Exploring nitrifier denitrification in *Nitrosomonas communis* and *Nitrosomonas europaea* through physiology and proteomic analysis

by

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Abstract

The current understanding of how ammonia oxidizing bacteria (AOB) produce the potent greenhouse gas nitrous oxide is incomplete and inaccurate since most studies are focused on the model organism for the group, *Nitrosomonas europaea*, which does not represent the physiological diversity of AOB. Since AOB are recognized as significant contributors to global nitrous oxide emissions, it is imperative that we study more species of AOB to refine our mechanistic understanding of how they produce nitrous oxide at the molecular level. Refining this knowledge is necessary for making informed decisions about increasing the sustainability of the processes on which AOB have a major impact, such as agriculture and wastewater treatment. The overall goal of this study was to uncover the enzymes involved in the production of nitrous oxide in the ammonia oxidizing bacterium, *Nitrosomonas communis,* which retains the ability to produce nitrous oxide despite lacking the canonical nitrite reductase (NirK) that was thought to be essential for this process. To confirm the ability of *N. communis* to produce nitrous oxide, the growth of the strain was observed in batch cultures where oxygen and the growth substrate, ammonium, were limited. Nitrite and nitrous oxide production were correlated with the consumption of ammonia and oxygen with nitrous oxide production starting at the onset of hypoxia. To ensure that the observed nitrous oxide production was from enzymatic nitrite reduction, resting cell assays were conducted where stationary phase cells were removed from growth medium and incubated in small vials with an external reductant (PMS + Ascorbic acid, or hydrazine) under anoxia. Nitrous oxide was observed only in vials containing live cells. While the nitrous oxide-producing ability of *N. communis* was confirmed, it was also observed that the amounts of N_2O produced were much less than the amounts produced by *N. europaea* under the same conditions, suggesting that the enzymology for nitrous oxide production was different between the two species. This was confirmed by examining the proteome of *N. communis* and *N. europaea* at mid-log phase and stationary phase. The canonical nitrite reductase, NirK, and nitric oxide reductase, NorB, were not present in the *N. communis* proteome in contrast to that of

N. europaea. Candidates for an alternate nitrite reductase were identified in the *N. communis* proteome along with several cytochromes that are speculated to contribute to nitrous oxide production. While further research is necessary to confirm the role of these candidate enzymes in nitrous oxide production, this study highlights the metabolic diversity of AOB and the need to study more of them to further understand the microbial processes that contribute to greenhouse gas production.

Preface

This thesis is an original work by Sujani Gomes-Yakiwchuk. No part of this thesis has been previously published.

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Firstly, I want to thank my supervisor Dr. Lisa Stein, who supported me throughout my master's program. She's celebrated my successes and cheered me onward through difficulties and believed in me always. I have so much admiration and respect for Lisa as a scientist and a mentor, and it has been an honor to study nitrification as Dr. Lisa Y. Stein's student.

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"But ask the animals, and they will teach you, or the birds in the sky, and they will tell you; or speak to the earth, and it will teach you, or let the fish in the sea inform you" – Job 12: 7-8

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List of Abbreviations

AMO: Ammonia monooxygenase

AOA: Ammonia Oxidizing Archaea

AOB: Ammonia Oxidizing Bacteria

BLAST: Basic Local Alignment Search Tool

c_{554}: Cytochrome C554

CH_4: Methane

c_{M552}: Cytochrome cM552

CO_2: Carbon dioxide

COG: Clusters of Orthologous Genes

cytL: cytrochrome P460

cytS: cytochrome c'-beta

h: hours

HAO: Hydroxylamine dehydrogenase

HURM: Hydroxylamine/Hydrazine-ubiquinone-redox-module

L: Liter

LC-MS/MS: Liquid chromatography tandem mass spectrometry

M: Molar

MBSU: Molecular biology services unit

min: minutes

mL: milliliter

mM: millimolar

N: Nitrogen

N_2: dinitrogen, nitrogen gas

N_2H_4: Hydrazine

N_2O: Nitrous oxide

NADH: Nicotinamide Adenine Dinucleotide

$NaNO_2$: Sodium nitrite

NCBI: National Center for Biotechnology Information

NH_2OH: Hydroxylamine

NH_3/ NH_4^+: ammonia/ ammonium ion

NH_3/$NH4^+$: Ammonia/Ammonium

$(NH_4)_2SO_4$: Ammonium sulphate

NIR: nitrite reductase

NirK/ *nirK:* copper containing nitrite reductase

NO: Nitric oxide

NO_2^-: Nitrite

NO_3^-: Nitrate

NOB: Nitrite Oxidizing Bacteria

NOO: Nitric oxide oxidase

NOR: Nitric oxide reductase

NorB: cytochrome pool oxidizing nitric oxide reductase

NorY: quinone oxidizing nitric oxide reductase

NOx: Nitrogen oxides

PCR: Polymerase Chain Reaction

PTIO: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO)

rRNA: Ribosomal Ribonucleic Acid

RuBisCo: Ribulose-1,5-bisphosphate carboxylase

SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis

TEA: Terminal electron acceptor

tRNA: Transfer Ribonucleic Acid

µl: Microlitre

CHAPTER 1 Introduction

1.1 The global nitrogen cycle

Nitrogen is one of the most essential elements for all life forms on planet Earth as it is a component of basic biomolecules such as amino acids and nucleotides. Pure nitrogen in its gaseous form as N_2 is the most abundant gas in the atmosphere. Nitrogen also exists in many compounds at various oxidation states. The conversion of nitrogen between these oxidation states is known as the nitrogen cycle and it is primarily run by microbial activity (Stein & Klotz, 2016). The inert N_2 gas is made bioavailable by the process of nitrogen fixation where the resulting compound is ammonia (NH_3) (Figure 1.1, 1), which is then assimilated by life forms (Figure 1.1, 2). Ammonia (NH_3) is aerobically oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) in the process of nitrification (Figure 1.1, 3 and 4) or anaerobically oxidized in the process known as anammox (Figure 1.1, 7). The reduction of nitrite (NO_2^-) and nitrate (NO_3^-) to gaseous forms, nitrous oxide (N_2O), nitric oxide (NO), and dinitrogen (N_2) is known as denitrification (Figure 1.1, 6). Each of these processes is carried out by different groups of microbes. All of these processes are also highly interconnected, especially as they often share reactive nitrogen species such as nitric oxide (NO) and hydrazine (N_2H_4) as intermediates and because oxidized nitrogen species can act as terminal electron acceptors (Kuypers et al., 2018; Stein & Klotz, 2016). While the processes and the corresponding microbial groups are often presented as separate, they occur and exist simultaneously in the same ecosystems and the interactions between these microbes are complex and difficult to individually define. Hence there is much to be discovered about the interconnected microbial processes that run the nitrogen cycle.

In the epoch of Anthropocene, the nitrogen cycle is perhaps the most altered biogeochemical system by anthropogenic activities (Rockström et al., 2009). The current state of imbalance in the global nitrogen cycle can be primarily attributed to ammonia synthesis by the Haber-Bosch process. In the early 1900s,

Fitz Haber discovered how to chemically produce NH_3 from combining N_2 and hydrogen (H_2) gases. The process was later developed to industrial scale by Carl Bosch. The goal for the process was to produce fertilizers to increase crop yields and food supply as the bioavailability of nitrogen was a limiting factor for plant growth (Erisman et al., 2008). Nitrogen fertilizers produced through the Haber-Bosch process drastically increased global agricultural outputs, and the increased food availability in turn supported population growth. It is estimated that the Haber-Bosh process is responsible for feeding nearly 50% of the world's population (Erisman et al., 2008). Unfortunately, the widespread application of the process for food production and other industries has also been detrimental to the environment over time due to low efficiency in nitrogen-use. As natural microbial processes cannot cycle reactive nitrogen fast enough to balance the high amounts applied, the majority of applied nitrogen in agriculture is lost to the environment where it remains in nitrogen reservoirs, often as highly reactive and toxic nitrogenous compounds (Erisman et al., 2008). The high availability of NH_3 in terrestrial and aquatic ecosystems increases nitrification and denitrification by microorganisms which lead to a slew of detrimental effects including soil acidification, formation of large oxygen-depleted zones in marine systems, and increase in greenhouses gases, most notably of nitrous oxide (Klotz & Stein, 2008).

1.1.1 Nitrous oxide as a greenhouse gas

Nitrous oxide (N_2O) is the third most abundant greenhouse gas in the atmosphere after carbon dioxide (CO_2) and methane (CH_4). While its atmospheric levels are low in comparison, its global warming potential over 100 years is 298 times that of CO_2. It also has a much longer lifetime in the atmosphere at 114 years (US EPA, 2015). Atmospheric concentration of this potent greenhouse gas has been steadily rising over the past several decades and is predicted to keep rising. N_2O production is greatly affected by rates of nitrification and denitrification in terrestrial and aquatic ecosystems. It is an intermediate in the denitrification process and also a by-product of nitrification.

It is widely recognized that agricultural soils are the largest source of N_2O emissions especially in the northern hemisphere (Tian et al., 2020; US EPA, 2015). High NH_3 availability in agricultural soil from fertilizer use stimulates nitrification and subsequently denitrification which leads to increased N_2O production. Eutrophication of other environments such as marine ecosystems and estuaries due to fertilizer run off has also lead to increases in N_2O emissions from these natural sources as well (Klotz & Stein, 2008; Tian et al., 2020). Due to its steady increase and high global warming potential, N_2O has been gaining global recognition as a key greenhouse gas to mitigate and control in recent years and there has been a rising interest to understand the biochemical processes that lead to its production.

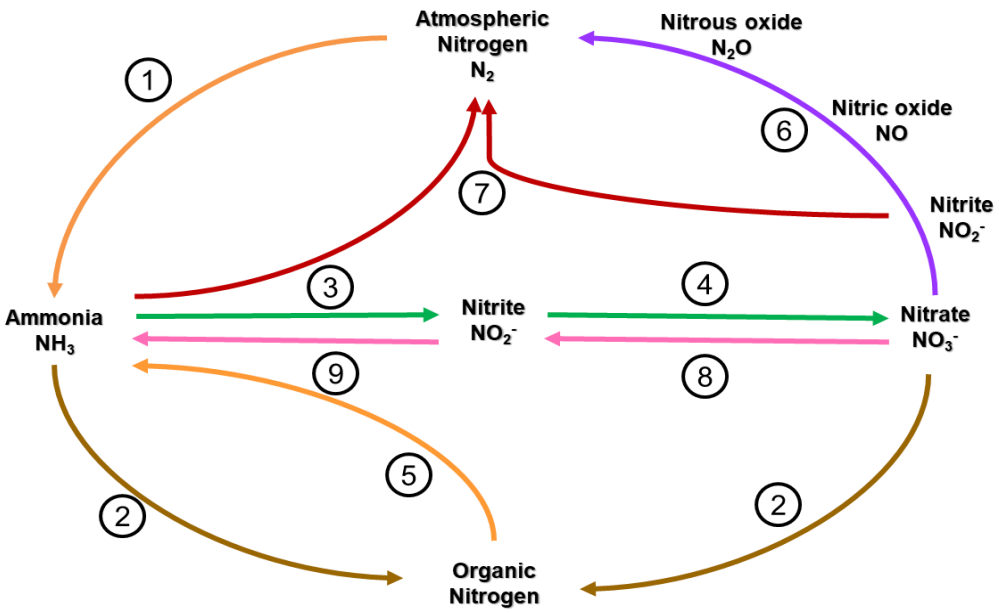


Figure 1.1 Major processes in the nitrogen cycle. The processes shown are (1) nitrogen fixation, (2) assimilation of ammonia to other organisms, (3, 4) nitrification consisting of (3) ammonia oxidation and (4) nitrite oxidation, (5) ammonification, (6) denitrification, and (7) anaerobic ammonia oxidation, and (8) nitrate reduction (9) dissimilatory nitrite reduction. Figure adapted from (Stein & Klotz, 2016)

1.2 Nitrification and Nitrifying Microorganisms

Nitrification is the aerobic oxidation of NH_3 or NH_4^+ to NO_2^- and NO_3^- by nitrifying microorganisms (Stein & Nicol, 2018). It is one of the primary processes in the nitrogen cycle with major implication for nitrogen balance in both natural and human-made ecosystems. Nitrification is particularly important for NH_3 removal in wastewater systems, NH_3 loss, and NO_3^- availability in agriculture. Until recently, nitrification was thought to be carried out in two steps by two distinct groups of organisms: i) oxidation of NH_3 to NO_2^- by ammonia oxidizing bacteria (AOB) and archaea (AOA), and ii) oxidation of NO_2^- to NO_3^- by nitrite oxidizing bacteria (NOB) (Stein & Nicol, 2018). Complete ammonia oxidizers (COMAMMOX) capable of fully oxidizing NH_3 to NO_3^- were discovered in 2015 (Daims et al., 2015; van Kessel et al., 2015). While nitrification is a necessary process, the intermediates and by products of nitrification by these organisms are unfavourable. The intermediates and products of ammonia oxidation, hydroxylamine (NH_2OH), nitric oxide (NO), and nitrite (NO_2^-), are highly reactive and toxic to many life forms. Additionally, nitrogenous greenhouse gases, NO and N_2O are also produced by nitrifiers, particularly AOB. Due to the high availability of NH_3 in soils due to agriculture and in aquatic ecosystems due to run off, N_2O production by AOB is a primary source of atmospheric N_2O (R. Liu et al., 2016; Wrage et al., 2001). Hence there is a dire need to better understand the metabolic processes involved in nitrification and the organisms that carry them out.

1.2.1 Ammonia oxidizing bacteria

Chemolithoautotrophic bacteria that rely on the oxidation of NH_3 as their primary energy deriving metabolism are known as ammonia oxidizing bacteria or AOB. These belong to Gammaproteobacteria and Betaproteobacteria classes with a majority of cultivated AOB belonging to the latter (Prosser et al., 2014). There are two known genera of betaproteobacterial AOB which are *Nitrosomonas* and *Nitrosospira.* Within *Nitrosomonas* there are seven identified lineages and several clusters that form across lineages (Prosser et al., 2014). *Nitrosomonas europaea,* which belongs to cluster 7, is the most

well studied and represents the model organism for AOB. Much of what is known about AOB metabolism and physiology have been based on *N. europaea*. Other known *Nitrosomonas* species that have been studied in some capacity, although not as deeply as *N. europaea*, include *N. eutropha, N. communis, N. ureae,* and *Nitrosomonas* sp. Is79A3 (Kozlowski, Kits, et al., 2016c; Stein et al., 2007; Zorz et al., 2018).The *Nitrosospira* genus encompasses four clusters but their phylogenetic relationships are not yet clearly understood. This genus includes three named species: *N. multiformis, N. briensis,* and *N. tenuis* (Prosser et al., 2014).

In AOB, ammonia (NH_3) is first oxidized to hydroxylamine (NH_2OH) by ammonia monooxygenase (AMO) which is encoded by the *amoCAB* operon (Figure 1.2). AMO is a multimeric transmembrane enzyme conserved across all ammonia oxidizers including AOA and comammox and is considered to be the hallmark enzyme for chemolithotrophic ammonia oxidation (Stein, 2019). NH_2OH was previously thought to be oxidized directly to NO_2^- by hydroxylamine dehydrogenase (HAO), but it was shown that the product of HAO is NO (Caranto & Lancaster, 2017). The enzyme for the subsequent step which would be a nitric oxide oxidase (NOO) for the oxidation of NO to NO_2^- is yet undiscovered (Figure 1.2). HAO is encoded by the *haoAB-cycAB* operon which also encodes cytochromes c_{554} and c_{M552} which transfer electrons from HAO to the quinone pool for generating proton motor force. Together this operon forms the hydroxylamine/hydrazine – ubiquinone redox module (HURM) (Chain et al., 2003; Klotz & Stein, 2008; Stein, 2019). These enzymes are conserved across all known AOB strains, although there are variations in the copy number of the operons and gene clusters depending on the strains and lineages (Prosser et al., 2014). AOB are found in a variety of ecosystems including soils, especially agricultural soils, marine and freshwater habitats and also in engineered ecosystems such as wastewater treatment plants.

