	AKP1	EKX56952.1
	Maritimibacter alkaliphilus	HTCC2654	WP_008332234.1

Table S 2 *Bacteroidetes* specific *nirK* primers designed in this study and their degree of degeneracy.

Gene	Primer [a]	Sequence (5'-3')	Degeneracy
nirK	Bact4F	CCWGTDSSHWTGCAYAT	288
	Bact4Fa	GCKCCWGTRSSHWTGC	192
	Bact4R	GAYCAYKCYATYTTYMGDGC	384
	Bact3R	CAYKCYATYTTYMGDGC	192
	Bact1R	CNTTYAAYAARGGDGC	96

[a] New *Bacteroidetes* primers designed using the 20 *Bacteroidetes nirK* gene sequences included in this study. *In silico* assessment of the six primer sets (forward primer Bact4Fa or Bact4F with reverse primer Bact4R, Bact3R or Bact1R) on our dataset indicated that the use of Bact4Fa compared to Bact4F as forward primers covered more *Bacteroidetes nirK* sequences. The level of matches with non-*Bacteroidetes nirK* sequences was very low (1.5-3.8%) for all newly designed primer sets.

Table S 3 Comparison of qualitative performance of traditional and novel *nirK* primers on *Bacteroidetes* and non-*Bacteroidetes* strains.

		PCR product [a]							
		F1aCu-R3Cu [b]	nirK1F-nirK5R [c]	Bact4Fa-Bact4R 55°C	Bact4Fa-Bact3R 60°C	Bact4Fa-Bact1R 58°C	Bact4F-Bact4R 49°C	Bact4F-Bact3R 53°C	Bact4F-Bact1R 49°C
Species	Strain	(472)	(514)	(469)	(469)	(484)	(466)	(466)	(481)
Paracoccus denitrificans [d]	LMG 4049T	-	-	-	-	-	-	-	-
Achromobacter denitrificans [e]	LMG 1231T	+	+	n/d	n/d	n/d	n/d	n/d	n/d
Aequorivita sublithincola	DSM 14238	-	-	-	-	+	-	-	-
Belliella baltica	DSM 15883	-	-	+	+	+	+	+	+
Capnocytophaga sputigena	ATCC 33612	-	-	+	+	-	+	+	+
Capnocytophaga gingivalis	ATCC 33624	-	-	-	+	+	+	+	+
Capnocytophaga granulosa	ATCC 51502	-	-	-	-	-	-	-	-
Chryseobacterium gleum	ATCC 35910	-	-	-	+	+	-	-	+
Marivirga tractuosa	DSM 4126	-	-	+	+	+	+	-	+
Muricauda ruestringensis	DSM 13258	-	-	+	+	+	+	-	-
Environmental samples [f]		+	n/d	-	-	-	-	-	-

[a] +, PCR product of expected size, -, no PCR amplification, n/d, not determined. Expected size of PCR product is shown in parentheses. Total genomic DNA from a subset of eight strains was extracted according to the guanidium-thiocyanate-EDTA-sarkosyl method (Pitcher et al. 1989) and amplification was performed in 25 µl reactions containing 2.5 µl PCR buffer (10x), 2.5 µl dNTPs (2 mM), 2 µl primer (10 µM), 0.5µl Taq polymerase (1u/µl) and 25 ng DNA extract. Temperature-time profiles were as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycli of 95 °C for 30 sec, variable T_a depending on primer set for 1 min and 72 °C for 1 min. Final extension was performed at 72 °C for 10 min.

[b] *nirK* primer pairs previously described by Hallin and Lindgren (1999)

[c] *nirK* primer pairs previously described by Braker et al. (1998)

[d] negative control, organisms known to contain *nirS*

[e] positive control used in *nirK* gene amplification with traditional primers

[f] Environmental samples included are soil, estuarine sediments and activated sludge. Identical temperature-time profiles and PCR mixes were used, with exception of the addition of BSA (1.26 µl of 2 mM concentration).

Reflection & discussion

Motivated by the well-known underestimation of environmental NirK-type denitrifiers (Penton et al., 2013), NirK sequence divergence was examined in the light of existing structural classes and phylogenetic clades using a taxonomically diverse NirK dataset. In addition, the potential to partition nitrate between denitrification and DNRA was explored and the limited performance of current *nirK* primers was evaluated. Copper-dependent nitrite reductase, NirK, encoded by the *nirK* gene catalyses the key step of the denitrification pathway, *i.e.* the reduction of fixed nitrite to the gas nitric oxide. Structurally different NirK classes (Ellis et al., 2007) have been observed and previous phylogenetic analysis revealed NirK division into two distinct clades (Jones et al., 2008). Physiological or ecological differences between these different clades are currently unknown due to primer limitations and ambiguous NirK classification. We confirmed the existence of two distinct clades of NirK sequences and propose a systematic usage of these clades in future environmental surveys to evaluate potential ecophysiological differences. The occurrence of both denitrification and DNRA within a single organism was shown to be more widespread than previously anticipated.

A shortcoming of our study is the non-exhaustive search performed to gather NirK sequences. The main reason for this is the manual compilation and subsequent analysis of the NirK database used in this study. In an attempt to automate the extraction of publically available (full length) NirK sequences from NCBI, a set of diverse NirK annotation names (i.e. *nirK*, AniA, CuNir, Cu-type dissimilatory nitrite reductase, etc.) was used, however this resulted in only a limited number of NirK sequences. As a consequence, NirK sequences were retrieved manually from NCBI through (i) a pBLAST using a diverse set of well-characterized NirK query sequences and (ii) a gene term search in the Integrated Microbial Genome (IMG) database using diverse NirK annotation terms. Similarly, screening of NirK containing genomes for other dissimilatory nitrogen reduction pathways was based on reference query databases in CLC genomics Wb 7.5. The accuracy of these gene searches will be defined by the initially included query sequences. Hidden Markov modelling of dissimilatory nitrogen reductase genes and a subsequent PSI-BLAST search of genome databases would have been a valid alternative and more sensitive approach for both types of screening (Sanford et al., 2012;Graf et al., 2014). In my opinion, such an approach in future functional gene studies will, however, only be feasible when multidisciplinary collaborations are established.

The utopian aim of designing a universal primer set capable of targeting full *nirK* diversity is outdated, but this is certainly not unique to *nirK* genes (Sanford et al., 2012). Even designing primers that target complete taxonomic groups or a specific *nirK* clade are not that straightforward. *In silico* analysis of the *Bacteroidetes* primers showed that not all *Bacteroidetes nirK* sequences included were targeted. Hitherto, no information is available on the relative importance of Clade I versus Clade II NirK in

diverse environments, although some first insights thereon might be gained from analyzing currently available whole-genome shotgun metagenomes. This was done previously for NosZ (Orellana et al., 2014), where the new NosZ Clade II (Sanford et al., 2012) outnumbered Clade I in diverse environmental samples. A similar conclusion might well be reached for NirK Clades I and II as the latter comprises much more diverse NirK sequences from more diverse taxa. However, the method of NirK sequence retrieval from the metagenome dataset (Blast or the use of HMM and the applied parameters), the NirK fragment size and location on the gene, will greatly influence phylogenetic analysis.

Wei and colleagues (2015) recently succeeded in designing new primers covering previously missed *nirK*-type denitrifiers and showed that *nirK* genes were 2-6 times more abundant compared to values obtained with conventional primer sets and Clade II *nirK* abundances exceeded those of Clade I. Nevertheless, even these higher environmental *nirK* abundances represent an underestimation of real *nirK* gene abundances as the Wei primers failed to cover *Firmicutes* completely and *in silico* analysis indicated that not all *nirK* sequences of our dataset were targeted by these new primers. The presence of the *nirK* gene does not necessarily imply gene expression or process activity, however it might nevertheless provide information on the distribution and diversity of different environmental NirK types. Despite many pitfalls related to primer specificity, new primer design efforts still remain valuable to quantitatively re-asses NirK-type denitrifiers. Such studies will undoubtedly disprove the previous assumption of NirK-type denitrifiers being less abundant in the environment compared to NirS-type denitrifiers. Specific research questions concerning denitrifiers in diverse environments (*e.g.* soil, marine sediment, …) still mainly depend on PCR-based surveys as shotgun approaches, while resulting in enormous amounts of data, may still potentially provide too little information on the process of interest. In my opinion, the most promising approach for future primer design is to design primers targeting specific subclades within Clade I and II using algorithms like CODEHOP (Rose et al., 1998) based on diverse *nirK* datasets, despite the fact that algorithm-based primer design will impose compromises in primer coverage.

To assess the occurrence of (in)complete denitrification pathways, presence of Nar, Nap, Nor and Nos was evaluated. The tricky thing about defining a bacterium as true denitrifier based on its genomic content is the occurrence of highly truncated versions of this pathway in numerous organisms. Variable definitions of denitrification *sensu stricto* have been proposed, with a bacterium being a denitrifier when its genome contains (i) nitrite and nitric oxide reductases (Zumft, 1997), (ii) nitric oxide reductase combined with either nitrite or nitrous oxide reductase (Graf et al., 2014) or (iii) nitrite, nitric oxide or nitrous oxide reductase (Shapleigh, 2013). We propose that a bacterium is a denitrifier if its genome contains a nitrite reductase as this enzyme is involved in the key step of denitrification resulting in the conversion of a fixed, nongaseous, and biologically preferred form of nitrogen, *i.e.* nitrite, to a gaseous form, *i.e.* nitric oxide. This diversity of definitions indicates the need for a uniform

and consistently applied definition of denitrification in genomic surveys as is available for physiological studies of denitrifiers (Mahne and Tiedje, 1995). The main reason therefore can be compared to the benefit of taxonomy, *i.e.* a clear definition allows researchers to communicate unambiguously.

The frequent occurrence of combined denitrification and DNRA pathways within a single organism indicates that it is more widespread compared to the limited number of strains currently used in ecophysiological research (Mania et al., 2014;Yoon et al., 2015b). Although our *nirK* search was incomplete, the high incidence of these combined pathways in *Actinobacteria*, *Proteobacteria* (*Alpha* and *Beta*), *Bacteroidetes* and *Firmicutes* probably results from a phylum specific bias in sequencing efforts (Land et al., 2015). Including more diverse organisms that simultaneously harbour pathways in ecophysiological studies would certainly benefit the unraveling of fine-scale regulation of these processes by environmental cues.

Multiple *nir* copies, either more than one *nirK* or the combination of *nirK* and *nirS*, were observed in phylogenetically diverse NirK-type denitrifiers. Speculation on potential ecological advantages of these organisms in diverse environmental conditions is currently based on the sole report on the presence of two *nirS* gene copies within *Thauera* sp. with one being expressed constitutively, while the other responded to environmental nitrate conditions (Etchebehere and Tiedje, 2005). In contrast, based on its location in the genome, the second *nirK* copy of *Ochrobactrum anthropi* ATCC 49188 was suggested not to be involved in denitrification (Shapleigh, 2013), which is plausible due to *nirK* gene involvement in other processes. Extensive *nirK* sequence divergence greatly affects diversity at the functional level, and therefore it can be expected that it also exerts an effect at the level of gene expression resulting in distinct catalytic performances (different optimal pH, temperature or substrate affinity). Experimental investigation of differential expression in correspondence to variable conditions (differences in pH, substrate affinity or temperature) will be useful to further shed light on the potential advantages of multiple *nir* copies for such organisms.

Chapter 4

Dissimilatory nitrate reduction in intertidal sediments of a temperate estuary: small scale heterogeneity and novel nitrate-to-ammonium reducers

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Author's contributions:

HD, KH and AW designed the experiments. HD performed the experiments. HD, KH and CVC analyzed the data. CVC contributed analysis tools. HD, KH and AW wrote the paper.

Summary

The estuarine nitrogen cycle can be substantially altered due to anthropogenic activities resulting in increased amounts of inorganic nitrogen (mainly nitrate). In the past, denitrification was considered to be the main ecosystem process removing nitrate from the estuarine ecosystem. However, recent reports on the contribution of dissimilatory nitrate reduction to ammonium (DNRA) to nitrate reduction in these systems indicated a similar or higher importance, although the ratio between both processes remains ambiguous. Compared to denitrification, DNRA has been underexplored for the last decades and the key organisms carrying out the process in marine environments are largely unknown. Hence, as a first step to better understand the interplay between denitrification, DNRA and reduction of nitrate to nitrite in estuarine sediments, nitrogen reduction potentials were determined in sediments of the Paulina polder mudflat (Westerschelde estuary). We observed high variability in dominant nitrate reducing processes over a short distance (1.6 m), with nitrous oxide, ammonium and nitrite production rates differing significantly between all sampling sites. Denitrification occurred at all sites, DNRA was either the dominant process (two out of five sites) or absent, while nitrate reduction to nitrite was observed in most sites but never dominant. In addition, novel nitrate-to-ammonium reducers assigned to *Thalassospira*, *Celeribacter* and *Halomonas*, for which DNRA was thus far unreported, were isolated, with DNRA phenotype reconfirmed through *nrfA* gene amplification. This study demonstrates high small scale heterogeneity among dissimilatory nitrate reduction processes in estuarine sediments and provides novel marine DNRA organisms that represent valuable alternatives to the current model organisms.

