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2. Abstract

With the recent discovery of the first microorganisms capable of complete ammonia oxidation to nitrate ('comammox'), the dogma on the division of labor in nitrification into two consecutive steps was proven invalid. Intriguingly all so far described comammox microorganisms are dispersed non-monophyletically within *Nitrospira* lineage II, which is well known for harboring some of the most predominant nitrite oxidizing bacteria on the planet. Comammox *Nitrospira amoA* genes are phylogenetically distinct from the variants of ammonium oxidizing bacteria (AOB), or archaea (AOA), thus suitable marker genes for its detection and enumeration. Previous metagenomic investigations revealed that comammox *amoA* genes are forming two monophyletic sister groups, now referred to as clade A and B. In this study, a molecular approach for the environmental detection of comammox *Nitrospira amoA* genes was developed and optimized, by the design of two highly specific PCR primer sets that target each clade individually.

In order to achieve a reasonable coverage of reference sequences, both primer pairs contain multiple ambiguous nucleotide positions. To counteract the consequences of primer degeneracy, I established equimolar primer mixtures of the individual primer variants with high coverages and showed their superior amplification efficiency in comparison to the conventional degenerated primer sets. Subsequently, I performed a proof of principle environmental screen by clone sequencing and thereby underlined the broad habitat spectra of comammox *Nitrospira*.

Since DNA extraction protocols also have an influence on the observed microbial community compositions, I evaluated the difference of two bead beating based methods to the subsequent amplification efficiency of comammox *amoA* genes. By the application on equal amounts of template DNA, the MoBio PowerSoil DNA extraction Kit intriguingly showed better results for one sample compared to a phenol/chloroform method. However for the majority of specimens, the phenol/chloroform method yields excessively more DNA.

This study paved the way for future PCR-based (e.g. next generation amplicon sequencing) studies about the global distribution of comammox *Nitrospira* and the understanding of niche partitioning between nitrifying microorganisms.

3. Zusammenfassung

Vor kurzem wurden die ersten Mikroorganismen entdeckt, die in der Lage sind Ammonium komplett zu Nitrat zu oxidieren (‘comammox’), und dadurch das Dogma, über die Arbeitsteilung in der Nitrifikation in zwei aufeinanderfolgenden Schritten, für ungültig erklärt. Interessanterweise gehören alle bisher beschriebenen comammox Mikroorganismen zu dem Genus *Nitrospira* und sind nicht monophyletisch innerhalb der *Nitrospira* Linie II verteilt, welche einige der am häufigsten vorkommenden Nitrit oxidierenden Bakterien der Welt beinhält. Comammox *Nitrospira amoA* Gene sind von den *amoA* Genen der Ammonium oxidierenden Bakterien und Archaeen unterscheidbar, und daher eignen sie sich als phylogenetische und funktionelle Markergene für die Detektion und Quantifizierung von comammox *Nitrospira*. Bisherige metagenomische Studien zeigten, dass comammox *amoA* Gene zwei monophyletische Schwester Gruppen bilden, welche mittlerweile clade A und B genannt werden. In dieser Studie wurde ein molekularbiologischer Ansatz für die Detektion von comammox *Nitrospira* in Umweltproben entwickelt, in dem zwei spezifische PCR Primer Paare entworfen wurden, die jede dieser Gruppe einzeln erfassen.

Um eine ausreichend hohe Abdeckungen der Referenz Sequenzen zu erzielen, enthalten beide Primer Paare an mehreren Positionen in ihren Sequenzen degenerierte Nukleotide. Um die daraus folgenden Konsequenzen zu minimieren, mischte ich äquimolar einzelne Varianten der Primer zu neuen Primer Paaren und zeigte ihre erhöhte Amplifikationseffizienz im Vergleich zu den gewöhnlichen degenerierten Primer Paaren. Anschließend bewies ich die Anwendbarkeit der Primer Paare auf Umweltproben mit Klon- Sanger Sequenzierung, und bestätigte die weite Verbreitung von comammox *Nitrospira* in der Umwelt.

Weil sich das Protokoll der DNA Extrahierung auf die beobachtete Zusammensetzung der mikrobiellen Gemeinschaft auswirkt, evaluierte ich den Unterschied zwischen zwei Methoden, welche auf mechanischer Zelllyse basieren, für die anschließende Amplifikations- Effizienz von comammox *amoA* Genen. Wenn gleiche Mengen von DNA angewandt werden, zeigt das „MoBio PowerSoil DNA Extraction Kit“ interessanterweise die besseren Resultate, verglichen zu einer Phenol/Chloroform Methode. Für die Mehrzahl an Proben jedoch erzielt die Phenol/Chloroform Methode einen höheren DNA Ertrag.

Mit dieser Studie ebnete ich den Weg für zukünftige PCR basierende Studien (wie zum Beispiel Amplikon Sequenzierung) über die globale Verbreitung von comammox *Nitrospira*, welche

das Verständnis der Nischenteilung zwischen nitrifizierenden Mikroorganismen fördern können.

4. Introduction

Nitrogen is ubiquitously needed by all living organisms, as it is a major constituent of proteins, DNA and vitamins. However, bioavailable forms of nitrogen are scarce in many environments and therefore often a growth-limiting factor (Vitousek and Howarth, 1991).

The global nitrogen cycle consists of various redox transformations for assimilatory, catabolic and detoxifying processes (Simon and Klotz, 2013). These reactions are usually microbially mediated, and link the nitrogen cycle to other biogeochemical cycles, such as the carbon and sulfur cycle (Boetius *et al.*, 2000; Raghoebarsing *et al.*, 2006).

4.1. The global Nitrogen cycle

4.1.1. Nitrogen fixation

$N_2 + 4\ H_2 \quad \rightarrow \quad 2\ NH_3 + H_2$

The majority of nitrogen on earth occurs as chemically inert nitrogen gas (N_2). By the energetically expensive process of nitrogen fixation, nitrogen gas is converted to ammonia and thereby introduced into the nitrogen cycle (Mortenson, 1964). The process occurs at high rates in nitrogen limited environments and is catalyzed by nitrogenase enzymes of diazotrophic microorganisms (Mohr *et al.*, 2010). These homodimeric Fe and heterotetrameric MoFe protein complexes are highly oxygen sensitive, as the Fe-S cofactors are destructed by oxidation (Kim and Rees, 1992). Therefore, diazotrophs living in oxygenated environments have several evolutionary adaptations for oxygen protection. Cyanobacteria, for example, form heterocysts – specialized cells with a degenerated photosystem II, which is responsible for the release of oxygen during oxidative photosynthesis, and additional cell walls to minimize O_2 diffusion into the cells (Thomas, 1970). Besides the biologically mediated, another natural way for the conversion of dinitrogen into nitrogen compounds are lightning strikes, however they contribute only little to the global nitrogen cycle (Noxon, 1976).

An industrial way for nitrogen fixation, now known as the Haber-Bosch process, was developed by Fritz Haber and Carl Bosch in the first half of the 20th century. This process requires large amounts of energy, since the fusion of nitrogen and hydrogen gas into ammonia requires the use of an iron containing catalyst, alongside with high pressures (>150 bar) and temperatures from 400 to 500 °C. Nowadays about 50% of the globally fixed nitrogen originates from this process (Erisman *et al.*, 2008). A large fraction of the thereby produced nitrogen compounds are used as fertilizer, most commonly in the highly bioavailable form of ammonia. Due to its

positive charge, ammonia fertilizers bind to the rather negatively charged soils, hence are not easily washed out by rainfall events. However in fertilized soils, still a large fraction of ammonia is oxidized by nitrifying microorganisms to nitrate, instead of being assimilation processes by plants (Gioacchini *et al.*, 2002). Nevertheless, this scientific achievement augmented the global agricultural productivity and enabled the exponential increase in human population.

4.1.2. Nitrification

$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2\ H^+$

$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$

Nitrification is the microbially mediated oxidation of ammonia to nitrite and subsequently to nitrate, which thereby interlinks the aerobic with the anaerobic processes of the nitrogen cycle. The bulk of environmental nitrification is performed by chemolithoautotrophic ammonia or nitrite oxidizers that are basically found in all oxygenated environments (Prosser, 1989).

In soils, the nitrification rates are usually determined by multiple factors, including substrate availability, aeration, pH and temperature (Dancer *et al.*, 1973; Breuer *et al*., 2002). The anthropogenic input of excess ammonia by fertilization facilitates nitrification and causes a pH decline, due to the release of H^+ ions during ammonia oxidation. Ultimately, this leads to a decrease of the cation exchange capacity and therefore to a severe loss in fertility of the soil (Barak *et al.*, 1997). The products of ammonia oxidation - nitrite and nitrate - are negatively charged, soluble and highly mobile, thus easily washed out by rainfall events into groundwater, rivers and ultimately the sea. This is the reason why specific nitrification inhibitors like 3,4-Dimethylpyrazole phosphate are added to fertilizers, leading to reduced nitrification rates and an increase of residence time of bioavailable nitrogen in soils (Amberger, 1989; Zerulla *et al.*, 2001; Moran *et al.*, 2012).

Not all nitrification processes are conducted by autotrophs. Heterotrophic nitrifiers (fungi, bacteria, archaea, and algae) convert various organic and inorganic nitrogen containing compounds to nitrate. However, it is still unclear whether these processes yields energy and how much they contribute to the global nitrogen cycle (Bock and Wagner, 2006).

While nitrification is not desired in agriculture, it is a key step in removing the excess nitrogen from sewage in wastewater treatment. In comparison to natural aquatic systems, wastewater is strongly enriched in diverse forms of nitrogen. A large fraction of nitrogen is present as

ammonia, which primarily originates from the breakdown of organic material (Wagner *et al.*, 2002; Schmidt *et al.*, 2003)

Without sewage treatment, excess nitrogen would be introduced into natural environments and lead to eutrophication events, such as toxic algae blooms and oxygen depletion events (Anderson *et al*., 2002). In order to prevent the perturbation of the environment with nitrogen, one strategy is sequential nitrification and denitrification, which results in the conversion of bioavailable nitrogen to N_2 (Dinçer and Kargi, 2000).

4.1.3. Denitrification

$$NO_3^- + 2\ H^+ \rightarrow NO_2^- + H_2O$$

$$NO_2^- + 2\ H^+ \rightarrow NO + H_2O$$

$$NO + 2\ H^+ \rightarrow N_2O + H_2O$$

$$N_2O + 2\ H^+ \rightarrow N_2 + H_2O$$

Microorganisms reducing nitrate to nitrite, nitric oxide, nitrous oxide and finally dinitrogen are consolidated to the functional guild of denitrifiers. Denitrifying capabilities are widely distributed among a phylogenetically diverse set of microorganisms, which are usually performing this process in a variety of oxygen depleted environments, i.e. soils, groundwater, wetlands, oxygen minimum zones and sediments (Braker *et al*., 1998; Jones *et al.*, 2008).

Generally, denitrification is perceived as the counterpart to nitrogen fixation, since it removes bioavailable and reactive nitrogen by its transformation into gaseous nitrogen compounds (Ward *et al.*, 2009). As each individual denitrification step is conducted independently, N_xO_x intermediates are released (Tavares *et al.*, 2006). Nitric and nitrous oxide are highly volatile substances and potential greenhouse gases. Their impact on the global climate is estimated to be about two orders of magnitude more severe than the effect of carbon dioxide. In the atmosphere, nitrous oxide is photo-chemically oxidized to nitric oxide, which reacts with ozone to nitrite, destroying the earth´s sensitive UV-protection layer (Lashof and Ahuja, 1990). Since the release of denitrification intermediates increases if electron donors become limiting, the amount of released denitrification intermediates in wastewater treatment, where denitrification is industrially utilized, is controlled by assuring the availability of sufficient electron donors, i.e. by the addition of ethanol, methanol or acetate (Isaacs and Henze, 1995).

4.1.4. Dissimilatory nitrate reduction to ammonia (DNRA)

$NO_3^- \rightarrow NO_2^-$

$NO_2^- \rightarrow NH_4^+$

Another respiratory pathway of reducing nitrate is dissimilatory nitrate reduction to ammonia (DNRA). It is a shortcut in the nitrogen cycle, because the thereby produced ammonia can subsequently be reused for assimilation or by nitrifiers and anammox organisms as a substrate. Hence DNRA prolongs the residence time of bioavailable nitrogen in the environment (An and Gardner, 2002).

Similar to the ability for denitrification, DNRA is relatively widespread among bacteria and also found in some fungi (Takaya, 2002). The whole process is conducted in two steps, first nitrate is reduced to nitrite and subsequently in the second step to ammonia by the nitrite reductase NrfA, without liberating any intermediates (Simon, 2002). Compared to denitrification, eight instead of five electrons are transferred onto nitrogen, making this metabolism especially suited for environments with a surplus of electron donors over limited electron acceptors (Tiedje *et al.*, 1983). DNRA accounts for up to 30% of the nitrate reduction in some environments, indicating a relatively high and previously unknown importance of this process for the global nitrogen cycle (Giblin *et al.*, 2013).

4.1.5. Anammox

$NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$

In his article about missing lithotrophs in 1977, Engelbert Broda predicted a potential chemolithotrophic lifestyle by the anaerobic oxidation of ammonia coupled to the reduction of nitrite - anammox (Broda, 1977). It took microbiologists two decades to find organisms capable of this process, although it is energetically more favorable than aerobic nitrification (Van de Graaf *et al.*, 1995). Phylogenetically, all so far discovered anammox organisms form the monophyletic group of *Candidatus* Brocadiales within the *Planctomycetes*. Due to the high toxicity and reactivity of hydrazine, a key intermediate of the anammox metabolism, anammox bacteria are compartmentalized, and anammox is conducted in the so called ‘anammoxosome’. To increase the impermeability of their membranes, all members of *Candidatus* Brocadiales possess ladderane lipids with cyclobutane rings in their head structure (Strous *et al.*, 2006). Furthermore, because of the oxygen sensitivity of hydrazine, anammox bacteria are restricted to anoxic environments (Kalvelage *et al.*, 2011). Since its discovery, anammox is heavily

applied in wastewater treatment plants in the form of e.g. DEMON© reactors. Compared to the prior mentioned sequential nitrification/denitrification process, anammox reactors are not dependent on an addition of electron donors, as in contrast to many denitrifiers, anammox bacteria are autotrophic. Additionally, energy for aeration is saved, since only half of the reduced nitrogen compounds have to be oxidized to nitrite (Kuenen, 2008).

4.2. Nitrification – processes and microorganisms

More than a century ago, Sergei Winogradsky described nitrification as a two-step process, conducted by two different functional guilds. He obtained the first pure cultures of *Nitroso*monas, an ammonium oxidizing bacteria (AOB) and *Nitro*bacter, a nitrite oxidizing bacteria (NOB) (Nitroso = ammonia oxidizers; Nitro = nitrite oxidizer). Those two model organisms shaped the research of nitrification for almost one hundred years (Meiklejohn, 1950; Zavarzin and Legunkova, 1959). The isolation of nitrifying microorganisms is difficult and very time consuming, due to their slow growth rates and high susceptibilities to contaminations. Consequently, there are still few cultured representatives of AOB and NOB, especially of those adapted to the low substrate concentrations occurring in nature (Ehrich *et al.*, 1995; Könneke *et al.*, 2005; Hatzenpichler *et al.*, 2008).

4.2.1. Ammonia oxidation

The oxidation of ammonia to nitrite was considered to be the rate limiting step of nitrification, which is one of the reasons why it has received more attention in the past than nitrite oxidation (Kowalchuk and Stephen, 2001). Chemolithoautotrophic ammonia oxidizing bacteria and ammonia oxidizing archaea (AOA) are conducting this process in practically all aerobic environments (Martens-Habbena *et al.*, 2009).

4.2.1.1. Ammonia oxidizing bacteria

AOB form two monophyletic clades within the *Betaproteobacteria* and the *Gammaproteobacteria.* While gammaproteobacterial AOB usually occur in marine environments, the betaproteobacterial *Nitrosomonadaceae* are found in diverse habitats such as soils, WWTPs, as well as marine and freshwater systems. The latter family consists of two genera, *Nitrosomonas* typically thriving in freshwater systems and *Nitrosospira* predominantly living in soils (Kowalchuk and Stephen, 2001).

