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Title: Transcriptome Responses of the Nitrifying Bacteria *Nitrosomonas europaea* and *Nitrobacter hamburgensis* to Low Oxygen.

Abstract Approved:

______________________________________________________________________________

Luis A. Sayavedra-Soto

Nitrification is the process within the global Nitrogen Cycle where ammonia (NH$_3$) is oxidized to nitrate (NO$_3^-$) and can be carried out by two distinct groups of bacteria. The ammonia-oxidizing bacteria (AOB) first oxidize NH$_3$ to nitrite (NO$_2^-$), and second, the nitrite-oxidizing bacteria (NOB) oxidize NO$_2^-$ to NO$_3^-$. In aerobic conditions, in either natural or engineered systems, nitrifying bacteria often compete for oxygen (O$_2$). In nitrification, low O$_2$ environments can cause the accumulation of NH$_3$, NO$_2^-$ and the greenhouse gases NO and N$_2$O, compounds that can be detrimental to the environment. This work examined the effects of O$_2$ limitation on the AOB *Nitrosomonas europaea* and the NOB *Nitrobacter hamburgensis*, and determined their transcriptome responses under replete and limiting O$_2$. When grown in co-culture with replete O$_2$, both *N. europaea* and *N. hamburgensis* were capable of consuming
~99% of the available NH$_3$, or NO$_2^-$, accordingly, and grew to 0.35 OD$_{600}$ in a steady state. Upon O$_2$ limitation in co-culture, *N. europaea* outcompeted *N. hamburgensis* for O$_2$. To consume 30 mM of the respective growth substrates, O$_2$ in the bioreactors was calculated at 9.25 µM O$_2$ for *N. europaea* and 54.8 µM O$_2$ for *N. hamburgensis*. In an O$_2$-limited co-culture bioreactor, *N. europaea* with higher O$_2$ affinity starved *N. hamburgensis* in 60 mM NH$_4^+$ medium. In single bioreactor cultures, *N. europaea* showed ~35% of its genome differentially expressed between treatments. Genes related to lipid metabolism, intracellular trafficking and secretion, cell motility, signal transduction, transcription and ribosomal structure and translation were at higher mRNA transcript levels under low O$_2$. Genes related to secondary metabolites, inorganic ion transport, coenzyme metabolism, nucleotide synthesis, amino acid transport, carbohydrate transport, energy production, post translational modification, cell envelope biogenesis, defense mechanisms, cell division and DNA replication were all at lower levels. *N. hamburgensis* in single culture differentially expressed ~15% of its genome between treatments. The transcripts of genes for lipid, coenzyme, nucleotide and amino acid metabolism, energy production, intracellular trafficking, cell envelope biogenesis, defense mechanisms, cell division, DNA replication, transcription and ribosomal biogenesis were at higher levels under low O$_2$. Genes related to inorganic transport, carbohydrate metabolism, post-translational modification, cell motility, and signal transduction pathways were expressed at lower levels under low O$_2$. The transcriptome data shows that *N. europaea* and *N. hamburgensis* utilize different compensatory strategies for maintaining growth rates in limited O$_2$ environments.
Transcriptome Responses of the Nitrifying Bacteria *Nitrosomonas europaea* and *Nitrobacter hamburgensis* to Low Oxygen

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Michael D. Dobie, Author
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Transcriptome responses of the nitrifying bacteria *Nitrosomonas europaea* and *Nitrobacter hamburgensis* to low oxygen