4.1 Introduction

The rate of terrestrial nitrogen input has more than doubled over the past century, mostly through fossil fuel combustion and increased use of agricultural fertilizers. When it is not biologically removed from streams and rivers, excess, anthropogenically-derived nitrogen ends up in estuaries and coastal areas, where it is implicated in eutrophication, alteration of food webs and hypoxia (Martinetto et al., 2006;Paerl et al., 2006;Diaz and Rosenberg, 2008). Nitrate can be lost from these systems via anaerobic ammonium oxidation (anammox) to dinitrogen gas or denitrification, *i.e*. the respiratory reduction of nitrate to either the potent greenhouse gas nitrous oxide or dinitrogen gas. Alternatively, it can be retained in the system as biologically available ammonium via dissimilatory nitrate reduction to ammonium (DNRA), with possible nitrogen losses via trace amounts of nitrous oxide (Smith, 1982;Cruz-Garcia et al., 2007;Giblin et al., 2013). For a long time, it was thought that denitrification was the main nitrate/nitrite removing process in coastal ecosystems (Burgin and Hamilton, 2007), outcompeting anammox and DNRA in dynamic, eutrophic estuaries (Trimmer et al., 2003;Rich et al., 2008;Dale et al., 2009;Giblin et al., 2013). Since the first reports on DNRA in estuarine environments approximately 40 years ago (Buresh and Patrick, 1978), an increasing number of studies indicated that DNRA is more relevant in nitrate/nitrite turnover in these systems than previously assumed (Gardner et al., 2006;Koop-Jakobsen and Giblin, 2010;Dong et al., 2011;Giblin et al., 2013). However in contrast to denitrification, DNRA has been underexplored for the last decades and, despite some attempts (Bonin, 1996;Yoon et al., 2015b), the key organisms carrying out this process in marine and estuarine environments, their response to varying environmental conditions and how the DNRA process itself relates to other nitrate reducing processes remain largely unknown.

DNRA is a facultative, two-step anaerobic process involving nitrate reduction to nitrite followed by the 6-electron reduction of nitrite to ammonium (Einsle et al., 1999), of which two different modes of energy conservation have been described. The respiratory mode generates a proton motive force by electron transport from non-fermentable organic substrates to nitrite resulting in ATP production (Simon, 2002), while in the fermentative mode, nitrite is an electron-sink allowing re-oxidation of NADH with the generation of one extra ATP by substrate level phosphorylation for each acetate produced (Cole and Brown, 1980;Polcyn and Podeszwa, 2009). Respiratory DNRA can also contribute to chemolithoautotrophic growth when coupled to the oxidation of reduced inorganic sulfur forms (hydrogen sulphide, sulphide or elemental sulfur) (Dalsgaard and Bak, 1994;Brunet and GarciaGil, 1996). Nitrite reduction to ammonium can be catalyzed by the cytoplasmic NADH-dependent nitrite reductase NirB or its to two-subunit variant NirBD (Harborne et al., 1992) and/or the periplasmic pentaheme cytochrome *c* nitrite reductase NrfA (Einsle et al., 1999), depending on the organism and growth conditions. *Escherichia coli* and *Bacillus vireti* were shown to harbor and express genes for both enzymes (Cole, 1996;Mania et al., 2014), while other DNRA organisms such as *Wollinella succinogenes* (Simon, 2002), *Bacillus subtilis* (Nakano and Zuber, 1998) and *Archaea* (Rusch, 2013) contain either *nrfA* or *nirB*. Furthermore, in *E. coli,* differential expression of *nrfA* and

nirB under low and high nitrate concentrations respectively was observed (Wang and Gunsalus, 2000). DNRA-related ecophysiology, enzymology, gene expression and regulation have been extensively studied in model organisms like *E. coli, W. succinogenes* and *B. subtilis* (Cole, 1996;Nakano and Zuber, 1998;Simon, 2002), and more recently also in *B. vireti* (Mania et al., 2014) and *Shewanella loihica* (Yoon et al., 2015a;Yoon et al., 2015b). Whole genome sequence analyses, however, demonstrated that DNRA, similar to denitrification, is phylogenetically very widespread, and can be found in members of *Bacteroidetes* (Mohan et al., 2004), *Proteobacteria* (*Gamma*-, *Delta*- and *Epsilon*) (Smith et al., 2007), *Actinobacteria*, *Firmicutes*, *Acidobacteria*, *Chloroflexi* and *Planctomycetes* (Welsh et al., 2014). Some DNRA bacteria have furthermore been shown to contain partial or complete suites of genes for both DNRA and denitrification in their genome (Heylen and Keltjens, 2012;Sanford et al., 2012;Yoon et al., 2013;Mania et al., 2014). Functional capacity to carry out both nitrate reducing processes and the environmental drivers of nitrate partitioning to either process, like carbon-to-nitrogen ratios and nitrite concentrations, have thus far only been demonstrated for the marine strain *Shewanella loihica* PV-4 (Yoon et al., 2015a;Yoon et al., 2015b). In addition to pure culture experiments, natural prokaryotic communities have also been used for examining these environmental drivers (Kraft et al., 2014;van den Berg et al., 2015). To date, only limited cultured DNRA bacteria, including only few from marine environments, are available (Cole et al., 1974;Bonin, 1996;Hoffmann et al., 1998;Mania et al., 2014;Yoon et al., 2015b), and no recent attempts have been made to isolate new (marine) members.

The Westerschelde estuary is an eutrophied system characterized by a nitrogen load of 5×10^9 mol N yr^{-1} (Soetaert and Herman, 1995) with nitrate being the predominant form of reactive nitrogen (Soetaert et al., 2006). Furthermore, denitrification and not DNRA was previously reported to be the predominant nitrate removing process (Dahnke et al., 2012;Van Colen et al., 2012). To better comprehend the relative importance of denitrification and DNRA in these estuarine sediments, nitrate reduction potentials were determined of sediments obtained from the Paulina polder mudflat (Westerschelde estuary, SW Netherlands). In addition, to increase the knowledge on the organisms involved, nitrate reducing bacteria were isolated from enriched sediment cultures.

4.2 Material and Methods

Sampling

Sediment samples were collected at the Paulina polder mudflat (51° 21' 24" N, 3° 42' 51" E) in collaboration with NIOZ, which provided the necessary permit for field sampling, issued by the 'Provincie Zeeland, The Netherlands; Directie Ruimte, Milieu en Water'. They were taken using a plexiglas corer (Ø 6.2 cm). Samples for isolation of nitrate reducing bacteria were collected in October 2011 and samples for determination of the nitrate reduction potential in October 2014. The latter samples were collected in triplicate at 5 different sampling sites over a distance of 1.6 m and stored at 4°C until further processing. Back in the lab, the upper cm of the sediment cores, containing the oxic-anoxic border (Van Colen et al., 2012;Decleyre et al., 2015) and main zone for dissimilatory nitrate reduction was sampled. Triplicate samples were pooled per sampling site to include as much spatial variation as possible and subsequently stored in sterile falcon tubes at -80°C. Physico-chemical characteristics of samples collected in October 2014 were determined as described previously (Decleyre et al., 2015). Statistical differences in physico-chemical parameters between all 5 sampling sites were evaluated using one-way ANOVA and post hoc tests in SPSS 21 (IBM SPSS Statistics for Windows, Version 21.0. Released 2012. Armonk, NY: IBM Corp.).

Determination of nitrate reduction potential

Nitrate reduction potentials were measured in triplicate using the acetylene inhibition technique according to Sørensen (Sorensen, 1978). Briefly, a 15 ml serum vial was filled with 2 g (ww) sediment (thawed at 37°C for 8 min) and 2 ml sterilized natural seawater (NSW). To prevent nitrogen limitation, the NSW was supplemented with 5 mM KNO_3[5]. Preliminary experiments (data not shown) including serum vials with and without addeditional carbon source (1/10 marine broth) demonstrated that sediment/NSW contained sufficient carbon to support anaerobic respiration and/or fermentation. Synthesis of new enzymes was inhibited using 0.1 mM chloramphenicol allowing potential activity measurement of *in situ* expressed nitrogen reducing enzymes (Murray and Knowles, 1999). The vials were sealed with black butyl stoppers and aluminum crimps, and flushed five times with helium to remove oxygen. After adding 10% or 101.3 hPa acetylene, the vials were incubated in the dark at 15°C and at a constant stirring rate of 100 rpm. The nitrous oxide and carbon dioxide concentrations of all replicates were measured every hour (T_1 to T_5). Initial (n=3) and final (n=3) nitrite/ammonium concentrations were determined for each vial at each time point. Denitrification, DNRA and net nitrite production rates were calculated using linear regressions (Table S1). No corrections were done for potential (i) overestimation of DNRA rates due to ammonium release by remineralization of organic matter during denitrification, and (ii) underestimation of denitrification rates due to incomplete

[5] Final [NO_3^-] for each sampling site (including pore water concentration) : (1) 5.00355 mM; (2) 5.00335 mM; (3) 5.00049 mM; (4) 5.00072 mM and (5) 5.00077mM.

inhibition of nitrous oxide reductase by acetylene (Groffman et al., 2006). Statistical differences in production rates between all 5 sampling sites were assessed using the non-parametric Kruskal-Wallis H test in SPSS 21.

Growth media

Growth conditions used in this study were defined by a set of variable and fixed parameters (Table 1) and growth media were prepared with sterile NSW collected form the Westerschelde estuary (Paulina polder) in an attempt to mimic natural conditions. They were based on the mineral medium of Stanier (Stanier et al., 1966) with slight modifications. Hepes (10 mM) was used as buffering agent, while phosphate was limited to 300 µM KH_2PO_4 based on the Redfield ratio (Redfield, 1934), to avoid decreased culturability as a consequence of high phosphate concentration (Bartscht et al., 1999). Iron, proven to be an essential element necessary for optimal growth of marine bacteria (D'Onofrio et al., 2010), was added as Fe(III)Na EDTA in a concentration (40 µM) mimicking the *in situ* concentrations found in the Westerschelde estuary (based on Schelde Monitoring database, http://www.scheldemonitor.be). Agarose (0.8%) was used as solidifying agent to eliminate potential growth inhibiting effects of agar (Tanaka et al., 2014). Other media components varied: signaling compound cyclic adenosine monophosphate (cAMP) at 0 or 10 µM; molar C/N ratio at 5 or 25, either with KNO_3 or a combination of KNO_3/KNO_2 as nitrogen source (always with a total N concentration of 5 mM); glucose (designated as DNR2 media), a combination of sodium succinate dibasic hexahydtrate/ethanol/glycerol (DNR3 media) or sodium pyruvate/ sodium acetate anhydrous (DNR4 media) as carbon source (Table 1). In addition, ten-fold diluted marine broth (MB) (BD Difco) (DNR1 media) supplemented with 5 mM of nitrate was also included as complex medium. Incubation temperature was set at 15°C as this approximates the yearly averaged temperature in the Westerschelde estuary. A detailed overview of all 26 growth media used in this study is given in Table S2.

Table 1 Fixed and variable parameters of the growth conditions.

Fixed parameter		Variable parameters	
Incubation temperature	15°C	Medium	1/10 MB
pH	7.2		Stanier mineral medium
Buffering agent	Hepes	C-sources	Glucose
NH_4^+ background concentration	4mM		Succinate-ethanol-glycerol
Fe(III)Na EDTA	40µM		Pyruvate-acetate
Vitamin solution	1ml/L	N-sources	KNO_3
Medium	NSW		KNO_3/KNO_2
N concentration	5mM	C:N ratio (Molar C:N)	5 or 25
Atmosphere	anaerobic	Signalling factor	cAMP

Enrichment, isolation and cryopreservation of marine isolates

Enrichment cultures were set up in liquid medium under anaerobic, nitrate-reducing conditions. Sediment (1 g) was vortexed with 9 ml NSW for 15 min, and subsequently diluted ten-fold up to 10^{-10} in 120 ml serum vials for each growth medium. The vials were sealed with black butyl stoppers and aluminum crimps, and flushed five times with helium to remove oxygen (overpressure of 0.3 bar). For each dilution series, an additional vial was prepared without inoculum to check for potential nitrosation reactions in sterile medium (Mania et al., 2014). After adding 10% acetylene and 10% carbon dioxide to the headspace, the vials were incubated in the dark at 15°C and at a constant stirring rate of 100 rpm. Headspace concentrations of nitrous oxide and carbon dioxide were determined weekly.

The two highest dilutions of each growth medium producing nitrous oxide for two consecutive weeks were used for isolation, because (i) DNRA bacteria also produce nitrous oxide as a side product, (ii) denitrifying DNRA bacteria were not to be excluded and (iii) ammonium production as proxy for DNRA in enrichments is hampered by remineralization of organic matter. For each enrichment, dilutions were made in sterile NSW (10^{-1}, 10^{-2} and 10^{-3} dilution, if necessary 10^{-4} and 10^{-5}) and 100 µl of diluted culture was plated on solid media. Incubation was done at 15°C in an anaerobic gas container (BD Gaspack Container System) with an anaerobic indicator (Microbiology Anaerotest) and anaerobic BD Gaspack sachets. Colony formation was checked weekly. If no additional colonies were formed for two consecutive weeks, five isolates with different colony morphology were picked from each medium, and subsequently purified on identical solid medium. Finally, isolates were dereplicated based on their 16S rRNA gene identity and the type of medium they were isolated from, *i.e.* a representative of each group of highly related isolates was retained for further analyses.

All isolates were preserved at -80°C as described previously (Vekeman and Heylen, 2015). In short, isolates obtained from defined media were preserved in 10% DMSO prepared with NSW. For DNR1 and DNR2 type media, 1/10 MB or glucose (4.17 mM or 20.83 mM) was additionally added to the respective 10% DMSO-NSW solution as an extra cryoprotectant.