Canonical AOB first oxidize ammonia to hydroxylamine by a membrane bound ammonia monooxygenase (AMO) and subsequently to nitrite with an periplasmic hydroxylamine

dehydrogenase (HAO) (Whittaker *et al.*, 2000). Similar to other monooxygenases, the AMO oxidizes not only ammonia, but also many other substrates such as phenol, which is the reason why AOB are also heavily applied in bioremediation processes (Rasche *et al.*, 1991). The HAO channels the four electrons, released by NH_2OH oxidation, into the respiratory chain of AOB (Igarashi *et al.*, 1997). Two of those electrons are needed for the activation of oxygen for the initial oxidation of NH_3 to NH_2OH, whereas the other two are available for energy conservation. Since the redox potential of NH_3/NO_2^- is below $NAD(P)^+$ /NAD(P)H, ammonia oxidizing microorganisms require an energy expensive reverse electron flow for the production of reduction equivalents (Klotz and Stein, 2008). Generally, this is achieved by utilizing the energy from the proton motive force, to channel low potential electrons in a reverse direction through the electron transport chain, to derive the required high potential electrons (Whittaker *et al.*, 2000). While AOB are autotrophs, the co-assimilation of small amounts of organic carbon i.e. in the form of glucose, acetate and pyruvate was observed in several independent studies on different AOB species (e.g. Martiny and Koops, 1982). Still, the bulk of carbon that is used for the production of cellular components is most probably fixated from CO_2 via the Calvin-Benson-Bassham cycle (Arp *et al*., 2007).

4.2.1.2. Ammonia oxidizing Archaea

With the help of metagenomics and extensive efforts in cultivation, one of the major paradigm shifts in AOM research happened only a decade ago – with the discovery of ammonia oxidizing archaea (AOA) (Könneke *et al.*, 2005). These organisms occur in relatively high abundances on both terrestrial as well as in marine environments, indicating their global importance for nitrogen cycling (Francis *et al.*, 2005; Leininger *et al.*, 2006; Park *et al.*, 2006).

The first cultured representative of AOA was *Nitrosopumilus maritimus* (Könneke *et al.*, 2005) In comparison to AOB, the ammonia monooxygenase of this organism has a relatively low K_m, thus underlining the environmental distribution of AOA to environments with low ammonia concentrations (Martens-Habbena *et al.*, 2009). The pathway of ammonia oxidation by AOA is still not completely understood, since no HAO homologue has been found so far within their genomes. Intriguingly, they are inhibited by NO scavengers, like PTIO (2-Phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide), suggesting a putative role of NO as intermediate for the catabolic ammonia oxidation pathway (Shen *et al.*, 2013). Recent models propose that hydroxylamine together with NO is oxidized by a “Copper Heme P460” like enzyme, which hypothetically channels 3 electrons instead of 2 by AOB into the quinone - quinol cycle.

Furthermore, the reaction of NO with NH_2OH may spontaneously form N_2O and therefore explain the increased nitrous oxide emissions of AOA (Kozlowski *et al.*, 2016).

Like AOB, AOA are also autotrophs, and use an altered version of the 3-hydroxypropionate / 4-hydroxybutyrate pathway, considered to be highly energy efficient, for carbon fixation (Berg *et al.*, 2007).

4.2.2. Nitrite oxidation

In the second step of nitrification, nitrite is oxidized to nitrate by the functional group of NOB, which are also chemolithoautotrophs. Often nitrite oxidizing bacteria are found in close association with AOM, explaining the rapid consumption and rare accumulation of nitrite in oxic environments (Gruber-Dorninger *et al.*, 2014). However nitrite is not only produced by AOM, but also during nitrate reduction, which is the reason why NOB also have an important regulatory role for nitrogen removal in the global nitrogen cycle (Füssel *et al.*, 2012).

4.2.2.1. Physiology of NOB

Generally, the oxidation of nitrite by chemolithoautotrophic organisms yields less energy than ammonia oxidation ($\Delta G^{\circ\prime} = -74.1$ kJ mol^{-1}). It is catalyzed by nitrite oxidoreductase (Nxr), a membrane associated protein complex, consisting of two to three subunits (alpha, beta, gamma) (Starkenburg *et al.*, 2006; Lücker *et al.*, 2010). The substrate binding site with the catalytic center of the holoenzyme is located in the alpha subunit, whereas the beta subunit channels the released two electrons to the gamma subunit or directly into the respiratory chain (Meincke *et al.*, 1992). The Nxr of *Nitrospira*, *Nitrospina* and *Candidatus* Nitromaritima is orientated towards the periplasmic space, releasing the H^+ ions of nitrite oxidation with H_2O on the "correct" site of the membrane. In the case of *Nitrobacter*, *Nitrococcus* and *Nitrolancea* the toxic nitrite must first be transported into the cytoplasm for the subsequent oxidation. The released protons are then transported by terminal oxidases to the periplasmic space, resulting in a relatively inefficient way for nitrite oxidation. Similar to AOM, all NOB need a reverse electron flow to generate redox equivalents, since the redox potential of NO_2^- / NO_3^- is below $NAD(P)^+$ /NAD(P)H (Daims *et al*., 2016).

4.2.2.2. Phylogenetic distribution

Phylogenetically the functional group of NOB is relatively widespread in the bacterial domain, as it has been found in seven different genera of four different phyla (Daims *et al*., 2016).

The first isolated and probably still best studied group of NOB are *Nitrobacter* (*Alphaproteobacteria*), because in contrast to other NOB´s, members of this genus are relatively easy cultivable. Yet, in the environment, *Nitrobacter* is only of minor importance, since they are only competitive under extremely high nitrite concentrations, only found i.e. in engineered systems or decomposition sites of carcasses (Boon and Laudelout, 1962; Van Belle *et al*., 2009). The recently discovered NOB genus *Nitrotoga* (*Betaproteobacteria*) was first enriched from a permafrost soil of Siberia. It was proposed that members of this genus are generally well adapted to cold conditions (Alawi *et al.*, 2007). In wastewater treatment plants, they often coexist in low numbers with other NOB and form aggregates with AOM (Lücker *et al.*, 2014). *Nitrococcus mobilis* was the first isolated gammaproteobacterial NOB and originated from the south pacific, with close relatives found in engineered ecosystems and soils (Watson and Waterbury, 1971; Daims *et al*., 2016). The only gram positive NOB that so far has been discovered belongs to the phylum of *Chloroflexi* and is named *Nitrolancea hollandica.* In comparison to most other NOB, it grows on agar plates. Furthermore, this microorganism is not restricted to nitrite oxidation and can use formate as an alternative energy source to nitrite (Sorokin *et al.*, 2014). In the marine environment, members of the genera *Nitrospina* and *Candidatus* Nitromaritima (*Nitrospinae*) are the most widely distributed NOB. They thrive especially well in oxygen minimum zones, where they account up to a third of the microbial community (Ngugi *et al.*, 2015).

4.2.3. The genus *Nitrospira*

The genus *Nitrospira* belongs to the phylogenetically deep branching phylum of *Nitrospira* and contains the most commonly found and diverse NOB according to our current knowledge (You *et al.*, 2003; Kanokratana *et al.*, 2004; Maixner *et al.*, 2006; Hongxiang *et al.*, 2008; Xia *et al.*, 2011). *Nitrospira* are classified into six different lineages, with cultured representatives in each lineage, except lineage III. The currently used similarity threshold of 16S rRNA genes to differentiate two lineages is 94% sequence identity (Daims *et al.*, 2001).

4.2.3.1. The cultivated lineages of *Nitrospira*

Strain ND1 and *Nitrospira defluvii* were isolated from a WWTP and belong to lineage I. No genes for the detoxification of oxygen radicals were found in the latter ones genome. This indicates that this species might have a dependence on symbiotic partners for oxygen radical detoxification, and might originate from microaerophilic or even anaerobic ancestors (Lücker *et al.*, 2010).

Nitrospira moscoviensis, *N. japonica* and *N. lenta* are affiliated with lineage II, which currently is considered to be the environmentally most widespread lineage among the *Nitrospira* (Ushiki *et al.*, 2013). Although *Nitrospira* were perceived to be metabolically restricted to nitrite oxidation, it was recently shown that *N. moscoviensis* is able to use formate or hydrogen as alternative substrates (Koch *et al.*, 2014, 2015).

Lineage IV contains the cultured representatives *N. marina* and *N. ecomares*, two marine NOB. The first species was isolated from the Gulf of Maine and described to be a curved rod that grows best in mixotrophic media (Watson *et al.*, 1986). The second species was initially thriving in a marine recirculating aquaculture system (RAS) filter and, similar to many other *Nitrospira*, has a tendency to biofilm formation (Keuter *et al.*, 2011).

Like *N. moscoviensis*, *Ca.* Nitrospira bockiana was isolated from corrosion deposits of the Moscow heating system and is the only cultured representative of lineage V (Lebedeva *et al.*, 2008).

Nitrospira calida originates from a geothermal mat and is affiliated with *Nitrospira* lineage VI (Lebedeva *et al.*, 2011). Closely related *Nitrospira* 16S rRNA gene sequences were retrieved from a similar geothermal spring, indicating that this lineage is putatively adapted to hot environments (Edwards *et al.*, 2013).

4.2.3.2. Interactions with other microorganisms

In various environments, different *Nitrospira* lineages have been shown to coexist. For example, co-occurrence of diverse *Nitrospira* affiliated with lineage I and II was observed in WWTPs, an at the first glance almost homogeneous habitat. Yet, activated sludge contains many so called microniches where similar species can co-exist (Gruber-Dorninger *et al.*, 2014). Another possible explanation for this co-occurrence is given by interactions with other microbes. For instance, many *Nitrospira* species have the enzymatic capability to produce ammonia from urea or cyanate, and thereby interact with AOM through reciprocal feeding (Koch *et al.*, 2015; Palatinszky *et al.*, 2015). In addition, they excrete organic compounds, which can facilitate the growth of heterotrophic microbes within their floc like colonies. These heterotrophs might engage in mutualistic interactions by reducing nitrate to nitrite, or simply detoxify superoxide radicals for the NOB (Kindaichi *et al.*, 2004). However not all interactions of *Nitrospira* with other microorganisms are mutualistic. For example, *Micavibrio*-like bacteria were shown to graze on *Nitrospira* in wastewater treatment plants (Dolinšek *et al.*, 2013).

4.2.3.3. Suspiciously high abundance of *Nitrospira* in certain habitats

The often observed high abundance of *Nitrospira* species, compared to known AOM in oligotrophic and oxygenated habitats like sand filtration systems and karst cave mantles, was enigmatic, since nitrite oxidation, as already mentioned, yields way less energy than ammonia oxidation (Martiny *et al.*, 2005). Also, these are oligotrophic and well oxygenated environments that are depleted in organic carbon, hence nitrate reduction as a source of nitrite might play only a minor role (Pronk *et al.*, 2009). Alternate metabolisms, like hydrogen oxidation or formate oxidation were only shown to occur under anaerobic conditions (Koch *et al.*, 2014, 2015). Thus, the most reasonable answer to this previously unresolved observation might be given by the recent discovery of complete ammonia oxidation to nitrate - comammox among the *Nitrospira* (Daims *et al.*, 2015; van Kessel *et al.*, 2015).

4.2.4. Prediction and discovery of comammox

The obligate division of nitrification into two consecutive steps, conducted by two different types of microorganisms was a riddle for a long time. Especially since complete ammonia oxidation to nitrate would result with more energy gain for the microbes. According to the kinetic theory of optimal pathway length, short pathways are preferential in nitrification, due to the relatively low energy gain in the second step (nitrite oxidation) and the high toxicity of all intermediates. It suggests that cross feeding nitrification leads to higher ATP production rates, whereas comammox organisms utilize ammonia more economically and have a higher growth yield per mol ammonia consumed. In diffusion and substrate limited cell clusters, like biofilms and oligotrophic environments, a comammox lifestyle might therefore be beneficial compared to two step nitrification (Costa *et al*., 2006).

More than a century after the pioneering studies of Sergei Winogradsky, the dogma on the obligate division of nitrification into two consecutive steps was ultimately declared invalid. With tremendous cultivation efforts, in combination with state of the art sequencing techniques, the first comammox organisms were found within the *Nitrospira*. Formerly, this genus was known for being among the most predominant nitrite oxidizing bacteria, a precept that led to overlooking their function as ammonia oxidizers for so many years. *Ca.* Nitrospira nitrificans, *Ca*. N. nitrosa and *Ca*. N. inopinata are the first cultivated comammox organisms. The first two species were enriched using a bioreactor and originated from filtration device of an recirculating aquaculture system, whereas *Ca*. N. inopinata was enriched by batch cultivation from an abundant oil drilling well in Aushiger (Russia) (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Cultivation independent approaches have also identified the presence of comammox *Nitrospira*

in drinking water systems, rapid gravity sand filters, soils and WWTPs (Daims *et al.*, 2015; van Kessel *et al*., 2015; Pinto *et al.*, 2015a; Palomo *et al.*, 2016).

4.2.4.1. Physiology of comammox *Nitrospira*

According to metagenomics, all three comammox *Nitrospira* species have a pathway for complete oxidation of ammonia to nitrite. The first step of nitrification is achieved by an ammonia monooxygenase (AMO) and a hydroxylamine dehydrogenase (HAO), both homologous and closely related to the enzymatic set of *Betaproteobacteria*. Subsequently, the oxidation of nitrite is achieved by a periplasmic, *Nitrospira* like nitrite oxidoreductase (Nxr). However *Ca*. Nitrospira inopinata cannot grow by solely oxidizing nitrite. The reason for this is most likely the lack of a second copy of respiratory complex III genes, needed for a high potential reverse electron flow to generate redox equivalents by oxidizing nitrite (Daims *et al.*, 2015).

4.3. Functional and phylogenetic marker gene assays

Marker gene based approaches rely on the detection and quantification of genes, encoding for key enzymes or phylogenetic marker genes of certain microbial groups (Leininger *et al.*, 2006; Meyer and Kuever, 2007). Functional gene based studies, especially in combination with next generation amplicon sequencing are powerful tools to investigate the importance and diversity of low abundant microorganisms. Additionally, functional genes which have not been heavily affected by horizontal gene transfer, are also good phylogenetic markers (Pester *et al.*, 2013).

The gene for the 16S rRNA is the most extensively used phylogenetic marker for studying microbial community compositions (Singleton *et al.*, 2001; Caporaso *et al.*, 2011). The beta subunit of the Nxr, encoded by *nxrB* gene showed a congruent evolution with the 16S rRNA genes of *Nitrospira* and due to less sequence conservation, has a higher phylogenetic resolution (Pester *et al.*, 2014). However all so far discovered comammox *Nitrospira* are classified into lineage II of *Nitrospira*, without forming a monophyletic clade. Since this lineage also harbors canonical NOB, neither the 16S rRNA gene nor the *nxrB* gene are suitable phylogenetic markers and cannot provide valuable ecological information on the environmental distribution of comammox *Nitrospira* (e.g. Pinto *et al.*, 2015).

4.3.1. The *amoA* - a functional and phylogenetic marker gene for comammox *Nitrospira*

The operon for the ammonia monooxygenase of comammox *Nitrospira* is structured, as in AOB, into *amoCAB* (Rotthauwe *et al*., 1997; Daims *et al.*, 2015). The alpha subunit is encoded

by the *amoA* gene which has been extensively used as a functional and phylogenetic marker gene in AOM research. The number of publicly available *amoA* sequences has thereby increased tremendously, since the publication of the first *amoA* sequence of *Nitrosomonas europaea* (McTavish *et al*., 1993). Therefore, the low resolution of this gene, due to its high conservation and short length of approximately 840 - 900 bp, is outweighed by the availability of huge reference datasets (Junier *et al.*, 2010).

A phylogenetic classification of available full length comammox *amoA* sequences within the *amoA* superfamily, showed that apparently two monophyletically clades are existing, currently referred to as comammox *Nitrospira* clade A and B. The closest relatives to this sister clades are canonical betaproteobacterial AOB *amoA* sequences, and genes encoding for the alpha subunits of particulate methane monooxygenases (*pmoA*). A screening of publicly available metagenomes for comammox *amoA* revealed that it is more widespread than initially predicted, as they have been found in a diverse set of soils, freshwater environments and in engineered systems (Daims *et al.*, 2015). However even deeply sequenced metagenomic datasets may underestimate the real environmental distribution of comammox *Nitrospira*, as the whole genus is generally low abundant and the cells are conceived to be difficult to lyse (Altmann *et al.*, 2003; Albertsen *et al.*, 2015).

