Elucidation of the microbial N-cycle in the subsurface – key microbial players and processes

Dissertation

To Fulfill the

Requirements for the Degree of

“doctor rerum naturalium” (Dr. rer. nat.)

FRIEDRICH-SCHILLER-UNIVERSITÄT JENA

Submitted to the Council of the Faculty

of Biology and Pharmacy

of the Friedrich Schiller University Jena

by

M.Sc. M.Tech.

Swatantar Kumar

born on 21-06-1984 in Baijnath Kangra, India

Jena, December 2017

Reviewers: 1. Prof. Dr. Kirsten Küsel (Friedrich Schiller University Jena)

2. Prof. Dr. Erika Kothe (Friedrich Schiller University Jena)

3. Prof. Dr. Marcus Horn (Leibniz University Hannover)

Day of the official disputation: 19.04.2018

Zugl. : Dissertation, Friedrich-Schiller-Universität Jena, [2018]

TABLE OF CONTENTS

LIST OF TABLES **vii**

LIST OF FIGURES **viii**

SUMMARY **1**

ZUSAMMENFASSUNG (German) **3**

1. INTRODUCTION **6**

1.1 The biogeochemical nitrogen cycle **6**

1.2 Excess reactive nitrogen and its ecological impacts **8**

1.3 Perspective on reactive nitrogen with groundwater **9**

1.4 How do karst oligotrophic aquifers respond to reactive nitrogen? **10**

1.5 Key microbial players for the attenuation of ammonium and nitrate to dinitrogen **12**

***1.5.1** Anammox bacteria; a piece in the lithotrophy puzzle* **12**

***1.5.2** Denitrifying microorganisms and their role in nitrogen cycle* **16**

1.6 Motivation of this thesis: understanding nitrogen loss in oligotrophic carbonate-rock aquifers **17**

***1.6.1** Biogeochemical role of chemolithoautotrophic anammox in groundwater* **18**

***1.6.2** Biogeochemical role of denitrifiers in oligotrophic groundwater* **19**

***1.6.3** The Collaborative Research Centre 1076 AquaDiva* **21**

1.7 Research hypotheses and experimental approach **23**

2. MATERIALS AND METHODS **26**

2.1 Characterization of the study site **26**

2.2 Groundwater sampling and characteristics **27**

***2.2.1** Groundwater samples for hydrochemistry* **27**

***2.2.2** Groundwater samples for microbial community analysis using molecular methods* **28**

***2.2.3** Groundwater samples for quantification of anammox-specific ladderane lipids* **28**

***2.2.4** Groundwater samples for quantification of anammox and denitrification rates* **29**

***2.2.5** Groundwater samples for microbial cultivation of planktonic denitrifiers* **29**

2.3 Rock chips sample (passive sampler) for microbial cultivation of attached denitrifiers **29**

2.4 Ladderane lipids quantification **29**

2.5 Anammox and denitrification rates measurements from groundwater **30**

2.6 Cultivation approaches for planktonic and attached denitrifying bacterial community **30**

***2.6.1** Groundwater based incubation and growth medium for enrichment of planktonic denitrifiers* **30**

2.6.1.1 *Growth medium for semi solid and solid Gelrite shake dilution and plates* **32**

2.6.1.2 *Gelrite shake dilution method* **32**

2.6.1.3 *Filter paper overlay method* **32**

2.6.2 *Rock chips (passive sampler) based incubations for cultivation of attached denitrifiers* **33**

2.7 Denitrification activity of an enriched chemolithoautotrophic consortium **34**

2.7.1 *Microcosm experiments set up M_I and M_II* **34**

2.7.2 *Analysis of denitrification products by Raman spectroscopy from microcosm set up M_I and M_II* **35**

2.7.3 *Analysis of reactants, intermediates and products of chemolithoautotrophic denitrification by ion chromatography from microcosm set up M_I and M_II* **36**

2.8 Nucleic acid extractions, PCR amplifications and cloning **37**

2.8.1 *Nucleic acids extractions from groundwater samples* **38**

2.8.2 *Nucleic acids extractions from enrichment consortia* **38**

2.8.3 *DNA extractions from pure denitrifying cultures* **39**

2.8.4 *Polymerase chain reaction assays and clone libraries* **39**

2.9 Quantitative-PCR and Reverse Transcription-qPCR based analysis of microbial abundances and potential gene transcripts from groundwater samples and enrichment culture **42**

2.10 Sample preparations for Illumina MiSeq amplicon sequencing **43**

2.10.1 *Illumina MiSeq amplicon sequencing of groundwater derived DNA and RNA* **43**

2.10.2 *Illumina sequencing based monitoring of the microbial community during the microcosm experiments* **44**

2.11 Sequence data analysis **45**

2.12 Statistical analysis **46**

3. RESULTS **47**

3.1 Groundwater chemistry of eight wells along the Hainich groundwater observation transect **47**

3.2 Co-occurrence of denitrifiers, anammox and aerobic ammonia oxidizing archaea and bacteria in groundwater reveled by quantitative PCR **49**

3.3 Anammox-*hzsA* transcriptional activity as an indicator of protein synthesis potential of anammox bacteria in Hainich aquifer assemblages **53**

3.4 Correlation with environmental parameters and observed gene abundances **55**

3.5 Ladderane lipids provide further support for the presence of an active anammox population **55**

3.6 Nitrogen loss from pristine carbonate-rock aquifers is primarily driven by chemolithoautotrophic anammox processes **56**

3.7 Presence of rRNA as indicative of protein synthesis potential of *Planctomycetes* organisms in limestone aquifers **57**

3.8 *Candidatus* Brocadia fulgida dominated the anammox bacterial community of the Hainich aquifer assemblages **60**

3.9 Denitrifying community composition based on *nirS*-genes revealed dominance of a chemolithotrophic community utilizing sulfur, hydrogen and iron as electron donors **62**

3.10 Taxonomic identification of denitrifying enrichments and bacterial cultures originating from the Hainich aquifer assemblages **64**

3.10.1 Community structure of a chemolithoautotrophic denitrifying bacterial enrichment originating from groundwater **64**

3.10.2 Chemolithotrophic bacterial enrichment originating from rock-chips and gelrite shake dilution (groundwater based) **66**

3.11 Chemolithoautotrophic denitrification coupled to the oxidation of thiosulfate and hydrogen **67**

3.11.1 *Microcosm experiment M_I: turnover of nitrogen and sulfur compounds by enriched consortium 1E* **67**

3.11.2 *Microcosm experiment M_II: complete denitrification ($^{15}N_2$ production) and hydrogen utilization was witnessed by Cavity Enhanced Raman Spectroscopy (CERS)* **67**

3.11.3 *Transcriptional activity of the enriched consortium during the two microcosm experiments* **69**

3.12 *Thiobacillus denitrificans as key transcriptionally active denitrifier in the consortium* **72**

3.13 Shifts in community composition during the microcosm experiments **73**

3.14 Taxonomic classification of pure mixotrophic and heterotrophic denitrifying isolates **75**

3.14.1 *Heterotrophic denitrification by pure culture of strain 2_Acidovorax defluvii* **77**

4 DISCUSSION **78**

4.1 Nitrogen loss is primarily driven by anammox processes in carbonate-rock aquifers of the Hainich CZE **79**

4.2 Co-occurrence of gene transcripts of aerobic and anaerobic ammonium oxidizers suggest potential coupling of aerobic and anaerobic ammonia oxidation in suboxic groundwater **81**

4.3 Transcriptionally active anammox bacteria in oxic and anoxic groundwater of the two aquifer assemblages **82**

4.4 Low denitrification rates in suboxic groundwater of Hainich aquifer assemblages **83**

4.5 Reoccurring large fractions of potential autotrophic denitrifiers oxidizing reduced sulfur compounds, hydrogen, or reduced iron in suboxic groundwater of Hainich aquifer assemblages **84**

4.6 Chemolithoautrophic enrichment culture obtained from groundwater representing key metabolic features of the natural groundwater denitrifier communities **85**

4.7 Denitrification by Chemolithotrophic consortium 1E, coupled to the oxidation of thiosulfate and hydrogen **88**

4.8 Growth of chemolithoautotrophic denitrifiers complemented with *nirS*- and *nosZ*-transcriptional activity during microcosm experiments **89**

4.9 Verification of Hypotheses and conclusions **91**

4.10 Strengths of the current study **93**

4.11 Limitations of study and considerations for future investigations **93**

5. CONCLUSIONS **95**

5.1 Anammox versus denitrification in carbonate-rock aquifers: Nitrogen loss from pristine carbonate-rock aquifers of the Hainich Critical Zone Exploratory (Germany) is primarily driven by chemolithoautotrophic anammox processes **95**

5.2 Physiological experiments with denitrifying microorganisms: Chemolithotrophic consortium provided insights into the complexities of denitrifiers in oligotrophic groundwater and their nitrate attenuation capacity **96**

REFERENCES **98**

APPENDIX **117**

ACKNOWLEDGEMENTS **126**

DECLARATION OF AUTHORSHIP **128**

PUBLISHED ARTICLE **129**

LIST OF TABLES

Table	Page
Table 1 | Stoichiometric reactions of anammox and denitrification.	12
Table 2 | PCR Primers used in this study for gene detection and quantification.	39-40
Table 3 | Detection of denitrification genes in groundwater isolates	75
Table 4 | Anammox and denitrification rates in marine and freshwater environments.	80
Table 5 | Composition of vitamin and trace element solution used in growth media (NTC).	117
Table 6 | Physicochemical parameters of groundwater samples from eight wells of the Hainich aquifer assemblages.	118
Table 7 | Correlation analysis (Spearman rank correlation coefficient) between physicochemical parameters and gene abundances of groundwater samples	118
Table 8 | Results of MiSeq Illumina amplicon sequencing of bacterial 16S rRNA genes (DNA-based).	119
Table 9 | Results of MiSeq Illumina amplicon sequencing of bacterial 16S rRNA genes (RNA-based).	120
Table 10 | Results of MiSeq Illumina amplicon sequencing of *nirS* genes in the groundwater of eight wells across the two aquifer assemblages.	121
Table 11 | Results of MiSeq Illumina amplicon sequencing of *nirS* genes in the groundwater of four wells across the two aquifer assemblages.	122

LIST OF FIGURES

Figures	Page
Figure 1 | Current view of biological nitrogen cycle.	**7**
Figure 2 | Anammox bacteria and potential biomarkers.	**14**
Figure 3 | Denitrifying bacteria and potential gene markers.	**16**
Figure 4 | Groundwater observation transect at the Hainich CZE	**26**
Figure 5 | Cultivation approaches for the planktonic denitrifying community.	**31**
Figure 6 | Cultivation approach for the attached denitrifying community.	**33**
Figure 7 | Microcosms set up with enrichment culture, 1E and subsequent chemical and molecular analysis for two microcosms, M_I and M_II	**35**
Figure 8 | Raman gas analysis and model of enrichment metabolism.	**36**
Figure 9 | Physical characteristics of the groundwater at Hainich aquifer assemblages.	**47**
Figure 10 | Chemical characteristics of the groundwater at Hainich aquifer assemblages.	**48**
Figure 11 | Bacterial gene quantification in the groundwater of the Hainich aquifer assemblages.	**50**
Figure 12 | Comparison of anammox bacteria abundances based on anammox specific 16S rRNA gene and functional gene, (*hzsA*) in groundwater.	**51**
Figure 13 | Abundances of genes and transcripts of (A) archaeal and bacterial ammonia oxidisers (*amoA*) (B) anammox bacteria in groundwater.	**52**
Figure 14 | Ratios of *hzsA*/archaeal + bacterial *amoA* on the gene and the transcript level in groundwater samples.	**54**
Figure 15 | Ratios of [*hzsA*/sum of archaeal and bacterial *amoA*] plotted against the oxygen concentration in the respective groundwater sample.	**54**
Figure 16 | Relative concentrations of ladderane lipids.	**55**
Figure 17 | Formation of $^{29}N_2$ (blue circles) and $^{30}N_2$ (red circles) over a 48 hours incubation period in anammox, denitrification rates assays.	**57**
Figure 18 | Bacterial community structure based on MiSeq Illumina amplicon sequencing of 16S rRNA genes in the groundwater of eight wells across the two aquifer assemblages.	**59**
Figure 19 | Community structure of anammox bacteria.	**60**
Figure 20 | Anammox bacterial community structure based on MiSeq Illumina amplicon sequencing of 16S rRNA genes in the groundwater of eight wells across the two aquifer assemblages	**61**

Figure 21 | Taxonomic affiliation of the *nirS*-type denitrifying bacterial communities in the groundwater of four wells across the two aquifer assemblages at Hainich CZE, analysis based on Illumina MiSeq amplicon sequencing of *nirS* genes. **63**

Figure 22 | Community composition of the enriched chemolithotrophic denitrifier consortium, Planktonic versus Attached denitrifiers **65**

Figure 23 | Community composition of groundwater based enrichment culture obtained by the Gelrite shake dilution technique under denitrifying conditions **66**

Figure 24 | Chemolithoautotrophic growth and metabolic activity of denitrifying enrichment culture 1E in batch cultures at 15 °C. **68**

Figure 25 | Transcript abundances of *nirS* and *nosZ*-containing denitrifiers over time during the two microcosm experiments, M_I and M_II. **70**

Figure 26 | Abundance and transcript/gene ratios.of functional marker genes for denitrification (*nirS* and *nosZ*) **71**

Figure 27 | Transcriptionally active denitrifiers based on *nirS*- and *nosZ*- sequences during microcosm experiment M_I **73**

Figure 28 | Changes of the denitrifying microbial community (1E; species level) growing under chemolithoautotrophic conditions with different nitrate/thiosulfate ratios **74**

Figure 29 | Unrooted neighbour-joining phylogenetic tree of 16S rRNA sequences of the groundwater denitrifying isolates. **76**

Figure 30 | Heterotrophic growth of pure culture strain 2_*Acidovorax defluvii* under denitrifying conditions at 15° C. **77**

Figure 31 | Control for microcosm assay M_I **123**

Figure 32 | pH and pressure were monitored during microcosm assay M_II. **123**

Figure 33 | SEM image of enrichment culture 1E showing cells at different time points during the M_II assay incubation. **124**

Figure 34 | Changes of the denitrifying microbial community (1E; class based) growing under chemolithoautotrophic conditions with different ratios of nitrate and thiosulfate **125**

SUMMARY

Our current understanding of the diversity and ecology of subsurface microorganisms is very limited. Oligotrophic environments are often dominated by bacterial lineages with no cultured representative and only limited insight from metagenomic studies. Despite the high relevance of anaerobic ammonium oxidation (anammox) for nitrogen loss from marine systems, its relative importance compared to denitrification in freshwater ecosystems has not received as much attention; our knowledge is especially scarce for groundwater environments, especially when considering oligotrophic and organic carbon limited pristine aquifers. In this PhD project, the anammox process was shown to contribute an estimated 83% to total nitrogen loss in suboxic groundwaters of the Hainich aquifer assemblages at rates of 3.5 to 4.7 nmol L^{-1} d^{-1}, presumably favoured over denitrification by low organic carbon availability. Abundances of anammox specific *hzsA* gene transcripts (encoding for hydrazine synthase subunit A) exceeded those of denitrifier *nirS* and *nirK* gene transcripts (encoding for nitrite reductase) by up to two orders of magnitude, providing further support of a predominance of anammox. Anammox bacteria closely related to *Candidatus* Brocadia fulgida were identified as a dominant group within the anammox bacterial community. Anammox bacteria constituted up to 10.6 % of the groundwater microbial community and were ubiquitously present across the two aquifer assemblages with an indication of potentially active anammox bacteria even in the presence of 103 µmol L^{-1} oxygen. Co-occurrence of *hzsA* transcripts and *amoA* transcripts encoding ammonia mono-oxygenase suggested coupling between aerobic and anaerobic ammonium oxidation under suboxic conditions. Here, more investigations would be needed to ultimately prove the co-occurrence of the respective processes and activities. Overall, these results clearly demonstrate the relevance of anammox as a key process driving nitrogen loss from suboxic groundwater, which might further be enhanced through coupling with incomplete nitrification.

The ability to fix inorganic carbon comes along with several biogeochemical potentials apart from providing a new source of carbon in the subsurface. The ability to oxidize reduced sulfur and hydrogen as part of a chemolithoautotrophic life style, with potential coupling to denitrification under anoxic/suboxic conditions, makes chemolithoautotrophic microorganisms exclusively successful in oligotrophic subsurface environments. Although a high genetic potential for autotrophic denitrification in these aquifers mediated by reduced sulfur, iron and hydrogen as potential electron donors had been reported earlier, first quantitative evidence for the denitrification process in the groundwater of the Hainich Critical

Zone Exploratory (CZE) was demonstrated by ^{15}N measurements together with the anammox rates. Assessment of *nirS*-type denitrifier community composition in the groundwater of four wells of both the upper and the lower aquifer assemblage at six different time points revealed constantly high fractions of potential chemolithoautotrophic denitrifiers. A successful enrichment of chemolithoautotrophic denitrifiers yielded microbial communities dominated by lineages closely related to the genera *Thiobacillus, Sulfuritalea,* and *Hydrogenophaga.* In two microcosm experiments, the enriched consortium's metabolic potential for denitrification was further validated through the quantitative assessment of metabolic intermediates and products of the denitrification process, using thiosulfate and hydrogen as electron donors and ^{15}N-labeled nitrate to faciliate the tracing of gaseous nitrogen compounds. Microcosm experiment M_I used a nitrate/thiosulfate ratio of 2.5 and focused on the analysis of dissolved intermediates and products along with the analysis of denitrifier transcriptional activity based on *nirS* and *nosZ*, encoding nitrite reductase and nitrogen oxide reductase, respectively. In the second microcosm experiment M_II, the nitrate/thiosulfate ratio was changed to 1 to ensure complete denitrification. Raman gas spectroscopy confirmed the nearly complete turnover of NO_3^- to N_2 by the end of the 19-days incubation period. No N_2O was formed, nor did concentrations of ammonium increase beyond initial background levels, indicating that nitrate was utilized by complete denitrification and not by dissimilatory nitrate reduction to ammonium (DNRA) by the enriched consortium. Under conditions of electron donor limitation (ratio of nitrate/thiosulfate 2.5), microcosm M_I did not meet the stoichiometric requirements of thiosulfate mediated denitrification, leading to incomplete denitrification with leftover nitrate but complete utilization of thiosulfate by the end of the incubation period.

Quantification of bacterial 16S rRNA genes as an approximation of bacterial population densities showed an increase by two orders of magnitude during the 14- and 19-days incubation periods of the two microcosm experiments, corresponding to an average doubling time of 28 hours. Moreover, 16S rRNA gene-targeted Illumina sequencing revealed shifts in community composition during the incubation experiment while *Thiobacillus denitrificans*-related denitrifiers were identified as the dominant transcriptionally active denitrifiers on the level of both *nirS* and *nosZ*. Although the enriched consortium did not exactly mirror the composition of the natural denitrifier communities, it well reflected its metabolic capacities and provided a useful model system to study chemolithoautotrophic denitrification and changes of its key players over time.

ZUSAMMENFASSUNG

Unser aktueller Kenntnisstand zur Diversität und Ökologie von Mikroorganismen in unterirdischen Lebensräumen ist begrenzt. In nährstoffarmen Lebensräumen herrschen oft bakterielle Gruppen vor, zu denen es keine kultivierten Repräsentanten und nur wenig Informationen aus metagenomischen Studien gibt. Trotz der hohen Relevanz der anaeroben Ammonium-Oxidation für Stickstoffverluste in marinen Systemen hat ihre Bedeutung relativ zur Denitrifikation in Süßwasserlebensräumen bisher weit weniger Beachtung gefunden. Speziell für Grundwasserlebensräume ist unser Wissen sehr begrenzt, insbesondere im Hinblick auf oligotrophe Aquifere mit nur geringer Verfügbarkeit von organischen Kohlenstoffverbindungen. In dieser Dissertation konnte gezeigt werden, dass der Anammox-Prozess geschätzt etwa 83% zu den gesamten Stickstoffverlusten im suboxischen Grundwasser der Hainich-Aquiferkomplexe beiträgt bei einer Prozessrate von 3,5 bis 4,7 nmol L^{-1} d^{-1}, wobei Anammox vermutlich durch das geringe Angebot an organischem Kohlenstoff gegenüber der Denitrifikation gefördert wird. Abundanzen von Transkripten des Anammox-spezifischen *hzsA* genes (kodiert für Hydrazine Synthase, Untereinheit A) überstiegen Abundanzen der Transkripte von *nirS* und *nirK*-Genen, die für Nitrit-Reduktase kodieren, um bis zu zwei Größenordnungen, wodurch die Dominanz des Anammox-Prozesses zusätzlich verdeutlicht wurde. Anammox-Bakterien mit enger Verwandtschaft zu *Candidatus* Brocadia fulgida wurden als dominante Gruppe innerhalb der Anammox-Gemeinschaft identifiziert. Anammox-Bakterien machten bis zu 10.6% der bakteriellen Gemeinschaften im Grundwasser aus und waren allgegenwärtig in beiden Aquiferkomplexen zu finden, zudem deuteten die Daten auf die Anwesenheit potentiell aktiver Anammox-Bakterien sogar bei Sauerstoffkonzentrationen von 103 µmol L^{-1} hin. Das gemeinsame Auftreten von *hzsA* Transkripten mit Transkripten des *amoA*-Gens, welches für Ammonium Monooxygenase kodiert, deutete auf eine mögliche Kopplung zwischen aerober und anaerober Ammonium-Oxidation unter suboxischen Bedingungen hin. Hier wären allerdings noch weiterführende Untersuchungen nötig, um das gemeinsame Stattfinden der beiden Prozesse und Aktivitäten ultimativ zu belegen. Insgesamt konnte durch die erzielten Ergebnisse deutlich gezeigt werden, dass Anammox einen Schlüsselprozess hinsichtlich der Stickstoffverluste in suboxischem Grundwasser darstellt, möglicherweise zusätzlich verstärkt durch die Verknüpfung mit unvollständiger Nitrifikation.

Die Fähigkeit, anorganischen Kohlenstoff zu fixieren, geht einher mit der Bereitstellung einer weiteren Kohlenstoffquelle in unterirdischen Lebensräumen sowie mit einer Reihe daran

geknüpfter biogeochemischer Potenziale. Die Fähigkeit, reduzierte Schwefelverbindungen oder Wasserstoff als Bestandteil eines chemolithoautotrophen Stoffwechsels zu oxidieren, möglicherweise auch gekoppelt mit Denitrifikation unter anoxischen oder suboxischen Bedingungen, verschafft chemolithoautotrophen Mikroorganismen in oligotrophen unterirdischen Lebensräumen einen Vorteil. Obwohl ein großes genetisches Potenzial für autotrophe Denitrifikation, basierend auf reduzierten Schwefelverbindungen, Eisen oder Wasserstoff als potenziellen Elektronendonatoren schon in früheren Studien für diese Aquiferkomplexe berichtet wurde, wurde der erste quantitative Nachweis des Denitrifikationsprozesses im Grundwasser des Hainich Critical Zone Exploratory durch die ^{15}N-basierten Messungen gemeinsam mit den Anammox-Ratenmessungen erbracht. Die Erfassung der Gemeinschaftszusammensetzung der *nirS*-Typ Denitrifikanten im Grundwasser von vier Brunnen des oberen und unteren Aquiferkomplexes über sechs Beprobungstermine zeigte einen konstant hohen Anteil an potenziell chemolithoautotrophen Denitrifikanten. Die erfolgreiche Etablierung einer Anreicherungskultur chemolithoautotropher Denitrifikanten führte zum Erhalt mikrobieller Gemeinschaften, die vorwiegend von Vertretern der Gattungen *Thiobacillus*, *Sulfuritalea* und *Hydrogenophaga* dominiert wurden. In zwei Mikrokosmen-Experimenten wurde das metabolische Potenzial zur Denitrifikation des angereicherten Konsortiums weitergehend untersucht durch eine quantitative Erfassung der Zwischen- und Endprodukte des Denitrifikationsprozesses, wobei Thiosulfat und Wasserstoff als Elektronendonatoren und ^{15}N-markiertes Nitrat verwendet wurden, um gasförmige Stickstoffverbindungen besser verfolgen zu können. Das Mikrokosmen-Experiment M_I verwendete ein Nitrat/Thiosulfat-Verhältnis von 2,5 und konzentrierte sich auf die Analyse gelöster Intermediate und Produkte, zusammen mit der Analyse der transkriptionellen Aktivität von Denitrifikanten basierend auf *nirS* und *nosZ*, welche für Nitritreduktase sowie Distickstoffoxid-Reduktase kodieren. In einem zweiten Mikrokosmen-Experiment M_II wurde das Nitrat/Thiosulfat-Verhältnis zu 1 geändert, um eine vollständige Denitrifikation sicherzustellen. Raman Gasspektroskopie bestätigte die nahezu vollständige Umsetzung des zugefügten Nitrats zu N_2 am Ende der 19-tägigen Inkubationsperiode. Eine Bildung von N_2O wurde nicht beobachtet, gleichfalls stiegen auch die Konzentrationen von Ammonium nicht gegenüber dem ursprünglichen Hintergrundwert an, was zeigte, dass das Nitrat durch vollständige Denitrifikation des angereicherten Konsortiums verbraucht worden war und nicht durch dissimilatorische Nitrat-Reduktion zu Ammonium (DNRA). Unter den Bedingungen einer Limitierung des Elektronendonors

(Verhältnis Nitrat/Thiosulfat 2,5), waren in Mikrokosmen-Experiment M_I nicht die stöchiometrischen Voraussetzungen der Thiosulfat-abhängigen Denitrifikation erfüllt, mit der Folge einer unvollständigen Denitrifikation mit verbleibenden Restmengen an Nitrat, aber der vollständigen Umsetzung des Thiosulfats am Ende der Inkubationsperiode.

Die Quantifizierung von bakteriellen 16S rRNA-Genen als Schätzwert bakterieller Populationsdichten zeigte einen Anstieg um zwei Größenordnungen während der 14- bzw. 19-tägigen Inkubationsperiode der zwei Mikrokosmen-Experimente, was einer durchschnittlichen Verdopplungszeit von 28 Stunden entspricht. Darüber hinaus deutete eine Illumina-Sequenzierung der 16S rRNA-Gene auf Verschiebungen in der Gemeinschaftszusammensetzung während des Inkubationsexperimentes hin, während *Thiobacillus denitrificans*-verwandte Denitrifikanten als dominate transkriptionell aktive Denitrifikanten auf dem Niveau von sowohl *nirS*- als auch *nosZ*-Genen identifiziert wurden. Obwohl das angereicherte Konsortium nicht exakt die Zusammensetzung der natürlichen Denitrifikanten-Gemeinschaften widerspiegelte, reflektierte es sehr gut deren Stoffwechselkapazitäten und stellte ein geeignetes Modellsystem dar, um die chemolithoautotorphe Denitrifikation und die Veränderungen der Hauptakteure über die Zeit zu verfolgen.

1. INTRODUCTION

1.1 The biogeochemical nitrogen cycle

The global biogeochemical cycling of nitrogen is central to the biogeochemistry on Earth, as a fundamental component of all living organisms (Gruber and Galloway, 2008; Fowler *et al*., 2013). The complexity of the nitrogen cycle is still being unravelled even after almost a century of studying, with recent breakthroughs in microbiology including new discoveries within the last two decades, such as anaerobic ammonium oxidation coupled to nitrite oxidation, (Anammox) by Mulder *et al*. (1995); van de Graaf *et al*. (1995), anaerobic methane oxidation coupled to nitrate reduction, (ANME) by Raghoebarsing *et al*. (2006) and complete ammonium oxidation to nitrate (Comammox) by Daims *et al*. (2015); van Kessel *et al*. (2015). The ecophysiology of novel microorganisms or consortia mediating these processes and interactions with classical types of nitrogen metabolism enable us to understand the microbial engines that drive the global nitrogen cycle itself and how it is linked with the cycles of carbon, sulfur and iron.

Unlike most elements, nitrogen can chemically bond in eight different ways- by donating one, two, three, four, or five electrons or accepting one, two, or three electrons - which makes nitrogen exceptionally versatile in joining with other atoms (Gorman, 2013). With this inherent reactive capacity, nitrogen occupies pivotal association in nature's most crucial building blocks such as amino acids (basic components of all proteins) and nucleotides (sequences of which serve as the basis of all genetic material).

Indeed, the nitrogen requirements for life are immense, something that can be inferred from element abundances. Depending on the life form, for every 100 atoms of the carbon incorporated into cells, between 2 and 20 atoms of nitrogen follow (Sterner and Elser, 2002). Despite its overwhelming abundance on Earth, weighed at *ca.* 4 x 10^{15} metric tons (t) (1t = 1,000 grams, or approximately 2,204 pounds) - more than the mass of four other elements combined (carbon, phosphorous, oxygen and sulfur), atmospheric dinitrogen is unavailable for most organisms (except nitrogen-fixing bacteria) (Mackenzie, 1998). Hence, fixed inorganic nitrogen availability such as nitrate (NO_3^-) and ammonium (NH_4^+) ions often limits primary production in both terrestrial and marine ecosystems (Falkowski *et al*., 1997, 2008; Galloway *et al*., 2003; Canfield *et al*., 2010).

The transformation of nonreactive N_2 into reactive nitrogen is indeed essential for life. In the environmental context, apart from NO_3^- and NH_4^+, other forms of reactive nitrogen include oxidized nitrogen species, nitric oxide (NO), nitrogen dioxide (NO_2), nitrous oxide (N_2O), nitrite (NO_2^-) as well as the reduced nitrogen species, ammonia (NH_3) and organically bound N, such as in the intermediate products of organic matter decomposition, humus and metabolism of nitrogen-containing compounds by animals, urea. The interchange between inert dinitrogen gas (N_2) and nitrogen compounds that support, or are products of, cellular metabolism and growth is significantly controlled by microbial activities and listed as intermediates in the nitrogen cycle (Stein and Klotz, 2016). **Figure 1**, illustrating the current view of microbial processes involved in the nitrogen cycle.

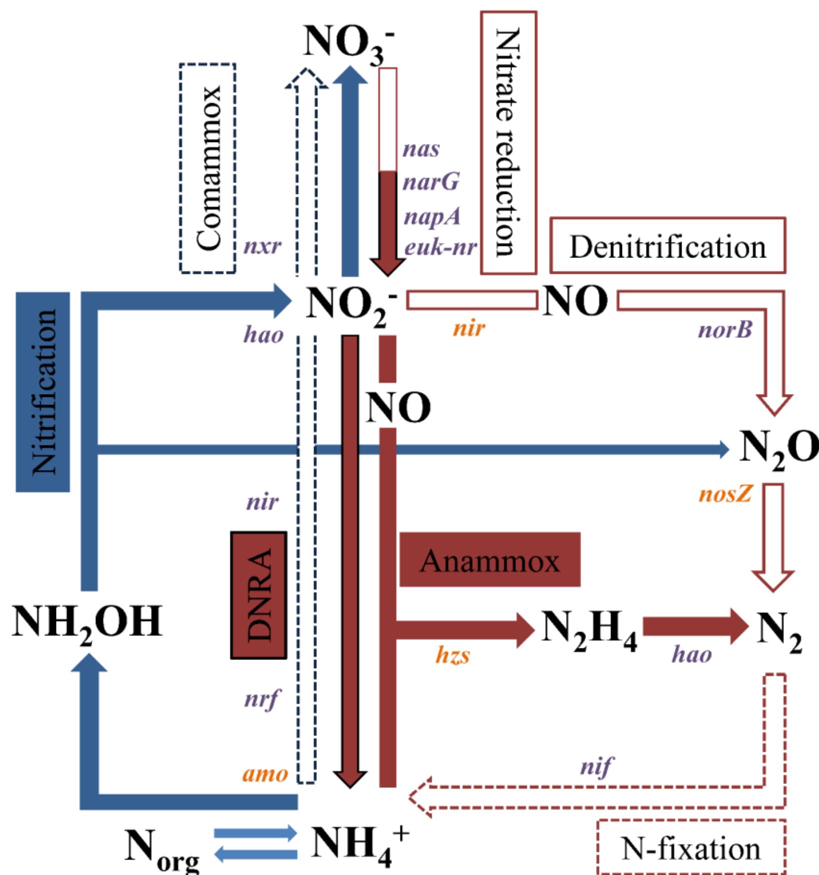


Figure 1 | Current view of biological nitrogen cycle. Biological nitrogen cycle pathways driven by associated enzymes. Gene encoding enzymes that conduct the important transformations including those for various nitrate reductases (*nas*, *euk-nr*, *narG*, *napA*), nitrite reductases (*nir*, *nrf*), nitric oxide reductase (*norB*), nitrous oxide reductase (*nosZ*), nitrogenase (*nif*), ammonium monooxygenase (*amo*), hydroxylamine oxidoreductase (*hao*), nitrite oxidoreductase (*nxr*), and hydrazine synthase (*hzs*). (Figure adapted from Canfield *et al*., 2010). Colour coding represents aerobic processes in blue, anaerobic processes in red and genes used as functional markers in this study in orange colour.

1.2 Excess reactive nitrogen and its ecological impacts

Although reactive forms of nitrogen are strongly related to energy and primary production, excess reactive nitrogen is a cause of environmental pollution. In response to biological evolution and varying environmental redox conditions, it has become apparent from the historical nitrogen isotope record that the nitrogen cycle has changed across the geological timeframe (Stüeken *et al*., 2016). Therefore, under current situations, when we use the term "nitrogen cycle", we are imposing a pattern on nature that suits our interests and is limited to our current understanding about the activity of a very dynamic and complex interplay of abiotic and biotic processes (Gorman, 2013).

As we are proceeding further through the twenty-first century, it is obvious that we humans have turned the tables on nature, with over 40% of the Earth's land surface now fully dominated by us and much of the rest significantly influenced by our activities (Steffen *et al*., 2004; Steffen, 2008). Apart from lightning discharges, which create biologically available reactive nitrogen (ca. 5 Tg N yr^{-1} globally), an active biosphere ultimately requires incorporation of nitrogen into biological molecules through biological nitrogen fixation, a process where prokaryotes in the bacterial and archaeal domains reduce nitrogen gas (N_2) to ammonium (ca. 198 Tg N yr^{-1} globally) (Fowler *et al*., 2013). With the advent of agriculture about 10,000 YA, the human-environment relationship changed in fundamental ways. This involves the clearing of forests to create managed grasslands or croplands, which started to modify the fluxes of elements, at least at local and regional scales. With the growing human population, the requirement for reactive nitrogen also increased to improve productivity. With the inception of cultivation-induced biological nitrogen fixation such as legume crops production, two more methods, the Haber-Bosch process (industrial fixation of N_2 to NH_3), and fossil fuel combustion (high temperature conversion of N_2 and fossil organic N to NO_x) and by the 1970s, anthropogenic processes have overtaken natural terrestrial processes in reactive nitrogen creation on a global scale (Galloway *et al*., 2004). By the combination of above-mentioned processes, reactive nitrogen compounds are now accumulating in the environment and resulting in a very significant alteration of the nitrogen cycle at local, regional and global scales.

Over the past 200 years, since our first discovery of nitrogen as an element, our knowledge of reactive nitrogen creation and its cascade through ecosystems has increased dramatically. Given the fundamental role that nitrogen has on ecosystem productivity, it is not surprising that these disturbances in the nitrogen cycle have profound impacts on the health of both ecosystems and

humans. Some of the well-known impacts are eutrophication of soils and water bodies, nitrate pollution in drinking water leading to health problems, reductions in ecosystem biodiversity, atmospheric acidification, stratospheric ozone depletion, and contribution to the global climate change problem (Fowler *et al*., 2013; Erisman *et al*., 2013). These impacts are linked in the reactive nitrogen cascade. Once a reactive molecule such as NO_X or NH_3 is created, it can move through the environment and contribute to all of those issues in sequence until either stored in long-term reservoirs such as the subsurface or converted back to its inert form as N_2 (Galloway *et al*., 2003).

1.3 Perspective on reactive nitrogen with groundwater

There is no doubt that terrestrial aquatic ecosystems all over the world are facing a high exposure to excessive reactive nitrogen, therefore affecting the Earth's Critical Zone for life that extends from the top of forest canopies to the depths reached by the groundwater, ultimately affecting the biogeochemical cycles of Earth (Fields *et al*., 2004; Akob *et al*., 2011, Li *et al*., 2017).

As discussed in the previous section, much evidence exists for the effects of increased concentration of reactive nitrogen mediating eutrophication of coastal zones, leading to biodiversity loss, as well as ozone depletion in the stratosphere, increasing the greenhouse effect from continuously increasing N_2O gas into the atmosphere. However, our understanding about its fate in dark, subsurface ecosystems is insufficient and the existing uncertainties, owed to the spatial and temporal variability, further demand better quantitative assessments of these components in the reactive nitrogen cascade and the associated microbial players. Similar to air, soil and surface waters, reactive nitrogen builds up in the subsurface through which it can percolate into aquifers and ultimately contaminate groundwater (Rivett *et al*., 2008; Thayalakumaran *et al*., 2008). As groundwater flows throughout the Earth's upper crust, microbial diversity plays an important role in influencing the groundwater's chemistry and associated biogeochemical processes.

Foremost, properties of groundwater bodies distinguish them from surface water bodies; the relatively slow movement of water through the underground means that residence times in aquifers are generally orders of magnitude (months to millions of years) longer than in surface waters (Gleeson *et al*., 2015). Once reactive nitrogen, such as nitrate or ammonium, makes its way into the groundwater, it is subjected to physical, chemical, and biological processes which further determine its fate trajectories. Without the natural attenuation processes, which remove reactive

nitrogen back into its inert form N_2, such contaminants can accumulate and be transported for tens of years (Stewart *et al*., 2011; Han *et al*., 2015). Despite immense geological variability from one geographical region to another, one striking physical feature of the shallow subsurface is the similarity of the types of strata in which freshwater aquifers are found (Freeze and Cheery, 1979; Bouwer, 1984). These strata have relatively constant physical and chemical properties that will determine the type, abundance, and activities of microorganisms (Hug *et al*., 2015). Porosity and permeability of the subsurface material affect water availability and determine the pore space, which in turn can limit the types and sizes of organisms present. The size of these interstices can range from minute pores to cracks, fissures and large cavities or cave structures and provide ecological niches to various microorganisms (Longley, 1981).

The chemical and biological reactive nitrogen removal processes are generally known to exhibit stringent regulation upon each other. Hug and colleagues (2015) proposed that the "aquifer environment selects for microbial species cohorts in sediment and groundwater". The delivery and fate of reactive nitrogen to the groundwater is highly dependent on concentrations of electron donors such as dissolved organic carbon (DOC), inorganic electron donors such as reduced iron, reduced sulfur or iron-sulfide minerals and ammonium. Apart from the land use type, distance from contamination point sources, groundwater recharge site, lithology of surface rocks, aquifer's geological structure, sediment pore size, presence of surface-dipping beds cavities such as sinkholes and hydrochemical parameters such as redox potential (Eh), pH, temperature and obviously, dissolved oxygen (Kölle *et al*., 1987; Korom, 1992; Pinay *et al*., 1993; DeSimone *et al*., 1998; Jacinthe *et al*., 1998; Devito *et al*., 2000; Wrage *et al*., 2001; Pabich *et al*., 2003; Rivett *et al*., 2008; Thyalakumaran *et al*., 2008; Lasagna *et al*., 2016; Nemčić-Jurec and Jazbec, 2017).

1.4 How do karst oligotrophic aquifers respond to reactive nitrogen?

With the increasing reactive nitrogen footprints, i.e. "the total amount of reactive nitrogen released to the environment as a result of an entity's consumption patterns: Galloway *et al*. (2014)" the current estimates suggested that 75% of the reactive nitrogen created on land by human action such as agriculture which is three – fold greater than natural terrestrial creation of reactive nitrogen (Galloway *et al*., 2013; Fowler *et al*., 2013; Vitousek *et al*., 2013). This unprecedented increase of reactive nitrogen is negatively affecting freshwater resources, especially Karst aquifers, which is

of growing concern on a global scale (Burgin and Hamilton, 2007, Schlesinger, 2009; Galloway 2014). Globally, a quarter of the human population relies on karstic aquifers for freshwater requirements, which are undoubtedly vulnerable to nitrate contamination due to their potential for rapid infiltration and temporary inflow of oxygenated water (Auckenthaler *et al*., 2002, Ford and Williams, 2007, Huebsch *et al*., 2014).

Moreover, in older limestone or shallow karstified aquifers, apart from intensive water exchange, contaminants in recharge might move rapidly through, with minimal opportunity for attenuation by adsorption, ion exchange, chemical breakdown and with limited time available for remedial action (Stevanovoić, 2015). Due to the presence of cavities and large conduits, these pollutants can be quickly transported a long distance, thus increasing the pollution risks such as observed by Ruggieri *et al*. (2017). By continued implementation of land use control measures, such as protection zones and reliance on natural attenuation processes, it could be possible to mitigate reactive nitrogen loading in the subsurface (Fields, 2004). To better understand the natural attenuation process, a focus on biogeochemical processes that control the loss of nitrate or ammonium (the two most available forms of reactive nitrogen in groundwater) is prerequisite.

For nitrate removal in nature, denitrification and dissimilatory nitrate reduction to ammonium (DNRA) were being considered as key microbiological processes (Korom, 1992; Sgouridis et al., 2011), whereas for ammonium removal, canonical nitrification (aerobic ammonium oxidation and nitrite oxidation), Comammox, and Ananmmox have been revealed as important players. But unlike denitrification and Anammox leading to the formation of N_2, DNRA and canonical nitrification/Comammox conserve nitrogen in the ecosystem in the form of ammonium and nitrate respectively which already have major implications for our understanding of how karst aquifers will respond to increase in the nitrogen loads **(Figure 1)**. Although these processes are important in nature, in my doctoral studies, I specifically focused on denitrification and Anammox which transform reactive nitrogen all the way to N_2.

1.5 Key microbial players for the attenuation of ammonium and nitrate to dinitrogen

In the quest for global sustainability of contamination-free freshwater and to understand the active microbial players helping do so, scientific inquiry has always been aimed in unravelling who's doing what, in natural microbial communities. The significance of nitrogen to the biosphere and cellular life is indisputable; however, our fundamental knowledge of the microorganisms and enzymatic processes that transform nitrogen into its various oxidation states is still evolving such as unearthing of new players such as anammox and comammox. Anammox and denitrification as mentioned below are two microbial processes known until now, which can successfully transform reactive nitrogen to inert dinitrogen gas **(Table 1)**.

Table 1 | Stoichiometric reactions of anammox and denitrification in presence of different electron donors and changes in Gibbs free energy under standard conditions (ΔG˚ Kilojoules per mole electron donor). Stoichiometric reactions and values for ΔG˚ adapted from the given references.

Pathway *Reaction stoichiometry*	*Model organism*	*ΔG° (kJ mol⁻¹)*	*Reference*
Anammox			
$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$	*Candidatus* Brocadia	-357	Strous *et al.*, 1998
Denitrification			
$CH_3COOH + 1.6\ NO_3^- \rightarrow 0.8\ N_2 + 2\ CO_2 + 1.6\ OH^- + 1.2\ H_2O$	*Acidovorax defluvii*	-843	López *et al.*, 2009
$H_2 + 2/5\ NO_3^- + 2/5\ H^+ \rightarrow 1/5\ N_2 + 6/5\ H_2O$	*Sulfuritalea hydrogenivorans*	-240	Blodau, 2011
$S_2O_3^{2-} + 1.6\ NO_3^- + 0.2\ H_2O \rightarrow 0.8\ N_2 + 2\ SO_4^{2-} + 0.4\ H^+$	*Thiobacillus denitrificans*	-728	Anantharaman *et al.*, 2016

1.5.1 Anammox bacteria; a piece in the lithotrophy puzzle

Before the discovery of anammox bacteria, Richard, (1965) mentioned ammonium deficits in anoxic marine basins and proposed this loss to be mediated by unknown microbes which were, later on, coined as "lithotrophs missing in the nature" by Broda, (1977). In 1995, Mulder and colleagues provided direct evidence for anaerobic ammonium oxidation, and hence termed the

process. However, since the discovery of anammox in 1995, no pure cultures have been achieved, yet. Van de Graff *et al*. (1995, 1997) and Strous *et al*. (1999) shed light on the most possible hypothetical metabolic mechanism and intermediates of the anammox process, which involves 1 mol of NH_4^+ getting oxidized by 1 mol of NO_2^- to produce N_2 gas via hydrazine (N_2H_4) as an intermediate under strictly anoxic conditions **(Figure 1 and Table 1)**.

Since the anammox process produces N_2 from taking one atom of nitrogen from NH_4^+ and one from NO_3^- / NO_2^-, it can clearly be distinguished from denitrification which generally combines nitrogen from two NO_3^- molecules to form N_2 **(Figure 2)** and therefore serve as the basis for ^{15}N-based approaches to distinguish anammox from denitrification rates in activity assays.

Although anaerobic ammonium oxidation coupled to nitrite reduction was only relatively recently recognized, it appears to play a significant role in nitrogen cycling in contemporary oxygen minimum zones (Kalvelage *et al.,* 2013; Lam *et al.,* 2009). With no pure isolate cultures available as of yet, five *Candidatus* genera of anammox bacteria have so far been described: Kuenenia (Schmid *et al*., 2000; Strous *et al*., 2006), Scalindua (Kuypers *et al*., 2003; Speth *et al*., 2017), Anammoxoglobus (Kartal *et al*, 2007), Brocadia (Kartal *et al*., 2008; Oshiki *et al*., 2011, Park *et al*., 2017), and Jettenia (Hu *et al*., 2012; Ali *et al*., 2015). All five genera share the same anammox metabolism within a specialized organelle called the anammoxosome (**Figure 2**) and form a separate monophyletic cluster branching off deep within the phylum *Planctomycetes* in the order *Brocadiales* (Strous *et al*., 1999; Jetten *et al*., 2010).

The anammoxosome constitutes 50-70% of the total cell volume of anammox bacteria, which utilize this unique organelle-like structure, lined with ladderane lipids, to carry out the anammox process with the volatile, toxic hydrazine as an intermediate (Jetten, 2001; Sinninghe Damste *et al*., 2005). Due to the unique characteristic of ladderane lipids, they have been also used as a biomarker to reveal the presence of anammox bacteria (Kuypers *et al*., 2003).

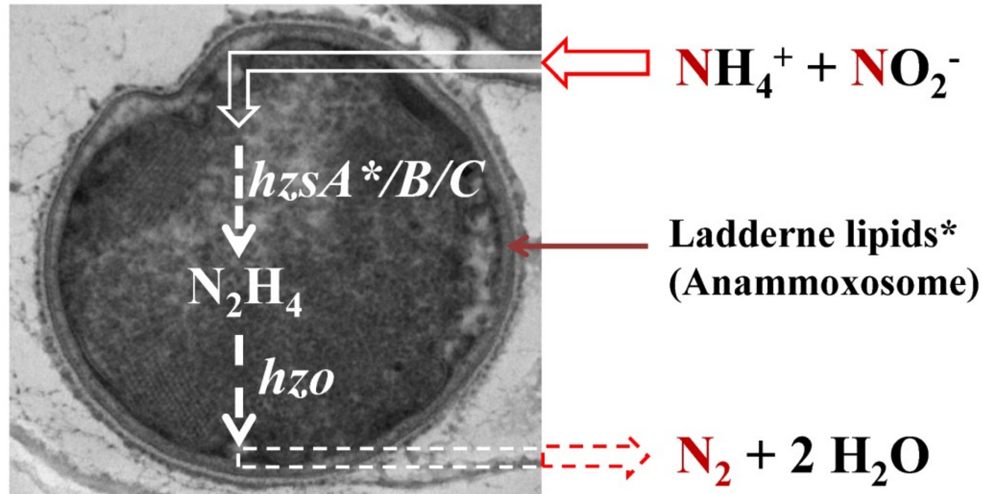


Figure 2 | Anammox bacteria and potential biomarkers. Transmission electron micrographs of anammox bacteria showing the anammoxosome membrane and key genes mediating anaerobic ammonium oxidation inside anammoxosome (Ladderane lipids and *hzsA* genes used as biomarker in this study)* (TEM image is adapted from van Niftrik *et al*., 2008; reproduced with permission). The anammox-*nirS* gene is not included in this figure.