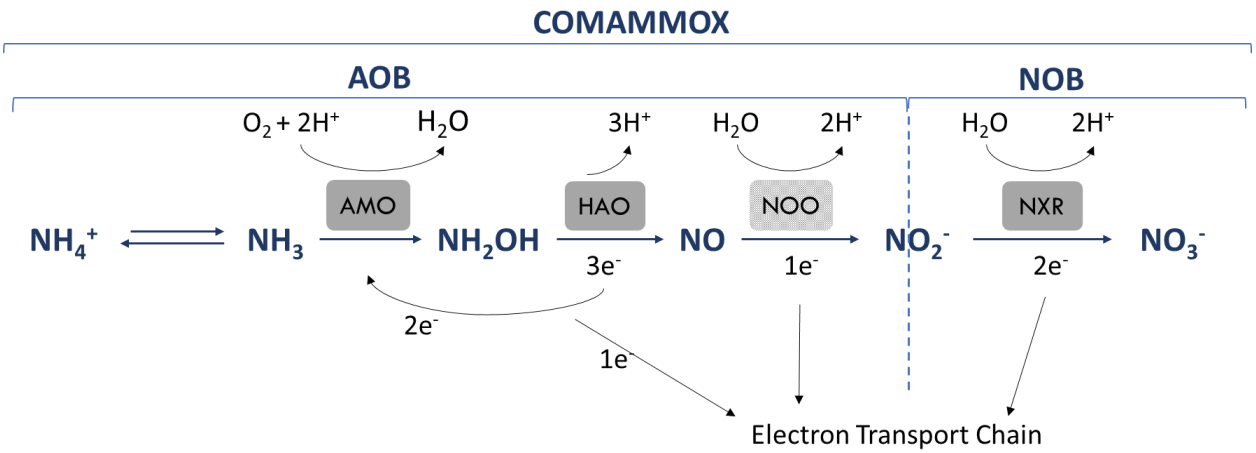


Figure 1.2 Energy deriving reactions of ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), and complete ammonia oxidizing bacteria (COMAMMOX). AMO = ammonia monooxygenase, HAO = hydroxylamine dehydrogenase, NOO = nitric oxide oxidoreductase, NXR = nitrite oxidoreductase. NOO is a yet uncharacterized enzyme. Figure redrawn from (Stein, 2019)

1.2.2 Ammonia oxidizing Archaea

Ammonia **o**xidizing **a**rchaea belong to the subphylum Thaumarchaeota and were discovered relatively recently with the first isolate being described in 2005 (Könneke et al., 2005). Similar to AOB, AOA are chemolithotrophs that derive energy from the oxidation of NH_3 to NO_2^- albeit through a different metabolic pathway. The first step of NH_3 oxidation (the oxidation of NH_3 to NH_2OH) catalysed by AMO is the same as in AOB, but the pathway for the oxidation of NH_2OH to NO_2^- has two proposed models (Figure 1.3). Both models identify NO as an important intermediate that is required for ammonia oxidation and implicates copper-based enzymes to catalyse the oxidation steps since heme based enzymes are not found in AOA genomes (Stein, 2019; Stein & Nicol, 2018). The first model, which was developed based on the observed effects of the NO scavenger PTIO on the production and/or consumption of O_2, NO_2^-, NO, and N_2O by the AOA *Nitrososphaera viennensis*, proposes that the oxidation of NH_2OH to NO_2^- requires NO, and is catalyzed by one novel copper-based enzyme complex with activity similar to that of Cytochrome P460 (Figure 1.3, A). It also suggests that the origin of NO is

the reduction of NO_2^- by nitrite reductase NirK, rather than the oxidation of NH_2OH (Kozlowski et al., 2016). The second model suggests that the pathway is more similar to that of AOB with NH_2OH being oxidized to NO_2^- in two steps, first to NO and then to NO_2^- by membrane bound cupredoxins and NirK functioning in reverse as a NO oxidase (Figure 1.3, B). This model is based on Thaumarchaea-enriched metatranscriptome data, where high *nirK* expression was observed (Carini et al., 2018). However there is currently no biological evidence that NirK is capable of operating in reverse as an oxidase for NO_2^- or NH_2OH as suggested in this second model, and many AOA strains do not encode NirK enzymes (Stein, 2019). Despite being discovered more recently than AOB, AOA occupy a more diverse range of habitats than AOB and are the numerically dominant ammonia oxidizers in many of them (Stahl & de la Torre, 2012).

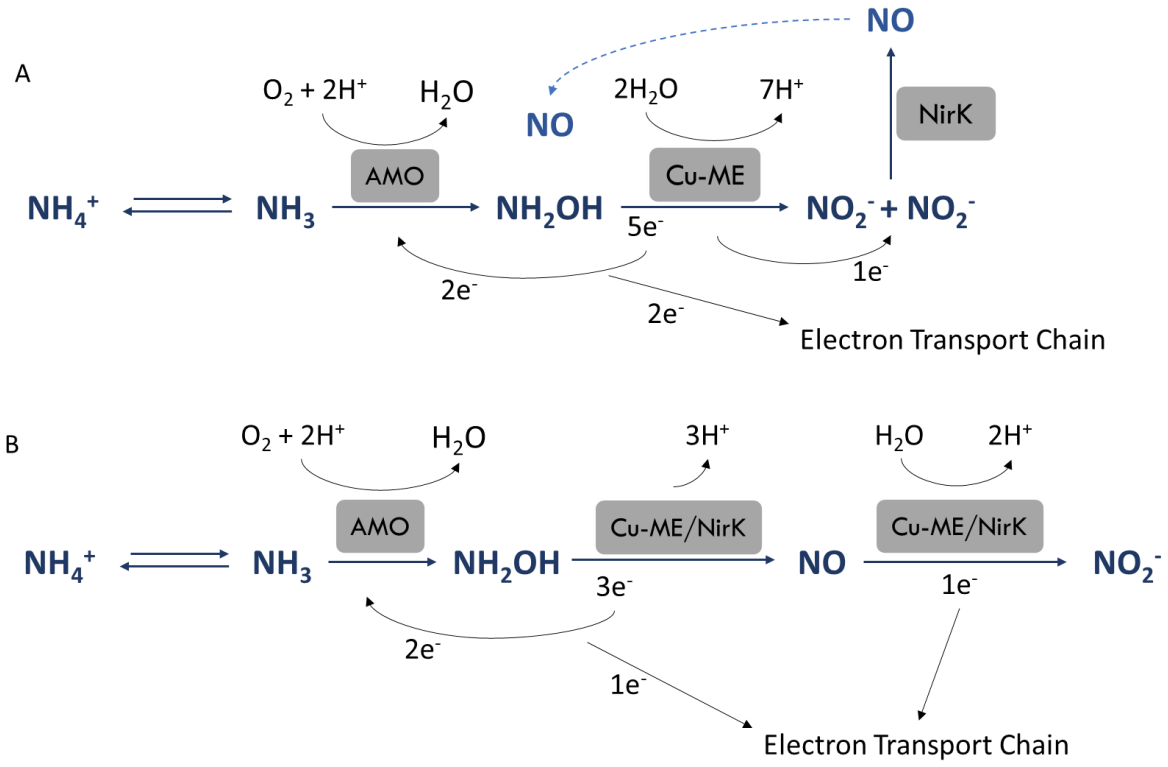


Figure 1.3 Proposed models for ammonia oxidation in ammonia oxidizing archaea (AOA). AMO= ammonia monooxygenase, NirK= Copper containing nitrite reductase, Cu-ME= Copper based metalloenzyme. In the first and more likely model, (A) a single, copper-based enzyme complex co-

oxidizes NH_2OH and NO to NO_2^-, and NirK reduces NO_2^- to NO which is returned to Cu-ME for co-oxidation in a cyclic pattern. In model (B) NH_3 oxidation is similar to AOB, NH_2OH is oxidized to NO_2^- in two steps by two enzymes. NirK either oxidizes NH_2OH to NO or oxidizes NO to NO_2^- in a reverse function, which is yet unconfirmed. A Cu-ME is proposed to fulfil the other role that NirK does not, as a NH_2OH oxidoreductase or NO oxidoreductase. Figure redrawn from (Stein, 2019)

1.2.3 Nitrite oxidizing bacteria

The second step of nitrification, the oxidation of NO_2^- to NO_3^-, is carried out by **n**itrite **o**xidizing **b**acteria or NOB. These are a diverse group of chemolithotrophic organisms that derive energy from the one electron oxidation of NO_2^- to NO_3^-. This reaction is catalysed by nitrite oxidoreductase, NXR, which is the key enzyme for all NOB (Figure 1.2) (Daims et al., 2016). There are seven genera that known NOB belong to, and they are found in a multitude of habitats in both natural and engineered ecosystems, and often are in communities with AOA and AOB (Stein & Nicol, 2018). NOB are known to be metabolically diverse with different lineages and species adapting to different environmental parameters, which allows for niche differentiation in the same ecological habitats (Daims et al., 2016). At present, nitrite oxidizing archaea have not been identified.

1.2.4 COMAMMOX

The existence of **com**plete **amm**onia **ox**idizers or comammox had been hypothesized many years before their discovery due to the confusing division of metabolic labour between AOB and NOB (Costa et al., 2006). Costa et al. argued that an organism that combines NH_3 and NO_2^- oxidation would maximize ATP and growth yield, rather than growth rate and rate of ATP production, and would be competitive in environments with low substrate flux (Costa et al., 2006). In 2015, two groups reported complete NH_3 oxidation activity and growth by a single *Nitrospira* genus (Daims et al., 2015; van Kessel et al., 2015). One of these, *Nitrospira inopinata*, was the first comammox organism to be isolated and further studied. Its genome carries genes for NXR for NO_2^- oxidation as well as homologues for AMO, and HAO for NH_3 and NH_2OH oxidation (Figure 1.2) (Daims et al., 2015). Kinetic analysis of *N. inopinata* revealed that as predicted, it was adapted to oligotrophic environments with a higher substrate affinity for NH_4^+ than

AOB, but had slower growth rate but higher growth yield than AOB (Kits et al., 2017). Since then, the presence of comammox organisms have been identified in a variety of diverse habitats based on sequence analyses of the *amo*A subunit of AMO. The genus *Nitrospira* has been further examined and two clades of comammox bacteria have been identified within this genus (Koch et al., 2019).

1.3 Nitrous oxide production by nitrifiers.

There are at least three ways that nitrifying microorganisms generate N_2O (Figure 1.4). Firstly, the process of ammonia oxidation produces highly reactive intermediates, NH_2OH, NO and NO_2^-, which can interact abiotically with reduced metals such as iron and copper to produce N_2O (Figure 1.4, 1). This process, known as chemodenitrification is observed in NO_2^--rich environments and has also been observed in pure cultures of AOA (Kozlowski et al., 2016; Otte et al., 2019). Secondly, N_2O is produced during aerobic NH_3 and NH_2OH oxidation (Figure 1.4, 2). In AOB, NH_2OH is oxidized to NO, which can be reduced by NOR enzymes and cytochromes. The NOR homologs NorB and NorS, and possibly cytochrome c554, are candidates for this reduction (Stein, 2011). In *N. europaea* cytochrome P460, or CytL, converts NH_2OH directly to N_2O under anoxic conditions as well (Caranto et al., 2016). In AOA, N_2O is produced during aerobic NH_3 oxidation by hybrid formation where N atoms that form N_2O originate from NH_4^+, or a derivative intermediate, and from NO_2^- (Stieglmeier et al., 2014). The amount of N_2O produced during aerobic ammonia oxidation is minimal compared to the levels produced by the third mechanism which is termed 'nitrifier denitrification'. This refers to the enzymatic reduction of NO_2^- to NO and then to N_2O under low O_2 conditions (Figure 1.4, 3) (Stein & Nicol, 2018).

Abiotic production of N_2O during aerobic ammonia oxidation has been observed with cultures of AOB and AOA as well as COMAMMOX (Kits et al., 2019; S. Liu et al., 2017). In habitats where AOA and comammox are the dominant nitrifiers, such as marine ecosystems, this may lead to substantial amounts of N_2O release. Enzymatic NH_2OH oxidation under anoxic conditions by cytochrome P_{460}

leading to N_2O production was found in *N. europaea* and the same enzyme is found other AOB but only appears sporadically in comammox *Nitrospira* genomes (Caranto et al., 2016; Kits et al., 2019).

Among the nitrifiers, enzymatic denitrification activity has only been confirmed in AOB. So far there is no reported evidence that a nitrifier denitrification pathway exists in AOA since they lack NOR enzymes to reduce NO (Kozlowski et al., 2016; Stein et al., 2021; Stieglmeier et al., 2014). Similarly, the comammox isolate *N. inopinata* lacks NO reductases and does not produce N_2O under hypoxia; hence, it also lacks nitrifier denitrification activity (Kits et al., 2019). It is unclear if and how much NOB contribute to global N_2O levels. NOB are not known to produce N_2O during NO_2^- oxidation, and while the potential for NO_2^- reductase activity to produce NO has been reported in some NOB of the *Nitrobacter* genus, the further reduction to N_2O has not been confirmed (Starkenburg et al., 2006). It is possible that other mechanisms to produce N_2O that are yet unidentified also exist given the metabolic diversity of nitrifiers. However it has already been recognized that nitrifier denitrification in AOB is one of the most significant contributors to global N_2O emissions, especially in agricultural soils (Wrage-Mönnig et al., 2018). Hence refining our knowledge of this pathway in AOB outside of *N. europaea* is the focus of this work.

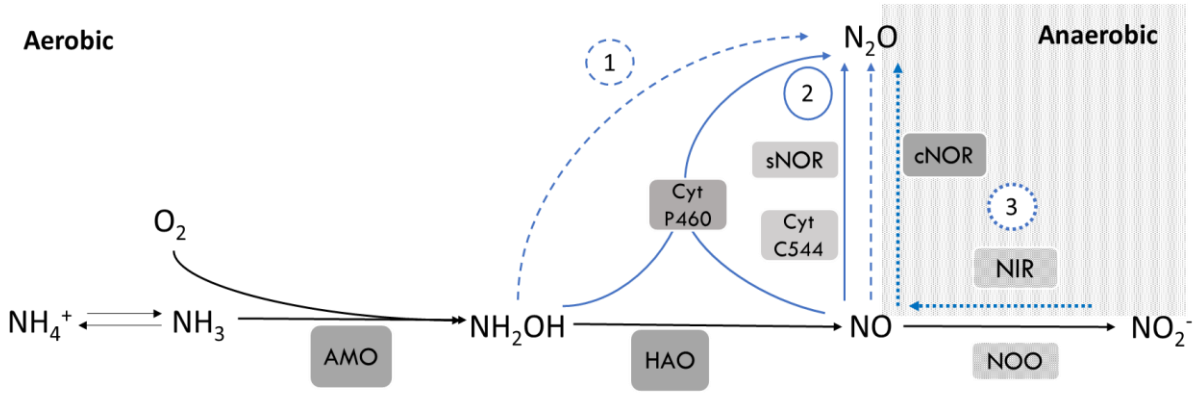


Figure 1.4 A simplified schematic of nitrous oxide production pathways in AOB. 1) abiotic N_2O production/chemodenitrification 2) N_2O production during aerobic NH_3 oxidation (hybrid formation in AOA not shown) 3) nitrifier denitrification. AMO: ammonia monooxygenase, HAO: hydroxylamine

dehydrogenase, NOR: nitric oxide reductase. NIR (nitrite reductase) and NOO (Nitric oxide oxidase) are postulated enzymes that have not yet been conclusively determined.

1.3.1 Nitrifier denitrification pathway

Nitrifier denitrification is the process where NO_2^- is reduced to NO and N_2O by nitrifying organisms. It accounts for almost all N_2O emissions derived from NH_3 and is the most significant source of N_2O in soils (Kool et al., 2011; Wrage-Mönnig et al., 2018). The increase in N_2O emissions from agricultural soils due to the application of urea or ammonia fertilizers can be attributed primarily to nitrifier denitrification (Wrage-Mönnig et al., 2018). N_2O production by AOB were reported as early as the 1960s (Goreau et al., 1980; Ritchie & Nicholas, 1972) and the pathways for N_2O production has been well studied in *N. europaea*.

In AOB, nitrifier denitrification occurs in two steps: the reduction of NO_2^- to NO by nitrite reductase (NIR), and the reduction of NO to N_2O by nitric oxide reductase (NOT) (Figure 1.4, 3). Since many AOB carry homologues to the copper containing nitrite reductase, NirK, this enzyme was thought to be the primary nitrite reductase carrying out the reduction of NO_2^- to NO in nitrifier denitrification. However, there is mounting evidence that a yet unidentified enzyme is carrying out nitrite reduction in AOB especially at low oxygen tensions. Firstly, it was found that NirK is not essential for N_2O production in *N. europaea* since NirK-deficient mutant strains actually produce more N_2O than the wildtype cells (Beaumont et al., 2002; Kozlowski et al., 2014). It was also found that NirK played a bigger role in driving NH_3 oxidation and NO_2^- removal to mitigate NO_2^- toxicity (Beaumont et al., 2002; Cantera & Stein, 2007). Additionally, *nirK* expression is upregulated in response to higher concentrations of NO_2^- rather than low O_2 and is regulated by the NO_2^- sensitive transcription repressor NsrR (Beaumont, Lens, et al., 2004; Sedlacek et al., 2020), which is consistent with its primary role being in NO_2^- removal and driving NH_3 oxidation rather than denitrification. The *nirK* gene cluster also has three other genes, *ncgA, ncgB,* and *ncgC,* which are under the same promoter, and play a role in NO_2^- tolerance in *N. europaea* (Beaumont

et al., 2005). Furthermore, *Nitrosomonas communis*, an AOB strain that lacks homologues to *nirK,* was still able to produce N_2O via nitrifier denitrification (Kozlowski, Kits, et al., 2016c). Hence, it is hypothesized that an unidentified enzyme(s) in AOB acts as a nitrite reductase for nitrifier denitrification at low oxygen tension.