It is important to keep in mind the neither the presence, nor the absence of functional marker genes are a guarantee for the metabolic activity of the respective pathway. One good example for this was found during a study of *Thaumarchaeota* in WWTPs. Despite the high abundance and expression of thaumarchaeal *amoA* genes, only AOB were oxidizing ammonia and performing carbon fixation (Mussmann *et al.*, 2011). Furthermore, as functional gene studies are based on the previous amplification of the marker genes by PCR, the resolution of these approaches are limited by the coverage of the respective primer pairs and hardly detect novel genes distantly related to the reference sequences on which the primers are based (Bourne *et al*., 2001).

4.4. Aims

To get a more detailed perspective on the actual environmental distribution of comammox *Nitrospira*, two highly specific PCR primer pairs were designed, targeting comammox *Nitrospira* clade A and B individually (see also: Pjevac *et al.*, 2016). In this study, I evaluated and applied these primer sets on various environmental DNA extracts, to gather novel comammox *Nitrospira amoA* sequences and increase our knowledge about their environmental distribution and diversity.

Primer design for protein encoding genes is often difficult, due to the degenerated code and the lack of a highly variable region between two conserved periphery regions (Rose *et al.*, 1998). To counteract this and increase the coverage of primer sets, so called degenerated primer sets are used that include ambiguous nucleotides within their sequence (Gaby and Buckley, 2012). However, these wobble positions lead to uncertainties of individual primer variant concentrations, alongside with a series of nonsense primer variants that are not covering any known reference sequence and ultimately lead to a dilution of the primer variants with high coverages. Therefore I established equimolar pools of all primer variants with high coverages to the reference dataset and compared them to degenerated primer sets with proportionate coverages by quantitative PCR.

The protocol used from DNA extraction is also known to alter the observed microbial community composition (Albertsen *et al.*, 2015). This is of interest here, since *Nitrospira* cells might be affected, due to biofilm formations, floc like colony structures and rigid layers of extracellular polymeric substance formed around their cell envelopes (Ehrich *et al.*, 1995). Thus, I evaluated the influence of two DNA extraction protocols with different bead beating intensities on the observed comammox *Nitrospira* abundance by qPCR.

5. Material and Methods

5.1. Solutions and Material

All solutions and buffers which have been used in this study were prepared with MQ water and if possible autoclaved at 121 °C for 20 min.

5.1.1. Solutions for total nucleic acid (TNA) extraction:

Table 1: PEG 8000, 30% (w/v)

Ingredient	Manufacturer / Additional Information
PEG 8000 300 g/L	Sigma-Aldrich, 81268
NaCl 93.5 g/L	Roth, 99.5%, 3957.2

Table 2: Phosphate Buffer, 120 mM, pH ~ 8

Ingredient	Manufacturer / Additional Information
Na_2HPO_4 x $2H_2O$ 20.09 g/L	Roth, 99.5%, 4984.1
NaH_2PO_4 x H_2O 0.9824 g/L	Roth, 98%, T878.1

Table 3: Tris/EDTA (TE) buffer, pH ~ 8

Ingredient	Manufacturer / Additional Information
Tris 1.2 g/L	Roth, 99.3%, AE15.3
EDTA 0.37 g/L	Roth, 99%, 8043.2

Table 4: Tris/NaCl/SDS (TNS) buffer, pH ~ 8

Ingredient	Manufacturer / Additional Information
Tris x HCl 78.8 g/L	Roth, 99%, 9090.1
NaCl 5.844 g/L	Roth, 99.5, 3957.2
SDS 100 g/L	Roth, 99%, 4360.2

Table 5: Other solutions

Name	Manufacturer / Additional Information
Ethanol, 70%	Merck, molecular grade, 1.08543.0250
Glycogen, RNA grade (20 mg/ml)	Fermentas
Roti-Phenol pH 8,	Roth, 0038.2
Roti-phenol/chloroform/isoamyl-alcohol 25/24/1, pH 8	Roth, A156.2
Roti-Chloroform/Isoamyl-alcohol 24/1, pH 8,	Roth, X984.3

5.1.2. Reagents and solutions for PCR and gel electrophoresis

Table 6: PCR / qPCR reagents

Name	Manufacturer / Additional Information
Bovine Serum Albumin, 20 mg/mL	BSA, Fermentas, B14
ddH_2O (PCR water)	Frensenius water, Kabi
dNTPs, 2mM	Fermentas, R0242
diverse primer sets	Biomers
DreamTaq Green DNA Polymerase, 5 U/µL	Thermo Fisher Scientific, EP0712
DreamTaq Green Buffer, 10 x	Thermo Fisher Scientific, B71
PerfeCTa SYBR Green FastMix, 2 x	Quanta Bioscience Inc, 733-1249

Table 7: Tris/Borate/EDTA (TBE) buffer, pH ~ 8.5

Ingredient	Manufacturer / Additional Information
Tris 89 mM/L	Roth, 99.3%, AE15.3
Boric acid 89 mM/L	Roth, 99.8%, 6943.1
Na_2EDTA x 2H_2O 2 mM/L	Roth, 99%, 8043.2

5.1.2.3. Table 8: Other solutions

Name	Manufacturer / Additional Information
GelRed Nucleic Acid Stain 10.000 x in DMSO	Biotium, 41002
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific, SM0313
LE Agarose	Biozym, 840004

5.1.3. Antibiotics and Media for cloning

Table 9: Antibiotics

Name	Manufacturer / Additional Information
Ampicillin 100 mg/ml	Sigma-Aldrich, A0166-5g
Kanamycin 100 mg/ml	Sigma-Aldrich, K4000-5g

Table 10: Lysogeny broth (LB) medium

Ingredient	Manufacturer / Additional Information
NaCl 5 g/L	(Roth, 99.5%, 3957.2)
Tryptone 10 g/L	Oxoid, LP0042B
Yeast Extract 5 g/L	Oxoid, LP0021
Agar 20 g/L	Bacto, 214010

5.1.4. Consumables

Table 11: Consumables

Name	Manufacturer / Additional Information
50 ml tubes, PP, DNAse and RNAase free	Greiner
96 well plates, flat bottom, transparent	Greiner
Eppendorf tubes, PP, various sizes	Biozym, Eppendorf, Greiner
Hard-Shell Thin-Wall 96-Well Skirted PCR Plates	Bio-Rad
Lysing matrix tubes	MP Biomedicals
Microseal 'B' Adhesive Seals	Bio-Rad
PCR plates, 96 well, skirted	Eppendorf
Petri dish, 90mm diameter	Sterilin
Pipette tips, various sizes, with filter	Bio-Rad

5.1.5. Instruments, Kits and Software

Table 12: Instruments

Name	Manufacturer / Additional Information
BL3100 (scale)	Sartorius
C 1000 CFX96 Touch™ Real-Time PCR Detection System	Bio-Rad
Centrifuge 5804R	Eppendorf
Eppendorf Pipettes, various volumina	Eppendorf
NanoDrop ND-1000	Thermo Fisher Scientific
ProfiLine pH 3110 (pH-meter)	WTW
RCT Basic (magnetic stirrer/hot plate)	IKA-Werke
Sub-Cell GT (agarose gel electrophoresis system)	Bio-Rad
T100 PCR (thermocycler)	Bio-Rad
UST-C30M-8R UV Transilluminator	Biostep
UV Sterilizing PCR Workstation	PEQLAB
Vortex Genie 2	Scientific Industries
Infinite M200 Plate reader	TECAN
Gel Doc XR+	Bio-Rad
MQ Biocel (water source for ddH_2O)	Merck Millipore

Table 13: Kits

Name	Manufacturer / Additional Information
PowerSoil DNA Isolation Kit	Mobio
QIAquick PCR purification kit	Qiagen
Quant-iT PicoGreen dsDNA Assay Kit	Thermo Fisher Scientific
TOPO TA Cloning Kit, pCR 4-TOPO Vector	Thermo Fisher Scientific
DreamTaq Green DNA Polymerase	Thermo Fisher Scientific

Table 14: Software

Name and Version	Webpage and Reference
ARB v6.0.5	http://www.arb-home.de/
Chromas lite 2.5.0	http://technelysium.com.au/wp/chromas/
CFX Manager v.3.1	http://www.bio-rad.com/en-us/product/cfx-manager-software
Image Lab 5.2	http://www.bio-rad.com/en-us/product/image-lab-software

5.1.6. Primer sequences and sets

Table 15: Comammox amoA clade A primer set

Primer Name	Head sequence	Primer sequences 5' - 3'
Comammox clade A *amoA* forward primers		
H_ComaA_F_a	GCTATGCGCGAGCTGC	TACAACTGGGTGAACTA
H_ComaA_F_b	GCTATGCGCGAGCTGC	TATAACTGGGTGAACTA
H_ComaA_F_c	GCTATGCGCGAGCTGC	TACAATTGGGTGAACTA
H_ComaA_F_d	GCTATGCGCGAGCTGC	TACAACTGGGTCAACTA
H_ComaA_F_e	GCTATGCGCGAGCTGC	TACAACTGGGTCAATTA
H_ComaA_F_f	GCTATGCGCGAGCTGC	TATAACTGGGTCAATTA
Comammox clade A *amoA* reverse primers		
H_ComaA_R_a	GCTATGCGCGAGCTGC	AGATCATGGTGCTATG
H_ComaA_R_b	GCTATGCGCGAGCTGC	AAATCATGGTGCTATG
H_ComaA_R_c	GCTATGCGCGAGCTGC	AGATCATGGTGCTGTG
H_ComaA_R_d	GCTATGCGCGAGCTGC	AAATCATGGTGCTGTG
H_ComaA_R_e	GCTATGCGCGAGCTGC	AGATCATCGTGCTGTG
H_ComaA_R_f	GCTATGCGCGAGCTGC	AAATCATCGTGCTGTG

Table 16: Comammox *amoA* clade B primer set

Primer Name	Head sequence	Primer sequences 5' - 3'
Comammox clade B *amoA* forward primers		
H_ComaB_F_a	GCTATGCGCGAGCTGC	TAYTTCTGGACGTTCTA
H_ComaB_F_b	GCTATGCGCGAGCTGC	TAYTTCTGGACATTCTA
H_ComaB_F_c	GCTATGCGCGAGCTGC	TACTTCTGGACTTTCTA
H_ComaB_F_d	GCTATGCGCGAGCTGC	TAYTTCTGGACGTTTTA
H_ComaB_F_e	GCTATGCGCGAGCTGC	TAYTTCTGGACATTTTA
H_ComaB_F_f	GCTATGCGCGAGCTGC	TACTTCTGGACCTTCTA
Comammox clade B *amoA* reverse primers		
H_ComaB_R_a	GCTATGCGCGAGCTGC	ARATCCAGACGGTGTG
H_ComaB_R_b	GCTATGCGCGAGCTGC	ARATCCAAACGGTGTG
H_ComaB_R_c	GCTATGCGCGAGCTGC	ARATCCAGACAGTGTG
H_ComaB_R_d	GCTATGCGCGAGCTGC	ARATCCAAACTGTGTG
H_ComaB_R_e	GCTATGCGCGAGCTGC	AGATCCAGACTGTGTG
H_ComaB_R_f	GCTATGCGCGAGCTGC	AGATCCAAACAGTGTG

Table 17: Equimolar pooled primer sets

Pool name	Variants used
H_ComaA_F equi	a,b,c,d,e,f
H_ComaA_R equi	a,b,c,d,e,f
H_ComaB_F equi	a,b,c,d,e,f
H_ComaB_R equi	a,b,c,d,e,f
ComaA_F abd	a,b,d (w.o. head)
ComaA_R abcd	a,b,c,d (w.o. head)
ComaA_F equi	a,b,c,d,e,f (w.o. head)
ComaA_R_equi	a,b,c,d,e,f (w.o. head)

Table 18: Degenerated primer sets

Primer Name	Primer sequences 5' - 3'
ComaA 1F	**TACAATTGGGTGAACTA**
ComaA 1R	**ARATCATSGTGCTRTG**
ComaA 2F	**TAYAAYTGGGTSAAYTA**
ComaA 2R	**ARATCATSGTGCTRTG**
H_ComaA 2F	(GCTATGCGCGAGCTGC) **TAYAAYTGGGTSAAYTA**
H_ComaA 2R	(GCTATGCGCGAGCTGC) **ARATCATSGTGCTRTG**

Table 19: M13 primer set

Primer Name	Primer sequences 5' - 3'
M13 F	GTAAAACGACGGCCAG
M13 R	CAGGAAACAGCTATGAC

5.2. Sample collection

For this study, I used an iron sludge sample recovered from the riser pipe of a groundwater well (GWW) in Wolfenbüttel (Germany) that has been previously characterized by metagenomics, and was shown to contain comammox *Nitrospira* affiliated both to clade A and clade B (Daims *et al*., 2015).

In addition, Dr. Bernd Bendinger (DVGW-Forschungsstelle TUHH) provided filter material from two drinking water treatment plants (DWTP). The first samples originated from the DWTP Friedrichshof (Germany) and consisted of quartz sand covered with manganese-oxides and microbial biomass, whereas the sample from DWTP Spiekeroog (Germany) contained Styrofoam beads that were covered by a thin layer of microbial biofilm.

Furthermore, I used activated sludge samples from the wastewater treatment facility of the University of Veterinary Medicine, Vienna (VetMed, Austria) and the municipal full scale WWTP in Ingolstadt (Germany). These samples were previously analyzed by Daims *et al*., 2015 (VetMed) and Gruber-Dorninger *et al*., 2014 (VetMed and Ingolstadt).

Further samples analyzed in this study were provided by several colleagues from the division of microbial ecology (DOME). Rice bulk soil and rice rhizosphere are two samples obtained from an experiment with rice plants growing on rice field soil from Vercelli (Italy). Rice bulk soil was sample material of the anaerobic and root free compartment of a box experiment stored at -20°C prior to analysis. In order to separate the rhizosphere from the bulk soil the roots were extracted and cut off. Immediately afterwards, the roots were shaken in a 50 ml tube containing 45 ml of phosphate buffer, orientated in a horizontal position (4 °C, 200 rpm, 10 min). Subsequently, the roots were removed and the tube centrifuged (8000 rpm, 10 min, 4 °C). After discarding the supernatant, the rhizosphere pellet was frozen and used for further analysis. These two samples were prepared and provided by Dr. Hannes Schmidt. A litter free soil sample from the top 5 cm of a beech forest in Klausen-Leopoldsdorf (Austria) was provided by Dr. Stephanie Eichorst. This soil was sieved through 2 mm mesh and stored at -20 °C as described in Eichorst *et al*. (2015). Furthermore, the top 10 cm of a sediment core from the alkaline lake Herrensee (Austria) were provided by Dr. Anne Daebeler. The sediment was also sieved through a 2 mm mesh and subsequently incubated for 7 days under nitrifying conditions in a 1:10 slurry of sediment and sterile filtered lake water. In addition, a sample from an enrichment culture, established by incubating river sediment of the Schwarza river (Austria) with 1 mM/L NH_4Cl as substrate, was provided by Dr. Julia Vierheilig.

5.3. DNA extraction

5.3.1. Phenol/Chloroform total nucleic acid (TNA) extraction

The majority of DNA extractions were done by using a bead beating based phenol/chloroform protocol (see Angel, 2012). For this approximately 0.3-0.6 g of sample was weight into lysis tubes and set on ice. Subsequently, 375 µl of Buffer PB, 125 µl of TNS and 400 µl of saturated Roti-Phenol were added and immediately processed by bead beating at a speed of 6.5 m/s for 30 seconds, using a dry ice cooled adapter. In order to separate the cell lysate and DNA from the sample was centrifuged (20.817 g, 3 min, RT) and the whole liquid phase was transferred into a fresh 2 ml Eppendorf tube (pp-tube). Subsequently, the first part of the protocol was repeated with the remaining sample in the lysis tube, in order to extract DNA from microorganisms with more rigid cell envelopes. In this study, the second bead beating step extract is referred to as the second fraction and was treated in the same way during the downstream process as the first fraction of the extraction. After recovering the liquid phases containing the total cell lysates into new pp-tubes, 800 µl of phenol/chloroform/isoamylalcohol was added to each of the pp-tubes and inverted several times, prior centrifugation (20.817 g, 3

min, RT). The overlying phases were again transferred into a new 2 ml pp-tube, mixed with 800 µl chloroform/isoamylalcohol and centrifuged (20.817 g, 3 min, RT). Again, the overlaying phase was transferred into a new 2 ml pp-tube and mixed with 1 ml of PEG-8000 solution and 1 µl of glycogen. Subsequently the tubes were centrifuged, in order to precipitate and pellet the DNA (20.817 g, 1 h, 4 °C). Supernatant was removed by pipetting and the pellet washed with 1 ml of ice-cold 70% ethanol. The DNA was then again pelleted by centrifugation (20.817 g, 20 min, 15 °C) and the ethanol removed by pipetting. In order to dry the DNA pellets, the pp-tubes were opened and vented under a DNA-sterile PCR hood until no visible liquid remained in the tubes. Finally, the DNA was dissolved in 30-100 µl of TE buffer or PCR grade water and stored at -20 °C.

