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ON THE ECOLOGY OF DISSIMILATORY NITRATE REDUCTION TO AMMONIUM

ON THE ECOLOGY OF DISSIMILATORY NITRATE REDUCTION TO AMMONIUM

Proefschrift

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aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus prof. ir. K. C. A. M. Luyben,
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door

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ingenieur, geboren te Leiderdorp, Nederland

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CONTENTS

List of Abbreviations and Definitions

Abbreviations

Ac	acetate
ADI	applikon dependable instruments
ATP	adenosinetriphosphate
CSTR	continuous stirred tank reactor
C-mol	carbon-mol
DGGE	denaturing gradient gel electrophoresis
DNRA	dissimilatory nitratre reduction to ammonium
e-mol	electron-mol
FISH	fluorescent *in situ* hybridization
Lac	lactate
N	nitrogen
PCR	polymerase chain reaction
P/O-ratio	phosphate / oxygen ratio
VSS	volatile suspended solids

SUMMARY

The anthropogenic nitrogen inputs in the environment exceed the input by natural processes and impact the global nitrogen cycle considerably [33, 34]. Human meddling in the N-cycle occurs mainly in agricultural ecosystems. Loss of nitrogen from the agricultural soils, other than crop harvest, can have polluting effects on other environments. The three main processes through which the losses occur are ammonia volatilization, the production of gaseous nitrogen compounds and leaching of nitrate [15], contributing to acid rain, ozone depletion and eutrophication respectively. To reduce N-pollution and improve mitigation strategies, we need to expand our understanding of the metabolic and environmental controls of the nitrogen cycle processes [63, 103].

This thesis focuses on the microbial competition for nitrate between two dissimilatory nitrate reduction processes in the nitrogen cycle, as the different end-products entail important biogeochemical consequences for nitrogen retention in aquatic ecosystems such as wastewater treatment plants [14, 64], as well as the successful operation of wastewater treatment systems. Nitrate can be reduced to nitrogen gas in the denitrification process, removing the nitrogen from the environment, which is desired for alleviation of eutrophication or treatment of waste water. Alternatively, in the process of dissimilatory nitrate reduction to ammonium (DNRA), ammonium is the end product, and the nitrogen is conserved in the environment, which can be beneficial in fertilizer management.

A more quantitative understanding of this microbial competition for nitrate can be obtained using enrichment cultures in a laboratory reactor, yet no successful DNRA enrichment culture had been described before starting this study. We set-up an enrichment culture based on the hypotheses that nitrate limitation is the dominant factor in selecting for DNRA, using nitrate as electron acceptor and the non-fermentable substrate acetate as the electron donor (Chapter 2). First, a conventional denitrifying culture was enriched from activated sludge, with acetate and nitrate as substrates. Then, the acetate concentration in the medium was increased to obtain nitrate limiting conditions. As a result, conversions shifted from denitrification to DNRA. In this selection of a DNRA culture two important factors were the nitrate limitation and a relatively low dilution rate (0.026 h^{-1}). The culture was a highly enriched population of *Deltaproteobacteria* most closely related to *Geobacter lovleyi*, based on 16S-rRNA gene sequencing (97% similarity). We established a stable and reproducible cultivation method for the enrichment of DNRA bacteria in a continuously operated reactor system. Because of the systems simplicity, both regarding conversions and population, it is particularly suited for a mechanistic study of the DNRA ecology and concomitant microbial competition for nitrate.

The successful enrichment of DNRA confirmed the electron acceptor –nitrate– limiting conditions as an important selecting factor. In extension to the electron acceptor limitation, an often proposed factor in the success of DNRA is the ratio of electron donor over electron acceptor. We choose to study the impact of this ratio in our enrichment system using acetate and nitrate and therefore expressed this ratio as Ac/N

(acetate/nitrate-nitrogen) (Chapter 3). In the experiment, the conditions were changed stepwise from nitrate limitation to nitrate excess in the system by applying a variable Ac/N ratio in the feed. We observed a clear correlation between Ac/N ratio and DNRA activity and the DNRA population in our reactor. The DNRA bacteria dominated under nitrate limiting conditions in the reactor and were outcompeted by denitrifying bacteria under limitation of acetate. Interestingly, in a broad range of influent Ac/N ratios a dual limitation of acetate and nitrate occurred with co-occurrence of DNRA bacteria and denitrifying bacteria. To explain these observations, the system was described using a kinetic model. The model illustrates that the Ac/N effect and concomitant broad dual limitation range related to the difference in stoichiometry between both processes, as well as the differences in electron donor and acceptor affinities. Population analysis showed that the presumed DNRA-performing bacteria were the same under nitrate limitation and under dual limiting conditions, whereas the presumed denitrifying population changed under single and dual limitation conditions.

With the use of a mineral medium containing the non-fermentable acetate and nitrate, we created a simple system, but also a very selective environment. To understand to what extent the mechanism of the Ac/N ratio on the completion between denitrification and DNRA for nitrate can be extrapolated, the impact of the nature of the used substrates was tested. Nitrite was used as alternative electron acceptor to nitrate, and as alternative to the electron donor acetate we choose to use the fermentable substrate lactate.

Chapter 4 describes the role of nitrite versus nitrate as terminal electron acceptor on the competition. Initially, a mixed culture chemostat was operated under nitrate limitation and performed DNRA. Stepwise, the influent nitrate was replaced with nitrite until nitrite was the sole electron acceptor and N-source present. Despite changing the electron acceptor from nitrate to nitrite, the dominant process remained DNRA and the same dominant organism closely related to *Geobacter lovleyi* was identified. Contrary to previous studies conducted with a complex substrate in marine microbial communities [65], the conclusion of this work is that nitrate versus nitrite as electron acceptor does not generally control the competition between DNRA and denitrification. In combination with results of previous studies, our results suggest that the effect of this ratio must be interpreted in combination with other environmental factors.

Chapter 5 illustrates how the outcome of the competition for nitrate between DNRA and denitrification is greatly affected when the lactate and concomitant fermentation processes were introduced in the system. This was investigated for varying ratios of lactate and nitrate in the influent, termed Lac/N ratio. The study was conducted in an open chemostat culture, enriched from activated sludge, under anoxic conditions. The mechanistic explanation of the conversions observed was based on integration of results from specific batch tests with biomass from the chemostat, molecular analysis of the biomass enriched, and a computational model. At high Lac/N ratio (2.97 mol/mol) both fermentative and respiratory nitrate reduction to ammonium occurred, coupled to partial oxidation of lactate to acetate, and to acetate oxidation respectively. Remaining lactate was fermented to propionate and actetate. At a decreased Lac/N ratio (1.15 mol/mol), the molar percentage of nitrate reduced to ammonium decreased to 58%, even though lactate was supplied in adequate amounts for full ammonification and nitrate remained the growth limiting compound. Data evaluation at this Lac/N ratio suggested conversions were comparable to the higher Lac/N ratio, except for lactate oxidation to acetate that was

coupled to denitrification instead of ammonification. Respiratory DNRA on acetate likely is catalysed by two *Geobacter* species related to *G. luticola* and *G. lovleyi*. Two *Clostridiales* members were likely responsible for lactate fermentation and partial lactate fermentation to acetate coupled to fermentative DNRA. An organism related to *Propionivibrio militaris* was identified as the organism likely responsible for denitrification. The results of this study clearly show that not only the ratio of available substrates, but also the nature of the electron donor influences the outcome of competition between DNRA and denitrification. Apparently, fermentative bacteria are competitive for the electron donor and thereby alter the ratio of available substrates for nitrate reduction.

In Chapter 6 further steps towards a better mechanistic understanding of the DNRA success factors and the DNRA culture in our system are discussed, as well as other research perspectives regarding the role of DNRA in the environment.

SAMENVATTING

De hoeveelheid door mensenhanden gegenereerde (antropogene) stikstof die in het milieu terechtkomt, is vele malen groter dan wat er door natuurlijke processen wordt geproduceerd en dit heeft grote gevolgen voor de globale stikstofcyclus [33, 34]. De impact is het grootst in de landbouw, waar het weglekken van overmatige stikstofverbindingen uit de bodem kan zorgen voor vervuiling in andere delen van het milieu. Drie processen spelen hierin een grote rol: ammoniak vervluchtiging, het uitlogen van nitraat en de productie van gasvormige stikstofverbindingen [15]. Respectievelijk dragen deze processen bij aan zure regen, eutrofiëring en ozon depletie. Om nieuwe stikstofvervuiling terug te dringen en bestaande vervuiling te compenseren, is het belangrijk om onze kennis van alle processen en omgevingsfactoren die een rol spelen in de stikstofcyclus uit te breiden [63, 103].

Dit proefschrift gaat over de microbiële competitie voor nitraat tussen twee dissimilatieve nitraat reducerende processen in de stikstofcyclus. De verschillende eindproducten van deze processen hebben belangrijke biogeochemische consequenties voor stikstofophoping in waterige ecosystemen [14, 64]. Een voorbeeld hiervan zijn afvalwaterzuiveringsinstallaties, waar deze eindproducten van invloed zijn op het functioneren. Door het denitrificatieproces kan nitraat worden gereduceerd tot stikstofgas, waardoor stikstof uit het milieu wordt gehaald. Hierdoor kan dit proces bijdragen in het verminderen van eutrofiëring, maar ook gebruikt worden in de behandeling van afvalwater. Het tweede proces, genaamd dissimilatieve nitraatreductie naar ammonium (DNRA), resulteert in ammonium als eindproduct. Dit zorgt juist voor behoud van stikstof in het milieu, wat de hoeveelheid benodigde bemesting in de landbouw kan verminderen.

Een meer kwantitatief inzicht in de microbiële competitie voor nitraat kan worden verkregen door gebruik van verrijkingscultures in een laboratoriumreactor, maar voor het begin van deze studie was er nog geen succesvolle DNRA verrijkingsculture beschreven. Wij hebben daarom een verrijkingsculture opgezet, welke gebaseerd was op de hypothese dat nitraatlimitatie de bepalende factor is in een selectie voor DNRA. In deze culture fungeerde nitraat als elektronacceptor en het niet-fermenteerbare substraat acetaat als elektrondonor (Chapter 2). Eerst werd een conventionele denitrificerende culture verrijkt uit actief slib. Vervolgens werd de acetaatconcentratie in het medium verhoogd om nitraatgelimiteerde condities te verkrijgen, wat resulteerde in een verschuiving van denitrificatie naar DNRA. In de selectie voor de DNRA culture was, behalve de nitraat-gelimiteerde condities, ook de relatief lage verdunningssnelheid ($0.026 h^{-1}$) een belangrijke factor. Doormiddel van 16S-rRNA sequencing werd bepaald dat de culture voornamelijk bestond uit een populatie van *Deltaproteobacteriën* die sterk verwant is aan de soort *Geobacter lovleyi* (97% overeenkomst). We hebben een stabiele en reproduceerbare kweekmethode ontwikkeld voor de verrijking van DNRA bacteriën in een continu reactorsysteem. Omdat dit een eenvoudig systeem betreft, zowel in de omzettingen als populatie, is het zeer geschikt voor mechanistische studies naar DNRA ecologie en bijbehorende microbiële

competitie voor nitraat.

De succesvolle verrijking van DRNA bevestigde dat het limiteren van de elektronacceptor, nitraat in dit geval, een belangrijke selecterende factor is. In het verlengde hiervan wordt vaak ook de ratio van elektrondonor ten opzichte van elektronacceptor aangevoerd als mogelijke factor in de selectie voor DNRA. Wij hebben ervoor gekozen deze ratio te onderzoeken met ons verrijkingssysteem en omdat dit systeem gebruik maakt van acetaat en nitraat, wordt de donor/acceptor ratio uitgedrukt als Ac/N (acetaat/nitraat-stikstof) (Chapter 3). Gedurende het experiment werden de condities in het systeem stapsgewijs aangepast van nitraatlimitatie naar nitraatoverschot, doormiddel van het variëren van de Ac/N ratio in de invoer. We constateerden dat er een duidelijke correlatie was tussen de Ac/N ratio, DNRA activiteit en de DNRA populatie in onze reactor. De DNRA bacteriën hadden de overhand gedurende nitraat-gelimiteerde condities in de reactor, maar werden weggeconcurreerd door de denitrificeerders tijdens acetaatlimitatie. Opvallend was dat in een breed bereik van Ac/N ratio's er een dubbele limitatie optrad van zowel acetaat als nitraat. Dit had tot gevolg dat DNRA bacteriën en denitrificeerders samen voorkwamen in de reactor. Om deze observaties te verklaren werd het systeem beschreven doormiddel van een kinetisch model. Het model laat zien dat het Ac/N effect, en het bijbehorende brede bereik van dubbele limitatie, zijn gerelateerd aan zowel het verschil in stoichiometrie tussen beide processen, als het verschil in affiniteit voor elektrondonor en -acceptor. Doormiddel van populatieanalyse werd duidelijk dat de veronderstelde DNRA-uitvoerende bacteriën hetzelfde waren onder zowel nitraatlimitatie als dubbele limitatie. De veronderstelde denitrificerende populatie daarentegen, verschilde wel degelijk tussen beide condities.

Doormiddel van een medium dat nitraat en het niet-fermenteerbare acetaat bevatte, hebben we niet alleen een eenvoudig systeem gecreëerd, maar ook een zeer selectieve omgeving. Om te begrijpen in hoeverre het mechanisme van de Ac/N ratio en de competitie tussen denitrificatie en DNRA voor nitraat geëxtrapoleerd kunnen worden, bestudeerden we het effect van de gebruikte substraten op de competitie. Nitriet werd in plaats van nitraat gebruikt als elektronacceptor, en het fermenteerbare substraat lactaat werd gekozen als alternatieve elektrondonor.

Chapter 4 beschrijft de rol van nitriet tegenover nitraat als uiteindelijke elektronacceptor in de competitie tussen de twee processen. Eerst werd een chemostaat met gemengde culture gedraaid onder nitraatlimitatie en DNRA vond plaats. Het ingevoerde nitraat werd stapsgewijs vervangen door nitriet, totdat dit de enige aanwezige elektronacceptor en N-bron was. Ondanks het vervangen van de elektronacceptor bleef DNRA het dominante proces en werd hetzelfde dominante organisme, gerelateerd aan *Geobacter lovleyi*, geïdentificeerd. In tegenstelling tot voorgaande studies, die een complex substraat gebruikten in een marine microbiële populatie [65], is de conclusie van dit project dat nitraat tegenover nitriet als elektronacceptor is op zichzelf niet bepalend in competitie tussen DNRA en denitrificatie. Samen met resultaten uit voorgaande studies laten onze resultaten zien dat het effect van deze verhouding geïnterpreteerd moeten worden in combinatie met andere omgevingsfactoren.

Chapter 5 laat zien dat introductie van lactaat, en de bijbehorende fermentatieprocessen, in het systeem de uitkomst van de competitie voor nitraat tussen DNRA en denitrificatie sterk beïnvloed. Dit was onderzocht voor verschillende verhoudingen tussen lactaat en nitraat in de invoer, hierna Lac/N ratio genoemd. De studie werd uitgevoerd in een

open chemostaat culture, verrijkt uit actief slib en onder anoxische condities. De mechanistische verklaring van de geobserveerde omzettingen werd gebaseerd op de integratie van resultaten verkregen uit specifieke batch testen met de biomassa uit de chemostaat, alsmede door moleculaire analyse van de verrijkte biomassa en een computermodel. Bij een hoge Lac/N ratio (2.97) vond zowel fermentatieve als respiratiore nitraatreductie naar ammonium plaats, gekoppeld aan respectievelijk partiele oxidatie van lactaat naar acetaat en acetaatoxidatie. Het overgebleven lactaat werd gefermenteerd tot propionaat en acetaat. Bij een lagere Lac/N ratio (1.15) verminderde het molaire percentage nitraat dat werd gereduceerd tot ammonium naar 58%. Dit gebeurde terwijl lactaat in voldoende mate aanwezig was voor volledige ammonificatie en nitraat nog steeds de groei-limiterende factor was. Onderzoek van de data bij deze Lac/N ratio suggereerde dat bijna alle omzettingen vergelijkbaar waren met die bij hogere Lac/N ratio, behalve de lactaat oxidatie naar acetaat die was gekoppeld aan denitrificatie in plaats van ammonificatie. De respiratoire DNRA werd uitgevoerd door *Geobacter* stammen die zeer verwant zijn aan *G. luticola* en *G. lovleyi*. Twee leden van de *Clostridiales* waren waarschijnlijk verantwoordelijk voor de lactaatfermentatie en de partiele lactaatfermentatie naar acetaat, die was gekoppeld aan fermentatieve DNRA. Een organisme gerelateerd aan *Propionivibrio militaris* werd geïdentificeerd als waarschijnlijk verantwoordelijk voor denitrificatie. De resultaten van deze studie laten duidelijk zien dat niet alleen de verhouding tussen beschikbare substraten, maar ook de aard van de elektrondonor de uitkomst van de competitie tussen DNRA en denitrificatie beïnvloeden. Blijkbaar concurreren de fermentatieve bacteriën voor de elektrondonor en zorgen ze daarbij voor een verandering in de verhouding van beschikbare substraten voor nitraatreductie.

In Chapter 6 worden de volgende stappen bediscussieerd die genomen kunnen worden richting een beter mechanistisch begrip van zowel de DNRA succesfactoren, als de DNRA culture in ons systeem. Ook wordt er ingegaan op andere onderzoeksmogelijkheden aangaande de rol van DNRA in het milieu.

1

INTRODUCTION

Eveline M. VAN DEN BERG

1

1.1. INTRODUCTION

NITROGEN is one of the primary elements in cell matter, usually taken up in the form of nitrate or ammonium. As these easily soluble forms of nitrogen are available in limiting quantities, nitrogen is an important factor controlling plant, animal and microbial growth [63]. To alleviate this limitation for food production, large scale industrial production of soluble forms of nitrogen is taking place for use as fertilizer for crop growth, using the Haber Bosch process, whereby gaseous dinitrogen is converted to ammonia ($N_2 + 3H_2 \rightarrow 2NH_3$). This supports food production for approximately 48% of the global human population [103]. However, part of the input nitrogen is lost from the agricultural ecosystem, thereby causing overfertilization and hence polluting other environments. The anthropogenic nitrogen inputs exceed the input by natural processes and impact the global nitrogen cycle considerably (Figure 1.1) [33, 34], thus bringing the natural nitrogen cycling out of balance. This is augmented by dislocated recycling due to massive international transport of agricultural products [34, 68]. Stein and Klotz [103] formulated: "*The fate of humanity is intertwined with our ability to control the nitrogen cycle*".

Loss of nitrogen from the agricultural soils occurs through three main processes [15], all of which can have polluting effect. Probably the most harmful of the three is the nitrogen pollution as result from leaching of nitrate via the groundwater into rivers and lakes, and into the seas. This can cause eutrophication, resulting ultimately in considerable damage or even destruction of fresh water ecosystems and increasing dead zones in coastal areas [103]. Substantial nitrate and ammonium contamination also stems from industrial and wastewater streams. Together they are directly responsible for contamination of groundwater. This augments problems in obtaining nitrate concentration in drinking water below the established health standards.

Another harmful process is the production of gaseous nitrogen compounds – nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen gas. Whereas the production of dinitrogen gas can be beneficial for removal of excess nitrogen, emissions of the greenhouse gasses nitric and nitrous oxide are hazardous, as they strongly contribute to ozone-depletion [63, 87]. The third process is ammonia volatilization, which contributes to acid rain and eutrophication of (oligotrophic) natural environments.

Restriction and management of fertilizer use and the artificial recycling in wastewater treatment systems have already reduced nitrogen pollutions. For further reduction and mitigation, we need to expand our understanding of the metabolic and environmental controls of the nitrogen cycle processes [63, 103].

This thesis focuses on the microbial dissimilatory nitrate reduction processes in the nitrogen cycle, as the different end products can have important implications for the ecosystem [14, 64], and moreover is relevant for the successful operation of wastewater treatment systems. Nitrate can be reduced to nitrogen gas in the denitrification process, removing the nitrogen from the environment, which is desired for alleviation eutrophication or treatment of waste water. However, incomplete denitrification may result in emission of the gaseous intermediates nitric and nitrous oxide. In the process of dissimilatory nitrate reduction to ammonium (DNRA), ammonium is the end product, and the nitrogen is conserved in the environment, which can be beneficial in fertilizer management. The positively charged ammonium-ion is generally retained in soil and sediments by absorption to the negatively charged clay, whereas the negatively charged nitrate and

nitrite anions easily leach away [96]. DNRA impairs the wastewater treatment process, since nitrogen is retained in the water in its original form, ammonium, instead of being converted in dinitrogen gas. In both nitrate reducing processes the reduction equivalents or electron donors usually originate from the oxidation of organic compounds, but both reactions can also be driven by inorganic oxidation processes such as hydrogen and sulfur oxidation. In a third process, the autotrophic anaerobic ammonium oxidation (anammox) bacteria oxidize ammonium with available nitrite to nitrogen gas. Additionally these bacteria are capable of performing DNRA [17, 57], but this is not considered relevant in carbon source abundant enrichments.

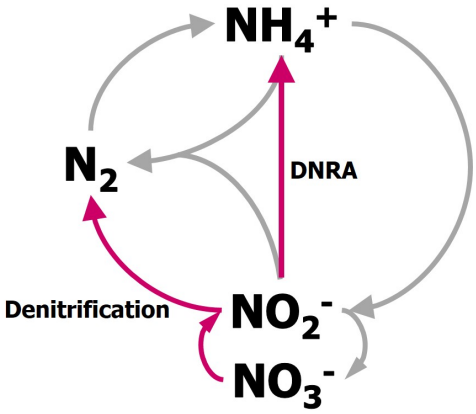

Figure 1.1: Simplified overview of the inorganic nitrogen cycle. Biological or industrial fixation of dinitrogen leads to ammonium, which under aerobic conditions can be converted by nitrifying Bacteria or Archaea to nitrite and nitrate. Under anaerobic/anoxic conditions, nitrite can be combined with ammonium by anammox bacteria to give dinitrogen. In the absence of oxygen nitrate and nitrite can also be converted to dinitrogen by denitrifying Bacteria or to ammonium by bacteria capable of dissimilatory nitrate or nitrite reduction (DNRA)

Nitrate reduction

Many denitrifying bacteria are facultative denitrifiers, which prefer use of oxygen as an electron acceptor, because of the higher energy yield. Nevertheless, some bacteria denitrify under both aerobic and anoxic conditions, which is termed aerobic denitrification [89]. DNRA, unlike denitrification, has no aerobic counterpart. DNRA bacteria are mostly facultative anaerobes. Under anaerobic conditions they are often able to ferment, and potentially also couple re-oxidation of NADH to direct reduction of nitrite [110].

Phylogenetically the ability to perform denitrification occurs in diverse groups of bacteria, but most of the studied and isolated denitrifiers are of the phylum *Proteobacteria* (Kraft et al. [64] and references therein). Also, the trait of DNRA has been found amongst gram-negatives as of the phylum *Proteobacteria*, and additionally in *Bacteroides* and members of the gram-positive *Bacilli* and a variety of taxa belonging to the *Firmicutes* [126]. For a long time, bacteria were thought to be only capable of either of the two processes [130]. In recent years, several bacteria have been found harboring the genes for both pathways (e.g. Heylen and Keltjens [50]), and one bacterium was confirmed to perform both denitrification and DNRA [127].

1

In both the denitrification and DNRA process, the first step of the pathway is the reduction of nitrate to nitrite. In this step, two electrons are transferred per mole of nitrate. The conversion can take place either in the cytoplasm or the periplasm of the (gram-negative) bacteria, each catalyzed by a different enzyme system (Figure 1.2). Both systems receive electrons from NADH dehydrogenase, when using an organic electron donor, thereby generating a proton motive force across the bacterial membrane, which is used for ATP synthesis. In the cytoplasm, a membrane bound nitrate reductase, Nar, catalyzes the reduction of nitrate to nitrite (Figure 1.2b). The heterodimeric complex of Nar translocates two protons by oxidizing ubiquinone, generating proton motive force. This conversion requires transport of nitrate into the cell and of the formed nitrite to the periplasm for further reduction, e.g. by a nitrate/nitrite antiporter or a nitrate/proton symporter (Figure 1.2a) [64]. Nitrate reduction in the periplasm is catalyzed by the periplasmic nitrate reductase, Nap (Figure 1.2c) [130]. This periplasmic enzyme is found almost exclusively in the phylum *Proteobacteria*, and many of them encode both the Nar and Nap system [98]. Electron transfer to Nap is mediated by membrane-bound quinol-oxidizing subunits, which has not been shown to result in generation of a proton motive force [98]. Nar and Nap enzyme systems have a different physiological role, which was demonstrated by Potter et al. [85] for enteric bacteria. Nar is an energy-efficient enzyme, with high activity, but low affinity for nitrate and requires nitrate transport, whereas Nap exhibits a high nitrate affinity but low activity. Therefore Nar is used when nitrate is in excess, i.e. non-limiting and Nap is useful for conditions where nitrate availability is limiting [75].

Denitrification

Denitrification from nitrite consist of three further reduction steps ($NO_2^- \rightarrow NO \rightarrow \frac{1}{2}\ N_2O \rightarrow \frac{1}{2}\ N_2$). In each of the steps one electron is transferred (per mol N), hence in denitrification a total of 5 electrons are transferred per nitrate. Each step is catalyzed in the periplasm or outer membrane space, by a different enzyme (Figure 1.3a). For use of an organic electron donor, the enzymes receive electrons from NADH dehydrogenase via cytochrome *bc1* complex, which is mediated by (ubi)quinones (UQ), and subsequently by a periplasmic pool of electron-transferring proteins [98], in Figure 1.3a a cytochrome *c* like protein is depicted. The transfer from the quinol via the cytochrome *bc1* complex generates proton motive force. In this way, despite that (most of) the enzymatic conversions in itself are electron neutral processes [98, 120], the three reduction steps contribute to the buildup of proton motive force.

First of the three steps is the nitrite reduction to nitric oxide in the periplasm, by one of two different nitrite reductases. One is a *cd1*-type NiR, which is a dimeric protein with *d1*-type hemes at the active site. The second is a copper-type NiR (CuNiR), a trimer with two types of copper centers.

The nitric oxide is subsequently reduced by the nitric oxide reductase, Nor, a cytoplasmic enzyme reducing the nitric oxide to nitrous oxide. Three prokaryotic classes of Nor have been identified. Of these, the cNor and qNor types are the most known nitric oxide reductase. The cNor consist of two subunits, one larger and the smaller subunit anchored to the membrane, while qNor are single subunit enzymes that are reactive with quinones. Despite the qNor genetic potential, no translocation of protons is shown to occur [4, 98].

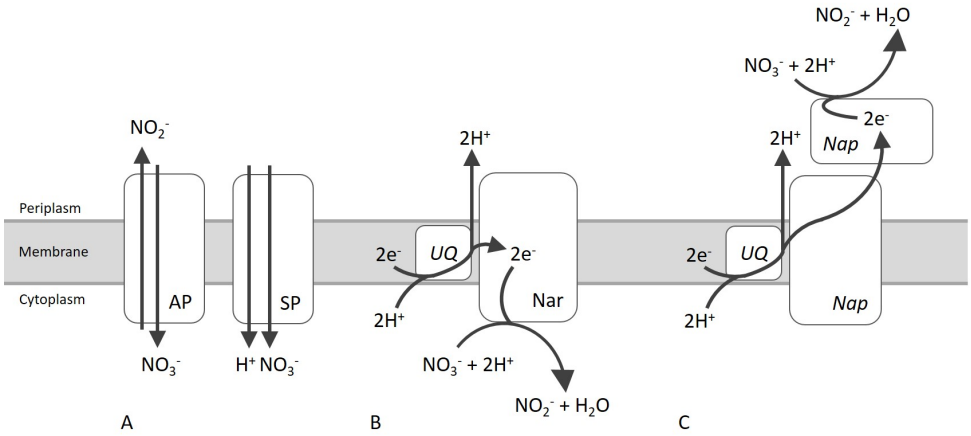

Figure 1.2: Compilation of electron transport in nitrate reduction to nitrite, adapted from Kraft et al. [64] and Torres et al. [112]. In these conversions a proton gradient is produced, which is required for subsequent ATP production. Ubiquinone (UQ) is depicted as redox mediator, which receives electrons from e.g. a NADH-dehydrogenase and donates electrons to the nitrate reductases. (A) Nitrate transport over the membrane is depicted via antiport with nitrite (AP) and symport with a proton (SP). (B) The membrane bound cytoplasmic nitrate reductase, Nar. (C) The periplasmic nitrate reductase, Nap, which has a subunit anchored to the membrane.

A third type, is the Nor from *Bacillus azotoformans* (CuANor), a hetero-trimeric protein with a di-copper site (CuA). CuANor is exceptional because it translocates one proton per NO and hence, generates additional proton motive force [4].

