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Developing a framework for microbial community analysis for wastewater treatment systems

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Vom Fachbereich Bau- und Umweltingenieurwissenschaften
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zur Erlangung des akademischen Grades eines
Doktors (Dr. rer. nat.) genehmigte Dissertation

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DISSERTATION

von
Laura Orschler, M.Sc.

Darmstadt, August 2020

“Ach, die Welt ist so geräumig, und der Kopf ist so beschränkt.”

(Wilhelm Busch)

Für meine Eltern.

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Abstract

PCR-based methods have caused a surge in the integration of eco-physiological approaches into research on partial nitritation anammox (PNA). PNA systems have been characterized as fine-tuned biological nitrogen removal (BNR) process with a very complex ecosystem. Therefore, molecular methods, which offer a wide range of approaches comparable to a workman's toolbox, have been intensively used to understand these PNA systems and achieve a stable process. On the one hand, quantitative PCR (qPCR) became the most common method to quantify target microorganisms in engineered systems such as PNA and other ecological studies and is therefore the so-called gold standard for a fast and reliable quantification. On the other hand, next-generation sequencing (NGS) as a new and advanced approach enabled in-depth analysis, provided new genomes in public databases, and resulted in a more conscious look on the PNA microbiome.

Hypervariable (*hv*) regions of the 16S rRNA gene, which is the so-called "fingerprint gene", are most commonly sequenced when using NGS to study the bacterial composition of the PNA systems as well as other ecosystems. With this approach, even poor quality or low concentrations of deoxyribonucleic acid (DNA) can be used for sequencing of the whole microbial community of a sample without prior selection for microbes of interest (as in the case of culture-based approaches). However, the *hv* region targeted and primer pair used can impact the results achieved and thereby impair the ability of researchers to compare different studies using different primer pairs and targeting different *hv* region. There is a vast collection of primers from different *hv* regions for both qPCR and NGS, which complicate the selection of appropriate primer pair. In my doctoral study, I used a 16S rRNA amplicon sequencing approach, which includes the parallel sequencing of six 16S rRNA *hv* regions using multiple primer pairs. The results revealed that there is no perfect hv region for PNA microbial communities. I observed that the community composition varies highly based on the chosen hv region and primer pair. Notably, using only one *hv* region for the analysis with subsequent normalization to relative fractions of target microorganisms and further comparison with other reactor studies is strongly discouraged.

In general, there are not only primers based on the 16S rRNA gene, but also for functional genes. For engineered ecosystems, this is of high interest as the role of target microorganisms in the process can be explicitly monitored. The ammonium oxidation, which is performed by ammonium oxidizing bacteria (AOB), is the first key step in PNA systems as it provides the nitrite for the anaerobic ammonium oxidizing bacteria (AnAOB). The role of AOB is known already for a long time, as they play an important role in the global nitrogen cycle. Therefore, there are primers available for the ammonium monooxygenase (*amoA*) gene that are supposed to cover all taxonomic clusters of the AOB. However, current genome entries in public databases demonstrate the opposite.

In my doctoral study, I used shotgun sequencing to determine the composition of the AOB in PNA and conventional activated sludge systems (CAS) in Germany to not only reevaluate available primers for the amoA gene, but to design highly specific primers for *Nitrosomonas eutropha* and *Nitrosomonas europaea* based on the amoA gene. The results showed that the coverage of the existing and most used primer pair (amoA1-amoA2r) is very poor, particularly

for N. eutropha and N. europaea commonly found in the N-DN and PNA systems (Purkhold et al., 2000; Vlaeminck et al., 2010). Therefore, in this study new specific primers and Taqman probes were designed for N. eutropha and N. europaea. These specific primers and probes allow gaining knowledge about the existence of niche separation between *N. eutropha* and *N. europaea* in PNA as well as CAS. In the last decade, many studies based on the 16S rRNA gene amplicon analysis revealed the presence of putative heterotrophic denitrifiers (HB) in the PNA microbiome. However, it is not very easy to determine the composition of HB based on the 16S rRNA gene, because denitrifiers are not typically linked to phylogeny. Therefore, in this study targeted metagenomic sequencing approach was employed to determine the composition of the HB based on the functional genes associated with the denitrification pathway. Both the PNA systems and the CAS systems were analyzed for the composition of the HB. Also, the community composition of the samples based on the 16S rRNA gene and denitrification pathway functional genes were compared. The results based on targeted metagenomics disclosed the diversity among HB in PNA and CAS systems, which was not observed based on the 16S rRNA gene amplicon sequencing. The use of targeted metagenomics also revealed that none of the members have the whole gene set for carrying out complete denitrification. The results demonstrate the need to look into this functional group more in detail, especially for a better understanding of PNA systems.

All research work combined in this study revealed a framework to overcome challenges for better integration of the molecular methods in wastewater microbiome studies - which is mainly about understanding the current biases in molecular methods, standardization, and selection of the right combination of molecular methods. In general, data consistency and accuracy strongly depend on the primer selection and data interpretation. The reevaluation of existing primers and the design of a more specific primer will improve the respective molecular studies and support our understanding, which then leads to an improved assessment of nitrification-denitrification (N-DN) and PNA systems.

The combination of traditional microbiology and the modern molecular biological methods has received only marginal attention in this work but will be the non-plus ultra-method for further insights into complex microbiomes.

Kurzfassung

PCR-basierte Methoden haben die Integration ökophysiologischer Ansätze in der Forschung besonders im Hinblick auf partielle Nitritation/Anammox (PNA)-Systeme stark vorangetrieben. Das PNA System ist definiert als biologischer Stickstoffentfernungsprozess (BNR), der durch ein sehr komplexes, aber fein abgestimmtes Ökosystem charakterisiert ist. Daher werden molekulare Methoden, die eine breite Palette von Ansätzen bieten, vergleichbar mit dem Werkzeugkasten eines Handwerkers, eingesetzt, um PNA-Systeme zu verstehen und einen stabilen Prozess zu garantieren. Diese Methoden weisen jedoch einen naturgemäßen Bias auf, sowie fehlenden Konsens für die Standardisierung und Durchführung. Trotzdem hat sich die quantitative PCR (qPCR) zur gebräuchlichsten Methode entwickelt, um Zielmikroorganismen in technischen Systemen wie dem PNA Prozess und anderen Ökosystemen zu quantifizieren und ist der sogenannte der Goldstandard für eine schnelle und zuverlässige Quantifizierung in allen ökologischen Studien. Zusätzlich hat sich durch Next Generation Sequencing Methoden (NGS) einen neuer und fortschrittlicher Ansatz durch eine sogenannte ‚in-depth' Analyse entwickelt und durch die Veröffentlichung neuer Genom-Sequenzen in öffentlichen Datenbanken zu einer kritischeren Betrachtung des PNA-Mikrobioms führen.

Bei den NGS-Methoden wird am häufigsten eine oder mehrere der neun hypervariablen (hv) Regionen des 16S rRNA Gens, das sogenannte „Fingerabdruck-Gen", sequenziert, um die bakterielle Gemeinschaft in PNA-Systemen sowie anderer Ökosysteme zu analysieren. Mit diesem Ansatz können sogar niedrige Desoxyribonukelinsäure (DNA)-Konzentrationen oder schlechte DNA-Qualität zur Sequenzierung der gesamten mikrobiellen Gemeinschaft verwendet werden, ohne eine vorherige Auswahl der Zielmikroorganismen (wie im Fall von kulturbasierten Ansätzen). Die ausgewählte *hv* Region und das jeweilige Primerpaar können jedoch die Ergebnisse beeinflussen und dadurch die Möglichkeit beeinträchtigen, Daten aus verschiedenen Studien zu vergleichen. Sowohl für qPCR als auch für NGS gibt es eine große Auswahl von Primern für verschiedenen *hv* Regionen des 16S rRNA Gen, die eine finale Entscheidung für ein geeignetes Primerpaars erschweren. In meiner Doktorarbeit verwende ich einen 16S rRNA Amplikon-Sequenzierungsansatz, der die parallele Sequenzierung von sechs 16S rRNA *hv* Regionen unter Verwendung mehrerer Primerpaare ermöglicht. Die Ergebnisse zeigten, dass es keine optimale hv Region für die Analyse von mikrobiellen Gemeinschaften in PNA- und konventionellen Belebtschlammsystemen (CAS) gibt und die Zusammensetzung der mikrobiellen Gemeinschaft je nach *hv* Region sowie Primerpaar stark variiert. Grundsätzlich wird dringend davon abgeraten nur eine hv Region für die Analyse zu verwenden, sowie eine anschließende Normalisierung auf die relativen Anteile der jeweiligen funktionellen Zielgruppen, um diese Ergebnisse für einen Vergleich mit anderen Reaktorstudien zu verwenden.

Für molekulare Analysen existieren nicht nur Primer, die auf dem 16S rRNA Gen basieren, sondern auch Primer, die die jeweiligen funktionellen Gene abdecken. Für technische Ökosysteme ist dies von großem Interesse, da die Rolle der Zielmikroorganismen im Prozess explizit überwacht werden kann. Die Ammoniumoxidation, die von ammoniumoxidierenden Bakterien (AOB) durchgeführt wird, ist der erste Schlüsselschritt in PNA-Systemen, da sie das

Nitrit für die anaeroben ammoniumoxidierenden Bakterien (AnAOB) liefert. Die Rolle der AOB ist seit langem bekannt, da sie im globalen Stickstoffkreislauf eine wichtige Rolle spielen. Daher gibt es bereits seit über 30 Jahren ein Primerpaar für das Ammoniak-Monooxygenase (*amoA*) -Gen, das alle taxonomischen Cluster der AOB abdecken soll, jedoch zeigen aktuelle Genomeinträge in öffentlichen Datenbanken das Gegenteil.

In meiner Doktorarbeit habe ich die Zusammensetzung der AOB in PNA- und CAS-Systemen in Deutschland mithilfe der Shotgun-Sequenzierung bestimmt, um nicht nur die verfügbaren Primer für das amoA-Gen neu zu bewerten, sondern auch spezifische Primer für Nitrosomonas eutropha und Nitrosomonas europaea zu entwerfen, basierend auf dem amoA-Gen. Die Ergebnisse zeigten, dass die Abdeckung des am häufigsten genutzten Primerpaars (amoA1-amoA2r) sehr schlecht ist, insbesondere für die häufig in den N-DN und PNA-Systemen vorkommenden *N. eutropha* und *N. europaea* (Purkhold et al., 2000; Vlaeminck et al., 2010). Daher wurden in dieser Studie neue spezifische Primer und Taq-Sonden für N. eutropha und N. europaea entwickelt, die für das Verständnis und die anschließende Schlussfolgerung zur Leistung von AOB in PNA-Systemen unerlässlich sind. Darüber hinaus ermöglichen die spezifischen Primer und Sonden das Studium einer Nischentrennung zwischen *N. eutropha* und *N. europaea* in PNA-Systemen sowie in herkömmlichen CAS-Systemen.

In den letzten zehn Jahren haben viele Studien, die auf der 16S rRNA Genamplikonanalyse basieren, eine Bandbreite an mutmaßlich heterotrophen Denitrifikanten (HB) im PNA-Mikrobiom gezeigt. Es ist jedoch komplex die Zusammensetzung der HB basierend auf dem 16S rRNA Gen zu bestimmen, da nur wenige Gene vorhanden sind, die mit dem Denitrifikationspfad assoziiert sind. Daher wurde in dieser Studie ein gezielter metagenomischer Sequenzierungsansatz verwendet, um die Zusammensetzung der HB basierend auf den funktionellen Genen zu bestimmen, die mit dem Denitrifikationspfad assoziiert sind. Sowohl PNA- als auch CAS-Systeme wurden auf die Zusammensetzung der HB analysiert. Außerdem wurde die Zusammensetzung der mikrobiellen Gemeinschaft sowohl basierend auf dem 16S rRNA Gen als auch auf funktionellen Genen des Denitrifikationspfads verglichen. Die Ergebnisse zeigten die Diversität zwischen HB in PNA- und CAS-Systemen, die aufgrund der 16S rRNA Genamplikonsequenzierung nicht detektiert wurden. Die Verwendung gezielter Metagenomik ergab auch, dass keines der Mitglieder über einen vollständigen Gensatz für die Durchführung einer vollständigen Denitrifikation verfügt. Die Ergebnisse zeigen, dass diese Funktionsgruppe genauer untersucht werden muss, insbesondere um Nitrifizierung-Denitrifizierung (N-DN)- und PNA-Systeme besser zu verstehen.

Die Kombination der Forschungsarbeiten in dieser Doktorarbeit ergaben die Rahmenbedingung, um die bekannten Herausforderungen zu bewältigen für eine bessere Integration von molekularen Methoden in PNA-, sowie CAS-Studien. Dabei geht es hauptsächlich um das Verständnis der aktuellen Biase bei molekularen Methoden, die Standardisierung der entsprechenden Methoden sowie die richtige Kombination molekularer Methoden. Denn im Allgemeinen hängen Datenkonsistenz und -genauigkeit stark von der Primerauswahl und Dateninterpretation ab. Die Neubewertung bestehender Primer und das Design spezifischerer Primer werden molekulare Studien verbessern und unser Verständnis unterstützen und dadurch zu einer verbesserten Bewertung von PNA -Reaktorstudien führen.

Die Kombination von traditioneller Mikrobiologie und modernsten molekularbiologischen Methoden hat in dieser Arbeit nur marginale Beachtung gefunden, wird aber in den kommenden Jahrzehnten die Non-Plus-Ultra-Methode sein, um weitere Einblicke in Mikrobiome zu erhalten.

Inhaltsverzeichnis

Abbreviations

Amo	Ammonium monooxygenase
amoA	Ammonium monooxygenase gene encoding subunit a
Anammox	Anaerobic ammonium oxidation
AnAOB	Anaerobic ammonium oxidizing bacteria
ANOVA	Analysis of variance
AOB	Ammonium oxidizing bacteria
ASV	Amplicon sequencing variant
Bp	Base pair
BNR	Biological nitrogen removal
CANON	Complete Autotrophic Nitrogen Removal Over Nitrite
CAS	Conventional activated sludge
CLR	Continuous long read
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DNB	Denitrifying bacteria
dPCR	Digital PCR
Ds-DNA	Double stranded DNA
EUB	Total bacterial abundance
FISH	Fluorescence in situ hybridization
FRET	Fluorescence resonance energy transfer
Hao	Hydroxylamine oxidoreductase
HB	Putative heterotrophic denitrifiers
hdh	Hydrazine dehydrogenase
Hv	Hypervariable regions of the 16S rRNA gene
hzs	Hydrazine-synthase
LH-PCR	Length heterogeneity-PCR
nap	Periplasmic nitrate reductase
napA	Periplasmic nitrate reductase encoding subunit A
nar	Respiratory nitrate reductase
narG	Respiratory nitrate reductase encoding subunit G
N-DN	Nitrification-Denitrification
nirK	Copper containing nitrite oxidoreductase
nirS	Cytochrome cd1 containing nitrite oxidoreductase
NGS	Next Generation Sequencing
NMDS	Non-metric multidimensional scaling
NOB	Nitrite oxidizing bacteria
nor	Cytochrome C-dependent nitric oxide reductase
norB	Cytochrome C-dependent nitric oxide reductase encoding subunit B
nos	Nitrous oxide reductase
nosZ	Nitrous oxide reductase encoding subunit Z
OTU	Operational taxonomic unit
PCR	Polymerase Chain Reaction
PM	Perfect Match
PNA	Partial nitritation/anammox
qPCR	Quantitative polymerase chain reaction
SBR	Sequencing batch reactors
SMRT	Single-molecule real-time sequencing
TGGE	Temperature gradient gel electrophoresis
t-RFLP	Terminal-restriction fragment length polymorphism
WWT	Wastewater treatment

WWTP	Wastewater treatment plant
ZMW	Zero Mode Waveguides

List of Figures and Tables

1 Introduction

In 1977, our understanding of bacterial taxonomy was revolutionized by the detection of the small ribosomal subunit of the 16S rRNA gene by Woese and Fox (1977) and its special role. Since then, the most common target for molecular identification of bacteria is the small ribosomal subunit of the 16S rRNA gene, as it is present in all bacteria, and their special conserved regions can be targeted by universal or specific primers. So far, microbial communities were considered as black boxes, and incomplete knowledge about their physiology and interactions in complex natural environments, impeded obtaining pure cultures of most microorganisms from the respective natural habitats (Sanz and Kochling, 2007). Ward et al. (1990) suggested that the analytical methods based on the 16S rRNA gene would help to understand the composition of microbial communities, because new culture independent methods are able to reveal some of the unexplored diversity within the microbial world.

For understanding biotechnological processes or to explain natural biological processes it is essential to understand the interrelationships between bacteria and their environment by studying the structural and functional diversity of bacterial communities and their response to various natural or man-made disturbances. Especially, the diversity of bacterial wastewater communities gained interest in the last years, because isolating and culturing microorganisms out of wastewater, which is a complex habitat, only led to the identification of few microorganisms (Daims et al., 2006). Nowadays, it is generally accepted that culture based microbial community analyses are known for their selectivity and are not considered representative of the extent of the bacterial community diversity (Cydzik-Kwiatkowska and Zielinska, 2016).

1.1 Background

Nitrogen is the fourth most abundant element on earth and essential for the synthesis of nucleic acids and proteins. 78% of Earth's atmosphere is nitrogen gas and the interchange between inert dinitrogen gas (N_2) and 'reactive' nitrogen compounds is entirely controlled by microbial activities. Before the development of the Haber-Bosch process (the industrial fixation of N_2 into ammonia, NH_3) in 1909, all of the reactive nitrogen in the biosphere was generated and recycled by microorganisms (Erisman et al., 2008; Stein and Klotz, 2016). But the Haber-Bosch process led to a man-made interference to the natural nitrogen cycle by quadrupling the productivity of agricultural crops and chemical fertilizers. This anthropogenic overproduction of nitrogen damages environmental systems, ranging from eutrophication of terrestrial and aquatic systems to global acidification (Galloway et al., 2008; Gruber and Galloway, 2008; Stein and Klotz, 2016), which led to the concern for discharge of high nitrogen load wastewater to

water bodies. Therefore, nitrification and denitrification in conventional activated sludge systems (CAS) has been widely applied for wastewater treatment.

1.2 A brief introduction into the nitrogen cycle and its significance for wastewater treatment

1.2.1 Nitrification

Looking one step closer, nitrification is the biological oxidation of ammonium to nitrate, which is subdivided into two steps: nitritation (oxidation of ammonium to nitrite) and nitratation (oxidation of nitrite to nitrate) (Figure 1). Nitritation is the first step of nitrification which is carried out by ammonium oxidizing bacteria (AOB) and the first AOB were isolated in 1890. Phylogenetically AOB are divided into two distinct groups: *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus* belong to the β-Proteobacteria subclass and *Nitrosococcus* to the γ-Proteobacteria subclass. Due to their gram-negative, multilayered cell wall and their motility due to the presence of flagella, AOB can be distinguished by their cell morphology (Koops and Pommerening-Röser, 2001; Purkhold et al., 2000).

Nitratation is the second step of nitrification which is carried out by chemolithoautotrophic nitrite oxidizing bacteria (NOB), belonging phylogenetically to α-Proteobacteria, γ-Proteobacteria and δ-Proteobacteria: *Nitrobacter*, *Nitrococcus*, *Nitrospira* and *Nitrospina*. For a long time, *Nitrobacter* spp. were assumed to be the dominant nitrite oxidizers in wastewater treatment plants (WWTPs), but 16S rRNA gene based molecular analysis disclosed the dominance of *Nitrospira*-like bacteria (Daims et al., 2001). *Nitrobacter* is an r-strategist NOB, relatively fast-growing with low affinity to nitrite and oxygen, whereas *Nitrospira* is a k-strategist having a low maximum specific growth rate, with high affinity to nitrite and oxygen. Therefore, *Nitrospira* has competitive advantage in oxygen-limited environments over *Nitrobacter* (Daims et al., 2001; Gilbert et al., 2014a).

In general, nitrifying bacteria are extremely slow-growing microorganisms and recalcitrant to cultivation attempts. Due to the sensitivity of nitrifying bacteria to disturbances like pH- and temperature shifts, breakdown of the nitrification process is frequently reported from municipal and especially industrial WWTPs (Wagner et al., 2002).

1.2.2 Denitrification

Denitrification is the second step after nitrification and involves the reduction of nitrate to dinitrogen gas via anaerobic respiration of nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) (Figure 1) (Zumft, 1997). Denitrification is a facultative anaerobic microbial process and is executed by heterotrophic microorganisms belonging to diverse groups of phylogenetically unrelated bacteria, including members of the Chloroflexi, Firmicutes, Actinobacteria, Bacteroides, and Proteobacteria phyla (Lu et al., 2014). Furthermore, denitrification can be considered as a community process, because most of the denitrifying microorganisms do not possess the complete suite of enzymes for complete denitrification, but have potential for partial or intermediate steps of the denitrification pathway (Wallenstein et al., 2006; Zumft, 1997). Heterotrophic microorganisms associated with denitrification can be divided into four subgroups, i.e. complete denitrifiers (capable of reducing nitrate to N_2), partial denitrifiers (reducing nitrite to N_2), incomplete denitrifiers (reducing nitrate or nitrite to nitrogen oxide intermediates instead of N_2) and nitrogen oxide reducers (capable of reducing NO and N_2O to N_2) (Lu et al., 2014; Stein and Klotz, 2016). Microorganisms with the ability to couple all of the denitrification pathway are known as classical or canonical denitrifiers (Stein and Klotz, 2016).

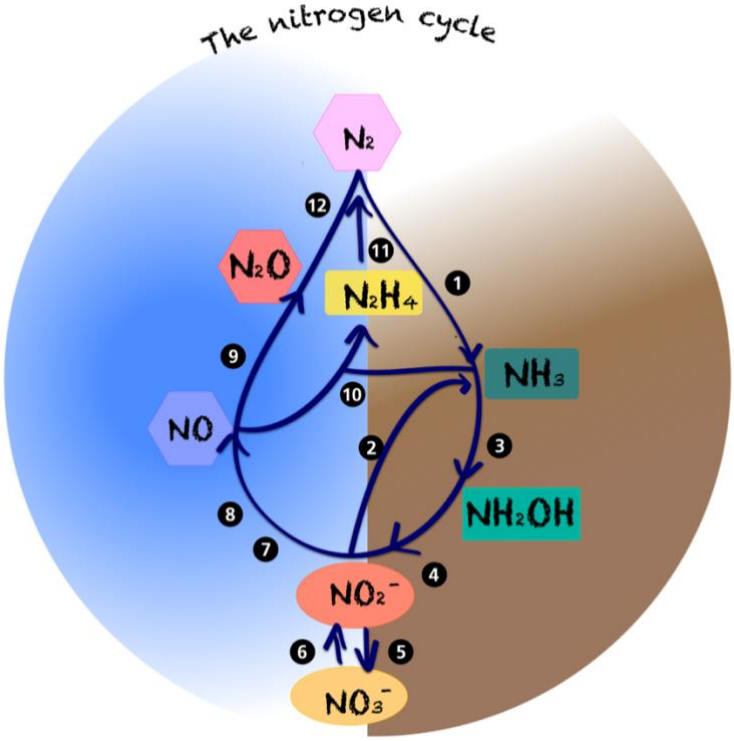

Figure 1: Overview of the nitrogen cycle in wastewater treatment plants; Major processes of the nitrogen cycling: 1. Reduction of dinitrogen (also referred as nitrogen fixation), 2. Dissimilatory nitrite reduction to ammonium (also known as

DNRA), Nitrification involving oxidation of ammonia to nitrite (3 & 4, nitritation) and oxidation of nitrite to nitrate (5, nitratation). Reduction of nitrate to nitrite can be coupled to DNRA, denitrification (8,9 & 12) and anammox (7,10 & 11).

The early research on the denitrifiers was limited to few isolates, which were generally believed to be the key denitrifying organisms in WWTPs due to their dominance in culturing methods. But the use of molecular methods revealed, that a vast diversity of denitrifiers exists, thereby highlighting the complex network of denitrifying microorganisms (Cheneby et al., 2004; Priemé et al., 2002; Scala and Kerkhof, 2000; Throback et al., 2004).

1.2.3 Anaerobic ammonium oxidizing bacteria (AnAOB)

For a long time, the anaerobic autotrophic oxidation of ammonia (anammox) was assumed to be biochemically impossible, until its occurrence was predicted by Broda (1977) followed by the discovery of the anaerobic ammonium oxidizing bacteria (AnAOB) in a denitrifying fluidized bed reactor in a WWTP 20 years later in Delft (Harhangi et al., 2012; Strous et al., 1999). The enrichment cultures and purified cells of the first AnAOB "*Brocadia anammoxidans*" enabled to identify the anammox process: converting ammonia and nitrite into dinitrogen gas in the absence of oxygen and fixing CO_2 into cellular carbon (Jetten et al., 2005; Jetten et al., 2009). AnAOB which belong to the Planctomycetes phylum, are characterized by slow growth and their dependence on a nearby source of nitrite (Jetten et al., 2005). Five genera of AnAOB have been identified so far, including *Kuenenia*, *Brocadia*, *Anammoxoglobus* and *Jettenia* mostly detected in activated sludge, whereas *Scalindua* is found in marine environments. They are all with the 'Candidatus' status, because it is possible to grow them in highly enriched cultures, but they do not exist in pure culture yet (Kartal et al., 2013; Oshiki et al., 2013).

These AnAOB possess a coccoid cell morphology with a diameter less than 1 µm. Similar to the other Planctomycetes, AnAOB lack peptidoglycan (Kartal et al., 2011a; van Niftrik et al., 2004). Moreover, they possess an intracytoplasmic compartment bounded by a single ladderane lipid-containing membrane, which is known as the anammoxosome (van Niftrik et al., 2004). Studies revealed one of the key enzymes of the anammox reaction- the hydrazine-synthase enzyme (*hzs*)- is encoded in the anammoxosome, indicating that the anammox catabolism is located inside the anammoxosome (Kartal et al., 2011b; Lindsay et al., 2001). The main difference between other Planctomycetes and AnAOB is the anaerobic chemolithoautotrophic metabolism potential of the AnAOB. Early studies reported, that AnAOB are slow growing microorganisms with doubling times between 15-30 days (Jetten et al., 2005), but recently Zhang et al. (2017) also

reported a doubling time of 2.1 and 3.7 days for Candidatus *Brocadia sinica* and *Candidatus Jettenia Caeni* in sodium alginate gel beads.

1.2.4 Nitrogen Removal in WWTPs

The most widely used method for biological nitrogen removal (BNR) from wastewater is the combination of aerobic autotrophic nitrification followed by anoxic denitrification, generally known as conventional nitrification-denitrification (N-DN) (Jenkins and Wanner, 2014). The conventional activated sludge (CAS) process is based on two-step N-DN.

Immediately after the discovery of AnAOB, the idea of an energy- and cost-effective BNR was proposed as "partial nitritation/anammox process (PNA)" (Jetten et al., 1997). PNA is a combination of (1) partial nitritation, where a part of the ammonium is oxidized to nitrite by AOB, (2) and anammox, where AnAOB convert the other part of ammonium together with nitrite to nitrogen gas in absence of oxygen. Despite several years of experience with PNA processes we are still facing challenges. One of the main challenges is the complexity of the PNA microbial ecosystem, which consists of desired as well as undesired microbial interactions: (1) the desired interaction for ammonium between AOB and AnAOB and (2) undesired interaction for nitrite between NOB, AnAOB and heterotrophic denitrifiers which is a limiting factor for the anammox process (Agrawal et al., 2018).

1.2.5 Functional genes of the nitrogen cycle in WWTPs

To assess the performance of the BNR based on molecular methods, functional genes are more suitable than the 16S rRNA marker gene, because they allow direct estimation of the functional capabilities of each metabolic pathway relevant for N-DN and PNA. Nitritation is carried out by two enzymes: ammonium monooxygenase (*amoA*) oxidizing NH_4^+ to H_3NO which is afterwards oxidized to NO_2^- by hydroxylamine oxidoreductase (*hao*). Nitratation is carried out by nitrite oxidoreductase (*nxrAB*) oxidizing NO_2^- to NO_3. In case of denitrifying bacteria, the use of functional genes also helps to capture the phylogenetic diversity better than the 16S rRNA gene, because denitrifiers are associated to multiple phyla. Therefore, most studies on denitrifiers in natural habitats target the functional genes coding for enzymes involved in denitrification (Wallenstein et al., 2006). Denitrification is coupled to four enzymatic conversions: (1) respiratory nitrate reductase (*nar*) or periplasmic nitrate reductase (*nap*) reducing nitrate to nitrite; (2) cytochrome cd1 containing nitrite oxidoreductase (*nirS*) or copper containing nitrite oxidoreductase (*nirK*) reducing nitrite to nitric oxide; (3) cytochrome *c*-dependent nitric oxide reductase (*nor*) reducing nitric oxide to nitrous oxide; (4)

and nitrous oxide is reduced to dinitrogen gas via nitrous oxide reductase (*nosZ*) (Zumft, 1997).

AnAOB use three enzymes: nitrite oxidoreductase (*nirS*) reducing NO_2^- to NO, while oxidizing minor amounts of NO_2^- to NO_3^- ; hydrazine synthase (*hzs*) oxidizing ammonium to hydrazine (N_2H_4) using NO and hydrazine dehydrogenase (*hdh*) oxidizing N_2H_4 to dinitrogen gas (Kartal et al., 2011b).

1.3 Microbiome analysis of wastewater treatment samples

1.3.1 DNA extraction

An efficient and effective method for nucleic acid extraction is often overlooked in the microbial ecologist's toolbox. It is not sufficient to simply extract nucleic acids from an environmental sample, but one needs to extract good quality and high yield nucleic acids, because freeing deoxyribonucleic acid (DNA) from contaminants and inhibitors such as humic substances, organic salts, or detergents for best possible downstream analysis is a main challenge in DNA extraction (Boesenberg-Smith et al., 2012). The major steps in DNA extraction include disruption or lysis of the cell, protein removal, chemical removal and redissolving the DNA in DNA-free water or a protective buffer (van Loosdrecht et al., 2016).

Several studies have investigated the influence of different DNA extraction protocols for activated sludge, comparing the yield and the ability to obtain reproducible PCR products (Vanysacker et al., 2010; Yu and Mohn, 1999). Gou and Zhang (2013) used next generation sequencing (NGS) to evaluate different DNA extraction kits for samples from WWTP, showing that DNA extraction without bead beating underestimates the presence of bacteria that are typically hard to lyse. Bead beating implies physical extraction using different kinds of beads such as glass or steel beads, where the high-speed agitating movement of the beads is used to lyse the cells (de Boer et al., 2010). A general recommendation for WWT samples is to use the FastDNA spin kit for soil (MP biomedicals), which is a bead beating method, to achieve high yield and purity (Albertsen et al., 2015; Guo and Zhang, 2013; Vanysacker et al., 2010).

1.3.2 Molecular methods in biological nitrogen removal in wastewater

Biological wastewater treatment is among the most important biotechnological applications, and as drivers of the key processes, microorganisms are key contributor to its success (Daims et al., 2006). Microorganisms are living as

microbial communities based on complex interrelationships and are therefore an important resource for improving bioprocesses like activated sludge and PNA systems.

The first step towards a better understanding of BNR process in WWTPs is the characterization of the microbiome present (N-DN as well as PNA) to attain an overview of the bacterial community, and apart from that to identify the key players. Modern molecular techniques, including environmental genomics, have identified unexpected microbial players involved in N-DN and PNA, and provided many exciting insights into the diversity, functions and niche differentiations (Agrawal et al., 2018; Bassin et al., 2018). In the last decade, advances in molecular methods and high throughput sequencing have given the opportunity to identify bacteria at high resolution by using the 16S rRNA gene as 'fingerprint' (van Loosdrecht et al., 2016).

1.3.3 Fingerprinting methods

Before the development of high throughput sequencing methods, the diversity of a microbial community was investigated using various molecular fingerprinting techniques: denaturing and temperature gradient gel electrophoresis (DGGE/TGGE), where denatured DNA-fragments of the same size are differentiated based on their variable mobility on a gel, which is induced due to different nucleic acid sequences and generates patterns that directly reflect the genetic biodiversity of a sample (Marzorati et al., 2008; Muyzer et al., 1993); length heterogeneity-PCR (LH-PCR) (Suzuki et al., 1998), which exploits the sequence length hyper-variability in different domains that exist within the 16S rRNA genes or intergenic spacer regions; terminal-restriction fragment length polymorphism (t-RFLP) (Liu et al., 1997), where the 16S rRNA gene is amplified with universal primers, one of them being fluorescently labelled and the product is digested with frequently cutting restriction enzymes; 16S rRNA gene clone libraries (Ward et al., 1990) imply the extraction of nucleic acids, amplification and cloning of the respective 16S rRNA gene, followed by sequencing (Gilbride et al., 2006; Sanz and Kochling, 2007; Smalla et al., 2007; van Loosdrecht et al., 2016) (Figure 2); and Fluorescence in-situ hybridization (FISH) which is a staining method, where short DNA sequences are labeled with a fluorescent dye, avoiding the drawbacks of DNA extraction (Wagner et al., 1993). This molecular method enables physiological and phylogenetic information and is a popular in-situ analysis for spatial organization of microorganisms in biofilms (Figure 2).

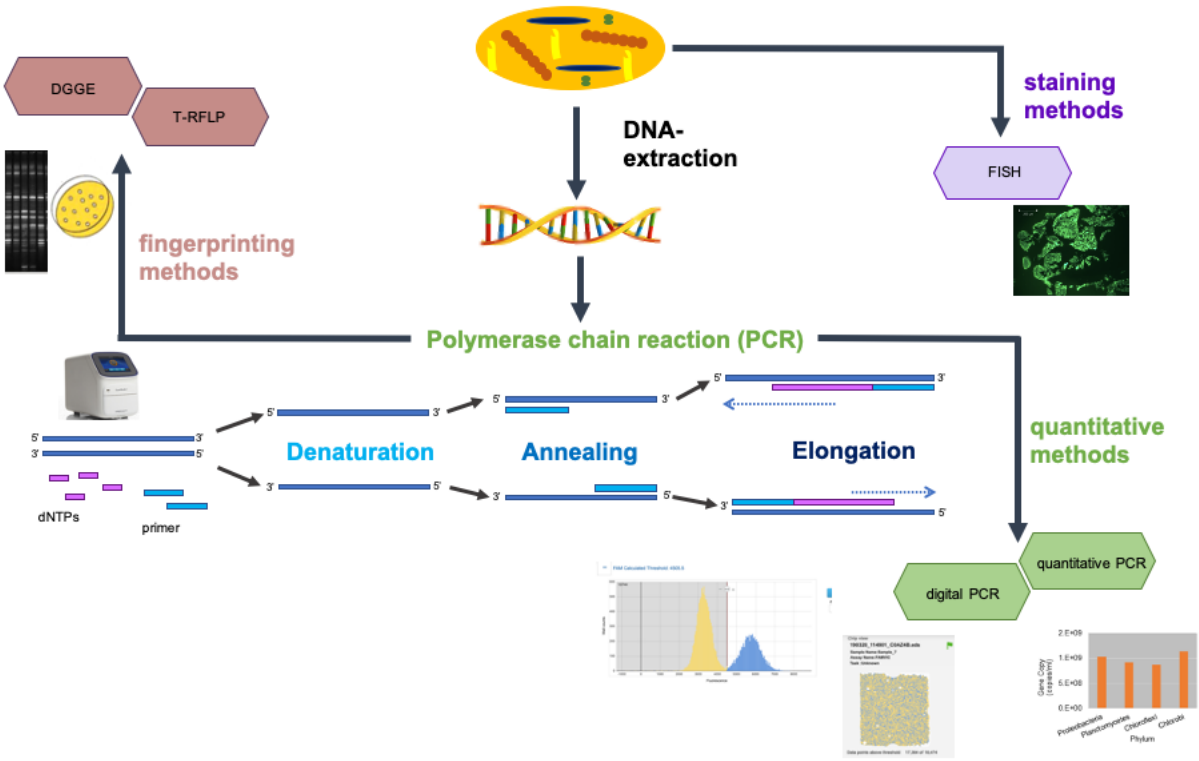

Figure 2: Overview of the molecular methods, that have been developed in the last 30 years. These methods are divided into fingerprinting methods, quantitative methods and staining methods. The chosen molecular methods belong to the most commonly used methods in the wastewater treatment sector. Additionally, it is divided into PCR based methods and non PCR based methods, and the basic principle of PCR (Denaturation, Annealing, Elongation) is illustrated in the middle.