Determination of dissimilatory nitrate metabolism

To test whether the obtained isolates were strictly dependent on nitrate/nitrite as electron acceptor in the absence of oxygen or, alternatively, could use other non-defined electron acceptors present in NWS, media without added nitrogen were used. To determine the nitrate reducing metabolism of each individual isolate, standardized growth experiments were performed in duplicate for each isolate with start- and endpoint determination of concentrations of nitrite, ammonium, nitrous oxide and carbon dioxide. A 120 ml serum vial containing 19.8 ml liquid medium was inoculated with 200 µl cell suspension of OD 0.1 from each selected isolate (for slow growing isolates OD 0.05 was used). All isolates were tested in both complex (1/10 MB) and mineral media (for DNR1 isolates, DNR3 mineral

media were used) to take into account the effect on our measurements of undefined N-compounds in marine broth. Blanks for each medium type were also included to detect potential nitrosation reactions (Mania et al., 2014). Positive controls for denitrification (*Paracoccus denitrificans* LMG 4049) and DNRA (*E. coli* LMG 5584) were included for all media. Incubation was performed at 15°C under anaerobic headspace with 10% acetylene and 10% carbon dioxide. Time of endpoint sampling was determined based on visual assessment of growth. An isolate was considered a denitrifier when 80% conversion of nitrate to nitrous oxide coincided with growth (Mahne and Tiedje, 1995) and a DNRA bacterium if the sum of nitrate reduction products (nitrite and nitrous oxide) was less than 70% of the consumed nitrate (Bonin, 1996) with concomitant ammonium production.

Analytical methods

Nitrous oxide and carbon dioxide were detected and quantified using a Compact GC (Global Analyzer Solutions, Belgium) equipped with two columns (oxygen/nitrogen and carbon dioxide/nitrous oxide separation) connected to a thermal conductivity detector. The change in pressure due to nitrous oxide/carbon dioxide production was monitored with an infield 7 pressure meter (UMS, Germany). Values obtained by gas chromatography were converted to µmol gas L_{Liquid}^{-1} by compensating for change in gas pressure (measured with the Infield 7 pressure meter) and taking the solubility of the gases into account. Samples for colorimetrics (500 µl of liquid culture) were pretreated using KCl to avoid inhibition of amines (Keeney and Nelson, 1987). Nitrite was determined using the Griess reaction (Griess, 1879) and ammonium using the salicylate-nitroprussidine method (Baethgen and Alley, 1989).

16S rRNA and *nrfA* gene sequence analyses

DNA was extracted from each isolate by the guanidium-thiocyanatelectronEDTA-sarkosyl method (Pitcher et al., 1989). Amplification and sequencing of the complete 16S rRNA gene was performed as described previously (Heyrman and Swings, 2001). Sequences were assembled using the BioNumerics 7.0 software (Applied Maths). Finally, the EzTaxon server (http://www.ezbiocloud.net/eztaxon; (Kim et al., 2012)) was used to taxonomically assign each isolate to a genus. Maximum likelihood analyses of 16S rRNA genes of the isolates obtained in this study together with previously identified DNRA bacteria (phenotypically characterized or based only on the presence of the *nrfA* gene) were performed to assess the diversity of DNRA bacteria obtained. Therefore the *nrfA*_Welsh data set in the Fungene database containing *nrfA* sequences obtained from whole genomes was used to select representatives of each genus of the currently known taxonomic diversity (Fish et al., 2013). After checking the *nrfA* genes for the presence of the key KXRH or KXQH motifs and 5 heme groups - this to prevent inclusion of closely related octaheme nitrite reductase (ONR) or other multiheme cytochrome *c* proteins - the corresponding 16S rRNA gene sequence of each representative was obtained from the

NCBI database for inclusion in the comparison. A profile-based multiple sequence alignment of the obtained 16S rRNA gene sequences was subsequently achieved using the SILVA Incremental Aligner (SINA v1.2.11) (Pruesse et al., 2012). Maximum likelihood analysis was performed in RaxML 7.4.2 using a general time reversible model with gamma distributed rates (GTR+G) (Stamatakis, 2006;Ott et al., 2010).

In addition, *nrfA* gene amplification was performed on all isolates using primer sets F1-7R1 (Mohan et al., 2004), F2-7R1 (Mohan et al., 2004) and nrfAF2aw-nrfAR1 (Welsh et al., 2014). To prevent interference of non-specific amplification during sequencing, amplicons obtained with Mohan primers (Mohan et al., 2004) were extracted from an agarose gel and subsequently used for sequencing. The *nrfA* identity of obtained amplicons was verified by checking for the presence of NrfA diagnostic motifs, *i.e*. KXRH or KXQH, as all three primer sets targeted the region between the third and the fourth heme binding motif (Mohan et al., 2004;Welsh et al., 2014).

Nucleotide accession numbers

The nucleotide sequences of the 16S rRNA and *nrfA* data generated in this study have been deposited in the GenBank database under accession numbers KT185111-KT185193 and KT159169-KT159180 respectively.

4.3 Results and discussion

Nitrate reduction potential of Westerschelde sediment at meter scale

Despite their ecological importance, knowledge on DNRA processes in marine environments remains scarce. In the past, denitrification was considered the dominant marine nitrate reduction pathway, while DNRA contributions were minimalized or even ignored (Burgin and Hamilton, 2007). Recent studies in marine and estuarine environments, however, have demonstrated that DNRA can also be the predominant nitrate reduction pathway. Giblin *et al*. (2013) showed DNRA dominated total nitrate reduction in approximately one-third of 55 coastal sediment sites examined. Similarly, Song *et al*. (2014) found benthic DNRA to be responsible for almost half of the nitrate reduction across the New River estuary, with DNRA rates exceeding those of denitrification (Lisa et al., 2014). Spatial variation in nitrate reduction rates is often assessed on a regional or local scale (Song et al., 2014;Smith et al., 2015), but not on meter or even smaller scale. Here, potential nitrate reduction rates, *i.e*. nitrate reduction to nitrite, denitrification (nitrous oxide measured with the acetylene method as a proxy) and DNRA, were measured across a 1.6 m scale, with five sampling sites approximately 9 cm from each other (Figure 1). Strikingly, we found significant differences in rate and dominance of the three processes at this small scale. Denitrification was observed at all sites, with significantly different rates ($p < 0.05$). In contrast, DNRA was limited to sites 1 and 5, located at a distance of 1.6 m, and appeared to be the dominant nitrate reducing process, with higher rates than denitrification (sites 1 and 5) and nitrate reduction to nitrite (site 1). Nitrite production was observed at all sites except site 5, with rates differing significantly between the five sampling sites ($p < 0.05$) (Figure 1). The averaged nitrite, ammonium and nitrous oxide production rates were 0.0047 ± 0.0013 µmol $N\text{-}NO_2^-$/g.h, 0.01 ± 0.002 µmol $N\text{-}NH_4^+$/g.h and 0.0058 ± 0.0003 µmol $N\text{-}N_2O$ /g.h respectively (individual rates in Table S4). Production rates of $N\text{-}N_2O$ observed in this study were not consistent with previous reports in marine sediments, they were either approximately one order of magnitude higher (Dul'tseva et al., 2000;Magalhaes et al., 2011) or nearly three orders of magnitude lower (Stock et al., 2014). Differences in the experimental set-up in these studies compared to ours, such as the non-inhibition of enzyme synthesis (Murray and Knowles, 1999) or the addition of extra carbon source, both leading to overestimation of denitrification rates (Bernot et al., 2003), are plausible explanations for the lower potential rates observed here. Furthermore, seasonal variability in time of sampling might also contribute to these observed differences. Nevertheless, potential rates of DNRA observed in sampling site 5 agreed with previous observations based on isotopic labelling experiments in estuarine sediments, while those of sampling site 1 were approximately two-fold higher (Kelly-Gerreyn et al., 2001;An and Gardner, 2002;Song et al., 2014).

In addition to significantly different rates between the five sites for all three nitrate reducing processes, their occurrence was also site-dependent. Carbon to nitrogen ratio has long been considered the determining factor for nitrate partitioning to either DNRA or denitrification (Tiedje, 1988), which

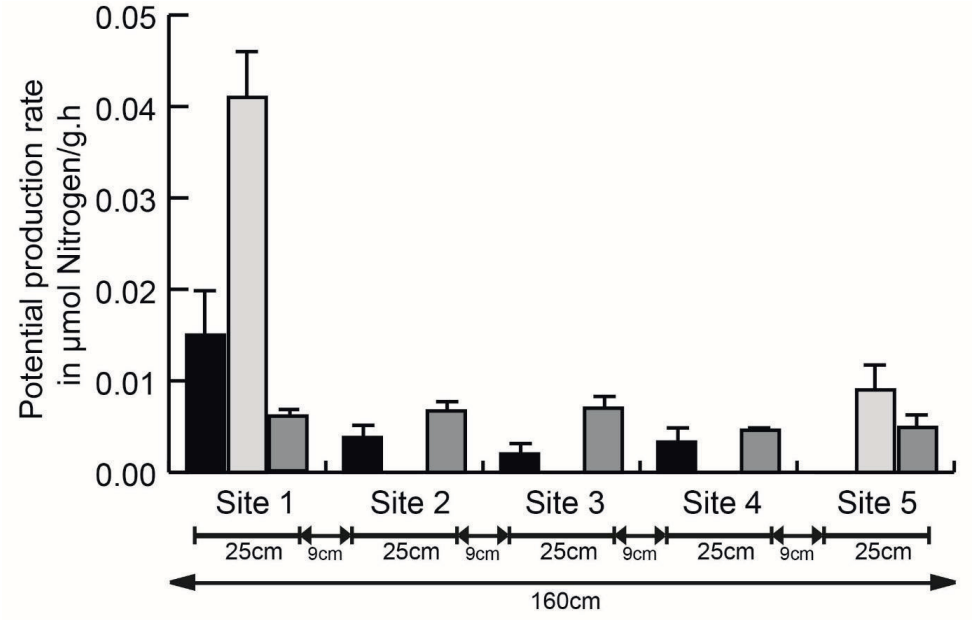

Figure 1 Averaged nitrite, ammonium and nitrous oxide production rates (± SD) per sampling site (n=3) over a period of 5 hours (=T_1-T_5). For clarity, one sided error bars are shown. Black bars: net nitrite production, light grey bars: dissimilatory nitrate reduction to ammonium (DNRA), dark grey bars: denitrification. Total distance between all 5 sampling sites (1.60 m) and individual distance between all sampling sites is represented. Detailed information on the physico-chemical composition of these five sites is found in Table S3.

was recently confirmed in long-term incubations of marine sediments (Kraft et al., 2014) as well as chemostat experiment with *S. loihica*, a gammaproteobacterium containing the gene inventory for both DNRA and denitrification (Yoon et al., 2015b). Based on the significant differences in nitrate concentration in pore water and similar total organic carbon content (Table S3), denitrification is expected to be favored at sites 1 and 2 (low C:N ratio) while DNRA would dominate the three other sites. In contrast, DNRA was limited to sites 1 and 5, with low and high C:N ratio respectively and denitrification contributed to nitrate reduction at all sites. While nitrate sufficiency (bulk addition of 5 mM at the start of the experiment) may explain the overall occurrence of denitrification, C:N ratio was clearly not the main driver differentiating between both processes. Previous reports on the effect of pH on DNRA and denitrification were inconclusive (Stevens et al., 1998;Rutting et al., 2011), although DNRA was favored over denitrification at elevated pH in *S. loihica* (Yoon et al., 2015b). We did not monitor the pH on-site, but know from previous work that pH can be variable at the meter scale in the Paulina tidal flat (Decleyre et al., 2015). Additional key environmental controls that could explain the observed small scale variation were either not relevant, such as microbial generation time (Kraft et al.,

2014) and supply of nitrite relative to nitrate (Kraft et al., 2014;Yoon et al., 2015a), or undetermined, such as the presence of free sulfides (hydrogen sulfide, sulfide) or elemental sulfur (Burgin and Hamilton, 2007). Site 1 with the highest DNRA rate did, however, contain the highest chl *a* concentration (Table S3), *i.e.* proxy for diatom biomass, although it was not significantly different from the four other sites. Nevertheless, it is plausible that diatoms, which are known to store nitrate intracellularly and us it as a dark survival strategy (Kamp et al., 2011), contribute to the high DNRA rate at that site. In addition, higher trophic levels like meiofauna can influence reduction processes and rates but are rarely considered (Stock et al., 2014) (here for example because of limited sample size). Nevertheless, the presence of meiofauna can directly (Frangoulis et al., 2005) or indirectly (Nascimento et al., 2012) increase organic matter, with subsequent stimulation of sulfate reduction (Berner and Westrich, 1985) resulting in hydrogen sulfide production, known to favor DNRA and autotrophic denitrification (Burgin and Hamilton, 2007;Moraes et al., 2012). Taken together, we expect that the combination of complex interactions between different trophic levels, the resulting microscale physico-chemistry and the highly dynamic nature of intertidal sediments might contribute to the observed significant variation in nitrate reducing processes at the meter scale.