5.3.2. MoBio PowerSoil Kit DNA extraction

The cell lysis of the MoBio PowerSoil DNA extraction Kit is also based on mechanical cell disruption. During this protocol, the lysis tubes of the Kit, containing lysis beads, extraction buffer, 0.3 gram of sample and 60 µl solution C1, were vortexed (Vortex Genie 2) at maximum speed for 10 min. Generally, the recommended protocol of the manufacturer was followed and the DNA finally eluted in 40-100 µl of solution C6 (which equals TE buffer) and stored at -20 °C. This method was applied to extract DNA from the river Schwarza enrichment culture, and for comparing the extraction methods on filter material from the DWTP Friedrichshof., VetMed and forest soil (Klausen-Leopoldsdorf).

5.4. DNA quantification

To get an estimate on DNA concentration and quality, 1 µl of sample was applied on the NanoDrop ND-1000. For more precise measurements, the DNA sample was then diluted to fall within the standard range of the Quant-IT PicoGreen Kit, which was used according to the manufacturer's instructions. Briefly, the DNA staining solution (1:200 PicoGreen-DMSO solution in TE buffer) was mixed with both standards and samples in a ratio of 10:1 and incubated for 5 minutes at room temperature in the dark. Fluorescence was then measured by using NanoDrop ND-3300, or the Infinite M200 plate reader (excitation: 480 nm; emission: 520 nm) and DNA concentrations calculated.

5.5. Data mining and *amoA* gene database construction in ARB

Dr. Craig Herbold and Dr. Petra Pjevac extracted bacterial *amoA* and *pmoA* sequences from publicly available full-length sequence depositories, by using functional profiler tool against *amoA*/*pmoA* pfam PF02461 (IMG-ER and –MER) and amended them with *amoA*/*pmoA*

sequences, recovered from NCBI Genbank during a previous study (Daims *et al.*, 2015). Subsequently, the sequences were filtered by hmmsearch (http://hmmer.janelia.org/ Expect<0.0001). Clustering the sequences at 90% identity was done with USEARCH (Edgar, 2010) and the resulting centroids aligned with MAFFT (Katoh *et al.*, 2002). A first phylogenetic classification of the centroids was performed by using the FastTree tool (Price *et al.*, 2009) and subsequently the cluster centroids expanded. Again, USEARCH was applied for re-clustering at 99% identity and phylogenetic affiliation the sequences (comammox / betaproteobacterial *amoA*) re-assessed using FastTree. A selection of nearly full-length comammox *amoA* and proteobacterial *amoA* and *pmoA* sequences were aligned by using MUSCLE (Edgar, 2004) and frameshifts manually corrected. Based on this alignment, a maximum likelihood reference tree was reconstructed with RAxML and 1000x bootstraps (Stamatakis, 2006). Subsequently, the tree was imported into the ARB software package (Ludwig *et al.*, 2004) and additional sequences added by maximum parsimony, without allowing changes to the main tree topology. Based on this phylogenetic tree, degenerated primer pairs (Table 18) targeting both comammox *amoA* clades specifically were designed with the 'probe design' function of ARB (Ludwig *et al.*, 2004; Pjevac *et al.*, 2016).

5.6. Polymerase Chain Reaction protocol

5.6.1. PCR Protocol

To reduce the risk of contaminations, PCR was prepared under a UV- sterilizing and clean PCR-hood. PCR reactions contained PCR graded water and PCR reagents with the respective concentrations of 0.2 mM dNTPs, 0.5 pmol/µl of forward and reverse primer, one unit of DreamTaq Green buffer (includes 2 mM $MgCl_2$), 0.025U DreamTaq DNA polymerase and 0.1 mg/ml Bovine Serum Albumin (BSA). For screening and gradient PCR, I used a total volume of 20 µl, whereas for clone sequencing triplicates with 50 µl per reaction. Positive controls were supplemented in the form of purified plasmids and negative controls in the form of PCR water. The run conditions within the thermocycler are displayed in Table 20, unless specified differently.

Table 20: PCR program for comaA F / R, comaB F / R

Time	Temperature	
3 min	95 °C Denaturation	
30 s	95 °C Denaturation	25 - 35 cycles
45 s	52 °C Annealing	
60 s	72 °C Elongation	
10 min	72° Final elongation	
hold	4 °C Storage	

5.6.2. Gel Electrophoresis

For evaluation of the PCR products, the amplicons were visualized by agarose gel electrophoresis. Gels were made using 2% (w/v) LE agarose in 1x TBE buffer and heated in a microwave. Subsequently 1.75 µl of 10,000 x GelRed (DNA stain) was added to the molten agarose and mixed by pivoting. The hot gel was then filled into the gel caster (SubCell GT System; Bio-Rad) and left for solidification at room temperature (20 min). Subsequently, the gel was loaded with 5 µl of sample and 3 µl of 1 kb ladder (GeneRuler) as a length standard. The electrophoresis was run at either 90 and 120 V for about 25 minutes and images were taken afterwards with the Gel Doc XR+ with automated exposure times, aiming for intense bands.

5.7. Primer optimization

5.7.1. Gradient PCR:

For determining the optimal annealing temperature of the novel primer sets, a vertical gradient PCR with 8 different annealing temperatures was performed on environmental DNA extracted from the drinking water well of “Wolfenbüttel” (Germany) (Daims *et al*., 2015). Based on theoretical melting temperatures calculated with CFX Manager (v.3.1), an annealing temperature range between 42 and 52°C was chosen, performing 25 cycles of amplification in total. I applied four technical replicates for each temperature by using four different DNA extracts from the same sample. The cycling conditions of the gradient PCR with the individual annealing temperatures is displayed in Table 21. After the amplification procedure, the

intensities of the bands with the anticipated product lengths and unspecific bands were compared by conventional Gel electrophoresis.

Table 21: Gradient PCR program

Time	Temperature	
3 min	95 °C Denaturation	
30 s	95 °C Denaturation	25 cycles
45 s	42; 42.6; 44; 45.9; 48.3; 50.3; 51.4; 52 °C Annealing	
45 s	72 °C Elongation	
10 min	72° Final elongation	
hold	4 °C Storage	

5.7.2. Equimolar primer mixing

To generate equimolar primer mixtures, PCR graded water and individual primer variants were pooled to an equimolar primer mix with 50 pmol/µl of total primer concentration. The individual primer sequences are displayed in Table 15 and 16, whereas the variants within the equimolar pools are shown in Table 17.

5.8. Cloning and sequencing

5.8.1. PCR product purification

Amplicon DNA was either purified directly by using the PCR product or in the case of double bands, cut out of an agarose gel. For the first one, I used the Qiaquick PCR purification Kit, for which I pooled the triplicates into sterile and DNA / RNA free 1.5 ml pp-tubes and subsequently proceeded according to the manufacturer's protocol. The cut bands from the agarose gel electrophoresis were processed by using the Qiaquick Gel extraction Kit again following the manufacturer's recommendation and eluted finally into 30 µl instead of 50 µl of TE buffer, to increase the resulting DNA concentration.

5.8.2. Ligation

The purified amplicons were ligated into the pCR4-TOPO vector. For this 1 µl of amplicon product was mixed with 4 µl of salt solution and 1 µl of vector, spinned down and incubated in the dark (45 min, room temperature). Subsequently the whole mix was added to TOP10 *E. coli* competent cells and the transformation procedure started.

5.8.3. Transformation

The entire ligation mix (6 µl) was added to defrosted chemically competent TOP10 *E.coli* cells and incubated on ice for 5 minutes. Immediately afterwards, a heat shock at 42 °C was applied for 30 s in a water bath. Subsequently the cells were put shortly on ice and 250 µl of preheated S.O.C. media added (37 °C). The transformants have then been incubated at 37 °C while gently shaking the tube in a horizontal position (200 rpm, 2 h).

5.8.4. Plating

Agar plates with 100 mg/L of ampicillin were preheated to 37 °C until all water residues that evolved during plate pouring were evaporated. From transformed *E.coli* cell mixtures, 100 µl and 150 µl have been spread on the agar plates, by using sterile glass beads. The plates were incubated at 37 °C overnight and stored at 4 °C.

5.8.5. Clone picking and inoculation to 96 well plates

Sterile LB medium containing 100 mg/L of ampicillin was filled into transparent and sterile 96 well cultivation plates. *E. coli* colonies were picked with autoclaved toothpicks, inoculated into the 96 well plates and incubated at 37 °C overnight. The assessment of positive clones with the correct insert lengths was based upon the result of a M13 PCR (60 °C annealing temperature). For sequencing, either 96 well plates of the manufacturer were filled with sterile 150 µl of LB media (100 mg/L kanamycin), incubated with target clones for 4 hours at 37 °C and sent to the sequencing facility (Microsynth). Alternatively purified M13 PCR products were sent in pp-tubes to Microsynth.

5.8.6. Cryostocks of clone libraries

For preserving the plasmids within the *E.coli* clone libraries, I transferred the picked clones into 96 well plates containing 200 µl LB media (12% of glycerol, 100 mg/L ampicillin). After approximately 4 hours of incubation at 37 °, the plates were shock frozen and stored at -80 °C. For a proof of principle, a selection of these clones were resuscitated after several weeks,

inoculated into new plates containing 200 µl LB (100 mg/L ampicillin) and incubated at 37 °C overnight. The presence of plasmids with the correct amplicon size was tested by PCR, using either M13 primer sets or comammox specific primers in combination with gel electrophoresis.

5.8.7. Sequencing and quality control

Sanger sequencing of purified M13 PCR products or *E.coli* transformants has been done by Microsynth (Balgach, Switzerland) using the M13 forward primer. All primer sequences were trimmed off the retrieved amplicons by using the batch processing option for sequence trimming of Chromas lite (v2.5.0). First quality assessment was then performed using Blastx (Camacho *et al*., 2009) with standard parameters and all as *amoA* classified sequences included into our further analysis.

5.8.8. Addition of cloned sequences

All sequences that passed the quality check were then imported into the ARB software (v6.0.5) package and added to a guide tree by maximum parsimony, without allowing changes in the overall tree topology.

5.8.9. Sample overview

A summary of the used environmental samples for the cloning experiment with the respective habitat type, Number of PCR amplification cycles and DNA extraction method is displayed in table 22.

Table 22: Sample information

Sample name	Habitat type	Gene amplified	Amplification cycles	DNA extraction method
Wolfenbüttel	Engineered (GWW)	Clade A *amoA* Clade B *amoA*	25 25	phenol/chloroform
Friedrichshof	Engineered (DWTP)	Clade A *amoA* Clade B *amoA*	25 25	phenol/chloroform
Spiekeroog	Engineered (DWTP)	Clade A *amoA* Clade B *amoA*	25 25	phenol/chloroform
VetMed	Engineered (WWTP)	Clade A *amoA*	30	phenol/chloroform
Ingolstadt	Engineered (WWTP)	Clade A *amoA*	30	phenol/chloroform
Rice bulk soil (Vercelli)	Rice paddy soil	Clade A *amoA* Clade B *amoA*	35 35	phenol/chloroform
Rice rhizosphere (Vercelli)	Rhizosphere	Clade A *amoA* Clade B *amoA*	35 35	phenol/chloroform
Forest soil (Klausen-Leopoldsdorf)	Forest soil	Clade B *amoA*	35	phenol/chloroform
Saline lake sediment (Herrensee)	Brackish lake sediment	Clade A *amoA*	30	phenol/chloroform
River enrichment (Schwarza)	Freshwater sediment	Clade A *amoA*	35	MoBio PowerSoil Kit

5.9. Quantitative Polymerase Chain Reaction

5.9.1. qPCR Protocol

The qPCR reagent premix was prepared under the DNA sterile conditions of a PCR-Hood and consisted of water, 0.1 mg/ml BSA and the respective amount for 1x Bio Rad iQ SYBR Green Supermix containing 50 U/ml iTaq DNA polymerase, 0.4 mM dNTPs, 100 mM KCl, 40 mM Tris-HCl, 6 mM $MgCl_2$, 20 mM fluorescein and stabilizers. For the primer comparison this premix was distributed into new pp-tubes and the different primers sets added to a final concentration of 0.5 pmol/µl. Subsequently, the resulting mixtures were distributed into a 96 well plate and 1 µl of template DNA added in triplicates.

To compare the DNA extraction methods I amended the aforementioned Supermix with the primer set comaA equimolar 2F/2R and 0.1 mg/ml BSA. Additionally, the concentrations of the three different DNA extracts from Friedrichshof were determined by using the aforementioned PicoGreen Kit. Subsequently I applied 0.68 ng of each DNA extract in triplicates with either 1 µl (1^{st} phenol fraction), 4.08 µl (2^{nd} phenol fraction) or 4.62 µl (Kit extract) to the qPCR assay and adjusted the required amount of added ddH_2O to the amount of template.

All qPCR plates were sealed by using special transparent films for qPCR assays and put into the Bio Rad C 1000 CFX96 Real-Time PCR thermocycler. The full protocol for the qPCR assay is listed in Table 23, whereas the corresponding thermocycling conditions with fluorescence measurements are displayed in Table 24.

Table 23: Reagent mix for quantitative PCR

Reagent	Amount
PerfeCTa SYBR Green FastMix, 2X	25 µl
primer F (50 pmol/µl)	0.5 µl
primer R (50 pmol/µl)	0.5 µl
BSA (20 mg/ml)	0.5 µl
ddH_2O	22.5 /19.42 /18.88 µl
Template	1 / 4.08 /4.62 µl

Table 24: qPCR Program

Time	Temperature	
3 min	95 °C Denaturation	
30 s	95 °C Denaturation	45 - 60 cycles
30 s	52 °C Annealing	
60 s	72 °C Elongation	
-	72 °C Plate read	
30 s	95 °C Denaturation	
30 s	38 °C Annealing / Equilibration	
10s	38 - 95 °C Melt curve, increment 0.5 °C / 10s + Plate Read	

6. Results

6.1. Primer evaluation

With the probe match function of the ARB software environment the coverage of each individual primer variant to all known *amoA* sequences of the reference database was evaluated and the primer specificity tested *in silico*. Subsequently, a DNA extract from the drinking water well of Wolfenbüttel (Germany), previously analyzed by metagenomics and known to harbor a relatively high abundance of both comammox *Nitrospira* clades (Daims *et al.*, 2015), was used for the initial *in situ* evaluation of the new primers (comaA 1F/1R, comaB 1F/1R).

6.1.1. Clade A primer sets

The forward primer of the clade A primer set (ComaA 1F/1R) covers 70 of the 106 publically available clade A *amoA* sequences, and the corresponding reverse primer 87. Both the forward and the reverse primer sets contain 2 ambiguous positions in their sequence, resulting in 4 different primer versions, respectively.

Two additional ambiguous positions in the forward primer (16 variants in total) and one further ambiguity in the reverse (8 variants in total) increased the matches to the reference sequence database to respective 101 and 94 hits (ComaA 2F/2R). Furthermore, this enhanced primer set enables the amplification of the *Ca*. Nitrospira inopinata *amoA* gene as a positive control for future studies.

6.1.2. Clade B primer set

The forward primer of the initial clade B primer set (ComaB 1F/1R) contains 3 ambiguous positions (8 primer versions) and is able to target 227 of 270 sequences of the reference dataset. The associated reverse primer also contains 3 ambiguous positions (12 different primer versions) and matches 225 of all known clade B comammox *amoA* sequences.

6.1.3. Specificity of both primer sets

Each of the primer pair contains minimally 4 mismatches to the respective other comammox *Nitrospira* clade. In addition, *in silico* analysis revealed that both primer pairs have at least 3 mismatches to almost all of the 14,019 betaproteobacterial *amoA* and 10,389 *pmoA* sequences from the reference database. Only a small subset (<30) of this *pmoA* and betaproteobacterial *amoA* contain two mismatches. No betaproteobacterial *amoA* or *pmoA* sequences with a single or no mismatches was detected.

