MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master‘s Thesis

„Ecophysiology and dynamics of nitrite oxidizers in saline-alkaline lakes“

verfasst von / submitted by

Queralt Güell Bujons

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2017 / Vienna 2017

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 830
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Molekulare Mikrobiologie, Mikrobielle Ökologie und Immunbiologie
Betreut von / Supervisor:	Univ.-Prof. Dipl.-Biol. Dr. Holger Daims

To my friends and familiy. For everything.
„Gràcies“

TABLE OF CONTENTS

ABSTRACT...8

ZUSAMMENFASSUNG..10

1. INTRODUCTION..12

1.1 Overview of the nitrogen cycle..12

1.2 Nitrite-oxidizing bacteria (NOB)...12

1.3 Environmental conditions and ecological mechanisms shaping NOB communities............................15

1.4 Microbial communities of saline-alkaline environments...17

1.5 The saline-alkaline lakes of the National Park "Neusidler see-Seewinkel"..18

1.6 Methodology: culture-dependent vs culture-independent approaches...19

1.7 Aims of the study...20

2. MATERIAL AND METHODS..22

2.1 Seasonal study of NOB communities from two saline-alkaline lakes..22
> Environment description and sampling...22
> DNA extraction...23
> Screening PCRs...23
> Gradient PCR and cloning...24
> Illumina MiSeq library preparation..24
> Illumina MiSeq computational analysis...25
> Data analysis. Statistics and phylogeny...25

2.2 Optimization, purification and study of three NOB enrichment cultures..26
> Environment description and sampling...26
> Enrichment, purification and optimization of the cultures...26
> Screening PCRs and metagenome analysis..27
> Fluorescence In Situ Hybridization (FISH)...28
> Scanning Electron Microscopy (SEM)..29
> Flow cytometry cell sorting...29

3. RESULTS..30

3.1 Seasonal study of NOB communities from two saline-alkaline lakes..30
3.1.1 Optimization of culture-independent methods for the study of nitrite oxidizers from saline-alkaline lake sediments...30
> Environmental data 30
> DNA extraction...32
> Screening PCRs...32

3.1.2 Seasonal study of NOB communities' composition and dynamics from two saline-alkalin1e lakes applying NGS techniques……34
> Illumina MiSeq library preparation……34
> Identification and phylogenetic affiliation of NOB OTUs……34
> Community structure and temporal dynamics……38
> Microbial community responses to the abiotic context……40

3.2 Optimization, purification and study of three NOB enrichment cultures……46
3.2.1 *Nitrospira* lineage IV enrichment ("KS")……46
> Culturing conditions……46
> Fluorescence In Situ Hybridization (FISH)……47
3.2.2 *Nitrospira* lineage II enrichment ("MixS")……47
> Fluorescence In Situ Hybridization (FISH)and scanning electron microscopy (SEM)……48
> Cell sorting……48
3.2.3 Unidentified nitrifier enrichment ("LL")……49
> Tetracycline (TET) resistance and solid media plates……50
> Cell sorting……51

4. DISCUSSION……52

4.1 Seasonal study of NOB communities from two saline-alkaline lakes……52
4.1.1 Optimization of culture-independent methods for the study of nitrite oxidizers from saline-alkaline lake sediments……52

4.1.2 Seasonal study of NOB communities' composition and dynamics from two saline-alkaline lakes applying NGS techniques……55
> Identification and phylogenetic affiliation of NOB OTUs……55
> Microbial community structure and responses to the abiotic context……57

4.2 Optimization, purification and study of three NOB enrichment cultures……64
3.2.1 *Nitrospira* lineage IV enrichment ("KS")……64

3.2.2 *Nitrospira* lineage II enrichment ("MixS")……66

3.2.3 Unidentified nitrifier enrichment ("LL")……69

5. CONCLUSIONS AND OUTLOOK……72

ACKNOWLEDGMENTS……74

REFERENCES……76

ANNEX I……88

ABSTRACT

Nitrite-oxidizing bacteria (NOB) perform the second nitrification step, the oxidation of nitrite to nitrate. Recently, a high diversity and great ecophysiological versatility of canonical NOB and even complete ammonia oxidizers to nitrate (comammox), belonging to a genus classically defined as nitrite-oxidizers, have been discovered. The high complexity of NOB communities found in natural systems strongly suggests that niche partitioning and niche specialization support the coexistence of sympatric NOB. However, current knowledge of nitrite-oxidizing communities and of comammox from extreme natural systems is scarce. Here we studied NOB and comammox organisms from saline-alkaline lakes of the National Park "Neusiedler See-Seewinkel" located at the border between Austria and Hungary. This interesting and unique ecosystem is strongly affected by intense seasonal fluctuations in temperature, salinity, pH, and inorganic nitrogen concentrations. The main aim of this M.Sc. project was the study of the ecophysiology of nitrifiers from this extreme system resolving their identity, adaptations and seasonal dynamics. The two approaches pursued, focused on the ecology and on the physiology of the NOB and provided novel and valuable information about particular nitrifiers.

Community composition and structure was disclosed by culture-independent identification of nitrite oxidizers and comammox in two different lake sediments, sampled monthly over more than a year, by barcoded next generation sequencing. Only canonical *Nitrospira* and comammox *Nitrospira* nitrifying genes (*nxrB* and *amoA*) were clearly detected. A high diversity of *Nitrospira* representatives, which belonged to different lineages of this NOB genus, was observed. Our results showed the predominance of few *Nitrospira* phylotypes throughout the year. Intriguingly, comammox *Nitrospira* of clade A were detected in both lakes and during most periods of the year. In contrast, comammox *Nitrospira* of clade B were found only stochastically. To our knowledge, this is the first detection of comammox organisms in aquatic systems with a strong alkaline pH and salinity far above common levels in freshwater. Additionally, we obtained strong indications for a significant influence of environmental parameters such as salinity and inorganic nitrogen concentrations on the community composition and dynamics of both target groups. Furthermore, evidences for unrecognized niche preferences and the probable existence of different phylotypes of comammox *Nitrospira* clade A were suggested by the obtained results.

The second approach, focused on the physiology of nitrite oxidizers, encompassed three enrichment cultures from saline-alkaline lakes of the same geographical area. Two of these cultures contained new *Nitrospira* species: one enrichment with a lineage IV *Nitrospira*, whose members were previously found only in marine environments, and one enrichment with a novel lineage II *Nitrospira.* Interestingly, the third enrichment culture showed a high nitrite-oxidizing activity but did not contain any known NOB according to molecular screenings for nitrite oxidoreductase genes and 16S rRNA-targeted FISH. Optimized culturing conditions and increased enrichment levels were accomplished for these cultures during the course of the project. Additionally, new morphologies and habitats could be described for canonical *Nitrospira*.

The genomic and physiological characterization of these novel organisms and the community dynamics followed for over a year provided insights into the unique adaptations to an extreme habitat and valuable information on the large diversity and physiological potential of canonical NOB and commamox *Nitrospira*.

ZUSAMMENFASSUNG

Nitrit-oxidierende Bakterien (NOB) führen den zweiten Schritt der Nitrifikation durch, die Oxidation von Nitrit zu Nitrat. Kürzlich wurde eine enorme Diversität und ökophysiologische Flexibilität von herkömmlichen NOB und sogar von Bakterien, die Ammoniak vollständig zu Nitrat oxidieren (Comammox) und zu einer Gattung von klassischerweise als NOB definierten Organismen gehören, entdeckt. Die große Komplexität von NOB Gemeinschaften in natürlichen Systemen legt nahe, daß Nischenaufteilung und -differenzierung die Koexistenz von sympatrischen NOB ermöglicht. Allerdings ist das verfügbare Wissen über Nitrit-oxidierende Gemeinschaften und über Comammox in extremen natürlichen Systemen stark eingeschränkt. Für diese Arbeit wurden NOB und Comammox-Organismen aus salzig-alkalischen Seen des Nationalparks "Neusiedler See – Seewinkel" an der Grenze zwischen Österreich und Ungarn untersucht. Dieses interessante und einzigartige Ökosystem is stark von saisonalen Schwankungen in Temperatur, Salzgehlat, pH und anorganischen Stickstoffkonzentrationen beeinflusst. Das Ziel dieser M.Sc. Arbeit war es vorrangig die Ökophysiologie der Nitrifikanten dieses extremen Systems zu erforschen und ihre Identität, Anpassungen und saisonale Dynamiken zu erschliessen. Die zwei zur Anwendung gebrachten Ansätze hatten die Ökologie und die Physiologie der NOB im Fokus and lieferten neue und wertvolle Informationen über einzelne Nitrifikantenarten.

Die Zusammensetzung und Struktur der Gemeinschaften on Nitritoxidierern und Comammox in Sedimenten von zwei verschiedenen Seen, welche für mehr als ein Jahr monatlich beprobt wurden, wurde mittels kulturunabhängiger Identifizierung durch "Next Generation" Sequenzierungen erschlossen. Nur Nitrifikationsgene (*nxrB* und *amoA*) von herkömmlichen Nitrospiren und Comammox Nitrospiren wurden eindeutig detektiert. Es wurde eine große Diversität von Nitrospirenarten, welche zu verschiedenen Linien dieser NOB Gattung gehörten, entdeckt. Die Ergebnisse zeigten die Dominanz weniger Nitrospiren im Verlauf des gesamten Jahres. Faszinierenderweise wurden Comammox der Gruppe A in beiden Seen während des ganzen Jahres detektiert, während Comammox der Gruppe B nur stochastisch präsent waren. Soweit bislang bekannt, ist dies der erste Fund von Comammox Organismen in aquatischen Systemen mit einem stark alkalischen pH und einem Salzgehalt weit über den für Frischwasser normalen Konzentrationen. Zusätzlich wurden starke Hinweise auf einen signifikanten Einfluß von Umweltfaktoren wie Salzgehalt und Konzentrationen von anorgansichen Stickstoffverbindungen auf die Zusammensetzung und Dynamiken von NOB und Comammoxgemeinschften gefunden. Schließlich legten die Ergebnisse dieser Studie bislang unentdeckte Nischenpräferenzen und die wahrscheinliche Existenz von verschieden Comammox Organismen der Gruppe A nahe.

Der zweite Ansatz, der die Physiologie von Nitritoxidierern im Fokus hatte, nutzte drei Anreicherungskulturen von salzig-alkalischen Seen des selben geografischen Gebietes. Zwei dieser Kulturen enthielten neue Nitrospirenarten: eine neue Art der Linie IV, dessen Mitgleider bislang nur in marinen Habitaten gefunden wurden, und eine neue Art der Linie II. Interessanterweise zeigte die dritte Anreicherungskultur stets eine hohe nitritoxidierende Aktivität, enthielt aber keine bekannten NOB gemäß molekularen Suchmethoden nach Nitritoxidoreduktasegenen und 16S rRNA-gerichteten FISH-Methoden. Verbesserte Kulturbedingungen und erhöhte Anreicherungslevel wurden im Laufe des Projektes etabliert.

Des weiteren konnten neue Morphologien und Habitate für herkömmliche Nitrospirenarten beschrieben werden.

Die genomische und physiologische Charakterisierung dieser neuen Organismen und die Dynamiken der Nitrifikationsgemeinschaften im Verlauf von mehr als einem Jahr lieferten Einsichten in die einzigartigen Anpassungen an extreme Habitate und wertvolle Informationen zu der großen Diversität und dem physiologischen Potential von herkömmlichen NOB und Comammox Nitrospira..

1. INTRODUCTION

1.1 OVERVIEW OF THE NITROGEN CYCLE

Nitrogen (N) is one of the essential elements for life. Via the biogeochemical nitrogen cycle N-compounds are fixed from the atmosphere, converted to organic forms and finally recycled anaerobically to gaseous N(Hermann, Ferguson, & Newton E, 2006). Thereby, the N cycle is responsible for delivering N for assimilatory and dissimilatory processes which are essential for the proper function of all ecosystems. Microorganisms are the key players of the N cycle and by regulating N concentration and conversion into distinct organic and inorganic forms they are responsible for the N cycle balance. Nitrification is one of the main processes of the global N cycle. It is defined as the sequential oxidation of ammonia (NH_4^+) via nitrite (NO_2^-) to nitrate (NO_3^-). This process supplies oxidized forms of nitrogen for assimilation and anaerobic respiration, significantly contributing to the primary production of ecosystems. Chemoautotrophic archaea and bacteria, termed microbial nitrifiers, are the responsible organisms for this conversion using ammonia or nitrite as their energy source and electron donor.

Until recently, the two separate but linked oxidative steps were assumed to be catalysed by distinct microbial functional groups. Ammonia-oxidizing archaea (AOA) and bacteria (AOB) were thought to perform the conversion of ammonia to nitrite and consecutively nitrite oxidizing bacteria (NOB) were assumed to execute the final transformation to nitrate. However, the recent discovery of organisms belonging to the classical NOB genus *Nitrospira* that perform complete nitrification changed the understanding of nitrification (Holger Daims et al., 2015; van Kessel et al., 2015). The complete oxidation of ammonia to nitrate performed by these organisms, called “comammox”, is possible with the full set of the genomic repertoire needed for both ammonia and nitrite oxidation. In comammox *Nitrospira,* ammonia oxidation is genetically encoded by homologous genes of the two key gene clusters found in AOB, coding for ammonia monooxygenase (AMO) and hydroxylamine dehydrogenase (HAO). However, the comammox genes for nitrite oxidation (encoding the nitrite oxidoreductase, NXR) found in comammox *Nitrospira* are highly similar to the ones found in the classical, strictly nitrite-oxidizing *Nitrospira* species(Holger Daims, Lücker, & Wagner, 2016). The detection of comammox Nitrospira-like genes in various environments suggests that comammox organisms are widespread in different natural and engineered systems, such as soil and freshwater, waste water treatments plants (WWPTs) and drinking water treatment systems (DWTPs) (Palomo et al., 2016; Pinto et al., 2015).

1.2 NITRITE-OXIDIZING BACTERIA (NOB)

The known NOB belong to seven genera in four bacterial phyla unequally spread in the environment. The habitats include terrestrial systems such as distinct soil types and geothermal surfaces and aquatic ones encompassing freshwater, marine, hypersaline and engineered ecosystems(Holger Daims et al., 2016).

The study of the NOB distribution in the environment relays on the identification of *nxrA* and *nxrB* genes, coding for subunits of the nitrite oxidoreductases, since they are powerful

functional and phylogenetic markers(Pester et al., 2014). The phylogenetic diversity and potential habitats for each known NOB group are summarized in FIGURE 1. *Nitrospira* is the most diverse NOB genus nearly ubiquitous in nature(Holger Daims et al., 2016). It consists of at least six phylogenetic sub lineages. Lineage I can be found in soil, engineered, freshwater and subsurface systems. Lineage II habitats include all terrestrial and aquatic systems described above with the exception of marine and hypersaline environments. The remaining lineages have a more reduced distribution. Lineage III *Nitrospira* has only been found in soil and subsurface samples and lineage IV members are restricted to marine and hypersaline systems. Lineage V is only present in soil and engineered habitats. Finally, Lineage VI *Nitrospira* is confined to geothermal ecosystems.

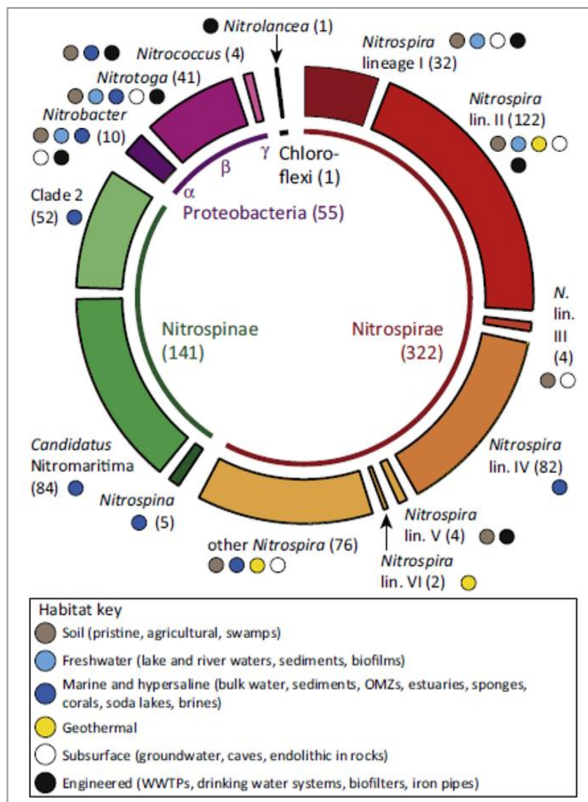

FIGURE 1. Summary of the potential habitats and phylogenetic diversity of nitrite-oxidizing bacteria (NOB). The inner targets identify the NOB regarding its phyla affiliation. The outer labels state the different genus and in the case of *Nitrospira* also the six lineages described. The wedges are drawn considering the amount of OTUs obtained for each NOB group or phyla assessed by clustering all 16S rRNA sequences of known NOB from the SIVLA Ref NR 99 database (July 2015). The habitats for each groups are illustrated by coloured circles. Figure obtained from Daims et al., 2016.

Due to the widespread presence of the genus *Nitrospira* and its relevant function in natural and engineered systems, the knowledge and studies regarding its distribution and adaptations are substantial(Cébron & Garnier, 2005; H. Daims et al., 2006; Holger Daims et al., 2015; Gruber-Dorninger et al., 2015; Koch et al., 2015; Kruse et al., 2013; Nowka, Off, Daims, & Spieck, 2015). Until recently, lineages I and II were thought to be the key NOB in WWTPs(H. Daims et al., 2006; Datta, Racz, Kotay, & Goel, 2011; Gruber-Dorninger et al., 2015; Hüpeden et al., 2016). However, another NOB has been shown to be important in these systems; representatives of the *Nitrotoga* genus coexists with *Nitrospira* in WWTP but they are also present in natural habitats like permafrost soils(Mashal Alawi, Lipski, Sanders, Eva-Maria-Pfeiffer, & Spieck, 2007; Hüpeden et al., 2016; Lucker et al., 2015; Saunders, Albertsen, Vollertsen, & Nielsen, 2016). Such findings imply that, temperature, pH and possibly other environmental factors influence niche partitioning and allow the presence of complex NOB communities(M. Alawi, Off, Kaya, & Spieck, 2009; Holger Daims et al., 2016; Hüpeden et al., 2016; Nowka, Daims, & Spieck, 2015; Palomo et al., 2017).

In the marine environment, *Nitrospinae* members are the dominant NOB group, present in high abundance(Lücker, Nowka, Rattei, Spieck, & Daims, 2013; Spieck, Keuter, Wenzel, Bock, & Ludwig, 2014; Watson W & Waterbury B, 1971). Together with *Candidatus* Nitromaritima, they are the two groups restricted to marine habitats(Ngugi, Blom, Stepanauskas, & Stingl, 2016). *Nitrococcu*s is also a typical marine nitrifier, but it can be found in soil or in engineered systems too(Holger Daims et al., 2016; Watson W & Waterbury B, 1971). *Nitrolancetus hollandicus* is the only known NOB representative of the *Chloroflexi* phyla, isolated from a nitrifying reactor(Dimitry Y Sorokin et al., 2012; Dimitry Y. Sorokin, Vejmelkova, et al., 2014). Finally, *Nitrobacter* genus, placed within the α-proteobacteria class, is another extensively studied and widespread nitrite oxidizer found in all habitats described with the exception of geothermal environments(Cébron & Garnier, 2005; Dimitry Y. Sorokin, Muyzer, Brinkhoff, Gijs Kuenen, & Jetten, 1998).

Apart from the influence of environmental factors, metabolic versatility or interactions with other organisms, as well as differences in kinetics and biochemistry of nitrite oxidation have an important role in niche partitioning of NOB(M. Alawi et al., 2009; Holger Daims et al., 2016; Hüpeden et al., 2016; Koch, Galushko, Albertsen, Schintlmeister, Gruber-Dorninger, et al., 2014; Lücker et al., 2010; Nowka, Daims, et al., 2015; Palatinszky et al., 2015; Princic, Mahne, Megusar, Eldor, & Tiedje, 1998b). For example, their affinity for nitrite and the localization of the NXR cluster has been shown to play a role in determining their optimal substrate concentration and preferred environment(Holger Daims et al., 2016). NOB with cytoplasmic-oriented NXR, like *Nitrobacter*, are adapted to high nitrite concentrations and out-compete *Nitrospira,* which contain a periplasmic-oriented NXR, under such conditions(Kim & Kim, 2006; Nogueira & Melo, 2006). *Nitrospira* species are adapted to lower nitrite levels, which explains their ubiquitous distribution in the environment, as in most natural systems, nitrite concentrations are close to the detection limit(Holger Daims, Nielsen, Nielsen, Schleifer, & Wagner, 2001; Kim & Kim, 2006). However, adaptations to nitrite concentrations can also vary between species and strains of *Nitrospira* and *Nitrobacter*(Gruber-Dorninger et al., 2015; Nowka, Daims, et al., 2015).

The newly discovered organisms known as *Nitrospira* comammox have introduced new topics and questions increasing the complexity of NOB communities(Holger Daims et al., 2015; Palomo et al., 2016; Pinto et al., 2015; van Kessel et al., 2015). Until now, there is still only a limited amount of knowledge available about comammox nitrifiers. All identified comammox organisms belong to the *Nitrospira* sub lineage II but they do not form a monophyletic group based on the 16S rRNA or *nxrB* gene(Holger Daims et al., 2015; Palomo et al., 2016; Pinto et al., 2015; van Kessel et al., 2015). However, the ammonia monooxygenase gene (*amoA*) allows the disclosure of two monophyletic related lineages, referred as clade A and clade B, and the detection and differentiation of comammox members in the environment(Holger Daims et al., 2015; Pjevac et al., 2017; van Kessel et al., 2015). The possibility to detect them *in situ* with molecular techniques has enabled recent studies about their distribution in the environment. The data gathered suggest that comammox is present in diverse soil types, freshwater habitats and engineered systems (WWTPs and DWTPs)(Holger Daims et al., 2015; Palomo et al., 2016; van Kessel et al., 2015)showing that Comammox *Nitrospira* coexist with other NOB in the environment. In consequence, studies regarding their niche and adaptations are essential for understanding their role in nature and their competitive abilities. Mechanisms influencing niche specialization could be different optimal ammonia concentrations, other speculated metabolisms such as hydrogen oxidation, dissimilatory nitrate reduction to ammonia (DNRA),

or the usage of alternative substrates like formate and a competitive advantage at low oxygen and/or microaerophilic conditions(Koch, Galushko, Albertsen, Schintlmeister, Gruber-Dorninger, et al., 2014; Koch et al., 2015; Palomo et al., 2017; Pjevac et al., 2017; van Kessel et al., 2015). In addition, the tight clustering of comammox *Nitrospira* in anaerobic reactors together with anammox bacteria proposes a functional cooperation with microorganisms from the genus *Brocadia,* potentially resulting from a selective benefit affecting comammox distribution in complex communities(van Kessel et al., 2015). Comammox *Nitrospira* are more similar in terms of their ammonium metabolism to some AOA than to AOB resulting from their adaptation to low ammonia concentrations in oligotrophic systems(Kits et al., 2017; Palomo et al., 2017). These facts are in agreement with the theory that the comammox process would thrive in microbial species with low growth rates but high growth yields(Costa, Pérez, & Kreft, 2006; van Kessel et al., 2015). The latest study on ammonia oxidation kinetics in *Nitrospira inopinata*, the only pure culture available so far, also supports the theories described above(Kits et al., 2017). Except for *Nitrosopumilus maritimus*, comammox was the organism tested with the highest affinity for ammonium(Kits et al., 2017). Deduced from a high affinity for ammonium, a low maximum rate of ammonia oxidation and a high growth yield, the authors conclude that comammox organisms thrive under oligotrophic and dynamic conditions(Kits et al., 2017; Palomo et al., 2017; Pjevac et al., 2017). Still, studies about the metabolic versatility and the effects of and responses to environmental conditions are necessary to disclose and understand the adaptations and roles of different *Nitrospira* comammox species in the environment. In summary, coexisting complex communities of NOB are brought about through complex niche specializations depending on multiple factors that need to be studied thoroughly.

1.3 ENVIRONMENTAL CONDITIONS AND ECOLOGICAL MECHANISMS SHAPING NOB COMMUNITIES

The natural co-existence of complex NOB communities suggests ecophysiological versatility leading to niche partitioning and specialization between different species and strains(Gruber-Dorninger et al., 2015; Pester et al., 2014). Distinct ecological niches enhance the development of different adaptations to specific environmental conditions, ecological strategies, microbe-microbe interactions, metabolisms other than nitrite oxidation and even complete nitrification(Holger Daims et al., 2016; Koch et al., 2015; Koch, Galushko, Albertsen, Schintlmeister, Spieck, et al., 2014; Palatinszky et al., 2015). Consequently, environmental factors such as pH, oxygen, temperature and substrate concentration have been shown to control nitrite oxidation rates(M. Alawi et al., 2009; H. Daims et al., 2006; Hüpeden et al., 2016; Princic, Mahne, Megusar, Eldor, & Tiedje, 1998a).

Studies conducted in WWTPs already suggest niche differentiation leading to temporal and/or spatial succession caused by shifts in environmental factors that favour certain NOBs or closely-related strains(M. Alawi et al., 2009; Holger Daims et al., 2016; Gruber-Dorninger et al., 2015). As introduced in the chapter above, low temperature together with slightly acidic pH can influence the nitrifying community enhancing the success of *Nitrotoga* species over *Nitrospira*(Hüpeden et al., 2016). Other determinants, like nitrite concentration can affect the predominance of some specific strains of *Nitrobacter* over *Nitrospira*, since *Nitrobacter* tends to thrive under high nitrite concentration and *Nitrospira* in nitrite-limiting conditions(Holger Daims et al., 2016). In addition, comammox organisms are likely to dominate under oligotrophic and/or

microaerophilic conditions(Kits et al., 2017; Palomo et al., 2017). However, extrapolation of results from a few studies, mostly from engineered systems, cannot be used to deduce the unique roles of different NOB species in natural habitats with certainty. Furthermore, analysis of the environment heterogeneity including the consideration of micro-environments and micro-niches is needed in microbial ecology studies(Prosser & Nicol, 2012).

In order to understand the functioning of a microbial guild in an ecosystem the influences of abiotic variables, which have a strong impact on the structure of microbial communities, should be taken into account. Several mechanisms play important roles in this relationship. Temporal and spatial dynamics, defined by shifts of environmental conditions, are important factors shaping microbial communities. Such fluctuations enable the generation of niches and the destruction of others, determining the populations present and their functional potential, affecting the overall dynamics of the ecosystem. The distribution and dynamics of ammonia oxidizing microbes (AOM) and how they are affected by temporal and spatial environmental patterns have been studied previously(Carini & Joye, 2008; Dini-Andreote, Brossi, van Elsas, & Salles, 2016; Shen, Zhang, Zhu, Zhang, & He, 2008; Smith, Mosier, & Francis, 2015; Dimitry Y. Sorokin, Banciu, & Muyzer, 2015; Wankel, Mosier, Hansel, Paytan, & Francis, 2011). The comparison between AOB and AOA niches and the impact of spatio-temporal dynamics on numerical dominances has helped understanding their coexistence and behaviour(Prosser & Nicol, 2012; Shen et al., 2008; Sher, Zaady, & Nejidat, 2013; Wankel et al., 2011). However, nitrite-oxidizing communities have received less attention and little information is available.

Micro-diversity is an important characteristic of microbial communities and populations. It is defined as the diversity between phylogenetically close organisms with potentially physiologically distinctive features that differ in the genetic repertoire and may occupy particular niches(Jaspers & Overmann, 2004; Nelson, Maezato, Wu, Romine, & Lindemann, 2016). This concept of micro-diversity shows those differences masked by highly similar or identical 16S rRNA sequences that enable the coexistence of complex communities and populations. Micro-diversity may range from different genes present or absent between distinct strains to allelic variations between clonal strains(Gruber-Dorninger et al., 2015; Kashtan et al., 2014). It reflects environmental adaptations, contributing to ecological differentiation and in consequence to niche specialization. Moreover, it may provide an extensive pool of adaptations and traits that are selected by different ecological conditions and overall ensures the resilience of the species and the ecological function(Biller, Berube, Lindell, & Chisholm, 2014). Actually, some studies have already postulated micro-diversity as a strategy of highly diverse communities of closely related members to coexist in the same habitat(Acinas et al., 2004; Gruber-Dorninger et al., 2015; Woebken et al., 2008). Nitrifying communities are not an exception. In order to succeed in WWTP over long operational periods, *Nitrospira* seem to follow the same mechanisms(Gruber-Dorninger et al., 2015). As shown by Gruber-Dorninger and colleagues, a high diversity of closely related *Nitrospira* strains could coexist in the same habitat over several years due to spatial heterogeneity, strong gradients and micro-niches plus temporal variations of environmental factors(Gruber-Dorninger et al., 2015).

Finally, the exploration of the rare biosphere is also required when studying microbial communities. Rare taxa, constituting the so-called rare biosphere, are low abundant taxa collectively named rare viable or dormant organisms encompassing high phylogenetic and functional diversity in particular environments at specific time points(Lynch & Neufeld, 2015). Although in many techniques and studies it is not analysed, it should be taken into account when

discussing and drawing conclusions about the dynamics and functions of a system. Rare-biosphere taxa can have important ecological roles and act as reservoirs of genetic and functional diversity. Resilience of an ecological function or of a guild also depend on conditionally rare taxa that may thrive when their niche becomes available. Biotic but also abiotic factors play an important role controlling the presence of rare microorganisms and their abundance(Lynch & Neufeld, 2015).

1.4 MICROBIAL COMMUNITIES OF SALINE-ALKALINE ENVIRONMENTS

The dictionary defines extreme as “of a character or kind farthest removed from the ordinary or average”. Accordingly, extreme conditions and systems could be described as unusual environments requiring particular adaptations that restrict the possible inhabitants. Two typical extreme environments are the ones characterized by alkaline pH or hypersaline conditions. Different systems with both features but singular properties can be found in the natural environment. Soda pans, hypersaline systems and saline-alkaline lakes are very different within each other. The study of microbial communities and functional microbiology from these systems has received quite some attention(Lanzén et al., 2013; Paul Antony et al., 2013; Dimitry Y. Sorokin et al., 2015; Dimitry Yu Sorokin et al., 2004a; Dimitry Yu Sorokin & Kuenen, 2005; Vavourakis et al., 2016). Saline-alkaline and soda lakes are prominent widely distributed alkaline systems formed by evaporation and particular geographical, geological and climatic conditions. High ambient temperatures and light intensities plus unlimited source of carbon dioxide via the $HCO_3^-/CO_3^{2-}/CO_2$ equilibrium enable high rates or primary production(Grant, 2006; Lanzén et al., 2013). Several microbial adaptations to the extreme conditions of salt and pH have been identified (Banciu & Muntyan, 2015; Carini & Joye, 2008; Dimitriu, Pinkart, Peyton, & Mormile, 2008; Humayoun, Bano, & Hollibaugh, 2003; Lanzén et al., 2013; Paul Antony et al., 2013; Dimitry Y. Sorokin, Berben, et al., 2014; Dimitry Y. Sorokin et al., 2015; Dimitry Yu Sorokin et al., 2004a, 2004b; Dimitry Yu Sorokin & Kuenen, 2005; Tourova, Grechnikova, Kuznetsov, & Sorokin, 2014; Vavourakis et al., 2016). As shown by a few studies, specialized nitrifiers contribute to an active cycling of nitrogen in such lakes(Dimitry Y. Sorokin, Berben, et al., 2014; Dimitry Y. Sorokin et al., 2015; Dimitry Yu Sorokin et al., 2004b; Dimitry Yu Sorokin & Kuenen, 2005). Although their biosphere encompass organisms and adaptations linked to halo-alkaliphilic conditions, soda lakes and saline-alkaline lakes encompass different communities(Dimitry Y. Sorokin et al., 2015; Dimitry Yu Sorokin et al., 2004a). Differently from saline-alkaline lakes, soda pans are typically characterized by low Mg^{2+} and Ca^{2+} and stable pH values above 11.5(Grant, 2006). Accordingly, saline-alkaline lakes need to be distinguished from soda lakes concerning its genesis, dynamics, microbial richness, diversity and microbial activity patterns.

The importance of environmental conditions such as salinity, sediment C/N ratio and ammonia availability in dictating the spatial distribution, activity and community composition of ammonia oxidizers has been demonstrated (Wankel et al., 2011). *Nitrosomonas halophilus* is recognized as the main AOB in alkaline lakes, adapted to grow up to pH 11 in low salinity conditions(Dimitry Y. Sorokin, Berben, et al., 2014; Dimitry Y. Sorokin et al., 2015). Ammonia oxidizing archaea have also been detected in saline-alkaline lakes and their activity was confirmed by active transcription of the archaeal *amoA* gene(Lanzén et al., 2013). The study of the NOB distribution in saline and/or alkaline environments is less extensive. *Nitrospira* presence

in alkaline environments and especially in saline-alkaline lakes remains almost unknown. Evidences for the abundance of *Nitrospira* species in high pH soil has been observed, but the correlation with pH was not significant(Rousk et al., 2010). To date, only a single *Nitrobacter* species, *Nitrobacter alkalikus*, has been successfully identified and characterized in saline-alkaline and soda lakes. The described organism does not rely on alkaline conditions for growth and is low salt tolerant. The isolated strains from soda lake sediments were able to grow well at sodium bicarbonate concentrations of 5-20g/l and a pH range from 6.5 to 10.2, with an optimum at 9.5(Dimitry Y. Sorokin et al., 1998). Concomitantly, the maximum salinity for nitrification in saline-alkaline lakes is observed to be 1M of total Na^+. Therefore, this is assumed to be the upper limit of salt tolerance for the oxidative part of the nitrogen cycle in hypersaline lakes(Dimitry Y. Sorokin et al., 2015). More detailed studies are necessary to fully disclose nitrite oxidation dynamics and key players in such systems.