1.3.4 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is a simple method for amplifying a target DNA fragment, based on three temperature depending steps: denaturation, annealing and elongation. Based on a temperature profile, the double stranded DNA is denaturated, specific primer pairs are annealed and with help of the polymerase enzyme elongated (Figure 2). In every PCR cycle (denaturation, annealing and elongation) the number of copies increases exponentially (Mullis, 1990). All previous introduced methods (in section 1.3.2.1) are based on the principle of PCR, except cloning and FISH.

Quantitative real time PCR (qPCR) is the quantitative detection of microorganisms on the basis of the PCR method, which was developed in the early 1990s. qPCR enables the detection of PCR amplicons during the early exponential phase of the amplification reaction allowing the detection and quantification of the PCR products (Figure 2) (Baker, 2012). The target is detected either by incorporation

of a double-stranded DNA (dsDNA)-specific dye or by the release of a TaqMan FRET (fluorescence resonance energy transfer) probe through polymerase 5'-3' exonuclease activity (Goodwin et al., 2016). The conceptual and practical simplicity of qPCR, together with its combination of speed, sensitivity, and specificity in a homogeneous assay, have made it the touchstone for nucleic acid quantification (Bustin et al., 2009). But results of qPCR may be influenced by the quality of the standard curve and inhibiting substances in the DNA sample (Bustin and Nolan, 2017; Pabinger et al., 2014).

Since the development of qPCR, it was refined or rather extended by inventing new technologies based on the PCR principle. For the identification of several targets it is possible to amplify several sequences in a single reaction, by using multiple primer pairs simultaneously, which is called multiplex PCR (Gilbride et al., 2006). This is a time- and effort-saving method as it analyzes different target regions of the 16S rRNA or functional genes depending on the research question. However, it can be restrictive since all the combinations of primer pairs must be able to function in a single set of reaction conditions. Additionally, primer dimer formation between primer pairs can occur. This leads to poor sensitivity and preferential amplification of certain targets. Despite its drawbacks, multiplex PCR can be a rapid and convenient screening assay for the detection of microorganisms in samples (Gilbride et al., 2006).

Since rapid advances in nanofabrication and microfluidics in the beginning of the 1990s', PCR became digital. The strategy for digital PCR (dPCR) is a highly diluted sample containing DNA, which is partitioned into hundreds of separate reaction chambers so that each one contains either one copy or no copy of the target sequence (Figure 2). Comparing the positive compartments (copy of target DNA detected) versus the negative compartments (no copy of target DNA detected), it is possible to determine exactly how many copies of the target DNA molecule were in the initial sample (Baker, 2012). Digital PCR uses the same primers and probes like its more familiar cousin qPCR, but is capable of higher sensitivity and precision (Baker, 2012). The reason is, that qPCR is not able to distinguish gene expression differences or copy number variants less than double, whereas dPCR can measure a 30% or smaller difference in gene expression. Additionally, dPCR does not require calibration and internal controls, like it is necessary for qPCR (Baker, 2012).

1.3.5 Primer development

Primers are vital to the specificity, sensitivity, and efficiency of all PCR-based methods (Smith and Osborn, 2009). Primers are basically short single-stranded nucleotide sequences (around 15-30 bp). They are classified as either broad range primers available for general bacteria (known as universal primers) associated to

phylogenetically highly conserved regions of the 16S rRNA gene or narrow range primers for various microbial groups, when focusing on the nitrogen cycle (Sipos et al., 2007).

It is a common practice in the wastewater field to use already published primers or commercially available ready-to-use primers. The usage of such primers is based on the mere assumption that primers have been validated and optimized. However, studies have shown that this assumption does not hold always true, due to variation in parameters (such as experimental conditions, thermal cycler, nucleic acid extraction method or assay conditions) a primer set will not generate the same results (Albertsen et al., 2015; Bustin et al., 2009; Orschler et al., 2019; Osborne et al., 2005). Therefore, it is very important to validate the primers before using them. The last decade of research also revealed, that different molecular methods require different types of primers, but the use of PCR primers for qPCR is still common. This is not advisable, because for qPCR the optimal product length is 50-150 bp, whereas PCR products are often longer (Dechesne et al., 2016).

One common approach for primer designing includes three steps:

(1) Selection of target genes – It is based on the research question, whether the need is to determine the phylogeny or also the functional association.

(2) Primer and probe design – While designing primers, the objective is usually pre-determined. A "specific" primer which represents a unique sequence or a "degenerate" primer which represents a collection of unique sequences for the same target gene. Operatively, the use of a degenerate primer implies the use of a group of specific primers that cover all the possible combinations of nucleotide sequences coding for a given protein sequence. Full complementarity between primer and template sequences is generally considered crucial for the specific amplification of a nucleic acid sequence, but can be difficult to achieve, in particular for applications depending on highly heterogenic nucleic acid input for amplification (Stadhouders et al., 2010). On the other hand, use of degenerate primers increase the chance of unspecific annealing of the designed primers, it also increases the probability of finding unknown divergent variants of a sequence family (Iserte et al., 2013).

(3) Experimental evaluation of specificity and efficiency – Designed primers are validated for PCR and qPCR analysis, either using pure culture DNA or environmental DNA.

1.3.6 Next generation sequencing methods

Since the discovery of the structure of DNA, technical advances used this discovery to approach a better understanding of the human genome, as well as in

clinical areas with new and complex methods. In 1977, Sanger sequencing was established as new method to determine nucleotide sequences (Sanger et al., 1977). But the traditional method was only capable of sequencing specimens separately and was therefore not suitable for complex environmental microbiomes (Shokralla et al., 2012). Since then, advances in the technology led to the first high-throughput sequencing platform in the mid 2000s, but high costs and limited throughput were still a major drawback (Goodwin et al., 2016). In the last decade, next-generation sequencing (NGS), which mainly means sequencing with high speed and high throughput, evolved quickly (Figure 3). The increasing capacity also provided longer read length and implied lower costs for sequencing (Ansorge, 2009; Goodwin et al., 2016).

The growing power and reducing cost sparked an enormous range of applications of the NGS technology. At present different sequencing technologies are available, ranging from emulsion PCR, solid phase bridge amplification, solid-phase template walking and in-solution nanoball generation (Goodwin et al., 2016). All companies present on the market, are aiming to provide the longest reads with their respective sequencing platform. For environmental purposes, two sequencing platforms are commonly used: (1) the Illumina platform based on the sequencing-by-synthesis approach coupled with bridge amplification on the surface of a flow cell (Shokralla et al., 2012). (2) The ION-Torrent platform Ion S5 based on emulsion PCR combined with semiconductor sequencing, meaning rather than using an enzymatic cascade to generate a signal, the technology detects the H^+ ions that are released as each dNTP is incorporated (Goodwin et al., 2016).

NGS not only offered new technologies and information, it also introduced a new vocabulary. The direct genetic analysis of genomes in an environmental sample is defined as metagenomics, whereas the study of gene expression in microbial communities is known as metatranscriptomics. Metaproteomics is the study of proteins in a microbial community and metabolomics the metabolite profiling and analysis of metabolic fluxes (Handelsman et al., 1998; Rodriguez et al., 2015; Thomas et al., 2012).

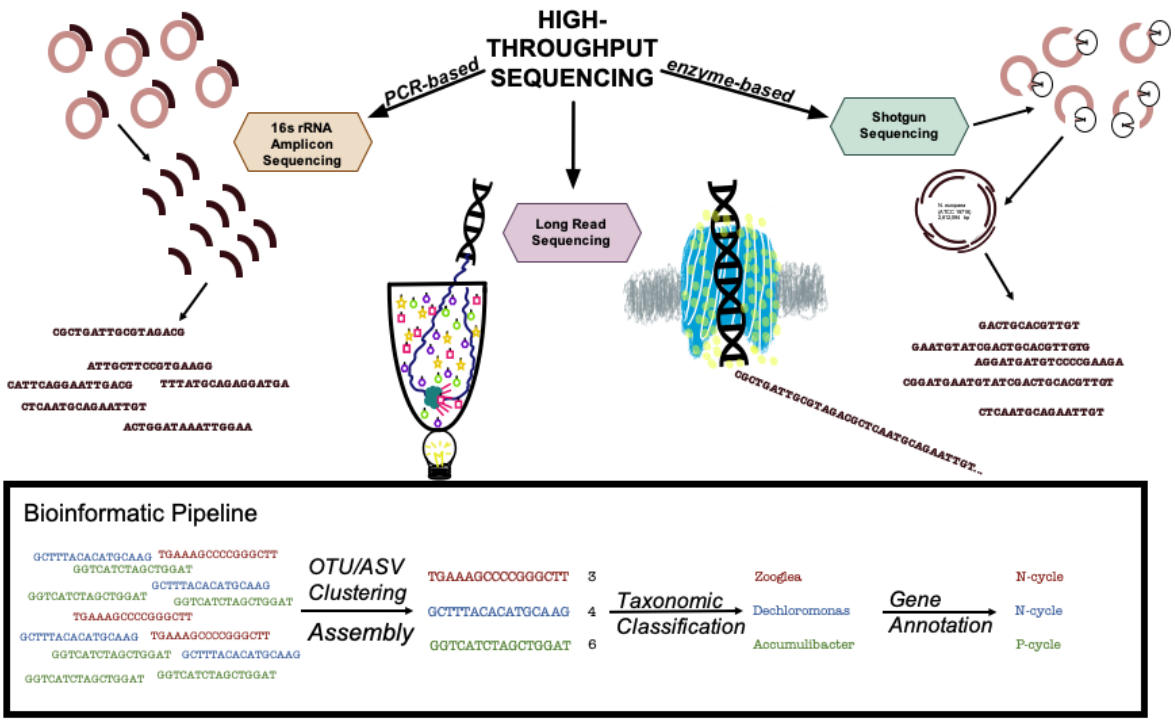

Figure 3: Schematic presentation of the most common high throughput sequencing methods; on the left 16s rRNA amplicon sequencing based on the PCR method, on the right side: shotgun sequencing based on enzymatic fragmentation of the DNA and in the middle the new long read sequencing methods: Single-molecule real time (SMRT) sequencing (left) and Nanopore sequencing (right); modified from van Loosdrecht et al. (2016).

Two approaches are generally adopted for characterizing taxonomic diversity of metagenomes. The most common sequencing approach is amplicon sequencing of the 16S rRNA gene. In this method one or more *hv* regions of the 16S rRNA gene are amplified by primers and afterwards sequenced (Sanz 2019) (Figure 3). The variation in the gene sequence of the amplicons among species enable their use as 'species-specific taxonomic barcodes', that can be used for obtaining insights into the taxonomic diversity of microbial communities (Mande et al., 2012). A second approach is *de novo* sequencing (or "shotgun sequencing") which uses enzymes to randomly shear the DNA into smaller fragments before amplifying the fragments with random primers to sequence overlapping regions of a genome (Figure 3). This method enables to generate longer reads, based on randomly overlapping sequences, without primer bias (Quince et al., 2017). But *de novo* sequencing is more expensive and requires more extensive data analysis and is therefore used less in environmental microbial ecology studies (Mande et al., 2012; Tyson et al., 2004).

The sequencing step typically generates millions of sequences, also referred to as 'reads'. Analyzing these reads with bioinformatic tools provides insights into the microbial community composition (Mande et al., 2012). In amplicon sequencing unique sequences (down to single nucleotide variation are detected) are identified and all identical unique sequences are assigned within each amplicon sequencing variant (ASV) (Callahan et al., 2017). Further the ASVs are compared against public databases (such as SILVA) for taxonomic assignment (Quast et al., 2012). In comparison shotgun sequencing involves construction of long reads from short sequencing reads, known as "assembly". These long reads are known as contigs, and contigs are grouped into scaffolds. Scaffolds are defined as huge amount of contig clusters, they are also called supercontigs or metacontigs. Further, these metacontigs are grouped together into individual genomes (Liao et al., 2019).

NGS technologies have developed rapidly in the last two decades and have become a robust method for routine use. But the shorter reads (400-600 bp), generated by either the Illumina or Ion Torrent platform, pose challenges in taxonomic classification to the lowest rank, i.e. species (Amarasinghe et al., 2020). Therefore, recently the NGS technology has undergone further development, enabling "long read sequencing" (several hundred kilobyte bp at once), thereby, extending possibilities to classify down to species level. At present, two long read sequencing platforms are available: (1) Nanopore sequencing developed by Oxford Nanopore Technologies, (2) and Single-molecule real-time sequencing (SMRT) developed by Pacific Biosciences (Amarasinghe et al., 2020).

The principle of the Oxford Nanopore sequencing platform is based on an engineered protein, also known as nanopore, that is embedded in an electrically resistant membrane made from a synthetic polymer. This sequencing technology is also available in a handy and small device known as MinION, which works with every computer via USB (Plesivkova et al., 2019). It is the first sequencing platform allowing sequencing on site and was initially used for the analysis of the Ebola virus in Africa (Quick et al., 2016). The big advantage of the Nanopore sequencing is that it provides longer reads at lower costs. But the routine use of Nanopore sequencing is still not possible and the performance still struggles due to high error rates (Sevim et al., 2019).

In comparison, SMRT sequencing is based on a SMRT cell with thousands of zero-mode waveguides (ZMW). ZMW are very small chambers, illuminated by laser light from below, so that the chamber can be seen as small, but powerful light microscope. Corresponding to each ZMW continuous light pulses are generated by fluorophore nucleotides which are interpreted as a sequence (called a continuous long read, CLR) (Rhoads and Au, 2015). Similar to nanopore, SMRT also provide lower per read accuracy than short-read sequencing (Illumina and ION Torrent) (Amarasinghe et al., 2020).

2 Research Gaps and Objective

Modern molecular tools have revolutionized the integration of microbial ecology studies into research on N-DN and PNA systems by circumventing the limitations of cultivation-based approaches (Gilbride et al., 2006). The use of qPCR enabled environmental engineers to quantify the desired microorganisms which supported the evaluation of PNA reactor systems (Gilbert et al., 2014b; Persson et al., 2017). The use of high-throughput sequencing in PNA systems revealed that their microbial composition is far beyond AOB and AnAOB (Agrawal et al., 2017; Speth et al., 2016). Overall, the molecular toolbox has always accompanied microbial ecology studies especially in case of PNA systems.

Even more, the advancement of molecular techniques has supported our progress in understanding the microbiome of PNA reactors. For example, from conventional cloning and Sanger sequencing for the characterization of the microbial community composition → DGGE for community dynamics and composition to → qPCR for quantification. qPCR is commonly used to quantify target microorganisms in BNR studies, but standardization is still not achieved (Figure 4) (Orschler et al., 2019). Although NGS is a more advanced molecular technique and provides in-depth information on microbiomes, it comes along with further methodological variables compared to qPCR, including sequencing technology, sequencing chemistry version, read length, insert size, and analysis pipelines, amongst others. This increase in variability affects both reproducibility and the comparability of the results from different NGS studies (Clooney et al., 2016; Gihring et al., 2012; Sinclair et al., 2015). Nevertheless, we use them extensively for analyzing the structure and dynamic of the microbial communities in WWTPs. Nowadays, we are at the edge of deciding, whether we find a solution for basic problems of the former addressed molecular methods to gain standardization and reproducibility or if we just go along with the new advances in molecular techniques.

My initial experiences during method establishment for qPCR and NGS, a literature assessment of- and the use of- these methods in studying microbiomes associated with BNR, led to the following open questions for my work:

a) Is technological progress of molecular techniques moving forward in parallel with usage, knowledge or the experiences with the respective method over the years?

b) Is it sufficient only to trust the progress in technology or to rely on only one method, which is in most cases the newest?

c) Can we find the perfect molecular method to analyze microbial communities in N-DN and PNA systems?

d) Is it possible to standardize methods, similar to chemical analytics?

e) Which method is most suitable for which scientific issue/question? Does it always have to be NGS?

f) Can we outcompete the weaknesses of all molecular methods by using the strengths of several methods together?

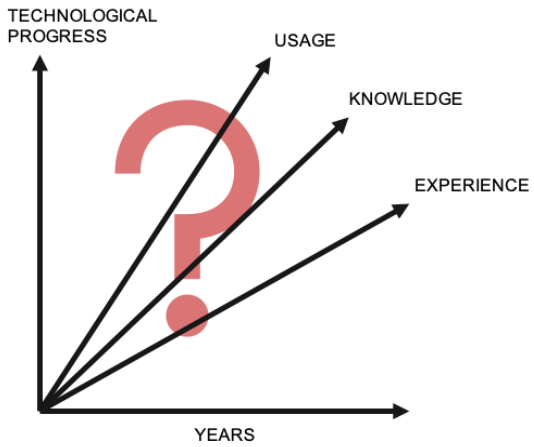

Figure 4: Relationship between technological progress, usage, knowledge and experience throughout the years

These questions are addressed as follow:

- In **chapter 3.1**, 16S rRNA gene amplicon sequencing was performed to determine the impact of primers on the result. Additionally, the impact of primers on the qPCR analysis of the AnAOB, AOB and NOB present in different PNA systems, was analyzed.
- In **chapter 3.2**, in-depth *in silico* analysis was performed to determine the coverage of previously published primers targeting the ammonium monooxygenase gene (*amoA*) of the AOB. Moreover, whether usage of broad-spectrum or narrow spectrum primers is a better approach for precise quantification of AOB population in the PNA biomasses, was determined.
- In **chapter 3.3**, an alternate approach for determining the complex composition of putative denitrifiers in PNA systems is presented, to overcome the problem associated with the 16S rRNA gene amplicon sequencing approach for studying putative denitrifiers. This approach also helps to overcome the challenges associated with primers for genes of the denitrification pathway.

3 Results

3.1 Microbial community analysis in PNA systems based on 16S rRNA *hv* regions (Chapter 3.1)

PCR-based methods have caused a surge for integration of eco-physiological approaches into research on partial nitritation anammox (PNA). However, a lack of rigorous standards for molecular analyses resulted in widespread data misinterpretation and consequently lack of consensus. Data consistency and accuracy strongly depend on the primer selection and data interpretation. An *in silico* evaluation of 16S rRNA gene eubacterial primers used in PNA studies from the last ten years unraveled the difficulty of comparing ecological data from different studies due to a variation in the coverage of these primers. The 16S amplicon sequencing approach used in this study, which includes parallel sequencing of six 16S rRNA hypervariable regions, showed that there is no perfect hypervariable region for PNA microbial communities. Using qPCR analysis, this study emphasize the significance of primer choice for quantification and caution with data interpretation. This study also provides a framework for PCR based analyses that will improve and assist to objectively interpret and compare such results.

Chapter 3.1

On resolving ambiguities in microbial community analysis of partial nitritation anammox reactors

Laura Orschler, Shelesh Agrawal, Susanne Lackner

3.1.1 Introduction

Partial nitritation anammox (PNA), a significant breakthrough as an energy- and cost-saving alternative to conventional biological nitrogen removal (Jetten et al., 1998; Mulder et al., 1995; Vlaeminck et al., 2010), demands a fine balance of operational conditions that support the characteristic microbial composition of ammonium oxidizing bacteria (AOB) and anaerobic ammonium-oxidizing bacteria (AnAOB). Researchers are adopting the combination of microbial ecology and physiology, also known as eco-physiological approach (Agrawal et al., 2017; Burgmann et al., 2011; De Clippeleir et al., 2013; Park et al., 2010; Park et al., 2015; Persson et al., 2014), to gain a more fundamental understanding and to optimize PNA processes.

Modern molecular tools have revolutionized the integration of microbial ecology studies into research on PNA systems by circumventing the limitations of cultivation-based approaches (Gilbride et al., 2006). The use of high-throughput 16S rRNA amplicon sequencing in PNA systems also revealed a microbial composition reaching far beyond AOB and AnAOB (Agrawal et al., 2017; Speth et al., 2016). In PNA studies, 16S amplicon sequencing is performed on the one hand for microbial community characterization and on the other hand - based on the relative abundance of reads - for quantification. Although recently developed ultrahigh-throughput sequencing technologies now overshadow quantitative polymerase chain reaction (qPCR) method, the ability of qPCR to target microorganisms down to strain level with particular taxonomic or functional markers and the ability for accurate enumeration is indispensable (Agrawal et al., 2017). Therefore, qPCR is used in parallel to validate the quantification results of 16S amplicon sequencing (Bagchi et al., 2016; Guo et al., 2016).

For engineering purposes, the quantification of the desired microorganisms is often more relevant than the inventory of species present in the reactor, and therefore, qPCR is an invaluable method in the molecular microbial ecologist's toolbox (Smith and Osborn, 2009). Moreover, the interpretation of qPCR results with subsequent translation into reactor performance is the most critical point, because these results support the evaluation of a reactor system. We introduce three PNA studies with similar objectives (application of PNA in the main wastewater treatment line) as examples for comparison, to explain how diverse results are interpreted and translated. These studies compared ecological data and reactor performance to understand which reactor operation strategy might be best applicable for mainstream PNA. Hu et al. (2013) investigated a lab-scale sequencing batch reactor (SBR) system and interpreted the reactor turnover based on qPCR results. For AnAOB quantification the primer pair hzsA526F/hzsA1829R was used instead of the previously recommended primer pair hzsA1597F/hzsA1857R (Harhangi et al., 2012). Persson et al. (2014) quantified microorganisms in a pilot-scale moving bed biofilm reactor (MBBR)

with qPCR and stated a high percentage of anammox by normalizing it with the total bacterial abundance captured using primer 1055f-1392r (V7-V8 hypervariable region). This study also compared AnAOB abundance with Hu et al. (2013), even though the primers differed – 16S rRNA gene and hzsA (hydrazine synthase) in latter. Gilbert et al. (2015) quantified target microbial members using qPCR and compared the results with Hu et al. (2013) and Persson et al. (2014), even though other primers were used.

Comparing reactor studies with each other is already challenging due to inherent ecological variability. Additionally, biases pervade PCR based analyses. Therefore, in PCR based methods (like qPCR and 16S rRNA amplicon sequencing) primer selection is the most critical step as also reported in several studies (Armougom, 2009; Klindworth et al., 2013; Schloss et al., 2011). Using primers with wide coverage can lead to overrepresentation, whereas primers with high specificity can lead to underrepresentation (Albertsen et al., 2015; Klindworth et al., 2013; Throback et al., 2004). Thus, PCR based analysis needs a framework, where methods and parameters are kept same to compare different studies, similar to the analytical chemistry framework for wastewater treatment plants (WWTP) (Association et al., 1915) (for example chemical oxygen demand, total suspended solids, pH analyses).

By now, there are some guidelines available for PCR based methods, known as MIQE (minimum information for publication of quantitative real-time PCR experiments), guidelines, which emphasize on better transparency in reporting of experimental data (Bustin, 2010; Bustin et al., 2009). These guidelines help to deal with some critical aspects in research fields such as medicine; food processing; and environmental studies, with respect to the reliability of PCR based methods (Dijkstra et al., 2014); false positive signals (Wolffs et al., 2005); reproducibility; and lack of comparability (Bustin and Nolan, 2017; Bustin, 2014; Ebentier et al., 2013). However, MIQE guidelines do not include information about experimental protocols, the influence of primer choice and subsequent data interpretation (Dijkstra et al., 2014). In the research field of wastewater treatment, experienced users, therefore, developed standardized step-wise protocols for PCR based methods (such as qPCR and 16S amplicon sequencing), primarily focused on wastewater treatment microbial ecology, addressed to non-specialists to shed light on the dark side of the PCR based experiments (van Loosdrecht et al., 2016). For non-specialists, these protocols are useful, however, detailed information about the impact of primer choice, and microbial community matrices on the data and interpretation of that data in PNA studies, which present their own hurdles, is still missing.

We, therefore, systematically provide insight into how to deal with two major questions: (1) What if selected primers do not tell us everything about the PNA microbial community? (2) Can we compare one PNA system with another based on ecological analysis, even when we select different primers for the same query?

We assessed previous PNA literature to determine the commonly used primers and approaches for the interpretation of the results with the link to reactor operation. The impact of primers targeting different hypervariable regions of the 16S rRNA gene was investigated by simultaneously sequencing six of the hypervariable regions of the 16S rRNA gene.

The significance of choosing the right data interpretation approach was evaluated by testing different approaches found in previous literature. Further, we developed a decision tree framework for the standardization of PCR-based analysis for PNA systems.

3.1.2 Materials and Methods

Scientific literature assessment

To evaluate previous studies, we conducted an internet search using the Web of Science platform (v.5.27.2) by Thomson Reuters and collected research papers using a search query with the following keywords: “anammox and pcr” or “partial nitri* and pcr or nitritation” and “pcr or anaerobic ammoni* and pcr” (Figure 5). The use of keywords with asterisk helped to find all the studies that shared at least the same root word with the same five or six letters in the beginning. The search considered papers between 2006 and 2016 and found the total of 582 studies based on the keywords. Out of these 582 studies, 70 studies remained focusing on partial nitritation anammox (PNA), partial nitritation (PN) and/or anammox (A) reactor systems and performed qPCR analysis (Figure 5, Annexure I A.Table 3). Information about the type of the reactor systems that were used in these studies is provided in the Annexure I (A.Figure 1).

The primer information extracted from these studies was sorted based on the target microbial group and the usage frequency (hits) (Figure 5). For every single study no hits or more than one hit is possible for each target microbial group depending on the experimental aims of the respective study. Further, *in silico* PCR analysis was performed for the 16S rRNA gene primers from the literature, targeting the total eubacterial population. *In silico* PCR analysis was performed to determine the coverage of the primer pairs, respectively. The coverage of primers was tested using the SILVA test prime function based on the version SILVA132 (https://www.arb-silva.de/search/testprime/).

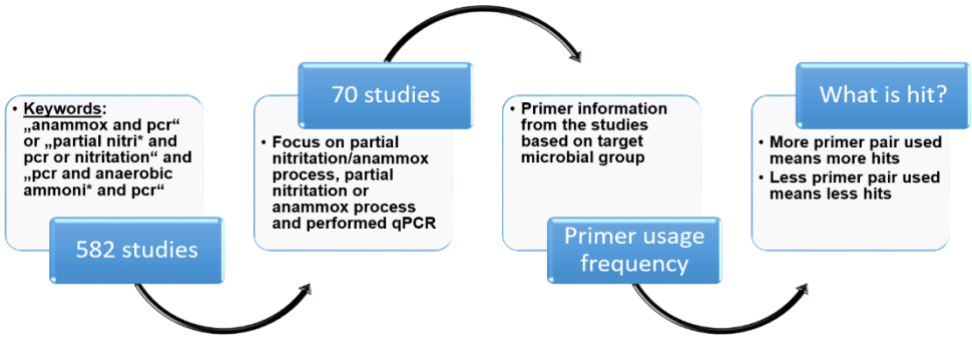

Figure 5: Schematic showing the approach used to extract PNA studies, which were used for the assessment.

Additionally, 16S rRNA gene primers were aligned using Unipro UGENE (Okonechnikov et al., 2012) a multiplatform, open-source application as a sequence alignment tool, with 16S rRNA gene sequences chosen of representative microbial members in PNA systems.

16S amplicon sequencing

Biomass samples were collected from three different PNA reactors: a full-scale single stage sidestream PNA (TUD1), a lab scale single stage PNA (TUD2) and a full-scale anammox stage sidestream PNA (TUD3). Total genomic DNA was extracted using the Fast DNA Spin kit for soil (MP Biomedicals) according to a modified manufacturer's protocol. The quality of the DNA was checked using gel electrophoresis, and the concentration was measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific). For each sample, multiple hypervariable regions of 16S rRNA genes were amplified with the 16S Ion Metagenomics Kit™ (Thermo Fisher Scientific) by two separate PCR reactions, amplifying the V2, V4, V8 and V3, V6-7, V9 hypervariable regions, according to the kit protocol (Agrawal et al., 2017). Equal volumes of V2, V4, V8 and V3, V6-7, V9 amplicons were combined. 100 nanograms of pooled amplicons were processed to the amplicon library using the Ion Xpress Plus Fragment Library Kit™, and each sample was tagged using the Ion Xpress Barcodes Adapters™ (Thermo Fisher Scientific), according to the manufacturer's protocol. Each sample was adjusted to a 10 picomolar concentration. All three samples were pooled, in equal volumes, and processed with One-Touch 2 and One-Touch ES systems (Thermo Fisher Scientific) according to the manufacturer's instructions.

Sequencing was performed on the Ion Torrent (ION Torrent Ion S5) using the 400-bp kit and 530 chip. Base calling and run demultiplexing were conducted by Torrent Suite version 4.4.2 (Thermo Fisher Scientific) with default parameters. The Ion Reporter^{TM} software (Thermo Fisher Scientific) is a bundle of bioinformatics tools, which uses QIIME ver. 1.9.1 to process 16S metagenomic data (Caporaso et al., 2010). QIIME was implemented for separating sequences based on their respective targeted regions and OTU (operational taxonomical unit) picking with its default settings. Overall, the *de novo* clustering of OTUs was done with 97% identity, corresponding to species level. The sequences were classified based on the taxonomy in the Silva database (97% confidence threshold, version 132) (Quast et al., 2012). The sequencing data were analyzed in R, using ggplot2 (v0.9.3.1) and two-way analysis of variance (ANOVA) to test significance of the results.

Quantitative PCR

Total genomic DNA was extracted from biomass samples using the Fast DNA Spin kit for soil (MP Biomedicals). DNA concentration and its integrity were analyzed using Qubit 3.0 Fluorometer with Qubit dsDNA HS kit (Thermo Fisher Scientific). The abundance of total bacterial abundance (EUB) was quantified targeting the V3-4 region of the 16S rRNA gene (primer pair 338f-518r and primer pair 341f-543r) and V7-8 region (primer pair 1055f-1392r). The abundance of AOB, AnAOB, and NOB was quantified targeting the ammonia monooxygenase (*amoA*) gene (primer pair amoA1f/amoA2r), and the 16S rRNA genes for AnAOB (primer pair Amx809f-Amx1066r), for Nitrobacter (primer pair Nitro1198f-Nitro1423r), and for Nitrospira (primer pair NSR1113f-NSR1264r). qPCR analysis was performed for each sample and primer pair as technical triplicate runs. Each qPCR run was then performed in triplicates for a 25 µL reaction mixture containing 12,5 µL of PerfeCTa SYBR® Green SuperMix 2X (QuantaBio), 0,5 µL of each primer, 5 µL of DNA (5 ng/µL) and PCR grade water. Thermal profiles for each primer pair are available in the Annexure I (A.Table 4). The qPCR abundance data were analyzed in R, using ggplot2 (v0.9.3.1) and one-way ANOVA.

Percentage dissimilarity was calculated to determine the impact of primer pair on the measured total eubacterial abundance. The percentage dissimilarity attributed to each primer pair, was calculated using a similarity percentage (SIMPER) analysis. The dissimilarity between the measured absolute abundance using three different primer pairs (pp.1 1055f-1392r, pp.2 338f-518r, pp.3 341f-543r) is reported as a percentage.

Data availability

OTU representative sequences were submitted to the GenBank under the accession numbers MH682261 - MH683001.

3.1.3 Results

Coverage assessment of known 16S rRNA gene universal primers

We retrieved details about the primers from previous studies, which performed microbial abundance quantification in PNA reactors, to determine the most frequently used primers. We found eight different universal primer pairs, targeting different hypervariable regions of the 16S rRNA gene, which were used in the evaluated studies (Table 1). The most frequently used EUB primer pair targets the hypervariable region V7-V8, i.e., primer pair 1055f-1392r (12 hits). The second and third most commonly used primer pairs belong to the hypervariable region V3-V4, primer pair 338f-518r (6 hits) and primer pair 341f-543r (4 hits), respectively. Moreover, there is huge variability in PCR product size, ranging from product sizes of 123 bp (1396F-1492R) as shortest, to 566 bp (341F-907R) as longest. The size of the PCR product also influences the qPCR results (Denman and McSweeney, 2006). This assessment revealed that a diverse set of primers had been used to quantify the microbial composition in PNA systems which raises the question whether the selection of the primer pair affects quantification and comparability?

To answer this question, the three most frequently used eubacterial primer pairs in all evaluated studies (primer pair 1:1055f-1392r; primer pair 2: 338f-518r; primer pair 3: 341f-543r) were selected from Table 1for the *in silico* PCR analysis (Figure 6). Based on the current 16S rRNA gene sequence database SILVA (silva132) we studied the total coverage of every primer pair, indicating how much information the respective primer pair provides of the known total eubacterial diversity.

Table 1: List of 16S rRNA gene primer pairs that were used in the evaluated studies (based on the literature assessment), with the respective hypervariable (HVR) regions and length in base pairs (bp). Hits refer to the frequency of the respective primer pair found in the evaluated studies, no hits as well as more than one hit is possible.

Primer pair	HVR- region	length [bp]	HITS
338f-518r	**V3-V4**	**180**	**6**
341f-543r	**V3-V4**	**202**	**4**
341f-907r	**V3-V5**	**566**	**2**

519f-907r	**V4-V5**	**391**	**2**
515f-806r	**V4-V5**	**291**	**1**
907f-1110r	**V6-V7**	**203**	**1**
1055f-1392r	**V7-V8**	**337**	**12**
1369f-1492r	**V8-V9**	**123**	**3**

Starting with primer pair 1, the database evaluation showed a total coverage of 44.5% for the eubacterial population (Figure 6). Additionally, the coverage for AOB was about 82.9%, for AnAOB 83.0%, the NOB coverage was 85.7% for *Nitrobacter* and 71.6% for *Nitrospira*. Primer pair 2 had a total coverage of 70.0%, with 41.0% for AOB, no coverage for AnAOB, 36.0% for *Nitrobacter* and 37.0% for *Nitrospira*. Primer pair 3 had a 51.2% coverage for total EUB, 94.0% for AOB, no coverage for AnAOB, 86.0% for *Nitrobacter* and 0.7% *Nitrospira*. These results prove that the qPCR data differs between various PNA studies using different primer pairs.