The last step in denitrification is the reduction of nitrous oxide to dintrogen gas by the nitrous oxide reductase, Nos. Although this step is energetically (i.e. thermodynamically) the most favored one, only a small part of the potential energy is harvested. Canonical Nos is a homodimeric enzyme with a copper center. In some bacteria Nos is a subunit of a membrane-bound quinol-oxidizing complex [98], which is an alternative to electron transport from the quinone pool to Nos via the cytochrome *bc1* complex.

A different denitrification pathway is used when it is coupled to anaerobic methane oxidation, which also known as 'nitrate/nitrite-dependent anaerobic methane oxidation'(n-damo) [31, 86]. In this conversion two molecules of the intermediate nitric oxide, which are normally converted to nitrous oxide in the canonical denitrification, are converted to dinitrogen and oxygen. Oxygen is subsequently used to oxidize methane with an oxygenase [32].

DNRA

Reduction of nitrite to ammonium in the DNRA process is performed in one step, in which six electrons are transferred by one enzyme, without the release of any intermediate. The pentaheme cytochrome *c* nitrite reductase Nrf, is the best known nitrite reductase generating ammonium (Figure 1.3b) [97]. The enzyme occurs either as a soluble protein or as a subunit of a membrane-bound menaquinol-reactive complex. As in the periplasmic denitrification steps, the Nrf-catalyzed conversion itself is electron neutral and contri-

1

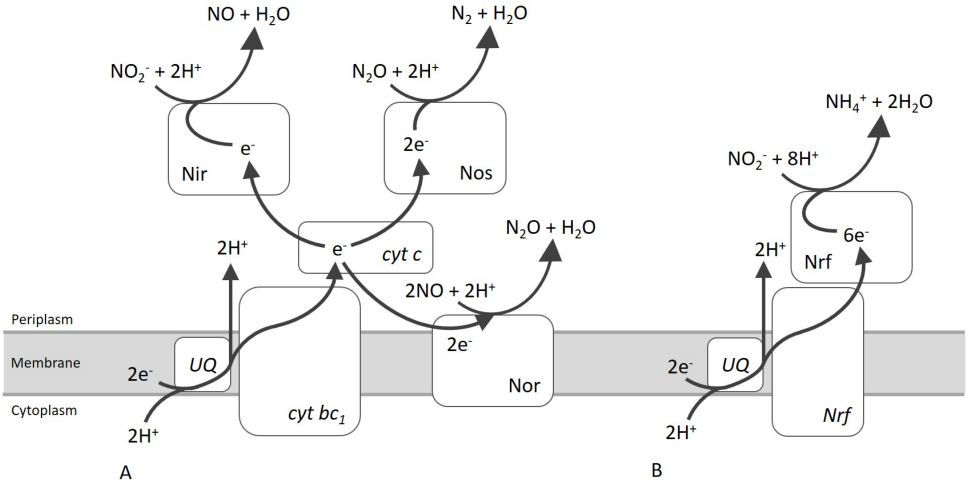

Figure 1.3: Compilation of the electron transport chains of nitrite reduction by denitrification (A) and DNRA (B), adapted from Kraft et al. [64] and Torres et al. [112]. In these processes a proton gradient is produced, which is used for subsequent ATP production. The amount of electrons shown transferred at the cytochromes is per step. Ubiquinone (UQ) is depicted as redox mediator, which receives electrons from e.g. a NADH-dehydrogenase. (A) In the denitrification electrons are transported from the redox mediators, via the cytochromes (cyt), here depicted are complexes *bc1* and *c*, to either the nitrite reductase Nir, nitric oxide reductase Nor (with membrane anchor) or the nitrous oxide reductase Nos. Note that in each step, one electron is transferred per nitrogen; a total of three electrons per NO_2^-. (B) In the DNRA using Nrf, electrons are transported from the redox mediators to the ammonium forming nitrite reductase Nrf, which is anchored to the membrane. In this one conversion step six electrons are transferred.

bution to proton motive force is achieved by quinol-mediated electron transfer from the NADH dehydrogenase [98]. Furthermore, the Nrf enzyme can commonly also convert other substrates like hydroxylamine, nitric oxide, sulfite and hydrogen peroxide and is therefore suggested to have a function in cell detoxification [98] and references therein). Another ammonium-generating nitrite reductase is NirB, which detoxifies nitrite formed by nitrate reduction in the cytoplasm without conserving energy [84]. Furthermore, octaheme cytochromes have been described to, amongst other substrates, convert nitrite to ammonium [99]. Alternatively, a reversely operating hydroxylamine oxidoreductase is suggested to function as a nitrite reductase [43].

Competition for nitrate

Denitrification and DNRA can occur in similar conditions in absence of oxygen or at low oxygen concentrations [65, 111]. Denitrification was long assumed to be the dominant nitrate reduction process in the environment. Recently, N-labeling experiments have indicated that DNRA may also contribute significantly [11, 14, 64, 91]. While the denitrification process is very well studied and understood to a great extent [52, 64], DNRA has received relatively little attention in the analysis and description compared to the other processes of the nitrogen cycle [104]. Although the physiology and bioenergetics of DNRA are relatively well studied in a selected number of pure cultures, the (quantitative) role of

DNRA in both engineered and natural ecosystems and its contribution to the nitrogen cycle received relatively little attention. As a result, little is known about the role of DNRA in the nitrogen cycle and the factors controlling its success [14, 64].

Lab- and field studies have generated several similar hypotheses on promoting conditions for DNRA. The suggested dominant selecting condition is low or limiting nitrate availability [64, 111]. This is generally attributed to the capacity of DNRA to accept eight electrons per nitrate molecule, compared to five by denitrification. However, one should also consider the potential change per electron in the electron acceptor reaction, which is lower for DNRA. The catabolic Gibbs energy gain per nitrate suggests a slightly higher yield for DNRA over denitrification [105, 111], but with such small differences probably the biochemistry rather than the available Gibbs energy will define the differences in growth yields.

Laboratory studies mainly consist of batch tests with environmental samples (e.g. [44, 59, 101]), in which, as in field studies, the system and microbial community were insufficiently defined. They also include pure culture studies (e.g. [22, 29, 88, 105, 127]), but these bacterial cultures have usually not been enriched and isolated on the basis of their DNRA capacity. An enrichment culture experiment specific for DNRA bacteria has been described by Kraft et al. [65], where they obtained DNRA bacteria in a marine mesocosm continuous system. The substrate in this system was a complex mixture of different carbohydrate and amino acids. When nitrate was provided as the exclusive electron acceptor, DNRA bacteria were enriched in the system, but denitrifiers were enriched when nitrite was fed. Therefore they postulated nitrate vs nitrite is an important selecting factor and that this is probably caused by a slightly higher apparent affinity for nitrite of the cytochrome *cd1* nitrite reductases of denitrification. They additionally established that denitrification is dominant at shorter generation times, and hypothesize that the ammonium producing nitrite reductase is slower than the NO producing nitrite reductase, and therefore not able to keep up with the denitrification at higher growth rates. Furthermore, they confirmed that limitation of the electron acceptor nitrate was necessary for successful selection of DNRA bacteria. Caution should be taken when extrapolating these results as other factors could have (strongly) contributed to the selective pressures in the complex marine culture growing on a mixture of fermentable and non-fermentable substrates [65].

We concluded that in order to study the base of the controlling factors of the DNRA process, it would be desirable to enrich for bacteria based on their DNRA capacity under simulated environmental conditions which are better defined in terms of carbon and nitrogen-turnover. Therefore, we aimed to set up a simple system and used the non-fermentable substrate acetate, which is an important substrate in anaerobic systems. In addition, we decided to investigate a fresh water ecosystem, which is more relevant for terrestrial/agricultural systems as well as common wastewater treatment systems, in which sulfate and sulfur cycling are less prominent.

In batch cultures the outcome of the competition between different microorganisms is determined by the maximum specific growth rate. In continuous systems, the competition is based on affinity for the growth limiting substrate [37, 66]. As nitrate limitation is an important factor for the selection of DNRA, it is essential to create this condition for a study into the nitrate reduction processes. The best experimental tool appears to be cultivation under nitrate limitation in continuous culture, either with relevant pure cultures or with

enrichment cultures [65]. Additionally, the use of a substrate limited continuous culture yields more reproducible and dependable data for the study of microbial competition than the batch cultivation mode [66].

Outline of this thesis

To gain more insight in the ecophysiology of DNRA bacteria and their competition for nitrate with denitrifiers, a lab chemostat DNRA enrichment culture was developed (Chapter 2), using nitrate as the only electron acceptor and a single non-fermentable substrate, i.e. acetate, as the carbon and energy source/electron donor. Because of the systems simplicity, both regarding conversions and population, it is particularly suited for a mechanistic study of the DNRA ecology and concomitant microbial competition for nitrate. We used this enrichment culture set-up as basis for the further studies described in this thesis. The successful enrichment of DNRA confirmed the electron acceptor –nitrate– limiting conditions as an important selecting factor. In extension to the electron acceptor limitation, an often adduced factor in the success of DNRA is the ratio of electron donor over electron acceptor. Chapter 3 describes the underlying mechanisms of this effect as observed in our simple system. To understand to what extent this mechanism can be extrapolated, the impact of the nature of the used substrates was tested. Use of nitrite as alternative electron acceptor to nitrate did not alter the mechanistic observations, as presented in Chapter 4. As alternative to the non-fermentable electron donor acetate we choose to use the fermentable substrate lactate. Chapter 5 illustrates how the outcome of the competition is greatly affected when the lactate and concomitant fermentation processes were introduced in the system. In Chapter 6 further steps towards a better mechanistic understanding of the DNRA success factors and the DNRA culture in our system are discussed, as well as other research perspectives regarding the role of DNRA in the environment and in particular in wastewater treatment systems.

2

ENRICHMENT OF DNRA BACTERIA IN A CONTINUOUS CULTURE

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Abstract

Denitrification and dissimilatory nitrate reduction to ammonium (DNRA) are competing microbial nitrate reduction processes. The occurrence of DNRA has been shown to be effected qualitatively by various parameters in the environment. A more quantitative understanding can be obtained using enrichment cultures in a laboratory reactor, yet no successful DNRA enrichment culture has been described. We showed that a stable DNRA dominated enrichment culture can be obtained in a chemostat system. The enrichment was based on the hypotheses that nitrate limitation is the dominant factor in selecting for DNRA. First, a conventional denitrifying culture was enriched from activated sludge, with acetate and nitrate as substrates. Then, the acetate concentration in the medium was increased to obtain nitrate limiting conditions. As a result, conversions shifted from denitrification to DNRA. In this selection of a DNRA culture two important factors were the nitrate limitation and a relatively low dilution rate (0.026 h^{-1}). The culture was a highly enriched population of Deltaproteobacteria most closely related to Geobacter lovleyi, based on 16S-rRNA gene sequencing (97% similarity). We established a stable and reproducible cultivation method for the enrichment of DNRA bacteria in a continuously operated reactor system. This enrichment method allows to further investigate the DNRA process and address the factors for competition between DNRA and denitrification or other N-conversion pathways.

2.1. INTRODUCTION

DISSIMILATORY reduction of nitrate is a well-studied microbial process, which is embodied in three main pathways in the nitrogen cycle: denitrification, anaerobic ammonium oxidation and dissimilatory nitrate reduction to ammonium (DNRA) [64]. All three processes compete for nitrate and nitrite. In this paper we will focus on the competition between denitrification and DNRA. During denitrification, nitrate is reduced to nitrogen gas, while in DNRA, ammonium is the end product. The denitrification process is very well studied and understood to a great extent [52, 64]. On the other hand, little is known about the role of DNRA in the nitrogen cycle and the factors controlling its success [14, 64].

A number of field studies report the occurence of DNRA in soils, sediments, anoxic zones in waters, and other sites [14, 64, 91]. These studies indicate that DNRA bacteria are generally found in anoxic, electron donor-rich zones with a low nitrate availability. Lab- and field studies generated several similar hypotheses on promoting conditions for DNRA. The dominant suggested selecting condition is low or limiting nitrate availability, which is mostly conveyed as a high mass ratio of available electron donor (Chemical Oxygen Demand (COD) equivalents) over nitrate-nitrogen (COD:N) [3, 59, 91, 111]. However none of the selective conditions have been experimentally substantiated and little is known about the underlying mechanisms.

Laboratory studies mainly consist of batch tests with environmental samples (e.g. [59, 101]), in which, as in field studies, the system and microbial community was insufficiently defined. They also include a few pure culture studies (e.g. [88, 105]), but in how far those represent environmental populations is unclear. These bacterial cultures have usually not been enriched and isolated on the basis of their DNRA capacity. Enrichment culture experiments specific for DNRA bacteria have not been described. Yet, performing this kind of experiments is essential to acquire better understanding of the DNRA process. Bacteria that are competitive based on their DNRA capacity are enriched and the environmental conditions can be simulated reasonably well while the system is quantitatively defined in terms of carbon and nitrogen-turnover. Nitrate limited growth conditions can for instance not be achieved in a batch culture, but can be easily achieved in a chemostat reactor. Recently, [65] used such an approach to study the role of DNRA in the nitrogen conversions of a marine sediment environment. Due to the complex substrate used, a complex microbial community of fermentative denitrifying and DNRA bacteria was enriched making it difficult to identify and study the DNRA organisms as such.

This study aimed to develop a cultivation method for the enrichment of a highly enriched population of DNRA bacteria in a mixed, open culture, the nutrient-limited chemostat. A conventional denitrifying culture was enriched from activated sludge, with acetate and nitrate as substrates. Then, based on the proposed hypotheses, the COD:N ratio in the medium was gradually increased to shift conversions from denitrification to DNRA. The enrichment culture is well suited to systematically study the DNRA process, and its competition with denitrification or other N-conversion pathways.

2.2. MATERIALS AND METHODS

Chemostat reactor operation

A double-jacket glass bioreactor with a working volume of 2 L (Applikon, Delft, The Netherlands) was used for the cultivation of a denitrifying culture. The reactor was operated as an open continuous stirred-tank reactor (CSTR, i.e. a flow controlled chemostat) and inoculated with activated sludge from the wastewater treatment plant (WWTP) Leiden-Noord, The Netherlands. The reactor was operated at 400 rpm with a stirrer that contained two standard geometry six-blade turbines. The flow of nitrogen gas to the reactor was kept at 50 Nml/min using a mass flow controller (Brooks Instrument, Ede, The Netherlands) and the reactor temperature was controlled at 20 °C by means of a water jacket and a cryostat bath (Lauda, Lauda-Königshofen, Germany). The concentration of dissolved oxygen (DO) in the reactor was measured using a DO electrode (Mettler Toledo, Tiel, The Netherlands) as percentage of air saturation. The pH of the reactor liquid was monitored with a pH electrode (Mettler Toledo) and was maintained at 7.1 ± 0.05 using 0.5 M HCl and 0.5 M NaOH. The pH pumps and the pH were controlled by an ADI 1030 biocontroller (Applikon). MFCS/win (Sartorius Stedim Systems, Bohemia, NY, U.S.A.) was used for data acquisition of the online measurements (dissolved oxygen (DO), pH, temperature, acid dosage, base dosage).

The dilution rate of the system was controlled at 0.026 ± 0.001 h^{-1} and the influent and effluent were pumped using peristaltic pumps (Masterflex, Vernon Hills, IL, USA). The effluent pump was controlled by a level sensor. The influent pumps, using L/S® 14 mm tubes, were set to pump 26 ml/h. The medium was supplied in two separate flows of a mineral medium (A) and substrate medium (B), thus a total of 52 ml/h influent was pumped in.

The culture media was autoclaved before use and sparged with a small flow of nitrogen gas while connected to the chemostat. Medium A contained per liter (day 0-271): 7.4 mmol KH_2PO_4, 0.41 mmol $MgSO_4{\cdot}7H_2O$, 0.37 mmol NaOH, 0.02 mmol yeast extract, 4 ml trace element solution [123], with only 2.2 g $ZnSO_4{\cdot}7H_2O$, and $NaNO_3$ and NH_4Cl. The concentration of $NaNO_3$ was 6.7 mM (day 0 untill 39), or 5.9 mM (from day 39). NH_4Cl concentrations were 0.01 mM (day 26-68), 0.02 mM (day 1-26, 68-82 and 94-122), 0.04 mM (day 82-94 and 122-186) and was finally omitted (from day 186). Medium B contained, per liter, initially 2.8 mM $NaCH_3COO{\cdot}3H_2O$; this was gradually increased to 4.4 mM (day 26 untill 39), 5.1 mM (day 39 untill 47), 6.3 mM (day 47 untill 122) or 9.9 mM (day 122-271).

Balances were set up over the reactor conversions. The nitrogen not accounted for in ammonium, nitrate, nitrite or biomass was assumed to be converted to N_2. The concentration of volatile suspended solids (VSS) was used for the biomass. For the computation of the CO_2 production rate from the off gas partial pressure we used the molar gas volume 24.5 l/mol. Losses by wash out of dissolved CO_2 and ionized species are included in the balancing.

Analytical procedures

Oxygen, carbon dioxide, nitric oxide and nitrous oxide concentrations in the headspace of the reactor were monitored in dried gas using a gas analyzer (NGA 2000, Rosemount, Chanhassen, MN, U.S.A.). To obtain a sufficient gas flow in the analyzer for quick response, gas was circulated in a closed loop between the analyzer and the head space at a rate of

400 ml/min. The headspace volume of the reactor set up was 1 l.

Samples taken from the reactor for analysis of acetate and nitrogen compounds were immediately filtered through a 0.45 µm pore size filters (PVDF membrane, Merck Millipore, Carrigtohill, Ireland). Initially, the acetate concentration in the liquid phase was measured as chemical oxygen demand (COD). After 3 weeks the acetate concentration was measured with a Chrompack CP 9001 gas chromatograph (Chrompack, Middelburg, The Netherlands). Samples were separated on a HP Innowax column (Aligent Technologies, Santa Clara, CA, USA) and compounds were detected with a flame ionization detector (FID). An indication of the nitrite- and nitrate concentration in the reactor liquid was obtained with test strips. When this was not zero, the concentrations were measured more accurately. COD-, nitrate-, nitrite- and ammonium-concentrations were determined spectrophotometrically with commercial cuvette test kits (Hach Lange, Düsseldorf, Germany).

The biomass concentration was measured by filtration and drying according to standard methods [108] for the denitrifying biomass. For the DNRA bacteria the biomass was centrifuged (10 000 rpm for 20 min) and the pellet dried at 105 °C. To compute VSS concentration an ash content in the biomass of 10% was assumed.

DGGE and sequence analysis of PCR amplified 16S genes

The microbial composition of the culture was analyzed by denaturing gradient gel electrophoresis (DGGE). Biomass samples were collected from the reactor and centrifuged and stored at -20 °C. The genomic DNA was extracted using the UltraClean Microbial DNA isolation kit (MO BIO, Carlsbad, CA, U.S.A.), following manufacturer's instructions. The extracted DNA products were evaluated on 1% (w/v) agarose gel. The extracted DNA was used as for PCR amplification of the 16S rRNA gene. The set of primers used is the 341F (containing a 40-bp GC clamp) and 907R [92]. The used PCR thermal profile started with a pre-cooling phase at 4 °C for 1 min, followed by initial denaturation at 95 °C for 5 min, 32 cycles of 95 °C for 30s, 55 °C for 40s, 72 °C for 40s, followed by an additional extension step at 72 °C for 30 min.

DGGE band isolation and DNA sequencing were performed as described by Bassin et al. [7] for 16S rRNA. The obtained 16S rRNA gene sequences were manually corrected using the program Chromas Lite 2.1.1 (http://technelysium.com.au). The corrected sequences were compared to those stored in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/blast). The sequences have been deposited in the GenBank under accession number KM403199 to KM403205.

FISH and microscopic analysis of the culture

Fluorescent *in situ* hybridization (FISH) was performed as described by Johnson et al. [55], using a hybridization buffer containing 35% (v/v) formamide. The applied probes are listed in Table 2.1. The general probe mixture EUB338 labeled with Cy5 was used to indicate all eubacteria species in the sample. No hybridization result was obtained with a probe specific for Beta- (Beta42a [72]) and Gammaproteobacteria (Gamma42a [72]), but was with a probe for *Deltaproteobacteria* (Delta495) (not shown). In the shown result, we used the EUB338 (Cy5), the Beta42a probe, labeled with FLUOS (plus an unlabeled Gamma42a probe, to minimize erroneous hybridizations of Beta42a) and a probe labeled

Table 2.1: Probes used in FISH analysis of the culture

Probe	Sequence (5'-> 3')	Dye	Specificity	Reference
EUB338mix	gcwgccwcccgtaggwgt	Cy5	Most bacteria	[5, 24]
Beta42a	gccttcccacttcgttt	Fluos	*Betaproteobacteria*	[72]
Gamma42a	gccttcccacatcgttt	none	*Gammaproteobacteria*	[72]
GeoBac464	agcctctctacacttcgtc	Cy3	Specific for 16S of *Geobacter* sp. in enrichment culture	This chapter

with Cy3 specifically designed for the detection of the 16S rRNA of the enriched microorganism, i.e. based on the DGGE obtained sequence under GenBank accession number KM403205. Probes were synthesized and 5'-labeled with either the FLUOS or with one of the sulfoindocyanine dyes Cy3 and Cy5 (Thermo Hybaid Interactiva, Ulm, Germany). Slides were observed with an epifluorescence microscope (Axioplan 2, Zeiss, Sliedrecht, The Netherlands), and images were acquired with a Zeiss MRM camera and compiled with the Zeiss microscopy image acquisition software (AxioVision version 4.7, Zeiss) and exported as TIFF format.

2.3. RESULTS

Reactor operation

A chemostat reactor was operated under non-sterile conditions, with acetate as electron donor and nitrate as electron acceptor. The reactor was kept anaerobic by flushing with 50 ml/min CO_2 gas. During the experiments acetate concentrations in the medium were changed with respect to nitrate (COD:N mass ratio) (Table 2.2). The dilution rate was 0.026 h^{-1}, which is reported as proper for growth of both denitrifiers and DNRA bacteria [88].

First, a denitrifying culture was enriched to establish denitrifying conditions. Acetate limited growth was applied and ammonium was supplied in the medium for biomass growth (period A, Table 2.2). When a stable culture was established, medium acetate concentrations were increased gradually, increasing the COD:N ratio (mg/mgN), to enrich a DNRA culture (Table 2.2). In period D, nitrate had become the limiting nutrient, but denitrification still prevailed. The culture was stable and performed full denitrification, emitting neither NO nor N_2O. There was also no nitrite accumulated in the medium.

The conversions shifted towards production of ammonium, when the COD:N ratio was further increased to 7.7 (period E, Table 2.2). Up to 90% of all nitrate was converted to ammonium, which includes the presumed assimilatory use of ammonium. In this steady state culture NO and N_2O were not detectable. The biomass concentration was 84 ± 9 mg VSS/l (0.63 ± 0.02 mg protein/mg VSS) and the nitrogen content of the biomass was 123 ± 11 mgN/gVSS.

To confirm that the enrichment of the DNRA microorganisms was solely based on the culture conditions, a second reactor was started up during period E (Table 2.2). Applying the same high COD:N ratio conditions, a similar culture was obtained directly from an activated sludge inoculum. This confirmed that these operating conditions select for a DNRA culture and that the role of the history in the first reactor was not important for the selection.

Table 2.2: List of chemostat operational conditions

Period		Medium					
Reference to text	[No. of days]	CH_3COO^- (mg/l)	$NO_3{}^-$ (mgN/l)	COD:$NO_3{}^-$-N (mg/mgN)	$NO_3{}^-$-N to $NH_4{}^+$ [a] (%)	Biomass (mg VSS/l)	Limiting nutrient
A	0 - 26	160	93	1.8	-	33	Ac^-
B	27 - 38	265	82	3.4	-	[b]	Ac^-
C	39 - 47	309	82	4.0	-	41	Ac^-
D	48 - 122	375	82	4.9	-	60	$NO_3{}^-$
E	123 - 230	595	82	7.7	90[c]	90	$NO_3{}^-$

a) Includes both the dissimilatory- and the presumed assimilatory conversion.
b) No data on biomass concentration is available for period.
c) Ammonium was supplied in the medium. This is corrected for in the calculations.

Table 2.3: Average conversion rates in the denitrification (DEN; day 70 till 90) and DNRA (DNRA; day 137 till 160) processes in the reactor (dilution rate 0.026 h^{-1}). During both periods no NO or N_2O was emitted

	Compound conversion rates [mmol/h]				
	Ac^-	$NO_3{}^-$	$NH_4{}^+$	$CH_{1.8}O_{0.5}N_{0.2}$ [b]	CO_2
DEN	-0.32 ±0.02	-0.31 ±0.01	-0.04 ±0.00	0.15 ±0.03	0.51 ±0.06
DNRA	-0.40 ±0.03	-0.31 ±0.01	0.25 ±0.01	0.18 ±0.01	0.51 ±0.02

a) Ammonium was present in the influent, also during DNRA. This is taken into account in the calculations.
b) Calculated from the measured volatile suspende solids

The conversion rates of denitrification and DNRA were averaged over a period and shown in Table 2.3. For denitrification period D was used, for DNRA period E (Table 2.2). The biomass yields during denitrification and DNRA periods were 0.47 and 0.45 respectively (Table 2.6). In the denitrification steady state reactor the analyzed data showed a closed carbon balance, while the electron balance closed with 87 ± 12%. For the DNRA process the electron balance was closed, but only 86 ± 3% of the incoming carbon was recovered in the C-balance. N_2 was not measured explicitly, thus the N-compounds could not be balanced. During DNRA, 90 ± 4% of N is recovered in ammonium and biomass; the missing fraction of nitrogen is assumed to be emitted N_2, produced by a still present small fraction of denitrifiers in the community.

Microbial population

DGGE analysis of the culture (Figure 2.1) shows the population change over time. The lanes A and B show the culture composition in the reactor in period D (Table 2.2) when denitrification was dominant. The microbial population consisted of a variety of ribotypes, five of which were clearly more abundant. The samples in lanes C, D, E and F (Figure 2.1) cover a period of 3 weeks at the start of period E (Table 2.2) in which the population composition is visibly shifting. The bacteria represented in band 3 and 4 in Figure 2.1 disappeared quickly. Gradually the other bands also disappeared, except one. One ribotype, which was only marginally present when denitrification was dominant (band 1), became more and more abundant (band 7). After the population shift, a stable, seemingly almost pure culture of bacteria was present in the reactor (lanes G, H, I, Figure 2.1). The

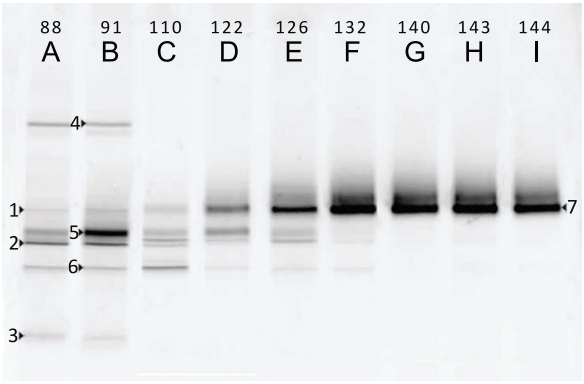

Figure 2.1: Photograph of DGGE gel of bacterial 16S rRNA gene PCR products amplified from the chemostat culture. The numbers above the lanes indicate the day on which the sample is taken (Table 2.1).

Table 2.4: BLASTn result for the 16S sequences. Sequences with the most similarity to those of band 1-6 indicated in Figure 2.1.

band #	Description	Identity [%]	Isolation site	Enrichment
1	*G. lovleyi* SZ strain SZ	95	creek sediment	PCE reduction using acetate
2	*Azospira restricta* SUA2	98	groundwater	general isolation
3	*Bacterium* GPB6	99	WWTP activated sludge	dinitrodoluene degradation
4	*Acinebacter* sp. ZH-14	98	WWTP activated sludge	degradation of pyrethoriods
5	*Magnetospirillum magneticum* AMB-1	100	fresh water pond water	magnetic + aerobic growth
6	*Acidovorax caeni*	99	anoxic tank activated sludge	denitrification

bands were excised from the gel and sequenced. The sequence represented by band 1 in lane A was the same as the sequence of the dominant band (7) in lane G, H and I, indicating that the same ribotype was already present when denitrification prevailed in period D.

The sequences of the PCR amplified excised DGGE gel bands were analyzed using the NCBI BLASTn algorithm. The bacteria most closely related to the abundant denitrifiers, represented by band 2-6 (Figure 2.1), are shown inTable 2.4. During DNRA, only one bacterium appeared to be abundant on the DGGE gel (lane G, H and I, Figure 2.1). This ribotype (band 1 and 7, Figure 2.1) relates most closely (97% 16S sequence similarity) to the *Deltaproteobacteria Geobacter lovleyi* and *Geobacter thiogenes* (Table 2.5). The culture composition of the second chemostat was the same as that of the first, with dominance of the same ribotype (data not shown).