A functional nitric oxide reductase, *norB,* from the *norCBQD* gene cluster is present in some AOB strains and encodes the main enzyme NorB carrying out the second step of nitrifier denitrification, which is the reduction of NO to N_2O (Kozlowski et al., 2014; Kozlowski, Kits, et al., 2016c). NorB deficient mutant cells of *N. europaea* can still produce N_2O at levels comparable to wildtype under atmospheric oxygen levels, but this ability is lost under hypoxic or anoxic conditions (Beaumont, van Schooten, et al., 2004; Kozlowski et al., 2014). Furthermore, AOB strains that lack NorB, such as *Nitrosomonas sp*. Is79A3 and *N. ureae,* are unable to produce N_2O enzymatically (Kozlowski, Kits, et al., 2016c). An alternate nitric oxide reductase, sNOR, is also present in some AOB and is encoded by the gene cluster *norSY-senC-orf1,* which contains the low oxygen NO-sensor, *senC* (Klotz & Stein, 2008). Even though sNOR is not the primary nitric oxide reductase involved in nitrifier denitrification, this gene cluster is transcribed at a higher level during prolonged oxygen limitation, which suggests sNOR may also function as an alternate terminal oxidase (Sedlacek et al., 2020).

There are other genes and corresponding proteins that are speculated to have a role in nitrifier denitrification in AOB, especially since current evidence strongly suggest that other enzymes are active in this pathway. One of these is Nitrosocyanin (*NcyA)*, a red copper protein with potential for electron transfer initially identified and purified from *N. europaea* and since found in genomes of nearly all AOB (Arciero et al., 2002; Klotz & Stein, 2008). It was also found to be highly expressed under oxygen-limited or ammonia-limited growth conditions in several *Nitrosomonas* species (Kartal et al., 2012; Sedlacek et al., 2020; Zorz et al., 2018). Additionally *NcyA* is not found in *Nitrospira* comammox species, which are incapable of performing nitrifier denitrification (Daims et al., 2015; van Kessel et al., 2015). All of these

suggest that Nitrosocyanin plays an important role in the energy-generating mechanism in AOB; however, the specifics of this role have not yet been experimentally determined.

Another group of candidate proteins that are suspected to be involved in nitrifier denitrification, especially as potential nitrite reductases, are multicopper blue proteins (MCBP). Nitrite reductases from denitrifying microorganisms and NirK are two-domain multicopper blue proteins with a type 1 copper binding site and an interdomain copper binding site (Nakamura & Go, 2005). The highly related multicopper oxidases (MCO) are three or six domain proteins also with type 1 copper binding sites and type 2 and/or type 3 interdomain copper binding sites, which include laccases and ceruloplasmin-type proteins (Nakamura & Go, 2005). A two domain MCO from *N. europaea* was identified in 1984 by DiSpirito et. al. This protein had p-phenylenediamine-oxidizing capacity and due to its nature was named 'blue copper protein' or BCO (DiSpirito et al., 1985). It has since been found that BCO is encoded by the first gene in the *nirK* gene cluster, the *ncgA* gene, and is under the same promoter P_{nir}. BCO also has some NO_2^- reducing capacity and plays a role in NO_2^- tolerance of *N. europaea* (Beaumont et al., 2005). The crystal structure of BCO revealed that it has structural similarity to nitrite reductases as well as laccases (Lawton et al., 2009). Interestingly, while *N. communis* lacks the entire *nirK* gene cluster, several sequences with predicted multicopper oxidase domain containing proteins can be found in its genome (Kozlowski, Kits, et al., 2016a).

1.4 The study of Ammonia Oxidizing Bacteria

1.4.1 *Nitrosomonas europaea* as a model organism

As previously mentioned, most of what is known about AOB physiology is based on studies of the model organism, *N. europaea*. It was first isolated by Sergei Winogradsky in 1890 and was the first AOB to be isolated (Stein, 2019). The complete genome sequence of *N. europaea* (ATCC 19718) was analysed and published in 2003 by Chain et al. (Chain et al., 2003). This study shed light on other metabolic processes occurring in AOB such as carbon fixation and cellular transport systems. It has also served as a

comparison point for the analysis of other AOB genomes that have since been sequenced. Most studies of AOB metabolic pathways and enzymes involved in ammonia oxidation and nitrifier denitrification have been based on *N. europaea* (Beaumont et al., 2002, 2005; Beaumont, Lens, et al., 2004; Beaumont, van Schooten, et al., 2004; Cantera & Stein, 2007; Caranto et al., 2016; DiSpirito et al., 1985; Kozlowski et al., 2014). *N. europaea* has been studied in terms of its proteomic and transcriptomic response to ammonia and oxygen availability (Pellitteri-Hahn et al., 2011; Sedlacek et al., 2020; Yu et al., 2018; Zorz et al., 2018). All of these studies have allowed us to understand the metabolic pathways of AOB and to make models that are the basis for further research of other species of AOB. Hence, the knowledge gathered from *N. europaea* is invaluable in the field of nitrification.

However, it is necessary to recognize that *N. europaea* is not a perfect representative for the multitude of named and unnamed species of AOB within both *Nitrosospira* and *Nitrosomonas* genera that are adapted to various trophic levels in a range of diverse ecosystems. For instance, the *N. europaea/ N. mobilis* lineage are eutrophic and are adapted to ammonia rich, high salinity environments such as wastewater; the members of the *N. marina* lineage are from marine environments; the *N. oligotropha* lineage comprises species that are oligotrophic; and the first *N. communis* was isolated from soil (Koops et al., 1991; Prosser et al., 2014). Genomic inventory for processes other than aerobic ammonia oxidation to nitrite also varies across species of AOB. *N. communis* lack the *nirK* gene*; N. multiformis, N. ureae,* and *Nitrosomonas* sp. Is79A3 lack the transcriptional regulator *nsrR;* and *N. ureae,* and *Nitrosomonas* sp. Is79A3 lack the nitric oxide reductase operons, *norCBQD* and *norSY-senC*-orf1 (Kozlowski, Kits, et al., 2016c). These genomic differences suggest that information gathered from studies on *N. europaea*, especially about the pathways for N_2O production and nitrifier denitrification, is likely not physiologically or ecologically relevant, especially since most N_2O emissions come from AOB in soils where *N. europaea* is not the dominant type of ammonia oxidizer. This leaves us with a need to

examine other AOB species to better understand AOB physiological diversity to get more accurate and relevant information regarding N_2O production.

In recent years, this need has been somewhat addressed with comparative studies of different species of AOB. Cua et. al. reported on the comparative physiological and gene regulation response to NO_2^- for *N. europaea, N. eutropha, and N. multiformis* (Cua & Stein, 2011). Zorz et. al. published a study on comparative proteome analysis also of three AOB species, *N. europaea, N. multiformis*, and *N. ureae* (Zorz et al., 2018). Kozlowski et. al. examined the nitrogen oxide metabolism among five species of AOB, which showed that the mechanisms for NO and N_2O production varies across AOB species, and also reinforced the hypothesis that an alternate nitrite reductase (other than NirK) is active during nitrifier denitrification (Kozlowski, Kits, et al., 2016c). These studies were facilitated by the increased availability of published annotated genomes of AOB species in the past two decades. Currently there are at least eight species of AOB with published complete genome sequences. This includes *N. eutropha* C91 (Stein et al., 2007), *Nitrosomonas* sp. AL212 (Yuichi et al., 2011), *Nitrosomonas* sp. Is79 (Bollmann et al., 2013), *N. multiformis* ATCC 25196T (Norton et al., 2008), *N. ureae* Nm10 (Kozlowski, Kits, et al., 2016b), *N. communis* Nm2 (Kozlowski, Kits, et al., 2016a), *Nitrosococcus oceani* ATCC19707 (Klotz et al., 2006), and *N. cryotolerans* ATCC 49181 (Rice et al., 2017).

1.4.2 Nitrosomonas communis

Nitrosomonas communis NM2 was first isolated and characterized in soil samples from Corfu, Greece in 1991 (Koops et al., 1991). This study described the *N. communis* species as non-motile coccoid cells around 2 µm in size that did not contain carboxysomes, could not use urea as a substrate, and had a low salt tolerance (200mM to 300mM) (Koops et al., 1991). *N. communis* NM2 belongs to the *Nitrosomonas communis* lineage (cluster 8) within the *Nitrosomonas* genus along with *N. nitrosa* and other unnamed isolates (Prosser et al., 2014).

The annotated genome sequence of *N. communis* NM2 was published in 2016 (Kozlowski, Kits, et al., 2016a). The 4.07 Mbp genome contains 3,189 protein coding genes which include multiple copies of genes encoding proteins used for NH_3 oxidation (ammonia monooxygenase *amoCAB*, hydroxylamine dehydrogenase (*haoAB-cycAB*), genes for proteins associated with nitrifier denitrification (nitric oxide reductases: *norCBQD* and *norSY-senC-orf1*, NO-responsive regulator *NnrS*, cytochrome P460: *cytL*, and cytochrome *c'-beta*), as well as genes for carbon fixation, copper transport, and iron acquisition and transport. Only the urea carboxylase and putative allophanate hydrolase genes were found with regards to urea metabolism confirming the observation of its inability to use urea as a growth substrate.

Notably, the genome does not contain homologs for the copper containing nitrite reductase, *nirK*, or the cytochrome cd1 nirS nitrite reductases that could be implicated in the nitrifier denitrification pathway. This is a defining feature of *N. communis* NM2, which makes it particularly interesting on the subject of how AOB aside from *N. europaea* produce N_2O.

Despite the lack of the known nitrite reductases, NO and N_2O production by *N. communis* NM2 was reported in a comparative study of the production of nitrogen oxides by various AOB (Kozlowski, Kits, et al., 2016c). In micro-respiratory experiments, instantaneous NO and N_2O production during NH_3 oxidation and NH_2OH oxidation and through anoxia was observed for *N. communis*. The N_2O production profile observed for *N. communis* was suggestive of enzymatic reduction of NO_2^- to N_2O since a linear production of N_2O was observed upon complete oxygen depletion when NH_2OH was provided (Kozlowski, Kits, et al., 2016c). This observation, along with the observation of N_2O production by the *N. europaea* NirK-deficient mutant (Kozlowski et al., 2014), are compelling evidence of yet unidentified enzymology for nitrifier denitrification in AOB. This also makes *N. communis* a particularly suitable candidate to examine alternative enzymology for nitrifier denitrification.

At the time of publication of the above-mentioned studies, the pathway for NH_3 oxidation implicated HAO as the enzyme oxidizing NH_2OH directly to NO_2^- rather than NO; hence the production of NO by *N. communis* during NH_3 oxidation in the above study was suggestive of NO_2^- reduction. The NH_3 oxidation pathway has since been updated to show that NO is an obligatory intermediate produced by the oxidation of NH_2OH by HAO for NH_3 oxidation (Caranto & Lancaster, 2017). Since this could provide an explanation for how *N. communis* was able to produce NO and N_2O without known nitrite reductases, one of the considerations for the current study was to confirm that *N. communis* retained NO_2^- reducing ability and to determine if N_2O is indeed produced via nitrifier denitrification rather than as a by-product of NH_3 oxidation.

1.4.3 Comparative proteomics as a tool for studying AOB enzymology

As with most areas of microbiology, the study of AOB is evolving from the discovery of new species, experiments on their physiology, single gene and protein studies to multiple omics approaches. As mentioned above, at least eight AOB species have published and annotated genomes which facilitate transcriptomic and proteomic studies.

In previous studies, comparative proteomics has been used as a tool to gain insight into the biochemical response to certain stresses. In *N. europaea*, changes in protein expression were observed in response to long term NH_3 starvation and as an adaptation to sustained oxic-anoxic cycling (Pellitteri-Hahn et al., 2011; Yu et al., 2018). The proteomic response to nitrogen dioxide and anoxia has also been examined in another AOB species, *N. eutropha* (Kartal et al., 2012). Furthermore, comparative proteomics have also been used to assess the diversity of protein expression across different AOB species (Zorz et al., 2018).

All of these studies used one dimensional liquid chromatography tandem mass spectrometry (LC-MS/MS) for protein identification, and only one used an isotope-labelled quantification approach. Isotope labelled mass spectrometry is more accurate in quantification than label-free methods, and

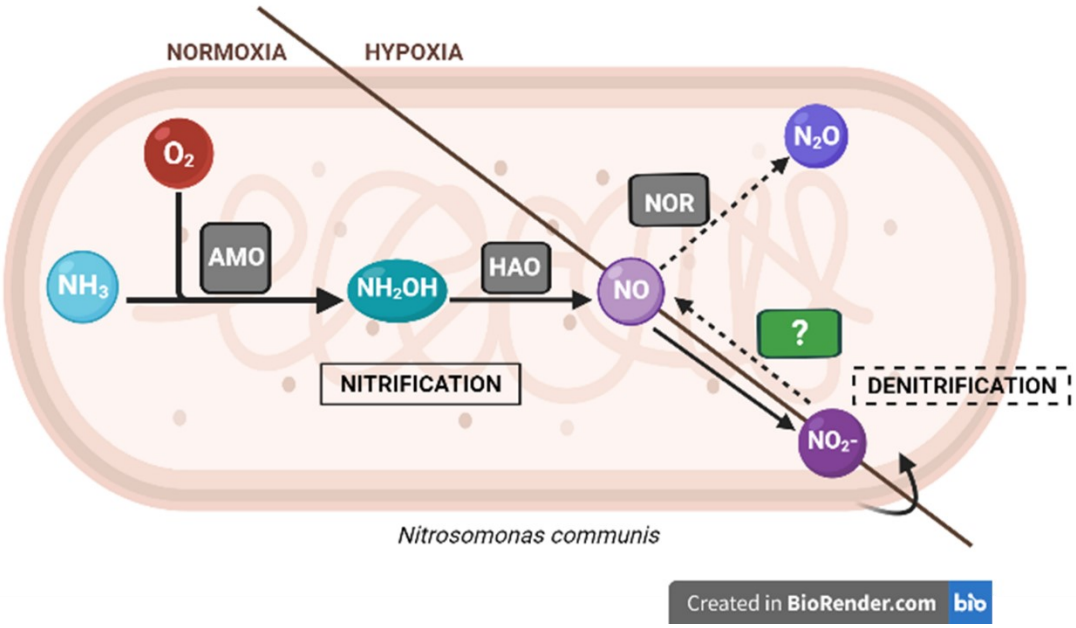
would be the more suitable approach for comparative studies of protein abundances to asses proteomic responses to certain conditions (Otto et al., 2014). The majority of proteome studies in AOB have used label free quantification approaches which rely on spectral counts or area under the curve (AUC) determinations to quantify proteins (Otto et al., 2014). Mass spectrometry based proteomics is widely used in a number of approaches in microbiology including identification of proteins in cell components such as membranes, cell walls and outer membrane vesicles, differential protein expression in stress responses, and post translational modifications, and even in studying bacterial virulence and host-pathogen interactions (Graham et al., 2007; Pérez-Llarena & Bou, 2016; Soares et al., 2016). Proteome analysis of more species of AOB would contribute to the field of nitrification by revealing more of the diversity in gene expression, enzymology, and biochemical responses to changing environments.

1.4.4 My project

The goal of this present study is to explore the pathways in AOB that contribute to N_2O production with a focus on nitrifier denitrification by studying the physiology and proteome of an understudied species *N. communis* in comparison to the model species, *N. europaea*. The specific goals were to confirm its ability to produce N_2O enzymatically and to identify candidate proteins that would be involved in nitrifier denitrification based on comparatively analysing its proteome at mid-log and stationary phases. In order to have a reference point for comparison to existing literature, the model organism for AOB, *N. europaea* was included in the study since there is very little literature on *N. communis* presently. Ultimately the purpose of this study is to further the understanding of the process of nitrification and the microbial metabolic pathways that lead to production of the greenhouse gas N_2O.