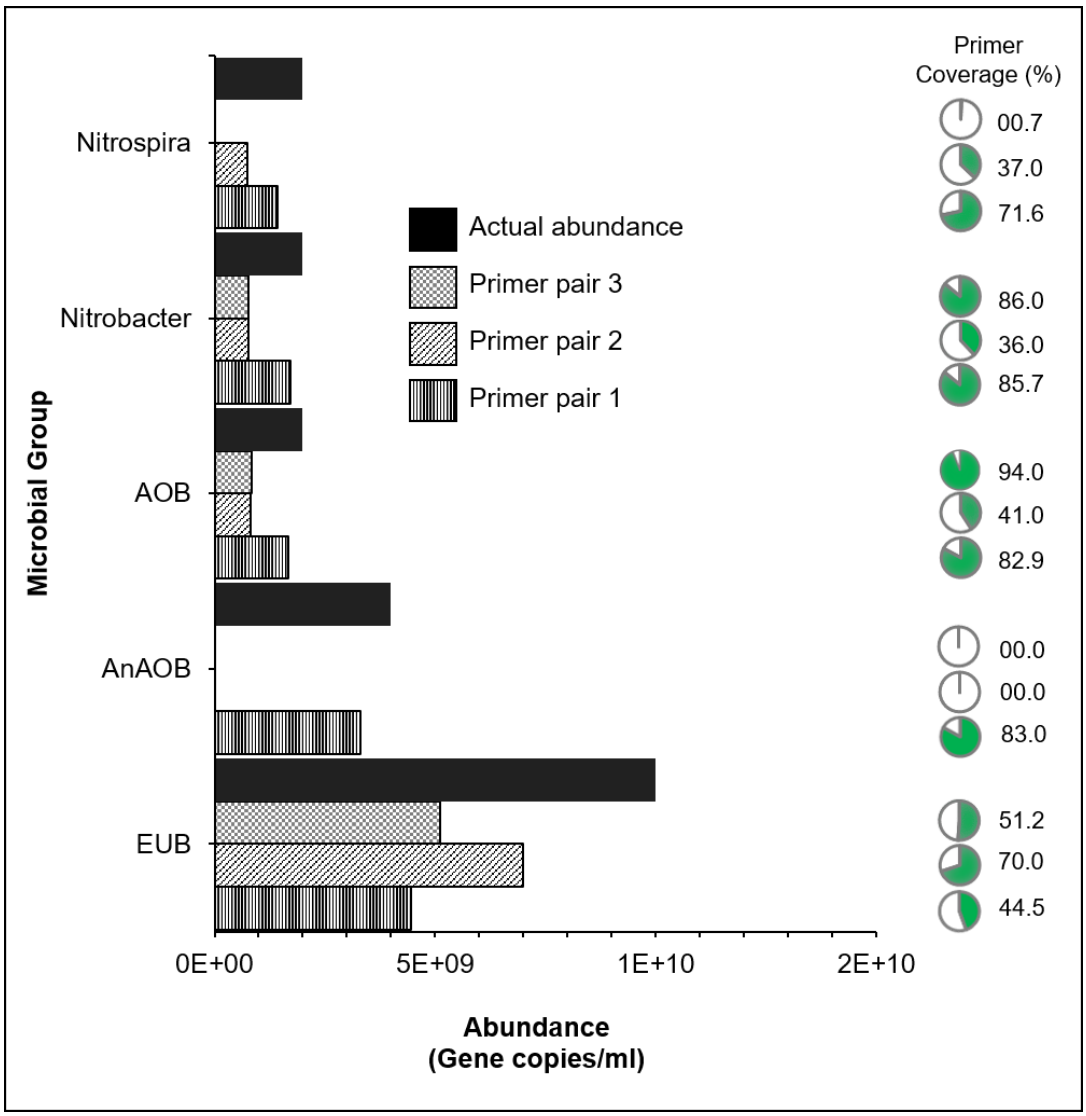

Figure 6: Comparison of the three most widely used EUB primer pairs based on the literature assessment, primer pair 1 (1055f-1392r), primer pair 2 (338f-518r) and primer pair 3 (341f-543r) for the primer coverage and abundance of the microbial groups most relevant for the PNA process (abundance is defined as theoretically calculated value). AnAOB (anaerobic ammonium oxidizing bacteria), (AOB) ammonium oxidizing bacteria, *Nitrobacter* and *Nitrospira* (nitrite oxidizing bacteria) and EUB (total eubacteria).

Previous studies have primarily highlighted that primer selection has a different influence on taxonomic assignments at different taxonomic levels (Albertsen et al., 2015; Guo et al., 2013). However, here we try to emphasize that primer selection also influences abundance quantification using a theoretical example. Let us consider a hypothetical biomass composition which contains 1.00E+10 16S rRNA gene copies/mL associated with the eubacterial population, 4.00E+09 16S rRNA gene copies/mL associated with AnAOB, and 2.00E+09 16S rRNA gene copies/mL associated with AOB, *Nitrobacter* and *Nitrospira*, respectively. Using

the different EUB primers resulted in significant, different theoretical abundances (p-value <0.01, two-way analysis of variance (ANOVA) analysis) (Figure 6). Based on the current SILVA database, the total eubacterial population is under-represented using either of the three most commonly used primer pairs. It is even more critical for specific microbial groups in PNA systems, as lack of appropriate primers can lead to false negative results, for example, primer pair 2 does not cover AnAOB and primer pair 3 does not cover AnAOB and *Nitrospira* (similar outcome in multiple sequence alignment, Annexure I A.Figure 2).

Coverage of primers that are microbial group-specific

The challenge to compare qPCR results from different PNA studies is not just limited to the EUB universal primers. It extends to microbial group-specific primers, too. Similar to EUB primers, a wide range of group-specific primers are used in PNA studies, hampering the comparison of PNA studies that used different group-specific primers. We, therefore, looked at the distribution of different primer pairs that were used in the evaluated studies, and obtained 213 hits for different group-specific primers (including 16S rRNA and functional genes) from the 70 studies (Figure 7). This survey resulted in the following diversity in primer usage: AOB<DNB (denitrifying bacteria)<NOB<AnAOB. The most commonly used primer pair for AOB was amoA1f-amoA2r; for Anammox it was Amx809f-Amx1066r; Nitro1198f-Nitro1423r for *Nitrospira*, and NTSPAf-NTSPAr for *Nitrobacter,* for heterotrophic denitrifiers as *nirS* (cytochrome cd1 type nitrite reductase) gene (nirScd3af-nirSR3cd).

Particularly for AnAOB, the extent of differences in primer pairs was extreme, with 24 different primer pairs in 70 studies. Further sequence alignment verified that all reported AnAOB 16S rRNA gene primers were not suitable for qPCR analysis of a biomass sample, where the AnAOB community composition is unknown, because some primers are genus specific (Kartal et al., 2011a). Therefore, the usage of such primers requires previous knowledge about the AnAOB population. Undertaking a ‘rule-out’ analysis using multiple AnAOB primers is another way to avoid under-representation or false negative qPCR results. For example, one study used primer pair Amx368f-Amx820r (specific for *Ca.* Brocadia anammoxidans and *Ca.* Kuenenia stuttgartiensis) for qPCR based quantification of their AnAOB population (Suto et al., 2017). However, the same study reported the presence of *Ca.* Brocadia anammoxidans, *Ca.* Kuenenia stuttgartiensis and *Ca.* Jettenia. This difference indicates an under-representation of the AnAOB population based on qPCR results.

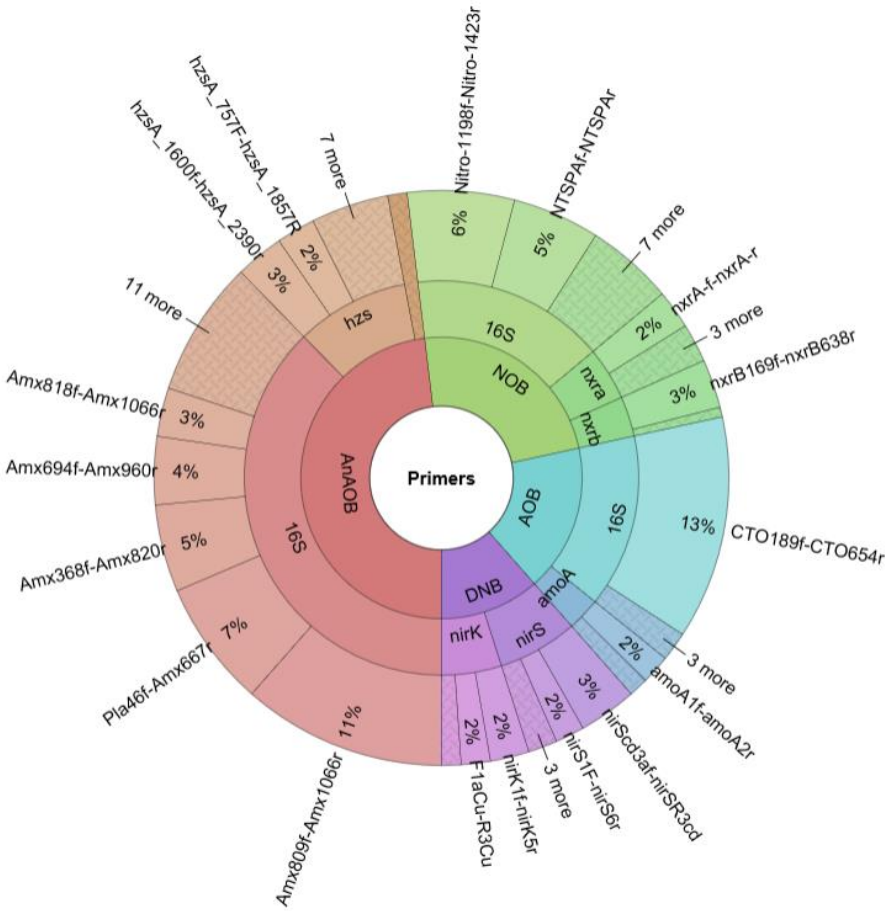

Figure 7: Distribution of the diversity of primer pairs based on the literature survey using certain keywords (i.e., "qPCR", "anammox", "wastewater"), for different microbial groups and the percentage of usage. (1) AnAOB: Anaerobic ammonium oxidizing bacteria; (2) AOB: ammonium oxidizing bacteria; (3) NOB: nitrite oxidizing bacteria; and (4) DNB: denitrifying bacteria. (1) 16S: 16S rRNA gene; (2) *amoA*: ammonia monooxygenase; (3) *hzs*: hydrazine synthase; (4) *nirK*: copper-containing nitrite reductase; (5) *nirS*: cytochrome cd1 type nitrite reductase; (6) *nxra*: nitrite oxidoreductase, alpha subunit; and (7) *nxrb*: nitrite oxidoreductase, beta subunit.

Influence of primer selection on next-generation 16S amplicon sequencing

Although a number of reports had revealed that primer choice introduces biases in 16S amplicon sequencing (Engelbrektson et al., 2010; Klindworth et al., 2013; Soergel et al., 2012; Wang and Qian, 2009), no study is available yet that specifically looked at the extent of primer selection and its influence on determining the microbial communities in PNA systems. Overall, very few studies have investigated the influence of primer choice in WWTP microbial ecological studies (Albertsen et al., 2015; Guo et al., 2013). Therefore, this study used 16S amplicon sequencing of multiple hypervariable regions to determine the influence of primer selection on the sequencing results in different PNA biomasses. Three

different samples were selected to determine if a similar variation was observable in various samples due to primer selection. Each primer pair associated with a respective hypervariable region presented significantly different ($p<0.001$; Annexure I A.Table 1) comprehensive information of the microbial community composition (Figure 8). Primers for the V9 regions amplified mainly Proteobacteria; Acidobacteria were more represented by the V2 region; Firmicutes by the V3 region; Chlorflexi by the V3 and V8 regions; Bacteriodetes by the V2, V3, V4 and V6-7 regions; Nitrospira by the V6-7 region; and Planctomycetes by the V4, V6-7 and V8 regions. This implies that primers significantly influence the profiling of the total community composition. The experimental results were in consensus with the *in silico* analysis conducted on known primers.

Some studies even made suggestions about which hypervariable region primers to use to capture certain microbial groups. Guo et al. (2013) suggested using the V1 and V2 region primer pairs, whereas, Albertsen et al. (2015) (Albertsen et al., 2015) recommended V1-V3 region primers for activated sludge. However, our results show that, at least in case of PNA biomasses, there is no general "best" primer because the influence of the primers varied between the samples (Annexure I A.Figure 3). For example, 16S rRNA gene primers for the V6-7 and V8 hypervariable regions over-represented the AnAOB in TUD2, only. This influence of primer also affects validation of 16S amplicon sequencing data using qPCR in PNA research (Annexure I A.Figure 4), which is in consensus with previous study (Guo et al., 2016). Therefore, it is important to test different primers for the respective samples and select primer pairs from multiple hypervariable regions to attain maximum coverage of the microbial community composition.

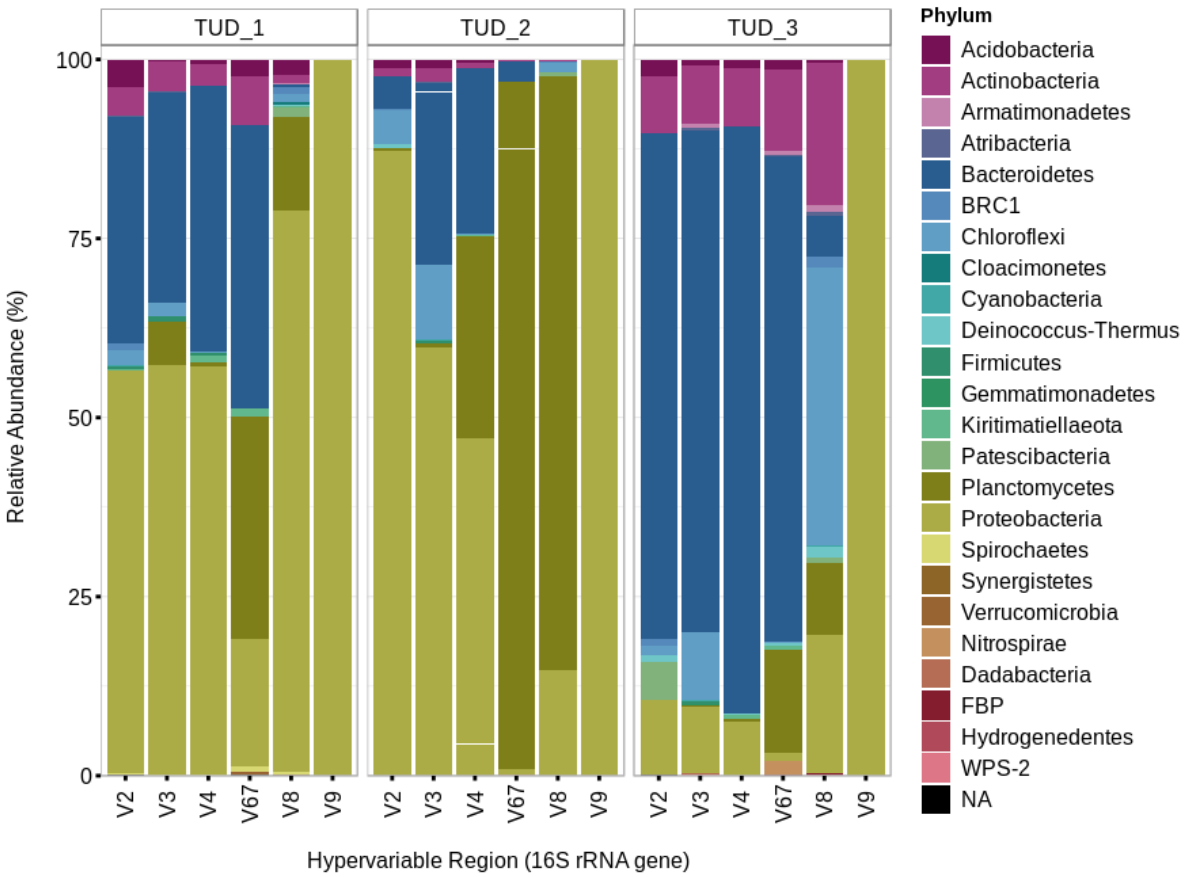

Figure 8: Relative abundance profiling of three samples: (1)TUD_1, (2) TUD_2, and (3) TUD_3, based on the 16S rRNA amplicon sequencing, targeting multiple hypervariable regions of 16S rRNA gene.

Influence of primer selection on quantification: relative or absolute

In wastewater engineering, molecular tools are primarily used to monitor the growth of microorganisms for better process understanding and optimization (Podmirseg et al., 2015; Suto et al., 2017; Tsushima et al., 2007a; Winkler et al., 2011; Yin et al., 2015; Zhang et al., 2017). Moreover, a recent study Podmirseg et al. (2015) has recommended using qPCR as a validation method for other simple quantification methods used in anammox based systems. Therefore, it is essential to understand that the primers influence the quantitative nature of the PCR based methods for relative and absolute quantification.

Absolute quantification, based on qPCR, was performed using the three most frequently used EUB primer pairs found by the literature survey. We conducted one-way ANOVA to assess the impact of the respective primer pair on the sample. The one-way ANOVA revealed high significance of primer pair on the measured EUB microbial groups (Annexure I A.Table 2).

However, the effect of the choice of a particular primer pair varied with the sample. Figure 9 shows the percentage dissimilarity between the absolute copy numbers of 16S rRNA genes. Dissimilarity was measured at a scale of 0-100%, the higher the percentage, the greater the difference between the measured absolute abundance. We calculated the dissimilarity based on the abundance difference between the respective eubacterial primer pairs for every sample. The percentage dissimilarity for sample TUD1 was in a range of 45 to 60%, whereas, it was between 25 and 75% for TUD2. In case of TUD3 dissimilarities were in a range of 45 to 65%. These results underline how dramatically absolute quantification data varies depending on the primers and due to variations in 16S rRNA gene copy numbers. Therefore, it is advisable that the abundance data for certain microbial groups should not be directly compared between different studies, unless the same set of primers was used. Also, the dissimilarity is greater between primers from different hypervariable regions of the 16S rRNA gene compared to primers belonging to the same region (Figure 9). These findings are in consensus with another study (Engelbrektson et al., 2010), which also reports that different primer pairs targeting the same region provide more comparable quantitative data.

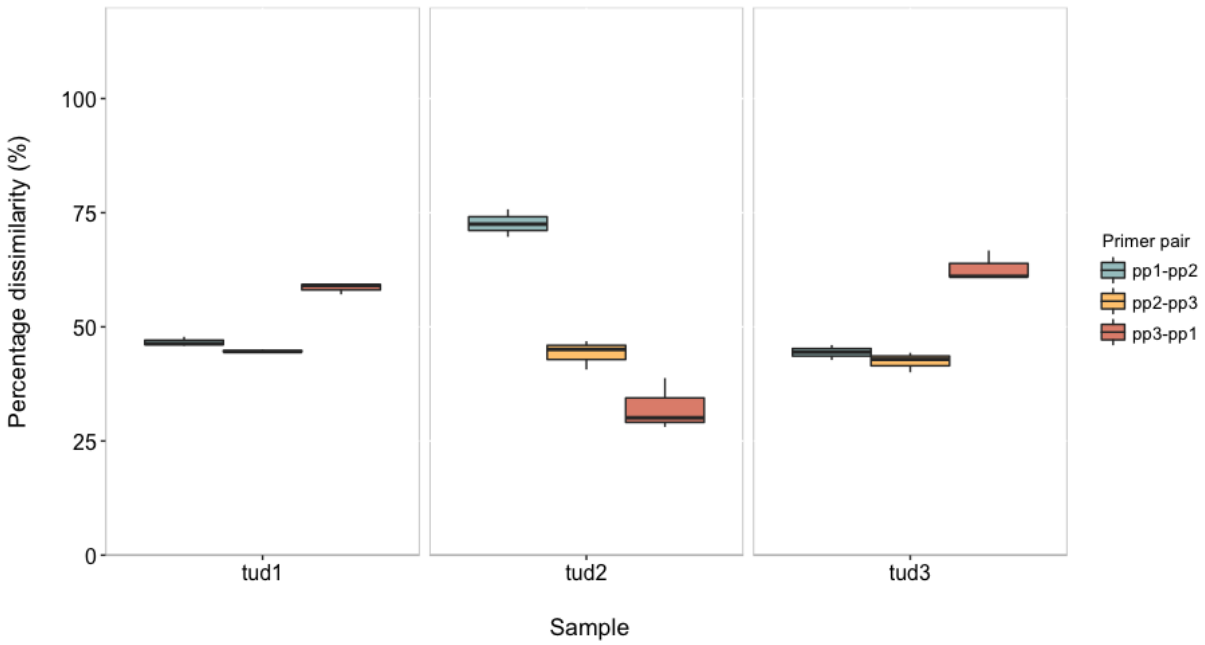

Figure 9: Percentage dissimilarity based on qPCR between absolute abundance of eubacterial population measured using three different primer pairs: (1) pp1 (1055f-1392r), (2) pp2 (338f-518r), and (3) pp3 (341f-543r). In legend pp1-pp2: percentage dissimilarity between pp1 and pp2, pp2-pp3: percentage dissimilarity between pp2 and pp3, pp3-pp1: percentage dissimilarity between pp3-pp1 (percentage dissimilarity was measured between a range of 0-100%, the higher the percentage greater the difference between measured absolute abundance).

In the above section, we already showed, how the relative abundance will vary in 16S rRNA amplicon sequencing data depending on primer selection. We also investigated, if similar inconsistencies occur in the relative abundances calculated from qPCR data. Depending on the 16S rRNA EUB primer pairs used for targeting the total eubacterial abundance (which also differed for different primer pairs, Annexure I A.Figure 5), the calculated relative abundance of AnAOB, AOB and NOB (Nitrobacter and Nitrospira) varied for all the samples (Figure 10). After normalization of the absolute abundance of AnAOB with the absolute abundance of total eubacteria, the relative abundance varied between 10 and 15% between the three primer pairs for sample TUD1; 20 – 60% for sample TUD2; and 1 – 5% for TUD3. Based on *in silico* analysis, the coverage of both primer pairs of region V3 – V4 for AnAOB is low, which explains the observed low relative abundance of AnAOB for primer pairs pp2 and pp3. Similar variations arose from the calculated relative abundances of AOB and NOB (Figure 10).

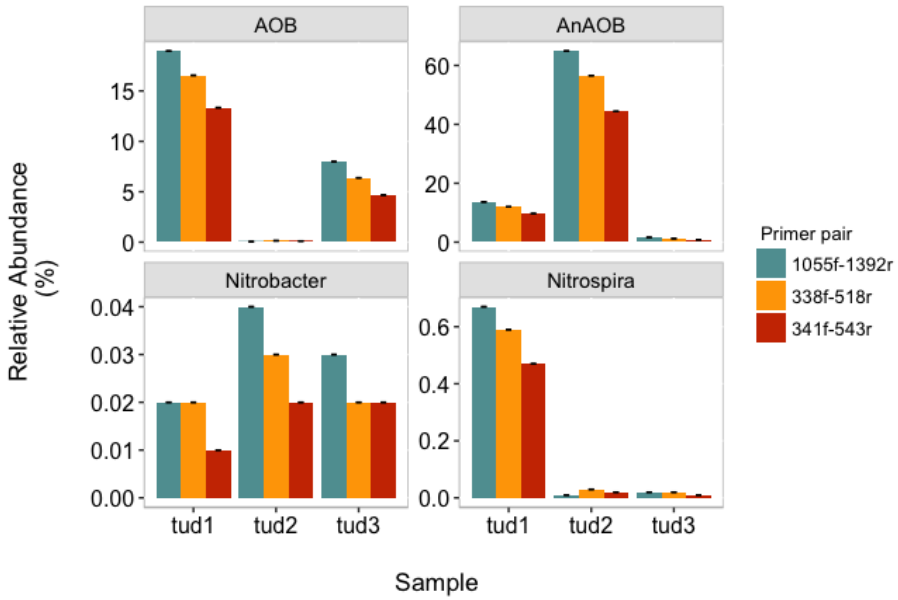

Figure 10: Relative abundance based on qPCR of anaerobic ammonium oxidizing bacteria (AnAOB), ammonium oxidizing bacteria (AOB), Nitrobacter (NOB) and Nitrospira (NOB) which is normalized to the abundance of total eubacteria (EUB), measured using three different primer pairs targeting two different hypervariable regions; error bar represents the standard deviation between qPCR technical triplicate runs.

PNA research, employing qPCR methods, emphasizes the quantification of the key microorganisms, based on either specific primers targeting 16S rRNA genes or functional genes, rather than the total bacterial population. However, it is a general practice to report results as relative abundance (i.e., the fraction of the total eubacterial population) in qPCR based studies (Bagchi et al., 2016; Guo et al.,

2016; Winkler et al., 2011). Based on our results, the relative abundance approach is not advisable for PNA systems, irrespective of sample type and target microorganisms.

3.1.4 Discussion

Designing a good pair of primers for qPCR is a critical factor – often highlighted in previous studies (Dechesne et al., 2016; Ye et al., 2012). Therefore, primer designing has drawn much attention but mainly focused on the re-evaluation and design of new primers for specific microbial groups (Dechesne et al., 2016; Meinhardt et al., 2015; Sonthiphand and Neufeld, 2013; Throback et al., 2004; Tsushima et al., 2007a) present in the PNA biomass. The rapid integration of the eco-physiological approach to study PNA systems has caused a backlog in mechanistically understanding the influence of primers on microbial ecology data. Additionally, there is a lack of guidance for the correct interpretation of such data.

In comparison to ecological diversity surveys, the objective of qPCR and/or 16S rRNA amplicon sequencing in wastewater engineering is different. The information generated serves as the basis for reactor operation and optimization, which demands comparability of quantitative data between different PNA studies. However, this is not possible unless analytical methods share common protocols (Dechesne et al., 2016). Based on our results and previous literature, there is no single best primer pair, which can be recommended for PNA systems. Therefore, we recommend using a combination of multiple primer pairs. In addition, we need a best practice approach that can improve data interpretation and further simplifies the comparison of the results of different studies, in addition to following the MIQE guideline (Bustin et al., 2009).

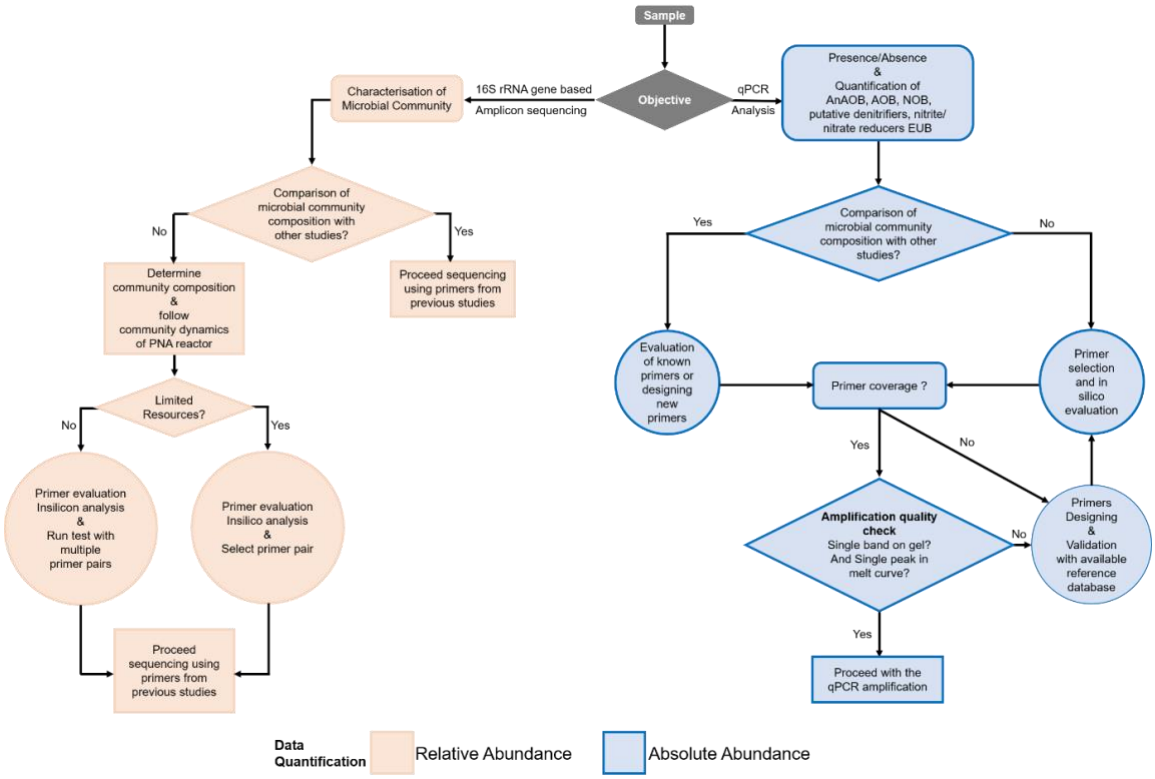

Figure 11: Decision tree framework for qPCR analysis and 16S amplicon sequencing for PNA systems.

Figure 11 presents a decision tree framework based on our literature assessment and experimental results. Before setting up a PCR-based approach, it is important to clarify which question needs to be answered: (1) Is the target microorganism present in the reactor system? (2) How many different microorganisms (species richness) are present in the reactor system? or (3) How many of each group of microorganisms (species evenness) are present? If the research objective is established, we suggest deciding whether the results should be provided as relative abundance using 16S rRNA based amplicon sequencing or absolute abundance using qPCR (as represented in Figure 11 with different colors).

Apart from defining the ecological question, it is also important to define whether the focus of the study is (1) to compare the results with other PNA studies, and/or (2) to study the community composition and dynamics of the PNA system. If the objective is to compare results with other PNA studies, based on growth rates and turnover, we recommend choosing the same primer pairs as used in these other studies. The use of different primers may introduce biases in community profiling (as shown in Figure 8) and thus reduces confidence in the comparison of studies. Nevertheless, before using the reported primer pairs, always verify the quality of the PCR product (whether it is a single band or multiple bands) with gel electrophoresis, except for degenerated primers. Although gel electrophoresis might seem an old-school method, it is still the only method to verify the quality of a PCR product visually. If the focus is to study the community composition and

dynamics of a PNA reactor, selected primers should be evaluated with *in silico* analysis to determine the coverage of the primer pair. It is also important to remember that the obtained results might not provide information about the whole microbial community composition. Therefore, studies focusing on temporal dynamics of the microbial community should interpret results relative to a reference sample belonging to the same PNA reactor. The mentioned set of questions will help to decide which primer pairs can be used for the study. We strongly advise against the normalization of the measured abundances for AnAOB, AOB, NOB and putative heterotrophs to the total eubacterial abundance. This interpretation of results might lead to false positive or false negative results. For instance, we observed different abundances of AnAOB based on normalized data (Figure 10).

Regardless of the objective, an *in silico* analysis for choosing the appropriate primer pair is an essential step due to the range of primer sets of the respective target group. There are also non PCR-based methods like FISH offering complementary information, which can also be useful to design new primers and probes. Despite the hurdles being stated here, PCR based methods are positively acknowledged to determine microbial composition in PNA systems because they are sensitive and fast techniques (Joss et al., 2011). A wise choice of primers and the mentioning of the information about the coverage of the primers will then further boost the confidence in such results.

3.2 Primer Influence on AOB (Chapter 3.2)

The choice of primer and TaqMan probes to quantify ammonia-oxidizing bacteria (AOB) in environmental samples is of crucial importance. The reevaluation of primer pairs, based on current genomic sequences, used for quantification of the *amoA* gene revealed significant misrepresentations of the AOB population in the environmental samples and lack of perfect match primer pairs for *Nitrosomonas europaea* and *Nitrosomonas eutropha*. We designed two new *amoA* cluster 7 specific primer pairs and TaqMan probes to quantify *N. europaea* (nerF/nerR/nerTaq) and *N. eutropha* (netF/netR/netTaq). Specificity and quantification biases of the newly designed primer sets were compared with the most popular primer pair (amoA1f/amoA2r) using DNA from various AOB cultures as individual templates as well as DNA mixtures and environmental samples. Based on the qPCR results, we found that newly designed primer pairs and the most popular one performed similarly for individual templates but differed for the DNA mixtures and environmental samples. Using the popular primer pair introduced a high underestimation of AOBs in environmental samples, especially for *N. eutropha*. Thus, there is a strong need for more specific primers and probes to understand the occurrence and competition between *N. europaea* and *N. eutropha* in different environments.

Chapter 3.2

Lost in translation: the quest for *Nitrosomonas* cluster 7 specific *amoA* primers and TaqMan probes

Laura Orschler, Shelesh Agrawal, Susanne Lackner

3.2.1 Introduction

The chemolithotrophic aerobic ammonia-oxidizing bacteria (AOB) carry out the first step of nitrification, the biological oxidation of ammonium to nitrite. AOB are an integral component of the nitrogen cycle in natural environments as well as wastewater treatment systems, and therefore, significant attention has been given to determine the quantitative dynamics of AOB. It is known, that the 16S rRNA gene sequence-based approach (using AOB targeting CTO primer pair) may result in co-amplification of sequences belonging to other microbial groups (Baptista et al., 2014; Rotthauwe et al., 1997; Sekido et al., 2008). Therefore, AOB are routinely quantified with real-time PCR (qPCR) targeting the ammonia monooxygenase alpha subunit (*amoA*) gene in both natural and engineered systems (Aakra et al., 2001; Orschler et al., 2019; Sharma et al., 2007) .

Two decades back, Rotthauwe *et al.* (1997) designed the popular primer set amoA1f/amoA2r (hereafter called "Rott pp") with two degenerate positions to generate a 491 bp long PCR product for broader coverage of the AOB. This primer pair is, in most cases the first choice (a commonly used primer pair), although covering the entire bandwidth of all AOB with a universal *amoA* primer pair is challenging (Rotthauwe et al., 1997). Since the development of the Rott pp, there has been rapid development in molecular biology, leading to continuous expansion of our understanding of complex microbiomes. Also, more genomic sequences have become available for the diverse AOB. Consequently, lack of 100% sensitivity and specificity (i.e. a perfect match (PM) primer set) of the Rott pp for all β-subclass AOB has been pointed out recurrently (Dechesne et al., 2016; Dionisi et al., 2002; Harms et al., 2003; Hornek et al., 2006; Junier et al., 2009; Purkhold et al., 2000). Also, the Rott pp has a number of mismatches with the *amoA* gene of some AOB, which affect their quantification in unknown microbiomes (Meinhardt et al., 2015; Okano et al., 2004). The Rott pp was designed with the idea of a "universal primer to maximize coverage of AOB diversity", instead of fine-scale quantification of diverse AOB subgroups, which is more useful for understanding and controlling an engineered ecosystem. In an attempt to circumvent this problem, Harms et al., (2003), designed a primer pair targeting the *amoA* gene and a TaqMan probe for specific quantification of *Nitrosomonas oligotropha*-type, which belongs to cluster 6A (Koops and Pommerening-Röser, 2001). Layton et al., (2005) developed new primer sets for *N. nitrosa*, to identify AOB population dynamics in a wastewater treatment plant. However, specific primers for *N. europaea* and *N. eutropha* are missing, even though *N. europaea* and *N. eutropha* have been reported as the most abundant AOB in several environments like freshwater (Stehr et al., 1995) or WWTPs (Siripong and Rittmann, 2007). Nitrosomonas cluster 7 members (*N. europaea* and

N. eutropha) are predominantly found in WWTP due to their environmental niches of nitrogen-rich environments (Bollmann et al., 2002; Purkhold et al., 2000; Wagner et al., 1995). This includes conventional biological nitrogen removal systems, as well as partial nitritation/anammox (PNA) systems (Vlaeminck et al., 2010).

Understanding the role of AOB in various environments adequately and studying their response to dynamic changes in the environment, requires a robust method with highly specific primer pairs. The lack of perfect match bacterial *amoA* primers can lead to over- or under-estimation of the AOB population (Dechesne et al., 2016; Dionisi et al., 2002), which is critical for understanding inter- and intra-microbial group competition. With degenerated primers, it is possible achieve perfect match primer pairs for multiple representatives of a microbial group. However, during the synthesis of degenerate primers, it is unlikely that equimolar proportions of all the individual primers will be produced. As a result, non-equimolar proportions of the primers in the mixture may also result in disproportional representation of the general AOB in the sample (Green et al., 2015). Additionally, there is a high specificity required, to understand if and why ammonia-oxidizing archaea (AOA) outcompete AOB in various environments like soil (Leininger et al., 2006), freshwater (Bollmann et al., 2014) or wastewater (Gwak et al., 2019), and how we put these results in scientific context.