Marine dissimilatory reducers of nitrate to ammonium

With the advance of whole genome sequencing, the ability to carry out DNRA was shown to be phylogenetically more widespread than originally thought. Still, key organisms involved in DNRA in marine ecosystems and their ecophysiology remain largely unknown. Macfarlane and colleagues were the first to report DNRA capabilities of a *Vibrio* sp. and a *Clostridium butyricum* strain obtained from estuarine sediments (Keith et al., 1982;Macfarlane and Herbert, 1982). Years later, Bonin (1996) confirmed the DNRA capability of two other Gammaproteobacterial strains isolated from estuarine sediments and reported that nitrate limitation (1 mM) resulted in ammonium production while high nitrate levels (10 mM) caused nitrite accumulation and less efficient ammonium production. Recent studies on the marine strain *S. loihica* PV-4 that is able to perform both denitrification and DNRA, indicated that nitrate limitation (high C:N), high nitrite-to-nitrate ratio, alkaline pH and high temperatures favor DNRA over denitrification activity (Yoon et al., 2015a;Yoon et al., 2015b). These Gammaproteobacterial strains form the basis for our current knowledge of DNRA in marine ecosystems, yet their limited number underline the urgent need for new cultured marine representatives to further explore the ecophysiology of phylogenetically distinct DNRA organisms. Therefore, we enriched estuarine sediments under anaerobic, nitrate reducing conditions using 26 different growth media (under 10% acetylene), mimicking *in situ* physico-chemical conditions, and subsequently performed isolations. In total, 83 isolates, belonging to 27 genera of *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Alpha-* and *Gammaproteobacteria* (Table S5) were obtained. This partially agreed with available 16S rRNA pyrosequencing data from the same site (Decleyre et al., 2015), in which *Gammaproteobacteria* and *Bacteroidetes* were found to be most dominant. Although we did not

apply an exhaustive isolation approach and only picked up 5 isolates per medium after elective enrichment, it is striking that only four of the 27 genera (*Martellela*, *Pseudoruegeria*, *Roseovarius* and *Vibrio*) were found via both isolation and pyrosequencing. As for denitrifiers (Heylen et al., 2006b), elaborate medium optimization is necessary to increase cultivated representatives for DNRA bacteria. Nevertheless, our study clearly showed that either diluted complex medium (representatives of 8 genera) or combined non-fermentable carbon sources (9 genera) are preferred over glucose (2 genera) (Table S5). Furthermore, addition of KNO_3 as electron acceptor yielded twice as much diverse isolates compared to media supplemented with KNO_3/KNO_2 as nitrogen source (18 vs. 9 genera), the latter probably caused by organism-dependent nitrite intolerance (Table S5) (Tiedje, 1988). Nevertheless, inclusion of nitrite (at low concentrations, *i.e.* 2 mM in this study) as electron acceptor is necessary to target bacteria lacking the genes for nitrate reductase but capable of nitrite reduction to ammonium or denitrification. *Paraoerskovia, Citrobacter*, *Shigella* and *Halomonas* were only isolated from media containing both KNO_3 and KNO_2 (Table S5). None of the isolates appeared solely dependent on nitrate or nitrite as electron acceptor in the absence of oxygen. Growth was still observed without added electron acceptors suggesting that natural seawater, used to prepare the growth media to mimic *in situ* physico-chemical conditions, provided all isolates with alternative electron acceptors (*e.g.* manganese, iron, sulfate) to support growth. This made it impossible for us to recognize dissimilatory nitrate reducers, *i.e.* isolates that are capable of nitrate or nitrite reduction to ammonium or dinitrogen, based solely on growth in nitrogen oxide amended media.

Therefore, unique representatives of each closely related group of isolates were selected based on their 16S rRNA gene identity and isolation medium, yielding 35 isolates for detailed determination of their dissimilatory nitrogen metabolism. In batch experiments, isolates were grown in their isolation medium and ten-fold diluted marine broth supplemented with 5 mM KNO_3 or KNO_3/KNO_2 (depending on original isolation conditions). Concentrations of potential end-products nitrite, ammonium and nitrous oxide were determined at end-point. Fifteen out of the 35 isolates were shown to actually reduce nitrate as electron acceptor. No denitrifying bacteria were isolated (confirmed by negative results of *nirK* and *nirS* PCR (data not shown)), but rather all isolates had a DNRA phenotype, capable of producing ammonium from nitrate. For twelve out of fifteen nitrate reducers, the DNRA phenotype was re-confirmed with the detection and sequencing of the *nrfA* gene (Table 2 and Figure S1). The three remaining isolates either contained divergent *nrfA* genes not targeted by the primers used or harbored *nirB*. The *nirB* gene is unfortunately a poor marker gene because of its role in both assimilatory and dissimilatory nitrate reduction to ammonium and general primers are currently lacking. The lack of denitrifiers among the isolates was initially surprising, as growth media were nitrate sufficient (> 1 mM) and nitrous oxide producing dilutions were selected for isolation (note that nitrous oxide production was used as a selection criterion because DNRA bacteria also produce nitrous oxide as side product, denitrifying DNRA bacteria were not to be excluded and ammonium production

as proxy for DNRA in enrichments is hampered by remineralization of organic matter). When looking at the data in more detail however, this makes sense. The amount of nitrous oxide produced by the enrichment cultures ranged from 0.09 to 0.6 mM, *i.e*. between 3.6 and 24% of all nitrate was converted to nitrous oxide. This is higher than one would expect from a pure culture DNRA bacterium (from 0.1 to 5%, depending on the organism) (Streminska et al., 2012), but much lower than expected for a denitrifier (80-100%) (Mahne and Tiedje, 1995). So, this range of nitrous oxide production from the enrichment cultures suggested a mix of denitrifiers and DNRA bacteria. The exclusive isolation of DNRA bacteria might point towards numerical dominance of DNRA bacteria in the enrichments, but this was not verified in additional tests.

Our data reconfirms the ability of members of *Vibrio* (Liu et al., 1988), *Shewanella* (Yoon et al., 2015b) and *Citrobacter* (Smith, 1982) to perform DNRA, while demonstrating for the first time this capability for members of *Halomonas*, *Thalassospira* and *Celeribacter*, previously only reported to perform nitrate reduction and/or (partial) denitrification (Peyton et al., 2001;Liu et al., 2007;Gonzalez-Domenech et al., 2010). Llamas and colleagues suspected DNRA in *Halomonas maura* (Llamas et al., 2006), but did not test it physiologically. *NrfA* amplicons were obtained from *Halomonas* sp. R-52914 and in *Celeribacter* sp. R-52651, while this was not the case for *Thalassospira* sp. R-52913 and R-52699. *In silico* analysis of all six publically available genome sequences of *Thalassospira* strains revealed *nirB* genes instead of *nrfA*, which might explain why all three *nrfA* primer sets failed to render an amplicon. This might also be the case for *Vibrio* sp. R-52669, although the closely related R-52688 did render a *nrfA* amplicon. Still, strain-dependent differences in dissimilatory nitrate reduction geno- and phenotype are not uncommon (Kloos et al., 2001;Falk et al., 2010;Liu et al., 2013).

All DNRA isolates obtained in this study belonged to the *Gammaproteobacteria* and *Alphaproteobacteria* (Table 2). An overview of diverse phyla reported to harbor DNRA bacteria, either tested phenotypically or by *nrfA* gene amplification, can be found in Figure 2. In contrast to previous reports of DNRA phenotype predominantly being found in *Gammaproteobacteria* (Forsythe et al., 1988;Liu et al., 1988;Bonin, 1996;Yoon et al., 2015b), here a wide variety was found of phylogenetically unrelated microorganisms belonging to 11 different phyla harboring the potential to perform DNRA. Such a broad taxonomic distribution was also previously observed for denitrifying organisms (Philippot, 2002). Furthermore, the observed diversity contrasts enormously with the number of physiologically tested representatives, *i.e*. limited to *Gammaproteobacteria* (Smith, 1982;Keith and Herbert, 1983;Liu et al., 1988;Yoon et al., 2015b), *Firmicutes* (Keith et al., 1982;Hoffmann et al., 1998;Mania et al., 2014) and *Alphaproteobacteria* (this study), underlining the previous underestimation of DNRA organism diversity.

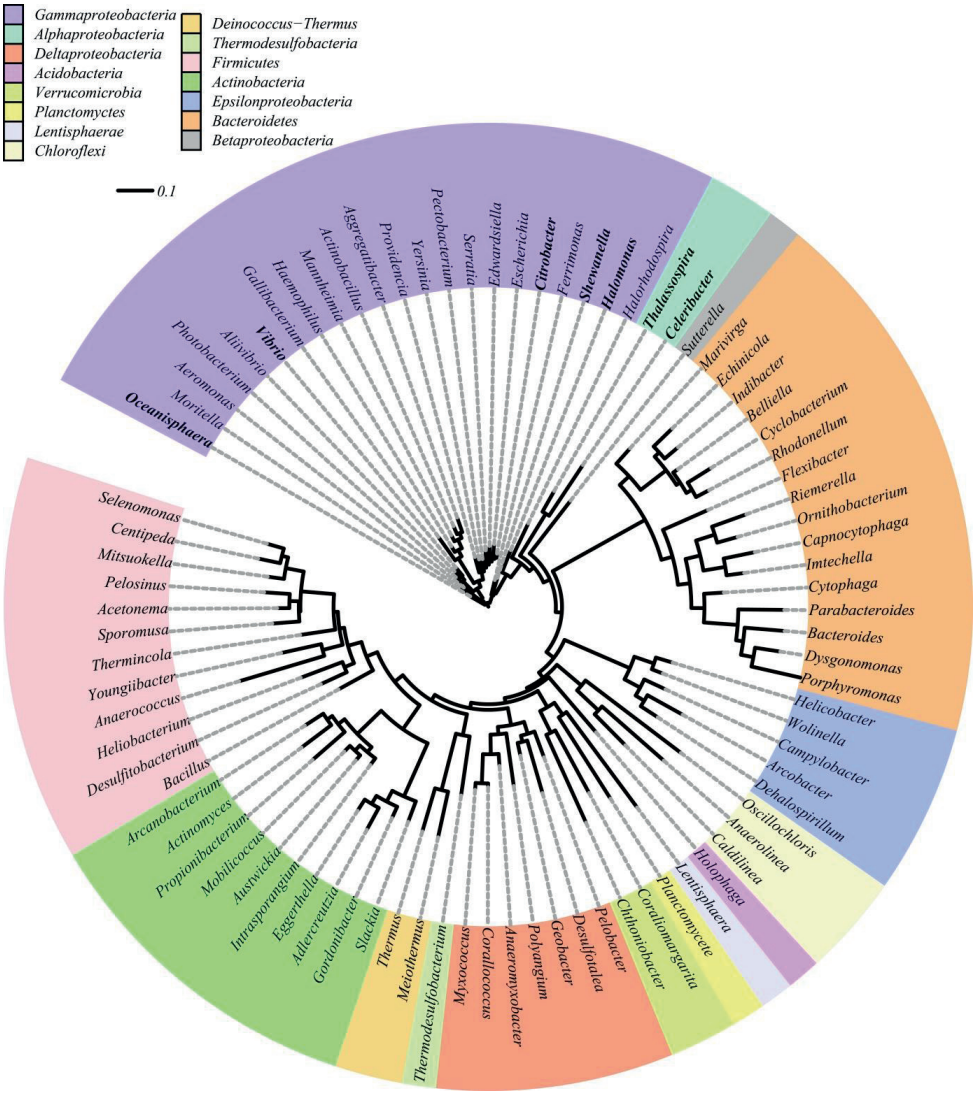

Figure 2 Maximum likelihood phylogenetic analysis of 16S rRNA genes of previously known DNRA bacteria as determined by the presence of a *nrfA* gene. Genera found during this study are indicated in bold.

Table 2 Identification of cultured nitrate/nitrite ammonifiers retrieved from estuarine sediments. Taxonomic assignment to genus level based on the 16S rRNA gene sequence analysis, observed dissimilatory reduction of nitrogenous compounds, amounts of nitrous oxide produced and *nrfA* amplification results are represented. Reduction of nitrate to nitrite and DNRA have been tested in 2 different growth conditions (complex or mineral medium).

Taxonomy	Isolate number	Type strain with the highest 16S rRNA gene sequence similarity to query sequences			Nitrate metabolism				*nrfA* gene amplification		
		Species name	% sim.	Accession number	NO_3^- reduction to NO_2^-	Denitrification	DNRA	N_2O production (%)[a)]	F1-7R1 [b)]	F2-7R1 [b)]	F2aw-7R1 [c)]
Alphaproteobacteria											
Rhodobacterales	R-52651	*Celeribacter baekdonensis* L-6^T	100	HM997022	+	-	+	0-1.1	-	+	-
Rhodospirillales	R-52913	*Thalassospira lucentensis* DSM	99.6	AM294944	+	-	+	0-0.3	-	-	-
	R-52699		99.6	AM294944	+	-	+	0-0.7	-	-	-
Gammaproteobacteria											
Aeromonadales	R-52674	*Oceanisphaera donghaensis* BL1^T	99.77	DQ190441	+	-	+	2-2.1	+	+	-
Alteromonadales	R-52649	*Shewanella colwelliana* ATCC	100	AY653177	+	-	+	0.3-1.3	-	+	-
	R-52673	*Shewanella marisflavi* SW 117^T	100	AY485224	+	-	+	0.8-1.4	+	+	-
Enterobacteriales	R-52910	*Citrobacter gillenii* CDC 4693-86^T	99.9	AF025367	+	-	+	0.6-2.9	+	+	+
Oceanospirillales	R-52914	*Halomonas denitrificans* M29^T	98.9	AM229317	+	-	+	2.4-2.9	+	+	+
Vibrionales	R-52677	*Vibrio alginolyticus* NBRC 15630^T	99.7	CP006718	+	-	+	0.8-2	+	+	+
	R-52915		99.4	CP006718	+	-	+	0.9-2.1	+	+	+
	R-52696		99.4	CP006718	+	-	+	1.2-1.7	+	+	+
	R-52683	*Vibrio diabolicus* HE800^T	99.4	X99762	+	-	+	1-1.9	-	+	-
	R-52669	*Vibrio neocaledonicus* NC470^T	99.4	JQ934828	+	-	+	0.7-2.4	-	-	-
	R-52688		99.79	JQ934828	+	-	+	0.7-2.4	+	+	+
	R-66650	*Vibrio rumoiensis* S-1^T	100	AB013297	+	-	+	0.3-2	+	+	-

[a)] Percentage of trace amounts of nitrous oxide detected in both 1/10 marine both and mineral media supplemented with 5 mM nitrate, [b)] *nrfA* gene amplification primers (505 bp and 231 bp amplicon respectively) from Mohan et al. 2004, [c)] *nrfA* gene amplification primers (269 bp amplicon) from Welsh et al. 2014.