CHAPTER 2 Insights into the production of nitrous oxide from ammonia oxidation and nitrite reduction by *N. communis* through comparative proteome analysis

2.1 Graphical abstract



2.2 Introduction

Ammonia oxidizing bacteria (AOB) are an important component of the global nitrogen cycle as they carry out the first step in the process of nitrification. Although aerobic ammonia (NH_3) oxidation is the primary energy deriving metabolism for AOB, they also encode enzymes for other N-conversions including the production of nitrogen oxides (Klotz & Stein, 2008; Stein & Klotz, 2016). AOB are significant contributors to the production of the potent greenhouse gas nitrous oxide (N_2O) which they produce through both biotic and abiotic processes. The most significant avenue of N_2O production by AOB is the process of nitrifier denitrification (Kool et al., 2011; Wrage et al., 2001; Wrage-Mönnig et al., 2018). This refers to the process whereby AOB reduce nitrite (NO_2^-) and nitric oxide (NO) as alternative electron acceptors under oxygen limitation, the end product of which is N_2O (Stein, 2011).

The study of N_2O producing pathways in AOB, and also most studies on the genes, physiology, enzymology and metabolism of AOB have been focussed on the model organism for this group,

Nitrosomonas europaea (Beaumont et al., 2004; Caranto & Lancaster, 2017; Chain et al., 2003; Kozlowski et al., 2014; Pellitteri-Hahn et al., 2011; Sedlacek et al., 2020). However AOB are very diverse, consisting of two genera, *Nitrosomonas*, and *Nitrosospira*, with multiple lineages within each of them, and adapted to a multitude of terrestrial, aquatic, marine, and engineered environments (Prosser et al., 2014). Hence *N. europaea* is not a perfect representative of all AOB. Additionally, recent studies of other AOB species have shown that genes, enzymology and metabolism of different AOB often vary from what is known from *N. europaea,* especially in terms of NOx metabolism (Kartal et al., 2012; Koops et al., 1991; Kozlowski, Kits, et al., 2016a, 2016c; Stein et al., 2007).

The overarching goal of this study is to gain insight into the nitrifier denitrification pathway in AOB by studying a previously unstudied strain, *N. communis,* in comparison to the model organism for AOB, *N. europaea*. As evidenced by previous studies, the pathways for NOx production varies among diverse species of AOB (Kozlowski, Kits, et al., 2016c). The species *N. communis* NM2 is a particularly interesting model to study the pathway for nitrifier denitrification due to its reported ability to produce N_2O despite lacking the genes for the known nitrite reductases. The genome of *N. communis* NM2 has previously been sequenced and annotated (Kozlowski, Kits, et al., 2016a) which allows for analysis of genes of interest and predicted protein outcomes. The physiology of *N. communis* NM2 has not been reported on previously, therefore all experiments were done in tandem with *N. europaea* to serve as a reference point and to allow for comparisons to existing data and literature on AOB, most of which report on *N. europaea*.

The specific objectives of the study were to: 1) study the growth of *N. communis* NM2 in comparison to *N. europaea* under oxygen limitation to confirm its ability to produce N_2O, 2) confirm that N_2O production in *N. communis* NM2 is from NO_2^- reduction from the nitrifier denitrification pathway rather than as a by-product of NH_3 oxidation, and 3) identify candidate enzymes for NO_2^- reduction in *N. communis* NM2 from comparative analysis of its proteome at mid-log and stationary phase. It was

necessary to confirm that NO_2^- was actively being reduced to NO and then to N_2O by this species to show that nitrifier denitrification was active, because AOB produce N_2O in multiple ways. As *N. communis* NM2 lacks the canonical nitrite reductase NirK, NO_2^- reduction by this strain would be indicative of the presence of alternative enzymes with nitrite reductase activity. The sampling points for comparative proteomic analysis were chosen as the points on the growth curve where ammonia oxidation would be the primary energy deriving mechanism (mid-log) and when primary metabolism would switch to nitrifier denitrification (stationary). At this point, the oxygen available to the culture has been mostly consumed. It was hypothesized that proteins involved in nitrifier denitrification would be most abundant at stationary phase. By comparing the abundances of the same proteins at mid-log phase, candidate proteins involved in nitrifier denitrification could thus be identified.

Although the study only involves these two strains of AOB, findings from this study could be applicable to nitrifier denitrification in other AOB strains as well, since it is likely that these alternative nitrite reducing enzymes are present in other AOB and are actively contributing to N_2O production via nitrifier denitrification.

2.3 Materials and Methods

2.3.1 Strain cultivation and maintenance.

Two AOB strains, *Nitrosomonas* communis NM2 and *Nitrosomonas europaea* ATCC 19718 were grown and maintained as batch cultures in a HEPES buffered mineral medium (modified HK media) containing per liter: 10 mM $(NH_4)_2SO_4$, 10 mM NaCl, 0.2 mM $MgSO_4.7H_2O$, 1.0 mM $CaCl_2.2H_2O$, 1.0 mM KCl, 0.02% Phenol red, 1 mL Trace solution (which contains per liter Mili-Q water: 11.5 mM NA_2-EDTA (Tripriplex III), 10.0 mM $FeCl_2.4H_2O$, 0.50 mM $MnCl_2.2H_2O$, 0.10 mM $NiCl_2.6H_2O$, 0.10 mM $CoCl_2.6H_2O$, 0.10 mM $CuCl_2.2H_2O$, 0.50 mM $ZnCl_2$, 0.10 mM $Na_2MoO_4.2H_2O$, and 1.0 mM H_3BO_3), 30 mM HEPES buffer, in 1000 mL total Milli-Q water. NaOH was added to adjust the pH of the media to pH 7.7. Media was then poured as 100 mL aliquots into clean 250 mL Wheaton bottles with butyl rubber caps, and then

autoclaved. A 1 M solution of KH_2PO_4 was autoclaved separately, after which, 40 µL was added aseptically to each 100 mL aliquot of the prepared media (for a final concentration of 0.4 mM KH_2PO_4) prior to inoculation. Media was inoculated with 5% inoculum from a late log-phase culture, initially revived from freezer stocks. The cultures were incubated at 30°C on a rotational shaker at ~150 rpm. When the phenol red indicator turned yellow indicating the acidity of the culture (typically at 24 hours for *N. europaea* cultures and 48 hours for *N. communis* cultures), the pH of the cultures were maintained by adding sterile 10% $NaHCO_3$ solution using sterile syringes and 0.2 µm syringe filter until the colour of the indicator was consistent with the pink colour expected for a pH of 7.7 (by comparison to cell free controls or sterile media). At early stationary phase, a 5% inoculum was transferred to a fresh batch of media for further propagation of the culture. At each transfer step, the cultures were checked for heterotrophic contaminants by spotting inoculum onto nutrient agar media plates and incubating the plates at 30°C for at least four days. Absence of growth on NA plates indicated the lack of heterotrophic contaminants. Strain identities were confirmed periodically using 16S rRNA sequencing to ensure that cross contamination between the two strains was avoided for all reported experiments. Briefly, the V4 region of the 16S rRNA genes was amplified with PCR using a concentrated cell suspension as template and the universal primers 26F and 1492R or 515F and 825R. The amplicons were purified using the Qiagen PCR clean up kit, then sequenced using Sanger sequencing at the Molecular Biology Services Unit and the Univ. Alberta. Sequence results were compared to known 16S rRNA sequences for bacterial species using a BLAST search and the strain identity was confirmed if the top hit was *N. communis* or *N. europaea* for the matching culture.

2.3.2 Growth assays of batch cultures

Strains were grown in triplicates (n=3) in modified HK media as described above. Cell-free controls were included in triplicate, but did not require adjustments to pH since they were free of biological agents to change the acidity of the media. As the turbidity of these cultures are too low to measure growth using

optical density, growth was measured by the production of nitrite using the acidic-Griess assay. This is a colorimetric assay that detects the presence of nitrite ions based on the chemical reaction of sulfanilamide and N-1-naphthylethylenediamene dihydrochloride (NNEQ) under acidic conditions. Briefly, 0.5 mL of a sulfanilamide solution (5 g sulfanilamide in 1.5 M HCl) and 0.5 mL NNEQ solution (100 mg NNEQ in 1.5 M HCl) were mixed with 2.5 µL of a culture sample in flat bottom 48 microwell plates. The change in colour was measured as absorbance at 540 nm using a spectrophotometer and converted to the specific concentration of nitrite using standard curves prepared using a dilution series of $NaNO_2$ standards.

The levels of oxygen and nitrous oxide in the headspace were measured using gas chromatography (thermal conductivity detector: Shimadzu GC-8A Gas Chromatograph). A 250 µL sample of the gas headspace was injected onto the Molecular sieve 5A column (Restek packed column) for measuring oxygen levels and a 500 µL sample was injected onto a HayeSep Q column to measure nitrous oxide. The peak area measurements were converted to volume of gas in the headspace using standard curves made using the respective pure gases as based on the ideal gas law formula (22.4 L/mol).

Data points for nitrite concentration, oxygen and nitrous oxide levels were collected at 12-hour intervals over 84 hours until the cultures reached stationary phase. At the end of the experiment (84 hours) the total biomass was measured as a dry weight measurement. Cells were collected onto pre weighed 0.45 µM sterile filter discs using vacuum filtration. The discs were allowed to air dry at 30°C for several days and weighed daily until their weight had stabilized. The difference between the weight of the discs before and after cell collection was calculated as the dry weight of cells in the culture.

Calculations and data analysis was done using Microsoft Excel which was also used to generate the figures depicting growth curves.

2.3.3 Resting cell assays

Batch cultures of each strain were grown to stationary phase as 250 mL cultures in 500 mL Wheaton bottles, or as 500 mL cultures in 1 L Wheaton bottles with butyl rubber septum caps using a 5% inoculum as described above. The larger volumes of cultures and bottles were used to maximize biomass.

Cells were collected on 0.22 µM sterile filter discs using vacuum filtration, then washed off into sterile 50 mL falcon tubes with ammonia-free media buffer (modified HK media without $(NH_3)_2SO_4$, or phenol red). Cells were then aliquoted into microcentrifuge tubes, washed with ammonia free buffer 3 times, then concentrated using microcentrifugation to a suspension of 10^9 or 10^{10} cells/mL. Cell counts were performed directly under phase-contrast light microscopy.

The cell suspension was added to 12 mL glass vials for a total volume of 1 mL containing cells, 10 mM NO_2^-, and either 0.1 mM PMS and 1 mM ascorbic acid, or 2 mM hydrazine hydrochloride as an electron source to facilitate nitrite reduction (n=3). This concentration of nitrite was within the range produced by each strain of bacteria in batch culture, hence determined to be physiologically relevant. PMS and ascorbic acid are a widely used artificial electron-donor system with ascorbic acid as an electron donor and PMS as an intermediate electron shuttle. Hydrazine (N_2H_4) is a competitive substrate for HAO and therefore an efficient electron donor for NO_2^- reduction, but its oxidation does produces N_2 instead of NO, and therefore it does not compound the observations of N_2O production. The serum vials were sealed with Wheaton rubber stoppers and aluminum crimp seals and sparged with N_2 gas for 10 minutes until anoxia was established. Oxygen levels were measured at 0 hours to confirm that cells were under anoxia. The aluminum seals on the vials were taped down and sealed with parafilm, then the vials were incubated at 30°C upside down to prevent gas leakage in order to maintain anoxia and to accumulate N_2O in the headspace. After 96 hours, N_2O levels were measure using the Haysep Q column on a TCD-gas chromatograph as described above. Measurements were converted to volume of gas in the

headspace using standard curves made using the respective pure gases based on the ideal gas law formula using Microsoft excel. In another experiment, measurements were made at multiple time points: 1 hour, 2 hours, 4 hours, 6 hours, and finally 72 hours, to examine the rate of N_2O production.

To ensure that N_2O was enzymatically produced by the cells, several controls were included (n=3). For killed cell controls, the cell suspension was incubated in a water-bath at 95°C for 10 minutes to heat kill the cells, then added to the glass serum vials with NO_2^- and reductant. Reductant free controls contained live cells and NO_2^- without a reducing agent to test if there was abiotic reduction of NO_2^- to N_2O. Since N_2H_4 is a competitive substrate for HAO and is a highly reactive chemical, one set of controls contained cells and N_2H4 without NO_2^-. For controls lacking NO_2^- or N_2H_4, the equivalent volume of buffer was added in place of NO_2^-, or the reductant, respectively.

2.3.4 Biomass collection for proteome analysis

The growth conditions were scaled up to maximize the amount of biomass collected for proteome analysis. Each sample for proteome analysis consisted of cells from 5 liters of culture. Samples were prepared in triplicate for each strain and phase. Cells were grown as 500 mL cultures in 1 L Wheaton bottles with butyl rubber seal caps as described above. To decide on sampling times, growth and N_2O production were measured as described above (Supplementary figure 1).

For mid-log phase samples, which was determined as the time point immediately prior to when N_2O was detected, *N. europaea* cells were collected after 24 hours of growth, and *N. communis* cells were collected after 48 hours of growth (supplementary). For stationary phase samples, cells were collected from both strains after 84 hours of growth.

Cells were collected by filtering the culture through 0.22 µm PES membrane Stericup® Vacuum Filters. Cells were washed off the filter disc using ammonia-free HEPES buffer (described above). Cell suspensions were aliquoted to Eppendorf® tubes, washed with buffer, and concentrated using

microcentrifugation. After centrifugation, all buffer was removed from the cell pellet and cell pellets were flash frozen in liquid nitrogen, stored at -80°C, and transported on dry ice to the Proteomics Core Facility at the University of British Columbia.

2.3.5 Sample preparation for mass spectrometry

The sample preparation and protein identification by mass spectrometry was carried out by Jeanne Yuan and Jason Rogalski at the Proteomics Core Facility as described below.

Cell pellets were lysed with lysis buffer (2% SDS, 100 mM Tris, pH 6.8), and boiled at 95°C for 10 minutes. The protein concentration of each sample was measured using the BCA assay.

From each sample, 20 µg protein was run on a SDS-PAGE gel at constant 200 V for 15 min. The gel was fixed for 35 min, stained for 1 h, and washed in water overnight. Bands were excised from the gel and cut into pieces (~1-2 mm per side). These were then washed with de- stain buffer (50 mM NH_4HCO_3/100% EtOH – 6:4), then dehydrated in EtOH. Reduction of disulfide bonds was done by incubation in 10 mM DTT for 45 min at 56°C, followed by alkylation in 55 mM IAA for 30 min at room temperature in the dark. Gel pieces were then dehydrated in EtOH, rehydrated in digestion buffer (50 mM NH_4HCO_3 – pH 8), then dehydrated again. The samples were digested with the addition of 0.45 – 0.5 µg trypsin and incubated at 37°C overnight.

Digestion was stopped with 10% TFA and samples were extracted twice with extraction solution (acidified water with acetonitrile – 40% ACN, 0.1% TFA). Samples were then concentrated via vacuum centrifugation.

Extracted peptide samples were cleaned up via STAGE-tip purification. Briefly, the resolubilized, acidified sample was forced through a conditioned and equilibrated column with 10 mm of C18 packing, washed with 0.2% TFA twice, and eluted into clean tubes by buffer containing 40% ACN, 0.1% TFA, and were then desiccated.

2.3.6 Protein identification by mass spectrometry

Samples were reconstituted in 0.5% ACN, 0.1% formic acid, and their concentration was measured using NanoDrop One (Thermo Scientific) with the A205 Scope method (absorbance at 205 nm, baseline correction at 340 nm). The peptides were analyzed using a quadrupole – time of flight mass spectrometer (Impact II; Bruker Daltonics) on-line coupled to an EasyLC 1200 HPLC (ThermoFisher Scientific) using a Captive spray nanospray ionization source (Bruker Daltonics) including Aurora Series Gen2 (CSI) analytical column, (25 cm x 75 µm 1.6 µm FSC C18, with Gen2 nanoZero and CSI fitting; Ion Opticks, Parkville, Victoria, Australia). The analytical column was heated to 40°C using tape heater (SRMU020124, Omega.com and in house build microprocessor temperature controller). Buffer A consisted of 0.1% aqueous formic acid and 2 % acetonitrile in water, and buffer B consisted of 0.1% formic acid in 80 % acetonitrile. Samples were run with 90 min gradient, in which the gradient started from 5% B to 13% B over 45 min, then to 35% B from 45 to 90 min, then to 90% B over 2 min, held at 90% B for 13 min. Before each run the analytical column was conditioned with 4 µL of buffer A. The LC thermostat temperature was set at 7°C. The analysis was performed at 0.40 µL/min flow rate. Impact II was run with OTOF Control v. 5.2 (Bruker). LC and MS were controlled with HyStar 5.0 SR1 (5.0.37.0, Bruker). The Impact II was set to acquire in a data-dependent auto-MS/MS mode, fragmenting the 20 most abundant ions (at 18 Hz rate) after each full-range scan from m/z 200 Th to m/z 2000 Th (at 5 Hz rate). The isolation window for MS/MS was 1 to 3 Th depending on parent ion mass to charge ratio and the collision energy ranged from 23 to 65 eV depending on ion mass and charge. Parent ions were then excluded from MS/MS for the next 0.4 min and reconsidered if their intensity increased more than 4 times. Singly charged ions were excluded since in ESI mode peptides usually carry multiple charges. Strict active exclusion was applied. Mass accuracy: error of mass measurement is typically within 5 ppm and is not allowed to exceed 10 ppm. The nano ESI source was operated at 1900 V capillary voltage, 0.25 Bar pressure with methanol in the nanoBooster, 3 L/min drying gas and 150°C drying temperature. Acquired data was then searched against either *Nitrosomonas europaea* fasta (reviewed + unreviewed downloaded from uniprot) or *Nitrosomonas communis* (unreviewed, from uniprot) depending on the species using the Byonic search Algorithm (v4.0.12) from Protein Metrics Inc. Full digestion

specificity with a maximum of one missed cleavage, 20 ppm and 30 ppm mass accuracies for precursors, and product ion masses with a 1% false discovery rate cut-off were used. Carbamidomethyl at Cysteine was added as a fixed modification and Deamidation at glutamine and oxidation at Methionine were added as variable modifications.