The use of multiple primer sets targeting the *amoA* gene has already been recommended (Bru et al., 2008; Dechesne et al., 2016; Meinhardt et al., 2015). Considering multiple primer sets when exploring AOB in unknown communities is a labor and time-intensive suggestion, but indispensable. For example, Stein et al., 2007 revealed several properties of the *N. eutropha* genome, which are distinct from the closely related *N. europaea* and support niche specialization, such as different chemoorganotrophic growth of *N. eutropha* and *N. europaea* under anoxic conditions with nitrite as terminal electron acceptor (Schmidt, 2009). It is also reported that *N. eutropha* can better tolerate nitrite accumulation than *N. europaea* (Zart and Bock, 1998). This highlights how *N. eutropha* and *N. europaea* may respond differently to a variety of WWTP operational conditions. Therefore, it is important to perform differential quantification of *N. europaea* and *N. eutropha*, which is not possible with universal primer sets like the Rott pp.

Here, we compared previously published *amoA* primers (Annexure II, A.Table 1) with the currently available *amoA* gene nucleotide sequences from the Functional Gene Repository (http://fungene.cme.msu.edu), to determine the coverage and specificity for group-specific AOB. Based on this information we designed two primer sets, including forward and reverse primer with Taqman probe for *N. europaea* and *N. eutropha* (Annexure II, A.Table 2, A.Figure 1). These primer sets were evaluated in both PCR and qPCR and compared with the Rott pp for pure culture DNA and environmental samples. Shotgun sequencing gave an overall

insight into the *amoA* gene sequences that were found in the environmental samples.

3.2.2 Material and Method

Reassessing the coverage and specificity of available AOB *amoA* gene primers and Taqman probes

For the reevaluation of the primers and Taqman probes, which target *amoA* gene of ammonium-oxidizing bacteria (AOB), we collected information about the previously designed primers and Taqman probes in different studies (Annexure II A.Table 1). For *in silico* analysis, reference sequences for AOB *amoA* gene were downloaded from the RDP FunGene repository (http://fungene.cme.msu.edu/). It was required that sequences share 97% amino acid sequence coverage to established HMM protein models.

The sequences were clustered at 99% nucleotide similarity using CD-HIT (v4.6.1c). Then, for each representative sequence in the cluster previously published primer pairs and Taqman probes were matched to determine the number of mismatches between the target and the primer's sequences. We used two approaches to determine the mismatches: (1) using a customized bash script to perform blastn between the cluster database and the primer sequences, (2) and the ProbeMatch function of FunGene. For a perfect match (PM) primer pair, both forward and reverse primers were required to share 100% nucleotide similarity over a minimum of 17 bp of the primer length.

Primer design and validation

Primers and Taqman probes were designed to target *N. eutropha* and *N. europaea* (Annexure II A.Table 2). Multiple AOB amoA gene reference sequences were aligned in UGENE to manually design PM primer pair and Taqman probe for *N. eutropha* and *N. europaea* (Annexure II A.Figure 1) respectively. Primers and probe set designed to target *N. europaea* are called as "ner" and for *N. eutropha* are called as "net". The specificity of both new primer sets and TaqMan probes was confirmed via *in silico* analysis (Figure 1) and PCR analysis. For each primer set PCR was performed with a set of target and non-target DNA (for potential false detections). Gel electrophoresis of PCR products showed no amplification for chosen false positives and high amplification for pure cultures (Annexure II A.Figure 2 and A.Figure 3).

PCR and qPCR analysis

Total genomic DNA was extracted from biomass samples using the Fast DNA Spin kit for soil (MP Biomedicals). DNA concentration and its integrity were analyzed using Qubit 3.0 Fluorometer with Qubit dsDNA HS kit (Thermo Fisher Scientific).

PCR was performed with peqGold Taq-DNA-Polymerase 'all inclusive' with a volume of 28 µL with 0.18 µM forward/reverse primer, 0.7 µM dNTPs and 0.02 u/µL Taq DNA Polymerase.

qPCR for the Rott pp was performed in triplicates for a 20 µL reaction mixture containing 1X PerfeCTa SYBR Green SuperMix 2X Low Rox (QuantaBio), 0.4 mM of each primer, 2 ng of DNA and PCR grade water according to manufacturer's specifications (Annealing T= 55°C, 40 cycles). TaqMan Assays for the ner/net primer/probe set were performed in triplicates for a 20 µL reaction mixture containing 1X TaqMan Fast Advanced MasterMix 2X (Thermofisher), 1X of TaqMan Gene Expression Assay (20X), 2 ng of DNA and PCR grade water according to manufacturer's specifications (Annealing ner T=65°C, net T=60°C; 40 cycles).

Comparative qPCR analysis was performed for pure DNA templates: (1) 100% *N. europaea*; (2) 100 % *N. eutropha*; (3) Mixture 1: *N. europaea*: *N.eutropha* – 3 : 1; (4) Mixture 2: *N. europaea*: *N.eutropha* – 1 : 3; (5) Mixture 3: *N. europaea*: *N.eutropha*: others – 1 : 1: 2; and (6) Mixture 4: *N. europaea: N.eutropha* – 1 : 1, and four samples from wastewater treatment plants. The wastewater treatment plant samples included both samples from conventional nitrification/denitrification systems and partial nitritation/anammox systems. Each qPCR run was performed in triplicate for every sample.

Shotgun sequencing analysis

For each environmental biomass sample from wastewater treatment samples shotgun sequencing was performed. Enzymatic shearing was performed with the Ion Shear™ Plus Reagents Kit with a fragment size of 600 bp. Each sample was tagged using the Ion Xpress Barcode Adapters™ (Thermo Fisher Scientific), according to the manufacturer's protocol. Amplification was performed with the Ion Plus Fragment Library Kit™ (Thermo Fisher Scientific), and afterward, the concentration of each sample was adjusted to 80 pM. All samples were pooled in equal volumes and processed with the Ion 520™ & Ion™ ExT Kit on the Ion Chef™.

Sequencing was performed on the Ion Torrent (ION Torrent Ion S5) using the 530 chip. *amoA* gene-targeted assembly was performed using Xander assembler (Wang et al., 2015). Seed sequences with minimum 95% HMM (Hidden Markov Model) coverage from FunGene repository (http://fungene.cme.msu.edu/) were downloaded.

3.2.3 Results & Discussion

We performed systematic *in silico* PCR analysis to determine the coverage of previously published primers and TaqMan probes targeting the *amoA* gene (Figure 12). To consider previously published primer pairs and TaqMan probes to be a perfect match (PM) with the *amoA* gene diversity, both, the primers and TaqMan probes (if available), were required to share 100% nucleotide identity over a minimum of 15 bp of the primer length. We found that the foremost primer pair (i.e. A189-A682) is not a PM for any cluster of beta-proteobacteria AOB, except for Nitrosospira sp. NpAV. This primer pair contains degeneracy in both primers, which can result in four variants of forward and 16 variants of reverse primer. Our analysis revealed that the coverage of the most commonly used primer pair, the Rott pp (Rotthauwe et al., 1997), is different for different clusters of AOB (Orschler et al., 2019). In case of cluster 7 (*N. europaea* lineage), it is not a PM primer pair (Figure 12). The forward primer (amoA1F) has one base pair (bp) mismatch (MM) to *N. europaea* and the reverse primer (amoA-2r), which is a degenerate primer, has one bp MM to *N. eutropha*. Also, for cluster 6A (*N. oligotropha* lineage) it is not a PM primer set. However, for some members of cluster 3 (Nitrosospira lineage) it is a PM primer pair. Afterwards, a modified amoA-2r was designed having degenerated bases which resulted in 18 sequence variants, to better capture AOB diversity (Hornek et al., 2006). A decade after the Rott pp was designed, the TaqMan probes (1) amoA-NM3 having degeneracy for cluster 7; (2) amoA-NM4 for cluster 6A; (3) and amoA-Ns having degeneracy for cluster 3, were designed for improved specific quantification of the respective AOB (Regan et al., 2007). We found that amoA-NM3 and amoA-Ns are PM probes for cluster 7 and cluster 3 due to the presence of degeneracy, but amoA-NM4 is a MM probe for cluster 6a (Figure 12). It was realized very early that it is difficult to design a single primer pair and TaqMan probe targeting the *amoA* gene to detect all AOB. Therefore, Harms et al. (2003) designed a primer pair (i.e. amoNo550D2f containing two degenerate bases and amoNo754r) and TaqMan probe (i.e. amoNoTaq729) for quantification of *N. oligotropha* like bacteria. Primer pair amoNo550D2f and amoNo754r combined with TaqMan Probe amoNoTaqD729 have high coverage for cluster 6A. However, only one possible forward primer was a PM primer, although three sequence variants are possible due to degenerated bases. Similarly, Layton et al. (2005) designed a primer pair and TaqMan probe (amoRI27542f, amoRI2767r, and amoARIbhq651r) for quantification of only *N. nitrosa* having MM for cluster 6A, 7 and 3 (Annexure II, A.Table 1). The recently designed primer pair amoA-1Fmod/GenAOBr without degeneracy (Meinhardt et al., 2015) is a PM primer pair for members of cluster 3 but not for cluster 6A (Figure 12).

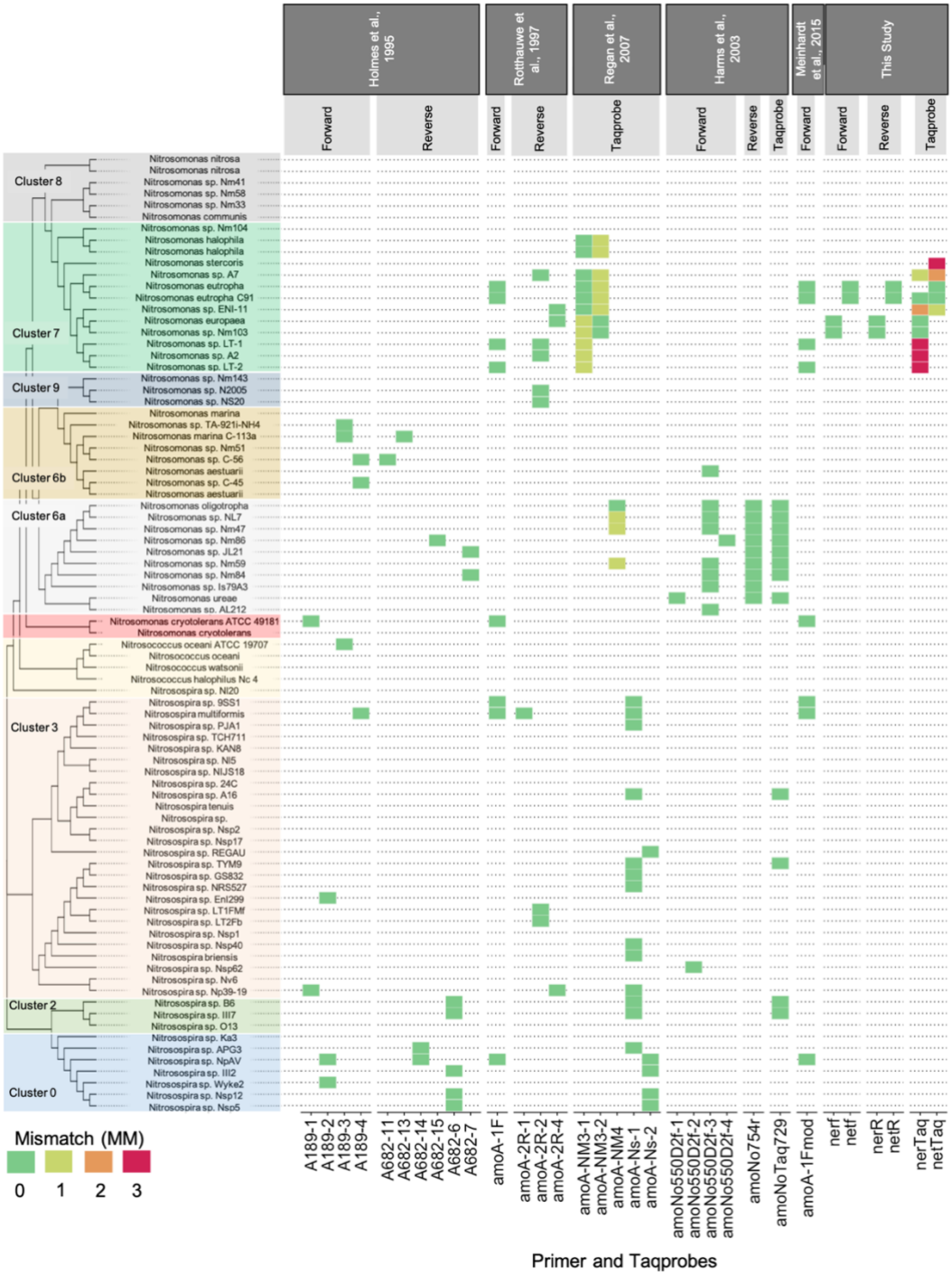

Figure 12: *In silico* PCR of all known *amoA* primer pairs/TaqMan probes represented in a neighbor-joining phylogenetic tree inferred from AOB *amoA* gene sequences; the heatmap represents the amplification for the respective primer pair/TaqMan probe; dark green shows perfect match (PM) primer pairs, olive green represents one mismatch (MM) pp, orange represents two MM pp, red represents three MM pp, and no colour denotes more than 3 MM pp. Clustering was based on Koops (1992).

This study even reported less efficient amplification of *N. europaea*. We designed primer pairs and TaqMan probes without introducing degeneracy for specific quantification of *N. europaea* (nerF/nerR combined with nerTaq as TaqMan probe) and *N.eutropha* (netF/netR with netTaq probe) (Figure 12), respectively. In addition, PCR followed by gel electrophoresis showed no amplification for a set of non-target microorganisms and high amplification for target microorganisms (Annexure II, A.Figure 2, A.Figure 3).

The effect of internal primer-template mismatch is highly variable and can lead to dramatic effects. It may lower PCR efficiency depending on the position of the mismatch and detection sensitivity if the annealing temperature is elevated to compensate the primer MM (Sekido et al., 2008; Wu et al., 2017). These mismatches introduce bias in determining community composition.

We performed comparative qPCR analysis using *N. europaea* and *N. eutropha* as pure cultures as well as in different mixtures, for Rott pp and new developed ner and net primer pairs/TaqMan probe (Figure 13, Annexure II A.Figure 4). The abundance measured in the PCR reaction mix containing 100% *N. europaea* DNA template was highly similar between the Rott pp and the ner primer/probe set. Less similarity for reactions containing 100% *N. eutropha* was observed between the Rott pp and the net primer/probe set. A significant difference between the abundance measured using the Rott pp and both newly designed primer/probe sets was observed for mixed DNA templates (i.e. not 100% target DNA in the qPCR reaction) (Figure 13, Annexure II A.figure 4). Based on percentual discrepancy, the Rott pp showed an underestimation of 50.78% for mixture 1 and 48.66% for mixture 2. Mixture 3 with four different AOB pure cultures resulted in an underestimation of 50.78%, and 48.64% for mixture 4. These results clearly show that presence of more than one target AOB in the PCR reaction impacts the performance of the Rott pp. The abundance measured using the Rott pp differed by almost half compared to the abundance measured using the newly developed primer/probe sets. Our results are in consensus with the previously expressed opinion about an underestimation of *amoA*-targeted quantification using the Rott pp (Dechesne et al., 2016; Layton et al., 2005; Meinhardt et al., 2015).

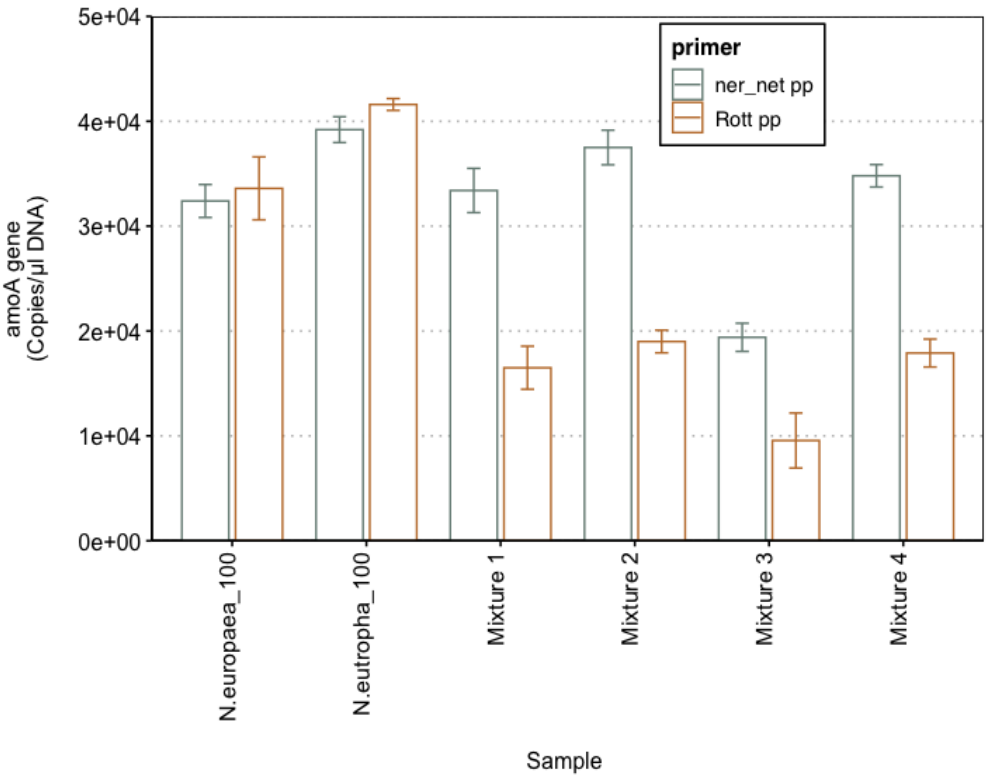

Figure 13: Abundance of *amoA* genes for 4 different DNA mixtures of different AOB: Mixture 1: *N. europaea:N. eutropha* – 3:1; Mixture 2: *N. europaea:N. eutropha* - 1:3; Mixture 3: *N. europaea:N. eutropha*: Others – 1:1:2; Mixture 4: *N. europaea:N. eutropha* – 1:1.

Metagenomics was performed to determine the composition of the AOB community in unknown environmental samples. *AmoA* gene-targeted assembly was performed for the environmental samples. The composition of AOB differed for each sample (Figure 14), containing AOBs from clusters 6a/7/ *N. cryotolerans* lineage.

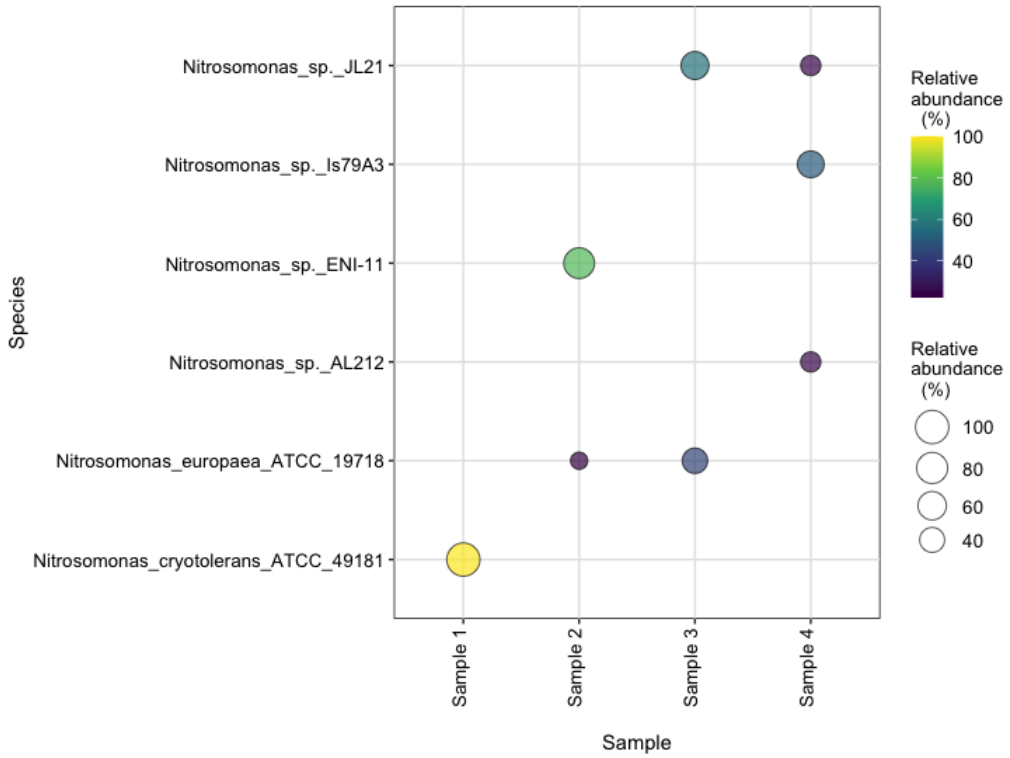

Figure 14: Community composition of AOB from wastewater treatment plant samples based on shotgun sequencing. Color bar and circle size represent the relative abundance in percentage.

To compare the performance of the Rott pp with ner and net primer/probe sets, the abundance of AOB in these environmental samples was quantified using respective primer/probe sets (Figure 15). *N. eutropha* was not found in environmental samples using net primer/probe set. These results fit very well with the metagenomics composition, too. In case of the ner primer/probe set, 4.02 ± 0.18 log copies/ng DNA in sample 2 and 3.2 ± 0.05 log copies/ng DNA in sample 3 were detected, whereas, 4.51 ± 0.03 log copies/ng DNA in sample 2 and 3.43 ± 0.11 log copies/ng DNA in sample 3 were detected based on the Rott pp. No detection of *N. europaea* in sample 1 and sample 4 was also in consensus with metagenomic composition. The abundance estimate based on the Rott pp was only 0.5 log copies/ng DNA for sample 2 and 0.3 log copies/ng DNA for sample 3 more than estimates based on ner primer/probe set, though the samples contained more of other AOBs as shown in Figure 3. These results suggest that use of Rott pp can

lead to misinterpretation in terms of the total abundance of the AOB population because in general (especially for WWTP operators and environmental engineers) it is assumed that use of universal primer pair like Rott pp, allows for the detection of all the AOBs present in the WWTP.

Moreover, a significant (p-value < 0.01, one-way analysis of variance (ANOVA) analysis) difference was observed in AOB concentration when all samples were spiked with a defined concentration of 5 log copies/ng DNA of *N. eutropha* to evaluate coverage of the Rott pp in comparison to the ner and net primer/probe sets. No significant change in the measured concentrations of AOB was observed using the Rott pp in comparison to the unspiked samples (Figure 15, Annexure II A.Figure 4). Using the net primer/probe set it was possible to measure the abundance of the spiked *N. eutropha* (Figure 15).

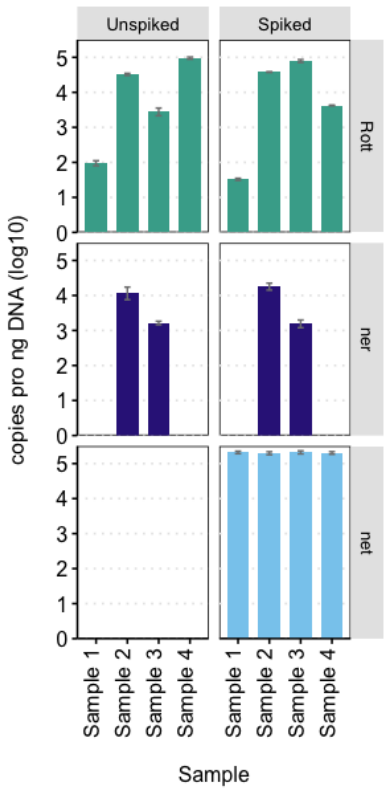

Figure 15: AmoA abundance analyzed with qPCR for the ner (dark blue) and net (light blue) primer sets and the Rott primer pair (amoA1f/amoA2r) (green); the two graphs on the left side (with "Unspiked") are the original environmental samples from the wastewater treatment plants, the two graphs on the right side (with "Spiked") show the results after spiking the samples with *N. eutropha*.

Because of the high specificity of the primers and probe sets to detect specific AOB, the principle of nesting (adding abundance of each specific AOB) can be applied for quantification of total AOB as suggested previously (Layton et al., 2005; Lim et al., 2008; Sekido et al., 2008). This helps to overcome the major problem of under- or over-estimating AOB abundances in environmental samples. For example, nitrite accumulation in engineered environments may disproportionately affect N. europaea and N. eutropha populations, at each AOB differs in their ability to tolerate increased nitrite conditons (Cua and Stein, 2011; Tan et al., 2008; Zart and Bock, 1998). The primers designed in this study, in contrast to the Rott primers, would be able to detect these species-specific population trends. This approach can further be used to analyze competitive dynamics amongst different AOB present in samples. The primer pairs and TaqMan probes developed in this study will additionally help to (1) have a closer look at environments where competition between N. europaea and N. eutropha are expected and (2) overcome underestimation of AOB, together with use of primers and probes developed for N. oligotropha cluster (Harms et al., 2003; Meinhardt et al., 2015) and Nitrospira cluster.

3.3 Analysis of denitrifying bacteria in wastewater treatment plants based on functional genes (Chapter 3.3)

Substantial presence of denitrifiers has already been reported in partial nitritation anammox (PNA) systems using the 16S rRNA gene, but little is known about the phylogenetic diversity based on denitrification pathway functional genes. Therefore, metagenomic analysis was performed to determine the distribution of denitrification genes and the associated phylogeny in PNA systems and whether a niche separation between PNA and conventional activated sludge (AS) systems exists. The results revealed a distinct abundance pattern of denitrification pathway genes and their association to the microbial species between PNA and AS systems. In contrast, the taxonomic analysis, based on the 16S rRNA gene, did not detect notable variability in denitrifying community composition across samples. In general, *narG* and *nosZa2* genes were dominant in all samples. While the potential for different stages of denitrification was redundant, variation in species composition and lack of the complete denitrification gene pool in each species appears to confer niche separation between PNA and AS systems. This study suggests that targeted metagenomics can help to determine the denitrifying microbial composition at a fine-scale resolution while overcoming current biases in qPCR approaches due to a lack of appropriate primers.

Chapter 3.3

Targeted metagenomics reveals extensive diversity of the denitrifying community in partial nitritation anammox and activated sludge systems

Laura Orschler, Shelesh Agrawal, Susanne Lackner

3.3.1 Introduction

The tremendous anthropogenic influence of increasing nitrogen loads to feed the world's population has a significant effect on the global nitrogen cycle (Gruber and Galloway, 2008). As only a small proportion of the nitrogen is captured in our bodies, the main fraction is excreted with urine. If not treated properly, these nitrogen compounds are discharged into open water bodies, resulting in eutrophication. The removal of nitrogen from wastewater is, therefore, of extreme environmental importance especially in densely populated areas (Kowalchuk and Stephen, 2001). Typical biological treatment technologies like the conventional activated sludge (AS) process are based on two-step nitrification/denitrification. Since the discovery of autotrophic anaerobic ammonium oxidizing bacteria (AnAOB) (Strous et al., 1999), there has been a shift from conventional nitrification/denitrification to anaerobic ammonium oxidation combined with partial nitritation (PNA) for certain applications over the last two decades. PNA is the completely autotrophic oxidation of ammonium with nitrite as electron acceptor, converting nitrite and the remaining ammonium from aerobic ammonium oxidizing bacteria (AOB) to nitrogen gas. Driven by the much higher energy-efficiency due to less aeration demands, today, more than 150 PNA installations are already successfully operated worldwide (Lackner et al., 2014).

Initially, only very limited number of microorganisms, i.e. AOB, AnAOB and nitrite oxidizing bacteria (NOB), were considered as key players in PNA systems. However, with time many studies reported that denitrifiers are common and usually dominant in PNA systems. Thus, these bacteria may contribute to the overall nitrogen removal potential of the PNA microbiome (Agrawal et al., 2017; Chen et al., 2019; Chu et al., 2015; Pereira et al., 2017; Persson et al., 2017; Wang et al., 2019b). Denitrification is a facultative respiratory pathway reducing nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) to nitrogen gas (N_2) catalyzed by four types of nitrogen reductases in sequence: nitrate reductase (*N*ar), nitrite reductase (*Nir*), nitric oxide reductase (*Nor*) and nitrous oxide reductase (*Nos*) (Zumft, 1997). Denitrification is not typically linked to phylogeny because denitrifiers are taxonomically diverse, therefore most studies about denitrification focused on functional genes rather than the 16S rRNA gene (Philippot, 2006). But, in case of PNA systems, most of the previous studies reported the occurrence of denitrifiers in PNA systems based on 16S rRNA gene amplicon sequencing (Agrawal et al., 2018; Cao et al., 2017; Pereira et al., 2017).

There are few studies (Pellicer-Nacher et al., 2014; Shu et al., 2018; Wang et al., 2019b), which used quantitative polymerase chain reaction (qPCR) for quantitative analysis of denitrifying functional genes in PNA systems. However,

for all denitrifying genes, several primer pairs have been published and continuously reassessed, especially for nitrite reductase (*nirK* and *nirS*) (Braker and Tiedje, 2003; Casciotti and Ward, 2001; Throback et al., 2004). Ma et al. (2019) evaluated the coverage of existing primer pairs *in silico* based on a metagenomic study. The results pointed out that the existing *nirK*, *nirS* and *norB* primers have low coverage over the entire gene pool, and in consequences are hardly suitable for molecular methods to investigate denitrifiers. Also for *narG* and *nosZ* primers, there are serious issues for both, coverage and specificity (Ma et al., 2019). This implies that the use of currently available primers for assessing denitrifiers can introduce bias in the results. A similar situation appears with primer pairs for the 16S rRNA gene and other functional genes (Orschler et al., 2019).

The use of metagenomics can circumvent the problems associated with PCR-based approaches in understanding the role of denitrifiers, which carry one or more denitrifying genes (Agrawal et al., 2018). However, very few metagenomic studies are available on PNA systems, and that limits the focus primarily to AnAOB and few selected denitrifiers (Bhattacharjee et al., 2017; Guo et al., 2016; Lawson et al., 2017; Speth et al., 2016). It is necessary to understand the abundance, structure, and activity of the denitrifiers to comprehend their role in the PNA. Additionally, to understand whether there are (dis)similarities in the denitrifying community between the PNA and AS systems.

In this study, we investigated differences and similarities of the denitrification genes and microorganisms associated with them, between two sidestream PNA systems and also compared with the conventional activated sludge (AS) processes of the respective WWTP. The *de novo* metagenomic assembly tends to assemble dominant microorganisms (Namiki et al., 2012; Wang et al., 2015), because it is reliant on reference sequence databases containing an unknown amount of extant microbial diversity. Thus, it could result in limited recovery of denitrification genes. Therefore, we analyzed samples from two different wastewater treatment plants (WWTP) employing a targeted metagenomic assembly approach.

3.3.2 Material and Methods

Sample Collection

Biomass samples were collected from two different wastewater treatment plants in Germany: two samples from conventional activated sludge (AS) nitrification-denitrification processes (AS1 and AS2) and two samples from sidestream PNA processes (PNA1 and PNA2) (Annexure III A.Table 1). Total genomic DNA was extracted using the Fast DNA Spin kit for soil (MP Biomedicals) according to a modified manufacturer's protocol (Orschler et al., 2019). The quality of the DNA

was checked using gel electrophoresis, and the concentration was measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific).

Library preparation and sequencing

Sample preparation and library construction were performed with the Ion Xpress™ Plus Fragment library kit (Thermo Fisher Scientific). Enzymatic shearing was used to prepare fragment libraries from genomic DNA for downstream template preparation and was handled according to the manufactures protocol. Purified DNA was tagged using the Ion Xpress Barcodes Adapters™ (Thermo Fisher Scientific) and size selection of the library for 600 base-pair reads was performed with E-Gel™ Size Select™ II Agarose Gel. Each sample was adjusted to a 60 pM concentration. Template preparation was performed on the ION chef system with Ion 520™ & Ion 530™ ExT Kit. Sequencing was performed on the Ion Torrent (ION Torrent Ion S5) using the 530 chip. Base calling, demultiplexing and initial quality control were conducted by Torrent Suite version 4.4.2 (Thermo Fisher Scientific) with default parameters. Trimmed and quality filtered reads were used for downstream analyses.

Targeted metagenomics for functional analysis

To evaluate the abundance and phylogenetic affiliation of genes associated with nitrogen metabolism, specific genes were assembled using the Xander assembler (Wang et al., 2015). To assemble the sequences, Xander requires the protein profile Hidden Markov Model (HMM) built from reference set of target genes. For narG, napA, nirK, nirS, norB, nosZ, nosZa1, and nosZa2 genes, nucleotide and amino acid sequences were downloaded from the Functional Gene Repository (http://fungene.cme.msu.edu). The minimal cutoff was set to 100 amino acids. For each gene, a table of operational taxonomic unit (OTU) counts was made based on k-mer (set value 45) coverage of the representative sequences. The OTU tables were further analyzed in R.

Taxonomic affiliation based on the 16S rRNA gene

From whole metagenome dataset, 16S rRNA gene sequences were extracted with Metaxa2 (version 2.0) using default settings (Bengtsson-Palme et al., 2015). Genus assignment was performed at >95% identity with the reference 16S rRNA gene sequence and reported as relative abundance.

Statistical analysis

All statistical tests were performed in R. NMDS analysis was performed using the metaMDS function of the 'vegan' package to create an ordination based on the sample dissimilarity. Heatmaps and barplots diagrams were all generated in R (http://www.R-project.org/). Venn diagrams were created using the 'VennDiagram' package in R. Proportionality correlation was performed using the 'propr' package in R and p-values for the correlation analysis were also calculated.

3.3.3 Results

The four samples (two samples from each WWTP: sidestream PNA and AS samples; for more information see Annexure III A.Table 1) produced an average of twelve million quality filtered, merged reads, at an average length of 460 bp. The four samples were grouped into two categories by process design for analysis and discussion. The first category summaries the AS processes (referred to as AS1 and AS2), whereas the second group includes the sidestream PNA systems (PNA1 and PNA2) with no external inoculum for process start-up. To focus on the denitrification pathway, reads were extracted from the full metagenome data set and analyzed. From here on, the read abundance is presented as mean read per million reads which is abbreviated as rpm.

The results revealed that the aggregate abundance of genes associated with nitrate (*narG* and *napA*) and nitrous oxide reduction (*nosZ*, *nosZa1* and *nosZa2*) were higher than the genes associated with the nitrite and nitric oxide reduction (Figure 16) (Annexure III A. Figure 1). The total abundance of the nitrate reductase encoding genes in PNA2 (217 rpm) was highest, followed by PNA1 (164 rpm) > AS2 (87 rpm) > AS1 (73 rpm). However, looking at individual genes (i.e. *napA* and *narG*), the respiratory nitrate reductase gene (*narG*) was higher in abundance than the periplasmic nitrate reductase gene (*napA*), especially in PNA systems.