6.1.4. *In situ* primer evaluation

The initially tested annealing temperature of 46 °C for both primer sets was based on theoretical melting temperature calculations and led to the amplification of two products, when the primers were applied to DNA extracts from GWW Wolfenbüttel. One amplicon had the anticipated length of approximately 415 bp, whereas the second amplification product was around 250 bp long. Both amplification products were separately excised from agarose gels, purified, cloned and sequenced. Only amplicons with the anticipated length were comammox *amoA* sequences, while sequencing products retrieved from cloning the second band were not related to the *amoA* superfamily. In order to minimize the formation of this unspecific second band, I tested different annealing temperatures by gradient PCR. The formation of a second band is eliminated or at least severely diminished at an annealing temperature of 52 °C. Consequently, by using this higher annealing temperature, I was able to directly purify the PCR product, without a previous separation by gel electrophoresis. The addition of the retrieved sequences to the *amoA* / *pmoA* reference tree by maximum parsimony, ensured that the primers are specific for each clade comammox *amoA* clade (Figure 1).

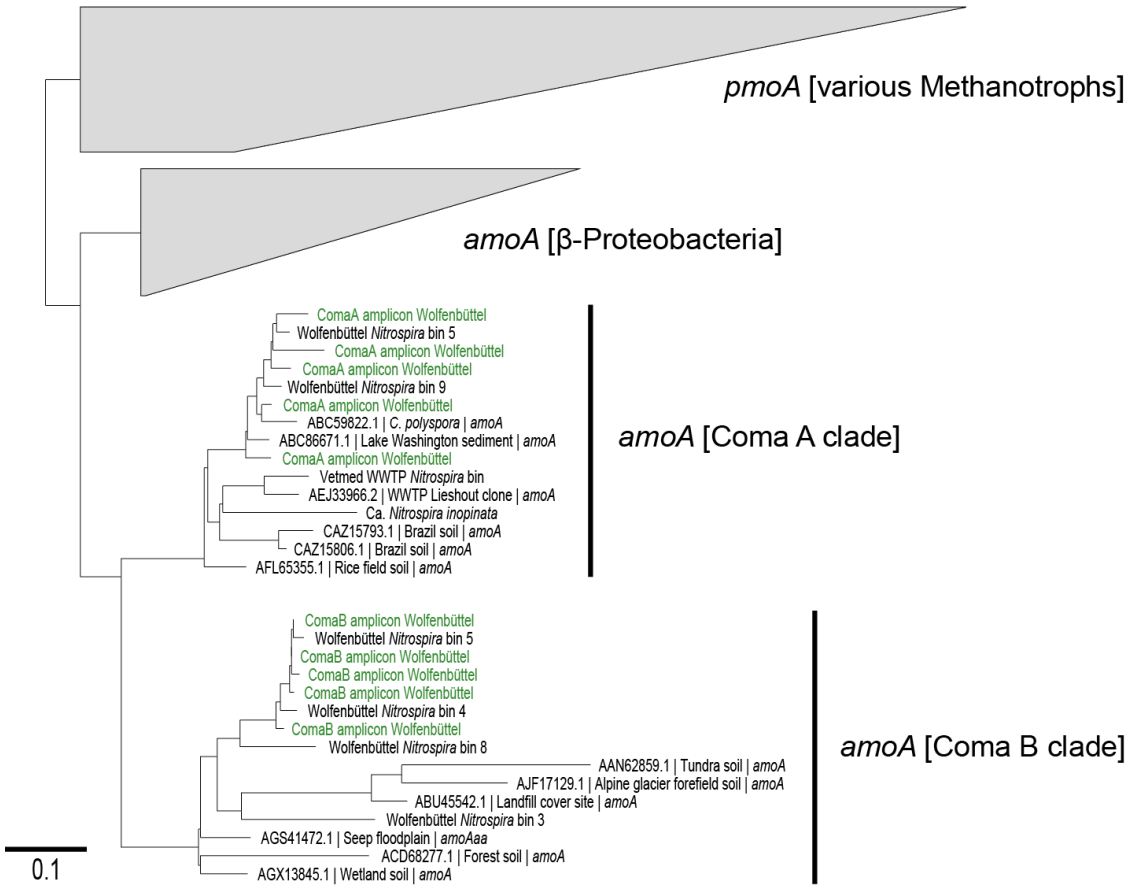

Figure 1: Phylogenetic tree of *amoA*/*pmoA* genes, including comammox amoA sequences obtained during the first evaluation of the primer sets on a sample from GWW Wolfenbüttel (in green). Sequences were obtained by using primer set comaA 1F/1R and comaB 1F/1R. The scale bar represents the estimated change per nucleotide.

6.2. Equimolar primer pools

The ambiguous nucleotides of the primer sets result in a number of oligonucleotide variants, present in the primer mixes, which do not match any reference sequences in our comprehensive *amoA* gene database. The known effects of such primer degeneracy are an impaired specificity and decreased amplification efficiency (e.g. Frank *et al.*, 2008). Ultimately, the presence of such oligonucleotide variants leads also to a dilution of the actual primers. Furthermore, in any degenerate primer, the concentration distribution of the individual oligonucleotide variants is not specified and likely not stable from synthesis to synthesis.

6.2.1. Equimolar pools of comammox *amoA* primers

To resolve potential problems caused by primer degeneracy, I established equimolar pools of all individual primer variants from ComaA 1F/1R and 2F/2R or ComaB 1F/1R that match at least one clade specific reference sequence in the database.

The equimolar version of primer set ComaA 1F/1R is named ComaA equimolar 1F/1R and reduced by the elimination of one nonsense primer variant. With the equimolar pooled version of the primer set ComaA 2F/2R, named ComaA equimolar 2F/2R, I was able to exclude 18 nonsense primer variants in total, while maintaining the high coverage of the primer set.

The amplification efficiencies of equimolar pooled primers were then compared by qPCR with the two degenerated primer pairs of the clade A primer set. As template I applied equal amounts of DNA, extracted from a comammox *Nitrospira* containing sand filter of a drinking water treatment plant in Friedrichshof (Germany).

The equimolar pool of the most important primer variants, (ComaA 1F/1R) showed the fastest increase of signal intensity, followed by the degenerated ComaA 1F/1R. The equimolar pool with 6 forward and reverse variants (ComaA equimolar 2F/2R) contains each primer variant in a lower concentration than the first two primer sets and requires 1 to 2 more amplification cycles, yet has a higher coverage (Figure 2). The degenerated primer pair (comaA 2F/2R) did not yield an amplicon in this experiment (Figure 2). Thus, I reevaluated the result with the degenerated head primers H_comaA 2F/2) and an equimolar pooled version of the primer set H_comaA equimolar 2F/2R. The head sequence of these primers enables the easy and cost efficient barcoding of amplicons by a second PCR step with the application of Barcode primers, thus are suited for next generation amplicon sequencing (Herbold *et al.*, 2015). The difference between amplification by these two primer sets is approximately 4 amplification cycles (Figure 3). All melting curves of primer comparison qPCR showed only one peak between 84 °C and 88 °C and are clearly distinguishable from the negative controls that are forming peaks at random temperatures (Figure 3).

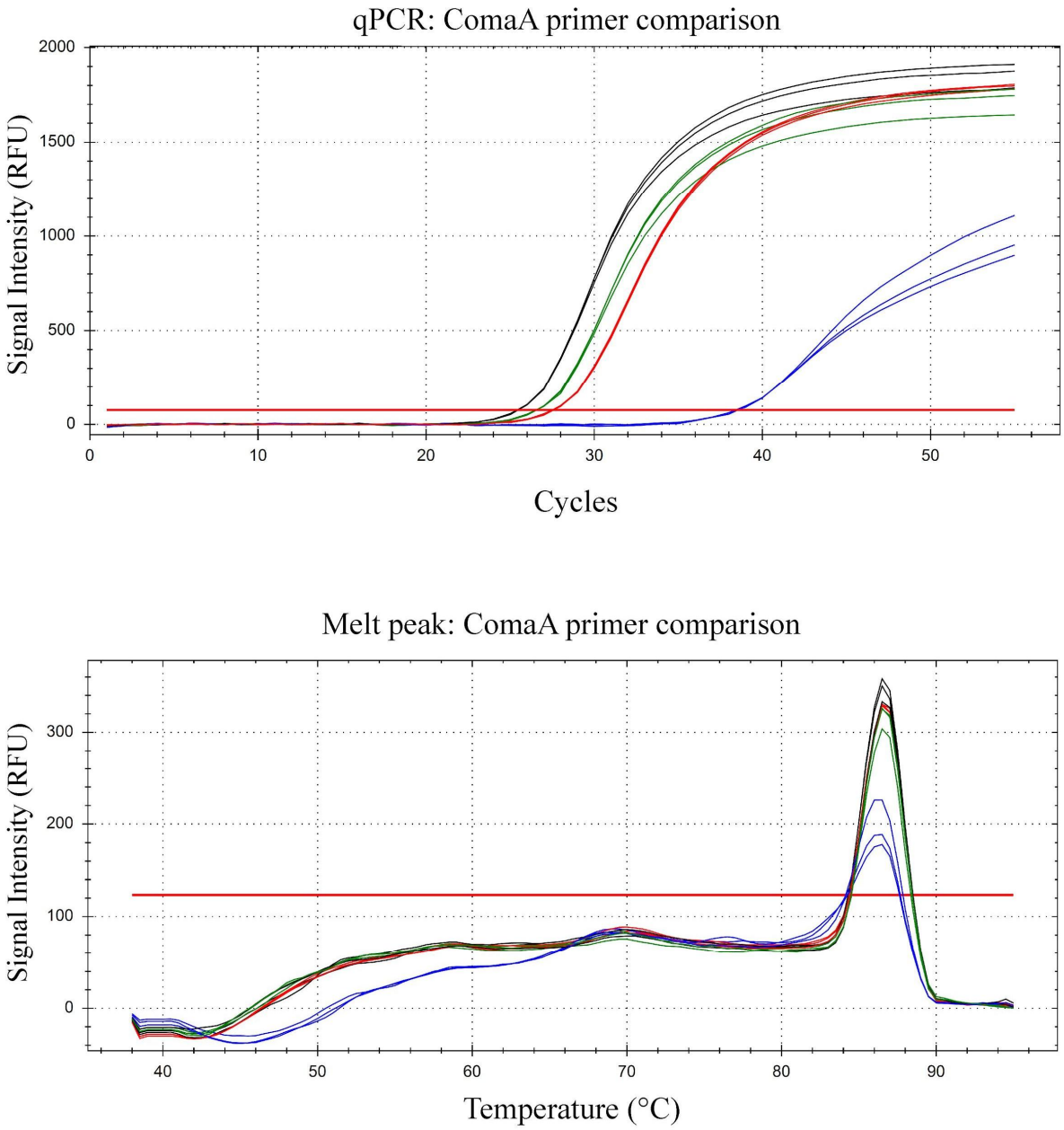

Figure 2: Comparison of comaA primer sets by qPCR. ComaA 1F/1R in black, ComaA equimolar 1F/1R in green, ComaA equimolar 2F/2R and the graph of degenerated ComaA 2F/2R in blue. The upper panel shows the increase of signal intensities per cycle, whereas the lower panel shows all melting curves for the respective PCR products.

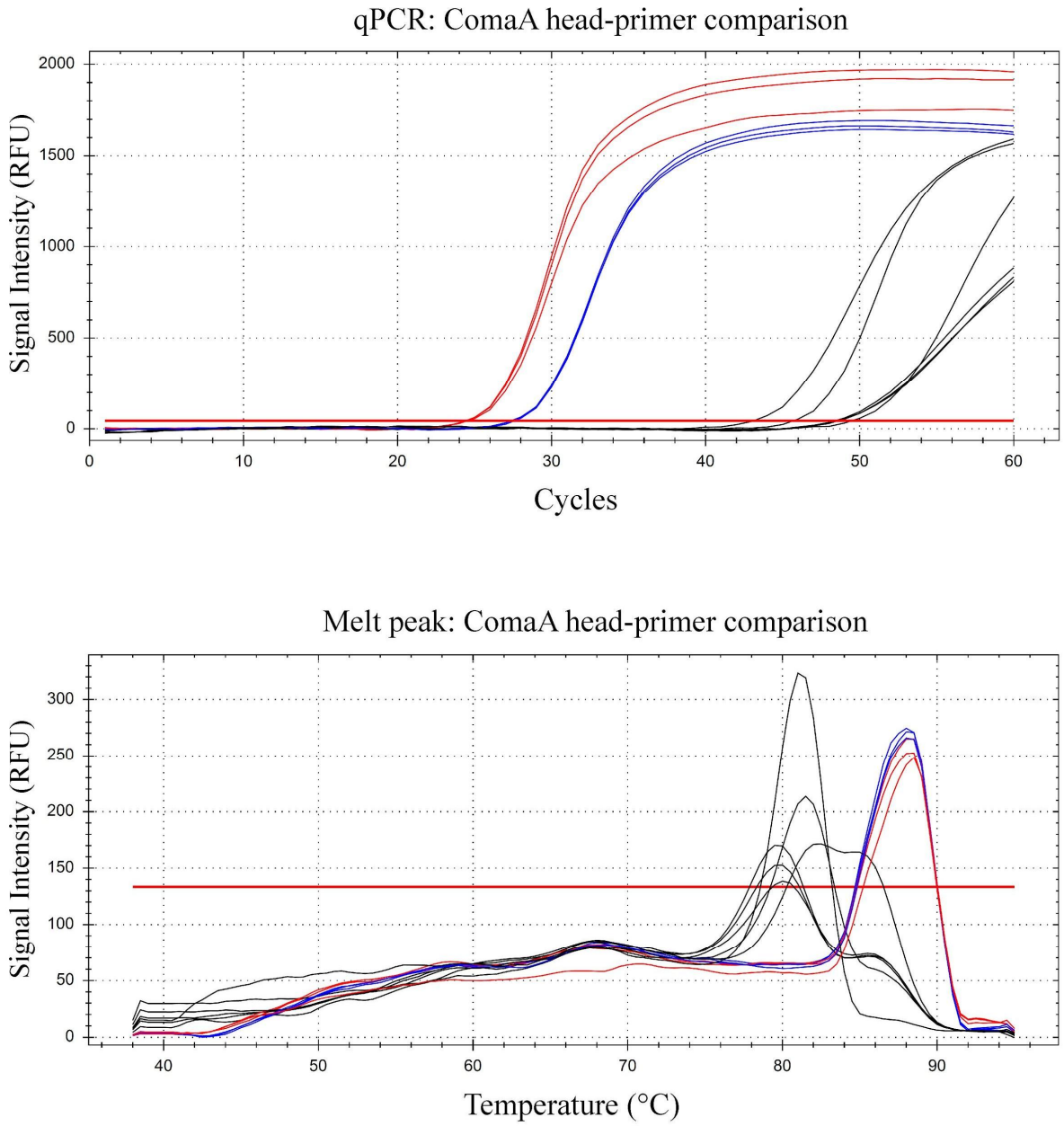

Figure 3: Comparison of head ComaA primer sets by qPCR. H_ComaA equimolar 2F/2R is displayed in red, H_ComaA 2F/2R in blue and negative controls in black. The upper panel shows the increase of signal intensities per cycle, whereas the lower panel shows all melting curves for the respective PCR products.

Similar to the comammox *amoA* clade A primer set, an equimolar pooled comammox *amoA* clade B primer set was designed and established. Generally, clade B has a higher sequence diversity in the reference database, especially in the binding region of the oligonucleotides. Consequently, some of the template sequences are hardly covered by degenerated primer sets, as it would require additional nucleotide ambiguities and thereby an further decrease in the primer specificity (Linhart and Shamir, 2002; Frank *et al.*, 2008). To circumvent this problem, a divergent oligonucleotide variant was added to the equimolar forward primer mixture, which increased the coverage for the forward primer set from 84% to 94%.

6.3. Amplification and sequencing of comammox *amoA* genes from environmental samples

To test the applicability and specificity of the novel primer pairs, and to obtain additional data on the distribution and diversity of comammox *Nitrospira*, I performed an environmental screening for comammox *amoA* genes in a broad selection of sample types. I recovered 5 to 59 clone *amoA* sequences from each sample with positive amplification results and calculated the number of OTUs based on a sequence identity threshold of 97%.

In the iron-rich sludge of the riser pipe from the GWW Wolfenbüttel, I detected both comammox clades and recovered 19 comammox *amoA* clade A and 16 comammox *amoA* clade B sequences. These *amoA* amplicons were subsequently grouped in six clade A, and two clade B OTUs, respectively.