2.3.7 Proteome data analysis

The excel output from the Byonic software identifies proteins with a description and a unique protein database (DB) number and ranks the proteins based on the absolute value of the log base 10 of the protein P-value, named |Log Prob|. The protein P-value is the likelihood that the peptide-spectrum matches (PSM) to this protein arises by chance, so a higher |Log Prob| value indicates a higher level of confidence in the identification of a given protein from the spectral matches. The Byonic output was analyzed using Microsoft excel. For each dataset (mid-log phase from *N. europaea,* stationary phase from *N. europaea*, mid-log phase from *N. communis,* and stationary phase from *N. communis*) the mean protein rank and mean |log prob| values were calculated from the triplicate samples by matching the corresponding values to the appropriate proteins. Only proteins for which rank and |log prob| values were found in all three replicates were considered for analysis as the absence of these values in any one replicate indicated that that the specific protein was not detected in that respective sample.

The goal of the proteomic analysis was to determine which proteins are reasonable candidates for performing nitrifier denitrification in *N. communis* based on what proteins are more abundant during stationary phase in comparison to mid-log phase (before and during nitrous oxide production). However, since the Byonic software does not quantify the abundance of proteins, the rank and |log prob| values of certain proteins of interest from *N. europaea* were examined to detect any predictable patterns of change between mid-log and stationary phase. The proteins examined in N. europaea were nitrite reductases and nitric oxide reductases, multicopper oxidases, and cytochrome p460, all proteins which have been biochemically demonstrated to have nitrite reducing capacity or nitrous oxide

producing capacity, and also nitrosocyanin, a copper-based redox protein that is unique and highly expressed in AOB. The same set of proteins were examined in the *N. communis* datasets when present. Since no predictive patterns were identified, other proteins of interest were identified based on their presence vs absence between the two growth phases.

Proteins that were present only in stationary phase of *N. europaea* and *N. communis* samples, respectively, were identified as proteins that did not yield a rank or |log prob| value in any of the triplicates in the mid-log phase samples, but were present in triplicates of the stationary phase samples. The accession numbers were first isolated in the excel files and were then were used on the UniProt ID mapping tool to retrieve the corresponding FASTA sequences. The FASTA sequences were used to retrieve the COG categories of these proteins using EggNOG mapper v2. Based on the COG categories and the protein descriptors, proteins of interest for nitrifier denitrification were identified from the *N. communis* stationary phase samples.

2.4 Results

2.4.1 Batch culture growth

The growth patterns of *N. communis* and *N. europaea* as determined by NO_2^- production show similar trends, but *N. communis* produces less NO_2^- than *N. europaea* (Figure 2.1, A). Both sets of cultures reach log phase at 24 hours and stationary phase at 60 hours. During the lag and log phases, *N. communis* cultures produce much less NO_2^-, but by stationary phase, both sets of cultures produce similar amounts of NO_2^- at 13 ± 0.087 mM for *N. europaea* and 12 ± 0.92 mM for *N. communis*. Biomass from both sets of cultures reached a dry weight of 2.5 mg. This gives a total of 0.52 mmol NO_2^- produced per mg of *N. europaea* cells and 0.48 mmol NO_2^- produced per mg of *N. communis* cells. For both strains, ~60% of the starting NH_3 concentration was consumed and oxidized to NO_2^-.

O_2 consumption by each strain is inversely related to their NO_2^- production (Figure 2.1, B). In *N. europaea* cultures, O_2 was consumed faster. Within 48 hours, the mean amount of O_2 in the headspace

had dropped to 0.68 ± 0.038 mmol, whereas in *N. communis* cultures, the O_2 levels were at 1.2 ± 0.030 mmol at 60 hours. The biggest difference between the two strains were observed for N_2O production during NH_3 oxidation (Figure 2.1, B). Measurable amounts of N_2O were observed starting at 36 hours for *N. europaea* and at 48 hours for *N. communis. N. europaea* cells were capable of producing nearly twice the amount of N_2O as *N. communis* cells at the assay end point with 12 ± 0.016 µmol N_2O and 7.5 ± 0.0030 µmol N_2O produced by each strain respectively. While N_2O levels increased as O_2 was consumed, it was still being produced during aerobic NH_3 oxidation. Therefore, observed N_2O was not solely due to nitrifier denitrification. Cell free controls were free of NO_2^- and N_2O and did not show a decreasing trend for O_2.

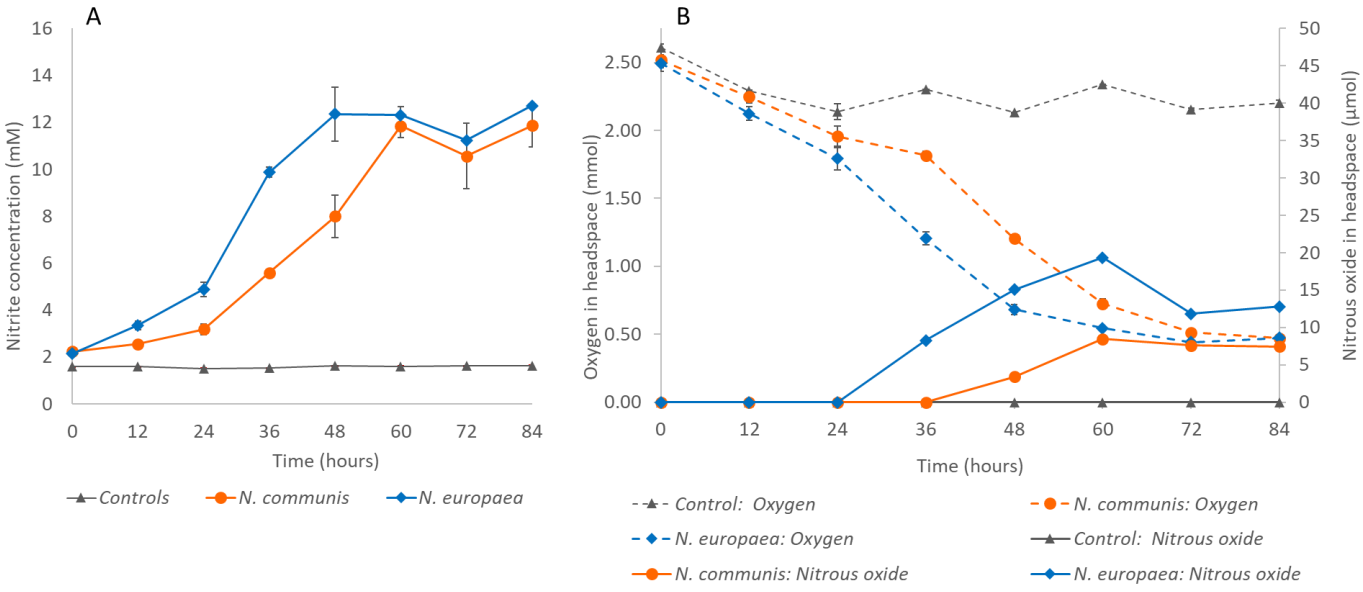


Figure 2.1 Batch culture growth of *N. communis* NM2 and *N. europaea* ATCC 19718. Nitrite production (A) as a measure of growth. (B) Oxygen consumption and nitrous oxide production measured in 100 mL cultures grown with 20mM ammonia to an average endpoint biomass of 2.5 mg/L. Error bars represent standard deviation of measurements across 4 replicate cultures for bacterial strains and 2 for controls.

2.4.2 Resting cell assays

Anoxic resting cell assays measure N_2O produced from NO_2^- reduction as opposed to during aerobic NH_3 oxidation. While measurable amounts of N_2O were produced by both strains after 96 hours, *N. communis* cells consistently produced less N_2O than *N. europaea* cells with either reductant source (Table 2.1). Hydrazine as a reductant was favored for NO_2^- reduction over PMS and ascorbate, since more N_2O was observed when cells were incubated with hydrazine. In fact, *N. communis* produced less than a tenth of the N_2O with PMS and ascorbate, as with hydrazine. In the controls containing only NO_2^-, where no reductant was provided to the cells, background N_2O was observed, but with less than two orders of magnitude compared to when cells were provided with a reductant, and there was no difference between the two strains. No N_2O was observed from vials containing killed cells or zero nitrite. N_2O observed in these assays from *N. communis* cells indicate that the cells have NO_2^- reducing ability, albeit with less efficiency than *N. europaea*.

Table 2.1 Resting cell assays measuring nitrous oxide produced from nitrite reduction. Nitrous oxide measured by TCD-GC from stationary phase cells after 96 hours of incubation under anoxia with nitrite and a reductant without growth substrate. Data presented as means (n=3).

	Nitrous oxide produced (µmol/ 10^9 cells)	
	N. europaea	*N. communis*
Experimental assays		
10mM Nitrite 0.1mM PMS & 1mM Ascorbic acid	2.39	0.118
10 mM Nitrite 2mM Hydrazine hydrochloride	4.54	3.38
Controls		
10 mM Nitrite (no reductant)	0.0274	0.0275
(no substrate) 2mM Hydrazine hydrochloride	0	0
Killed cell control 10mM Nitrite 0.1mM PMS & 1mM Ascorbic acid	0	0
Killed cell control 10 mM Nitrite 2mM Hydrazine hydrochloride	0	0

In the assays where the accumulation of N_2O was measured periodically, *N. europaea* cells were observed to reduce NO_2^- quite rapidly. Within an hour, 1.92 µmol of N_2O was measured, and the maximum amount measured was 3.10 µmol at 4 hours (Figure 2.2). In comparison, N_2O was not observed in vials with *N. communis* cells until 4 hours, and only accumulated to a maximum of 0.475 µmol at 72 hours. The decreased measurements in the *N. europaea* samples at 72 hours was likely due to gas leakage.

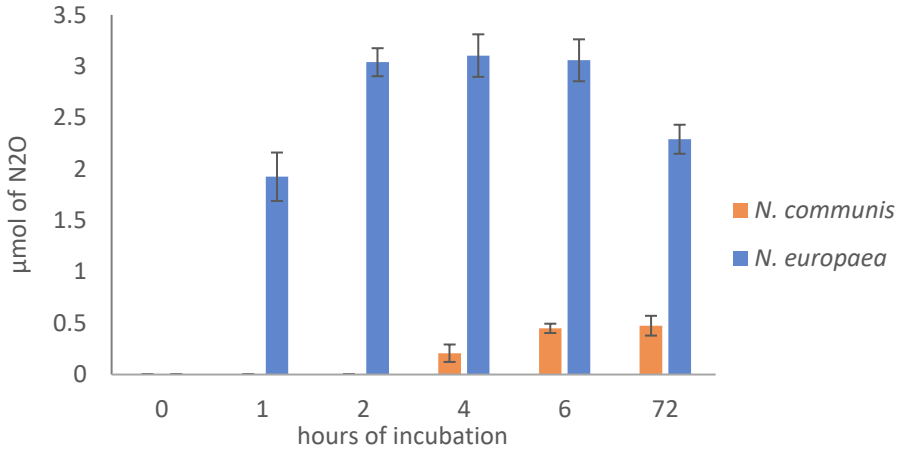


Figure 2.2 Nitrous oxide production in resting cell assays. Stationary phase cells from each strain were incubated in glass vials (n=3) under anoxia with 10mM nitrite, 2mM hydrazine hydrochloride, and nitrous oxide was measured periodically using TCD-GC.

2.4.3 Proteome analysis

A total of 1175 proteins were detected in the *N. communis* samples, with 1009 proteins detected in all replicates of mid-log phase samples, 1135 proteins detected in all replicates of stationary phase samples, and 969 proteins detected across all samples. In *N. europaea* samples, a total of 1098 proteins were detected, with 1088 proteins in all replicates of mid-log phase samples, 1070 proteins in all replicates of stationary phase samples, and 1060 proteins detected across all samples (both phases).

For *N. europaea,* several proteins that implicated in N_2O production and NO_2^- reduction were detected in both mid-log phase and stationary phase samples without a clear trend of increase or decrease in rank between the two phases. The copper containing NO_2^- reductase NirK, which is known to be necessary for effective NH_3 oxidation (Cantera & Stein, 2007) was ranked higher in stationary phase by approximately 2 standard deviations (Table 2.2). NO reductase NorQ is part of the *norCBQD* gene cluster which encodes the cNOR NO reductase, which is considered an essential enzyme for nitrifier denitrification (Klotz & Stein, 2008; Kozlowski et al., 2014). NorQ and NorBC were also detected in both phases without much difference in their rankings between the phases. For *N. communis,* a NO_2^- reductase was detected in similar ranks in both phases. Even though the *N. communis* genome contains a *norCBQD* gene cluster(Kozlowski et al., 2016a), no NOR proteins were detected in either phase. Cytochrome P460 converts NH_2OH to N_2O directly under anoxic conditions. This enzyme was detected in both phases in both strains in similar ranks. Nitrosocyanin, which is a protein that is known to be highly expressed in AOB and have been proposed as a candidate for the unknown nitric oxide oxidase (NOO) (Arciero et al., 2002; Whittaker et al., 2000) was also present in both strains. Furthermore, two multicopper oxidases were detected in *N. europaea,* both of which were ranked much higher in stationary phase than in mid log phase (by more than 5 standard deviations). A multicopper oxidase was also present in both phases for *N. communis* although the rankings were similar (Table 2.2).

Table 2.2 Proteins of interest based on previous literature

		Mid-Log Phase		Stationary Phase	
Accession number	Description	Mean Rank	Mean |Log Prob|	Mean Rank	Mean |Log Prob|
N. europaea					
A0A837WYT9	Copper-containing nitrite reductase	38.0	175.9	16.7	249.3
Q82TG8	Nitric oxide reductase NorQ protein	50.3	154.2	73.3	131.8
Q82TA2	NorQ protein	1047.3	7.7	1101.3	6.3
A0A837WTE6	Nitric-oxide reductase (NorBC) subunit	1227.0	4.0	1059.3	7.3
Q820S6	Chain A, Red Copper Protein Nitrosocyanin	316.7	37.3	249.3	68.2
H2VFU9	Cytochrome P460	274.0	61.0	288.3	61.5
Q82XF9	Possible multicopper oxidase	495.7	36.8	37.0	178.9
A0A837X4X8	Multicopper oxidase	126.7	97.9	64.0	138.8
N. communis					
A0A0F7KC46	Nitrite reductase	64.7	138.4	71.7	195.6
A0A5D3YAI0	Nitrosocyanin	201.3	78.0	239.3	114.1
A0A0F7KD97	Multicopper oxidase	282.7	61.0	265.0	106.6
A0A1H2YJH6	Cytocrome P460	205.7	75.7	275.3	105.8

For *N. communis,* 166 proteins were uniquely present in stationary phase. Out of these, EggNOG mapper returned annotations for 147 proteins (Supplementary table 1). Out of these, 17 were uncharacterized proteins. The proteins were categorized into 24 COG categories, however 39 of them were categorized as 'Function unknown'. The next most abundant category was 'Energy production and Conservation' which accounted for 10% of the proteins unique to stationary phase. Within this category, several proteins of interest were identified that might have some capacity to be involved in nitrifier denitrification, NO_2^- and/or NO reduction, or other energy producing pathways. This includes several cytochromes with oxidoreductase activity and a nitrite/nitrate sensor histidine kinase (Table 2.3). For *N. europaea,* only 11 proteins were uniquely present in stationary phase, and out of these only one, a hydroxylase, falls into the energy production and conservation category with oxidoreductase activity (Supplementary table 2). Another was a transposase involved in replication and repair, while the rest were unknown in function.