Conclusion

Denitrification in marine environments is generally accepted to contribute substantially to nitrate reduction. Reports on the comparable or higher contribution of DNRA to nitrate reduction have revived the scientific interest in DNRA, physico-chemical parameters determining nitrate partitioning to denitrification and DNRA, the relative importance of the key players *in situ* and their ecophysiology. Here, we demonstrate that small scale heterogeneity in intertidal sediments influences the occurrence and rates of dissimilatory nitrate reduction processes. Whereas denitrification rates were comparable at the cm to m scale, DNRA and nitrate reduction to nitrite was site-specific and could vary significantly within 25 cm. Key environmental drivers partitioning nitrate among these processes could not be identified but did not relate to carbon to nitrogen ratio. Furthermore, fifteen DNRA strains were obtained from estuarine sediments, including members of *Thalassospira*, *Celeribacter* and *Halomonas* previously unrecognized DNRA organisms. These novel environmental strains are now available for further ecophysiological studies on DNRA.

4.4 Acknowledgements

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4.5 Supplementary information

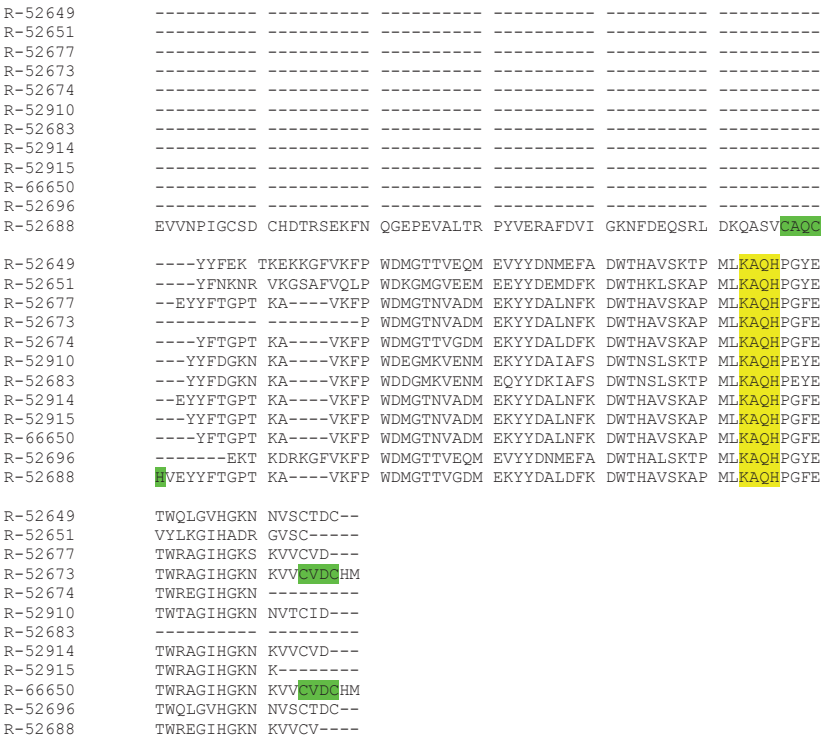

Figure S 1 Multiple sequence alignment of translated *nrfA* genes from twelve out of the fifteen isolates obtained in this study. Three different primer pairs were used, each targeting the region between the third and the fourth heme binding motif. NrfA identity of obtained amplicons was verified by the presence of a NrfA diagnostic motif (marked in yellow), *i.e.* KXRH or KXQH. In some cases, heme binding motifs were also detected as indicated in green.

Table S 1 Time series data of sampling site 1. Individual rates per replicate, the standard error, the R^2 obtained after regression analysis and individual measurements of nitrite, ammonium and nitrous oxide concentrations per vial per replicate for each hour are represented.

	Replicate no.	Individual rates	SE	R^2	Time serie data	
		(µmol N/ g.h)	(µmol N/ g.h)		h	(µmol N/ vial)
NO_2^-	Rep1	1.64E-02	4.84E-03	0.66	1	0.00E+00
					2	4.25E-03
					3	4.09E-02
					4	1.22E-01
					5	2.35E-01
	Rep2	9.05E-03		0.64	1	9.21E-05
					2	7.89E-03
					3	2.00E-02
					4	6.15E-02
					5	1.34E-01
	Rep3	1.82E-02		0.74	1	0.00E+00
					2	2.08E-02
					3	6.83E-02
					4	1.30E-01
					5	2.47E-01
NH_4^+	Rep1	4.21E-02	4.59E-03	0.90	1	1.07E-01
					2	1.08E-01
					3	2.60E-01
					4	2.86E-01
					5	4.71E-01
	Rep2	4.48E-02		0.88	1	9.84E-02
					2	2.40E-01
					3	2.51E-01
					4	2.91E-01
					5	4.81E-01
	Rep3	3.58E-02		0.61	1	1.49E-01
					2	1.88E-01
					3	2.11E-01
					4	2.53E-01
					5	3.49E-01
N_2O	Rep1	6.30E-03	1.89E-04	0.93	1	7.65E-03
					2	2.19E-02
					3	3.06E-02
					4	5.44E-02
					5	5.35E-02
	Rep2	6.00E-03		0.94	1	3.41E-03
					2	2.05E-02
					3	3.26E-02
					4	5.28E-02
					5	6.11E-02
	Rep3	5.95E-03		0.94	1	6.45E-03
					2	1.75E-02
					3	3.27E-02
					4	5.29E-02
					5	5.97E-02

Table S 2 Overview of variable parameters between all various growth media tested.

Medium	Type of medium		C-source			N-sources		C:N ratio (molar)		cAMP [(a)]
	Complex	Mineral	Glucose (mM)	Succinate; ethanol; glycerol (mM)	pyruvate; acetate (mM)	NO_3^- (mM)	NO_3^-/NO_2^- (mM)	Low (5)	High (25)	
DNR1P1	x					5				x
DNR1P2	x					5				
DNR2P1		x	4.17			5		x		
DNR2P2		x	4.17				3/2	x		
DNR2P3		x	20.83			5			x	
DNR2P4		x	20.83				3/2		x	
DNR2P5		x	4.17			5		x		x
DNR2P6		x	4.17				3/2	x		x
DNR2P7		x	20.83			5			x	x
DNR2P8		x	20.83				3/2		x	x
DNR3P1		x		2.08; 4.17; 2.78		5		x		
DNR3P2		x		2.08; 4.17; 2.78			3/2	x		
DNR3P3		x		10.42; 20.84; 13.89		5			x	
DNR3P4		x		10.42; 20.84; 13.89			3/2		x	
DNR3P5		x		2.08; 4.17; 2.78		5		x		x
DNR3P6		x		2.08; 4.17; 2.78			3/2	x		x
DNR3P7		x		10.42; 20.84; 13.89		5			x	x
DNR3P8		x		10.42; 20.84; 13.89			3/2		x	x
DNR4P1		x			4.17; 6.25	5		x		
DNR4P2		x			4.17; 6.25		3/2	x		
DNR4P3		x			20.83; 31.25	5			x	
DNR4P4		x			20;83; 31;25		3/2		x	
DNR4P5		x			4.17; 6.25	5		x		x
DNR4P6		x			4.17; 6.25		3/2	x		x
DNR4P7		x			20.83; 31.25	5			x	x
DNR4P8		x			20.83; 31.25		3/2		x	x

[(a)] Cyclic Adenosine MonoPhosphate

Table S 3 Physico-chemical parameters of the five sampling sites used for determination of denitrification potential (n=3). Significant differences in pore water nitrate concentration between site 1,2 (indicated by *) and 3,4,5 (indicated by $^{+}$) could be detected ($p < 0.05$). No significant differences between site 1 and 2 or sites 3,4 and 5 could be detected. All other parameters were found to be not significantly different between all five sampling sites.

Parameter	Sampling site 1	Sampling site 2	Sampling site 3	Sampling site 4	Sampling site 5
TOM (%)[a]	3.87 ± 0.07	3.26 ± 0.20	3.65 ± 0.31	3.79 ± 0.08	3.58 ± 0.40
[Chl *a*] (µg/g dw)	27.19 ± 4.51	19.03 ± 4.93	17.97 ± 3.40	19.05 ± 2.47	19.94 ± 5.15
[EPS] (µg/mg dry sediment)[b]	0.28 ± 0.03	0.26 ± 0.05	0.27 ± 0.03	0.24 ± 0.01	0.26 ± 0.02
% mud [c]	47.59 ± 2.24	51.41 ± 2.72	47.74 ± 3.16	48.39 ± 1.00	49.18 ± 2.73
[NH_4^+] (mg/l)[d]	2.76 ± 0.46	3.10 ± 0.52	3.71 ± 0.45	3.57 ± 0.48	3.51 ± 0.22
[NO_3^-] (µg/l)[d]	220.04 ± 16.43*	207.56 ±65.31*	30.42 ± 22.44^{+}	44.82 ± 7.01^{+}	47.55 ± 37.84^{+}
[NO_2^-] (µg/l)[d]	8.48 ± 1.59	11.70 ± 4.06	6.35 ± 1.72	6.96 ± 1.01	5.62 ± 0.55
[PO_4^{3-}] (mg/l)[d]	1.18 ± 0.15	1.14 ± 0.26	1.39 ± 0.34	1.42 ± 0.47	1.12 ± 0.07
[Si] (mg/l)[d]	4.23 ± 1.36	4.63 ± 0.23	6.53 ± 0.83	5.79 ± 1.21	5.95 ± 0.92

a. TOM, total organic matter

b. Extracellular polymeric substances

c. Percentage mud (particle size <63µm) determined using the Wenthworth grain size chart

d. Pore water concentrations.

Table S 4 Individual rates of nitrite, ammonium and nitrous oxide production per sampling site per replicate and the standard error.

Replicate n°	Individual rates (µmol/g.h)					
	NO_2^-	SE	NH_4^+	SE	N_2O	SE
1.1	1.64E-02	4.84E-03	4.21E-02	4.59E-03	6.30E-03	1.89E-04
1.2	9.05E-03		4.48E-02		6.00E-03	
1.3	1.82E-02		3.58E-02		5.95E-03	
2.1	3.85E-03	8.13E-04	0.00E+00	0.00E+00	6.45E-03	3.04E-04
2.2	2.80E-03		0.00E+00		6.95E-03	
2.3	4.40E-03		0.00E+00		6.40E-03	
3.1	2.25E-03	3.28E-04	0.00E+00	0.00E+00	6.40E-03	4.65E-04
3.2	2.00E-03		0.00E+00		7.15E-03	
3.3	2.65E-03		0.00E+00		7.25E-03	
4.1	2.55E-03	4.31E-04	0.00E+00	0.00E+00	4.70E-03	7.64E-05
4.2	3.40E-03		0.00E+00		4.55E-03	
4.3	3.10E-03		0.00E+00		4.65E-03	
5.1	0.00E+00	0.00E+00	6.25E-03	3.82E-03	4.95E-03	5.77E-04
5.2	0.00E+00		7.35E-03		4.30E-03	
5.3	0.00E+00		1.34E-02		5.45E-03	

Table S 5 Diversity of isolates retrieved from estuarine sediments. Taxonomic assignment to genus level based on the 16S rRNA gene sequence analysis, isolation conditions, number of isolates obtained per genus per type of medium and strain numbers are represented.