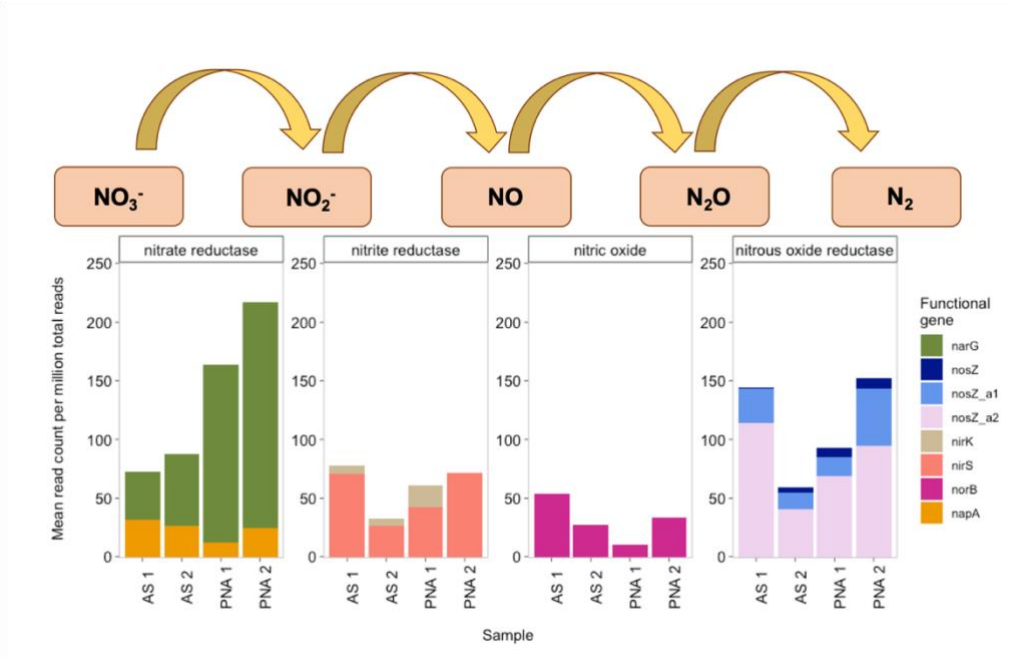

Figure 16: Abundance of reads associated with each denitrifying gene across the samples. (i.e. narG: respiratory nitrate reductase; napA: cytoplasmic nitrate reductase; nirK: copper containing nitrite reductase; nirS: cytochrome cd containing nitrite reductase; norB: nitritic oxide reductase; nosZ: typical nitrous oxide reductase; nosZa1: atypical nitrous oxide reductase clade1; nosZa2: atypical nitrous oxide reductase clade 2). AS1 and AS2: conventional activated sludge samples; PNA1 and PNA2: sidestream PNA samples.

The *narG*:*napA* ratio ranged from 12.0 for PNA1; 8.0 for PNA2; 2.0 for AS2 to 1.0 for AS1. In the AS samples, the *napA* gene was more abundant than in the PNA samples. In AS1, AS2, and PNA1 the cytochrome-cd containing nitrite reductase gene (*nirS*), as well as the copper containing nitrite reductase gene (*nirK*) were found. However, the *nirS* gene was dominant in all the samples. In PNA2 only *nirS* was found. The aggregate abundance of the nitrite reductase genes (i.e. *nirS* and *nirK*) was very similar for AS1 (77 rpm) and PNA2 (71 rpm), followed by PNA1 (61 rpm) and AS2 (32 rpm). The abundance of nitric oxide gene (*norB*) was higher in the AS than in the PNA samples (Annexure III A.Figure 1). The aggregate read abundance of the N_2O reductase genes (i.e., *nosZ*, *nosZa1*, and *nosZa2*), exhibited a similar trend to the *nirS* because *nosZ* gene abundances were higher in the AS and lower in the sidestream PNA in WWTP1 and vice versa in WWTP2. Of the total reads in all samples assigned to *nosZ*, the majority of the reads assigned to the atypical clade II *nosZ* gene, i.e. between 62 to 80% of the total reads.

Correlation analysis of denitrification genes

To investigate the association between the target genes, we assessed the proportionality between the read abundance of the genes across samples. Results are presented in a heatmap of the proportionality metric, ρ, between read counts from each gene in more detail (Figure 17). *NarG* and *napA* read counts were inversely proportional (ρ = -1), while the abundance of the *narG* was strongly proportional with *nirK* (ρ = 0.99) and *nosZ* (ρ = 0.96). *NapA* and *norB* abundances were also strongly proportional (ρ = 0.95). Proportionality clustering revealed that the abundances of the *nirS* and *nirK* had no significant association. However, *nirS*, *nosZa1*, and *nosZa2* have significant association, while *nirK* and *nosZ* abundances are significantly associated.

Taxonomic composition

Additionally, we performed taxonomic annotations of the denitrification target genes to determine the pattern in abundance of the microorganisms associated with denitrification pathway genes. Also, to determine whether the microorganisms associated with denitrification genes are ubiquitous across the samples, especially between sidestream PNA and AS samples. We used Xander software that uses a novel data structure combining de Bruijn graphs and Hidden Markov Models (HMM) to target assembly of specific protein-coding genes from metagenomic data (Wang et al., 2015). Using this data structure allowed us to apply powerful graph search techniques to assemble individual genes. Xander analysis disclosed all Match Names related to the analyzed gene set.

Among the annotated reads for all denitrification genes, Proteobacteria was the most abundant phylum, while the abundance varied between samples (Annexure III A.Figure 2).

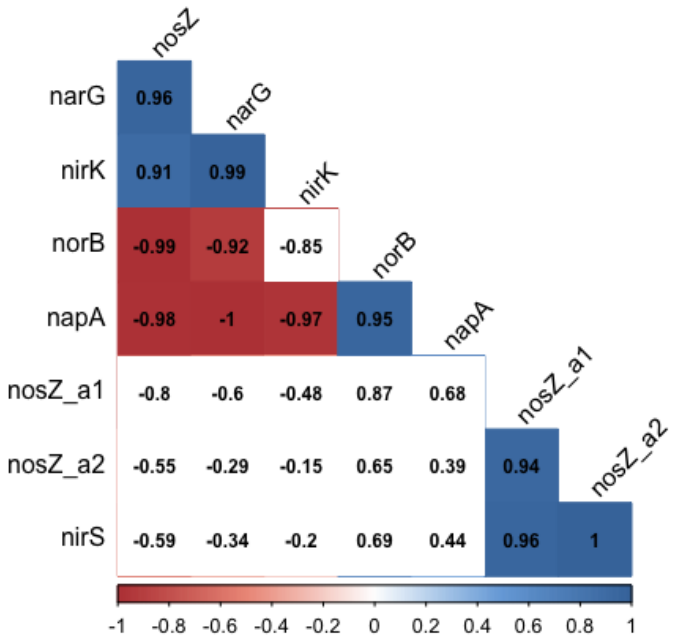

Figure 17: Heatmap showing the proportionality for the abundances of each denitrification gene. (i.e. *narG*: respiratory nitrate reductase*; napA*: cytoplasmic nitrate reductase; *nirK*: copper containing nitrite reductase; *nirS*: cytochrome cd containing nitrite reductase; *norB*: nitritic oxide reductase; *nosZ*: typical nitrous oxide reductase; *nosZa1*: atypical nitrous oxide reductase clade1; *nosZa2*: atypical nitrous oxide reductase clade 2)

The most abundant phyla in the sidestream PNA samples were Proteobacteria (32% in PNA1 and 38% in PNA2), Chloroflexi (18% in PNA1 and 4% in PNA2), Chlorobi (10% in PNA1 and 13% in PNA2), Bacteroidetes (8% in PNA1 and 12% in PNA2) and Ignavibacteriae (7% in PNA1 and 5% in PNA2). In comparison, in conventional activated sludge samples, Proteobacteria (50% in AS1 and AS2) and Bacteroidetes (33% in AS1 and 27% in AS2) were the dominant phyla. Besides, in AS2, Actinobacteria (10%) was also dominant (Annexure III A.Figure 2).

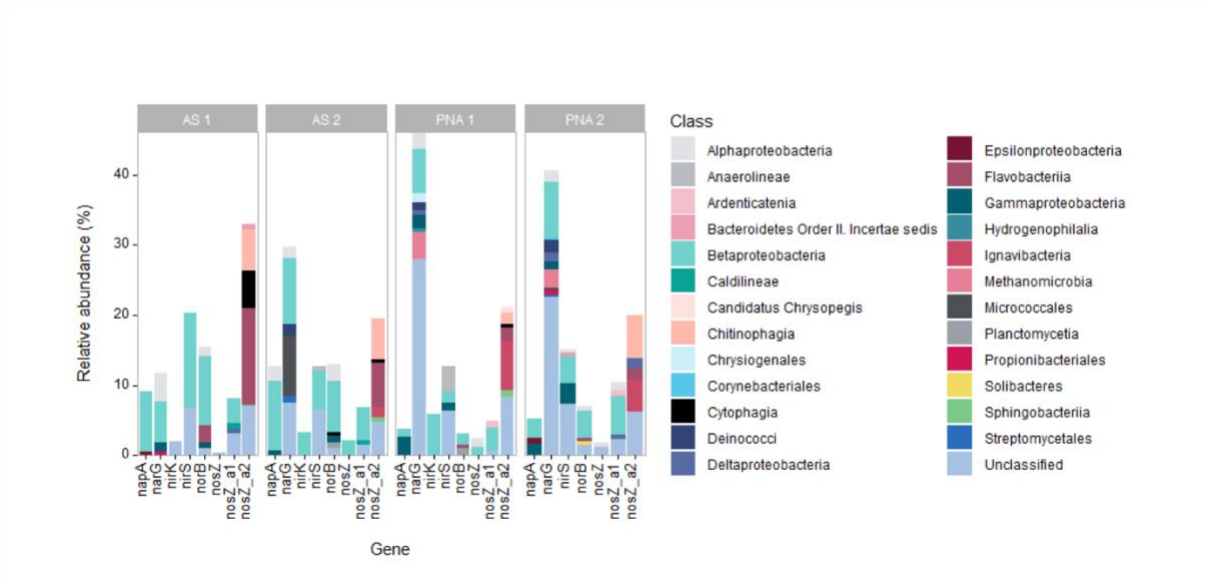

Figure 18: Relative abundance of the classes, associated with all denitrification genes, found in the samples.

At the class level, Betaproteobacteria, associated with at least one gene for each intermediate denitrification pathway, were dominant and ubiquitous across the samples (Figure 18). For other dominant classes, we observed differences between the PNA and AS samples (Figure 18). For example, in PNA samples, Gammaproteobacteria associated with *narG*, *napA*, *nirS* (6% in PNA1 and PNA2); unclassified Chlorobi associated with *narG* and *nosZa2* (10% in PNA1 and 15% in PNA2) and Ingnavibacteria associated with *nosZa2* (7% in PNA1 and 5% in PNA2), were dominant. Whereas, Alphaproteobacteria associated with *narG*, *napA*, *norB* (6% in AS1 and 7% in AS2); Flavobacteriia associated with *norB* and *nosZa2* (16% in AS1 and 8% in AS2) were dominant in AS samples.

In total, 192 species were detected, associated with the denitrification genes. Amongst the 50 most abundant species across the samples for each respective gene, none had the potential for complete denitrification, and the majority were not "generalists" (Figure 19). For nitrate reduction, *narG* carrying *Chloroflexi bacterium* OLB14 in PNA1 (28%) and Chlorobi bacterium OLB6 were most abundant in PNA2 (36%), whereas, *Dechloromonas denitrificans* carrying the *napA* gene was most abundant in AS1 (75%) and AS2 (65%). *NirK* reads associated mainly with *Nitrosomonas sp.* AL212 in AS2 and PNA1; and *Nitrosomonas* sp. Is79A3 in PNA1, which constitute 100% of the relative abundance. Apart from *Nitrosomonas europaea* ATC 19718 carrying the *norB* gene, no other dominant common species were present in the two PNA systems. In AS2 sample we found higher relative abundance (>80%) of *Simplicispira suum* associated with the *nosZ* gene. The *nosZa1* was carried by *Sulfuritalea hydrogenivorans* sk43H (35% relative abundance) in PNA1 and in both PNA samples by Ca. *Accumulibacter* sp. SK-11 (15-20%).

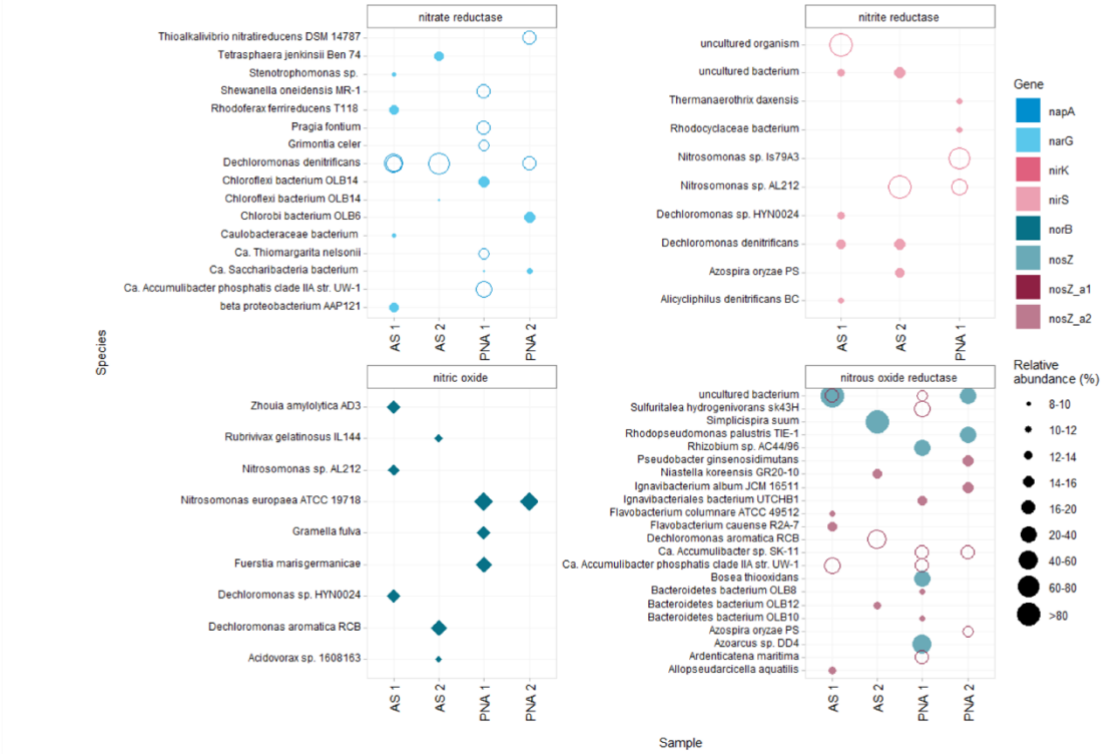

Figure 19: Relative abundance of top 50 abundant species found across the samples for each gene, respectively. Functional gene analysis was performed with Xander and adjustment of MatchNames with NCBI database.

It was also carried by *Ca. Accumulibacter phosphatis* clade II A str. UW-1 (AS1, 25% relative abundance and 16% in PNA1) and *Dechloromonas aromatica* RBC in AS2 (45% relative abundance).

Comparison of sidestream PNA vs. AS microbial composition: denitrifying members vs. whole community

We determined the extent of compositional (dis)similarities between the PNA and AS samples, based on the denitrifying community. Additionally, we compared whether compositional (dis)similarities observed for the denitrifying members extend to the whole microbial community. Therefore, we performed a non-metric multidimensional scaling (NMDS) to ascertain differences in the microbial community across the samples (Figure 20).

For the denitrifying community, the analysis disclosed no specific clustering among all four samples (Figure 20 A). The data points are widely distributed for every sample in one specific corner of the plot. No common species were found in the samples (Annexure III A.Figure 3). Interestingly, the most abundant species across the samples had the potential for nitrate reduction (Figure 20 A). However, in Figure 20 B which is based on the 16S rRNA gene for whole community composition, samples from the PNA system clustered together, whereas the

samples from the AS showed differences in community composition along the primary (horizontal) axis (Figure 20 B). Nevertheless, we found 43 common species across the samples (Annexure III A.Figure 4).

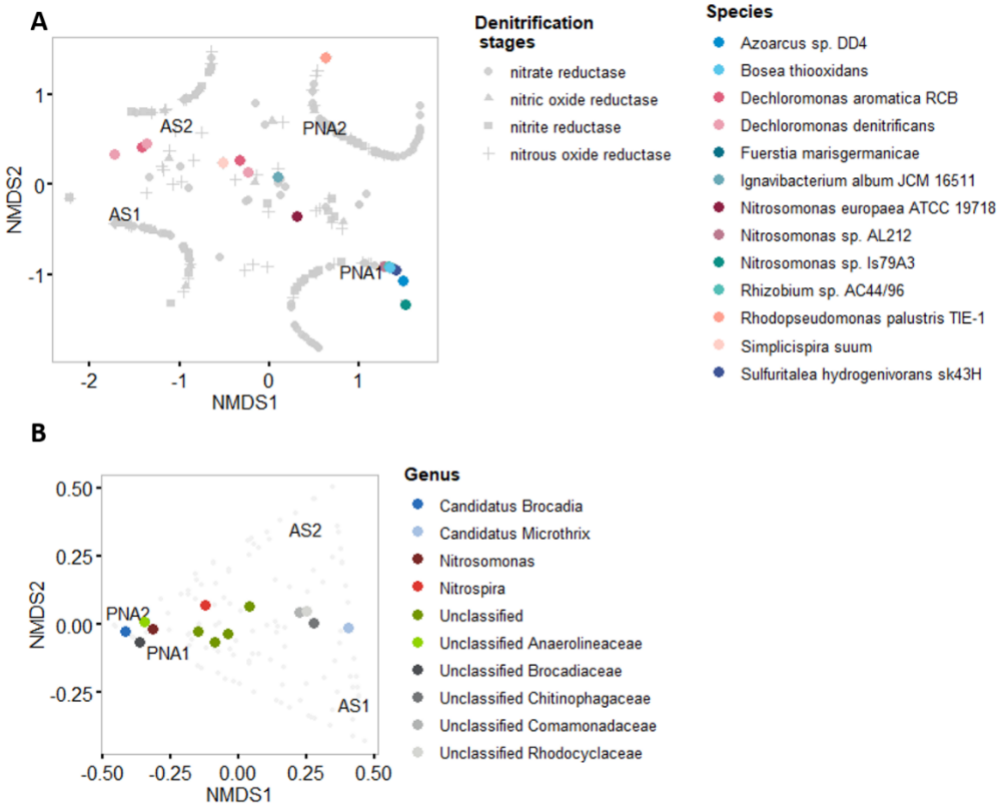

Figure 20: Non-metric multi-dimensional scaling (NMDS) ordination plots comparing the AS and sidestream PNA samples. NMDS plots were derived from Bray Curtis distance based on; (A) abundance of denitrifiers, which associate with different denitrifying genes, found in the samples, also showing the top 13 abundant species across the samples. (B) abundance of all microbial members detected in the samples based on the 16S rRNA gene sequence analysis performed using Metaxa, also showing the top 10 abundant genus across the samples.

3.3.4 Discussion

The objective of this study was to compare the structure of the denitrifiers community, associated with the denitrification genes (i.e., *narG*, *napA*, *nirS*, *nirK*, *nosZ*, *nosZa1*, and *nosZa2*), to infer patterns that may differ between the denitrifiers with one or multiple denitrification genes in sidestream PNA processes of two wastewater treatment plants, as well as, to compare pattern in AS processes of the respective WWTPs.

Metagenomic analysis based on Xander assembly revealed, that the most abundant denitrifiers found in each sample were *Chloroflexi bacterium* OLB14 (PNA1), *Chlorobi bacterium* OLB6 (PNA2), and *Dechloromonas denitrificans* (AS1 and AS2) respectively. *Dechloromonas denitrificans* is found in several WWTPs worldwide (Albertsen et al., 2012; Gonzalez-Martinez et al., 2016). We found that Chlorobi and Chloroflexi species associated with denitrifying genes were more abundant in PNA systems compared to AS systems (Annexure III A.Figure 2), though both of them have been reported as common members in the activated sludge microbiome based on 16S rRNA gene analysis (Kragelund et al., 2007; Nielsen et al., 2009b). Reason could be a lack of denitrifying genes in Chloroflexi and Chlorobi present in the AS samples. Kragelund et al. (2007) reported a lack of denitrifying capability of some Chloroflexi isolates from activated sludge samples (*napA*/*narG*, *nirK*/*nirS*, *norB* and *nosZ*). In recent years, several studies focused on the heterotrophic activity in anammox systems and the continuous presence of Chlorobi and Chloroflexi gained in interest (Agrawal et al., 2017; Bhattacharjee et al., 2017; Pereira et al., 2017; Speth et al., 2016). They possess *narG* und *nosZ* and presumably encode a nitrite loop with anammox and NOB, and therefore support anammox growth, as previously reported (Lawson et al., 2017).

The *napA* enzyme has a higher affinity for nitrate and is usually associated with nitrate-limited environments (Papaspyrou et al., 2014; Potter et al., 1999). However, PNA and AS systems have typically sufficient nitrate, which could explain the dominance of *narG* in all the samples (Figure 16). Additionally, we observed a clear separation of dominant *narG* and *napA* communities between PNA and AS samples (Figure 19), except *Dechloromonas denitrificans*, as it was detected in all four samples. A significant abundance of *narG* in PNA samples supports that partial denitrifiers could support the removal of nitrate through the nitrate-nitrite loop (Agrawal et al., 2017; Bhattacharjee et al., 2017; Speth et al., 2016).

The *nirS* gene was found dominant in all our samples (Figure 16), which is in line with prior studies reporting *nirS* being dominant in PNA and AS systems. Although *nirS* and *nirK* are functionally and physiologically equivalent, it has been reported that the dominance of *nirS* over *nirK* in environments with sufficient nitrite might be due to its higher efficiency for nitrite respiration (Graf et al., 2014; Nadeau et al., 2019). The *nirK* gene found in the samples associated with Nitrosomonas species (*Nitrosomonas europaea* ATC 19718 (PNA1/PNA2), *Nitrosomonas sp.* Is79A3 (PNA1), and *Nitrosomonas sp.* AL212 (AS1/AS2/PNA1) underlines as previously reported that Nitrosomonas uses the nitrite pathway only against nitrite toxicity and not for the respiration. There is presumably a key role for *nirK* and *norB* genes in nitrifier denitrification (Schmidt et al., 2004).

The presence of *norB* across all the samples with similar relative abundance might suggest its redundant nature, as nitric oxide reductase encoded by *norB* can reduce

both nitric oxide and oxygen (Chen and Strous, 2013). Moreover, many denitrifiers and non-denitrifiers contain *norB*, which is advantageous against nitrosative stress and microaerobic conditions (Heylen et al., 2007). Nitric oxide reductase showed a clear separation between PNA and AS systems, presumably due to differences in the environmental conditions (Annexure III A.Table 1), as *norB* was associated with Nitrosomonas, Fuerstia, Gramella, Bacteriodetes and Planctomycetes in the PNA system, and associated with Dechloromonas, Nitrosomonas, Zhoueia, Rubrivivax and Acidovorax in the AS system. For the *norB* gene, nitric oxide reducers, we also found a clear separation between PNA and AS systems.

We found that in all our samples, a significant fraction of *nosZa2* reads could be assigned to members of Bacteroidetes and Ignavibacteriae (Figure 16, Figure 19), similar to a previous study (Juhanson et al., 2017). Nevertheless, Ignavibacteriae was only predominant in PNA systems and exclusively assigned to the *nosZa2* gene, which agrees with previous studies, often reporting members of Ignavibacteriae in PNA systems (Juhanson et al., 2017; Pereira et al., 2017).

The presence and composition of denitrifiers in the PNA systems based on 16S rRNA gene amplicon sequencing has been extensively reported (Agrawal et al., 2017; Du et al., 2019; Laureni et al., 2015; Persson et al., 2017; Wang et al., 2019a). Based on the 16S rRNA gene taxonomic composition, it is difficult to accurately determine the denitrifying potential, because it may not denote whether all functional genes are involved in the denitrification process. For example, metagenomic studies on PNA systems revealed that none of the heterotrophic members carried all the genes of the denitrification pathway (Bhattacharjee et al., 2017; Guo et al., 2016; Speth et al., 2016). Moreover, it is known that taxonomic diversity based on denitrifying genes is not congruent with 16S rRNA gene phylogeny (Heylen et al., 2006; Kragelund et al., 2007). Similarly, our results demonstrate the compositional diversity of denitrifiers between samples, which is not visible based on the 16S rRNA gene phylogeny (Figure 20).

The results from this study show that denitrification pathway genes do show a distinct pattern of abundance and association to the microbial species between sidestream PNA and AS systems. To understand the complex trophic network and relationships between different heterotrophs carrying a particular set of denitrification genes (also counterpart gene for the same function) is not possible from this study alone. Thus, we suggest that more studies (using targeted metagenomic for better resolution or whole genomics) should focus on denitrifying microbial communities and the functional genes plus more, mainstream as well as sidestream PNA systems need to be examined to decipher the role of denitrifiers in the PNA systems.

4 Conclusion and Future Perspectives

4.1 What is the perfect molecular method to analyze microbial communities associated to BNR in CAS as well as PNA systems?

During my work, I found three important topics of the molecular toolbox, that require attention for improving the trust in the outcomes of the molecular methods in wastewater microbiome studies: (1) Standardization, (2) Focus of the study and (3) Usability of microbiome analysis (Figure 21).

(1) Standardization

A much wider appreciation of the utilization of molecular methods to address fundamental questions in microbial ecology now requires proper experimental designing and training, because in the field of WWT not only microbiologists focus on the molecular analysis, but also engineers with advanced knowledge in micro- and molecular biology. Therefore, standardization guidelines for PCR methods (MIQE guidelines) (Bustin et al., 2009) and experimental protocols from experienced research groups with focus on DNA extraction, PCR and NGS (Albertsen et al., 2015; van Loosdrecht et al., 2016) are available to support non-specialists for a better understanding and successful implementation of these methods in their own research labs. However, these protocols cannot give predefined choices on the right primer set, false positive signals or guidance for the correct interpretation of such data (Bustin, 2010). For example, van Loosdrecht et al. (2016) recommended the popular primer pair designed by Rotthauwe et al. (1997) to target the *amoA* gene. However, this study (chapter 3.2), as well as several other studies (Dechesne et al., 2016; Meinhardt et al., 2015), disclosed major weaknesses of this popular primer regarding the coverage of relevant *Nitrosomonas*-species in wastewater treatment systems.

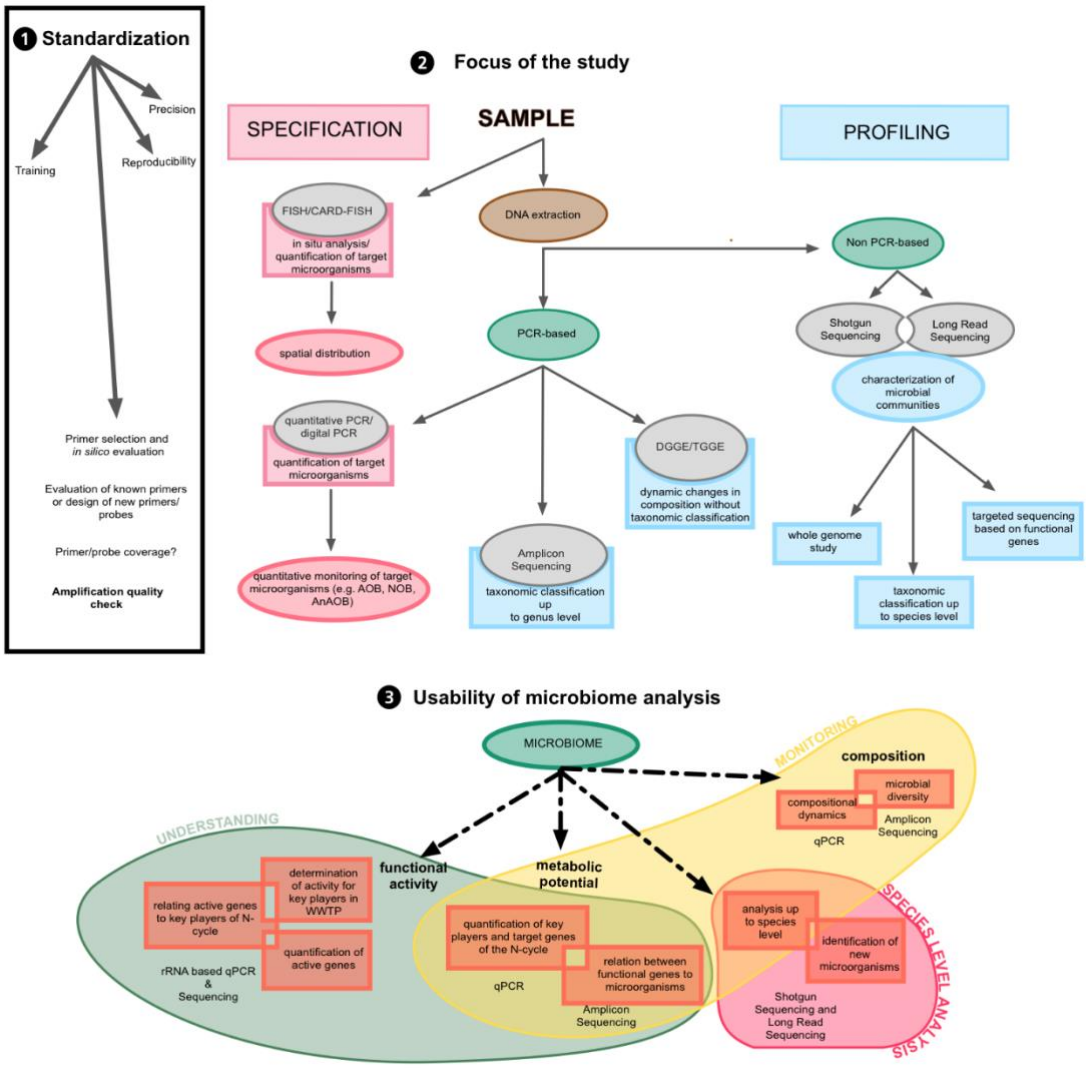

Figure 21: Framework based on (1) standardization, (2) knowing the focus of the study, (3) and understanding the usability of analytical methods, for better implementation of molecular methods to study wastewater microbiomes.

Also DNA extraction still needs special attention, because studies revealed highly variable results with inconspicuously small changes in the standard protocol of the same extraction method (Albertsen et al., 2015; Guo and Zhang, 2013). This implies substantially higher variability between different DNA extraction methods. An internal quality control and an understanding of the methods as well as the associated susceptibility to errors is vital for all molecular methods.

There are also recommendations on optimal *hv* regions of the 16S rRNA gene and associated primers to study WWTP samples with amplicon sequencing. In case of CAS, Albertsen et al. (2015) recommended the use of V3-4 region primer sets, whereas Guo et al. (2013) recommended V1-2 for amplicon sequencing. None of these studies considered all *hv* regions, but chose three or four different *hv* regions for their decision. In this work (chapter 3.1), parallel sequencing of all *hv* regions

was performed, to figure out, whether there is a suitable *hv* region for both qPCR and amplicon sequencing in case of PNA systems. The results revealed drastic variation even on phylum level (Figure 7, chapter 3.1), which consequently also showed, that there is no optimal *hv* region for PNA systems, but there is need for multi-*hv* region analysis.

(2) Focus of the study

The approach of using the molecular methods is based on the research field, as scientific questions e.g. in soil microbiology or medicine differ highly from the ones in wastewater microbiology. Addressing the right research focus is of main importance for further downstream analysis and correct data interpretation. It also includes, whether the focus of the study is (1) to compare the results with other studies, and/or (2) to study the community composition and dynamics of a certain system. If the objective is to compare results with other N-DN/PNA studies, it is highly recommended choosing the same primer pairs as used in the other studies, because selection of different primers can result in differences in the results, as found in chapter 3.1. Therefore, this work has developed a framework for PCR-based methods (chapter 3.1), which provides a specific guideline for PCR-based experiments in the field of PNA systems. It is important to mention, that the focus of this framework was very specific to WWTP, because a more generalized framework for methodological approaches is hardly possible due to the inherent complexity of the workflows. Nevertheless, I believe that this framework supports the standardization in the molecular analysis lab work routine between the microbiologists (specialists) and environmental engineers (non-specialists).

For the study of community composition and dynamics of N-DN or PNA systems, it is important to distinguish between specification and profiling. Specification is focusing mainly on key players in the nitrogen cycle (AOB, NOB, AnAOB and HB) and is mostly studied with qPCR. The choice of the right primer set is highly important, as emphasized in chapter 3.2, which focused on quantifying AOBs in environmental samples. The primer set, that is mostly used with the false assumption to cover nearly all AOBs was designed 20 years ago by Rotthauwe et al. (1997). *In silico* analysis revealed perfect match with *Nitrosospira multiformis* and *Nitrosomonas* sp. LT-1. The outcomes of chapter 3.2, also highlight that it is better to use specific primers for sub-groups of a microbial group rather than using generic primers for a microbial group, if it is not possible to have perfect match generic primers. Profiling the wastewater microbiome implies the characterization of the microbial community, including taxonomic information up to species level for all members of the microbiome and detailed insights into functional genes. Both 16S rRNA amplicon sequencing and shotgun sequencing can provide in-depth information, but conclusive results need a high standard bioinformatic analysis. The versatility of the NGS methods is shown in this work: 16S rRNA amplicon sequencing was used in chapter 3.1 to analyze the microbial community

composition of three different PNA samples. In chapter 3.2 shotgun sequencing revealed information about the AOB, based on the *amoA* gene, that were found in four different WWTPs to verify the qPCR results. The diversity and abundance of denitrifying microorganisms was analyzed in chapter 3.3, where the denitrifying community was compared based on the 16S rRNA marker gene and the functional genes of the denitrification pathway.

The results from this study underline that we have variety of molecular methods for different research questions to understand the wastewater microbiome, however each method has its own challenges which should be considered during its implementation.

(3) Usability of microbiome analysis

Microbiome research is a hot topic nowadays in every kind of research field, because it offers a holistic approach to study the microbial community composition in complex samples. For wastewater microbiomes, defining what is there using high-throughput sequencing is the first step, but will only document inter- and intraspecies linkage and certainly not prove causation. In the wastewater research field, the three main pillars are based on these topics: (1) composition and dynamics of the wastewater microbiome, (2) metabolic potential of the wastewater microbiome and (3) activity in the wastewater microbiome (Figure 21).

There must be a greater emphasis on compositional analysis based on functional genes of the BNR pathway in future studies for a better understanding of the complex microbial interactions in CAS as well as PNA systems. The analysis of the denitrifying community composition in CAS and PNA systems in chapter 3.3 revealed a better picture of the diversity of denitrifying bacteria, based on the functional genes of the denitrification pathway than based on the 16S rRNA gene. The high variation between community composition based on the 16S rRNA gene and the functional genes highlights the far-reaching impact of gene selection on community composition analysis.

Metabolomics, employing techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy, can identify molecules produced by the wastewater microbiota and help to define metabolic pathways. Other '-omics' approaches, such as metatranscriptomics and metaproteomics, have also the potential to further reveal the functions of the various members of the wastewater microbiota (Rodriguez et al., 2015).

4.2 Which method is suitable for which scientific issue/question?

In general, the culture-independent molecular methods revealed new insights of key microbial players in WWTPs, because in most cases the wrong bacteria were considered important for various processes (Lu et al., 2014). Besides the molecular methods revealed insights into the physiology and genomics of already cultured and uncultured bacteria far beyond what was known before (Agrawal et al., 2017; Speth et al., 2016). Molecular methods have gained tremendous utility and popularity for both engineers and microbiologists: on the one hand to study microbial communities to solve specific practical problems or to implement novel processes such as anammox for the engineering purposes, and on the other hand for the microbiologist to understand the highly interesting engineered microbiomes in WWTPs, regardless whether activated sludge or PNA systems (Daims et al., 2006). The modern molecular ecologist's toolbox is very powerful and versatile (Table 2). To study the spatial distribution of microorganisms in anammox granules or flocs or biofilms, FISH is a very popular method. But FISH is very time-consuming, because sample preparation is intensive, and an experienced person is needed for microscopy (Nielsen et al., 2009a). PCR provides information on the presence or absence of target microorganisms in a sample and combining PCR with DGGE opens up the possibility to monitor temporal changes in microbial communities. But without sequencing of the PCR products afterwards, there is no deeper insight into taxonomic classification or quantitative significance.