In the filter material of DWTPs Friedrichshof and Wolfenbüttel also both comammox clades were found, and 22 to 23 *amoA* sequences per sample and primer set were retrieved. In Friedrichshof, six comammox *amoA* clade A OTUs were present, whereas in Spiekeroog only two comammox *amoA* clade A OTUs were detected. The cloned *amoA* sequences of both samples with affiliation to clade B were clustered into two OTUs.

In activated sludge from the WWTPs VetMed and Ingolstadt, I only detected clade A comammox *Nitrospira.* From the established clone libraries, 20 comammox *amoA* sequences from VetMed and 5 from Ingolstadt were recovered, which could be assigned to five OTUs in VetMed, and a single OTU in Ingolstadt. Likewise, in an alkaline, saline lake sediment from Lake Herrensee, and in an ammonium-fed enrichment culture inoculated with a river biofilm sample, only comammox *amoA* clade A was detected. I clustered the 10 *amoA* clone sequences from the lake sediment sample and the 37 sequences from the enrichment culture into four and two OTUs, respectively.

A high number of comammox OTUs was found within a rice paddy soil sample and rice rhizosphere, whereby 22-31 *amoA* sequences were recovered. Seven OTUs with affiliations to comammox *amoA* clade A were recovered in the bulk soil, and 10 in the rhizosphere. The observed comammox *amoA* clade B diversity was equally high, with 8 OTUs in both samples.

Comammox *amoA* clade A was not detected within the forest soil of Klausen-Leopoldsdorf, but I was able to obtain 8 clade B OTUs out of 59 *amoA* sequences from this sample.

Overviews on the environmental screening results are displayed in Figure 5 and 6, whereas the phylogenetic affiliation of one comammox *amoA* clade A and B OTU representative is shown in Figures 7 and 8, respectively.

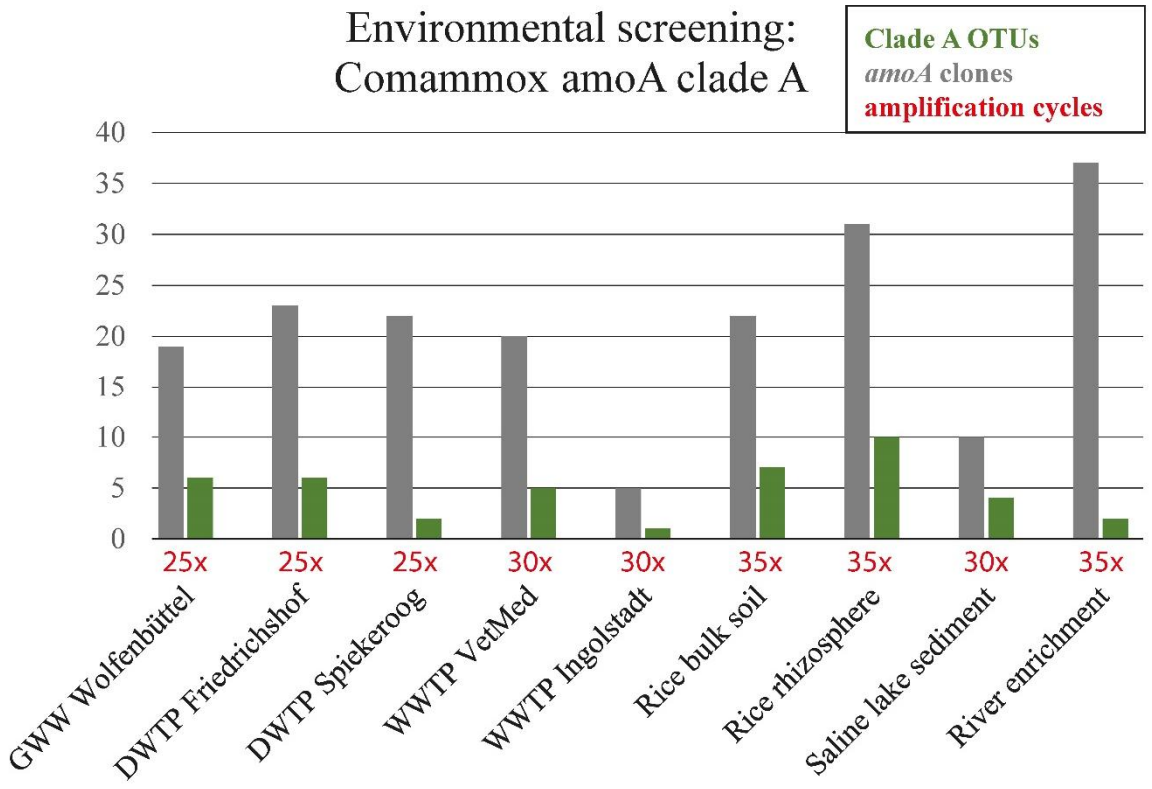

Figure 5: Results of environmental screening for comammox *amoA* clade A. The number of sequenced clones is indicated by the grey bars. All green bars are displaying the retrieved number of clade A OTUs, based on 97% sequence similarity. The number of amplification cycles is displayed below the bars in red.

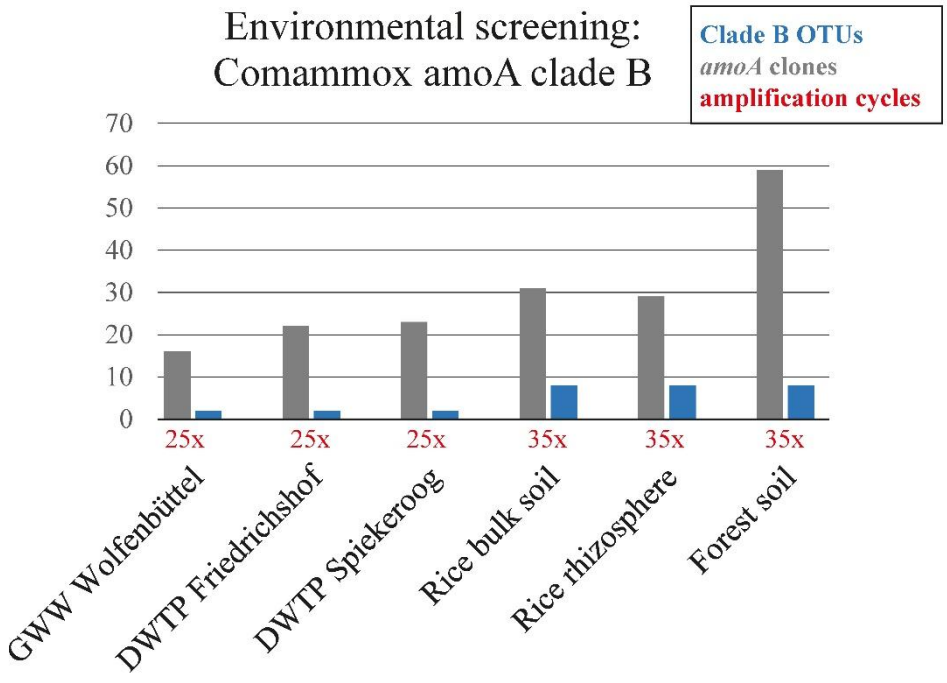

Figure 6: Results of environmental screening for comammox *amoA* clade B genes. The number of sequenced clones is indicated by the grey bars. All blue bars are displaying the retrieved number of clade B OTUs, based on 97% sequence similarity. The number of amplification cycles is displayed below the bars in red.

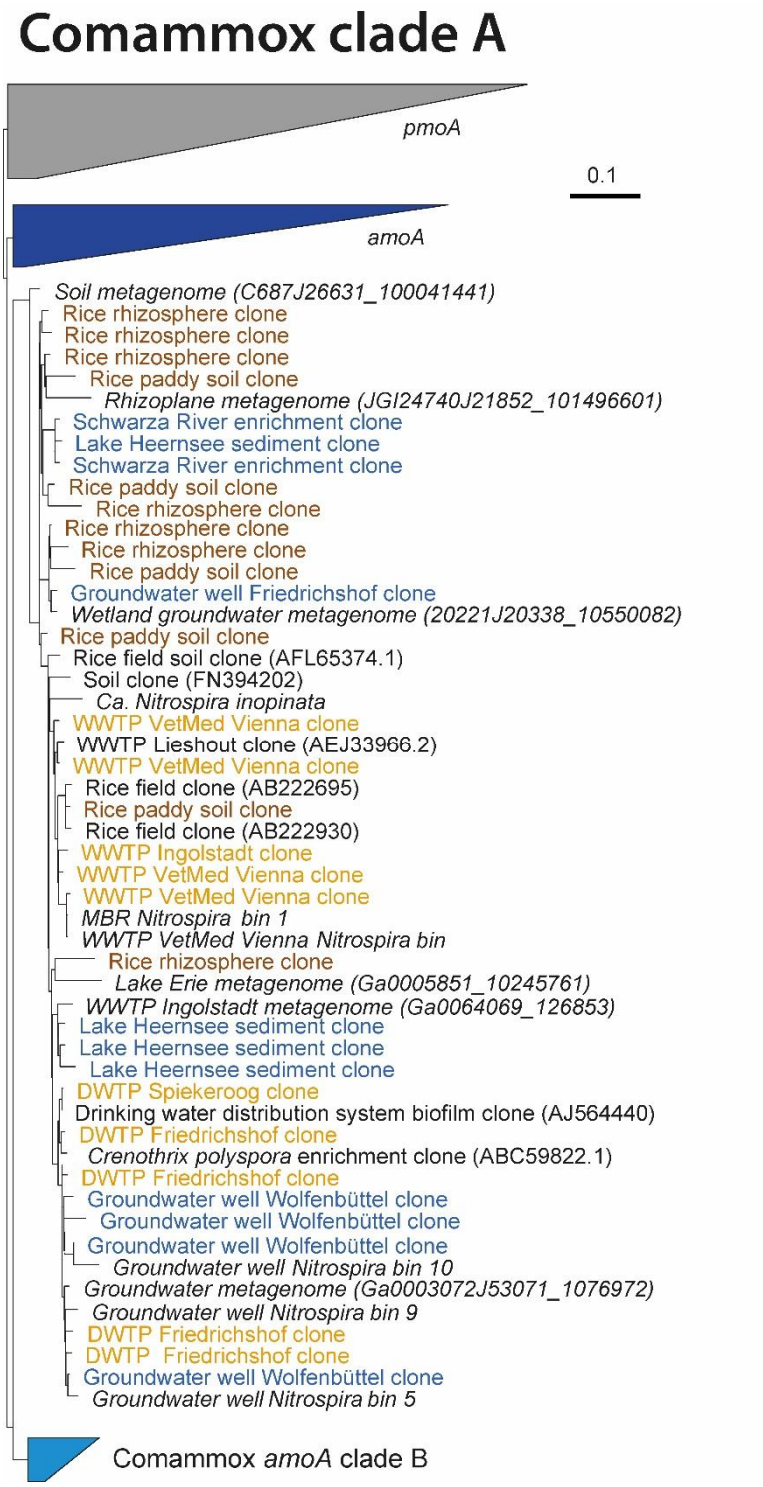

Figure 7: A maximum likelihood tree showing the phylogenetic affiliation of comammox *amoA* clade A amplicons by one OTU representative. In brown all amplicons retrieved from soils, in blue all freshwater systems, in yellow samples from engineered habitats, and in black a selection of reference sequences from the database. Betaproteobacterial *amoA* and *pmoA* sequences from various methanotrophs served as outgroup. The scale bar represents the estimated change per nucleotide.

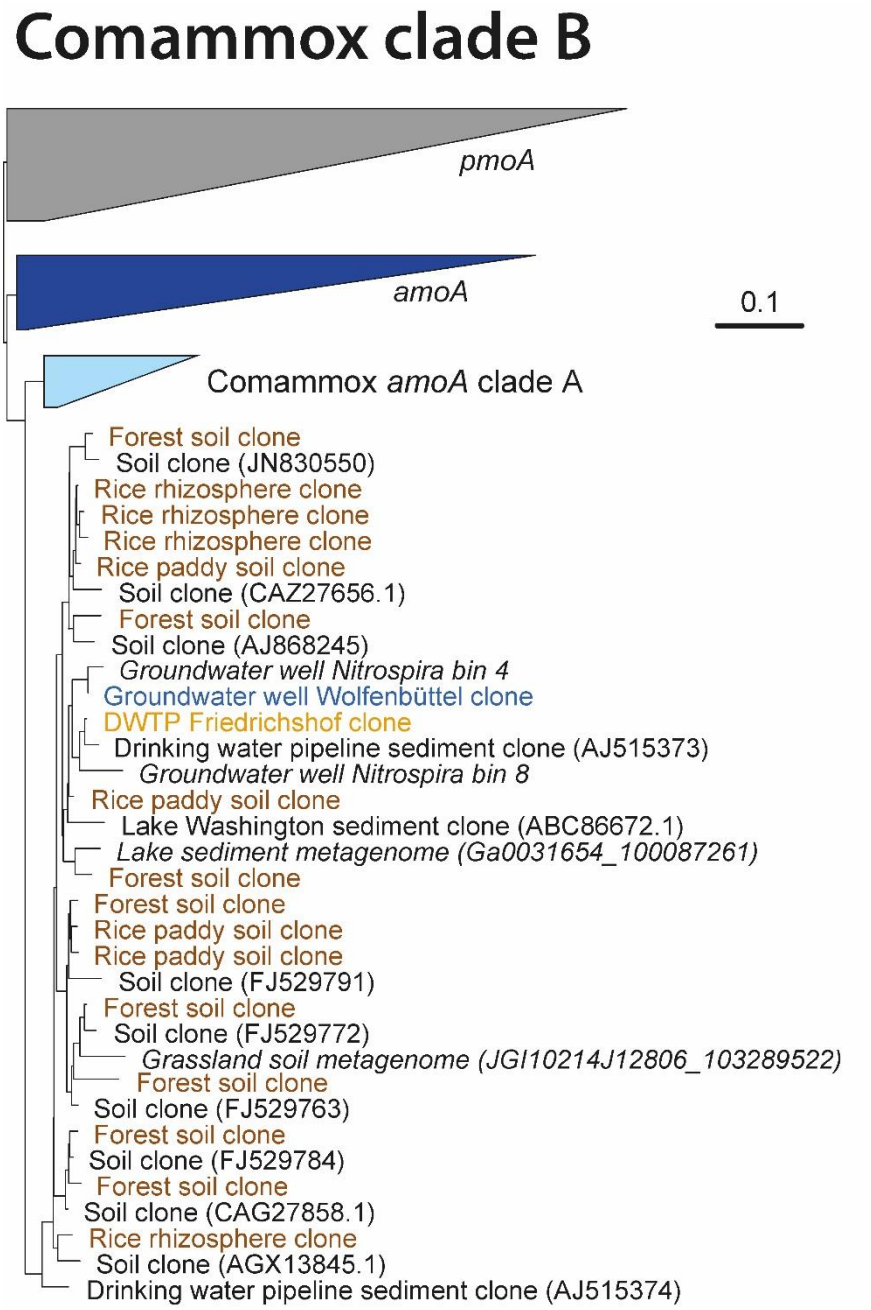

Figure 8: Maximum likelihood tree showing the phylogenetic affiliation of comammox *amoA* clade B amplicons by one OTU representative. In brown all amplicons retrieved from soils, in blue all freshwater systems, in yellow samples from engineered habitats, and in black a selection of reference sequences from the database. Betaproteobacterial *amoA* and *pmoA* sequences from various methanotrophs served as outgroup. The scale bar represents the estimated change per nucleotide.

6.4. Comparison of DNA extraction method effect on the detection of comammox *amoA* genes

For determining the influence of DNA extraction on the detection efficiency and observed abundance of comammox *Nitrospira amoA* genes, I extracted DNA from a trickling filter of the DWTP Friedrichshof (Germany) by two methods that differ in their intensity of physical force during cell lysis.

With first method (MoBio PowerSoil Kit), DNA was extracted from 0.2261 g of the filter material and eluted in 100 µl. The second protocol (phenol/chloroform extraction) was applied on 0.4673 g of sample and each of the resulting DNA pellets from the two bead beating steps, were dissolved in 30 µl TE buffer. Subsequently, the concentration of recovered DNA was determined fluorometrically. The Kit extract contained 0.15 ng/µl of DNA, the first fraction of the phenol/chloroform extraction 0.68 ng/µl and the second fraction 0.17 ng/µl. Normalized to the amount of sample, 65.08 ng/g of DNA was retrieved by the Kit extraction, 43.65 ng/g during the first bead beating step by the phenol/chloroform method and 10.68 ng/g from the second bead beating step.