1.5 THE SALINE-ALKALINE LAKES OF THE NATIONAL PARK "NEUSIDLER SEE-SEEWINKEL"

The Carpathian Basin, also referred to as the Pannonian Basin, is a large basin in Central Europe where soda lakes and saline-alkaline lakes are abundant(Boros, Horváth, Wolfram, & Vörös, 2014a). Today the region is divided among Austria, Hungary, Slovakia, Ukraine, Romania, Serbia, Croatia and Slovenia. A detailed description of the saline-alkaline lakes found in the region is explained by Boros and colleagues (2014)(Boros et al., 2014a). This protocol focuses only on the saline-alkaline lakes of the National park 'Neusiedler See/ Seewinkel', found east of the Lake Neusiedl, in the north of the province of Burgenland, Austria. The region encloses 45 saline lakes, the majority of them dominated by Na-HCO_3 ions. The special environment is defined by the increased salinity contents and strongly affected by high, fluctuating pH but also by several other abiotic factors like temperature and concentrations of inorganic N. They are shallow and temporary waterbodies that may dry out in summer. Therefore, they are naturally fishless with a vegetation-free bed and sparse vegetation on the shorelines. Salinity varies between lakes and seasons; from sub-saline (0.5-3g/l) to hypersaline (>50g/l), with a mean value in the hypo-saline range (3-20g/l)(Boros et al., 2014a).The occasional occurrences of magnesium and dissolved calcium as secondary dominant cations besides sodium are noteworthy. Since true soda lakes are characterized by low levels of magnesium and calcium the anionic composition of the Carpanthian-Seewinkel saline-alkaline lakes constitute a distinct subtype of soda waters. The presence of various geological substrates, specific climate and hydrologic environments determine the diverse ionic composition and dynamics in the lakes of the Carpathian Basin(Boros et al., 2014a). During the last interglacial arid period the climate conditions favoured the formation of soda-carrying soils. High annual average temperatures and low annual precipitation together with windy conditions and high sunshine incidence led to increased evaporation rates. The groundwater rose enabling the salts to reach the surface by capillary action. The salts, mainly sodium carbonate, dissolved in the water generating the so called soda pans or saline-alkaline lakes. Evaporation processes still play a role nowadays, changing local salinity levels of the lakes by affecting the groundwater level and the capillary rise of the salts. In addition, water level varies seasonally from 70 cm to total desiccation and local temperature exhibits high night-day variation. The lakes differ in chemical composition and soil substratum.

For this study two lakes have been selected, the Herrnsee (HS) and the Unterer Stinkersee (US). Both are characterized by brackish waters with an alkaline pH and temperature oscillating throughout the year. Detailed abiotic conditions of both lakes are described in TABLE 1. These seasonally fluctuating features allow the study of the impact of changing environmental conditions on microbial communities. In this project, the nitrite-oxidizing community is analysed over the year in order to increase the knowledge of NOB and the nitrogen cycle in these distinctive systems.

TABLE 1. Abiotic factors of lakes Herrnsee and Unterer Stinkersee.

Herrnsee (HS)		
Area (ha)	4.3	Metz and Forró (1989)
Salinity (g/L)	7.04 / 7.68-3.712	Metz and Forró (1989) / sampling data (2014-2015)
Temperature (ºC)	28ºmax 1.8ºmin	Sampling data (2014-2015)
pH	8.8 / 9.6 -8.52	Metz and Forró (1989) / sampling data (2014-2015)
Dominant ionic composition (e%)	0.99 K^+/ 79.76 Na^+/ 0.89 Ca^+ / 18.36 Mg^{2+} / 27.50 SO_4^{2-} / 21.67 Cl^- / 50.84 CO_3^{2-} / 40.48 HCO_3^- / 10.36 CO_3^{2-} /	Metz and Forró (1989)
Unterer Stinkersee (US)		
Area (ha)	35.6	Wolfram et al. (2004)
Salinity (g/L)	4.04 / 3.592-1.502	Wolfram et al. (2004) / sampling data (2014-2015)
Temperature (ºC)	25ºmax 0.8ºmin	Sampling data (2014-2015)
pH	9.7 / 9.47 - 8.65	Wolfram et al. (2004) / sampling data (2014-2015)
Dominant ionic composition (e%)	1.39 K^+/ 77.35 Na^+/ 2.09 Ca^+ / 19.16 Mg^{2+} / 5.56 SO_4^{2-} / 12.92 Cl^- / 81.52 CO_3^{2-} / 72.56 HCO_3^- / 8.96 CO_3^{2-} /	Wolfram et al. (2004)

1.6 METHODOLOGY: CULTURE-DEPENDENT VS CULTURE-INDEPENDENT APPROACHES

In any investigation the results obtained are partly dependent on the methodology applied. The techniques chosen will only answer some specific questions. In microbiology, the methods can be broadly classified between culture-dependent or culture-independent.

Cultivation and culture-dependent techniques were the first ones to applied in microbiology providing the first knowledge about microorganisms(Winogradsky, 1890). Nowadays they are not extensively applied since it is assumed that the cultivable fraction of any given sample or environment only represents less than 1% of the total diversity present(Amann, 1995). The cultivation process itself its extremely biased in the assessment of microbial diversity, providing only selected conditions for specific bacteria. Consequently, this approach may favour some microbes from the community allowing their growth over other members, even if they are not the most abundant species *in situ*. Additionally, cultivation of microbes in optimal conditions for long periods of time may cause domestication of the strains, enhancing the development of adaptations that would not evolve in the natural habitat(Eydallin, Ryall, Maharjan, & Ferenci, 2014). Despite all the disadvantages, there are many benefits of culturing microbes. Only in culture we can truly perform physiological characterization and understand the responses of microorganisms to changing abiotic conditions. The study of the interplay between physiological properties and environmental conditions is essential in order to understand a community and its role in the ecosystem. Cultures also enable the study of the link between functions and specific genes, the confirmation of hypotheses drawn using molecular techniques and facilitate

genome sequencing(Prosser & Nicol, 2012). Still, it is not right to state properties or behaviours of a whole community based only in the characteristics observed in few cultivated strains. A large number of molecular techniques have revolutionized the microbial ecology field, allowing scientist to study microbial communities and all their properties, functions, distribution and interactions *in situ* s(Su, Lei, Duan, Zhang, & Yang, 2012). However, also culture-independent methods have their drawbacks. They often involve DNA extraction and gene amplification. The biases of DNA extraction influencing the quantity and purity of the DNA have been demonstrated and together with primer choice they strongly affect the community composition observed(Albertsen, Karst, Ziegler, Kirkegaard, & Nielsen, 2015; Guo & Zhang, 2013; Vanysacker et al., 2010). Overall, because of the heterogeneity and uncontrolled nature of natural ecosystems, interpreting data and drawing conclusions with a single approach is not feasible. Combining distinct approaches and perspectives is the best way to answer biological questions.

1.7 AIMS OF THE STUDY

Increasing and encompassing the knowledge of the ecophysiology of nitrifiers is essential for understanding the nitrogen cycle and its alterations caused by the global change(Erisman et al., 2013; Gruber & Galloway, 2008). Studies about nitrite oxidizers ecology and physiology are undoubtedly needed in order to disclose the interplay between NOB communities and the natural environment.

The main aim of this M.Sc. project includes resolving the identity, adaptations and responses of NOB from saline-alkaline lakes from the National Park of Neusidler See-Seewinkel. The objective is divided in two sub-projects focusing on the ecology or the physiology of the nitrite oxidizing communities and individual organisms. In one hand, the NOB communities of two lakes of the National Park were sampled monthly during more than a year and analysed by culture-independent methods. Molecular methods based on DNA extraction and new generation sequencing (NGS) allowed the study of the community composition, the temporal dynamics and its correlation with abiotic determinants. On the other hand, samples from similar lakes from the same ecosystem were cultured and enriched for NOB. The resulting cultures enabled the analysis of the physiology of three different NOB. The main hypothesis for this project predicts a high diversity of NOB present in the lakes, with unknown adaptations to the local conditions, correlating with distinct environmental factors and defined by a temporal succession.

2. MATERIAL AND METHODS

Two main approaches were used in the project in order to unravel the ecophysiology of NOB communities of saline-alkaline lakes from the National Park "Neusiedler See/Seewinkel". The first subproject focuses on two saline-alkaline lakes analysed throughout fourteen months using culture-independent methods based on DNA extraction and Next Generation Sequencing (NGS) techniques. The second one encompasses three enrichment cultures of NOB originated from saline-alkaline lakes of the same area. In the following chapters, the two subprojects are described separately.

2.1 SEASONAL STUDY OF NOB COMMUNITIES FROM TWO SALINE-ALKALINE LAKES

We performed a culture-independent identification of the NOB communities in lake sediments by barcoded next generation sequencing, targeting two protein-coding genes involved in nitrification (*nxrB* and *amoA*). The different steps conducted in order to sequence and identify the organisms present are drawn in order of execution in FIGURE 2.

FIGURE 2. Diagram summarizing all the steps applied in the culture-independent seasonal study based on NGS techniques.

Environment description and sampling.

Herrnsee (HS) and Unterer Stinkersee (US) are two saline-alkaline lakes located in the "Neusiedler See-Seewinkel" national park. Temperature, pH and conductivity of the lake water were measured together with the sampling. The ammonia content, nitrate and nitrite were measured immediately after sampling in the lab. Both of these measurements were conducted by collaborators from the biological research station of the national park. The water and sediment samples used in this study were collected monthly between April 2014 and June 2015. A sampling overview is displayed in FIGURE 3. Water samples were obtained by filtering between 30-100 ml through 0.2 µm pore size filters for each lake. The amount filtered was dependent on the amount of colloidal material that clogged the filters blocking the filtration. Sediment cores were obtained in triplicates for each lake and samples were collected from the core surface (0-10 cm) and from a lower depth (15-25 cm). All filters and sediment samples were frozen and kept at -20ºC until DNA extraction.

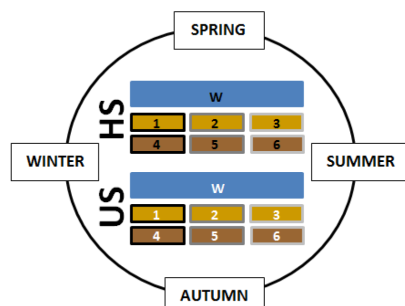

FIGURE 3. Graphic overview of the samples collected from two saline-alkaline lakes throughout a year (from April14 to June15). Sediment samples were collected in triplicate intact cores, from the upper layer (cores numbered 1-3) and the subsequent lower layer (cores numbered 4-6). Water samples were obtained from the water column. Abbreviations: HS (Herrnsee), US (Unterer Stinkersee), W (water sample).

DNA extraction.

Three different deoxyribonucleic acid (DNA) extraction methods were tested in order to detect the most suitable method for polymerase chain reaction (PCR)-based analysis of bacterial nitrite oxidizers from both water and sediment samples. Two distinct kits were used, the DNA Isolation Kit Power Soil (MO BIO-QIAGEN GmbH, Austria) and the Fast DNA Spin Kit for Soil (MPBIO, CA, USA) following the manufacture's recommended protocols. Furthermore, a phenol-chloroform extraction method was also performed as described in Angel et al, 2012 (Angel, Claus, & Conrad, 2012) and compared to the other two methods by analysing DNA quantity and quality and by testing for the ability to detect nitrite oxidizers by PCR. Quantification of the extracted DNA was performed using a Nano-Drop ND-1000 spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) and the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, CA, USA) on a fluorometric Nano-Drop ND-3300 (Peqlab Biotechnologie GmbH, Erlangen, Germany). Additionally, DNA extraction assessment was done by PCR with DNA from two sediment samples from November 2014 from both lakes. The generated PCR products were analysed using a 1.5 % agarose gel pre-stained with Gelred (Biotium, Inc. Fremont, CA, USA) and visualized with a Bio-Rad Gel Doc^{TM} XR+ (Bio-Rad Laboratories Ges.m.b.H, Wien, Austria).

Screening PCRs.

To check for the presence of known NOB, PCRs with specific primer pairs targeting the *nxrB* gene of NOB from the phylogenetic groups *Nitrospirae*, *Nitrobacter*, *Nitrococcous*, *Nitrotoga, Nitrospinaea*, Comammox *Nitrospira* clade A and clade B were performed. Detection of *Nitrotoga-like NOB* was also attempted using 16S rRNA specific primers. PCR mixes were subjected to amplification in a PCR thermal cycler T100 (BIO-RAD, Hercules, CA, USA). Detailed information about the primers and cycling conditions can be found in TABLE 2.

TABLE 2. Summary of the primer pairs and cycling conditions used in the screening PCRs

TARGET	GENE	PRIMER	SEQUENCE 5'-3'	REFERENCE	CYCLING CONDITIONS
Nitrospira	nxrB	F169	TAC ATG TGG TGG AAC A	Pester et al. 2014	5 min at 95°C, followed by 30 cycles of 40 s at 94°C, 30 s at 56°C and 60 s at 72 °C, and 10 min at 72°C
		R707	CGG TTC TGG TCR ATC A		
Nitrobacter, Nitrococcus	nxrB	F706	AAG ACC TAY TTC AAC TGG TC	Maixner (unpublished)	5 min at 95°C, followed by 30 cycles of 40 s at 95°C, 40 s at 56°C and 60 s at 72 °C, and 10 min at 72°C
		R1431	CGC TCC ATC GGY GGA ACM AC		
Nitrobacter	nxrB	Fb169	TAC ATG TAC TGG AAC A	Maixner (unpublished)	5 min at 95°C, followed by 35 cycles of 40 s at 95°C, 40 s at 52°C and 60 s at 72 °C, and 10 min at 72°C
		Rd638	CGC TCC TGG CTG ACC A	Lücker (unpublished)	
Nitrospinaea	nxrB	Fw1	CGTGAGTTCACNCAGTC	Müller (unpublished)	5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 52°C and 60 s at 72 °C, and 10 min at 72°C
		Re1	GGNACCATGTACTGRTC		
Nitrotoga	nxrB	F166	GAA ACS ATA TTC TGG AAT	Lücker (unpublished)	5 min at 95°C, followed by 35 cycles of 40 s at 95°C, 30 s at 52°C and 60 s at 72 °C, and 10 min at 72°C
		R551	CGG GAC GCA TCA ATC A		
Nitrotoga	16S rRNA	F124	ATC GGA ACG TAC CCG GAA A	Lücker et al. 2014	5 min at 94°C, followed by 45 cycles of 40 s at 94°C, 30 s at 63°C and 90 s at 72 °C, and 10 min at 72°C
		Rb1462	CGA ACC CTA CCG TGG CAA C		
Comammox *Nitrospira*, clade A	amoA	ComaA-244F	TAYAAYTGGGTSAAYTA	Pjevac et al. 2017	5 min at 94°C, followed by 35 cycles of 40 s at 94°C, 30 s at 52°C and 60 s at 72 °C, and 10 min at 72°C
		ComaA-659R	ARATCATSGTGCTRTG		
Comammox *Nitrospira*, clade B	amoA	ComaB-244F	TAYTTCTGGACRTTYTA	Pjevac et al. 2017	5 min at 94°C, followed by 35 cycles of 40 s at 94°C, 30 s at 52°C and 60 s at 72 °C, and 10 min at 72°C
		ComaB-659R	ARATCCARACDGTGTG		
All bacteria	16 rRNA	F616V	AGAGTTTGATYMTGGCTC	Juretschko et al. 1998	10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 54°C and 90 s at 72 °C, and 10 min at 72°C
		R1492	GGTTACCTTGTTACGACTT	Loy et al. 2002	

Because of the large number of samples, (n=98), a strategic selection was made for an initial screening (see TABLE S1, Annex I). Sediment and water samples from each season of the year were chosen and analysed. One of the three upper layer sediment cores (1-3) was selected for each month. In order to avoid PCR inhibition in samples from lake sediments by co-extracted substances such as humic acids, two dilutions (1/10 and 1/50) were tested. Water samples were diluted 1/10.

Gradient PCR and cloning.

Following the initial screening for NOB groups, PCR optimization of the detected groups was attempted. For this purpose, the gradient PCR technique was used to empirically determine the optimal annealing temperature and decrease the occurrence of non-specific secondary amplicons. This method was applied for PCR assays were unspecific binding hampered a clear detection of expected amplicons. The gradient of the annealing temperature ranged from 52ºC to 62ºC in eight increments. To verify the specific amplification of DNA from NOB, only selected PCR amplicons were subjected to cloning and sequencing. In brief, PCR products of the correct length were excised from the agarose gel with either a sterile scalpel or glass capillaries and purified with the ZR-96 Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) according to manufacturer’s instructions. Cloning of the purified PCR products was performed by ligation into a TOPO-TA cloning vector system according to the manufacturer’s protocols (Invitrogen, Carlsbad, CA, USA). Subsequently, selected clones containing an insert of the correct length on the plasmid were sequenced by the Sanger method(Sanger & Coulson, 1975). The obtained nucleotide sequences were analysed using the BLASTn program(S. F. Altschul, Gish, Miller, Myers, & Lipman, 1990).

Illumina MiSeq library preparation.

Since only amplicons from *Nitrospira* and comammox *Nitrospira* were found through the initial PCR screening in all samples tested, only these organisms were targeted for the NGS approach. The protocol used for amplicon sequencing using MiSeq Technology was performed following the Standard Operational Procedure (SOP) by Angel, R. 2016 based on the work of Herbold et al., 2015(Herbold et al., 2015). The protocol consisted of the following seven steps. First, a PCR was conducted with specific primers amplifying all *Nitrospira nxrB* and *Nitrospira* comammox *amoA* (see TABLE 2). Both forward and reverse primers included a 16 bp head sequence, essential for the second step PCR. Cycling conditions and head sequences can be found in Herbod et al., 2015(Herbold et al., 2015). Subsequently, the PCR products were cleaned-up in order to avoid carrying over primers, primer dimers and unspecific bands. The cleaning of the products obtained with the *Nitrospira nxrB*-specific primers was conducted with the ZR-96 DNA Clean&Concentrator$^{TM\text{-}5}$ Kit (Zymo Research, Irvine, CA, USA) according to manufacturer’s instructions. Due to the appearance of double bands in the PCR products targeting comammox *amoA* gene, the PCR products were first loaded onto a 1.5% agarose gel pre-stained with SYBRSafe (Invitrogen, Carlsbad, CA, USA). Afterwards, the correct bands were excised from the agarose gel with either a sterile scalpel or glass capillaries and purified with the ZR-96 Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) according to manufacturer’s protocol. After this, a second step PCR using the first step PCR products as a template was carried out. This PCR step attaches a specific 8 bp barcode sequence to the amplicons, unique for each DNA sample analysed. Thereby, this approach enables the

identification of the sequences obtained from each sample through their unique barcode and the specific gene primer. Cycling conditions and barcode sequences are listed in Herbod et al., 2015(Herbold et al., 2015). The obtained PCR products were evaluated with a 1.5 % agarose gel pre-stained with SYBRSafe (Invitrogen, Carlsbad, CA, USA), visualized with a Bio-Rad Gel Doc™ XR+ (Bio-Rad Laboratories Ges.m.b.H, Wien, Austria) and subsequently cleaned with the ZR-96 DNA Clean&Concentrator™-5 Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. The cleaned PCR products were quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, CA, USA) and a Infinite 200 Pro spectrophotometer (Tecan Group AG) exiting at 480nm reading at 520 nm emission. Finally, the samples were pooled in equimolar concentrations generating a library containing $20 \cdot 10^9$ copies for each amplicon. The pooled library was quantified with the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, CA, USA) and sent for sequencing to on a MiSeq system (Illumina) at Microsynth AG (Balgach, Switzerland).

Illumina MiSeq computational analysis.

The reads generated by Illumina MiSeq were processed and assembled into species-level operational taxonomic units (OTUs) using the pipeline described by Herbold et al., 2015(Herbold et al., 2015) , which includes a chimera check using the UPARSE pipeline(Edgar, 2010, 2013). The applied species threshold was 95% *nxrB* identity as inferred by Pester et al., 2014(Pester et al., 2014). The clustering criterion for the obtained comammox *amoA* sequences was also set at an identity threshold of 95%(Francis, O'Mullan, & Ward, 2003) in order to avoid an overestimation of species-level diversity. However, a validated identity cut-off for *Nitrospira* comammox *amoA* sequences is not known yet, since there is not enough genomic data to correctly match species-level 16S sequence difference to a *amoA* threshold infer(Pjevac et al., 2017).

Data analysis. Statistics and phylogeny.

All statistical analyses were computed with the open-source software R (R core team, 2015). Firstly, in order to confirm the identity of each of the OTUs detected and discard non-target sequences, all the representative sequences for each obtained OTU were aligned to the NCBI protein database using the BLASTx tool(S. Altschul, 1997). Secondly, the affiliation of the OTUs was validated by aligning the sequences to an existing protein-coding gene database (Daims et al 2016, Pester et al. 2013) with the ARB software(Ludwig, 2004; Westram et al., 2011). Afterwards the aligned sequences were added to reference trees published in Daims et al., 2015(Holger Daims et al., 2015) and Pester et al., 2014(Pester et al., 2014). The OTU libraries obtained for each sample were normalized applying the GMPR normalization method before the performance of any statistical analysis(Chen & Chen, 2017). The relative abundance of the normalized OTUs was graphically displayed with heat maps combining the functions *geom_tile* with the *scale_fill_gradientn* within the ggplot2 package(Wickham, 2009). In order to assess autocorrelation between the different environmental variables the Spearman's rank correlation test was applied with the *cor.test* formula(Best & Roberts, 1975). Multivariate analyses of variance were performed to test how changes in environmental factors effected OTU community composition. The R function *adonis*(Anderson, 2001), part of the vegan Package(Dixon, 2003; Oksanen et al., 2005), computed the coefficient of correlation and its significance. In order to test if there were statistically significant differences between the two lakes regarding salinity and the different inorganic nitrogen concentrations, the Anova analysis

of variances was applied with the *aov* formula(Chambers, Freeny, & Heiberger, 1992). Constrained analyses of principal coordinates were applied in order to resolve and display the community composition patterns in dependence of environmental gradients. The *capscale* function(Anderson & Willis, 2003; Legendre & Anderson, 1999) was used to generate a constrained ordination using only those environmental factors that were found to have a significant relationship with community composition as constraints. Significant correlations of environmental factors and normalized OTU abundances with the ordinations were determined by vector fitting with the function *envfit*(Anderson & Willis, 2003; Legendre & Anderson, 1999; Mcardle & Anderson, 2001) and subsequent permutation analysis (n = 999). Finally, the ordination plus the fitted vectors were plotted using the basic *plot* function in R. All formulas used in the ordination analysis belong to the packages vegan and MASS(Dixon, 2003; Oksanen et al., 2005; Venables & Ripley, 2003). The following complementary libraries were also used in the different scripts: RColorBrewer (Author: Erich Neuwirth, 07/12/2014) and reshape2 package(Wickham, 2007).

2.2 OPTIMIZATION, PURIFICATION AND STUDY OF THREE NOB ENRICHMENT CULTURES

Environment description and sampling.

The sampling and inoculation of the cultures was not performed within the course of this mater thesis. However, it is described here in order to provide all information available about the enrichment cultures. The samples used as inoculum for the three enrichment cultures were obtained from lake sediment and water located in the "Neusiedler See-Seewinkel" National Park. The culture named "MixS" was inoculated with shore sand from the Östliche Wörthenlacke and with sterile filtered water from 4 lakes (Östliche Wörthenlacke, Darscho, Westliche Wörthenlacke, Krautingsee). Both the inoculums for the second and third enrichment cultures, termed "KS" and "LL", were inoculated with water samples from the Krautingsee and from the Lange Lacke, respectively. All three cultures were incubated at 28°C in a medium that was designed to provide the chemical conditions of the saline-alkaline lakes. The medium with a pH around 10.2, consisted of the following components in g/l: 0.037 KCl, 0.053 CaCl, 0.74 Na_2SO_4, 0.32 $MgCl_2$, 0.15 KH_2PO_4, 0.7 Na_2CO_3 plus 1ml of autoclaved 1000x trace element solution (TES) encompassing 34.4 mg/L $MnSO_4 \times 1\ H_2O$, 50.0 mg/L H_3BO_3, 70.0 mg/L $ZnCl_2$, 72.6 mg/L $Na_2MoO_4 \times 2\ H_2O$, 20.0 mg/L $CuCl_2 \times 2\ H_2O$, 24.0 mg/L $NiCl_2 \times 6\ H_2O$, 80.0 mg/L $CoCl_2 \times 6\ H_2O$ and 1.000 g/L $FeSO_4 \times 7\ H_2O$ dissolved in MiliQ water with the later addition of 2.5ml of HCl. Unless otherwise stated this was the medium that used in all cultivation approaches. The three enrichments were established in February 2014.

Enrichment, purification and optimization of the cultures.

Cultures were grown in autoclaved Schott bottles of 100 ml volume (Sigma-Aldrich, Saint Louis, Missouri, USA) and sterile plastic culture flasks of 50 ml (Thermo Scientific, Nunclon TM Delta Surface, Roskilde, Denmark). The cultures were maintained at 28ºC and at a pH between 8.5 and 9.5. The nitrite-oxidizing activity was monitored weekly using Quantotix Semi-quantitative test strips (Macherey-Nagel, Düren, Germany) with a colorimetric reaction revealing the presence or absence of nitrate and/or nitrite. Nitrite concentrations supplied were 1 mM, 0.5

mM and 5 mM for the MixS, KS and LL enrichments respectively. Nitrite was re-supplied when the culture had completely used it. The pH was controlled and corrected monthly adding 2M NaOH solution as buffer when needed. Different traditional techniques were applied in order to increase the enrichment level of the containing NOB. Several dilution series into new flasks aimed to dilute the contaminants and enrich for the present NOB in both MixS and LL cultures. Subsequent 10^{-1} or 10^{-2} dilution factors were applied by diluting 2 ml or 200 µl, respectively, into new flasks with 20 ml of medium volume. The highest diluted flask, reaching 10^{-8} or 10^{-9} dilution factors, was fed with 0.5 mM NO_2^- in the MixS culture and with 2.5 mM NO_2^- for the LL enrichment. Simple transfers of old cultures to new media were performed by diluting 2 ml of the old culture in 20 ml of media supplemented with nitrite. In addition, filtration procedures through 3 µm filters were used with the LL culture to separate the planktonic population from the flocs. 2 ml of the filtrate was incubated in a new flask and the filter was placed into another one using the captured flocs as inoculum. Both flasks contained 20 ml of medium volume and were amended with 2.5 mM NO_2^-. In order to attempt purification of nitrifiers from the LL enrichment 10 µl/ml tetracycline were added to the enrichment obtained from the captured flocs in the filtration process. Media containing 4x and 8x more total salt content than the original cultivation medium (8.27g/L for the 4x medium and 16.55g/L for the 8x) was tested for the KS enrichment.

Solid media plates. Agar plates for the cultivation of novel nitrifiers from the saline-alkaline lakes were prepared as follows. The medium already described for NOB cultivation without Na_2CO_3 and the 1 ml of TES was amended with 15g/l of agar and cook during 15 minutes until the solution became clear. After autoclaving, 0.7 Na2CO3, 0.08 $C_2H_3NaO_2$ and 1 ml of the already described 1000x TES were added to the cooled agar medium. Finally, around 25 ml of the medium was poured and solidified in each plate. The plates amended with 10µl/mL tetracycline (TET) were produced by diluting 125µl of TET in sterile MiliQ water and spreading it into the plates using sterile glass beads. The plates were kept still for 10minutes in order to let the TET soak in. The plates were inoculated with 10, 50 or 100 µl of enrichment culture LL with the use of sterile glass beads and incubated at 28ºC for at least five days. The colonies grown were transferred to a new plate and incubated again at 28ºC for at least one week. Finally, a representative colony and of each morphological type was transferred to sterile 96-well plates filled with 200 µl of media supplemented with 1 mM NO_{2-}. Nitrite consumption was checked using the Sulfanilamide/NED reagent as described Miranda et al., 2001(Miranda, Espey, & Wink, 2001).

Screening PCRs and metagenome analysis.

To identify the microorganisms present and to test the purity of the enrichment cultures several PCRs were performed. Gene primers targeting the *nxrB* gene of genus *Nitrospira* (F169/R707) were used to confirm which *Nitrospira* was nitrifying in the KS culture. Detailed information about the primers and cycling conditions can be found in TABLE 2. General 16S rRNA primers (F616V and R1492, TABLE 2) were also used in all enrichments. The generated PCR products were analysed using a 1.5 % agarose gel pre-stained with Gelred (Biotium, Inc. Fremont, CA, USA) and visualized with a Bio-Rad Gel Doc™ XR+ (Bio-Rad Laboratories Ges.m.b.H, Wien, Austria). Selected products were sequenced by the Sanger method(Sanger & Coulson, 1975) and the obtained nucleotide sequences were analysed using the BLASTn tool(S. F. Altschul et al., 1990) by aligning the sequences to the NCBI database or to the representatives OTU sequences

obtained in the seasonal study conducted in this master thesis. The metagenomic analysis of the LL enrichment culture (analyzed by M. Albertsen) enabled the assembly of two genomes bins (produced by P. Pjevac). The bin affiliated with the genus *Thauera* was uploaded and annotated by the MGRast tool(Meyer et al., 2008).

Fluorescence In Situ Hybridization (FISH).

The *in situ* hybridization of the three cultures was performed in order to check the enrichment level and/or identify the nitrifiers present. The technique was applied according to a standard FISH protocol(H Daims, Stoecker, & Wagner, 2005). The oligonucleotide probe sequences, formamide concentrations, fluorophores used and extra information are given in TABLE 3. The probes were all purchased from (Biomers, GmBH, Ulm, Germany). Cells were non-specifically stained with the nucleic acid-targeting stain 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI, Sigma Aldrich, Saint Louis, Missouri, USA). Images were acquired with a CSLM Leica TCS SP8X (Leica, Wetzlar, Germany), exciting the fluorophores at their respective excitation wavelengths with a diode laser or the white light laser and captured by adjusted emission recording windows (see TABLE 3). The Leica application suite X software was used to record images.

TABLE 3. Detailed information about the oligonucleotide FISH probes and fluorophores applied in this study.

Oligonucleotide probes					
Probe	**Fluorophore**	**Specificity**	**Sequence (5'- 3')**	**FA %**	**Reference**
Ntspa712	Dope FLUOS	Phylum *Nitrospirae*	CGCCTTCGCCACCGGCCTTC C	35	Daims et al., 2001
Ntspa662	Dope FLUOS	Genus *Nitrospira*	GGAATTCCGCGCTCCTC	35	Daims et al., 2001
Ntspa1151	Dope Cy3	*Nitrospira* lineage II	TTCTCCTGGGCAGTCTCTCC	35	Maixner et al., 2006
Ntoga122	Cy3	Genus *Nitrotoga*	TCCGGGTACGTTCCGATAT	40	Lücker et al., 2015
c1Ntoga122	-	-	TCWGGGTACGTTCCGATAT		Lücker et al., 2015
c2Ntoga122	-	-	TCYGGGTACGTTCCGATGT		Lücker et al., 2015
NIT3	Cy3	Genus *Nitrobacter*	CCTGTGCTC CATGCT CCG		Wagner et al., 1996
<u>EUBmix</u> EUB338-I EUB338-II EUB338-III/ SBACTV 338	Dope Cy3	EUB338-I (most Bacteria) EUB338-II (*Planctomycetales*) EUB338-III (*Verrucomicrobia*)	EUB338-I (GCTGCCTCCCGTAGGAGT) EUB338-II (GCAGCCACCCGTAGGTGT) EUB338-III (GCTGCCACCCGTAGGTGT)	0-50	EUB338-I (Amann et al., 1990) EIB338-II (Daims et al .,1999) EUB338-III (Daims et al., 1999)

Dyes excitation and emission wavelengths			
Fluorophore	**λ_{max} (excitation)**	**λ_{max} (emission)**	**Detection window (nm)**
Cy3	554 nm	668 nm	550-570
FLUOS	492 nm	517 nm	510 - 530

Scanning Electron Microscopy (SEM).

For scanning electron microscopy, cells from the MixS enrichment culture were fixed on poly-L lysine coated slides with a filter-sterilized 2.5% glutaraldehyde fixation solution in cacodylate buffer (25 mM sodium cacodylate, 0.7 mM MgCl2, pH 7.0) or PBS buffer (130 mM NaCl in 5% [v/v] phosphate buffer mixture [20 to 80 v/v] of 200 mM NaH_2PO_4 and 200 mM Na_2HPO_4 ; filter sterilized with 0.2 µm filter) after letting the cells adhere to the slide by placing it into an active culture for 3 days. The fixed cells were washed three times for 10 min in cacodylate/ PBS buffer and were post-fixed with a 1% OsO4 solution in cacodylate/ PBS buffer for 40 min. The fixed cells were again washed three times in cacodylate/ PBS buffer and dehydrated in a 30 to 100% (v/v) ethanol series, washed in 100% acetone, and critical point dried with a CPD 300 unit (Leica, Wetzlar, Germany). Samples were mounted on stubs, sputter coated with gold using a sputter coater JFC-2300HR (JEOL, Peabody, Massachusetts, USA). Images were obtained with a JSM-IT300 scanning electron microscope (JEOL, Peabody, Massachusetts, USA).

Flow cytometry cell sorting.

Single cells or micro-colonies from both MixS and LL enrichment cultures were sorted with a MoFloAstrios (BeckmanCoulter, Brea, CA, USA) flow cytometer equipped with the following lasers: 488nm, 561nm, 640nm, 405nm and 355nm. The sorted cells or micro-aggregates were inoculated into sterile 96-well plates (VWR International, Radnor, Pennsylvania, USA) filled with 200 µl medium. For each enrichment culture two 96-well plates were used; one filled with regular cultivation medium and the second one with medium additionally supplemented with 4.2 µl of the vitamin mix. The vitamin solution contained in 1 l of volume: biotin (0.02g), folic acid (0.02g), pyridoxine (0.10g), thiamine (0.05g), riboflavin (0.05g), nicotinic acid (0.05g), DL pantothenic acid (0.05g), P aminobenzoic acid (0.05g), choline chloride (2g) and vitamin B12 (0.01g). Both culturing media were complemented with 0.5 mM of nitrite. The plates were incubated for at least two weeks at 28ºC. Transfer to a larger volume was done when the added 0.5 mM nitrite was consumed and converted to nitrate. After reaching 1 ml of volume, 1 mM nitrite was supplemented upon depletion in the same well without new media addition. Nitrite determination was performed with the Sulfanilamide/NED reagent as described Miranda et al., 2001(Miranda et al., 2001). Nitrite conversion to nitrate was checked with the Quantotix Semi-quantitative test strips (Macherey-Nagel, Düren, Germany) as described above. Finally, the cultures reaching a volume of 1 mL and consuming 1 mM nitrite/week were sampled for purity analysis. The identity and purity of the present microorganisms in the cultures was tested with general 16S rRNA PCR (primers F616V and R1492, see TABLE 2 above) and Sanger sequencing, as well as *Nitrobacter*-specific (NIT3, TABLE 3) and *Nitrospira*-specific (Ntspa662 and Ntspa712, TABLE 3) FISH and microscopy analysis.