The first report of the metabolic versatility of anammox bacteria was given by Strous *et al.* (2006) where the authors were able to reconstruct the genome of *Candidatus* "Kuenenia stuttgartiensis" in order to understand the anammox metabolic capacities and genes. This understanding of genomic potential was further improved by *in silico* analysis by Kartal *et al*., (2011) and other genome studies of *Ca.* Brocadia fulgida (Gori *et al*., 2011), *Ca.* Jettenia asiatica (Hu *et al*., 2012), *Ca.* Scalindua profunda (Vossenberg *et al*., 2013), *Ca.* Scalindua brodae (Speth *et al*., 2015), *Ca.* Brocadia sinica (Oshiki *et al*., 2015), *Ca.* Scalindua japonica (Oshiki *et al*., 2017) and *Ca.* Brocadia caroliniensis (Park *et al*., 2017).

The possible molecular mechanism of the anammox couples the oxidation of ammonium to the reduction of nitrite which includes, nitrite reductase (encoded by *nirS/K*) mediating nitrite reduction to nitric oxide, followed by a concurrent oxidation with ammonium to produce hydrazine (N_2H_4), by hydrazine synthase (encoded by *hzsABC* genes cluster), which further get oxidized to dinitrogen gas by hydrazine dehydrogenase (encoded by *hzo*) **(Figure 1, 2)** (Jetten *et al*., 2009; Haranghi *et al*., 2012 and Park *et al*., 2017). Although, recent studies are still considering anammox-specific 16S rRNA gene quantification for the detection of anammox bacteria in various environments (Hou *et al*., 2015; Cho *et al*., 2017; Han *et al*., 2017), the current paradigm focuses on quantification of anammox functional genes, especially *hzs* and *hzo,* as decisive genetic

biomarkers (Shehzad *et al*., 2016; Wang *et al*., 2017; Crowe *et al*., 2017). New estimates about doubling times of anammox bacteria, earlier estimated to be eleven days, suggest between 2.1 days to 3.9 days, achieved in a membrane bioreactor with high substrate loading rates at 37 °C (Jetten *et al*., 2005; Strous *et al*., 2006; Zhang *et al*., 2017).

To date, anammox bacteria have been detected worldwide in natural ecosystems. 16S rRNA sequences retrieved from marine ecosystems are exclusively dominated with *Ca*. Scalindua (Kuypers *et al*., 2003; Oshiki *et al*., 2017). In some studies related to oxygen minimum zone waters of Benguela, Chile, Peru, marine anammox bacteria are exclusively responsible for *ca*. 100% of total nitrogen production, also known as anammox hotspots (Kuypers *et al*., 2005; Thamdrup *et al*., 2006; Lam *et al*., 2009). In other marine water columns of Golfo Dulce (Dalsgaard *et al*., 2003), Black Sea (Kuypers *et al*., 2003; Lam *et al*., 2007) and Arabian Sea (Ward *et al*., 2009 and Jensen *et al*., 2011), the contribution of anammox to nitrogen loss was found to range from 10 – 35%. Anammox along with denitrification has therefore been suggested as the dominant process in the loss of nitrogen from marine ecosystems (Kuypers *et al*., 2003; Arrigo 2005). Contrary to marine ecosystems, Humbert and colleagues (2010) recognized that terrestrial ecosystems harbor more diverse anammox communities including the *Candidatus* Brocadia, Kuenenia, Scalindua, Jettenia, Anammoxoglobus, and similarly high diversity was also observed for aquatic realms such as land-freshwater interfaces, lakes, and aquifers (Hirsch *et al*., 2011; Zhu *et al*., 2013; Wang *et al*., 2017; Crowe *et al*., 2017) along with a contribution of anammox to N_2 loss varying from 13 – 50 % in freshwater lakes (Schubert *et al*., 2006; Wenk *et al*., 2013, Crowe *et al*., 2017) and from 0.5 – 40 % in ammonium-contaminated groundwater (Moore *et al*., 2011).

Recently, Smith *et al*. (2015) revealed that in ammonium-contaminated groundwater, anammox contributed up to 90 % to nitrogen loss, especially at the hotspot characterised with low dissolved organic carbon. Summarized by Oshiki and colleagues (2016), anammox activities are highly dependent on local environmental conditions such as dissolved oxygen, NO_x^- concentrations, temperature, the molar ratio of NH_4^+ to NO_x, and pH. Along with environmental factors, simultaneous activities of other microbial players could be competitive or supporting, such as ammonia oxidizing archaea or denitrifying bacteria which consume NH_4^+ or NO_2^- but could also supply the NO_2^-, originating from either aerobic ammonia oxidation or nitrate reduction,

respectively, and could be subsequently utilized in the anammox process (Lam *et al*., 2007, 2009; Pitcher *et al*., 2011; Zhou *et al*., 2014; Cao *et al*., 2016). Furthermore, anammox bacteria are known for having a high affinity for nitrite due to possession of multiple nitrite transporters (3-6), and can grow at concentrations of less than 5 µM (Strous *et al*., 1999; Park *et al*., 2017). In wastewater treatment plants and in marine sediments, anammox has been shown to compete effectively with denitrification, two environments where nitrate and ammonium often co-occur (Jetten, 2008). However, little is known about the potential importance of anammox activity in pristine aquifers, particularly relative to denitrification.

1.5.2 Denitrifying microorganisms and their role in nitrogen cycle

When oxygen is lacking from any ecosystem, nitrate, if available, becomes a very attractive electron acceptor for microorganisms that are able to respire it. Denitrification was considered exclusively performed by bacteria and archaea until reports suggested that even fungi and foraminifera also mediate this process and play an important role in the global geochemical nitrogen cycle (Zumft, 1992; Kobayashi *et al*., 1996; Philippot *et al*., 2002; Glock *et al*., 2013). Since the first detailed study of bacterial denitrification by Gayon and Dupetit in 1886, heterotrophic denitrifiers that can utilize organic compounds, has been considered as an integral part of the microbial nitrogen cycle; and due not least to the increasing nitrate contamination from intensive agriculture and organic contaminants; they have been the focus of research activities (Knowles, 1982; Zumft, 1997). In contrast, a comprehensive, quantitative understanding of denitrification rates across oligotrophic ecosystems and transcriptional activity mediated by autotrophic denitrifiers is still in its infancy (Davidson and Seitzinger, 2006; Beller *et al*., 2006).

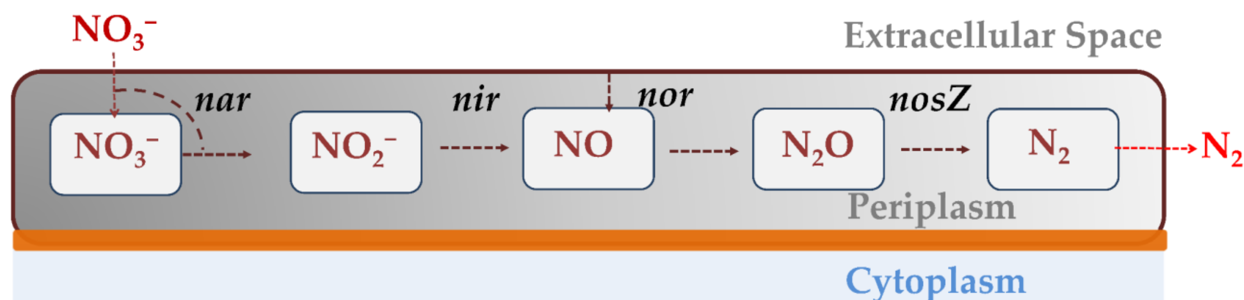


Figure 3 | Denitrifying bacteria and potential gene markers. Topological model of a typical denitrifying bacteria, illustrating stepwise reduction of nitrogen oxides up to dinitrogen, mediated by transcriptional activity of respective genes (according to Zumft, 1997).

Common in all prokaryotic denitrifiers is the stepwise reduction of nitrate via NO_2^-, NO and N_2O to N_2, employing four different enzymes: membrane bound/periplasmic nitrate-reductase (encoded by *nar*- or *nap*- genes), periplasmic nitrite-reductase (encoded by *nir* genes), membrane bound nitric oxide-reductase (encoded by *norB*), and periplasmic nitrous oxide-reductase (encoded by *nosZ*) (Zumft, 1997) **(Figure 3)**. While *narG* and *napA* genes can also be found in non-denitrifying nitrate reducers, all the other genes occur exclusively in denitrifiers. All these genes have been used in a large number of studies to investigate the diversity and abundance of denitrifiers in a broad range of environments including groundwater ecosystems (Kandeler *et al*., 2006; Barrett *et al*., 2013; Liu *et al*., 2014; Saarenheimo *et al*., 2015; Wei *et al*., 2015; Zeng *et al*., 2016).

As denitrifying bacteria are essentially ubiquitous in the subsurface, the critical limiting factors are oxygen and electron donor concentration and availability. The type of electron donor will determine the performance of the denitrifying process in terms of change in Gibbs free energy ($\Delta G^{o\prime}$) and consumption rate (Cuervo-López, *et al*., 2009). Denitrification, with three different electron sources and their respective ΔG^o values are given in **Table 1**. For chemolithautotrophs, the complete oxidation of thiosulfate to sulfate is among the most attractive metabolisms energetically **(Table 1)**. Brettar and Rheinheimer (1991), suggested that at oxic – anoxic interfaces, having the presence of reduced sulfur (both H_2S and $S_2O_3^{2-}$) and low organic carbon, reduced sulfur compounds could be valuable electron donors for chemolithoautotrophic denitrifiers. Furthermore, under such conditions, chemolithoautotrophic denitrifiers has been shown to outcompete heterotrophic denitrifiers both in numbers and denitrification activity by Brettar and Rheinheimer (1991) and therefore could be a leading player among denitrifiers in suboxic oligotrophic ecosystems as well.

1.6 Motivation of this thesis: understanding nitrogen loss in oligotrophic carbonate-rock aquifers

Generally, heterotrophic denitrification have been primarily attributed to nitrogen losses from freshwater environments, including aquifers (Seitzinger *et al*., 2006, Rivett *et al*., 2008). Having said that, in pristine aquifers, especially under conditions of limited organic carbon availability, autotrophic nitrate/nitrite reducing processes such as autotrophic denitrification or anaerobic ammonium oxidation are likely to become more competitive.

1.6.1 Biogeochemical role of chemolithoautotrophic anammox in groundwater

Despite the high significance of anammox process for nitrogen losses from oxygen minimum zones sitting at the heart of the marine nitrogen cycle (Thamdrup and Dalsgaard, 2002; Jensen *et al*., 2011), this process has also recently gaining the focus of nitrogen loss mediated from freshwater environments including lakes, rivers, groundwater and even extreme freshwater ecosystems (Schubert *et al*., 2006; Clark *et al*., 2008; Moore *et al*., 2011; Yoshinaga *et al*., 2011; Yang *et al*., 2015; Zhu *et al*., 2015; Shen *et al*., 2016). Characterised with low temperature, groundwater seemed to be a suitable environment for anammox to occur (Rysgaard and Glud, 2004, Isaka *et al*., 2008, Canion *et al*., 2014). Investigations from ammonium- and nitrate-contaminated groundwater assisted by isotope-based and molecular marker studies suggested anammox to be the responsible nitrogen attenuation process mediating up to 90 % of nitrogen loss (Clark *et al*., 2008, Smits *et al*., 2009, Humbert *et al*., 2010; Moore *et al*., 2011, Robertson *et al*., 2012, Hanson and Madsen, 2015, Smith *et al*., 2015).

Together with Starke *et al.* (2017) and Schwab *et al.* (2017), the researchers within AquaDiva, provided metaproteomic and ladderane lipids biomarkers based first evidence of active anammox bacteria in oligotrophic groundwater of the Hainich Critical Zone Exploratory. However, the activity of anammox and denitrifying organisms has yet to be confirmed with ^{15}N labelling incubations and functional gene biomarker-based evidence, which further elucidate the role of anammox, compared to denitrification for nitrogen loss from this oligotrophic aquifer system. Among the possible contributing factors included is the availability of electron acceptors and donors, as well as potential interactions with other key players of the nitrogen cycle. As an example, in natural ecosystems such as deep soils and marine systems, interactions between anammox bacteria and archaeal ammonia oxidising partners have been suggested (Woebken *et al*., 2007; Yan *et al*., 2012). In fact, the anammox studies in oxygen minimum zones suggested that nitrite originating from incomplete nitrification may fuel the anammox process under low oxygen conditions (Lam *et al*., 2007, 2009), the relevance of a potential coupling of these two processes from groundwater environments has not yet been addressed. Nevertheless, in the suboxic groundwater, cooperation between ammonia oxidizing archaea (AOA) and bacteria (AOB) could provide nitrite to anammox bacteria in a partnership similar to those proposed by Schmidt and colleagues (2002) for marine systems. Two of the main enzymes in aerobic ammonia oxidation

are ammonia monoxygenase (AMO; encoded by *amoA* and *amoB* gene) which catalyzes the oxidation of ammonium to hydroxylamine, and hydroxylamine oxidoreductase (HAO; encoded by *hao* gene) which oxidizes hydroxylamine to nitrite, and hence the genes involved can be used to detect those groups on a molecular level (Francis *et al*., 2005; Schmid *et al*., 2008; Opitz *et al*., 2014). Martens-Habbena and colleagues, in 2009, suggested that a very low affinity value (*Ks*) of AOA for ammonium (133 nM), and AOA could be operative at low oxygen availability to oxidize the ammonia to nitrite for anammox bacteria. Consequently, among other habitats, aquifers could provide suitable conditions for the coexistence of ammonia-oxidizing archaea, ammonia-oxidizing bacteria and anammox and hence, another question to see the relevance in groundwater ecosystem.

1.6.2 Biogeochemical role of denitrifiers in oligotrophic groundwater

Another process relevant for nitrogen loss in an oligotrophic environment could be autotrophic denitrification. Although, organic carbon availability is considered as the major driving force in heterotrophic denitrification, pristine aquifers are reportedly limited in organic carbon given that most of the readily metabolized compounds are consumed by surface microorganisms, before they reach the groundwater, restricting the overall contribution of the heterotrophic denitrifying microbial community (Alfoldi, 1988; Ghiorse and Wilson, 1988). Autochthonous autotrophic denitrifying bacteria can prevail in these organic carbon-limited ecosystems, utilizing inorganic electron donors such as reduced sulfur derived from the oxidation of iron sulfide minerals, and hydrogen (Table 1) (Chapelle, 1993; Nelson and Hagen, 1995; Ottley *et al*., 1997).

Often dominated by sulfur-driven autotrophic denitrifiers, gaining energy through concomitant S and H_2 oxidation, the synergistic effect of these electron donors for the survival of the microbial community from the oligotrophic deep-subsurface has also been highlighted, recently (Lau *et al*., 2016). There are also several lines of metatranscriptomic evidence suggesting that chemolithoautotrophic potential in aquifers is indeed highly relevant for C, S, N and Fe cycling (Jewell *et al*., 2016, 2017; Starke *et al*., 2017). Most of the chemolithotrophic denitrifying organisms isolated and described from freshwater environments, such as lakes and aquifers, belong to the *Proteobacteria* phylum, such as the genera *Thiobacillus, Sulfuricella*, *Sulfuritalea, Dechloromonas, Hydrogenophaga* and *Acinetobacter,* (Smith *et al*., 1994; Alfreider *et al*., 2003;

2009; Aburto *et al*., 2009; Kellermann *et al*., 2009, 2012; Kojima and Fukui, 2010, 2011; Zeng *et al*., 2016).

Although the presence of chemolithoautotrophic denitrifiers in oligotrophic, organic carbon limited subsurface environments, has been well documented, their capacity to utilize reduced sulfur compounds and hydrogen on the community level has rarely been demonstrated (Lau et al., 2016). Among inorganic electron donors for nitrate reduction that may typically occur in subsurface environments, thiosulfate is both a readily available and non-toxic sulfur compound while hydrogen-driven nitrate removal has relatively faster kinetics; the presence of both electron donors may synergistically support the effective reduction of nitrate to N_2 gas (Ergas and Reuss, 2001; Lee and Rittmann, 2002; Cardoso *et al*., 2006; Zhu and Getting, 2012). Even complete denitrifiers are equipped with all four reductases, it has been shown that any imbalances in the electron donor supply can potentially lead to incomplete denitrification and build up harmful nitrogen oxides intermediates (Townsend-Small *et al*., 2011; Chen and Strous, 2013). To date, our knowledge of denitrifier transcriptional activity is mostly based on heterotrophic or mixotrophic bacterial isolates (Baumann *et al*., 1996; Hartig and Zumft, 1999; Philippot, 2002; Bergaust *et al*., 2010; Bakken *et al*., 2012) while information is lacking for chemolithoautotrophic consortia.

The pursuit of understanding natural processes controlling nitrate in the aspect of environmental sustainability will require substantial knowledge of the potential mediated by natural microbial community. While the gene-based approaches in microbial ecology has provided evident understanding that diversity of microbial life in natural ecosystems, far exceeds that which has been revealed by cultivation-based approach, the metabolic function of most members remains speculative or completely unknown (Gagen et al., 2013). Cultivation-based approaches with growth on specific substrates, remains the final proof of metabolic activity (Gagen et al., 2013). The successful cultivation of dominant denitrifiers from oligotrophic groundwater, along with the combination of gene-based analysis will help to understand microbial life under oligotrophic conditions and further delineate their potential for denitrification.

1.6.3 The Collaborative Research Centre (CRC) 1076 AquaDiva

This research work is part of the Collaborative Research Centre 1076 AquaDiva, which is financially supported by the Deutsche Forschungsgemeinschaft (DFG) and is focusing on the important roles of water (Aqua) and biodiversity (Diva) in shaping and structuring the properties and functions of the subsurface (Küsel *et al*., 2016). Additional financial support was provided by the International Max Planck Research School for Global Biogeochemical Cycles, Jena, Germany. Even though microorganisms reside in the subsurface - about 3×10^{29} cells on a global scale, the energy flux available from buried organic carbon is less than 1 % of the carbon fixed by photosynthesis on the surface of our planet (Whitman *et al*., 1998; Kallmeyer *et al*., 2012). How these vast microbial communities can persist in conditions which provide only marginal energy for cell growth and activity, has always remained an interesting question (Hoehler and Jorgensen, 2013).

Numerous interdisciplinary studies and PhD projects with in the CRC AquaDiva and the integrated Research Training Group, respectively, are being carrying out at the Hainich Critical Zone Exploratory under the framework of CRC AquaDiva. Within AquaDiva, several studies have been conducted to characterize the microbial life under low energy flux, which appears to be more favorable to autotrophic microbial processes, though dominant metabolisms differ with groundwater well hydrochemistry (Herrmann *et al*., 2015, 2017; Starke *et al*., 2017; Schwab *et al*., 2017; Nowak *et al*., 2017). Among communities related to the nitrogen cycle (aerobic ammonia oxidizers) and total microbial communities in general, availability of oxygen, distance to surface environments and hydrological factors (heavy rain falls, snow melt) were revealed as main drivers to determine community composition and abundances (Opitz *et al*., 2014). In particular Herrmann *et al*. (2017) suggested the availability of suitable inorganic or organic electron donors as a key factor shaping both attached and suspended denitrifiers communities in these aquifers. Among the archaeal community, although Archaea represented a minor fraction of communities in groundwater, the majority of Archaea on aquifer rocks (anoxic) was dominated by lithoautotrophs and showed the capacity to fix CO_2 (Lazar *et al*., 2017). Nawaz and colleagues (2016) investigated the functional classification of eukaryotic microorganisms such as fungi in these aquifers which revealed an overwhelming majority of saprotrophs and suggested the possibility of surface to subsurface transport during the recharge episodes.

Not only limited to groundwater, research within the CRC AquaDiva has also been focusing on the understanding of the effect of different land use regimes in the Hainich CZO and their link to groundwater geochemistry, therefore, merging biodiversity with biogeochemistry. Among such studies, soil samples from the upper 10 cm in the close proximity of groundwater wells were studied for viral diversity by Narr and colleagues (2017), who revealed that soil specific factors such as pH and total nitrogen content played a significant role in shaping both soil viral abundance and community structure, in addition to temperature and rainfall. These two superimposed aquifer assemblages are largely pristine with low microbial biomass, very low concentrations of organic carbon, and limited impact of agricultural land use on groundwater nitrate concentrations (Küsel *et al.,* 2016). Kohlhepp *et al*. (2017) provided insights into the hydrogeochemical features of the thin-bedded carbonate-siliciclastic alternations of the Hainich CZE and suggested that the aquifer configuration (spatial arrangement of strata, valley incision/outcrops) and related geostructural links (enhanced recharge areas, karst phenomenon) control the groundwater quality. However, the contribution for nitrogen loss (rates) by anammox and denitrifiers in these aquifers has remained unexplored and was to be investigated in the project of this thesis.

Herrmann *et al*. (2015; 2017) suggested the predominant potential of chemolithoautotrophic denitrifiying communities oxidizing reduced iron and sulfur compounds, however, these studies were mainly based on a gene-targeted approach. With no cultured representatives, this further imparted limitation on our understanding of their physiologies and potential. Based on previously available knowledge of genomic data, I took the opportunity of culturing chemolithoautotrophic denitrifiers as well as different physiological groups of denitrifying bacteria from groundwater that, through their growth and metabolizing activities, might have been influencing the groundwater chemistry. Although subsurface ecosystems reflect the natural processes over timescales that are completely inaccessible in the laboratory, through the progress in understanding natural ecosystem coupled with laboratory research, I focused my research on exploring prokaryotic life in oligotrophic pristine aquifers, and its responsibility for the loss of reactive nitrogen to N_2. The Hainich groundwater observatory therefore provided a unique opportunity to study pristine freshwater ecosystems and to characterize the microbial life under low energy flux which might be more favorable to autotrophic microbial processes and players rather than their heterotrophic counterparts, particularly anammox and autotrophic/heterotrophic denitrifiers.

Since the Collaborative Research Centre 1076 AquaDiva is an interdisciplinary research project, with multiple groups working in close collaborations, I received data complementing my studies from several of collaboration partners. The ladderane lipids data used in this study was kindly provided by Dr. Valerie F. Schwab and colleagues (Friedrich Schiller University, Jena) (published in Kumar *et al*., 2017). The physical characteristics of the groundwater (dissolved oxygen, ORP, temperature, pH) as well as the hydrochemistry data for TOC, TIC, and major cations was kindly provided by Prof. Dr. Kai-Uwe Totsche and colleagues (Friedrich Schiller University, Jena) (published in Kumar *et al*., 2017). For the ^{15}N-isotope based assessment of anammox and denitrification rates, I received help from Prof. Dr. Bo Thamdrup and colleagues (University of Southern Denmark, Odense) (published in Kumar *et al*., 2017). For the culture based denitrification experiments, I received help for the Raman gas analysis from Dr. Torsten Frosch and colleagues (Leibniz Institute for Photonic Technology, Jena) while for ion measurements, I received analytical support from my collaborator, Ines Hilke, and colleagues (Max Planck Institute for Biogeochemistry, Jena).

1.7 Research hypotheses and experimental approach

In order to study the potential of microbial players responsible for nitrogen loss and groundwater derived chemolithoautotrophic denitrifying populations, the following five hypotheses were addressed with a focus to understand anammox and denitrifying processes at the Hainich aquifers.

1) Contrasting redox regimes and availability of dissolved inorganic nitrogen shape bacterial diversity and abundances of anammox and denitrifiers across the Hainich groundwater observation transect.

2) In anoxic groundwater with low organic carbon availability, anammox could significantly contribute to N_2 production, therefore being responsible for relatively more nitrogen loss than denitrification.

3) Aerobic and anaerobic ammonium oxidizers co-occur in suboxic groundwater and their coupled activities could be important for groundwater nitrogen cycling.

4) Oligotrophic groundwater in carbonate-rock aquifers supports high fractions of chemolithoautotrophic denitrifiers within the denitrifying microbial communities.

5) Denitrifying consortia originating from organic carbon limited groundwater could be used as a model community to understand the metabolic potential of denitrifiers within the natural groundwater bacterial communities.

In order to address these hypotheses, the work carried out in this PhD project had the following objectives:
1) Comparative assessment of anammox and denitrifying bacterial abundance and communities across the two aquifer assemblages using 16S rRNA sequencing and molecular markers.
2) Quantitative analysis of anammox and denitrification rates from suboxic groundwater using incubations with ^{15}N- labeled ammonium and nitrite.
3) Assessment of the transcriptional activity of aerobic and anaerobic ammonia oxidizers in the groundwater targeting functional genes.
4) Enrichment and isolation of chemolithoautotrophic bacteria from groundwater mediating denitrification by using inorganic electron donors such as thiosulfate and hydrogen.
5) Microcosm experiments with different ratios of thiosulfate and nitrate to analyse the metabolic response of the chemolithoautotrophic denitrifying communities and their transcriptional activity targeting *nirS* and *nosZ* genes.
I anticipate that, ultimately the combination of molecular approaches and cultivation and stable isotope based incubation methods such as those outlined here will shed light on the relative contributions to N_2 formation by denitrification and anammox, which is not well understood in freshwater environments, especially aquifers. Anammox bacteria could play a substantial role in oligotrophic aquifers with regard to their contribution to freshwater nitrogen budgets. In addition, groundwater denitrification in the Hainich CZO could potentially be efficiently driven by the oxidation of reduced sulfur compounds and hydrogen, affecting the turnover of sulfur, nitrogen, and carbon in aquifers by autotrophic denitrifiers.

This study is among the first investigations of chemolithoautotrophic anammox activity (nitrogen loss rates measurements) in pristine groundwater and denitrifying potential of chemolithoautotrophic bacterial communities derived from pristine groundwater.

In order to establish a framework for this study, two core perspectives or research work packages were investigated:

Anammox vs denitrification in carbonate-rock aquifers[1]	**Physiological experiments with denitrifying microorganisms**
Illumina MiSeq amplicon sequencing of functional genes for the analysis of groundwater denitrifying communities (*nirS*)	Establishment of chemolithoautotrophic bacterial cultures and enrichments from groundwater with the metabolic potential to oxidize inorganic electron donors coupled to denitrification
DNA and RNA based Illumina MiSeq amplicon sequencing of 16S rRNA genes for groundwater bacterial community analysis	Community analysis of the denitrifying enrichment by 16S rRNA gene-targeted amplicon sequencing (Illumina MiSeq)
Assessment of groundwater anammox and denitrification rates using ^{15}N-based approaches and IRMS	$^{15}N\text{-}NO_3^-$ based denitrification microcosm assays and analysis of metabolites by Raman Spectroscopy and ion chromatography
Functional gene-targeted clone libraries to assess anammox and denitrifying bacteria community structure (*nirS*, *nirK*, *hzsA*)	Q-PCR and RT-qPCR based quantification of denitrifier functional genes and transcripts derived from the microcosm experiments to follow growth and transcriptional activity
Q-PCR and RT-qPCR based quantification of genes and transcripts of anammox, aerobic ammonia oxidizing archaea/bacteria, and denitrifiers	Construction of functional gene transcripts targeted clone libraries and sequencing to identify transcriptionally active denitrifiers in the microcosm assays.

[1]Part of this thesis were published as an open-access article in Frontiers in Microbiology, 2017; 8:1951.
doi: 10.3389/fmicb.2017.01951
Nitrogen loss from pristine carbonate-rock aquifers of the Hainich Critical Zone Exploratory (Germany) is primarily driven by chemolithoautotrophic anammox processes.
Kumar S., Herrmann M., Thamdrup B., Schwab V.F., Geesink P., Trumbore S., Totsche K-U., Küsel K.

2. MATERIALS AND METHODS

2.1 Characterization of the study site

Groundwater samples were obtained from a groundwater-monitoring transect which offered access to two superimposed limestone aquifer assemblages, in the carbonate-rock terrain of the Hainich (Thuringia, Central Germany) The Hainich Critical Zone Exploratory (CZE) was established under the framework of the Collaborative Research Centre (CRC) AquaDiva (Küsel *et al*., 2016). The geological setting and groundwater wells construction have been described in more detail by Küsel *et al*. (2016) and Kohlhepp *et al*. (2017). The Hainich CZE comprises of surface and belowground observational sites, along a 5.4 km-long hillslope. A groundwater monitoring transect which features karst phenomena like sinkholes and consists of screened wells at five locations that span a land-use gradient from managed deciduous forest (H1), unmanaged woodland (H2), grassland/pasture (H3) to cropland agriculture (H4 and H5) of depths ranging from 5.1 m up to 88.5 m (Kohlhepp *et al*., 2017; Nowak *et al*., 2017; **Figure 4**).

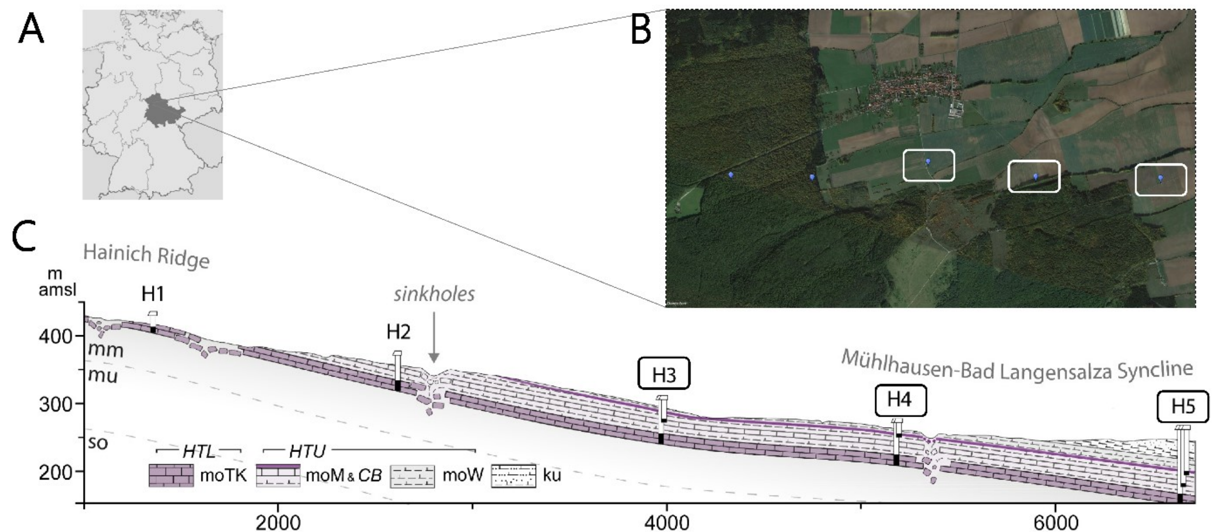


Figure 4 | Groundwater observation transect at the Hainich CZE (51.0804° N, 10.4342° E), Germany (A) Locator map of the state of Thuringia, Central Germany, (B) aerial features of the Hainich aquifer transect, (C) geological cross-section of the Hainich groundwater well transect with respective aquifer assemblages (HTU; Upper aquifer assemblage and HTL; Lower aquifer). Samples for this study were taken from locations H3, H4 and H5 (framed labels). (Upper Buntsandstein, mu, Lower Muschelkalk; mm, Middle Muschelkalk; mo, Upper Muschelkalk; moTK, Trochitenkalk formation; moM, Meissner formation; CB, Cycloides-Bank; moW; Warburg formation; ku, Lower Keuper; based on Küsel *et al*. (2016). Image credits: (A) Locator map was adapted from TUBS; assessed on 03.11.2017 (free media repository). (B) Aerial map was obtained via Google Earth Image © 2015 DigitalGlobe. (C) Geological cross section was adapted from Küsel *et al*., 2016.

Although all wells are in relatively close proximity, the Hainich groundwater observatory offers a unique opportunity to study contrasting groundwater biogeochemistry due to land use differences; wells situated at managed forested sites offer access to pristine groundwater of drinking water quality, whereas wells situated in agriculture and pasture land offer access to

groundwater characterised by little-to-moderate human activity (Küsel *et al*., 2016). Bedrocks housing the aquifers of the investigated area belong to the lithostratigraphic subgroup Upper Muschelkalk of the German Triassic (Kohlhepp *et al.,* 2017). Groundwater samples for this study were taken from locations H3, H4 and H5. The screened wells provide access to two main superimposed aquifer assemblages, which extend in alternating sequences of fractured limestones and marlstones, aggregated to the upper aquifer assemblage (HTU: wells H32, H42, H43, H52, H53) and the limestone-dominated lower aquifer assemblage (HTL: wells H31, H41, H51;), with recharge areas covered by forest, pasture, or cropland (HTU) or mostly forest (HTL) (Küsel *et al.,* 2016, Kohlhepp *et al.,* 2017).

2.2 Groundwater sampling and characteristics

Within the long-term monitoring program of the CRC AquaDiva, regular sampling of groundwater is being carried out, which allowed access to monthly groundwater samples from January 2014 to August 2015 and additionally November 2015 for this study. Before sampling groundwater, stagnant water was pumped out of the wells using submersible motor pumps (MP1, Grundfos, Denmark) until a steady state in the physico-chemical conditions was established for water temperature, dissolved oxygen concentration, pH and redox potential, which were measured simultaneously in a flow-through cell, in the field, using respective probes (Küsel *et al.,* 2016). Furthermore, the groundwater was subjected to follow-up chemical as well as micro- and molecular biological analyses.

2.2.1 Groundwater samples for hydrochemistry

For hydrochemical analysis, groundwater from eight wells was sampled in sterile glass bottles (1 L), covered to maintain dark conditions and transported at 4°C, followed by immediate analysis after reaching the research facility, to minimise any sample holding time effects. If immediate analysis was not possible, the samples were stored at 2-4 °C and analysed within 12 hours. For sulfide analysis, groundwater samples were fixed on-site with addition of 2 % (v/v) Zn-acetate and transported, followed by subsequent analysis in the lab using a standard colorimetric protocol with a modified methylene blue method (Trüper and Schlegel, 1964). Concentrations of sulfate and thiosulfate were determined from unfiltered groundwater samples by ion chromatography (IC 20 system equipped with an IonPac AS11-HC column and an IonPac AG11-HC precolumn, Dionex, Sunnyvale, CA) by Dr. Kai-Uwe Totsche and colleagues (Friedrich Schiller University, Jena) and by Ines Hilke and colleagues (Max Planck Institute for Biogeochemistry, Jena). Total inorganic carbon (TIC) and total

organic carbon (TOC) concentrations were determined by Dr. Kai-Uwe Totsche and colleagues (Friedrich Schiller University, Jena). Major cations were measured with ICP-OES (725 ES, Varian/Agilent, USA) after filtration through 0.45 µm pore size polyvinylidine fluoride (PVDF) membrane filters (Kohlhepp *et al.,* 2017). For nitrate, nitrite, ammonium, sulfate and iron (II) analysis, I received analytical help from Bernd Ruppe, Danny Schelhorn and Falko Gutmann at the Friedrich Schiller University, Jena. The groundwater samples (50 ml) for which, were filtered on-site through 0.2 µm-pore size sterile syringe filters in 50 ml sterile falcon tubes and transported for subsequent analysis in the laboratory using standard colorimetric procedures (DEV, 1975; Grasshoff *et al*., 1983).

2.2.2 Groundwater samples for microbial community analysis using molecular methods

Groundwater samples for molecular analysis were transferred to sterile glass bottles, filled up to maximum level, and transported to the laboratory at 4 °C. First sampling campaign was conducted in August 2014, involving DNA and RNA based community analysis in the groundwater of eight wells to characterize the total bacterial population and the population, with the potential for protein biosynthesis, targeting 16S rRNA gene from the same sample. Additional sampling campaigns in August and November 2015 were undertaken to confirm the observed community patterns targeting 16S rRNA gene in the groundwater of seven wells utilising DNA-based community analysis. The samples were filtered through 0.2 µm pore size polyether-sulfone filters (PES) (Supor, Pall Corporation, USA) for DNA extraction and through 0.2 µm pore size polycarbonate filters (Nuclepore, Whatman; Merck-Millipore) for RNA extraction within a few hours after sampling, with 5 to 6 litres of groundwater passing through each filter. The filters were then transferred to sterile 2 ml tubes and frozen on dry ice within 1 min of filtering, followed by storage at – 80 °C.

2.2.3 Groundwater samples for quantification of anammox-specific ladderane lipids

Sampling and subsequent collection of groundwater particulate organic matter (POM) for follow-up Ladderane phospholipid derived fatty acids (PLFA) analysis was carried out by my collaborators, Dr. Valerie F. Schwab and her colleagues (Friedrich Schiller University Jena). Concisely, *ca.* 1000 L of groundwater were filtered on site in November of 2015, using a stainless steel filter holder (diameter 293 mm; Millipore, USA) equipped with a removable pre-combusted (5 h at 500 °C) glass-fiber membrane filter (pore size 0.3 µm; Sterlitech, USA) (Schwab *et al.,* 2017).

2.2.4 Groundwater samples for quantification of anammox and denitrification rates

A ^{15}N-labelling approach (Dalsgaard *et al.,* 2012) was used to measure anammox and denitrification rates in the groundwater samples from well H53 in November 2015. During sampling, the groundwater sample was allowed to fill from bottom to top through a sterile pipette in 1 L sterile glass bottles, followed by overflow for three volume exchanges, leaving the bottles without headspace. The bottles were then sealed with rubber stoppers and transferred to the laboratory at 4 ºC in the dark, within 2 hours of collecting, for further processing.

2.2.5 Groundwater samples for microbial cultivation of planktonic denitrifiers

To target planktonic denitrifying bacteria, groundwater samples were collected in sterile glass bottles (July 2014 to July 2015). All samples were transported in dark conditions at *ca*. 8 °C and processed inside an anoxic chamber within a few hours of collection.

2.3 Rock chips sample (passive sampler) for microbial cultivation of attached denitrifiers

Passive samplers (or microtraps) containing sterilized aquifer rock chips (thimbles) up to 1 mm size and weighing around 1.4 – 2.45 kg were lowered into selected groundwater wells and exposed for a period of six to nine months, to allow for microbial colonisation (Küsel *et al*, 2016). In wells, the groundwater is expected to be in direct contact with passive material for months-long time spans and be colonised by groundwater-native microbes (Griebler *et al*., 2002; Flynn *et al*, 2008, 2013). To target the attached communities, rock chips from a microtrap, hanged at depth of 48m at site H53 (50 m depth) for approximately six months, were used as inocula for denitrifier enrichment cultures (November, 2014). Upon retrieval of the passive sampler, thimbles were immediately placed in a sterile glass bottle with anoxic atmosphere, transferred to the laboratory at *ca.* ~~8~~ 4 °C and processed inside an anoxic chamber within a few hours after sampling.

2.4 Ladderane lipids quantification

The following quantification of ladderane PLFA was carried out by my collaborator, Dr. Valerie F. Schwab and her colleagues (Friedrich Schiller University Jena). The detailed method has been explained in Kumar *et al*. (2017).

2.5 Anammox and denitrification rates measurements from groundwater

Groundwater samples were flushed with nitrogen gas, followed by immediate processing inside an anoxic chamber, where 30 ml of groundwater was dispensed into each serum bottles with 8 ml headspace volume. ^{15}N-labeled ammonium and nitrite were added as two separate treatments, the first with $^{15}NH_4^+$ (50 µM) and $^{14}NO_2^-$ (5 µM); while the second setup was spiked with only $^{15}NO_2^-$ (5 µM) without additional $^{14}NH_4^+$. The headspace of the serum bottles was purged with helium gas for 5 min followed by incubation at 15 °C in the dark. After 0, 14, 24, 36, and 48 hours, triplicate samples in serum bottles were destructively sampled by introducing 300 µl of saturated aqueous Zinc chloride solution. The headspace of each sample was analysed for the isotopic composition of the N_2 gas, by coupled gas chromatography isotope ratio mass spectrometry (Dalsgaard *et al.,* 2012). Rates of anammox and denitrification were calculated from the accumulation of the ^{15}N-labeled N_2 species $^{14}N^{15}N$ ($^{29}N_2$) and $^{15}N^{15}N$ ($^{30}N_2$) according to Thamdrup and Dalsgaard, (2002). For the experimental design, sample analysis and calculations, help was provided from my collaborator Prof. Dr. Bo Thamdrup and colleagues (University of Southern Denmark, Denmark). The dilution of the ^{15}N label by unlabelled (background) nitrite and ammonium already present was taken into account for the calculation of anammox and denitrification rates. I received additional help from Herr Jens Wurlitzer at the Friedrich Schiller University during the sample preparations under anoxic conditions. The labelled fraction determined as the ratio of the concentration of ^{15}N labelled nitrite or ammonium added to the total concentration of nitrite or ammonium, respectively (Kumar *et al*., 2017).

2.6 Cultivation approaches for planktonic and attached denitrifying bacterial community

To represent overall denitrifying communities associated with carbonate-rock aquifers, planktonic denitrifying bacteria as well as attached communities were targeted for cultivation-based approaches.

2.6.1 Groundwater based incubation and growth medium for enrichment of planktonic denitrifiers

Enrichment cultures of chemolithoautotrophic denitrifiers were set up in triplicates. Serum vials used for setting up the enrichment were acid-washed and rinsed with deionized/demineralized water (5x) and highly pure milli-Q water (18 Ω resistance) (2x).

Unless otherwise noted, preparation of the microcosms (serum bottles, butyl septa, aluminium crimps) was done under sterile conditions and all materials were allowed to degas in an anoxic chamber under an N_2 atmosphere for at least 1 day prior to use. The mineral medium utilized for the enrichment contained 0.94 mmol L^{-1} NH_4Cl, 0.08 mmol L^{-1} $MnCl_2$, 0.04 mmol L^{-1} $FeCl_3.6H_2O$, along with 10 ml of Chloride-solution (1.0 % $MgCl_2$ and 0.5 % $CaCl_2$), 10 ml of Phosphorous-solution (0.4 % KH_2PO_4) and 10 nmol L^{-1} $CuCl_2$ solution (part A) and was purged with 90 % N_2 - 10 % CO_2 using an MCQ gas blender (GB-103) for 15 min (modified medium after Kojima and Fukui, 2010). After autoclaving, part B was added consisting of filter-sterilized (0.2 µm pore size), anoxic solutions of $NaNO_3$ (0.01 mmol L^{-1}), $Na_2S_2O_3^{2-}$ (0.01 mmol L^{-1}), and $NaHCO_3$ (10 mmol L^{-1}), followed by the addition of a vitamin solution [DSMZ 461 Mineral medium (Nagel and Andreesen): http://www.dsmz.de] (5 ml L^{-1}) and a trace element solution [DSMZ 461 Mineral medium (Nagel and Andreesen): http://www.dsmz.de] (1 ml L^{-1}) **(Appendix; Supplementary Table 1)**. The resulting growth medium was collectively called "nitrate thiosulfate carbonate medium" NTC. The pH was adjusted to 7.3 with sterile solutions of 0.5 M HCl or 0.5 M NaOH. A headspace of 80 % N_2:10 % CO_2:10 % H_2 was always maintained in the serum bottles. 5 ml of groundwater was added to 60 ml of anoxic NTC medium in 120 ml butyl septum, aluminium crimped serum vials **(Figure. 5)**.

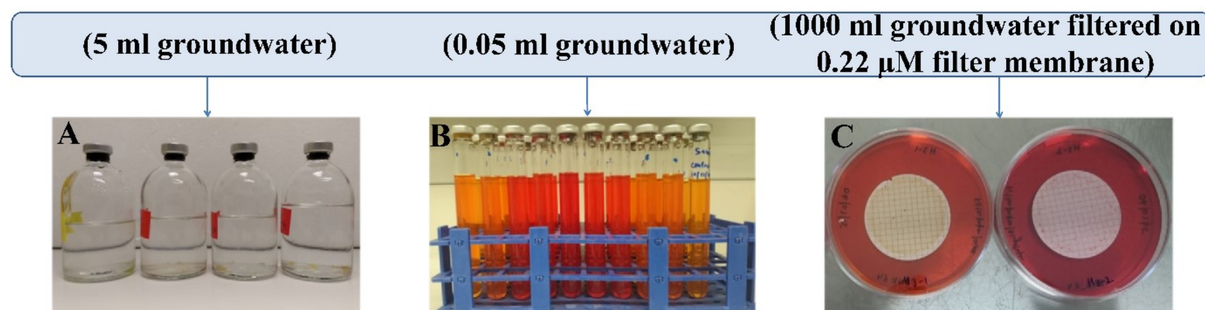


Figure 5 | Cultivation approaches for the planktonic denitrifying community. (A) Enrichment broth, (B) Gelrite shake dilution in Hungate tubes (0.5% Gelrite), and (C) Filter paper overlay Gelrite plates (1.5%).

Groundwater enrichment cultures were incubated in the dark at 15 °C for 5 months, shaking at 50 rpm. Blank controls consisted of non-inoculated medium. Five months later, 17 % (v/v) of liquid culture was inoculated to fresh NTC medium and incubated for 30 days in similar conditions, and was further sub-cultured every month in fresh medium with gradually increased concentrations of nitrate and thiosulfate, up to 5 and 2 mmol L^{-1} respectively.

Subsamples were taken at different time points to monitor nitrate concentration, followed by the addition of fresh medium, if necessary.

2.6.1.1 Growth medium for semi solid and solid Gelrite shake dilution and plates

The mineral medium included the following reagents per liter: 0.04 mmol $FeSO_4.7\ H_2O$, 0.08 mmol $MnCl_2$, 2.8 mmol NH_4Cl, 0.1 g phenol red, 10 ml of Cl-solution (1.0 % $MgCl_2$ and 0.5 % $CaCl_2$), 10 ml of P-solution (0.4 % KH_2PO_4), KNO_3 (1 to 5 mmol L^{-1}), prepared in N_2-flushed MQ water and pH set to *ca*. 7.8 followed by the addition of Gelrite (0.5 or 1.5 % for semi solid and solid medium, respectively) (modified from Taylor *et al*., 1971). After autoclaving, sterile filtered anoxic solution of $NaHCO_3$ (10 to 18 mmol), $Na_2S_2O_3^{2-}$ (1 to 4 mmol L^{-1}), vitamin solution [DSMZ 461 Mineral medium (Nagel and Andreesen): http://www.dsmz.de] (5 ml L^{-1}) and trace element solution [DSMZ 461 Mineral medium (Nagel and Andreesen): http://www.dsmz.de] (1 ml L^{-1}) were added. For the pure culture isolation, following two strategies were used (2.6.1.2 and 2.6.1.3) **(Figure 5; B and C)**.

2.6.1.2 Gelrite shake dilution method

In another set up, traditional agar-shake dilution approach was utilised, as described by Pfennig, 1978. Groundwater (50 µl) was directly inoculated into Hungate tubes containing 20 ml mineral medium with 0.5 % Gelrite under similar gas atmosphere as used for the enrichment culture and incubated under dark conditions at 15 °C. After 4 weeks of colonies development in the semisolid medium, they were picked with sterile Pasteur pipettes and inoculated into liquid NTC media (without Gelrite). All incubations were carried out at 15 °C in dark for a period of one month each and this process was repeated for 26 consecutive transfers with alternate semisolid and liquid mineral medium. Ultimately, all obtained isolates were phylogenetically analysed and fresh stocks of enrichment cultures were stored in glycerol (20% v/v) at – 80 °C.

2.6.1.3 Filter paper overlay method

Microbial cells suspended in groundwater were filtered using a sterile 47 mm Supor-R 200 membrane filter of 0.2 µm poresize (1 liter/filter paper) under mild vacuum pressure (approximately 5 lbf/in^2 (pound-force per square inch), in order to avoid damage to the bacteria) from one litre of groundwater (Myers, 2003). Filters were collected using sterilized forceps and placed carefully (avoiding air bubbles under the membrane) on the top of the Gelrite (1.5 %) medium surface in a petri dish. The plates were incubated inside an anaerobic

jar with a gas atmosphere of N_2, CO_2 and H_2 (80:10:10 % [v/v]) at 15 °C under dark conditions for a period of 4 weeks. Afterwards, 110 distinct colonies were randomly selected and streaked further in anoxic mineral Gelrite medium at least fifteen times to obtain pure cultures over a period of two years. The initial screening was done by colony PCR, targeting the bacterial 16S rRNA genes. Pure strains, which had been taxonomically characterized based on 16S RNA gene information, were stored in glycerol (20 % v/v) in triplicates at – 80 °C.