Table 2.3 Proteins involved in energy production and secondary metabolism in stationary phase in *N. communis.* Highlighted rows show proteins of interest.

Accession number	Protein Description	Mean Rank	Mean |log Prob|	COG Category
A0A0F7KAQ2	**Cytochrome C**	**925.0**	**28.63**	**Energy production and conservation**
A0A0F7KEK1	Catalase	1711.3	35.62	Energy production and conservation
A0A0F7KF98	Acetyl-CoA synthetase	1557.0	42.98	Energy production and conservation
A0A0F7KG07	**Cbb3-type cytochrome c oxidase subunit III**	**1599.3**	**7.66**	**Energy production and conservation**
A0A0F7KG39	Alcohol dehydrogenase	1470.7	25.22	Energy production and conservation
A0A0F7KH97	Bifunctional protein PutA	926.3	23.39	Energy production and conservation
A0A0F7KJ72	NAD-reducing hydrogenase large subunit	1440.3	21.07	Energy production and conservation
A0A0F7KJR9	**Cytochrome C**	**1241.7**	**17.04**	**Energy production and conservation**
A0A1H2TZP9	Glycerophosphoryl diester phosphodiesterase	1543.7	10.69	Energy production and conservation
A0A1I4JNZ8	**Decaheme c-type cytochrome, DmsE family**	**1475.0**	**2.77**	**Energy production and conservation**
A0A1I4PBG1	ATP synthase subunit delta	1459.7	88.23	Energy production and conservation
A0A5D3YF35	Glycine/D-amino acid oxidase-like deaminating enzyme	1175.3	61.96	Energy production and conversion, Amino acid metabolism and transport
A0A0F7KF70	Thiol-disulfide isomerase/thioredoxin	1420.0	5.76	Energy production and conservation, Posttranslational modifications, protein turnover, chaperones
A0A0F7KHB1	Glutaredoxin-like protein DUF836	1029.3	4.06	Energy production and conservation, Posttranslational modifications, protein turnover, chaperones
A0A5D3YF74	**Two-component system nitrate/nitrite sensor histidine kinase NarX/two-component system sensor histidine kinase UhpB**	**532.0**	**34.86**	**Signal Transduction mechanisms**

2.5 Discussion

2.5.1 Differences in growth, nitrite production and oxygen consumption

N. europaea and *N. communis* are both eutrophic, aerobic, Betaproteobacteria AOB strains belonging to the genus *Nitrosomonas* (Prosser et al., 2014). While *N. europaea* is well studied, not much is known about *N. communis* other than its genome and that it was observed to be capable of producing NO and N_2O (Kozlowski, Kits, et al., 2016a, 2016c). The observed growth results for *N. communis* batch cultures show that it is slower than *N. europaea* in oxidizing NH_4 to NO_2^-. *N. europaea* consistently produced more NO_2^- earlier in the growth curve than *N. communis* (Figure 2.1). At end point however, both strains produced similar amounts of NO_2^- per cell using up to 60% of the starting NH_3 concentration and reached the same dry-weight biomass. O_2 consumption by each strain is inversely corelated to NO_2^- production. *N. europaea* consumes O_2 faster than *N. communis* and the remaining O_2 levels in both sets

of cultures at the endpoint were around 0.47 mmol. Since O_2 availability is limited in this batch culture system, it is likely that stationary phase was reached due to the limitation of O_2. In systems where O_2 is unlimited, perhaps the differences in NO_2^- production between the two cultures would be more pronounced even at the endpoint. Measuring growth indirectly using NO_2^- concentration is a standard practice for AOB since cultures do not become turbid enough to accurately measure growth using optical density. However, it would have been more informative to also measure growth using cell counts to infer if the slower NO_2^- production is also reflected in a slower doubling time for *N. communis* cells. The genomes of both strains encode genes for AMO and HAO which catalyse the oxidation of NH_3 to NH_2OH and NH_2OH to NO respectively (Figure 1.4). There is no evidence to suggest that there are differences in these enzymes of these two strains that could result in slower NH_3 oxidation by *N. communis.* Both strains also encode genes for carbon fixation via the Calvin cycle, and the observation that similar amounts of NO_2^- produced resulted in similar biomass measurements is consistent with both strains having the same carbon fixation method.

One reason for less efficient NO_2^- production of *N. communis* in comparison to *N. europaea* could be the lack of the NirK enzyme in *N. communis*. In *N. europaea,* NirK plays a role in maximizing NH_3 oxidizing metabolism to speed up cell growth by acting as an electron sink that promotes NH_2OH oxidation (Cantera & Stein, 2007). This was observed in a NirK-deficient mutant of *N. europaea* where overall cell doubling time was significantly higher than wildtype cells, and NH_2OH consumption was dramatically decreased in under high and low O_2 tensions, more so than NH_3 consumption (Cantera & Stein, 2007). While NH_2OH consumption in *N. communis* was not specifically tested in the current study, *N. communis* was previously observed to be more sensitive to NH_2OH than other AOB strains, tolerating concentrations only up to 100 µM in comparison to 250 µM by other strains (Kozlowski et al., 2016c). This is consistent with the suggested role of NirK for promoting NH_2OH oxidation, since a strain lacking it would be ill equipped to tolerate higher concentrations of NH_2OH, as is seen for *N. communis*.

2.5.2 *N. communis* produces less nitrous oxide than *N. europaea* suggesting different enzymology for nitrifier denitrification

N. europaea consistently produces more N_2O than *N. communis* both during aerobic growth and in resting cell assays which are anoxic (Figure 2.1, Table 2.1). This implies that the enzymology for N_2O production during NH_3 oxidation and nitrifier denitrification between these two strains is different.

N_2O produced during aerobic NH_3 oxidation is from the reduction of intermediates NH_2OH and/or NO. Nitrifier denitrification is primarily active under low oxygen tension and typically the amounts of N_2O produced by nitrifier denitrification far exceeds the amounts produced during aerobic NH_3 oxidation. This is due to the limitation of O_2, which causes an "electron flow bottleneck" in the electron transport chain. At this point, NO_2^- and NO become electron sinks in the periplasm, which indirectly facilitates electron flow to the terminal oxidase by membrane-bound complexes for the generation of PMF. During oxygen limited batch culture growth, measurable amounts of N_2O were detected starting at 36 hours in *N. europaea* cultures and at 48 hours in *N. communis* cultures (Figure 2.1) before either was depleted of O_2, although more N_2O was measured in the *N. europaea* cultures. One reason for observing a higher rate of N_2O production in *N. europaea* cultures would be their faster rate of O_2 consumption, hence activating nitrifier denitrification at an earlier time point. Another reason would be its more efficient NH_3 oxidation, which would result in more substrates (NO and NO_2^-) available for N_2O production.

Even though the amounts of N_2O measured from *N. communis* cultures during aerobic growth was less than that from *N. europaea* cultures, the observation confirms what has been previously reported, which is that *N. communis* retains the ability to produce N_2O despite lacking the dominant NO_2^- reductase NirK (Kozlowski et al., 2016c). This study by Kozlowski et al., reported that in micro-respirometry experiments, *N. communis* produced NO during ammonia oxidation and N_2O upon O_2 depletion (Kozlowski, et al., 2016c). While the present study did not measure NO or use NH_2OH as a

substrate, the growth results show that *N. communis* does indeed produce N_2O during NH_3 oxidation. At the time of publication for the previous study, NO was not known to be an obligatory intermediate for NH_3 oxidation. It was previously thought that HAO oxidized NH_2OH directly to NO_2^-. Hence the production of NO by *N. communis* was sufficient evidence to suggest that an alternative NO_2^-reductase other than NirK must be present in *N. communis* and possibly in other AOB. However, according to the updated model, HAO oxidizes NH_2OH to NO, so the observed N_2O during growth could be primarily from NH_3 oxidation rather than NO_2^-reduction (Caranto & Lancaster, 2017). Considering that N_2O measured during growth was observed before O_2 depletion, it is likely produced both from NH_3 oxidation and, perhaps, nitrifier denitrification.

To specifically test if *N. communis* was capable of producing N_2O by NO_2^- reduction was the purpose of the resting cell assays. Since these assays are conducted under anoxia, and do not provide any NH_3 as a substrate, any N_2O produced would have to arise from NO_2^- reduction or nitrifier denitrification based on the lack of activity in killed-cell and no cell controls. Since there is no growth substrate or O_2 to act as a terminal electron acceptor, the cells would need to activate anaerobic metabolic pathways to sustain N_2O production. NO_2^- is provided as a substrate, and a reductant or electron source is provided to facilitate electron flow to reduce NO_2^-. If the enzymology for NO_2^- reduction is present in the cells, NO_2^- will be reduced to NO and then to N_2O. If NO_2^-reducing enzymology is absent, no N_2O would be observed.

Both AOB strains were able to produce N_2O in the resting cell assays with both types of reductants used, PMS & ascorbate or hydrazine (N_2H_4) (Table 2.1). This shows that both strains are capable of NO_2^- reduction under anoxia, implying that enzymology for NO_2^- reduction is activated under low O_2 tension in both species. In early iterations of the resting cell assays (data not shown), anoxia was not maintained in the vials due to O_2 leakage from the rubber stoppers. This resulted in little to no N_2O produced, which was indicative that the enzymatic reduction of NO_2^- was activated only under anoxia. No N_2O was

measured in the cell free controls, killed cell controls, and substrate free controls. Vials containing cells and NO_2^- without a reductant did produce small amounts of N_2O, but the measured amounts were at least two orders of magnitude smaller than the amounts measured from vials containing cells, NO_2^- and reductant. This set of experiments and controls confirms the validity of the assay that N_2O was indeed produced by enzymatic reactions from live cells.

N. europaea produced much more N_2O than *N. communis* with PMS & ascorbate as a reductant, while both strains had similar results when hydrazine was used as a reductant. The same strain also produced N_2O much faster than *N. communis*, where more than 50% of the total amount was produced within one hour of incubation for *N. europaea* cells, but 4 h incubation was required to first observe N_2O production by *N. communis* cells. Both observations suggest that the enzymology for nitrifier denitrification is different between the two strains and that nitrifier dentification enzymology in *N. europaea* is much faster and more efficient. In this instance, the difference could not be attributed to the lack of NirK in *N. communis* since NirK was previously shown to not be the active NO_2^- reductase in *N. europaea* either.

Both strains produced more N_2O when hydrazine was used as a reductant rather than the artificial electron donor-shuttle system PMS & ascorbate. Hydrazine (N_2H_4) is a competitive substrate for HAO, but unlike NH_2OH, its oxidation produces N_2 rather than NO (Hooper & Nason, 1965; Maalcke et al., 2014; Schatteman et al., 2022). Hence, the results of the resting cell assay wouldn't be confounded with multiple sources of NO. It is likely that NO_2^- and NO reduction is more efficient in AOB with hydrazine because it is a more natural electron source for AOB. The difference of using hydrazine as a reductant was more pronounced in *N. communis* where a mean of 3.38 µmol $N_2O/10^9$cells was observed with hydrazine as opposed to 0.11 µmol $N_2O/10^9$cells with PMS & ascorbate. In the study by Kozlowski, et al., (2016c), they reported that in *N. communis*, N_2O production was stronger when NH_2OH was provided as a substrate instead of NH_3, and suggested that an enzymatic pathway for N_2O formation under anoxia is

likely activated in the absence of NH_3. It is interesting that under the same conditions, having hydrazine as a substrate for HAO rather than NH_2OH seems to render similar results. Perhaps oxidation of either substrate by HAO is in itself a trigger for anaerobic N_2O production.

2.5.3 Proteins involved in nitrous oxide production

Some proteins known to be involved in nitrous oxide production in AOB were detected in the proteomes of *N. communis* and *N. europaea* in both mid-log and stationary phase samples (Table 2.2). In *N. europaea,* the copper containing NO_2^- reductase NirK, and the cNOR NO reductase consisting of NorQ, and NorBC proteins were detected in both phases. While NirK is not essential for nitrifier denitrification, it is known to be highly expressed in *N. europaea,* therefore its presence in the proteome is expected. NorB is considered to be essential for nitrifier denitrification, and its presence in the *N. europaea* proteome confirms that nitrifier denitrification is active in the batch cultures. Due to limitations of the present study, an alternate NO_2^- reductase could not be identified in the *N. europaea* proteomes.

In *N. communis*, whose genome does not encode *nirK,* a different NO_2^- reductase was detected in both phases (Table 2.2). UniProt identifies the protein additionally as a Sulfite reductase (NADPH) hemoprotein beta-component. It contains two NIR_SIR (nitrite reductase and sulfite reductase) domains and two NIR_SIR_FERR (ferredoxin-like) domains. Sulfite and nitrite reductases consist of a siroheme domain and catalyses the six-electron reduction of sulfite to sulfide and nitrite to ammonia. These enzymes are found in bacteria, archaea and eukarya and are essential for assimilation of sulfur and nitrogen, especially in plants. Some SIR and NIRs derive reducing equivalent from ferredoxin and hence contain ferredoxin like binding domains (Crane et al., 1995). In *Escherichia coli*, the NirB nitrite reductase also contains a siroheme, and is expressed in response to increased nitrate concentrations (Wang & Gunsalus, 2000). In the opportunistically pathogenic fungus *Aspergillus fumigatus,* the lack of siroheme in its nitrite reductase NiiA increases its sensitivity to NO (Dietl et al., 2018). While the presence of this enzyme in *N. communis* shows that the cells have NO_2^- reducing ability, it is unclear if this enzyme is

involved in N_2O production through nitrifier denitrification. If this enzyme is like other siroheme containing NIRs, it would reduce NO_2^- to NH_3 rather than to NO.

The genome of *N. communis* carries the operons *norCBQD* and *norSY-senC-orf1* which encode two NO reductases (Kozlowski et al., 2016a). Interestingly neither of these enzymes were detected in the proteome of *N. communis* in either mid-log or stationary phases. This bears some similarity to the protein expression of another AOB, *N. eutropha,* where none of the NorCB proteins were detected under the conditions tested, but in contrast, the NorY nitric oxide reductase was detected in oxic and anoxic conditions that also contained nitrogen dioxide (Kartal et al., 2012). The lack of NOR expression in *N. communis* may offer an explanation as to why the cells produced a small amount of N_2O in comparison to *N. europaea* both during both growth and in resting cell assays. While the resting cell assays confirm that *N. communis* can produce N_2O using NO_2^- as the only substrate, it is possible that the observed N_2O was a result of hybrid formation from excess NO. This is still consistent with what was previously reported by Kozlowski et al., 2016c, where an excess of NO was observed upon the depletion of O_2 after NH_3 oxidation, and in comparison the amount of N_2O produced was much smaller for *N. communis* than for *N. europaea*.

Both strains also expressed Cytochrome P460, which is known to convert NH_2OH directly to N_2O in *N. europaea* anaerobically (Caranto et al., 2016). It is likely that this activity is retained in *N. communis* as well, which would provide another source for N_2O produced during aerobic growth when NH_2OH was produced, but not during the resting cell assays as no NH_2OH was available for this reaction. The role of the red copper protein nitrosocyanin is yet to be determined, but it has been proposed to be the yet unidentified NO oxidase/oxidoreductase or to have a similarly essential role in NH_3 oxidation since it is expressed in AOB at similar levels to proteins involved in central metabolic pathways, and its sequence is homologous to mononuclear blue copper proteins and the type 1 copper binding region of nitrite

reductase (Arciero et al., 2002). Hence it is unsurprising that this protein was detected in the proteomes of both strains (Table 2.2).

Multicopper oxidases were also examined as proteins of interest since they have NO_2^- reducing capacity. Specifically, the blue-copper oxidase encoded by *ncgA* in *N. europaea* was reported to have NO_2^- reducing activity. Two multicopper oxidases were detected in the *N. europaea* proteome and they were ranked much higher in stationary phase (Table 2.2). One multicopper oxidase was also identified in *N. communis*, which was ranked similarly in both phases. Further investigation into these specific proteins will be necessary to determine their involvement, if any, in nitrifier denitrification, but they are reasonable candidates to examine for NO_2^- reductase activity.

2.5.4 Expected proteomic responses based on existing literature

The transcriptomic and proteomic response of *N. europaea* to anoxic/oxic cycling, energy starvation, oxygen and ammonia limitation has been previously studied in order to gain insight into the metabolic processes of AOB (Otto et al., 2014; Pellitteri-Hahn et al., 2011; Sedlacek et al., 2020; Wei et al., 2006; Yu et al., 2018; Zorz et al., 2018). While the conditions tested in these studies vary, they provide context as to what could be expected in the proteomic response from *N. europaea* cells. The transcriptomic and proteomic responses often seem to differ rather than align and it appears that changes in the proteome are more profound in long term adaptations whereas short term responses are regulated at the transcript level.