1. Chapter 1

Introduction

1.1: Nitrification

Nitrification is a process within the Nitrogen Cycle where ammonia (NH$_3$) is oxidized to nitrate (NO$_3^-$). The process is carried out by mostly chemolithoautotrophic microorganisms which derive energy for growth from the oxidation of NH$_3$ (Arp, Sayavedra-Soto et al. 2002). Aerobically, NH$_3$ is first oxidized to nitrite (NO$_2^-$) by ammonia-oxidizing bacteria (AOB), such as *Nitrosomonas* and *Nitrosospira* and ammonia-oxidizing archaea (AOA), such as *Nitrosopumilus* and *Nitrososphaera*. Generally, in balanced ecosystems, NO$_2^-$ is oxidized to NO$_3^-$ by nitrite-oxidizing bacteria (NOB), such as *Nitrobacter* and *Nitrospira*. These distinct groups of bacteria were thought the only ones to carry out these two separated steps in nitrification. The recent discovery that some *Nitrospira* are capable of the complete transformation of NH$_3$ to NO$_3^-$ in the process known as comammox has expanded nitrification (Daims et al. 2015). It is also possible to carry out the oxidation of NH$_3$ in the absence of oxygen (O$_2$) in a process known as anammox (Sliekers et al. 2005). This anaerobic oxidation is carried out by bacteria spanning at least five genera, where NH$_3$ and NO$_2^-$ are used to form nitrogen gas (N$_2$) and water.

In nature and in engineered systems, the two groups of microbes that perform two-step nitrification generally live in close association and compete for O$_2$ (Laanbroek and Gerards 1993, Sliekers 2005). Both groups of nitrifying bacteria have been found in oxic environments and in
oxic-anoxic interfaces where NH$_3$ is available (Sliekers et al. 2005; Jetten et al. 2003). Enrichments of AOB and NOB from these aerobic and low O$_2$ environments have been achieved (Sliekers et al. 2005).

During nitrification, 1.5 molecules of O$_2$ are necessary for the oxidation of NH$_3$ to NO$_2^-$ and 0.5 O$_2$ is necessary for the oxidation of NO$_2^-$ to NO$_3^-$ (see below). NO$_2^-$ tends to accumulate in O$_2$-limited environments, suggesting that NOB struggle more with O$_2$ limitation than AOB and show varied tolerances to sub-oxic conditions (Sliekers et al. 2005). For example, studies with *Nitrobacter winogradskyi* and *Nitrosomonas europaea* grown in low O$_2$ bioreactors result in NO$_2^-$ accumulation while *Nitrobacter hamburgensis* showed signs of adaption that prevented NO$_2^-$ accumulation (Laanbroek and Gerards 1993; Laanbroek, Bodelier, and Gerards 1994). Similarly, in mixed culture bioreactors of AOB and NOB where O$_2$ was limiting, Sliekers’ group (2005) showed that the NOB washed out of the bioreactor, but remained when NH$_3$ was the limiting growth factor (Sliekers et al. 2005). This could be explained by the relatively lower $K_s$ for O$_2$ for AOB compared to NOB described by Laanbroek, Bodelier, and Gerards (1994). Furthermore, AOB in anaerobic conditions have been shown to use alternative electron donors, such as hydrogen and pyruvate, however their growth in these conditions is limited (Clark and Schmidt 1966).

The effects of low dissolved O$_2$ on AOB and NOB is important in wastewater treatment (WWT) where the conversion of NH$_3$ to NO$_3^-$ and to N$_2$ gas is often the desired outcome (Jetten, Horn, and van Loosdrecht 1997). In most biological nutrient removal (BNR) systems, O$_2$ is in high demand, creating competition for all aerobes, not just nitrifying microorganisms. In WWT, the effects of low O$_2$ are commonly linked to NO$_2^-$ spikes further causing a decrease in bio-sludge transformation efficiency that leads to pollution and eutrophication of lakes and other
aquatic ecosystems (Sliekers et al. 2005). In engineered systems and other NH₃-rich environments with low O₂, such as nitrogen-amended soils, nitrifying populations have been linked to greenhouse gas emissions (N₂O and NO) and may contribute to climate change (Wrage et al. 2001). This is exacerbated by anthropogenic activities that imbalance the nitrogen (N) cycle and result in changes made to global weather patterns (Canfield, Glazer, and Falkowski 2010). It is essential that we gain a better understanding of the influence of nitrifying microorganisms to help ameliorate potential greenhouse gas emissions. For example, it is documented that O₂ limitation caused cultures of Nitrosomonas spp. to generate N₂O and NO (Kester 1997). Most studies on nitrification have been carried out under non-limiting O₂ conditions, however, in environments where ammonium is in excess, the NH₃-oxidizing microbes outcompete the NO₂⁻-oxidizing microbes, leading to the accumulation of NO₂⁻ which is toxic to human and animals and can lead to NOₓ gas production.