Taxonomy	Type strain with the highest 16S rRNA gene sequence similarity to query sequences			Isolation conditions		No isolates	Isolate strain number
	Species and strain	% similarity	Accession number	e- donor (conc. in mM)	e-acceptor (conc. in mM)		
Actinobacteria							
Paraoerskovia	*Paraoerskovia marina* DSM21750 T	100	JNIY01000001	succinate (2.08mM)/ethanol (4.17mM)/glycerol (2.78mM)	nitrate (3mM)/nitrite (2mM)	2	R-66634, R-66635
Alphaproteobacteria							
Labrenzia	*Labrenzia alba* CECT 5094 T	99.07	AJ878875	succinate (2.08mM)/ethanol (4.17mM)/glycerol (2.78mM)	nitrate (3mM)/nitrite (2mM)	6	R-66636, R-66637, R-66638, R-66639, R-66640, R-66641
	Labrenzia aggregata IAM 12614 T	99.86	AAUW01000037	pyruvate (4.17mM)/acetate (6.25mM)	nitrate (5mM)	3	R-52697, R-52691, R-52692
Martelella	*Martelella endophytica* YC6887 T	97.82-98.80	HM800924	succinate (10.42mM)/ethanol (20.84mM)/glycerol (13.89mM)	nitrate (3mM)/nitrite (2mM)	5	R-66642, R-66643, R-66645, R-66646, R-66647
				pyruvate (4.17mM)/acetate (6.25mM)	nitrate (5mM)	4	R-52687, R-52690, R-66644, R-66648
Celeribacter	*Celeribacter baekdonensis* L-6 T	98.7-100	HM997022	glucose (4.17mM)	nitrate (5mM)	4	R-52661, R-52662, R-52663, R-52664
				glucose (20.83mM)	nitrate (3mM)/nitrite (2mM)	4	R-52665, R-52666, R-52667, R-52668
				1/10 marine broth	nitrate (5mM)	1	R-52651
Pelagicola	*Pelagicola litorisedimines* D1-W8 T	99.4	KC708867	1/10 marine broth	nitrate (5mM)	3	R-52656, R-52658, R-52660
Phaeobacter	*Phaeobacter gallaeciensis* BS107 T	99.0-99.4	ABIF01000020	pyruvate (4.17mM)/acetate (6.25mM)	nitrate (5mM)	3	R-52693, R-52695, R-52698
Loktanella	*Loktanella rosea* Fg36 T	99.34	AY682199	1/10 marine broth	nitrate (5mM)	1	R-52657
Pseudoruegeria	*Pseudoruegeria lutimaris* HD-43 T	98.34	FJ374173	1/10 marine broth	nitrate (5mM)	1	R-52653
Roseovarius	*Roseovarius gaetbuli* YM-20 T	100	KF208688	1/10 marine broth	nitrate (5mM)	2	R-52654, R-52655
Thalassospira	*Thalassospira lucentensis* DSM 14000T	99.58	AM294944	pyruvate (4.17mM)/acetate (6.25mM)	nitrate (3mM)/nitrite (2mM)	1	R-52699
				pyruvate (4.17mM)/acetate (6.25mM)	nitrate (5mM)	1	R-52913
Gammaproteobacteria							
Oceanisphaera	*Oceanisphaera donghaensis* BL1 T	99.77	DQ190441	succinate (10.42mM)/ethanol (20.84mM)/glycerol (13.89mM)	nitrate (5mM)	2	R-52674, R-52676
Marinobacter	*Marinobacter vinifirmus* FB1 T	99.73	DQ235263	succinate (2.08mM)/ethanol (4.17mM)/glycerol (2.78mM)	nitrate (5mM)	1	R-52700

Shewanella	*Shewanella colwelliana* ATCC 39565 T	100	AY653177	1/10 marine broth	nitrate (5mM)	2	R-52649,R-66649
	Shewanella marisflavi SW 117 T	100	AY485224	succinate (10.42mM)/ethanol (20.84mM)/glycerol (13.89mM)	nitrate (5mM)	2	R-52673, R-52675
Citrobacter	*Citrobacter gillenii* CDC 4693-86 T	99.93	AF025367	succinate (10.42mM)/ethanol (20.84mM)/glycerol (13.89mM)	nitrate (3mM)/nitrite (2mM)	1	R-52910
Shigella	*Shigella flexneri* ATCC 29903	97.7	X96963	pyruvate (4.17mM)/acetate (6.25mM)	nitrate (3mM)/nitrite (2mM)	2	R-52920, R-52921
Halomonas	*Halomonas denitrificans* M29 T	98.96	AM229317	pyruvate (4.17mM)/acetate (6.25mM)	nitrate (3mM)/nitrite (2mM)	1	R-52914
Vibrio	*Vibrio alginolyticus* NBRC 15630 T	99.38-99.73	CP006718	succinate (2.08mM)/ethanol (4.17mM)/glycerol (2.78mM)	nitrate (5mM)	6	R-52677, R-52678, R-52679, R-52680, R-52681, R-52682
				pyruvate (4.17mM)/acetate (6.25mM)	nitrate (5mM)	2	R-52696, R-52911
				pyruvate (20.83mM)/acetate (31.25mM)	nitrate (5mM)	4	R-52915, R-52916, R-52917, R-52918
	Vibrio diabolicus HE800 T	99.15-99.36	X99762	pyruvate (4.17mM)/acetate (6.25mM)	nitrate (5mM)	4	R-52683, R-52684, R-52685, R-52686
				1/10 marine broth	nitrate (5mM)	1	R-52650
	Vibrio neocaledonicus NC470 T	99.43-99.79	JQ934828	succinate (10.42mM)/ethanol (20.84mM)/glycerol (13.89mM)	nitrate (5mM)	4	R-52669, R-52670, R-52671, R-52672
				pyruvate (20.83mM)/acetate (31.25mM)	nitrate (5mM)	2	R-52688, R-52689
	Vibrio rumoiensis S-1T	100	AB013297	pyruvate (20.83mM)/acetate (31.25mM)	nitrate (3mM)/nitrite (2mM)	1	R-66650
Bacteroidetes							
Marinifilum	*Marinifilum flexuosum* CECT 7448 T	95.49	HE613737	1/10 marine broth	nitrate (5mM)	1	R-52652
Formosa	*Formosa algae* KMMM 3553	99.86	AY228461	pyruvate (4.17mM)/acetate (6.25mM)	nitrate (5mM)	1	R-52912
Lutibacter	*Lutibacter agarilyticus* KYW566 T	97.43	JN864028	1/10 marine broth	nitrate (5mM)	1	R-52659
Yeousuana	*Yeousuana aromativorans* GW 1-1 T	96.84	AY682382	pyruvate (20.83mM)/acetate (31.25mM)	nitrate (5mM)	1	R-66651
Firmicutes							
Bacillus	*Bacillus drentensis* LMG 21831 T	99.58	AJ542506	glucose (4.17mM)	nitrate (5mM)	3	R-66632, R-66633, R-66652

Reflection & discussion

The aim of this study was two-fold: (i) examine nitrate reduction potentials (denitrification, DNRA and nitrate reduction to nitrite) of estuarine sediments and their variation at the meter scale and (ii) isolate nitrate reducing bacteria. In contrast to what was previously believed, denitrification can be rivaled by DNRA as predominating estuarine nitrate reducing pathway. Knowledge on the organisms involved is, however, mainly limited to in-depth studies on model organisms, while information on the key organisms involved, how they respond to changing environmental conditions and how these processes relate to each other is largely lacking. We observed small scale heterogeneity in the occurrence and the importance of dissimilatory nitrate reducing processes and obtained fifteen new DNRA strains that now can be used in marine ecophysiological studies. Some issues concerning the use of the acetylene inhibition technique, data analysis, cultivation-based studies and defining DNRA bacteria will, however, be further addressed.

Nitrate reduction potentials were assessed using the acetylene inhibition technique. A major difficulty encountered during comparison with other studies, was that different variants of the same technique are used. The inclusion of chloramphenicol, an antibiotic that inhibits *de novo* protein synthesis but does not inhibit existing enzymes except at high concentrations (Murray and Knowles, 1999), is not standardly used indicating that previously reported nitrogen reduction rates might be influenced by *de novo* synthesis of denitrifying enzymes as a consequence of microbial growth. The addition of various concentrations of carbon and/or nitrogen sources (here only 5 mM KNO_3 and no carbon was added) furthermore create ideal conditions (anoxic conditions and adequate N and C) for the stimulation of the growth of r-strategist denitrifying bacteria, and therefore the production of denitrifying enzymes, as these organisms are characterized by high maximal growth rates (μ_{max}) and high Ks values (low nitrate affinity). Addition of chloramphenicol would, however, prevents this issue as only *in situ* expressed enzymes would be interrogated in such assays. Generally, there is a need for a more consistent way of determining nitrate reduction potentials, *i.e.* standard inclusion of a fixed concentration chloramphenicol and nitrogen source, which would allow more elaborate comparison between potentials from similar or different environments. Determination of the optimal concentration chloramphenicol (sufficient inhibition of *de novo* enzyme synthesis without disruption of existing enzymes), however, requires further investigation as currently no consensus (range from 0.1-0.3mM, (Murray and Knowles, 1999;Qin et al., 2012)) thereon is available. Isotopic labelling experiments using ^{15}N-nitrite, ^{15}N-nitrate or ^{15}N-ammonium provide a valid alternative to the acetylene inhibition technique. This approach has as main advantage that it allows distinction between denitrification, anammox and DNRA without the need of colorimetric assays (Introduction § 1.3), however, it requires expensive laboratory equipment. The acetylene inhibition technique will provide a fast and

cheap alternative for measuring nitrate reduction rates of bacterial populations once a uniform protocol generally accepted by the scientific community is available.

No linkage between observed variation in nitrate reduction potentials and determined physico-chemical parameters could be found. The main reason therefore is that potential parameters such as pH (Simek and Cooper, 2002) and the presence of free sulfides (Burgin and Hamilton, 2007) were not evaluated in this study. Therefore, I recommend that future studies determining nitrate reduction potentials in marine environments should determine a standard minimal set of parameters in an attempt to explain observed patterns, including pH, [nitrate], [nitrite], [ammonium], oxic-anoxic border, [sulfide] and [organic carbon]. In addition, it is important that other interacting organisms (MPB, meio- and macrofauna) in such systems are also considered. Microphytobenthos abundances can be evaluated through determination of [chl *a*] while meio- and macrofauna can be sieved from the sediment and subsequently described. Similarly to determination of physico-chemical parameters, these parameters will, out of necessity (Reflection & discussion Chapter 2), be analyzed on additional samples taken in close proximity of the cores used for nitrate reduction rate determination.

The advent of next generation sequencing combined with the issue of the "Great plate count anomaly", resulted in a renewed interest in traditional culturing due to its crucial role in confirming functionality and, through whole genome sequencing of pure cultures, improvement of sequence databases used for interpretation of (meta)genomic data (Walker et al., 2014). The isolation approach used in this study was limited, *i.e*. five isolates were randomly picked up per type of medium, and it therefore resulted in a low taxonomic diversity of DNRA bacteria, despite earlier indications on the taxonomically widespread occurrence of DNRA (Welsh et al., 2014). The main reason therefore is the laborious culturing work which remains the major bottleneck in isolation based studies and imposes compromises to reduce logistical demands, *e.g*. combining different carbon sources, that might skew the assessment of denitrifying and DNRA bacteria present in estuarine sediment. Future high-throughput isolation procedures have now become feasible through the availability of a robotized colony picker at LM-UGent. Culturomics, *i.e*. high-throughput screening of different pretreatment procedures (*e.g*. antibiotics, cell extraction, pasteurization, …) and isolation media (Lagier et al., 2012), combined with high-throughput MALDI-TOF MS dereplication, identification, colorimetric assays and subsequent NOx compound analysis using gas chromatography of interesting strains will certainly increase the recovery of new taxonomically more diverse and potentially *in situ* relevant denitrifying and DNRA bacteria.

The DNRA phenotype of our isolates was additionally confirmed through positive amplification of the *nrfA* gene for twelve out of fifteen DNRA isolates. However, DNRA can also occur through the NADH-dependent nitrite reductase NirB encoded by the gene *nirB*, an enzyme also known to be involved in assimilatory nitrogen cycling. Similar as with denitrification this raises the semantic

though relevant question of when an organism should be considered as a DNRA bacterium. In my opinion, a bacterium can be defined as a DNRA bacterium when nitrite is converted to ammonium, irrespective of the energy metabolism, in a process not resulting in nitrogen assimilation. This means that organisms that perform detoxification of nitrite to ammonium under high nitrite conditions can also be considered as DNRA bacteria. In contrast to *nrfA* (Mohan et al., 2004;Welsh et al., 2014), the presence of the *nirB* gene can currently not be checked due to lack of *nirB* primers with exception of limited archaeal primers (Rusch, 2013). Therefore, it would be valuable to design *nirB* primers to allow more accurate assessment of potential DNRA bacteria, *i.e.* both *nrfA* and *nirB* containing organisms, in the environment.

Chapter 5

Concluding remarks and future perspectives

Increased terrestrial nitrogen input due to more extensive use of fertilizers and fossil fuel combustion have substantially altered nitrogen cycling on Earth. Estuarine ecosystems receive part of this anthropogenically produced nitrogen through riverine run off, and can remove it either via denitrification, dissimilatory nitrate reduction to ammonium (DNRA) or anammox. The findings of this PhD provide insights on (i) the effect of microphytobenthos (MPB) on estuarine total bacterial and *nirK*/*nirS* functional guild abundance and community structure, (ii) the enormous NirK sequence divergence and its consequences for PCR-dependent surveys, (iii) the variety of dissimilatory nitrate reducing pathways within taxonomically diverse NirK-type denitrifiers and (iv) the small scale heterogeneity of these processes within estuarine sediments. In addition, new estuarine DNRA strains are now available for future ecophysiological studies.

In the light of detrimental effects of eutrophication and global warming (where the nitrogen cycle is implicated in emission and mitigation of the greenhouse gas nitrous oxide), a main objective of nitrogen cycling research is to gain solid understanding on how this cycle works and is regulated (both abiotically and biotically) to subsequently apply this knowledge into system-based predictive models that can be used for ecosystem protection and management practices. However, in my opinion, progress in this field is significantly hampered by (i) the complexity of the interactions between different trophic levels, *i.e*. Prokaryotes, meiofauna and macrofauna, and their impact on the nitrogen cycle and (ii) the difficulty to unambiguously define denitrification and DNRA, and reliably monitor organisms involved.