The DNRA performing population was additionally studied with FISH (Figure 2.2) to validate the one species dominance observed in DGGE analysis. A FISH probe was developed specific for the 16S sequence of the dominant species obtained in DGGE (band 7, Figure 2.1). In the FISH picture (Figure 2.2) almost all fixed bacteria are colored purple

Table 2.5: BLASTn result for the 16S sequence of band 7 (Figure 2.1). List of the ten most closely related bacteria (>95%), from what environment they were isolated and on which characteristics their enrichment was based.

Description	Identity [%]	Isolation site	Enrichment
G. lovleyi SZ strain SZ	97	non contaminated creek sediment	PCE reduction using acetate
G. lovleyi strain Geo7.1A	97	soil impacted with TCA[a] and cis-DCE[b]	PCE[c]-to-cis-DCE-dechlorination
G. lovleyi strain Geo7.3B	97	soil impacted with TCA and cis-DCE	PCE-to-cis-DCE-dechlorination
G. lovleyi strain Geo7.2B	97	soil impacted with TCA and cis-DCE	PCE-to-cis-DCE-dechlorination
G. lovleyi strain Geo7.2A	97	soil impacted with TCA and cis-DCE	PCE-to-cis-DCE-dechlorination
G. lovleyi SZ	97	non contaminated creek sediment	PCE reduction using acetate
G. thiogenes strain K1	97	soil leached w/ chlorinated chemicals	TCA dechlorination
G. lovleyi	97	non contaminated creek sediment	PCE reduction using acetate
G. lovleyi strain Geo7.3C	96	soil impacted with TCA and cis-DCE	PCE-to-cis-DCE-dechlorination
G. sp. IFRC128	96	uranium contaminated ground water	Fe(III) reduction
G. thiogenes	96	freshwater sediment	Fe(III) reduction

a) TCA: trichloroacetate b) DCE: dichloroethene c) PCE: tetrachloroethene

and thus hybridized with both eubacterial probe (blue) and our specific probe (red). This confirms that an almost pure culture of the *Geobacter* species is present in the reactor. Furthermore, the microscopic images also show that the bacteria are rod shaped and about 2 μm long.

2.4. DISCUSSION

Dissimilative nitrate reduction

We managed for the first time to cultivate a highly enriched population of DNRA bacteria in an open culture. This provides a new opportunity to study the ecophysiology of the DNRA process. This study confirms nitrate limitation, a result of high COD:N ratio, as a factor promoting nitrate conversion to ammonium. This will strengthen the insight into the competition between the denitrification and DNRA process.

The COD:N ratio of available substrates is the most suggested controlling factor in previous studies and regarded as the dominant parameter that directs the competition between DNRA and denitrification. These studies highly varied in their set-up. In batch tests with sediment or sludge samples ammonium production for varying initial nitrate or C-source concentrations was observed especially at higher COD:N ratio's [3, 59, 111]. In field studies in soil and marine environments the change in end product of nitrate reduction upon addition of nitrate or C-source has been studied [14, 91]. In a chemostat reactor with a mixture of two pure cultures [88] a high COD:N ratio benefitted the DNRA culture. The observation in our chemostat enrichment culture that DNRA increases with increasing COD:N ratio of available substrates clearly confirms that this factor affects the

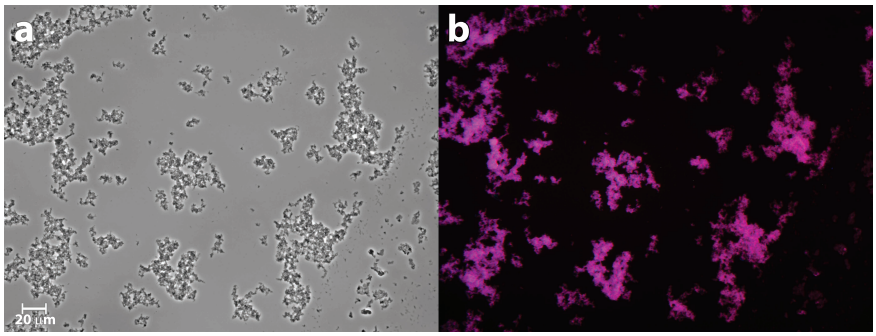

Figure 2.2: FISH microscopic photographs. (a) Image of the fixated cells of the DNRA preforming culture. (b) FISH image of the DNRA culture stained with Cy5 labeled probe for bacteria (EUB338 mix, blue), fluorescein-labeled probe for most *Deltaproteobacteria* (Delta495, not shown) and Cy3 labeled probe specific for the reactor species (GeoBac464, red). Blue color would indicate only EUB338mix hybridized. The purple color indicates both EUB338mix and GeoBac464 hybridized. The Delta495 probe hybridization was not shown to emphasize the GeoBac464 hybridization, as all cells hybridized with GeoBac464 hybridize with Delta495.

nitrate partition. Matheson et al. [73] argue that the change in COD:N ratio alters the oxidation state of the environment and claim that the oxidation state or prevailing redox potential is the actual key factor affecting the competition. Also Buresh and Patrick [13] state that it is the redox potential which influences the competition between DNRA and denitrification. They controlled the redox potential in sediment suspensions by sparging with different N_2/O_2 gas mixtures [81] and measured a higher DNRA activity at lower potentials. The COD:N ratio is inextricably linked to the oxidation state, but oxidation state can also be influenced by presence of reductants. To distinguish between these factors and to verify and address the importance of one or the other, further studies in well-defined enrichment cultures are required.

The nitrate limitation in our system, a result of the high COD:N feed to a chemostat reactor, promoted the success of DNRA. In many environments nitrate is generally limiting, and hence nitrate is a growth limiting substrate. DNRA is thought to have an advantage over denitrification under these nitrate limiting conditions for their ability to accept eight instead of five electrons per nitrate [65, 111]. Truly growth limiting conditions in the lab can only be obtained in a chemostat or fed batch system. In these systems microorganisms compete for the uptake of the growth limiting substrate, and the important competitive trait is the substrate affinity, $\frac{\mu^{max}}{K_S}$ [45, 66]. DNRA bacteria outcompeted regular denitrifiers under nitrate limiting conditions in our system. As these bacteria have a lower μ^{max} [65], we have to assume that the affinity constant (K_S value) for nitrate uptake is lower for DNRA organisms. An example is the K_S for nitrate uptake by the denitrifier *Paracocccus denitrificans*, which is about 200 µM [36], while the K_S for nitrate of *Escherichia coli*, which performs DNRA, is estimated 15 µM [85]. As described by Kuenen [66], the respective substrate saturation curves (Monod) of a denitrifier and a DNRA organism in the example would cross. Hence, at an adequately low dilution rate DNRA bacterium would be able to grow faster at the concentration of the growth limiting nitrate. Thus the nitrate limitation should be an effective condition to control the competition towards DNRA, as a result from high COD:N ratio, in our system.

In batch processes with high COD:N, thus relatively low, but not limiting nitrate conditions during growth, respiratory DNRA bacteria are not successful. Behrendt et al. [8] performed denitrifying batch experiments, with a high acetate:nitrate ratio in the medium, no DNRA was observed. Akunna et al. [2] performed mixed culture batch experiments for varying C-sources at similar initial amounts of COD. In both experiments, the main selective force was the μ^{max}. Akunna et al. [2] reported DNRA activity only when fermentative growth on glucose and glycerol occurred, but not for conversion of acetic acid, lactic acid and methanol. Possibly the ability to ferment at high rate, using nitrate as terminal electron acceptor for excess reduction equivalents gives an advantage for DNRA over the respiratory process of denitrification. Likely, the μ^{max} of organisms performing respiratory DNRA was not high enough to compete successfully under the nitrate excess conditions of batch cultivation. Kraft et al. [65] also indicated that supply of fermentable substrates to a nitrate limited system can lead to enrichment of DNRA. This underlines the requirement of nitrate limitation for successful selection of respiratory DNRA bacteria in mixed culture laboratory experiments. In the context of the work of Akunna et al. [2] it is unclear whether the DNRA in the work of Kraft et al. [65] was associated to fermentation or was performed by specialized DNRA bacteria, as they based their conclusions on molecular genetic analysis solely.

The yields for DNRA and denitrification are shown in Table 2.6. For acetic acid as a C-source, growth yields for denitrification have been limitedly reported [105] and not at all for DNRA. Yields are theoretically correlated to the Gibbs energy released in the catabolic reaction [46]. The catabolic energy gain from acetate is different when it is oxidized during DNRA and denitrification [105, 111]. Based on the Gibbs energy values (Table 2.6) a higher yield for denitrification per mole of acetate has been suggested, while in this study we observe a similar yield for denitrification and DNRA per mole of acetate. The theoretical catabolic energy gain would predict yields are similar per mole of nitrate for both processes, but they differ experimentally (Table 2.6). Strohm et al. [105] observed similar deviations in the practical yields for growth on formate compared to the theoretical values for both processes. They proposed denitirifiers have a lower biomass yield on ATP. Table 2.6 shows the net energy gain per electron is lower in the DNRA process. However the net energy dissipation is similar for both processes (900-1000 kJ/C-mol biomass produced) indicating that the growth efficiency is not influenced by the catabolic process. This would mean that a difference in growth yield is not related to a different (ATP) efficiency in the anabolism, but is due to the different energy gains in the catabolic process. For a chemostat as used here the growth yield is not influencing the competition outcome [39], but in field situations with irregular (batch wise) substrate supply or growth in biofilms a higher yield on the limiting substrate would indeed lead to a better competitiveness.

A high COD:N ratio of available substrates clearly affected the prevailing nitrate reduction process. The non-fermentative simple substrate acetate ensured an enrichment of specialized dissimilatory nitrate respiring bacteria. Most likely the nitrate limitation in combination with the adequately low dilution rate were the major factors in the selection of DNRA bacteria and the affinity for nitrate was the distinctive trait.

Table 2.6: Experimental parameters and calculated Gibbs energy values for the denitrification (DEN) and DNRA.

Parameter		Units	DEN	DNRA
Y_{SX}	Biomass yield on acetate	[C-mol X/mol Ac^-]	0.47 ±0.12	0.45 ±0.07
Y_{NX}	Biomass yield on acetate	[C-mol X/mol $NO_3{}^-$]	0.48 ±0.09	0.58 ±0.07
Y_{eX}	Biomass yield on e- transferred in catabolic process	[C-mol X/e^--mol]	0.1	0.07
ΔG^{01}_{CAT}	Catabolic energy change per mole donor[a]	[kJ/mol Ac^-]	-802	-505
ΔG^{01}_{CAT}	Catabolic energy change per mole acceptor	[kJ/mol $NO_3{}^-$]	-501	-505
ΔG^{01}_{e}	Gibbs energies per transferred electron	[kJX/e^--mol]	-100	-63

a) Calculated using the standard Gibbs free energy values defined by Thauer et al. [108].

Microbial population

In general it is expected that in chemostats with one limiting substrate one organism will become dominant [66]. However, during denitrification we observed several dominant species. Most likely, effectively at least two or possibly even four different limiting substrates (nitrate, nitrite, nitric oxide and nitrous oxide, respectively) are present in the nitrate limited denitrifying chemostat leading to the accumulation of a diverse population of partial denitrifyers [39]. However, also perturbations in period D (data not shown) could have prevented the accumulation of one dominant organism. In this research the role of each organism in the chemostat with denitrification was not investigated, this should be a topic of future research.

The DNRA performing culture in the reactor was an almost pure culture, as the results from DGGE and FISH analysis (lanes G, H, and I, Figure 2.1 and Figure 2.2) clearly showed. The bacteria appear to be most closely related to *Geobacter lovleyi* or *Geobacter thiogenes* (Table 2.5). Both *G. lovelyi* and *G. thiogenes* are reported rod shaped bacteria and both can reduce nitrate to ammonium using acetate as electron donor [79, 106]. However, *G. thiogenes* was reported to be non-motile, while *G. lovleyi* is a motile bacterium. Microscopic analysis of the chemostat DNRA culture showed motile cells. This indicated *G. lovleyi* bacteria are likely the closest relatives of our DNRA performing organism in the reactor.

Geobacter species appear to have significant environmental relevance and potential practical applications. The organisms are, for example, used in bioremediation of contaminated environments, microbial fuel cells and anaerobic sludge digesters. The *Geobacter* species are known for their physiological capacity to couple oxidation of organic compounds to the reduction of insoluble Fe(III) minerals. Furthermore, all *Geobacter* species are known to use acetic acid as an electron donor, among various others, but not glucose or glycerol. Apart from reduction using Fe(III), *Geobacter* species are also able to conserve energy from organic matter by reduction of various other e-acceptors, such as Mn(IV) and U(VI), anthraquinone-2,6-disulfonic acid and elemental sulfur [69]. Also, some species are capable of reductive dechlorination and reduction of nitrate to ammonium [26, 106]. Furthermore, some *Geobacter* species produce pili that are electrically conductive, allowing them to grow on cathodes or anodes. Most of the recent attention to this group is related to their ability for direct electron transfer to minerals. DNRA capability is reported in the characterization of these organisms when found (e.g. [106]), but has not been

further investigated.

2.5. CONCLUSION

We showed that a DNRA culture can be reproducibly enriched in a continuously operated reactor system. Nitrate limitation and a low dilution rate were the most important aspects in the competition between DNRA and denitrifying bacteria. The enriched culture was mainly consisting of *Deltaproteobacteria*, closely related to *Geobacter lovleyi*. These chemostat enrichment experiments represent the environmental selection conditions reasonably well, whereas batch enrichments are likely not selective for DNRA organisms. Future studies can use this method to further investigate the DNRA process and address the factors in its competition with denitrification.

2.6. ACKNOWLEDGEMENTS

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3

DNRA AND DENITRIFICATION COEXIST OVER A BROAD RANGE OF ACETATE/N-NO_3^- RATIOS, IN A CHEMOSTAT ENRICHMENT CULTURE

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Abstract

Denitrification and dissimilatory nitrate reduction to ammonium (DNRA) compete for nitrate in natural and engineered environments. A known important factor in this microbial competition is the ratio of available electron donor and elector acceptor, here expressed as Ac/N ratio (acetate/nitrate-nitrogen). We studied the impact of the Ac/N ratio on the nitrate reduction pathways in chemostat enrichment cultures, grown on acetate mineral medium. Stepwise, conditions were changed from nitrate limitation to nitrate excess in the system by applying a variable Ac/N ratio in the feed. We observed a clear correlation between Ac/N ratio and DNRA activity and the DNRA population in our reactor. The DNRA bacteria dominated under nitrate limiting conditions in the reactor and were outcompeted by denitrifiers under limitation of acetate. Interestingly, in a broad range of Ac/N ratios a dual limitation of acetate and nitrate occurred with co-occurrence of DNRA bacteria and denitrifiers. To explain these observations, the system was described using a kinetic model. The model illustrates that the Ac/N effect and concomitant broad dual limitation range related to the difference in stoichiometry between both processes, as well as the differences in electron donor and acceptor affinities. Population analysis showed that the presumed DNRA-performing bacteria were the same under nitrate limitation and under dual limiting conditions, whereas the presumed denitrifying population changed under single and dual limitation conditions.

3.1. INTRODUCTION

DENITRIFICATION and Dissimilatory Nitrate Reduction to Ammonia (DNRA) are two microbial anaerobic respiration processes that compete for nitrate and nitrite in the environment. When nitrate is reduced by denitrification, the nitrogen is released in the atmosphere as dinitrogen gas, and traces of the gaseous intermediates nitric and nitrous oxide, while DNRA will retain the nitrogen in the habitat in the form of ammonium. The different fates of the nitrogen due to these two different dissimilatory processes can have important implications [35]. For example, in wastewater treatment plants, denitrification is usually the desired process to remove the fixed nitrogen from the wastewater. DNRA can be important for nitrogen conservation in ecosystems because the ammonium-ion is generally retained in soil and sediments by absorption to the negatively charged clay minerals and therefore available for plant and microbial uptake. In contrast, the nitrate and nitrite anions are easily lost due to leaching [96].

Denitrification and DNRA can occur in similar conditions in absence of oxygen or at low oxygen concentrations [65, 111]. For a long time, DNRA received little consideration in studies of nitrate respiration in natural and man-made ecosystems, like wastewater treatment plants. In the last decade, interest in DNRA increased since N-labeling experiments have indicated that DNRA may play a significant role in the N-cycling [11, 14, 64, 91]. Although the physiology and bioenergetics of DNRA are relatively well studied in a selected number of pure cultures, the (quantitative) role of DNRA in the environment is one of the least described of the nitrogen cycle processes [104].

The general hypothesis is that DNRA may outcompete denitrification at low or limiting nitrate conditions and a surplus of available carbon, i.e. a high ratio of available electron donor over nitrate-nitrogen, often expressed as the molar C/N ratio [58, 65, 91, 111, 114]. These conditions occur for example in the rizosphere or rumen [111]. A low C/N ratio, i.e. low or limiting available organic carbon, has been suggested to promote denitrification. This effect of the C/N ratio has been widely observed in both aquatic and terrestrial ecosystems [14, 44, 91], but this phenomenom has rarely been reproduced in controlled mixed culture systems in the laboratory.

In batch cultures the outcome of the competition between different microorganisms is determined by the maximum specific growth rate. In continuous systems, the competition is based on affinity for the growth limiting substrate, as truly substrate limiting conditions can be applied [37]. The affinity is defined as the maximum specific growth rate over the affinity constant for the limiting substrate ($\frac{\mu^{max}}{K_S}$) [66]. This means that the competition will be won by the organism able to grow faster at certain dilution rate, at the given concentration of the growth limiting substrate [66]. As nitrate limitation is an important factor for the selection of DNRA, this configuration is essential to study the nitrate reduction processes in (continuous) enrichment culture [114] (or Chapter 2). Additionally, the use of a substrate limited continuous culture yields more reproducible and dependable data for the study of microbial competition than the batch cultivation mode [66].

Recently, two studies using lab continuous cultures addressed the effect of the C/N ratio on the end product of nitrate reduction [65, 127]. Kraft et al. [65] obtained a marine sediment mesocosm culture performing DNRA under nitrate limitation. When switching the system to carbon limiting conditions, DNRA activity ceased and the nitrate was

reduced to dinitrogen gas. In a pure culture of *Shewanella loihica* PV-4, which can catalyse both DNRA and denitrification, Yoon et al. [127] varied the ratio of electron donor over nitrate-nitrogen by varying influent lactate and nitrate concentrations. They observed DNRA under nitrate limiting conditions and denitrificaton under carbon-limitation, and additionally observed the simultaneous occurance of both processes, when the substrate ratio was such that both lactate and nitrate were limiting. These studies described the effect of the C/N ratio in a complex microbial community and a pure culture, which was not originally isolated for its DNRA capacity. To obtain additional insight in the underlying mechanisms of the microbial competition between denitrifiers and DNRA bacteria, we studied the effect of the ratio of available electron donor over nitrate-nitrogen in a simple enrichment culture grown on acetate mineral medium in continuous culture. Acetate was chosen to avoid fermentation processes. As this ratio is different for each substrate, dependent on the electrons it can transfer, we want to specifically refer to acetate and express this ratio as Ac/N. We hypothesized that the substrate affinities for the limiting substrates will be a major parameter in the effect of the Ac/N ratio on the competition between DNRA and denitrification.

A chemostat culture was inoculated with activated sludge and fed with acetate as electron donor and nitrate as electron acceptor at a low enough dilution rate to allow growth of both the denitrifying and DNRA bacteria. Initially, the culture was nitrate limited and performed DNRA [114]. Then the Ac/N ratio was changed in alternating steps. The relative contribution of denitrification and DNRA in the reactor was monitored by determining the amount of nitrate-N that was converted. Steady state populations were analyzed with denaturing gradient gel electrophoresis and fluorescent *in situ* hybridization. A kinetic model was used to describe the system and illustrate the underlying mechanisms in the competition.

3.2. MATERIAL AND METHODS

Chemostat operation

A double-jacket glass bioreactor with an operating volume of 2 L (Applikon, Delft, The Netherlands) was used for the cultivation of the culture. The reactor broth was continuously sparged with dinitrogen gas to maintain anaerobic conditions and operated as a continuous stirred-tank reactor (CSTR). The system was inoculated with activated sludge from the wastewater treatment plant (WWTP) Leiden-Noord, The Netherlands. Before the start of the current experiments the reactor had been running continuously for a year, as described in [114] (or Chapter 2). The reactor was operated at 400 rpm with a stirrer that contained two standard geometry six-blade turbines. The reactor temperature was controlled at 20 °C by means of a water jacket and a cryostat bath (Lauda, Lauda-Königshofen, Germany). The redox potential was monitored using a Redox electrode (Mettler Toledo, Tiel, The Netherlands). The pH of the reactor liquid was monitored with a pH electrode (Mettler Toledo) and was maintained at 7.1 ± 0.05 using 0.5 M HCl and 0.5 M NaOH. The pH pumps and the pH were controlled by an ADI 1030 biocontroller (Applikon). MFCS/win (Sartorius Stedim Systems, Bohemia, NY, U.S.A.) was used for data acquisition of the online measurements (redox, pH, temperature, acid dosage, base dosage). The dilution rate of the system was controlled at 0.026 ± 0.001 h^{-1} and the influent and effluent were pumped using Masterflex® pumps. The effluent pump was

Table 3.1: The acetate concentrations in medium B, used to obtain the different Ac/N ratios for the medium supplied to the chemostat, and the ammonium concentrations in medium A, added as N-source for the cultures lacking measurable ammonium production by DNRA.

Days	Ac/N (mol·mol^{-1})	Acetate (mM)	Ammonium (mM)
1-12	1.87	44.1	-
13-24	1.50	35.3	-
25-38	1.08	25.5	-
39-66	1.23	29.0	-
67-100	0.93	22.0	-
101-109	0.93	22.0	2.2
110-123	0.93	22.0	2.8
124-171	1.16	27.3	-
172-200	0.66	15.4	2.8

controlled by a level sensor. The medium was supplied in two separate flows of a mineral medium (A) and substrate medium (B). The influent pumps, using L/S® 14 mm tubes, were set to pump 26 ml/h, thus a total of 52 ml/h influent was pumped in. The culture media was autoclaved before use and sparged with a small flow of nitrogen gas while connected to the chemostat. Medium A contained per liter: 23.5 mmol $NaNO_3$, 22.0 mmol KH_2PO_4, 1.2 mmol $MgSO_4 \cdot 7H_2O$, 1.5 mmol NaOH, 1.5 mg yeast extract and 5 ml trace element solution [123], with the $ZnSO_4 \cdot 7H_2O$ concentration reduced to 2.2 g per liter. For the Ac/N ratios of 0.93 and 0.66, NH_4Cl was added to medium A (Table 3.1). Medium B contained varying concentrations of $NaCH_3COO \cdot 3H_2O$ (Table 3.1) in order to create the different Ac/N ratios.

Analytical procedures

Oxygen, carbon dioxide, nitric oxide and nitrous oxide concentrations in the headspace of the reactor were monitored in dried gas using a gas analyzer (NGA 2000, Rosemount, Chanhassen, MN, USA). The flow of nitrogen gas to the reactor was kept at 100 Nml min^{-1} using a mass flow controller (Brooks Instrument, Ede, The Netherlands), to maintain sufficient flow through the gas analyzer (80 ml min^{-1}).

Samples taken from the reactor were centrifuged and supernatants were used for analysis of acetate and nitrogen compounds. The acetate concentration in the liquid phase was measured by High Performance Liquid Chromatography using an Aminex HPX-87H column (T = 60 °C) from Bio-Rad Laboratories (Hercules, CA, USA) coupled to a UV and RI detector using phosphoric acid (0.01 M) as eluent, with a lower limit of detection of 0.1 mM. A rapid qualitative indication of the nitrite- and nitrate-concentration in the reactor was obtained with test strips (Merck Millipore, Carrigtohill, Ireland). When this was not zero, the qualitative measurements for nitrate-, nitrite- and also ammonium-concentrations were performed spectrophotometrically with commercial cuvette test kits (Hach Lange, Düsseldorf, Germany). Nitrate concentrations as low as 0.23 mgN/l (0.02 mM) could be measured with this method. Sulfide was not detectable (< 0-5 μmol/l).

To determine the biomass concentration, the reactor effluent was centrifuged (10 000 rpm for 20 min) and the pellet was dried at 105 °C. Subsequently the ash content was subtracted to obtain VSS concentration. The ash content was determined by burning the organic parts of the dried pellet at 550 °C. Protein content of the biomass was measured

Table 3.2: Probes used in FISH analysis of the culture

Probe	Sequence (5'-> 3')	Dye	Specificity	Reference
EUB338mix	gcwgccwcccgtaggwgt	Cy5	Most bacteria	[5, 24]
Beta42a	gccttcccacttcgttt	Cy3	*Betaproteobacteria*	[72]
Gamma42a	gccttcccacatcgttt	none	*Gammaproteobacteria*	[72]
Delta495	agttagccggtgcttcct	Fluos	*Deltaproteobacteria*	[70]

using the Uptima BC Assay Protein Quantitation Kit (Interchim, Montluçon, France). The heme content of the biomass was measured in cell suspensions, with 0.7 mg/ml protein for the DNRA biomass and 1.0 mg/ml protein for the denitrifying biomass. The absorption spectra for the heme content in the cells were recorded on an Olis DW2000 (Bogart, GA, USA) double beam spectrophotometer. Solid dithionite was used as the reductant to measure the reduced spectrum.

The biomass composition was calculated from the measured Total Organic Carbon (TOC) and Total Organic Nitrogen (TON) of washed biomass pellets, using a TOC-L CPH/CPN analyzer (Shimadzu Benelux, 's-Hertogenbosch, The Netherlands). TOC was determined as Total Carbon (TC) subtracted by Inorganic Carbon (IC) (TOC = TC - IC). Biomass composition was measured for several steady states and did not significantly differ for the different populations. In our calculations we used the average of 0.23 ± 0.01 mol N per C-mol biomass.

A balance of degree of reduction and a charge balance of incoming and exiting elements in the chemostat were set up to verify the consistency of our measurements. The ammonium production was attributed to nitrate reduction by DNRA. The nitrogen not accounted for in ammonium, nitrate, nitrite or biomass was assumed to be converted to N_2. The concentration of volatile suspended solids (VSS) was used as biomass concentration. Value comparisons were evaluated using an unpaired student t-test or linear regression analysis. For the computation of the CO_2 production rate from the off gas partial pressure we used the molar gas volume 24.5 l/mol. Losses by wash out of dissolved CO_2 and ionized species are included in the balancing.

Microbial population analysis

The microbial community structure was analyzed by denaturing gradient gel electrophoresis (DGGE). Biomass samples were collected from the reactor and centrifuged and stored at -20 °C. The genomic DNA was extracted and analyzed as described by Van den Berg et al. [114]. The set of primers used is the 341F (containing a 40-bp GC clamp) and 907R [92]. The obtained sequences were corrected using the program Chromas Lite 2.1.1 (http://technelysium.com.au) and then compared to sequences stored in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/blast). The sequences have been deposited in the GenBank under accession numbers KT317069 to KT317073, KX002073 and KX002074.

Fluorescent *in situ* hybridization (FISH) was performed as described by Johnson et al. [55], using a hybridization buffer containing 35% (v/v) formamide. The applied probes are listed in Table 3.2. The general probe mixture EUB338 labeled with Cy5 was used to indicate all eubacteria species in the sample [5, 24]. The Beta42a probe, labeled with Cy3

(plus an unlabeled Gamma42a probe, to minimize erroneous hybridizations of Beta42a [72], was used to target the denitrifiers and a probe for *Deltaproteobacteria* (Delta495) labeled with FLUOS was used to target the DNRA bacteria. Probes were synthesized and 5′-labeled with either the FLUOS or with one of the sulfoindocyanine dyes Cy3 and Cy5 (Thermo Hybaid Interactiva, Ulm, Germany). Slides were observed with an epifluorescence microscope (Axioplan 2, Zeiss, Sliedrecht, The Netherlands), and images were acquired with a Zeiss MRM camera and compiled with the Zeiss microscopy image acquisition software (AxioVision version 4.7, Zeiss) and exported as TIFF format. The relative abundances of the bacteria were based on a cell count of four randomly selected subsections of each picture, counting at least 100 cells per section.

Model description

A computational model was developed to describe the competition between nitrate reduction to dinitrogen gas (denitrification, DN) and nitrate reduction to ammonium (ammonification, AM) in a chemostat. The model was based on Monod-kinetics with potentially two limiting substrates, nitrate (NO3) and/or acetate (AC). The actual growth rate of the microorganisms catalyzing both conversions was consequently described as shown in equation 1.

$$\mu = \mu^{max} \cdot \frac{NO3}{NO3+K_{NO3}} \cdot \frac{AC}{AC+K_{AC}} \quad (1)$$

In this equation NO3 and AC are the concentrations for nitrate and acetate respectively, μ^{max} is the maximum specific growth rate ($molX \cdot molX^{-1} \cdot h^{-1}$) and K_{NO3} and K_{AC} (mM) are the affinity constants for nitrate and acetate respectively. To describe process stoichiometries in the kinetic model, the overall growth reactions for denitrification and DNRA obtained from measurements were used (equation 2 and 3). Thus, implemented is nitrate reduction to the pathway end-products, despite the branching of the nitrate reduction pathways at nitrite. The kinetic parameter values used are presented in Table 3.7.