Chandran's group (2010) used quantitative PCR (qPCR) to determine the mRNA changes of key nitrifier genes in response to low O₂ levels and to elevated NO₂⁻ concentration on N. europaea (Yu and Chandran 2010). The group looked specifically at the mRNA levels of amoA, hao, nirK, and norB, all of which play a role in NH₃ oxidation and nitrogen reductive pathways. Under low dissolved O₂ (DO) there were higher mRNA levels of amoA and hao. The mRNA levels of nirK and norB also increased under conditions where NH₃ was not the limiting growth factor. Their findings suggested that N. europaea has the capacity to alter gene expression to adapt to grow in low O₂ and elevated NO₂⁻ environments, although this might be occurring only under transient stages.

No study to date has evaluated the global transcriptomic response to O₂ limitation in steady-state growth for N. europaea or N. hamburgensis, both model organisms in the study of
nitrification. A full understanding of AOB and NOB physiology and their genetic responses to O2 limitations is needed for efficient nitrification in the reclamation of wastewater, management of soil amendments in agriculture, and slowing the release of greenhouse gasses.

1.1.1 Ammonia-oxidizing bacteria (AOB)

The role of the microbial process of nitrification (i.e. NH3 and NO2- oxidation) in the N cycle is well established and AOB have been studied extensively (Ward, Arp, and Klotz 2011; Arp, Chain, and Klotz 2007). The AOB belong to two classes of microorganisms, the Betaproteobacteria (e.g. Nitrosomonas and Nitrosospira) and the Gammaproteobacteria (e.g. Nitrosococcus) (Purkhold et al. 2000). The aerobic oxidation of NH3 is generally thought to be the rate-limiting step of nitrification and is impacted by environmental factors such as pH, salinity, NH3 concentration and O2 availability (Arp, Chain, and Klotz 2007). These factors in turn influence the distribution of nitrifying microorganisms in natural and engineered environments (Ward, Arp, and Klotz 2011). Nitrosomonas spp. in WWT facilities can tolerate NH3 concentrations that may exceed 300 mM (Campos et al. 1999). Furthermore, AOB can also be found in low NH3 environments- for example, Nitrosococcus spp. inhabit marine ecosystems where NH3 is < 50 nM, and Nitrosospira spp. can be found in soil ecosystems where NH3 levels are typically < 1 µM, with variable pH and inorganic mineral composition (Martens-Habbena et al. 2009).

N. europaea ATCC 19718 is a Gram-negative chemolithoorganotroph. This AOB is used as a model due to the ease of culturing and relatively fast doubling time (8 to 14 hours) compared to the other AOB. N. europaea has been shown to utilize a limited number of small chain organic compounds, although none has been found to support growth (Hommes, Sayavedra-Soto,
and Arp 2003). The *N. europaea* genome has been annotated and consists of a circular chromosome 2,812,094 bp (2.8 Mbp) in size and contains 2,460 protein-encoding genes averaging 1,011 bp in length with intragenic regions averaging 117 bp. Coding genes are fairly distributed with 47% transcribed from one strand and 53% from the complement (Chain et al. 2003). The G+C content of the *N. europaea* genome is ~50% and has no plasmids. Insertion sequences (IS) are abundant and has been suggested that the genome of *N. europaea* is decreasing in size (Chain et al. 2003), still the genome of *N. europaea* must contain all the necessary genes to carry out all necessary metabolic and assimilatory functions for growth with two simple molecules, NH₃ and CO₂.