This is evident in a study by Yu et al., 2018, which examined the physiological, transcriptomic, proteomic, and metabolomic response of *N. europaea* cells that were cycled through oxic and anoxic conditions daily for 13 days. The study reports that transcripts of *amo* and *hao* were decreased during anoxic states and reverted back to pre-anoxia levels upon transition to an oxic state, while the opposite trend was observed for *nirK* transcripts. However no significant differences were observed in

transcription levels in the long term. In contrast, intracellular protein concentration for AMO, HAO and NIR were not significantly changed during oxic-anoxic cycling but at the end of a 13 day adaptation period, overall protein concentration of both AMO and NIR had increased in the cells that were now adapted to the cyclic culture conditions (Yu et al., 2018). Although anoxia or hypoxia is reached in stationary phase of oxygen-limited batch cultures that were tested in the present study, it is quite different from transient anoxic-oxic cycling tested in the previous study. Hence, the proteome response in stationary phase would not be expected to be comparable.

The study by Sedlacek et al., 2020 reports the global transcriptomic response of *N. europaea* cells to steady state NH_3 limited and O_2 limited growth. They found no significant differences in the transcription levels of most core metabolic genes including *amo, hao, cycB* (Cyt c 552), *and ncyA* (nitrosocyanin) between the two steady states. Similarly, Zorz et al., 2018 reported that the corresponding proteins AMO, HAO, Cyt C552, and nitrosocyanin were expressed in the proteomes of three diverse AOB under ammonia replete sampling conditions, and that no significant differences were detected in these proteins for ammonia starved conditions. This is consistent with the results of the present study, where the same proteins were present in all samples of the *N. europaea* proteome without a notable difference in the protein ranks (Table 2.2, data not shown for AMO, HAO, and Cyt c552). The sampling conditions in the study by Zorz et al., would be the most similar to the mid-log phase sampling conditions in the present study, hence the similarities in the expression of the core metabolic proteins were expected.

A quantitative proteomic study by Pellitteri-Hahn et al., 2011 that examined the changes in protein abundances in response to ammonia starvation identified only 27 proteins that were significantly different in abundance between non-starved *N. europaea* cells and cells that were starved of ammonia for a period of 2 weeks, which is significantly longer than in the present study. Proteins involved in

nitrifier denitrification were not among these 27. Additionally, perhaps a longer incubation period under specific conditions is necessary to observe significant changes in the proteome.

The transcriptomic study by Sedlacek et al., 2020 is the only reported evidence of *N. europaea* cells response to prolonged oxygen limitation. While this incubation period in steady state growth was longer and different from the stationary phase conditions tested in the present study, the previous study does report on changes in the transcription of genes involved in N_2O production. The study reports that under O_2 limitation, *nirK* transcripts were significantly decreased, while transcripts for the blue copper protein *ncgA* were significantly increased. Transcription for the cNOR NO reductase, *norCBQD* were increased but not significantly, and transcription of *norSY-senC*, the alternate sNOR NO reductase were significantly increased (Sedlacek et al., 2020). In contrast, in the present study, the protein rank for NirK was increased in stationary phase, implying an increase of the protein during O_2 limited growth, while notable differences were not observed for the NorQ and NorBC proteins. Additionally, sNOR proteins and the blue copper oxidase encoded by *ncgA* were not detected in the proteome. As stated previously, it is possible that the response to oxygen limitation is regulated more at the transcription level than the protein level, or perhaps a longer incubation period is necessary for proteomic differences to be observed.

In contrast to *N. europaea*, *N. communis* is an understudied species of AOB. Since its discovery in 1991, its complete genome has been annotated, which allows for a predictive reference proteome. No previous studies have reported on its transcriptome, or proteome, under any condition. Hence, there is no existing literature for comparison or to verify our observations.

2.5.5 Proteins of interest in *N. communis* identified from proteins uniquely expressed in stationary phase

In order to identify candidate proteins that may be involved in nitrifier denitrification, we examined proteins that were uniquely expressed in stationary phase. For *N. europaea,* only 10 proteins were

found in all three replicates of stationary phase that were absent in all replicates of mid-log phase. For the majority of these, a COG category could not be identified. For *N. communis,* 166 proteins were present in stationary phase replicates that were absent in mid log phase. Since most of its physiology is yet unknown, it is unclear why such a large number of proteins were expressed in stationary phase for this organism. It is possible that it has a more elaborate stress response than its counterpart *N. europaea*. Furthermore, *N. communis* has been cultured in labs a much shorter duration than *N. europaea*, which likely plays a factor in how the cells respond to stress in batch culture.

Since its protein content has not been studied previously, its reference proteome is not well curated. Hence, only 147 out of 166 proteins could be matched to COGs, and a function could not be determined for a majority of them. Setting aside the uncharacterized proteins and those that could not be categorized, the most prominent COG category was energy production and conservation. Several cytochromes within this category were examined as proteins of interest due to their electron carrying capacity.

Two cytochrome c proteins were detected uniquely in stationary phase in *N. communis*, however several other cytochrome c proteins were detected in both mid-log and stationary phase proteomes as well (data not shown). Cytochrome c proteins are known to be involved in electron transport during ammonia oxidation and in nitric oxide reduction in AOB. In *N. europaea*, both types of NO reductases, cNOR and sNOR, are membrane bound cytochrome c oxidases, and four other cytochrome c proteins are involved in electron transport in NH_3 oxidation (Klotz & Stein, 2008; Sedlacek et al., 2020), so it is likely that cytochromes have similar roles in *N. communis* as well. Further studies of the sequences and structure of the cytochromes found in *N. communis* and *N. europaea* would be required to elucidate their role in stationary phase, and whether or not they are involved in electron transport to support nitrifier denitrification.

Cbb3-type cytochrome c oxidase subunit III was also detected in stationary phase of *N. communis* (**Error! Reference source not found.**), but multiple copies were also found in mid-log and stationary phase (data not shown). Out of the genome-sequenced AOB, *N. eutropha* encodes at least three of the four subunits for *cbb3* oxidase, and it was found to be expressed under oxic and anoxic conditions in the presence of nitrogen dioxide as an oxidant (Kartal et al., 2012; Stein et al., 2007). Cbb3 type cytochrome oxidases are expressed under anaerobic or microaerobic conditions and are high affinity oxidases (Pitcher & Watmough, 2004). NO reductase activity has been observed in the cytochrome cbb3 oxidase of *Pseudomonas stutzeri,* and the FixN catalytic subunit of the cytochrome cbb3 oxidase shares 21% identity in protein sequence with the catalytic subunit of cNOR, of which NorB is one type (Forte et al., 2001; Stein et al., 2007). Given that *N. eutropha* and *N. communis* both express Cbb3type cytochrome c oxidase, and do not express cNOR components, this protein is a promising candidate for an alternative NO reductase in these AOB strains.

Also expressed uniquely in the stationary phase of *N. communis* was a decaheme c-type cytochrome of the DMSE family. Decaheme c-type cytochromes are listed in the predicted proteomes of many species including *Nitrosomonas oligotropha, Nitrosomonas sp.* NM34 and *Nitrosomonas sp.* NM84, but its existence at the protein level is only confirmed in the extracellular iron oxide reducing bacterium *Shewanella oneidensis* (*Decaheme Cytochrome, DMSE Family in UniProtKB (573) | UniProt*, n.d.). In *S. oneidensis,* periplasmic proteins and outer membrane components form structures dubbed 'nanowires' that facilitate the transfer of electrons to external iron oxides (Pirbadian et al., 2014). MtrA, a protein critical to this process is a decaheme c-type cytochrome (Firer-Sherwood et al., 2011). MtrA, and the Decaheme c-type cytochrome in *N. communis* share the same paired CXXCH heme-binding domains. However, the potential role of such a protein in AOB is unknown, but offers an exciting prospect to examine further for its role in extracellular electron transport during anoxia.

From the other proteins that were expressed uniquely in stationary phase, the next most prominent categories were replication and repair and signal transduction mechanisms. Among the proteins in the signal transduction COG was one described 'Two-component system nitrate/nitrite sensor histidine kinase, NarX/two-component system sensor histidine kinase UhpB' (Table 2.3). NarX and NarQ are well studied nitrite/nitrate sensor histidine kinase two component systems in *Escherichia coli.* NarX responds to NO_3^- and, in combination with NarQ, regulates several operons including *nar* (nitrate reductase), *nir* (nitrite reductase), *nap* (periplasmic nitrate reductase), *frd* (fumarate reductase), *dcu* (dicarboxylate uptake), or *dms* (dimethylsulphoxide reductase) in *E. coli* (Gushchin et al., 2021). In *N. europaea*, NO_2^- reductase is regulated by the NO_2^- sensitive transcription repressor *nsrR*(Beaumont, Lens, et al., 2004; Klotz & Stein, 2008), but this transcription factor is absent from *N. communis*. Since AOB produce NO_2^- but not NO_3^-, the role of a NO_3^-regulating NarX protein component is puzzling especially since there is no evidence to suggest that *N. communis* has the other components of the NarXL and NarQP regulatory system.

2.5.6 Limitations of the proteome analysis and looking for candidate proteins

The initial approach for proteome analysis was to identify candidate proteins for nitrifier denitrification based on differences in protein expression levels. However, the type of analysis used in the current study including the process of sample preparation and injection as well as the Byonic software used to match spectra to protein databases are not suited to quantify the proteins that were detected. Although the |log prob| and protein rank values give some rough indication of how abundant a protein might be in a sample based on the logic that a more abundant protein would result in more spectral matches which would then be reflected in a higher |log prob| values, these values are not meant to quantify the proteins. Furthermore, proteins known to be involved in nitrifier denitrification such as NorQ and NorB, were not ranked higher in stationary phase proteomes as would be expected. Accounting for this limitation, the search for candidate proteins for nitrifier denitrification in *N. communis* had to be

simplified to consider only the proteins that were uniquely present in stationary phase. While this method was able to identify some proteins of interest, it is likely that other possible candidates and other proteins involved in nitrifier denitrification are not expressed uniquely in stationary phase, and hence would go undetected.

CHAPTER 3 Conclusions and future directions

The physiological and proteomic evidence gathered from *N. communis* in this study suggest that its enzymatic pathways for N_2O production is quite different from that in *N. europaea.* This study was able to confirm that *N. communis* is indeed able to produce N_2O during NH_3 oxidation and via NH_3 reduction. The latter implies that nitrifier denitrification must be at least partially active in this species. However, in both instances, *N. communis* produced less N_2O than *N. europaea* and at a slower rate. The absence of known NO reductases in its proteome may offer an explanation as to why less N_2O was produced by *N. communis* in contrast to *N. europaea*. However, the proteomic analysis did identify an alternative NO_2^- reductase in *N. communis,* as well as several cytochromes which may function as oxidases or reductases, including an intriguing multiheme cytochrome. The findings of this study suggest that *N. communis* is able to reduce NO_2^- enzymatically but may lack the ability to reduce NO enzymatically. The observed N_2O during anoxia could be produced from hybrid formation of excess NO, or through the action of cytochrome P460 in the presence of NH_2OH, although this would not account for the N_2O produced in the resting cell assay with hydrazine. However, the oxidation of NH_2OH or hydrazine via HAO appears to encourage N_2O production in *N. communis* even though the enzymology is unresolved.

Several further studies would be necessary to confirm the enzymatic pathways for N_2O production in *N. communis*. Firstly, it would be necessary to examine if *N. communis* has the ability to enzymatically reduce NO. The resting cell assays used in this study confirm NO_2^- reduction and N_2O production, but NO was not directly measured. Direct measurement of NO would further reveal which enzymatic activity is retained. Furthermore, the NO_2^- reductase and the potential candidates identified from its proteome would need to be further examined to confirm if they do indeed posses the expected enzymatic activity. This could be done by expression of candidate cytochromes in a heterologous host to test activity.

Little is known about *N. communis* outside of this study; hence a number of possibilities exist for future research. As observed in other studies of AOB proteomes and transcriptomes, it appears that short term responses to environmental changes are regulated more at the transcriptome level than the protein level. Hence, perhaps a comparative transcriptomic study would be better suited to identify changes in enzymology between mid-log and stationary phase cultures of AOB. It would also be interesting to explore the growth and physiology of *N. communis* in continuous cultures where it would be more productive for generating biomass and the responses to prolonged culture conditions could be tested. Ultimately, *N. communis* is only one of many diverse AOB that are vastly understudied. More research on more species of AOB is imperative to continue to widen our overall knowledge of nitrification and N_2O production by these important microorganisms.

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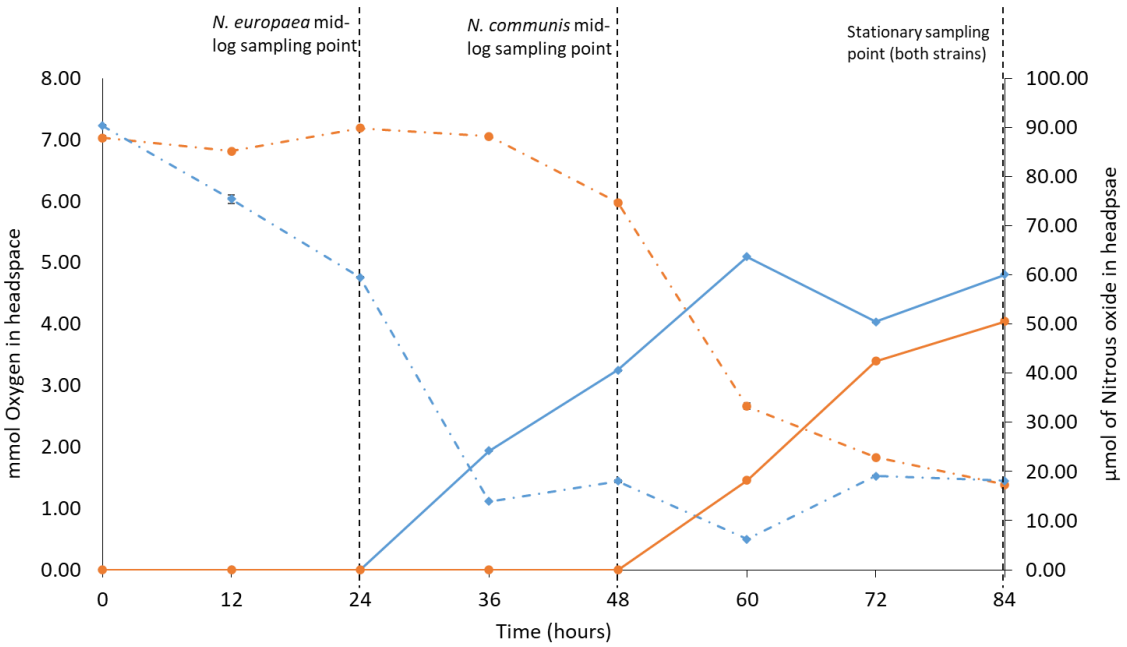
Appendix



Supplementary figure 1 Consumption of oxygen and production of nitrous oxide by large batch cultures of *N. communis* and *N. europaea*, and sampling scheme for proteomic analysis.

Supplementary table 1 Proteins expressed uniquely in stationary phase in *N. communis*. Highlighted rows show proteins of interest.