The resulting system was numerically solved using the steady state assumption μ=D where D equals the dilution rate ($L \cdot L^{-1} \cdot h^{-1}$) using a two-step approach. First the effluent concentrations acetate and nitrate were calculated assuming that only denitrification or ammonification occurred at Ac/N ratios in the feed ranging from 0.6 to 2.0. If the steady state effluent concentrations of acetate and nitrate were both lower for denitrification, this process will outcompete the ammonification process, and vice versa. If this is not the case ammonification and denitrification will coexist and there is a unique solution for the effluent acetate and nitrate concentrations where both the growth rates of denitrification and ammonification are equal to the dilution rate. The full model is made available in the supplementary materials.

Table 3.3: Biomass yield on acetate and nitrate for the different Ac/N ratios. Yields and average deviations were calculated using the average volatile suspended solids of steady state measurements and the average amount of substrate that was consumed.

Ac/N	Biomass yield (%)	
(mol·mol^{-1})	gVSS·mol acetate^{-1}	g VSS·mol nitrate^{-1}
0.66	11.3 ±1.8	11.6 ±1.4
0.93	10.7 ±1.1	9.9 ±0.8
1.08	12.5 ±1.2	13.6 ±0.9
1.16	9.9 ±1.1	11.5 ±0.9
1.23	10.3 ±1.2	12.7 ±1.1
1.50	12.3 ±1.4	18.4 ±1.6
1.87	12.3 ±1.6	18.0 ±1.1

3.3. RESULTS

Reactor operation

THE influence of the mass ratio of acetate and nitrate (Ac/N ratio) on the competition between denitrification and DNRA was analyzed using an anaerobic enrichment culture. Nitrate was used as electron acceptor and N-source and acetate as electron donor and carbon source. Variable Ac/N ratios were obtained by varying the acetate concentration in the influent. Ac/N-ratios were alternated non-linearly in time (Table 3.1) to avoid gradual adaptation. The culture was assumed to be in steady state when the conversions observed were constant for at least five volume changes.

Since nitrogen fixation is unlikely to occur in presence of ammonium, the ammonium production was attributed to nitrate reduction by DNRA. In steady state nitrite was not detected, and in the off-gas no nitric oxide or nitrous oxide could be detected (both detection limits of 5 ppm). Although 0.65 mM of sulfate was present in the influent, sulfate reduction was considered negligible, because no sulfide was detected (detection limit 0.5 μmol/l).

The initial culture was enriched and grown at a high Ac/N ratio of 1.87 mol/mol in the influent (Figure 3.1a). This resulted in nitrate limitation in the reactor, while acetate was in excess: 15 ± 2% of the nitrate was assimilated and 70 ± 3% was reduced to ammonium via DNRA. The remaining 15% of nitrate was assumed to be reduced to dinitrogen gas, and thus denitrified. However, this remains to be verified. At this high Ac/N ratio, the biomass yields were 18.0 ± 1.1 g VSS/mole nitrate and 12.3 ± 1.6 g VSS/mole acetate (0.62 ± 0.04 mg protein/mg VSS) and the C/N content of the biomass was 0.22 ± 0.1 molN/molC. The redox potential in the reactor under these conditions was -450 mV and the color of the mixed culture was pink/reddish, due to high heme content of the biomass (redox spectra in Figure 3.6). These observations showed a good reproducibility of the previous enrichment in the same conditions [114] (or Chapter 2).

A low Ac/N ratio of 0.66 resulted in acetate limiting conditions in the reactor (Figure 3.1a), with excess of nitrate. Under these conditions, no ammonium was measured, hence DNRA activity was undetectable. Here, ammonium was added to the medium for growth. The biomass yield of this culture was 11.6 ± 1.4 g VSS/per mole nitrate, which is lower than the yield of the culture dominated by the DNRA bacteria. The yield on acetate was comparable for both denitrification and DNRA (Table 3.3), as well as the C/N content

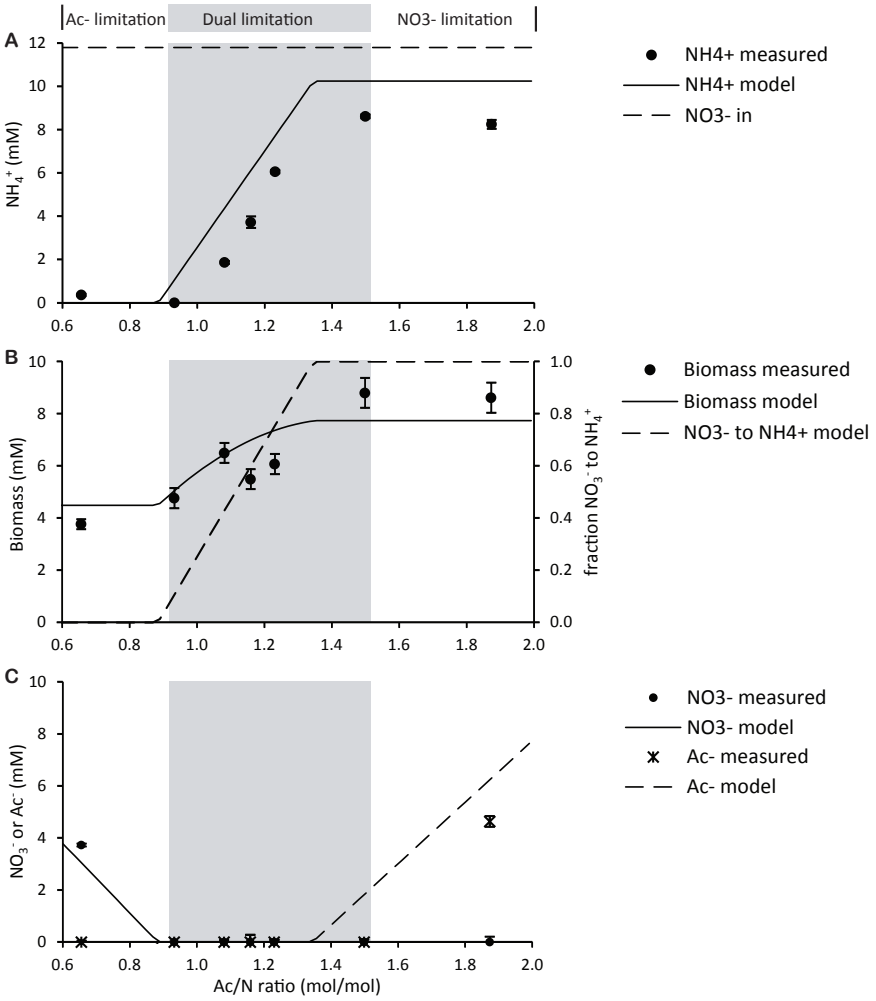

Figure 3.1: Steady state reactor concentrations were measured for each cycle. Average deviations were obtained from daily measurements during the steady state (n > 3). (A) Ammonium concentrations measured and modelled for different Ac/N ratios. As a reference, the influent nitrate concentration is shown. (B) Biomass concentrations measured and modelled. The modelled fraction of DNRA biomass is shown for reference. (C) Nitrate and acetate concentrations, measured and modelled.

Table 3.4: Steady state conversion rates and balances for the enrichment cultures at different Ac/N ratios. The conversion rates were calculated using the measured concentrations of the compounds. Standard deviations were obtained from daily measurements during the steady state (n = 3-4 for biomass, n = 4-5 for other compounds). When substrates were completely consumed standard deviations were estimated from concentration variations due to inaccuracy of medium preparation. The nitrogen and carbon balance are unavailable since N_2 and CO_2 were not measured.

Ac/N	Compound conversion rates ($\frac{mmol}{h}$)					Balance residuals (%)	
($\frac{mol}{mol}$)	Ac^-	NO_3^-	H^+	$CH_{1.8}O_{0.5}N_{0.2}$	NH_4^+	Reduction	Charge
0.66	-0.39 ±0.01	-0.44 ±0.08	-0.78 ±0.04	0.19 ±0.01	0.00 ±0.01	1	4
0.93	-0.55 ±0.02	-0.59 ±0.01	-1.07 ±0.02	0.24 ±0.02	0.00 ±0.01	7	6
1.08	-0.64 ±0.02	-0.59 ±0.01	-1.26 ±0.02	0.32 ±0.02	0.09 ±0.01	6	5
1.16	-0.55 ±0.03	-0.59 ±0.01	-1.45 ±0.04	0.27 ±0.02	0.19 ±0.01	12	0
1.23	-0.72 ±0.02	-0.59 ±0.01	-1.50 ±0.04	0.30 ±0.02	0.30 ±0.01	9	8
1.50	-0.88 ±0.02	-0.59 ±0.01	-1.75 ±0.03	0.42 ±0.03	0.43 ±0.01	10	9
1.87	-0.86 ±0.05	-0.59 ±0.01	-1.87 ±0.08	0.43 ±0.03	0.41 ±0.01	9	1

and protein content of the biomass. The redox potential in the reactor at denitrifying conditions was -160 mV and the color of the broth was yellowish, as the enrichment culture did not have a high heme content like the DNRA culture (redox spectra in Figure 3.6).

At Ac/N ratios between 0.93 and 1.50, a dual limitation of both acetate and nitrate was observed, as residual concentrations of both acetate and nitrate were not detected. The ammonium production decreased with the decreasing Ac/N ratios, indicating that DNRA became less dominant (Figure 3.1a). Other observations confirming a decrease in DNRA activity were the decrease in acid consumption and the change in color of the culture, which became less red and more yellow as the Ac/N ratio was decreased. Furthermore, the biomass yield on nitrate decreased with the diminishing of DNRA activity in the reactor, while the biomass yield on acetate did not change significantly (Table 3.3). Hence, as acetate conversion decreased with Ac/N ratios, the biomass concentration decreased as well (Figure 3.1b). An overview of all steady state conversion rates is shown in Table 3.4. The redox potential in the reactor for the steady states with both substrate limiting conditions ranged from -260 ± 50 to -350 ± 50 mV (Figure 3.2). Although the redox values are somewhat unstable, a trend in the redox potential could be observed.

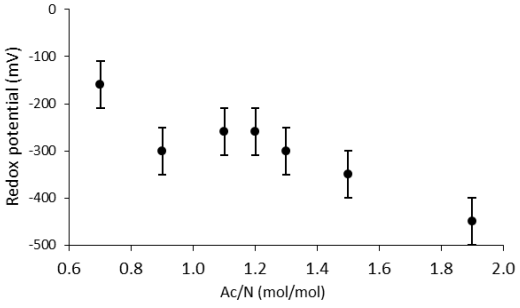

Figure 3.2: Redox potential measured in the culture at different Ac/N ratios. A linear correlation could be observed.

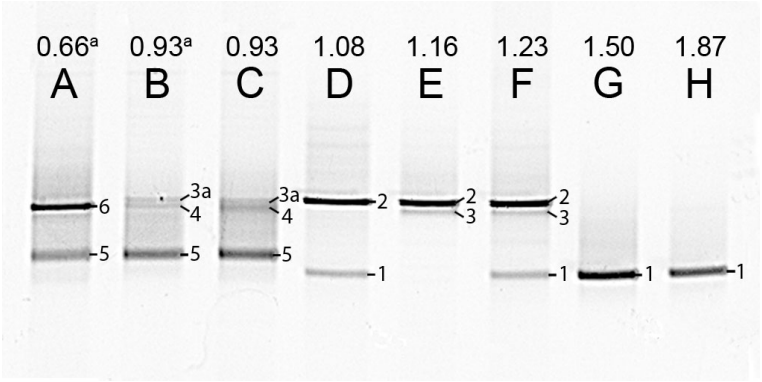

Figure 3.3: Photograph of DGGE gel with the PCR products of the 16S rRNA genes from steady state reactor samples, with different Ac/N ratios (cropped; no other bands were present in the lanes). The numbers on the right side of the bands correspond to the markers in Table 3.5. Bands labeled with the same number, contained the same sequence. Note that for the culture in lane A denitrification is dominant and in H DNRA is. The unprocessed DGGE photo is included in Figure 3.2. a) The influent was supplemented with 1.4 mM ammonium.

Table 3.5: BLASTn results'best alignments for the different band sequences Figure 3.3

band #	Description	Class	Identity (%)	Accession number
1	*Geobacter luticola*	*Deltaproteobacteria*	97	AB682759.1
2	*Azospira oryzae* strain N1	*Betaproteobacteria*	100	DQ863512.1
3	*Geobacter lovleyi* strain SZ	*Deltaproteobacteria*	97	NR_074979.1
3a	*Geobacter lovleyi* strain SZ	*Deltaproteobacteria*	96	NR_074979.1
4	*Sulfurisoma sediminicola*	*Betaproteobacteria*	94	AB842427.1
5	*Sulfurisoma sediminicola*	*Betaproteobacteria*	97	AB842427.1
6	*Variovorax boronicumulans*	*Betaproteobacteria*	99	JQ692103.1

Microbial community structure

The microbial populations in the reactor steady states were analyzed by DGGE and verified by FISH (Figure 3.3 and Figure 3.4, respectively). DGGE bands were sequenced and analyzed using BLASTn (Table 3.5). For the DNRA dominated community as obtained in nitrate limiting conditions, one dominant genotype was observed on the gel (lane A, Figure 3.3), which is most closely related to *Geobacter luticola*. This is the genotype corresponding to the organism performing DNRA, as is explained in the discussion. When both nitrate and acetate were limiting and denitrification and DNRA coexisted, two other dominant genotypes appeared (lane C-E, Figure 3.3). One of those genotypes is most closely related to *Geobacter lovleyi* (band 3, Figure 3.3), and 100% similar to the DNRA genotype found in our previous study [114] (or Chapter 2). Thus, this *Geobacter* genotype is assumed to be responsible for DNRA, just like the *G. luticola* related organism. Alignment of the sequences of the bands 1 and 3 showed 97% similarity. The other genotype that appeared when both nitrate and acetate were limiting (Ac/N 1.08-1.23 mol/mol) related to *Azospira oryzae* (band 2, Figure 3.3), which was most likely responsible for the denitrification. When the Ac/N ratio was 0.93 (lane F, Figure 3.3), a genotype related

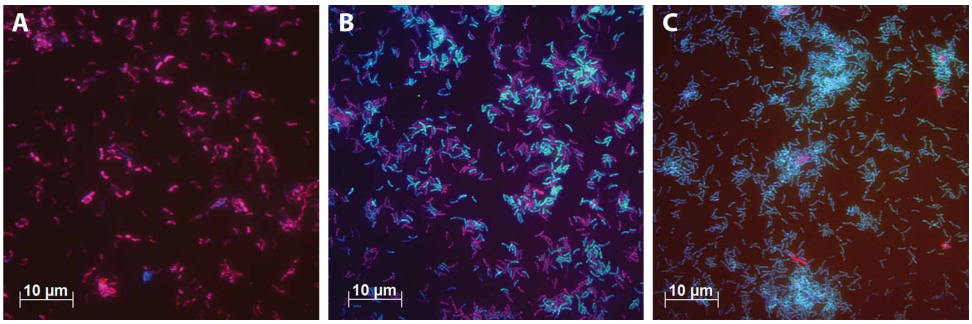

Figure 3.4: FISH microscopic photographs of steady state cultures. The cells were stained with Cy5-labeled probes for bacteria (EUB338mix, blue), Cy3-labeled probes for *Betaproteobacteria* (Beta42a, red) and FLUOS-labeled probes for *Deltaproteobacteria* (Delta495, green). Cells that are purple indicate cells to which the probes EUB338mix and Beta42a were hybridized. Cells that are light blue indicate cells to which the probes EUB338mix and Delta495 were hybridized. (A) The culture grown with Ac/N ratio of 0.66. (B) The culture grown with Ac/N ratio of 1.08. (C) The culture grown with Ac/N ratio of 1.87.

with 96% identity to *Geobacter lovleyi* (band 3a, Figure 3.3) remained, with 98% sequence similarity to genotype 3. The denitrifier with the *A. oryzae* genotype had disappeared and two other dominating genotypes were found, next to the *Geobacter* genotype. These genotypes (band 4 and 5, Figure 3.3) were highly similar, 98% similarity, and for both the closest cultivated relative was *Sulfurisoma sediminicola*. Under acetate limiting conditions (Ac/N 0.66, lane H, Figure 3.3), two genotypes dominated. The *Geobacter* genotype had disappeared and one of the *Sulfurisoma sediminicola* genotypes remained. A new genotype appeared (band 6, Figure 3.3), which was most closely related to *Variovorax boronicumulans*.

Because there was no DNRA performed at low Ac/N ratios (0.93 and 0.66 mol/mol), the ammonium concentration was below the detection limit in the reactor, and nitrate was also used for assimilation. To investigate if nitrate or ammonium availability affected the denitrifying community at low Ac/N ratios, the medium was supplemented with 1.4 mM ammonium for 20 volume changes. This was sufficient for growth, and an excess of 0.2 mM residual ammonium remained in the reactor. Supplementing the medium with ammonium resulted in no identifiable differences in functional performance of the system and in the DGGE analysis result (compare lane F and G, Figure 3.3). So the type of N-source for assimilation did not change the denitrifying population.

As artifacts occur in DGGE analysis Neilson et al. [78], FISH analysis was performed to confirm these results. The DNRA performing bacteria found with DGGE analysis belong to the class of *Deltaproteobacteria* and the bacteria identified in the denitrifying cultures all belong to the *Betaproteobacteria* (Table 3.5). Therefore the relative abundance of denitrifying and DNRA performing bacteria can be seen with FISH using probes for *Beta-* and *Deltaproteobacteria* respectively. For each steady state, the relative abundance of these bacteria was estimated (Table 3.6) to illustrate its correspondence with the trend observed with the culture conversions and DGGE result. Figure 3.4 shows FISH photos of three steady state populations. The culture grown with the highest Ac/N ratio consists of almost only *Deltaproteobacteria*. With the decrease of the Ac/N ratio, the relative abundance of the *Deltaproteobacteria* decreases and the relative abundance of

Table 3.6: Relative abundances of the *Betaproteobacteria* and *Deltaproteobacteria* in the steady state populations obtained from cell counts of the FISH analyses. The indicated average deviations relate to the cell counts and not to accuracy of the FISH analysis.

Ac/N (mol·mol^{-1})	*Betaproteobacteria* (%)	*Deltaproteobacteria* (%)
0.66	96 ±2	0 ±0
0.93	85 ±1	15 ±1
1.08	46 ±3	54 ±3
1.16	38 ±5	62 ±5
1.23	25 ±1	75 ±1
1.50	1 ±1	99 ±1
1.87	1 ±1	99 ±1

the *Betaproteobacteria* increases. The cultures grown with the lowest Ac/N ratio show almost only *Betaproteobacteria*.

Modeling the results

A mathematical model was developed to describe the experimental results obtained and to clarify co-occurrence of denitrification and ammonification at the intermediate Ac/N ratios investigated. As the model describes the overall growth reactions, branching of the nitrate reduction pathways at nitrite was not incorporated. To validate the model structure proposed in the material and methods section, the stoichiometry of both processes needs to be identified first. Given that at low Ac/N ratios denitrification was found to dominate the process, the stoichiometry of the denitrification (equation 2) was calculated from the measured biomass yield on acetate (-0.49 Cmol/mol).

$$-2.1C_2H_3O_2^- - 2.3NO_3^- - 4.4H^+ 1CH_{1.8}O_{0.5}N_{0.2} + 1.1N_2 + 3.1CO_2 + 4.4H_2O \quad (2)$$

At high Ac/N ratios ammonification was strongly dominant and the reaction stoichiometry for nitrate ammonification (equation 3) was derived from the biomass yield on acetate, which was on average found to be comparable to denitrification (-0.49 Cmol/mol).

$$-2.1C_2H_3O_2^- - 1.5NO_3^- - 4.9H^+ 1CH_{1.8}O_{0.5}N_{0.2} + 1.3NH_4^+ + 3.1CO_2 + 2H_2O \quad (3)$$

The maximum specific growth rate value for the ammonifying culture was estimated from exponential growth curves measured during transition from low to high Ac/N-ratios (data not shown). The affinity constant for nitrate of the DNRA bacterium was estimated from reactor nitrate concentrations that were measured under nitrate limiting conditions (data not shown). When acetate limitation was observed, residual acetate concentrations were at all times below the detection limit of the used methods. The values for the affinity constants of the denitrifying community were therefore obtained from literature [42, 93]. An overview of the kinetic parameter values is presented in Table 3.7.

Analysis of the affinity of both processes for nitrate and acetate in a chemostat, as identified by the value for $\frac{\mu^{max}}{K_S}$ in Table 3.7, shows that the model correctly describes the dominance of denitrification in acetate limiting conditions (Ac/N ratios smaller than 0.93), and that of DNRA at nitrate limiting concentrations (Ac/N ratios higher than 1.50). It should be noted that although, the affinity constants in the model were roughly estimated

Table 3.7: Parameter values used for modelling denitrification and ammonification in a chemostat culture. The origin of the different values in the table is explained in the text.

Parameter	Symbol	Unit	DNRA	DN
Maximum specific growth rate	μ^{max}	h^{-1}	0.052	0.086
Affinity constant for nitrate	K_NO3	μM	2	10
Affinity constant for acetate	K_AC	μM	10	10
Affinity for nitrate	$\frac{\mu^{max}}{K_NO3}$	$h^{-1} \cdot \mu M^{-1}$	26.2	8.6
Affinity for acetate	$\frac{\mu^{max}}{K_AC}$	$h^{-1} \cdot \mu M^{-1}$	5.2	8.6

from literature and preliminary experimental data, the model output in terms of the ratios of denitrification versus DNRA is largely independent of the absolute affinity constant values. The ratio of the affinity constants is the main factor determining the relative contribution of DNRA or denitrification in the conversions in the chemostat.

Also at the intermediate Ac/N ratios the model adequately describes the co-occurrence of denitrification and ammonification (Figure 3.1a). Total biomass concentrations as predicted from combined denitrification and ammonification correspond well to the measured biomass concentration (Figure 3.1b). Effluent ammonium concentrations due to DNRA are always overestimated by approximately 15% by the model due to partial reduction of nitrate to dinitrogen gas in our experiments as described previously.

3.4. DISCUSSION

Chemostat system

IN this study the influence of the Ac/N ratio on the competition for nitrate between denitrification and DNRA was investigated in an open continuous culture enrichment system. We used acetate as the single non-fermentable substrate. We observed in this system that within a remarkably wide range of Ac/N ratios dual substrate limitation and co-occurrence of both DNRA and denitrification occurred. To describe the basic behavior of our system, we made a kinetic model to describe substrate competition and co-occurrence of DNRA and denitrifiers, omitting nitrate reduction to nitrite as a possibility. As shown in Figure 3.1, the model correctly describes the experimentally observed co-occurrence of denitrification and DNRA at intermediate Ac/N ratios.

In the chemostat steady states with one limiting substrate we observed a domination of one of the two different nitrate respiration processes. The DNRA bacteria dominated during nitrate limitation, indicating they have a higher affinity for nitrate than the denitrifying bacteria. The denitrifiers dominated under acetate limiting conditions, indicating a higher affinity for acetate for the denitrifiers than for the DNRA bacteria.

The biomass yield of the DNRA culture was higher than the yield of the denitrification culture per mole nitrate, whereas these yields were similar per mole of acetate. A comparison of the DNRA and denitrification yields found in this system, with theoretically expected and other empirical yields can be found in the discussion of Van den Berg et al. [114] (or Chapter 2).

For a remarkably wide range of Ac/N ratios no nitrate or acetate could be detected in the effluent and both nitrate reduction processes co-existed. In case of one conversion,

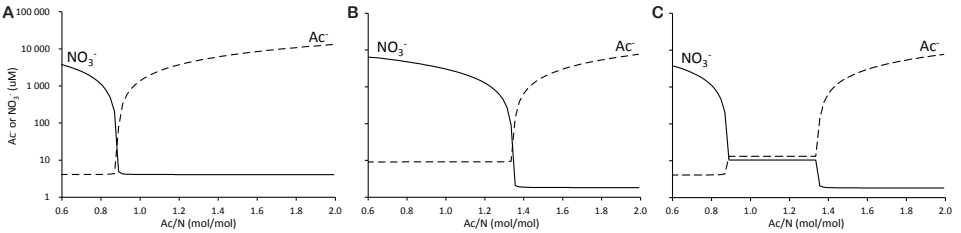

Figure 3.5: Calculated change in residual nitrate (solid line) and acetate (dotted line) concentrations for change of influent Ac/N ratio in a chemostat enrichment culture. (A) Denitrification only. (B) DNRA only. (C) Denitrification and DNRA.

with one electron donor and one electron acceptor, e.g. when only denitrification or DNRA occurs, this dual limitation range is expected to be very narrow. This is shown in Figure 3.5a and b, where the effluent concentrations of acetate and nitrate are calculated assuming that either DNRA or denitrification occurs. Furthermore, these graphs (Figure 3.5a, b) show that the Ac/N ratio where both carbon and nitrogen limitation occur is strongly different for denitrification (Ac/N=0.89) and DNRA (Ac/N=1.36). This is due to the difference in the number of electrons transferred per unit of nitrate converted. This difference in stoichiometry between both processes prompts the double limitation for nitrate and acetate and co-occurrence of DNRA and denitrification over a broad range of Ac/N ratios.

It should be noted that if one process, DNRA or denitrification, would have a higher affinity for both acetate and nitrate, only one of these processes would occur over the full range of Ac/N values. Combined with the higher affinity for nitrate of DNRA and the higher affinity for acetate of denitrification, the difference in stoichiometry facilitates the broad range of Ac/N ratios where DNRA and denitrification coexist as adequately described by the model.

The model predicts that residual limiting substrate concentrations are higher in the dual substrate limitation range than in the single substrate limitation range (Figure 3.5c). This is the result of competition for both substrates, in which microorganisms do not manage to keep the substrate concentrations as low as for the case of a single substrate limitation. Under nitrate limiting conditions, the DNRA bacteria keep the nitrate well below a level attainable for denitrifying bacteria. At decreasing Ac/N ratios, the acetate concentration becomes limiting for the DNRA bacteria and nitrate starts to accumulate. At these increased nitrate concentrations and low acetate concentrations, the denitrifiers have a competitive edge due to their higher affinity for acetate. They can establish in the system up to the point where nitrate gets limited for the denitrifiers, and denitrification and DNRA can co-occur. As both organisms are pulling at both substrates in the transition phase, neither manages to outcompete the other, and they will coexist, albeit at higher residual substrate concentrations (Figure 3.5c).

Other factors, which are not considered in the model, possibly contributed to our observations of the Ac/N effect. The apparent higher affinity of denitrifiers for acetate might be a result of their higher competitiveness in comparison to the DNRA bacteria at increased redox potential imposed at nitrate excessive conditions. The low redox

conditions at high Ac/N ratios might not only be influenced by the nitrate concentration but also facilitated by sulfate reducing bacteria, which are inevitably present in anoxic environments with sulfate and excess organic carbon present. Sulfide was not detected in the culture supernatant, but the small amounts produced could be directly consumed by the denitrifiers in autotrophic nitrate respiration. Micromolar amounts of sulfide are sufficient to strongly affect the redox potential of the system. Hence, the DNRA bacteria might depend on the sulfide producers to be able to persist in the reactor. A lower sulfide production in the reactor at lower Ac/N ratios in combination with the presence of nitrate would increase the redox potential. This increase might inhibit growth of the DNRA bacteria [13], and result in the domination of denitrifiers which are less sensitive to high redox.

The double substrate limitation described in this study deserves particular attention. For one, it underlines how hybrid the nitrate conversion is, with different conversion occurring simultaneously even in a simple system as a chemostat with one carbon- and energy source. Secondly, it differs from cases of double substrate limitations reported in literature. Most studies describing dual limitation of heterologous substrates, i.e. substrates that cannot be replaced by one another, study limitation of anabolic substrates in pure cultures [30, 129]. Very few studies describe a dual limitation of heterologous catabolic substrates [40, 62, 118]. In the dual limitation of anabolic substrates, the dual limitation range is a result of the biomass flexibility to change composition [129], which can be predicted by the biomass yields and is dependent on the dilution rate. In this study, the dual limitation is a result of the difference in stoichiometries, analogous to the yields in the anabolic substrate limitations, of denitrification and DNRA and not the biomass flexibility to change C/N content. As the C/N content of the biomass at different influent Ac/N ratios weres constant, we hypothesize that the observed dual limitation range is independent of the dilution rate. In the study of [118] on the dual limitation of the heterologous substrates glucose and oxygen in a mixture of two pure cultures, coexistence occurred as a result of the different susceptibilities of the cultures for substrate inhibition by oxygen. In our results, the occurrence of inhibition might be due to the millimolar excess of either acetate or nitrate, but appears unlikely since, at the pH used, the concentrations of candidates for toxicity, free acetate or nitrate, are very low.