### 1.1.1.1 Metabolism of AOB

AOB, including *N. europaea*, are capable of deriving all energy required for growth from the oxidation of NH₃ to NO₂⁻, which has a low energy yield (ΔG°' = -275 kJ mol⁻¹) (Daims et al. 2015). Aerobic ammonia oxidation by *N. europaea* is carried out as follows:

\[
\text{NH}_4^+ + 1.5 \text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + 2\text{H}^+
\]

This process releases two free protons and results in acidification of the growth medium that shifts the equilibrium of NH₃/NH₄⁺ to NH₄⁺ (pKₐ = 9.25). The substrate for AOB is thought to be NH₃, not NH₄⁺, and is oxidized in the cytoplasm to derive energy for growth. Many studies have demonstrated that most AOB have reduced growth rates at a lower pH range (Suzuki, Dular, and Kwok 1974). For the growth of AOB in bioreactors and continuous flow systems, maintaining the culture pH above 7 is crucial to ensure a steady supply of NH₃.

The oxidation of NH₃ by AOB has two biochemical steps. The first step is the oxidation of NH₃ by O₂ that is catalyzed by the copper-containing, membrane-bound enzyme, ammonia
monooxygenase (AMO). The resulting product is the intermediate hydroxalamine (NH₂OH). The second step takes place in the periplasmic space, where the Fe-containing, soluble enzyme, hydroxylamine dehydrogenase (HAO) further oxidizes NH₂OH to NO₂⁻, stripping 4 electrons in the process and releasing 5 protons. Of the 4 electrons, two electrons are fed back to AMO to promote further NH₃ oxidation and two electrons are passed downstream through cytochromes within the cell membrane to the terminal electron acceptor, O₂, producing H₂O. This process generates the membrane potential that drives energy production (ATP). Electrons are also used to create cell reductant (NADH) from NAD⁺ that is primarily used for carbon fixation and general biosynthesis (Arp, Chain, and Klotz 2007). AOB can produce N₂O and NO under certain growth conditions (Kool et al. 2011). The periplasmic enzyme NirK can function as a NO₂⁻ reductase, consuming electrons and generating NO gas. A membrane-bound nitric oxide reductase (NOR) transforms NO to N₂O. N. europaea has also demonstrated increased mRNA levels of nirK and norB during exponential growth phase of batch cultures under low dissolved O₂ and elevated NO₂⁻ concentrations (Yu and Chandran 2010).

1.1.2 Nitrite-oxidizing bacteria (NOB)

NOB are more phylogenetically diverse when compared to AOB. Nitrobacter, Nitrospina, Nitrococcus and Nitrospira are common genera and belong to their own classes, Alphaproteobacteria, Deltaproteobacteria, Gammaproteobacteria and Nitrospirae, respectively. Nitrobacter has been enriched from a multitude of terrestrial and aquatic systems while Nitrococcus and Nitrospina have been found exclusively in marine environments (Teske et al. 1994), and Nitrospira has been found to be an abundant NOB in WWT (Kraigher and Mandic-Mulec 2011). However, compared to their AOB counterparts, NOB have not been studied as
extensively. The NOB complete the second non-limiting step in nitrification oxidizing $\text{NO}_2^-$ to $\text{NO}_3^-$ as shown below:

$$\text{NO}_2^- + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + 2\text{H}^+ + 2\text{e}^-$$

*Nitrobacter hamburgensis* X14, also a focus of this work, was isolated from soil samples in Hamburg, Germany and has also served as a model organism in the study of nitrification. A member of the *Alphaproteobacteria*, *N. hamburgensis* is closely related to *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris*. The genome consists of one 4.4 Mbp chromosome and three plasmids (294, 188 and 121 kbp). Most of the plasmid-born genes are unique to *N. hamburgensis* including those involved in central metabolism, energy conservation, conjugation and heavy metal resistance (Starkenburg et al. 2008). Approximately 75% of an autotrophic DNA portion within the largest plasmid of *N. hamburgensis* is highly conserved in the genome of *N. winogradskyi*. Within the core genome, additional unique genes are found that confer the catabolism of aromatic, organic and one-carbon compounds, several of which have diverged significantly from the *Alphaproteobacteria* lineage (Starkenburg et al. 2008). The genome of *N. hamburgensis* is much larger than other sequenced *Nitrobacter* species such as *N. winogradskyi* and NB311A. Several features of the *N. hamburgensis* genome account for its relatively large size, mainly the large number of pseudogenes and paralogs contributing to ~20% of the total genome. Additionally, the majority of the genes carried on the *N. hamburgensis* plasmids do not have orthologs in the other annotated *Nitrobacter* genomes. The large plasmids harbor a number of metabolic functions that are unique to *N. hamburgensis* and clearly separate this bacterium from other nitrite oxidizers (Starkenburg *et al.* 2008).
1.1.2.1 Metabolism of NOB