2.6.2 Rock chips (passive sampler) based incubations for cultivation of attached denitrifiers

Under the anoxic chamber, 10 to 15 grams of rock chips from the microtrap thimble were transferred by sterilised spatula to sterile, anoxic NTC mineral media (60 ml) prepared in glass bottles (150 ml), sealed with butyl rubber and aluminium cap, followed by the establishment of a gas atmosphere of N_2, CO_2 and H_2 (80:10:10 % [v/v]) in the headspace **(Figure 6).** The incubations were kept at 15°C in the dark for three months. The glass bottles were fed with anoxic, filter sterilized (0.2 µm pore size) sodium thiosulfate (0.01 mM) and nitrate solution (0.01 mM) at the interval of one month during the incubation. After three months, 1 ml of the grown culture was transferred to a serum bottle containing 60 ml anoxic TNC medium, and was incubated for one month, followed by repeated subculturing. These passive samplers were deployed by Sebastian Opitz, Patricia Geesink (Friedrich Schiller University Jena) and Dr. Denise M. Akob (USGS, USA). I received additional help from Jens Wurlitzer (Friedrich Schiller University) during the retrieval and transport of the passive samplers under anoxic conditions.

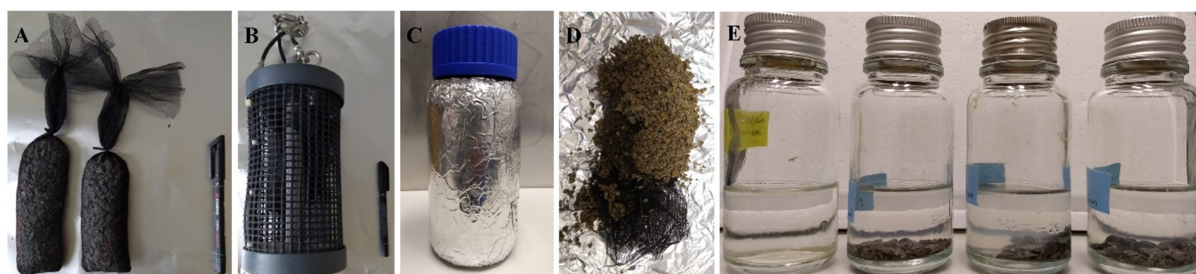


Figure 6 | Cultivation approach for the attached denitrifying community.
Passive sampler and rock material exposed to suboxic groundwater for in situ colonization and subsequent cultivation of attached denitrifying bacteria (Küsel *et al*., 2016). (A) Thimble containing sterile rock chips, (B) thimbles encased in microtrap (passive sampler), (C) retrieval of passive sampler in dark and anoxic bottle from the well after nine months, (D) rock chips opened inside the anoxic chamber (E) glass bottles with TNC growth medium incubated with rock chips.

2.7 Denitrification activity of an enriched chemolithoautotrophic consortium

Using the enriched chemolithoautotrophic consortium obtained from groundwater (1E), two microcosm experiments were set up at two different nitrate/thiosulfate ratios for time-resolved analysis of denitrification potential and substrate turnover, along with analysis of bacterial community composition and functional gene transcript analysis, which were performed as described below **(Figure 7)**.

2.7.1 Microcosm experiments set up M_I and M_II

In order to trace turnover reactions of electron donors and electron acceptor during the denitrification process, i.e., reduction of nitrate to nitrite and N_2 as well as the oxidation of thiosulfate and H_2, two microcosm experiments (M_I and M_II) were set up with enrichment 1E **(Figure 8 B)**.

Prior to setup of the first microcosm experiment (M_I), a pre-culture of the enrichment culture (36th subculture) was setup in triplicate 120 ml serum vials with butyl rubber stoppers and aluminium crimps. The medium composition was the same as used for the ongoing main enrichment culture 1E, except that part A of the medium was made under argon gas atmosphere to make the medium anoxic and lower the N_2 background. For part B of the medium, filter-sterilised $Na^{15}NO_3$ (99.2 atom% of $^{15}NO_3^-$) was used, corresponding to a final concentration of 5 mmol L^{-1} $^{15}NO_3^-$, and supplemented with $S_2O_3^{2-}$ at 2 mmol L^{-1} and bicarbonate at 10 mmol L^{-1}. 40 ml of $^{15}NO_3^-$-spiked NTC medium was filled in each serum vial and headspace gases were replaced with filter-sterilised (0.22 µm) argon gas and supplemented with a gas mixture of 4 % H_2 and 5 % CO_2 (In contrast to N_2, CO_2 and H_2 (80:10:10 % [v/v]) used in previous culturing conditions). Cells were pre-incubated with $^{15}NO_3^-$ for 10 days, centrifuged at 4000 x g for 6 min at 15 °C in 50 ml sterile falcon tubes, and washed two times with anoxic carbonate mineral medium (original medium without nitrate and thiosulfate) to minimize the nutrient carryover. The cell pellets obtained from this pre-incubated culture were then used to inoculate the denitrification microcosm experiments in anoxic ^{15}N-NTC medium.

Due to the flocculated growth behaviour of the enrichment, destructive sampling was carried out. Harvesting three biological replicates at each sampling time during the incubation along with one control provided a clear advantage for robust investigation. Microcosms were maintained as batch assays for an incubation period of 14 and 17 days during microcosm

experiment M_I and M_II, respectively with replicates randomly distributed in incubator maintained at a temperature of 15 ºC.

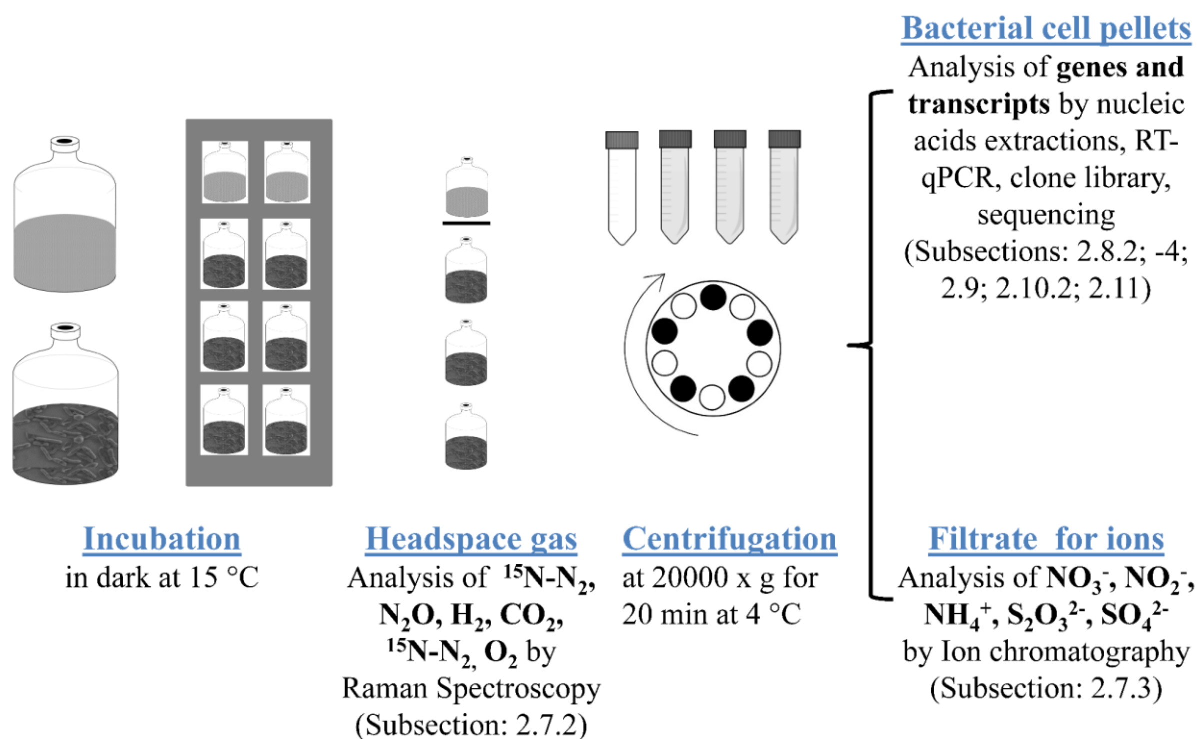


Figure 7 | Microcosms set up with enrichment culture, 1E and subsequent chemical and molecular analysis for two microcosms experiments, M_I and M_II

2.7.2 Analysis of denitrification products by Raman spectroscopy from microcosm set up M_I and M_II

To quantify the denitrification metabolic activity (gaseous components) exhibited by each enrichment culture, I received help to analyse the gas samples through Raman Spectroscopy from Annika Düver (Leibniz Institute of Photonic Technology, Jena). One control and three inoculated microcosms were destructively sampled every day from day 0 to day 11 and additionally at day 14. The headspace from the anoxic microcosms was analysed for $^{15}N_2$, $^{15}N_2O$, CO_2, H_2, ^{14}N-N_2 and O_2 using a specially designed Raman gas sensor based on cavity-enhanced Raman spectroscopy (CERS). This allows simultaneous measurements of these gases down to concentrations of approximately 100 ppm (parts per million) with sub-second time resolution (Frosch *et al.*, 2013; Jochum *et al.*, 2015a, 2015b, 2017). Calibration spectra were generated by flushing the device with pure CO_2, $^{15}N_2$, $^{15}N_2O$, N_2 and O_2 gases at fixed pressure and temperature. For hydrogen, a gas mixture of 5 % H_2 in N_2 was used. The peaks belonging to H_2 were scaled up to 100 % and the nitrogen peak was removed to generate the calibration spectrum. The concentrations of the gases were calculated via a least squares fit using the whole set of calibrated reference spectra and the measured spectrum from 500 to

3000 cm^{-1}, and by monitoring temperature, pressure and laser intensity with the help of additional sensors (Keiner *et al*., 2014). All analysed gases have a distinct spectral signature and could be quantified without cross-sensitivity.

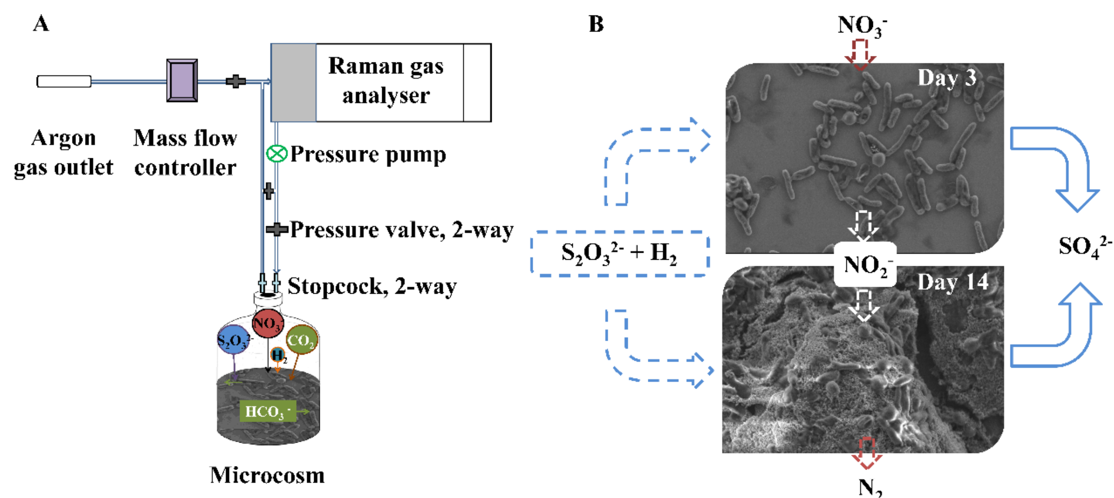


Figure 8 | Raman gas analysis and model of enrichment metabolism. (A) Schematic diagram of Raman gas analysis set up for headspace gas quantification of $^{15}N_2$, $^{15}N_2O$, H_2, CO_2 along with $^{14}N_2$. On the right, (B) model of chemolithotrophic denitrifying consortium mediating denitrification couple with the oxidation of thiosulfate and hydrogen. The SEM images were from enrichment culture at Day-3 and Day-14 during microcosm M_I.

For the measurement of the headspace, the measurement system was flushed with argon (Raman inactive) for several minutes until no gas was detectable anymore. At each sampling time prior to the destructive sampling, the serum bottle was connected and a closed loop was established using a pump integrated in the setup to circulate the gas **(Figure 8 A)**. That way, the headspace from the bottle was cycled through the spectrometer and back into the bottle, mixing it with the argon already present in the measurement setup. The original concentration of gases in the bottle headspace could be calculated, as the volume of the measurement cycle is exactly known and the gas composition is not altered during the measurement. To avoid diffusion of ambient air into the system, the measurement time was limited to five minutes, which was long enough to provide reliable results. Complete mixing of gases was already realized after less than a minute. Due to technical issues with the Raman gas analysis, there was no quantification data of nitrogenous gases (N_2O and N_2) and hydrogen from microcosm set up M_I.

2.7.3 Analysis of reactants, intermediates and products of chemolithoautotrophic denitrification by ion chromatography from microcosm set up M_I and M_II

To quantify the denitrification metabolic activity (utilization and production of ionic substrates) exhibited by enrichment culture, I received help to analyse the ion samples by ion

chromatography from Ines Hilke (Max Planck Institute for Biogeochemistry, Jena). After the Raman measurement of the headspace, the entire content of each vial was transferred to a 50 ml sterile falcon tube, followed by centrifugation at 20,000 x g for 20 min at 4 °C. The liquid supernatant was decanted into sterile 50 ml falcon tubes while the cell pellets were immediately transferred to dry ice and stored at – 80 °C until nucleic acid extractions were to be performed. The decanted supernatant was filtered through a 0.22 µm filter and processed immediately for tracking the concentrations of dissolved inorganic nitrogen (NO_3^-, NO_2^-, NH_4^+) and sulfur ($S_2O_3^{2-}$, SO_4^{2-}). Nitrate, nitrite, and sulfate measurements were performed on an ion chromatography system DX-500 (Thermo Fisher Scientific GmbH, Dreieich, Germany), while thiosulfate and ammonium were quantified on an ion chromatography system ICS-5000 (Thermo Fisher Scientific GmbH, Dreieich, Germany). The second microcosm experiment (M_II) was carried out using the 39th subculture of the enrichment culture, consisting of 40 test samples (30 inoculated samples and 10 non-inoculated controls), which were maintained under the same conditions as described for the first microcosm experiment. One control and three inoculated cultures were destructively sampled after 0, 7, 10, 12, 14, 17 and 19 days. To improve the Raman-based detection of gaseous nitrogen compounds, the concentration of thiosulfate as electron donor for denitrification was increased to 5 mmol L^{-1} and the volume of supplemented growth medium in each serum vial was increased to 60 ml. Additionally, enrichment cells were observed by scanning electron microscopy (SEM) at two different time points during microcosm experiment M_II at day 7 and day 17 of the incubation. Briefly, 1 ml of the liquid culture was centrifuged at 3000 x g for 5 min to collect the cell pellet which was then fixed with 2.5 % glutaraldehyde in 1 x PBS at pH 7.4, followed by three times washing step with 1 x PBS (Carmichael et al., 2013; Bohu et al., 2016). I received help for preparing the samples for SEM from Jens Wurlitzer (Friedrich Schiller University Jena) and SEM imaging was performed by Sylvio Hunger at the Friedrich Schiller University Jena on ULTRA PLUS scanning electron microscope (Zeiss).

2.8 Nucleic acid extractions, PCR amplifications and cloning

To analyse the genetic potential for processes involved in nitrogen loss and total bacterial community, in natural as well as bacterial cultures samples, different nucleic acid extraction techniques were utilized depending upon the biomass origin such as environmental (groundwater), enrichment consortia, pure bacterial cultures, which are presented below.

2.8.1 Nucleic acids extractions from groundwater samples

Genomic DNA and total RNA from groundwater samples were extracted using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., USA) and the PowerWater RNA Isolation Kit (MO BIO Laboratories Inc., USA), respectively, according to the manufacturer's protocol. Processing of RNA was performed as described previously (Schwab *et al.,* 2017). DNA and RNA extractions were performed by Falko Gutmann, Danny Schelhorn and Patricia Geesink Friedrich Schiller University.

2.8.2 Nucleic acids extractions from enrichment consortia

Nucleic acids extractions for the identification of four initial enrichment cultures (1E, 2E, 3E, 4E) of denitrifiers obtained from different culture strategies and later on from the samples obtained (1E enrichment) during the microcosm experiment M_I and M_II, were carried out to identify any changes in community shift in one of the enrichment culture subjected to different ratio of nitrate and thiosulfate.

Anoxic suspensions (60 ml) from four enrichment cultures (1E, 2E, 3E, 4E) were subjected to DNA isolation after 28 months for later 16S RNA gene-targeted clone libraries. 1E originated from a groundwater derived enrichment culture by the dilution method. 2E enrichment originated from the incubations from passive sampler material. Enrichment cultures 3E and 4E originated from the approach using the agar shake dilution method. Freshly grown (one week) cells of the enriched consortia were pelleted by centrifugation at 20000 x g for 20 mins at 4 °C (Eppendorf centrifuge 5810R). The pelleted cells were immediately processed for DNA extraction using the Sigma bacterial DNA extraction kit (GenElute^{TM} Bacterial Genomic DNA kit; NA2120-1KT) according to the manufacturer instructions.

As illustrated in **Figure 7**, cell pellets collected from both microcosm experiments **(M_I, M_II)** were subjected to simultaneous DNA and RNA extraction using the All prep bacterial DNA/RNA/Protein kit (Mo Bio Laboratories, CA, USA) according to the manufacturer's instructions. Bead-beating was performed using a Bio 101 FastPrep FP120 instrument (Thermo Electron Corporation, Milford, MA, USA) for three times 60 s at the speed setting 4.5. RNA extracts were treated with Ambion TURBO DNA-free (ThermoFisher Scientific, Waltham, MA, USA), followed by reverse-transcription PCR using ArrayScript reverse transcriptase following the manufacturer's instructions along with a control which was set up with RT-PCR grade water instead of reverse transcriptase to test for carry-over of non-digested DNA.

2.8.3 DNA extractions from pure denitrifying cultures

One hundred and one isolates obtained using the "filter paper overlay" method were subjected to colony PCR. Instead of DNA extraction from bacterial colonies, a modified protocol was used to perform colony PCR (Hiraishi, 1992). After 15 sub-transfers, freshly grown isolated colonies (after five days) were picked using a sterile toothpick, and suspended in 30 µl of nuclease-free water. To this suspension, 10 µl of a proteinase K solution (1 mg/ml) and 50 µl of 40 mM Tris buffer of pH (8.0) containing 1% Tween 20, 0.2 mM EDTA were added and vortexed for 2 minutes. The resulting suspension was incubated at 60 °C for 10 minutes followed by 95 °C for 10 minutes and stored at -20 ºC.

2.8.4 Polymerase chain reaction assays and clone libraries

The PCR for subsequent clone library construction to generate standards for quantitative PCR was carried out using the HotstarTaq Mastermix (Qiagen, Germany) with previously published primer combinations and cycling conditions (Table 2).

PCR reactions were performed in triplicates using a peqSTAR, VWR thermal cycler in a total volume of 15 µl containing Hotstar Taq Master mix (Qiagen) 7.5 µl, BSA 0.5 µl, forward and reverse primer of 1 µl each and 4 µl of PCR grade water and 1 µl template or water. The DNA obtained from enrichment cells (1E, 2E, 3E, and 4E), and from both microcosm (M_I, M_II) were subjected to examine microbial community composition based on 16S rRNA gene based clone library. Genomic DNA was subjected to PCR using the universal bacterial primers 8F and 907R (Lane *et al*., 1985 and Felske *et al*., 1997).

Table 2 | PCR Primers used in this study for gene detection and quantification. Optimised annealing temperature (AT) for PCR reaction is given in parenthesis, wherever it differed from already published information.

Target organism and gene	Primer	Sequence (5'→3')	Reference
Domain Bacteria, Bacterial 16S rRNA gene	**27F**	AGAGTTTGATCMTGGCTCAG	Lane *et al*., 1991
Domain Bacteria, Bacterial 16S rRNA gene	**8F**	AGAGTTTGATCCTGGCTCAG	Turner *et al*., 1999
Domain Bacteria, Bacterial 16S rRNA gene	**1492R**	TACGGYTACCTTGTTACGACTT	Lane *et al*., 1991
Domain Bacteria, Bacterial 16S rRNA gene	**907R**	CCGTCAATTCMTTTRAGTTT	Amann *et al*., 1992
Domain Bacteria, Bacterial 16S rRNA gene (qPCR)	**Bac8Fmod**	AGAGTTTGATYMTGGCTCAG	Nercessian et al., 2005
Domain Bacteria, Bacterial 16S rRNA gene (qPCR)	**Bac338Rabc**	GCWGCCWCCCGTAGGWGT	Daims et al., 1999

Denitrifiers, *nirS* gene (qPCR, AT: 57 °C)	**nirS cd3af**	GTSAACGTSAAGGARACSGG	Michotey *et al*., 2000
Denitrifiers, *nirS* gene (qPCR, AT: 57 °C)	**nirS R3cd**	GASTTCGGRTGSGTCTTGA	Throbäck *et al*, 2004
Denitrifiers, *nirS* gene (AT: 43 °C)	**nirS1F**	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T	Braker *et al*, 1998
Denitrifiers, *nirS* gene (AT: 43 °C)	**nirS3R-m**	CCGCCRTCRTGVAGRAA	Braker *et al*, 1998
Denitrifiers, *nirK* gene	**nirK F1aCu**	ATCATGGTSCTGCCGCG	Hallin and Lindgren. 1999
Denitrifiers, *nirK* gene	**nirK R3Cu**	GCCTCGATCAGRTTGTGGTT	Hallin and Lindgren. 1999
Denitrifiers, *nosZ* gene	**nosZ-F-1181**	CGC TGT TCI TCG ACA GYC AG	Rich *et al*., 2003
Denitrifiers, *nosZ* gene	**nosZ-R-1880**	ATG TGC AKI GCR TGG CAG AA	Rich *et al*., 2003
Anammox, *hzsA* gene (Qpcr, AT:49 °C)	**hzsA-1597F**	WTYGGKTATCARTATGTAG	Harhangi *et al*., 2012
Anammox, *hzsA* gene (qPCR)	**hzsA-526F**	TAYTTTGAAGGDGACTGG	Harhangi *et al*., 2012
Anammox, *hzsA* gene (qPCR)	**hzsA-1857R**	AAABGGYGAATCATARTGGC	Harhangi *et al*., 2012
Planctomycetes specific 16S rRNA gene (PCR)	**Pla46F**	GACTTGCATGCCTAATCC	Neef *et al*., 1998
Anammox specific 16S rRNA gene	**AMX-820-R**	AAAACCCCTCTACTTAGTGCCC	Schmid *et al*., 2000
Anammox specific 16S rRNA gene (qPCR standard)	**AMX-368-F**	TTCGCAATGCCCGAAAGG	Schmid *et al*., 2003
Anammox specific 16S rRNA gene (qPCR standard)	**AMX-1390-R**	GACGGGCGGTGWGTRCA	Schmid *et al*., 2000
Anammox specific 16S rRNA gene (qPCR)	**AMX-808-F**	CAGCCATGCAAACACCTGTRATA	Hamersley *et al*., 2007
Anammox specific 16S rRNA gene (qPCR)	**AMX-1040-R**	TCGCACAAGCGGTGGAGCATGTGGCTT	Hamersley *et al*., 2007
Anammox specific 16S rRNA gene (qPCR)	**Probe-AMX-931**	TCGCACAAGCGGTGGAGCATGTGGCTT A	Hamersley *et al*., 2007
Archaeal ammonium oxidizer, *amoA* gene (qPCR)	**Arch-AmoAF**	STAATGGTCTGGCTTAGACG	Francis *et al*., 2005
Archaeal ammonium oxidizer, *amoA* gene (qPCR)	**Arch-AmoAR**	GCGGCCATCCATCTGTATGT	Francis *et al*., 2005
Bacterial ammonium oxidizer, *amoA* gene (qPCR)	**AmoA-1F**	GGGGTTTCTACTGGTGGT	Rotthauwe *et al*., 1997
Bacterial ammonium	**AmoA-2R**	CCCCTCKGSAAAGCCTT	Rotthauwe *et*

oxidizer, *amoA* gene (qPCR)		CTTC	*al*., 1997

Furthermore, *nirS*- and *nosZ*- transcript based clone libraries from the microcosm experiment M_I were prepared using cDNA generated from samples collected during incubation experiment M_I. PCRs were carried out with previously published primer combinations, cd3aF/R3cd, and nosZ-F-1181/nosZ-R-1880 respectively, for *nirS,* and *nosZ* genes encoding cytochrome c-dependent nitrate reductase and nitrous oxide reductase, using cycling conditions as described in Rich *et al*. (2003); Throbäck *et al*. (2004); Kandeler *et al*. (2006). The detailed information of primers used in this study is given at **Table 2**. An additional *nirS* gene transcript-targeted clone library was constructed from microcosm experiment M_I using forward primer nirS1F (Braker *et al*., 1998) with a modified version of primer nirS3R (CCGCCRTCRTGVAGRAA) to also target *nirS*-type denitrifiers that had too many mismatches with the cd3aF/R3cd primer set and the nirS1F/nirS3R primer set. Regarding the DNA of pure isolates, an aliquot (1 µl) of protease treated colony suspension was then added to each 16S rRNA targeted PCR reaction (25 µl). All the PCR reactions were performed in triplicates using Hotstar Taq Mastermix (Qiagen, Hilden, Germany). Also relative fractions of OTUs of anammox bacteria based on deduced hzsA protein sequences was analysed from the groundwater of the two aquifer assemblages. A functional gene based *hzsA* clone library (169) from sampling campaign of August 2014 were analysed with the number of sequences per groundwater well ranging from 19 to 23.

The correct size of the amplification product was verified using electrophoresis on a 1.5 % agarose gel, visualized through ethidium bromide staining and purified using the NucleoSpin extract II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Purified PCR products were ligated into pGEM T-Easy cloning vector and transformation was performed using chemically competent *Escherichia coli* (JM109) in accordance with the manufacturer's protocols (Promega). Positive clones were screened using M13 primers. PCR products from positive clones were purified and sequenced at Macrogen (South Korea/The Netherlands) using M13 or T7 primers. Plasmids for generating qPCR standards were extracted using GeneJET plasmid miniprep kit (Thermo Fisher Scientific, Germany) and sequencing of cloned inserts was performed at Macrogen (The Netherlands). The trimmed sequences were analysed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database project (https://rdp.cme.msu.edu/) to find their closest relatives.

2.9 Quantitative-PCR and Reverse Transcription-qPCR based analysis of microbial abundances and potential gene transcripts from groundwater samples and enrichment culture

To quantify the abundance and transcriptional activity of taxonomic and functional gene markers within the natural environment as well as the microbial consortium, quantitative PCR (q-PCR) and reverse transcription-qPCR approach was utilised. This method combines traditional end-point detection PCR with fluorescent detection technologies to record the accumulation of amplicons in "real time" during each cycle of the PCR amplification (Smith and Osborn, 2009). These amplicons were detected during the early exponential phase of the PCR, which enables the quantification of the gene or transcript numbers proportional to the starting template concentration. With the careful consideration of assay design, standard preparation, template preparation, accurate gene quantification can be achieved whereas the choice of q-PCR primers can allow a wide spectrum of specificity.

DNA extracted from groundwater and enrichment cultures, was subjected to quantification of bacterial 16S rRNA genes, *nirS,* and additionally for *hzsA*, *nirK, amoA* genes from only groundwater by quantitative PCR (q-PCR) on a Mx3000P qPCR cycler (Agilent Technologies) using Maxima SYBR Green Mastermix (Thermo Fisher Scientific). For anammox specific 16S rRNA quantification, Maxima qPCR Probe Mastermix (2x) was used (Thermo Fisher Scientific). Q-PCR targeting bacterial 16S rRNA genes, *hzsA*, *nirK*, and *nirS* genes was performed for monthly samples from January 2014 to August 2015, while q-PCR targeting *amoA* genes and transcripts of *hzsA*, *nirK*, *nirS,* and *amoA* was only performed for samples obtained in August and November 2015.

Quantification of transcripts was only carried out for selected time points and (*nirS*, *nosZ*) genes from the enrichment experiment M_I and M_II, where *nirS* and *nosZ* transcripts were quantified using the same primer combinations as used for the cloning approach. The thermal cycling conditions for *nirS*, were followed as described in Throbäck *et al*. (2004). In detail, the following primer combinations were used: Bac8Fmod/Bac338Rabc (Loy *et al.,* 2002; Nercessian et al., 2005) for bacterial 16S rRNA genes following Herrmann *et al.* (2012), 1597F/1857R5 for *hzsA* genes (Harhangi *et al.,* 2012), Amx_808F8/1040_R along with probe Amx 931 for anammox 16S rRNA genes (Hamersley *et al*., 2007). Primers for archaeal and bacterial *amoA* genes, Arch-AmoAF/Arch-AmoAR (Francis *et al.,* 2005) and AmoA1F/AmoA-2R (Rotthauwe *et al.,* 1997) were used as described in Opitz *et al.* (2014). For denitrifiers specific functional genes, primers F1aCu/R3Cu, cd3aF/R3cd, nosZ-F-

1181/nosZ-R-1880, for *nirK*, *nirS* genes and *nosZ* were utilized respectively (Michotey *et al.,* 2000; Throbäck *et al.,* 2004, Hallin and Lindgren, 1999) with cycling conditions as given in Throbäck *et al.* (2004), and Rich *et al*. (2003). Non-linearized plasmids containing inserts of the respective target genes were serial diluted for standard curves and were linear from 5 x 10^1 to 5 x 10^8 copies per reaction with $R^2 > 0.99$, for the functional genes and from 5 x 10^2 to 5 x 10^8 copies per reaction for bacterial 16S rRNA genes. For the database archival, raw data from gene quantification obtained in this study have been submitted to the AquaDiva Data Portal repository (BExIS).

2.10 Sample preparations for Illumina MiSeq amplicon sequencing

Illumina MiSeq has provided a reliable platform for amplicon sequencing in microbial ecology studies due to its high-throughput, longer sequence reads and higher accuracy (Caporaso et al., 2012; Nelson et al., 2014; Wu et al., 2015). I used this sequencing platform for robust and reliable analysis of microbial community analysis in both natural and enrichment culture. Sample preparation for sequencing was done by my laboratory supervisor Dr. Martina Herrmann at the Friedrich Schiller University Jena.

2.10.1 Illumina MiSeq amplicon sequencing of groundwater derived DNA and RNA

Total bacterial and anammox community structure was analysed by MiSeq Illumina sequencing of 16S rRNA genes based on DNA (sampling campaigns August 2014, August 2015 and November 2015) and RNA (sampling campaign August 2014).

The structure of denitrifier communities based on Illumina MiSeq amplicon sequencing of *nirS* and *nirK* genes was analysed from groundwater samples obtained during six sampling campaigns in July, August, 2014 and January, March, June, August, 2015. Because of insufficient amplification of *nirK* genes for MiSeq Illumina sequencing at very low *nirK* gene abundances in the groundwater, a detailed analysis of denitrifier community composition was focused on *nirS*-type denitrifiers only.

Generation of barcoded amplicons and amplicon sequencing using the Illumina MiSeq platform was performed by LGC Genomics (Berlin). Overall, bacterial 16S rRNA genes were targeted using the primer combination Bakt_341F/Bakt_805R (Herlemann *et al.,* 2011), covering the V3-V5 region of the bacterial 16S rRNA gene. Amplicons of *nirS* and *nirK* genes were generated from DNA only (August 2014) using the same primer combinations as for cloning and qPCR. In addition to that, community composition of *nirS*-type denitrifiers in the natural groundwater samples was also assessed by *nirS*-targeted MiSeq Illumina amplicon

sequencing for wells H41, H43, H51, and H53 for six different time points. The PCR reactions included about 5 ng of DNA extract, 15 pmol of the respective forward and reverse primer in 20 µL volume of 1 x MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 µl of BioStabII PCR Enhancer (Sigma). The forward and reverse primers for each sample, had the same 10-nt barcode sequence. PCRs programs were carried out for 30 cycles using the following conditions: 2 min 96 °C predenaturation; followed by 96 °C for 15 s, 50 °C for 30 s, 70 °C for 90 s. Cycling conditions for *nirS* were similar except for annealing temperature at 56 °C. 20 ng amplicon DNA of each sample were pooled for up to 48 samples carrying different barcodes. Furthermore, to remove primer dimers and other small mispriming products, the amplicon pools were purified with one volume AMPure XP beads (Agencourt), followed by an additional purification on MinElute columns (Qiagen). About 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Samples of Illumina libraries were pooled, size selected by preparative Gelelectrophoresis and the sequencing was done on an Illumina MiSeq using V3 Chemistry (Illumina).

2.10.2 Illumina sequencing based monitoring of the microbial community during the microcosm experiments

To assess changes in the bacterial community structure of the enriched consortium during the two microcosm experiments, I used 16S rRNA gene-targeted Illumina MiSeq amplicon sequencing for four time points (M_I: Day 0 and 14; M_II: Day 0 and 17). 16S rRNA gene PCR products were generated using the primer combination Bakt_341F/Bakt_805R (Herlemann *et al.,* 2011), covering the V3-V5 region of the bacterial 16S rRNA gene and HotStar Taq Mastermix (Qiagen, Hilden, Germany). PCR products were purified using NucleoSpin Gel & PCR Clean-Up Kit (Macherey-Nagel, Germany). Amplicon libraries were prepared using NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs) and purified using AMPure XP Beads (Beckman Coulter) according to the manufacturer's instructions. Sequencing was performed on an Illumina MiSeq platform using v3 chemistry (Illumina) by Dr. Martina Herrmann and colleagues at the Friedrich Schiller University, Jena.

2.11 Sequence data analysis

I used Mothur (Schloss *et al*, 2009, v.1.39.1) sequence analysis pipeline for the analysis of microbial community in both natural samples and enrichment cultures. I received help for

working with Mothur and other software used in the pipeline from Dr. Martina Herrmann at the Friedrich Schiller University Jena.

For the bacterial 16S rRNA amplicons sequence analysis, screening for quality and chimera, alignment and operational taxonomic unit (OTU) assignment were performed using Mothur, following the Mothur MiSeq SOP (http://www.mothur.org/wiki/Schloss_SOP; Schloss *et al.*, 2011) utilising the SILVA bacteria reference alignment (Quast *et al.,* 2013). Briefly, for the taxonomic classification classify.seqs command was used in Mothur with a reference database based on SILVA release v128. Using a 0.03 distance cut-off, species-level operational taxonomic units (OTUs) were assigned. The library size of each sample was normalized to the same number of reads (16,346 reads) using the sub.sample command implemented in Mothur for comparisons of community structure across samples. Phylogenetic analysis of cloned *hzsA* fragments was done utilising, reference alignments of deduced hzsA protein sequences, generated in ARB. Sequences were translated to deduced protein sequences and aligned against the reference alignment. Based on a 0.03 distance cut-off on protein level, species-level OTUs were assigned.

Closest relatives were determined based on a BLAST search using blastx against the non-redundant protein sequence (nr) database at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and using phylogenetic tree construction in ARB. Due to very low *nirK* gene abundances in the groundwater followed by insufficient amplification of *nirK* genes for MiSeq Illumina sequencing, a detailed analysis of denitrifier community composition was focused only on *nirS*-type denitrifiers. *nirS* sequences were analysed using Mothur integrating BioEdit (Hall *et al.,* 1999), and the ARB package (Ludwig *et al.,* 2004) for the analysis of protein-encoding genes. Briefly, after removing low quality sequence reads following the standard settings of the Mothur MiSeq SOP, nucleic acid sequences were translated to deduced amino acid sequences using BioEdit, and sequences containing stop codons were removed. Reference alignments on nucleic acid level were generated by realignment of sequences according to the protein-based alignment in ARB (Herrmann *et al.,* 2017), and badly aligned sequences were excluded. Utilising a 0.18 distance cut-off on nucleic acid level, OTU assignment was done (Palmer *et al.,* 2012). Using nucleotide Basic Local Alignment Search Tool (blastn) of one representative sequence per OTU, closest relatives were determined against the Nucleotide collection (nr/nt) database at the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi). In order to evaluate the evolutionary relationships between pure groundwater isolates and other known strains of the genus they belong, the 16S rRNA gene sequences of all isolates were aligned using ClustalW, and a phylogenetic tree was

conducted using the neighbour-joining method implemented in MEGA 6.0 (Tamura *et al*., 2013). For the database archival, sequences obtained in this study have been submitted to Genbank for clone library-based sequencing (sample accession numbers KY887284-KY887452 for *hzsA* genes, KY887453-KY887461 for *nirS* genes, and KY887462-KY887471 for *nirK* genes) and to the European Nucleotide Archive (ENA) for results of MiSeq Illumina amplicon sequencing (study accession number: PRJEB20223, sample accession numbers ERS1645471 to ERS1645508).

2.12 Statistical analysis

Community diversity indices (Chao's richness estimator, the Shannon index and Simpson) were calculated by Mothur. Correlation analysis (Spearman rank correlation coefficient) between physicochemical parameters and bacterial gene abundances in groundwater samples across eight wells and two aquifers assemblages using the software IBM SPSS Statistics v. 21 were calculated to describe the relationship between anammox bacteria, denitrifying bacteria and environmental factors. For the hydrochemistry across the two-aquifer assemblages, box and whiskers plot were prepared in R environment (R Core Team, 2017) using the "ggplot2" package (Wickham, 2009). I received help for working with R environment and IBM SPSS from Constantinos Xenophontos, Dr. Carl-Eric Wegner and Dr. Martina Herrmann (Friedrich Schiller University, Jena).

3. RESULTS

3.1 Groundwater chemistry of eight wells along the Hainich groundwater observation transect

The two aquifer assemblages differ strongly in availability of dissolved oxygen, from suboxic (< 10 µmol oxygen L^{-1} at H52 and H53) to anoxic conditions (H42 and H43) in the upper aquifer assemblage (HTU) (except for H32 (62 µmol L^{-1}) and oxic conditions in the lower aquifer assemblage (HTL) (91–183 µmol L^{-1}) **(Figure 9A)**. Similar differences were encountered in their oxidation-reduction potential, or ORP. A positive ORP of 401–416 mv was observed for HTL and H32 of the HTU, which was about two-fold more oxidising than the groundwater of H42, H43, H52 and H53 (158–263 mv) **(Figure 9B)**. Confirming previous observations (Küsel *et al.,* 2016, Kohlhepp *et al.,* 2017), the groundwater was characterized by stable temperature of 10±0.53 (°C) and a slightly alkaline pH 7.2±0.1 across all wells **(Figure 9C and 9D)**.

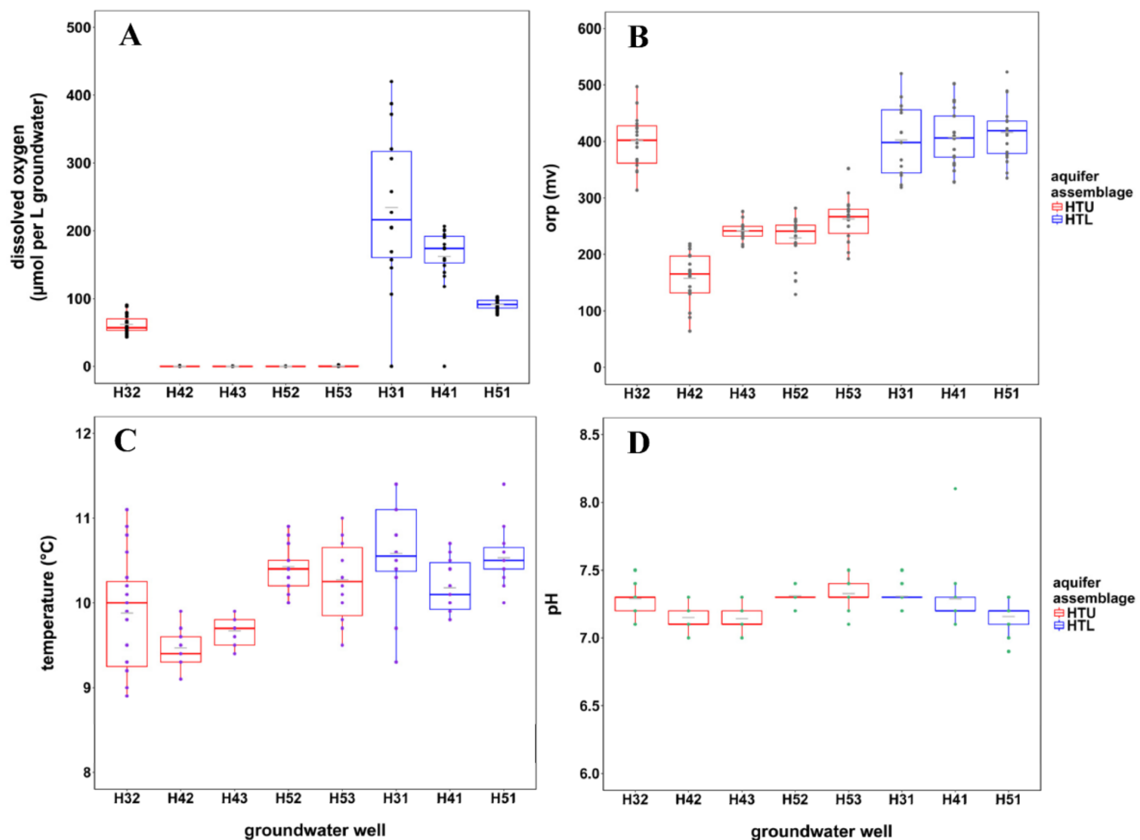


Figure 9 | Physical characteristics of the groundwater at Hainich aquifer assemblages. Sampling sites investigated in this study included five wells from the Hainich transect upper aquifer assemblage (HTU; in red) and three wells from the Hainich transect lower aquifer assemblage (HTL; in blue). Box-and-whisker plots display variation in physical parameters quantified in groundwater samples with upper and lower extremes along with upper and lower quartiles and median. Light grey line inside the box represents mean. Samples were obtained between January 2014 and June 2015 (n = 19). The data for physical characteristics was provided by Prof. Dr. Kai-Uwe Totsche and colleagues (Friedrich Schiller University, Jena) (Kumar *et al*., 2017).

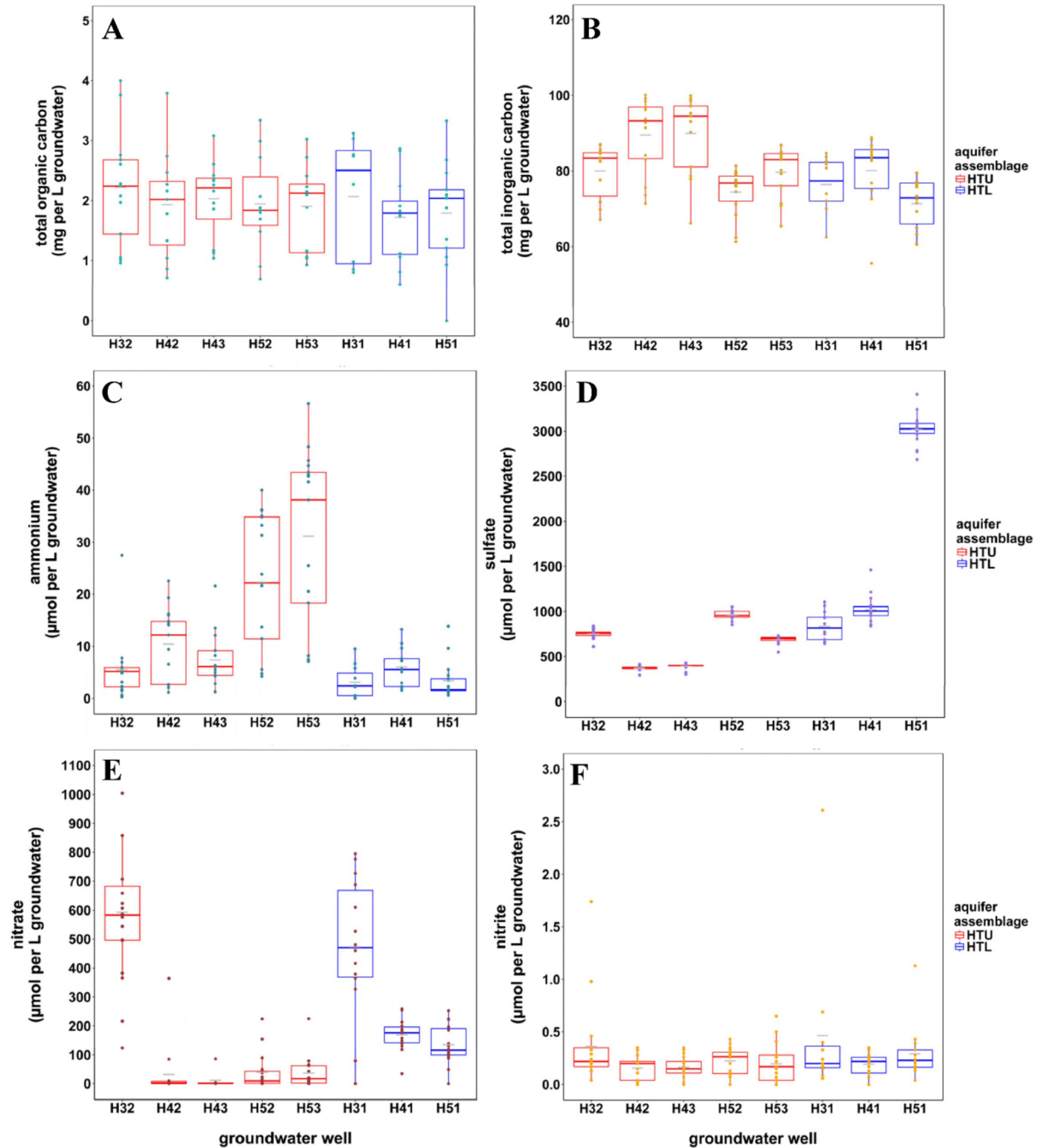


Figure 10 | Chemical characteristics of the groundwater at Hainich aquifer assemblages. Box-and-whisker plots displaying variations in hydrochemical properties quantified with upper and lower extremes along with upper and lower quartiles and median. Light grey line inside the box represents mean. Samples were obtained between January 2014 and June 2015 (n = 19). TOC and TIC values were provided by Prof. Dr. Kai-Uwe Totsche and colleagues (Friedrich Schiller University, Jena) (Kumar *et al.*, 2017). Detailed metadata (mean±SD) of groundwater physicochemical characteristics is included in **Appendix Table 6** and also available at BExIS.

Regarding hydrochemical characteristics, groundwater from all wells was characterized by low concentrations of TOC (1.95±0.25 mg L^{-1}) **(Figure 10A),** whereas TIC ranged from 71±32 to 90±40 mg L^{-1} **(Figure 10B)**.

Ammonium concentrations were higher in the groundwater from HTU (5.5±6 to 31±18 µmol L^{-1}), with highest concentrations of 31±18 µmol L^{-1} in the groundwater of well H53, compared to the wells of HTL (3.1±3 to 6.0±4 µmol L^{-1}) **(Figure 10C)**. Sulfate concentrations were in the range of 370±25 to 1020±282 µmol L^{-1} in the groundwater from HTU and HTL, compared to high concentrations in the H51 well of HTL (3019±711 µmol L^{-1}) **(Figure 10D)**.

Similarly to dissolved oxygen, nitrate concentrations were higher in the groundwater from the HTL wells (135±66 to 474±247 µmol L^{-1}) compared to the HTU wells (11±28 to 39±61 µmol L^{-1}) with the exception of well H32, which showed the highest nitrate concentration (594±233 µmol L^{-1}) **(Figure 10E)**. Concentrations of nitrite ranged from 0.16±0.11 to 0.46±0.69 µmol L^{-1} in both HTU and HTL **(Figure 10F)**.