3. RESULTS

3.1 SEASONAL STUDY OF NOB COMMUNITIES FROM TWO SALINE-ALKALINE LAKES

In order to generate optimal and valid results for the seasonal study using culture-independent techniques, an optimization of different steps was required. Accordingly, the first part of the project consisted in the optimization of the basic methods applied in order to minimize as much as possible the biases introduced. DNA extraction tests and screening PCRs revealed the best DNA extraction technique of the ones tested, and the presence of target organisms. Afterwards, library preparation for Illumina amplicon sequencing was done with the chosen optimal conditions.

3.1.1 Optimization of culture-independent methods for the study of nitrite oxidizers from saline-alkaline lake sediments.

Environmental data.

Temperature, pH and salinity varied seasonally in both lakes (FIGURE 4). Temperature was the factor displaying the highest fluctuations through the months, ranging from a maximum of 28°C in June and July to a minimum of 0.8°C in January (FIGURE 4A). However, it is the factor more conserved between the two lakes.

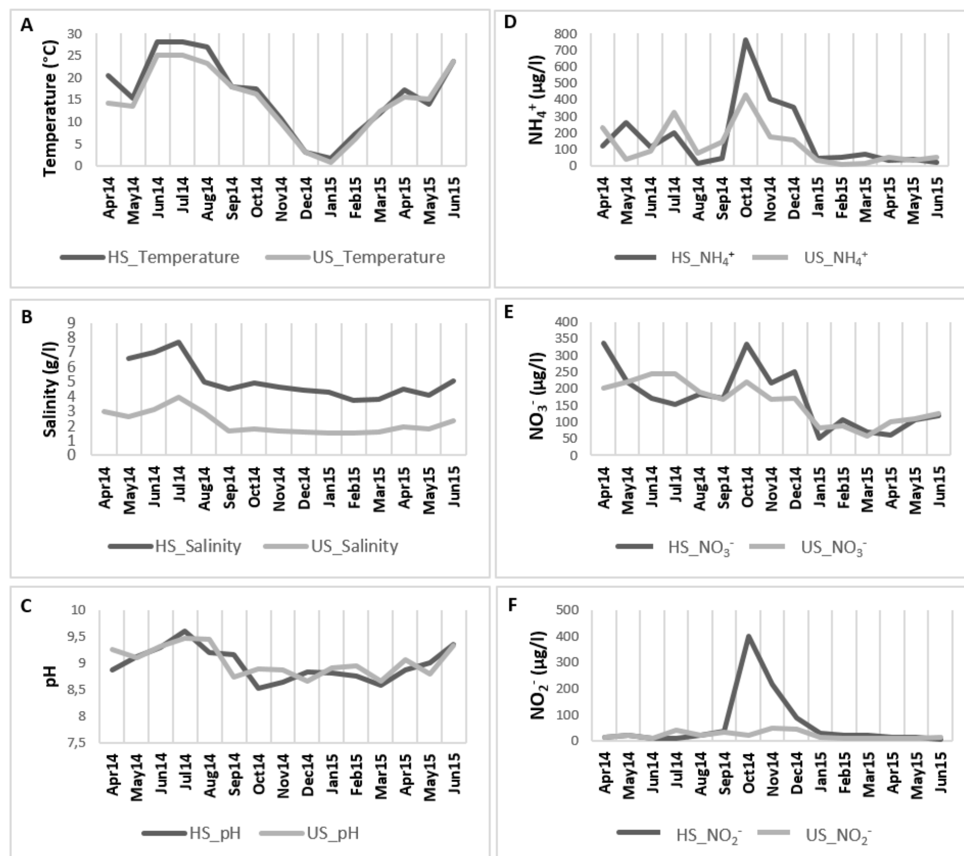

FIGURE 4. **Monthly data from the water column of lake Herrnsee, HS, (dark grey line) and Unterer Stinkersee, US, (light grey line) over the course of 15 months.** Data points represent single measurements. Temperature (ºC) is presented in panel A, salinity (g/L) in panel B and pH in panel C. Panel from D-F display nutrient measurements performed also from water column samples, calculated in µg/L. Ammonia (NH4+) data is shown in panel D, nitrate (NO3-) in panel E and nitrite (NO2-) in panel F.

Differently, the two lakes differ in salinity content. Herrnsee (HS) doubles the salinity content of the Unterer Stinkersee (US), having even three times more salt content in some specific months, like in May14 or Jun14. Nevertheless, both lakes salinity follows the same annual trend, increasing in spring and summer and decreasing in autumn and winter (FIGURE 4B). Both lakes have similar alkaline pH values between 8.52 and 9.6. Furthermore, both experience the same fluctuations through the year, resembling the salinity tendency to rise in spring and summer and decline in winter and autumn (FIGURE 4C). Ammonia (NH_4^+) content also fluctuates through the year, increasing in October, November and December 2014 in both lakes, but remarkably in the Herrnsee Lake (FIGURE 4D). A rise of NO_3^- and NO_2^- is also observed in the same months but only in the Herrnsee Lake (FIGURE 4E, 4F). However, nitrate content also oscillates in Unterer Stinkersee between the different months. Differently, nitrite content is maintained stable in low levels in Unterer Stinkersee (FIGURE 4F).

The Spearman's rank correlation test results are displayed in TABLE4. The strength of the correlation coefficient can vary between +1 and -1, indicating a positive relationship between the variables or a negative one, respectively. When the value is +/- 1, there is perfect degree of association. As the correlation goes towards 0, the relationship is weaker. Although correlation was detected between most of the variables, significant association was only found between a few environmental factors. Temperature associated positively with pH and salinity and negative correlation was found between pH and NO_2^- (TABLE 4A). The distinct nitrogen forms also showed significant and positive relationships within each other (TABLE 4B). The measurements conducted on the water column correlated all three of them between each other, with significant notable strength. Except for nitrate with ammonium, the other two relations were also significant and powerful in the data compiled from the sediment layer. Finally, only nitrate concentrations from the water layer and from the lake sediments correlated between each other. However, ammonium from the water column influenced significantly nitrite and nitrate concentrations on the sediment layer.

TABLE 4. Spearman's rank correlation test between the different environmental parameters measured. A) Correlation between temperature, pH and salinity versus inorganic nitrogen forms from the water column. B) Correlation results between the different nitrogen forms measured in the water column and in the sediment layer. Significant relations are marked with * (** p<0.001 / *p<0.05).

A

	Temp.	pH	Salinity	NH_4^+(W)	NO_2^- (W)	NO_3^- (W)
Temp.		**0.67****	**0.49****	0.10	-0.33	0.35
pH			0.31	-0.13	**-0.46***	0.23
Salinity				0.24	0.02	0.30

B

	NH_4^+(S)	NO_2^- (S)	NO_3^- (S)	NH_4^+(W)	NO_2^- (W)	NO_3^- (W)
NH_4^+(S)		**0.63****	0.61	0.18	0.28	0.32
NO_2^- (S)			**0.47****	**0.42***	0.30	0.36
NO_3^- (S)				**0.53***	0.30	**0.81****
NH_4^+(W)					**0.58****	**0.67****
NO_2^- (W)						**0.46***
NO_3^- (W)						

DNA extraction.

Three different methods were tested for DNA extraction from two sediment samples, one from each lake. The first kit used, DNA Isolation Kit Power Soil from MO BIO (Mo), extracted 18.5 ng/µl of DNA from the HS but no DNA was recovered from US. Additionally, the DNA obtained showed traces of contamination (260/280 ratio= 1,21 and 260/230=0,30). The 260/280 ratio is used to assess the purity of extracted DNA and RNA, values around 1.8 for DNA and 2.0 for RNA are expected for "pure" material. The 260/230 is a secondary measure of nucleic acid purity. Expected 260/230 values for "pure" nucleic acids are commonly in the range of 2.0-2.2. The second kit, Fast DNA Spin Kit for Soil from MPBIO (Mp), enabled the extraction of higher DNA amounts for both lakes (HS=66.6 ng/µl, US=44.8 ng/µl). However, it also carried some contaminants through the extraction (HS_260/280= 1.82 US_260/280=1.74 HS_260/230=0.11 US_260/230=0.08). Finally, a conventional phenol-chloroform extraction (Angel, Claus, & Conrad, 2012)(Angel, Claus, & Conrad, 2012) also enabled the extraction of sufficient DNA from both lakes (HS=85.05ng/µl US=28.4 ng/µl) but again with some contamination (HS_260/280= 1.44 US_260/280=1.31 HS_260/230=0.89 US_260/230=1.01). In addition, a second assessment of the methods was accomplished by PCR with specific primer pairs targeting the amoA gene of comammox *Nitrospira* clade A, an organism known to be present in the samples (A. Daebeler, personal communication). In order to decrease inhibition by contaminants two dilutions (1/10 and 1/50) were tested for each DNA extract. Firstly, the conventional phenol extraction did not allow the detection of comammox *Nitrospira* clade A in any extracted DNA (FIGURE 5, samples 1-6). On the contrary, the DNA Isolation Kit from MO BIO enabled the amplification in the DNA extracted from the HS in all the dilutions tested but not in the DNA from the US sample (FIGURE 5, samples 7-12). The DNA extracted with the Fast DNA Spin Kit from MP BIO allowed the detection of comammox clade A in both lakes and in all dilutions with the only exception of the US_1/50 sample (FIGURE 5 samples 13-18).

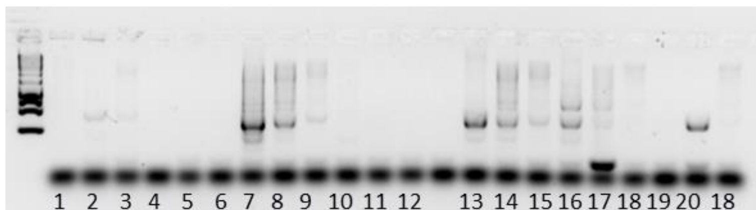

FIGURE 5. Agarose gel displaying the PCR products obtained with the primers targeting the *amoA* gene of Comammox *Nitrospira* clade A in the DNA diluted 1/10 and 1/50 extracted with the three distinct methods. Samples: **1**-P_HS **2**-P_HS1/10 **3**-P_HS1/50 **4**-P_US **5**-P_US1/10 **6**-P_US1/50 **7**-Mo_HS **8**-Mo_HS1/10 **9**-Mo_HS1/50 **10**-Mo_US **11**-Mo_US1/10 **12**-Mo_US1/50 **13**-Mp_HS **14**-Mp_HS1/10 **15**-Mp_HS1/50 **16**-Mp_US **17**-Mp_US1/10 **18**-Mp_US1/50 **19**-C- **20**-C+

Screening PCRs.

PCR screening tests with primers targeting the *nxrB* gene and the 16S rRNA of different phylogenetic groups of nitrite-oxidizing bacteria (NOB) provided a first insight into the diversity of nitrite oxidizers in the saline-alkaline lake sediments and water samples.

Nitrospira. The PCR targeting the bacteria of the entire genus *Nitrospira (*169F/707R) was the only one to give clear results that did not need further refining (see Figure S1, Annex I). The gene encoding the subunit B of the NXR in *Nitrospirae*, *nxrB*, was detected in all selected samples in both dilutions tested. However, negative results were obtained in all DNA extracted from water samples.

Nitrobacter and Nitrococcus. The detection of both *Nitrobacter* and *Nitrococcus* species was attempted with a specific primer pair targeting the *nxrB* gene (706F/1431R). The first PCR results were unclear, yielding double and triple bands in some samples in both dilutions next to the correct size band, which seemed to be present in between the unspecific bands (see Figure S2, Annex I). Similar to the *Nitrospira* PCR results, all water samples showed no amplification of *Nitrobacter* or *Nitrococcous nxrB* genes. Due to the double and triple bands observed, a gradient PCR was applied to tune the annealing temperature and decrease non-specific binding of the primers (706F/1431R). Surprisingly only the positive control displayed positive bands in the agarose gel. None of the annealing temperatures tested enabled the amplification of the target gene in all samples chosen. Since the first primer set failed to positively detect the targeted organisms, a second primer set specific only for *Nitrobacter nxrB* was tested (169Fb/638Rd). In order to screen distinct annealing temperatures, a gradient PCR was performed. Again, double and triple bands encompassing the right size band could be observed in the samples tested. The positive control allowed the confirmation of the annealing temperature at 52ºC, where the specific target was amplified and no unspecific bands could be observed. Parallel to the gradient PCR a conventional PCR with the primer set targeting both organisms (706F/1431R) was performed at 52ºC using the undiluted raw extracted DNA sample as template. Negative results were again obtained. Those samples with a potential positive band obtained with *Nitrobacter* specific primers were loaded again in a second gel. In order to get a clearer band, the first three temperatures replicates were combined in the same well adding all the PCR product volume left from each sample. Unfortunately, no concise band could be recognized. Therefore, a conventional PCR with higher reaction volume at 52°C with the 169Fb/638Rd primer set was performed using one of the samples with the potential positive band as template. All product volume was loaded into an agarose gel in order to have clear band enabling further cloning procedures. Unluckily multiple bands appeared again preventing visualization and the cutting of the correct band.

Comammox Nitrospira clade A and clade B. Comammox detection was accomplished with primer pairs targeting the *amoA* comammox gene. Despite the observation of positive bands with the same size as the positive control, double bands hindered a definite observation of clade A PCR products (see FIGURE 6, white numbers). Compared to clade A, clade B bands were much weaker. In addition, unspecific binding and amplification was also inferred aside from the expected band (FIGURE 6, grey numbers).

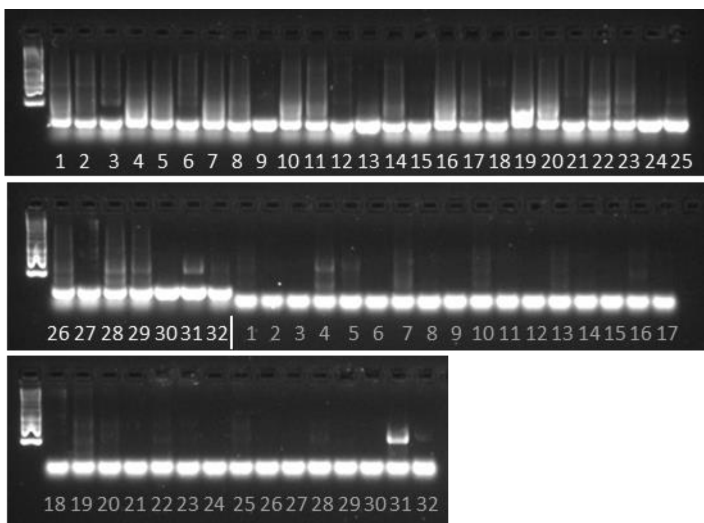

FIGURE 6. Agarose gel displaying the PCR *amoA* products amplified with specific primer pairs against *Nitrospira* Comammox. The first 32 samples correspond to clade A and the following 32 to clade B. Samples: **1**-H2.3 1/10 **2**-H2.3 1/50 **3-**H2.W 1/10 **4-**H5.2 1/10 **5-**H5.2 1/50 **6-**H5.W 1/10 **7-**H8.1 1/10 **8-**H8.1 1/50 **9-**H8.W 1/10 **10-**H11.1 1/10 **11-** H11.1 1/50 **12-** H11.W 1/10 **13-**H14.2 1/10 **14-**H14.2 1/50 **15-**H14.W 1/10 **16-**U2.1 1/10 **17-**U2.1 1/50 **18-**U2.W 1/10 **19-**U5.2 1/10 **20-**U5.2 1/50 **21-**U5.W 1/10 **22-**U8.3 1/10 **23-**U8.3 1/50 **24-**U8.W 1/10 **25-**U11.3 1/10 **26-**U11.3 1/50 **27-** U11.W 1/10 **28-**U14.2 1/10 **29-**U14.2 1/50 **30-**U14.W 1/10 **31-**C+ **32-**C-

Nitrotoga was firstly targeted using primers against the *nxrB* gene. Negative results were obtained. In order to double check the absence of Nitrotoga in the samples, a second PCR was performed with specific primers amplifying *Nitrotoga* rRNA 16S. Surprisingly bands matching the size of the positive control were observed in most of the samples and dilutions tested (Figure S3, Annex I). Subsequently the rRNA 16s PCR was repeated in higher reaction volumes to get enough product for cloning procedures. The cloning protocol was repeated several times until the colony PCR displayed few potential positive clones. The chosen clones were sent for Sanger sequencing. The obtained sequences form both clones were blasted using the BLAST online tool. Both clones were not identified as Nitrotoga with the standard nucleotide-nucleotide alignment. The closest matches to the cloned sequences were random rRNA 16S and regions amplified from the cloning vector.

Nitrospinae displayed clear negative amplifications where no band could be identified.

3.1.2 Seasonal study of NOB communities' composition and dynamics from two saline-alkaline lakes applying NGS techniques.

Illumina MiSeq library preparation.

The detection of *Nitrospira* genus with a specific primer pair targeting its *nxrB* was positively accomplished in all sediment samples as inferred in the screening PCRs. Accordingly, a total of 98 sediment samples from both lakes were included in the library. The library encompassed, for each lake, three biological core replicates (.1 .2 .3) of the surface layer from each month and one replicate core (.4) of the lower depth from July14, Oct14, Jan15 and April15. For comammox *Nitrospira* cladeA detection the correct size band had to be cut apart from double bands of the agarose gel. Not all samples displayed comammox presence. Therefore, the, comammox *Nitrospira* cladeA library consisted of 66 samples. Comammox *Nitrospira* cladeB signals were much weaker, without any clear coherent pattern even within biological replicate samples from the same month. Only 9 samples, for which the band was clear through all the library preparation process were included for sequencing. A detailed list of the samples included in each library is given in Table S2 Annex I.

Identification and phylogenetic affiliation of NOB OTUs.

The OTUs clustered at 95% sequence similarity were aligned to an existing *nxrB* or *amoA* gene database and placed in phylogenetic reference trees (Pester et al., 2014; Daims et al., 2015) The trees enabled the confirmation of the OTUs identity and their affiliation to distinct lineages, clades or subgroups. In addition, the sequences clustering close to OTUs generated in this study provided knowledge about potential environment preferences and distribution. The trees also suggested phylogenetic relations and evolution patterns that cause divergence between groups and clusters. This differentiation is represented by the horizontal branch length, proportional to sequence divergence(Nei, Tajima, & Tateno, 1983).

Canonical Nitrospira. The computational analysis clustered the *nxrB* sequences into 20 distinct OTUs. The phylogenetic analysis demonstrated a high diversity of Nitrospira representatives widespread among the six lineages (see FIGURE 7). Affiliation of OTUs from the present study with distinct *Nitrospira* lineages provided information about their possible habitats and closest relatives.

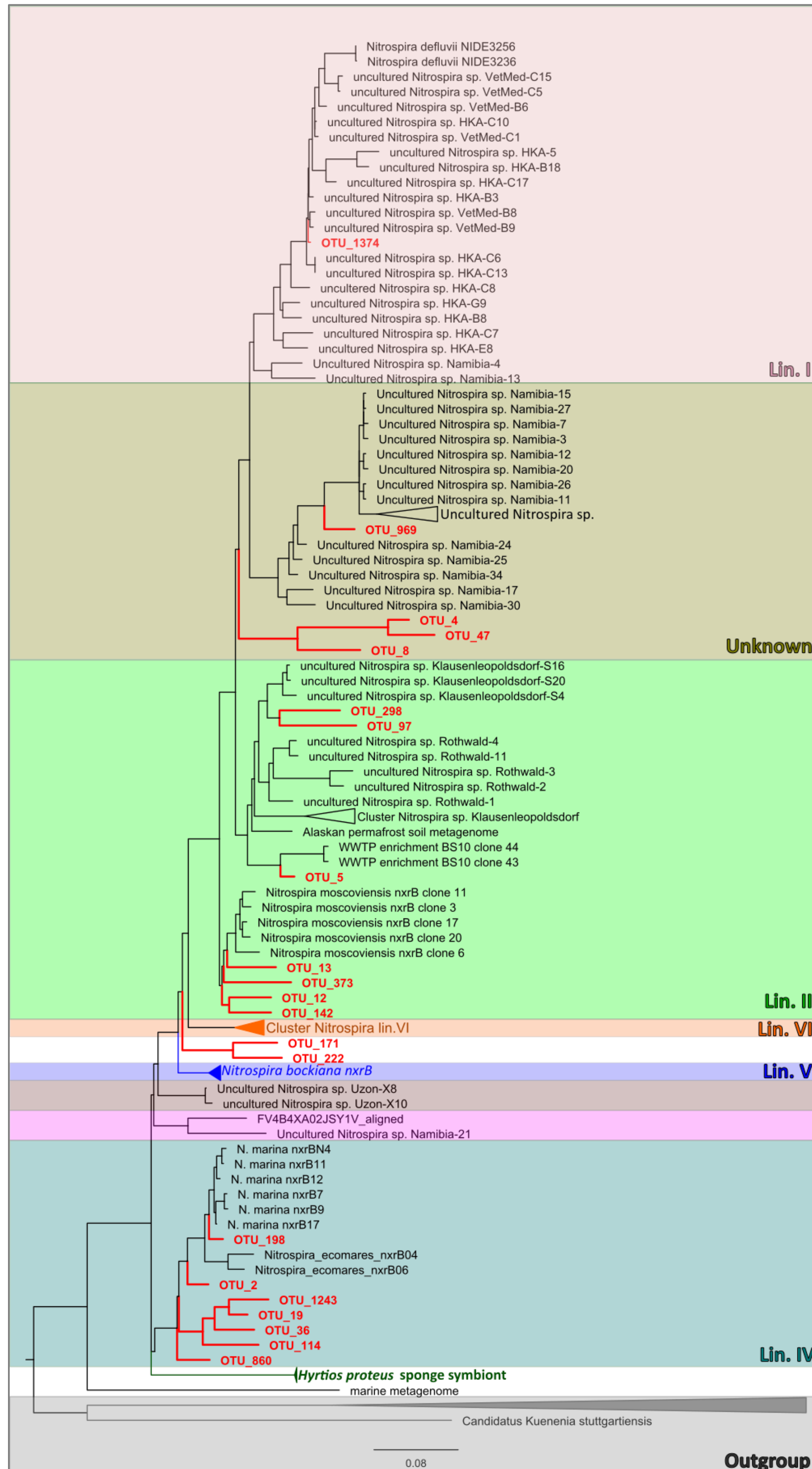

FIGURE 7. Phylogenetic affiliation of *Nitrospira nxrB* sequences from two saline-alkaline lakes. Phylogenetic tree showing the relationship between the OTUs obtained in this study and sequences from an extensive *nxrB* database from different environments and representing the distinct six known *Nitrospira* lineages. The OTUs from this study are displayed in red. Some clades have been collapsed for clarity and for reducing the tree dimensions. However, their identity is stated in the tree. The scale bar indicates an estimate of 8 changes per 100 nucleotides.

The two lineages encompassing the highest number of OTUs were Lin. II and Lin. IV, with seven OTUs each. Six OTUs were placed outside of the known lineages, and one (OTU 1374) within the lineage I. The majority of the taxa clustered as monophyletic groups, distant from the other sequences present already in the tree. In addition, fine-scale identification of most of the OTUs was not possible since the closest sequences belong to uncultured *Nitrospira* clones without any known species affiliation. However, two exceptions could be identified. OTU 198 fell within a cluster consisting only of N. *marina nxrB* sequences. Also, a group of OTUs consisting of OTU 13, OTU 373, OTU 12 and OTU 142 belonging to lineage II, clustered together with the *Nitrospira moscoviensis nxrB* clade.

Comammox *Nitrospira* clade A. Twelve comammox clade A OTUs were assembled during the data processing. Comammox sequences spread along the tree forming two distinct phylogenetic clusters placed apart from each other in the reference tree (see FIGURE 8). OTU 22 clustered alone within sequences originated from engineered systems. The clade observed on the top part of the tree, named Clade I in FIGURE 8, belongs within a larger group including two of the *Candidatus* species described (*Nitrospira inopinata* and *Ca. Nitrospira nitrosa* (Daims et al., 2015; van Kessel et al., 2015)). However, the OTUs form a separate clade together with an uncultured bacterium sequence. The clade II forms a separate group together with a sequence obtained from activated sludge.

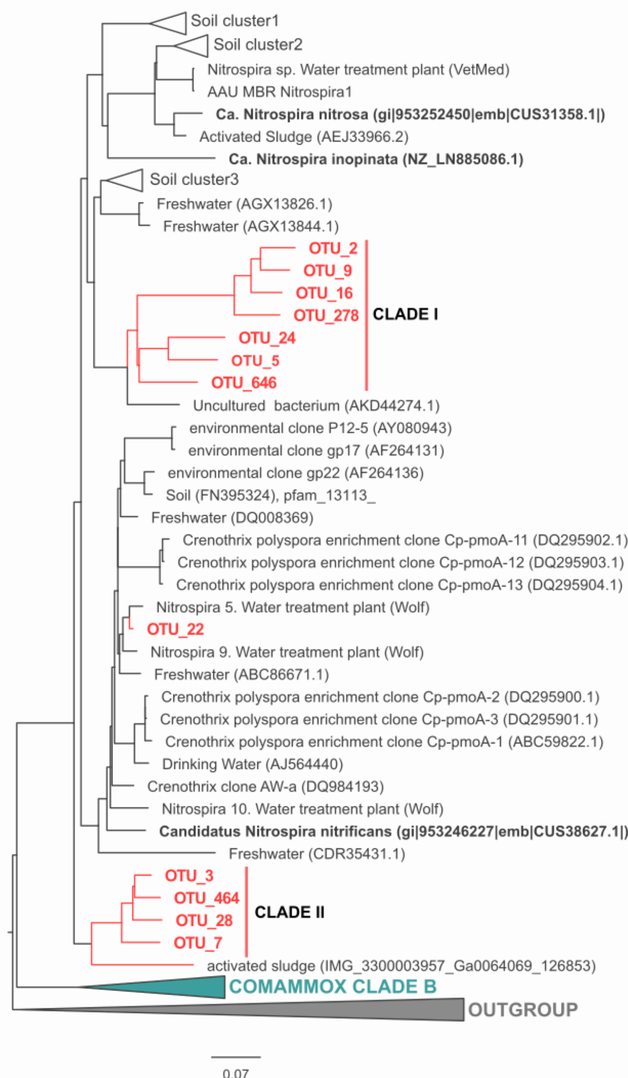

FIGURE 8. Phylogenetic affiliation of comammox clade A *amoA* sequences obtained in the seasonal study of two saline-alkaline lakes. Phylogenetic tree adapted from Daims et al. 2015 displaying the clustering and affiliation of the OTUs. Comammox *amoA* clade B (116 taxa) has been collapsed for clarity. The OTUs detected in this study are displayed in red. Some additional clades have been collapsed to reduce the tree dimensions. However, their identity is stated in the tree. Scale bar indicates estimate change per nucleotide.

The clades drawn in the tree do not reflect any phylogenetic association analysis. They are only used to facilitate the differentiation of groups of OTUs and the discussion of the results by providing a name to distinct sets of taxa.

Comammox Nitrospira clade B. The few obtained sequences with specific primers targeting the *amoA* gene of comammox clade B were assembled into eight distinct OTUs. Their clustering within known *amoA* clade B sequences from different environments confirmed their affiliation to comammox *Nitrospira* cladeB (FIGURE 9). Four of the OTUs (OTU 1, OTU 28, OTU 16 and OTU 156) formed a phylogenetic cluster within soil-originated sequences. OTU 9, OTU 30 and OTU 7 fell individually, but all close to samples from soils or lake sediments. Finally, OTU 139 was affiliated with a monophyletic cluster formed by sequences obtained from water treatment plants.

FIGURE 9. Phylogenetic affiliation of comammox clade B *amoA* sequences obtained in the seasonal study of two saline-alkaline lakes. Phylogenetic tree adapted from Daims et al., 2015 displaying the clustering and affiliation of the OTUs within a reference *amoA*-based tree from Daims et al. 2015. Comammox *amoA* clade A (224 taxa) have been collapsed for clarity. The representative sequences for OTUs from this study are displayed in red. Some clades have been collapsed to reduce the tree dimensions and their identity is stated in the tree. Scale bar indicates estimate change per nucleotide.

Community structure and temporal dynamics.

Illumina MiSeq sequencing of the functional genes *nxrB* and *amoA*, crucial for the nitrification process, revealed the canonical *Nitrospira* and comammox *Nitrospira* populations present in two saline-alkaline lakes. With the detection of the NOB communities present each month during more than a year in combination with monitoring of environmental factors, we aimed at disclosing a temporal succession defined by the fluctuation of environmental factors. The seasonal study was conducted with samples originated from two different lakes differentiated regarding their environmental parameters. Accordingly, the community composition is described in separate heat maps (FIGURE 10 and 11).

Canonical Nitrospira. The presence of *Nitrospira* in both lakes and all months was expected since they were already detected through all library preparation steps by PCR. Clustering of the sequences into OTUs and placement of the obtained taxa into a phylogenetic tree revealed a high diversity. However, the phylogenetic diversity of the *Nitrospira* OTUs did not reflect a clear temporal succession throughout the year of the study. Changes in the relative abundances over the months were not pronounced. As illustrated in FIGURE 10, both lakes, HS and US, showed a similar community structure depicting the typical rank-abundance curve(Lynch & Neufeld, 2015) with a predominance of few OTUs followed by a large tail of rare taxa present in low abundances.

The Herrnsee had a higher salinity than the US and only one single taxa, OTU 2, dominating throughout the whole monitoring period as illustrated in FIGURE 10A. OTU 2 was affiliated with *Nitrospira* lineage IV and was the most abundant OTU in every sample. It was followed in relative abundance by OTU 4 from an unknown *Nitrospira* lineage and OTU 5 from *Nitrospira* lineage II. The relative abundance of OTU 13, present in all 14monhts, suggested a possible temporal succession with increasing relative abundances during the summer season in 2014, followed by decreasing between September '14 and January '15 and increasing again from February '15 on, achieving the highest relative abundances in June'15. OTUs 8, 12,19 were also detected in all the months sampled but in lower abundances. All other detected Nitrospira lineages were represented by several OTUs, but in much lower abundance and only in some specific months.

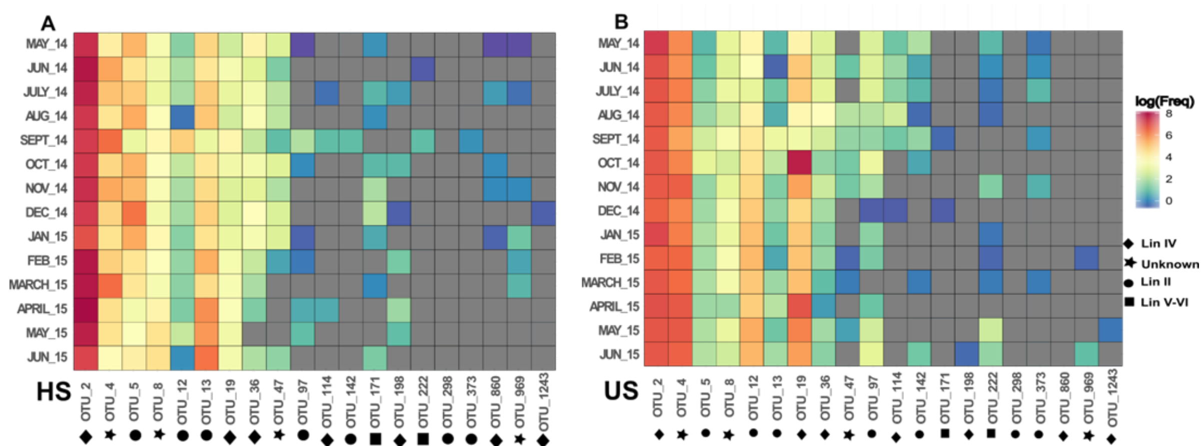

FIGURE 10. Heat maps describing the relative abundance of the canonical *Nitrospira* OTUs present in the two saline-alkaline lakes Herrensee (HS, panel A) and Unterer Stinker (US, panel B). Each heat map shows the relative abundance in the sampled months, from May '14 until June '15 on the y-axis. The different OTUs are depicted on the x-axis. The phylogenetic identity of each OTU is indicated by symbols.

The Unterer Stinkersee showed the same pattern of dominance of few OTUs over a diverse, rarer majority displayed in FIGURE 10B. Here, two taxa OTU 2 and OTU 4 from *Nitrospira* lineage IV and from the not further classified *Nitrospira* lineage, respectively, were dominant through all the year. OTU 12 increased in relative abundance after the winter months of 2014, resembling the same trend of OTU 13 in the HS. OTU 19 from *Nitrospira* lineage IV exhibited single high relative abundances in October '14 and April '15. The detection of OTUs 5, 8, 13 and 36 was possible during the whole monitoring period. Like for HS, the rest of the OTUs were present in low abundances, but most of them only in some months.

Comammox *Nitrospira* clade A. Unlike canonical *Nitrospira,* comammox clade A was not detected in all sediment samples.

Herrnsee. During the PCR the amplification of the comammox *Nitrospira amoA* gene was unsuccessful in some samples of HS. These samples were from December '14 until February '15 and from June'15. The heat maps of FIGURE 11 nevertheless illustrate these months in order to present all the results and facilitate data interpretation. In addition, the months of May '14, June '14, July '14, and August '14 were missing at least one of the three replicates, since no clear band was observed after attempted PCR amplification. Despite impaired interpretation due to the missing data from a few months, a similar pattern to that of canonical *Nitrospira* could be inferred (see FIGURE 10 and 11). One single taxa, OTU 2, was dominant throughout the year. It belongs to Cluster I described in FIGURE 11A and varied in frequency over the months, with the highest relative abundance in April '15. OTU 3 and OTU 464 were also in the high abundant range in all the samples. Two more OTUs, OTU 7 and OTU 9, were detected in all months but in much lower frequencies. The remaining seven OTUs were only detected stochastically and in low relative abundance.