The results described in this paper suggest that coexistence of DNRA and denitrification will occur in environments at a relative wide range of C/N supply ratios. Shifts in carbon or nitrate/nitrite loads may change the ratio of nitrate reduction products, but both processes will remain present. For example, in a wastewater treatment system with high organic carbon and nitrate in the influent (as in aquaculture and industrial wastewater) or nitrate presence in a recirculation stream which is mixed with the influent, DNRA bacteria can be active. Co-occurrence of denitrification and DNRA in artificial wastewater treatment wetlands and natural ecosystems, such as sediments, is often reported in literature [35, 44, 77]. To which extent the Ac/N ratio dependent co-occurrence of DNRA and denitrification is influenced by the nature of the electron donor/carbon source in the system [35, 82] and the oxidized nitrogen compound utilized (i.e. nitrite or nitrate), will be the topic of future studies. In addition, spatial heterogeneities in the environment could affect the co-occurrence [121]. Furthermore, one should not rule out the possibility that the organisms respond physiologically to the changing in situ concentrations of nitrate and acetate, by various metabolic mechanisms or regulatory

effects [38, 39].

Microbial population

For the nitrate limiting conditions, the FISH and DGGE results indicated dominance of one organism. This organism was most closely related to *Geobacter luticola*. Although *G. luticola* was shown to reduce nitrate to N_2O [124] and no DNRA activity was reported, the related organism found in this study is clearly performing the DNRA in our system.

The organism related to *G. lovleyi* strain SZ, which occurred after the shift of the Ac/N from 1.08 to 1.23 (Table 3.1), was assumed to perform the same conversions as the *G. luticola*-related strain in the reactor as it was 100% similar to the DNRA-performing dominant organism described previously by Van den Berg et al. [114] (or Chapter 2). Additionally, the *G. luticola* related genotype had 97% sequence identity to *G. lovleyi*-related sequence and 96% to the *G. lovleyi* SZ. Hence, both the presumed DNRA-performing organisms in the studied system are closely related. Shifts between the two strains were seen earlier in this reactor (before the experiments of this study), while the conditions remained the same and the conversions were unaffected. Additionally, in the current experiments these organisms interchange in time (compare Table 3.1, Figure 3.3). Therefore, most likely the shift is not an effect of the different Ac/N ratio but rather a shift between two very similar organisms with very close affinities possibly affected by minor fluctuation in substrate supply [38].

Although the population consists of >95% of the presumed DNRA organism under nitrate limitation, 15% of the nitrate was still reduced to dinitrogen gas. Either the DNRA bacteria are also capable of denitrification, like *Shewanella loihica* [127], or the side population was denitrifying but with a relatively low growth yield. The low yield could be the results of other unknown factors such as the possible production of NO or N_2O as by products by the DNRA bacteria [23, 112] with the denitrifiers growing on these compounds rather than nitrate. Besides direct competition for the substrates nitrate and acetate, the bacteria can interact, which can be inhibitory or stimulatory [39], or they might have an effect on the regulation of enzymes or nutrient uptake system of one another [38]. With the current experiments, the origin of the nitrogen formation was unidentifiable.

Under acetate limiting conditions, two dominant Betaproteobacterial genotypes were observed on the DGGE gel, while the *Geobacter* species had disappeared. One organism was most closely related to *Sulfurisoma sediminicola* and the other to *Variovorax boronicumulans*. *S. sediminicola* is a confirmed denitrifier [61], while *V. boronicumulans* is not [74]. However, closely related *Variovorax* species, for example *Variovorax paradoxus*, was shown to grow anaerobically with nitrate and acetate and its 16S RNA-gene is 98% similar to the genotype found in this study. The co-occurrence of the two denitrifiers under acetate limitation may be due to very close affinities and metabolic control of the two organisms. The sample for DNA extraction was collected relatively soon after the concentrations in the reactor became constant. A steady state situation in the concentrations in the chemostat does not necessarily mean a steady state in the population. If a bacterium has only a small net competitive advantage, it would need more time to outcompete others [38, 39]. As we did not run the steady state for more than five doubling times, we cannot disregard that possibility. Finally, it cannot be ruled out the two denitrifying

organisms each perform a part of the denitrification pathway [113].

When both nitrate and acetate were limiting, DNRA and denitrification coexisted and two organisms dominated in the microbial community, the *Geobacter* sp. and the *Azospira oryzae* (formerly *Dechlorosoma suillum* [107])-related strain. *A. oryzae* was most likely responsible for the denitrification in the reactor. This is supported by the reported characteristics which indicated that*A. oryzae* was able of nitrate reduction with acetate whereby nitrate was completely reduced to nitrogen gas [1]. As *A. oryzae* is most likely the *Betaproteobacterium* observed in FISH, it's abundance increased in the population with the increase in denitrification as a function of the Ac/N ratio in the feed (Table 3.5 and Table 3.6). For the steady state with dual limitation, but no ammonium production (Ac/N ratio of 0.93), the *A. oryzae* related organism was not observed and two other denitrifiers appeared to dominate. Both were related to *Sulfurisoma sediminicola*, as a closest cultured relative. Thus, the presumed DNRA bacteria dominant under nitrate limitation remained under dual limiting conditions, whereas the presumed denitrifying population changed as a result of the additional limiting factor.

At Ac/N ratio of 0.93, no ammonium production was observed, but the DNRA bacterium had remained, albeit at a low level, in the reactor, as was observed both with DGGE (band 3a, Figure 3.3) and FISH (data not shown). It could well be that all produced ammonia was so small that it was directly consumed and incorporated into the biomass of both organisms. However, the possibility remains that the *Geobacter*-related organism was performing mainly denitrification. When the feed was supplemented with an excess amount of ammonium, at Ac/N ratio of 0.93, the population did not change, not even after 20 doubling times. Although the use of nitrate instead of ammonium for biomass results generally in a lower yield, no change in yields was measured within our accuracy. Many bacteria can use nitrate as a nitrogen source for growth [41], while some obligatory depend on ammonia for growth, e.g. *Nitrolancetus hollandicus* [100]. Probably the dominant bacteria in our system were able to use nitrate as an N-source, while retaining a competitive advantage.

Conclusion

We showed a clear correlation between the Ac/N ratio and the prevalent dissimilatory nitrate reduction process in an open chemostat system using acetate as electron donor. Under nitrate limiting conditions DNRA was the dominant process while under acetate limiting conditions denitrification was dominant. Moreover, we demonstrated that for a substantial range of Ac/N supply ratios both substrates were limiting and denitrification and DNRA coexisted. The range of dual substrate limiting conditions can be explained as a result of both the stoichiometries of DNRA and denitrification and a higher affinity of the prevailing DNRA bacteria for nitrate and of the prevailing denitrifying bacteria for acetate. The presumed DNRA performing bacterium was most closely related to *Geobacter luticola* or *G. lovleyi* (*Deltaproteobacteria*). The presumed denitrifying population was dominated by three members of the *Betaproteobacteria*, belonging to *Azospira oryzae, Sulfurisoma sediminicola* and *Variovorax boronicumulans*. While the same DNRA bacteria were present under nitrate limitation as well as dual substrate limitation, the denitrifying community varied between acetate limited conditions and dual substrate limiting conditions. These insights into the mechanism of the competition between

denitrification and DNRA helps improve our understanding of the N-cycle processes. This will be useful to predict the fate of nitrogen in different environments and contribute e.g. to the ability to predict eutrophication trajectories in aquatic environments or to evaluate potential impaired contribution of DNRA in wastewater treatment plants.

3.5. SUPPLEMENTARY MATERIAL

The supplementary model can be found online at:
http://journal.frontiersin.org/article/10.3389/fmicb.2016.01842/full#supplementary-material

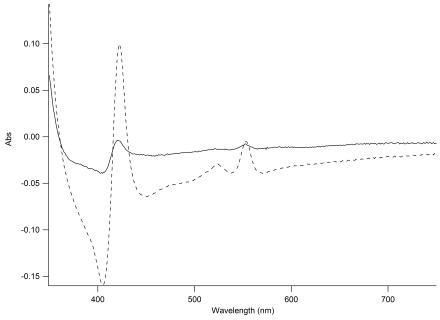

Figure 3.6: Redox adsorption spectra of the DNRA culture at Ac/N 1.87 (dotted line) and denitrifying culture at Ac/N 0.66 (solid line) to illustrate the difference in heme content.

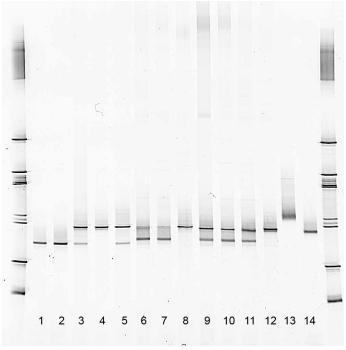

Figure 3.7: Full DGGE gel picture from which Figure 3.3 is adapted. Lane 1-7 are lane A-G in Figure 3.3. Lane 9 is lane H in Figure 3.3. In lane 8 is a culture sample taken between the steady state of Ac/N 1.16 and 0.66 mol/mol (Table 3.1). The sample in lane 10, like in lane 9, is a steady state sample of Ac/N 0.66. Lane 11 and 12 are two culture samples taken between the steady states of Ac/N 0.93 to 1.16 (Table 3.1). The samples in lane 13 and 14 belong to other research.

4

ROLE OF NITRITE IN THE COMPETITION BETWEEN DENITRIFICATION AND DNRA IN A CHEMOSTAT ENRICHMENT CULTURE

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Abstract

Denitrification and dissimilatory nitrate reduction to ammonium (DNRA) are two microbial processes that compete for oxidized nitrogen compounds in the environment. The objective of this work was to determine the role of nitrite versus nitrate as terminal electron acceptor on the competition between DNRA and denitrification. Initially, a mixed culture chemostat was operated under nitrate limitation and performed DNRA. Stepwise, the influent nitrate was replaced with nitrite until nitrite was the sole electron acceptor and N-source present. Despite changing the electron acceptor from nitrate to nitrite, the dominant process remained DNRA and the same dominant organism closely related to Geobacter lovleyi was identified. Contrary to previous studies conducted with a complex substrate in marine microbial communities, the conclusion of this work is that nitrate versus nitrite as electron acceptor does not generally control the competition between DNRA and denitrification. Our results show that the effect of this ratio must be interpreted in combination with other environmental factors, such as the type and complexity of the electron donor, pH, or sulfide concentrations.

4.1. INTRODUCTION

NITRATE reduction is an important process in the nitrogen cycle. Nitrate can be reduced to nitrogen gas by denitrification, which removes the nitrogen from the ecosystem. This process balances natural and anthropogenic nitrogen inputs and counteracts eutrophication. Alternatively, dissimilatory nitrate reduction to ammonium (DNRA) retains the nitrogen in the environment. For instance, in agricultural soils, this retention can lead to a more optimal use of nitrogen containing fertilizer and prevention of nitrate leaching [96]. Finally, the autotrophic anaerobic ammonium oxidation (anammox) bacteria can also reduce nitrate to nitrite and ammonium and subsequently to dinitrogen gas, but is not considered relevant in carbon source abundant enrichments. As the fate of nitrate can have important implications for the ecosystem [14, 64], as well as the successful operation of wastewater treatment systems, we want to understand the competition between DNRA and heterotrophic denitrification in order to allow manipulation of nitrate reduction towards the desired end product (N_2 or NH_4^+).

Denitrification was long assumed to be the dominant nitrate reduction process in the environment. DNRA had received relatively little attention, in particular with respect to its quantitative contribution to the nitrogen cycle. In the past decade, DNRA has become recognized to contribute significantly to nitrate reduction in the environment [11, 27, 35, 90]. We have limited understanding of the environmental factors that control the nitrate reduction processes [53, 64]. A known important factor in the competition for nitrate between DNRA and denitrification is the ratio of available electron donor (i.e. easily degradable carbon) and electron acceptor (i.e. nitrate or nitrite) [65, 115, 127]. Consistently, in organic carbon rich environments, where nitrate is limiting, DNRA dominates, and in environments with excess of nitrate and limiting carbon denitrification dominates [91, 111].

Kraft et al. [65] postulated that the terminal electron acceptor has a determinative effect on the competition. In DNRA and denitrification, nitrate and nitrite can both be terminal electron acceptors. Kraft and her colleagues enriched nitrite reducers from marine sediments on a complex carbon source, including amino acids, sugars and organic acids, in a continuous fed chemostat enrichment system and observed only conversion to nitrogen gas and no production of ammonia. When nitrate was the electron acceptor instead of nitrite, the mixed enrichment cultures showed combined fermentative and respiratory properties with a predominant conversion of nitrate to ammonia. These observations were attributed to a comparatively higher apparent affinity of denitrifiers for nitrite and a comparatively higher apparent affinity of DNRA bacteria for nitrate. Kraft et al. [65] concluded therefore that supply of nitrate/nitrite was a key controlling factor in the nitrate partition. Interestingly and alternatively, Yoon et al. [128] reported an opposite trend in *Shewanella loihica* chemostat cultures with nitrate or nitrite as electron acceptor. Nevertheless, they also conclude that nitrite is a determining factor in the choice between DNRA or denitrification.

As the effect of nitrite as a controlling factor in the competition between the two nitrate reducing processes is ambiguous, we wanted to verify the determinative effect of nitrate versus nitrite using an enrichment culture grown on acetate mineral medium in continuous culture. With our simplified system we can obtain additional and more quantitative insight in the DNRA process. Since the acetate in our culture is directly

oxidized to carbon dioxide, the system is better defined than the more complex, partly fermentative marine microbial community studied by Kraft et al. [65], which even showed significant turnover of sulfate and sulfide. Since we used enrichment cultures, our study is an important complement to the pure culture studies by Yoon et al. [128] with *Shewanella loihica*, which was not isolated based on DNRA capacity.

In this work we describe the results obtained with a chemostat culture inoculated with activated sludge and operated with freshwater-mineral medium containing acetate as electron donor and, initially, nitrate as electron acceptor as described by Van den Berg et al. [114] (or Chapter 2), operated at a dilution rate adequate for growth of both the denitrifying and DNRA bacteria. Throughout the study, the culture was operated under electron acceptor limiting conditions. The initial nitrate-only culture performed DNRA. Stepwise, the influent nitrate was replaced with nitrite until nitrite was the sole electron acceptor and N-source present. Steady state populations were analyzed with fluorescent *in situ* hybridization (FISH) probes.

4.2. MATERIALS AND METHODS

Chemostat operation

THE experiments were conducted using an open continuous-flow stirred-tank reactor (CSTR, i.e. a flow controlled chemostat). The basic reactor set up was the same as described by Van den Berg et al. [114]. The redox potential was monitored using a Redox probe (Mettler Toledo, Tiel, The Netherlands). Before the start of the experiments of this study the reactor had been running continuously for two years under the conditions described in this paper. Two separate media flows were supplied to the reactor in equal amounts. Both culture media were autoclaved before use and sparged with a small flow of nitrogen gas while connected to the chemostat to ensure anaerobic conditions. Medium A contained per liter: 22.0 mmol KH_2PO_4, 1.2 mmol $MgSO_4 \cdot 7H_2O$, 1.5 mmol NaOH, 1.5 mg yeast extract (as vitamin supplement) and 5 ml trace element solution [123], with the $ZnSO_4 \cdot 7H_2O$ concentration reduced to 2.2 g per liter and varying amounts of $NaNO_3$ and/or $NaNO_2$ (Table 4.1). Medium B contained varying concentrations of acetate, $NaCH_3COO \cdot 3H_2O$ (Table 4.1) to match the amount of electron acceptor provided. Note that acetate was always in excess, only the residual concentrations were decreased. Each time when the feed to the reactor was changed, 10 ml of activated sludge and 10 ml of an enriched denitrifier community on acetate were added to the reactor culture, to increase the potential for enriching the most competitive organism in the culture. Both media were pumped at 26 mL/h into the reactor so that the total influent was 52 mL/h. The effluent pump was controlled using a level sensor. The resulting dilution rate was 0.027 h^{-1}. The culture was assumed to be in steady state if conversions were constant for 5 doubling times, which was approximately 8 days.

Analytical procedures

Oxygen, carbon dioxide, nitric oxide and nitrous oxide concentrations in the headspace of the reactor were monitored in dried gas using a gas analyzer (NGA 2000, Rosemount, Chanhassen, MN, U.S.A.). The flow of nitrogen gas to the reactor was kept at 100 ml$\cdot min^{-1}$ using a mass flow controller (Brooks Instrument, Ede, The Netherlands), to maintain sufficient flow through the gas analyzer (80 ml$\cdot$ min^{-1}).

Table 4.1: Nitrate, nitrite and acetate concentrations in the influent as used in the different experimental periods.

Days	Concentration in the influent (mM)			N% as nitrite
	nitrate	nitrite	acetate	(%)
0-32	11.8	0.00	22.1	0
33-60	8.83	2.62	22.1	23
61-82	5.88	5.23	20.2	47
83-123	2.94	7.85	16.5	73
124-165	0.00	11.8	14.7	100

Samples taken from the reactor were centrifuged and supernatants were used for analysis of acetate and nitrogen compounds. The acetate concentration in the liquid phase was measured by High Performance Liquid Chromatography using an Aminex HPX-87H column (T = 60 °C) from Bio-Rad Laboratories (Hercules, CA, USA) coupled to a UV and RI detector using phosphoric acid (0.01 M) as eluent. An indication of the nitrite- and nitrate-concentration in the reactor was obtained with test strips (Merck Millipore, Carrigtohill, Ireland). When this was not zero, the concentrations were measured more accurately. Nitrate-, nitrite- and ammonium-concentrations were determined spectrophotometrically with commercial cuvette test kits (Hach Lange, Düsseldorf, Germany).

To determine the biomass concentration, the reactor effluent was centrifuged (10 000 rpm for 20 min) and the pellet was dried at 105 °C. Subsequently the ash content was subtracted to obtain VSS concentration. The ash content was determined by burning the organic parts of the dried pellet at 550 °C. Protein content of the biomass was measured using the Uptima BC Assay Protein Quantitation Kit (Interchim, Montluçon, France).

The biomass composition was calculated from the measured Total Organic Carbon (TOC) and Total Organic Nitrogen (TON) of washed biomass pellets, using a TOC-L CPH/CPN analyzer (Shimadzu Benelux, 's-Hertogenbosch, The Netherlands). TOC was determined as Total Carbon (TC) subtracted by Inorganic Carbon (IC) (TOC = TC - IC). Biomass composition was measured for several steady states and did not significantly differ for the different populations. In our calculations we used the average of 0.23 ± 0.01 mol N per C-mol biomass.

A balance of degree of reduction and a charge balance of incoming and exiting elements in the chemostat were set up to verify the consistency of our measurements. The concentration of volatile suspended solids (VSS) was used as biomass concentration. The growth in the system is relatively fast compared to e.g. soils. As a result biomass decay is not significant and immobilization/re-mineralization negligible. Hence, ammonium production was attributed to nitrate reduction by DNRA. As emissions of nitric and nitrous oxide were not detected, the nitrogen not accounted for in ammonium, nitrate, nitrite or biomass was assumed to be converted to N_2. Sulfide was not detectable (< 2 μmol/l). The dissolved CO_2 species, mostly HCO_3^-, which leave the reactor in the effluent, were estimated and taken into account. It was assumed Henry's law applies, using T = 298 K, p = 1 atm, $H^{cp}_{CO_2}$ = 3.4·10^{-4}mol· m^{-3}·Pa^{-1} (2), pK_a = 6.35 ($H_2CO_3 \rightleftharpoons HCO_3^- + H^+$), K_{HCO_2} = 1.7·10^{-3} [51].

Table 4.2: Probes used in FISH analysis of the culture

Probe	Sequence (5'-> 3')	Dye	Specificity	Reference
EUB338mix	gcwgccwcccgtaggwgt	Cy5	Most bacteria	[5, 24]
Beta42a	gccttcccacttcgttt	Fluos	*Betaproteobacteria*	[72]
Gamma42a	gccttcccacatcgttt	none	*Gammaproteobacteria*	[72]
GeoBac464	agcctctctacacttcgtc	Cy3	Specific for DNRA bacterium	[114]

Microbial population analysis

The microbial composition of the culture was analyzed with fluorescent *in situ* hybridization (FISH) as described by [55], using a hybridization buffer containing 35% (v/v) formamide. The applied probes are listed in Table 4.2. The general probe mixture EUB338 labeled with Cy5 was used to identify all eubacteria species in the sample. In the shown result, we used the EUB338 (Cy5), the Beta42a probe, labeled with FLUOS (plus an unlabeled Gamma42a probe, to minimize erroneous hybridizations of Beta42a) and GeoBac464, a probe labeled with Cy3 specifically designed for the detection of the 16S rRNA of the DNRA microorganism dominating the culture under nitrate limitation (Table 4.3, Table 4.4; [114]).

Probes were synthesized and 5'-labeled with either the FLUOS or with one of the sulfoindocyanine dyes Cy3 and Cy5 (Thermo Hybaid Interactiva, Ulm, Germany). Slides were observed with an epifluorescence microscope (Axioplan 2, Zeiss, Sliedrecht, The Netherlands), and images were acquired with a Zeiss MRM camera and compiled with the Zeiss microscopy image acquisition software (AxioVision version 4.7, Zeiss) and exported as TIFF format.

In addition, denaturing gradient gel electrophoresis (DGGE) was performed. Biomass samples were collected from the reactor and centrifuged and stored at -20 °C. The genomic DNA was extracted and analyzed as described by Van den Berg et al. [114]. The set of primers used is the 341F (containing a 40-bp GC clamp) and 907R [92]. The obtained sequences were corrected using the program Chromas Lite 2.1.1 (http://technelysium.com.au) and then compared to sequences stored in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm (http://www.ncbi.nlm.nih.gov/blast).

4.3. RESULTS

A chemostat based enrichment system was operated under electron acceptor (nitrate/nitrite) limiting conditions with acetate as electron donor and a dilution rate of 0.027 h^{-1}. Acetate was always detected in the effluent of the reactor and the redox potential was constant during the experimentation at minus 480 ± 50 mV. This confirmed electron acceptor limiting conditions. Initially, the electron acceptor was nitrate and the culture converted 70 ± 3% of the influent nitrate-N to ammonium, 15 ± 2% was incorporated into biomass (Table 4.5, Table 4.6), and 15% percent was presumably converted to dinitrogen gas. Stepwise, the influent nitrate was replaced by nitrite. In the first step 23% of the nitrate was replaced, and subsequently 47% and 73%, until all influent nitrogen was nitrite. When nitrate in the feed was changed to nitrite, the fraction of the influent N converted to ammonia did not change (Figure 4.1a). Thus, despite the change of electron

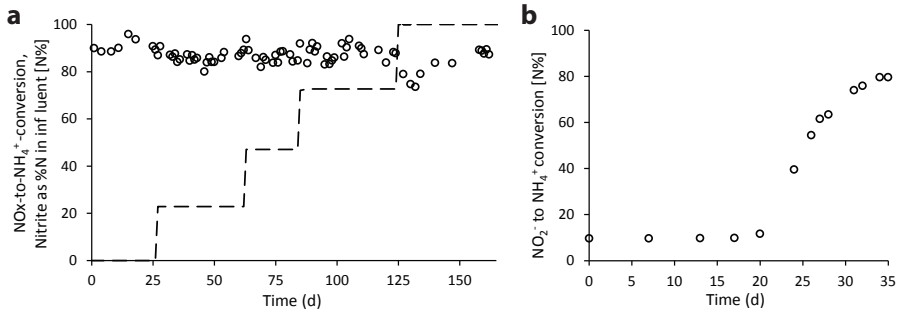

Figure 4.1: Ammonium production in the acetate fed chemostat systems, as a percentage of the NOx conversion in time. This includes both dissimilatory and assimilatory production of ammonium. (a) Ammonium production (open circles) for the varying percentages of NO_2^- in the influent nitrogen (dashed line). The other influent nitrogen was nitrate. (b) Ammonium formation in the enrichment inoculated with activated sludge with nitrite as electron acceptor.

acceptor from nitrate to nitrite, DNRA remained equally dominant in the reactor.

To confirm that DNRA bacteria can not only remain, but also outcompete the denitrifiers with nitrite as electron acceptor, a second reactor was started up in the same conditions as the nitrite-only system. Starting from an inoculum of activated sludge, a DNRA culture was enriched with nitrite as electron acceptor (Figure 4.1b). Thus, we confirmed that DNRA bacteria successfully outcompete denitrifiers when nitrite is the limiting electron acceptor in the chemostat enrichment culture.

Both in the nitrate-only and nitrite-only culture, the C/N ratio of the biomass was measured to be the same, 0.23 ± 0.01 molN/molC. The biomass yield was 12.3 ± 1.4 gVSS/mol NO_2^- for growth on nitrite, which was lower than for nitrate, 19.0 ± 0.3 gVSS/mol NO_3^- (Table 4.7). The protein content of the VSS was measured to be 0.60 ± 0.04 mg protein/mg VSS. When both yields are compared as per mole electron donor (acetate), the yields are similar for growth on nitrate and nitrite (13.4 ± 0.6 and 11.7 ± 1.5 gVSS/mol acetate respectively).

The microbial population analysis using fluorescent *in situ* hybridization (FISH) showed that also the population did not change when changing from nitrate to nitrite as electron acceptor in the system (Figure 4.2). In Figure 4.2 almost all fixed bacteria of both cultures are purple colored and thus almost all bacteria hybridized with a probe specific for one *Geobacter* ribotype (Table 4.3, Table 4.4), described by Van den Berg et al. [114]. Additional DGGE analysis showed the ribotypes were identical (Figure 4.4). The green colored bacteria (*Betaproteobacteria*) are not necessarily the same species in both cultures, but are present in the same low amount in both steady states and are therefore assumed not to be relevant for the major conversion stoichiometry.

4.4. DISCUSSION

IN our chemostat enrichment system provided with acetate as the simple non-fermentable carbon and energy source, the competition between DNRA and denitrification was unaffected by the type of electron acceptor. Despite changing the supply of nitrate to nitrite, DNRA remained the dominant N-reduction pathway in the reactor. This is in

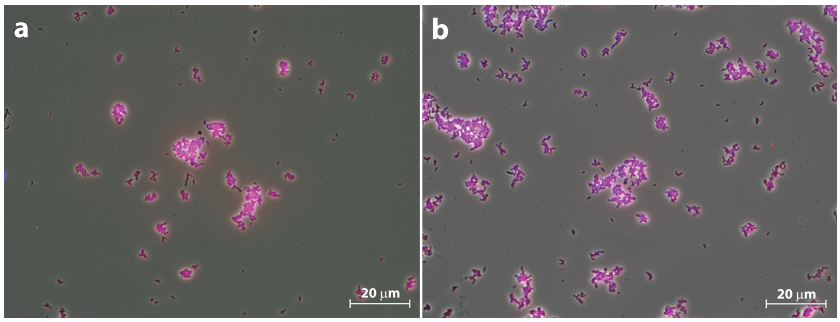

Figure 4.2: FISH microscopic photographs of steady state cultures. (a) The culture grown on nitrate only. (b) The culture grown on nitrite only. The cells were stained with Cy5-labeled probes for bacteria (EUB338mix, blue), FLUOS-labeled probes for Betaproteobacteria (Beta42a, green) and Cy3-labeled probes specific for the reactor species (GeoBac464, red). Cells that are green indicate cells to which the probes EUB338mix and Beta42a were hybridized. Cells that are purple indicate cells to which the probes EUB338mix and GeoBac464 were hybridized.

accordance with pure culture studies by Yoon et al. [128]. Our observations are clearly different from the observations in a chemostat enrichment culture by Kraft et al. [65]. They reported that in an electron acceptor limited marine enrichment culture, fed with glucose, acetate and amino acids, nitrate selected for a DNRA community whereas nitrite selected for a denitrifying community.

The enrichment of a DNRA culture under nitrite limitation with a nitrate based DNRA culture as inoculum was confirmed by starting a similar enrichment culture inoculated with activated sludge. In this case initially a denitrifying culture was obtained, likely due to their faster growth rate. The denitrification culture was rapidly replaced by a stable DNRA culture. This development from activated sludge inoculum replicates the enrichment of a DNRA culture under nitrate limiting conditions [114] (or Chapter 2). This emphasizes the similarity of nitrate or nitrite in the enrichment of DNRA bacteria.