This study was performed in a microbiology research lab and therefore mainly focused on bacteria. However, in complex environments such as marine ecosystems, it is difficult to extract meaningful biological information without taking into account the impact of higher trophic levels. The complexity and biological diversity within marine sediments results in many feedbacks and diverse potential interactions, most of which are not considered in traditional compartmentalized approaches focusing on one trophic level at a time. Examples of such interactions between different benthic inhabitants include (i) production of EPS (Riemann and Helmke, 2002;McKew et al., 2013) by diatoms and meiofauna which can affect the prokaryotic community, (ii) production of cytotoxins (Ianora and Miralto, 2010) by diatoms that can negatively affect both prokaryotes and meiofauna, (iii) excretion products of meiofauna provide sources of nitrogen and carbon that can potentially be used for bacterial growth (Frangoulis et al., 2005), (iv) bioturbation of the sediment by macrofauna (Braeckman et al., 2010), and to a lesser extent meiofauna (Martin et al., 2005), thereby influencing the general environmental physico-chemistry, and (v) grazing by higher trophic organisms, *e.g*. meiofauna can eat their own body weight equivalent in microorganisms every day (Montagna, 1984). Limited information is available on (in)direct effects of such trophic organisms and other environmental drivers on the total bacterial community, specific functional guilds or nitrate reduction processes and their rates. Our research indicated that there is indeed an effect of MPB on bacterial community

abundance, although we could not elucidate whether it was derived from MPB directly or indirectly through grazing of meio- and/or macrofauna on diatoms and/or bacteria (Chapter 2). In addition, small scale heterogeneity was observed in both importance and presence of denitrification, DNRA and nitrate reduction to nitrite, however, this variation could (again) not be linked to specific environmental drivers (Chapter 4). Future microbiological research on marine nitrate reducing processes should include different trophic levels (at least more than prokaryotes alone) and assess diverse physico-chemical parameters in an attempt to obtain a more complete picture on how nitrogen cycling proceeds *in situ*.

Despite many years of research on both DNRA and denitrification pathways, defining a bacterium as a denitrifier and/or a DNRA bacterium is, surprisingly, not straightforward. Therefore, we propose that a denitrifier should at least be capable of nitrite reduction to nitric oxide linked to energy conservation, *i.e.* it should thus contain NirK or NirS, while a DNRA organism has the ability to reduce nitrite to ammonium through either NrfA and/or NirB, regardless of it supports growth or not. The use of all these functional marker genes can provide valuable, more complete information on the diversity, abundance and composition of these communities in future studies. Culture-independent surveys are necessary for in-depth monitoring of these functional guilds in environmental samples. Amplicon-based sequencing studies allows deep analysis of these functional guilds compared to metagenome analyses, however, their efficiency is seriously compromised by primer biases (Chapter 3). In contrast, metagenome studies can provide a good overview of the genetic potential of the community studied and are independent of biases associated with library construction and PCR (*i.e.* primer limitations and chimera formation). Although metagenomics is an incredible powerful technique, it has several limitations: (i) impossibility of mapping obtained data onto reference databases (Teeling and Gloeckner, 2012), (ii) difficulty of unambiguous assignment of function based on sequence similarity alone (therefore resulting in misannotation (Schnoes et al., 2009)), and (iii) sequencing depth and length may currently limit the information obtained when interested in specific functional guilds. Common to both types of sequencing is that the initial DNA extraction method and its subsequent storage remain crucial in determining how representative the DNA is of the original microbial community. Cultivation-based analyses, despite the well-known 'Great plate count anomaly', are nevertheless still an essential part of microbial ecology as they provide a link between environmental observations and phenotypic properties. In addition, cultivation studies generate a knowledge base onto which -omics data can be mapped and linked. New marine DNRA strains were isolated in our study (Chapter 4) and can now be used in ecophysiological and genomic studies, whether or not linked to dissimilatory nitrate reduction processes. They will provide new relevant information as not all organisms behave like well-studied model organisms. In conclusion, it is of outmost importance that a researcher is aware of the weaknesses inherent to different techniques available and adjusts the methodological approach according to the research question.

Future perspectives

Research on dissimilatory nitrate reduction within estuarine (intertidal) systems has mainly focused on denitrification in the past as it was believed to be the dominant process. However, recent insights on the importance of DNRA in estuarine systems indicated that denitrification is not necessarily the dominant nitrate reducing process, which can have major consequences for the fate of excess anthropogenic nitrate. It is of utmost importance to understand what drives both processes in this environment to determine when nitrogen loss via denitrification or nitrogen retention via DNRA is dominant and apply this knowledge into system-based predictive models that can subsequently be used to design efficient ecosystem management practices during perturbation or permanently changed conditions (*e.g.* promoting N_2 loss and minimizing both N_2O emission and ammonium retention in nitrate-rich environments). Many mitigation strategies for nitrous oxide emission control (liming, plant rotation system, commercial nitrification inhibitors, controlled release fertilizers, etc.) have already been suggested and applied in agriculture. I believe that to determine the key drivers of denitrification and DNRA in these systems with the aim of their incorporation in predictive models, basic information and model parameters are needed on at least two fronts: (i) the interplay between different trophic levels and its influence on nitrate reduction, and (ii) the involvement of nitrite reduction enzymes in DNRA and the mechanisms and general occurrence of nitrous oxide emission.

Unravelling the effect of diverse benthic organisms on nitrate reduction processes

The high variability and biotic complexity of estuarine systems makes studying them as a whole very challenging. The presence of diverse trophic levels (Prokaryotes, microphytobenthos, meiofauna and macrofauna) and their interactions make it difficult to evaluate their effects on nitrogen cycling. Therefore experimental approaches investigating the influence of multi-trophic interactions on nitrogen cycling should focus on a reduced complexity approach in which defaunated mesocosms with natural sediment samples harbouring natural prokaryotic communities serve as starting point. The effect of each trophic level on nitrogen cycling can be gained by sequentially increasing complexity, *i.e.* first level of complexity includes prokaryotes, followed by microphytobenthos (diatoms), meiofauna and macrofauna. Dissimilatory nitrate reduction processes will be monitored via isotopic pairing through the addition of labelled ^{15}N-nitrate and measuring $^{30}N\text{-}N_2$ and $^{30}N\text{-}N_2O$ for denitrification, ^{15}N-ammonium for DNRA and $^{29}N_2O$ for anammox, allowing the determination of which process is dominant under each tested condition. With a correct experimental design, thorough statistical analyses will enable the quantification of the contribution of each trophic level to each process, which can then be considered as model parameter if efficient strategies exist to have realistic biomass quantification for each level. Although isotopic pairing has the advantage of measuring the process without detection biases as exist for molecular procedures (as explained throughout this dissertation), it has also some weaknesses, the major one being the difficulty to discriminate between anammox bacteria that produce their own ammonium from ^{15}N-nitrate and thus also produce $^{30}N\text{-}N_2$

(Kartal et al., 2007a). Still, as the anammox process is probably not important or dominant in intertidal estuarine sediments, this limitation is acceptable. In addition to biomass quantification for each trophic level, it has been suggested that gene/transcript/protein abundances rather than functional guild diversity might be more relevant to include as a model parameter (Hu et al., 2015). This also makes sense considering the existence of highly truncated versions of both the denitrification and DNRA pathways in diverse bacteria (Chapter 3). I also believe that in the near future it will be technically feasible to quantitatively determine relevant enzymes involved in the different processes via a metaproteomics approach, which can, when incorporated in the above-described mesocosm experiments, provide additional parameters for the predictive models.

Although *ex situ* experiments do not exactly mimic *in situ* complexity, well-designed mesocosm experiments can represent a relevant proxy for what might be happening in these complex systems. The main challenge in understanding nitrate reduction, or nitrogen cycling in general, within estuarine systems involves the need for more multidisciplinary collaborations. Specialists in different organisms and aspects of this complex system as well as experts in methodologies and statistics are essential for proper experimental design (*e.g.* ranges of faunal abundances, faunal cultivability, competition, etc) and data interpretation that might result in new hypothesis driven experiments which will ultimately contribute to a more in-depth understanding of these processes at a system level. To complement *ex situ* mesocosm experiments, the need exists to set up ambitious *in situ* monitoring studies assessing nitrogen fluxes of numerous big field plots within the intertidal environment, followed by the assessment of diverse abiotic environmental parameters (salinity, oxic-anoxic border, sediment type, etc) and the abundances of all trophic levels at those sites. This is technically very challenging but already feasible in less dynamic soil environments.

Detailed physiological studies of DNRA organisms

Currently available predictive models do not take into account nitrogen retention or nitrous oxide production by DNRA, as its environmental significance has been underestimated in the last decades. To increase our knowledge of the DNRA process in general and more specifically in estuarine/marine environments and discriminate potential informative parameters to include in ecosystem models, first, new attempts at isolating DNRA organisms should be made. Instead of focusing on trial-and-error cultivation attempts using diverse cultivation media, chemostat-based enrichment experiments should be performed in an attempt to obtain taxonomically diverse DNRA bacteria. Chemostats have been shown to be particular successful in enriching DNRA bacteria as a high C:N (strict nitrate limitation) can be maintained at a constant level in such experimental setups (van den Berg et al., 2015;Yoon et al., 2015b). Second, dissimilatory reduction of nitrite to ammonium involves two different enzymes: NrfA and NirB. Hitherto, gene/transcript based environmental surveys only included NrfA, thereby underestimating the potential relevance of this process. The main reason for this is the dual role of NirB in both dissimilatory and assimilatory processes. The involvement of NirB in assimilation makes

it very unlikely that energy will be conserved during dissimilatory functioning and points towards a difference in regulation depending on the environmental conditions. A more thorough understanding of the regulation of NirB in diverse DNRA bacteria is needed to better comprehend its role in DRNA. Based on this reasoning, one could expect that NrfA will be the enzyme involved in respiratory DNRA while the NirB enzyme will function in fermentative DNRA or nitrite detoxification, however, this still needs to be confirmed. The abundances of both enzymes/encoding genes/expressed genes could prove useful proxies for evaluating the dominance of the process *in situ*. Next, it needs to be resolved how, in what amounts and under which conditions DNRA bacteria can produce nitrous oxide. Studying *Escherichia coli* and *Salmonella typhimurium* showed the potential involvement of NarG, indicating that nitrous oxide might not be produced during DNRA, but during detoxification of high levels of nitrite (Gilberthorpe and Poole, 2008). As it is very likely that taxonomically diverse organisms do not all behave like an *E. coli*, it would be interesting to physiologically evaluate nitrous oxide production by other (non-Gammaproteo) bacteria in an attempt to understand the potential ecological relevance of nitrous oxide production by DNRA bacteria. One approach could be to perform mutagenesis experiments to ascertain which enzymes are involved in nitrous oxide production in different organisms, another way could be to perform proteomics in chemostats under different conditions to determine its potential drivers. In that respect, the study of marine bacteria having the ability to partition nitrate to either denitrification or DNRA, can provide interesting experimental systems to explore the regulation of dissimilatory NO_3^-/NO_2^- reduction pathways.

Finally, it is pivotal to assess the importance of *in situ* nitrous oxide production by DRNA bacteria. Despite the fact that nitrous oxide production has been observed for DNRA bacteria in culture, no data is available on if and how much nitrous oxide is produced in natural systems. A potentially interesting approach to distinguish nitrous oxide production by DNRA bacteria from other nitrous oxide producing bacteria (denitrifiers, nitrifiers) might be through the evaluation of the nitrous oxide isotopic signature. Distinct isotopic signatures have already been observed for nitrous oxide produced by ammonia oxidizing bacteria versus ammonia oxidizing Archaea (Santoro et al., 2011). Nitrous oxide production in nitrification, denitrification and DNRA proceeds in different ways and therefore is it very plausible that differences in isotopic signatures will be observed. The array of taxonomically diverse DNRA isolates that could be isolated via the above-mentioned approaches would prove useful to determine if indeed a specific isotopic signature is found in nitrous oxide produced by the DNRA process, regardless of the organism involved. If so, this would allow a very neat and relatively easy method to make the contribution of DNRA to nitrous oxide emission for specific ecosystems quantifiable and thus suitable for modelling.

Summary

Anthropogenic influences have altered the Earth's nitrogen cycle substantially over the past century. The rate of terrestrial nitrogen input has more than doubled, mostly through fossil fuel combustion and increased production and use of agricultural fertilizers. In addition, use of fertilizers increases atmospheric concentrations of nitrous oxide (N_2O), a major greenhouse gas contributing to ozone destruction and global warming. Increased inputs of fixed nitrogen through agriculture run off also impact more distant marine ecosystems, where fixed inorganic nitrogen is a key factor controlling primary productivity. Estuarine systems constitute major conduits for the transport of anthropogenically and terrestrially derived nitrogen from land to sea. When this excess nitrogen is not removed by biotic uptake or dissimilatory nitrate reduction in streams and rivers, it ends up in estuaries and coastal areas, where it is implicated in (i) eutrophication that can generate excessive biochemical oxygen demands resulting in hypoxic zones, and (ii) promotion of harmful algal blooms. Until recently, denitrification was considered the main nitrate removing process present in estuarine environments. Increasing evidence indicates, however, that dissimilatory nitrate/nitrite reduction to ammonium (DNRA) rivals denitrification as an important nitrate removing pathway. A major difference between both processes is that denitrification results in nitrogen loss while DNRA retains nitrogen in the system as ammonium which can subsequently be oxidized back to nitrate. The main bacterial players involved in these processes, their ecophysiology and how these processes relate in estuarine systems remain, however, largely unknown.

This PhD research focused on some of the above mentioned aspects and provided insights into (i) the diversity, abundance and composition of the total bacterial and denitrifier community in Westerschelde estuarine sediments, (ii) the effect of microphytobenthos presence on these community features, (iii) the phylogenetic division and divergence of whole genome NirK sequences, (iv) the diversity of the dissimilatory nitrogen cycling gene inventories present within diverse NirK-type denitrifiers and (v) the relative importance of distinct nitrate removing pathways within estuarine sediments over a short distance. Furthermore, novel estuarine DNRA strains are now available for further ecophysiological studies on DNRA.