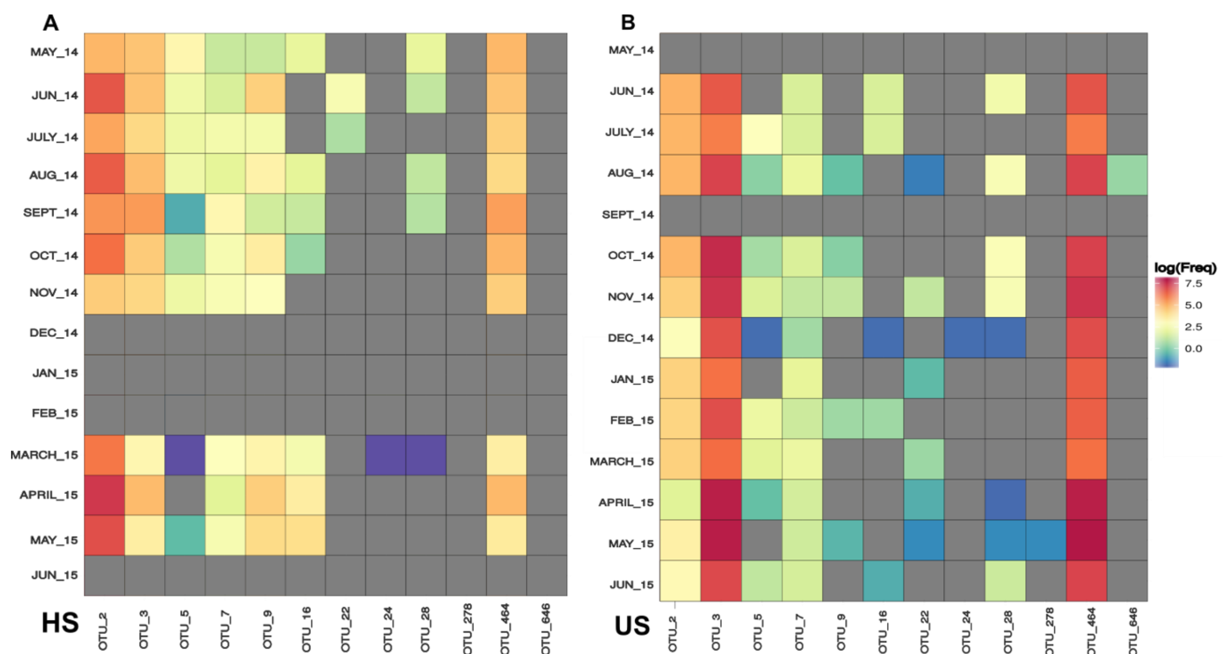

FIGURE 11. Heat maps describing the relative abundance of *Nitrospira* comammox clade A OTUs assembled with the sequences obtained from the two saline-alkaline lakes. Each heat maps illustrates the abundance in each sampled month, from May14 until June15 in the y-axis. The x-axis lists the different OTUs reconstructed. The relative abundance data was log transformed and displayed as frequency represented by different colours. The samples not included in the sequencing are also displayed in the heat map depicted in grey for all OTUs representing negative results. Figure. A depicts the community composition of the Herrnsee and the B the one detected from Unterer Stinkersee samples.

Unterer Stinkersee. Likewise, comammox clade A was not detected in each month in the US. May ‘14 and September ‘14 showed absence of comammox clade A for all the OTUs. In difference to HS. However, two different taxa, OTU 3 and OTU 464, from clade II (see FIGURE 11B) were the clear dominant ones in the Unterer Stinkersee in all samples as illustrated in FIGURE 11B. Furthermore, OTU 2 together with OTU 7 were present in all samples. The remaining eight OTUs were only randomly detected in some months without displaying any clear pattern.

Comammox Nitrospira clade B was found only stochastically. In the majority of the months they could not be detected. In addition, comammox clade B sequences were only retrieved in a subset of the biological replicates for some months.

Herrnsee. The presence of comammox organisms from clade B in the HS was only detected in August ‘14 and March ‘15. All the reconstructed OTUs were present in the HS community.

Unterer Stinkersee. Comammox clade B was detectable in October ‘14, December ‘14, January ‘15 and May ‘15. The three OTUs observed in all samples from both lakes were OTU 1, OTU 7 and OTU 9. This three fell widespread in the phylogenetic tree displayed in FIGURE 9. Additionally, the US did not reflect the presence of OTU 30, OTU 139 and OTU 156 at any sample being exclusively detected in the HS.

Microbial community responses to the abiotic context.

Community composition analysis illustrated the structure and dynamics of canonical *Nitrospira* and comammox *Nitrospira* populations. Significant correlation was found between identified taxa and environmental conditions. However, we cannot conclude if the factors influence the communities or are the organisms themselves that modify the conditions. This chapter focuses on the effect of the environmental fluctuations on the nitrifier community structures, providing data about the niche conditions preferred by the identified OTUs.

In order to discern the possible relationships between microbial community structure and environmental variables, a distance-based constrained ordination (dbRDA) analysis was applied(Anderson & Willis, 2003; Legendre & Anderson, 1999). Firstly, the effect of environmental factors on the community composition was tested using multivariate analysis of variance(Anderson, 2001; Legendre & Anderson, 1999). By this approach the factors with strong and significant impact were identified and taken as constrains in the ordination analysis. The ordination was complemented by fitting of relative phylotype abundances, which significantly correlated with the grouping of samples in the ordination space thereby disclosing OTUs that significantly differed between communities. The vectors reflect the direction of the gradient or relative OTU abundance, pointing to the communities with highest values. The length represents the gradient’s strengths, since it is proportional to the correlation between ordination and variable. Ordination results illustrate the variation of the data, represented by axes and computed in eigenvectors. In dbRDA eigenvalues are calculated for unconstrained and constrained axes, reflecting the differences only based on the species matrix or the ones related to environmental descriptors, respectively(Anderson & Willis, 2003; Legendre & Anderson, 1999). OTU vector fitting was only applied to the canonical *Nitrospira* and comammox *Nitrospira* clade A data. Clade B comammox data did not contain enough information to conduct valuable statistical analysis. Analysis were conducted with the data sets of the two lakes together and

from each lake individually. When studied the two datasets together, ANOVA analysis of variance was applied to test if specific factors significantly differ between the two lakes.

Abiotic and biotic context differences between Herrnsee and Unterer Stinkersee.

The factors detected by the Adonis test as significant descriptors of both lakes' communities were: lake, salinity, ammonium, nitrate and nitrite concentrations in the sediments and nitrite concentration in the water column. The ANOVA test results, detailed in TABLE 5, depicted the presence of statistically significant differences between the two lakes regarding the factors affecting the community composition.

TABLE 5. One-way ANOVA test results of the factors identified as significant descriptors. (S=sediment, W=water column)

FACTOR	One-way ANOVA
Salinity	p<0.001, Df= 1, F= 179.9
NH_4^+ (S)	p<0.001, Df= 1, F= 46.88
NO_3^- (S)	p<0.005, Df= 1, F= 11.41
NO_2^- (S)	p<0.001, Df= 1, F= 12.88
NO_2^- (W)	p<0.001, Df= 1, F= 12.62

Canonical *Nitrospira*. The Adonis analysis of variance revealed a total of five factors with significant ($p < 0.05$) effect on the OTU data matrix. Lake had the highest squared correlation coefficient ($R^2 = 0.45$) followed by salinity ($R^2 = 0.38$). Three other parameters, nitrogen concentration in the sediment, showed significant correlations but much lower influence (NH_4^+, $R^2 = 0.16$; NO_3^-, $R^2 = 0.10$; NO_2^-, $R^2 = 0.12$). Subsequently, ordination analysis with salinity and lake as constraints were performed to examine the direct relationships between these variables and the community structure. The remaining of constrained-ordinations plots are provided in AnnexI (FIGURES S4 - S6).

LAKE. CAP analysis using lake as a constraint was able to explain the 38.43% of the microbial community variance, illustrated in FIGURE 12A, disclosing all observed variance on the unconstrained axes (y-axis, MDS1) and all variance explicable by the factor lake on the x-axis (CAP1). The originating lake clearly separated the communities into two distant groups (see FIGURE 12A). The *envfit* formula allowed the disclosure of 13 OTUs significantly ($p < 0.001$) differing in their relative abundance between these groups and thereby affecting the ordination. OTU 8, 47, 5, 171, 13, 198 and 2 correlated with communities from lake HS. On the contrary, lake US communities correlated significantly with the following taxa: OTU 12, 19, 4, 97, 373 and 142.

SALINITY. Analysis of variance also depicted salinity as a powerful factor differentiating the communities (32.38% of variance explained, see FIGURE 12B). Two main discernible clusters could clearly be identified as in the lake-constrained ordination. A total of 9 taxa were established as significantly differing between the groups ($p < 0.001$) and were plotted in the constrained ordination graphic. The salinity eigenvector illustrated the direction of the gradient affecting the ordination structure. OTU 13, 171, 5 and 36 vectors followed the same direction as the salinity eigenvector. On the contrary, vectors representing OTU 4, 19 and 12 were drawn in the opposite direction. In addition, OTU 2 also contributed significantly to the ordination structure but it did not follow neither of the two directions of the salinity gradient.

Comammox *Nitrospira* clade A. The factor with the strongest influence on *Nitrospira* comammox community composition was the lake. Adonis multivariate analysis of variance computed a squared correlation coefficient of 0.41, with a $p < 0.001$. However, salinity also showed a high significant correlation with a coefficient of 0.35. Other abiotic factors with a significant minor correlation ($p < 0.05$) were the inorganic N forms, listed from higher to lower regarding their correlation coefficient: NH_4^+ ($R^2 = 0.15$), NO_2^- ($R^2 = 0.12$) and NO_3^- ($R^2 = 0.11$)

concentrations in the sediment layer and finally NO_2^- concentration in the water column (R^2 = 0.10). The ordination analysis applying salinity and lake as constraints are described in the following sections. The remaining of ordination plots are provided in Annex I (FIGURES S7).

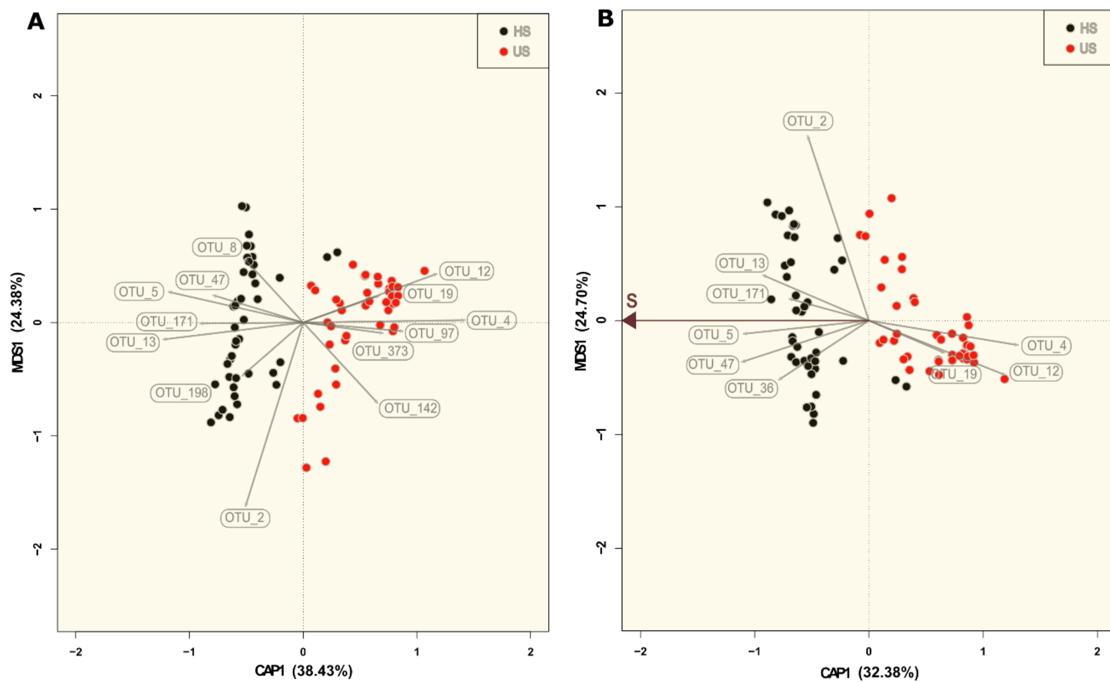

FIGURE 12. Ordination plots based on distance-based redundancy analysis (db-RDAs) applied to the canonical *Nitrospira* dataset. Lake-constrained ordination is represented in panel A and salinity-constrained one in panel B. The y-axis (MDS1) represents the first of the unconstrained axes and the x-axis the constrained one (CAP1) displaying also the percentage explained by each axis. The ordination allows the differentiation of two communities. The filled black dots represent the samples from the Herrnsee (HS) and the red ones from the Unterer Stinkersee (US). The significant ($p<0.001$) OTUs eigenvectors influencing the community structure are illustrated by grey lines and tags.

LAKE. Lake-constrained ordination was able to explain 39.10% of the total variation (FIGURE 13A). Although one sample from each lake (U3.1 and H6.2) clustered with the opposing group, divergent communities from each lake were observed. The *envfit* formula revealed 7 significantly different OTUs ($p < 0.001$) that were also plotted in the ordination. OTU 2, 9, 16 clearly had higher relative abundances in the HS. Two other taxa (OTU 5 and OTU 7) indicated a less pronounced preference for the HS. OTU 3 and 464 showed the opposite trend, since their gradient increased towards the US samples.

SALINITY. The high correlation coefficient obtained by the Adonis analysis was matched by a value of 33.58% of community variance explained in the salinity-constrained ordination (FIGURE 13B). Again, the same individual samples (U3.1 and H6.2) associated within the opposing cluster. The differentiation of two communities was also revealed in FIGURE 13B, illustrating most of the variation on the first constrained axis (CAP1, x-axis) vs the first unconstrained axis (MSD1, y-axis). Three different gradients were inferred by plotting the OTUs, significantly ($p < 0.001$) influencing the community structure. As observed in lake-constrained ordination, OTU 2, 9 and 16 correlated as a group, increasing in relative abundance in HS samples. Also OTU 5 depicted higher abundances in the HS, but in another cluster of samples. The two gradients correlating with the HS showed a slight preference towards salinity. The third gradient, represented by OTU 464 and OTU3, was drawn in the opposite direction of the salinity gradient, illustrating also higher abundances of these two taxa in US samples.

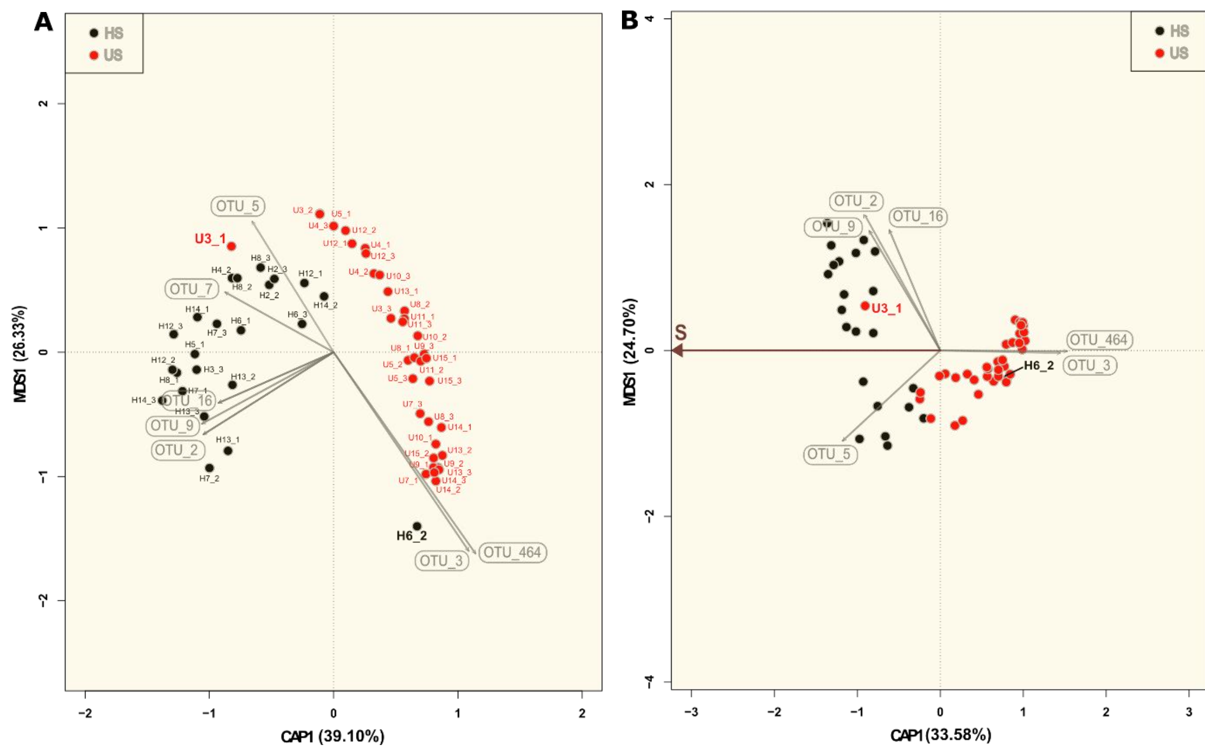

FIGURE 13. Ordination plots based on distance-based redundancy analysis (db-RDAs) applied to the comammox clade A *Nitrospira* dataset. Lake-constrained ordination is represented in panel A and salinity-constrained one in panel B. The y-axis (MDS1) represents the first of the unconstrained axes and the x-axis the constrained one (CAP1) displaying also the percentage explained by each axis. The ordination allows the differentiation of two communities. The filled black dots represent the samples from the Herrnsee (HS) and the red ones from the Unterer Stinkersee (US). The significant ($p<0.001$) OTUs eigenvectors influencing the community structure are illustrated by grey lines and tags.

Individual influence of each lake conditions on the contained communities.

Canonical *Nitrospira*. Only the month was found to be as significant factor with strong effects on the community structure in both of the lakes. In the Herrnsee, month was the strong and only significant descriptor detected, explaining in total 49.65% of the observed community variation divided into 13 constrained axes. The ordination graph in FIGURE 14A displays the two first constrained axis (CAP1 and CAP2) representing a 27,50% and 11.34%, respectively, of community variation. The significant ($p < 0.001$) four OTUs vectors fitted depicted three different gradients. OTU 13 and OTU 2 were individually plotted in distinct orientation. OTU 19 and OTU 4 were drawn in the same direction, illustrating the third gradient inferred.

The Unterer Stinkersee analysis was more complex. Although month was also the stronger influencing factor explaining 60.64% of the total variation, six more environmental factors showed minor, but significant effects. Salinity explained 13.54% of the US community variation. The rest of significant descriptors were the different inorganic nitrogen forms measured. Nitrate from the sediment layer had the stronger coefficient, explaining the 14.61% of the total variance, followed by nitrite with a 12.84%. Nitrate data from the water column explained the 12.30% of the total variation. Finally, ammonia from the water column was also a significant minor descriptor with an explanatory value of 8.55%. Considering the obtained results, only a month-constrained ordination was plotted together with fitted vectors representing OTUs with significantly ($p < 0.001$) different relative abundances in correlation with the ordination in FIGURE 14B. The ordination graph exposes the two first constrained axis (CAP1 and CAP2) representing a 31,77% and 12.57%, respectively, of the total variation split into 13

constrained axes. Again, three different gradients represented by distinct OTUs correlated with the community composition patterns. OTU 19 was individually representing one of the gradients proposed. Two groups of two taxa, OTU 2 and 142 vs OTU 12 and 4 were plotted with divergent directions, revealing the second and third gradients observed.

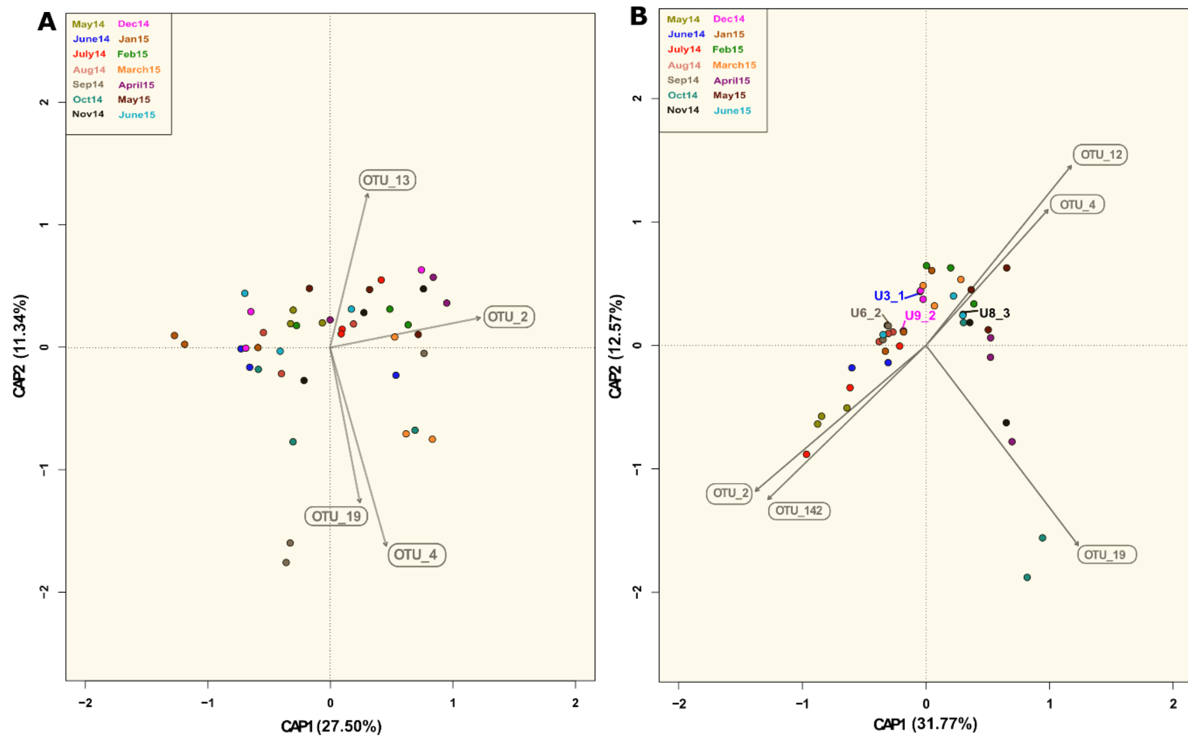

FIGURE 14. Ordination plots based on distance-based redundancy analysis (db-RDAs) constrained to month factor and applied to the comammox clade A *Nitrospira* dataset. Panel A reflect the ordination of the Herrnsee (HS) samples and panel B the one with the samples from the Unterer Stinkersee (US). The x-axis represents the first constrained eigenvalue (CAP1) and the y-axis the second one (CAP2). Both display also the percentage explained by each axis. The colour palette identifies each dot regarding its month. The significant ($p<0.001$) OTUs eigenvectors influencing the community structure are illustrated by grey lines and tags.

Comammox *Nitrospira* clade A None of the Herrnsee abiotic factors showed significant correlation with the dissimilarity community matrix. In consequence, no constrains were available for dbRDA analysis.

Unlike the HS, the US community was significantly influenced by four abiotic factors. Month was by far the main influencing factor, with a squared correlation coefficient of 0.62. Salinity correlation coefficient was 0.16 with an explanatory value of 16%. The other two parameters were nitrite and nitrate concentrations in the sediment, with coefficients of 0.14 and 0.1 respectively. The month-constrained ordination explained a total of 60.7% of the variation, divided into 11 constrained axes. The ordination graph in FIGURE 15 represents the two first constrained axis eigenvalues (CAP1 and CAP2) representing a 51,60% and 5.93%, respectively, of the total variation. Four OTUS were significantly ($p < 0.001$) influencing the community structure. Two gradients encompassing two taxa each (OTU 2 and 7 vs OTU 3 and 464) were oriented towards different clusters of samples, reflecting differences between communities of particular months.

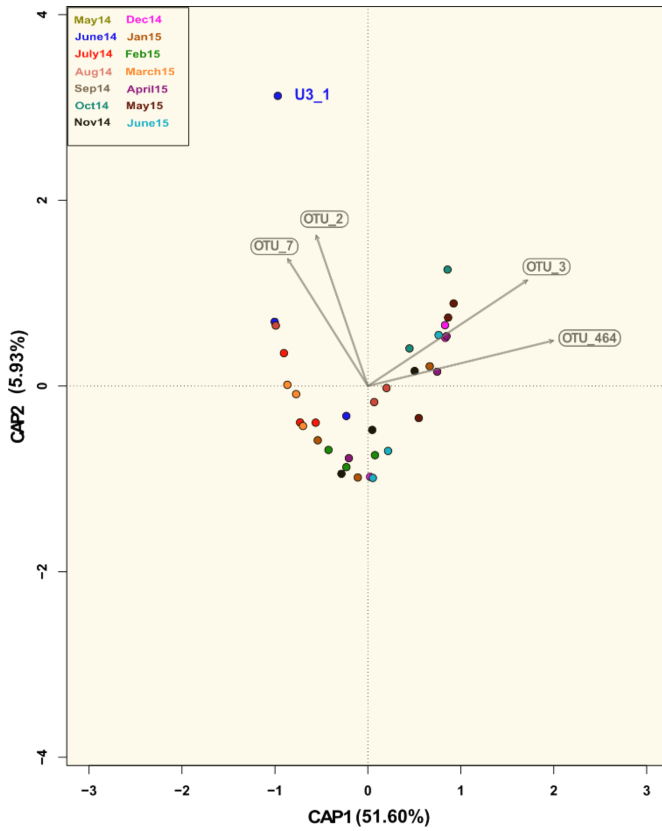

FIGURE 15. Ordination plots based on distance-based redundancy analysis (db-RDAs) constrained to month factor and applied to the US samples from comammox clade A *Nitrospira* dataset*.* The x-axis represents the first constrained eigenvalue (CAP1) and the y-axis the second one (CAP2). Both display also the percentage explained by each axis. The colour palette identifies each dot regarding its month. The significant ($p<0.001$) OTUs eigenvectors influencing the community structure are illustrated by grey lines and tags.

3.2 OPTIMIZATION, PURIFICATION AND STUDY OF THREE NOB ENRICHMENT CULTURES

In microbial ecology, the impact of interactions between abiotic and biotic surroundings on microbial communities is crucial. In consequence, cultured representatives of the microbial guild under study are needed for controlled physiological characterization. Here, NOB from saline-alkaline lakes in the National Park "Neusiedler See-Seewinkel" were enriched. The inoculation with lake sediment and water and incubation under nitrite-oxidizing conditions resulted in three enrichment cultures exhibiting nitrifying activity. They were maintained in oligotrophic, saline medium at 28ºC and a pH between 8.5-9.5. Within the frame of this master thesis no enrichment was started, but the existing ones were maintained and further cultivated from November 2016 until July 2017.

3.2.1 *Nitrospira* lineage IV enrichment ("KS").

The aim proposed for this enrichment culture was to optimize the culturing conditions promoting the growth of *Nitrospira* lineage IV members, which were known to be a stable member in the enrichment (Anne Daebeler, oral communication). The continued identity and presence of *Nitrospira* lineage IV as the only nitrifier was confirmed by Sanger sequencing of *nxrB* PCR amplicons. The obtained nucleotide sequences were analysed using the BLASTn program that classified the organisms as *Nitrospira marina*-related, with a 94% *nxrB* nucleotide sequence identity. The analysis of the 16S rRNA sequence with the BLASTn alignment tool also revealed *Nitrospira marina* as the closest relative, with a 98% sequence similarity. Additionally, the alignment of the KS *nxrB* sequence to the *nxrB* dataset obtained in the seasonal study classified the KS nitrifier and OTU 2 within the same species due to a 99% *nxrB* sequence identity. Besides OTU 2, the closet relative from the seasonal dataset was OTU 860 with a 95% sequences similarity. The enrichment level of the culture was assessed several times during the project with genus *Nitrospira* specific FISH probes. The first FISH analysis provided an approximate enrichment level of 40%. Several approaches were used in order to fulfil the objective declared. Transfer to new media and new culture flasks enabled dilution of contaminants and the waste products accumulated.

Culturing conditions.

The lineage IV *Nitrospira* in the enrichment had slow activity rates, only consuming 1 mM of nitrite in month or month and a half. In order to avoid inhibition by nitrite excess and optimize the culturing conditions, the feeding amount was decreased to 0.5 mM NO_2^-. The new treatment resulted in faster nitrite depletion consuming 0.5 mM NO_2^- weekly. Also, the pH was checked and adjusted every month to 8.5-9.5. In previous studies, not included in this master thesis, the genome of the N. *marina*-related *Nitrospira* species was reconstructed from a metagenome of the culture. Several genes for adaptations to high salinity environments were detected in the genome (Daebeler et al., manuscript in preparation). However, when the culture was transferred to medium containing eight and four times more salt content (comparable to about half and one quarter of the level of sea water, respectively), no activity or growth could be detected after four months of incubation.

Fluorescent in situ hybridization (FISH).

Phylum Nitrospirae and genus *Nitrospira*-specific probes (Ntspa712 and Ntspa662, respectively) were applied to control and analyse the development of *Nitrospira* abundance. The culture consisted of a few planktonic cells but mostly of large flocks encompassing individual cells, thick clusters and more disperse aggregations of cells. The specific FISH signals of interest appeared only in aggregated cells located inside the flocks (FIGURE 16). The latest FISH analysis conducted on 12/07/17 evinced the presence of *Nitrospira* but no improvement in the enrichment level.

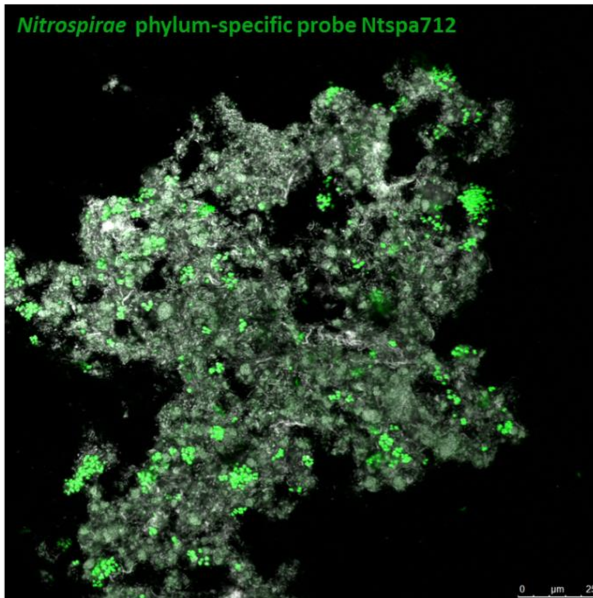

FIGURE 16. Confocal image of FISH-stained *Nitrospira marina*-like bacteria in fixed samples from the KS enrichment culture. *Nitrospira* cell aggregates are hybridized to Ntspa712 (green) probes. All DNA is DAPI stained (grey). Probe details are given in TABLE 3. The scale bar represents 25 µm.

3.2.2 *Nitrospira* lineage II enrichment ("MixS").

Achievement of a pure culture of alkalitolerant *Nitrospira* lineage II was the main objective stated for the enrichment 'MixS'. The nitrifying activity of this enrichment culture was stable and required 1mM of NO_2^- per week. *Nitrospira* lineage II members were the only NOB of the enrichment but some contaminants were still present as revealed by FISH analysis. Different dilutions and enrichments postulated an enrichment level around 80%. However, in the latest dilutions obtained we could not infer a higher enrichment level than 60%, illustrated by the FISH image of FIGURE 17A. Phylogenetic analysis of Sanger sequenced 16S rRNA PCR amplicons confirmed its belonging within lineage II *Nitrospira* species, clustering between *Nitrospira moscoviensis* and *Nitrospira japonica*. The nucleotide sequence, analysed by the BLASTn alignment tool, revealed *Nitrospira moscoviensis* at the closest cultured relative with a 98% 16S rRNA gene sequence identity.

Fluorescent in situ hybridization (FISH) and scanning electron microscopy (SEM).

FISH analyses were used to confirm the identity applying a lineage II-specific *Nitrospira* probe (Ntspa1151) and assess and follow the development of the enrichment level. Confocal microscopy, used for the observation of FISH trails, demonstrated the planktonic growth of the culture with some small sparse flocks. Since the *Nitrospira* cells were also growing planktonically, several dilutions series were executed in order to dilute the contaminants. Highly enriched culture of alkalitolerant *Nitrospira* lineage II was obtained after consecutive dilution series. FISH analysis together with DAPI staining proposed the presence of only one morphotype next to the target cells (FIGURE 17A). The *Nitrospira* cells had a short, thick rod shape and the contaminant, appeared as longer and thinner rods. No typical spiral *Nitrospira* morphology could be found in the enrichment. Scanning electron microscopy was used to confirm the morphologies detected with the FISH images using a highly enriched culture. The obtained high resolution images displayed in FIGURE 17B confirmed the proposed morphologies. Two types of rods could be differentiated in the enrichment culture: A longer and thinner rod morphology marked with a X and a shorter and bulkier rod type tagged with an arrow, both in FIGURE 17B. No spiral shaped cells were observed with the SEM either.

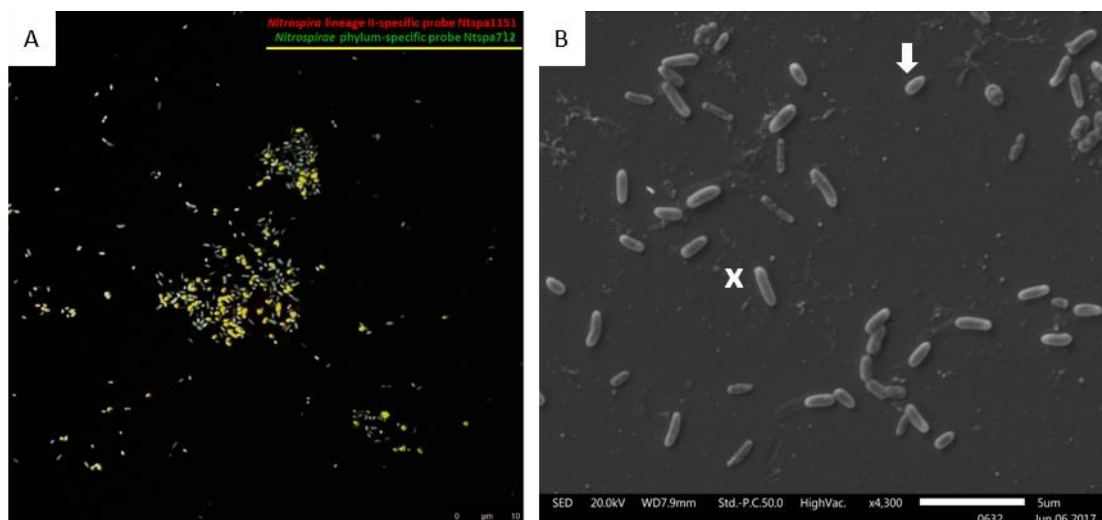

FIGURE 17. Microscopy images of fixed samples from the MixS enrichment culture. A) Confocal image of FISH-stained Lineage II *Nitrospira* members. *Nitrospira* cells are double hybridized to Ntspa712 phylum specific probe (green) and to Ntspa1151, lineage II-specific probe (red). In consequence, *Nitrospira* cells appear in yellow. All DNA is DAPI stained (grey). Probe details are given in TABLE 3. The scale bar represents 10 µm. B) Scanning electron microscope image from the MixS culture. The two different morphologies stated in the main text are marked with two symbols. One of the thicker, short rods is marked with an arrow. The cross symbol points at one of the longer, thinner rods. The microscope and imaging parameters are stated in the image legend. The scale bar represents 5 µm.