3.2 Co-occurrence of denitrifiers, anammox and aerobic ammonia oxidizing archaea and bacteria in groundwater reveled by quantitative PCR

Quantitative PCR (qPCR)-based approach facilitated the quantification of total bacteria abundances and genetic potential of denitrifiers, anammox and aerobic ammonia oxidisers in the oligotrophic groundwater of the two carbonate-rock aquifer assemblages investigated in this study. Abundances of total bacteria ranged from 1.7 x 10^7 to 3.9 x 10^8 bacterial 16S rRNA genes L^{-1} for both aquifer assemblages. Abundances of denitrifier-associated *nirK* and *nirS* genes ranged from 1.1 x 10^3 to 6.5 x 10^5 and 1.1 x 10^4 to 8.5 x 10^7 genes L^{-1}, respectively, across all sites and time points (**Figure 11 A**).

A comparison of anammox bacterial abundances based on anammox-related 16S rRNA and functional genes (*hzsA*), in groundwater samples, revealed the difference of nearly one order of magnitude between each other. Abundances of anammox bacteria, based on anammox specific 16S rRNA genes, ranged from 4.8 x 10^5 to 2.2 x 10^7 genes L^{-1} while *hzsA* gene-based abundances ranged from 8.4 x 10^4 to 8.4 x 10^6 genes L^{-1} across all sites and time points **(Figure 12)**. Contemporary evidence indicates the existence of a single copy of the *hzsA* gene per anammox bacterial genome, according to Strous *et al*. (2006) and Kartal *et al*. (2011). Based on that, the estimated fraction of organisms within the total community harboring the genetic potential for the anammox process ranged from 1.8 to 10.6 % in HTU and 5.3 to 8.3% in HTL. This estimation was made by assuming the value of 4.05 as the average 16S rRNA gene operon number for all bacteria (rrndb database, Fogel *et al*., 1999, Stoddard *et al*., 2014).

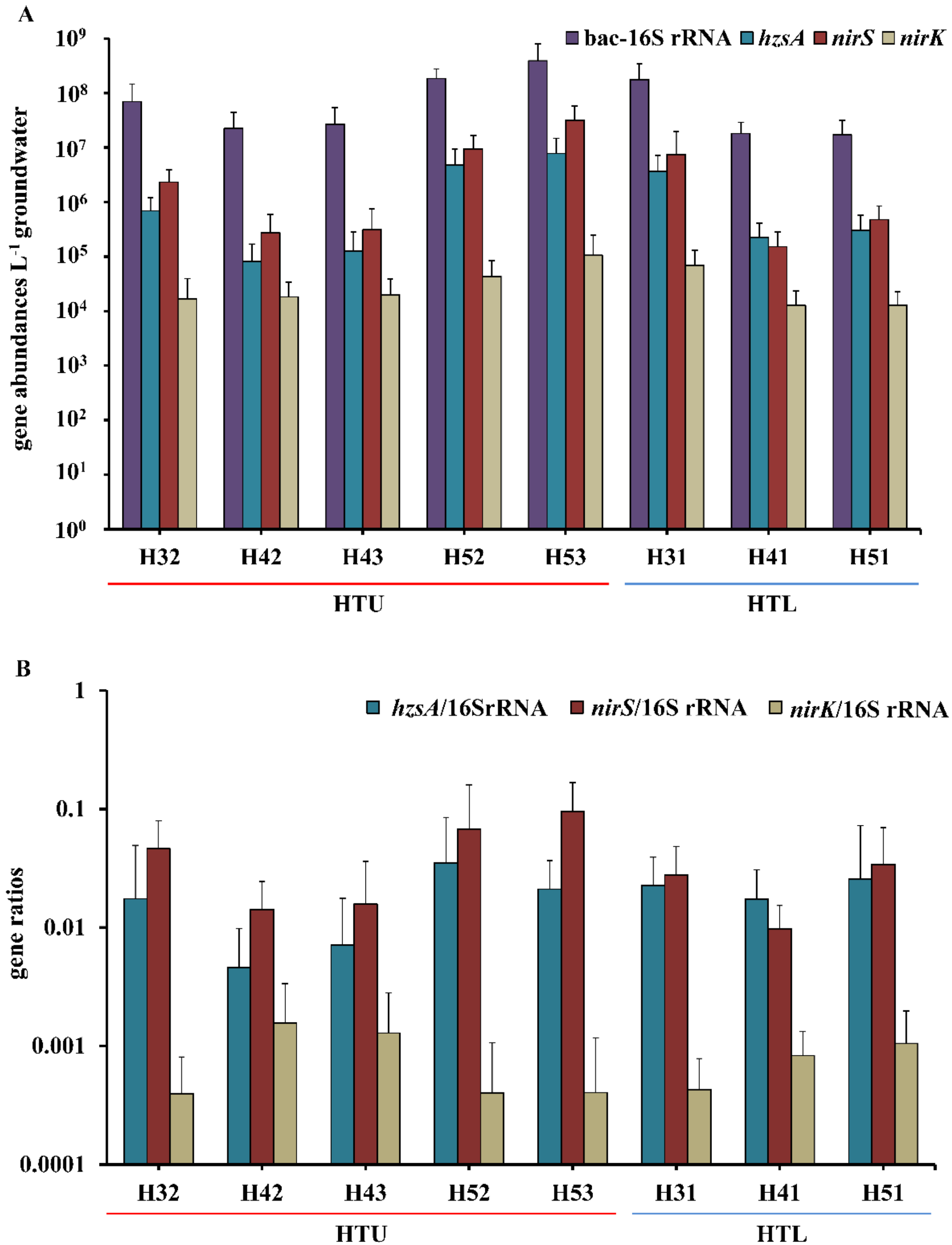


Figure 11 | Bacterial gene quantification in the groundwater of the Hainich aquifer assemblages. (A) Total abundances of bacterial 16S rRNA genes, anammox-related *hzsA*, denitrifier-related *nirS* and *nirK* genes and (B) gene ratios of functional genes relative to bacterial 16S rRNA genes in groundwater samples obtained from eight wells of the upper and lower aquifer assemblage. Bars represent mean (± standard deviation) of monthly measurements from January 2014 to June 2015 (Kumar *et al.*, 2017).

In the suboxic wells H52 and H53, relative proportions of denitrifiers, approximated by *nirS*/16S rRNA gene ratios, followed a similar trend as observed for the anammox

population, with maximum gene ratios of 0.068 and 0.095, respectively. Lower ratios of 0.014 to 0.015 and 0.028 to 0.046, were observed for anoxic wells (H42, H43) and oxic wells (H31, H32, H41, H51), respectively **(Figure 11 B)**. Transcript abundances of denitrifier-related *nirS* and *nirK* genes remained below the quantification limit of 10^3 transcripts L^{-1} groundwater for all wells.

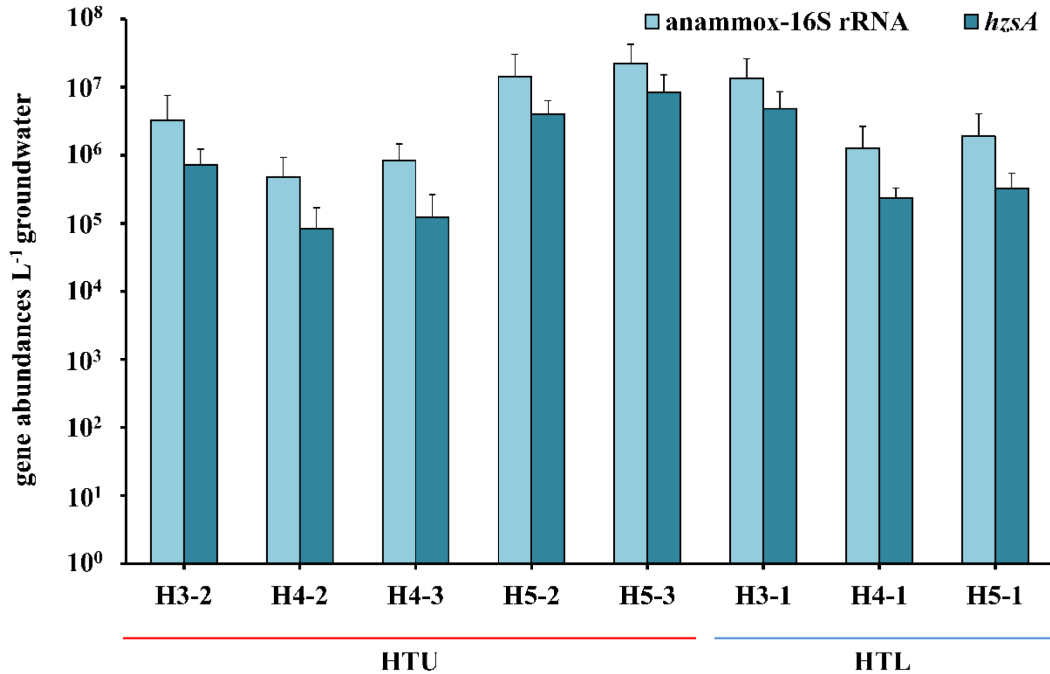


Figure 12 | Comparison of anammox bacteria abundances based on anammox specific 16S rRNA gene and functional gene, (*hzsA*) in groundwater. Samples obtained from eight wells of the upper and lower aquifer assemblage. Bars represent mean (± standard deviation) of monthly measurements from January 2014 to September 2014.

Genes and transcripts of ammonia-oxidizing archaea and bacteria (*amoA,* encoding for ammonia monooxygenase-subunit A) were quantified to target potentially co-occurring activities of aerobic and anaerobic ammonium oxidation. Groundwater samples obtained during August and November of 2015 from seven groundwater wells were analysed for *amoA* gene abundances. The *amoA* quantification pointed to similar or only slightly lower total abundances of aerobic ammonia oxidizing bacteria in suboxic wells H52 and H53 (6.2×10^4 and 3.9×10^5 *amoA* genes L^{-1}, respectively), compared to the oxic wells H41 and H51 (1.5×10^6 and 9.7×10^4 *amoA* genes L^{-1}, respectively). Similarly, abundance of archaeal aerobic ammonia oxidizers in suboxic and oxic wells was observed ranging from 3.2×10^5 and 1.7×10^5 at suboxic wells H52, H53 respectively and 7.3×10^5 and 1.1×10^5 *amoA* genes L^{-1} at oxic wells H41, H51, respectively. At anoxic wells H42 and H43, one-fold low archaeal *amoA* gene abundance was observed **(Figure 13 A)**.

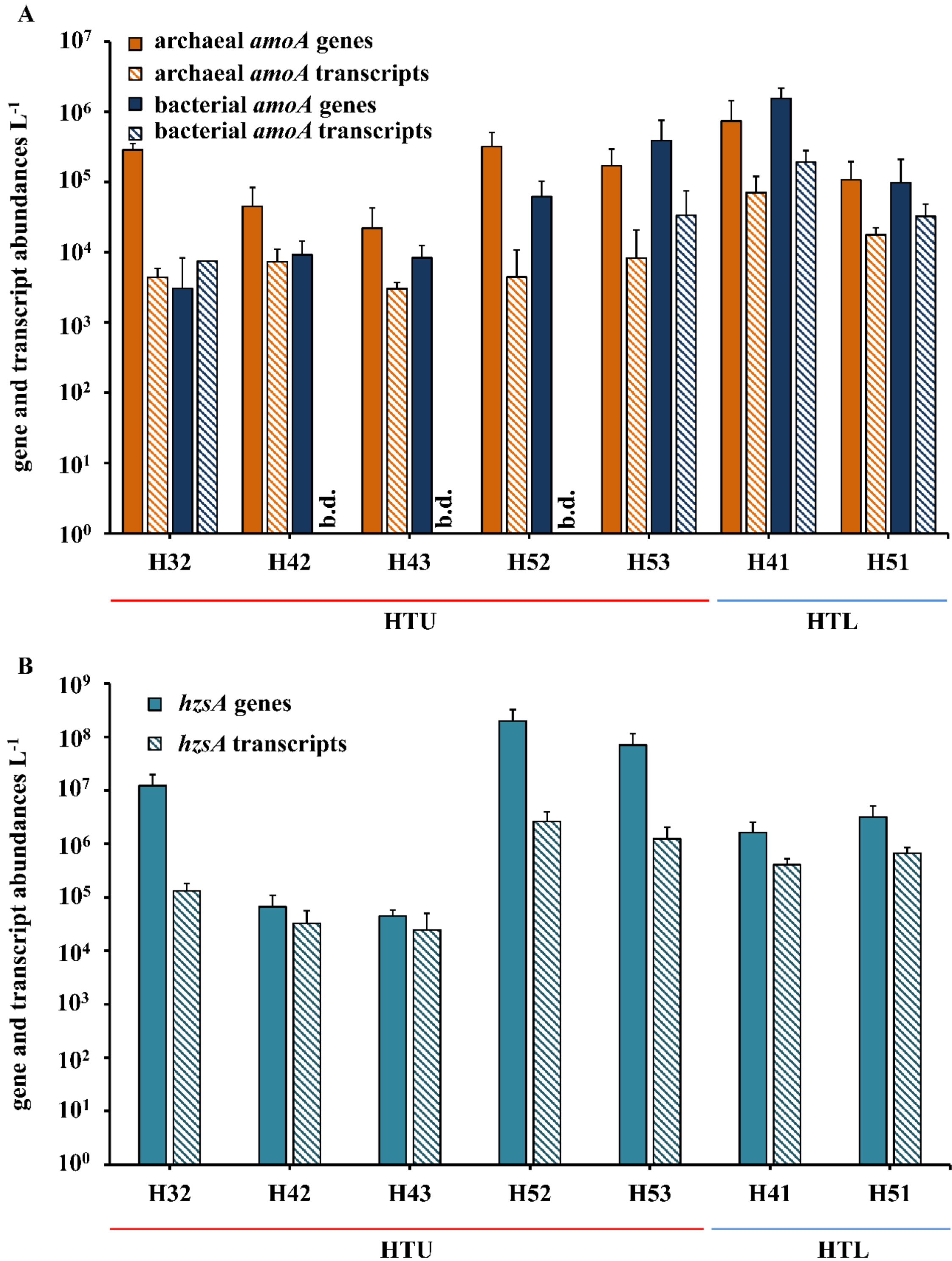


Figure 13 | Abundances of genes and transcripts of (A) archaeal and bacterial ammonia oxidisers (*amoA*) (B) anammox bacteria in groundwater. Samples obtained from seven wells of the upper and lower aquifer assemblage in August and November 2015. Bars represent mean (± standard deviation) of two time points and each of the three technical replicates from the qPCR analysis. b.d., below detection (Kumar *et al*., 2017).

Transcripts of both bacterial and archaeal *amoA* genes were detectable in the oxic groundwater of HTL but also at suboxic groundwater at H53. Per liter bacterial *amoA* transcripts were detected as 1.9×10^5 and 3.2×10^4 at oxic wells H41 and H51, respectively.

In the suboxic well H53, bacterial *amoA* transcripts were 3.4 x 10^4 per liter. Transcripts of archaeal *amoA* genes in oxic wells H41 and H51 were 7.1 x 10^4 to 1.7 x 10^4 L^{-1}, respectively, while one-fold low archaeal *amoA* transcripts number were observed at H52 and H53 (4.4 x 10^3 and 8.3 x 10^3 L^{-1}, respectively) **(Figure 13 A)**.

3.3 Anammox-*hzsA* transcriptional activity as an indicator of protein synthesis potential of anammox bacteria in Hainich aquifer assemblages

The distribution patterns of total and potentially active anammox bacteria suggested by quantitative analysis targeting *hzsA* genes and transcripts, revealed maximum *hzsA* gene and transcript abundances in the suboxic groundwater of wells H52 and H53 of the upper aquifer assemblage. Transcript abundances of *hzsA* determined in August and November 2015 varied substantially across sites with the highest abundances in the groundwater of wells H52 and H53 (2.6 x 10^6 - 1.2 x 10^6 L^{-1}) and the lowest abundances in the groundwater of wells H42 and H43 (3.3 x 10^4 - 2.5 x 10^5 L^{-1}). Surprisingly, transcripts of anammox *hzsA* genes were also detectable in oxic groundwater of wells H32, H41, and H51 (1.3 x 10^5, 4.1 X 10^5, - 6.7 x 10^5 L^{-1}, respectively **(Figure 13 B)**.

Furthermore, gene and transcript ratios of (*hzsA*/sum of archaeal and bacterial *amoA*) calculated across sites was used as an indicator of a predominance of either anaerobic or aerobic ammonium oxidation in the genetic potential or transcriptional activity of the groundwater microbial communities. The ratio of *hzsA*/*amoA* ranged from 0.1 to 444.7 and from 1.2 to 1087.6 on the gene and transcript level, respectively **(Figure 14)**. Highest *hzsA*/*amoA* gene and transcript ratios were observed for wells H52 and H53, pointing to a clear dominance of anammox although aerobic ammonia oxidation likely still co-occurred.

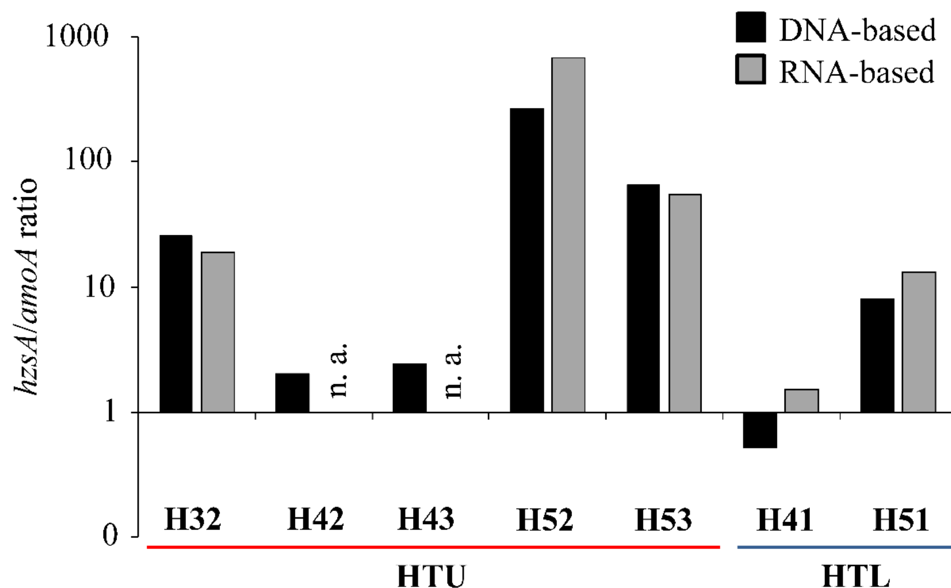


Figure 14 | Ratios of *hzsA*/archaeal + bacterial *amoA* on the gene and the transcript level in groundwater samples (excluding H31). Data are means of two time points (August and November 2015). n. a. = not analysed (Kumar *et al*., 2017).

Furthermore, when gene and transcript data was excluded from wells H42 and H43 (wells showing *hzsA* and *amoA* transcript numbers close to the detection limit), *hzsA*/*amoA* ratios were negatively correlated to groundwater oxygen concentrations across sites (Spearman rank correlation coefficient −0.85 and −0.875 for gene and transcript-based analysis, respectively, $p<0.01$) **(Figure 15)**.

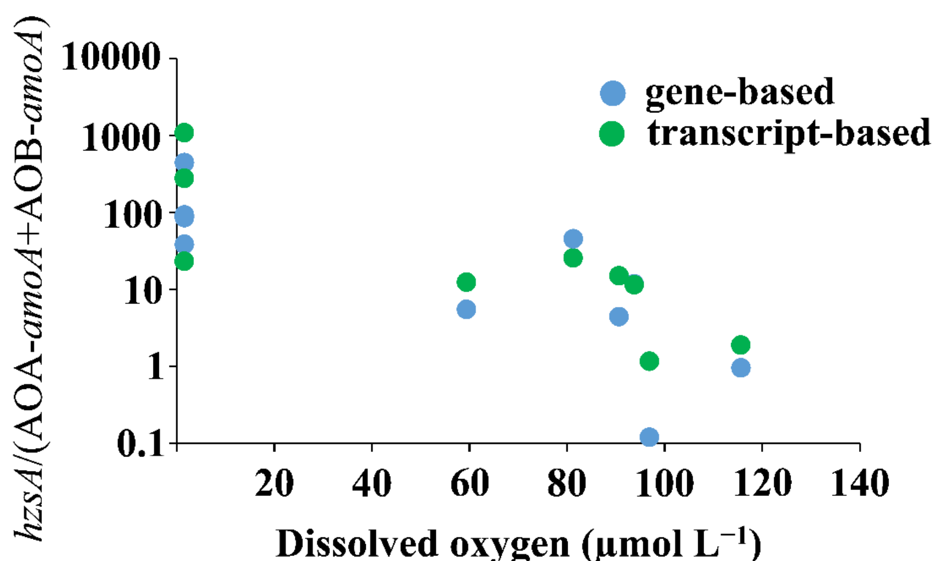


Figure 15 | Ratios of [*hzsA*/sum of archaeal and bacterial *amoA*] plotted against the oxygen concentration in the respective groundwater sample. Data are based on ten groundwater samples obtained from five groundwater wells in August and November 2015. Blue dots: DNA based analysis. Green dots: Transcript-based analysis (Kumar *et al*., 2017).

3.4 Correlation with environmental parameters and observed gene abundances

Across all wells and time points, abundances of bacterial 16S rRNA genes were significantly positively correlated with abundances of *hzsA* (Spearman rank correlation coefficient 0.806), *nirS* (0.847), and *nirK* genes (0.662) **(Appendix; Table 7)**. Similarly, *nirS*, *nirK*, and *hzsA* gene abundances were significantly positively correlated with each other. Abundances of all genes showed significant positive correlations ($P\leq0.01$) with potassium concentrations and pH (except *nirK*) and significant negative correlations ($P\leq0.01$) with concentrations of calcium. *hzsA* abundances were positively correlated with sulfate ($P\leq0.01$). Nitrate, nitrite and ammonium, important for denitrification and anammox, showed no clear correlation to the *nirS* and *hzsA* gene abundance, but was significantly (negative) correlated with *nirK* gene abundance ($P\leq0.01$ and $P\leq0.05$).

3.5 Ladderane lipids provide further support for the presence of an active anammox population

As a distinct feature among anammox bacteria, ladderane lipids offer a rare opportunity in providing complementary data to support gene based information, as measured by my collaborator Dr. Valerie F. Schwab and colleagues (Friedrich Schiller University Jena). Due to the absence of commercially available standards, absolute quantification of phospholipids derived [5]- and [3]-ladderanes was not possible and only changes of their relative concentration between wells have been shown in **Figure 16.**

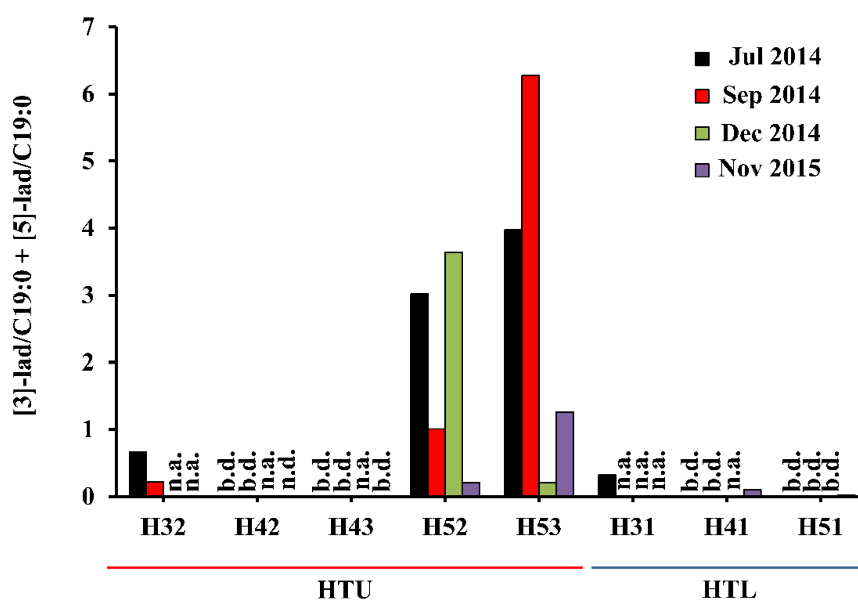


Figure 16 | Relative concentrations of ladderane lipids (sum of ladderane-[3]-FAME and ladderane-[5]-FAME peak area relative to C19:0 internal standard peak area) in the groundwater. b.d., below detection (relative concentrations < 0.01); n.a., not analyzed. Data of July, September, and December 2014 were also subject of analyses published in Schwab *et al.* (2017) (Kumar *et al.*, 2017).

Schwab *et al.* (2017) observed the highest relative concentrations of ladderanes in the suboxic groundwater of wells H52 and H53 with maxima observed at well H53 for most of the time points, obtained in July, September and December 2014, corroborating the *hzsA* quantification analysis. To confirm these patterns for the time point when anammox rate measurements were performed, additional ladderane data of November 2015 were analysed by Dr. Valerie F. Schwab and colleagues (Friedrich Schiller University) (Kumar *et al.*, 2017). In the suboxic groundwater at well H53, ladderane concentrations were exceeded by a factor of 35 than those observed in oxic groundwater at H51.

Temporal fluctuations of ladderane concentrations at wells H52 and H53 were more than one order of magnitude but did not show any correlation with temporal fluctuations of ammonium or oxygen concentrations. Furthermore, low relative concentrations of ladderanes were also detectable in the oxic groundwater of wells H31, H41, and H51.

3.6 Nitrogen loss from pristine carbonate-rock aquifers is primarily driven by chemolithoautotrophic anammox processes

Groundwater samples from well H53 were selected for rate measurements of anammox and denitrification activity as this site provided the most favorable environmental conditions for the anammox and denitrification process, i.e., suboxic conditions and co-occurrence of ammonium and nitrate **(Figure 10C, E)** along with maximum abundances of functional genes (Figure 11A) and high relative concentrations of ladderane lipids as an indicator of potential active anammox bacteria **(Figure 16)**.

Incubations with $^{15}NH_4^+$ and $^{14}NO_2^-$ or $^{15}NO_2^-$ and natural NH_4^+ background revealed a progressive increase in $^{29}N_2$ formation, a signature of the active anammox process, whereas $^{30}N_2$ production was considered as a result from denitrification (**Figure 17 A)**.

The production rates of $^{29}N_2$ from anammox varied between 3.5 nmol N_2 L^{-1} d^{-1} and 4.7 nmol N_2 L^{-1} d^{-1}, respectively. The production rates of $^{30}N_2$ from denitrification showed an activity of 0.7 nmol N_2 L^{-1} d^{-1}. Based on the measured activities in the incubations with added $^{15}NO_2^-$, we observed a total N_2 production activity of 4.2 nmol N_2 L^{-1} d^{-1} to which anammox contributed an estimated 83 % (**Figure 17 B)**.

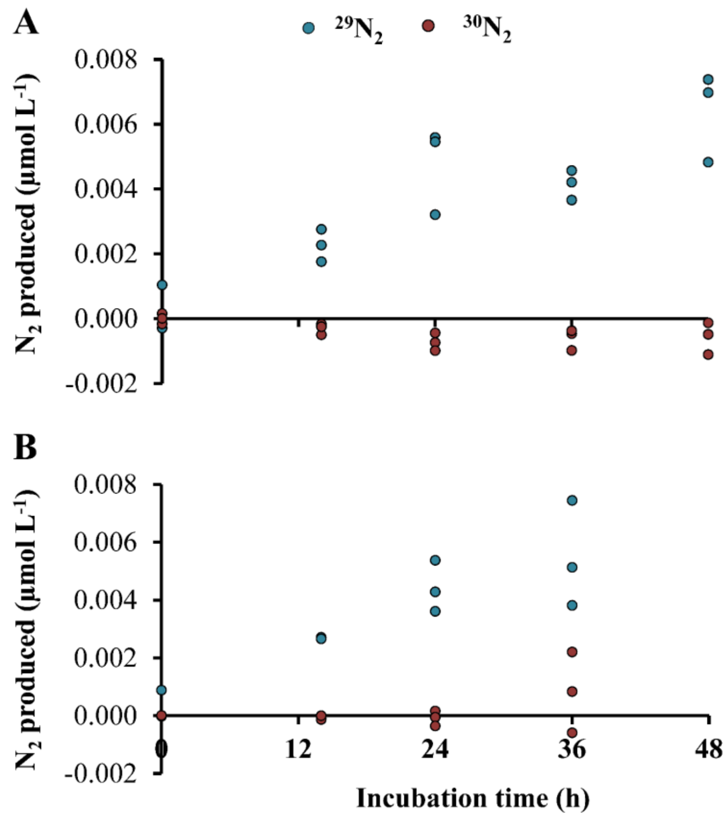


Figure 17 | Formation of $^{29}N_2$ (blue circles) and $^{30}N_2$ (red circles) over a 48 hours incubation period in anammox, denitrification rates assays. (A) Incubation with $^{15}N\text{-}NH_4^+$ and $^{14}N\text{-}NO_2^-$ (B) Incubation with $^{15}N\text{-}NO_2^-$ and natural $^{14}N\text{-}NH_4^+$ background. Assays were run in triplicates. (Kumar *et al*., 2017)

Based on *hzsA* gene abundances in the groundwater of well H5-3 at the same time point when samples were taken for rate measurements (4.59 x 10^6 L^{-1}), specific cellular activities were calculated, assuming one *hzsA* gene copy per anammox bacterial cell (Strous *et al*., 2006; Kartal *et al*., 2011), which yielded estimated per-cell activities between 0.77 fmol d^{-1} and 1.03 fmol d^{-1}.

3.7 Presence of rRNA as indicative of protein synthesis potential of *Planctomycetes* organisms in limestone aquifers

Groundwater bacterial community structure was assessed based on 16S rRNA gene-targeted MiSeq Illumina amplicon sequencing for DNA and RNA samples obtained in August, 2014 from eight wells to identify the overall groundwater community along with potential key players responsible for nitrogen loss in the groundwater.

Additionally, DNA based analysis was carried out for two time points in August and November 2015 from seven wells. Groundwater bacterial communities across both aquifer assemblages were primarily composed of members of *Parcubacteria* (14-38 % of sequence

reads) followed by *Nitrospirae* (1-32 %), *Betaproteobacteria* (3-17 %), *Deltaproteobacteria* (4-12 %) and *Alphaproteobacteria* (1-13 %) **(Figure 18 A)**.

Anammox bacteria belong to the bacterial phylum *Planctomycetes*, the sequence reads to which ranged from 1-9 %with highest fractions in the groundwater of suboxic well H52 (5-9 %) followed by 5-6 % in oxic well H51. Within the *Planctomycetes*, lowest fraction of sequence reads affiliated with anammox bacteria were observed in the oxic aquifer (26-33 %) and at site H4 of the anoxic upper aquifer assemblage (16-27 %) and showed maximum values of 93-96 % at wells H53 and H52, indicating that the *Planctomycetes* community in the groundwater of these wells was almost exclusively composed of anammox bacteria.

Representing the bacterial population with protein biosynthesis potential on the 16S rRNA level, high sequence reads affiliated with *Planctomycetes* were observed especially in the groundwater of wells H52 (27 %) and H53 (15 %), out of which anammox-affiliated reads accounted for 95 to 96 % **(Figure 18 B)**. Besides the potentially active members of *Planctomycetes*, the RNA based data also revealed the microbial potential from the most abundant microorganisms being *Nitrospirae* (average abundance: 22 %), followed by the presence of *Betaproteobacteria* and *Deltaproteobacteria* (12.5 %).

Observed numbers of OTUs and estimated numbers using Chao1 richness estimator on the DNA and RNA level ranged from 1402 to 5181 and from 1597 to 4115, respectively, across groundwater wells. The lowest observed and estimated diversity for both the DNA- and RNA-based analyses was observed in the groundwater of wells H53, reflected by a lower Shannon diversity index for these samples (3.74 and 4.07) compared to the other groundwater wells (7.19 and 6.2; maximum at H43) **(Appendix Table 8, Table 9)**.

Representing the bacterial population with protein biosynthesis potential on the 16S rRNA level, high sequence reads affiliated with *Planctomycetes* were observed especially in the groundwater of wells H52 (27 %) and H53 (15 %), out of which anammox-affiliated reads accounted for 95 to 96 % **(Figure 18 B)**.

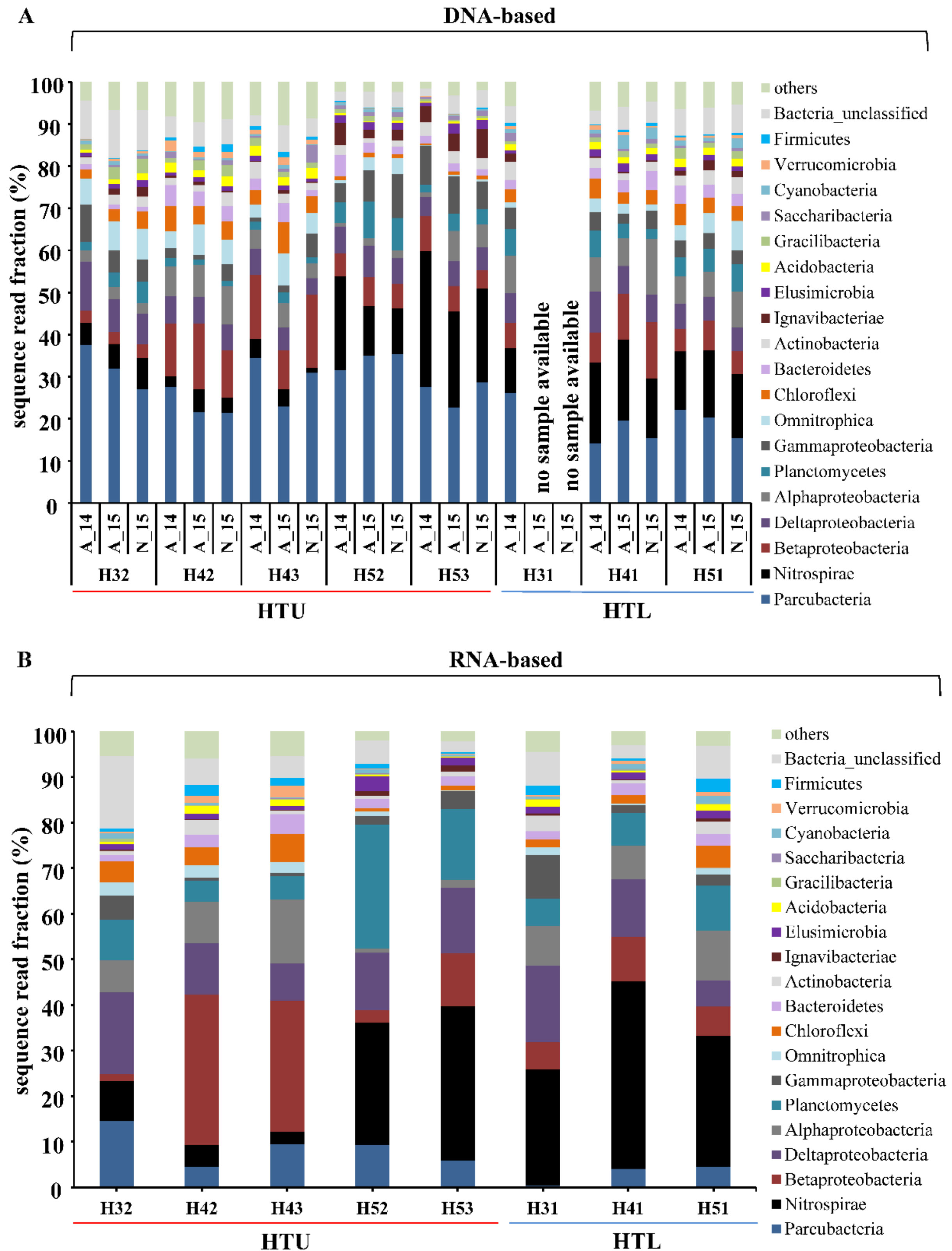


Figure 18 | Bacterial community structure based on MiSeq Illumina amplicon sequencing of 16S rRNA genes in the groundwater of eight wells across the two aquifer assemblages, analysis based on (A) DNA, samples from [August 2014 (A_14), August (A_15) and November (N_15) 2015] and (B) RNA, samples from August 2014 (Kumar *et al.*, 2017).

Besides the potentially active members of *Planctomycetes*, the RNA based data also revealed the microbial potential from the most abundant microorganisms being *Nitrospirae* (average abundance: 22 %), followed by the presence of *Betaproteobacteria* and *Deltaproteobacteria* (12.5 %).

3.8 *Candidatus* Brocadia fulgida dominated the anammox bacterial community of the Hainich aquifer assemblages

Based on the functional gene marker indicative of anammox bacteria targeted in this study, *hszA*, a cloning approach yielded nine different OTUs, with most of them being affiliated with the *Candidatus* genus Brocadia, with sequence identities of deduced hzsA protein sequences to those of *Ca*. Brocadia fulgida ranging from 90 to 92 % **(Figure 19).**

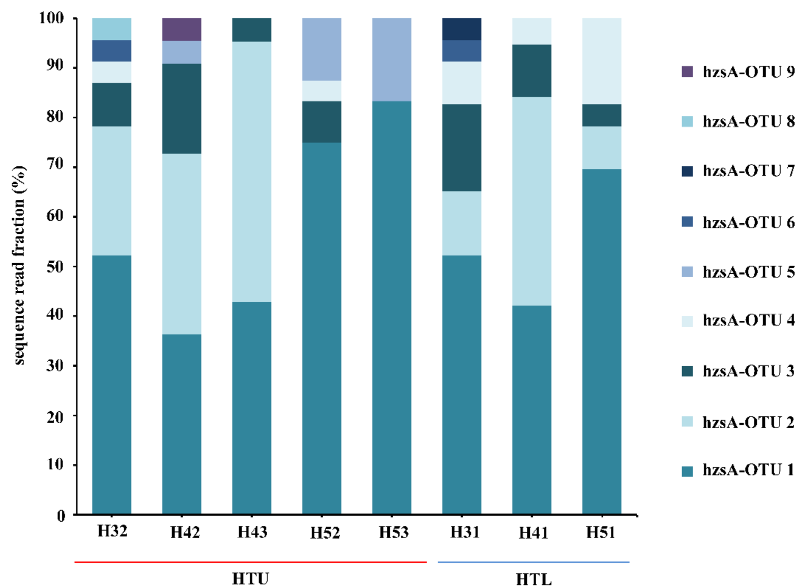


Figure 19 | Community structure of anammox bacteria.
Relative fractions of OTUs of anammox bacteria based on deduced hzsA protein sequences in the groundwater of the two aquifer assemblages.

Sequence identities (protein level) to closest cultured relatives are: OTU1, OTU2, OTU3, OTU4: *Candidatus*. Brocadia fulgida (92%), OTU5: *Ca.* Brocadia fulgida (91%), OTU 6: *Ca.* Jettenia asiatica (91%), OTU7: *Ca.* Jettenia asiatica (88%), OTU8: *Ca.* Brocadia fulgida (90%), OTU9: *Ca.* Jettenia asiatica (91%). A total of 169 cloned *hzsA* sequences were analyzed with the number of sequences per groundwater well ranging from 19 to 23.

Furthermore, these results were complimented by 16S rRNA gene and transcript-targeted Illumina MiSeq amplicon sequencing, which identified four *Candidate* genera of anammox bacteria. Among the sequence reads affiliated with *Brocadiaceae*, up to 78 % were affiliated

with *Candidatus* Brocadia, followed by *Ca.* Kuenenia and *Ca.* Jettenia (Figure 20 A) with similar results for DNA- and RNA-based sequencing in August 2014 (**Figure 20 A, B)**.

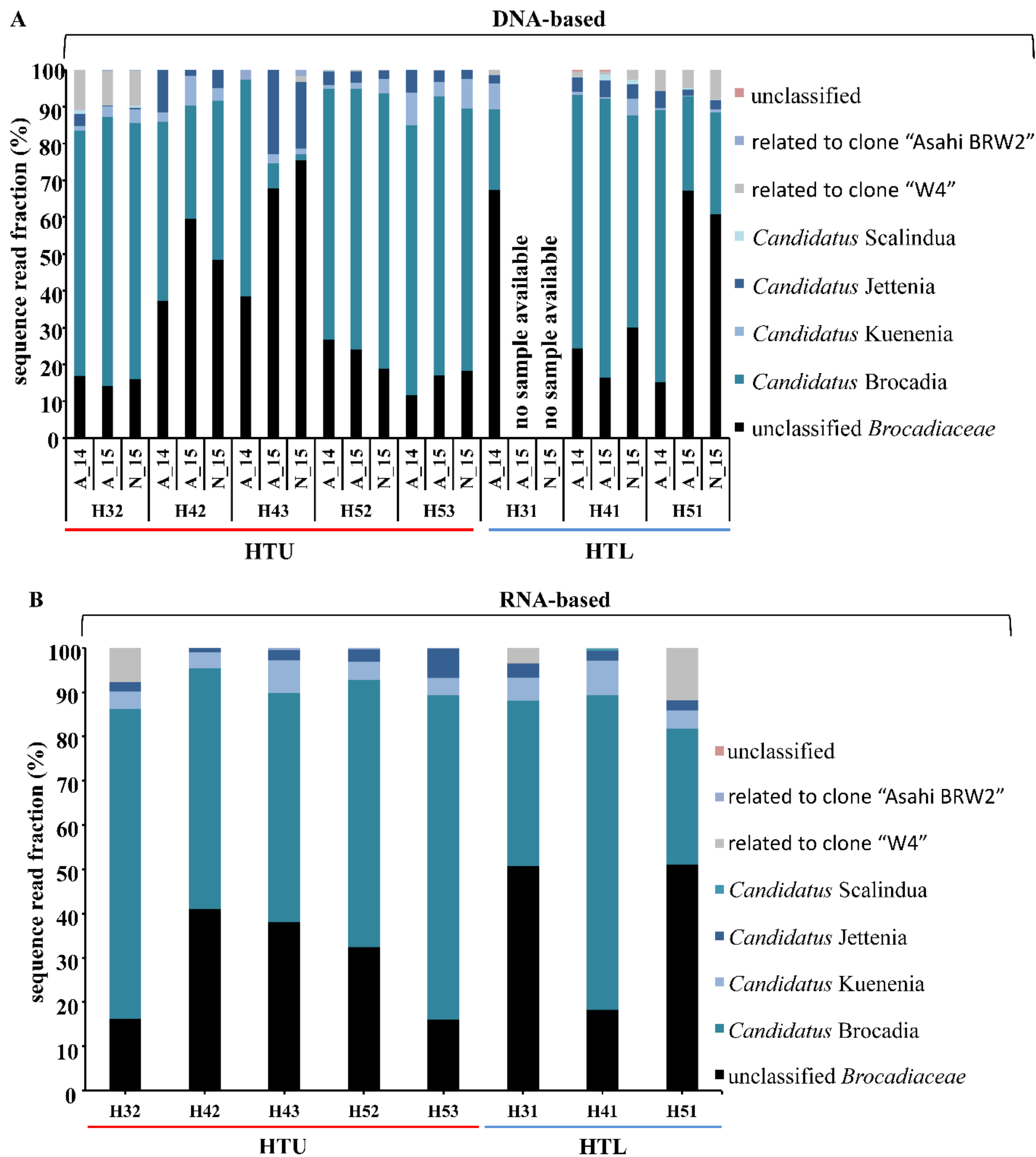


Figure 20 | Anammox bacterial community structure based on MiSeq Illumina amplicon sequencing of 16S rRNA genes in the groundwater of eight wells across the two aquifer assemblages, analysis based on (A) DNA, samples from [August 2014 (A_14), August (A_15) and November (N_15) 2015] and (B) RNA, samples from August 2014. Bars represent fractions of sequences assigned to different Candidatus genera of anammox bacteria (corresponding to 39 up to 4,264 sequence reads out of 16,383 total bacterial 16S rRNA sequence reads per well) (Kumar *et al*., 2017).

3.9 Denitrifying community composition based on *nirS*-genes revealed dominance of a chemolithotrophic community utilizing sulfur, hydrogen and iron as electron donors

A detailed analysis of denitrifier community composition focused on *nirS*-type denitrifiers for the all eight sites in August 2014 and additionally from four selected sites in 2014 and 2015 (July, August; 2014 and January, March, June, August; 2015).

Samples from August 2014 revealed OTU numbers (0.18 distance cut-off on nucleic acid level) and estimated numbers using the Chao1 richness estimator on the DNA level ranged from 44 to 107 across groundwater wells. The lowest observed and estimated diversity was observed in the suboxic groundwater of wells H53, reflected by a lower Shannon diversity index for these samples (1.58) compared to the other groundwater wells (3.19; maximum at H51) **(Appendix Table 10)**.

nirS-type denitrifier communities were dominated by one OTU distantly related to *Sulfurifustis variabilis* (nirS-OTU1: 85 % sequence identity), and two OTUs related to the genus *Azospirillum* (nirS-OTU2, nirS-OTU3: 82 % to 83 % sequence identity), except for wells H42 and H43.

For wells H32, H52, and H53, these OTUs accounted for more than 70 % of the sequence reads, while at sites H42 and H43, they were only represented by few sequence reads **(Figure 21 A).** Here, *nirS*-type denitrifier communities were mainly composed of denitrifiers closely related to *Sulfuritalea hydrogenivorans* (90 % to 95 % sequence identity), *Ideonella* sp. (83 % sequence identity), and poorly characterized *nirS*-type denitrifiers (nirS-OTU4, sequence identity < 80%), with the latter accounting for 20 to 28 % of all the *nirS* sequence reads detected at wells H42 and H43.

Subsequently, potential denitrifiers were additionally identified based on the 16S rRNA sequence information from the same samples (August 2014), as the *nirS* primer set used in this study may discriminate against some denitrifying genera due to mismatches in the primer binding region (Herrmann *et al.,* 2017). This further confirmed the presence and distribution patterns of the genera *Sulfurifustis*, *Sulfuritalea*, and *Sulfuricella* across sites. Additionally the denitrifying genera *Hydrogenophaga* and *Sideroxydans* were also identified, which were especially abundant in the groundwater of wells H42 and H43 (*Hydrogenophaga*: 0.09 - 1.6 %; *Sideroxydans*: 0.1 - 0.8 % of all 16S rRNA gene sequence reads) **(Figure 21 A)**.