Studying the population dynamics of key players in the nitrogen cycle (AOB, NOB, AnAOB and HB) in reactor systems or WWTPs is mostly done with qPCR as it is a fast and robust technique. The costs to study environmental samples vary between 5-7 € for qPCR and 12-15€ for dPCR Table 2: Summary of all available molecular methods in microbiome analysis in WWTP (Table 2). DPCR is a chip-based system without the need of a standard curve, which is one of the known drawbacks of the qPCR method. Reproducibility and precision are extremely high due to the parallel amplification of 20.000 wells, which leads to a distinction between five or six gene copies. Nevertheless, all PCR-based methods require a primer set.

The upcoming NGS methods, regardless of the approach (amplicon sequencing or shotgun sequencing), provide in-depth insights into complex microbial communities. Both approaches are very time- and work-intensive and very costly. All NGS methods require experienced lab personnel and several days for sample preparation as well as library preparation.

Amplicon sequencing can be ranked as cheapest and most adapted method for routine analysis in the NGS method block (100 – 200 €), but is based on the conventional PCR method, and therefore also limited to primer choice (Table 2).

Shotgun sequencing is a delicate enzyme-based approach to generate random DNA fragments in different sizes, requiring a well-trained personnel and additionally comes with higher costs (200 – 300 €). Both approaches provide deep insight information about microbial communities down to genus level, and in addition targeted metagenomics reveals information about specific genes or microbial groups of interest. Shotgun sequencing provides the highest flexibility, because it not only offers all previously enumerated options, but also the ability for whole genome sequencing, information down to species level and additional information on the metabolic potential.

The choice of molecular methods should depend upon the researcher's hypothesis or question. Every molecular method has its advantages and disadvantages, as well as its strengths and weaknesses (Table 2). To understand complex microbial communities, we should not focus too much on the easiest or latest technologies, but combine all available methods in the best manner, similar to building a house with all available tools in our toolbox and not only using the hammer.

Table 2: Summary of all available molecular methods in microbiome analysis in WWTP

Molecular Methods	Benefits	Limitations	Cost per sample (€)	Estimated time (hrs)	Necessary preliminary work	Example of usage
Fluorescence in situ hybridization (FISH)	Advantageous in analyzing biofilms, granules or flocs in WWTPs.	Probe bias; Time and labour intensive; Depended on a microscope; Difficult to distinguish various target microorganisms due to lack of distinct fluorescence signal.	10	48 – 55	Design of probes.	Spatial distribution of AnAOB in biofilms. Presence and absence of microorganisms NOB in biofilm granules.
DGGE	Overview over microbial community composition in complex environments.	Primer bias; Not quantitative; Limited resolution.	5	30 – 40	*In silico* PCR analysis; Design of primers.	To follow microbial community temporal dynamics as a response to changes in temperature of a PNA reactor.
Polymerase chain reaction (PCR)	Presence or absence of target organism in a sample.	Primer bias; Not quantitative.	3 – 5	8 – 10	*In silico* analysis; Design of primers.	Presence or absence of target microorganisms such as AOB, NOB, AnAOB in the PNA reactor.
Quantitative PCR	Quantitative analysis of target genes associated with different microorganisms.	Primer bias; Variability in amplification efficiency; Dependence upon standard curve.	5 – 7	5 – 8	*In silico* analysis; Design of primers.	Quantitative measurement of target microorganisms such as AOB, NOB, AnAOB in the PNA reactor.

Molecular Methods	Benefits	Limitations	Cost per sample (€)	Estimated time (hrs)	Necessary preliminary work	Example of usage
Digital PCR	Quantitative analysis; High specificity based on TaqMan Probes; No need to rely on standard curves.	Primer bias; Time intensive.	12 – 15	5 – 7	Design of primers and TaqMan Probes.	Quantitative measurement of target microorganisms such as AOB, NOB, AnAOB in the PNA reactor.
Multiplex PCR	Rapid and simultaneous detection of several targets.	Primer bias.	Depends on method	5 – 8	Design of primers.	Detection of presence or absence of several target microorganisms in one sample.
Amplicon Sequencing	Insights into complex microbial communities.	Primer bias; Intensive hands-on; Expensive; Bioinformatic analysis required; Dependence upon reference databases.	100-200	3-5 days without bioinformatic analysis	Primer design; *in silico* PCR analysis.	Microbial Community composition, based on 16S rRNA gene, in a PNA reactor.
Shotgun Sequencing	Not PCR based; Insights into complex microbial communities and their metabolic potential; Whole genome.	Intensive hands-on; Expensive Shorter reads; Extensive bioinformatic analysis required.	300-400	3-5 days without bioinformatic analysis	Defining the bioinformatic workflow.	Microbial Community Analysis based on functional genes involved in nitrogen cycle. Whole genome sequencing of non-isolated AnAOB.

Molecular Methods	Benefits	Limitations	Cost per sample (€)	Estimated time (hrs)	Necessary preliminary work	Example of usage
Targeted Sequencing	Contributing to a better understanding of microbial interactions and functional genes at higher resolution.	Primer bias; Bioinformatic analysis required.	300-400	3-5 days without bioinformatic analysis	Development and validation of long list of primers; Developing a bioinformatic analysis pipeline.	Determining the denitrifying microbial community composition based on functional genes in a PNA reactor.
SMRT-Sequencing and Nanopore	Insights into complex microbial communities; Better coverage for whole-genome sequencing.	Base calling; Expensive; High error rate; Bioinformatic analysis workflows under development.	400-500	3-5 days without bioinformatic analysis	Developing a bioinformatic analysis pipeline; validation of sequencing workflows.	'on site' sequencing at a WWTP. Long read sequencing to complete genomes generated using Illumina or ION Torrent platform.

4.3 Future challenges in the field of molecular methods for understanding microbiomes in wastewater treatment

Future challenges in wastewater treatment will focus on the standardization of molecular methods like qPCR/dPCR and NGS, with the main topics of training, precision, reproducibility and primer evaluation (Figure 21). Although high-throughput sequencing methods have revolutionized our understanding of the wastewater microbiome, qPCR still remains the most sensitive technique for quantification of target DNA. But the qPCR method has its bias as found in chapter 3.1 and previously reported by Smith and Osborn (2009), therefore there is a need to develop a reliable quantification method for determining absolute copy numbers (Figure 21).

Metagenomic sequencing offers increased information content and reduced biases related to amplification and gene copy numbers as benefits compared to amplicon sequencing. However it is currently not determined what sequencing depth is required for the respective purpose e.g. whole genome sequencing (Clooney et al., 2016). In general, NGS methods still need more standardization for data processing and analysis, which involves converting raw data and classification based on phylogenetic information from databases. Freely available pipeline programs such as QIIME or mothur simplify the handling of sequence data considerably, but still require a thorough understanding of the individual steps of the pipeline and their relationships in order to achieve meaningful results in the form of a taxonomic classification, composition, genome constructions and the graphical representation of the results (Sanz and Köchling, 2019). In addition to understanding the pipelines, there is also a great lack of harmonization in implementation of the pipelines (Clooney et al., 2016).

Moreover, metagenomics increases the available information for taxonomical characterization and functional potential, it provides little information on metabolic and enzymatic activity of the wastewater microbiome. Metatranscriptomic analysis based on RNA sequencing provides information on the active functions of PNA microbial communities, however until now only few studies have been performed (Bagchi et al., 2016; Lawson et al., 2017; Yang et al., 2020). It is important to note that genomic-based approaches do not reveal the extent of protein expression. Proteins can catalyze the synthesis of certain metabolites that regulate the physiological process of an organism or mediate its biological function directly. To understand microbe-microbe und microbe-molecule interactions, metaproteomics and metabolomics are inevitable, but are still underrepresented in wastewater treatment studies due to their analytical complexity. For future studies, it is important to understand that metagenomics, metatranscriptomics, metaproteomics, and metabolomics are closely linked, and

metaproteomics might play a critical part (Narayanasamy et al., 2015; Rodriguez et al., 2015; Wilmes and Bond, 2006).

In terms of the sequencing technology, the NGS platforms for long read sequencing technologies (SMRT, e.g. Pacific Biosciences and Oxford Nanopore Technologies) generate sequences with reading lengths of more than 1000 bp and exceed the known sequencing platforms Illumina or IonTorrent. But to date, these technologies have not been used extensively for studies of diverse bacterial communities due to the high rate of randomly distributed sequencing errors that would lead to artificially inflated diversity in the community (Schloss et al., 2015). PacBio sequencing is often used in parallel with other NGS platforms (e.g. Illumina, Roche 454 and SoLiD) to enable scaffolding and phishing to produce ready / tight genomes with high sequence quality (Koren et al., 2013). Furthermore, online real-time detection using NGS will bridge the gaps in real-time monitoring of genetic parameters for water quality as various NGS technologies advance towards improved sequence chemistry for longer read length with higher throughput and reduced error rate (Venkatesan and Bashir, 2011). For sure, these sequencing approaches offer high specificity compared to other platforms and especially with focus on Nanopore will replace the bigger systems in the distant future, but the technology is still at the very beginning and needs more empirical values and improvement, due to high error rates.

The future will decide, whether new technologies or new instruments will point the way of research in BNR or if the standardization of the already popular molecular methods improve our molecular toolbox. Despite the benefit of advanced sequencing the basis for understanding the complex microbiomes is also depending on culturing individual species forms in wastewater. Only the combination of traditional cultivation and new *-omics* approaches together with systematic measurements and experimental validation will pave the path for a holistic characterization of microbial communities (Ferrera and Sanchez, 2016).

5 Literature

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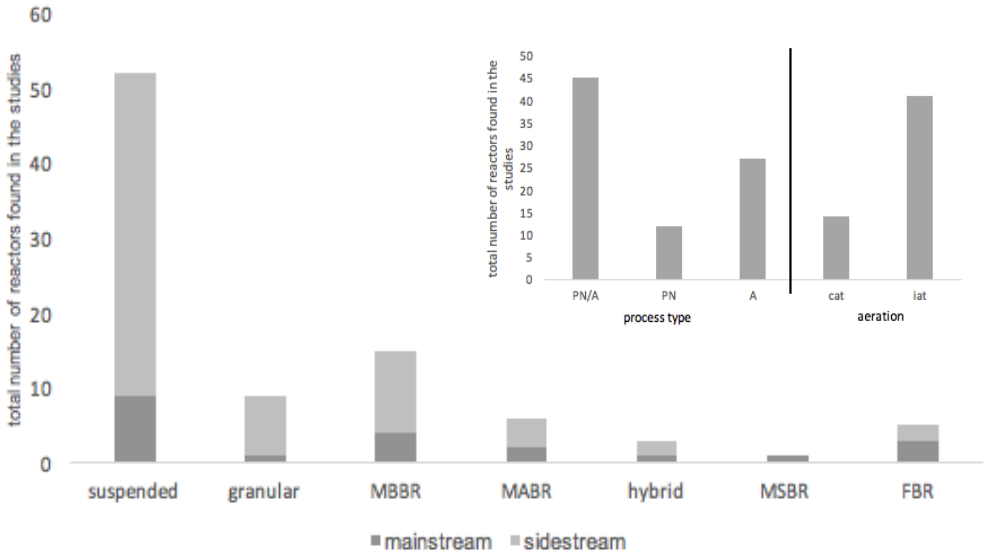
Annexure I

Types of reactor systems

In the surveyed literature, reactors with suspended biomass were the most studied group, the second most group were MBBRs, followed by MABRs and granular sludge reactors (A.Figure 1). More than 80% of the suspended biofilm reactors were set up for side-stream treatment, which is comparable with the proportional distribution of the granular biofilm reactors, MBBR and MABR.

PNA reactors were the most common reactors used in all studies and studied three-times more than PN reactors. More than half of the studied reactors were operated with intermittent aeration, and only one third was operated with continuous aeration.



A.Figure 1: Data evaluation for the reactor types divided into seven groups: suspended biomass, granular sludge, moving bed biofilm reactor (MBBR), membrane aerated biofilm reactor (MABR), hybrid, membrane sequencing batch reactor (MSBR) and fluidized bed reactor (FBR); further subcategorization into process type: partial nitritation/anammox (PNA), partial nitritation (PN) and anammox (A) and continuously aerated (cat) and intermittently aerated (iat).

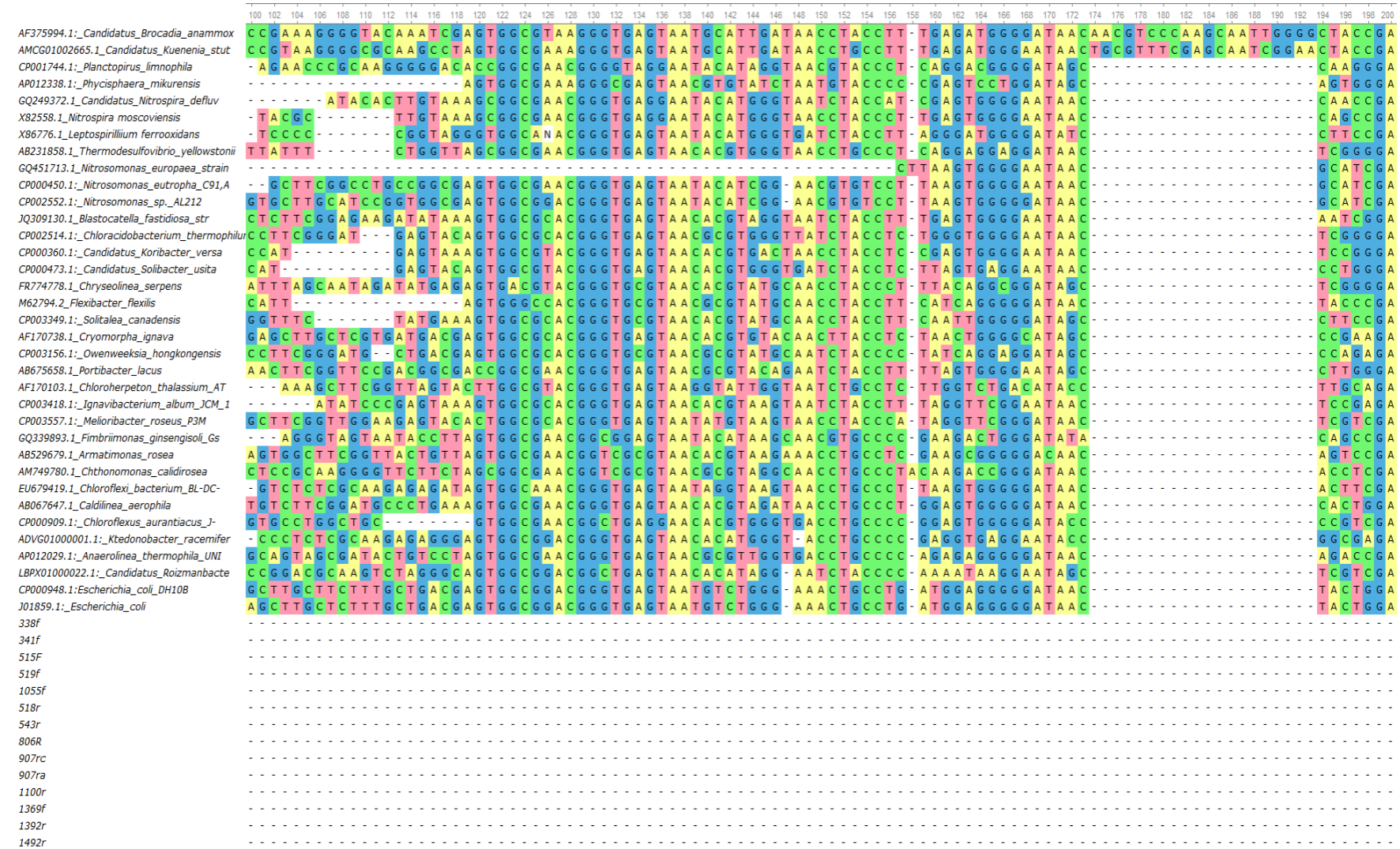
Multiple sequence alignment

We performed alignment of the multiple 16S rRNA gene sequences associated to the known representatives of the PNA community, as reported in a previous study (Speth et al., 2016), with the eubacterial 16S rRNA gene primer pairs found in the literature assessment (Chapter 3.1, Table 1). Additionally, we aligned two Escherichia coli sequences for the verification of the primer pairs. It is important to note that the 16S rRNA gene sequences used for the alignment represented a

small fraction of the total PNA community. Especially in case of heterotrophic members of the micobrial communtiy, which have wide diversity.

We evaluated eight primer pairs belonging to various hypervariable regions of the 16S rRNA gene, two for the V3/4 region, one for the V3/V5 region, two for the V4/5 region, one for the V6/7 region, one for the V7/8 region and one for the V8/9 region. Only, primer pair 1055f-1392r covered all members relevant for the PNA process, i.e. Planctomycetes and γ-Proteobacteria (A.Figure 2), however it did not cover the whole microbial community in PNA systems e.g. Bacteriodetes. Primer pair 519f-907r do not cover any of the representative sequences. On the other hand, none of the primer pairs covered the sequence of *Cryomorpha ignava*, *Fimbriimonas ginsengisoli Gsoil 348* and *Candidatus Roizmanbacteria bacterium GW2011_GWC2_35_12 UR63_C0022*.

AF375994.1:_Candidatus_Brocadia_anammox
AMCG01002665.1_Candidatus_Kuenenia_stut
CP001744.1:_Planctopirus_limnophila
AP012338.1:_Phycisphaera_mikurensis
GQ249372.1_Candidatus_Nitrospira_defluv
X82558.1_Nitrospira_moscoviensis
X86776.1_Leptospirillium_ferrooxidans
AB231858.1_Thermodesulfovibrio_yellowstonii
GQ451713.1_Nitrosomonas_europaea_strain
CP000450.1:_Nitrosomonas_eutropha_C91,A
CP002552.1:_Nitrosomonas_sp._AL212
JQ309130.1_Blastocatella_fastidiosa_str
CP002514.1:_Chloracidobacterium_thermophilu
CP000360.1:_Candidatus_Koribacter_versa
CP000473.1:_Candidatus_Solibacter_usita
FR774778.1_Chryseolinea_serpens
M62794.2_Flexibacter_flexilis
CP003349.1:_Solitalea_canadensis
AF170738.1_Cryomorpha_ignava
CP003156.1:_Owenweeksia_hongkongensis
AB675658.1_Portibacter_lacus
AF170103.1_Chloroherpeton_thalassium_AT
CP003418.1:_Ignavibacterium_album_JCM_1
CP003557.1:_Melioribacter_roseus_P3M
GQ339893.1_Fimbriimonas_ginsengisoli_Gs
AB529679.1_Armatimonas_rosea
AM749780.1_Chthonomonas_calidirosea
EU679419.1_Chloroflexi_bacterium_BL-DC-
AB067647.1_Caldilinea_aerophila
CP000909.1:_Chloroflexus_aurantiacus_J-
ADVG01000001.1:_Ktedonobacter_racemifer
AP012029.1:_Anaerolinea_thermophila_UNI
LBPX01000022.1:_Candidatus_Roizmanbacte
CP000948.1:Escherichia_coli_DH10B
J01859.1:_Escherichia_coli
338f
341f
515F
519f
1055f
518r
543r
806R
907rc
907ra
1100r
1369f
1392r
1492r