Cell sorting.

Despite of several further dilutions performed, no additional increase of the enrichment level was possible. Therefore, sterile single cell sorting of a live, highly enriched culture was applied. Two 96-well plates were inoculated with randomly sorted single cells from the enrichment culture. After two weeks of incubation in the regular cultivation medium and medium supplemented with vitamins at 28ºC, 43 and 48 wells from regular and vitamin supplemented medium respectively showed complete nitrite depletion and were presupposed active. In a week 15 and 6 wells more from each plate, respectively, also showed full depletion of nitrite. Nitrate formation was qualitatively confirmed in randomly selected wells with the Quantotix Semi-quantitative test strips. The active wells were transferred to new plates with larger well

volumes. Finally, aliquots from active, single cell grown cultures were used as a template for general 16S rRNA PCR amplification. Additionally, DAPI staining and direct observation under the microscope was performed. The Sanger sequencing of the obtained general 16S PCR products revealed the presence of more than one species through ambiguous, low quality sequences that impeded identification by the BLASTn alignment tool. Observation of DAPI-stained cells under the microscope was hampered because of low biomass and presence of debris that prevented the assessment of the number of morphologies present.

3.2.3 Unidentified nitrifier enrichment (“LL”).

The main objective proposed for this enrichment was the identification of the nitrifier/s responsible for nitrite consumption. The LL culture had the highest nitrification rates of the three enrichments, consuming 5 mM NO_2^- per week. Several identification attempts were already performed before the start of this master thesis. Negative results were obtained in distinct trials with *Nitrobacter*-specific FISH probes (Anne Daebeler, oral communication). During the course of this thesis, a *Nitrotoga*-specific probe (Ntoga122, see TABLE3) was applied on the culture with negative results. The only FISH signals observed were obtained with *Nitrospira*-targeting probes, phylum and genus specific (Ntspa712 and Ntspa662, respectively). However, the signals detected were present in very low numbers, only observed in some fields of the microscope and always as a minority group amounting to maximally 3%.

Despite all the negative results, DAPI staining of the fixed samples enabled the assessment of the morphologies and structure of the culture (see FIGURE 18). Samples from early stage cultures displayed huge thick flocks with great diversity of morphologies and sizes, including rods, coccoid cells and a lot of filaments. Dilution series were unsuccessful since only one of three diluted cultures became active and only after four months of incubation. This results indicate that dilution series were not the most suitable method regarding its low and retarded success. In order to separate the planktonic cells from the flocks, filtration of an enrichment sample through sterile 3 µm filters was successfully achieved. Two active cultures were obtained, one inoculated with the flocks blocked by the filter and the other one with the flow through. The flock-inoculated culture was used to test TET as selective element, result explained below. This second one displayed mostly planktonic cells and small flocks, which enabled the isolation strategy of dilution series resulting in fewer morphologies (FIGURE 18). The more homogeneous morphologies present in the culture suggested an increase of the enrichment level.

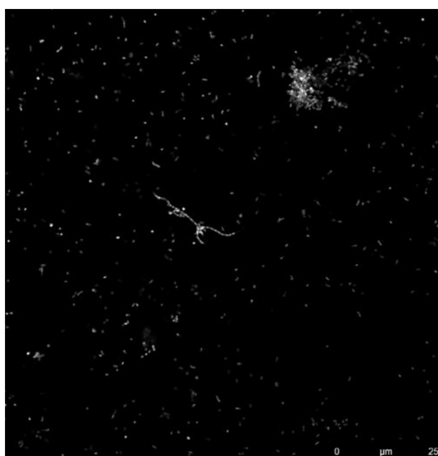

FIGURE 18. Confocal image of cells with DAPI-stained DNA from the LL enrichment culture. The scale bar represents 25 µm.

Tetracycline (TET) resistance and solid media plates.

Metagenomic analysis of the culture (conducted by M. Albertsen), displayed in FIGURE 19, were performed before the start of this master thesis.

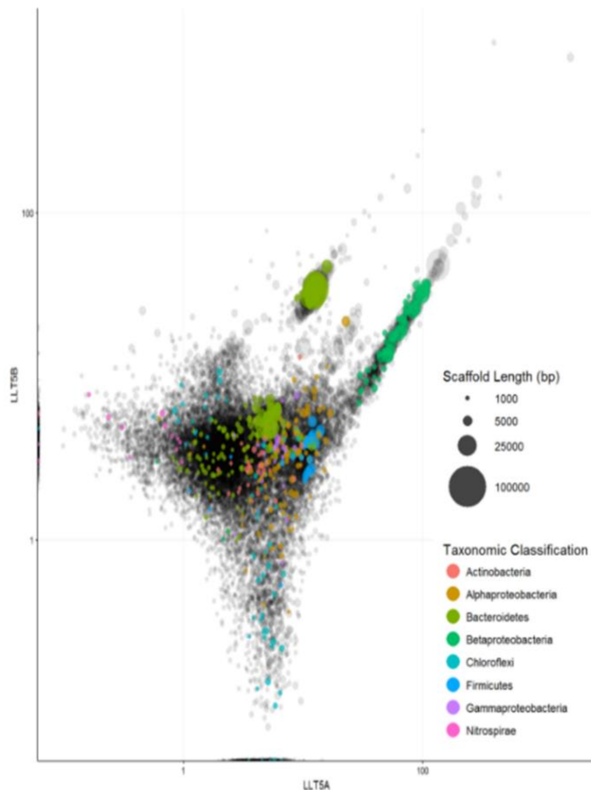

FIGURE 19. Binning of scaffolds using metagenome coverage from the two duplicate LL cultures analysed. Taxonomic identification is illustrated by a palette of colours. Legends regarding the scaffold lengths and the taxonomic classification are provided in the same graph. Figure provided by M. Albertsen.

The metagenome obtained demonstrated the presence of *Nitrospira* in low abundance and two main abundant groups belonging to the Saprospiracea family (Bacteroidetes phylum) and to the Rhodocyclaceae family (β-Proteobacteria class). In order to gain more knowledge about the unidentified nitrifier, the bin belonging to the Rhodocyclaceae family (produced by P. Pjevac) and more specifically to the *Thauera* genus was analysed with the MGRast annotation tool. No nitrite oxidizing potential was clearly annotated in the reconstructed genome. However, tetracycline resistance genes were present within the bin, suggesting a TET-resistance of this group. In consequence, TET was added to the regular cultivation medium in order to increase the selectivity towards the Rhodocyclaceae members. The flask with the TET amendment showed visual media differences with huge flocks and intense turbidity. Additionally, while nitrite was still consumed, nitrate did not accumulate. When transferred to new media without TET, regular nitrite oxidation with nitrate production could be detected again. With solid agarose plates supplemented with sodium acetate and TET, we attempted to grow the *Thauera* organisms as colonies in solid media. Such growth would easily enable isolation. Multiple colonies, with different morphologies and sizes grew in both plates, the ones supplemented with only sodium acetate or also with TET after only five days incubated at 28ºC. We could differentiate small circular transparent colonies covering most of the plates, small circular ones also transparent but with shiny appearance, big irregular swarming colonies, transparent circular ones present in bigger sizes and with a white halus around them and finally white round colonies easily detectable. Representative colonies were transferred to the regular LL cultivation liquid medium without organics, but no nitrite consumption was detected.

Cell sorting.

In order to obtain highly enriched or pure cultures, sterile single cell sorting was applied to this enrichment. Randomly sorted single cells were inoculated into two 96-well plates containing regular medium with and without supplemented vitamins incubated at 28ºC for at least two weeks. 61 wells from the plate amended with vitamin mix and 56 from the plate with only salty NOB media became active after the two weeks displaying nitrite depletion. Nitrate formation was qualitatively confirmed in randomly selected wells. After the third week on incubation 8 and 10 more wells from the plates were presupposed active because of depletion of nitrite. These cultures were transferred to higher volumes and higher nitrite amendments. Active wells were selected for purity tests via 16S rRNA PCR and Sanger sequencing, but failed to provide confirmation of purity and identification of the organism/s sorted. FISH trials for *Nitrobacter* and *Nitrospira* with fixed sorted cells showed no positive FISH signals.

4. DISCUSSION

4.1 SEASONAL STUDY OF NOB COMMUNITIES FROM TWO SALINE-ALKALINE LAKES

The culture-independent approach targeted two specific lakes form the National Park "Neusidler See-Seewinkel". Monthly samples were analyzed in order to get insights into the microbial ecology of nitrite oxidizers within the extraordinary abiotic context. Molecular methods and next generation sequencing (NGS) allowed the study of the community composition and their temporal dynamics in correlation with environmental parameters. The results obtained provided new information about *Nitrospira* adaptations and habitats and suggested new theories regarding *Nitrospira* comammox ecology.

4.1.1 Optimization of culture-independent methods for the study of nitrite oxidizers from saline-alkaline lake sediments.

Previous studies on soda lakes dynamics have provided insights into distinct biogeochemical cycles and the overall microbial diversity. However, even though the nitrogen cycle has been given attention in some studies (Dimitry Y. Sorokin et al., 2014; Dimitry Y. Sorokin, Banciu, & Muyzer, 2015; Dimitry Yu Sorokin et al., 2004a; Dimitry Yu Sorokin & Kuenen, 2005), knowledge on nitrifiers and their activity is still limited in these systems. Here, I discuss the optimization of culture-independent methods to study nitrifiers in saline-alkaline lakes and place preliminary results using the methods into context.

The Spearman's rank correlation test displayed significant correlation between different parameters. Temperature showed to be positively related to pH and salinity. As described in the introduction, higher temperature increases the evaporation rate, affecting different abiotic parameters of the lakes. Lower water levels might cause the accumulation of ions increasing the salinity content and possibly also the pH. The different nitrogen forms measured were also positively related to each other. The current information does not allow the complete explanation of the correlation. However, since they are the main forms involved in the nitrification process, their positive relation is expected. The negative relation between pH and NO_2^- could be due to biotic and/or abiotic reactions not identified in the current study.

Obtaining high quantity and quality DNA is the first key step for culture-independent environmental studies of microbial communities. Extraction of DNA that represents the true diversity is often a challenge in samples with complex microbial matrices carrying high concentration of PCR inhibitors. In addition, different methods have different impacts on DNA purity and quantity but also on the recovered communities (Albertsen, Karst, Ziegler, Kirkegaard, & Nielsen, 2015a; Guo & Zhang, 2013a; Vanysacker et al., 2010a). Therefore, sample characteristics, the purpose of the extracted DNA and the community targeted should influence the selection of the DNA extraction method(Guo & Zhang, 2013b). In this study three different methods were tested: two distinct kits and the phenol-chloroform protocol described by Angel et al(Angel, Claus, & Conrad, 2012). DNA quantity and quality was used as one of the evaluation criteria. Detection of one of the target functional group, *Nitrospira* Comammox, by PCR was applied as a second criteria since specific microorganisms can be underestimated by some methods(Guo & Zhang, 2013b). The Fast DNA Spin Kit for Soil from MPBIO (Mp) and the phenol-

chloroform protocol rely on physical disruption by bead beating combined with chemical lysis. Bead beating has been demonstrated crucial for obtaining high DNA amounts and not losing low abundant members of the community (Albertsen, Karst, Ziegler, Kirkegaard, & Nielsen, 2015b; Guo & Zhang, 2013b). The DNA Isolation Kit Power Soil from MO BIO (Mo) also relies on chemical and physical disruption, but exchanges the bead beating step by vortexing at maximum speed. The first method tested, the MO BIO kit, enabled the extraction of DNA from the Herrnsee (HS) but not from the Unterer Stinkersee (US) impeding the detection of *Nitrospira* Comammox by PCR in the US Lake. Low extraction efficacy could be due to the missing bead beating step. Although, the phenol-chloroform protocol extracted sufficient DNA amount from both lakes, no positive results were obtained in the PCR. The quality of the extracted DNA displayed the presence of contaminants that likely inhibited the PCR reaction(Vanysacker et al., 2010b). In addition, the DNA purification step of this method is accomplished by several transfers of the DNA phase to a new tube. Each transfer implies unavoidable loss of DNA (Vanysacker et al., 2010b); that could have led to loss of low abundance organisms such as *Nitrospira* Comammox. Finally, the third method, the MPBIO kit, succeeded in obtaining DNA from both lakes and enabling the amplification of the target group by PCR. The Mp Kit has been tested and recommended for the extraction of DNA from environmental samples, like activated sludge, in studies comparing different extraction methods(Guo & Zhang, 2013b; Vanysacker et al., 2010b). The main difference suggested as responsible for higher efficiency of this method is the usage of a unique and highly specific DNA binding matrix (Guo & Zhang, 2013b). Taking into account both evaluation criteria, the Mp Kit was the optimal one for our setup and was the chosen one for further DNA extractions.

Due to the big set of samples, the screening PCRs which were performed to determine which nitrite oxidizers were present were done using a representative subset of all samples. The set included sediment samples tested in two dilutions (1/10 and 1/50) and water samples from each season. Negative results were obtained for all nitrifiers targeted in all the DNA samples extracted from the water column. Accordingly, only sediment samples are further analyzed and discussed.

Members of the genus *Nitrospira* were present in all samples and dilutions, demonstrating its broad distribution and ubiquity in the saline-alkaline lake sediments. Their detection in these saline-alkaline lakes, together with other studies suggesting its presence in high pH soils, supports the notion that *Nitrospira* organisms are able to inhabit alkaline systems(Rousk et al., 2010). In addition to canonical *Nitrospira*, Comammox *Nitrospira* clade A organisms were present in lake sediments from some months despite the present of double bands. These results are in concordance with data obtained from the same lakes where Comammox *Nitrospira* was present and active at the RNA level (A. Daebeler, personal communication). Although clade B Comammox *Nitrospira* signals were also observed in the screening PCRs, these signals were much weaker and unclear. In order to validate or disprove its presence, PCR products of the clade B Comammox *Nitrospira* assay were included in the subsequent Illumina Miseq analysis together with clade A and the general genus of *Nitrospira*.

The detection trials for *Nitrobacter, Nitrococcus* and *Nitrotoga* were more complex and ambiguous. The first attempt to target both *Nitrobacter* and *Nitrococcus* with the 706F/1431R primer set showed potential positive bands. The presence of at least *Nitrobacter* was expected, since it is the only nitrite oxidizer shown to live in soda lakes and under alkaline conditions (Grant, 2006; Paul Antony et al., 2013; Dimitry Y. Sorokin et al., 2015; Dimitry Yu Sorokin et al., 2004b;

Dimitry Yu Sorokin & Kuenen, 2005). Furthermore, *Nitrobacter alkalicus* was firstly isolated and characterized from soda lake sediments and soda soil samples(Dimitry Y. Sorokin, Muyzer, Brinkhoff, Gijs Kuenen, & Jetten, 1998). *Nitrococcus* presence was also feasible since it is commonly found in marine environments, especially in OMZ (Oxygen Minimum Zones) where together with *Nitrospina* are the most abundant NOB detected(Holger Daims, Lücker, & Wagner, 2016; Füssel et al., 2012; Watson W & Waterbury B, 1971). *Nitrococcus* adaptations to slightly alkaline marine pH and salinity would enable its presence in saline-alkaline lakes. Due to the presence of double bands, optimization of the PCR conditions to achieve specific amplification of target genes only was attempted. Influence and biases of PCR in bacterial community analysis are widely recognized (Albertsen et al., 2015b; Sipos et al., 2007). Primer choice and annealing temperature are the main factors influencing the output data(Albertsen et al., 2015b). Despite several trials trying to optimize the annealing temperature with two different primer sets, no specific amplification could be achieved. Consequently, further cloning procedures failed to verify the presence of *Nitrobacter* and/or *Nitrococcus.* Hence, they were not included in further analysis. However, the failed detection does not necessarily mean that *Nitrobacter* and *Nitrococcus* are absent in the saline-alkaline lake system. Ecophysiological versatility and niche specialization of NOB induce spatial distribution patterns and temporal dynamics. One of the main factors affecting its physiology is the nitrite oxidation kinetics and biochemistry. *Nitrobacter* might thrive in nitrite rich conditions due to the cytoplasmic localization of its nitrite oxidation genes (NXR)(Holger Daims et al., 2016). Consequently, the high nitrite concentration of the lakes sediments would promote its presence. However, this feature cannot be always assumed, since it has been shown that adaptations to nitrite levels are strain dependent(Holger Daims et al., 2016; Nowka, Daims, & Spieck, 2015). In addition, other factors such as temperature, pH or dissolved oxygen contribute to the complex temporal and spatial dynamics of NOB in the environment (M. Alawi, Off, Kaya, & Spieck, 2009; H. Daims et al., 2006; Hüpeden et al., 2016; Princic, Mahne, Megusar, Eldor, & Tiedje, 1998). Nonetheless, cause-effect relations were not analyzed in this preliminary study and multiple abiotic and biotic factors could have cause the unsuccessful detection of *Nitrobacter*.

Nitrotoga spp are another group of NOB, found firstly in cold, oligotrophic environments(Mashal Alawi, Lipski, Sanders, Eva-Maria-Pfeiffer, & Spieck, 2007a). Recently, they have also been described to be relevant nitrite oxidizers in eutrophic systems and wastewater treatment plants (WWTP)(Lucker et al., 2015; Saunders, Albertsen, Vollertsen, & Nielsen, 2016). In addition, they have been detected in brackish environments and under slightly acidic conditions(Hüpeden et al., 2016; Kruse et al., 2013). In consequence, *Nitrotoga* detection was attempted firstly by targeting the *nxrB* gene and secondly using specific primers against the 16S rRNA gene. Neither the *nxrB* nor the 16S rRNA gene could be certainly detected in the sediment samples. The current knowledge about *Nitrotoga* suggests that it prefers low temperatures, between 10 and 17ºC(M. Alawi et al., 2009; Mashal Alawi, Lipski, Sanders, Eva-Maria-Pfeiffer, & Spieck, 2007b; Hüpeden et al., 2016; Lücker et al., 2015). Although, they are still active at higher temperatures, *Nitrospira* strains might outcompete *Nitrotoga* in these conditions(M. Alawi et al., 2009). However, the coexistence of *Nitrospira* and *Nitrotoga* was been shown in waste water treatment plants (WWTPs) supported by seasonal temperature shifts and temporally optimal conditions provided by other factors shaping the available niches(Holger Daims et al., 2016; Lücker et al., 2015). Because of the inability to confirm *Nitrotoga* presence in our system, its detection was not covered in further analysis.

Finally, *Nitrospinae* was clearly absent from the lake samples. In consequence, its detection was not included in further analysis. The presence of *Nitrospinae* strains was feasible since its only known habitats are marine systems(Holger Daims et al., 2016; Lücker, Nowka, Rattei, Spieck, & Daims, 2013).

In conclusion, a broad range of nitrite oxidizers were targeted to get a first insight in the diversity and dynamics occurring in two saline-alkaline lakes. At the end, only canonical *Nitrospira* and Comammox *Nitrospira* presence was verified. The dynamics and extreme conditions of the system might cause a lower diversity of NOB compared to regular freshwater systems. Further steps will analyze this two NOB groups in detail together with environmental parameters fluctuations.

4.1.2 Seasonal study of NOB communities' composition and dynamics from two saline-alkaline lakes applying NGS techniques.

Identification and phylogenetic affiliation of NOB OTUs.

Illumina MiSeq library preparation allowed the sequencing of genus *Nitrospira nxrB* and both *Nitrospira* comammox clade A and clade B *amoA* genes. Unlike comammox organisms, canonical *Nitrospira* was present in all samples analyzed. Before discussing biological implications of the results, technical biases should be examined. DNA extraction and PCR biases are fully recognized, evaluated and discussed in the chapter 1.1(Albertsen et al., 2015b; Guo & Zhang, 2013b; Vanysacker et al., 2010a; von Wintzingerode, Göbel, & Stackebrandt, 1997). The main technical difference between canonical *Nitrospira* and comammox *Nitrospira* library preparation protocols was the gel cutting step required for the comammox *amoA* PCR products. The presence of double bands implied a higher exposition time to UV light in the transilluminator, causing damage and affecting DNA quality and quantity(Gründemann & Schömig, 1996; Hartman, 1991; Pääbo, Irwin, & Wilson, 1990). DNA recovery from the agarose gel was maximized using both scalpel and glass capillaries, still it might have entailed irregular loss of DNA. The purification step from both *Nitrospira* 1st PCR products and comammox gel bands was performed with two commercial kits, both based on binding resins that might imply low recovery yields (Abraham, Miguel, Inocencio, & Blondy, 2017; Kurien & Scofield, 2002). Besides manual gel cutting techniques, both purification steps after the first and second step PCR should have affected equally each sample due to identical treatment following manufacturer's instructions. In consequence, relative abundance data should not be influenced by DNA purification steps.

Aside from methodical biases, ecophysiology of both NOB certainly influences its presence in the samples. While canonical *Nitrospira* identification in all samples was not a surprise considering its widespread existence in the natural environment (Holger Daims et al., 2016), *Nitrospira* populations of saline-alkaline environments remain understudied(Dimitry Y. Sorokin et al., 2014, 2015, 1998; Dimitry Yu Sorokin et al., 2004a; Dimitry Yu Sorokin & Kuenen, 2005). Surprisingly, the known alkaline nitrite oxidizers from the *Nitrobacter* genus (Dimitry Y. Sorokin et al., 2015, 1998) were undetected in this environment. Latest studies on *Nitrospira* comammox started to provide clues about its distribution in the environment, suggesting its adaptations and niche preferences(Kits et al., 2017; Palomo et al., 2017; Pjevac et al., 2017; van Kessel et al., 2015). Studies regarding metabolic potential, habitat and responses to environmental conditions are necessary. This study increases the knowledge of comammox ecology providing the first evidence as clade A being a stable member of the nitrifier community

in saline-alkaline systems. The sequences of comammox clade B PCR products confirmed the presence of these organisms, still they were detected stochastically in singular months and only in some of the biological replicates. Accordingly, clade B data discussion was hampered and further analysis and conclusions were dismissed.

The identity threshold for the OTU clustering was set at 95% as stated by Pester et al.,2014(Pester et al., 2014). The threshold applied determines the number of OTUs detected, defining the diversity present. It is crucial to discuss here the existence and implications of multiple paralogous *nxrB* copies in single *Nitrospira* genomes(Pester et al., 2014). In order to confirm the diversity levels observed, a second clustering with a 90% threshold could be computed.

Canonical Nitrospira nxrB sequences were grouped in 20 distinct OTUs widespread along the different lineages. However, the clustering of similar OTUs as monophyletic clades suggests that each OTU could represent one *nxrB* copy instead of a different *Nitrospira* organism(Pester et al., 2014). Monophyletic groups can alternatively be explained by being significantly different form the rest of sequences and closely related between them. This interpretation makes sense in the context of extreme systems where environmental conditions can strongly select for peculiar adaptations like unique enzymes and taxa (Kamekura, 1999; van den Burg, 2003; Russell H. Vreeland & Rosenzweig, 1999). Despite the diversity observed, two lineages (II and IV) encompassed most of the OTUs. Lineage II members have the broadest natural distribution, living in terrestrial, aquatic and engineered systems (Holger Daims et al., 2016). Its range of habitats include freshwater systems but not marine environments(Holger Daims, Nielsen, Nielsen, Schleifer, & Wagner, 2001). Alkaliphilic/alkalitolerant potential of some lineage II members, enabling its life in saline-alkaline lakes, is demonstrated in this study. The close phylogenetic relationship of some OTUs with the cultured species *Nitrospira moscoviensis* postulates that they may belong within this species. Lineage IV contains the same number of OTUs as lineage II. Since all known habitats for lineage IV organisms are marine(Holger Daims et al., 2016, 2001; Kruse et al., 2013; Watson, Bock, Valois, Waterbury, & Schlosser, 1986), its large presence in saline-alkaline lakes was probable. Living in marine environments requires acclimatization and maintenance of the osmotic equilibrium (Oren, 2008; Vargas et al., 2008; R H Vreeland, 1987; Russell H. Vreeland & Rosenzweig, 1999). The carrying of some of these adaptations in lineage IV *Nitrospira* cells likely provides an important advantage that enables their dominance in saline-alkaline lakes communities(Haaijer et al., 2013; Watson et al., 1986). The close phylogenetic relation of OTU_198 with *Nitrospira marina* sequences, enables the inference of its identity. Although the typical habitat for this species is considered the ocean, its presence in brackish systems was already observed (Kruse et al., 2013). Furthermore, this results are in agreement with the identified nitrifiers in the enrichment cultures, originating from lakes of the same saline-alkaline area. The nitrifiers identified in two enrichment cultures belonged exactly to these two *Nitrospira* lineages. In summary, predominance of these NOB in saline-alkaline environments is supported by the two approaches applied in his project.

Comammox *Nitrospira* clade A *amoA* sequences were classified within twelve OTUs assembled in two phylogenetic clusters and one individual branch, all spread throughout the tree. Both cluster I and II are depicted by long horizontal branches, representing notable sequence divergence with the rest of the sequences of the tree. As discussed for *Nitrospira nxrB* sequences, extreme conditions can enhance the rise of dissimilar features and strains(Kamekura, 1999; van den Burg, 2003; Russell H. Vreeland & Rosenzweig, 1999). The recent discovery of

comammox organisms and the limited efficient detection methods restricts the amount of environments samples and in consequence the volume of available sequences(Holger Daims et al., 2015; Pjevac et al., 2017; van Kessel et al., 2015). This study is the first one pursuing and accomplishing the detection of comammox in saline-alkaline systems. OTU 22 branches between water treatment plant *Nitrospira* sequences. Also, cluster II forms a monophyletic clade including engineered sequences from activated sludge. Further information about the conditions defining these two environments is needed to understand why their sequences cluster together. Since no reconstructed OTU branches close to any of the three *Candidatus* species, no accurate identification could be inferred. The clear distinction of two main clusters could reflect the environment impact on the specie's phylogeny, causing the differentiation into possible ecotypes. The influence of environmental conditions on the phylogeny and ecology of some microbial groups has been clearly demonstrated (Biller, Berube, Lindell, & Chisholm, 2014; Gruber-Dorninger et al., 2015).

Even though **Comammox clade B** detection was stochastic and irregular, phylogenetic analysis validated the presence of these microorganisms in the studied saline-alkaline lakes. Four of the reconstructed OTUs branched individually without any clustering and displayed considerable sequence divergence from the rest. The other four taxa formed two different adjacent clades. Except from one individual taxon (OTU 139) that clustered with sequences from engineered systems, the majority of obtained sequences fell close to soil or freshwater originated samples. Soil systems and particularly forest soil is the known environment where comammox clade B usually dominates over clade A organisms (Pjevac et al., 2017). The preference of comammox clade B for forest soils, often characterized by low pH(Nugroho, Röling, Laverman, & Verhoef, 2007; Rousk, Brookes, & Baath, 2009), would be in agreement with the stochastic, unstable presence detected in saline-alkaline lakes in this study.

The detection of comammox is unavoidable when applying *nxrB*-specific primers in PCR-based methods. In consequence, some of the identified *Nitrospira nxrB* OTUs were indeed comammox *Nitrospira.* However, is not possible nowadays to differentiated whether yet uncharacterized *Nitrospira* bacteria are comammox or strict NOB since *16S rRNA* or *nxrB*-based phylogenetic studies cannot differentiate between the two groups. Comammox sequences intersperse with strict NOB and don not form a monophyletic clade in *16S* or *nxrB*-based phylogenetic trees(Pjevac et al., 2017). Further studies should include correlation analysis of the *nxrB* with the *amoA* OTUs, illustrating possibly the same trends for sequences belonging to the same comammox organism.

Microbial community structure and responses to the abiotic context.

Coexistence of NOB populations in natural and engineered systems involving niche partitioning and spatial and temporal succession events have already been demonstrated (M. Alawi et al., 2009; Holger Daims et al., 2016; Hüpeden et al., 2016; Lucker et al., 2015). Additionally, fine scale specialization is also known within the *Nitrospira* genus, illustrated by a high diversity of coexisting *Nitrospira* (Gruber-Dorninger et al., 2015; Kruse et al., 2013; Maixner et al., 2006). Niche differentiation, of uncultured microbes playing key roles in essential biogeochemical processes like the nitrogen cycle, is understudied(Maixner et al., 2006). This project contributes to elucidate the ecophysiological flexibility and the abiotic and biotic factors influencing *Nitrospira* and comammox *Nitrospira* population structure and dynamics.

Canonical *Nitrospira*. Despite the existence of a complex *Nitrospira* population encompassing different ecophysiological potentials, no apparent temporal succession could be inferred. Competition and niche differentiation shape microbial communities at different resolutions. Fluctuations of environmental parameters, like nitrite concentration, have been shown to select for the best adapted organisms resulting in spatial and temporal patterns (Kruse et al., 2013; Maixner et al., 2006). The fluctuating conditions recorded in the two saline-alkaline lakes were expected to generate a temporal succession between different *Nitrospira* lineages and/or phylotypes.

The log transformed data depicted in the heat maps allowed the observation of large and subtler differences in relative abundance. Both analyzed lakes presented similar rank-abundance curves, defined by a predominance of a few OTUs over the rest during the whole year. OTU 2 from *Nitrospira* lineage IV was the top abundant one in HS and within the dominant taxa in the US. As already discussed above, lineage IV members are acclimatized to marine environments and provided with adaptations that might facilitate their dominance in saline-alkaline lakes (Haaijer et al., 2013; Watson et al., 1986). The US community encompasses also OTU 4 from an unknown lineage within the highest abundant ones. The rest of the sequences placed within this unclassified group in the phylogenetic tree were obtained from Namibian soil samples(Pester et al., 2014). No information is available about members of this lineage, which would allow to place the detection of these organisms in saline-alkaline systems in context. The remaining OTUs, which were stable members of the community with notable abundances, belong to the two already listed lineages and lineage II, characterized by a widespread distribution (Holger Daims et al., 2016).

The community structure observed in both lakes resembled the typical rank-abundance curve illustrated by few dominant species followed by a long tail of low relative abundance taxa known as the rare biosphere (Lynch & Neufeld, 2015). Rare members, defined by low abundance and extensive sequence diversity, assemble a genetic and functional reservoir that contributes to community ecology and resilience capacity (Lynch & Neufeld, 2015). The determination of taxa as dominant or rare community members should consider the following three biases: I) Relative abundance results may be influenced by different numbers of *nxrB* copies in individual *Nitrospira* cells (Pester et al., 2014). Therefore, an OTU population could be incorrectly presented as highly abundant due to multiple *nxrB* copies contained in the cell. On the other hand, the assessment of phylotype diversity could be skewed due to the presence of several *nxrB* copies with an identity of less than the 95% threshold which was applied for OTU clustering in a single cell. II) Unrecognized sequencing errors can cause the generation of inexistent rare OTUs (Huse, Welch, Morrison, & Sogin, 2010; Lynch & Neufeld, 2015) III). Sequencing of extracellular DNA from dead cells is usually detected and assembled as rare OTUs too (Lynch & Neufeld, 2015).

The low-abundant OTUs detected were expected to behave as conditionally rare taxa, increasing in abundance under some conditions and establishing a succession event. The trend observed did not match the hypothesis proposed. Several explanations can be raised. Simply, the desired conditions might not have occurred during the monitoring time or the rare taxa were just inactive, unable to grow and revive. Other reasons could be based on the already postulated overestimation of rare taxa, affecting the illustrated dynamics and masking differences in relative abundance between real taxa (Huse et al., 2010; Lynch & Neufeld, 2015). We observed a stable community dominated by the best adapted taxa during the whole year. Possibly, the extreme conditions, that define the studied system, could have diminished the effect of the

environmental fluctuations and selected a few resilient taxa able to thrive under such conditions. However, it needs to be considered that temperature, salinity and pH data were recorded from the lake water, but samples for DNA extraction were taken from the first layer of sediment. In consequence, the abiotic data recorded might not be directly applicable, since the sediment could buffer the recorded fluctuations and exhibit different abiotic conditions(Atkinson, Jolley, & Simpson, 2007; Ben-Yaakov, 1973). The low correlation between inorganic nitrogen concentration from the water column and from the sediments supports the presence of divergent abiotic conditions in the sediment layer. Even if the conditions in the water were maintained in the sediment, presence of micro-niches with other abiotic gradients and conditions might define the community composition at a smaller scale(Bertics & Ziebis, 2009; Paerl & Pinckney, 1996). Furthermore, stronger fluctuations that significantly affect the structure and enhance succession of taxa could occur in longer or shorter time periods not detected with the approach applied (Brussaard et al., 2016; Pickett, 1976; Zhou et al., 2014). The last argument discussed refers to the resolution chosen when studying microbial taxa, proposing the occurrence of the expected succession but in another scale. Niche partitioning leading to spatial and temporal succession between different nitrite oxidizers was discarded since no other NOB presence could be confirmed in the screening PCRs. However, since the analysis of micro-diversity was not in the objectives of the project, its importance was understudied. Phylogenetically close organisms can differ in the genetic and functional potential enabling the occupation of different niches(Biller et al., 2014; Jaspers & Overmann, 2004; Nelson, Maezato, Wu, Romine, & Lindemann, 2016). Such micro-diversity and niche partitioning between close related strains has been already demonstrated for a diversity of microbes including *Nitrospira*(Gruber-Dorninger et al., 2015).

The accurate analysis of the community composition inferred a temporal pattern for at least some particular OTUs. In HS, a gradual increase of OUT 13 was observed within the course of the months. OTU 12 displayed the same pattern in the US. This temporal pattern could be dependent on a progressive change of biotic or abiotic parameters. Both taxa correlated significantly towards and against salinity, respectively. A gradual stabilization of the salinity levels in both lakes from July 2014 on displayed in FIGURE 4 might have allowed the recorded increase in relative abundance. The rare OTUs only detected sporadically were hypothesized to not really respond to the environmental parameters. The low or undetectable abundance could reflect an external origin, different from the saline-alkaline lakes. Immigration events from neighboring environments, such as groundwater aquifers, lake surroundings or the atmosphere would justify their occasional presence in the community(Ford & Harvey, 2007; Kellogg & Griffin, 2006; Lynch & Neufeld, 2015). The arrival to the extreme habitat would also inhibit their growth and ecological success, causing extinction and consequent absence until new dispersal events (Pointing et al., 2009; Wall & Virginia, 1999). The immigration – extinction hypothesis conforms with the rank-abundance curve dynamics that was observed (Lynch & Neufeld, 2015).