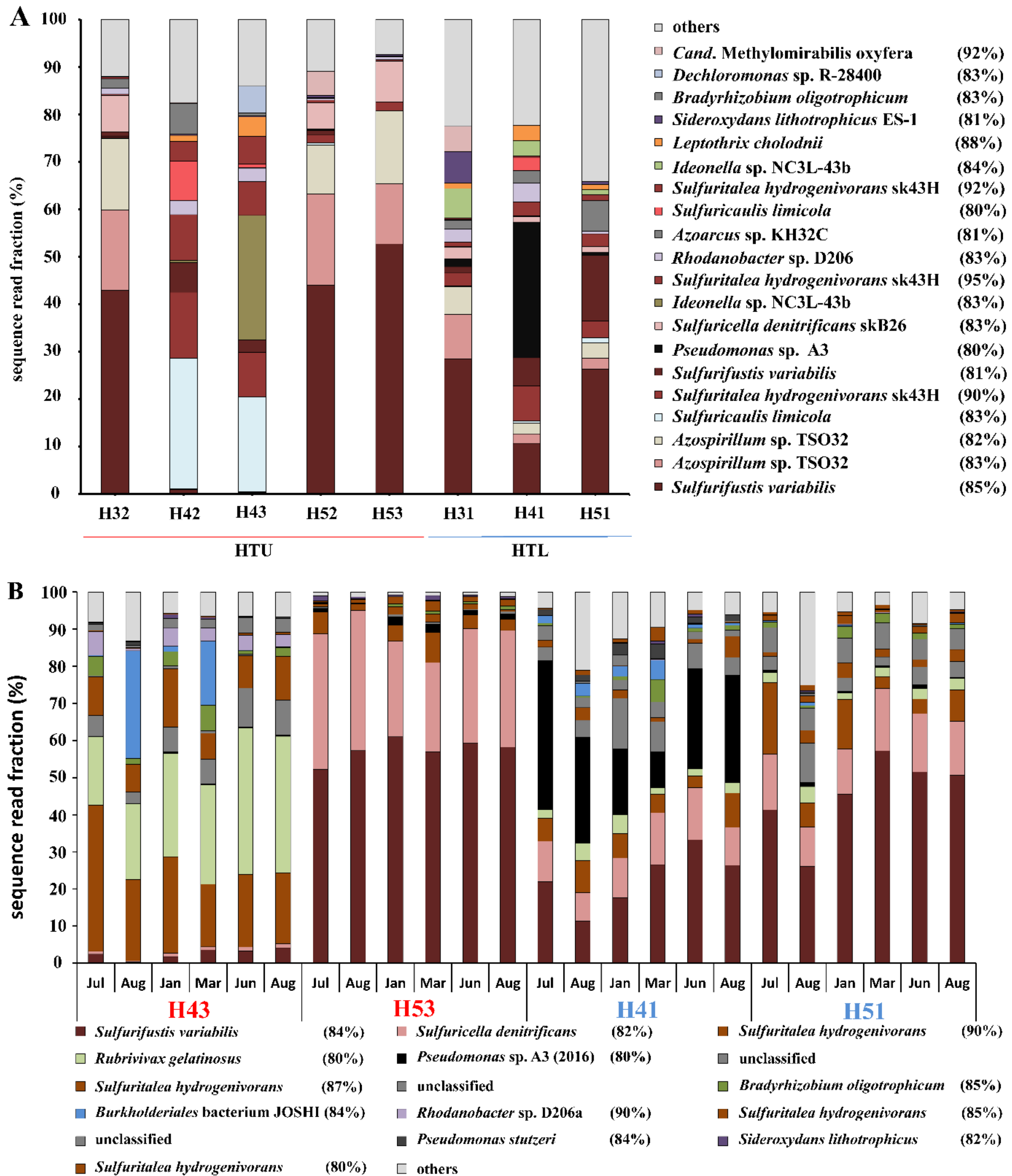


Figure 21 | Taxonomic affiliation of the *nirS*-type denitrifying bacterial communities in the groundwater of four wells across the two aquifer assemblages at Hainich Critical Zone Exploratory, analysis based on Illumina MiSeq amplicon sequencing of *nirS* genes. (A). Eight wells during one sampling time point August 2014 (Kumar *et al*., 2017) (B). Four selected wells during six sampling time points in 2014-2015. Sequences showing less than 80% *nirS*- sequence identity with cultured denitrifiers are referred to as "unclassified".

Illustrated in **Figure 21 B**, a robust temporal analysis of *nirS*-type denitrifiers facilitated to analyse a total of 1,822,947 sequences from four wells (H43, H53 and H41 and H51) of the two aquifer assemblages, to understand the observed lowest and highest *nirS*-type denitrifying bacterial diversity at H53 and H51 respectively. Samples from six sampling

campaigns (July, August; 2014 and January, March, June, August; 2015) revealed numbers of OTUs and estimated numbers using Chao1 richness estimator on the DNA level ranged from 47 to 123 across groundwater wells. The lowest observed and estimated diversity corroborated to be found again in the groundwater of well H53, reflected by a lower Shannon diversity index for these samples (0.91) compared to the other groundwater wells (2.84; maximum at H51) **(Appendix Table 11)**.

The community structure of groundwater *nirS*-type denitrifying bacteria showed less fluctuation over time in the suboxic groundwater of well H53 compared to the other wells. Here, sequences related to *Sulfurifistis variabilis*, chemolithoautotrophic bacteria (with 84 % sequence identity) constituted more than half (56±6 %) of the sequence reads. Remaining reads were associated with *Sulfuricella denitrificans* (with 82 % sequence identity) (30±8 % of the total sequence reads) and *Sulfuritalea hydrogenivorans* (with 90 % sequence identity) (8±5 %), which are representatives of a chemolithoautotrophic and chemolithotrophic denitrifier community, respectively. At well H43 of the upper anoxic aquifer assemblage, most of the *nirS*-type denitrifier community was constituted by organisms related to *Sulfuritalea hydrogenivorans* (50±20 %), followed by bacteria related to the mixotrophic denitrifier *Rubrivivax gelatinosus* (38±20 %).

In the groundwater of HTL, of well H51, the most abundant OTUs were affiliated with *Sulfurifustis variabilis* (41±16 %), followed by *Sulfuritalea hydrogenivorans* (13±8 %) and *Sulfuricella denitrificans* (14±3 %) while at well H41, most of the sequence reads were affiliated with *Pseudomonas* sp. (25±15 %), followed by denitrifiers related to *Sulfurifustis variabilis* ((22±11 %) and *Sulfuricella denitrificans* (11±3 %). Since community analysis across six time points confirmed that suboxic well H53 of HTU consistently harbored a stable community of potential chemolithoautotrophic denitrifiers, the enrichment culture obtained from groundwater of this well was further used to delineate its taxonomic identity.

3.10 Taxonomic identification of denitrifying enrichments and bacterial cultures originating from the Hainich aquifer assemblages

The phylogenetic relatedness of denitrifying enrichments obtained from groundwater, from passive sampler rock chips and bacterial cultures was determined, and further delineated.

3.10.1 *Community structure of a chemolithoautotrophic denitrifying bacterial enrichment originating from groundwater*

To analyse the 16S rRNA gene based community composition of the enriched consortium after 22 months (35th-subtransfer), DNA was extracted from the enrichment culture 1E. A 16S rRNA gene-targeted clone library (136 clones) was sequenced and revealed that more than half (59 %) of the sequences were assigned to the genus *Thiobacillus* with closely related sequences identified as *T. thiophilus, T. thioparus* and *T. denitrificans*, followed by *Hydrogenophaga* related sequences (21 %) on the taxonomic level. Apart from that, 7 % of the clone sequences were closely related to *Sulfuritalea hydrogenivorans*, and *Ferribacterium limneticum*, while 4 % sequences were affiliated to *Dechloromonas aromatica*. *Sulfuricella denitrificans* related sequences formed a marginal group (2 %) within the initial consortium (**Figure 22 A**).

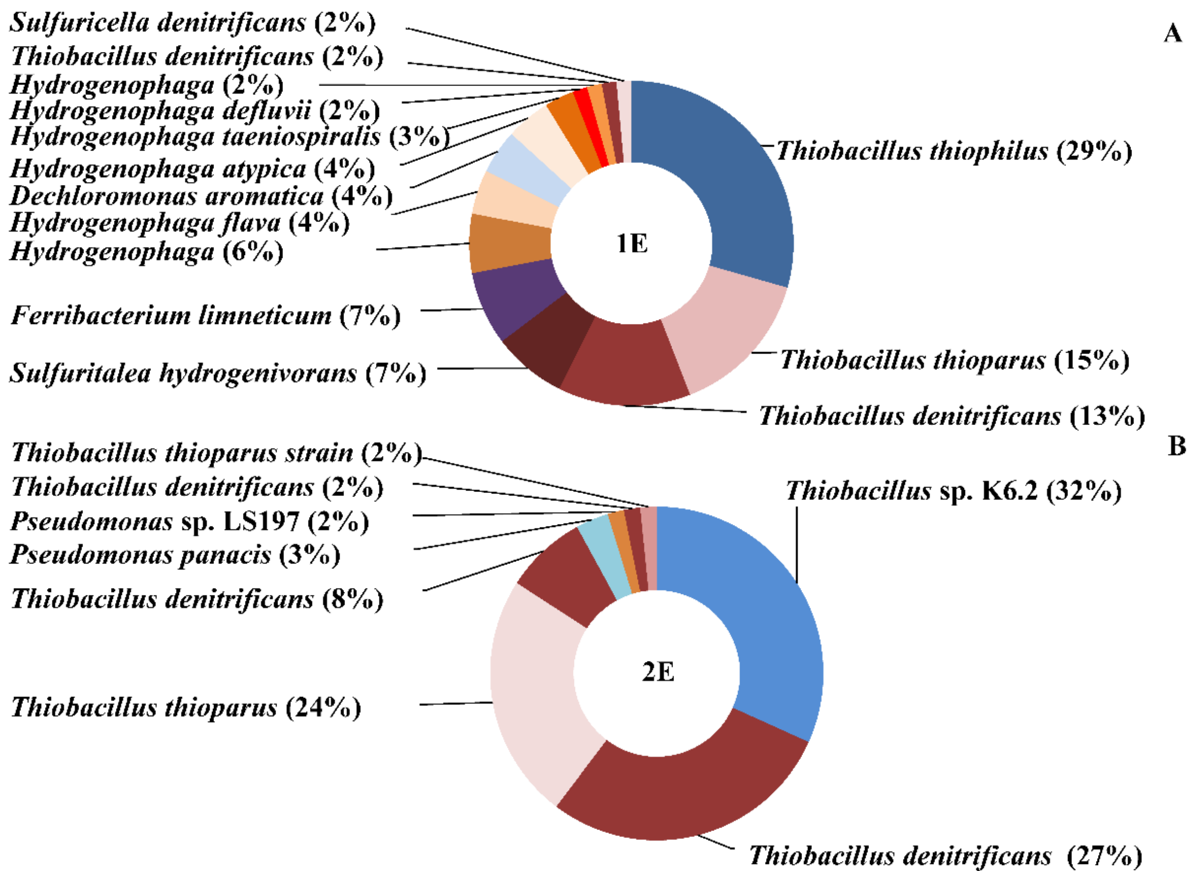


Figure 22 | Community composition of the enriched chemolithotrophic denitrifier consortium, Planktonic versus Attached denitrifiers (A) 1E (groundwater based consortium, 136 clones) and (B) 2E (rock chips based consortium, 63 clones). Doughnut chart represents the percentage of related sequences indicating their taxonomic affiliation (sequence similarity 93-99 %) among the sequenced 16S rRNA clones, originating from extracted enrichment culture DNA (35th subculture).

3.10.2 *Chemolithotrophic bacterial enrichment originating from rock-chips and gelrite shake dilution (groundwater based)*

While the groundwater-obtained enriched consortium (planktonic) showed successful enrichment of chemolithotrophs, the enrichment obtained from crushed rock material exposed in a passive sampler, (2E) revealed that 95 % of clone sequences were affiliated to *Thiobacillus* genus, consisting of sequences closely related to *Thiobacillus* sp. K6.2, *T. denitrificans* and *T. thioparus* along with the presence of representatives of the genus *Pseudomonas* (5%) consisting of *P. panacis* and *P*. sp. LS197 **(Figure 22 B)**.

On the other hand, the enrichment cultures obtained from Gelrite shake dilutions showed that overall community was each dominated by two members. Enrichment culture 3E was dominated by organisms closely related to *Pseudomonas extremaustralis* strain 14-3 (75%) and *P. veronii* strain CIP 104663 (25%), while enrichment culture 4E consisted of *Simplicispira pshychrophila* strain CA 1 (96%) and *Thermomonas fusca* strain R-10289 (4%) **(Figure 23 A and B)**.

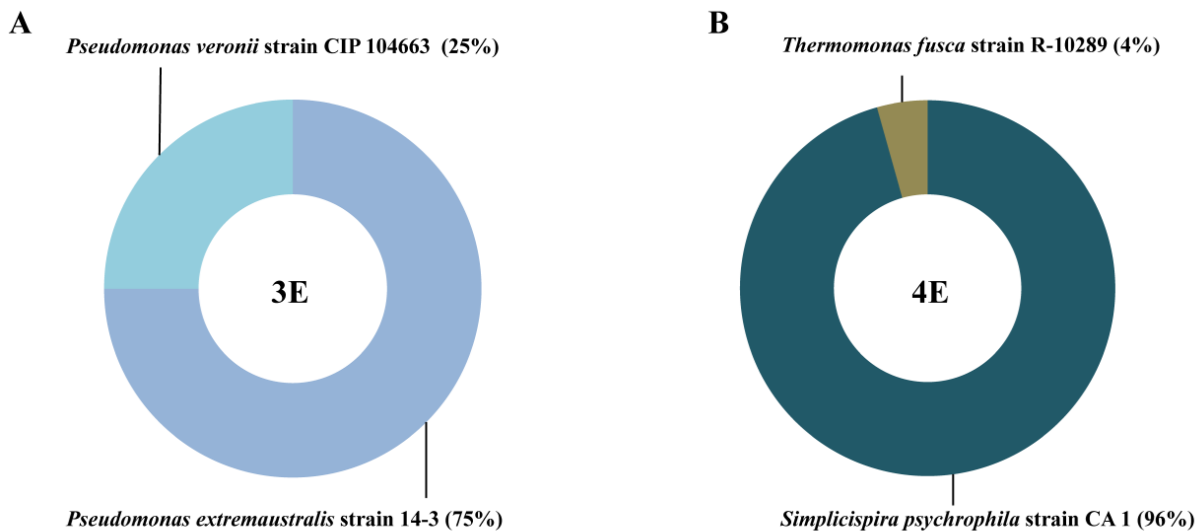


Figure 23 | Community composition of a groundwater based enrichment culture obtained by the Gelrite shake dilution technique under denitrifying conditions (A) 3E from well H31 and (B) 4E from well H41. Sequence identities with cultured representatives ranged from 97 to 100%.

3.11 Chemolithoautotrophic denitrification coupled to the oxidation of thiosulfate and hydrogen

Denitrification metabolic potential of the microbial community (enrichment 1E) enriched from the groundwater under chemolithoautotrophic conditions, was successfully demonstrated, and further addressed as outcome of two microcosm experiments.

3.11.1 *Microcosm experiment M_I: turnover of nitrogen and sulfur compounds by enriched consortium 1E*

In the first microcosm experiment, M_I, up to 93 % of the nitrate was readily reduced on the expense of thiosulfate and hydrogen oxidation by the end of the 14-days-incubation period. The initially gradual depletion of nitrate up to day 8 was followed by a rapid decrease in nitrate concentration until the end of the incubation, where only 370 µmol of nitrate remained.

During microcosm experiment M_I, with a nitrate/thiosulfate/nitrate ratio of 2.5, growth was observed using 16S rRNA gene quantification. Simultaneously, thiosulfate (2 mM) and nitrate (5 mM) consumption during the time course was quantified along with the concomitant production of sulfate and nitrite (**Figure 24A**). Accumulation of nitrite was observed but never exceeding 912 µmol NO_2^- L^{-1}. Thiosulfate was consumed completely by day 10 of the incubation, resulting in the accumulation of 4495±60 µmol SO_4^{2-} L^{-1}.

Ammonium concentrations showed negligible variations in the microcosms during the time course, averaging 2600±200 µmol NH_4^+ L^{-1}. Nitrite was not detected in the non-inoculated controls, nor were any changes observed in ammonium, thiosulfate and nitrate concentrations **(Appendix Figure 31)**.

The rates of nitrate reduction and sulfate production were calculated as 220 µmol L^{-1} d^{-1} and 292 µmol L^{-1} d^{-1} respectively based on the near-linear change of concentrations between day 1 and day 7.

3.11.2 *Microcosm experiment M_II: complete denitrification ($^{15}N_2$ production) and hydrogen utilisation was witnessed by Cavity Enhanced Raman Spectroscopy (CERS)*

In the microcosm experiment M_II, with a nitrate/thiosulfate ratio of 1; cells grown on increased thiosulfate amendment (5 mmol L^{-1}) and supplemented hydrogen (4 % corresponding to 1.7 mmol L^{-1}), nearly complete (98%) consumption of the initially supplied 5 mmol $^{15}N\text{-}NO_3^-$ L^{-1} was observed with subsequent accumulation of $^{15}N\text{-}N_2$ up to 2.4 mmol

L^{-1} at day 19 of the microcosm experiment. **Figure 24 B** showing $^{15}N-N_2$ formation from $^{15}N-NO_3^-$ and H_2 depletion, which was followed by Raman gas spectroscopy. The concentration of hydrogen decreased down to 519 µmol L^{-1} by day 7, and hydrogen was completely consumed by the end of the incubation.

The rates of $^{15}N-N_2$ production and H_2 oxidation under denitrifying conditions were 243 µmol L^{-1} d^{-1} and 22 µmol L^{-1} d^{-1}, respectively, calculated based on the near-linear change in concentrations between day 7 and day 12 of the experiment for $^{15}N-N_2$ and between day 0 and day 10 for H_2.

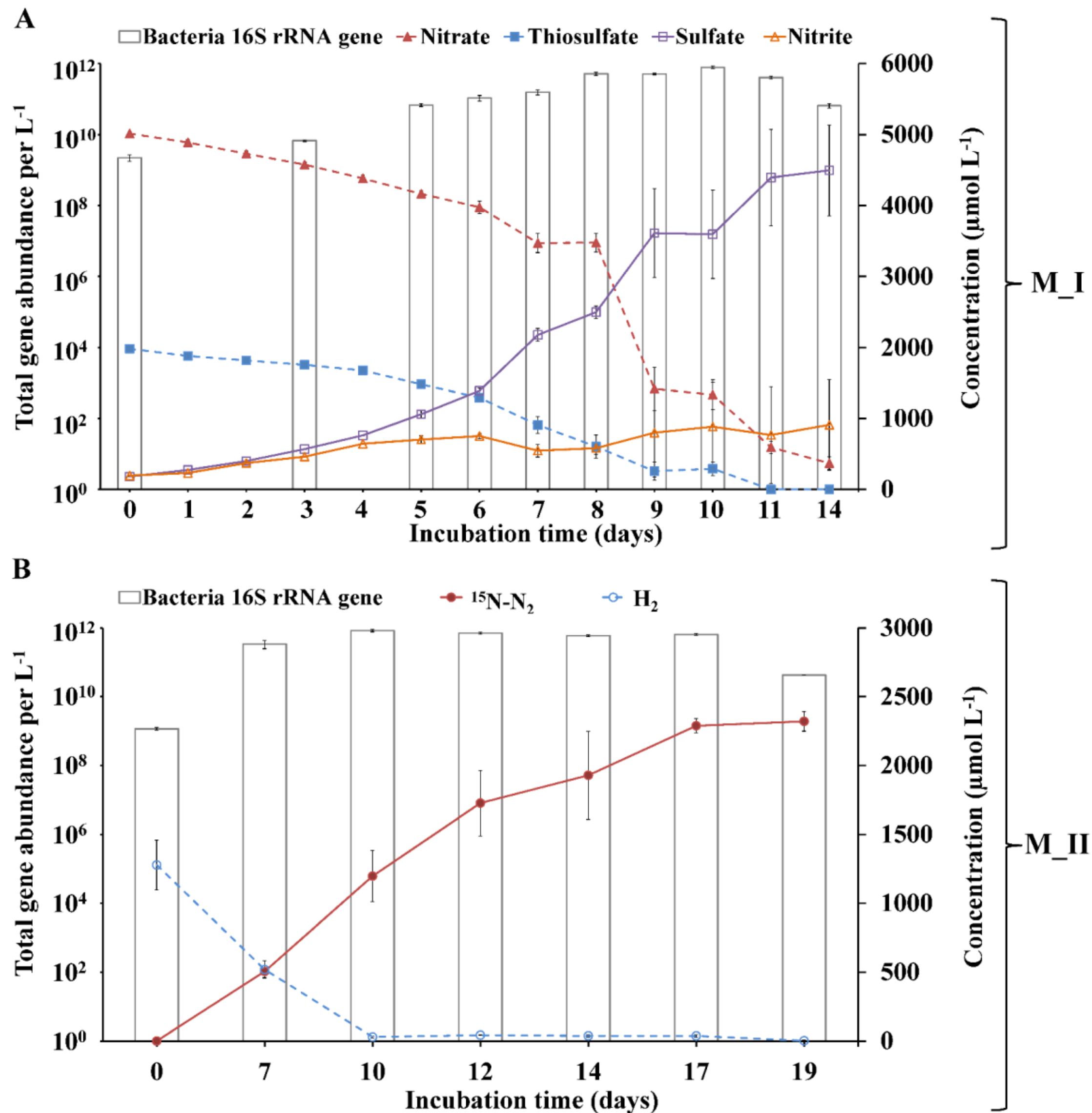


Figure 24 | Chemolithoautotrophic growth and metabolic activity of denitrifying enrichment culture 1E in batch cultures at 15 °C. Microcosm experiments (A) M_I and (B) M_II. All datapoints for observed chemistry and gases shown in (A) and (B) are the means of three biological replicates where as bacterial 16S rRNA gene abundance data shown are the means of 9 replicates (3 biological with 3 technical each); error bars are SEMs.

Nitrous oxide was not detected nor were significant changes in the CO_2 concentrations detected during the incubation period. The pH of the microcosms remained in the range of 7.5±0.2 while the pressure was in the range of 1.2 – 1.4 bar in all serum bottles throughout the incubation period **(Appendix Figure 32)**. I did not observed any formation of ^{15}N-N_2 or N_2O in the non-inoculated controls.

The total bacterial population (**Figure 24 A, B**) increased by about two orders of magnitude during the two incubation experiments (14 and 19 days, respectively). During microcosm experiment M_1, bacterial 16S rRNA gene abundances showed increasing abundances from 2.2 x 10^9 genes L^{-1} culture on day 0 to maxima of 7.8 x 10^{11} genes L^{-1} on day 10. A similar two-fold increase was observed during microcosm experiment M_II having1.2 x 10^9 genes L^{-1} culture on day 0 to maxima of 8.4 x 10^{11} genes L^{-1} on day 10.

Although no significant morphological plan could be attributed to specific single microbial cell in enrichment culture, SEM micrographs of enrichment culture under chemolithoautotrophic denitrifying conditions revealed less dense cocci cells of 556 nm in size co-occurring with highly abundant rod cells. SEM micrographs corresponding to day 7 (A-D) and day 17 (E-H) of the enrichment culture showed less dense and less EPS encapsulated cells in the beginning of the microcosm experiment (M_II) (after 7th day), while after 17th day, the cells were densely packed with extracellular polymeric substance (EPS) **(Appendix Figure 33)**.

3.11.3 Transcriptional activity of the enriched consortium during the two microcosm experiments

Functional gene based denitrifying transcripts (*nirS* and *nosZ*) were also quantified during both microcosm experiments (M_I and M_II), which suggested strong control of electron acceptor/donor ratio on transcriptional activity.

During microcosm experiment M_I, a maximum of relative transcripts for *nirS*-containing denitrifiers was observed on day 9 (1536-fold increase compared to day 0). An increase in relative transcripts for *nosZ*-containing denitrifiers during microcosm experiment M_I, was also observed on day 8, albeit only 10-fold (maximum) compared to day 0 **(Figure 25A)**.

In contrast to that, during microcosm experiment M_II, relative transcripts for *nosZ*-containing denitrifiers showed strong transcriptional increase of 32000-fold on day 17 compared to day 0. Similarly relative transcription of *nirS*-containing denitrifiers also showed a maximum increase of 7862-fold on day 10 compared to day 0 **(Figure 25 B).**

Furthermore, a better measure for relative transcriptional activity analysis was calculated as cDNA/DNA ratio of the functional genes (**Figure 26**).

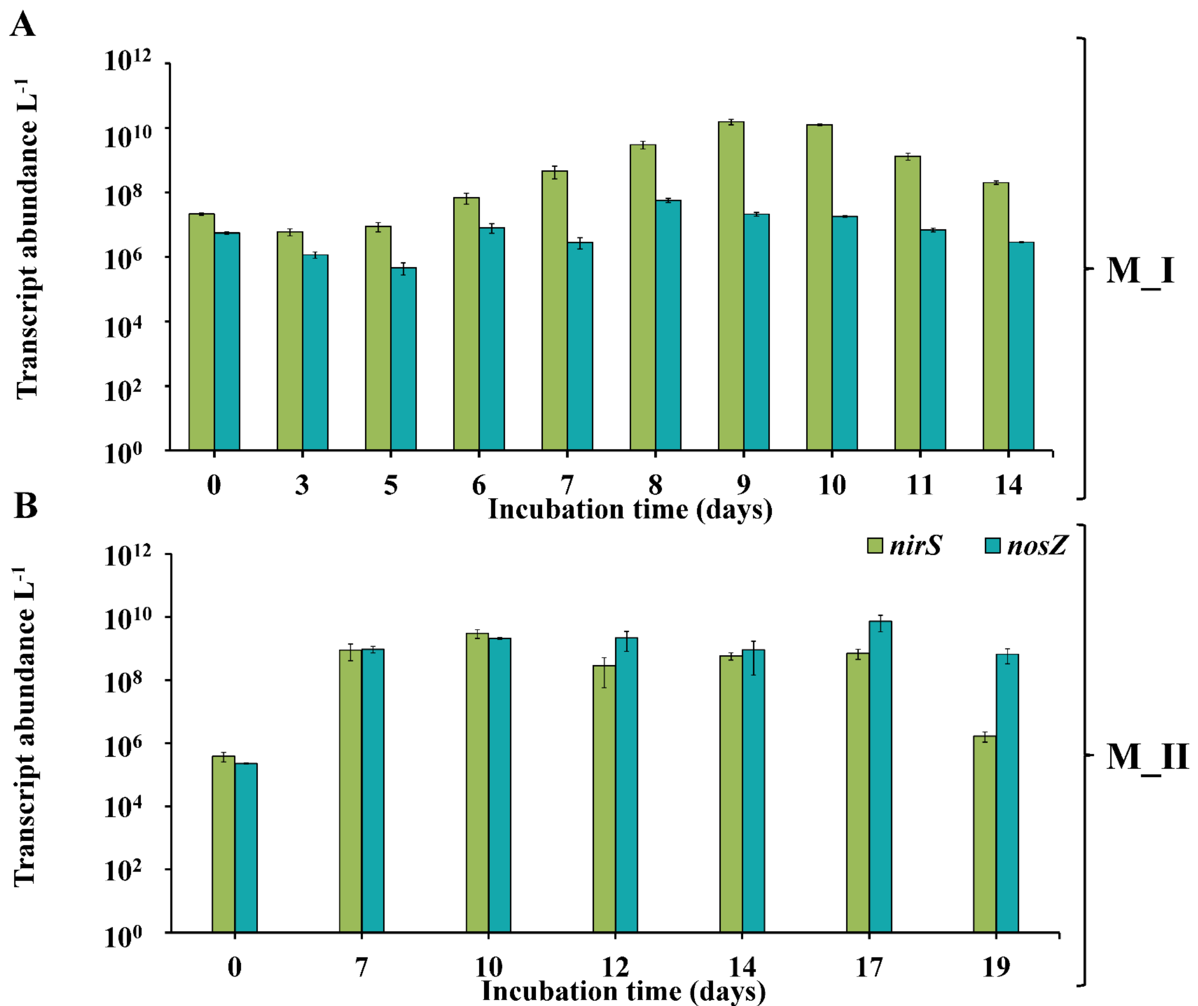


Figure 25 | Transcript abundances of *nirS* and *nosZ*-containing denitrifiers over time during the two microcosm experiments, M_I and M_II. Microcosm experiment (A) M_I and (B) M_II. All data points for observed transcripts are the means of nine replicates (3 biological with 3 technical each); error bars are SEMs.

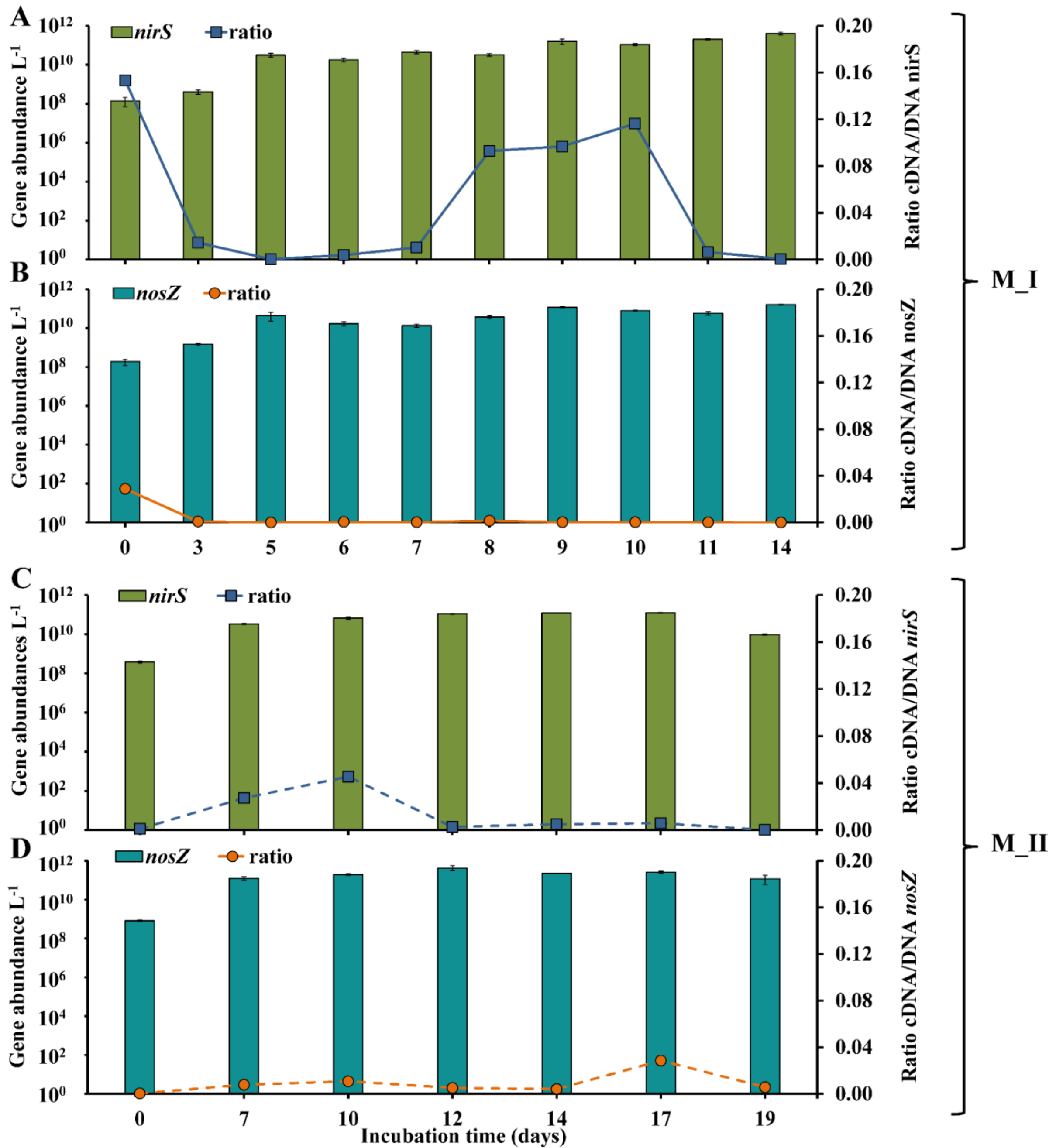


Figure 26 | Abundance and transcript/gene ratios of functional marker genes for denitrification (*nirS* and *nosZ*). On the primary vertical y-axis (left), total gene abundances represented by bars white on the secondary vertical right axis (right), transcript/gene ratios were represented by the line. (A) *nirS*; (B) *nosZ* based analysis from the microcosm experiment M_I (C) *nirS*; (D) *nosZ* from the microscosm experiment M_II. All datapoints for observed gene abundances are the means of 9 replicates (3 biological with 3 technical each); error bars are SEMs.

Based on the qPCR data, these enrichment cultures exhibited an estimated doubling time of *ca.* 28 hours. Gene abundances for *nirS*- and *nosZ*- containing denitrifiers ranged from 1.4 x 10^8 to 4.0 x 10^{11} and 2.0 x 10^8 to 1.6 x 10^{11} genes L^{-1} culture, respectively during the first microcosm experiment (M_I) **(Figure 26A, B)**. Similarly, during the second microcosm

experiment (M_II), gene copy numbers for *nirS*- and *nosZ*- containing denitrifiers ranged from 3.9 x 10^8 to 1.2 x 10^{11} and 8.2 x 10^8 to 4.6 x 10^{11} genes L^{-1} (**Figure 26 C, D**).
nirS transcript/gene ratios showed a 723-fold increase from day 5 to day 9 (from 2.8 x 10^{-4} to 2.0 x 10^{-1} corresponding to 8.8 x 10^6 transcripts L^{-1} at day 5 to a maximum of 3.3 x 10^{10} transcripts L^{-1} at day 9 respectively (**Figure 25 A, 26 A**). On the other hand, *nosZ* transcript/gene ratios were highest on day 0 (2.9 x 10^{-2}) and remained on a low level throughout the microcosm experiment (**Figure 25A, 26 B**).

Transcriptional activity of *nirS* during the microcosm experiment M_II showed a 258-fold (maximum) increase at day 10, corresponding to an increase in transcript numbers from 3.8 x 10^5 transcripts L^{-1} at day 0 to 3.02 x 10^9 transcripts L^{-1} at day 10 (**Figure 25 B, 26 C**). Transcriptional activity of *nosZ* revealed a gradual increase from 2.8 x 10^{-4} (day 0) to 2.9 x 10^{-2} (day 17) corresponding to 2.3 x 10^5 transcripts L^{-1} and 7.3 x 10^9 transcripts L^{-1}, cumulating to a maximum of 103-fold increase at day 17 (**Figure 25 B, 26 D**).

3.12 *Thiobacillus denitrificans as key transcriptionally active denitrifier in the consortium*

Analysis of metabolic activity exhibited by the denitrifying consortium was complimented with the identification of the transcriptionally active organisms at day 3 and day 9 of the first microcosm experiment (M_I), determined by cDNA-based sequence analysis of the *nirS*- and *nosZ*-transcripts clone library (211 clones in total).

T. denitrificans-related sequences accounted for 65 % of the potentially active *nirS*-type denitrifying community in the early stage of the incubation, while this fraction decreased to 24 % of the potentially active population on day 9 (**Figure 27**).

Fractions of *nirS* transcripts related to *S. denitrificans* decreased slightly whereas those related to *S. hydrogenivorans* increased more than five times by day 9. Similarly, fractions of *nirS* transcripts related to *D. suillum* PS increased. In contrast, *nirS*-transcripts related to *Dechlorospirillum* sp. Bh37 (2% at day 3) were absent at day 9 of the incubation while *D. aromatica* RCB like sequences were absent on day 3 but appeared (17 %) on day 9.

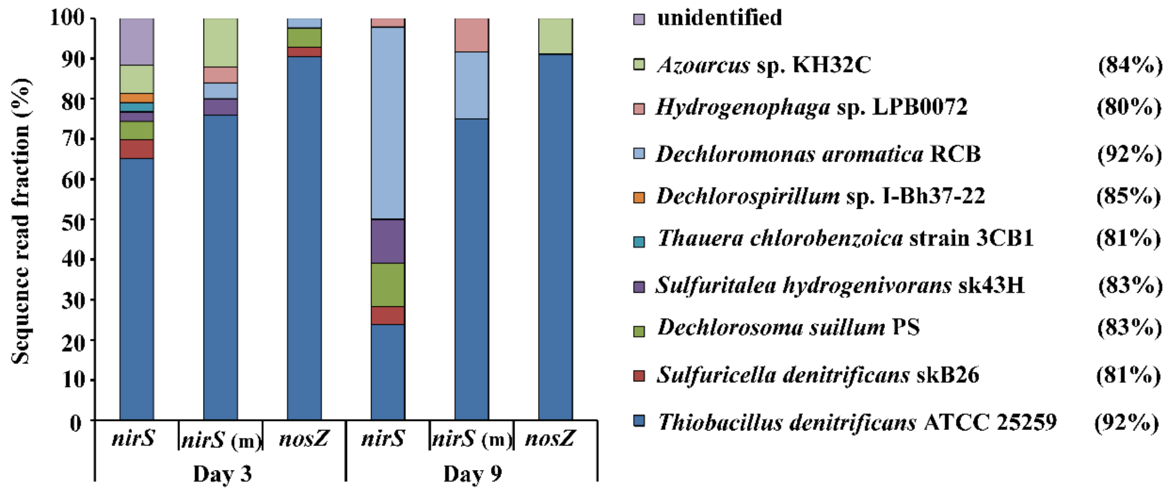


Figure 27 | Transcriptionally active denitrifiers based on *nirS*- and *nosZ*- sequences during microcosm experiment M_I Sequence identity to closest cultured relative given in parentheses. Bar labelled as [*nirS* (m)] represents *nirS* sequences obtained with a modified *nirS* primer set (nirS1F and nirS3R-m; Table 2).

Results obtained with a modified *nirS* primer set confirmed the predominance of *T. denitrificans*-related *nirS* transcripts (83 % and 75% at day 3 and day 9, respectively). *Azoarcus*-related *nirS* transcripts accounted for 13% at day 3 but were not detectable on day 9. *D. aromatica*-related sequences were found to increase more than four times (from 4% to 17%). *Hydrogenophaga*-affiliated sequences showed a two-fold increase from 4 % to 8 %. Transcript clone libraries based on *nosZ* further confirmed *T. denitrificans* as transcriptionally active member in the denitrifier community at both day 3 and day 9 (90 % and 91% of all sequenced *nosZ* transcripts resspectively) while *Azoarcus*-related sequences were not detected at day 3 but accounted for 9 % on day 9. Sequences related to *D. suillum*, *D. aromatica*, *S. denitrificans* corresponded to 5, 2 and 2 %, respectively, at day 3 but were not detected any more on day 9.

3.13 Shifts in community composition during the microcosm experiments

In order to check if the communities in the enrichment changed substantially during the two microcosm experiments or between the two microcosm experiments, the consortium community was analysed by 16S rRNA Illumina sequencing and clone library construction. At day 0 of both microcosms, 52 % of the community was dominated by members of the *Betaproteobacteria*, whose fraction increased to 87 and 71 % at day 14 and day 19 respectively **(Appendix Figure 34)**. The second most abundant class was *Alphaproteobacteria*, which decreased from 21 to 10 % during first microcosm experiment

and from 28 to 18 % during the second experiment, respectively. Organisms affiliated to *Bacteroidetes* changed from 23 to 1 % and 7 to 9 % during microcosm experiments M_I and M_II, respectively. Furthermore, genus level investigation revealed that nearly 60 % of the sequences were affiliated with sulfur oxidising bacteria from the genus *Thiobacillus*, consisting of *T. thioparus* and *T. denitrificans* species (**Figure 28**). During microcosm experiment M_I, fractions of *T. thioparus* related sequences were on the wane from 37 to 9 % at day 0 and day 14 respectively. On the contrary, the fractions between two time points during experiment, M_II were nearly similar (43 to 45 %). Sequence fractions related to *T. denitrificans* always showed an increase in both microcosm experiments, where they radically increased from 2 to 41 % during M_I and from 2 to 12 % during M_II. *Rhizobium selenitireducens* showed a decrease of sequence fraction from 18 to 7 % during the M_II, 16 to 12 % during the M_II experiment. Percentage fractions of the sequences related to *Lutibacter maritimus* showed a reduction from 21% to 1 % during M_I, whereas during M_II, they showed a moderate increase from 2 to 7 %. Contrasting results were revealed for *Hydrogenophaga* sp. related sequences, which showed an increase of 7 to 16 % during microcosm experiment M_I, while sequence fractions decreased from 3 to 0 % during M_II. *Ferribacterium limneticum* like sequences showed a moderate increase in both experiments M_I, and _II, with sequence fractions increasing from 1 to 11 % and 1 to 6%.

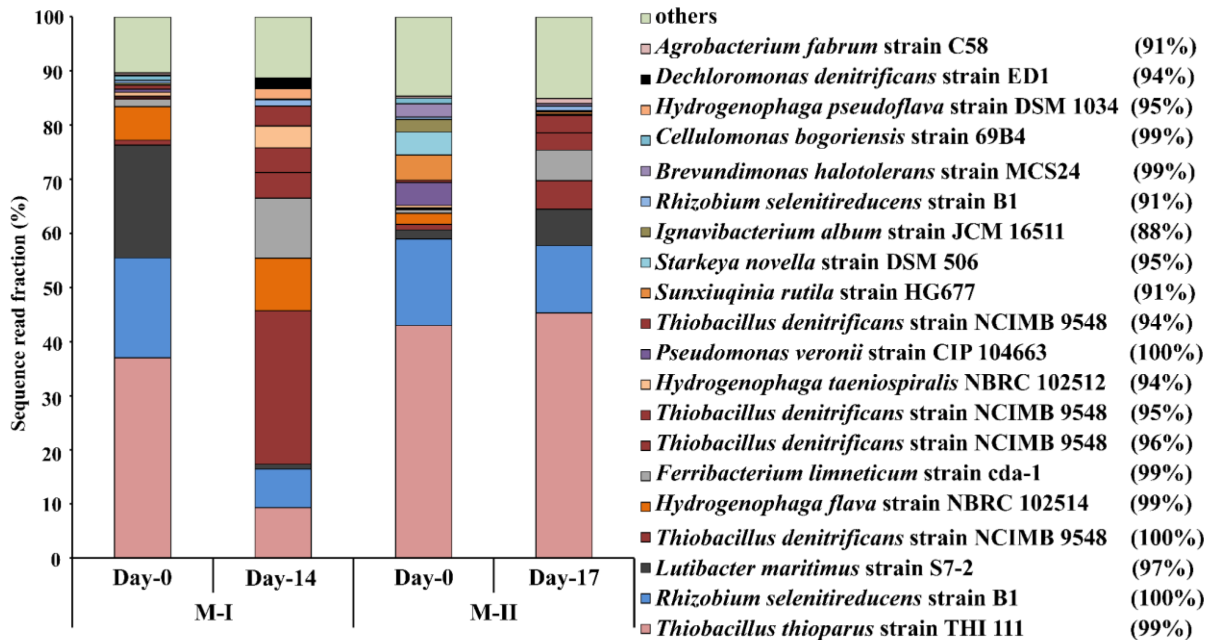


Figure 28 | Changes of the denitrifying microbial community (1E, species level) growing under chemolithoautotrophic conditions with different nitrate/thiosulfate ratios: 2.5 and 1, utilised in microcosm experiment M_I and M_II, respectively. MiSeq amplicon sequencing based on 16S rRNA genes. Each bar represents the community composition of the denitrifying consortium at a particular time point where the DNA was extracted from triplicate cultures.

3.14 Taxonomic classification of pure mixotrophic and heterotrophic denitrifying isolates

Based on 16S rRNA gene sequence analysis, 100 pure denitrifying strains were classified as belonging to three classes of the *Proteobacteria* phylum (*Gamma>Alpha>Betaproteobacteria*; the number of isolates 69, 18, 14 respectively) and only one was classified as *Actinobacteria* phylum **(Figure 29).** 63% of the pure culture isolates were classified as *Gammaproteobacteria*, were members of the genus *Pseudomonas* (within the order *Pseudomonadales*, along with genus *Stenotrophomonas* within the order *Xanthomonadales*, *Aeromonas* within the order *Aeromonadales*. Among *Alphaproteobacteria*, the isolates belonged to two genera within two orders: *Brevundimonas* (*Caulobacterales*) and *Rhizobium* (*Rhizobiales*). *Betaproteobacteria* isolates were belonged to two genera within one order: *Acidovorax* and *Janthinobacterium* (*Burkholderiales*). All isolates showed 97-100% sequence similarities to cultured representatives on the 16S rRNA gene sequence level. Furthermore, denitrification genotype was identified for selected groundwater isolates as shown in the table below.

Table 3 | Detection of denitrification genes in groundwater isolates

Isolate	Origin	Denitrification gene[a]		
		nirS	*nirK*	*nosZ*
[*]2_*Acidovorax defluvii*	**H41**	+	-	+
7_*Micrococcus aloeverae*	H51	+	-	+
16_*Acidovorax delafieldii*	H32	+	-	+
18_*Pseudomonas veronii*	H31	+		+
21_*Stenotrophomonas rhizophilla*	H41	+	-	-
35_*Pseudomonas chloritidismutans*	H32	+	-	+
48_*Rhizobium selenitireducens*	H42	+	+	+
113_*Acidovorax defluvii*	H32	+	-	+
129_*Acidovorax defluvii*	H41	+	-	+

[a] (+) positive reaction; (-) negative reaction

[*]strain further used as a model organism for heterotrophic denitrification

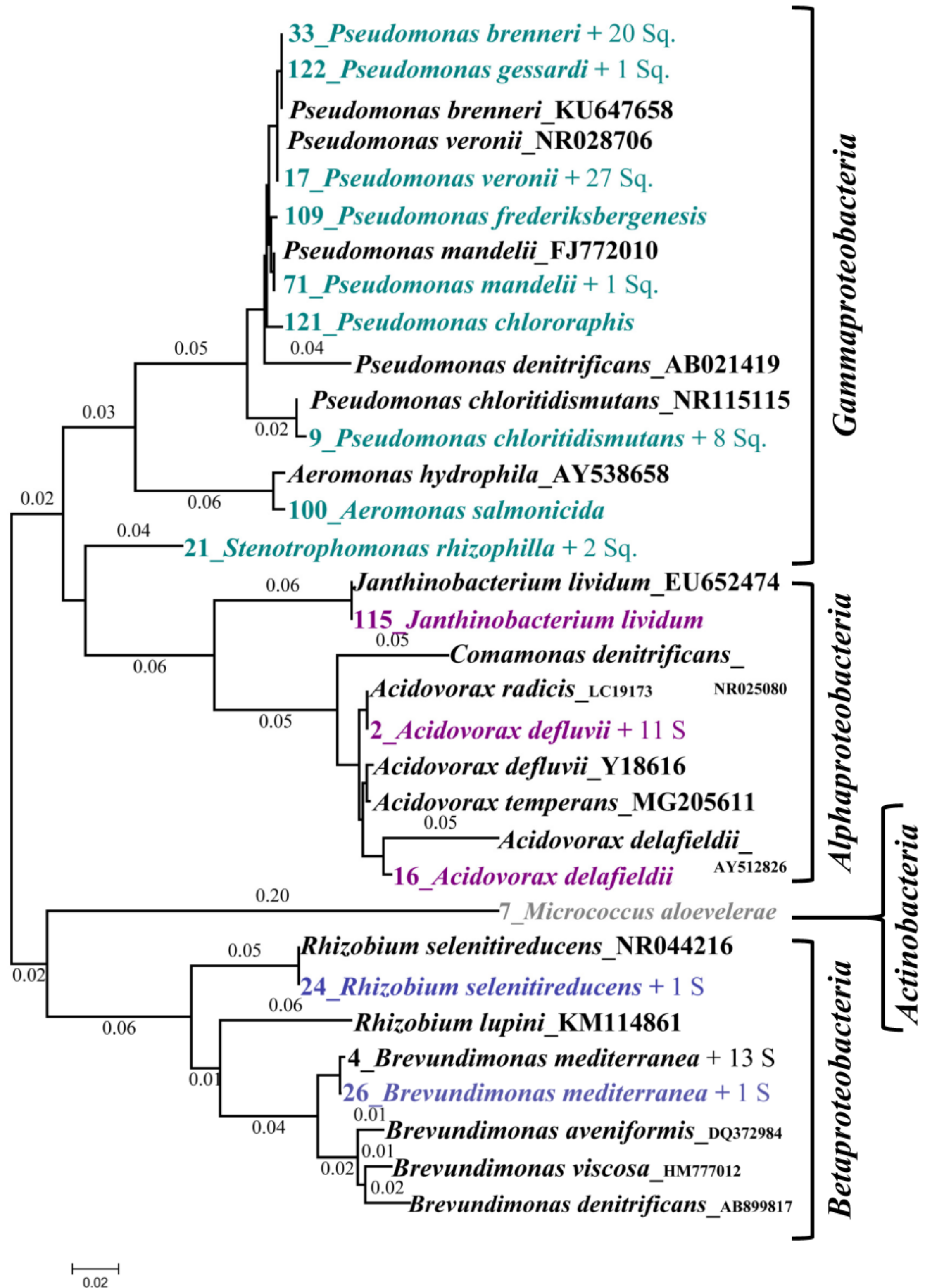


Figure 29 | Unrooted neighbour-joining phylogenetic tree of 16S rRNA sequences of the groundwater denitrifying isolates. Isolated strains from groundwater are in colour and "+ X S" represents the number of additional similar sequences (isolates with the same taxonomy). The scale bar indicates the number of changes per sequence position.

3.14.1 Heterotrophic denitrification by pure culture of strain 2_Acidovorax defluvii

Denitrification potential of a pure culture of 2_*Acidovorax defluvii* **(Table 3)** was assessed in an approach similar to the test conditions used for microcosm experiments M_I and M_II. The only difference to the previous microcosm experiments was the utilization of butyrate (2 mmol L^{-1}) as electron donor as well as carbon source with a N/C ratio of 1 (2 mmol Nitrate L^{-1}).