As an exemplary contrast to this result, the first phenol/chloroform fractions from the forest soil sample and VetMed contained respective 62.1 µg DNA and 181.25 µg DNA, whereas the MoBio PowerSoil Kit obtained from approximately the same amount of these two samples only respective 0.704 µg and 0.496 µg of DNA.

For the comparison of the extraction efficiency for comammox *Nitrospira* cells, I applied equimolar amounts of template DNA from all Friedrichshof DNA extracts and performed qPCR using the primer set ComaA equimolar 2F/2R. The best result was yielded with the MoBio PowerSoil Kit extract, followed by the first fraction of the phenol/chloroform method and slowest signal intensity increase was observed on the recovered DNA from the second bead beating step. The melting curves of this qPCR indicate the formation of one specific amplification product (Figure 9).

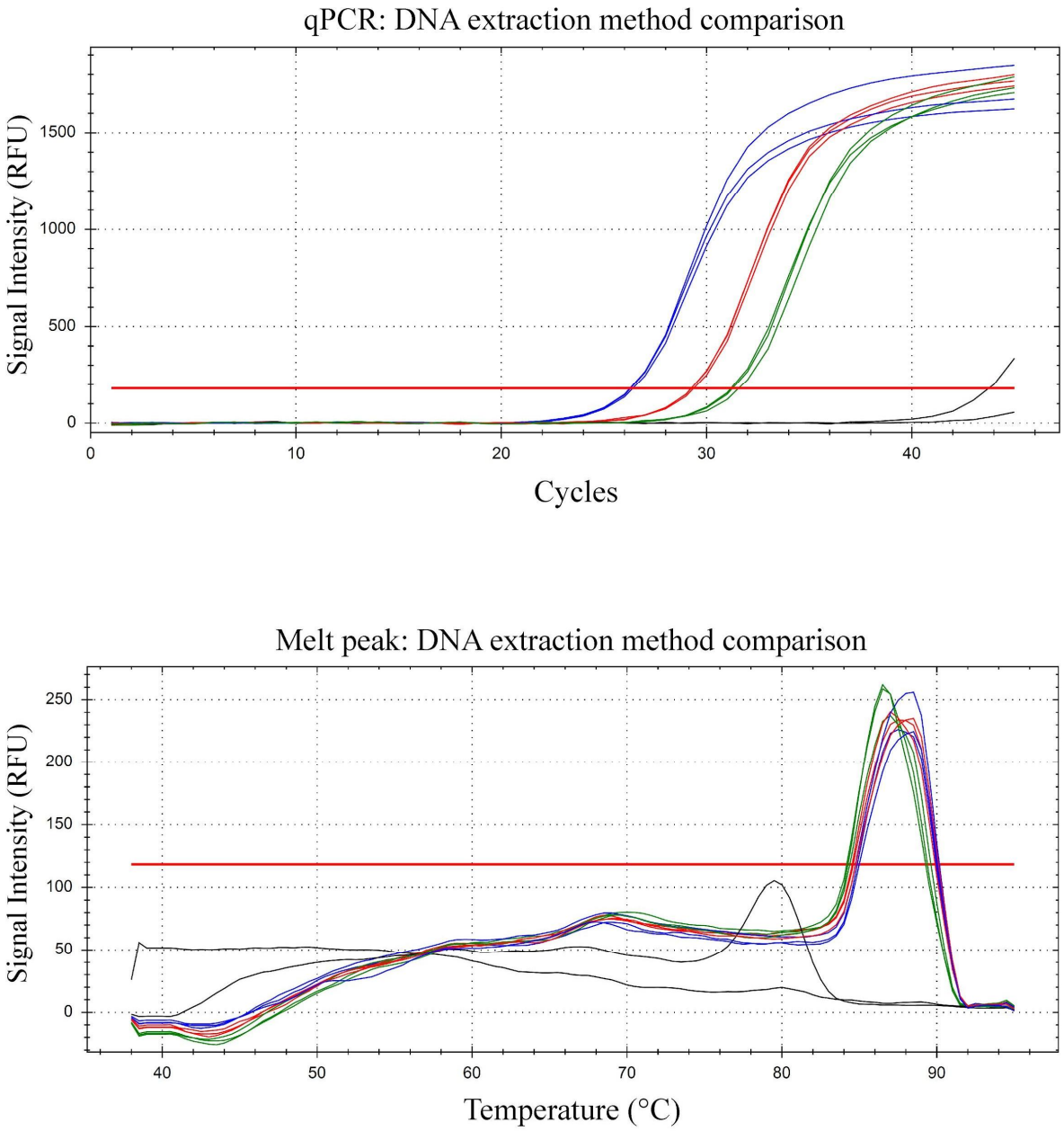

Figure 9: Comparison of DNA extraction methods. The MoBio PowerSoil DNA extract is indicated in blue, first phenol fraction red and second fraction green (negative controls in black). The upper panel shows the increase of signal intensities per cycle, whereas the lower panel shows all melting curves for the respective PCR products.

7. Discussion

The labor division in nitrification was a riddle for a long time. The first scientific paper that selectively examines this topic, even though only theoretical, was published in 2006 and predicted comammox microorganisms to be preferentially thriving in oligotrophic systems and biofilms (Costa *et al*., 2006). Despite the tremendous advances of sequencing techniques and elaborate metagenomic studies about nitrifying microorganisms, it took nine more years to the actual discovery of comammox within *Nitrospira* lineage II (Daims *et al*., 2015, Van Kessel *et al*., 2015). Intriguingly, as soon as the first enrichment cultures and full genomes of comammox *Nitrospira* were published, functional and phylogenetic marker genes for comammox, such as the *amoA* gene*,* were found within several metagenomic *Nitrospira* bins, extracted from engineered systems, soils and also freshwaters (Daims *et al.*, 2015; Pinto *et al.*, 2015; Palomo *et al.*, 2016). This hinted that the "inside the box thinking" eventually led to overlooking one of the most decisive scientific discoveries about nitrification, since the pioneering studies of Sergei Winogradsky. In addition this, metagenomic analyses indicated that comammox might be environmentally widespread and could be substantially contributing to nitrification, and the global nitrogen cycle (Daims *et al*., 2015). In order to enable investigations of the diversity and distribution of comammox *Nitrospira*, novel PCR primer sets that specifically target the *amoA* genes of both clades respectively were developed and their applicability evaluated on a range of environmental samples (see also: Pjevac *et al.*, 2016).

7.1. Deducing function from phylogeny

The recent discovery of comammox within the genus *Nitrospira* - a widespread bacterial group, commonly perceived to be strict nitrite oxidizers, has again shown the common misconception of direct links between phylogeny and function (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Therefore, predictive tools such as PICRUSt, that anticipate the functional composition of a community by the analysis of its phylogenetic composition, have to be used with caution. Additionally, their results should not be over interpreted, and applied at most for hypothesis generation. Otherwise, incorrect conclusions might be drawn (Langille *et al.*, 2013). For example, a recent study concluded comammox *Nitrospira* are not important in wastewater treatment, because only few retrieved 16S rRNA gene sequences were very closely related to 16S rRNA genes of known comammox *Nitrospira* (Gonzalez-Martinez *et al.*, 2016). However, as pointed out beforehand, comammox *Nitrospira* do not form a monophyletic group within the *Nitrospira* lineage II and therefore the conventionally used phylogenetic markers for *Nitrospira*

- 16S rRNA and *nxrB* genes - are not suited for determining presence or absence of these microorganisms (Daims *et al.*, 2015). Both in previous database mining, and experimentally in this study, comammox *Nitrospira amoA* genes were found in wastewater treatment plants, thus indicating they might be more abundant in WWTPs than estimated by Gonzalez-Martinez *et al*., (2016).

7.2. Detection of comammox *Nitrospira* in the environment

At the moment, there are only few suitable methods available to detect comammox *Nitrospira* in biological systems. An accurate, but complicated approach is by cultivation. How complicated such approaches can turn out to be is illustrated by the fact that it took more than 130 years from the discovery of the first nitrifying microorganisms to the enrichment and isolation of the first comammox organism, *Ca*. Nitrospira inopinata (Daims *et al.*, 2015; Daims, unpublished). Even with elaborate cultivation techniques and tremendous *a priori* knowledge, the enrichment and isolation of nitrifying microorganisms remains tedious, time consuming and also stochastic (Tourna *et al.*, 2011). Therefore, for profound ecological statements the foundation and application of cultivation independent methods with high sensitivities are necessary to determine the presence of comammox *Nitrospira*.

Fluorescence *in situ* hybridization of labelled oligonucleotides, targeting the 16S subunits of rRNA from microorganisms has revealed fundamental insights into microbial community structures and associations between different types of phylogenetic groups and putative functional guilds (Schramm *et al.*, 1999; Wagner *et al*., 2003). However, also the canonical FISH approach is subject to the problematic non monophyletic affiliation of comammox *Nitrospira,* thus not a valuable option. Bioorthogonal non-canonical amino acid tagging (BONCAT) FISH is a new method that enables the *in situ* detection of microbial activity in combination with conventional fluorescence *in situ* hybridization (Hatzenpichler *et al.*, 2014). This technique and the use of a specific inhibitor for nitrite oxidation by *Nitrospira*, could potentially enable the *in situ* detection and quantification of comammox *Nitrospira*. One putative inhibitor for strictly nitrite oxidizing *Nitrospira* is tungstate - the elemental analogue to molybdate. The inhibitory mechanism is most probably based on the incorporation of tungstate instead of molybdate into the catalytic center of the nitrite oxidoreductase, thereby inactivating the enzyme (Spieck *et al.*, 1998). However, *Nitrospira* might also use alternative metabolisms to derive energy (Koch *et al.*, 2014, 2015) and remain active during the incubation with ammonia, tungstate and a non-canonical amino acid such as L-azidohomoalanine, despite not being comammox *Nitrospira*. Thus, BONCAT-FISH would only provide an estimate for

the proportion of comammox to canonical *Nitrospira.* Furthermore, the expected low abundance of comammox *Nitrospira* in most environments may impair the discriminability between false positives and actual FISH signals.

A more direct approach with a higher resolution is provided by metagenomics (e.g. Handelsman, 2004). In recent years, metagenomic approaches greatly benefited from improvements in sequencing techniques, facilitating the work of microbiologists in numerous aspects (Metzker, 2010). For instance, it revealed the comammox potential of *Nitrospira* and was also useful for a first glance into the environmental distribution of these microorganisms (Daims *et al.*, 2015). Furthermore, long length *amoA* and *pmoA* reference sequences, derived from metagenomic studies, were crucial for the reconstruction of the phylogenetic guide tree of this study. Yet, this technique also has its limitations, as it is often not sensitive enough to cover low abundant microorganisms, despite the possibility and affordability of deeply sequenced metagenomes (Metzker, 2010). In addition, current assembly algorithms struggle with microdiversity, and assemblies of microbial communities harboring closely related canonical and comammox *Nitrospira* are troublesome (e.g. Palomo *et al*., 2016).

Ultimately, at this stage the most suitable method sensitive enough for the environmental detection and investigation of low abundant microorganisms, is the amplification of functional and phylogenetic marker genes in combination with next generation sequencing (e.g. Pester *et al.*, 2013). This offers the possibility for cost efficient, fast and deep insights about the phylogeny and diversity of microbial communities and will also facilitate research on the environmental distribution of comammox *Nitrospira* in the near future.

7.3. Previous *amoA* and *pmoA* primer sets

The AMO of betaproteobacteria and comammox *Nitrospira* are closely affiliated with the particulate methane monooxygenases (PMO, Holmes *et al.*, 1995). For this reason, the first amplicons of comammox *amoA* were falsely annotated as *pmoA* genes (Daims *et al*., 2015). One prominent example is the 'unusual *pmoA*' of *Ca*. Crenothrix polyspora, which likely originated from a comammox *Nitrospira* and was mislinked to the methanotrophic microorganism (Stoecker *et al.*, 2006; Oswald, unpublished).

The first primer set, that co-amplifies comammox *amoA* sequences along with *pmoA* and betaproteobacterial *amoA* fragments, was published by Holmes and colleagues in 1995 (A189 and A682). This highly degenerated primers have a restricted coverage to the individual *pmoA/amoA* clades, but a broad coverage over the whole superfamily of copper-containing

membrane-bound monooxygenases (CuMMOs, Holmes *et al*., 1995). Recently, an updated version of this primer set was published with an increased amount of sequence ambiguities to enlarge its target spectrum (Wang *et al.*, 2016). However for the easy detection alongside with a fairly good coverage over both comammox clades the design of novel and specific primer sets was necessary. In this study, two primer pairs with adequate properties and coverages compared to previous *amoA* primer sets targeting betaproteobacterial (amoA 1F/2R; Rotthauwe *et al*., 1997) or thaumarchaeal (CamoA 19F/616R; Pester *et al.*, 2012) gene variants were evaluated and their applicability to environmental samples tested.

7.3.1. Primer design on a limited number of reference sequences

One of the most limiting factors for primer design was the limited amount of reference comammox *amoA* sequences. An imaginable way to increase the database without metagenomics, would have been amplification of long regions within the *amoCAB* operon. A similar approach was used to increase the amount of *nxrB* sequences of *Nitrospira*. Almost full length *nxrB* sequences from several pure and enrichment cultures of *Nitrospira* were thereby amplified and sequenced, for the subsequent design of primer pairs, that target a shorter regions of *Nitrospira nxrB* (Pester *et al.*, 2014). Yet, in comparison to canonical *Nitrospira*, only 3 evidenced comammox *Nitrospira* are in culture, hence this method is not possible at the moment.

Ultimately, I consider the reference database to be state of the art, because it includes all publicly available comammox *amoA* sequences. Since many of the comammox *amoA* reference sequences were only partially complete, primer design was restricted to regions of the sequence alignment, in which approximately 80% of the reference comammox *amoA* sequences contained nucleotide information. Consequently, the resulting short amplicons of 415 bp have a restricted phylogenetic resolution. However, in return they have a high and reliable coverage over the individual clades.

A general strategy for primer design on limited reference sequences and not highly conserved genetic regions is the introduction of ambiguous positions (Linhart and Shamir, 2002). For the analysis of nitrifying microorganisms, several degenerated primer sets are currently published and used (i.e. Pester *et al*., 2012). However, due to the close affiliation of comammox *amoA*, betaproteobacterial *amoA* and *pmoA* sequences, highly degenerated primer sets would lead to unwanted co-amplifications (Daims *et al*., 2015). So in this case it is a fine line between the desired specificity and achieving a high coverage. ComaA 2F/2R and ComaB F/R contain

enough ambiguous nucleotide positions to capture most of the currently known comammox *amoA* sequences, and still contain sufficient mismatches to *pmoA* and betaproteobacterial *amoA* sequences to assure a high specificity.

7.4. Optimization of the comammox *amoA* targeted PCR protocol

Functional gene primer sets with nucleotide ambiguities are prone to impaired specificities, due to the myriad of genetic variants which are present in environmental samples and are also known for decreased amplification efficiencies (Linhart and Shamir, 2002; Frank *et al.*, 2008). Thus, the two novel primer sets were evaluated and optimized in detail, to be confidently applicable in future studies to the accurate environmental detection of comammox *Nitrospira*.

7.4.1. Annealing temperature

At the initially tested annealing temperature of 46 °C, a co-amplification of shorter, unspecific DNA fragments occurred. Similar problems have also been observed with other primer sets (Pester *et al.*, 2012, 2014; Wang *et al*., 2013). By increasing PCR stringency through increasing annealing temperature to 52°C, the unspecific co-amplification could be circumvented. This approach is less time intensive than a selection of the target amplicon by electrophoresis and gel band excision. However, an increased annealing temperature reduces the amplification efficiency and affects the ability of primer sets to seize single mismatch sequences (Sipos *et al.*, 2007). While the majority of reference sequences are already covered without any mismatch by the primer sets and increased stringency was selected to prevent the unwanted co-amplification of *pmoA* or betaproteobacterial *amoA* genes, one could conduct exploratory investigations with lower annealing temperatures, as it potentially allows the detection of novel – distantly related comammox *amoA* sequence variants.

7.4.2. Equimolar Pools

The introduction of ambiguous nucleotides to the primer sequences results in higher coverage. However, it also has the aforementioned disadvantages. By equimolar pooling of individual primer variants I was able to improve the amplification efficiency, erase stochastic primer concentrations and even enhance the coverage of the clade primer B primer set through the addition of a single oligonucleotide variant.