Two distinct *Nitrospira* communities were present in each lake, as suggested by the heat map and ordination analysis. The differences in relative abundance responsible for this result were higher abundances of OTU 8, 47, 5, 171, 13 and 196 for the HS and OTU 12, 19, 4, 97, 373 and 142 in the US. Some of these OTUs were top taxa identified in the heat maps. Such different community composition may reflect the presence of distinct niches, that select for particular taxa (Dumbrell, Nelson, Helgason, Dytham, & Fitter, 2009; Fraser, Alm, Polz, Spratt, & Hanage, 2009). Accordingly, the distinct abiotic context of each lake actively affected the dissimilarities

observed. Analysis of variance identified salinity as an important descriptor for *Nitrospira* community structure as well. Moreover, Anova analysis stated strong significant differences in the salinity content between the two lakes. Specifically, the HS showed higher salinity levels than the US all year long. Constrained ordination analysis to salinity demonstrated its significant impact, specifically explaining 32.38% of total recorded variation, and showed a similar structure as the one found by the lake-constrained ordination. Clear divergent patterns could be observed, where the HS samples clustered in a group towards the higher end of the salinity gradient and the US representatives grouped in the opposite direction. This analysis confirmed salinity as an important selective factor that partially explains the assembly of singular communities (Oren, 2008; Vargas et al., 2008). OTU 13, 171, 5, 47 and 36 had a positive relation with salinity explaining their higher abundance in HS as shown in the lake-constrained ordination. Surprisingly, only OTU 36 belongs to lineage IV, the typical marine lineage. The positive relation between salinity and OTU 5 and 13 confirms the existence of haloalkaliphilic or haloalkalotolerant taxa within lineage II, postulating brackish saline environments as another potential habitat. The taxa displaying a negative correlation with salinity (OTU 12, 19, 4) were partially the ones with significantly higher relative abundance in US, likely due to the lower salinity levels maintained throughout the year. The negative correlation with salinity was unexpected for OUT 19 considering its affiliation to lineage IV, whose only known habitats are marine ecosystems. Despite temperature showed no significant influence on the community structure, correlation tests illustrated positive relation between salinity and temperature. Therefore, the impact of salinity on the community variation could partially depend on temperature fluctuations. High temperatures affect multiple abiotic factors, such as low water level causing accumulation of ions and increasing the salinity content(Boros, Horváth, Wolfram, & Vörös, 2014; Krachler, Korner, & Kirschner, 2012).

However, salinity only explained the 32.38% of the variation, indicating the existence of other factors and gradients inducing community patterns. The sediment concentration of the three inorganic nitrogen forms measured also showed significant influence on the community composition. Despite the minor individual explanatory potential, the total explained variation accounted for a 28.41% of the total observed variation. The Spearman's rank correlation test demonstrated the positive correlation between the three inorganic nitrogen forms. In consequence, the study of each nitrogen form individual effect is hampered and the discussion describes inorganic nitrogen as one main descriptor of the community variation. However, since nitrite is the main substrate for NOB and its role in niche partitioning of nitrifying communities has been already demonstrated(H. Daims et al., 2006; Kim & Kim, 2006; Nogueira & Melo, 2006), nitrite concentration is inferred as the main N form influencing the community composition. Accordingly, the differences in nitrogen content were expected as community descriptors but more pronounced than observed. Although the identification of the samples regarding their origin also resembled the two different lake communities (see FIGURES S4-S6 in Annex I), the division was less strict due to the lower impact of the constrains on the community variation. Nevertheless, statistically significant differences between the two lakes regarding ammonium, nitrate and nitrite concentrations in the sediment and nitrite concentration from the water column were confirmed. Clustering of most of the HS samples in the same direction of the inorganic N gradients postulated a higher concentration of inorganic nitrogen in the sediment of the HS. According to the constrained ordination analysis OTU 47, 171, 5 and 2 tended to correlate with the *Nitrospira* community samples from HS, suggesting a preference for higher N

concentrations. On the other hand, OTU 4, 12 and 19 tended to correlate with the ordination of the US communities, the lake with lower ammonia, nitrate and nitrite concentrations. However, none of the OTUs vectors clearly correlated with or against the different nitrogen gradients. In consequence, the low explanatory potential and the uncertain correlation only confirms a subtle role of nitrite and nitrogen forms' in shaping NOB communities. Finally, other undetected factors accounting for the remaining observed variance must influence the differentiation of the two communities. Further analysis of the system should include the study of additional elements like oxygen concentration and/or presence of key organisms and their interplay in saline-alkaline lakes.

HS and US were also studied independently in order to disclose the patterns observed within each lake. The results obtained did not answer the questions raised by the relative abundance analysis. The only detected strong descriptor of the two communities was the month. None of the other abiotic factors had considerable effects that could help explain the patterns described in the HS. The US community was however significantly affected by five other minor descriptors. Salinity analysis of the US lake confirmed the patterns observed with the two data sets together, with the OTUs 36 and 2 displaying a higher relative abundance at higher salinity also in the US. The other four factors were the different inorganic N forms from the sediment and the water column. Although no clear results were obtained when analyzing both lakes together, the US lake alone displayed differences in N concentration between months that contributed in differentiating the *Nitrospira* communities present each month. In addition, in US OTUs 114 and 2 showed inclinations towards high nitrogen concentration and OTU 12 suggested again a preference for lower nitrite and nitrate amounts. In conclusion, the measured inorganic nitrogen forms were found to be a minor factor contributing to the monthly *Nitrospira* community variation in US. The overall results support the above mentioned theory of an underestimation of the biotic context and/or an inefficient measurement of the abiotic factors.

***Nitrospira* comammox clade A.** The recent discovery of organisms performing complete nitrification has increased the complexity of nitrification (Holger Daims et al., 2015; van Kessel et al., 2015). Nowadays, just hypotheses, theories and little confirmed information is available regarding comammox community ecology (Kits et al., 2017; Palomo et al., 2017; Pjevac et al., 2017; van Kessel et al., 2015). In addition, since it was discovered in engineered systems, most of the studies are focused on these environments and less on natural habitats. In consequence, the discussion, comparison and corroboration of the results is hindered by a shortage of information.

The number of different OTUs reconstructed should be discussed carefully, since the applied threshold in the computational data analysis is not yet confirmed for comammox *Nitrospira amoA* sequences. The utilization of a 95% minimum identity threshold is more conservative than suggested by previous studies(Pjevac et al., 2017), in order to avoid overestimation of the diversity. The detection of a few OTUs in most of the months suggests comammox as a stable member of the nitrifier community. The observed persistence defines *Nitrospira* comammox as a resilient population adapted to salinity and alkaline pH. Detailed genomic studies of singular phylotypes and analysis of the pangenome should disclose adaptations and mechanisms for resilience (Biller et al., 2014; Palomo et al., 2017). Differences in dominating OTUs between the two lakes also shows the response of singular phylotypes to abiotic and/or biotic conditions presented by each lake system.

The community structure was composed by a few OTUs dominating over the rest. The rare, low abundant, community and its implications is difficult to interpret since multiple biases, described above, are taking place. Consequently, the rare biosphere influence on community structure and dynamics is not further discussed. Nonetheless, particular results were observed for each lake community. The abundant taxa dominating in each lake belong to different phylogenetic clades, demonstrating a selective effect of the lake on the phylotypes abundances. OTU 2 from clade I was the dominant taxa in HS with the highest relative abundance in the majority of months. In the US, OTU 3 and OUT 464, both from clade II had the highest relative abundances throughout the year. These results provide the first evidence that phylogenetically distant comammox taxa may also behave ecologically different. The two lakes, significantly differing in some abiotic conditions like salinity and several inorganic N compounds, see TABLE 5, might provide distinct niches occupied by particular phylotypes. Niche partitioning would demonstrate ecophysiological versatility in *Nitrospira* comammox organisms, a feature already established for canonical *Nitrospira* (Gruber-Dorninger et al., 2015; Koch et al., 2015; Koch, Galushko, Albertsen, Schintlmeister, Gruber-Dorninger, et al., 2014; Palatinszky et al., 2015). Since OTU 2 had a considerable relative abundance not only in HS, but also in US, the overall dominance of the three mentioned taxa can be confirmed in these specific saline-alkaline environments.

Dominance of singular OTUs, peaks in abundance in specific months or varying temporal abundance can be explained by connecting biological adaptations with abiotic parameters and their fluctuations. The structure of the comammox *Nitrospira* communities was strongly defined by the factor lake. This may be explained by the significantly different salinity between the two lakes, clearly affecting each lake's community composition. Again, demonstrated correlation between salinity and temperature should be taken into account since salinity explanatory potential could be partly dependent on temperature. Besides salinity, inorganic N concentration also influenced the comammox population structure, as shown for canonical *Nitrospira* and other NOB communities (H. Daims et al., 2006; Gruber-Dorninger et al., 2015; Kim & Kim, 2006; Nogueira & Melo, 2006; Nowka, Off, Daims, & Spieck, 2015; Princic et al., 1998), but with lower impact. As described in canonical *Nitrospira,* positive correlation between the three inorganic nitrogen forms influences their impact on the community variation and suggests its discussion as one main descriptor. Significant different inorganic N concentrations between the two lakes were demonstrated. The two following samples, U3_1 and H6_2, clustered in all ordination analysis within the group of the other lake, indicating similar composition and/or biotic and abiotic context to the opposite lake. The OTUs significantly responsible for the differences in community structure were identified. Two taxa (OTU 3 and 464) had a significantly higher relative abundance in US. The opposite direction of their vectors regarding the salinity gradient, postulates antagonism against salinity as the main factor determining their abundances in the lakes. On the other side, five other taxa (OTU 2, 5, 7, 9 and 16) were relatively more abundant in HS and tended to positively correlate with salinity. Besides OTU 7, the taxa significantly discriminating the two lakes also belong to different phylogenetic clades. Accordingly, this study postulates that different phylotypes could be characterized by different environmental adaptations, for example towards salinity, demonstrating a niche partitioning and the existence of comammox ecotypes as described for canonical *Nitrospir*a (Gruber-Dorninger et al., 2015).

Aside from salinity, no other significant abiotic factor correlated clearly with any of the plotted OTUs. The absence of clear results hindered the discovery of other preferences and/or

niche conditions for each phylotype that would have helped unraveling the reasons behind the different communities. We cannot answer why certain phylotypes predominate since no other parameter besides salinity for the OTU 3 and 464, showed clear influence on the taxa. These results evinced the existence of other important factors defining the niches available and the population structure as discussed above.

Individual analysis of HS and US communities had the objective of unraveling the patterns and dynamics observed in each lake. However, no abiotic factor significantly influenced the HS community structure. Missing data from some months could have affected the ordination analysis. The absence of the samples from some months in the library preparation could be due to technical biases, already referred to in chapter 1.2, or real biological absence or presence under the detection limit. Still, the most feasible explanation is the oversight of the main abiotic or biotic descriptors of the community and the presence of micro-niches (Dumbrell et al., 2009; Fraser et al., 2009; Zhou et al., 2014). In addition, the existence of an unexplored succession of taxa dependent on environmental parameters could have occurred in shorter or longer time periods (Pickett, 1976; Zhou et al., 2014). On the contrary, the US community was affected by several parameters. The most important one was the month, suggesting the existence of monthly fluctuations that strongly affected the niches available and the community composition in response (Dumbrell et al., 2009; Fraser et al., 2009). Month-constrained ordination suggested the existence of two different sub communities. Salinity and sediment nitrate and nitrite concentrations were also detected as significant descriptor of US community variation but with a much weaker potential. These results indicated that the different salinity, nitrate and nitrite concentrations between months contributed to shaping the communities Within the *Nitrospira* comammox communities, several OTUs showed a preference for lower (OTU 3 and 464) and higher salinity (OTU 16). Furthermore, OTU 16 correlated positively with nitrate and nitrite concentrations. The opposite trend was found for OTU 3 and 464. Thereby, we have confirming results for the theory that nitrite but also nitrate concentrations define comammox niches(Kits et al., 2017; Palomo et al., 2017) and the differences between phylotypes.

The overall analysis proposes different comammox ecotypes adapted to different conditions. Clade I represents comammox ecotypes adapted to brackish environments and higher inorganic N concentrations, dominating in the HS. On the other side, clade II encompasses ecotypes with the known adaptations to low nitrite concentrations and oligotrophic environments (Kits et al., 2017; Palomo et al., 2017) plus an antagonism towards salinity that causes their success in the US. In further studies the measurement of other abiotic factors known to influence comammox presence, like oxygen concentration (Palomo et al., 2017), should be included. Regarding the importance of micro-niches in microbial ecology (Fraser, Alm, Polz, Spratt, & Hanage, 2009; Paerl & Pinckney, 1996), their detection and analysis should be performed in future analyses to further characterize Nitrospira comammox ecotypes and their dynamics.

A common drawback of the seasonal approach was the only targeting of the community DNA. In consequence, activity shifts between *Nitrospira* OTUs and comammox taxa that might have defined the expected temporal succession could not be detected. Different temporal patterns in gene expression between phylotypes has been observed for other groups and genes related to the nitrogen cycle(Church, Short, Jenkins, Karl, & Zehr, 2005).

4.2 OPTIMIZATION, PURIFICATION AND STUDY OF THREE NOB ENRICHMENT CULTURES

4.2.1 *Nitrospira* lineage IV enrichment ("KS").

We proposed as objective for the KS culture the optimization of the culturing conditions since it displayed low and unstable activity. The nitrification reaction was performed by members of the genus *Nitrospira*, lineage IV. The confirmation of the identity was obtained with a *Nitrospira nxrB*-specific PCR, that amplified one single *nxrB* type. The BLASTn alignment tool identified the sequence as *Nitrospira marina*-related with a 94% *nxrB* similarity. The 16S rRNA alignment also revealed *Nitrospira marina* as the closest relative with a 98% sequence similarity. The accepted threshold for *Nitrospira nxrB*, set at 95% sequence identity(Pester et al., 2014), and the new propose cut-off species threshold at 98.7% in 16S rRNA identity(Konstantinidis, Rosselló-Móra, & Amann, 2017; Whitman & Keswani, 2001) are the main indicators suggesting a new *Nitrospira* species. Phylogeny information was complemented with the alignment of the KS nitrifier *nxrB* with the *nxrB* dataset obtained with the seasonal study. The 99% *nxrB* sequence similarity between the N.*marina*-related strain and OUT 2, postulated their belonging to a new species. Phylogenetic tree depicted OUT 2 with an individual branch without any clear clustering within *Nitrospira marina* or *Nitrospira ecomares* clades. Additionally, the KS growth on alkaline pH and the unique haloalkalotolerant adaptations described in the reconstructed genome (Anne Daebeler, manuscript in preparation) constitute new phenotypic properties that contribute in the proposal of a new species.

The presence of *Nitrospira* lineage IV members coheres in brackish environments, since all lineage IV typical habitats are marine(Holger Daims et al., 2016, 2001; Kruse et al., 2013; Watson et al., 1986). In addition, the seasonal culture-independent study also confirms the presence and predominance of lineage IV members through all the 14 months analyzed. The two approaches present salinity as an important environmental selective factor. Nitrospira lineage IV members, as *Nitrospira marina*, are known to have specific adaptations for salt tolerance, that enable the maintenance of the osmotic balance(Haaijer et al., 2013; Watson et al., 1986). The nitrifier cultured here also has some of such adaptations and other unique ones (Anne Daebeler, manuscript in preparation) that possibly enable its success in these saline-alkaline lakes. These results contribute to the postulation of the original environment and conditions as strong influences on the community composition, defining the niches available and selecting the best adapted microorganisms (Carini & Joye, 2008; Kraemer & Boynton, 2017; Smith, Mosier, & Francis, 2015).

Despite the culturing of the enrichment in slightly saline medium, the nitrifying activity was unstable, reflected by feeding rates of 1 mM nitrite per month. Therefore, it was assumed that the enriching conditions were perhaps not optimal. One possible argument causing the unstable activity could be that the nitrite supplied exceeded the optimal concentration for the present nitrifier(Kim & Kim, 2006; Nowka, Daims, et al., 2015). Members of the *Nitrospira* genus are the most widespread NOB, present in multiple and diverse environments (Holger Daims et al., 2016). Their extensive success is not fully understood, but their adaption to low nitrite concentrations could play a major role(Maixner et al., 2006). Actually, the demonstration of a high affinity for nitrite, from nanomolar concentrations until below detection limit amounts, coheres with *Nitrospira* behavior as a K-strategist thriving in oligotrophic environments(Holger

Daims et al., 2016; Nowka, Daims, et al., 2015; Schramm, de Beer, van den Heuvel, Ottengraf, & Amann, 1999). In the line of this theory, the feeding amount was decreased from 1 to 0.5 mM nitrite.

In addition, a low enrichment level also diminishes nitrification activity. Possibly, low abundance of *Nitrospira* members caused the low nitrite oxidation rates. Indeed, FISH analysis demonstrated their presence as a minority group, only present in big flocks as relatively small agglomerated clusters in earlier enrichments. By optimizing the culturing conditions and promoting the growth of *Nitrospira* lineage IV organisms, we hoped to also increase the level of enrichment.

Adjusting the preferred conditions for culturing microorganisms is an essential part in an enrichment process. The original habitat is specifically and notably defined by an alkaline pH, suggesting an alkaliphilic or alkalitolerant lifestyle of the present organisms. In consequence, we started to control and adjust the pH to 8.5 - 9.5 regularly. The influence of pH on nitrification has already been studied and demonstrated(Hüpeden et al., 2016; Princic et al., 1998; Dimitry Y. Sorokin et al., 2014, 2015). The first studies already suggested pH as a selective environmental force causing changes in the community structure(Princic et al., 1998). The importance of adjusting the preferred pH for culturing nitrifiers stated in this study agrees with the demonstrated role of pH in defining the ecological niches of NOB(Hüpeden et al., 2016). Sorokin et al, 2014 and 2015 postulated *Nitrobacter alkalicus* as the solely nitrite oxidizer present in soda lakes because of the selective potential of alkaline pH(Dimitry Y. Sorokin et al., 2014, 2015). Therefore, this project is the first one postulating other nitrite oxidizers as specific *Nitrospira* strains as the main NOB in saline-alkaline lakes. This statement is endorsed by the enrichments obtained but also by the seasonal culture-independent study. The corrected feeding rates together with the pH adjustment enabled higher nitrifying activity, that enabled weekly addition of nitrite to the culture. Nonetheless, the higher activity did not correspond with an increase of the enrichment level or the structure of the culture. *Nitrospira* continued to be present only in big flocks as a minority population. This observed fact rejects the theory that biomass restriction was the cause of low nitrite oxidation. Although, the amount of nitrite fed and the pH cannot be confirmed as the main problem hampering further enrichment, an improvement of the culturing conditions was achieved.

The FISH analysis enabled the observation of the community's spatial structure. The majority of the biomass was grouped in huge flocks encompassing multiple morphologies and clusters. Since the flocks impede the enriching by dilution series, new strategies were attempted. From the genome it was known that the strain contained specific genes possibly enabling adaptions to elevated salt concentrations and pH (Anne Daebeler, oral communication). Therefore, new media were designed with four times and eight times more salinity than the usual cultivation medium and inoculated with a KS culture sample. However, presence of nitrite and absence of nitrate demonstrated inexistent nitrifying activity after four months of incubation. Several arguments must be considered for explaining the failed results. Two main strategies are described for maintaining the osmotic equilibrium(Oren, 2008; Vargas et al., 2008; R H Vreeland, 1987). The first one involves the internal accumulation of KCl, implying an adaptation of cellular enzymes and internal machinery. The second system is based on the expulsion of salt from the cytosol and the accumulation of organic/compatible solutes such as betaine or choline without impeding the enzymatic reactions. Some cells are also able to synthesize itself some compatible solutes(Oren, 2008; Vargas et al., 2008). The absence of the

all the required adaptations could cause the fail of both systems. The lack of organic solutes in the media could also explain why no nitrification activity could be observed. In addition, *Nitrospira* members displayed already a slow growth and activity in the original enrichment culture. Hence, the absence of nitrification activity might be influenced by a slow adaptation process that impede *Nitrospira* cells to react to the new media even within four months. Considering the low nitrifier biomass detected in the culture and its presence in only big flocks, we need to recognize also the possibility that insufficient biomass was transferred as inoculum to the new media.

The culture-independent approach detected lineage IV *Nitrospira* in both lakes through all the year as dominant compared to other *Nitrospira*. Despite both lakes are described to lie within the hyposaline range (3-20 g/l), the Herrnsee (HS) reached salinity amounts close to 8 g/l. The predominance of lineage IV *Nitrospira* through all the months demonstrates the ability of some taxa to tolerate and actually thrive in four time more salinity conditions than the medium used for enrichment cultivation. The alignment of the N. *marina*-related *nxrB* with the *nxrB* dataset from the seasonal study revealed a 99% similarity between the KS nitrifier *nxrB* and the OTU 2, suggesting their classification within the same species. Actually, OTU 2 was the predominant one in the HS lake all year long and was present in the US within the top abundant taxa throughout all sampled period. Accordingly, the ability of OTU 2 to thrive in higher salt contents is demonstrated and suggest that the KS nitrifier also has the capacity to grow under the conditions tested.

In summary the aim proposed for this enrichment was fulfilled partially. Optimization of the culturing conditions was accomplished, manifested by higher nitrifying activity. Still, no increase in the enrichment level corresponded with the improved conditions and the higher activity. Further isolation approaches, targeting the nitrifying population should be used. In order to capture the flocks containing *Nitrospira* clusters, filtration could be applied as described for the LL culture. Another procedure based on sonication would allow the disruption of flocks, generating micro-colonies of nitrifying cells that could be sorted and isolated from the rest of the culture(Fujitani, Kumagai, Ushiki, Momiuchi, & Tsuneda, 2015; Fujitani, Ushiki, Tsuneda, & Aoi, 2014; Ushiki, Fujitani, Aoi, & Tsuneda, 2013). Instead of randomized cell sorting, micromanipulation or Raman-activated sorting techniques follow the same idea by selecting individual target cells(Li, 2012; Raghunathan et al., 2005; Y. Song, Yin, & Huang, 2016). Furthermore, since the reconstructed genome is available the detailed analysis of its genetic potential, adaptations and/or resistant genes could be used in the design of new, more selective media.

4.2.2 *Nitrospira* lineage II enrichment ("MixS").

The main objective presented for the MixS enrichment was the obtaining of a pure culture of alkalitolerant *Nitrospira* lineage II. Phylogenetic analysis illustrated its affiliation within lineage II, branching between *Nitrospira moscoviensis* and *Nitrospira japonica*. General 16S rRNA PCR and BLASTn alignment of the nitrifier 16S sequence showed a 98% 16S sequence identity to *Nitrospira moscoviensis*.

The culture already showed stable activity with weekly turnover of substrate. The planktonic growth and the identity of the nitrifier was confirmed by FISH. Although some members of *Nitrospira* lineages are proposed to be specialized and adapted to certain habitats,

lineage II encompasses nitrite oxidizers able to succeed in multiple environments(Holger Daims et al., 2016, 2001; Koch et al., 2015). Lineage II includes the cultured species *Nitrospira moscoviensis* but mostly uncultured bacteria originating from a diversity of systems such as bioreactors, soil, geothermal habitats and freshwater systems, but not marine ecosystems(Holger Daims et al., 2016, 2001). Their widespread distribution is explained by different adaptations and ecophysiological traits that enable their ecological success(Holger Daims et al., 2001; Koch et al., 2015). However, up to now saline-alkaline environments were not listed and a alkaliphilic/alkalitolerant potential for *Nitrospira* was not known. The presence of *Nitrospira* lineage II taxa in high relative abundance in these systems was confirmed by the culture-independent seasonal study conducted also in the course of this master thesis.

Due to the planktonic growth observed, several dilution series were performed to increase the enrichment level. The use of this technique enabled the dilution of superior faster (heterotrophic) growers and the enrichment of the *Nitrospira* population, which may have been growing slower (Jackson, Roden, & Churchill, 1998). Accordingly, the contaminants decreased in abundance and the *Nitrospira* cells became the most abundant group detected. Despite this initial success, further dilutions series did not enable the obtaining of a pure culture due to some technical or/and biological limitations. The random sampling by pipetting could include contaminants already in the inoculum for the dilution series. The presence of contaminants in the first dilution compromises the rest of the series, diluting the contaminants but also the nitrifying members. Additionally, the selection of a flock with both populations could lead to the undiluted presence of contaminants observed in the last dilution series due to the contaminant's higher growth rates s(Jackson et al., 1998).

Regardless of the unsuccessful dilutions attempts, the enrichment level was estimated between a 70-90% dependent on the sample and on the field of the microscope. FISH analysis could only distinguish between two morphologies, one of them a rod-shaped *Nitrospira* and the other a second rod-shaped contaminant. No spiral-shape cell was detected by the FISH signals. Historical classification of nitrite oxidizers genera was based on cell morphology and intracytoplasmic membrane structure(Spieck & Bock, 2005). *Nitrospira* cells were characterized by a spiral shape and inexistent intracytoplasmic membranes(Spieck & Bock, 2005). The isolation in pure culture of *Nitrospira* species and *in situ* detection analysis allowed a more concise study of their morphology, confirming that the most common morphology was the spiral(Ehrich, Behrens, Lebedeva, Ludwig, & Bock, 1995; Lebedeva, Alawi, Jozsa, Daims, & Spieck, 2008; Watson et al., 1986). Since lineage II cultured representative *Nitrospira moscoviensis* is still defined by the spiral shape, the observation in this study of rod *Nitrospira* lineage II cells was unexpected(Ehrich et al., 1995). However, cell shape variations during a life cycle and morphological variety in planktonic cells has already been demonstrated in *Nitrospira bockiana* (lineage V), whose cell shape ranges from spirals, slightly curve cells, coccoid members and even straight rods(Lebedeva et al., 2008). In order to confirm the morphology inferred through FISH studies, samples of a high enriched MixS culture were observed by Scanning Electron Microscopy. Again, only rod cells were detected with high resolution in an enrichment containing *Nitrospira* lineage II as the majority group according to prior FISH analysis, supporting a new morphology within *Nitrospira* lineage II. Nonetheless, two distinct groups of rod organisms could be differentiated regarding their length and width. The suggested morphology for the nitrifying cells is characterized by a shorter and thicker shape than the contaminant. The reason/s below for the adoption of a new morphology can be discussed but not confirmed with the current

information. Besides cellular cycle influence, as described for *Nitrospira bockiana*, other physical, environmental and biological factors strongly impact cell morphology(Lebedeva et al., 2008; Yang, Blair, & Salama, 2016; Young, 2006). Since the habitat conditions affect most of the parameters selecting for a morphology, such as nutrient access, attachment surfaces, dispersion and mobility, the extreme saline-alkaline environment of the original habitat may play a role in influencing cell morphology(Yang et al., 2016; Young, 2006). The discussion of the novel characteristic should also question if the morphology adopted in the culture is also the adopted one in the natural habitat. Cultured organisms experience a dramatic and inevitable change of conditions, impeding the direct assumption that the behavior and characteristics observed in the culture are the same ones occurring in the natural ecosystem(Eydallin, Ryall, Maharjan, & Ferenci, 2014; Seckbach, n.d.). The last point included in this discussion is based on the possibility that we could exclusively be observing the contaminant with the SEM. The discernment between a rod or a spiral-shaped cell with simply FISH images is complicated and dubious. The observation of only contaminant rod organisms with the electron microscope is thinkable as a result of a failed sample preparation. The slide was directly placed on an actively growing culture in order to allow the cells to attach independently to the glass. If the contaminant had faster attaching and growing rates or the nitrifying cells could not even attach and grow on the surface provided, the images would only display the contaminant organism. Therefore, other sample preparations protocols that directly spread the culture on the slide should be applied. However, in order to fully confirm the new morphology of *Nitrospira* lineage II, isolation in pure culture is necessary.

The most widely used assay for taxonomic identification is the sequencing of the 16S rRNA and its alignment to an extensive database(Chan, Halachev, Loman, Constantinidou, & Pallen, 2012). However, the application of a polyphasic approach defines a species as “a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property”(Rosselló-Mora & Amann, 2001). Despite the traditional applied species threshold of 97% 16S rRNA similarity, recent investigations propose a cut-off value of 98.7% 16S rRNA identity(Konstantinidis et al., 2017; Whitman & Keswani, 2001). In agreement with the polyphasic approach, three main arguments are used in this study to set up and define the proposal of a new species. Firstly, the demonstration of an entirely different habitat characterized by saline-alkaline conditions, differing from the neutral pH and stable 37ºC temperature preferred by N.*moscoviensis*(Ehrich et al., 1995). The second reason is based on 16S ribosomal RNA identity with N.*moscoviensis.* Considering the latest species definition, a 98% sequence identity infers that the *Nitrospira* organism belongs to a new species. Finally, the new morphology suggested, unnoticed for the rest of *Nitrospira* lineage II organisms, is postulated as the third evidence.

The last approach used to isolate the nitrifying cells was sterile flow cytometry single cell sorting. The first results obtained pointed at an initial success. Due to the high enrichment level, most of the sorted wells from both vitamin-amended and not vitamin-amended plates showed nitrifying activity. The results confirmed the presence of nitrifying cells in the wells, but not the absence of the contaminant. Unfortunately, the first wells tested with purity analysis based on general 16S rRNA PCR suggested the presence of contaminants. The random process of cell sorting includes the possibility of selecting micro-colonies instead of single cells that may encompass non-nitrifying organisms(Fujitani et al., 2015). We also need to take into account probable contamination events between wells of the same plate.

Finally, the fulfillment of the proposed objective cannot be stated yet. The majority of the active enrichments after sorting need to be checked with purity analysis. The worst-case scenario would be the presence of contaminants in all the active wells. Subsequently, higher enriched cultures derived from not pure wells should be used in dilution series or in other sorting techniques as listed in the KS chapter above.

4.2.3 Unidentified nitrifier enrichment ("LL").

The third enrichment, named LL, was the most unexplored one. Due to the multiple questions and difficulties arising from this culture, the only objective proposed was the identification of the nitrifying organism. The outstanding feature was the high nitrite-oxidizing rates, consuming 5 mM nitrite per week, possibly capable of more. Regarding its structure and composition, high diversity of morphologies was observed, grouped in big and small flocks hindering any enrichment. Identification of the nitrifier present was attempted several times with multiple FISH probes targeting different NOBs. Despite the positive FISH signals for *Nitrospira* cells, their low abundance as a minority group and generally slower nitrite oxidation even in pure culture, placed their role as the major contributor to the nitrifying activity observed into question. Accordingly, the hypothesis of the presence of a second, unidentified NOB responsible for the larger part of the nitrite oxidation was investigated.

The subsequent efforts were focused on increasing the enrichment level of the unidentified NOB by decreasing the amount of morphologies differentiated. Due to the presence of huge, thick flocks performing dilution series was not the only technique pursued. Filtration through 3 µm filters enabled the separation of the flocks form the planktonic community. Both resulting cultures showed nitrifying activity and delivered a planktonic nitrifying culture that potentially allowed for successful dilution series. The approach decreased the diversity of morphologies observed, illustrated by more homogenous cultures. The planktonic cultures were used for sterile flow cytometry single cell sorting during the last month of the project. The amount of active wells obtained from both vitamin amended and not vitamin-amended plates suggested a high enrichment level in the original culture. However, the few wells tested for purity returned the same results as already discussed in the MixS enrichment chapter. In the case of the LL culture, further analyses were applied to identify the sorted, active cells. A FISH approach targeting *Nitrobacter* and *Nitrospira* was applied at the end of the project, but the results were non-conclusive and repetition was discouraged for time and biomass constraints. The FISH analysis discarded the presence of *Nitrobacter* and *Nitrotoga* by displaying negative results through all the enriching time. However, despite it being highly unlikely, *Nitrospira* cannot be fully rejected as the nitrifying organism.

Metagenomic analysis of the enrichment culture (analyzed by M. Albertsen) provided new clues to be investigated. Two bins were recovered from the metagenome, belonging to the Saprospiracea and Rhodocyclaceae families. The Saprospiracea are known as common contaminants in many NOB and AOA enrichments (unpublished results), so its role as a contaminant organism in this enrichment was feasible. Two main arguments for a detailed analysis of the second family listed are given. First, the Rhodocyclaceae family is closer phylogenetically to other NOB. It belongs to the β-proteobacteria group that encompasses nitrite oxidizers of the *Nitrotoga* genus. Secondly, the metabolic versatility described within this family included denitrifying activities(Kerkhof, Palleroni, Häggblom, & Song, 2001; Liu, Mao, Bergaust,

Bakken, & Frostegård, 2013). In addition, this bin had the highest read number in the metagenome, being ten times more abundant than all the other organisms. The genome bin (produced by P. Pjevac) affiliated with the genus *Thauera* was uploaded and analyzed with the MGRast tool. The genomic potential annotated did not provide any clear evidence of a nitrification potential. However, tetracycline resistance genes were detected and applied as selective element for isolating *Thauera* from the LL enrichment.

The addition of TET caused increased turbidity of the medium and possible switches back and forth from denitrification to nitrification activity in some cultures. Transfers of the TET-amended cultures to new medium without TET recovered a stable nitrifying activity. A powerful degrading potential of halobenzoate compounds under denitrifying conditions has been postulated for the *Thauera* genera(Kerkhof et al., 2001; B. Song, Kerkhof, & Häggblom, 2002). Considering its metabolic versatility, it can be hypothesized that the addition of organics stimulated denitrifying activity that changed back to strict nitrification under autotrophic conditions. However, in order to disclose the behavior described, genomic and functional information is required. Nonetheless, the experiment confirmed that the nitrifier present is resistant to tetracycline, a property that might be useful as selective tool.

Thauera organisms are able to grow on solid plates with minimum media supplemented with organics(Kerkhof et al., 2001). Accordingly, solid plates supplemented with sodium acetate and TET expected the isolation of colonies with nitrite oxidizing metabolism when transferred to liquid cultivation medium. The approach failed since no nitrite consumption was observed by any of the transferred colonies. These results were not surprising since the cultivable fraction represents less than 1% of the total microbial diversity(Amann, Ludwig, & Schleifer, 1995). Besides the fact that the overall percentage is even lower when counting the ones also growing on solid media, nitrite oxidizing bacteria are described as difficult to cultivate and isolate(Fujitani et al., 2014; Kamagata, 2015; Nowka, Off, et al., 2015). Slow adaptation to the new conditions, reluctant growth, loss of autotrophic properties or the wrong postulation of *Thauera* as potential nitrite oxidizers could also play a role in the negative results obtained.