Isolated bacterial strain, 2_*Acidovorax defluvii* clearly showed utilization of nitrate (100 %) along with the production of nitrite during the microcosm assay during a 10 days incubation period **(Figure 30)**. Complete utilization of nitrate and nitrite production upto 2 mmol was observed at the end of incubation period of 10 days. Rates of nitrate reduction and nitrite production were calculated as 192 µmol L^{-1} d^{-1} and 215 µmol L^{-1} d^{-1}, respectively using the least square method from the data points of day 1, 3 and 5 of the incubation time.

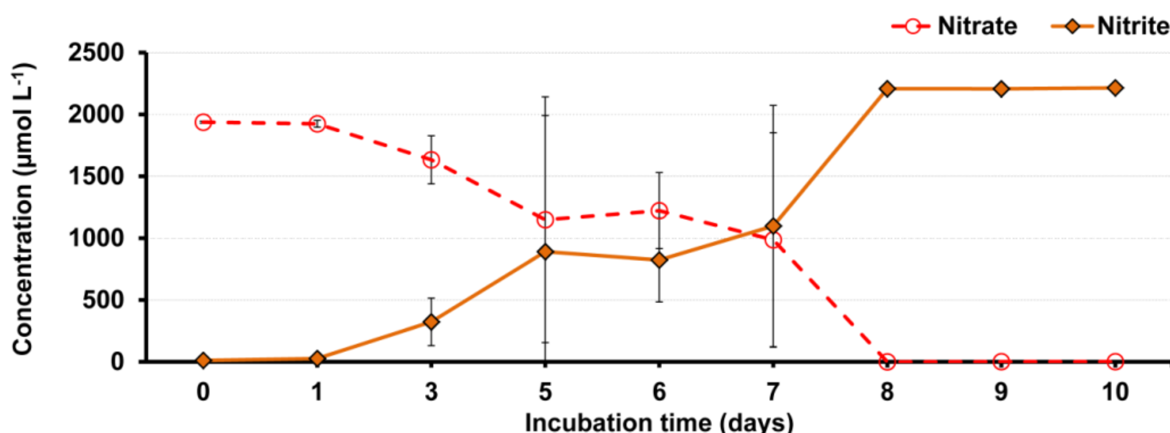


Figure 30 | Heterotrophic growth of pure culture strain 2_*Acidovorax defluvii* under denitrifying conditions at 15° C. Graph showing the consumption of nitrate and production of nitrite when bacteria were grown on nitrate as electron acceptor (2 mmol L^{-1}) and butyrate as electron donor as well as carbon source (2 mmol L^{-1}). All datapoints for observed chemistry are the means of three biological replicates; error bars are SEMs.

4. DISCUSSION

In this doctoral research work, in order to get a first overview over the natural potential of anammox and denitrification, two key processes responsible for nitrogen loss, in the form of N_2, were targeted in groundwater sampled from oligotrophic and organic carbon limited carbonate–rock aquifers. I utilized ^{15}N based stable isotope labeling incubations, quantification of molecular markers and diversity analysis using functional genes and transcripts based evidence to address their potential role under natural conditions. Furthermore, the possibility of aerobic and anaerobic ammonium oxidizer co-occurrence in suboxic aquifers was also addressed by targeting functional genes and transcripts from the groundwater samples. First part of the discussion build on the argumentation, that chemolithoautotrophic anammox could be the dominant process for nitrogen loss in oligotrophic aquifers. These results were complemented with ladderane lipid based biomarker studies by Schwab *et al*. (2017) and reconfirmed in Kumar *et al*. (2017), demonstrating not only the presence but also the active role of anammox bacteria in mediating nitrogen attenuation in groundwater under natural conditions. This study provided anammox rate measurements complemented with functional gene based molecular evidence for the presence of a diverse assemblage of anammox bacteria at the Hainich site, and marks the first combined isotope- and molecular-based confirmation of reactive nitrogen attenuation activity mediated by anammox communities in pristine aquifers (Kumar *et al*., 2017).

In the second part of the discussion, I utilised traditional microbiological approaches complemented with functional gene and transcripts based molecular analytical tools. Although, a high relevance for autotrophic denitrification in these aquifers mediated by reduced sulfur, iron and hydrogen has been reported by Herrmann *et al*. (2017), first evidence for the importance of the denitrification process was demonstrated by isotopic ^{15}N measurements together with anammox measurements. Further lines of evidence include:

(i) The cohort and reoccurring presence of chemolithoautotrophic denitrifiers in anoxic as well as oxic wells followed by targeting *nirS*-containing denitrifiers at six sampling time points during the study period,

(ii) Successful enrichment of chemolithoautotrophic microbial communities with the metabolic potential for denitrification revealed through quantitative detection of denitrification specific metabolic products utilising reduced sulfur and hydrogen as electron donors and complemented with *nirS* and *nosZ* gene and transcript analysis.

4.1 Nitrogen loss is primarily driven by anammox processes in carbonate-rock aquifers of the Hainich CZE

The relative concentrations of ladderane lipids and reoccurring distribution patterns pointed to the existence of potential active sites of anammox in suboxic to anoxic groundwater of the wells H52 and H53 of the Hainich CZE (Schwab *et al.,* 2017). These patterns not only complement the occurrence of the active anammox process at rates of 3.5 to 4.7 nmol N_2 L^{-1} d^{-1} in the suboxic groundwater of well H53 but also supported my hypothesis of its high relevance compared to denitrification for the removal of fixed nitrogen at an estimated contribution of 83 %. Neighbouring well H52, sharing a high similarity of hydrochemical characteristics with the suboxic groundwater of well H53, was also characterized to strongly support anammox with 31% of the identified proteins found being, related to *Candidatus* Brocadiales (Starke *et al.*, 2017). Together with 16S rRNA-targeted Illumina sequencing, where anammox-bacteria represented the high fraction among *Planctomycetes phylum, two* orders of magnitude higher *hzsA* compared to *nirS* gene transcripts, suggested a strong support for these wells being an anammox hotspot within carbonate-rock aquifers. Nitrite, an electron acceptor utilised in the anammox process, usually remained below the detection limit of 0.1 µM throughout the study period, as was also reported by Opitz *et al.* (2014) in earlier studies from these aquifers, suggesting that nitrite was most likely subjected to high turnover and could be a limiting factor for in situ anammox activity. That being said, I cannot rule out that addition of nitrite in the anammox and denitrification assays might have additionally stimulated anammox but also denitrification activity.

Anammox rates observed in our study are similar to average rates reported from off-shore marine oxygen minimum zones (e.g., 1.9 nmol N_2 L^{-1} d^{-1} and 3.0 nmol N_2 L^{-1} d^{-1}; Dalsgaard *et al.,* 2012, Kalvelage *et al.,* 2013) but were noticeably lower than anammox activities measured in groundwater contaminated with nitrate or ammonium (Table 3). The close similarities in anammox contributions and rates observed to some of these anoxic water regimes are also reflected by abundances of anammox bacteria (10^6-10^7cells L^{-1}) which were found quite comparable to the cell numbers found in our study (Kuypers *et al*, 2003, 2005; Schubert *et al*, 2006; Galán *et al*, 2009). Other freshwater anoxic regimes, like lake water, have been reported to exhibit higher anammox rates ranging from 16-540 nmol N_2 L^{-1} d^{-1}, contributing an estimated 13-50% of the total N_2 production, whereas the remainder was attributed to denitrification (Schubert *et al*, 2006; Hamersley *et al*, 2009). While the

contribution of anammox was found to be lower (18-41 %) in groundwater at DOC concentrations up to 30 mg L^{-1} and surface flow constructed wetlands as observed by Moore *et al.* (2011) and Smith *et al.* (2015), a high contribution of anammox to nitrogen loss under oligotrophic conditions agrees with findings from oceanic oxygen minimum zones (Lam and Kuypers, 2011) and with reports from ammonium-contaminated aquifers at low organic carbon availability (<2.6 mg L^{-1} DOC, Smith *et al.,* 2015) (**Table 4)**.

Table 4 | Anammox and denitrification rates in marine and freshwater environments summarized in Kumar *et al*. (2017).

Study site	NH_4^+	NO_3^-	Anammox	Denitrification	References
	(µmol L^{-1})		(nmol N_2 L^{-1} d^{-1})		
marine anoxic basin	0.2	7.2	24-480 (19-35%)	12-2,568	Dalsgaard et al., 2003
marine oxygen-deficient water	<0.05	<40	0.4-27 (74-100%)	0.4	Thamdrup et al., 2006
marine oxygen minimum zone	<0.1	<30	1-21 (35%)	3-190	Dalsgaard et al., 2012
marine oxygen minimum zone (coastal)	0.25-0.5	<50	2.8-227 (30%)	2.2-5.4	Kalvelage et al., 2013
freshwater lake	0-53	<0.1-10	24-240 (9-13%)	498-2,322	Schubert et al., 2006
nitrate-contaminated groundwater	10.6-145	3,000-7,172	N/D	387,000-465,000	Tobias et al., 2001
Fertilizer-contaminated groundwater (DOC up to 30 mg L^{-1})	0.5- 19,680	3.2-3,854	~319-751, (18-36%)	N/D	Moore et al., 2011
ammonium-contaminated groundwater (DOC <1.0 mg L^{-1})	0-47	0-209.4	9.1-458, (39-90%)	1.0-662	Smith et al., 2015
Carbonate-rock aquifers (DOC <1.8 mg L^{-1})	3.4-30	12-572	3.5-4.7, (83%)	0.7	Kumar et al., 2017

Therefore, the observed high relevance of anammox versus denitrification in the oligotrophic carbonate-rock aquifers of the Hainich CZE was most likely shaped by low availability of organic carbon. In fact, carbon isotope-based studies in the groundwater of wells H52 and H53 point to a tight internal cycling of carbon including oxidation of sedimentary old organic matter depleted in both ^{13}C and ^{14}C, and chemolithoautotrophically mediated isotopic DIC signatures of a subsequent refixation of ^{13}C- and ^{14}C-depleted CO_2 (Nowak *et al.,* 2017). Similarly, a substantial contribution of anammox bacteria and their mediated processes to in situ autotrophic CO_2-fixation was confirmed in the same wells by Schwab *et al.* (2017), reflected by a strong depletion of phospholipid [3]- and [5]-ladderanes in ^{13}C ($\delta^{13}C$ values ranging from −48.0 ± 10.5 ‰ to −45.9 ± 11.7 ‰), indicative of active CO_2-fixation via the acetyl-CoA-pathway and characteristic of anammox bacteria (Schouten *et al.,* 2004). Furthermore, disconnection of these suboxic groundwater wells from surface-derived organic carbon input was emphasized due to thick overlying soils, low infiltration potential, and low hydraulic conductivities with estimated groundwater travel times up to 587 years (Kohlhepp *et al.,* 2017, Nowak *et al.,* 2017). Given that rate of water passage through aquifers is

sufficiently slow, this might permit equilibration of CO_2 with the surrounding rock matrix as well (Baines and Worden, 2004). Sieburg and colleagues (2017) also revealed higher $\Delta CO2$ concentrations (ranging from 0.80 to 1.21 vol%) than those in ambient air at Hainich aquifers, especially at site H5 and suggested the lateral transport of carbon dioxide by water. Furthermore, Sieburg *et al*. (2017) also proposed that gas atmosphere in these subsurface layers was mostly determined by the corresponding carbonate-rock aquifers and soil/rock properties. Thus, anammox bacteria, being autotrophic, make use of this inorganic carbon as the carbon source for the production of biomass.

4.2 Co-occurrence of gene transcripts of aerobic and anaerobic ammonium oxidizers suggest potential coupling of aerobic and anaerobic ammonia oxidation in suboxic groundwater

Nitrite and nitrate are the products of partial and complete nitrification, respectively, serving as substrates for anammox and denitrification. Partial bacterial nitrification and archaeal nitrification are considered in several ecosystems, especially marine ecosystems, as the source from which anammox receives nitrite (Zhu *et al*., 2011; Kuypers *et al*., 2005; Lam *et al*., 2007, 2009). Under suboxic conditions, nitrite can be available to the anammox process from nitrate reduction by anammox bacteria or other nitrate reducers but also from incomplete nitrification. Groundwater of suboxic well H53 showed oxygen concentrations of 0.73 ± 0.7 µmol L^{-1}, which might provide supportive conditions for the coupling between aerobic and anaerobic ammonium oxidation as shown for similar hypoxic/suboxic ecosystems such as marine oxygen minimum zones (Lam *et al.,* 2007, 2009, Lam and Kuypers, 2011) or recently also for intertidal sediments (Fernandes *et al.,* 2016) and aquifer soil cores obtained from the phreatic zone (Wang *et al*., 2017). Transcripts of archaeal and bacterial *amoA* genes detected in the suboxic groundwater of well H53, further suggested ongoing aerobic ammonia oxidation *in situ*. Previously, Opitz *et al*., 2014 reported nitrification rates of 10.4 to 14.4 nmol NO_x L^{-1} d^{-1} from the oxic well at H41, which agrees with relatively high *amoA* transcriptional activity observed in the present study. Negative correlation of anammox and aerobic ammonia oxidizers related gene and transcripts to groundwater oxygen concentration across sites further posited that oxygen availability is an important factor driving the predominance of anammox versus aerobic ammonia oxidation. Anammox being favoured over aerobic ammonia oxidation at wells H52, H53 and the least at well H41, also comes in support. This study could have been improved with the inclusion of additional time points for targeted functional gene transcripts as well as ^{15}N isotopic labeling

rate measurements and future studies assessing nitrification and anammox activity in parallel are needed to get a more comprehensive insight into the potential coupling between aerobic and anaerobic ammonium oxidation.

4.3 Transcriptionally active anammox bacteria in oxic and anoxic groundwater of the two aquifer assemblages

In contrast to previous studies from groundwater ecosystems (mostly organically contaminated sites), which provide limited functional gene biomarker based evidence of anammox bacteria (Smits *et al*., 2009; Moore *et al*., 2011), its clearly demonstrated, here, the ubiquitous presence of anammox bacteria along the two limestone aquifer assemblages of the Hainich CZE, albeit at a variation of *hzsA* gene abundances by four orders of magnitude across all groundwater wells of *hzsA* gene abundances. Although anammox bacteria in marine waters, dominated by *Ca.* Scalindua are reported to be reversibly inhibited by oxygen concentrations higher than ~10 µmol L^{-1} (Jensen *et al.,* 2011, Dalsgaard *et al.,* 2014), surprisingly, this study revealed *hzsA* gene abundances in the range of 2.3 x 10^5 – 3.7 x 10^6 L^{-1} and high *hzsA* transcriptional activity also in the oxic groundwater of wells of the lower aquifer assemblage. Although anammox bacteria are not famous for ubiquitous presence but rather reflect the specific ecological requirements of oxygen limited environments, they also depend on the concomitant presence of both oxidised and reduced inorganic nitrogen compounds, to which Humbert *et al*. (2010) suggested that oxic/anoxic interfaces in terrestrial ecosystems could provide suitable niches for anammox bacteria. It is not yet clear though, if freshwater and particularly groundwater anammox bacteria could really flourish and carry out anaerobic ammonium oxidation in the presence of considerable concentrations of dissolved oxygen (111-429 µmol L^{-1}). Association with aerobic heterotrophs or close proximity to aerobic ammonia oxidisers might provide microenvironments at reduced oxygen concentrations such as in the CANON (Completely autotrophic nitrogen removal over nitrite) process and in natural ecosystems (Third *et al*., 2005; Fernandes *et al.,* 2016). Also, contributions of anammox up to 58 % to in situ N_2 production in permeable riverbeds at an oxygen concentration of 134 ± 14 µmol L^{-1} were reported by Lansdown *et al.* (2016), providing evidence against the restriction of the anammox process to anoxic sites only. Among the four candidate genera of anammox bacteria detected in the Hainich aquifer assemblages, *Ca.* Brocadia, mostly described from terrestrial habitats (Humbert *et al.,* 2010, Hirsch *et al.,* 2011), was encountered as the dominant anammox representative in the communities. Closely related organisms to *Ca.*

Brocadia, *Ca.* Brocadia sinica has been reported to cope with elevated oxygen concentrations of up to 63 µmol L^{-1} (Oshiki *et al.,* 2011), which could be the reason of their presence in oxic water.

4.4 Low denitrification rates in suboxic groundwater of Hainich aquifer assemblages

Low denitrification rates in the present study (0.7 nmol N_2 L^{-1} d^{-1}) were similar to rates reported from labile carbon limited groundwater from contaminated aquifers (1.0 nmol N_2 L^{-1} d^{-1}) containing high inorganic nitrogen (209.4 µM NO_3^-, 471 µM NH_4^+). Higher rates (1–662 nmol N_2 L^{-1} d^{-1}) compared to the observed denitrification rates, were observed in other sites' samples not limited with labile carbon (Smith *et al*, 2015) **(Table 3)**. Depending upon the low spatial subsidies of DOC (3.09 mg L^{-1}) reported from UK aquifers, Gooddy and Hinsby, (2008) suggested that autotrophic and lithotrophic opportunists may dominate over the heterotrophic microbial community and therefore processes may primarily be mediated by them. Given the availability of reducing conditions and inorganic nitrogen in the Hainich, limited organic carbon is mainly responsible for constraining denitrification potential. Transcript numbers of *nirS* being up to three orders of magnitude (10^3) lower than *nirS* gene copy numbers (10^6) also indicate that the *nirS* type denitrifiers were abundant but not highly transcriptionally active. Observed rates of denitrification fell in the lower range of reported rates, ranging between 100-350 nmol N_2 L^{-1} d^{-1} from contaminated groundwater (DeSimone and Howes, 1996; Tobias *et al*, 2001), 74 nmol N_2 L^{-1} d^{-1} from the lake water (Hamersley *et al*, 2009) and 2.2 – 5.4 nmol N_2 L^{-1} d^{-1} from OMZ water (Kalvelage *et al*, 2013). Observed anammox rates and its relative importance agree with observations in other anoxic regimes, too, where the driving force was availability of organic matter (Trimmer and Nicholls, 2009, Moore *et al*, 2011, Ward, 2013; Smith *et al*, 2015). In fact, large fractions of potential autotrophic denitrifiers oxidizing reduced sulfur compounds, hydrogen, or reduced iron such as the genera *Sulfurifustis* (Kojima *et al.,* 2015), *Sulfuritalea* (Kojima *et al.,* 2011), *Sulfuricella* (Kojima *et al.,* 2010), or *Sideroxydans* (Emerson and Moyer, 1997), were observed in the groundwater *nirS*-type denitrifier communities, confirming previous observations of a high genetic potential for sulfur-driven autotrophic denitrification in the aquifers of the Hainich CZE as suggested by Herrmann and colleagues (2015, 2017). However, the low *nirS* transcriptional activity in the groundwater of wells H52 and H53 pointed to low in situ denitrification activity. Similar to our results of higher transcriptional activity of the anammox *hzsA* genes compared to denitrifying *nirS* genes, prevalent transcriptional activity of the genes involved in anammox (hydrazine oxidoreductase, *hzo*)

compared to denitrification (nitrous oxide reductase, *nosZ*) in ammonium contaminated aquifers was detected by Smith *et al*. (2015). There, anammox was reported to contribute up to 90% of potential N_2 production at an organic carbon limited site. In addition to low availability of suitable organic carbon, a second factor favoring anammox over denitrification was most likely the constantly low groundwater temperature around 10 °C. A study by Dalsgaard and Thamdrup (2002) and Rysgaard and Glud (2004) revealed that the temperature optimum of anammox is lower that of denitrification and anammox could be favoured in cold environmental conditions. The adaption to lower temperatures of anammox bacteria in lab-scale bioreactors (Dosta *et al*, 2008; Hu *et al*, 2013; Lotti *et al*, 2014) revealed a pronounced effect after performing a temperature gradient assay in the range of 10-35°C to analyse anammox activity. Low temperature of the groundwater therefore could support anammox bacterial communities and might be one reason of high anammox to denitrification activity in our groundwater system.

4.5 Reoccurring large fractions of potential autotrophic denitrifiers oxidizing reduced sulfur compounds, hydrogen, or reduced iron in suboxic groundwater of the Hainich aquifer assemblages

Hypoxic groundwater at the upper aquifer assemblage (well H53) was found to harbor a stable consortium of sulfur oxidizing obligate chemolithoautotrophs related to *Sulfurifustis variabilis* and *Sulfuricella denitrificans.* Genomic data previously indicated the nitrate reduction potential of *S. variabilis* and its ability to fix inorganic carbon under different concentrations of carbon dioxide and/or oxygen based on the presence of *rbcL* and *rbcS* genes (Umezawa *et al*., 2016). *S. denitrificans* is known to oxidize reduced sulfur-compounds as well as H_2 while mediating complete denitrification. Moreover, it possesses the genes for two forms of RubisCO, encoded by *cbbL* and *cbbM* genes, providing further support for its autotrophic metabolism (Kojima and Fukui, 2010; Watanabe *et al*., 2014). *Sulfuritalea hydrogenivorans,* which dominated the *nirS*-type denitrifier community in anoxic well H43, was described as a facultative chemolithoautotroph, capable of growing on reduced sulfur compounds and H_2 under denitrifying conditions (Kojima and Fukui, 2011). Similarly, *Rubrivivax gelatinosus* (36 %), has been reported to oxidize thiosulfate, H_2 and diverse organic acids (Hu *et al*., 2012; Nagashima *et al*., 2012; Wawrousek *et al*., 2014). In habitats such as oligotrophic groundwater, few chemolithotrophs mediating nitrate reduction along with the oxidation of inorganic sulfur and hydrogen have been reported to grow mixotrophically as well (Kuenen and Robertson, 1984, Canfield *et al*., 2010; Kojima *et al*.,

2011; Anantharaman *et al*., 2013; Lau *et al*., 2016). *Sulfurifustis variabilis* and *Sulfuritalea hydrogenivorans*-related denitrifiers were less abundant in the oxic lower aquifer assemblage while denitrifiers related to *Pseudomonas* sp. A3 were more dominant, denitrifiers known to perform aerobic denitrification (Lawal *et al*., 2016; Xu *et al*., 2017). Previous investigations suggested faster water infiltration as well as high flow rates along with surface input signals of freshly introduced organic carbon for wells H41, H51, and H43 compared to well H53 (Kohlhepp *et al*., 2017; Nowak *et al*., 2017), which might result in conditions supportive of a fluctuating, mixotropic and heterotrophic denitrifier community in wells H41, H51, and H43. In contrast, DIC isotopes indicated the oxidation of organic matter derived from chemolithoautotrophic organisms and thus a stronger internal C cycling with a higher contribution of chemolithoautotrophy for groundwater well H53 (Nowak *et al*., 2017).

Thiosulfate is usually involved in reduction, oxidation, and disproportionation pathways keeping its concentration in the environment relatively low (Jørgensen BB. 1990; Stoffels *et al*., 2012). Similarly, hydrogen may be subject to rapid turnover *in situ*. While its natural concentrations have not been yet assessed in the groundwater of the Hainich CZE, its concentration may vary from 2.5–240 nM in aquifers (Alter and Steiof, 2005; Flynn *et al*., 2013). Molecular H_2 is an important product of fermenters (Laanbroek and Veldkamp, 1982), also a by-product of the nitrogenase reaction by nitrogen-fixing microbes (Dixon *et al*., 1978) members of which have been recognized ubiquitously in the Hainich aquifers and could provide H_2 to chemolithoautotrophic denitrifiers (Nowak *et al*., 2017; Lazar *et al*., 2017; Starke *et al*., 2017).

4.6 Chemolithoautrophic enrichment culture obtained from groundwater representing key metabolic features of the natural groundwater denitrifier communities

Successful enrichment of chemolithotrophic microbial communities with the metabolic potential for denitrification was confirmed through the quantitative analysis of denitrification specific metabolic products when growing cells with reduced sulfur and hydrogen as electron donors, complemented with *nirS* and *nosZ* gene and transcript-targeted analysis, for both of which transcriptional activity was detected denitrification conditions.

Initial taxonomic identification by a 16S rRNA gene based clone library and Illumina sequencing data during the two microcosm assays revealed that the enrichment culture established in this study was dominated by obligate chemolithotrophic bacteria of the genus *Thiobacillus* (*T. thiophilus*, *T. thioparus,* and *T. denitrificans*) along with members of the

natural denitrifying community such as *Sulfuritalea* and *Sulfuricella*. *T. thiophilus*, a strict chemolithoautotroph, present in the initial enrichment has been cultured earlier from aquifer samples by Kellerman and Griebler, (2009) and is known for its potential to fix carbon dioxide via the Calvin cycle under anoxic conditions, using nitrate as electron acceptor and thiosulfate as electron donor. *T. thioparus* has been reported in groundwater and is known for its trait as a nitrate-reducing sulfur-oxidizing bacterium (Katayama and Kuraishi, 1978; Vlasceanu *et al*., 1997; Zhang *et al*., 2017). *T. denitrificans* is also known as an obligate chemolithoautotroph, which is capable of effective reductive fixation of carbon dioxide by the Calvin-Benson-Bassham cycle under anoxic conditions using nitrate as electron acceptor and thiosulfate as electron donor (Beijerinck 1904; Taylor and Hoare, 1971; Beller *et al*., 2006). *Hydrogenophaga* sp. RAC07 is known to represent a mixotrophic and facultative anaerobic lifestyle for which hydrogen and thiosulfate oxidation has as of yet only been known from genomic information (Fixen *et al*., 2016). *D. aromatica* RCB is a facultative chemolithotroph, and a facultative anaerobe, known to possess the capability of oxidation of H_2, H_2S and Fe (II) along with anaerobic aromatic degradation coupled to nitrate reduction (Coates *et al*., 2001; Chakraborty *et al*., 2005; Shrout *et al*., 2005). Genetic evidence pointed to the existence of a $S_2O_3^{2-}$ oxidation pathway, in *D. aromatica*, but so far, experimental confirmation is lacking (Salinero *et al*., 2009). *R. selenitireducens*, is known to exhibit heterotrophic denitrification (Hunter *et al*., 2007). The presence of facultative alongside obligate chemolithoautotrophic bacteria represents the status of natural groundwater bacterial communities where they exist not exclusively in isolation but as interacting microbial species, reflected in their syntrophic metabolisms (Schink *et al*., 2006), which has also recently been described for oligotrophic subsurface communities as well (Lau *et al*., 2016).

Few studies strongly argue that merely groundwater samples do not reflect bacterial densities and their activity in subsurface systems (Alfreider and colleagues; 1997 and Flynn *et al*., 2008). However, recent studies showed that microbial densities within rock-cores samples, from these pristine aquifers are rather low (Lazar *et al*., 2017). Similarly, uncontaminated core samples from other pristine aquifers were also reported to be nearly devoid of biomass, while the surrounding groundwater hosted nearly 10^5 cells per ml (Lehman *et al*., 2010). Another enrichment "2E", obtained from rock-chips or surrogate solid media, hence representing attached microbial communities, showed presence of mixotrophic community members and but also share the representatives from groundwater based planktonic community enrichment. The additional groups of *Pseudomonas* detected in the attached community enriched in this PhD work also reported to have the potential to mediate nitrate

reduction (Park *et al*., 2005), have a propensity to colonize surfaces (Read and Costerton, 1987), and have often been observed as members of planktonic and attached communities in other pristine aquifers studies (Boyd *et al*., 2007; Herzyk *et al*., 2014). Additionally, clone sequences originated from enrichment cultures 3E and 4E, obtained from Gelrite shake dilution using groundwater as inoculum represented limited diversity (each consortium consisted of two members only), mostly belonging to extremophiles (specially psychrophiles) (Mergaert *et al*., 2003; Grabovich *et al*., 2006; López *et al*., 2009; Morales *et al*., 2016). Tribelli *et al*. (2012) revealed that *P. extremaustralis*, isolated from Antarctica have genes involved in nitrate, nitric oxide, and nitrous oxide reduction while lacking genes encoding for nitrite reductases. The partner microbe in enrichment 3E, *P. veronii,* is a well-known denitrifier and could supply nitrite to *P. extremaustralis, helping* to mediate denitrification under anoxic conditions. Enrichment 4E consisted of bacteria related to *Simplicispira psychrophila* and *Thermomonas fusca*, whose denitrification potential has already been acknowledged under low nutrient and temperature conditions (Mergaert *et al*., 2003; Grabovich *et al*., 2006). Although grown under chemolithoautotrophic conditions, the presence of heterotrophs in enrichment and pure cultures might be, due to the carryover of dead bacteria or organic carbon.

Although we did not test our enrichments in either oxic conditions or amended organic carbon conditions, Herrmann *et al*. (2017) observed nitrate reduction rates using incubations of attached denitrifiers in these aquifers. Incubations from an oxic well, revealed similar denitrification rates using inorganic (thiosulfate) and organic (Acetate/fumarate) electron donor, whereas higher rates were observed with thiosulfate compared to organic carbon in incubations from an anoxic well (Herrmann *et al*., 2017). Among 101 pure denitrifying isolates obtained in this PhD work, strain 2_*Acidovorax defluvii* was subjected to assays of denitrification activity with butyrate as preferred carbon source and tested positive for the presence of both *nirS* and *nosZ* gene. *Acidovorax defluvii* -related strains are known to mediate hydrogen-based autotrophic denitrification and have been isolated from various environments previously (Willems *et al*., 1990; Heylen *et al*., 2006; Ehsani *et al*., 2015). The isolation of denitrifying strains and the establishment of enrichment cultures showed that heterotrophic and mixo/-autotrophic denitrifiers were ubiquitously distributed, also suggested in other aquifer studies (Zeng *et al*., 2017). Although the pure culture of *Acidovorax defluvii* showed the complete nitrate reduction to nitrite, this study would have yielded more information if I had observed the gaseous denitrification products, such as N_2O or N_2 as well.

To assess the feasibility of such an approach, a current study has begun to examine its denitrification potential, and will apply Raman based and qPCR techniques described in this thesis, as well as using combination of double stable isotope, ^{15}N and ^{13}C, to understand denitrification under heterotrophic, mixotrophic as well as autotrophic conditions in parallel.

4.7 Denitrification by chemolithotrophic consortium 1E, coupled to the oxidation of thiosulfate and hydrogen

Both thiosulfate and hydrogen were oxidized by the enriched consortium under chemolithotrophic denitrifying conditions during an incubation period of 14 and 19 days. While microcosm experiment M_I represented electron donor limited conditions, having a nitrate/thiosulfate ratio of 2.5 instead of a stoichiometric molar ratio of 1.6 as suggested by Robertson and Kuenen, (2006), in the second microcosm experiment with a nitrate/thiosulfate molar ratio of 1, we achieved almost complete denitrification to N_2 with very low nitrite accumulation and formation of sulfate up to 8 mmol L^{-1}. Similar to our studies, Manconi *et al*. (2007) reported an optimum nitrate/thiosulfate ratio of 1 for a complete nitrate removal and also observed similar patterns for the sulfate produced, which formed from almost 90% of the oxidized thiosulfate. Similar to published studies with sulfur oxidizing microorganisms, no ammonium was detected at a level above the initial background, nor nitrous oxide (N_2O), which reflected that the consortium was able to mediate complete denitrification and no dissimilatory reduction to ammonium (Cardoso *et al*., 2006; Harrold *et al*., 2016).
A nitrate reduction rate of 220 µmol L^{-1} d^{-1} was observed using 2 mmol L^{-1} of thiosulfate and 1.7 mmol L^{-1} of hydrogen collectively as electron donor and 5 mmol L^{-1} of nitrate as electron acceptor. Autotrophic denitrification using sulfur compounds and hydrogen as electron donor is quite efficient and well recognized from pure culture as well as enrichment cultures from sludge based bioreactors where nitrate reduction rates (3871-7560 µmol L^{-1} d^{-1} utilizing thiosulfate) and (1742-39016 µmol L^{-1} d^{-1} utilizing hydrogen) have been observed (Cardoso *et al*., 2006; Manconi *et al*., 2007; Lee *et al*., 2010; Chen *et al*., 2014). Higher temperature (30°C) and higher substrate availability (20-fold higher for nitrate, thiosulfate and even with continuous supply of hydrogen) used in these studies may provide an explanation why these rates were substantially higher than the rates observed in our study.

4.8 Growth of chemolithoautotrophic denitrifiers complemented with *nirS*- and *nosZ*-transcriptional activity during microcosm experiments

We estimated a doubling time of 28 hours for the denitrifying enrichment culture under chemolithoautotrophic conditions, which agreed well with doubling times of 1-15 days reported for sulfur and hydrogen oxidizing chemolithoautotrophs (Kodama and Watanabe; 2003) and represents about ten times faster growth compared to rates reported for aquifers (15 days, Mailloux and Fuller, 2003). The slow but steady increase in 16S rRNA, *nirS* and *nosZ* gene abundances over time coincided with increased oxidation of thiosulfate and hydrogen coupled with N_2 production under denitrifying conditions. While *nirS* mediate the second step of denitrification, enabling the reduction of soluble nitrite to gaseous nitric oxide, this reaction also distinguishes denitrification from the dissimilatory nitrate reduction to ammonium (Zumft, 1997). The multi-copper homodimeric nitrous oxide reductase (*nosZ*) mediates the last step of the denitrification pathway involving the reduction of nitrous oxide gas to dinitrogen gas, a key signature of complete denitrification (Zumft, 1997). The observed relative expression levels tended to decrease in the order *nirS* > *nosZ* during our first microcosm experiment, M_I, while the opposite observation (*nirS* < *nosZ*) was made for the second microcosm experiment, M_II. Our results from M_I, corroborate with an exemplary expression level reported for pure *T. denitrificans* bacteria under anoxic conditions (Beller *et al*., 2006). Although Beller *et al*., 2006 used a similar nitrate/thiosulfate ratio of 1, relative expression levels for the second microcosm, M_II, were different (*nirS* < *nosZ*) than reported (*nirS* > *nosZ*) for pure culture studies of *T. denitrificans.* The plausible explanation for the upregulation of *nosZ* expression during microcosm experiment, M_II, could be the optimized nitrate/thiosulfate ratio along with the availability of hydrogen (4%) as additional electron donor, supporting complete denitrification. Similar observations of N/S ratio as a key factor enabling complete denitrification were made using a thiosulfate-based reactor with *T. denitrificans* (Manconi *et al*., 2007).

Limitation of electron donor in the first microcosm experiment resulted in incomplete denitrification that did not proceed beyond the step of nitrite reduction, which was also reflected on the low level of *nosZ* transcriptional activity. NO is a toxic intermediate of the denitrification pathway and known as an inducer molecule for transcription of the *nos* genes (Arai *et al*., 2003). Although under anoxic conditions, the microcosm experiment M_I revealed a relatively high transcriptional activity of *nirS* genes, which most likely led to the production of NO. However, due to the limitation of electron donors, the further metabolic

cascading was most likely disturbed, providing also a plausible explanation for the low *nosZ* transcripts.

It is also evident that among denitrifiers, regulation of gene expression can be diverse, even in closely related organisms (Arai *et al*., 2003; Spiro 2012). Current data suggest inconsistent trends for gene expression in autotrophic denitrification mediated by *T. denitrificans* compared to heterotrophic counterparts such as *Pseudomonas stutzeri*, *P. denitrificans* and *P. pantotrophus*, where upregulation of *nos* genes under denitrifying conditions has been revealed (Korner *et al*., 1989; Moir *et al*., 1995; Baumann *et al*., 1996) which is not the case for *T. denitrificans* (Beller *et al*., 2006). Only single study available for transcriptional regulation with chemolithoautotrophic bacteria, Beller and colleagues suggested that except for *nos* genes, other genes associated with denitrification were found upregulated under denitrifying conditions in the following range: *nar*, 54- to 95-fold; *nir*, 10- to 21-fold; *nor*, 4- to 10-fold; *nos*, 0.5- to 0.9-fold in the order *nar* > *nir* > *nor* > *nos*. Utilising cDNA/DNA ratio, enrichment at M_II showed a maximum of 258-fold increase in *nirS* transcriptional activity, which is twelve time more than observations made (21-fold) by Beller and colleagues, 2006. Similarly, for *nosZ* upregulation, the consortium showed a 138-fold increase, which is 153-fold high compared to 0.9-fold (maximum) increase observed by Beller *et al*. (2006). Justin and Kelly, 1974 reported that the levels of nitrate and nitrite reductase enzymes in *T. denitrificans* growing with thiosulfate are regulated in response to oxygen tension, and significant activities of nitrate reductase were detected in anaerobic or microaerophilic conditions. Furthermore, enrichment at microcosm M_II also showed increased *nosZ* transcriptional activity of about a 103-fold, which is hundred time more than earlier observations (0.9-fold for *nosZ*) (Beller *et al*., 2006). The close accounting between transcriptionally active *Thiobacillus denitrificans* (both *nirS*- and *nosZ*-) and the overall denitrifier community (16S rRNA) suggested that it was likely a dominant player, representing a significant fraction of the active population in the consortium throughout the microcosm assay. Overall, shifts in community composition did not result in impairment of the denitrifying function of the community and consistent with other denitrifiers related studies (Brenzinger *et al*., 2015).

Combined with *nirS* and *nosZ* transcript-targeted clone libraries and Illumina sequencing data from four time points, I could confirm a broad metabolic repertoire of groundwater originated sulfur and hydrogen oxidizing bacteria such as of the genus *Thiobacillus.* Obligate chemolithoautotrophic *T. denitrificans* have a higher affinity for thiosulfate than for other

sulfur sources and appeared to be favoured by the high concentration of thiosulfate provided in the enrichment culture (Trouve *et al*., 1998; Manconi *et al*., 2007). *Thiobacillus* lineages have been shown to play important roles in aquifers because of their denitrifying capacity (Rödelsperger, 1989) and are also known for exceptional denitrifying ability where several psychrophilic strains mediated highest nitrate removal capacity at 10 °C, providing another explanation why they might have been successful at an incubation temperature of 15 °C used in this study (Trouve *et al*., 1998; Broman *et al*., 2017). A preferential utilization of reduced sulfur compounds over organic compounds as electron donors has been reported from natural systems at the oxic-anoxic interface, where a *Thiobacillus* related community was shown to outcompete heterotrophic denitrifiers (Brettar and Rheinheimer, 1991). Compared to obvious differences observed in the community in natural *nirS*-denitrifier population and chemolithoautotrophic culture "1E", these studies suggested a strong link of chemolithotrophic microbial processes in the natural ecosystem.

4.9 Verification of hypotheses and conclusions

Hypothesis I.
Contrasting redox regimes and availability of dissolved inorganic nitrogen shape bacterial diversity and abundances of anammox and denitrifiers across the Hainich groundwater observation transect.

Co-occurrence of nitrate and ammonium, in the absence of oxygen, facilitated conducive conditions for anammox and denitrifiers in the upper anoxic aquifer complemented with gene abundances up to 10^6 and 10^7 (L^{-1}), respectively. Along with the high representation of anammox-bacteria based on 16S rRNA-targeted Illumina sequencing, maximum abundances of *hzsA* genes, and two orders of magnitude higher *hzsA* compared to *nirS* gene transcripts, these findings provided strong support for H52, H53 wells being an anammox hotspot within the heterogeneous carbonate-rock aquifer system of the Hainich CZE. The estimated fraction of organisms within the total community harboring the genetic potential for the anammox process ranged from 1.8 to 10.6% in upper aquifer assemblage and from 5.3 to 8.3% in low aquifer assemblage. Surprisingly, ~~we~~ I found *hzsA* gene abundances in the range of 2.3 x 10^5 - 3.7 x 10^6 L^{-1} and high *hzsA* transcriptional activity (10^5 L^{-1}) also in the oxic groundwater of wells of the lower aquifer assemblage. While anammox in marine waters, dominated by *Cand*. Scalindua, is inhibited by oxygen levels ≤ ~10 µmol L^{-1}, it remains unclear if

groundwater anammox bacteria could actually thrive and carry out anaerobic ammonium oxidation in the presence of considerable concentrations of oxygen (43-420 µmol L^{-1}).

Hypothesis II.

In anoxic groundwater with low organic carbon availability, anammox could significantly contribute to N_2 production, therefore being responsible for relatively more nitrogen loss than denitrification.

Reoccurring patterns of ladderane lipids and *hzsA* transcripts pointed to the existence of a potential hotspot of anammox at well H53 which was confirmed by ^{15}N-isotope enabled data indicating the anammox process to contribute an estimated 83% to total nitrogen loss in suboxic groundwater with a rate of 3.5-4.7 nmol N_2 L^{-1} d^{-1}, while the denitrification rate was limited to 0.7 nmol N_2 L^{-1} d^{-1}.

Hypothesis III.

Aerobic and anaerobic ammonium oxidizers co-occur in suboxic groundwater and their coupled activities could be important for groundwater nitrogen cycling.

Detection of transcripts of archaeal and bacterial *amoA* genes in the groundwater of well H53 suggested ongoing aerobic ammonia oxidation at suboxic conditions. Highest *hzsA*/*amoA* gene and transcript ratios were observed for wells H52 and H53, pointing to a clear dominance of anammox although aerobic ammonia oxidation likely still co-occurred. Under in situ conditions, nitrite fueling the anammox process could originate from nitrate reduction by anammox bacteria or other nitrate reducers but also from incomplete nitrification.

Hypothesis IV.

Oligotrophic groundwater in carbonate-rock aquifers supports high fractions of chemolithoautotrophic denitrifiers within the denitrifying microbial communities.

Analysis of the groundwater denitrifier community across the two aquifer assemblages confirmed the potential role of large fractions of an autotrophic denitrifier population oxidizing reduced sulfur compounds, hydrogen, or reduced iron. The denitrifying populations were found to be dominated by chemolithoautotrophic denitrifiers such as *Sulfurifustis variabilis*, *Sulfuricella denitrificans* and *Sulfuritalea hydrogenivorans*, which have potential to mediate denitrification using reduced sulfur and hydrogen. Since these bacteria can also grow under aerobic conditions, their presence seems to be limited by the availability of electron donors and not completely on the dissolved oxygen.

Hypothesis V.

Denitrifying consortia originating from organic carbon limited groundwater could be used as a model community to understand the metabolic potential of denitrifiers within the natural groundwater bacterial communities.

Despite long doubling time of nearly 28 hours, chemolithoautotrophic microbial communities in the enriched consortium obtained from groundwater were efficiently able to transcribe nitrite reductase and nitrous oxide reductase genes, mediating oxidation of reduced sulfur and hydrogen coupled with complete denitrification under simulated conditions of low temperature and darkness. Chemolithotrophy therefore proved as a very useful strategy for living in oligotrophic cold conditions of the subsurface, and suggested to control denitrifying capacities of organisms thriving at carbon-limited aquifers. The ratio between nitrate and inorganic electron donor is important for complete denitrification and seems to be a crucial factor for influencing denitrification activity.

4.10 Strengths of the current study

Groundwater contamination with reactive nitrogen has the potential to negatively affect health of both natural ecosystems and human health. Microorganisms mediating anammox and denitrifying processes are involved in losses of reactive nitrogen from several aquatic and terrestrial ecosystems across the globe. Having said that, this study is the first to identify not only dominant anammox bacterial communities but also their activity demonstrated by rate measurements from pristine oligotrophic groundwater. Although the anammox processes obviously, wins the race against denitrification for mediating nitrogen loss in the Hainich aquifer assemblages, this study could demonstrate the possibility for complete denitrification by natural chemolithoautotrophic communities.

4.11 Limitations of study and considerations for future investigations

Anammox rates were only measured for an anammox hotspot site among Hainich aquifer assemblages. Future research should also consider to assess the quantification of anammox bacteria by utilizing another anammox specific functional gene such as *hzsB* and *hzsC* encoding for hydrazine synthase as well as the *hzo* gene encoding for hydrazine oxidoreductase reported from other aquifer studies (Smith *et al*., 2015; Wang *et al*., 2017). Moreover, the denitrifier enrichment approach does not directly demonstrate that apart from *Thiobacillus denitrificans*, which organisms among the detected populations were directly involved in the denitrification activities. Moreover, PCR based methods may suffer from

unavoidable primer biases (Saarenheimo *et al*., 2015), investigations of denitrifying organisms recently reported the existence of two clades for *nosZ* (Jones *et al*., 2013) and *nirK* (Helen *et al*., 2016) as well as limited comprehensiveness of *nirS* gene primers (Murdock and Juniper, 2017), and challenges involved with highly unstable RNA (McGrath *et al*., 2008). These are among the key limitations, which in further studies should be addressed for better understanding the relationships between denitrifying-anammox-aerobic ammonium oxidising microorganisms and their potential in natural ecosystems.

Alternative methods such as DNA stable-isotope probing (DNA-SIP) (Radajewski *et al*., 2000, 2003), fluorescence in situ hybridization-SIP (FISH-SIP) (Huang *et al*., 2007) or nanoscale secondary ion mass spectrometry or (Nano-SIMS) (Kopf *et al*., 2015) could effectively complement these studies and might provide an informative tool to detect chemolithoautotrophic organism mediating anammox and denitrification at *in situ* natural conditions. These improved and novel technologies will assist in exploring the surging scientific questions of microbial ecology but apart from challenging, they are quite limited and need to adjusted for understanding microbial processes in diverse ecosystems including oligotrophic subsurface, where the biomass concentration are usually quite low (Singer *et al*., 2017; Ravindran, 2017).

5. CONCLUSIONS

In the introduction section, I gave a brief overview of the two core perspectives or research work packages of this PhD thesis, the outcome of which has been enlisted below:

5.1 Anammox versus denitrification in carbonate-rock aquifers: Nitrogen loss from pristine carbonate-rock aquifers of the Hainich Critical Zone Exploratory (Germany) is primarily driven by chemolithoautotrophic anammox processes

The current findings indicate that the contribution of anammox in pristine aquifers is quite similar to habitats depleted in labile carbon (freshwater) and dissolved oxygen-depleted water at different OMZs, which also share properties of long-term declines in oxygen concentrations along with intensive exchange of nitrogen compounds. Together with a strong functional resemblance regarding anammox activity, its high contribution to nitrogen loss, and its potential coupling with aerobic ammonia oxidation, these observed similarities and the identification of potential drivers of these patterns are key research outcomes of this thesis. This study provided first insight into the quantitative relevance of anammox versus denitrification in pristine groundwater. Together with knowledge gained from previous PLFA- and proteomics-based studies, the results obtained in my thesis point to the existence of an anammox hot spot within the heterogeneous carbonate-rock aquifer system of the Hainich groundwater observatory. Apart from the predominance of anammox over denitrification and its importance within nitrogen cycling in suboxic to anoxic groundwater, these chemolithoautotrophs could also make a substantial contribution to autotrophic CO_2-fixation under conditions of strong organic carbon limitation. Since anammox bacteria are making a living by fixing inorganic carbon available in the carbonate-rock aquifers while living on the scarce resources of suitable electron donors and acceptors, these bacteria seem to be ideally equipped (such as high affinity for nitrite) for life in anoxic groundwater where they have the unprecedented potential to influence inorganic nitrogen concentrations under rather stable physico-chemical conditions and long groundwater residence times. Concomitant presence of anammox bacteria in anoxic/suboxic as well as oxic groundwater, supported by transcripts and ladderane based data, points to the fact that anammox in these limestone aquifers might not be restricted to completely anoxic zones only. Future studies should also address the coupling between anammox and the first step of nitrification, which might enhance nitrogen loss from oligotrophic groundwater environments and knowledge of

which might also contribute to the elucidation of potential mechanisms that allow anammox bacteria to thrive in oxic groundwater.

5.2 Physiological experiments with denitrifying microorganisms: Chemolithotrophic consortium provided insight into the complexities of denitrifiers in oligotrophic groundwater and their nitrate attenuation capacity

As distinct from anammox, a relatively broad range of bacteria can accomplish denitrification. The combined data presented here on *nirS*-type denitrifier diversity in anoxic and oxic groundwater, complemented with growth studies using an enriched chemolithotrophic consortium under denitrifying conditions provided comprehensive insight into how chemolithotrophic denitrifiers co-occur and likely mediate denitrification in carbonate-rock oligotrophic aquifers. Two important features observed for all four groundwater wells at which *nirS*-type denitrifier communities were analyzed for several time points were (i) the repeatedly high fraction of potential chemolithoautotrophic denitrifiers and (ii) their potential metabolic versatility to use either multiple inorganic electron donors or to be also capable of a mixotrophic lifestyle.