Under single substrate limiting conditions in a chemostat, Yoon et al. [128] observed no effect of nitrate versus nitrite on the end-product of the nitrate reduction process in their pure culture of *Shewanella loihica* strain PV-4, using partial lactate oxidation to acetate. Like in our study, the end-product of the nitrogen conversion was predominantly ammonium under electron acceptor limiting conditions. Yoon et al. [128] did observe an effect of nitrite when the C/N ratio of the influent substrates was such that both electron donor and acceptor were limiting. When nitrite instead of nitrate was used under these conditions, a higher fraction of the influent nitrogen was converted to ammonium, i.e. increase of the lactate that was used for DNRA and decrease for denitrification. In our system we would expect a similar observation, due to the lower electron acceptor capacity of NO_2^- compared to NO_3^-. In other words, more moles of electron donor are required per mole nitrate compared to nitrite. The effect of C/N ratio essentially is a result of the ratio of electrons that can be donated over electrons that can be accepted. To exemplify this, an extrapolation was done using the data and model of our previous study with the same chemostat enrichment culture system on the C/N effect [115] (or Chapter 3). For use of nitrite instead of nitrate, both the stoichiometry of DNRA and denitrification change. As a result the dual limitation range shifts and slightly broadens, as described in

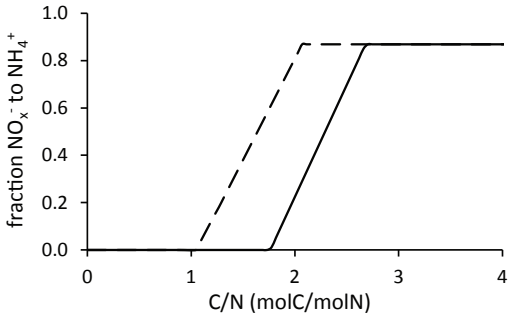

Figure 4.3: Predicted ammonium production at different influent acetate:nitrogen ratios in a chemostat fed with nitrite (dashed line) or nitrate (solid line) as electron acceptor. The ammonia production using nitrate is obtained from the model for our previous study [115]. The shown ammonia concentrations for use of nitrite are an extrapolation of the model data.

Figure 4.3. For example, at a C/N ratio of 2 molC/molN with nitrate-N, the system will result in a steady state in the dual limitation phase with coexistence of both processes, whereas at this ratio of 2 molC/molN for nitrite-N, in the steady state only nitrite will be limiting and DNRA dominates. Thus, at the same C/N ratio, a change in electron acceptor from nitrate to nitrite will result in more reduction to ammonia and less denitrification to dinitrogen gas. Thus nitrite, replacing nitrate, affects the competition by changing the electron accepting capacity and thereby making the conditions more electron acceptor limited. This effect would presumably also be observed in our system, when tested under dual substrate limited conditions.

In the electron acceptor limited chemostat enrichments of Kraft et al. [65], nitrite was predominantly reduced to dinitrogen gas, whereas the main product of nitrate reduction was ammonium. The authors observed that despite additional factors which might favor DNRA (e.g. increase of C/N ratio of the substrates, addition of sulfide, lower pH, or the use of non-fermentative electron donors), denitrification remained dominant when nitrite was supplied as electron acceptor. Therefore, Kraft et al. [65] proposed nitrite versus nitrate as one of the key factors in the competition between denitrification and DNRA in their marine system and furthermore suggest that denitrifiers have a higher affinity for nitrite and DNRA bacteria for nitrate. The results of this study and of Yoon et al. [128] illustrate that the effect of nitrite/nitrate supply per se is not a universal controlling factor in the competition between denitrification and DNRA. At the same time, the ambiguity shows that a combination of environmental factors can have more significant differentiating effects. As already stated by [9] we first need to establish the effect of separate environmental factors using simple systems to understand behavior in more complex lab systems.

Despite the supplementary inoculation of our established enrichment chemostat culture with fresh activated sludge from an existing wastewater treatment plant and denitrifier communities, the change of electron acceptor from nitrate to nitrite did not change the dominant ribotype in the electron acceptor limited chemostats described in this work. In all experiments the same *Geobacter lovleyi* related ribotype dominated the microbial community. Apparently, under the used conditions, this species has highest

affinity (mumax/Ks) for both electron acceptors.

Our results suggest that nitrite and nitrate fluctuations in an environment will have limited influence on the dominant nitrate reducing process when acetate is the electron donor. This implies that other competition affecting factors, such as pH, sulfide concentrations, or the type and complexity of the electron donor, may have a decisive effect on the nitrate reducing pathway that dominates, rather than, as suggested by Kraft et al. [65], via direct control of either nitrite or nitrate as electron acceptor.

In summary, we show nitrite is not a controlling factor in the competition between DNRA and denitrification in a fresh water mixed culture chemostat with acetate as electron donor. In our experiments no changes were observed in the nitrogen reducing pathway when nitrate was replaced by nitrite as electron acceptor. The dominant process remained DNRA and the same *Geobacter* species was the dominant enriched organism, independent of the supply of nitrite or nitrate as electron acceptor. When starting from a fresh inoculum with nitrite as electron acceptor, DNRA outcompeted denitrification.

4.5. SUPPLEMENTARY MATERIAL

Table 4.3: Test with SILVA TestProbe (database SSU 128, sequence collection REFNR).

Mismatches allowed	Matches	
0	0	-
1	0	-
2	1	uncultured in genus *Geobacter*

Table 4.4: Test with RDP ProbeMatch.

Mismatches allowed	Matches	
0	2	uncultured in genus *Geobacter*[1]
1	4	uncultured in genus *Geobacter*
2	7	uncultured in genus *Geobacter* (6), order of unclassified *Desulfuromonadales* (1)

[1] These are two sequences of the ribotype the probe specifically targets, which were deposited in our previous study [114].

Table 4.5: Steady state conversion rates in the reactor with average deviation.

Influent	Compound conversion rates (mmol·h^{-1})						
NO_2^- (N%)	Ac^-	NO_3^-	NO_2^-	H^+	Biomass	NH_4^+	CO_2
0	-0.85 ±0.04	-0.60 ±0.01	-	-1.97 ±0.05	0.46 ±0.04	0.44 ±0.08	1.04 ±0.06
23	-0.80 ±0.03	-0.45 ±0.01	-0.13 ±0.00	-1.77 ±0.03	0.39 ±0.04	0.43 ±0.01	0.92 ±0.06
47	-071 ±0.03	-0.30 ±0.01	-0.27 ±0.01	-1.53 ±0.04	0.34 ±0.03	0.45 ±0.01	0.88 ±0.06
73	-0.63 ±0.02	-0.15 ±0.00	-0.40 ±0.01	-1.59 ±0.02	0.33 ±0.03	0.43 ±0.01	0.81 ±0.05
100	-0.63 ±0.04	-	-0.60 ±0.01	-1.49 ±0.11	0.30 ±0.06	0.44 ±0.02	n.a.

Table 4.6: Balances over the steady state conversion rates in the reactor.

Influent	Balance residuals (%)		
NO_2^- (N%)	Carbon	Reduction	Charge
0	7	3	6
23	11	6	1
47	8	5	10
73	5	3	1
100	-	8	12

Table 4.7: Growth yield values in the reactor steady states, calculated from values in Table 4.6.

Influent	Biomass yield (%)	
NO_2^- (N%)	gVSS·mol acetate^{-1}	g VSS·mol N^{-1}
0	13.4 ±0.6	19.0 ±0.3
23	12.2 ±0.5	16.6 ±0.4
47	11.8 ±0.5	14.8 ±0.3
73	13.0 ±0.5	14.9 ±0.3
100	11.7 ±1.5	12.3 ±1.4

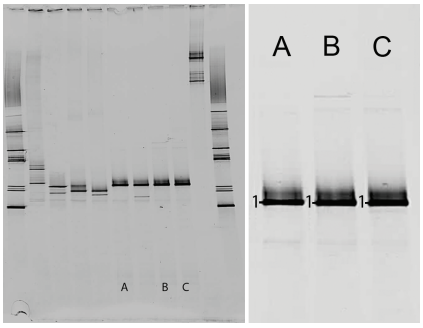

Figure 4.4: (a) DGGE image zoomed and processed using Adobe Photoshop. (b) Full DGGE gel picture. The sample in lane A is from the steady state with nitrite limitation, after stepwise change from nitrate. The sample in lane B is from the steady state with limiting nitrate. The sample in lane C is from the steady state with limiting nitrite, enriched directly from activated sludge. The DGGE bands sequences of the bands indicated with 1 were identical. Additionally, they were similar to the ribotype sequences found in our previous studies (identical to NCBI accession KT317071, one gap with KM403205), which were closely related to *Geobacter lovleyi* strain SZ [114, 115] (or Chapter 2, Chapter 3).

5

FERMENTATIVE BACTERIA INFLUENCE THE COMPETITION BETWEEN DENITRIFIERS AND DNRA BACTERIA

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Abstract

Denitrification and dissimilatory reduction to ammonium (DNRA) are competing nitrate-reduction processes that entail important biogeochemical consequences for nitrogen retention/removal in natural and man-made ecosystems. The nature of the available carbon source and electron donor have been suggested to play an important role on the outcome of this microbial competition. In this study, the influence of lactate as fermentable carbon source on the competition for nitrate was investigated for varying ratios of lactate and nitrate in the influent (Lac/N ratio). The study was conducted in an open chemostat culture, enriched from activated sludge, under strict anoxia. The mechanistic explanation of the conversions observed was based on integration of results from specific batch tests with biomass from the chemostat, molecular analysis of the biomass enriched, and a computational model. At high Lac/N ratio (2.97 mol/mol) both fermentative and respiratory nitrate reduction to ammonium occurred, coupled to partial oxidation of lactate to acetate, and to acetate oxidation respectively. Remaining lactate was fermented to propionate and acetate. At a decreased Lac/N ratio (1.15 mol/mol), the molar percentage of nitrate reduced to ammonium decreased to 58 %, even though lactate was supplied in adequate amounts for full ammonification and nitrate remained the growth limiting compound. Data evaluation at this Lac/N ratio suggested conversions were comparable to the higher Lac/N ratio, except for lactate oxidation to acetate that was coupled to denitrification instead of ammonification. Respiratory DNRA on acetate was likely catalysed by two Geobacter species related to G. luticola and G. lovleyi. Two Clostridiales members were likely responsible for lactate fermentation and partial lactate fermentation to acetate coupled to fermentative DNRA. An organism related to Propionivibrio militaris was identified as the organism likely responsible for denitrification. The results of this study clearly show that not only the ratio of available substrates, but also the nature of the electron donor influences the outcome of competition between DNRA and denitrification. Apparently, fermentative bacteria are competitive for the electron donor and thereby alter the ratio of available substrates for nitrate reduction.

5.1. INTRODUCTION

NITRATE can be reduced by different dissimilatory nitrogen cycle processes. The processes of denitrification and anaerobic ammonium oxidation (anammox) remove nitrogen from the environment by converting nitrate to dinitrogen gas [64]. Removal of nitrate is essential to counteract pollutions as a result of anthropogenic N inputs, for example, from wastewaters and brines, prior to its discharge in oceans or rivers [14, 103]. Alternatively the dissimilatory nitrate reduction to ammonia (DNRA) retains the nitrate-nitrogen in the ecosystem as ammonium and therefore DNRA does not alleviate eutrophication [54]. Conversion of nitrate to ammonium can also be beneficial, as the ammonium-ion is retained in soils and sediments by absorption, whereas the nitrate anion is easily lost due to leaching [96]. The DNRA process has received markedly less attention compared to denitrification, and it is the least well described of the nitrogen cycle processes [104]. Although contributions have increased in the past decade, our understanding of the role of DNRA in the environment is limited. As a result, the environmental factors directing the nitrate reduction competition are limitedly understood. To enable control of the nitrate reduction towards the desired end product (N_2 or NH_4^+), we need to improve this understanding. We focus on the competition between heterotrophic denitrification and DNRA in particular, since autotrophic denitrification and anammox are not considered relevant in organic carbon abundant enrichments.

An environmental factor well reported to direct the competition between denitrification and DNRA is the C/N ratio of available substrates [65, 91]. DNRA bacteria have a competitive advantage in nitrate limiting conditions and excess of electron donor, whereas denitrifiers are more competitive when electron donor is limiting [65, 115]. This was shown qualitatively in both aquatic and terrestrial environments. Lab cultures provided more insight in the mechanism of this selection by the ratio of available substrates (e.g. [3, 111, 115, 127]). Tiedje et al. [111] proposed that DNRA could be more favorable under nitrate limiting conditions, because of the capacity of DNRA to accept eight electrons per nitrate, whereas in denitrification five electrons are accepted, even though thermodynamics suggest that the free energy change per nitrate reduced is comparable. Van den Berg et al. [115] studied the effect of available substrates in a continuous enrichment system, using (non-fermentable) acetate and nitrate as substrates at variable acetate concentrations in the influent to alter the electron donor and acceptor in the influent (i.e. Ac/N ratio). For a wide range of substrate ratios, a steady state was established where denitrification and DNRA coexisted, and both acetate and nitrate were limiting. A model showed that this behavior could be attributed to the differences in the process stoichiometries of DNRA and denitrification, i.e. use of acetate per nitrate in the metabolism [115] (or Chapter 3).

Ecological niches allowing DNRA to occur have typical an excess of carbon substrate, this will also give the possibility for fermentative bacteria to be active at the same time. Fermentative conversions will have an influence on the type of carbon source available for nitrate reduction and thereby potentially affect the relative occurrence of DNRA and denitrification. Differences in use of electron donors for the reduction of nitrate are limitedly understood. For some pure cultures, yields have been reported for different substrates (e.g. [105]). Rehr and Klemme [88] studied denitrifying and DNRA pure cultures competing for nitrate using lactate and different additional amounts of glucose in a chemostat

system [88]. They suggest the bacterium performing fermentation and DNRA, as opposed to denitrification only, had a competitive advantage, because it could obtain energy from both fermentation and electron donor oxidation coupled to acetate production. Akunna et al. [2] reported batch cultivations with sludge from an anaerobic digester and showed that nitrate reduction to ammonia occurs only for the fermentable substrates glucose and glycerol, but not for lactate, acetate and methanol. These observations suggest that DNRA can be more competitive when the organic electron donors available are more reduced, because of additional occurrence of fermentative DNRA. This 'fermentative DNRA' is bioenergetically advantageous compared to pure fermentation, because DNRA allows more acetate production. Hence, using fermentative DNRA more substrate level ATP can be produced from acetyl-CoA, without compromising the required redox balance, as the reduction equivalents are channeled off to reduce nitrate [22, 64, 83]. In fermentative DNRA, the electrogenic yield of the nitrate reduction can be absent or lower compared to the respiratory DNRA, and varies for different conditions [21, 25, 80, 84].

Previous we reported on the effect of C/N ratio with a non-fermentable substrate [115] (or Chapter 3), and in the present study we have extended the complexity by using a fermentable substrate to test the influence of fermentative conversions on the competition between DNRA and denitrification. Lactate was chosen as a "model"-fermentable energy- and C-source, as for this substrate fermentation pathway options are relatively limited compared to carbohydrates like glucose, thereby minimizing the additional complexity of the system. We hypothesized that lactate fermentation only occurs when nitrate is depleted, as observed previously in acetate - nitrate studies [115] and that C/N effect will have the same stoichiometric basis.

We studied the effect of lactate/N (Lac/N) in a continuous enrichment (i.e. mixed) culture grown on mineral medium with lactate as electron donor and nitrate as electron acceptor. The reactor was operated at a low enough dilution rate to allow growth of both the denitrifying and fermentative and respiratory DNRA bacteria [22, 65, 114]. Lactate concentrations were adapted to create different ratios of lactate per nitrate (Lac/N ratio) in the influent, comparable in terms of electron equivalents to the acetate/N (Ac/N) ratios used in our previous study (Table 5.1) [115]. As the additional complexity in the system, compared to acetate use, obscured direct interpretation of the conversions, batch tests were performed with the steady state cultures to identify the potential capacities for pathways of the relevant e-donors and e-acceptors involved in the steady states. In addition, a model was developed to evaluate the possible pathway contributions in the overall conversions. Furthermore, the steady state microbial communities were analyzed using amplicon sequencing, verified by denaturing gradient gel electrophoresis (DGGE), and fluorescent *in situ* hybridization (FISH).

5.2. MATERIALS AND METHODS

Chemostat operation

CONTINUOUS culture experiments were performed in an anoxic chemostat reactor, a double-jacket glass reactor with a working volume of 2 l (Applikon, Delft, the Netherlands). The bioreactor was inoculated with a sample of 2 l of activated sludge (3 to 3.5 g dry matter/l) from the Wastewater Treatment Plant Harnaschpolder (Delft, The Netherlands). The reactor was operated in anoxic conditions by sparging a constant flow

Table 5.1: Lactate/nitrate influent ratios translated to C/N ratios. As the results are compared with acetate influent, in the third column the influent acetate/N ratio representing the same amount of influent electron equivalents as the Lac/N is listed. Lactate can donate twelve electrons and acetate eight, so they both donate 4 electrons per C-mol.

Day	Lac/N (mol/mol)	C/N (C-mol/N-mol)	Comparable Ac/N (mol/mol)
0-45	2.97	8.92	4.46
46-110	1.15	3.45	1.87
111-135	0.63	1.88	1.94

of 100 ml min^{-1} of nitrogen gas, by means of a mass flow controller (Brooks Instrument, The Netherlands). The stirring speed was kept at 400 rpm, a stirrer with two standard geometry blades was used. The pH of the culture was monitored by a pH electrode (Mettler Toledo, USA) and controlled to a set point of 7.1 ± 0.05 with 0.5 M NaOH and 0.5 M HCl by a pH biocontroller, ADI 1030 (Applikon, Delft, The Netherlands). To monitor acid and base consumption the respective bottles were periodically weighted. The redox potential of the culture was monitored by a redox electrode (Mettler Toledo, USA). Data acquisition of online measurements (redox potential, pH, acid and base dosage) was accomplished by MFCS/win (Sartorius Stedium Systems, USA). A water jacket and cryostat bath (Lauda, Germany) was used to maintain the reactor temperature at 22 °C.

Peristaltic pumps (Masterflex®, USA) were used to supply influent and remove effluent, controlling the dilution rate of the system to 0.027 ± 0.001 h^{-1}. The effluent pump was controlled by a volume level sensor. The influent pump was calibrated to deliver two separate medium flows at equal rates to a total constant rate of 53 ml h^{-1}, which corresponds to the mentioned dilution rate. Both culture media were autoclaved before use and sparged with a small flow of nitrogen gas while connected to the chemostat to ensure anaerobic conditions. The pump tubing was Noroprene Masterflex®, all other tubing was Noroprene. The substrate medium (A) contained lactate prepared from a sodium DL-lactate solution syrup, 60% (w/w) to obtain a concentration of 35.0 mM for Lac/N 2.97, 13.5 mM for Lac/N 1.15 and 7.38 mM for Lac/N 0.63 (Table 5.1). The mineral medium (B) contained per litre: 23.5 mmol $NaNO_3$ as nitrogen source and electron acceptor, 22.0 mmol KH_2PO_4, 1.2 mmol $MgSO_4 \cdot 7H_2O$, 1.5 mmol NaOH , 1.5 mg yeast extract and 5 ml trace element solution [123], with the $ZnSO_4 \cdot 7H_2O$ concentration reduced to 2.2 g per liter and use of sodium molybdate instead of ammonium molybdate. In a parallel reactor with identical set up the Lac/N 2.97 mol/mol steady state and Lac/N 1.15 steady state were reestablished from the chemostat culture effluent (2 l) from another steady state, Lac/N 1.15 and Lac/N 0.63 respectively, and, in addition, 10 ml of fresh activated sludge.

Batch experiments

One liter of chemostat effluent was collected on an ice bath under anoxic conditions by continuously flushing with a low flow of dinitrogen gas. Prior to the batch tests the biomass concentration was determined as volatile suspended solids (VSS). The effluent was centrifuged during 20 min at 10 000 rpm and 4 °C. The cell pellet was resuspended in a phosphate buffer (26.8 mM, pH 7.00), which was flushed with nitrogen gas for 30 min to minimize dissolved oxygen concentration. The batch experiments were performed in 20

Table 5.2: List of batch tests performed in the culture of Lac/N ratio of 2.97 and respective combination of electron donor and acceptor. Initial concentrations of electron donor were always 5 mM and electron acceptor 4 mM, in a batch volume of 10 ml.

Test	Electron donor	Electron acceptor
A	Lactate	-
B	Lactate	Nitrate
C	Lactate	Nitrite
D	Acetate	Nitrate
E	Acetate	Nitrite
F	Propionate	Nitrate
G	Propionate	Nitrite

Table 5.3: List of batch tests performed in the culture of Lac/N ratio of 1.15 and respective combination of electron donor and acceptor. Initial concentrations of electron donor were always 5 mM and electron acceptor 4 mM, in a batch volume of 20 ml.

Test	Electron donor	Electron acceptor	Acetylene concentration (%(v/v))
H	Lactate	-	-
I	Lactate	Nitrate	-
J	Lactate	Nitrite	-
K	Acetate	Nitrate	-
L	Acetate	Nitrite	-
M	Propionate	Nitrate	-
N	Propionate	Nitrite	-
O	Lactate	Nitrate	5
P	Acetate	Nitrate	5
Q	Propionate	Nitrate	5

or 30 ml serum bottles equipped with rubber stoppers and aluminum cap sealers. Batch tests were not duplicated. The carbon source and electron acceptor were added in varying combinations according to Table 5.2 and Table 5.3. Initial concentrations of electron donor were always 5 mM and electron acceptor 4 mM, in a batch volume of 10 ml or 20 ml. To estimate production of dinitrogen gas in the batch tests, in particular cases the cells were additionally incubated with 10% acetylene in the gas phase to block the nitrous oxide reductase. The observed N_2O production in these batch tests is an indication of the denitrifying potential of the biomass. The bottles were sequentially sealed, flushed with dinitrogen gas with a syringe tip through the rubber lid and submitted to vacuum to release dissolved gasses. During the experiments a slightly positive pressure was maintained in the vials to avoid oxygen leakage into the bottles and to facilitate sampling. Incubation times varied between 3 and 6 hours and the sampling interval varied from 45-90 minutes (Table 5.7, Table 5.8).

Analytical procedures

Either for chemostat or batch experiments, periodic samples were taken, respectively, from the reactor or vials and centrifuged for 4 min at 13 000 rpm. The supernatant was collected to measure nitrogen compounds (ammonium, nitrite and nitrate), lactate

and volatile fatty acids concentrations. Test strips (Merck Millipore, Germany) were used to test qualitatively the presence of nitrate and nitrate. Ammonium concentrations were quantified spectrophotometrically with a commercial cuvette test kit (Hach Lange, Germany), with a lower detection limit of 1 μM. In case of a test strip positive result nitrate and nitrite were tested with a similar test kit, with lower detection limits of respectively 0.02 and 0.01 mM. Lactate and volatile fatty acids, such as acetate and propionate, were determined with high-performance liquid chromatograph using a BioRad Animex HPX-87H column. Together with the set of samples, standards for acetate and lactate (5mM) and propionate (5 and 18 mM) were analysed in HPLC to validate the calibration curve and results. Oxygen, carbon dioxide, nitric oxide and nitrous oxide partial pressure in the off-gas of the chemostat were monitored using a gas analyser (NGA 2000, Rosemount, USA). The gas flow through the reactor of 100 ml min^{-1} was chosen to maintain sufficient flow through the gas analyzer (80 ml min^{-1}). Nitrous oxide partial pressure in the headspace of the batch vials was measured off-line on an Agilent 6890 Gas Chromatograph, with reported protocol [56]. The system was considered to be in steady state when conversion rates were stable for at least 7 days, i.e. 5 volume changes.

Biomass concentrations of the chemostat culture were measured by determination of the volatile suspended solids (VSS) concentrations using reported methods for DNRA bacteria [114] (or Chapter 2). To determine the biomass concentration, the reactor effluent was centrifuged (10 000 rpm for 20 min) and the pellet was dried at 105 °C. Subsequently the ash content was subtracted to obtain VSS concentration. The ash content was determined by burning the organic parts of the dried pellet at 550 °C. Protein concentrations were measured by the bicinchoninic acid method using BC Assay Protein Quantification Kit (Interchim, France) following manufacturer's instructions.

A balance of degree of reduction and a charge balance of incoming and exiting elements in the chemostat were set up to verify the consistency of our measurements. The concentration of volatile suspended solids (VSS) was used as biomass concentration. The system has a relatively high dilution rates, compared to e.g. soils. As a result biomass decay is not significant and immobilization/re-mineralization negligible. Hence, ammonium production was attributed to nitrate reduction by DNRA. As emissions of nitric and nitrous oxide were not detected, the nitrogen not accounted for in ammonium, nitrate, nitrite or biomass was assumed to be converted to N_2. Sulfide was not detectable with the methylene blue method [20], with a lower limit of 1 μM. To calculate the concentration of bicarbonate species in the chemostat solution, the electro-neutrality equation for the charged species in the chemostat was solved with pKa values listed in Table 5.7 . In the batch conversions, the end product concentrations are used to calculate percentages of N-conversion as a percentage of the consumed nitrate or nitrite. In the batches with acetylene, the N_2 production was estimated from the end product concentration of nitrous oxide, subtracted by the nitrous oxide produced in the batch without acetylene.

DGGE and amplicon sequence analysis of PCR amplified 16S genes

The microbial community structure of the culture was analyzed by amplicon sequence analysis. To verify the results, the DNA extracts were additionally analyzed using denaturing gradient gel electrophoresis (DGGE), as in both methods a different PCR protocol is applied. Biomass samples were collected from the reactor, and centrifuged and stored at

-20 °C. The sample 2.97a was taken on day 36, 1.15a on day 107, 0.63 on day 134, 2.97b on day 40 of the parallel reactor and 1.15b on day 25 after restarting the parallel reactor. The genomic DNA was extracted using the UltraClean Microbial DNA isolation kit (MO BIO, Carlsbad, CA, USA), following manufacturer's instructions. The extracted DNA products were evaluated on 1% (w/v) agarose gel.

In amplicon sequencing the extracted DNA was processed by Novogene Bioinformatics Technology (Beijing, China). Amplification of part of 16S rRNA gene was performed using a paired-end Illumina HiSeq platform to generate 450 bp pair-end reads (Raw PE), which were trimmed to 250 bp. Amplicons were generated targeting hypervariable regions (V3-4) of 16S rRNA genes using specific primers (341F-806R) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Quantification and qualification of the PCR products was done by electrophoresis on a 2% agarose gel. PCR products were mixed in equidensity ratios and then purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on an IlluminaHiSeq2500 platform and 250 bp paired-end reads were generated (Table 5.8). The data was split by assigning pair-end reads to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. The paired-end reads were merged using FLASH (V1.2.7), and the splicing sequences were called raw tags. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags according to the Qiime (V1.7.0) quality controlled process. Chimeras were detected by comparing with the reference database (Gold database) using UCHIME algorithm (UCHIME Algorithm) and subsequently removed to obtain 'Effective Tags'. Sequences analysis were performed by Uparse software (Uparse v7.0.1001). Sequences with ≥97% similarity were assigned to the same consensus sequences. The consensus sequences were classified using the Greengene Database, based on the RDP classifier algorithm (Version 2.2). Alpha diversities were calculated for the different consensus sequence abundances (Table 5.9), which were normalized using a standard of sequence number corresponding to the sample with the least sequences, were used.

In DGGE analysis the extracted DNA was used as for PCR amplification of the 16S rRNA gene. The set of primers used was the 341F (containing a 40-bp GC clamp) and 907R [92]. The used PCR thermal profile started with a pre-cooling phase at 4 °C for 1 min, followed by initial denaturation at 95 °C for 5 min, 32 cycles of 95 °C for 30 s, 55 °C for 40 s, 72 °C for 40 s, followed by an additional extension step at 72 °C for 30 min. DGGE band isolation and DNA sequencing were performed as described by Bassin et al. [7] for 16S rRNA. The obtained 16S rRNA gene sequences were manually corrected using the program Chromas Lite 2.1.1 (http://technelysium.com.au). The corrected sequences bands and consensus sequences from amplicon sequencing analysis were compared with those stored in GenBank using the Basic Local Alignment Search Tool algorithm (http://www.ncbi.nlm.nih.gov/blast). The sequences have been deposited in the GenBank under accession numbers MF445187-MF445194 and MF445197-MF445207.

Table 5.4: Probes used in the FISH analysis.

Probe	Sequence (5'-> 3')	Dye	Specificity	Reference
EUB338mix	gcwgccwcccgtaggwgt	Cy5	Most bacteria	[5, 24]
Beta42a	gccttcccacttcgttt	Cy3	*Betaproteobacteria*	[72]
Gamma42a	gccttcccacatcgttt	none	*Gammaproteobacteria*	[72]
GeoBac464	agcctctctacacttcgtc	Cy3	*Geobacter* ribotype	[114]
GeoBacII464	aacctccgtacacttcgcc	Cy3	*Geobacter* ribotype	This study

FISH and microscopic analysis of the culture

Fluorescent *in situ* hybridization (FISH) was performed as described by [55], using a hybridization buffer containing 35% (v/v) formamide. The applied probes are listed in Table 5.4. The general probe mixture EUB338 labeled with Cy5 was used to indicate all eubacteria species in the sample. In the shown result, we used combined this probe with either the Beta42a probe, labeled with Cy3 (plus an unlabeled Gamma42a probe, to minimize erroneous hybridizations of Beta42a) or a mixture of two Cy3 labeled probes specifically designed for the detection of the 16S rRNA of the enriched *Geobacter* related microorganisms (GeoBac464 and GeoBacII464). The specificity for the GeoBacII464 probe, presented in this study, is included in Table 5.10. Probes were synthesized and 5'labeled with either the FLUOS or with one of the sulfoindocyanine dyes Cy3 and Cy5 (Thermo Hybaid Interactiva, Ulm, Germany). Slides were observed with an epifluorescence microscope (Axioplan 2, Zeiss, Sliedrecht, The Netherlands), and images were acquired with a Zeiss MRM camera and compiled with the Zeiss microscopy image acquisition software (AxioVision version 4.7, Zeiss) and exported as TIFF format.