In summary, important progress was accomplished with the LL culture. Within the course of this master thesis we gained more information about its genomic and physiologic potential. In addition, more homogenous cultures and highly enriched nitrifying sorted wells were obtained. However, the objective proposed was not fulfilled. The outlook encompasses several approaches described right after. The first steps would be focused on the rest of the active wells, expecting the obtaining of a pure nitrifying culture that could be identified by a general 16S rRNA PCR. Following analysis should include the growth of biomass to run several activity tests, single-cell genome reconstruction, transcriptomic and metabolic analysis(Koch, Galushko, Albertsen, Schintlmeister, Spieck, et al., 2014; Koch et al., 2015; Palatinszky et al., 2015). The FISH-MAR technique is a useful method used already with nitrifiers to link activity and identification(H. Daims et al., 2006). If none of the wells hosts a nitrifying pure culture, the low-contaminated wells could be used for FISH-MAR, further dilutions or different sorting methods, already described and proposed for the other two enrichments.

5. CONCLUSIONS AND OUTLOOK

Nitrite oxidizers are an essential functional group for all ecosystems, with important regulatory functions affecting the global biogeochemical nitrogen cycle. Nevertheless, their reputation as physiologically limited and difficult to cultivate causes a lack of knowledge of this group compared to the rest of nitrogen-cycling microbes. Surprisingly, recent studies of NOB communities demonstrated higher metabolic versatility and diversity than expected.

The main hypothesis for the seasonal study of this M.Sc. project predicted high diversity of NOB in the two saline-alkaline lakes studied, correlating with distinct environmental factors and defined by a temporal succession. The culture-independent study illustrated the unequivocal presence of canonical and comammox *Nitrospira* but postulated the absence of the other known nitrite oxidizers. Furthermore, the seasonal analysis illustrated the stable dominance of few taxa during fourteen months, rejecting the expectations of temporal patterns of *Nitrospira* OTUs. However, the restricted analysis to only DNA prevented the detection of probable activity shifts defining an overlooked temporal succession.

The project expanded the knowledge about *Nitrospira* and comammox *Nitrospira* distribution in the natural environment. Both described sub-projects confirmed the ubiquity of canonical *Nitrospira* even in the extreme habitats studied. Specifically, the seasonal study identified lineage IV and lineage II as the dominant members of the nitrite-oxidizing community, discovering *Nitrospira* as the main NOB in saline-alkaline lakes. Accordingly, two of the three nitrite-oxidizing enrichment cultures obtained from saline-alkaline lakes of the same area encompassed *Nitrospira* organisms from lineage IV and II as nitrifiers. The seasonal project also described for the first time the existence of comammox *Nitrospira* clade A in brackish environments with alkaline pH. In addition, two distant phylogenetic clades were recognized, detected in significantly different abundances in the two lakes, postulating niche differentiation and providing novel information about comammox ecology.

Different communities were found in the two lakes, which seemed to respond differently to particular environmental factors. Salinity and inorganic N concentrations significantly correlated with the community differences, likely shaping the relative abundance of the different OTUs by providing preferred or undesirable conditions. Besides the differences between lakes, the community composition also changed over time, indicating the presence of some factor/s differing between the months and affecting the members of the ecosystem. The unravelling of the reasons behind the observed correlations of singular taxa with higher or lower salinity or nitrogen levels needs future physiological studies, requiring preferably cultivation and isolation of the strains. The KS enrichment, had previously allowed for the reconstruction of the genome and the identification of unique genetic adaptations to the saline-alkaline lakes conditions. However, physiological tests did not confirm a preference of this NOB towards elevated salinity beyond the culturing conditions. Nonetheless, the cultures provided new valuable information. Lineage II members, dominant in the MixS enrichment culture, demonstrated the nitrifying activity of novel *Nitrospira* strains at alkaline pH in saline environments. Finally, the LL culture suggested the existence of a novel group and/or enzymes able to oxidize nitrite to nitrate. In summary, potentially novel organisms with previously unknown adaptations and new morphologies increased our knowledge of NOB diversity and their responses in extreme environments.

Multiple questions remain unanswered and need further investigation. Pure cultures from each of the enrichments are needed to identify novel nitrite oxidizers, confirm the new morphology observed for *Nitrospira* and test the inferred adaptations to elevated salinity and pH. The seasonal monitoring sub-project lacks quantification and activity data. Analysis of the overall abundance of the identified nitrifiers and their potential activity would contribute to illustrating the importance of these groups in the ecosystem. Due to the failed detection of other NOB groups by PCR, *in situ* analysis like FISH could be applied to corroborate the results and confirm their absence from the community. Comparisons between *Nitrospira* comammox and other ammonia oxidizers activity and abundance are required to disclose the predominant ammonia oxidizing group. Finally, additional samples, complemented taxonomic studies in finer resolutions and measurement of other abiotic and biotic factors are required to draw more conclusions and understand better the dynamics of NOB in saline-alkaline lakes.

ACKNOWLEGMENTS

I would like to specially thank Professor Holger Daims, for giving me the possibility to be part of his research group and allow me to learn much more than expected, to grow as a student and as a researcher. He motivated me for keep answering questions and rethinking the results obtained. I also owe my sincerely gratitude to Dr. Anne Daebeler, for providing continuous, great supervision and always being available and willing to help me in every doubt and every step. I would further like to thank all the other members of my research group and all Domies for helping me in everything they could and providing a great atmosphere.

REFERENCES

Abraham, O.-S. J., Miguel, T.-S., Inocencio, H.-C., & Blondy, C.-C. (2017). A quick and effective in-house method of DNA purification from agarose gel, suitable for sequencing. *3 Biotech*, *7*(3), 180. https://doi.org/10.1007/s13205-017-0851-1

Acinas, S. G., Klepac-Ceraj, V., Hunt, D. E., Pharino, C., Ceraj, I., Distel, D. L., & Polz, M. F. (2004). Fine-scale phylogenetic architecture of a complex bacterial community. *Nature*, *430*(6999), 551–554. https://doi.org/10.1038/nature02649

Alawi, M., Lipski, A., Sanders, T., Eva-Maria-Pfeiffer, & Spieck, E. (2007). Cultivation of a novel cold-adapted nitrite oxidizing betaproteobacterium from the Siberian Arctic. *The ISME Journal*, *1*(3), 256–264. https://doi.org/10.1038/ismej.2007.34

Alawi, M., Off, S., Kaya, M., & Spieck, E. (2009). Temperature influences the population structure of nitrite-oxidizing bacteria in activated sludge. *Environmental Microbiology Reports*, *1*(3), 184–190. https://doi.org/10.1111/j.1758-2229.2009.00029.x

Albertsen, M., Karst, S. M., Ziegler, A. S., Kirkegaard, R. H., & Nielsen, P. H. (2015a). Back to Basics – The Influence of DNA Extraction and Primer Choice on Phylogenetic Analysis of Activated Sludge Communities. *PLOS ONE*, *10*(7), e0132783. https://doi.org/10.1371/journal.pone.0132783

Altschul, S. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, *25*(17), 3389–3402. https://doi.org/10.1093/nar/25.17.3389

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Amann, R. I. (1995). In situ identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. In A. D. L. Akkermans, J. D. Van Elsas, & F. J. De Bruijn (Eds.), *Molecular Microbial Ecology Manual* (pp. 331–345). Dordrecht: Springer Netherlands. https://doi.org/10.1007/978-94-011-0351-0

Amann, R. I., Krumholz, L., & Stahl, D. A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology*, *172*(2), 762–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1688842

Amann, R. I., Ludwig, W., & Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, *59*(1), 143–169. https://doi.org/0146-0749/95/$04.00+0

Anderson, M. J. (2001). A new method for non parametric multivariate analysis of variance. *Austral Ecology*, *26*(2001), 32–46. https://doi.org/10.1111/j.1442-9993.2001.01070.pp.x

Anderson, M. J., & Willis, T. J. (2003). Canonical analysis of principal coordinates: A useful method of constrained ordination for ecology. *Ecology*, *84*(2), 511–525. https://doi.org/10.1890/0012-9658(2003)084[0511:CAOPCA]2.0.CO;2

Angel, R., Claus, P., & Conrad, R. (2012). Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions. *The ISME Journal*, *6*(4), 847–862. https://doi.org/10.1038/ismej.2011.141

Atkinson, C. A., Jolley, D. F., & Simpson, S. L. (2007). Effect of overlying water pH, dissolved oxygen, salinity and sediment disturbances on metal release and sequestration from metal contaminated marine sediments. *Chemosphere*, *69*(9), 1428–1437. https://doi.org/10.1016/j.chemosphere.2007.04.068

Banciu, H. L., & Muntyan, M. S. (2015). Adaptive strategies in the double-extremophilic prokaryotes inhabiting soda lakes. *Current Opinion in Microbiology*, *25*, 73–79. https://doi.org/10.1016/j.mib.2015.05.003

Ben-Yaakov, S. (1973). pH Buffering of pore water of recent anoxic marine sediments. *Limnology and Oceanography*, *18*(1), 86–94. https://doi.org/10.4319/lo.1973.18.1.0086

Bertics, V. J., & Ziebis, W. (2009). Biodiversity of benthic microbial communities in bioturbated coastal sediments is controlled by geochemical microniches. *The ISME Journal*, *3*, 1269–1285. https://doi.org/10.1038/ismej.2009.62

Best, D. J., & Roberts, D. E. (1975). Algorithm AS 89: The Upper Tail Probabilities of Spearman's Rho. *Applied Statistics*, *24*(3), 377. https://doi.org/10.2307/2347111

Biller, S. J., Berube, P. M., Lindell, D., & Chisholm, S. W. (2014). Prochlorococcus: the structure and function of collective diversity. *Nature Reviews Microbiology*, *13*(1), 13–27. https://doi.org/10.1038/nrmicro3378

Boros, E., Horváth, Z., Wolfram, G., & Vörös, L. (2014b). Salinity and ionic composition of the shallow astatic soda pans in the Carpathian Basin. *Annales de Limnologie - International Journal of Limnology*, *50*, 59–69. https://doi.org/10.1051/limn/2013068

Brussaard, C. P. D., Peperzak, L., Beggah, S., Wick, L. Y., Wuerz, B., Weber, J., … van der Meer, J. R. (2016). Immediate ecotoxicological effects of short-lived oil spills on marine biota. *Nature Communications*, *7*, 11206. https://doi.org/10.1038/ncomms11206

Carini, S. a., & Joye, S. B. (2008). Nitrification in Mono Lake, California: Activity and community composition during contrasting hydrological regimes. *Limnology and Oceanography*, *53*(6), 2546–2557. https://doi.org/10.4319/lo.2008.53.6.2546

Cébron, A., & Garnier, J. (2005). Nitrobacter and Nitrospira genera as representatives of nitrite-oxidizing bacteria: detection, quantification and growth along the lower Seine River (France). *Water Research*, *39*(20), 4979–92. https://doi.org/10.1016/j.watres.2005.10.006

Chambers, J. M., Freeny, A., & Heiberger, R. M. (1992). Chapter 5. Analysis of variance; designed experiments. In J. M. Chambers & T. J. Hastie (Eds.), *Statistical Models in S* (p. 608). Wadsworth & Brooks/Cole Advanced Books & Software. Retrieved from https://books.google.es/books/about/Statistical_Models_in_S.html?id=uyfvAAAAMAAJ&redir_esc=y

Chan, J. Z.-M., Halachev, M. R., Loman, N. J., Constantinidou, C., & Pallen, M. J. (2012). Defining bacterial species in the genomic era: insights from the genus Acinetobacter. *BMC Microbiology*, *12*(1), 302. https://doi.org/10.1186/1471-2180-12-302

Chen, J., & Chen, L. (2017). GMPR: A novel normalization method for microbiome sequencing data. *bioRxiv*, *0*(0), 1–2. https://doi.org/10.1101/112565

Church, M. J., Short, C. M., Jenkins, B. D., Karl, D. M., & Zehr, J. P. (2005). Temporal Patterns of Nitrogenase Gene (nifH) Expression in the Oligotrophic North Pacific Ocean. *Applied and Environmental Microbiology*, *71*(9), 5362–5370. https://doi.org/10.1128/AEM.71.9.5362-5370.2005

Costa, E., Pérez, J., & Kreft, J. U. (2006). Why is metabolic labour divided in nitrification? *Trends in Microbiology*, *14*(5), 213–219. https://doi.org/10.1016/j.tim.2006.03.006

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. *Systematic and Applied Microbiology*, *22*(3), 434–444. https://doi.org/10.1016/S0723-2020(99)80053-8

Daims, H., Lebedeva, E. V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., … Wagner, M. (2015). Complete nitrification by Nitrospira bacteria. *Nature*, *528*(7583), 504–509. https://doi.org/10.1038/nature16461

Daims, H., Lücker, S., & Wagner, M. (2016). A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. *Trends in Microbiology*, *24*(9), 699–712. https://doi.org/10.1016/j.tim.2016.05.004

Daims, H., Maixner, F., Lücker, S., Stoecker, K., Hace, K., & Wagner, M. (2006). Ecophysiology and niche differentiation of Nitrospira-like bacteria, the key nitrite oxidizers in wastewater treatment plants. *Water Science & Technology*, *54*(1), 21. https://doi.org/10.2166/wst.2006.367

Daims, H., Nielsen, J. L., Nielsen, P. H., Schleifer, K. H., & Wagner, M. (2001). In Situ Characterization of Nitrospira-Like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants. *Applied and Environmental Microbiology*, *67*(3–12), 5273–5284. https://doi.org/10.1128/AEM.67.11.5273

Daims, H., Stoecker, K., & Wagner, M. (2005). Fluorescence in situ hybridisation for the detection of prokaryotes. In A. Osborn & C. Smith (Eds.), *Molecular Microbial Ecology* (pp. 213–219). Abingdon, UK: Bios-Garland.

Datta, T., Racz, L., Kotay, S. M., & Goel, R. (2011). Seasonal variations of nitrifying community in trickling filter-solids contact (TF/SC) activated sludge systems. *Bioresource Technology*, *102*(3), 2272–2279. https://doi.org/10.1016/j.biortech.2010.10.043

Dimitriu, P. A., Pinkart, H. C., Peyton, B. M., & Mormile, M. R. (2008). Spatial and Temporal Patterns in the Microbial Diversity of a Meromictic Soda Lake in Washington State. *Applied and Environmental Microbiology*, *74*(15), 4877–4888. https://doi.org/10.1128/AEM.00455-08

Dini-Andreote, F., Brossi, M. J. de L., van Elsas, J. D., & Salles, J. F. (2016). Reconstructing the Genetic Potential of the Microbially-Mediated Nitrogen Cycle in a Salt Marsh Ecosystem. *Frontiers in Microbiology*, *7*(JUN), 1–13. https://doi.org/10.3389/fmicb.2016.00902

Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science*, *14*(6), 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x

Dumbrell, A. J., Nelson, M., Helgason, T., Dytham, C., & Fitter, A. H. (2009). Relative roles of niche and neutral processes in structuring a soil microbial community. *The ISME Journal Advance Online Publication*. https://doi.org/10.1038/ismej.2010.48

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, *10*(10), 996–998. https://doi.org/10.1038/nmeth.2604

Ehrich, S., Behrens, D., Lebedeva, E. V, Ludwig, W., & Bock, E. (1995). A New Obligately Chemolithoautotrophic, Nitrite-Oxidizing Bacterium, Nitrospira-Moscoviensis Sp-Nov and Its Phylogenetic Relationship. *Archives Of Microbiology*, *164*(1), 16–23. https://doi.org/10.1007/s002030050230

Erisman, J. W., Galloway, J. N., Seitzinger, S., Bleeker, A., Dise, N. B., Petrescu, A. M. R., … de Vries, W. (2013). Consequences of human modification of the global nitrogen cycle. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1621), 20130116–20130116. https://doi.org/10.1098/rstb.2013.0116

Eydallin, G., Ryall, B., Maharjan, R., & Ferenci, T. (2014). The nature of laboratory domestication changes in freshly isolated Escherichia coli strains. *Environmental Microbiology*, *16*(3), 813–828. https://doi.org/10.1111/1462-2920.12208

Ford, R. M., & Harvey, R. W. (2007). Role of chemotaxis in the transport of bacteria through saturated porous media. *Advances in Water Resources*, *30*(6–7), 1608–1617. https://doi.org/10.1016/j.advwatres.2006.05.019

Francis, C. A., O'Mullan, G. D., & Ward, B. B. (2003). Diversity of ammonia monooxygenase (amoA) genes across environmental gradients in Chesapeake Bay sediments. *Geobiology*, *1*(2), 129–140. https://doi.org/10.1046/j.1472-4669.2003.00010.x

Fraser, C., Alm, E. J., Polz, M. F., Spratt, B. G., & Hanage, W. P. (2009). The Bacterial Species Challenge: Making Sense of Genetic and Ecological Diversity. *Science*, *323*(5915), 741–746. https://doi.org/10.1126/science.1159388

Fujitani, H., Kumagai, A., Ushiki, N., Momiuchi, K., & Tsuneda, S. (2015). Selective isolation of ammonia-oxidizing bacteria from autotrophic nitrifying granules by applying cell-sorting and sub-culturing of microcolonies. *Frontiers in Microbiology*, *6*(OCT), 1–10. https://doi.org/10.3389/fmicb.2015.01159

Fujitani, H., Ushiki, N., Tsuneda, S., & Aoi, Y. (2014). Isolation of sublineage I Nitrospira by a novel cultivation strategy. *Environmental Microbiology*, *16*(10), 3030–3040. https://doi.org/10.1111/1462-2920.12248

Füssel, J., Lam, P., Lavik, G., Jensen, M. M., Holtappels, M., Günter, M., & Kuypers, M. M. (2012). Nitrite oxidation in the Namibian oxygen minimum zone. *The ISME Journal*, *6*(6), 1200–1209. https://doi.org/10.1038/ismej.2011.178

Grant, W. . (2006). Alkaline environments and biodiversity. In C. Gerday & N. Glansdorff (Eds.), *Extremophiles (Life under extreme environmental conditions)*. Eolss Publishers, Oxford, UK. https://doi.org/10115582565

Gruber-Dorninger, C., Pester, M., Kitzinger, K., Savio, D. F., Loy, A., Rattei, T., … Daims, H. (2015). Functionally relevant diversity of closely related Nitrospira in activated sludge. *The ISME Journal*, *9*(3), 643–655. https://doi.org/10.1038/ismej.2014.156

Gruber, N., & Galloway, J. N. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature*, *451*(7176), 293–296. https://doi.org/10.1038/nature06592

Gründemann, D., & Schömig, E. (1996). Protection of DNA during preparative agarose gel electrophoresis against damage induced by ultraviolet light. *BioTechniques*, *21*(5), 898–903. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8922632

Guo, F., & Zhang, T. (2013a). Biases during DNA extraction of activated sludge samples revealed by high throughput sequencing. *Applied Microbiology and Biotechnology*, *97*(10), 4607–4616. https://doi.org/10.1007/s00253-012-4244-4

Haaijer, S. C. M., Ji, K., van Niftrik, L., Hoischen, A., Speth, D., Jetten, M. S. M., … Op den Camp, H. J. M. (2013). A novel marine nitrite-oxidizing Nitrospira species from Dutch coastal North Sea water. *Frontiers in Microbiology*, *4*(MAR), 1–12. https://doi.org/10.3389/fmicb.2013.00060

Hartman, P. S. (1991). Transillumination can profoundly reduce transformation frequencies. *BioTechniques*, *11*(6), 747–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1809328

Herbold, C. W., Pelikan, C., Kuzyk, O., Hausmann, B., Angel, R., Berry, D., & Loy, A. (2015). A flexible and economical barcoding approach for highly multiplexed amplicon sequencing of diverse target genes. *Frontiers in Microbiology*, *6*(JUL), 1–8. https://doi.org/10.3389/fmicb.2015.00731

Hermann, B., Ferguson, S., & Newton E, W. (2006). *Biology of the Nitrogen Cycle*. (B. Hermann, S. Ferguson, & W. Newton E, Eds.) (1st editio). Elsevier Science.

Humayoun, S. B., Bano, N., & Hollibaugh, J. T. (2003). Depth Distribution of Microbial Diversity in Mono Lake, a Meromictic Soda Lake in California. *Applied and Environmental Microbiology*, *69*(2), 1030–1042. https://doi.org/10.1128/AEM.69.2.1030-1042.2003

Hüpeden, J., Wegen, S., Off, S., Lücker, S., Bedarf, Y., Daims, H., … Spieck, E. (2016). Relative Abundance of Nitrotoga spp. in a Biofilter of a Cold-Freshwater Aquaculture Plant Appears To Be Stimulated by Slightly Acidic pH. *Applied and Environmental Microbiology*, *82*(6), 1838–1845. https://doi.org/10.1128/AEM.03163-15

Huse, S. M., Welch, D. M., Morrison, H. G., & Sogin, M. L. (2010). Ironing out the wrinkles in the rare biosphere through improved OTU clustering. *Environmental Microbiology*, *12*(7), 1889–1898. https://doi.org/10.1111/j.1462-2920.2010.02193.x

Jackson, C. R., Roden, E. E., & Churchill, P. F. (1998). Changes in bacterial species composition in enrichment cultures with various dilutions of inoculum as monitored by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, *64*(12), 5046–5048.

Jaspers, E., & Overmann, J. (2004a). Ecological Significance of Microdiversity : Identical 16S rRNA Gene Sequences Can Be Found in Bacteria with Highly Divergent Genomes and Ecophysiologies. *Applied and Environmantal Microbiology*, *70*(8), 4831–4839. https://doi.org/10.1128/AEM.70.8.4831

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K. H., Pommerening-Röser, A., Koops, H. P., & Wagner, M. (1998). Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. *Applied and Environmental Microbiology*, *64*(8), 3042–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9687471

Kamagata, Y. (2015). Keys to Cultivating Uncultured Microbes: Elaborate Enrichment Strategies and Resuscitation of Dormant Cells. *Microbes and Environments*, *30*(4), 289–290. https://doi.org/10.1264/jsme2.ME3004rh

Kamekura, M. (1999). Alkaliphilic Microorganisms. In *Enigmatic Microorganisms and Life in Extreme Environments* (pp. 479–485). Dordrecht: Springer Netherlands. https://doi.org/10.1007/978-94-011-4838-2_37

Kashtan, N., Roggensack, S. E., Rodrigue, S., Thompson, J. W., Biller, S. J., Coe, A., … Chisholm, S. W. (2014). Single-Cell Genomics Reveals Hundreds of Coexisting Subpopulations in Wild Prochlorococcus. *Science*, *344*(6182), 416–420. https://doi.org/10.1126/science.1248575

Kellogg, C. A., & Griffin, D. W. (2006). Aerobiology and the global transport of desert dust. *Trends in Ecology & Evolution*, *21*(11), 638–644. https://doi.org/10.1016/j.tree.2006.07.004

Kerkhof, L. J., Palleroni, N. J., Häggblom, M. M., & Song, B. (2001). Characterization of halobenzoate-degrading, denitrifying Azoarcus and Thauera isolates and description of Thauera chlorobenzoica sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *51*(2), 589–602. https://doi.org/10.1099/00207713-51-2-589

Kim, D.-J., & Kim, S.-H. (2006). Effect of nitrite concentration on the distribution and competition of nitrite-oxidizing bacteria in nitratation reactor systems and their kinetic characteristics. *Water Research*, *40*(5), 887–894. https://doi.org/10.1016/j.watres.2005.12.023

Kits, K. D., Sedlacek, C. J., Lebedeva, E. V., Han, P., Bulaev, A., Pjevac, P., … Wagner, M. (2017). Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature*. https://doi.org/10.1038/nature23679

Koch, H., Galushko, A., Albertsen, M., Schintlmeister, A., Spieck, E., Richter, A., … Daims, H. (2014). Growth of nitrite-oxidizing bacteria by aerobi hydrogen oxidation. *Science*, *345*(6200), 1052–1054.

Koch, H., Lücker, S., Albertsen, M., Kitzinger, K., Herbold, C., Spieck, E., … Daims, H. (2015). Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus Nitrospira. *Proceedings of the National Academy of Sciences*, *112*(36), 11371–11376. https://doi.org/10.1073/pnas.1506533112

Konstantinidis, K. T., Rosselló-Móra, R., & Amann, R. (2017). Uncultivated microbes in need of their own taxonomy. *The ISME Journal*, 1–8. https://doi.org/10.1038/ismej.2017.113

Krachler, R., Korner, I. (Arge V. und L. G., & Kirschner, A. (2012). *Die Salzlacken des Seewinkels*.

Kraemer, S. A., & Boynton, P. J. (2017). Evidence for microbial local adaptation in nature. *Molecular Ecology*, *26*(7), 1860–1876. https://doi.org/10.1111/mec.13958

Kruse, M., Keuter, S., Bakker, E., Spieck, E., Eggers, T., & Lipski, A. (2013). Relevance and Diversity of Nitrospira Populations in Biofilters of Brackish RAS. *PLoS ONE*, *8*(5), e64737. https://doi.org/10.1371/journal.pone.0064737

Kurien, B. T., & Scofield, R. H. (2002). Extraction of Nucleic Acid Fragments from Gels. *Analytical Biochemistry*, *302*(1), 1–9. https://doi.org/10.1006/abio.2001.5526

Lanzén, A., Simachew, A., Gessesse, A., Chmolowska, D., Jonassen, I., & Øvreås, L. (2013). Surprising Prokaryotic and Eukaryotic Diversity, Community Structure and Biogeography of Ethiopian Soda Lakes. *PLoS ONE*, *8*(8), e72577. https://doi.org/10.1371/journal.pone.0072577

Lebedeva, E. V., Alawi, M., Jozsa, P. G., Daims, H., & Spieck, E. (2008). Physiological and phylogenetic characterization of a novel lithoautotrophic nitrite-oxidizing bacterium, "Candidatus Nitrospira bockiana." *International Journal of Systematic and Evolutionary Microbiology*, *58*(1), 242–250. https://doi.org/10.1099/ijs.0.65379-0

Legendre, P., & Anderson, M. J. (1999). Distance-Based Redundancy Analysis : Testing Multispecies Responses in Multifactorial Ecological Experiments. *Ecological Monographs*, *69*(1), 1–24. https://doi.org/doi:10.1890/0012-9615(1999)069[0001:DBRATM]2.0.CO;2

Li, M. (2012). *Developing Raman sorting and imaging techniques to characterise and differentiate unculturable microbial single cells*. University of Sheffield.

Liu, B., Mao, Y., Bergaust, L., Bakken, L. R., & Frostegård, Å. (2013). Strains in the genus Thauera exhibit remarkably different denitrification regulatory phenotypes. *Environmental Microbiology*, *15*(10), n/a-n/a. https://doi.org/10.1111/1462-2920.12142

Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., … Wagner, M. (2002). Oligonucleotide Microarray for 16S rRNA Gene-Based Detection of All Recognized Lineages of Sulfate-Reducing Prokaryotes in the Environment. *Applied and Environmental Microbiology*, *68*(10), 5064–5081. https://doi.org/10.1128/AEM.68.10.5064-5081.2002

Lücker, S., Nowka, B., Rattei, T., Spieck, E., & Daims, H. (2013). The Genome of Nitrospina gracilis Illuminates the Metabolism and Evolution of the Major Marine Nitrite Oxidizer. *Frontiers in Microbiology*, *4*(FEB), 1–19. https://doi.org/10.3389/fmicb.2013.00027

Lücker, S., Schwarz, J., Gruber-Dorninger, C., Spieck, E., Wagner, M., & Daims, H. (2015). Nitrotoga-like bacteria are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants. *The ISME Journal*, *9*(3), 708–720. https://doi.org/10.1038/ismej.2014.158

Lücker, S., Wagner, M., Maixner, F., Pelletier, E., Koch, H., Vacherie, B., … Daims, H. (2010). A Nitrospira metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(30), 13479–84. https://doi.org/10.1073/pnas.1003860107

Ludwig, W. (2004). ARB: a software environment for sequence data. *Nucleic Acids Research*, *32*(4), 1363–1371. https://doi.org/10.1093/nar/gkh293

Lynch, M. D. J., & Neufeld, J. D. (2015). Ecology and exploration of the rare biosphere. *Nature Reviews Microbiology*, *13*(4), 217–229. https://doi.org/10.1038/nrmicro3400

Maixner, F., Noguera, D. R., Anneser, B., Stoecker, K., Wegl, G., Wagner, M., & Daims, H. (2006). Nitrite concentration influences the population structure of Nitrospira-like bacteria. *Environmental Microbiology*, *8*(8), 1487–1495. https://doi.org/10.1111/j.1462-2920.2006.01033.x

Mcardle, B. H., & Anderson, M. J. (2001). Fitting Multivariate Models to Community Data : A Comment on Distance-Based Redundancy Analysis. *Ecology*, *82*(1), 290–297.

Metz, H., & L, F. (1989). Contributions to the knowledge of the chemistry and crustacean zooplankton of sodic waters: the Seewinkel pans revisited.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E., Kubal, M., … Edwards, R. (2008). The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics*, *9*(1), 386. https://doi.org/10.1186/1471-2105-9-386

Miranda, K. M., Espey, M. G., & Wink, D. A. (2001). A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. *Nitric Oxide*, *5*(1), 62–71. https://doi.org/10.1006/niox.2000.0319

Nei, M., Tajima, F., & Tateno, Y. (1983). Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution*, *19*(2), 153–170. https://doi.org/10.1007/BF02300753

Nelson, W. C., Maezato, Y., Wu, Y.-W., Romine, M. F., & Lindemann, S. R. (2016). Identification and Resolution of Microdiversity through Metagenomic Sequencing of Parallel Consortia. *Applied and Environmental Microbiology*, *82*(1), 255–267. https://doi.org/10.1128/AEM.02274-15

Ngugi, D. K., Blom, J., Stepanauskas, R., & Stingl, U. (2016). Diversification and niche adaptations of Nitrospina-like bacteria in the polyextreme interfaces of Red Sea brines. *The ISME Journal*, *10*(6), 1383–1399. https://doi.org/10.1038/ismej.2015.214

Nogueira, R., & Melo, L. F. (2006). Competition betweenNitrospira spp. andNitrobacter spp. in nitrite-oxidizing bioreactors. *Biotechnology and Bioengineering*, *95*(1), 169–175. https://doi.org/10.1002/bit.21004

Nowka, B., Daims, H., & Spieck, E. (2015). Comparison of Oxidation Kinetics of Nitrite-Oxidizing Bacteria: Nitrite Availability as a Key Factor in Niche Differentiation. *Applied and Environmental Microbiology*, *81*(2), 745–753. https://doi.org/10.1128/AEM.02734-14

Nowka, B., Off, S., Daims, H., & Spieck, E. (2015). Improved isolation strategies allowed the phenotypic differentiation of two Nitrospira strains from widespread phylogenetic lineages. *FEMS Microbiology Ecology*, *91*(3), 1–11. https://doi.org/10.1093/femsec/fiu031

Nugroho, R. A., Röling, W. F. M., Laverman, A. M., & Verhoef, H. A. (2007). Low Nitrification Rates in Acid Scots Pine Forest Soils Are Due to pH-Related Factors. *Microbial Ecology*, *53*(1), 89–97. https://doi.org/10.1007/s00248-006-9142-9

Oksanen, J., Guillaume-Blanchet, F., Kindt F, R., Legendre, P., Minchin, P. ., OHara, R. ., & Al., E. (2005). Vegan: Community Ecology Package. R package version 2.2-1.

Oren, A. (2008). Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline Systems*, *4*(1), 2. https://doi.org/10.1186/1746-1448-4-2

Pääbo, S., Irwin, D. M., & Wilson, A. C. (1990). DNA damage promotes jumping between templates during enzymatic amplification. *The Journal of Biological Chemistry*, *265*(8), 4718–21. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2307682

Paerl, H. W., & Pinckney, J. L. (1996). A mini-review of microbial consortia: Their roles in aquatic production and biogeochemical cycling. *Microbial Ecology*, *31*(3), 225–247. https://doi.org/10.1007/BF00171569

Palatinszky, M., Herbold, C., Jehmlich, N., Pogoda, M., Han, P., von Bergen, M., … Wagner, M. (2015). Cyanate as an energy source for nitrifiers. *Nature*, *524*(7563), 105–108. https://doi.org/10.1038/nature14856

Palomo, A., Jane Fowler, S., Gülay, A., Rasmussen, S., Sicheritz-Ponten, T., & Smets, B. F. (2016). Metagenomic analysis of rapid gravity sand filter microbial communities suggests novel physiology of Nitrospira spp. *The ISME Journal*, *10*(11), 2569–2581. https://doi.org/10.1038/ismej.2016.63

Palomo, A., Pedersen, A. G., Fowler, S. J., Dechesne, A., Sicheritz-Pontén, T., & Smets, B. F. (2017). Comparative Genomics Sheds Light On Niche Differentiation And The Evolutionary History Of Comammox Nitrospira. *bioRxiv*, 1–25. https://doi.org//10.1101/138586

Paul Antony, C., Kumaresan, D., Hunger, S., Drake, H. L., Murrell, J. C., & Shouche, Y. S. (2013). Microbiology of Lonar Lake and other soda lakes. *The ISME Journal*, *7*(3), 468–476. https://doi.org/10.1038/ismej.2012.137

Pester, M., Maixner, F., Berry, D., Rattei, T., Koch, H., Lücker, S., … Daims, H. (2014). NxrB encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing N itrospira. *Environmental Microbiology*, *16*(10), 3055–3071. https://doi.org/10.1111/1462-2920.12300

Pickett, S. T. A. (1976). Succession: An Evolutionary Interpretation. *The American Naturalist*, *110*(971), 107–119. https://doi.org/10.1086/283051

Pinto, A. J., Marcus, D. N., Ijaz, U. Z., Bautista-de lose Santos, Q. M., Dick, G. J., & Raskin, L. (2015). Metagenomic Evidence for the Presence of Comammox Nitrospira -Like Bacteria in a Drinking Water System. *Applied and Environmental Science*. https://doi.org/10.1128/mSphere.00054-15

Pjevac, P., Schauberger, C., Poghosyan, L., Herbold, C. W., van Kessel, M. A. H. J., Daebeler, A., … Daims, H. (2017). AmoA-Targeted Polymerase Chain Reaction Primers for the Specific Detection and Quantification of Comammox Nitrospira in the Environment. *Frontiers in Microbiology*, *8*(AUG), 1–11. https://doi.org/10.3389/fmicb.2017.01508

Pointing, S. B., Chan, Y., Lacap, D. C., Lau, M. C. Y., Jurgens, J. A., & Farrell, R. L. (2009). Highly specialized microbial diversity in hyper-arid polar desert. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(47), 19964–9. https://doi.org/10.1073/pnas.0908274106

Princic, A., Mahne, I., Megusar, F., Eldor, A., & Tiedje, J. M. (1998a). Effects of pH and Oxygen and Ammonium Concentrations on the Community Structure of Nitrifying Bacteria from Wastewater. *Applied and Environmantal Microbiology*, *64*(10), 3584–3590.