The denitrifying capacity of the enriched consortium reflected the common metabolic potential of large fractions of the natural denitrifier community, including primarily hydrogen and reduced sulfur driven chemolithoautotrophy coupled with denitrification. Microcosm experiments confirmed that complete denitrification can be carried out by this model consortium if correct stoichiometry of electron donors and acceptor is provided. Most of these groundwater chemolithotrophs appeared to be quite diverse in their metabolic requirements and capabilities, being able to oxidize thiosulfate and hydrogen completely. In natural systems such as oligotrophic groundwater, the chemolithotrophic denitrifying community could act as an effective natural barrier attenuating dissolved inorganic nitrate at moderate concentrations by utilizing the available inorganic electron donors. The work carried out in this thesis has therefore significantly broadened our understanding of which microbial groupsmake up the groundwater denitrifying community in limestone aquifers and what role their metabolic repertoire may play in nitrogen loss under organic carbon limited and oligotrophic conditions.

REFERENCES

Akob DM, Küsel K (2011). Where microorganisms meet rocks in the Earth's Critical Zone. *Biogeosciences* **8:** 3531-3543.

Alfreider A, Krössbacher M, Psenner R (1997). Groundwater samples do not reflect bacterial densities and activity in subsurface systems. *Water Res* **31:** 832-840.

Ali M, Oshiki M, Awata T, Isobe K, Kimura Z, Yoshikawa H *et al* (2015). Physiological characterization of anaerobic ammonium oxidizing bacterium '*Candidatus* Jettenia caeni'. *Environ Microbiol* **17:** 2172-2189.

Alter MD, Steiof M (2005). Optimized method for dissolved hydrogen sampling in groundwater. *J Contam Hydrol* **78:** 71-86.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56:** 1919-1925.

Anantharaman K, Breier JA, Sheik CS, Dick GJ (2013). Evidence for hydrogen oxidation and metabolic plasticity in widespread deep-sea sulfur-oxidizing bacteria. *Proc Natl Acad Sci U S A* **110:** 330-335.

Anantharaman K, Brown CT, Hug LA, Sharon I, Castelle CJ, Probst AJ *et al* (2016). Thousands of microbial genomes shed light on interconnected biogeochemical processes in an aquifer system. *Nat Commun* **7:** 13219.

Arai H, Mizutani M, Igarashi Y (2003). Transcriptional regulation of the *nos* genes for nitrous oxide reductase in Pseudomonas aeruginosa. *Microbiol* **149:** 29-36.

Arrigo KR (2005). Marine microorganisms and global nutrient cycles. *Nature* **437:** 349.

Auckenthaler A, Raso G, Huggenberger P (2002). Particle transport in a karst aquifer: natural and artificial tracer experiments with bacteria, bacteriophages and microspheres. *Water Sci Technol* **46:** 131-138.

Bakken LR, Bergaust L, Liu B, Frostegård Å (2012). Regulation of denitrification at the cellular level: a clue to the understanding of N_2O emissions from soils. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367:** 1226-1234.

Barrett M, Jahangir MMR, Lee C, Smith CJ, Bhreathnach N, Collins G *et al* (2013). Abundance of denitrification genes under different peizometer depths in four Irish agricultural groundwater sites. *Environ Sci Poll Res* **20:** 6646-6657.

Baumann B, Snozzi M, Zehnder AJ, Van Der Meer JR (1996). Dynamics of denitrification activity of *Paracoccus denitrificans* in continuous culture during aerobic-anaerobic changes. *J Bacteriol* **178:** 4367-4374.

Beijerinck MW (1904). Ueber die Bakterien, welche sich im Dunkeln mit Kohlensäure als Kohlenstoffquelle ernähren können. *Centralbl Bakteriol Parasitenkd Infektionskr Hyg Abt II* **11:** 593-599.

Beller HR, Letain TE, Chakicherla A, Kane SR, Legler TC, Coleman MA (2006). Whole-genome transcriptional analysis of chemolithoautotrophic thiosulfate oxidation by *Thiobacillus denitrificans* under aerobic versus denitrifying conditions. *J Bacteriol* **188:** 7005-7015.

Bergaust L, Mao Y, Bakken LR, Frostegård Å (2010). Denitrification response patterns during the transition to anoxic respiration and posttranscriptional effects of suboptimal pH on nitrogen oxide reductase in *Paracoccus denitrificans*. *Appl Environ Microbiol* **76:** 6387-6396.

Blodau C (2011). Thermodynamic control on terminal electron transfer and methanogenesis. *Aquatic Redox Chemistry*. American Chemical Society. pp 65-83.

Bohu T, Akob DM, Abratis M, Lazar CS, Küsel K (2016). Biological Low-pH Mn(II) Oxidation in a manganese deposit influenced by metal-rich groundwater. *Appl Environ Microbiol* **82:** 3009-3021.

Boyd ES, Cummings DE, Geesey GG (2007). Mineralogy influences structure and diversity of bacterial communities associated with geological substrata in a pristine aquifer. *Microb Ecol* **54:** 170-182.

Braker G, Fesefeldt A, Witzel K-P (1998). Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Appl Environ Microbiol* **64:** 3769-3775.

Brenzinger K, Dörsch P, Braker G (2015). pH-driven shifts in overall and transcriptionally active denitrifiers control gaseous product stoichiometry in growth experiments with extracted bacteria from soil. *Front Microbiol* **6**.

Broda E (1977). Two kinds of lithotrophs missing in nature. *Z Allg Mikrobiol* **17:** 491-493.

Broman E, Jawad A, Wu X, Christel S, Ni G, Lopez-Fernandez M *et al* (2017). Low temperature, autotrophic microbial denitrification using thiosulfate or thiocyanate as electron donor. *Biodegr* **28:** 287-301.

Burgin AJ, Hamilton SK (2007). Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. *Front Ecol Environ* **5:** 89-96.

Canfield DE, Glazer AN, Falkowski PG (2010). The evolution and future of Earth's nitrogen cycle. *Science* **330:** 192-196.

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

Canion A, Overholt WA, Kostka JE, Huettel M, Lavik G, Kuypers MM (2014). Temperature response of denitrification and anaerobic ammonium oxidation rates and microbial community structure in Arctic fjord sediments. *Environ Microbiol* **16:** 3331-3344.

Cao S, Li B, Du R, Ren N, Peng Y (2016). Nitrite production in a partial denitrifying upflow sludge bed (USB) reactor equipped with gas automatic circulation (GAC). *Water Res* **90:** 309-316.

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N *et al* (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal* **6:** 1621-1624.

Cardoso RB, Sierra-Alvarez R, Rowlette P, Flores ER, Gómez J, Field JA (2006). Sulfide oxidation under chemolithoautotrophic denitrifying conditions. *Biotechnol Bioeng* **95:** 1148-1157.

Carmichael MJ, Carmichael SK, Santelli CM, Strom A, Bräuer SL (2013). Mn(II)-oxidizing bacteria are abundant and environmentally relevant members of ferromanganese deposits in caves of the upper Tennessee river basin. *Geomicrobio J* **30:** 779-800.

Chakraborty R, O'Connor SM, Chan E, Coates JD (2005). Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* Strain RCB. *Appl Environ Microbiol* **71:** 8649-8655.

Chen J, Strous M (2013). Denitrification and aerobic respiration, hybrid electron transport chains and co-evolution. *Biochim Biophys Acta (BBA) - Bioenergetics* **1827:** 136-144.

Chen D, Yang K, Wang H (2014). Effects of important factors on hydrogen-based autotrophic denitrification in a

bioreactor. *Desalin Water Treat* **57:** 3482-3488.

Cho K, Choi M, Jeong D, Lee S, Bae H (2017). Comparison of inoculum sources for long-term process performance and fate of ANAMMOX bacteria niche in poly(vinyl alcohol)/sodium alginate gel beads. *Chemosphere* **185:** 394-402.

Clark I, Timlin R, Bourbonnais A, Jones K, Lafleur D, Wickens K (2008). Origin and Fate of Industrial Ammonium in Anoxic Ground Water—15N Evidence for Anaerobic Oxidation (Anammox). *Ground Water Monitor Remed* **28:** 73-82.

Coates JD, Chakraborty R, Lack JG, O'Connor SM, Cole KA, Bender KS *et al* (2001). Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. *Nature* **411:** 1039-1043.

Crowe SA, Treusch AH, Forth M, Li J, Magen C, Canfield DE *et al* (2017). Novel anammox bacteria and nitrogen loss from Lake Superior. *Sci Rep* **7:** 13757.

Daims H, Brühl A, Amann R, Schleifer K-H, Wagner M (1999). The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe Set. *Syst Appl Microbiol* **22:** 434-444.

Daims H, Lebedeva EV, Pjevac P, Han P, Herbold C, Albertsen M *et al* (2015). Complete nitrification by *Nitrospira* bacteria. *Nature* **528:** 504.

Dalsgaard T, Thamdrup B (2002). Factors controlling anaerobic ammonium oxidation with nitrite in marine sediments. *Appl Environ Microbiol* **68:** 3802-3808.

Dalsgaard T, Canfield DE, Petersen J, Thamdrup B, Acuna-Gonzalez J (2003). N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. *Nature* **422:** 606-608.

Dalsgaard T, Thamdrup B, Farías L, Revsbech NP (2012). Anammox and denitrification in the oxygen minimum zone of the eastern South Pacific. *Limnol and Oceanogr* **57:** 1331-1346.

Dalsgaard T, Stewart FJ, Thamdrup B, De Brabandere L, Revsbech NP, Ulloa O *et al* (2014). Oxygen at nanomolar levels reversibly suppresses process rates and gene expression in anammox and denitrification in the oxygen minimum zone off northern Chile. *MBio* **5:** e01966.

Davidson EA, Seitzinger S (2006). The enigma of progress in denitrification research. *Ecol Appl* **16:** 2057-2063.

DeSimone LA, Howes BL (1998). Nitrogen transport and transformations in a shallow aquifer receiving wastewater discharge: A mass balance approach. *Water Resour. Res* **34:** 271-285.

DEV **(**1975). Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung. Physikalische, chemische, biologische und bakteriologische Verfahren. ed Fachgruppe Wasserchemie in der Gesellschaft Deutscher Chemiker in Gemeinschaft mit dem Normenausschuß Wasserwesen (NAW) im DIN Deutsches Institut für Normung e.V. VCH Verlagsgesellschaft, Weinheim, Germany.

Devito KJ, Creed IF, Rothwell RL, Prepas EE (2000). Landscape controls on phosphorus loading to boreal lakes: implications for the potential impacts of forest harvesting. *Can J Fish Aquat Sci* **57:** 1977-1984.

Dixon ROD (1978). Nitrogenase — Hydrogenase interrelationships in Rhizobia. *Biochimie* **60:** 233-236.

Dosta J, Fernández I, Vázquez-Padín JR, Mosquera-Corral A, Campos JL, Mata-Álvarez J *et al* (2008). Short- and long-term effects of temperature on the Anammox process. *J Hazard Mater* **154:** 688-693.

Ehsani E, Jauregui R, Geffers R, Jarek M, Boon N, Pieper DH *et al* (2015). First Draft Genome Sequence of the *Acidovorax caeni* sp. nov. Type Strain R-24608 (DSM 19327). *Gen Announc* **3:** e01378-01315.

Emerson D, Moyer C (1997). Isolation and characterization of novel iron-oxidizing bacteria that grow at circumneutral pH. *Appl Environ Microbiol* **63:** 4784-4792.

Ergas SJ, Reuss AF (2001). Hydrogenotrophic denitrification of drinking water using a hollow fibre membrane bioreactor. *J Water Sup: Res Tech - Aqua* **50:** 161-171.

Erisman JW, Galloway JN, Seitzinger S, Bleeker A, Dise NB, Petrescu AMR *et al* (2013). Consequences of human modification of the global nitrogen cycle. *Philos Trans Royal Soc B: Bio Sci* **368:** 20130116.

Falkowski PG (1997). Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. *Nature* **387:** 272.

Falkowski PG, Fenchel T, Delong EF (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science* **320:** 1034-1039.

Felske A, Rheims H, Wolterink A, Stackebrandt E, Akkermans ADL (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class *Actinobacteria* in grassland soils. *Microbiology* **143:** 2983-2989.

Fernandes SO, Javanaud C, Michotey VD, Guasco S, Anschutz P, Bonin P (2016). Coupling of bacterial nitrification with denitrification and anammox supports N removal in intertidal sediments (Arcachon Bay, France). *Estuarine, Coastal and Shelf Science* **179:** 39-50.

Fields S (2004). Global Nitrogen: Cycling out of Control. *Environ Health Perspect* **112:** A556-A563.

Fixen KR, Starkenburg SR, Hovde BT, Johnson SL, Deodato CR, Daligault HE *et al* (2016). Genome sequences of eight bacterial species found in coculture with the haptophyte *Chrysochromulina tobin*. *Genome Announcements* **4:** e01162-01116.

Flynn TM, Sanford RA, Bethke CM (2008). Attached and suspended microbial communities in a pristine confined aquifer. *Water Resour Res* **44**

Flynn TM, Sanford RA, Santo Domingo JW, Ashbolt NJ, Levine AD, Bethke CM (2012). The active bacterial community in a pristine confined aquifer. *Water Resour Res* **48:** n/a-n/a.

Flynn TM, Sanford RA, Ryu H, Bethke CM, Levine AD, Ashbolt NJ *et al* (2013). Functional microbial diversity explains groundwater chemistry in a pristine aquifer. *BMC Microbiol* **13:** 146.

Ford D, Williams P, Ford D, Williams P (2007). Introduction to Karst. *Karst Hydrogeology and Geomorphology*. John Wiley & Sons Ltd,. pp 1-8.

Fowler D, Coyle M, Skiba U, Sutton MA, Cape JN, Reis S *et al* (2013). The global nitrogen cycle in the twenty-first century. *Philos Trans Royal So B: Bio Sci***368**.

Francis CA, Roberts KJ, Beman JM, Santoro AE, Oakley BB (2005). Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci U S A* **102:** 14683-14688.

Freeze RA and Cherry JA (1979). Groundwater: Englewood Cliffs, NJ, Prentice-Hall, 604 p.

Frosch T, Keiner R, Michalzik B, Fischer B, Popp J (2013). Investigation of Gas Exchange Processes in Peat Bog Ecosystems by Means of Innovative Raman Gas Spectroscopy. *Anal Chem* **85:** 1295-1299.

G. BIaR (1991). Denitrification in the central Baltic – evidence for H_2S-oxidation as motor of denitrification at the

oxic–anoxic interface. *Mar Ecol Prog Ser* **77:** 157-169.

Gagen EJ, Huber H, Meador T, Hinrichs K-U, Thomm M (2013). Novel cultivation-based approach to understanding the miscellaneous *crenarchaeotic* group (MCG) archaea from sedimentary ecosystems. *Appl Environ Microbiol* **79:** 6400-6406.

Galán A, Molina V, Thamdrup B, Woebken D, Lavik G, Kuypers MMM *et al* (2009). Anammox bacteria and the anaerobic oxidation of ammonium in the oxygen minimum zone off northern Chile. *Deep Sea Res Part II: Topical Studies in Oceanography* **56:** 1021-1031.

Galloway JN, Aber JD, Erisman JW, Seitzinger SP, Howarth RW, Cowling EB *et al* (2003). The Nitrogen Cascade. *Bioscience* **53:** 341-356.

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP *et al* (2004). Nitrogen Cycles: Past, Present, and Future. *Biogeochemistry* **70:** 153-226.

Galloway JN, Leach AM, Bleeker A, Erisman JW (2013). A chronology of human understanding of the nitrogen cycle(). *Philos Trans Royal Soc B: Biol Sci* **368:** 20130120.

Garba L, Mohamad Ali MS, Oslan SN, Rahman RNZRA (2016). Molecular Cloning and Functional Expression of a Δ9- Fatty Acid Desaturase from an Antarctic *Pseudomonas* sp. A3. *PLoS One* **11:** e0160681.

Gayon, V and Dupetit G (1886). Recherches sur la réduction des nitrates par les infiniment petits. Soc. Sci. phys. naturelles Bordeaux, Series 3, 2, 201-307.

Gleeson T, Befus KM, Jasechko S, Luijendijk E, Cardenas MB (2015). The global volume and distribution of modern groundwater. *Nature Geoscience* **9:** 161.

Glock N, Schönfeld J, Eisenhauer A, Hensen C, Mallon J, Sommer S (2013). The role of benthic foraminifera in the benthic nitrogen cycle of the Peruvian oxygen minimum zone. *Biogeosciences* **10:** 4767-4783.

Gooddy DC, Hinsby, K (2008). Organic quality of groundwaters. In: Edmunds, W.M.; Shand, P., (eds.) *Natural groundwater quality.* Oxford, Blackwell, 59-70, 11pp.

Gori F, Tringe SG, Kartal B, Machiori E, Jetten MSM (2011). The metagenomic basis of anammox metabolism in *Candidatus* 'Brocadia fulgida'. *Biochem Soc Trans* **39:** 1799-1804.

Gorman HS (2013). Introduction. In: Gorman HS (ed). *The Story of N*. Rutgers University Press. pp 1-8.

Grabovich M, Gavrish E, Kuever J, Lysenko AM, Podkopaeva D, Dubinina G (2006). Proposal of *Giesbergeria voronezhensis* gen. nov., sp. nov. and *G. kuznetsovii* sp. nov. and reclassification of [*Aquaspirillum*] *anulus*, [A.] *sinuosum* and [*A.*] *giesbergeri* as *Giesbergeria anulus* comb. nov., *G. sinuosa* comb. nov. and *G. giesbergeri* comb. nov., and [*Aquaspirillum*] *metamorphum* and [A.] psychrophilum as *Simplicispira metamorpha* gen. nov., comb. nov. and *S. psychrophila* comb. nov. *Int J Syst Evol Microbiol* **56:** 569-576.

Grasshoff K, Kremling K, Ehrhardt M (1983). Methods of Seawater Analysis. *Verlag Chemie Weinheim*, Weinheim.

Griebler C, Mindl B, Slezak D, Geiger-Kaiser M (2002). Distribution patterns of attached and suspended bacteria in pristine and contaminated shallow aquifers studied with an *in situ* sediment exposure microcosm *Aquat. Microbial Ecol.*, **28**, pp. 117-129

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

H. J. Laanbroek HV (1982). New dimensions in microbiology: mixed substrates, mixed cultures and microbial communities - Microbial interactions in sediment communities. *Philosophical Transactions of the Royal Society of*

London B, Biological Sciences **297:** 533-550.

Hallin S, Lindgren P-E (1999). PCR detection of genes encoding nitrite reductase in denitrifying bacteria. *Appl Environ Microbiol* **65:** 1652-1657.

Hamersley MR, Lavik G, Woebken D, Rattray JE, Lam P, Hopmans EC *et al* (2007). Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. *Limnol Oceanograp* **52:** 923-933.

Hamersley MR, Woebken D, Boehrer B, Schultze M, Lavik G, Kuypers MM (2009). Water column anammox and denitrification in a temperate permanently stratified lake (Lake Rassnitzer, Germany). *Syst Appl Microbiol* **32:** 571-582.

Han D, Cao G, McCallum J, Song X (2015). Residence times of groundwater and nitrate transport in coastal aquifer systems: Daweijia area, northeastern China. *Sci Total Environ* **538:** 539-554.

Han P, Klümper U, Wong A, Li M, Lin J-G, Quan Z *et al* (2017). Assessment of molecular detection of anaerobic ammonium-oxidizing (anammox) bacteria in different environmental samples using PCR primers based on 16S rRNA and functional genes. *Appl Microbiol Biotechnol* **101:** 7689-7702.

Hanson BT, Madsen EL (2015). In situ expression of nitrite-dependent anaerobic methane oxidation proteins by *Candidatus* Methylomirabilis oxyfera co-occurring with expressed anammox proteins in a contaminated aquifer. *Environ Microbiol Rep* **7:** 252-264.

Harhangi HR, Le Roy M, van Alen T, Hu BL, Groen J, Kartal B *et al* (2012). Hydrazine synthase, a unique phylomarker with which to study the presence and biodiversity of anammox bacteria. *Appl Environ Microbiol* **78:** 752-758.

Harrold ZR, Skidmore ML, Hamilton TL, Desch L, Amada K, van Gelder W *et al* (2016). Aerobic and anaerobic thiosulfate oxidation by a cold-adapted, subglacial chemoautotroph. *Appl Environ Microbiol* **82:** 1486-1495.

Härtig E, Zumft WG (1999). Kinetics of *nirS* expression (cytochrome cd(1) nitrite reductase) in *Pseudomonas stutzeri* during the transition from aerobic respiration to denitrification: evidence for a denitrification-specific nitrate- and nitrite-responsive regulatory system. *J Bacteriol* **181:** 161-166.

Helen D, Kim H, Tytgat B, Anne W (2016). Highly diverse *nirK* genes comprise two major clades that harbour ammonium-producing denitrifiers. *BMC Genomics* **17:** 155.

Herlemann DPR, Labrenz M, Jurgens K, Bertilsson S, Waniek JJ, Andersson AF (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J* **5:** 1571-1579.

Herrmann M, Hädrich A, Küsel K (2012). Predominance of thaumarchaeal ammonia oxidizer abundance and transcriptional activity in an acidic fen. *Environ Microbiol* **14:** 3013-3025.

Herrmann M, Rusznyak A, Akob DM, Schulze I, Opitz S, Totsche KU *et al* (2015). Large fractions of CO_2-fixing microorganisms in pristine limestone aquifers appear to be involved in the oxidation of reduced sulfur and nitrogen compounds. *Appl Environ Microbiol* **81:** 2384-2394.

Herrmann M, Opitz S, Harzer R, Totsche K, Küsel K (2017). Attached and suspended denitrifier communities in pristine limestone aquifers harbor high fractions of potential autotrophs oxidizing reduced Iron and Sulfur compounds. *Microb Ecol***:** 1-14.

Herzyk A, Maloszewski P, Qiu S, Elsner M, Griebler C (2014). Intrinsic potential for immediate biodegradation of toluene in a pristine, energy-limited aquifer. *Biodegradation* **25:** 325-336.

Heylen K, Vanparys B, Wittebolle L, Verstraete W, Boon N, De Vos P (2006). Cultivation of denitrifying bacteria: Optimization of isolation conditions and diversity study. *Appl Environ Microbiol* **72:** 2637-2643.

Hiraishi A (1992). Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. *Lett Appl Microbiol* **15:** 210-213.

Hirsch MD, Long ZT, Song B (2011). Anammox bacterial diversity in various aquatic ecosystems based on the detection of Hydrazine Oxidase genes (*hzoA*/*hzoB*). *Microb Ecol* **61:** 264-276.

Hoehler TM, Jørgensen BB (2013). Microbial life under extreme energy limitation. *Nature Reviews Microbiology* **11:** 83.

Hou L, Zheng Y, Liu M, Li X, Lin X, Yin G *et al* (2015). Anaerobic ammonium oxidation and its contribution to nitrogen removal in China's coastal wetlands. *Sci Rep* **5:** 15621.

Hu P, Lang J, Wawrousek K, Yu J, Maness P-C, Chen J (2012). Draft Genome Sequence of *Rubrivivax gelatinosus* CBS. *J Bacteriol* **194:** 3262-3262.

Hu Z, Speth DR, Francoijs K-J, Quan Z-X, Jetten MSM (2012). Metagenome analysis of a complex community reveals the metabolic blueprint of anammox bacterium "*Candidatus* Jettenia asiatica". *Front Microbiol* **3:** 366.

Hu BL, Shen LD, Liu S, Cai C, Chen TT, Kartal B *et al* (2013). Enrichment of an anammox bacterial community from a flooded paddy soil. *Environ Microbiol Rep* **5:** 483-489.

Huang WE, Stoecker K, Griffiths R, Newbold L, Daims H, Whiteley AS *et al* (2007). Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence in situ hybridization for the single cell analysis of identity and function. *Environ Microbiol* **9:** 1878-1889.

Huebsch M, Fenton O, Horan B, Hennessy D, Richards KG, Jordan P *et al* (2014). Mobilisation or dilution? Nitrate response of karst springs to high rainfall events. *Hydrol Earth Syst Sci* **18:** 4423-4435.

Hug LA, Thomas BC, Brown CT, Frischkorn KR, Williams KH, Tringe SG *et al* (2015). Aquifer environment selects for microbial species cohorts in sediment and groundwater. *The ISME Journal* **9:** 1846-1856.

Humbert S, Tarnawski S, Fromin N, Mallet MP, Aragno M, Zopfi J (2010). Molecular detection of anammox bacteria in terrestrial ecosystems: distribution and diversity. *ISME J* **4:** 450-454.

Hunter WJ, Kuykendall LD, Manter DK (2007). *Rhizobium selenireducens* sp. nov.: A selenite-reducing α-*Proteobacteria* isolated from a bioreactor. *Curr Microbiol* **55:** 455-460.

IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.

Isaka K, Date Y, Kimura Y, Sumino T, Tsuneda S (2008). Nitrogen removal performance using anaerobic ammonium oxidation at low temperatures. *FEMS Microbiol Lett* **282:** 32-38.

Jacinthe P-A, Groffman PM, Gold AJ, Mosier A (1998). Patchiness in microbial nitrogen transformations in groundwater in a riparian forest. *J Environ Qual* **27:** 156-164.

James NG, Wilfried W, Adrian L, Allison ML, Albert B, Jan Willem E (2014). Nitrogen footprints: past, present and future. *Environmental Research Letters* **9:** 115003.

Jensen MM, Lam P, Revsbech NP, Nagel B, Gaye B, Jetten MS *et al* (2011). Intensive nitrogen loss over the Omani Shelf due to anammox coupled with dissimilatory nitrite reduction to ammonium. *ISME J* **5:** 1660-1670.

Jetten MSM (2001). New pathways for ammonia conversion in soil and aquatic systems. *Plant and Soil* **230:** 9-19.

Jetten MSM, Wagner M, Fuerst J, van Loosdrecht M, Kuenen G, Strous M (2001). Microbiology and application of the anaerobic ammonium oxidation ('anammox') process. *Curr Opin Biotechnol* **12:** 283-288.

Jetten MSM, Cirpus I, Kartal B, van Niftrik L, van de Pas-Schoonen KT, Sliekers O *et al* (2005). 1994–2004: 10 years of research on the anaerobic oxidation of ammonium. *Biochem Soc Trans* **33:** 119-123.

Jetten MSM (2008). The microbial nitrogen cycle. *Environ Microbiol* **10:** 2903-2909.

Jetten MS, Niftrik L, Strous M, Kartal B, Keltjens JT, Op den Camp HJ (2009). Biochemistry and molecular biology of anammox bacteria. *Crit Rev Biochem Mol Biol* **44:** 65-84.

Jochum T, Michalzik B, Bachmann A, Popp J, Frosch T (2015). Microbial respiration and natural attenuation of benzene contaminated soils investigated by cavity enhanced Raman multi-gas spectroscopy. *Analyst* **140:** 3143-3149.

Jochum T, von Fischer JC, Trumbore S, Popp J, Frosch T (2015). Multigas leakage correction in static environmental chambers using sulfur hexafluoride and Raman spectroscopy. *Anal Chem* **87:** 11137-11142.

Jochum T, Fastnacht A, Trumbore SE, Popp J, Frosch T (2017). Direct Raman spectroscopic measurements of biological nitrogen fixation under natural conditions: An analytical approach for studying Nitrogenase activity. *Anal Chem* **89:** 1117-1122.

Jones CM, Graf DRH, Bru D, Philippot L, Hallin S (2013). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *The ISME Journal* **7:** 417-426.

Jørgensen BB (1990). A Thiosulfate Shunt in the Sulfur Cycle of Marine Sediments. *Science* **249:** 152-154.

Justin P, Kelly DP (1978). Metabolic changes in *Thiobacillus denitrificans* accompanying the transition from aerobic to anaerobic growth in continuous chemostat culture. *Microbiology* **107:** 131-137.

Kallmeyer J, Pockalny R, Adhikari RR, Smith DC, D'Hondt S (2012). Global distribution of microbial abundance and biomass in subseafloor sediment. *Proceedings of the National Academy of Sciences* **109:** 16213-16216.

Kalvelage T, Lavik G, Lam P, Contreras S, Arteaga L, Loscher CR *et al* (2013). Nitrogen cycling driven by organic matter export in the South Pacific oxygen minimum zone. *Nature Geosci* **6:** 228-234.

Kandeler E, Deiglmayr K, Tscherko D, Bru D, Philippot L (2006). Abundance of *narG*, *nirS*, *nirK*, and *nosZ* genes of denitrifying bacteria during primary successions of a glacier foreland. *Appl Environ Microbiol* **72:** 5957-5962.

Kartal B, Rattray J, van Niftrik LA, van de Vossenberg J, Schmid MC, Webb RI *et al* (2007). *Candidatus* "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. *Syst Appl Microbiol* **30:** 39-49.

Kartal B, Van Niftrik L, Rattray J, Van De Vossenberg JLCM, Schmid MC, Sinninghe Damsté J *et al* (2008). *Candidatus* 'Brocadia fulgida': an autofluorescent anaerobic ammonium oxidizing bacterium. *FEMS Microbiol Ecol* **63:** 46-55.

Kartal B, Maalcke WJ, de Almeida NM, Cirpus I, Gloerich J, Geerts W *et al* (2011). Molecular mechanism of anaerobic ammonium oxidation. *Nature* **479:** 127.

Katayama Y, Kuraishi H (1978). Characteristics of *Thiobacillus thioparus* and its thiocyanate assimilation. *Can J Microbiol* **24:** 804-810.

Keiner R, Frosch T, Massad T, Trumbore S, Popp J (2014). Enhanced Raman multigas sensing - a novel tool for control and analysis of $^{13}CO_2$ labeling experiments in environmental research. *Analyst* **139:** 3879-3884.

Kellermann C, Griebler C (2009). *Thiobacillus thiophilus* sp. nov., a chemolithoautotrophic, thiosulfate-oxidizing bacterium isolated from contaminated aquifer sediments. *Int J Syst Evol Microbiol* **59:** 583-588.

Knowles R (1982). Denitrification. *Microbiol Rev* **46:** 43-70.

Kobayashi M, Matsuo Y, Takimoto A, Suzuki S, Maruo F, Shoun H (1996). Denitrification, a Novel Type of Respiratory Metabolism in Fungal Mitochondrion. *J Biol Chem* **271:** 16263-16267.

Kodama Y, Watanabe K (2003). Isolation and characterization of a sulfur-oxidizing chemolithotroph growing on crude oil under anaerobic conditions. *Appl Environ Microbiol* **69:** 107-112.

Kohlhepp B, Lehmann R, Seeber P, Küsel K, Trumbore SE, Totsche KU (2017). Aquifer configuration and geostructural links control the groundwater quality in thin-bedded carbonate–siliciclastic alternations of the Hainich CZE, central Germany. *Hydrol Earth Syst Sci* **21:** 6091-6116.

Kojima H, Fukui M (2010). *Sulfuricella denitrificans* gen. nov., sp. nov., a sulfur-oxidizing autotroph isolated from a freshwater lake. *Int J Syst Evol Microbiol* **60:** 2862-2866.

Kojima H, Fukui M (2011). *Sulfuritalea hydrogenivorans* gen. nov., sp. nov., a facultative autotroph isolated from a freshwater lake. *Int J Syst Evol Microbiol* **61:** 1651-1655.

Kojima H, Shinohara A, Fukui M (2015). *Sulfurifustis variabilis* gen. nov., sp. nov., a sulfur oxidizer isolated from a lake, and proposal of *Acidiferrobacteraceae* fam. nov. and *Acidiferrobacterales* ord. nov. *Int J Syst Evol Microbiol* **65:** 3709-3713.

Kölle W, Strebel O, Böttcher J (1987). Reduced sulfur compounds in sandy aquifers and their interactions with groundwater. Proceedings of the International Symposium on Groundwater Monitoring and Management, 23–28 March 1987, Dresden, pp. 3-14

Kopf SH, McGlynn SE, Green-Saxena A, Guan Y, Newman DK, Orphan VJ (2015). Heavy water and 15N labelling with NanoSIMS analysis reveals growth rate-dependent metabolic heterogeneity in chemostats. *Environ Microbiol* **17:** 2542-2556.

Körner H, Zumft WG (1989). Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. *Appl Environ Microbiol* **55:** 1670-1676.

Korom SF (1992). Natural denitrification in the saturated zone: A review. *Water Resources Research* **28:** 1657-1668.

Kuenen JG, and Robertson LA (1984). Competition among chemolithotrophic bacteria under aerobic and anaerobic conditions. *In: M J Klug and C A Reddy (Eds) Current Perspectives in Microbial Ecology* American Society for Microbiology, Washington, D.C.: 306–313.

Kumar S, Herrmann M, Thamdrup B, Schwab VF, Geesink P, Trumbore SE *et al* (2017). Nitrogen loss from pristine carbonate-rock aquifers of the Hainich critical zone exploratory (Germany) is primarily driven by chemolithoautotrophic anammox processes. *Front Microbiol* **8**.

Küsel K, Totsche KU, Trumbore SE, Lehmann R, Steinhäuser C, Herrmann M (2016). How deep can surface signals be traced in the critical zone? Merging biodiversity with biogeochemistry research in a central German muschelkalk landscape. *Frontiers in Earth Science* **4**.

Kuypers MM, Sliekers AO, Lavik G, Schmid M, Jørgensen BB, Kuenen JG *et al* (2003). Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* **422:** 608-611.

Kuypers MM, Lavik G, Woebken D, Schmid M, Fuchs BM, Amann R *et al* (2005). Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. *Proc Natl Acad Sci U S A* **102:** 6478-6483.

Lam P, Jensen MM, Lavik G, McGinnis DF, Muller B, Schubert CJ *et al* (2007). Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. *Proc Natl Acad Sci U S A* **104:** 7104-7109.

Lam P, Lavik G, Jensen MM, van de Vossenberg J, Schmid M, Woebken D *et al* (2009). Revising the nitrogen cycle in the Peruvian oxygen minimum zone. *Proc Natl Acad Sci U S A* **106:** 4752-4757.

Lam P, Kuypers MM (2011). Microbial nitrogen cycling processes in oxygen minimum zones. *Ann Rev Mar Sci* **3:** 317-345.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci U S A* **82:** 6955-6959.

Lane DJ (1991). 16S/23S rRNA sequencing. In: E. Stackebrandt M. Good fellow. Nucleic acid techniques in bacterial systematics. New York, N.Y.: John Wiley & Sons, Inc.. pp. 115-176.

Lansdown K, McKew BA, Whitby C, Heppell CM, Dumbrell AJ, Binley A *et al* (2016). Importance and controls of anaerobic ammonium oxidation influenced by riverbed geology. *Nature Geosci* **9:** 357-360.

Lasagna M, De Luca DA, Franchino E (2016). The role of physical and biological processes in aquifers and their importance on groundwater vulnerability to nitrate pollution. *Environmental Earth Sciences* **75:** 961.

Lau MCY, Kieft TL, Kuloyo O, Linage-Alvarez B, van Heerden E, Lindsay MR *et al* (2016). An oligotrophic deep-subsurface community dependent on syntrophy is dominated by sulfur-driven autotrophic denitrifiers. *Proc Natl Acad Sci U S A* **113:** E7927-E7936.

Lazar CS, Stoll W, Lehmann R, Herrmann M, Schwab VF, Akob DM *et al* (2017). Archaeal diversity and CO_2 fixers in carbonate-/siliciclastic-rock groundwater ecosystems. *Archaea* **2017:** 2136287.

Lee K-C, Rittmann BE (2002). Applying a novel autohydrogenotrophic hollow-fiber membrane biofilm reactor for denitrification of drinking water. *Water Research* **36:** 2040-2052.

Lee JW, Lee KH, Park KY, Maeng SK (2010). Hydrogenotrophic denitrification in a packed bed reactor: Effects of hydrogen-to-water flow rate ratio. *Biores Tech* **101:** 3940-3946.

Lehman RM, O'Connell SP, Banta A, Fredrickson JK, Reysenbach A-L, Kieft TL *et al* (2004). Microbiological comparison of core and groundwater samples collected from a fractured basalt aquifer with that of dialysis chambers incubated *in situ*. *Geomicrobiology Journal* **21:** 169-182.

Li L, Maher K, Navarre-Sitchler A, Druhan J, Meile C, Lawrence C *et al* (2017). Expanding the role of reactive transport models in critical zone processes. *Earth-Sci Rev* **165:** 280-301.

Liu J, Sun F, Wang L, Ju X, Wu W, Chen Y (2014). Molecular characterization of a microbial consortium involved in methane oxidation coupled to denitrification under micro-aerobic conditions. *Micro Biotech* **7:** 64-76.

López NI, Pettinari MJ, Stackebrandt E, Tribelli PM, Põtter M, Steinbüchel A *et al* (2009). *Pseudomonas extremaustralis* sp. nov., a Poly(3-hydroxybutyrate) producer isolated from an Antarctic environment. *Curr Microbiol* **59:** 514-519.

Longley G (1981). The Edwards aquifer: Earth's most diverse groundwater ecosystem? *International Journal of Speleology*, **11**: 123-128.

Lotti T, Kleerebezem R, Lubello C, van Loosdrecht MCM (2014). Physiological and kinetic characterization of a suspended cell anammox culture. *Water Res* **60:** 1-14.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar *et al* (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363-1371.

M. R (1989). Natural denitrification processes in the aquifer. *In Contaminant Transport in Groundwater (Edited by Kobus H E and Kinzelbach W)* Balkema, Rotterdam: 159-161.

Mackenzie FT (2011). Our Changing Planet: *An Introduction to Earth System Science and Global Environmental Change.* Prentice Hall.

Mailloux BJ, Fuller ME (2003). Determination of *in situ* bacterial growth rates in aquifers and aquifer sediments. *Appl Environ Microbiol* **69:** 3798-3808.

Manconi I, Carucci A, Lens P (2007). Combined removal of sulfur compounds and nitrate by autotrophic denitrification in bioaugmented activated sludge system. *Biotechnol Bioeng* **98:** 551-560.

Marín AI, Andreo B (2015). Vulnerability to contamination of karst aquifers. In: Stevanović Z (ed). *Karst Aquifers—Characterization and Engineering*. Springer International Publishing: Cham. pp 251-266.

McGrath KC, Thomas-Hall SR, Cheng CT, Leo L, Alexa A, Schmidt S *et al* (2008). Isolation and analysis of mRNA from environmental microbial communities. *J Microbiol Methods* **75:** 172-176.

Mergaert J, Cnockaert MC, Swings J (2003). *Thermomonas fusca* sp. nov. and *Thermomonas brevis* sp. nov., two mesophilic species isolated from a denitrification reactor with poly(ε-caprolactone) plastic granules as fixed bed, and emended description of the genus *Thermomonas*. *Int J Syst Evol Microbiol* **53:** 1961-1966.

Michotey V, Méjean V, Bonin P (2000). Comparison of methods for quantification of cytochrome cd(1)-denitrifying bacteria in environmental marine samples. *Appl Environ Microbiol* **66:** 1564-1571.

Moir JWB, Richardson DJ, Ferguson SJ (1995). The expression of redox proteins of denitrification in *Thiosphaera pantotropha* grown with oxygen, nitrate, and nitrous oxide as electron acceptors. *Arch Microbiol* **164:** 43-49.

Moore TA, Xing Y, Lazenby B, Lynch MD, Schiff S, Robertson WD *et al* (2011). Prevalence of anaerobic ammonium-oxidizing bacteria in contaminated groundwater. *Environ Sci Technol* **45:** 7217-7225.

Morales M, Sentchilo V, Bertelli C, Komljenovic A, Kryuchkova-Mostacci N, Bourdilloud A *et al* (2016). The genome of the toluene-degrading *Pseudomonas veronii* strain 1YdBTEX2 and its differential gene expression in contaminated sand. *PLoS One* **11:** e0165850.

Mulder A, van de Graaf AA, Robertson LA, Kuenen JG (1995). Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiol Ecol* **16:** 177-183.

Mulder A, van de Graaf AA, Robertson LA, Kuenen JG (1995). Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiol Ecol* **16:** 177-183.

Murdock SA, Juniper SK (2017). Capturing compositional variation in denitrifying communities: a multiple-primer approach that includes *Epsilonproteobacteria*. *Appl Environ Microbiol* **83:** e02753-02716.

Nagashima S, Kamimura A, Shimizu T, Nakamura-Isaki S, Aono E, Sakamoto K *et al* (2012). Complete genome sequence of phototrophic *Betaproteobacterium Rubrivivax gelatinosus* IL144. *J Bacteriol* **194:** 3541-3542.

Narr A, Nawaz A, Wick LY, Harms H, Chatzinotas A (2017). Soil viral communities vary temporally and along a land use transect as revealed by virus-like particle counting and a modified community fingerprinting approach (fRAPD). *Front Microbiol* **8**.

Nawaz A, Purahong W, Lehmann R, Herrmann M, Küsel K, Totsche KU *et al* (2016). Superimposed pristine limestone aquifers with marked hydrochemical differences exhibit distinct fungal communities. *Front Microbiol* **7:** 666.

Neef A, Amann R, Schlesner H, Schleifer K-H (1998). Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology* **144:** 3257-3266.

Nelson MC, Morrison HG, Benjamino J, Grim SL, Graf J (2014). Analysis, optimization and verification of Illumina-generated 16S rRNA gene amplicon surveys. *PLoS One* **9:** e94249.

Nemčić-Jurec J, Jazbec A (2017). Point source pollution and variability of nitrate concentrations in water from shallow aquifers. *Applied Water Science* **7:** 1337-1348.

Nercessian O, Fouquet Y, Pierre C, Prieur D, Jeanthon C (2005). Diversity of Bacteria and Archaea associated with a carbonate-rich metalliferous sediment sample from the Rainbow vent field on the Mid-Atlantic Ridge. *Environ Microbiol* **7:** 698-714.

Nowak ME, Schwab VF, Lazar CS, Behrendt T, Kohlhepp B, Totsche KU *et al* (2017). Carbon isotopes of dissolved inorganic carbon reflect utilization of different carbon sources by microbial communities in two limestone aquifer assemblages. *Hydrol Earth Syst Sci* **21:** 4283-4300.

Opitz S, Küsel K, Spott O, Totsche KU, Herrmann M (2014). Oxygen availability and distance to surface environments determine community composition and abundance of ammonia-oxidizing prokaroytes in two superimposed pristine limestone aquifers in the Hainich region, Germany. *FEMS Microbiol Ecol* **90:** 39-53.

Oshiki M, Shimokawa M, Fujii N, Satoh H, Okabe S (2011). Physiological characteristics of the anaerobic ammonium-oxidizing bacterium '*Candidatus* Brocadia sinica'. *Microbiology* **157:** 1706-1713.

Oshiki M, Shinyako-Hata K, Satoh H, Okabe S (2015). Draft genome sequence of an anaerobic ammonium-oxidizing bacterium, "*Candidatus* Brocadia sinica". *Genome Announcements* **3:** e00267-00215.

Oshiki M, Satoh H, Okabe S (2016). Ecology and physiology of anaerobic ammonium oxidizing bacteria. *Environ Microbiol* **18:** 2784-2796.

Oshiki M, Mizuto K, Kimura Z-i, Kindaichi T, Satoh H, Okabe S (2017). Genetic diversity of marine anaerobic ammonium-oxidizing bacteria as revealed by genomic and proteomic analyses of '*Candidatus* Scalindua japonica'. *Environ Microbiol Rep* **9:** 550-561.

Pabich WJ, Valiela I, Hemond HF (2001). Relationship between DOC concentration and vadose zone thickness and depth below water table in groundwater of Cape Cod, U.S.A. *Biogeochemistry* **55:** 247-268.

Palmer K, Biasi C, Horn MA (2012). Contrasting denitrifier communities relate to contrasting N_2O emission patterns from acidic peat soils in arctic tundra. *ISME J* **6:** 1058-1077.

Park Y-D, Lee HB, Yi H, Kim Y, Bae KS, Choi J-E *et al* (2005). *Pseudomonas panacis* sp. nov., isolated from the surface of rusty roots of Korean ginseng. *Int J Syst Evol Microbiol* **55:** 1721-1724.

Park H, Brotto AC, van Loosdrecht MCM, Chandran K (2017). Discovery and metagenomic analysis of an anammox bacterial enrichment related to *Candidatus* "Brocadia caroliniensis" in a full-scale glycerol-fed nitritation-

denitritation separate centrate treatment process. *Water Research* **111:** 265-273.

Pfennig N (1978). *Rhodocyclus purpureus* gen. nov. and sp. nov., a ring-shaped, vitamin B12-requiring member of the family *Rhodospirillaceae*. *Int J Syst Evol Microbiol* **28:** 283-288.

Philippot L (2002). Denitrifying genes in bacterial and Archaeal genomes. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1577:** 355-376.

Pinay G, Roques L, Fabre A (1993). Spatial and temporal patterns of denitrification in a riparian forest. *J Appl Ecol* **30:** 581-591.

Pitcher A, Villanueva L, Hopmans EC, Schouten S, Reichart G-J, Sinninghe Damste JS (2011). Niche segregation of ammonia-oxidizing archaea and anammox bacteria in the Arabian Sea oxygen minimum zone. *ISME J* **5:** 1896-1904.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P *et al* (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41:** D590-D596.

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Baines JS, Worden RH (2004). Geological storage of carbon dioxide. Geological Society, London, Special Publications **233**, pp. 129-145.

Radajewski S, Ineson P, Parekh NR, Murrell JC (2000). Stable-isotope probing as a tool in microbial ecology. *Nature* **403:** 646.

Radajewski S, McDonald IR, Murrell JC (2003). Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. *Curr Opin Biotechnol* **14:** 296-302.

Raghoebarsing AA, Pol A, van de Pas-Schoonen KT, Smolders AJP, Ettwig KF, Rijpstra WIC *et al* (2006). A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* **440:** 918.

Ravindran S (2017). Inner Workings: Bacteria work together to survive Earth's depths. *Proceedings of the National Academy of Sciences* **114:** 788-790.

Read RR, Costerton JW (1987). Purification and characterization of adhesive exopolysaccharides from *Pseudomonas putida* and *Pseudomonas fluorescens*. *Can J Microbiol* **33:** 1080-1090.

Rich JJ, Heichen RS, Bottomley PJ, Cromack K, Myrold DD (2003). Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. *Appl Environ Microbiol* **69:** 5974-5982.

Rivett MO, Buss SR, Morgan P, Smith JW, Bemment CD (2008). Nitrate attenuation in groundwater: a review of biogeochemical controlling processes. *Water Res* **42:** 4215-4232.

Robertson WD, Moore TA, Spoelstra J, Li L, Elgood RJ, Clark ID *et al* (2012). Natural attenuation of septic system nitrogen by anammox. *Groundwater* **50:** 541-553.

Rotthauwe JH, Witzel KP, Liesack W (1997). The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* **63:** 4704-4712.

Ruggieri R, Forti P, Antoci ML, De Waele J (2017). Accidental contamination during hydrocarbon exploitation and the rapid transfer of heavy-mineral fines through an overlying highly karstified aquifer (Paradiso Spring, SE Sicily).

Journal of Hydrology **546:** 123-132.

Rysgaard S, Glud RN (2004). Anaerobic N_2 production in Arctic sea ice. *Limnol and Oceanog* **49:** 86-94.

Saarenheimo J, Tiirola MA, Rissanen AJ (2015). Functional gene pyrosequencing reveals core proteobacterial denitrifiers in boreal lakes. *Front Microbiol* **6**.

Salinero KK, Keller K, Feil WS, Feil H, Trong S, Di Bartolo G *et al* (2009). Metabolic analysis of the soil microbe *Dechloromonas aromatica* str. RCB: indications of a surprisingly complex life-style and cryptic anaerobic pathways for aromatic degradation. *BMC Genomics* **10:** 351-351.

Schlesinger WH (2009). On the fate of anthropogenic nitrogen. *Proceedings of the National Academy of Sciences* **106:** 203-208.

Schink B, Stams AJM (2006). Syntrophism among prokaryotes. In: Dworkin, M.; Falkow, S.; Rosenberg, E.; Schleifer, KH.; Stackebrandt, E., editors. *The Prokaryotes: an evolving electronic resource for the microbiological community.* Vol. 3. Vol. 2. Springer-Verlag; 2006. p. 309-335.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB *et al* (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75:** 7537-7541.