Model

A simple model was used to deduce the contributions of different possible conversions for the reactor steady states. First, for each pathway considered the conversion stoichiometry was established. The lactate fermentation stoichiometry (equation 1) was based on thermodynamic state analysis as described by Kleerebezem and Van Loosdrecht [60] combined with the Gibbs energy dissipation concept proposed by Heijnen and Van Dijken [47] and Heijnen et al. [48] to estimate biomass yields and use of Gibbs energies of formation as established by Thauer et al. [108]. The resulting biomass yield was similar to measured yields for lactate fermentation by Seeliger et al. [94].

$$-8.88Lac^- - 0.20NO_3^- - 0.53H^+ + 1.00CH_{1.8}O_{0.5}N_{0.2} + 3.15Ac^- + 5.40Prop^- \\ +3.15CO_2 + 3.35H_2O \quad (1)$$

In equation 1 the abbreviation *Lac* is used for lactate, *Ac* for acetate and *Prop* for propionate. Assuming that in fermentative DNRA using lactate the same amount of ATP is harvested per acetate produced as in lactate fermentation, and nitrate reduction is not electrogenic, the stoichiometry was written as equation 2.

$$-3.51Lac^- - 1.55NO_3^- - 3.24H^+ + 1.00CH_{1.8}O_{0.5}N_{0.2} + 3.15Ac^- + 1.33NH_4^+ \\ +3.15CO_2 + 2.03H_2O \quad (2)$$

The measured metabolic stoichiometry for respiratory DNRA using acetate described by

[115] (or Chapter 3) was used (equation 3), as a very similar experimental setup was used in that study.

$$-1.96Ac^- - 1.44NO_3^- - 4.63H^+ + 1.00CH_{1.8}O_{0.5}N_{0.2} + 1.24NH_4^+ + 2.92CO_2 + 1.89H_2O \quad (3)$$

For the stoichiometry of denitrification coupled to partial lactate oxidation, the result of thermodynamic calculations was combined with the described yield per mole nitrate for denitrification using acetate in a similar system [115], as there is a discrepancy between theoretical and practical energy gain [105, 114]. The yield per mol nitrate was assumed comparable for partial lactate oxidation to acetate and acetate oxidation, because these processes have a similar ATP yield per electron mole. This resulted in a stoichiometry shown in equation 4.

$$-3.44Lac^- - 2.33NO_3^- - 2.66H^+ + 1.00CH_{1.8}O_{0.5}N_{0.2} + 3.11Ac^- + 1.06N_2 + 3.11CO_2 + 4.37H_2O \quad (4)$$

In the model, the contribution of the individual reaction rates to the overall reaction observed was estimated using an optimization procedure. The differences between the computed and the measured rates per compound were weighted by a factor equal to the inverse of the standard deviation of the compound measurements. Subsequently, the computed sum of the squared errors was minimized to obtain the optimal pathway contributions to describe the data. Biomass fractions for the contributing processes were extracted from this result by taking the separate computed biomass production rates and dividing this by the summed biomass production rate. Note that the biomass yield in fermentative DNRA would be higher, when the nitrate reduction was electrogenic. However, this would hardly affect the model outcome, as biomass was a less important parameter in the evaluation due to the relatively high standard deviation.

5.3. RESULTS

Chemostat operation

To explore the C/N effect in the competition between denitrification and DNRA with lactate as carbon source and electron donor and nitrate as e-acceptor and N source, a chemostat system was inoculated with activated sludge as mixed microbial community. Initially, the supplied Lac/N ratio was 2.97 mol/mol, which was subsequently decreased to 1.15 and 0.63 mol/mol. The system was considered in steady state when conversions were stable for at least 5 retention times. In all steady states, nitrate concentrations were always below the detection limit (<0.02 mM) and therefore considered limiting. Furthermore, no nitrite was detected in the culture (<0.01 mM), neither were nitric oxide or nitrous oxide in the off-gas (both detection limits of 5 ppm). Steady state conversions and balances are shown in Table 5.5. For all steady states, the protein content of the biomass was 0.59 ± 0.03 mg protein/mg VSS and the redox potential was -380 ± 50 mV.

Starting from activated sludge, a culture was enriched and grown at a high influent Lac/N ratio of 2.97 mol/mol. The high excess of the electron donor lactate enabled the possibility for nitrate reduction using partial lactate oxidation to acetate. In the obtained steady state, 23% of the nitrate was incorporated into biomass and 69% of the nitrate was reduced to ammonium, which was attributed to DNRA activity. No residual lactate was observed and the culture contained significant amounts of the lactate fermentation

Table 5.5: Net conversion rates (mmol/h) in the reactor steady states for the different influent Lac/N ratios (mol/mol). Calculations for the bicarbonate concentration are included in the supplementary materials.

Lac/N	Lac^-	$NO_3{}^-$	H^+	Biomass	Ac^-	Prop	$NH_4{}^+$	CO_2
2.97	-1.77 ±0.06	-0.59 ±0.02	-1.78 ±0.02	0.69 ±0.05	0.32 ±0.02	0.76 ±0.03	0.41 ±0.02	1.87 ±0.07
1.15	-0.69 ±0.02	0.60 ±0.02	-1.55 ±0.02	0.54 ±0.04	0	0.07 ±0.00	0.23 ±0.01	1.24 ±0.06
0.63	-0.37 ±0.06	-0.59 ±0.02	n.d.	n.d.	0	0	0	n.d.

Table 5.6: Balance residuals (%) for the conversions in the reactor steady states calculated from the conversion rates. As no biomass, carbon dioxide and proton consumption measurements were available for the steady state receiving 0.63 Lac/N, balances could not be evaluated for this

Lac/N	Carbon	Charge	Degree of reduction
2.97	3	12	2
1.15	4	16	11

products acetate and propionate. Approximately 18% of the lactate was converted to acetate and 42% was converted to propionate. The biomass yield in this culture was 9.6 ± 0.5 g VSS/mol lactate.

Subsequently, the excess influent lactate was reduced (but still in excess) to achieve the Lac/N ratio of 1.15. In this case only 10% of lactate was converted to propionate and there was no residual acetate. 58% of influent nitrate was converted to ammonium, and 20% was used for biomass production. The part of converted nitrate unaccounted for, 38%, was assumed to be converted to dinitrogen gas, with an estimated rate of 0.13 ± 0.02 mmol/h. In addition, acid consumption was lower as less acid was consumed in the nitrate reduction to dinitrogen gas as compared to DNRA (Table 5.11). For this decreased Lac/N ratio of 1.15, the yield on lactate was increased to 19.2 ± 1.0 g VSS/mol lactate, since a larger fraction of lactate was respired.

To validate conversions at dual limitation of electron donor and $NO_3{}^-$-N, the influent Lac/N ratio was further decreased to 0.63 mol/mol. At this steady state, all influent nitrate was denitrified, apart from assimilation, and the effluent contained no residual lactate or fermentation products. Hence, both the DNRA and fermentative bacteria were outcompeted and only denitrification remained.

Batch experiments

To estimate the possible catabolic processes occurring in the reactor steady states, simultaneous batch tests were performed using resting cell suspension obtained from the steady state reactor biomass. Additionally, the consumption rates of different substrates were evaluated. Different combinations of carbon sources and electron acceptors were tested. Lactate, acetate and propionate were tested separately as electron donors since these were available in the different steady states of the chemostat experiment. Carbon sources were always supplied in higher initial concentrations (5 mM) than electron acceptor (4 mM) to assure electron-excessive conditions. A full overview of the batch results can be found in Figure 5.4 and the resulting conversion rates in Table 5.11and Table 5.12.

In Figure 5.1 the concentration profiles of the four most relevant the batch tests

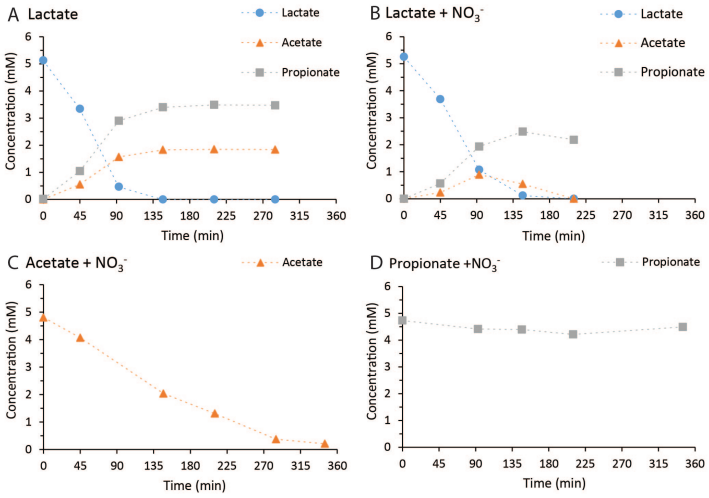

Figure 5.1: The concentration profiles of the batch tests performed with the culture operated at Lac/N ratio 2.97. The tested substrate combinations shown are (A) lactate in the absence of an electron acceptor, (B) lactate with nitrate, (C) acetate with nitrate and (D) propionate with nitrate.

performed with the culture operated at Lac/N ratio 2.97 are shown. In the absence of an electron acceptor, one mole lactate was fermented to 0.37 mole of acetate and 0.69 mole of propionate (Figure 5.1a), with no measurable production of H_2. Batch tests using lactate together with nitrate or nitrite as electron acceptor showed similar rate of propionate production and a transient acetate accumulation (Figure 5.1b). The acetate accumulation was lower with nitrite as electron acceptor compared to nitrate. For both electron acceptors, lactate and acetate were consumed simultaneously and the production rate of propionate was lower than in the absence of an electron acceptor.

With acetate as electron donor, the conversion appeared to be slower than lactate depletion for the same concentrations of respective electron acceptor (Figure 5.1c). Propionate, when used as an electron donor, was only consumed for the conversion of nitrate into nitrite but at an insignificant rate (Figure 5.1d).

In the batch tests with the cells from the chemostat culture operated at Lac/N ratio 1.15, when only 58%-N was converted to ammonium, the lactate fermentation stoichiometry observed was similar to the high Lac/N culture. Also, the lactate consumption rates were similar, both for using nitrate as electron acceptor and for pure fermentation. However, consumption of lactate was slower when nitrite was used as electron acceptor instead of nitrate. The propionate consumption rate with nitrate as electron acceptor was much higher compared to the Lac/N 2.97 culture. However, no propionate conversion was observed with nitrite. In the incubations with acetate, the relative amounts of ammonium and nitrous oxide produced were slightly higher for use of nitrite than nitrate.

Overall only 3-17% of the converted nitrogen could be recovered as ammonium or nitrous oxide. To estimate the production of dinitrogen gas, in additional batch tests, cells were incubated with acetylene in the gas phase (Table 5.12). These tests were performed with the nitrate incubations only and showed a great denitrifying potential, as 58-84% of

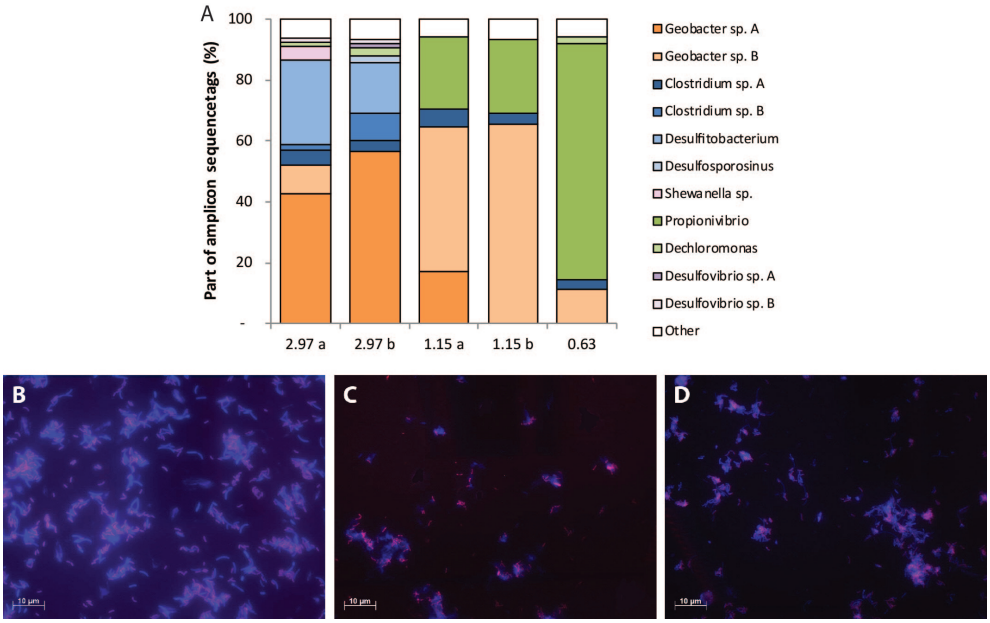

Figure 5.2: (A) Amplicon sequencing results, including consensus sequences which make up ≥1% of amplicon sequences. For the steady states of ratio 2.97 and 1.15 two samples were analyzed. (B) FISH micrograph of the steady state population receiving 2.97 Lac/N influent. (C), (D) FISH micrograph of the steady state population receiving 1.15 Lac/N influent. In (B), (C) and (D) the cells were stained with Cy5-labeled probes for bacteria (EUB338mix, blue), and was in (B) and (C) combined with Cy3-labeled probes specific for the *Geobacter* species (GeoBac464 and GeoBacII464). There, cells colored purple indicate cells to which the probes EUB338mix and GeoBac464 or GeoBacII464 were hybridized. Whereas in (D) Cy3-labeled probes for *Betaproteobacteria* (Beta42a) were used and cells colored purple indicate cells to which the probes EUB338mix and Beta42a were hybridized.

the nitrate was converted to nitrous oxide.

Microbial population

The microbial community structure for the different chemostat steady states was analyzed by amplicon sequencing, and additional DGGE for verification, and fluorescent in situ hybridization (FISH) (Figure 5.2). The consensus sequences (250 bp) which were made up of ≥1% of amplicon sequences were analyzed using BLASTn. Alpha diversities for the different samples are included in Table 5.9. Samples indicated with 'b' (Figure 5.2, 2.97b and 1.15b) were taken from the reestablished steady states. For the steady state at high Lac/N ratio of 2.97, where fermentation of lactate and DNRA were the main conversions, three predominant consensus sequences were observed in the amplicon result (Figure 5.2, 2.97a and 2.97b). On the basis of a limited identification with the 250 bp only, two of the dominant taxa were a member of the genus *Clostridium*, another related to a *Desulfitobacterium* species (blue, Figure 5.2), both described to be capable of fermentation of lactate. The other two dominant consensus sequences related most closely to the *Geobacter* species (orange, Figure 5.2), both related 100% to a ribotype

identified in a DNRA enrichment culture [115] (or Chapter 3). One was identical to the *G. luticola* related (97%) ribotype (sp. A in Figure 5.2) and the other was identical to the *G. lovleyi* related (97%) ribotype (sp. B in Figure 5.2).

When the Lac/N ratio was reduced to 1.15 and fermentation, denitrification and DNRA appeared to co-exist, the two *Geobacter* ribotypes remained dominant in the chemostat and, in addition, one of the *Clostridium* species (A) remained present (Figure 5.2a). Furthermore, a consensus sequence for bacteria closely related to *Propionivibrio militaris* was present (green, Figure 5.2a), which was assumed to be responsible for the denitrification [109].

In the culture receiving the influent Lac/N of 0.63, only denitrification was observed and the presumed denitrifier consensus sequence, closely related to *Propionivibrio militaris*, was dominant in the population (Figure 5.2a). Also the *Geobacter* sp. A and *Clostridium* sp. A consensus sequence were detected in this culture. For each of the samples, similar results were obtained using DGGE profiling (Figure 5.5).

To verify the results of the amplicon analysis and to estimate the relative abundance of predominant organisms, the populations of the steady states with Lac/N 2.97 and 1.15 were analyzed using FISH (Figure 5.2b). The relative abundance of the *Geobacter* population was analyzed using a combination of the very specific FISH probes developed for each of both *Geobacter* ribotypes [115] and Table 5.10). As *Propionivibrio militaris* belongs to the *Betaproteobacteria*, and was the only dominant *Betaproteobacterium* observed in the sequencing analysis, its relative abundance was assumed to be covered by FISH probes for *Betaproteobacteria*. The *Clostridiales* and *Desulfitobacterium* members found in amplicon sequencing were not targeted with a (group-)specific probe and therefore largely made up the population only hybridizing with the probe for eubacteria (blue colored cells, Figure 5.2b). A FISH probe for *Gammaproteobacteria* was used to determine the relative abundance of the *Shewanella* species observed in Figure 5.2, 2.97a, but no hybridization was observed (not shown).

For the steady state with Lac/N ratio 2.97, about half of the cells hybridized with the specific probes for the *Geobacter* species, colored in purple in the FISH image (Figure 5.2b), and therefore identified as the *Geobacter* related biomass. Separate probing of the two species is included in the supplementary materials. The remaining cells, colored in blue, were assumed to belong the consensus sequences of the *Clostridiales*, and there was no signal of *Betaproteobacteria*. In the population of the steady state with Lac/N ratio 1.15 (Figure 5.2c and d), the relative abundance of the two combined *Geobacter* species remained. However, a significant part of the population was then identified as *Betaproteobacteria*, most likely of the consensus sequence related to *Propionivibrio militaris*.

Model based evaluation

The contribution of the different metabolic pathways to the overall conversion in the system was estimated using the mixed culture model proposed in the materials and methods section. The error between measured and calculated residual concentrations in the system was minimized by optimizing the contribution of the individual pathways proposed. The following assumptions were used in defining the different metabolic pathways occurring:

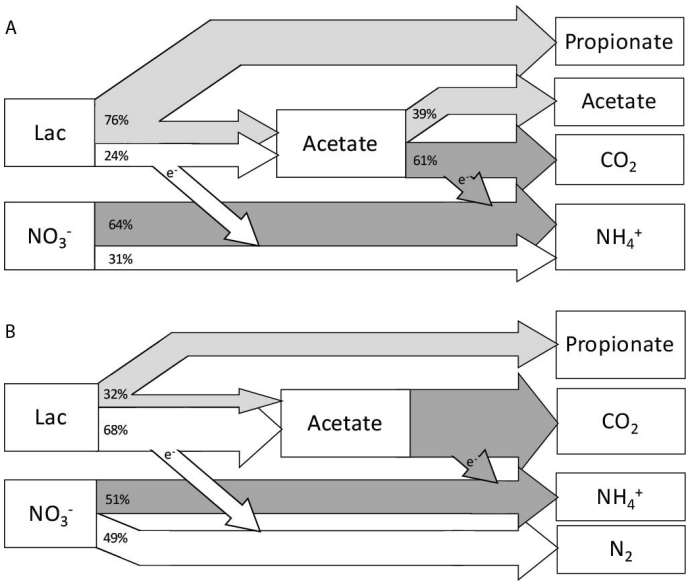

Figure 5.3: Schematic depiction of the results from the modeled pathway contributions to the steady state conversions. (A) Contributing conversions for the Lac/N 2.97 steady state: lactate fermentation (light grey), fermentative DNRA with partial oxidation of lactate to acetate (white) and respiratory DNRA with acetate (dark grey). Indicated is only 95% of nitrate consumption, the other 5% was assimilated in the biomass of the bacteria fermenting lactate. (B) Contributing conversions for the Lac/N 1.15 steady state: lactate fermentation (light grey), denitrification with partial oxidation of lactate to acetate (white) and respiratory DNRA with acetate (dark grey). Here, the nitrogen assimilated by the fermentative bacteria amounted to less than 1% of influent nitrate.

- Propionate consumption was negligible in the batch tests, therefore the residual propionate concentration was used as a measure for lactate fermentation in the system.
- Ammonium production (including assimilatory consumption) in the system was attributed to DNRA. Respiratory DNRA was assumed to be coupled to acetate oxidation, because the presumed DNRA bacteria were the *Geobacter* species observed in the microbial community. Both species were tested for their capacity to convert lactate. A *Geobacter* sp. B enrichment grown on acetate (unpublished data) showed no activity on lactate. The *Geobacter* sp. A has been isolated as pure culture (manuscript in preparation) and was not able to grow on lactate.

For the steady state of Lac/N 2.97, the nitrogen balance had a relatively small gap (8%, from Table 5.5a), and all nitrate was assumed to be converted into organic nitrogen in biomass and ammonium. Consequently denitrification was assumed not to play a significant role. Initially, nitrate reduction to nitrite coupled to partial oxidation of lactate to acetate was considered as a separate conversion. However, calculations demonstrated that nitrate reduction to nitrite could not account for all electrons donated by lactate oxidation. Therefore we assumed the partial lactate oxidizing bacteria convert nitrate to ammonium. As a result, the three processes used to estimate the contribution to the overall conversions were: (i) lactate fermentation, (ii) fermentative DNRA with lactate

oxidation to acetate and (iii) respiratory DNRA using acetate (Figure 5.3a). The estimated biomass fractions of these processes were 50% for the fermentative bacteria (of which 22% was related to fermentative DNRA), and 50% for the biomass performing respiratory DNRA with acetate.

For the steady state with influent Lac/N 1.15, i.e. still with substantial electron donor excess, nitrate was converted to both ammonia and dinitrogen gas. When respiratory DNRA was assumed to be coupled only to acetate oxidation, ammonium could be accounted for provided that partial lactate oxidation to acetate occurred. This directly implied that hypothetical partial oxidation of lactate to acetate coupled to nitrate denitrification should be included to account for removal of the remaining nitrate. No other known pathway applicable for the system could be used to obtain a correct description of the observed conversions, including pathway segregation over nitrite. Including this process resulted in a model output that described our observations adequately (Figure 5.3b) with a computed biomass composition of 6% lactate fermenters, 35% denitrifiers and 59% DNRA bacteria.

In the steady state receiving 0.63 Lac/N influent all lactate was converted by denitrification and concomitant assimilation, and the modeled biomass consisted fully of denitrifiers.

5.4. DISCUSSION

We have previously presented a mechanistic insight on the effect of the C/N ratio on the competition for nitrate between denitrification and DNRA using the non-fermentable carbon source acetate [115] (or Chapter 3). With acetate, under nitrate limiting conditions the DNRA activity was dominant .The extent to which the factors governing the competition hold true for use of the fermentable carbon source lactate was investigated in this work. Also with lactate in great excess, Lac/N 2.97, DNRA was dominant for nitrate reduction. When the influent lactate was decreased to lactate limiting conditions, Lac/N 0.63, all lactate and nitrate were used for denitrification. Herewith the competition between DNRA and denitrification in the lactate system was comparable to the acetate experiments described previously. At the intermediate Lac/N ratio, denitrification and ammonification coexisted, but no double limitation was observed like in the former acetate study. Instead, a complex mixture of conversions was observed. A probable network of metabolic reactions is proposed on the basis of the in silico fit, and could be aligned with the microbial community structure observed.

High lactate to nitrate ratio

For nitrate limiting conditions at Lac/N ratio of 2.97 mol/mol, with a substantial excess of lactate, 92% of the nitrate was converted to ammonium by DNRA, and partially used for assimilation. Since lactate was supplied in stoichiometric excess compared to nitrate, lactate not used for DNRA was expected to be used fully by fermentative bacteria. However propionate to acetate product ratio's in combination with batch tests and molecular community analysis indicated that a more complex conversion had to occur. Batch tests performed with cells from the culture, showed that in the absence of electron acceptor lactate was fermented to acetate and propionate in a molar ratio of 1:2. This stoichiometry was also described by [94], who had performed batch tests on pure cultures of lactate fer-

menting bacteria. Presumably the observed *Clostridium* and *Desulfitobacterium* species were responsible for lactate fermentation, as the observed *Geobacter* species are unlikely to consume lactate. The typical *Clostridia* members are obligate anaerobes, capable to ferment a wide range of substrates. For example, *Clostridium propionicum* is capable to ferment lactate to acetate, propionate and CO_2, with the same stoichiometry found in our batch test experiments [71].

Acetate was not only a product of fermentation, but also an electron donor for the nitrate reduction, thereby being oxidized to CO_2. The Geobacter ribotypes were the identical to the ribotypes described responsible for DNRA activity in previous studies with acetate as electron acceptor [114, 115]. Therefore, they were assumed to perform respiratory DNRA using acetate and not to consume lactate.

Furthermore, we observed that oxidation of propionate coupled with the reduction of nitrate to nitrite only occurred at a very low rate compared to acetate oxidation. In the chemostat culture propionate was therefore assumed not to be consumed by the nitrate reducers at significant rate and its production was used as a measure for the amount of lactate fermentation in the model evaluation. Apparently nitrate reducers oxidizing the propionate were not competitive in the system and lactate and acetate were preferred as electron donors. Using the stoichiometry for propionate production by fermentation of lactate indicated lactate use by a second process besides fermentation. Probably lactate was used directly by bacteria performing fermentative DNRA. Here, we use this term because the partial oxidation of lactate to acetate is a fermentative step (leading to substrate level ATP formation via acetylCoA), however we do not know whether or not the nitrate reduction was electrogenic. Several fermentative bacterial species have been demonstrated to perform this conversion such as *Enterobacteria* and *Clostridia* [10, 16, 119]. In addition, species of the *Desulfitobacterium* were also able to ferment lactate and some were capable of nitrate reduction [19, 122]. Therefore we hypothesized that the bacteria related to the *Clostridium* and *Desulfitobacterium* species are responsible for fermentative DNRA with lactate oxidation to acetate in this steady state.

Implementing these assumptions, the model based evaluation suggested a combination of three different conversions to match the overall conversions at the Lac/N ratio of 2.97 (Figure 5.3a); lactate fermentation, fermentative DNRA using lactate and respiratory DNRA using acetate. The bacteria performing respiratory DNRA using acetate are likely the specific *Geobacter* species, which were computed to consume 64% of the nitrate and make up 50% of the biomass, which is confirmed by the dominance of *Geobacter* identified by FISH. The fermentative bacteria were computed to consume 75% of lactate by fermentation to acetate and propionate and 25% in fermentative DNRA. These functions are assigned to the other two dominant taxa in this culture: a *Clostridium* and a *Desulfitobacterium* species. These two taxa are both capable of fermentation of lactate producing propionate and fermentative DNRA. As we cannot distinguish with the current results, we can only conclude that these two organisms were performing the two fermentative conversions. Either each performs one process, or they both perform both processes.

Moderately high lactate to nitrate ratio

When the chemostat was operated at the decreased Lac/N ratio of 1.15, the fermentative activity decreased. Despite the supply of electron donor in adequate amounts for full

nitrate reduction to ammonium, a decrease of DNRA activity was observed. Only 61% of nitrate was reduced to ammonium by DNRA or used for assimilation. The remaining nitrate was reduced to dinitrogen gas by denitrifiers, as their presence was indicated in batch tests performed with the culture enriched at Lac/N ratio of 1.15 and acetylene. With the assumption that respiratory DNRA occurred only via acetate oxidation in our system, we could not find a system based on reported pathways for anaerobic/anoxic lactate oxidation which could describe our data. Therefore we hypothesized that partial lactate oxidation to acetate coupled to denitrification occurred in our enrichment culture. This proposed process was presumably performed by the ribotype related to *Propionivibrio militaris* strain MP, which was dominant in the culture at Lac/N ratio 1.15 next to the previous found *Clostridales* bacteria and *Geobacter* species. This *Betaproteobacterium* has been described as a non-fermentative, strictly respiring facultative anaerobe capable of nitrate and nitrite denitrification with acetate, propionate or lactate [109]. Therefore it was presumed to perform the denitrification with partial oxidation of lactate in the culture.

Assuming that no denitrification using acetate occurs, in the steady state culture receiving 1.15 Lac/N the three parallel processes modelled were lactate fermentation to acetate and propionate, denitrification using lactate oxidation to acetate and DNRA using acetate. Fermentation of lactate and DNRA using acetate were again attributed to the *Clostridiales* and *Geobacter* members respectively. The denitrifiers were presumed to relate to the *P. militaris* ribotype. With this interpretation, the model suggests that half of the nitrate is denitrified and the denitrifying *Betaproteobacterium* makes up 35% of the total population. The acetate-using ammonifying *Geobacter* species would make up 59% of the population, and the fermenting bacteria 6%, which is confirmed by the dominance of *Geobacter* identified by FISH.