AF375994.1:_Candidatus_Brocadia_anammox
AMCG01002665.1_Candidatus_Kuenenia_stut
CP001744.1:_Planctopirus_limnophila
AP012338.1:_Phycisphaera_mikurensis
GQ249372.1_Candidatus_Nitrospira_defluv
X82558.1_Nitrospira moscoviensis
X86776.1_Leptospirillium ferrooxidans
AB231858.1_Thermodesulfovibrio_yellowstonii
GQ451713.1_Nitrosomonas_europaea_strain
CP000450.1:_Nitrosomonas_eutropha_C91,A
CP002552.1:_Nitrosomonas_sp._AL212
JQ309130.1_Blastocatella_fastidiosa_str
CP002514.1:_Chloracidobacterium_thermophilu
CP000360.1:_Candidatus_Koribacter_versa
CP000473.1:_Candidatus_Solibacter_usita
FR774778.1_Chryseolinea_serpens
M62794.2_Flexibacter_flexilis
CP003349.1:_Solitalea_canadensis
AF170738.1_Cryomorpha_ignava
CP003156.1:_Owenweeksia_hongkongensis
AB675658.1_Portibacter_lacus
AF170103.1_Chloroherpeton_thalassium_AT
CP003418.1:_Ignavibacterium_album_JCM_1
CP003557.1:_Melioribacter_roseus_P3M
GQ339893.1_Fimbriimonas_ginsengisoli_Gs
AB529679.1_Armatimonas_rosea
AM749780.1_Chthonomonas_calidirosea
EU679419.1_Chloroflexi_bacterium_BL-DC-
AB067647.1_Caldilinea_aerophila
CP000909.1:_Chloroflexus_aurantiacus_J-
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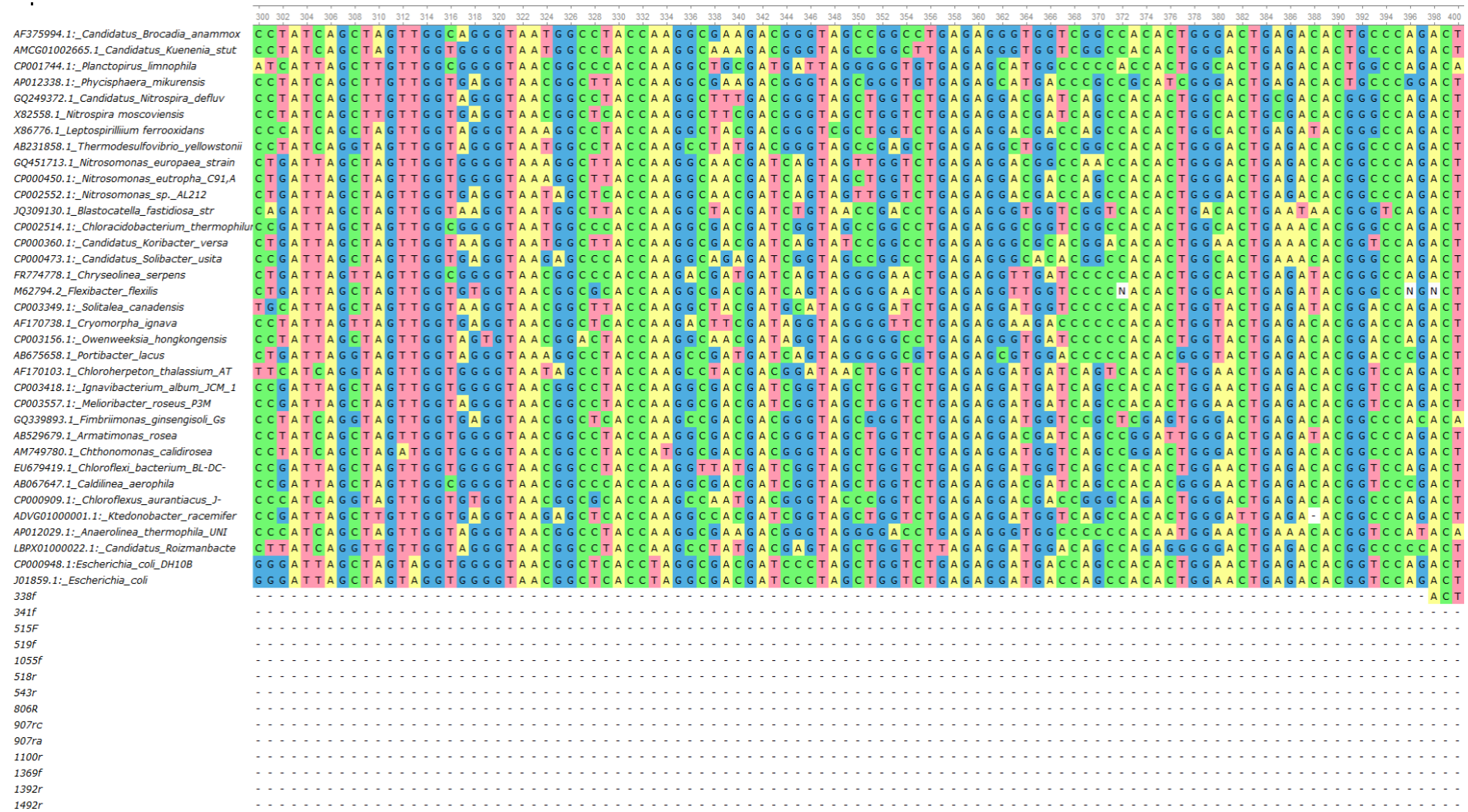
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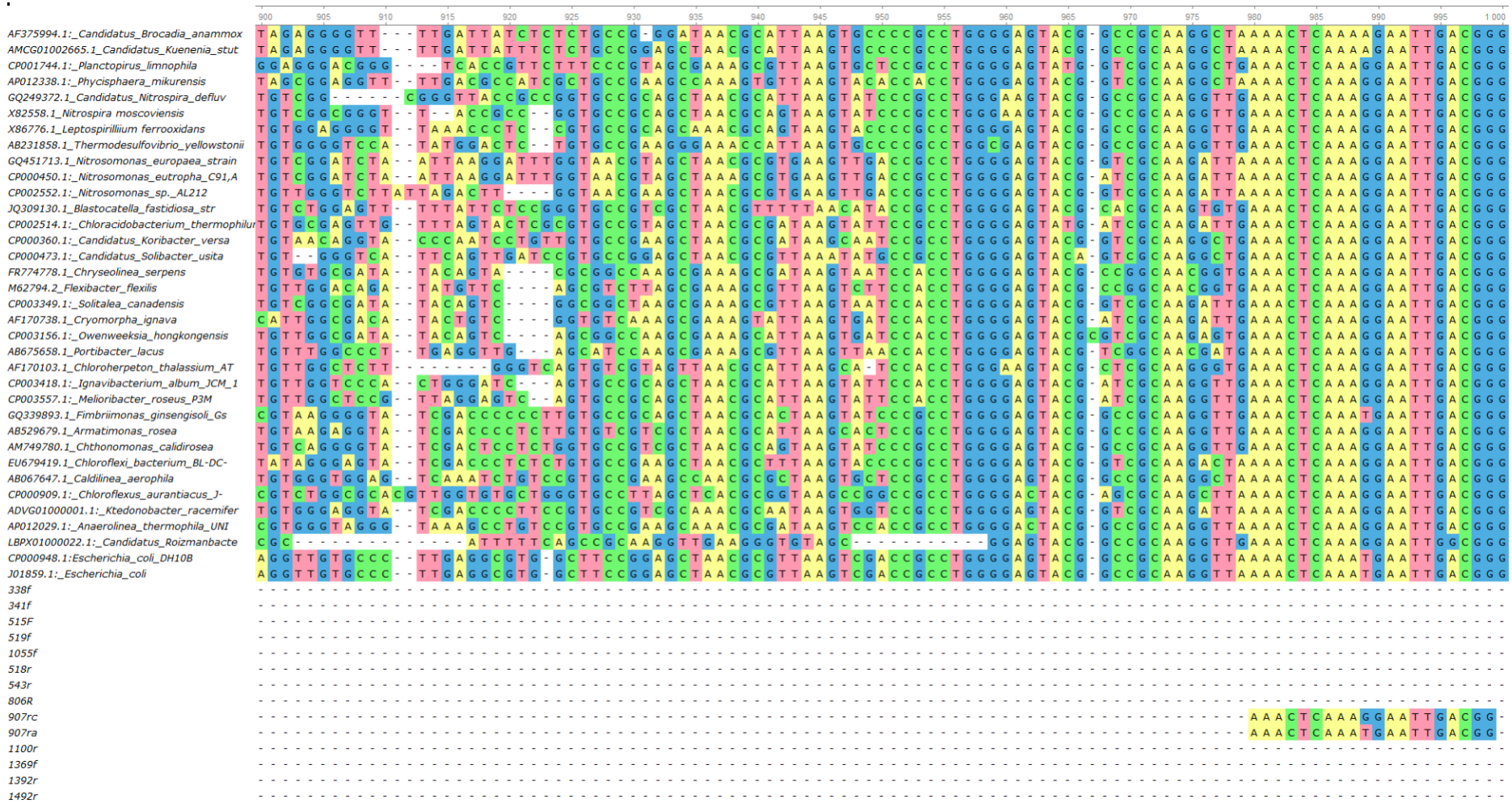
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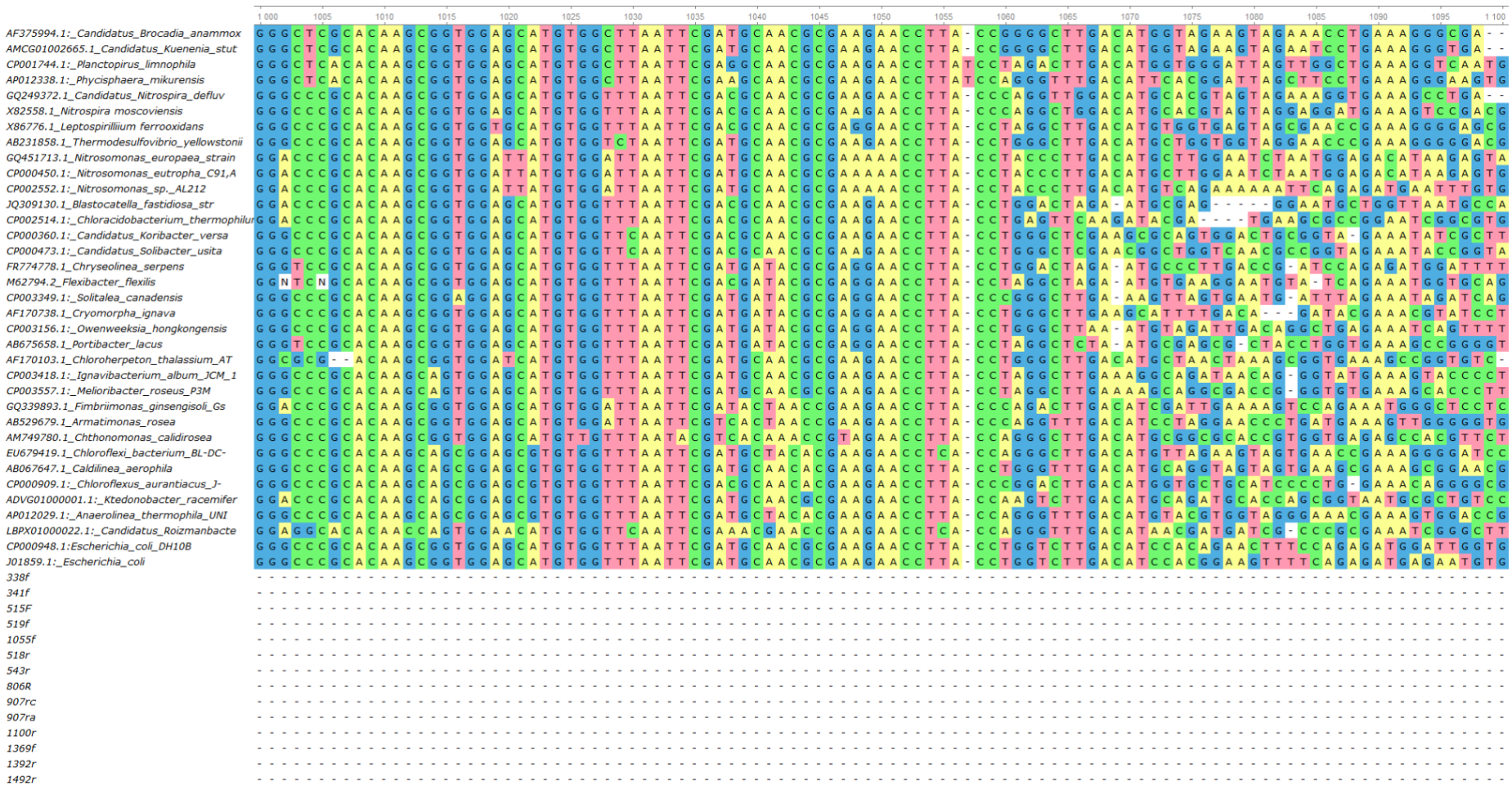
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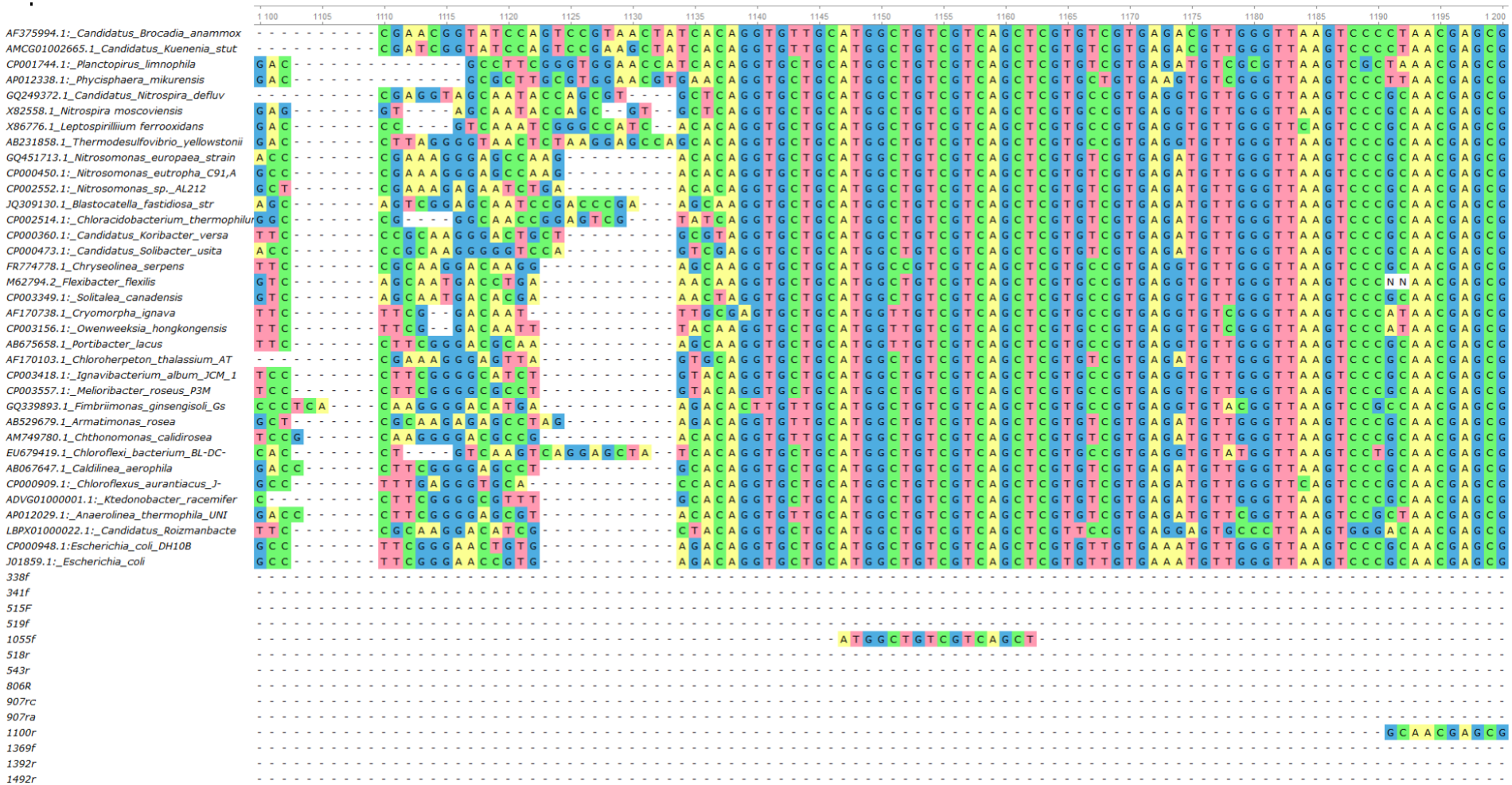
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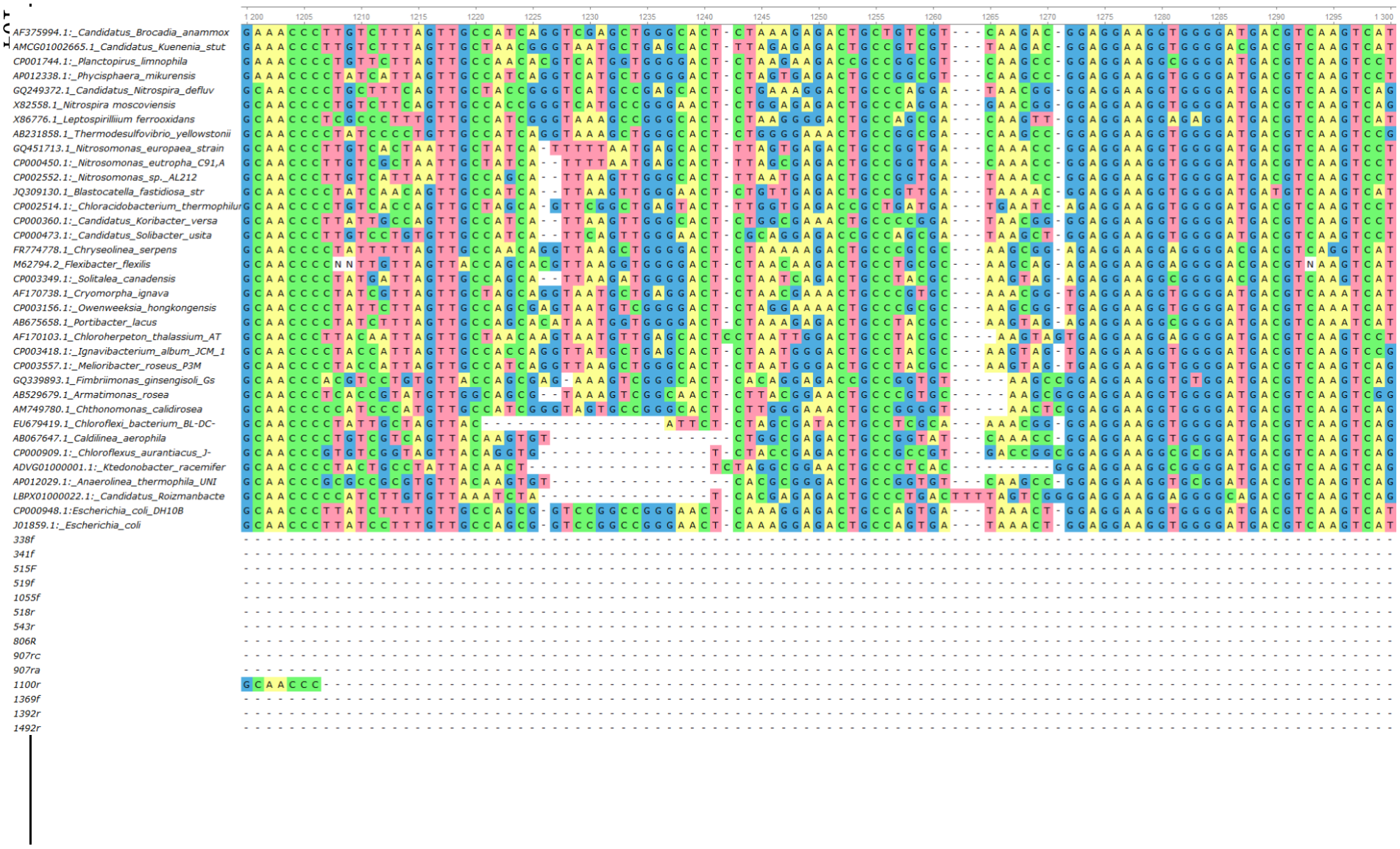
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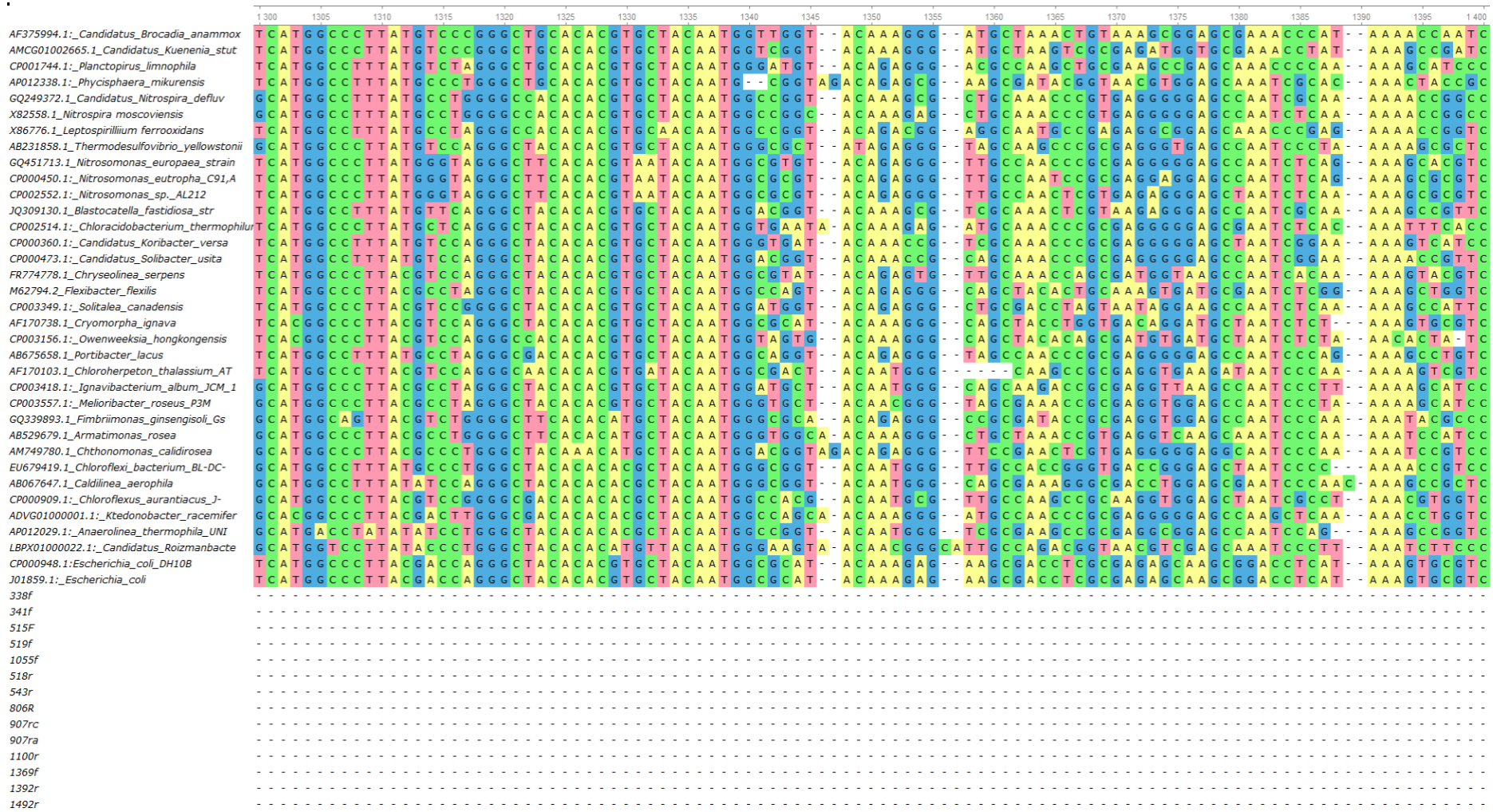
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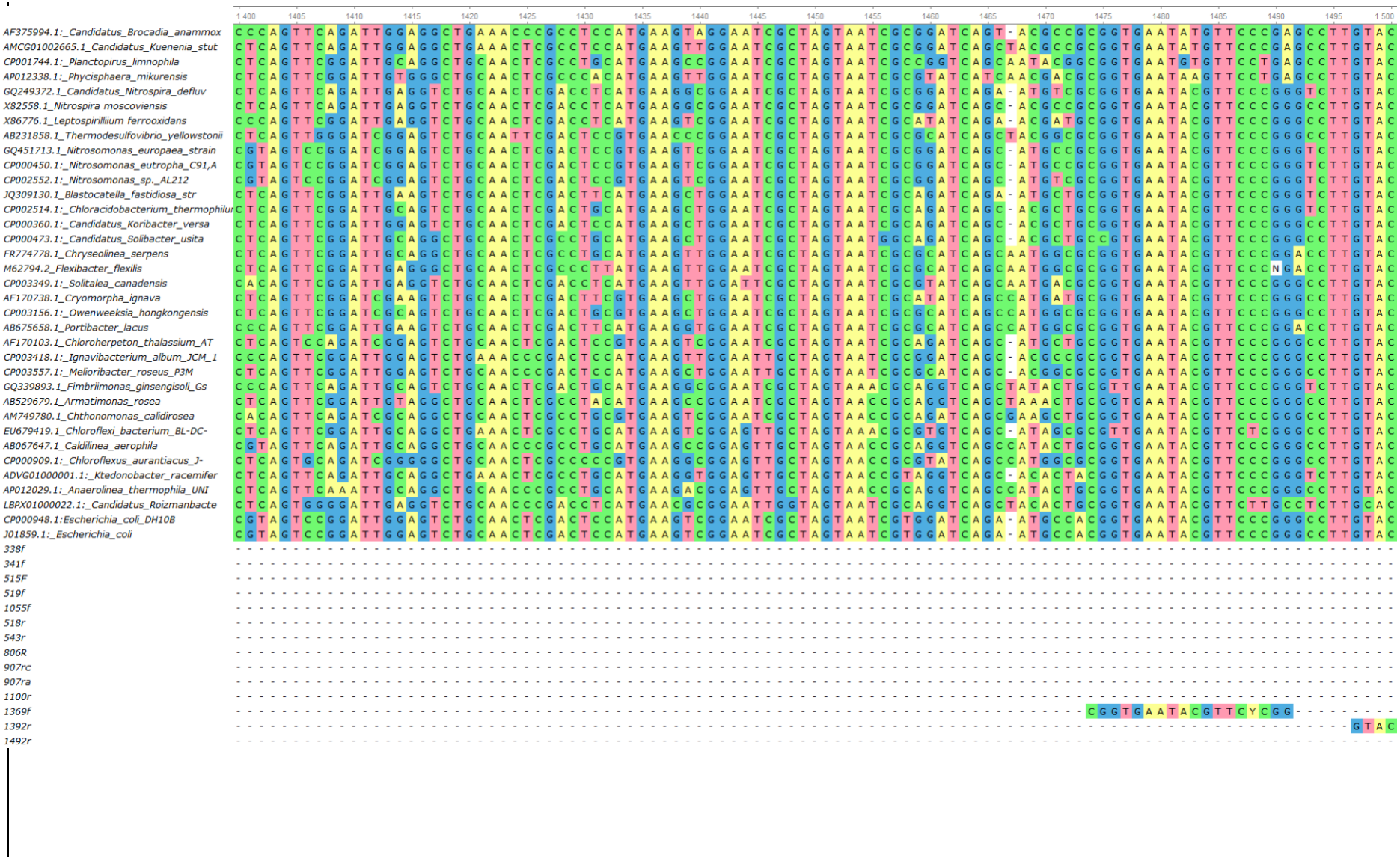
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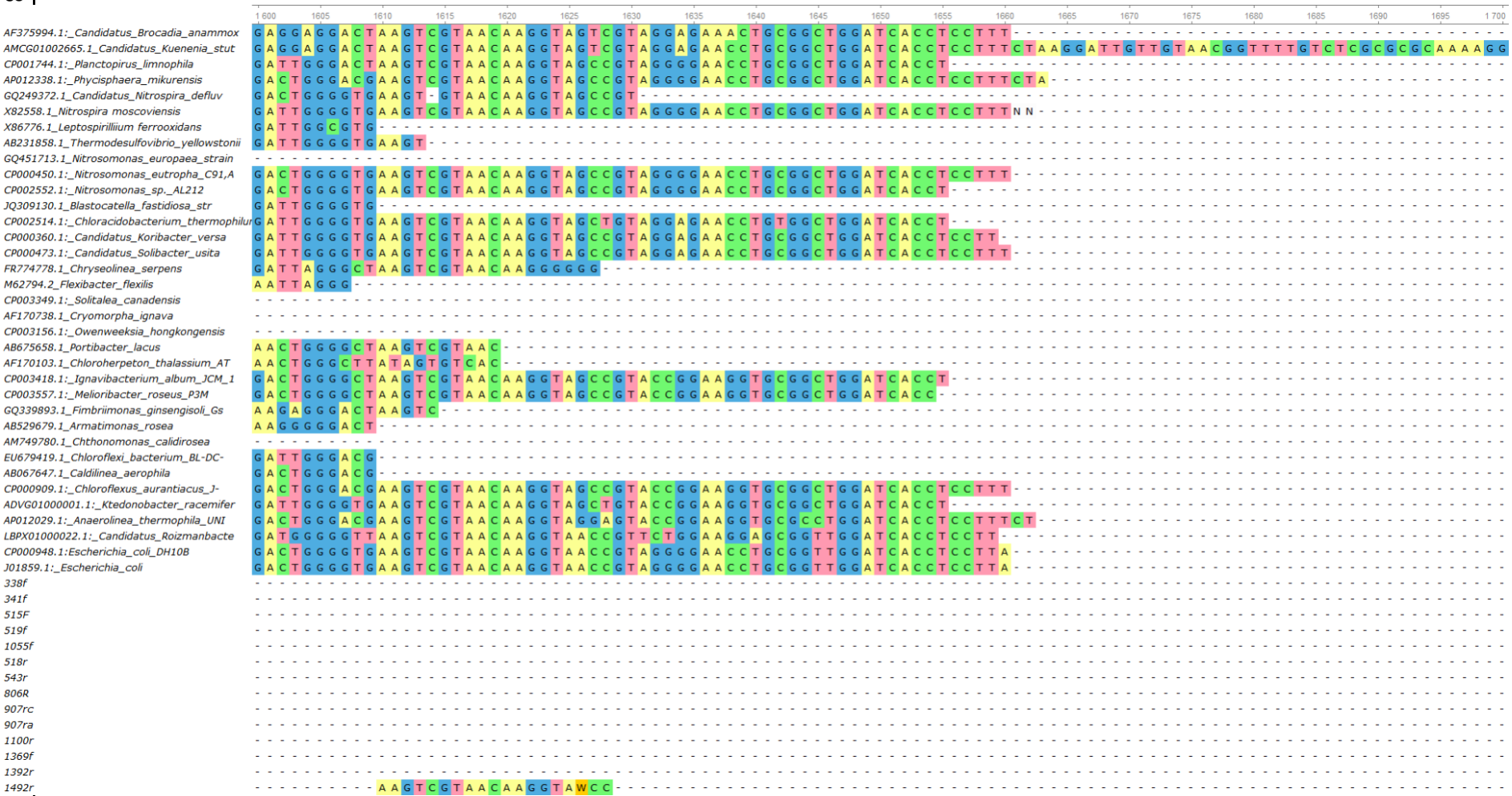
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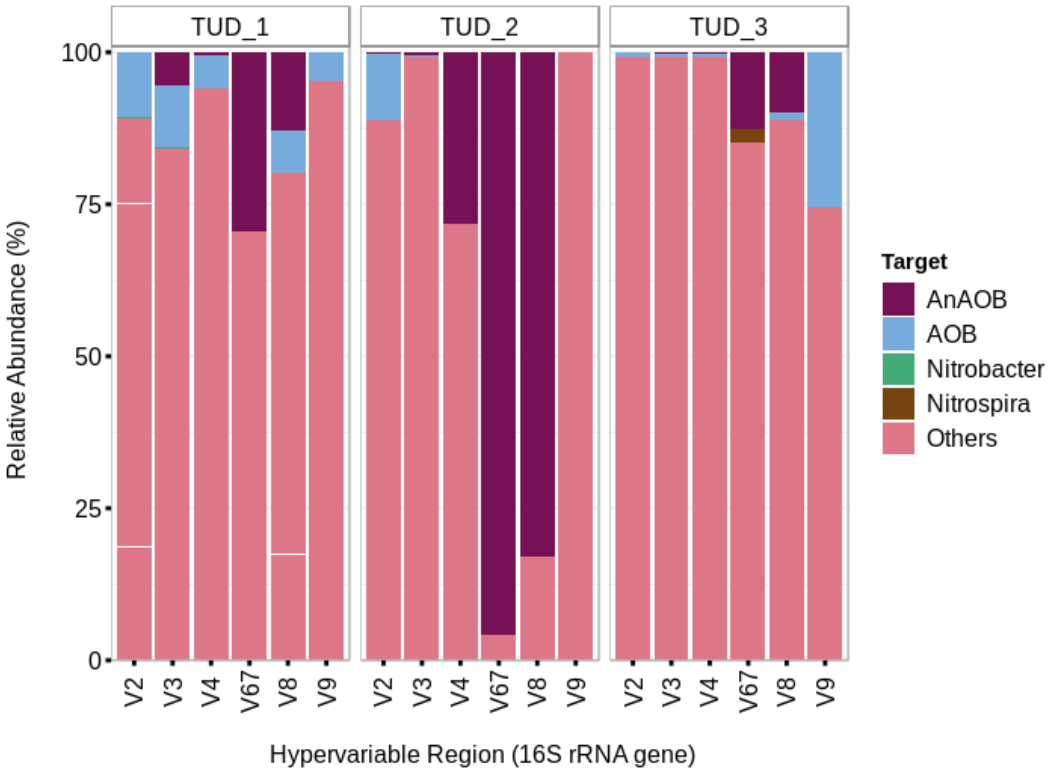
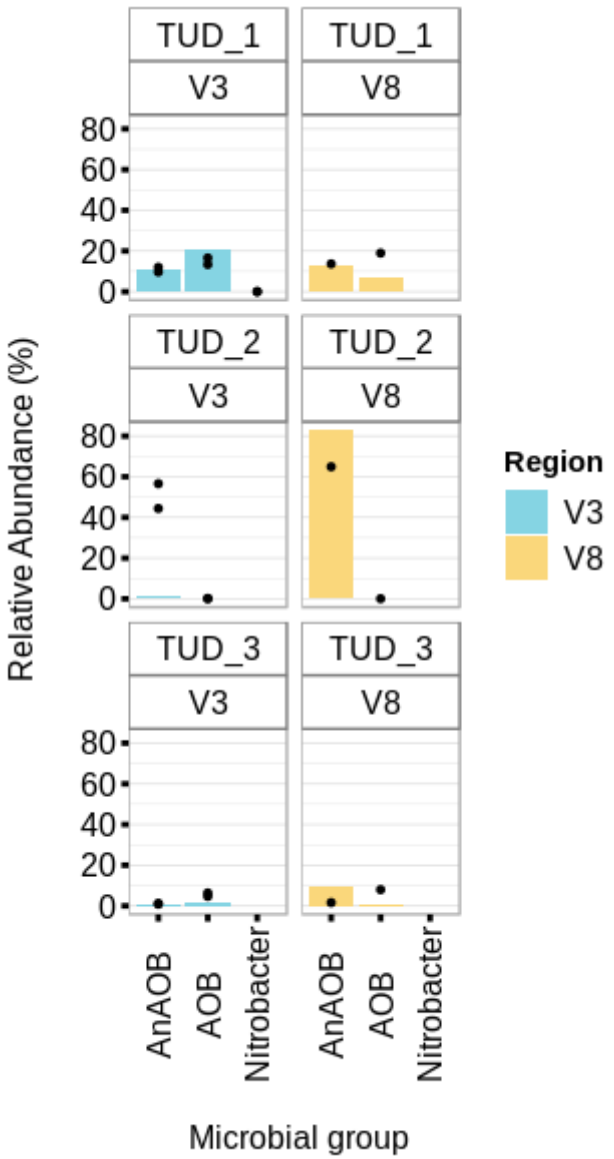
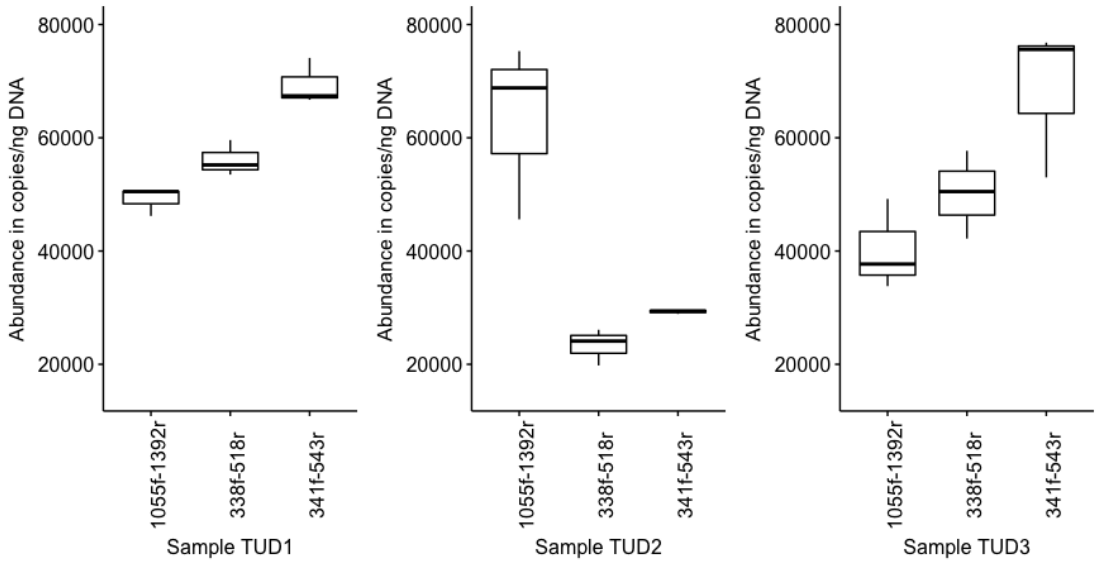
A.Figure 2: Sequence alignment of the 16S rRNA gene from representatives of the PNA community according to Speth et al. (2017)1 and two E.coli sequences for verification of the primer position with all eubacterial primer sets found in literature assessment (Table 1). List of microbial members that were considered for the sequence alignment: (1) Planctomycetes – *Candidatus Brocadia anammoxidans* (AF375994.1), *Phycisphaera mikurensis* (AP012338.1), *Planctopirus limnophila* (CP001744.1), *Candidatus Kuenenia stuttgartiensis* (AMCG01002665.1); (2) γ-Proteobacteria – *Nitrosomonas europaea strain ATCC 25978* (GQ451713.1), *Nitrosomonas eutropha ATCC 19718* (CP000450.1); *Nitrosomonas sp. AL212* (CP002552.1); (3) Nitrospira – *Candidatus Nitrospira defluvii* (GQ249372.1), *Nitrospira moscoviensis* (X82558.1), *Thermodesulfovibrio yellowstonii* (AB231858.1), *Leptospirillum ferroxidans* (X86776.1); (4) Acidobacteria – *Blastocatella fastidiosa strain A2-16* (JQ309130.1), *Chloracidobacterium thermophilum* (CP002514.1), *Candidatus Koribacter versatilis Ellin345* (CP000360.1), *Candidatus Solibacter usitatus Ellin6076* (CP000473.1); (5) Bacteriodetes – *Chryseolinea serpens* (FR774778.1), *Flexibacter flexilis* (M62794.2), *Solitalea canadensis* (CP003349.1), *Cryomorpha ignava* (AF170738.1), *Owenweeksia hongkongensis* (CP003156.1), *Portibacter lacus* (AB675658.1); (6) Chlorobi – *Chloroherpeton thalassium ATCC 35110* (AF170103.1), *Ignavibacterium album JCM 16511* (CP003418.1), *Melioribacter roseus P3M* (CP003557.1); (7) Armatimonadetes– *Fimbriimonas ginsengisoli Gsoil 348* (GQ339893.1), *Armatimonas rosea* (AB529679.1), *Chthonomonas calidirosea* (AM749780.1); (8) Chloroflexi– *Chloroflexi bacterium BL-DC-9* (EU679419.1), *Caldilinea aerophila* (AB067647.1), *Chloroflexus aurantiacus J-10-fl* (CP000909.1), *Ktedonobacter racemifer strain SOSP1-21* (ADVG01000001.1), *Anaerolinea thermophila UNI-1* (AP012029.1); (9) Parcubacterium – *Candidatus Roizmanbacteria bacterium GW2011_GWC2_35_12 UR63_C0022* (LBPX01000022.1); (10) Escherichia coli – *Escherichia coli* (J01859.1), *Escherichia coli str. K12 substr. DH10B* (CP000948.1)



A.Figure 3: Relative abundance of different microbial groups based on 16S rRNA gene amplicon sequencing, focusing on the microbial groups associated to PNA systems, i.e. anaerobic ammonium oxidizing bacteria (AnAOB), ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (Nitrobacter and Nitrospira) and others (rest of the microbial groups detected in the samples).



A.Figure 4: comparison of relative abundance based on qPCR analysis and 16S rRNA amplicon sequencing. • represent the qPCR based relative abundance and the barplots represent the 16S rRNA based relative abundance. The represented microbial groups are associated to the PNA process, i.e. anaerobic ammonium oxidizing bacteria (AnAOB), ammonium oxidizing bacteria (AOB), nitrite oxidizing bacteria (Nitrobacter and Nitrospira).



A.Figure 5: qPCR based absolute abundance of samples TUD1, TUD2 and TUD 3 for eubacterial primer sets 1055f-1392r, 338f-518r and 341f-543r in copies/ng DNA; comparison of total eubacterial abundance measured using three different primer pairs (primer sets: (1) 1055f-1392r, (2) 338f-518r and (3) 341f-543r) for each sample.

A.Table 1: Two-way ANOVA analysis to determine the effect of primer pair on the relative abundance based on16S rRNA gene amplicon sequencing analysis

two-way ANOVA for 16S rRNA sequencing	
F-value	p-value
109	p<0.001

A.Table 2: One-way ANOVA analysis to determine the effect of primer pairs on the measured abundance of EUB using qPCR analysis

Sample	**F-value**	**p-value**
TUD 1	29.08	p<0.001

TUD 2	16.39	$p<0.01$
TUD 3	6.074	$p<0.05$

S.No.	List of Studies
1	Hu, B. L., Zheng, P., Tang, C. J., Chen, J. W., van der Biezen, E., Zhang, L., ... & Kartal, B. (2010). Identification and quantification of anammox bacteria in eight nitrogen removal reactors. *Water research*, *44*(17), 5014-5020.
2	Suto, R., Ishimoto, C., Chikyu, M., Aihara, Y., Matsumoto, T., Uenishi, H., ... & Waki, M. (2017). Anammox biofilm in activated sludge swine wastewater treatment plants. *Chemosphere*, *167*, 300-307.
3	Ni, B. J., Hu, B. L., Fang, F., Xie, W. M., Kartal, B., Liu, X. W., ... & Yu, H. Q. (2010). Microbial and physicochemical characteristics of compact anaerobic ammonium-oxidizing granules in an upflow anaerobic sludge blanket reactor. *Applied and environmental microbiology*, *76*(8), 2652-2656.
4	An, P., Xu, X., Yang, F., Liu, L., & Liu, S. (2013). A pilot-scale study on nitrogen removal from dry-spun acrylic fiber wastewater using anammox process. *Chemical engineering journal*, *222*, 32-40.
5	Han, P., Huang, Y. T., Lin, J. G., & Gu, J. D. (2013). A comparison of two 16S rRNA gene-based PCR primer sets in unraveling anammox bacteria from different environmental samples. *Applied microbiology and biotechnology*, *97*(24), 10521-10529.
6	Bae, H., Park, K. S., Chung, Y. C., & Jung, J. Y. (2010). Distribution of anammox bacteria in domestic WWTPs and their enrichments evaluated by real-time quantitative PCR. *Process Biochemistry*, *45*(3), 323-334.
7	Park, H., Rosenthal, A., Jezek, R., Ramalingam, K., Fillos, J., & Chandran, K. (2010). Impact of inocula and growth mode on the molecular microbial ecology of anaerobic ammonia oxidation (anammox) bioreactor communities. *water research*, *44*(17), 5005-5013
8	Ke, Y., Azari, M., Han, P., Görtz, I., Gu, J. D., & Denecke, M. (2015). Microbial community of nitrogen-converting bacteria in anammox granular sludge. *International Biodeterioration & Biodegradation*, *103*, 105-115.
9	Van der Star, W. R., Abma, W. R., Blommers, D., Mulder, J. W., Tokutomi, T., Strous, M., ... & van Loosdrecht, M. C. (2007). Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam. *Water research*, *41*(18), 4149-4163.
10	Yang, Y. A. N. G., Zuo, J. E., Quan, Z. X., Lee, S., Shen, P., & Gu, X. (2006). Study on performance of granular ANAMMOX process and characterization of the microbial community in sludge. *Water science and technology*, *54*(8), 197-207.
11	Tsushima, I., Kindaichi, T., & Okabe, S. (2007). Quantification of anaerobic ammonium-oxidizing bacteria in enrichment cultures by real-time PCR. *Water Research*, *41*(4), 785-794.
12	Pathak, B. K., Kazama, F., Tanaka, Y., Mori, K., & Sumino, T. (2007). Quantification of anammox populations enriched in an immobilized microbial consortium with low levels of ammonium nitrogen and at low temperature. *Applied microbiology and biotechnology*, *76*(5), 1173-1179.
13	Tsushima, I., Ogasawara, Y., Shimokawa, M., Kindaichi, T., & Okabe, S. (2007). Development of a super high-rate Anammox reactor and in situ analysis of biofilm structure and function. *Water science and technology*, *55*(8-9), 9-17.
14	Li, X. R., Du, B., Fu, H. X., Wang, R. F., Shi, J. H., Wang, Y., ... & Quan, Z. X. (2009). The bacterial diversity in an anaerobic ammonium-oxidizing (anammox) reactor community. *Systematic and applied microbiology*, *32*(4), 278-289.
15	Ni, S. Q., Lee, P. H., Fessehaie, A., Gao, B. Y., & Sung, S. (2010). Enrichment and biofilm formation of Anammox bacteria in a non-woven membrane reactor. *Bioresource technology*, *101*(6), 1792-1799.
16	Yapsakli, K. (2010). Co-occurrence of nitrogen-converting organisms in full-scale treatment plants. *Journal of Environmental Science and Health Part A*, *45*(9), 1060-1070.

A.Table 3: Overview of the evaluated studies conducted for the review the following keywords: "anammox and pcr" or "partial nitri* and pcr or nitritation" and "pcr or anaerobic ammoni* and pcr"

17	Terada, A., Lackner, S., Kristensen, K., & Smets, B. F. (2010). Inoculum effects on community composition and nitritation performance of autotrophic nitrifying biofilm reactors with counter-diffusion geometry. *Environmental microbiology*, *12*(10), 2858-2872.
18	Ni, S. Q., Fessehaie, A., Lee, P. H., Gao, B. Y., Xu, X., & Sung, S. (2010). Interaction of anammox bacteria and inactive methanogenic granules under high nitrogen selective pressure. *Bioresource technology*, *101*(18), 6910-6915.
19	Zhang, T., Yan, Q. M., & Ye, L. (2010). Autotrophic biological nitrogen removal from saline wastewater under low DO. *Journal of chemical technology and biotechnology*, *85*(10), 1340-1345.
20	Ozdemir, B., Mertoglu, B., Yapsakli, K., Aliyazicioglu, C., Saatci, A., & Yenigun, O. (2011). Investigation of nitrogen converters in membrane bioreactor. *Journal of Environmental Science and Health, Part A*, *46*(5), 500-508.
21	Harhangi, H. R., Le Roy, M., van Alen, T., Hu, B. L., Groen, J., Kartal, B., ... & den Camp, H. J. O. (2012). Hydrazine synthase, a unique phylomarker with which to study the presence and biodiversity of anammox bacteria. *Applied and environmental microbiology*, *78*(3), 752-758.
22	Bürgmann, H., Jenni, S., Vazquez, F., & Udert, K. M. (2011). Regime shift and microbial dynamics in a sequencing batch reactor for nitritation/anammox treatment of urine. *Applied and environmental microbiology*, AEM-02986.
23	Ni, S. Q., Ni, J. Y., Hu, D. L., & Sung, S. (2012). Effect of organic matter on the performance of granular anammox process. *Bioresource technology*, *110*, 701-705.
24	Daverey, A., Su, S. H., Huang, Y. T., & Lin, J. G. (2012). Nitrogen removal from opto-electronic wastewater using the simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) process in sequencing batch reactor. *Bioresource technology*, *113*, 225-231.
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A.Table 4: Real-time Primer sequences with reaction conditions used in this study

Primer Pair	**Annealing Temperature (°C)**	**Primer name/Sequence (5'-3')**	**References**
1055f-1392r	55	1055f (ATGGCTGTCGTCAGCT) 1392r (ACGGGCGGTGTGTAC)	(Ferris et al., 1996)
341f-543r	62	341f (CCTACGGGAGGCAGCAG) 543r (TTACCGCGGCTGCTGGCAC)	(Koike et al., 2007)
338f-518r	64	338f (ACTCCTACGGGGAGGCAGCA) 518r (ATTACCGCGGCTGCTGG)	(Muyzer et al., 1993)
amoA1f-amoA2r	55	amoA1f (GGGGTTTCTACTGGTGGT) amoA2r CCCCTCKGSAAAGCCTTCTTC)	(Rotthauwe et al., 1997)
Amx809f-Amx1066r	65	Amx809f (GCCGTAAACGATGGGCACT) Amx1066r (AACGTCTCACGACACGAGCTG)	(Tsushima et al., 2007b)
NSR1113f-NSR1265r	68	NSR1113f (CCTGCTTTCAGTTGCTACCG) NSR1265r (GTTTGCAGCGCTTTGTACCG)	(Kindaichi et al., 2006)
Nitro1198f-Nitro1423r	68	Nitro1198f (ACCCCTAGCAAATCTCAAAAACCG) Nitro1423r (CTTCACCCCAGTCGCTGACC)	(Knapp and Graham, 2007)

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- **Annexure II**

A.Table 1: Published primers/TaqMan probes to target ammonium-oxidizing bacteria for *amoA* gene with the respective target group and the sequence.