Schloss PD, Gevers D, Westcott SL (2011). Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-Based Studies. *PLoS One* **6:** e27310.

Schmid M, Twachtmann U, Klein M, Strous M, Juretschko S, Jetten M *et al* (2000). Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. *Syst Appl Microbiol* **23:** 93-106.

Schmid M, Walsh K, Webb R, Rijpstra WI, van de Pas-Schoonen K, Verbruggen MJ *et al* (2003). *Candidatus* “Scalindua brodae”, sp. nov., *Candidatus* “Scalindua wagneri”, sp. nov., two new species of anaerobic ammonium oxidizing bacteria. *Syst Appl Microbiol* **26:** 529-538.

Schmid MC, Hooper AB, Klotz MG, Woebken D, Lam P, Kuypers MMM *et al* (2008). Environmental detection of octahaem cytochrome c hydroxylamine/hydrazine oxidoreductase genes of aerobic and anaerobic ammonium-oxidizing bacteria. *Environ Microbiol* **10:** 3140-3149.

Schmidt I, Sliekers O, Schmid M, Cirpus I, Strous M, Bock E *et al* (2002). Aerobic and anaerobic ammonia oxidizing bacteria – competitors or natural partners? *FEMS Microbiol Ecol* **39:** 175-181.

Schouten S, Strous M, Kuypers MMM, Rijpstra WIC, Baas M, Schubert CJ *et al* (2004). Stable carbon isotopic fractionations associated with inorganic carbon fixation by anaerobic ammonium-oxidizing bacteria. *Appl Environ Microbiol* **70:** 3785-3788.

Schubert CJ, Durisch-Kaiser E, Wehrli B, Thamdrup B, Lam P, Kuypers MMM (2006). Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). *Environ Microbiol* **8:** 1857-1863.

Schwab VF, Herrmann M, Roth VN, Gleixner G, Lehmann R, Pohnert G *et al* (2017). Functional diversity of microbial communities in pristine aquifers inferred by PLFA- and sequencing-based approaches. *Biogeosciences* **14:** 2697-2714.

Seitzinger S, Harrison JA, Böhlke JK, Bouwman AF, Lowrance R, Peterson B *et al* (2006). Denitrification across landscapes and waterscapes: a synthesis. *Ecol Appl* **16:** 2064-2090.

Sgouridis F, Heppell CM, Wharton G, Lansdown K, Trimmer M (2011). Denitrification and dissimilatory nitrate reduction to ammonium (DNRA) in a temperate re-connected floodplain. *Water Research* **45:** 4909-4922.

Shehzad A, Liu J, Yu M, Qismat S, Liu J, Zhang X-H (2016). Diversity, Community composition and abundance of anammox bacteria in sediments of the north marginal seas of China. *Microbes Environ* **31:** 111-120.

Shen L-d, Wu H-s, Gao Z-q, Cheng H-x, Li J, Liu X *et al* (2016). Distribution and activity of anaerobic ammonium-oxidising bacteria in natural freshwater wetland soils. *Appl Microbiol Biotechnol* **100:** 3291-3300.

Shrout JD, Scheetz TE, Casavant TL, Parkin GF (2005). Isolation and characterization of autotrophic, hydrogen-utilizing, perchlorate-reducing bacteria. *Appl Microbiol Biotechnol* **67:** 261-268.

Sieburg A, Jochum T, Trumbore SE, Popp J, Frosch T (2017). Onsite cavity enhanced Raman spectrometry for the investigation of gas exchange processes in the Earth's critical zone. *Analyst* **142:** 3360-3369.

Singer E, Wagner M, Woyke T (2017). Capturing the genetic makeup of the active microbiome in situ. *The ISME Journal* **11:** 1949-1963.

Sinninghe Damsté JS, Rijpstra WIC, Geenevasen JAJ, Strous M, Jetten MSM (2005). Structural identification of ladderane and other membrane lipids of *planctomycetes* capable of anaerobic ammonium oxidation (anammox). *FEBS Journal* **272:** 4270-4283.

Smith CJ, Osborn AM (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol Ecol* **67:** 6-20.

Smith RL, Bohlke JK, Song B, Tobias CR (2015). Role of anaerobic ammonium oxidation (Anammox) in nitrogen removal from a freshwater aquifer. *Environ Sci Technol* **49:** 12169-12177.

Smits THM, Hüttmann A, Lerner DN, Holliger C (2009). Detection and quantification of bacteria involved in aerobic and anaerobic ammonium oxidation in an ammonium-contaminated aquifer. *Bioremed J* **13:** 41-51.

Speth DR, Lagkouvardos I, Wang Y, Qian P-Y, Dutilh BE, Jetten MSM (2017). Draft genome of Scalindua rubra, obtained from the interface above the discovery deep brine in the Red sea, sheds light on potential salt adaptation strategies in anammox bacteria. *Microb Ecol* **74:** 1-5.

Spiro S (2012). Nitrous oxide production and consumption: regulation of gene expression by gas-sensitive transcription factors. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367:** 1213-1225.

Stahl DA, Hullar M, and Davidson S (2006). The structure and function of microbial communities, in The Prokaryotes, edited by M. Dworkin et al., pp. 299 – 327, Springer, New York.

Starke R, Müller M, Gaspar M, Marz M, Küsel K, Totsche KU *et al* (2017). *Candidate* Brocadiales dominates C, N and S cycling in anoxic groundwater of a pristine limestone-fracture aquifer. *J Proteom* **152:** 153-160.

Steffen W (2008). Looking Back to the Future. *AMBIO: A Journal of the Human Environment* **37:** 507-513.

Steffen W, Sanderson A, Tyson PD, Jäger J, Matson PA, Moore III et al., (2004). Matson PA, Richardson K, Oldfield F, Schellnhuber HJ, Turner II BL, Wasson RJ. *Global Change and the Earth System: A Planet under Pressure* Springer, Heidelberg, Germany

Stein LY, Klotz MG (2016). The nitrogen cycle. *Curr Biol* **26:** R94-R98.

Sterner RW, Elser JJ, Vitousek P (2002). *Ecological stoichiometry: The biology of elements from molecules to the biosphere*. Princeton University Press.

Stevanović Z (2015). Karst environment and phenomena. In: Stevanović Z (ed). *Karst aquifers—characterization*

and engineering. Springer International Publishing: Cham. pp 19-46.

Stewart MK, Stevens G, Thomas JT, van der Raaij R, Trompetter V (2011). Nitrate sources and residence times of groundwater in the Waimea Plains, Nelson. *Journal of Hydrol (New Zealand)* **50:** 313-338.

Stoffels L, Krehenbrink M, Berks BC, Unden G (2012). Thiosulfate Reduction in *Salmonella enterica* is driven by the proton motive force. *J Bacteriol* **194:** 475-485.

Strous M, Heijnen JJ, Kuenen JG, Jetten MSM (1998). The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Appl Microbiol Biotechnol* **50:** 589-596.

Strous M, Fuerst JA, Kramer EHM, Logemann S, Muyzer G, van de Pas-Schoonen KT *et al* (1999). Missing lithotroph identified as new planctomycete. *Nature* **400:** 446-449.

Strous M, Pelletier E, Mangenot S, Rattei T, Lehner A, Taylor MW *et al* (2006). Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature* **440:** 790.

Stüeken EE, Kipp MA, Koehler MC, Buick R (2016). The evolution of Earth's biogeochemical nitrogen cycle. *Earth-Science Reviews* **160:** 220-239.

Hall, TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucl Acids Symp Ser* **41**:95-98

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* **30:** 2725-2729.

Taylor BF, Hoare DS, Hoare SL (1971). *Thiobacillus denitrificans* as an obligate chemolithotroph. *Arch Mikrobiol* **78:** 193-204.

Thamdrup B, Dalsgaard T (2002). Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. *Appl Environ Microbiol* **68:** 1312-1318.

Thamdrup B, Dalsgaard T, Jensen MM, Ulloa O, Farías L, Escribano R (2006). Anaerobic ammonium oxidation in the oxygen-deficient waters off northern Chile. *Limnol and Oceanogr* **51:** 2145-2156.

Thayalakumaran T, Bristow KL, Charlesworth PB, Fass T (2008). Geochemical conditions in groundwater systems: Implications for the attenuation of agricultural nitrate. *Agricul Water Manag.* **95:** 103-115.

Third KA, Paxman J, Schmid M, Strous M, Jetten MSM, Cord-Ruwisch R (2005). Enrichment of Anammox from Activated Sludge and Its Application in the CANON Process. *Microb Ecol* **49:** 236-244.

Throbäck IN, Enwall K, Jarvis A, Hallin S (2004). Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol Ecol* **49:** 401-417.

Tobias CR, Macko SA, Anderson IC, Canuel EA, Harvey JW (2001). Tracking the fate of a high concentration groundwater nitrate plume through a fringing marsh: A combined groundwater tracer and in situ isotope enrichment study. *Limnol Oceanogr* **46:** 1977-1989.

Townsend-Small A, Pataki DE, Czimczik CI, Tyler SC (2011). Nitrous oxide emissions and isotopic composition in urban and agricultural systems in southern California. *Journal of Geophysical Research: Biogeosciences* **116:** n/a-n/a.

Tratnyek PG, Grundl TJ, Haderlein SB (eds) (2011). *Aquatic Redox Chemistry*. American Chemical Society.

Tribelli PM, Raiger Iustman LJ, Catone MV, Di Martino C, Revale S, Méndez BS *et al* (2012). Genome sequence of the polyhydroxybutyrate producer *Pseudomonas extremaustralis*, a highly stress-resistant Antarctic bacterium. *J Bacteriol* **194:** 2381-2382.
Trimmer M, Nicholls JC (2009). Production of nitrogen gas via anammox and denitrification in intact sediment cores along a continental shelf to slope transect in the North Atlantic. *Limnology and Oceanography* **54:** 577-589.

Trouve C, Chazal PM, Gueroux B, Sauvaitre N (1998). Denitrification by new strains of *Thiobacillus Denitrificans* under non-standard physicochemical conditions. Effect of temperature, pH, and sulphur Source. *Environ Technol* **19:** 601-610.

Trüper HG, Schlegel HG (1964). Sulphur metabolism in *Thiorhodaceae* I. Quantitative measurements on growing cells of *Chromatium okenii*. *Antonie Van Leeuwenhoek* **30:** 225-238.

Turner S, Pryer KM, Miao VPW, Palmer JD (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis1. *J Eukaryot Microbiol* **46:** 327-338.

Umezawa K, Watanabe T, Miura A, Kojima H, Fukui M (2016). The complete genome sequences of sulfur-oxidizing Gammaproteobacteria *Sulfurifustis variabilis* skN76(T) and *Sulfuricaulis limicola* HA5(T). *Standards in Genomic Sciences* **11:** 71.

van de Graaf AA, Mulder A, de Bruijn P, Jetten MS, Robertson LA, Kuenen JG (1995). Anaerobic oxidation of ammonium is a biologically mediated process. *Appl Environ Microbiol* **61:** 1246-1251.

van de Graaf AA, de Bruijn P, Robertson LA, Jetten MSM, Kuenen JG (1997). Metabolic pathway of anaerobic ammonium oxidation on the basis of 15N studies in a fluidized bed reactor. *Microbiology* **143:** 2415-2421.

van de Vossenberg J, Woebken D, Maalcke WJ, Wessels HJCT, Dutilh BE, Kartal B *et al* (2013). The metagenome of the marine anammox bacterium '*Candidatus* Scalindua profunda' illustrates the versatility of this globally important nitrogen cycle bacterium. *Environ Microbiol* **15:** 1275-1289.

van Kessel MAHJ, Speth DR, Albertsen M, Nielsen PH, Op den Camp HJM, Kartal B *et al* (2015). Complete nitrification by a single microorganism. *Nature* **528:** 555.

van Niftrik L, Geerts WJC, van Donselaar EG, Humbel BM, Yakushevska A, Verkleij AJ *et al* (2008). Combined structural and chemical analysis of the anammoxosome: A membrane-bounded intracytoplasmic compartment in anammox bacteria. *J Struct Biol* **161:** 401-410.

Vitousek PM, Menge DNL, Reed SC, Cleveland CC (2013). Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. *Philosophical Transactions of the Royal Society B: Biological Sciences* **368:** 20130119.

Vlasceanu L, Popa R, Kinkle BK (1997). Characterization of *Thiobacillus thioparus* LV43 and its distribution in a chemoautotrophically based groundwater ecosystem. *Appl Environ Microbiol* **63:** 3123-3127.

Wang S, Radny D, Huang S, Zhuang L, Zhao S, Berg M *et al* (2017). Nitrogen loss by anaerobic ammonium oxidation in unconfined aquifer soils. *Sci Rep* **7:** 40173.

Ward BB, Devol AH, Rich JJ, Chang BX, Bulow SE, Naik H *et al* (2009). Denitrification as the dominant nitrogen loss process in the Arabian Sea. *Nature* **461:** 78-81.

Ward BB (2013). How nitrogen is lost. *Science* **341:** 352-353.

Watanabe T, Kojima H, Fukui M (2014). Complete genomes of freshwater sulfur oxidizers *Sulfuricella denitrificans* skB26 and *Sulfuritalea hydrogenivorans* sk43H: Genetic insights into the sulfur oxidation pathway of

betaproteobacteria. *Syst Appl Microbiol* **37:** 387-395.

Wawrousek K, Noble S, Korlach J, Chen J, Eckert C, Yu J *et al* (2014). Genome annotation provides insight into carbon monoxide and hydrogen metabolism in *Rubrivivax gelatinosus*. *PLoS One* **9:** e114551.

Wei W, Isobe K, Nishizawa T, Zhu L, Shiratori Y, Ohte N *et al* (2015). Higher diversity and abundance of denitrifying microorganisms in environments than considered previously. *The ISME Journal* **9:** 1954.

Wenk CB, Blees J, Zopfi J, Veronesi M, Bourbonnais A, Schubert CJ *et al* (2013). Anaerobic ammonium oxidation (anammox) bacteria and sulfide-dependent denitrifiers coexist in the water column of a meromictic south-alpine lake. *Limnology and Oceanography* **58:** 1-12.

Whitman WB, Coleman DC, Wiebe WJ (1998). Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences* **95:** 6578-6583.

Wickham H (2009) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Willems A, Falsen E, Pot B, Jantzen E, Hoste B, Vandamme P *et al* (1990). *Acidovorax*, a new genus for *Pseudomonas facilis*, *Pseudomonas delafieldii*, E. Falsen (EF) group 13, EF group 16, and several clinical isolates, with the species *Acidovorax facilis* comb. nov., *Acidovorax delafieldii* comb. nov., and *Acidovorax temperans* sp. nov. *Int J Syst Bacteriol* **40:** 384-398.

Woebken D, Fuchs BM, Kuypers MM, Amann R (2007). Potential interactions of particle-associated anammox bacteria with bacterial and archaeal partners in the Namibian upwelling system. *Appl Environ Microbiol* **73:** 4648-4657.

Wrage N, Velthof GL, van Beusichem ML, Oenema O (2001). Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biol and Biochem* **33:** 1723-1732.

Wu L, Wen C, Qin Y, Yin H, Tu Q, Van Nostrand JD *et al* (2015). Phasing amplicon sequencing on Illumina Miseq for robust environmental microbial community analysis. *BMC Microbiol* **15:** 125.

Xu Y, He T, Li Z, Ye Q, Chen Y, Xie E *et al* (2017). Nitrogen removal characteristics of *Pseudomonas putida* Y-9 capable of heterotrophic nitrification and aerobic denitrification at low temperature. *BioMed Research International* **2017:** 1429018.

Yan J, Haaijer SCM, Op den Camp HJM, Niftrik L, Stahl DA, Könneke M *et al* (2012). Mimicking the oxygen minimum zones: stimulating interaction of aerobic archaeal and anaerobic bacterial ammonia oxidizers in a laboratory-scale model system. *Environ Microbiol* **14:** 3146-3158.

Yang X-R, Li H, Nie S-A, Su J-Q, Weng B-S, Zhu G-B *et al* (2015). Potential Contribution of Anammox to Nitrogen Loss from Paddy Soils in Southern China. *Appl Environ Microbiol* **81:** 938-947.

Yoshinaga I, Amano T, Yamagishi T, Okada K, Ueda S, Sako Y *et al* (2011). Distribution and diversity of anaerobic ammonium oxidation (Anammox) bacteria in the sediment of a eutrophic freshwater lake, lake Kitaura, Japan. *Microbes Environ* **26:** 189-197.

Zeng X, Hosono T, Matsunaga M, Ohta H, Niidome T, Shimada J *et al* (2017). Spatial distribution of microbial communities in the alluvial aquifer along the Oyodo river, Miyakonojo basin, Japan. *J Water Environ Technol* **15:** 152-162.

Zhang L, Narita Y, Gao L, Ali M, Oshiki M, Okabe S (2017). Maximum specific growth rate of anammox bacteria revisited. *Water Research* **116:** 296-303.

Zhang Y, Yu M, Guo J, Wu D, Hua Z-S, Chen G-H *et al* (2017). Spatiotemporal heterogeneity of core functional bacteria and their synergetic and competitive interactions in denitrifying sulfur conversion-assisted enhanced biological phosphorus removal. *Sci Rep* **7:** 10927.

Zhou S, Borjigin S, Riya S, Terada A, Hosomi M (2014). The relationship between anammox and denitrification in the sediment of an inland river. *Sci Total Environ* **490:** 1029-1036.

Zhu B, Sánchez J, van Alen TA, Sanabria J, Jetten MSM, Ettwig KF *et al* (2011). Combined anaerobic ammonium and methane oxidation for nitrogen and methane removal. *Biochem Soc Trans* **39:** 1822-1825.

Zhu I, Getting T (2012). A review of nitrate reduction using inorganic materials. *Environ Tech Rev* **1:** 46-58.

Zhu G, Wang S, Wang W, Wang Y, Zhou L, Jiang B *et al* (2013). Hotspots of anaerobic ammonium oxidation at land–freshwater interfaces. *Nature Geoscience* **6:** 103.

Zhu G, Xia C, Shanyun W, Zhou L, Liu L, Zhao S (2015). Occurrence, activity and contribution of anammox in some freshwater extreme environments. *Environ Microbiol Rep* **7:** 961-969.

Zumft WG (1997). Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61:** 533-616.

APPENDIX

Appendix Table 5 | Composition of vitamin and trace element solution used in growth media (NTC).

Components	Quantity
Vitamin B_{12}	50.00 mg
Pantothenic acid	50.00 mg
Riboflavin	50.00 mg
Pyridoxamine-HCl	10.00 mg
Biotin	20.00 mg
Folic acid	20.00 mg
Nicotinic acid	25.00 mg
α-lipoic acid	50.00 mg
p-aminobenzoic acid	50.00 mg
Thiamine-HCl x $2H_2O$	50.00 mg
Distilled water	1000.00 ml
Stir for some hours, filter sterilize the solution.	

Composition of trace element solution SL-10

Components	Quantity
HCl (25%; 7.7 M)	10.00 ml
$FeCl_2$ x 4 H_2O	1.50 g
$ZnCl_2$	70.00 mg
$MnCl_2$ x 4 H_2O	100.00 mg
H_3BO_3	6.00 mg
$CoCl_2$ x 6 H_2O	190.00 mg
$CuCl_2$ x 2 H_2O	2.00 mg
$NiCl_2$ x 6 H_2O	24.00 mg
Na_2MoO_4 x 2 H_2O	36.00 mg
Distilled water	990.00 ml
Stir for some hours, filter sterilize the solution.	

[Adopted from DSMZ 461 Mineral medium (Nagel and Andreesen); http://www.dsmz.de]

For trace element preparation, $FeCl_2$ was first dissolved in the HCl, then diluted in 100 ml water, followed by the addition of other salts. Finally the volume was make up to 1000 ml. Both solutions (trace element solution and vitamin solution) were flushed with sterile Argon using 0.22 µm pore size sterile filter for 15 minutes to make them anoxic and the solutions were stored in covered serum bottle at 4 °C.

Appendix Table 6 | Physicochemical parameters of groundwater samples from eight wells of the Hainich aquifer assemblages. Data represent mean (± standard deviation) of samples obtained between Jan 2014 and Jun 2015 (n = 19). Distinct red and blue colour of well numbers represents Hainich transect upper aquifer assemblage (HTU) and Hainich transect lower aquifer assemblage (HTL), respectively. Cells are colored with hues of light grey and dark grey to indicate the lowest and highest value observed, respectively. Data for dissolved H_2S obtained between Mar 2015 to Sep 2016 (n=19).

Well	H32	H42	H43	H52	H53	H31	H41	H51
depth (m)	22.5	12.7	12	65	50	47	48	88
pH	7.3±0.1	7.1±0.1	7.1±0.1	7.3±0.1	7.3±0.1	7.3 ± 0.1	7.3±0.2	7.2±0.1
temperature (°C)	9.9±0.7	9.5±0.2	9.6±0.2	10.4±0.2	10.2±0.5	10.4 ± 0.9	10.2±0.3	10.5±0.3
oxygen (µmol L^{-1})	111±15	0.1±0.2	0.03±0.1	0.09±0.1	0.73±0.7	429±81	301±26	162±8
redox potential (mv)	401.2±47.9	157.5±15.8	241.8±15.9	228.9±39.4	262.8±37.8	402.5±66.3	407.2±49.2	416.2±49.2
DOC (mg L^{-1})	1.8±0.7	1.7±0.8	1.8±0.6	1.6±0.8	1.6±0.6	1.7±0.8	1.5±0.7	1.8±0.7
TOC (mg L^{-1})	2.2±0.9	1.9±0.8	2.0±0.6	1.9±0.9	1.9 ± 0.7	2.1±1.0	1.7±0.7	1.8±0.9
TIC (mg L^{-1})	80±37	90±36	90±40	74±34	80±37	76±38	80±35	71±33
NH_4^+ (µmol L^{-1})	6.0±6.6	11.0±6.9	7.8±5.6	22.2±13.9	30.0±17.3	3.0±3.5	6.0±4.2	3.4±3.8
NO_3^-(µmol L^{-1})	572.3±247.7	14±27.9	11.7±28.1	29.6±29.1	31.3±28.4	425.4±138.2	150±47.1	117.9±45.1
NO_2^-(µmol L^{-1})	0.3±0.2	0.3±0.5	0.1±0.2	0.2±0.2	0.2±0.2	0.2±0.2	0.3±0.6	0.3±0.5
SO_4^{2-}(µmol L^{-1})	633.7±177.4	319.1±74.3	325.2±63.4	843.7±255.4	596.4±196.6	1016.6±29.9	1079.7±335.9	2525.1±692.7
H_2S (µmol L^{-1})	0.4±0.7	0.2±0.4	0.2±0.4	0.2±0.3	0.6±1.2	0.2±0.5	0.2±0.3	0.2±0.4
Fe^{2+} (µmol L^{-1})	0.6±0.8	2.3±1.1	0.9±0.6	0.4±0.3	0.8±0.5	2.2±2.4	0.2±0.2	0.5±0.4
K (µmol L^{-1})	71.3±2.4	168.7±4.0	143.9±3.3	253.5±6.8	353.5±26.8	77.2±10.2	116.0±29.2	47.1±4.8
Ca (mmol L^{-1})	2.35±0.06	2.13±0.21	2.21±0.16	1.72±0.03	1.54±0.11	2.42±0.11	2.80±0.23	4.55±0.15

Appendix Table 7 | Correlation analysis (Spearman rank correlation coefficient) between physicochemical parameters and gene abundances of groundwater samples across eight wells and two aquifer assemblages. Analyses are based on monthly samples obtained between January 2014 and August 2015. Levels of significance are indicated as p≤0.05*, p≤0.01**, n.s.= not significant. Cells are colored with hues of blue and red to indicate the strength of positive or negative correlation coefficient, respectively.

Gene abundances	pH	O_2	NH_4^+	NO_2^-	NO_3^-	SO_4^{2-}	K	Ca	*nirS*	*nirK*	*hzsA*
Bac 16S rRNA	0.349**	n.s.	n.s.	-0.171*	n.s.	n.s.	0.484**	-0.438**	0.847**	0.662**	0.806**
nirS	0.451**	n.s.	n.s.	n.s.	n.s.	n.s.	0.442**	-0.446**	-	0.509**	0.877**
nirK	n.s.	n.s.	-0.173*	n.s.	-0.238**	n.s.	0.437**	-0.326**	-	-	0.618**
hzsA	0.407**	n.s.	n.s.	n.s.	n.s.	0.238**	0.379**	-0.315**			-
***nirS*/16S rRNA**	0.349**	n.s.	n.s.	n.s.	n.s.	n.s.	0.226**	-0.236**	-	-	-
***nirK*/16S rRNA**	-0.375**	n.s.	-0.198*	-0.243**	n.s.	n.s.	n.s.	0.321**	-	-	-
***hzsA*/16S rRNA**	0.220**	0.219**	n.s.	n.s.	n.s.	0.364**	n.s.	n.s.	-	-	-

Appendix Table 8 | Results of MiSeq Illumina amplicon sequencing of bacterial 16S rRNA genes (DNA-based). Values are given for data sets after subsampling to 16383 sequence reads per sample. OTUs were assigned on a 0.03 distance cut-off level. n before subsampling = number of sequence reads before subsampling; Chao1 = Chao's richness estimator; Shannon = Shannon diversity index. (A_14: August, 2014; A_15: August, 2015 and N_15: November, 2015). Distinct red and blue colour of well numbers represents Hainich transect upper aquifer assemblage (HTU) and Hainich transect lower aquifer assemblage (HTL), respectively.

sample	Month	Nucleic acid	n before subsampling	Observed OTUs	Chao1	Coverage (%)	Shannon
H32	A_14	DNA	38428	2871	5640	0.901	6.18
H32	A_15	DNA	20015	3389	7038	0.882	6.68
H32	N_15	DNA	67178	3536	7429	0.873	6.75
H42	A_14	DNA	44170	3746	6611	0.880	6.94
H42	A_15	DNA	33198	3188	8213	0.877	6.26
H42	N_15	DNA	43871	3094	7447	0.885	6.42
H43	A_14	DNA	21585	2490	4641	0.919	6.05
H43	A_15	DNA	74402	2596	4395	0.931	6.63
H43	N_15	DNA	60855	5181	15062	0.780	7.19
H52	A_14	DNA	34006	1579	5394	0.933	4.36
H52	A_15	DNA	18689	1472	4277	0.943	4.75
H52	N_15	DNA	56633	1972	6415	0.916	4.96
H53	A_14	DNA	37392	1402	4732	0.940	3.74
H53	A_15	DNA	61972	1609	4081	0.938	4.64
H53	N_15	DNA	56546	1826	5043	0.926	4.60
H31	A_14	DNA	34495	3219	9192	0.874	6.40
H41	A_14	DNA	16383	2433	3339	0.940	6.24
H41	A_15	DNA	34384	2464	4475	0.921	6.00
H41	N_15	DNA	30368	3061	7373	0.882	6.18
H51	A_14	DNA	17902	2277	2792	0.961	6.61
H51	A_15	DNA	33360	3205	5882	0.896	6.55
H51	N_15	DNA	43984	3055	5265	0.906	6.44

Appendix Table 9 | Results of MiSeq Illumina amplicon sequencing of bacterial 16S rRNA genes (RNA-based). Values are given for data sets after subsampling to 16,383 sequence reads per sample. OTUs were assigned on a 0.03 distance cut-off level. n before subsampling = number of sequence reads before subsampling; Chao1 = Chao's richness estimator; Shannon = Shannon diversity index. Distinct red and blue colour of well numbers represents Hainich transect upper aquifer assemblage (HTU) and Hainich transect lower aquifer assemblage (HTL), respectively.

sample	Nucleic acid	n before subsampling	Observed OTUs	Chao1	Coverage (%)	Shannon
H32	RNA	21610	3227	8510	0.871	6.03
H42	RNA	24888	2424	6391	0.905	5.54
H43	RNA	19446	4115	14741	0.814	6.21
H52	RNA	24244	2058	7576	0.908	4.24
H53	RNA	34332	1597	4708	0.933	4.07
H31	RNA	20500	2500	10828	0.894	5.66
H41	RNA	32378	3323	14364	0.843	5.07
H51	RNA	46416	2766	7683	0.893	5.82

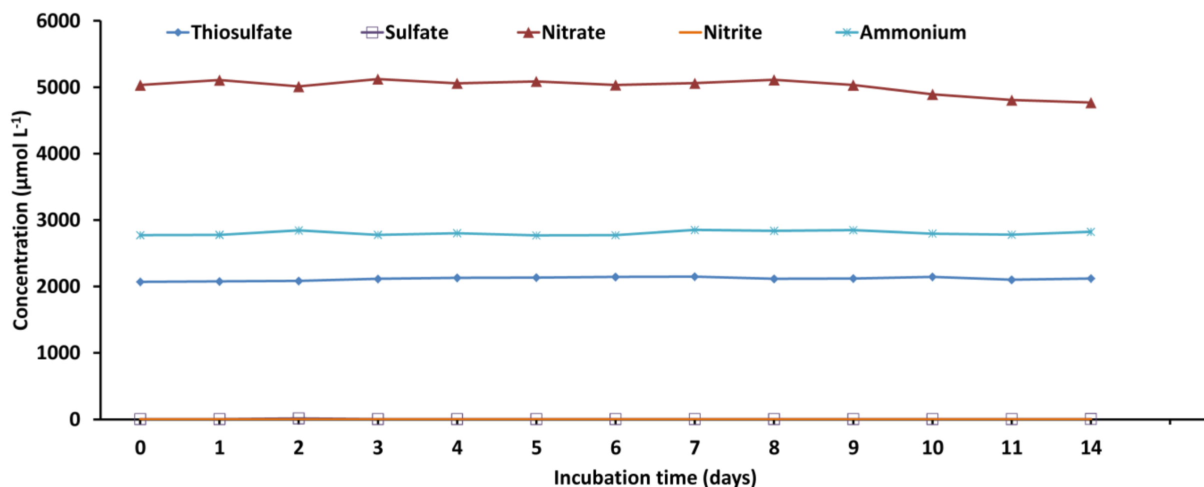
Appendix Table 10 | Results of MiSeq Illumina amplicon sequencing of *nirS* genes in the groundwater of eight wells across the two aquifer assemblages. Values are given for data sets after subsampling to 3005 sequence reads per sample. OTUs were assigned on a 0.18 distance cut-off level. n before subsampling = number of sequence reads before subsampling; Chao1 = Chao's richness estimator; Shannon = Shannon diversity index. (Samples from August 2014). Distinct red and blue colour of well numbers represents Hainich transect upper aquifer assemblage (HTU) and Hainich transect lower aquifer assemblage (HTL), respectively.

sample	Nucleic acid	n before subsampling	Observed OTUs	Chao1	Coverage (%)	Shannon
H32	DNA	15507	67	84	0.992	2.01
H42	DNA	9777	69	77	0.995	2.65
H43	DNA	3113	66	87	0.993	2.56
H52	DNA	6889	68	91	0.992	1.89
H53	DNA	17974	44	57	0.995	1.58
H31	DNA	3005	107	160	0.987	2.95
H41	DNA	3308	88	100	0.994	3.01
H51	DNA	5409	95	108	0.994	3.19

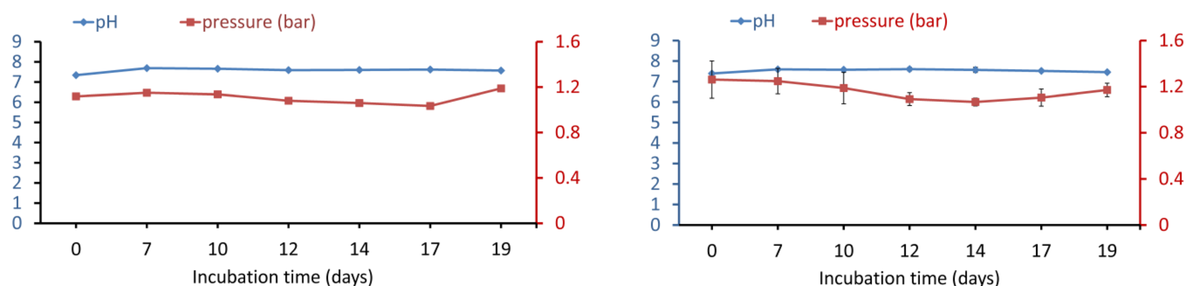
Appendix Table 11 | Results of MiSeq Illumina amplicon sequencing of *nirS* genes in the groundwater of four wells across the two aquifer assemblages. Values are given for data sets after subsampling to 3113 sequence reads per sample. OTUs were assigned on a 0.2 distance cut-off level. n before subsampling = number of sequence reads before subsampling; Shannon = Shannon diversity index. (Samples from 6 sampling events July, August; 2014, January, March, June, August 2015; at 4 sites H43, H53, H41 and H51). Distinct red and blue color of well numbers represents Hainich transect upper aquifer assemblages (HTU) and Hainich transect lower aquifer assemblages (HTL), respectively.

sample	Month	Nucleic acid	n before subsampling	Observed OTUs	Coverage (%)	Shannon
H43	Jul_14	DNA	103621	88	0.9998	2.07
H43	Aug_14	DNA	3113	57	0.9939	2.18
H43	Jan_15	DNA	102316	78	0.9998	2.17
H43	Mar_15	DNA	73392	85	0.9997	2.35
H43	Jun_15	DNA	93434	74	0.9999	2.07
H43	Aug_15	DNA	77476	80	0.9997	2.12
H53	Jul_14	DNA	134580	85	0.9998	1.17
H53	Aug_14	DNA	17982	47	0.9991	0.95
H53	Jan_15	DNA	120857	67	0.9998	0.91
H53	Mar_15	DNA	91030	74	0.9998	1.20
H53	Jun_15	DNA	106533	95	0.9997	1.36
H53	Aug_15	DNA	149290	93	0.9998	1.12
H41	Jul_14	DNA	173087	103	0.9998	1.16
H41	Aug_14	DNA	3308	58	0.9985	2.75
H41	Jan_15	DNA	72281	110	0.9996	2.66
H41	Mar_15	DNA	66455	123	0.9995	2.64
H41	Jun_15	DNA	67115	123	0.9995	2.08
H41	Aug_15	DNA	80622	117	0.9996	2.21
H51	Jul_14	DNA	95293	119	0.9997	1.98
H51	Aug_14	DNA	5409	61	0.9991	2.84
H51	Jan_15	DNA	42456	113	0.9992	2.02
H51	Mar_15	DNA	44694	86	0.9994	1.64
H51	Jun_15	DNA	53974	118	0.9995	1.94
H51	Aug_15	DNA	44629	117	0.9993	1.91

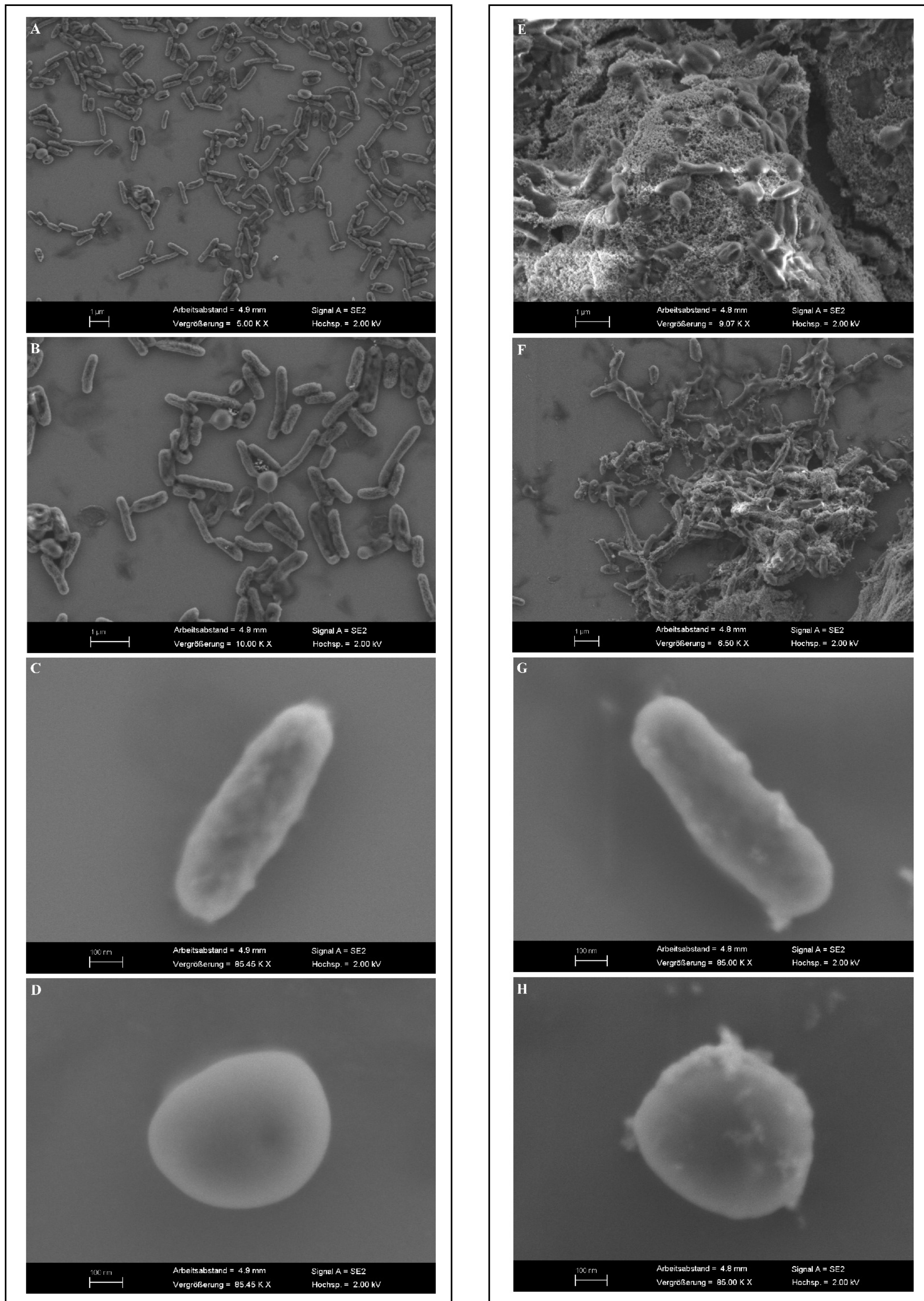
Appendix Figure 31 | Control for microcosm assay M_I



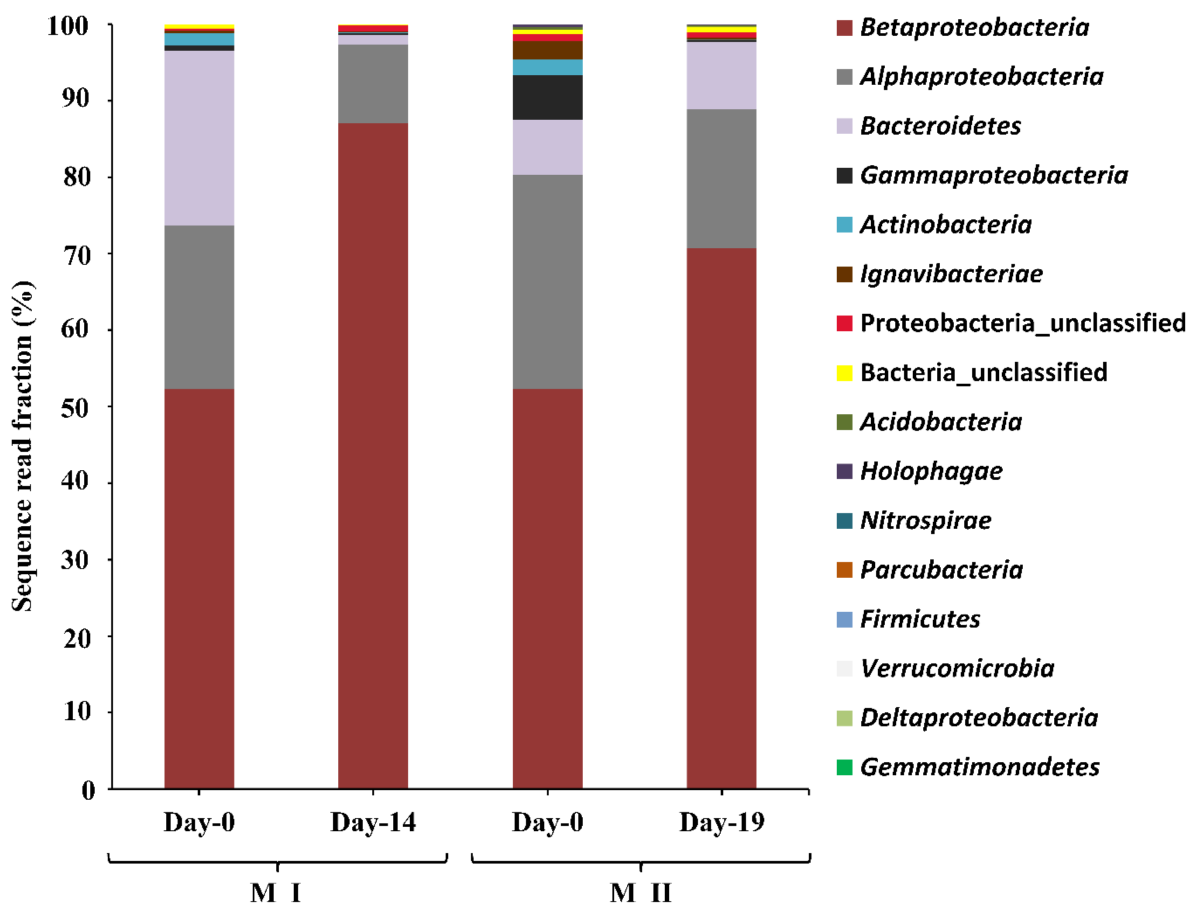
Appendix Figure 32 | pH and pressure were monitored during microcosm assay M_II. On left, values are from control, while on right, values are from triplicate samples (Bars represent, mean±standard deviation). Distinct blue and red colour represents pH of the growth medium and pressure inside the serum bottle, respectively.



Appendix Figure 33 | SEM image of enrichment culture 1E: Cells at different time points during the M_II assay incubation. Left panel, A-D showing cells at day-7 while right panel, E-H showing cells at day-17 of the incubation. Scale bars correspond to 1 µm (A, B, E, F) and 100 nm (C, D, G, H).



Appendix Figure 34 | Changes of the denitrifying microbial community (1E; class based) growing under chemolithoautotrophic conditions with different ratios of nitrate and thiosulfate: 2.5 and 1 utilized in microcosm experiment M_I and M_II, respectively. MiSeq amplicon sequencing based on 16S rRNA genes. Each bar represents the community composition (class based) of the denitrifying consortium at particular time point where the DNA was extracted from triplicate cultures.



ACKNOWLEDGEMENTS

Research work in the critical zone exploratory relies heavily on collaborators and support in order to get a clear understanding of interdisciplinary interactive processes in the biosphere, especially pristine groundwater ecosystems – neither an easy environment to work with or sample. Much of this work, therefore, is a result of teamwork. It involved encouragement, support, suggestions and guidance from many people and I wish to express here my sincere thanks to all of them for their invaluable help.

First, I would like to thank Prof. Dr. Kirsten Küsel, for providing me the opportunity to work in her research group and in addition to her constant support and interest in the interdisciplinary approach of microbiology, molecular biology and biogeochemistry. I appreciate Prof. Dr. Erika Kothe and Prof. Dr. Marcus Horn for their interest in the reviewing of this thesis.

My sincere thanks to Dr. Martina Herrmann for her mentoring, trust, patience and tolerance through all the haps and mishaps during the course of my research work. I am very grateful to her for continuing inspiration and her efforts in polishing my research skills. Also, she helped me to correct the complete thesis for technical and language errors.

I also wish to thank Prof. Dr. Susan Trumbore and Prof. Dr. Kai Uwe Totsche for their helpful advice and dedicated support on my work. Thanks must also go to all of my research collaborators for their contributions as co-authors in the published and pending manuscripts: Dr. Valerie F. Schwab, Dr. Torsten Frosch and Ines Hilke. I must greatly thank Prof. Dr. Bo Thamdrup, Dr. Peter Stief, Susanne Moller and Ann Sofie for giving me the opportunity for my short research visit in their group and training me on ^{15}N isotope analysis. I would also like to thank Prof. Dr. Mike Jetten for his suggestions regarding anammox quantification through functional genes.

I sincerely want to acknowledge the financial support from International Max Planck Research School for Global Biogeochemical Cycle Jena, the Deutsche Forschungsgemeinschaft (DFG); Collaborative Research Centre AquaDiva, Jena School of Microbial Communication; Jena and the Thuringian Landesgraduiertenstipendien.

Special thanks to Robert Lehmann, Heiko Minkmar, Bernd Ruppe, Jens Wurlitzer, Danny Schelhorn, Falko Gutmann, Sonja Rosenloecher for groundwater sampling and providing me with excellent laboratory assistantship. Also, I extend my appreciation to Dr. Maria Fabisch, Dr. Anna Spathe, Dr. Christine Steinhäuser, John Kula, Dr. Steffi Rotthardt, Dr. Julia Rödiger, Dr. Katja Praefcke, Karina Liebsch, and people at the Graduate Academy, Jena for their tremendous support as graduate school coordinators and constant help with university and administration obligations. My thanks to Kirsten Heinrich and Linda Maack at the library of Max Planck Institute for Biogeochemistry, for their constant support regarding excellent library services.

A warm heartfelt thanks to my colleagues in the Aquatic Geomicrobiology group, which has been welcoming and supportive. In particular, I would like to thank Constantinos, Patricia, Dominik, Annika, and Stefan for the lively discussions, allowing me to keep pushing forward.

My great gratitude is also given to Dr. Maria Judith Gonsalves and Dr. Sonal Malhotra. I would also like to thank my former colleagues and friends at the CSIR NIO Goa and the MoES NCAOR Goa, India. Their expertise in microbiology and interdisciplinary research fields incented me to widen my research from various perspectives.

I am very grateful to my parents and siblings for allowing me to choose my path, and constantly showering me with unconditional love and support that makes me feel so blessed and secure. Last, but not least, I wish to express my gratitude to my wife Pooja, who agreed to move halfway around the world and then back again so I could pursue the research of my dreams, and her support throughout all good and bad.
Only truth prevails.

DECLARATION OF AUTHORSHIP

I hereby affirm that I composed this dissertation by myself and only with the assistance and literature cited in the text. Those who provided assistance for the experiments, data analysis and writing of the manuscript are listed as coauthors or mentioned in the acknowledgements.

I confirm that I have read and dully understood the 'Course of Examination for Doctoral Candidates' (Promotionsordnung) by the Faculty of Biology and Pharmacy of the Friedrich Schiller University Jena.

I did not obtain any assistance from a consultant for doctoral theses, and no third parties have received any indirect or direct financial rewards in relation with the contents of this dissertation.

This dissertation has not been previously submitted for scientific survey to the Friedrich Schiller University Jena or to any other university.

Jena, 2017 ______________________________

Swatantar Kumar

PUBLISHED ARTICLE

The data included in this thesis were published in an international peer-reviewed journal. My contribution to the manuscript preparation is documented below.

Kumar S., Herrmann M., Thamdrup B., Schwab V.F., Geesink P., Trumbore S., Totsche K-U., Küsel K. (2017) Nitrogen loss from pristine carbonate-rock aquifers of the Hainich Critical Zone Exploratory (Germany) is primarily driven by chemolithoautotrophic anammox processes. ***Frontiers Microbiology***. 8:1951. doi: 10.3389/fmicb.2017.01951

MH, KK, and ST designed the work. I performed most of the molecular work, contributed to chemical analysis and field work, analyzed all the data, and wrote the first version of the manuscript. ST and KT contributed substantially to the biogeochemical, transect-oriented interpretation of the results. Analysis of sequence data was carried out by me and MH. PG performed a large part of the RNA-based work. BT carried out the ^{15}N based assessment of anammox and denitrification rates. VS performed the analysis of [3]-ladderane and [5]-ladderane phospholipid derived fatty acids. All authors contributed to the writing of the manuscript.

Confirmation of the individual contribution to the manuscript preparation:

Jena, ___________________________________

Kirsten Küsel