In a first study, high-throughput sequencing combined with the quantitative assessment of the total bacterial community within estuarine sediments revealed a disproportional tenfold increase when MPB (microphytobenthos) biomass doubled. Unexpectedly, this tenfold increase in bacterial abundance did not translate to significant differences in total bacterial community structure. Similar observations were obtained for both *nirK* and *nirS* denitrifying guilds indicating that there is no competition for nitrate between denitrifiers and MPB. Therefore, it was suggested that MPB probably acts as a general determinant of estuarine bacterial communities. However, the environmental variable(s) accounting for the doubling in MPB biomass could not be deduced, and therefore no causal relation between MPB biomass and bacterial abundance could be inferred.

Driven by the generally acknowledged limitations of *nirK* primer coverage, the second study involved an in-depth analysis of a taxonomically diverse set of full-length NirK sequences. Previously observed phylogenetic division of NirK into two distinct clades was confirmed with Clade I NirK harbouring mainly *Alphaproteobacteria* and the structural Class I NirK, while Clade II contained a more diverse set of both taxonomically and structurally different NirK. Hitherto, because of ambiguous classification of NirK types, no information on potential different ecological strategies or environmental distributions of these distinct NirK Clades is available. Therefore, we propose a systematic usage of Clade I and Clade II designation in future NirK environmental studies. In PCR-based surveys, this enormous *nirK* sequence divergence, probably resulting from variable *nirK* gene evolutionary trajectories, will remain a continuing problem for *nirK* primer coverage with even clade or taxon-specific primer design being a challenge. Evaluation of the dissimilatory nitrate reducing gene inventory of diverse NirK-type denitrifiers demonstrated that the simultaneous presence of both the denitrification and the DNRA pathway is more widespread than originally anticipated as 67% of all NirK-type denitrifiers were shown to contain both pathways in their genome.

In contrast to what was previously assumed, the contribution of DNRA to marine nitrate reduction appears to be comparable to that of denitrification. Therefore, nitrate reduction potentials were determined from estuarine sediments which demonstrated small scale (1.6 m) heterogeneity in the occurrence and rates of denitrification, DNRA and nitrate reduction to nitrite. Denitrification rates were found to be similar at the cm to m scale, while DNRA and nitrate reduction to nitrite were site-specific and could vary significantly within 25 cm. The key environmental drivers partitioning nitrate among these processes remained unidentified, but carbon-to-nitrogen ratio could be excluded. In addition, novel, thus far unreported nitrate-to-ammonium reducers belonging to *Thalassospira*, *Celeribacter* and *Halomonas* were isolated and their DNRA phenotype was reconfirmed through *nrfA* gene amplification.

Taken together, this PhD thesis illustrates that both denitrification and DNRA are important nitrate removing processes in a temperate estuarine ecosystem. Nevertheless, knowledge on environmental parameters (both physico-chemistry and benthic organisms) driving denitrification and DNRA processes, and those that shape their respective communities, is still limited. The inferred potentially important drivers together with the novel estuarine DNRA strains can now be used as a starting point for further in-depth research on the determination of the key parameters for each process in such systems. Furthermore, it is important to take into account technical limitations of the experimental approach applied. This thesis specifically highlighted the primer coverage issue involved in PCR-dependent surveys.

Door menselijk toedoen is de stikstofcyclus op aarde sterk veranderd tijdens de afgelopen eeuw. De invoer van terrestrisch stikstof verdubbelde, grotendeels ten gevolge van de verbranding van fossiele brandstoffen, en een verhoogd produceren en gebruiken van landbouwmeststoffen. Daarenboven resulteert het gebruik van deze meststoffen ook in verhoogde atmosferische concentraties lachgas (N_2O), een belangrijk broeikasgas betrokken in de vernieling van de ozonlaag en opwarming van de aarde. Verhoogde toevoer van gefixeerd stikstof vanuit landbouwgronden heeft daarnaast ook een invloed op verder weg gelegen mariene ecosystemen waar het vaak een limiterende factor is voor primaire productiviteit. Estuariene systemen vormen belangrijke toevoerkanalen waarlangs terrestrisch en door de mens geproduceerd stikstof van land naar zee vloeit. Wanneer dit teveel aan stikstof niet verwijderd wordt door biotische opname of dissimilatorische stikstof reductie in stromen en rivieren, dan komt het terecht in estuaria en kustgebieden waar het betrokken is in (i) eutrofiëring dat een overmatige biochemische zuurstofgebruik in de hand werkt wat resulteert in hypoxische zones, en (ii) bevordering van de groei van toxische algen. Tot voor kort werd denitrificatie gezien als het belangrijkste nitraat verwijderend proces in de estuariene omgeving. Toenemend bewijs toont echter aan dat de dissimilatorische reductie van nitraat/nitriet tot ammonium (DNRA) concurreert met denitrificatie als belangrijk nitraat verwijderend proces. Het voornaamste verschil tussen beide processen is dat denitrificatie resulteert in stikstof verwijdering terwijl DNRA stikstof weerhoudt in het systeem als ammonium dat vervolgens weer geoxideerd kan worden tot nitraat. De belangrijkste spelers betrokken in deze processen, hun ecofysiologie en hoe deze processen zich onderling verhouden in estuariene systemen zijn echter grotendeels ongekend.

Deze doctoraatsthesis focuste zich op sommige van bovenvermelde aspecten en resulteerde in inzichten over (i) de diversiteit, de abundantie en de samenstelling van de volledige bacteriële en de denitrificerende gemeenschap aanwezig in Westerschelde estuariene sedimenten, (ii) het effect van *microphytobenthos* op deze gemeenschapskenmerken, (iii) de fylogenetische opsplitsing en de sequentie divergentie van volledige NirK genen, (iv) de diversiteit in dissimilatorische stikstof cyclus genen aanwezig in diverse NirK denitrificeerders en (v) het relatieve belang van verschillende vormen van nitraat verwijderende processen in estuariene systemen over een korte afstand (1.6 m). Daarnaast werden nieuwe estuariene DNRA stammen bekomen die nu beschikbaar zijn voor verder DNRA ecofysiologisch onderzoek.

Een eerste studie, waarbij *high-throughput sequencing* gecombineerd met de kwantitatieve evaluatie van de volledige bacteriële gemeenschap aanwezig in estuariene sedimenten, toonde een onevenredige tienvoudige toename aan wanneer de MPB (*microphytobenthos*) biomassa verdubbelde. Onverwacht resulteerde diezelfde tienvoudige toename in bacteriële abundantie niet in een significant verschil in de structuur van de volledige bacteriële gemeenschap. Vergelijkbare resultaten werden bekomen voor zowel *nirK* als *nirS* denitrificeerders wat aantoont dat er geen competitie bestaat voor nitraat tussen denitrificeerders en MPB. Daarom werd vooropgesteld dat MPB waarschijnlijk eerder fungeren als

een algemene determinant van de bacteriële gemeenschap. De omgevingsvariabelen verantwoordelijk voor deze verdubbeling in MPB biomassa konden echter niet bepaald worden, waardoor geen causaal verband tussen MPB biomassa en bacteriële abundantie kon worden afgeleid.

Gemotiveerd door de algemeen erkende beperkingen van *nirK* primer *coverage*, werd in de tweede studie een gedetailleerde analyse uitgevoerd op een taxonomische diverse set van volledige NirK sequenties. De eerder geobserveerde opsplitsing van NirK in twee duidelijke fylogenetische clades werd herbevestigd waarbij NirK Clade I voornamelijk *Alphaproteobacteria* en de structurele Klasse I NirK bevatte, terwijl Clade II een meer diverse set van taxonomisch en structureel verschillende NirK omsloot. Tot nu toe is er geen informatie beschikbaar over de mogelijke verschillende ecologische strategieën of verspreiding van deze verschillende NirK clades, en net daarom wordt een systematische gebruik van de benamingen Clade I en Clade II gesuggereerd in toekomstig omgevingsonderzoek waarin NirK ingesloten wordt. In PCR afhankelijke studies zal de immense *nirK* sequentie divergentie, vermoedelijk veroorzaakt door variabele *nirK* gen evolutionaire routes, een blijvend probleem vormen voor *nirK* primer *coverage*, en dit zelfs voor clade of taxon specifiek primer design. Bepaling van de dissimilatorische stikstof reductie genen aanwezig in diverse NirK denitrificeerders toonde aan dat de simultane aanwezigheid van de denitrificatie en DNRA pathway meer algemeen voorkomend was dan oorspronkelijk gedacht aangezien in 67% van alle NirK denitrificeerders beide pathways in hun genoom werden teruggevonden.

In tegenstelling tot oorspronkelijk aangenomen, blijkt de bijdrage van DNRA in mariene stikstof verwijdering vergelijkbaar te zijn met die van denitrificatie. Stikstof reductiepotentialen van estuariene sedimenten werden daarom bepaald en toonden kleinschalige heterogeniteit aan in de aanwezigheid en snelheid van denitrificatie, DNRA en nitraatreductie tot nitriet. Denitrificatie was gelijkaardig op een cm tot meter schaal, terwijl DNRA en nitraatreductie tot nitriet locatie afhankelijk bleken te zijn en significant varieerden binnen 25 cm. De cruciale omgevingsparameters betrokken in de verdeling van nitraat tussen denitrificatie en DNRA blijven ongekend, maar omvatten alleszins niet de C:N ratio. Daarnaast werden nieuwe ongekende nitaat-tot-ammonium reduceerders geïsoleerd behorende tot *Thalassospira*, *Celeribacter* en *Halomonas*, en hun DNRA fenotype werd bevestigd door middel van *nrfA* gen amplificatie.

Samengevat toont deze doctoraatsthesis aan dat denitrificatie en DNRA beiden belangrijk zijn in nitraatverwijdering in gematigde estuariene ecosystemen. Desalniettemin, blijft kennis omtrent omgevingsparameters (zowel de fysicochemie als benthische organismen) die denitrificatie en DNRA en hun respectievelijke gemeenschappen bepalen, nog steeds beperkt. Potentieel belangrijke parameters afgeleid uit deze studie, samen met de nieuwe estuariene DNRA stammen vormen nu een goed startpunt voor verder onderzoek betreffende de bepaling van de belangrijke parameters voor elk proces in deze systemen. Het is daarnaast ook belangrijk rekening te houden met beperkingen van de

toegepaste techniek. Deze doctoraatsthesis toonde dit specifiek aan voor primer *coverage* beperkingen in PCR afhankelijke studies.

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Educational background

2011-Present	PhD – LM-UGent, Faculty of Science, Ghent University Dissertation: Functional diversity of dissimilatory nitrate reducers in estuarine sediments. Promotors: Prof. Anne Willems, Dr. Kim Heylen
2009-2011	Ghent University, Ghent, Belgium Degree of Master of Science in Biology Dissertation: Bacterial endosymbionts within the green algae *Bryopsis*: facultative versus obligate
2006-2009	Ghent University, Ghent, Belgium Degree of Bachelor of Science in Biology Dissertation: Isolation and identification of endosymbiotic bacteria in in *Bryopsis* sp.

Scientific output

A1 publications

Decleyre H., Heylen K., Tytgat B. and Willems A. (2015) Highly diverse *nirK* genes comprise two major clades that harbor ammonium-producing denitrifiers. Submitted to BMC Genomics.

Decleyre H., Heylen K., Van Colen C. and Willems A. (2015) Dissimilatory nitrogen reduction in intertidal sediments of a temperate estuary: small scale heterogeneity and novel nitrate-to-ammonium reducers. Front. Microbiol. 6:1124. doi: 10.3389/fmicb.2015.01124

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Oral presentations

Decleyre H., Heylen K., Willems A. (2015). Dissimilatory nitrogen reduction in intertidal sediments of a temperate estuary: small scale heterogeneity and novel nitrate to ammonium reducers. The 20th European Nitrogen cycle meeting, September 28th-30th, Aberdeen, Scotland.

Decleyre H., Heylen K., Tytgat B., Sabbe K., Van Colen C., Willems A. (2014). Microphytobenthos increases denitrifier and total bacterial abundances in intertidal sediments of a temperate estuary. The 19th European Nitrogen cycle meeting, September 10th-12th, Ghent, Belgium.

Decleyre H., Heylen K., Tytgat B., Van Colen C., Willems A. (2013). Diversity and abundance of dissimilatory nitrate reducers present in estuarine sediments of the Paulina Polder tidal flat, The Netherlands. The 18th European Nitrogen cycle meeting, September 18th-20th, Darmstadt, Germany.

Poster presentations

Decleyre H., Heylen K., Tytgat B., Sabbe K., Van Colen C., Willems A. (2014). Microphytobenthos affects abundance of denitrifying guild and total bacterial communities in a temperate intertidal estuary. The 15th International symposium on microbial ecology (ISME), August 24th-29th, Seoul, South-Korea.

Decleyre H., Heylen K., Tytgat B., Van Colen C., Willems A. (2013). Diversity and abundance of dissimilatory nitrate reducers present in estuarine sediments of the Paulina Polder tidal flat, The Netherlands. BSM symposium - Microbial diversity for science and industry, November 26th-27th, Brussels, Belgium.

Decleyre H., Vekeman B., Willems A., De Vos P., Heylen K. (2013). Bacterial sources and sinks of important greenhouse gases in marine sediments. PhD symposium, March 20th, Ghent, Belgium.

Decleyre H., Heylen K., Tytgat B., Van Colen C., Willems A. (2012). Diversity and abundance of dissimilatory nitrate reducers present in estuarine sediments of the Paulina Polder tidal flat, The Netherlands. The 17th European Nitrogen cycle meeting, September 26th-28th, Oslo, Norway.