In the steady state receiving influent Lac/N ratio of 0.63 mol/mol, a dual limitation of electron donor and acceptor was expected. All influent nitrate and lactate were used in denitrification and the DNRA and fermentative bacteria were outcompeted. However, in the community next to the dominant denitrifier, related to the *P. militaris* ribotype, also the *Geobacter* was present. For a similar steady state of dual limitation with acetate and nitrate, where only denitrification seemed to occur, the presumed DNRA bacterium was also present [115]. It was speculated that this organism might produce less ammonia than the denitrifiers consume, and that as a result no residual ammonia had been detected. Adapting the model for denitrifiers to use ammonium for growth showed that the DNRA biomass could amount up to 15%, without net ammonia production in the culture.

Nature of the carbon source

The overall results suggest that use of a fermentable carbon source affects the competition for nitrate between DNRA and denitrification compared to a non-fermentable source. For the non-fermentable substrate acetate, when provided in excess, the nitrate was reduced to ammonium [115] (or Chapter 3). In contrast, for supply of lactate at a comparable amount of electron equivalents (at Lac/N ratio 1.15, comparable to Ac/N 1.87 (Table 5.1)) a lower DNRA activity of 58% of the nitrate reduction was observed, even though nitrate was limiting and the electron donor lactate was provided in excess. Only for high excess of lactate, at the Lac/N ratio of 2.97, all nitrate was converted to ammonia.

Qualitatively, the decrease of DNRA activity with the decrease of Lac/N ratio is similar for acetate and lactate, and was also observed for the fermentable substrate glucose [3]. However, for acetate a direct mechanistic coupling of conversions at a certain Ac/N to metabolic Ac/N stoichiometries was derived, which does not apply in the case of lactate. Possibly the lactate consumption by the fermenters limited electron donor availability for nitrate reduction. Fermenters are fast consumers and growers and could therefore have a sufficiently high affinity ($\frac{\mu_{max}}{K_S}$) for lactate to be competitive with the dissimilatory processes, despite their lower ATP yield per lactate converted [64]. Hence, fermentative lactate consumption creates a dual substrate limitation for the nitrate reducers. Just as for acetate grown enrichments, the dual limitation at lower Lac/N ratio also resulted in coexistence of denitrification with DNRA. However, the fermentative bacteria are only competitive for the energy source to a certain extent, because they were outcompeted by the denitrifiers in the steady state at Lac/N 0.63, where both lactate and nitrate influent concentrations were limiting. It is remarkable to see that at the intermediate Lac/N ratio denitrification and DNRA coexisted and both lactate and acetate were limiting. Only propionate remained in the reactor effluent. Apparently, the type of organic carbon limiting the conversion has an impact on the nitrate reduction pathway obtained. It remains unclear why acetate limitation as obtained at Lac/N=1 .15 favours lactate oxidation to acetate coupled to denitrification, and acetate excess favours fermentative DNRA.

Studies in the environment or with environmental slurries regard the C-source mostly as labile carbon forms and non-labile forms, of which the latter are harder to degrade than the former [35, 82]. In some studies different labile energy sources are compared, e.g. Morley and Baggs [76] described 4% of nitrate converted to ammonium for the fermentable glucose, the highest formation in their studies. Other studies on the impact of the nature of the carbon source focused on nitrate removal efficiency in wastewater treatment systems. Batch test have been reported with e.g. biofilms, aerobic and anaerobic granular sludge comparing substrates as glucose and acetate. Generally, glucose and acetate showed similar efficiency for removal, only for glucose some nitrite and/or ammonia accumulation is observed (<4%) [18, 49, 102]. The result of this study establishes the difference in the competition for nitrate between DNRA and denitrification for use of lactate, compared to acetate. The presence of fermentative bacteria, in addition to the nitrate reducers, increases the range of apparent available substrate C/N ratios, for which the denitrifiers and DNRA bacteria coexist. As a consequence, the amount of DNRA activity expected based on electron donor availability, as suggested by Van den Berg et al. [115], is probably lower in practice when (part of) the available electron donors are fermentable. As it implies higher nitrate removal through denitrification, this is a positive result for wastewater treatment.

5.5. CONCLUSION

IN this study we showed that the C/N effect on the nitrate competition between DNRA and denitrification in enrichment chemostat cultures for acetate is qualitatively similar for lactate as electron donor. However, the coupling of the range of dual substrate limitation to the process Ac/N stoichiometry cannot readily be extrapolated. Apparently, fermentative bacteria are competitive for lactate and can thereby limit the availability for the preferred carbon source(s) for the obligate nitrate reducing bacteria. The altered

ratio of apparent substrates available affects the competition between denitrifiers and DNRA bacteria for nitrate in favor of the denitrification. Furthermore, for the obtained steady states we were able to identify the pathways likely responsible for the overall system function and couple this to the community structure. In the steady state receiving influent Lac/N of 2.67, three processes co-occurred: fermentation of lactate to acetate and propionate and fermentative DNRA, performed by two species of *Clostridia*, and respiratory DNRA using acetate, performed by two Geobacter species. For the Lac/N 1.15 mol/mol steady state, fermentation and DNRA, coupled to the same taxa, had decreased and denitrification played a significant role in the conversions, which was presumably linked to the presence of the *Betaproteobacterium* related to *Propionivibrio militaris*. The results improve our understanding for the C/N effect on the competition between nitrate reducers and helps predict DNRA or denitrification contributions in aqueous environments, e.g. wetlands or wastewater treatment systems.

5.6. SUPPLEMENTARY MATERIAL

Table 5.7: Acid/base equilibrium and respective pKa considered in calculation of the bicarbonate concentration in the chemostat for the different steady states. Equilibrium between H_3PO_4 and $H_2PO_4^-$ was not taken into consideration since the correspondent pka (equal to 2.3) is so low comparing with the working pH. T, the electro-neutrality equation for the charged species in the chemostat solved was written as $[H^+]+[Na^+]+[K^+]=[HCO_3^-]+2\cdot[CO_3^{2-}]+[OH^-]+[Ac^-]+[Prop^-]+[Cl^-]+[H_2PO_4^-]+2\cdot[HPO_4^{2-}]+3\cdot[PO_4^{3-}]$.

Equilibria	pKa
$H_2CO_3 \rightleftharpoons HCO_3^- + H^+$	6.37
$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+$	10.36
$HAc \rightleftharpoons Ac^- + H^+$	4.76
$HProp \rightleftharpoons Prop^- + H^+$	4.88
$H_2PO_4^- \rightleftharpoons HPO_4^{2-}\ H^+$	7.21
$HPO_4^{2-} \rightleftharpoons PO_4^{3-} + H^+$	12.32
$NH_4^+ \rightleftharpoons NH_3 + H^+$	9.26

Table 5.8: Sequences generated in the amplicon sequencing, 250bp paired-end raw reads (Raw PE), and read numbers in subsequent processing steps.

Sample	Raw PE(#)	Combined (#)	Qualified (#)	Nochime (#)	AvgLen (nt)	Effective%
2.97a	73,325	69,096	61,146	59,936	429	81.74
2.97b	74,349	69,926	61,762	60,477	427	81.34
1.15a	62,44	58,809	52,202	51,293	429	82.15
1.15b	71,999	67,506	60,012	59,021	429	81.97
0.63	76,412	71,041	62,192	61,58	428	80.59

Table 5.9: Alpha diversities calculated for the different samples analyzed by amplicon sequencing. In the calculations normalized consensus sequence abundances, which were normalized using a standard of sequence number corresponding to the sample with the least sequences, were used.

Sample	Observed species	Shannon	Simpson	Chao1	ACE	Goods coverage
2.97a	333	2.7	0.7	423	447	0.998
2.97b	281	2.5	0.6	347	364	0.998
1.15a	317	2.3	0.7	403	420	0.998
1.15b	272	1.7	0.5	317	344	0.998
0.63	353	1.5	0.4	454	475	0.997

Table 5.10: Test with SILVA TestProbe (database SSU 128, sequence collection REFNR) for FISH probe GeobacII_464.

Mismatches allowed	Matches	
0	0	-
1	0	-
2	1	uncultured in genus *Geobacter*

Table 5.11: Test with RDP ProbeMatch for FISH probe GeobacII_464.

Mismatches allowed	Matches	
0	0	-
1	3	uncultured in genus *Geobacter*
2	5	uncultured in genus *Geobacter* (4), family of unclassified *Desulfuromonadales* (1)

Table 5.12: Conversion rates of the different substrates and products (mM/h) observed in the batch tests performed with the steady state biomass from culture receiving Lac/N ratio 2.97 mol/mol. Rates were calculated by linear regression of the different concentrations over time and respective standard deviations by the function LINEST in Microsoft Office Excel.

Substrates	Ref to fig S1	Conversion rates (mmol· h^{-1}· L^{-1})				N to NH_4^+ (%)
		Lac	Ac[a]		Prop	
Lac.	A	-3.02 ±0.35	1.01 ±0.15	-	1.87 ±0.27	0
Lac. + NO_3^-	B	-2.46 ±0.34	0.58 ±0.15	-0.52 ±0.00	1.08 ±0.15	83.5
Lac. + NO_2^-	C	-2.12 ±0.22	0.16 ±0.15	-0.15 ±0.00	1.09 ±0.10	85.5
Ac. + NO_3^-	D	-	-	-0.97 ±0.07	-	86.0
Ac. + NO_2^-	E	-	-	-0.82 ±0.14	-	84.9
Prop. + NO_3^-	F	-	-	-	-0.14 ±0.04	10.8
Prop. + NO_2^-	G	-	-	-	-0.01 ±0.13	7.5

[a] When acetate was first produced and later used as a substrate, positive and negative rates are presented corresponding to production and consumption, respectively.

Table 5.13: Conversion rates of the different substrates and products (mM/h) observed in the batch tests performed with the steady state biomass from culture receiving Lac/N ratio 1.15 mol/mol. Rates were calculated by linear regression of the different concentrations over time and respective standard deviations by the function LINEST in Microsoft Office Excel.

Substrates	Ref to	Conversion rates (mmol· h^{-1}· L^{-1})			N-conversion end products (N%)		
	fig S2	Lac	Ac	Prop	NH_4^+	N_2O	to N_2 [a]
Lac.	A	-1.50 ±0.07	0.50 ±0.01	0.98 ±0.03	-	-	n.d.
Lac. + NO_3^-	B	-1.55 ± 0.04	0.51 ±0.00	0.90 ±0.19	2	0.4	77
Lac. + NO_2^-	C	-0.68 ± 0.08	0.26 ±0.00	0.13 ±0.00	3	4	n.d.
Ac. + NO_3^-	D	-	-1.28 ±0.11	-	2	-	84
Ac. + NO_2^-	E	-	-0.90 ±0.12	-	10	7	n.d.
Prop. + NO_3^-	F	-	-	-1.51 ±0.04	3	4	56
Prop. + NO_2^-	G	-	-	-0.01 ±0.02	3	-	n.d.

[a] Percentage estimated based on measurements of N_2O fraction in the headspace of the control batch vials, which were inoculated with 5% (v/v) acetylene. n.d. Not detected

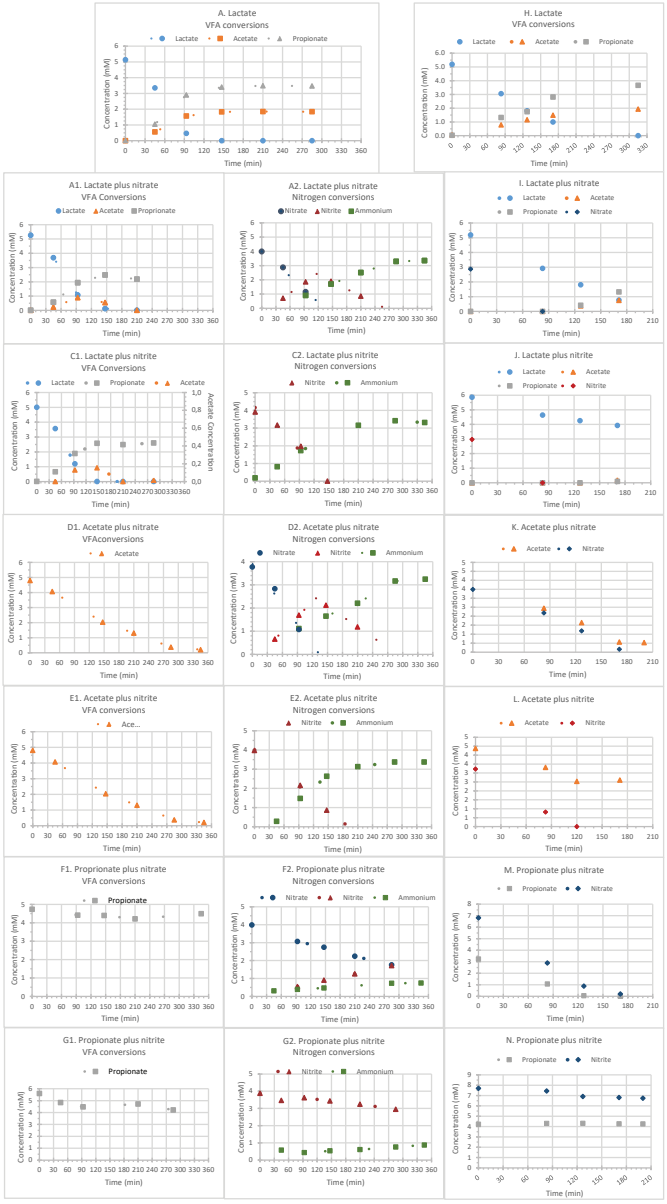

Figure 5.4: Concentration profiles of the simultaneous batch tests. Initial electron donor concentrations were 5 mM of and electron acceptor concentrations were 4 mM. (A-I) Tests performed with the steady state biomass from culture receiving Lac/N ratio 2.97 mol/mol. Note that transient accumulation of nitrite occurred when nitrate was the electron acceptor with either lactate or acetate as carbon source. (H-N) Tests performed with the steady state biomass from culture receiving Lac/N ratio 1.15 mol/mol.

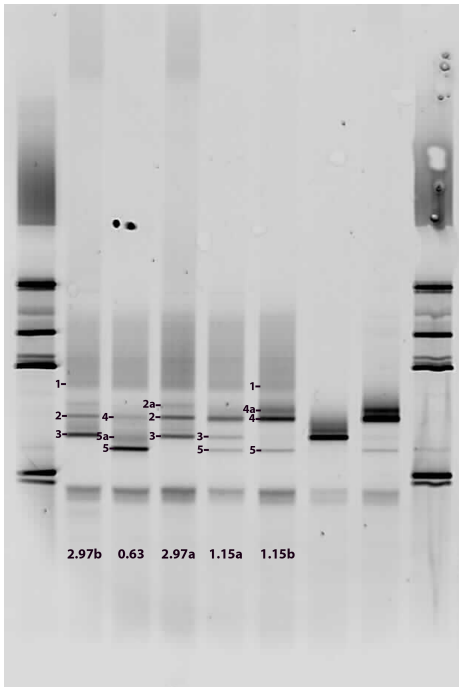

Figure 5.5: DGGE gel picture. The same DNRA extracts were analyzed as in the amplicon sequencing (figure 2) and the sample names in the lane are the same as in the amplicon result. The other lanes belong to other research. Bands labeled with the same number contained the same sequence, and the sublabel 'a' was given to slightly different sequences which related to the same species. BLASTn result for closest related species and identities: band 1 *Clostridium* sp. SW001 (99%); band 2 *Desulfitobacterium hafniense* (99%); band 3 *Geobacter luticola* (97%); band 4 *G. lovleyi* (97%); band 5 *Propionivibrio militaris* (99%).

6

OUTLOOK

Eveline M. VAN DEN BERG

THE study described in this thesis aimed to extend our insight in the ecophysiology of bacteria performing dissimilatory nitrate reduction to ammonium (DNRA) and their competition for nitrate with denitrifying bacteria. Nitrate leaching, and subsequent eutrophication, is a main negative consequence of fertilization in agriculture. With a drive towards a bio-based society agricultural activities will certainly increase further, making management of nutrient cycles increasingly important. Nitrate present in groundwater or surface water complicates the production of drinking water from these sources. The end product of nitrate reduction impacts these natural environments as well as wastewater treatment efficiency. Hence, improved understanding of the metabolic and environmental controls the dissimilatory nitrate reduction processes will help in a better management of nutrient cycles in anthropogenic influenced ecosystems.

6.1. PHYSIOLOGY AND COMPETITIVENESS

DNRA was until now mainly studied in natural ecosystems and no pure cultures isolated on their capacity to perform DNRA have been reported. To make DNRA easier to study in a controlled environment we developed a reproducible cultivation method for the enrichment of DNRA bacteria in a continuously operated reactor system under nitrate limiting conditions, which was hypothesized to be the dominant factor in selection for DNRA (Chapter 2). The non-fermentable substrate acetate was chosen as carbon and energy source to keep the system as simple as possible. The enrichment culture was mainly consisting of *Deltaproteobacteria* (>95%), closely related to *Geobacter lovleyi* (97% 16S rRNA similarity). Despite this dominance of a DNRA bacterium in the population, 15% of converted nitrate was unaccounted for and presumably denitrified. For such a contribution to the nitrate conversions a denitrifying bacterium is expected to be more abundant in the population, based on general growth yields. Therefore it does not explain how nitrogen is produced in the culture. Possibly, the bacteria performing DNRA are also capable of denitrification, as was recently described for *Shewanella loihica* [127]. Alternatively the side population (<5%) was denitrifying, but with a relatively low growth yield. The low yield could be the result of unknown factors such as growth of the denitrifiers on the possible byproducts of the DNRA bacteria (NO or N_2O; [23, 112]) rather than nitrate. Isolation of a pure culture of the dominant DNRA bacterium, the *Geobacter* species, would allow testing of these hypotheses. It would additionally enable us to study its versatility and genomic inventory, as *Geobacter* was considered an unusual organism to dominate in a competitive environment for such an oxidized electron acceptor as nitrate.

Furthermore in this thesis studies we have observed two different *Geobacter* ribotypes dominating in the enrichment culture with no apparent difference in the conversions. The two *Geobacter* species are supposedly very similar and affected by minor fluctuations in in the system [38]. A comparative study of these *Geobacter* strains could help discover the reason(s) for the interchange of these two organisms. This would expand our understanding of microbial competitiveness and possible function of the distinctive trait in the environment.

In Chapter 3 we observed a clear correlation between influent acetate/nitrate (Ac/N) ratio and DNRA activity and the DNRA population in our reactor. The DNRA bacteria dominated under nitrate limiting conditions in the reactor and were outcompeted by denitrifying bacteria under limitation of acetate. In our explanation we posed that at

an adequately low dilution rate, DNRA bacteria would be able to outcompete regular denitrifiers when nitrate is the growth limiting substrate. This also implies that the μ^{max} of organisms performing respiratory DNRA is not high enough to compete successfully under the nitrate excess conditions of batch cultivation. Heijnen [46] proposed a hypothesis that the electron transport system is the rate limiting step in microbial growth. Since the growth yield per electron for DNRA is lower than for denitrification (Table 3.6) it is in line with this theory that the maximal growth rate of the DNRA organisms is lower than for regular denitrifiers. It was also confirmed in the chemostat experiments of Kraft et al. [65], where dentrifiers outcompeted the DNRA bacteria at higher growth rates under nitrate limiting conditions. Therefore we had to assume that the affinity constant (K_S value) for nitrate uptake is lower for DNRA organisms, to explain their competitive advantage in a nitrate limiting chemostat. The reported K_S-values of DNRA for nitrate are not consistently lower than of denitrification. These values are however obtained in pure cultures that were not isolated on their DNRA capacity, i.e. non-specialists. It would be interesting to determine the K_S value for a *Geobacter* species isolate, and unravel how it achieves a lower K_S value when compared to denitrification, despite that the first step in the conversion of nitrate in DNRA or denitrification is the same; nitrate reduction to nitrite. With additional transcriptome and proteome studies on pure cultures we could observe regulatory differences and differences in enzyme systems used by the bacteria. Different enzyme systems could additionally play a role in the achievement of a lower K_S. Next to steady state observations, it would be relevant to preform batch tests to expand the kinetic understanding and obtain information on the maximum biomass specific uptake rates (q-rates) and system response to perturbations.

6.2. ENVIRONMENTAL CONTROLS

While DNRA bacteria dominated under nitrate limiting conditions and denitrifying bacteria under limitation of acetate, in Chapter 3 we also showed that DNRA bacteria and denitrifiers coexisted for a broad range of influent Ac/N ratios at a dual limitation of acetate and nitrate. A kinetic model was used to describe the system. Based on the model evaluation we put forward that the Ac/N effect and concomitant broad dual limitation range were a result of the difference in metabolic Ac/N stoichiometry between both processes, as well as the differences in electron donor and acceptor affinities. Others argue that the change in oxidation state of the environment due to the altered substrate ratio is essentially the condition affecting the competition [13, 73]. The derived question that drives more microbial ecology questions is to what extent the redox potential directly influences microbial conversions, and to what extent the conversions are the resultant of prevailing conditions with the redox potential being mainly an indicator for the environment experienced by the bacteria. The challenge is to find a redox active compound that is inert in the microbial conversions. For example, the addition of sulfide in the enrichment culture system could alter the redox potential, maintaining low redox potentials even at dual limitation and nitrate excess conditions. However, the interpretation can be complicated by the denitrifiers use of the sulfide as electron donor. Pure cultures might help in setting up such conditions.

When we replaced the electron acceptor nitrate with nitrite, the conversions and population in the system remained the same (Chapter 4). This was contradictory to the

results reported by Kraft et al. [65], who observed in an electron acceptor limited marine enrichment culture, fed with glucose, acetate and amino acids, that nitrate selected for a DNRA community whereas nitrite selected for a denitrifying community. Presumably not nitrite in itself, but a combination with other factors, such as pH, salinity, sulfide concentrations, or the type and complexity of the electron donor, had a decisive effect on the completion between the nitrate reduction pathways. To understand the behavior in the more complex laboratory system of Kraft et al. [65] we would need to establish the effects of the separate environmental factors using a simple system like ours [9]. A first step was the use of the fermentable carbon source lactate instead of acetate (Chapter 5). More complex fermentable substrates like glucose will have to be tested as well as the influence of elevated salinity, sulfide presence or high influent amino acid concentrations. In the enrichment cultures with lactate (Chapter 5) no microbe using propionate had appeared. To understand why this occurred, nitrate reduction using propionate is a candidate for future research. Another question regards the interaction of sulfide with the nitrate reduction processes. In marine sediments, the presence of sulfide was described to correlate positively with DNRA [6, 11, 12]. However, other studies report an opposite trend or no significant effect on the competition [28, 65], a discrepancy we might be able to unravel with separate enrichment system studies. Additionally, an enrichment with sulfide (or thiosulfate) as electron donor could increase our insight in autotrophic nitrate reduction and the interaction of nitrogen and sulfur cycle.

Instead of the non-fermentable substrate acetate, in Chapter 5 the fermentable substrate lactate was the electron donor and C-source. In this system fermentative bacteria were present besides nitrate reducers under lactate excess conditions, converting the lactate to propionate and acetate. The fermentative bacteria appeared competitive for the electron donor and thereby altered the ratio of available substrates for nitrate reduction. While the electron donor was supplied in excess and was expected to lead to DNRA prevalence, the influence of the fermentative bacteria resulted in equal nitrate reduction contributions of DNRA and denitrification. Apparently, the type of organic carbon limiting the conversion has an impact on the nitrate reduction pathway obtained. As a consequence, the amount of DNRA activity expected based on electron donor availability, as suggested from our study with acetate in Chapter 3, is probably lower in practice when (part of) the available electron donors are fermentable. This result is advantageous for wastewater treatment as it implies higher nitrate removal through denitrification. The mechanisms behind the observation have yet to be elucidated.

6.3. WASTEWATER TREATMENT

In Chapter 4 we showed DNRA was also enriched with nitrite as electron donor instead of nitrate. This creates a window of cooperative coexistence of DNRA and anaerobic ammonium oxidation, abbreviated as anammox. The DNRA process could convert part of the nitrite and nitrate present to ammonia for subsequent use by anammox, which combines equimolar amounts of ammonia and nitrite to form dinitrogen gas. This potential collaboration was proposed to occur in the Peruvian oxygen minimum zone [67], where the measured DNRA ammonium production rates would suffice to supply a great part of the ammonium needed by anammox. Cooperation of DNRA and anammox also holds the potential to enhance efficiency of wastewater treatment using anammox.

In a first stage of such a system, organic carbon is removed using oxygen. Then part of the residual influent ammonium is converted in aerobic ammonium oxidation to nitrite, while the anammox uses the leftover ammonium and produced nitrite to form dinitrogen gas. An obstacle in operation of this technology is the elimination of bacteria oxidizing the nitrite to nitrate from the system. With addition of the DNRA process, this nitrate could be converted to ammonium and used by anammox. Alternative to cooperation with a separate group of DNRA bacteria, anammox itself can perform nitrate reduction to ammonium. DNRA by anammox would be more desired as it reduces the nitrate without conserving any potential energy, hence no biomass is produced [17].

On the other hand, DNRA bacteria may be active in conventional wastewater treatment systems, in which ammonia is converted via aerobic nitrification and anoxic denitrification to dinitrogen gas. Until now this has received very little attention in literature. DNRA activity would impair the treatment process, with potential increase of effluent ammonium concentrations and waste of energy input. DNRA is selected under nitrate limiting conditions, with excess of (organic) electron donor. Regarding these conditions, DNRA may occur in a wastewater treatment system with high organic carbon and nitrate in the influent (as in aquaculture and industrial wastewater), anaerobic digesters or nitrate presence in a recirculation stream which is mixed with the influent. Or it could occur in the flocks of activated sludge, which can easily have nitrate limitations while the bulk concentration is high. A first grasp of the DNRA potential in wastewater treatment systems can be obtained by studying its genetic potential, analyzing the abundance of the marker gene *nrfA*. The microbiological potential of DNRA has already been suggested in metagenomics studies [95, 125]. To know in which system DNRA could play a role, substrate ratios and fluctuations in the systems need to be characterized. Ultimately, physiological tests need to be designed to verify DNRA contributions in a wastewater treatment system. Additional knowledge gained by further microbial studies will help determine the role and use of DNRA in these engineered and other environments and will result in process improvement.

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List of Publications

Journal articles

4. **E.M. van den Berg**, Marina P. Elisário, J. Gijs Kuenen, Robbert Kleerebezem and Mark C. M. van Loosdrecht, *Fermentative bacteria influence the competition between denitrifiers and DNRA bacteria*, Frontiers in Microbiology, **8**: 1684 (2017)

3. **E.M. van den Berg**, Julius L. Rombouts, J. Gijs Kuenen, Robbert Kleerebezem and Mark C. M. van Loosdrecht, *Role of nitrite in the competition between denitrification and DNRA in a chemostat enrichment culture*, AMB Express, **7**: 91 (2017)

2. **E.M. van den Berg**, Marissa Boleij, J. Gijs Kuenen, Robbert Kleerebezem and Mark C. M. van Loosdrecht, *DNRA and Denitrification Coexist over a Broad Range of Acetate/N-NO_3^- Ratios, in a Chemostat Enrichment Culture*, Frontiers in Microbiology, **7**: 1842 (2016)

1. **E.M. van den Berg**, Udo van Dongen, Ben Abbas and Mark C. M. van Loosdrecht, *Enrichment of DNRA bacteria in a continuous culture*, The ISME Journal, **9**(10): 2153-2161 (2015)

Selected oral presentations

5. **E.M. van den Berg**, J. Gijs Kuenen, Robbert Kleerebezemand Mark C. M. van Loosdrecht, (2015) *Denitrification vs DNRA*, Microbial Ecology and Water Engineering, Copenhagen, Denmark

4. **E.M. van den Berg**, J. Gijs Kuenen, Robbert Kleerebezemand Mark C. M. van Loosdrecht, (2015) *Denitrification vs DNRA; The effect of COD/N ratio*, European Nitrogen Cycle meeting, Aberdeen, UK

3. **E.M. van den Berg**, J. Gijs Kuenen, Robbert Kleerebezem and Mark C. M. van Loosdrecht, (2015) *DNRA versus denitrification*, KNVM Spring meeting, Arnhem, The Netherlands (invited)

2. **E.M. van den Berg**, J. Gijs Kuenen and Mark C. M. van Loosdrecht, (2014) *Enrichment of DNRA bacteria in a continuous culture*, NESSC and SIAM symposium, Noordwijkerhout, The Netherlands

1. **E.M. van den Berg**, Udo van Dongen, Ben Abbas and Mark C. M. van Loosdrecht, (2014) *Enrichment of DNRA bacteria in a continuous culture*, European Nitrogen Cycle meeting, Ghent, Belgium

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