Name	Function	Target group	Sequence (5'-3')	Reference
amoA-1F amoA-2R amoAr-new A189 A682 amoA-Nm3 amoA-Nm4 amoA-Ns	F primer R Primer R primer F primer R primer TaqManProbe TaqManProbe TaqManProbe	*amoA* gene *N. europaea* group *N. oligotropha* Nitrosospira group	GGGG TTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC CCCCTCBGSAAAVCCTTCTTC GGNGACTGGGACTTCTGG GAASGCNGAGAAGAASGC TGTCGATGGCTGAYTACATGGG ATCATGTTGCTGACCGGTAACTGGC CCGACSCACCTGCCGCTGG	(Rotthauwe et al., 1997; Hornek et al., 2006)
amoA-1Fmod GenAOBr	F primer R Primer	Nm. Europaea & Nsp. Briensis amoA gene	CTG GGG TTT CTA CTG GTG GTC GCA GTG ATC ATC CAG TTG CG	(Meinhardt et al., 2015a)
amoNo550D2f amoNo754r amoNoTaq729	F primer R Primer	N. oligotropha amo	TCAGTAGCYGACTACACMGG	(Harms et al., 2003)

	TaqManProbe	A gene	CTTTAACATAGTAGAAAGCGG [FAM]CCAAAGTACCACCATACGCAG[TAM]	
AmoNN542f AmoNN676r	F primer R Primer	N. Nitrosa amo A gene	TATTGCTTTCAATGGCAGACTACA CCGCAAAGAACGCAGCAATC	(Layton et al., 2005)
amoRI27542f amoRI27679r amoRI27bhq651r	F primer/ R Primer/ TaqManProbe	*N. sp.* Strain RI-27 *amoA*	CATTGTTATCGATGGCTGACTATA ACGCTGAGAAGAATGCTGCAAT [FAM]TGTATGACCACCGAACGTACGCAGTGAG[BHQ]	(Layton et al., 2005)
βAMOf βAMOr	F primer/ R Primer/ TaqManProbe	*N. sp.* Strain RI-27 *amoA*	CATTGTTATCGATGGCTGACTATA ACGCTGAGAAGAATGCTGCAAT [FAM]TGTATGACCACCGAACGTACGCAGTGAG[BHQ]	(McCaig et al., 1994)
amoA-F3 amoB-R4	F primer/ R Primer/ TaqManProbe	*N. sp.* Strain RI-27 *amoA*	CATTGTTATCGATGGCTGACTATA ACGCTGAGAAGAATGCTGCAAT [FAM]TGTATGACCACCGAACGTACGCAGTGAG[BHQ]	(Lim et al., 2008)
AMOF1 (f9) AMOR2 (r1826) AMOF2 (f104) AMOR2R (r1800)	F primer R Primer F primer R Primer	Nm. europaea amo A	CCCGTTATTCCAATCTGACCG CCACCCCATACCAGCGCCA GCAGAAGTTGCGCTTGGGGTAC CAGAATGGCAAGTACCCAGGTG	(Hastings et al., 1997)

amoNo550D2f amoNo754r amoNoTaq729	F primer R Primer TaqManProbe	N. oligotropha amoA gene	TCAGTAGCYGACTACACMGG CTTTAACATAGTAGAAAGCGG [FAM]CCAAAGTACCACCATACGCAG[TAM]	(Harms et al., 2003)

FAM: 6-FAM (6-carboxylfluorescein)

TAM: TAMRA (Carboxy-Tetramethyl-Rhodamine)

BHQ: Black Hole Quencher

HEX: hexachloro-fluorescein

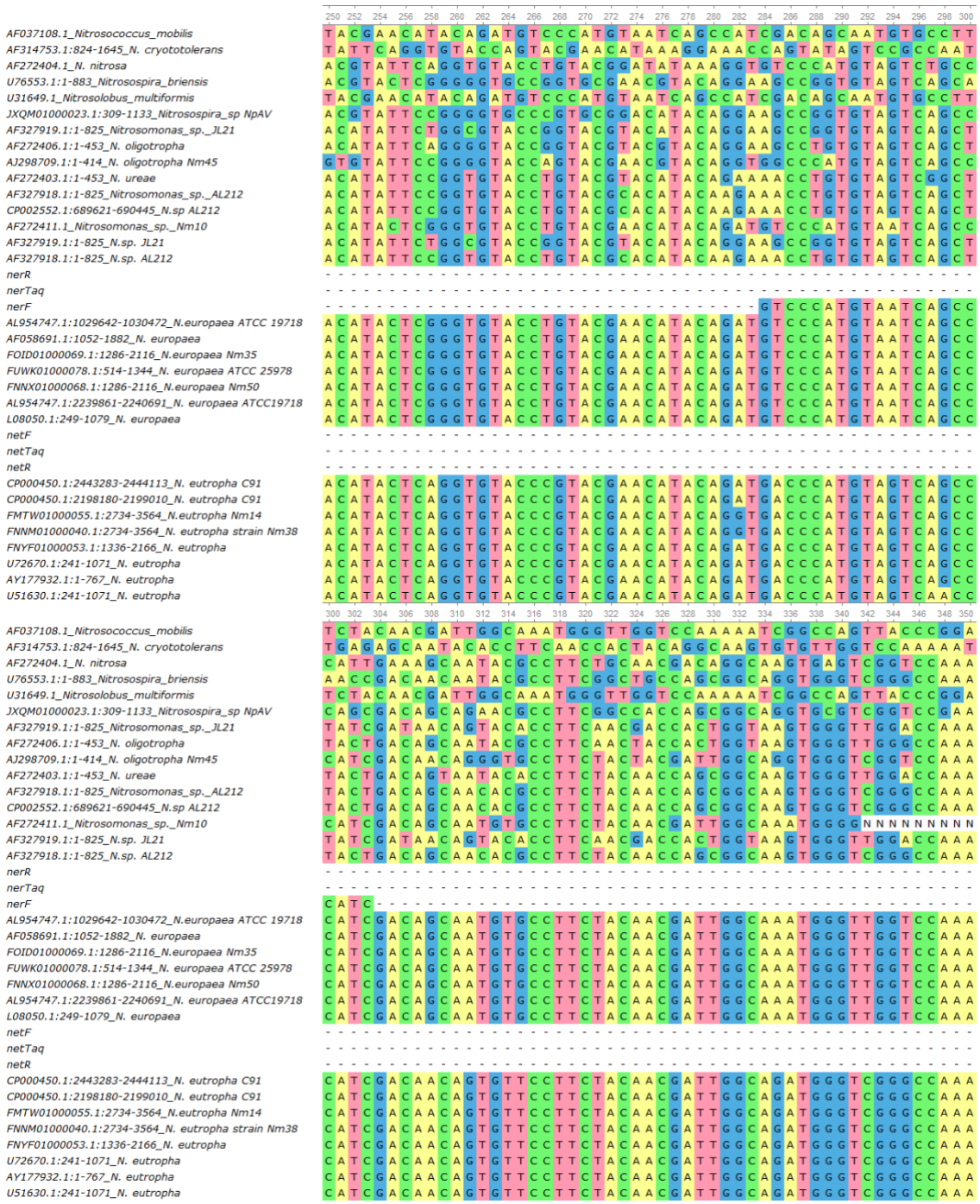
A.Table 2: Primer and TaqMan Probes for *N. europaea* and *N. eutropha* designed in this study.

Name	**Function**	**Sequence (5'-3')**	**Target**	**Size (bp)**
nerF/ nerR	F primer R Primer TaqMan Probe	GTCCCATGTAATCAGCCATC CACACTACCCCATCAACTTC [FAM]ATAGAACAGCAGACCGAAGAATCCACCTCCAACCA[TAM]	N.europaea *amoA* gene	221
netF/ netR	F primer R Primer TaqMan Probe	ATCAGGCCAAAGAATCCACC TCCACTCAATTTTGTAACCCC [HEX]CAACCAGTTACGTGTCAGATACATTGTGAAATCC[TAM]	N.eutropha *amoA* gene	122

A.Table 3: PCR Protocol: TaqMan Fast Advanced MasterMix.

	HOLD	PCR (40 cycles)	
95°C	95°C	95°C	Annealing T
	00:00:20	00:00:01	00:00:20

Annealing Temperature: ner T=65°C, net T=60°C, Rott pp =55°C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AF272404.1_N. nitrosa
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U31649.1_Nitrosolobus_multiformis
JXQM01000023.1:309-1133_Nitrosospira_sp NpAV
AF327919.1:1-825_Nitrosomonas_sp._JL21
AF272406.1:1-453_N. oligotropha
AJ298709.1:1-414_N. oligotropha Nm45
AF272403.1:1-453_N. ureae
AF327918.1:1-825_Nitrosomonas_sp._AL212
CP002552.1:689621-690445_N.sp AL212
AF272411.1_Nitrosomonas_sp._Nm10
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AF327918.1:1-825_N.sp. AL212
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nerTaq
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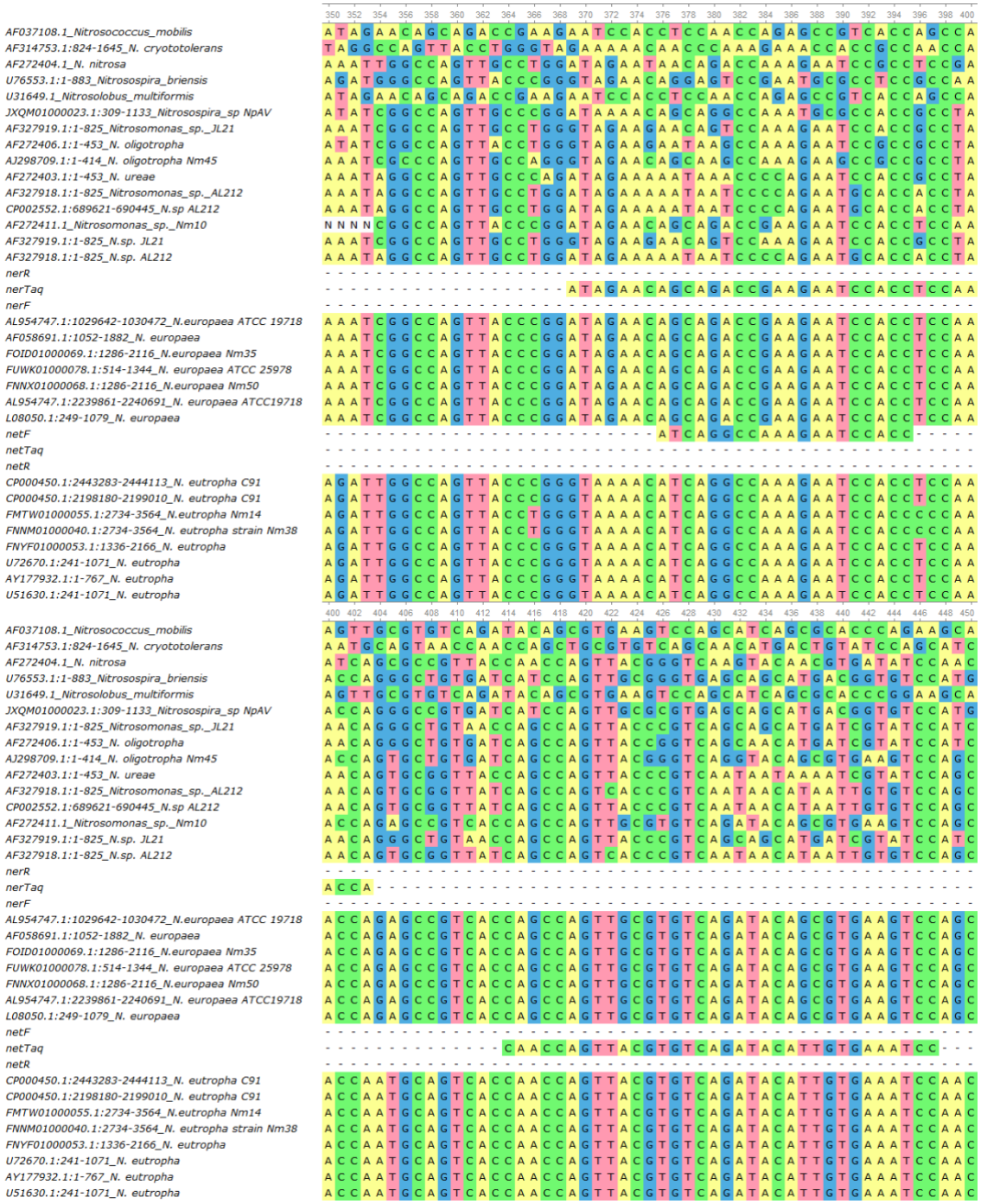
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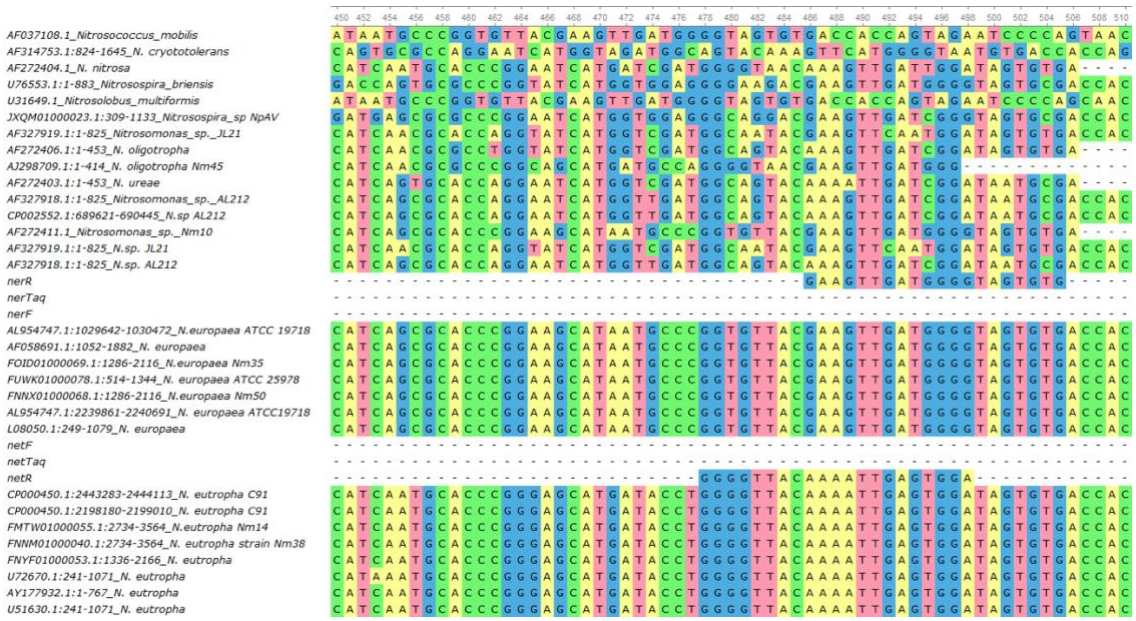
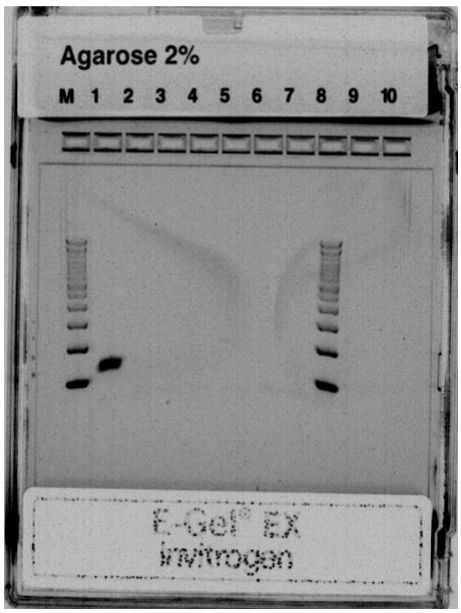
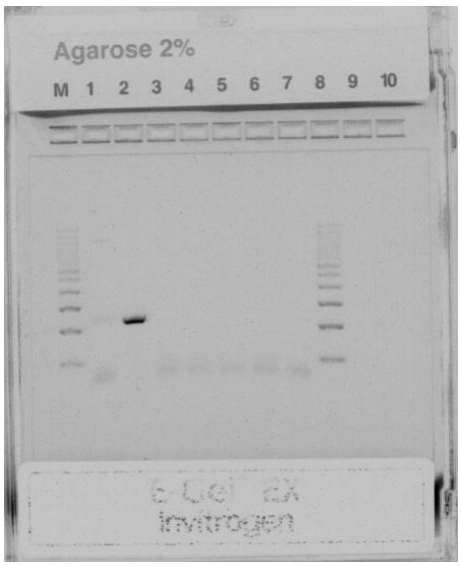


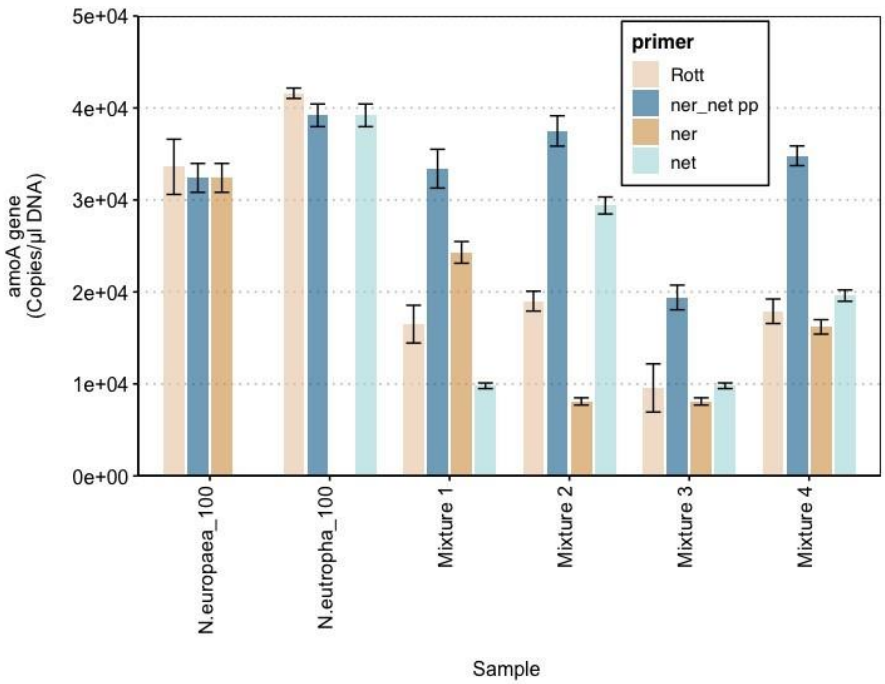
A.Figure 1: Sequence alignment of the *amoA* gene sequences of various AOB. List of microbial members that were considered for the sequence alignment: *Nitrosococcus mobilis* (AF037108.1), *Nitrosomonas cryotolerans* (AF314753.1), *Nitrosomonas nitrosa* (AF272404), *Nitrosospira briensis* (U76553.1), *Nitrosolobus multiformis* (U31649.1), *Nitrosospira sp.* NpAV (JXQM0100023.1), *Nitrosomonas sp.* JL21 (AF327919.1), *Nitrosomonas oligotropha* (AF272406.1), *Nitrosomonas oligotropha* Nm45 (AJ298709.1), *Nitrosomonas ureae* (AF272403.1), *Nitrosomonas sp.* AL212 (AF327918.1), *Nitrosomonas sp.* AL212 (CP002552.1), *Nitrosomonas sp.* Nm10 (AF272411.1), *Nitrosomonas sp.* JL21 (AF327919.1), *Nitrosomonas sp.* AL212 (AF327918.1), *Nitrosomonas europaea* ATCC 19718 (AL954747.1), *Nitrosomonas europaea* (AF058692.1), *Nitrosomonas europaea* Nm35 (FOID01000069.1), *Nitrosomonas europaea* strain ATCC 25978 (FUWK01000078.1), *Nitrosomonas europaea* strain Nm50 (FNNX01000068.1), *Nitrosomonas europaea* ATCC 19718 (AL954747.1), *Nitrosomonas europaea* (L08050.1), *Nitrosomonas eutropha* C91 (CP000450.1), *Nitrosomonas eutropha* strain Nm14 (FMTW01000055.1), *Nitrosomonas eutropha* strain Nm38 (FNNM01000040.1), *Nitrosomonas eutropha* strain Nm56 (FNYF01000053.1), *Nitrosomonas eutropha* (U72670.1), *Nitrosomonas eutropha* (AY177932.1), *Nitrosomonas eutropha* (U51630.1)



A.Figure 2: PCR product of net primer pair visualized on a precast 2% agarose E-Gel with Annealing Temperature of 65 °C. Lane M=100 bp ladder, Lane 1= DSM 101675 (*Nitrosomonas eutropha*), Lane 2= DSM 28437 (*Nitrosomonas europaea*), Lane 3 = DSM 28438 (*Nitrosomonas nitrosa*), Lane 4= DSM 28436 (*Nitrosomonas communis*), Lane 5= DSM 428 (*Cupriavidus necator*), Lane 6= DSM 1650 (*Pseudomonas nitroreducens*), Lane 7= NC, Lane 8=100 bp ladder.

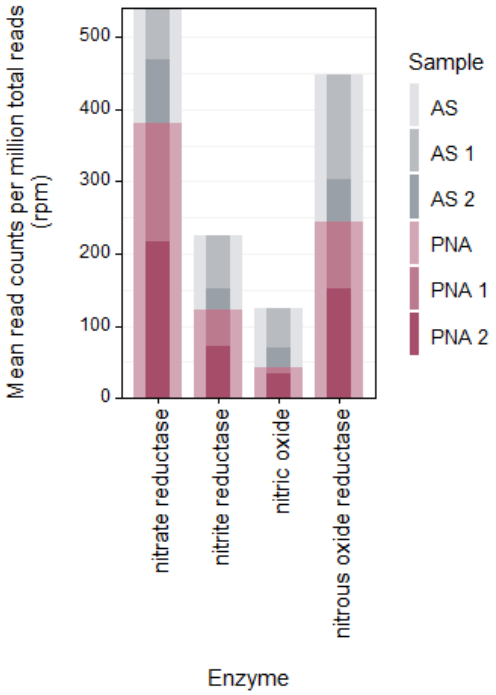


A.Figure 3: PCR product of ner primer pair visualized on a precast 2% agarose E-Gel with Annealing Temperature of 65 °C. Lane M=100 bp ladder, Lane 1= DSM 101675 (*Nitrosomonas eutropha*), Lane 2= DSM 28437 (*Nitrosomonas europaea*), Lane 3 = DSM 28438 (*Nitrosomonas nitrosa*), Lane 4= DSM 28436 (*Nitrosomonas communis*), Lane 5= DSM 428 (*Cupriavidus necator*), Lane 6= DSM 1650 (*Pseudomonas nitroreducens*), Lane 7= NC, Lane 8=100 bp ladder.

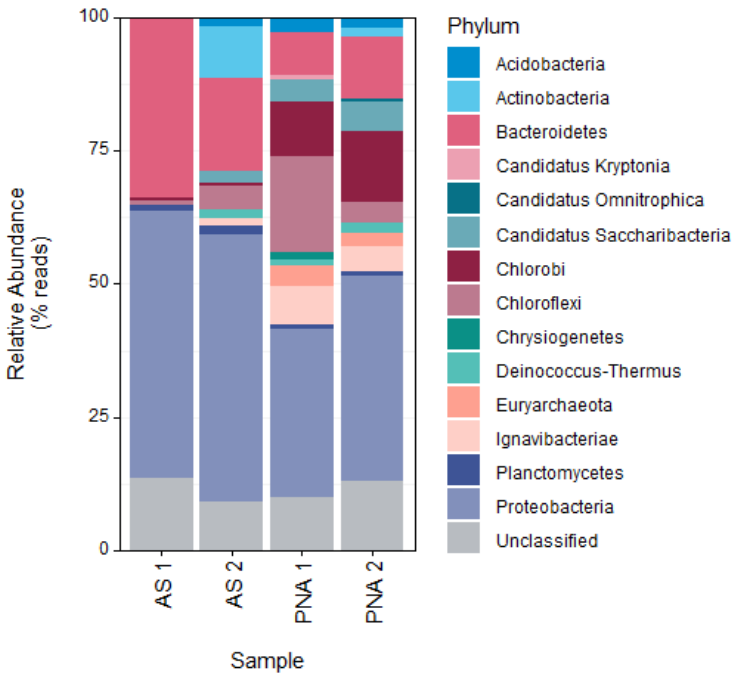


A.Figure 4: Abundance of *amoA* genes for 4 different mixtures of different AOB: Mixture1: *N. europaea: N.eutropha* – 3 : 1; Mixture 2: *N. europaea: N.eutropha* – 1 : 3; Mixture 3: *N. europaea: N.eutropha:* others – 1 : 1: 2; Mixture 4: *N. europaea: N.eutropha* – 1 : 1. And, 100% *N. europaea*, 100 % N. eutropha.

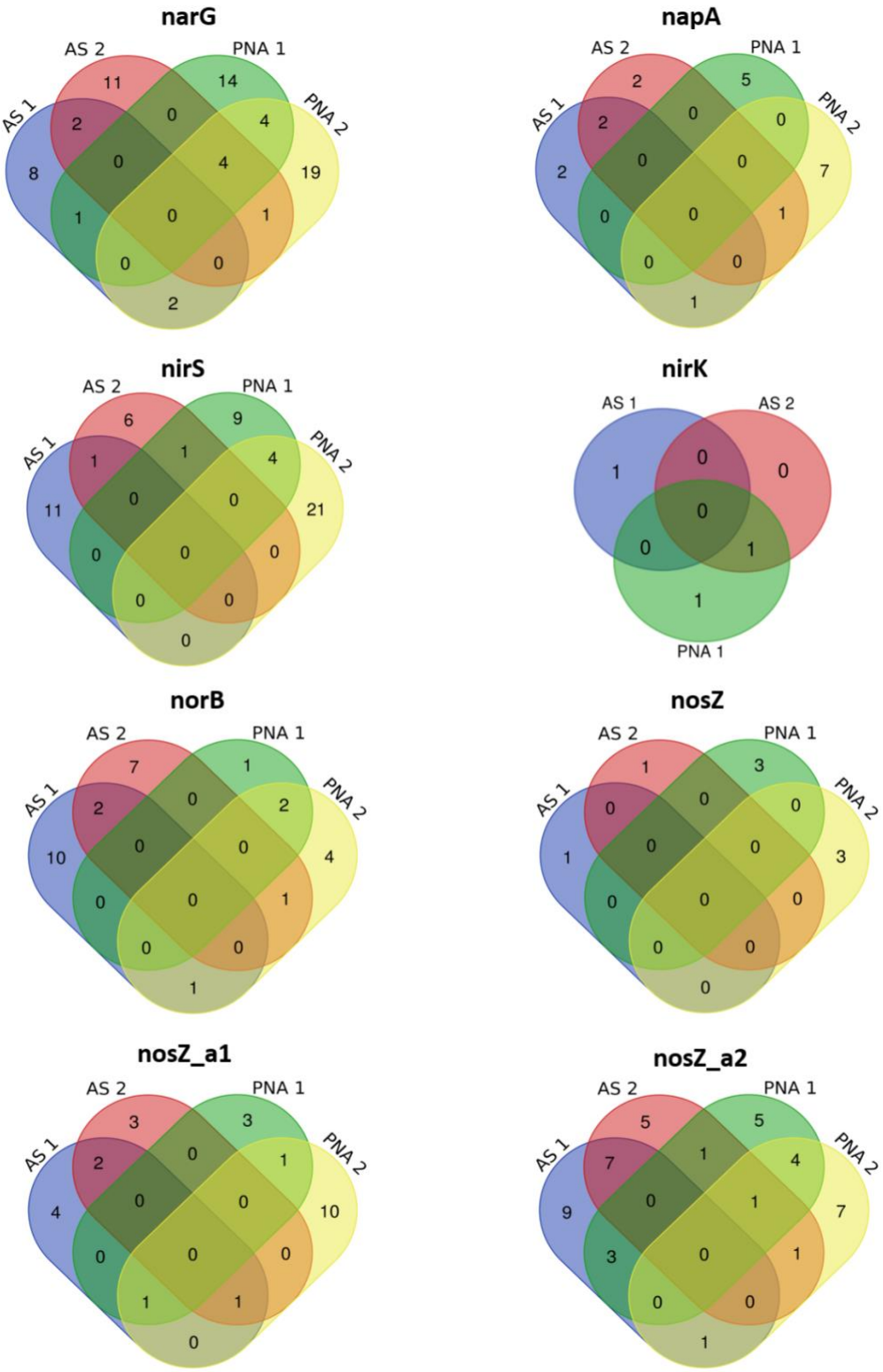
- **Annexure III**



A.Figure 1: Aggregate abundance of reads associated with denitrifying genes coding different enzymes across the samples. AS1 and AS2: conventional activated sludge samples; PNA1 and PNA2: sidestream PNA samples.

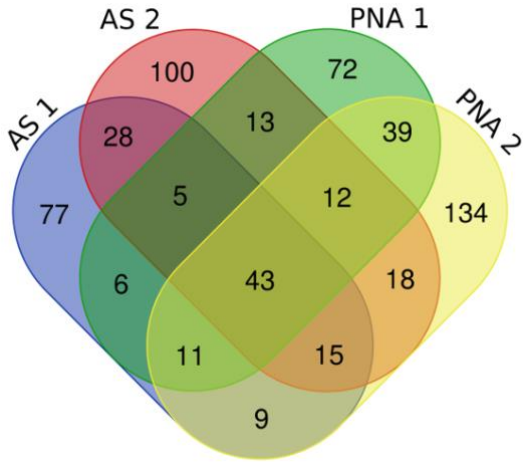


A.Figure 2: Relative abundance of the phyla, associated with all denitrification genes, found in the samples.



A.Figure 3: Venn diagrams comparing denitrifiers associated with respective denitrifying genes are compared between the samples to determine the number of (un)shared members. (i.e. *narG*: respiratory nitrate reductase; *napA*: cytoplasmic nitrate reductase; *nirK*: copper containing nitrite reductase; *nirS*: cytochrome cd

containing nitrite reductase; *norB*: nitritic oxide reductase; *nosZ*: typical nitrous oxide reductase; *nosZa1*: atypical nitrous oxide reducatse clade1; *nosZa2*: atypical nitrous oxide reductase clade 2). AS1 and AS2: mainstream activated sludge samples; PNA1 and PNA2: sidestream PNA samples.



A.Figure 4: In the Venn diagram, based on 16S rRNA gene detected microbial species are compared between the samples to determine the number of (un)shared members. AS1 and AS2: conventional activated sludge samples; PNA1 and PNA2: sidestream PNA samples.

A.Table 1: Overview of the WWTPs from which biomass samples were obtained.

WWTP		WWTP 1		WWTP 2	
Sample type		AS 1	PNA 1	AS 2	PNA 2
NH4-N (mg/L)	Influent	33	972	24.3	690
	Effluent	0.18	85	1.7	33.8
NO2-N (mg/L)	Influent	0.29	0.07	0.5	na
	Effluent	0.16	4.3	0.11	2
NO3-N (mg/L)	Influent	1.24	1.1	1.4	na
	Effluent	7.1	64	3.8	2.2
tCOD (mg/L)	Influent	353	359	359	na
	Effluent	24	38.8	38.8	na
Temperature (^{o}C)		16.6	33.5	14.7	31.2

AS: Conventional activated sludge system; PNA: sidestream partial nitritation anammox system.

na: Data not available

TCOD: total chemical oxygen demand

6 Publications

Publications authored or co-authored that are closely related to the topic, but are not included in the thesis are listed below and include peer-reviewed journal publications and conference contributions.

Peer-reviewed Journal articles

Orschler, L., Agrawal, S., & Lackner, S. (2019). On resolving ambiguities in microbial community analysis of partial nitritation anammox reactors. Scientific reports, 9(1), 1-10.

Orschler, L., Agrawal, S., & Lackner, S. (2020). Lost in translation: the quest for Nitrosomonas cluster 7-specific amoA primers and TaqMan probes. Microbial Biotechnology.

Yasuda, S., Suenaga, T., **Orschler, L.**, Agrawal, S., Lackner, S., & Terada, A. (2020). Identification of a Metagenome-Assembled Genome of an Uncultured Methyloceanibacter sp. Strain Acquired from an Activated Sludge System Used for Landfill Leachate Treatment. Microbiology Resource Announcements, 9(32).

Lackner, S., **Orschler, L.**, Sinn, J., Fundneider, T. & Agrawal, S. (2020). Elimination von Antibiotikaresistenzen – weitergehende Verfahren für kommunale Kläranlagen. 252 Essen, Gesellschaft zur Förderung des Instituts für Sie

Conference Presentation and Posters

Orschler, L., Agrawal, S., & Lackner, S. (2019). Impact of microbial community composition on the start-up of PNA MABR: Activated sludge vs. DEMON® biomass. Microbial Ecology and Water Engineering Conference (MEWE) 2019, Hiroshima (Japan).

Orschler, L., Agrawal, S., & Lackner, S. (2019). Species richness or abundance in activated sludge vs. partial nitritation anammox systems? Primer selection is a critical determinant of community analysis at lower taxonomic rank. Microbial Ecology and Water Engineering Conference (MEWE) 2019, Hiroshima (Japan).

Agrawal, S., **Orschler, L.**, Sinn, J., & Lackner, S. (2019). Antibiotic Resistance in Wastewater Treatment Plant Deciphered by Metagenomics Analysis: Germany vs. Namibia. Microbial Ecology and Water Engineering Conference (MEWE) 2019, Hiroshima (Japan).

Agrawal, S., **Orschler, L.**, Sinn, J., & Lackner, S. (2019). Occurrence and fate of antibiotic resistance genes in wastewater treatment plants: Pond system in Namibia

vs. activated sludge system in Germany. 20th International Symposium on Health Related Water Microbiology, Vienna (Austria).

Orschler, L., Agrawal, S., & Lackner, S. (2018). Linking microbial contamination of the river to the operational strategies of a wastewater treatment plant. 17th Symposium on Microbial Ecology (ISME17), Leipzig (Germany).

Zur Autorin:

Laura Orschler wurde 1990 in Frankfurt am Main geboren. Im Oktober 2011 begann sie ihre wissenschaftliche Laufbahn an der Universität Duisburg-Essen mit dem Bachelor-Studiengang Water Science. Nach dem Bachelor setzte sie in Essen den gleichnamigen Master mit einem Auslandsaufenthalt an der University of Washington fort. Im November 2016 begann sie ihre Promotion am Institut IWAR an der Technischen Universität Darmstadt.

Dort arbeitet sie am Fachgebiet Abwasserwirtschaft im Bereich Molekularbiologie und hat sich mit dem Schwerpunkt der Methodenetablierung und –evaluierung für ein besseres Verständnis von mikrobiellen Gemeinschaften in Systemen zur Stickstoffentfernung in Kläranlagen beschäftigt.

Zum Inhalt:

PCR-basierte Methoden haben die Integration ökophysiologischer Ansätze in der Forschung besonders im Hinblick auf partielle Nitritation/Anammox (PNA)-Systeme stark vorangetrieben. Das PNA System ist definiert als biologischer Stickstoffentfernungsprozess (BNR), der durch ein sehr komplexes, aber fein abgestimmtes Ökosystem charakterisiert ist. Daher werden molekulare Methoden, die eine breite Palette von Ansätzen bieten, vergleichbar mit dem Werkzeugkasten eines Handwerkers, eingesetzt, um PNA-Systeme zu verstehen und einen stabilen Prozess zu garantieren. Diese Methoden weisen jedoch ein naturgemäßes Bias auf, sowie fehlenden Konsens für die Standardisierung und Durchführung. Trotzdem hat sich die quantitative PCR (qPCR) zur gebräuchlichsten Methode entwickelt, um Zielmikroorganismen in technischen Systemen wie dem PNA Prozess und anderen Ökosystemen zu quantifizieren und ist der sogenannte der Goldstandard für eine schnelle und zuverlässige Quantifizierung in allen ökologischen Studien. Zusätzlich hat sich durch Next Generation Sequencing Methoden (NGS) ein neuer und fortschrittlicher Ansatz durch eine sogenannte ‚in-depth' Analyse entwickelt und durch die Veröffentlichung neuer Genom-Sequenzen in öffentlichen Datenbanken zu einer kritischeren Betrachtung des PNA-Mikrobioms führen.

Ziel dieser Doktorarbeit war die Entwicklung von Rahmenbedingungen, um die bekannten Herausforderungen zu bewältigen für eine bessere Integration von molekularen Methoden in PNA-, sowie CAS-Studien. Dabei geht es hauptsächlich um das Verständnis der aktuellen Biase der molekularen Methoden, die Standardisierung der entsprechenden Methoden sowie die richtige Kombination molekularer Methoden.

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