Accession number	Protein description	Mean Rank	Mean |log prob|	COG Categories
A0A1I4K7M2	Acetoin utilization deacetylase AcuC	1721.3	10.56	Chromatin structure and dynamics, Secondary metabolites biosynthesis, transport and catabolism
A0A0F7KAQ2	**Cytochrome C**	**925.0**	**28.63**	**Energy production and conservation**
A0A0F7KEK1	Catalase	1711.3	35.62	Energy production and conservation
A0A0F7KF98	Acetyl-CoA synthetase	1557.0	42.98	Energy production and conservation
A0A0F7KG07	**Cbb3-type cytochrome c oxidase subunit III**	**1599.3**	**7.66**	**Energy production and conservation**
A0A0F7KG39	Alcohol dehydrogenase	1470.7	25.22	Energy production and conservation
A0A0F7KH97	Bifunctional protein PutA	926.3	23.39	Energy production and conservation
A0A0F7KJ72	NAD-reducing hydrogenase large subunit	1440.3	21.07	Energy production and conservation
A0A0F7KJR9	**Cytochrome C**	**1241.7**	**17.04**	**Energy production and conservation**
A0A1H2TZP9	Glycerophosphoryl diester phosphodiesterase	1543.7	10.69	Energy production and conservation
A0A1I4JNZ8	**Decaheme c-type cytochrome, DmsE family**	**1475.0**	**2.77**	**Energy production and conservation**
A0A1I4PBG1	ATP synthase subunit delta	1459.7	88.23	Energy production and conservation
A0A5D3YF35	Glycine/D-amino acid oxidase-like deaminating enzyme	1175.3	61.96	Energy production and conversion, Amino acid metabolism and transport
A0A0F7KF70	Thiol-disulfide isomerase/thioredoxin	1420.0	5.76	Energy production and conservation, Posttranslational modifications, protein turnover, chaperones
A0A0F7KHB1	Glutaredoxin-like protein DUF836	1029.3	4.06	Energy production and conservation, Posttranslational modifications, protein turnover, chaperones
A0A0F7KE78	Tyrosine recombinase XerD	732.7	4.09	Cell cycle control and mitosis
A0A0F7KFW1	Uncharacterized protein	832.0	11.71	Cell cycle control and mitosis
A0A0F7KGS6	Uncharacterized protein	1015.0	7.48	Cell cycle control and mitosis
A0A0F7KIS0	Uncharacterized protein	802.7	19.2	Cell cycle control and mitosis
A0A0F7KD43	Arginine decarboxylase	1420.7	8.32	Amino acid transport and metabolism
A0A0F7KF26	Alanine acetyltransferase	898.7	58.02	Amino acid transport and metabolism
A0A0F7KFK7	Aminotransferase	1247.3	17.37	Amino acid transport and metabolism
A0A0F7KJI2	Ornithine cyclodeaminase	1307.3	2.57	Amino acid transport and metabolism
A0A0F7KL71	Ornithine cyclodeaminase	1398.0	7.78	Amino acid transport and metabolism
A0A5D3YPR6	Choline dehydrogenase	1599.3	27.51	Amino acid transport and metabolism
A0A0F7KE52	Phosphopantetheine adenylyltransferase	1246.0	7.53	Nucleotide metabolism and transport
A0A1H2TY11	F-type H+-transporting ATPase subunit beta	1328.0	8.03	Nucleotide metabolism and transport
A0A1H2TYQ7	Phosphoribosyl-ATP pyrophosphatase	1469.7	5.94	Nucleotide metabolism and transport
A0A5D3Y8A9	Gluconokinase	770.7	16.78	Nucleotide metabolism and transport
A0A5D3YFY9	dITP/XTP pyrophosphatase	830.0	8.69	Nucleotide metabolism and transport
A0A0F7KGE6	Malto-oligosyltrehalose trehalohydrolase	1399.0	6.78	Carbohydrate transport and metabolism
A0A0F7KH73	Phosphoenolpyruvate-protein phosphotransferase	1582.7	34.68	Carbohydrate transport and metabolism
A0A0F7KJX2	Cellobiose phosphorylase	1491.7	13.07	Carbohydrate transport and metabolism
A0A0F7KK54	BT1 family protein	1374.0	7.88	Carbohydrate transport and metabolism
A0A5D3YH45	Multiple sugar transport system substrate-binding protein	1536.0	4.84	Carbohydrate transport and metabolism
A0A0F7KD32	Gamma-glutamyl: cysteine ligase YbdK (ATP-grasp superfamily)	1084.3	8.94	Coenzyme transport and metabolism
A0A0F7KH75	S-adenosylmethionine synthetase	819.7	28.69	Coenzyme transport and metabolism
A0A0F7KI16	Malonyl CoA-acyl carrier protein	1613.7	28.86	Coenzyme transport and metabolism
A0A0F7KJQ8	Riboflavin biosynthesis protein RibD	1737.0	14.2	Coenzyme transport and metabolism
A0A1H2ZX39	Threonylcarbamoyl-AMP synthase	1088.7	7.87	Coenzyme transport and metabolism
A0A1I4KYA4	Riboflavin synthase	1350.3	4.8	Coenzyme transport and metabolism
A0A5D3YEH4	Nicotinate phosphoribosyltransferase	1382.3	16.95	Coenzyme transport and metabolism
A0A0F7KBY3	Glutathione synthase	1149.7	19.18	Coenzyme transport and metabolism, Translation

A0A0F7KE59	Squalene--hopene cyclase	1506.7	40.99	Lipid transport and metabolism
A0A1H2Q126	Pimeloyl-ACP methyl ester carboxylesterase	1600.7	5.36	Lipid transport and metabolism
A0A1H2UPX2	Stearoyl-CoA desaturase (Delta-9 desaturase)	967.7	26.65	Lipid transport and metabolism
A0A5D3YDZ0	Diacylglycerol kinase family enzyme	1479.3	9.72	Lipid transport and metabolism
A0A0F7KK39	Oxidoreductase	1412.7	10.13	Lipid transport and metabolism, Secondary metabolites biosynthesis, transport and catabolism
A0A0F7KC79	tRNA N6-adenosine threonylcarbamoyltransferase	1081.7	20.62	Translation, Ribosomal biogenesis
A0A0F7KCG1	tRNA pseudouridine synthase B	1640.7	4.23	Translation, Ribosomal biogenesis
A0A0F7KFZ8	Ribosomal RNA small subunit methyltransferase	1538.3	4.97	Translation, Ribosomal biogenesis
A0A1H2RMS0	Metallo-beta-lactamase family protein	1431.3	13.14	Translation, Ribosomal biogenesis
A0A1H2VD86	tRNA (cytidine(34)-2'-O)-methyltransferase	1617.0	4.05	Translation, Ribosomal biogenesis
A0A1I4RT98	Methionine aminopeptidase	1572.3	18.07	Translation, Ribosomal biogenesis
A0A1I4TF06	30S ribosomal protein S12	1332.0	3.99	Translation, Ribosomal biogenesis
A0A5D3YCI4	Protein-L-isoaspartate O-methyltransferase	1167.3	8.89	Translation, Ribosomal biogenesis
A0A0F7KD46	CRP-like cAMP-binding protein	1428.0	5.28	Transcription
A0A0F7KFR2	Transcriptional regulator MraZ	585.3	14.3	Transcription
A0A0F7KIA3	COQ9 protein	893.7	21.87	Transcription
A0A0F7K910	Ribonuclease H	977.7	25.21	Replication and repair
A0A0F7K965	Holliday junction ATP-dependent DNA helicase RuvA	1174.3	16.58	Replication and repair
A0A0F7KGF1	Helicase SNF2	1518.3	4.44	Replication and repair
A0A0F7KJB6	DNA polymerase III subunit	1107.3	15.8	Replication and repair
A0A0F7KDE1	Membrane assembly protein	1283.3	56.88	Cell wall/membrane/envelope biogenesis
A0A0F7KEF6	Nucleoside-diphosphate-sugar epimerase	1645.3	6.41	Cell wall/membrane/envelope biogenesis
A0A0F7KFX3	Alanine racemase	1323.7	6.19	Cell wall/membrane/envelope biogenesis
A0A1H2RXI6	OmpA-OmpF porin, OOP family	1275.3	9.83	Cell wall/membrane/envelope biogenesis
A0A1H2T2H2	Efflux transporter, outer membrane factor (OMF) lipoprotein, NodT family	1701.7	12.41	Cell wall/membrane/envelope biogenesis
A0A1H2UC98	Murein hydrolase A	1357.7	25.98	Cell wall/membrane/envelope biogenesis
A0A5D3YDL0	OmpA family protein	1430.7	7.21	Cell wall/membrane/envelope biogenesis
A0A5D3YNU0	HlyD family secretion protein	1313.7	4.92	Cell wall/membrane/envelope biogenesis
A0A0F7KD75	Cobalt-zinc-cadmium efflux system outer membrane protein	1582.3	13	Cell wall/membrane/envelope biogenesis, Intracellular trafficking, secretion, and vesicular transport
A0A5D3Y9Q5	Outer membrane protein TolC	1654.0	12.56	Cell wall/membrane/envelope biogenesis, Intracellular trafficking, secretion, and vesicular transport
A0A0F7KFS9	General secretion pathway protein E	1508.3	34.13	Cell motility, Intracellular trafficking, secretion and vesicular transport
A0A0F7KIR6	Uncharacterized protein	557.0	36.92	Cell motility, Intracellular trafficking, secretion and vesicular transport
A0A5D3Y8Q5	DNA repair protein RadA	1161.3	26.85	Posttranslational modification, protein turnover, chaperones
A0A0F7KD44	Carbonate dehydratase	1437.3	8.77	Inorganic ion transport and metabolism
A0A0F7KF66	Cation transporter	1032.3	9.29	Inorganic ion transport and metabolism
A0A0F7KGT4	Arachidonate 15-lipoxygenase	1491.7	8.46	Inorganic ion transport and metabolism
A0A1H2YLY6	Uncharacterized protein involved in oxidation of intracellular sulfur	1602.7	5.48	Inorganic ion transport and metabolism
A0A1I4UJH1	Bacterioferritin	1653.3	5.78	Inorganic ion transport and metabolism
A0A5D3YJ46	Uncharacterized protein	1605.0	8.29	Inorganic ion transport and metabolism
A0A0F7KG51	Methyltransferase family protein	978.0	6.5	Secondary metabolites biosynthesis, transport and catabolism
A0A0F7KG95	Homospermidine synthase	1530.7	9.61	Secondary metabolites biosynthesis, transport and catabolism
A0A0F7KIU5	ATP-binding cassette, subfamily B, MsbA	929.3	13.25	Secondary metabolites biosynthesis, transport and catabolism
A0A1H2SJS4	1,4-dihydroxy-2-naphthoyl-CoA hydrolase	1687.7	55.64	Secondary metabolites biosynthesis, transport and catabolism

A0A1I4P7C0	Hemolysin-type calcium-binding repeat-containing protein	1496.0	8.02	Secondary metabolites biosynthesis, transport and catabolism
A0A0F7K8V7	Osmotically-inducible protein OsmY	1247.3	14.33	Function Unknown
A0A0F7KA01	SAM-dependent MidA family methyltransferase	955.7	26.64	Function Unknown
A0A0F7KBS8	Membrane protein	1581.0	5.35	Function Unknown
A0A0F7KCE2	Urea carboxylase	1476.7	7.3	Function Unknown
A0A0F7KCQ7	Membrane protein	1484.3	7.64	Function Unknown
A0A0F7KCX0	CNP1-like family protein	829.3	34.22	Function Unknown
A0A0F7KDF5	Sodium-dependent bicarbonate transporter	572.7	29.02	Function Unknown
A0A0F7KE40	Cytokinin riboside 5'-monophosphate protein	1343.3	14.22	Function unknown
A0A0F7KEH5	Uncharacterized protein DUF1840	1549.7	2.88	Function unknown
A0A0F7KEZ6	General stress protein	1395.3	29.7	Function unknown
A0A0F7KF62	Aromatic ring-opening dioxygenase catalytic subunit (LigB family)	569.0	22.55	Function unknown
A0A0F7KFC8	Glycine cleavage system protein	724.7	13.89	Function unknown
A0A0F7KFQ3	RNA-splicing ligase RtcB	1549.0	54.31	Function unknown
A0A0F7KFS2	OmpA family protein	1227.7	6.55	Function unknown
A0A0F7KGG5	Peptidase M20	1629.7	9.89	Function unknown
A0A0F7KGK1	Membrane protein	1373.7	4.76	Function unknown
A0A0F7KGQ9	YmgG-like glycine-zipper protein	1612.7	23.93	Function unknown
A0A0F7KHY1	Uncharacterized protein DUF4194	1651.7	4.67	Function unknown
A0A0F7KI44	PRC-barrel domain protein	1017.3	30.26	Function unknown
A0A0F7KJD4	BON domain-containing protein	1195.3	11.91	Function unknown
A0A0F7KJW5	Esterase/lipase OS=Nitrosomonas	1199.0	6.93	Function unknown
A0A0F7KK14	Putative DCC family thiol-disulfide oxidoreductase YuxK	1251.7	8.98	Function unknown
A0A1H2PWP2	CBS domain-containing protein	1434.7	7.68	Function unknown
A0A1H2QCL8	Dynamin family protein	1738.3	10.4	Function unknown
A0A1H2QZB0	Patatin-like phospholipase	1410.3	5.81	Function unknown
A0A1H2R960	Nicotinamide-nucleotide amidase	1549.7	8.59	Function unknown
A0A1H2T1M8	Putative membrane protein	1584.0	3.32	Function unknown
A0A1H2WS83	Hydrolase_4 domain-containing protein	1659.3	9.03	Function unknown
A0A1H2X2K5	Cupin domain protein	1405.3	3.48	Function unknown
A0A1H2XAC2	Uncharacterized protein	1329.0	5.04	Function unknown
A0A1H2XYT9	Uncharacterized protein	1539.0	21.57	Function unknown
A0A1H2YJN9	Putative membrane protein	1397.7	5.2	Function unknown
A0A1H2Z5V1	Putative Zn-dependent protease, contains TPR repeats	1569.3	3.14	Function unknown
A0A1H2ZD16	Uncharacterized protein	1694.7	20.22	Function unknown
A0A5D3Y7L1	Uncharacterized protein (DUF2267 family)	1556.3	38.13	Function unknown
A0A5D3Y9Q0	Uncharacterized protein DUF421	1586.0	3.9	Function unknown
A0A5D3YBD4	ElaB/YqjD/DUF883 family membrane-anchored ribosome-binding protein	1293.3	13.94	Function unknown
A0A5D3YH42	Putative toxin-antitoxin system antitoxin component (TIGR02293 family)	1420.3	6.15	Function unknown
A0A5D3YMH9	Sel1 repeat-containing protein	1447.0	12.4	Function unknown
A0A0F7KEQ2	Diguanylate cyclase	805.7	9.75	Signal Transduction mechanisms
A0A0F7KIU4	PhoH-like ATPase	1111.7	28.28	Signal Transduction mechanisms
A0A0F7KJ14	Histidine kinase	1265.7	8.37	Signal Transduction mechanisms
A0A1H2PWN0	Two-component system, response regulator RegA	1467.3	8.76	Signal Transduction mechanisms
A0A1H2PZX9	Histidine kinase	1485.3	11.54	Signal Transduction mechanisms
A0A1I4QH76	Bis(5'-nucleosyl)-tetraphosphatase, symmetrical	352.7	5.47	Signal Transduction mechanisms
A0A5D3YCA5	Diguanylate cyclase (GGDEF)-like protein	538.3	16.74	Signal Transduction mechanisms
A0A5D3YF74	**Two-component system nitrate/nitrite sensor histidine kinase NarX/two-component system sensor histidine kinase UhpB**	**532.0**	**34.86**	**Signal Transduction mechanisms**
A0A0F7KBN3	Uncharacterized protein	1167.0	18.17	Defense mechanisms
A0A0F7KIC3	AmpD protein	1055.3	3.25	Defense mechanisms

A0A1H2SQT5	ATP-binding cassette, subfamily B/ATP-binding cassette, subfamily B, multidrug efflux pump	571.7	5.32	Defense mechanisms
A0A1H2X467	Putative ABC transport system ATP-binding protein	1688.7	11.56	Defense mechanisms
A0A5D3YBZ4	ATP-binding cassette subfamily B protein	1248.0	59.3	Defense mechanisms
A0A0F7KBU7	Uncharacterized protein	1358.0	11.15	-
A0A0F7KE09	Uncharacterized protein	720.3	11.16	-
A0A0F7KIL0	Uncharacterized protein	1433.7	57.36	-
A0A0F7KKM7	Uncharacterized protein	1460.0	9.31	-
A0A0F7KLC7	Uncharacterized protein	1453.7	8.2	-
A0A1H2S5B7	Uncharacterized protein	1677.7	3.31	-
A0A1H2XQI7	Uncharacterized protein	1599.3	6.08	-
A0A1H2Y9Q3	Uncharacterized protein	1082.3	9.98	-

Supplementary table 2 Proteins expressed uniquely in stationary phase in *N. europaea*.

Accession number	Protein Description	Mean Rank	Mean |log prob|	COG_category
A0A837X0J2	Hydroxylase	955.0	10.4	Energy production and conversion
Q82VZ1	S-formylglutathione hydrolase	1016.7	8.7	Function unknown
Q81ZJ8	Transposase	1137.3	5.6	Replication and repair
A0A837WVK5	Peptidase	1181.0	5.2	Function unknown
A0A837WWE0	Transposase	1228.3	3.7	Function unknown
A0A837WTB6	NtrP Protein	1231.0	3.5	Function unknown
A0A837WUY3	Osmotically inducible protein Y	1359.0	2.1	Function unknown
A0A837X187	STAS domain-containing protein	1337.3	1.9	Function unknown
A0A837X2R0	Esterase/lipase/thioesterase family protein	1374.7	1.6	Function unknown
A0A837X3D0	Uncharacterized protein	1361.0	1.7	-