Prosser, J. I., & Nicol, G. W. (2012). Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends in Microbiology*, *20*(11), 523–531. https://doi.org/10.1016/j.tim.2012.08.001

Raghunathan, A., Ferguson, H. R., Bornarth, C. J., Song, W., Driscoll, M., & Lasken, R. S. (2005). Genomic DNA Amplification from a Single Bacterium. *Applied and Environmental Microbiology*, *71*(6), 3342–3347. https://doi.org/10.1128/AEM.71.6.3342-3347.2005

Rosselló-Mora, R., & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiology Reviews*, *25*(1), 39–67. https://doi.org/10.1111/j.1574-6976.2001.tb00571.x

Rousk, J., Bååth, E., Brookes, P. C., Lauber, C. L., Lozupone, C., Caporaso, J. G., … Fierer, N. (2010). Soil bacterial and fungal communities across a pH gradient in an arable soil. *The ISME Journal*, *4*(10), 1340–1351. https://doi.org/10.1038/ismej.2010.58

Rousk, J., Brookes, P. C., & Baath, E. (2009). Contrasting Soil pH Effects on Fungal and Bacterial Growth Suggest Functional Redundancy in Carbon Mineralization. *Applied and Environmental Microbiology*, *75*(6), 1589–1596. https://doi.org/10.1128/AEM.02775-08

Sanger, F., & Coulson, A. R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology*, *94*(3), 441–448. https://doi.org/10.1016/0022-2836(75)90213-2

Saunders, A. M., Albertsen, M., Vollertsen, J., & Nielsen, P. H. (2016a). The activated sludge ecosystem contains a core community of abundant organisms. *The ISME Journal*, *10*(1), 11–20. https://doi.org/10.1038/ismej.2015.117

Schramm, A., de Beer, D., van den Heuvel, J. C., Ottengraf, S., & Amann, R. (1999). Microscale Distribution of Populations and Activities of Nitrosospira and Nitrospira spp . along a Macroscale Gradient in a Nitrifying Bioreactor : Quantification by In Situ Hybridization and the Use of Microsensors Microscale Distribution of Populations. *Applied and Environmental Microbiology*, *65*(8), 3690–3696.

Seckbach, J. (Joseph). (n.d.). *ESeckbach, J. (Joseph). (n.d.). Enigmatic microorganisms and life in extreme environments. Retrieved from https://books.google.es/books?id=gmVsCQAAQBAJ&pg=PA440&lpg=PA440&dq=environment+affecting+morphology+microorganisms&source=bl&ots=RL-48CzE7X&sig=I5Af*.

Shen, J., Zhang, L., Zhu, Y., Zhang, J., & He, J. (2008). Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. *Environmental Microbiology*, *10*(6), 1601–1611. https://doi.org/10.1111/j.1462-2920.2008.01578.x

Sher, Y., Zaady, E., & Nejidat, A. (2013). Spatial and temporal diversity and abundance of ammonia oxidizers in semi-arid and arid soils: indications for a differential seasonal effect on archaeal and bacterial ammonia oxidizers. *FEMS Microbiology Ecology*, *86*(3), 544–556. https://doi.org/10.1111/1574-6941.12180

Sipos, R., Székely, A. J., Palatinszky, M., Révész, S., Má¡rialigeti, K., & Nikolausz, M. (2007). Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis. *FEMS Microbiology Ecology*, *60*(2), 341–350. https://doi.org/10.1111/j.1574-6941.2007.00283.x

Smith, J. M., Mosier, A. C., & Francis, C. A. (2015). Spatiotemporal Relationships Between the Abundance, Distribution, and Potential Activities of Ammonia-Oxidizing and Denitrifying Microorganisms in Intertidal Sediments. *Microbial Ecology*, *69*(1), 13–24. https://doi.org/10.1007/s00248-014-0450-1

Song, B., Kerkhof, L. J., & Häggblom, M. M. (2002). Characterization of bacterial consortia capable of degrading 4-chlorobenzoate and 4-bromobenzoate under denitrifying conditions. *FEMS Microbiology Letters*, *213*(2), 183–188. https://doi.org/10.1016/S0378-1097(02)00804-2

Song, Y., Yin, H., & Huang, W. E. (2016). Raman activated cell sorting. *Current Opinion in Chemical Biology*, *33*, 1–8. https://doi.org/10.1016/j.cbpa.2016.04.002

Sorokin, D. Y., Banciu, H. L., & Muyzer, G. (2015). Functional microbiology of soda lakes. *Current Opinion in Microbiology*, *25*, 88–96. https://doi.org/10.1016/j.mib.2015.05.004

Sorokin, D. Y., Berben, T., Melton, E. D., Overmars, L., Vavourakis, C. D., & Muyzer, G. (2014). Microbial diversity and biogeochemical cycling in soda lakes. *Extremophiles*, *18*(5), 791–809. https://doi.org/10.1007/s00792-014-0670-9

Sorokin, D. Y., Gorlenko, V. M., Namsaraev, B. B., Namsaraev, Z. B., Lysenko, A. M., Eshinimaev, B. T., … Kuenen, J. G. (2004a). Prokaryotic communities of the north-eastern Mongolian soda lakes. *Hydrobiologia*, *522*(1–3), 235–248. https://doi.org/10.1023/B:HYDR.0000029989.73279.e4

Sorokin, D. Y., & Kuenen, J. G. (2005). Chemolithotrophic haloalkaliphiles from soda lakes. *FEMS Microbiology Ecology*, *52*(3), 287–295. https://doi.org/10.1016/j.femsec.2005.02.012

Sorokin, D. Y., Lücker, S., Vejmelkova, D., Kostrikina, N. A., Kleerebezem, R., Rijpstra, W. I. C., … Daims, H. (2012). Nitrification expanded: discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum Chloroflexi. *The ISME Journal*, *6*(12), 2245–2256. https://doi.org/10.1038/ismej.2012.70

Sorokin, D. Y., Muyzer, G., Brinkhoff, T., Gijs Kuenen, J., & Jetten, M. S. M. (1998). Isolation and characterization of a novel facultatively alkaliphilic Nitrobacter species, N. alkalicus sp. nov. *Archives of Microbiology*, *170*(5), 345–352. https://doi.org/10.1007/s002030050652

Sorokin, D. Y., Vejmelkova, D., Lucker, S., Streshinskaya, G. M., Rijpstra, W. I. C., Sinninghe Damste, J. S., … Daims, H. (2014). Nitrolancea hollandica gen. nov., sp. nov., a chemolithoautotrophic nitrite-oxidizing bacterium isolated from a bioreactor belonging to the phylum Chloroflexi. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY*, *64*(Pt 6), 1859–1865. https://doi.org/10.1099/ijs.0.062232-0

Spieck, E., & Bock, E. (2005). The Lithoautotrophic Nitrite-Oxidizing Bacteria. In G. Garrity (Ed.), *Bergey's Manual® of Systematic Bacteriology* (pp. 149–153). Boston, MA: Springer US. https://doi.org/10.1007/0-387-28021-9_19

Spieck, E., Keuter, S., Wenzel, T., Bock, E., & Ludwig, W. (2014). Characterization of a new marine nitrite oxidizing bacterium, Nitrospina watsonii sp. nov., a member of the newly proposed phylum "Nitrospinae." *Systematic and Applied Microbiology*, *37*(3), 170–176. https://doi.org/10.1016/j.syapm.2013.12.005

Su, C., Lei, L., Duan, Y., Zhang, K.-Q., & Yang, J. (2012). Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. *Applied Microbiology and Biotechnology*, *93*(3), 993–1003. https://doi.org/10.1007/s00253-011-3800-7

Tourova, T. P., Grechnikova, M. a., Kuznetsov, B. B., & Sorokin, D. Y. (2014). Phylogenetic diversity of bacteria in soda lake stratified sediments. *Microbiology*, *83*(6), 869–879. https://doi.org/10.1134/S0026261714060186

Ushiki, N., Fujitani, H., Aoi, Y., & Tsuneda, S. (2013). Isolation of Nitrospira belonging to sublineage II from a wastewater treatment plant. *Microbes and Environments / JSME*, *28*(3), 346–53. https://doi.org/10.1264/jsme2.ME13042

Van den Burg, B. (2003). Extremophiles as a source for novel enzymes. *Current Opinion in Microbiology*, *6*(3), 213–218. https://doi.org/10.1016/S1369-5274(03)00060-2

Van Kessel, M. A. H. J., Speth, D. R., Albertsen, M., Nielsen, P. H., Op den Camp, H. J. M., Kartal, B., … Lücker, S. (2015). Complete nitrification by a single microorganism. *Nature*, *528*(7583), 555–559. https://doi.org/10.1038/nature16459

Vanysacker, L., Declerck, S. A. J., Hellemans, B., De Meester, L., Vankelecom, I., & Declerck, P. (2010a). Bacterial community analysis of activated sludge: an evaluation of four commonly used DNA extraction methods. *Applied Microbiology and Biotechnology*, *88*(1), 299–307. https://doi.org/10.1007/s00253-010-2770-5

Vargas, C., Argandoña, M., Reina-Bueno, M., Rodríguez-Moya, J., Fernández-Aunión, C., & Nieto, J. J. (2008). Unravelling the adaptation responses to osmotic and temperature stress in Chromohalobacter salexigens, a bacterium with broad salinity tolerance. *Saline Systems*, *4*, 14. https://doi.org/10.1186/1746-1448-4-14

Vavourakis, C. D., Ghai, R., Rodriguez-Valera, F., Sorokin, D. Y., Tringe, S. G., Hugenholtz, P., & Muyzer, G. (2016). Metagenomic Insights into the Uncultured Diversity and Physiology of Microbes in Four Hypersaline Soda Lake Brines. *Frontiers in Microbiology*, *7*(FEB), 1–18. https://doi.org/10.3389/fmicb.2016.00211

Venables, W. N., & Ripley, B. D. (2003). Modern Applied Statistics With S. *Technometrics*, *45*(1). https://doi.org/10.1198/tech.2003.s33

von Wintzingerode, F., Göbel, U. B., & Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews*, *21*(3), 213–29. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9451814

Vreeland, R. H. (1987). Mechanisms of halotolerance in microorganisms. *Critical Reviews in Microbiology*, *14*(4), 311–356. https://doi.org/10.3109/10408418709104443

Vreeland, R. H., & Rosenzweig, W. D. (1999). Survival of Halophilic Bacteria in Ancient Salts: Possibilities and Potentials. In *Enigmatic Microorganisms and Life in Extreme Environments* (pp. 387–398). Dordrecht: Springer Netherlands. https://doi.org/10.1007/978-94-011-4838-2_30

Wagner, M., Rath, G., Koops, H. P., Flood, J., & R, A. (1996). In situ analysis of nitrifying bacteria in sewage treatment plants. *Water Science & Technology*, *34*, 237–244.

Wall, D. H., & Virginia, R. A. (1999). Controls on soil biodiversity: insights from extreme environments. *Applied Soil Ecology*, *13*(2), 137–150. https://doi.org/10.1016/S0929-1393(99)00029-3

Wankel, S. D., Mosier, A. C., Hansel, C. M., Paytan, A., & Francis, C. A. (2011). Spatial Variability in Nitrification Rates and Ammonia-Oxidizing Microbial Communities in the Agriculturally Impacted Elkhorn Slough Estuary, California. *Applied and Environmental Microbiology*, *77*(1), 269–280. https://doi.org/10.1128/AEM.01318-10

Watson, S. W., Bock, E., Valois, F. W., Waterbury, J. B., & Schlosser, U. (1986). Nitrospira marina gen. nov. sp. nov.: a chemolithotrophic nitrite-oxidizing bacterium. *Archives of Microbiology*, *144*(1), 1–7. https://doi.org/10.1007/BF00454947

Watson W, S., & Waterbury B, J. (1971). Characteristics of Two Marine Nitrite Oxidizing Bacteria, Nitrospina gracilis nov. ten. nov. sp. and Nitrococcus mobilis nov. ten. nov. sp. *Arch. Mikrobiol.*, *77*, 203–231.

Westram, R., Bader, K., Prüsse, E., Kumar, Y., Meier, H., Glöckner, F. O., & Ludwig, W. (2011). ARB: A Software Environment for Sequence Data. In *Handbook of Molecular Microbial Ecology I* (Vol. I, pp. 399–406). Hoboken, NJ, USA: John Wiley & Sons, Inc. https://doi.org/10.1002/9781118010518.ch46

Whitman, W. B., & Keswani, J. (2001). Relationship of 16S rRNA sequence similarity to DNA hybridization in prokaryotes. *International Journal of Systematic and Evolutionary Microbiology*, *51*(2), 667–678. https://doi.org/10.1099/00207713-51-2-667

Wickham, H. (2007). Reshaping Data with the reshape Package. *Journal of Statistical Software*, *21*(12), 1–20. https://doi.org/10.1016/S0142-1123(99)00007-9

Wickham, H. (2009). ggplot2: Elegant Graphics for Data Analysis. New York, NY: Springer-Verlag. Retrieved from http://ggplot2.org

Winogradsky, S. (1890). Sur les organismes de la nitrification. C.R. *Acad. Sci.*, *110*, 1013–1016.

Woebken, D., Lam, P., Kuypers, M. M. M., Naqvi, S. W. A., Kartal, B., Strous, M., … Amann, R. (2008). A microdiversity study of anammox bacteria reveals a novel Candidatus Scalindua phylotype in marine oxygen minimum zones. *Environmental Microbiology*, *10*(11), 3106–3119. https://doi.org/10.1111/j.1462-2920.2008.01640.x

Wolfram, G., Schagerl, M., Donabaum, K., & Riedler, P. (2004). *Untersuchung der räumlichen und zeitlichen Verteilung benthischer Evertebraten in den Salzlacken des Seewinkels und ihre Rolle als Nahrungsgrundlage für Wasservögel. Teil I: Abiotische Charakterisierung.*

Yang, D. C., Blair, K. M., & Salama, N. R. (2016). Staying in Shape: the Impact of Cell Shape on Bacterial Survival in Diverse Environments. *Microbiology and Molecular Biology Reviews*, *80*(1), 187–203. https://doi.org/10.1128/MMBR.00031-15

Young, K. D. (2006). The Selective Value of Bacterial Shape. *Microbiology and Molecular Biology Reviews*, *70*(3), 660–703. https://doi.org/10.1128/MMBR.00001-06

Zhou, J., Deng, Y., Zhang, P., Xue, K., Liang, Y., Van Nostrand, J. D., … Arkin, A. P. (2014). Stochasticity, succession, and environmental perturbations in a fluidic ecosystem. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(9), E836-45. https://doi.org/10.1073/pnas.1324044111

ANNEX I

MATERIAL AND METHODS

TABLE S1. Descriptive summary of the sample selection used for the initial screening PCRs

Herrnsee (HS)		Unterer Stinkersee (US)	
H2.3 1/10 1/50	May2014. SedimentCore3	**U2.1 1/10 1/50**	May2014. SedimentCore1
H2. W 1/10	May2014. Water column	**U2.W 1/10**	May2014. Water column
H5.2 1/10 1/50	August2014. SedimentCore2	**U5.2 1/10 1/50**	August2014. SedimentCore2
H5. W 1/10	August2014. Water column	**U5. W 1/10**	August2014. Water column
H8.1 1/10 1/50	November2014.SedimentCore1	**U8.3 1/10 1/50**	November2014.SedimentCore3
H8.W 1/10	November2014. Water column	**U8. W 1/10**	November2014. Water column
H11.1 1/10 1/50	February2015. SedimentCore1	**U11.3 1/10 1/50**	February2015. SedimentCore3
H11. W 1/10	February2015. Water column	**U11.W 1/10**	February2015. Water column
H14.2 1/10 1/50	May2015. SedimentCore2	**U14.2 1/10 1/50**	May2015. SedimentCore2
H14.W 1/10	May2015. Water column	**U14.W 1/10**	May2015. Water column

RESULTS

SEASONAL STUDY OF NOB COMMUNITIES FROM TWO SALINE-ALKALINE LAKES

Optimization of culture-independent methods for the study of nitrite oxidizers from saline-alkaline lake sediments.

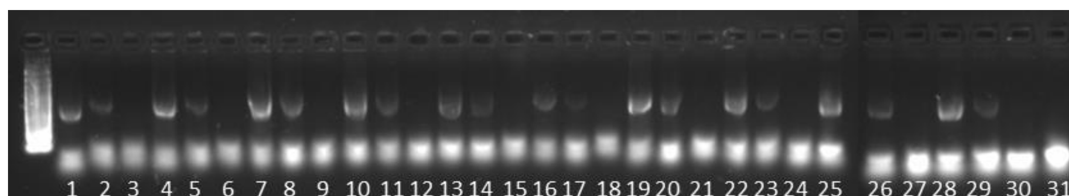

FIGURE S1. **Agarose gel displaying the PCR products obtained with the primer set (169F/707R) targeting the *nxrB* of all *Nitrospira* in DNA extracts from saline-alkaline lake sediment and water**. Samples: **1**-H2.3 1/10 **2**-H2.3 1/50 **3-**H2.W 1/10 **4-**H5.2 1/10 **5-**H5.2 1/50 **6-**H5.W 1/10 **7**-H8.1 1/10 **8-**H8.1 1/50 **9-**H8.W 1/10 **10-**H11.1 1/10 **11**- H11.1 1/50 **12**- H11.W 1/10 **13**-H14.2 1/10 **14**-H14.2 1/50 **15**-H14.W 1/10 **16**-U2.1 1/10 **17**-U2.1 1/50 **18**-U2.W 1/10 **19**-U5.2 1/10 **20**-U5.2 1/50 **21**-U5.W 1/10 **22**-U8.3 1/10 **23**-U8.3 1/50 **24**-U8.W 1/10 **25**-U11.3 1/10 **26**-U11.3 1/50 **27-** U11.W 1/10 **28**-U14.2 1/10 **29**-U14.2 1/50 **30-C**+ **31**-C-

---

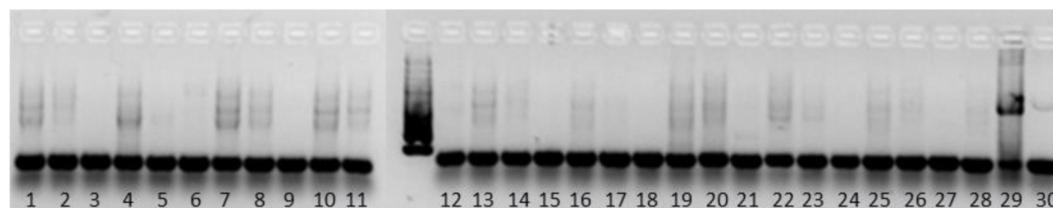

FIGURE S2. Agarose gel displaying the PCR products obtained with the primer set 706F/1431R against *Nitrobacter* and *Nitrococcus* nxrB in sediment and water DNA samples. Samples: **1**-H2.3 1/10 **2**-H2.3 1/50 **3**-H2.W 1/10 **4-**H5.2 1/10 **5**-H5.2 1/50 **6**-H5.W 1/10 **7**-H8.1 1/10 **8**-H8.1 1/50 **9**-H8.W 1/10 **10**-H11.1 1/10 **11**- H11.1 1/50 **12**- H11.W 1/10 **13**-H14.2 1/10 **14**-H14.2 1/50 **15**-H14.W 1/10 **16**-U2.1 1/10 **17**-U2.1 1/50 **18**-U2.W 1/10 **19**-U5.2 1/10 **20**-U5.2 1/50 **21**-U5.W 1/10 **22**-U8.3 1/10 **23**-U8.3 1/50 **24**-U8.W 1/10 **25**-U11.3 1/10 **26**-U11.3 1/50 **27-** U11.W 1/10 **28**-U14.2 1/10 **29**-C+ **30**-C-

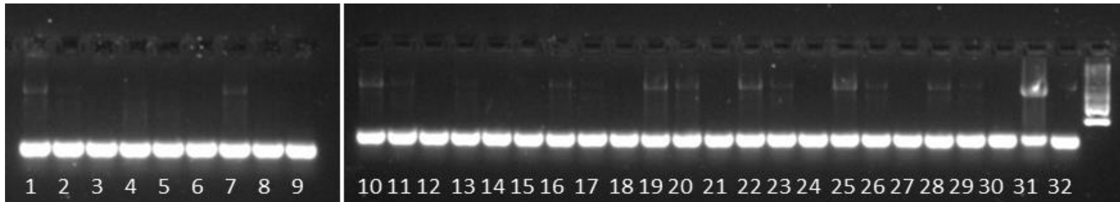

FIGURE S3. Agarose gel loaded with the PCR products obtained with the primer pair targeting the rRNA 16S of *Nitrotoga.* Samples: **1**-H2.3 1/10 **2**-H2.3 1/50 **3-**H2.W 1/10 **4-**H5.2 1/10 **5-**H5.2 1/50 **6-**H5.W 1/10 **7-**H8.1 1/10 **8-**H8.1 1/50 **9-**H8.W 1/10 **10**-H11.1 1/10 **11**- H11.1 1/50 **12**- H11.W 1/10 **13-**H14.2 1/10 **14-**H14.2 1/50 **15-**H14.W 1/10 **16-**U2.1 1/10 **17-**U2.1 1/50 **18-**U2.W 1/10 **19-**U5.2 1/10 **20-**U5.2 1/50 **21-**U5.W 1/10 **22-**U8.3 1/10 **23-**U8.3 1/50 **24-**U8.W 1/10 **25-**U11.3 1/10 **26-**U11.3 1/50 **27-** U11.W 1/10 **28-**U14.2 1/10 **29-**U14.2 1/50 **30-**U14.W 1/10 **31-**C+ **32-**C-

---

TABLE S2. Summary of the samples included in each of the three libraries sent for sequencing. Dot (•) reflects inclusion and cross (x) exclusion from the library.

		LIBRARIES		
SAMPLE	**SAMPLE ID**	***Nitrospira* (*nxrB*)**	**Comammox clade A (*amoA*)**	**Comammox clade B (*amoA*)**
April14. HS. US. Top	H1.1 H1.2 H1.3 / U1.1 U1.2 U1.3	• •• // • ••	• •• // • • •	x x x // x x x
May14. HS. US. Top	H2.1 H2.2 H2.3 / U2.1 U2.2 U2.3	• •• // • ••	x • • // x x x	x x x // x x x
June14. HS. US. Top	H3.1 H3.2 H3.3 / U3.1 U3.2 U3.3	• •• // • ••	x x • //• • •	• x x// x x x
July14. HS. US. Top	H4.1 H4.2 H4.3 / U4.1 U4.2 U4.3	• •• // • ••	x • x //• • •	x x x // x x x
July14. HS. US. Low	H4.4 / U4.4	• •• // • ••	x // •	x // x
Aug14. HS. US. Top	H5.1 H5.2 H5.3 / U5.1 U5.2 U5.3	• •• // • ••	x • • // • • •	x • x// x x x
Sep14. HS. US. Top	H6.1 H6.2 H6.3 / U6.1 U6.2 U6.3	• •• // • ••	• • • // x x x	x x x // x x x
Oct14. HS. US. Top	H7.1 H7.2 H7.3 / U7.1 U7.2 U7.3	• •• // • ••	• • • // • x •	x x x // • x •
Oct14. HS. US. Low	H7.4 / U7.4	• •• // • ••	•// x	x // x
Nov14. HS. US. Top	H8.1 H8.2 H8.3 / U8.1 U8.2 U8.3	• •• // • ••	• •• // • • •	x x x // x x x
Dec14. HS. US. Top	H9.1 H9.2 H9.3 / U9.1 U9.2 U9.3	• •• // • ••	x x x// • • •	x x x // x x •
Jan15. HS. US. Top	H10.1 H10.2 H10.3 / U10.1 U10.2 U10.3	• •• // • ••	x x x// • • •	x x x //• x x
Jan15. HS. US. Low	H10.4 / U10.4	• •• // • ••	x // x	x // x
Feb15. HS. US. Top	H11.1 H11.2 H11.3 / U11.1 U11.2 U11.3	• •• // • ••	x x x// • • •	x x x // x x x
Mar15. HS. US. Top	H12.1 H12.2 H12.3 / U12.1 U12.2 U12.3	• •• // • ••	• •• // • • •	• x • // x x x
April15. HS. US. Top	H13.1 H13.2 H13.3 / U13.1 U13.2 U13.3	• •• // • ••	• •• // • • •	x x x // x x x
April15. HS. US. Low	H13.4 / U13.4	• •• // • ••	x // x	x // x
May15. HS. US. Top	H14.1 H14.2 H14.3 / U14.1 U14.2 U14.3	• •• // • ••	• •• // • • •	x x x //• x x
June15. HS. US. Top	H15.1 H15.2 H15.3 / U15.1 U15.2 U15.3	• •• // • ••	x x x// • • •	x x x // x x x

Seasonal study of NOB communities' composition and dynamics from two saline-alkaline lakes applying NGS techniques.

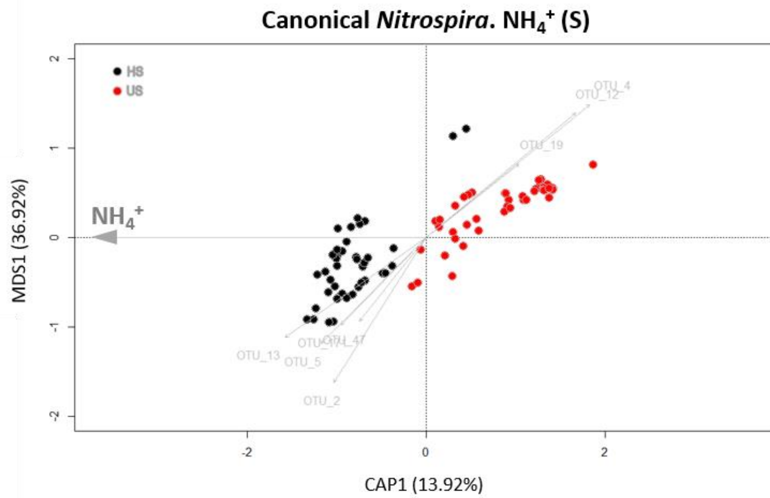

FIGURE S4. Ordination plot based on distance-based redundancy analysis (db-RDAs) constrained to ammonium (NH_4^+) concentration in the sediment (S). Applied to the canonical *Nitrospira* dataset. The y-axis (MDS1) represents the first of the unconstrained axes and the x-axis the constrained one (CAP1) displaying also the percentage explained by each axis. The filled black dots represent the samples from the Herrnsee (HS) and the red ones from the Unterer Stinkersee (US). The significant ($p<0.001$) OTUs eigenvectors influencing the community structure are illustrated by grey lines and tags.

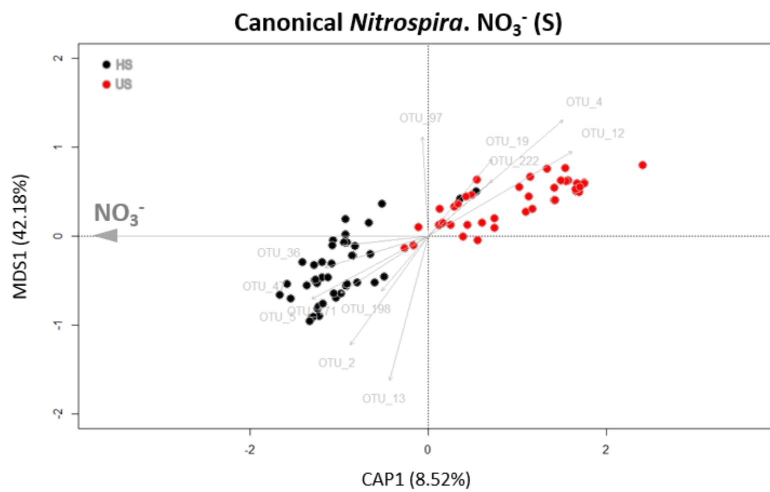

FIGURE S5. Ordination plot based on distance-based redundancy analysis (db-RDAs) constrained to nitrate (NO_3^-) concentration in the sediment (S). Applied to the canonical *Nitrospira* dataset. The y-axis (MDS1) represents the first of the unconstrained axes and the x-axis the constrained one (CAP1) displaying also the percentage explained by each axis. The filled black dots represent the samples from the Herrnsee (HS) and the red ones from the Unterer Stinkersee (US). The significant ($p<0.001$) OTUs eigenvectors influencing the community structure are illustrated by grey lines and tags.

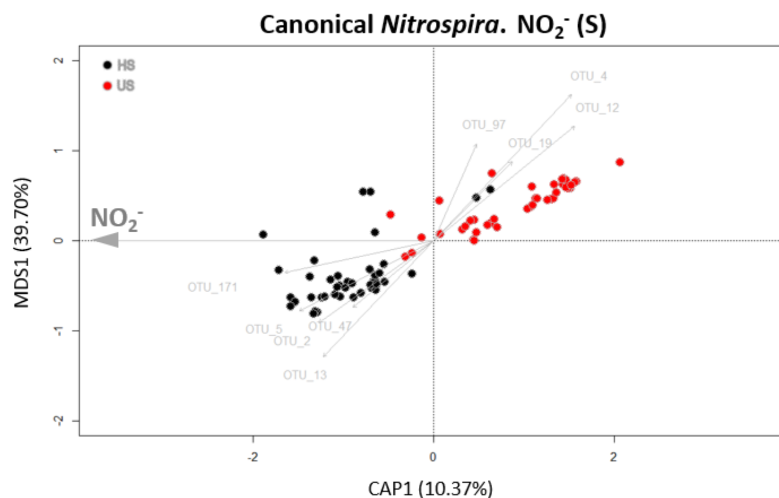

FIGURE S6. Ordination plot based on distance-based redundancy analysis (db-RDAs) constrained to nitrite (NO_2^-) concentration in the sediment (S). Applied to the canonical *Nitrospira* dataset. The y-axis (MDS1) represents the first of the unconstrained axes and the x-axis the constrained one (CAP1) displaying also the percentage explained by each axis. The filled black dots represent the samples from the Herrnsee (HS) and the red ones from the Unterer Stinkersee (US). The significant ($p<0.001$) OTUs eigenvectors influencing the community structure are illustrated by grey lines and tags.

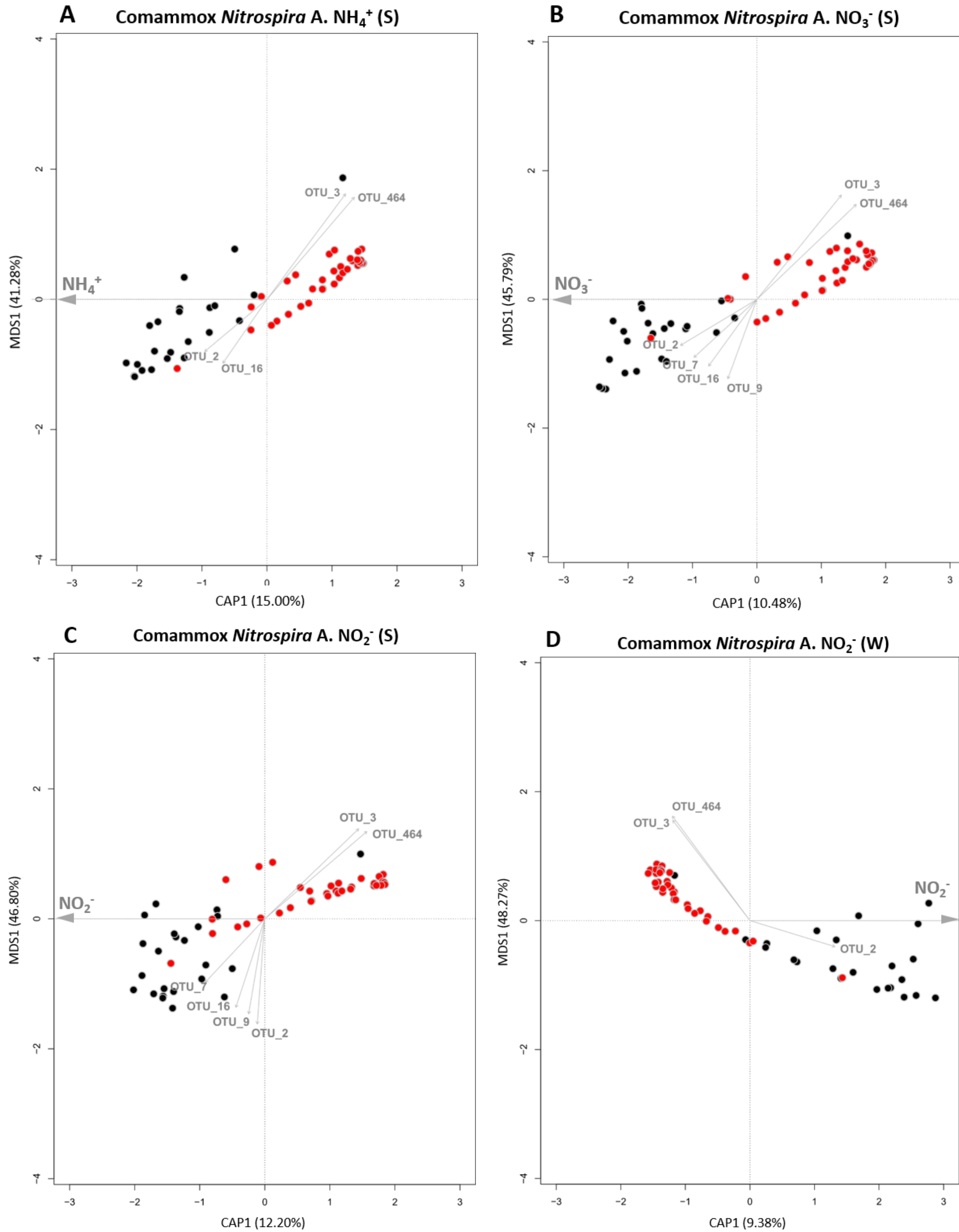

FIGURE S7. Ordination plot based on distance-based redundancy analysis (db-RDAs) applied to the comammox *Nitrospira* clade A dataset. A) Ordination constrained to ammonium (NH_4^+) concentration in the sediment (S). B) Ordination constrained to nitrate (NO_3^-) concentrationed in the sediment (S). C) Ordination constrained to nitrite (NO_2^-) concentration in the sediment (S). D) Ordination constrain to nitrite (NO_2^-) concentration in the water column (W). The y-axis (MDS1) represents the first of the unconstrained axes and the x-axis the constrained one (CAP1) displaying also the percentage explained by each axis. The filled black dots represent the samples from the Herrnsee (HS) and the red ones from the Unterer Stinkersee (US). The significant ($p<0.001$) OTUs eigenvectors influencing the community structure are illustrated by grey lines and tags.