Since comammox *Nitrospira*, like other nitrifiers, are probably low abundant in many environments, often up to 35 PCR cycles were needed to achieve comammox *amoA* amplification, increasing the risk of false positives by unwanted co-amplification of non-target

sequences. Thus, strategies to increase the amplification efficiency of target genes, like equimolar pooled primer sets, are useful.

Besides equimolar pooling, a second possibility for enhancing the detection efficiency of PCR while reducing the risk for false positives, is nested PCR (Gundersen and Lee, 1996). In these two-step PCR approaches, a shorter and more specific amplification product is nested within a longer and more unspecific amplicon from the first amplification. However this would either result in an even shorter specific amplicon, or require the design of primers within genetic regions that are currently poorly covered and therefore would result with low coverages.

As soon as more full length sequences of the comammox *amoCAB* operon are available, the here proposed primer pairs need to be reevaluated, and if necessary redesigned to increase their coverage. Currently, the here proposed primer sets are able to reveal fundamental insights into the distribution of comammox *Nitrospira* and will help to elucidate the niche separation of nitrifying microorganisms in more detail.

7.4.3. OTU sequence similarity threshold

After establishing a suitable PCR protocol, I performed an environmental screen by clone sequencing to proof the applicability of the novel primers to different types of habitats and thereby show the occurrence of comammox *Nitrospira*. I clustered the retrieved *amoA* sequences in OTUs based on a 97% sequence similarity. While higher thresholds are prone to errors by Taq polymerases during PCR and also sequencing, lower thresholds might underestimate the species richness, since the amplified region is highly conserved. Ideally a OTU threshold would be based upon a linear regression of 16S and *amoA* sequence similarities of individual species, which is currently not feasible for comammox *Nitrospira* due to data limitation (Pester *et al.*, 2014; Müller *et al.*, 2015). I also expect that the OTUs sequence similarity threshold will be adapted in future studies to the typical error rate of the respective sequencing method and the applied type of DNA polymerase, in order to confidently delineate individual comammox *Nitrospira* species.

7.5. Environmental distribution of comammox *Nitrospira*

State of the art ecological assertions are usually based upon next generation sequencing data with more than 4000 reliable amplicons per sample and several biological replicates. The here presented dataset is far more limited. Still, it is the first of its kind and thus it is tempting to deduce the following two patterns from the clone sequencing approach: (i): Clade A is primarily

found in the aquatic habitats. (ii): Clade B is preferentially thriving in soils and has a more restricted habitat spectra to oligotrophic systems. The following indices for the presence of comammox *Nitrospira* in the respective environments illustrate these two assertions.

Both clades were found within a groundwater well and in drinking water treatment plants. These are oligotrophic systems with high flow-through rates, in which microorganisms tend to form biofilms and the attachment on surfaces to circumvent washout. Some of these biofilms are dominated by the phylogenetic group of *Nitrospira,* despite the only presence of ammonia and no clear source of nitrite (Martiny *et al.*, 2003). Hence, some of these *Nitrospira* are likely comammox, which was already indicated by previous metagenomic investigations and endorsed during this study (Daims *et al*., 2015, Pinto *et al*., 2015; Palomo *et al*., 2016). Furthermore, the actual presence of comammox *Nitrospira* within this niche was predicted by the kinetic theory of optimal pathway length and underlines its validity (Costa *et al.,* 2006).

Clade A of comammox *Nitrospira* was also found in wastewater treatment plants, a rather unexpected habitat, since these systems have high washout rates and, in contrast to drinking water facilities hypertrophic conditions. Furthermore, the majority of microorganisms is free living, instead of forming biofilms (Wagner *et al*., 2002). Under these circumstances, especially *Nitrospira* lineage I and II are forming floc like colonies and often live in close association with ammonia oxidizing microorganisms. Intriguingly the comammox containing lineage II was observed to be more independently localized to AOM in WWTPs than lineage I (Gruber-Dorninger *et al*., 2014). Contingently, comammox may play a role for this niche differentiation, which is the reason why it will be crucial for future studies to understand how many of these floc like colonies are indeed comprised by comammox *Nitrospira*

The highest relative comammox richness was found within the rhizosphere and bulk soil of rice plants. Even though rice plant roots have aerenchyma and aerate the adjacent rhizosphere, the oxygen concentration decreases rapidly with an increasing distance to the root (Revsbech *et al.*, 1999). Comammox *Nitrospira* might benefit from this low oxygen concentrations in two ways, firstly their carbon fixation pathway (rTCA) is potentially oxygen sensitive, despite possible modifications of all associated O_2-sensitive enzymes (Lücker *et al.*, 2010; Erb, 2011). Secondly the rate of nitrogen fixation is facilitated under micro-aerobic conditions and might release some excessive ammonia which may be scavenged by *Nitrospira* (Fay, 1992). However the presence of comammox *Nitrospira* in in anoxic compartment of the bulk soil is puzzling as oxygen is crucial for their lifestyle, unless alternative energy generating pathways, such as H_2 or formate oxidation, are used, (Koch *et al.*, 2014, 2015). Generally, *Nitrospira* is persistent to

long periods of starvation, potentially because of its metabolic versatility, and might have been remnant or even in a dormant state (Martiny *et al.*, 2005; Koch *et al.*, 2015).

The presence of only comammox clade B in the litter free topsoil of a beech forest is congruent with our assumption that this clade is predominantly living in soils. In contrast to this stands our discovery of clade A in aquatic environments such as lake and river sediments, as well as in eutrophic systems, like wastewater treatment plants. Moreover the detection of comammox *Nitrospira* in the saline lake sediment opens the possibility for its occurrence within the largest habitat on earth - the ocean.

Ultimately, it is important not to over interpret the here presented results at the moment as the amount of data about comammox *Nitrospira* is too limited. As soon as these datasets are expanded by amplicon NGS of several biological replicates, the distribution patterns will become more apparent and the here presented hypotheses verified or refuted.

7.6. The Influence of DNA extraction on the detection of comammox *Nitrospira*

In this study, qPCR was used to determine the influence of two different DNA extraction methods to the observed comammox clade A abundance of a filter from a drinking water treatment plant. Instead of normalizing to 16S rRNA genes, I determined the DNA concentrations of the extracts and applied equimolar amounts of template DNA. Notably, in this sample the best result was delivered by the MoBio PowerSoil Kit, as the extract was not only amplified more efficiently, but also had the highest yield of total DNA. Furthermore, the first fraction of bead beating recovered relatively more *Nitrospira amoA* sequences than the second bead beating fraction, which is in congruence to the observed lysis pattern of *Nitrospira* during another study on WWTPs (Albertsen *et al.*, 2015). In these systems *Nitrospira* is forming floc like colonies and tends to the formation of thick extracellular polymeric substance layers (Gruber-Dorninger *et al*., 2014), a pattern which was not observable by FISH in the filtration material of Friedrichshof. Therefore, the less intense mechanical cell lysis during the MoBio PowerSoil Kit probably was sufficient or even beneficial as the resulting DNA is less sheared.

In addition the phenol/chloroform method is prone to the carryover of extraction solutions that might hinder the subsequent downstream processing, such as PCR. Despite the in this case good result of the MoBio PowerSoil kit, phenol based protocols are usually superior in their DNA yields. For example, from wastewater treatment plants I recovered 365 times more DNA by using the phenol/chloroform protocol, thus would probably also estimate higher and more

accurate abundances of target genes. In order to reduce the risk of qPCR inhibition, the amount of BSA can be increased and the DNA purified with DNA-cleanup Kits or simply diluted with additional TE buffer. Conclusively phenol/chloroform probably provide more accurate results for qPCR than Kit extractions. However, it is generally important that all extracts are derived by one single method, to prevent effects introduced by extraction biases (see also Albertsen *et al*., 2015).

8. Outlook

The newly designed primer pairs for comammox *amoA* clade A and B of this study are not restricted to amplicon sequencing studies, but offer the possibility for various applications. Two of them are illustrated and discussed in the following section.

8.1. Abundance of comammox *Nitrospira*

Not only the diversity and occurrence of comammox is pivotal for our understanding of nitrification, but also the abundance of these microorganisms in comparison to other ammonia oxidizers. First insights were revealed by placing the raw reads of metagenomes into our reference tree, showing that comammox *Nitrospira amoA* genes are either low abundant or not even detected by metagenomics (Pjevac *et al*., unpublished). A fundamental understanding of niche differentiation between nitrifying microorganisms requires the application of highly sensitive and robust methods such, as quantitative polymerase chain reaction (Leininger *et al.*, 2006). Preliminary data has already shown the applicability of both primer sets and revealed the abundances of comammox *amoA* sequences for selected habitats (Figure 10) (Pjevac *et al*., 2016). Similar to canonical PCR, also quantitative PCRs are prone to inhibitory substances that either origin from the sample or are introduced during the DNA extraction. For instance all phenol/chloroform DNA extracts needed up to 1000 fold dilutions, until all inhibitory substances were in sufficiently low concentrations. In combination with the generally low abundance of comammox *Nitrospira*, this may result in a high uncertainty of false negative results. Therefore, I suggest for future studies the application of DNA-cleanup Kits in combination with phenol/chloroform extractions to enable an efficient and accurate enumeration of comammox *Nitrospira*.

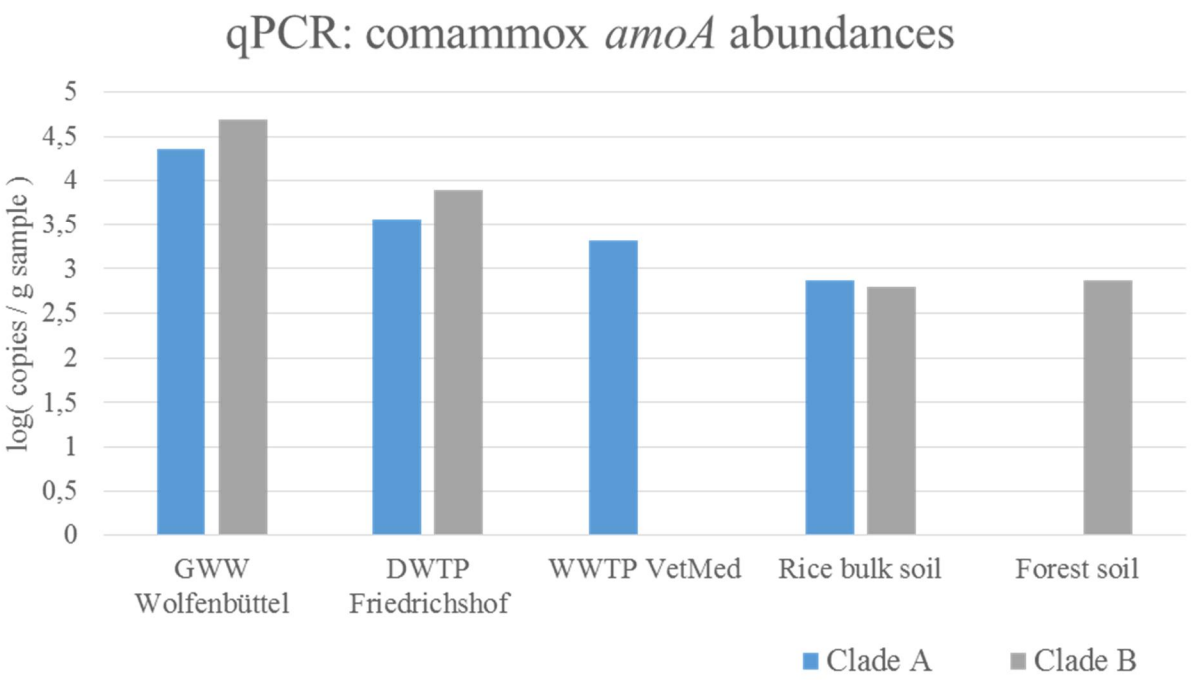

Figure 10: *amoA* abundances of comammox *Nitrospira* in selected DNA extracts of this study, estimated by qPCR (Pjevac *et al.*, 2016).

8.2. Nitrification rates of comammox *Nitrospira*

The abundance of microorganisms does not always correlate with the substrate utilization rates (Bernhard *et al*., 2009). Thus, to investigate the relative contribution of different phylogenetic groups to nitrification, pool dilution assays in combination with specific inhibitors are widely applied (Martens-Habbena *et al.*, 2015). Intriguingly, nitrifying activity of comammox *Nitrospira* is inhibited by the NO scavenging molecule PTIO and also by Octine (Vierheilig *et al*., unpublished). While Octine should therefore result with the potential nitrification rates of AOA, PTIO should reveal the relative contribution of AOB to this process. These two values combined and subtracted from the full nitrification rate should then result with the nitrification rates of comammox *Nitrospira.* Certainly this approach will have some methodological challenges. To mention one of them, the two inhibitors are tested only on one comammox pure culture - *Ca.* Nitrospira inopinata, and might not work well with environmental samples (Vierheilig *et al.*, unpublished). In order to infer the applicability of the two inhibitors to a wide range of comammox *Nitrospira,* without time intensive previous cultivation, reverse transcription qPCR targeting comammox *amoA* transcripts may be applied. If the nitrifying activity of comammox *Nitrospira* is really disabled by those two substrates, the cells should

enter a starvation phase and eventually shut down the transcription machinery. The resulting decrease of detectable *amoA* transcripts would then give first insights about the feasibility of the selective pool dilution assay in combination with the currently known inhibitors or indicate the necessity of new strategies to estimate the actual contribution of comammox *Nitrospira* to the global nitrogen cycle.

8.3. Are *Nitrospira* the only comammox microorganism?

One of the most pivotal questions of comammox research remains to be answered - are *Nitrospira* lineage II the only phylogenetic group capable of full nitrification, or is this capability more widespread among the genus of *Nitrospira* and beyond? Functional gene assays certainly will not provide answers to these questions as they are limited to known genes. Also by pure metagenomic studies novel physiologies are rarely discovered, because these microorganisms are either in too low abundances, have enzymes with no homologous similarity to already annotated genes or are simply overlooked (Könneke *et al*., 2005; Daims *et al*., 2015).

Thus, still the probably best way to discover novel physiologies is cultivation. In addition to modern molecular methods, fast and valuable results can be accomplished without the intricate complete isolation of the target microorganisms (van Kessel *et al*., 2015). A good starting point for inoculums of future enrichment cultures is again the theoretical niche of comammox, since it is not only occupied by *Nitrospira*. For instance *Thaumarchaeota* dominate numerous oligotrophic systems and tend to the formation of biofilms (Kerou *et al.*, 2016). The common assumption that those *Thaumarchaeota* are only oxidizing ammonia to the level of nitrite is based upon a limited set of cultured representatives, coming from a defined set of environments, while the full metabolic capacity of this phylum remains virtually unexplored (Könneke *et al.*, 2005; Hatzenpichler *et al.*, 2008; Tourna *et al.*, 2011).

In the end and after more than one hundred years of nitrification research, it is still a vast playground for innovative thoughts and bears numerous unresolved mysteries for future studies to discover.

9. Literature

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10. Abbreviations

Table 25: A list of all used Abbreviation and the corresponding full designations.

Abbreviation	Full designation
AMO	Ammonia monooxygenase
amoA	Ammonia monooxygenase gene - alpha subunit
Anammox	Anaerobic ammonia oxidation
AOA	Ammonia oxidizing Archaea
AOB	Ammonia oxidizing Bacteria
AOM	Ammonia oxidizing Microorganisms
BSA	Bovine Serum Albumin
Ca.	Candidatus species
Comammox	complete oxidation of ammonia to nitrate
DNA	Deoxyribonucleic acid
DNRA	Dissimilatory nitrate reduction to ammonia
EDTA	Ethylenediaminetetraacetic acid
GWW	Groundwater well
H_2O_2	Hydrogen peroxide
N2O	Nitrous oxide
NGS	next generation sequencing
NH_4^+	Ammonia
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite oxidizing Bacteria
Nxr	Nitrite oxidoreductase
nxrB	Nitrite oxidoreductase gene - beta subunit

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PMO	Particulate methane monooxygenase
PP	Polypropylen
PTIO	2-Phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide
qPCR	quantitative Polymerase Chain Reaction
RAS	Recirculating aquaculture system
RNA	Ribonucleic acid
RT	Room Temperature
TE	Tris/EDTA buffer
TNA	Total Nucleic Acids
TNS	Tris/NaCl/SDS buffer
UV	Ultraviolet
w.o.	without
WWTP	Wastewater treatment plant