The energy derived from the oxidation of NO$_2^-$ is $\Delta G^0 = -74$ kJ mol$^{-1}$, that is nearly 4-fold less than that of the oxidation of NH$_3$ to NO$_2^-$ (Daims et al. 2015). The entire process produces 2 electrons, identical to the net electron yield of AOB. NOB are thought to require an AOB partner as a source of substrate and are typically co-isolated with one another (Wagner et al. 1996).

In NOB the oxidation of NO$_2^-$ is by a membrane-bound, iron-sulfur-containing nitrite oxidoreductase (NXR). NXR produces NO$_3^-$ from the addition of NO$_2^-$ and H$_2$O and releases 2 electrons used for the reduction of O$_2$ to H$_2$O. Membrane potential is created and used to generate ATP. It has been postulated that NOB make use of reverse electron flow to overcome low energy yields of this process and create NADPH/FADH required for biosynthesis (Arp, Chain, and Klotz 2007). Researchers have not verified the pathways or the roles of organic electron donors that NOB may be capable of using. Regardless, when the reductant pool swells as a result of low O$_2$ environments as suggested in Starkenburg et.al 2008, excess electrons can be utilized to make polyhydroxybutyrate (PHB) granules, a feature not seen in characterized AOB (Freitag, 1987). In general, NOB are more metabolically diverse than AOB. For example, select Nitrobacter spp. have been shown to use and metabolize carbon compounds (e.g. pyruvate, acetate, α-ketoglutarate, and glycerol) in the absence of NO$_2^-$ (Starkenburg et al. 2008). For example, Starkenburg found that, N. hamburgensis can utilize D-lactate for growth, but not L-lactate, under NO$_2^-$-limiting conditions. This mixotrophic behavior may give the organism an advantage in NO$_2^-$-limited environments but remains to be studied in detail.
1.2 Carbon fixation by AOB and NOB

AOB and NOB are capable of autotrophic growth utilizing a modified Calvin-Benson Pathway, relying on CO₂ or aqueous bicarbonate (HCO₃⁻) for carbon assimilation. In both AOB and NOB, CO₂ is reduced to glyceraldehyde-3-phosphate (G3P) that is then used a precursor for biosynthesis. Ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) is the enzyme that fixes the carbon from one CO₂ molecule into organic carbon using 3 ATP and 2 NADPH derived from oxidative phosphorylation (Arp, Chain, and Klotz 2007). Both *N. europaea* and *N. hamburgensis* genomes encode carbonic anhydrase (CA), a membrane bound enzyme that converts HCO₃⁻ to gaseous CO₂. Additionally, most NOB, including *N. hamburgensis* synthesize carboxysomes, organelle-like structures that facilitate carbon fixation by concentrating CO₂ and protecting RuBisCO from oxidative stress. Genes required for carboxysome biosynthesis are absent in most AOB, including *N. europaea*. Due to the large energy cost associated with fixation via the Calvin Cycle, AOB and NOB specifically, show evidence of the uptake and metabolism of small carbon molecules (Hommes, Sayavedra-Soto, and Arp 2003; Starkenburg, Arp, and Bottomley 2008a). An alternative source of carbon for electron donation and assimilation could greatly reduce the energy required for normal autotrophic growth. The growth of *N. hamburgensis* and *N. winogradskyi* on yeast extract, peptone, casamino acids, pyruvate, acetate, α-ketoglutarate, lactate and glycogen has been documented and in most cases resulted in higher growth rates and yields (Starkenburg, Arp, and Bottomley 2008a; Laanbroek and Gerards 1993). *N. hamburgensis* has been described as having a greater organotrophic potential than *N. winogradskyi* and other NOB. Many studies have examined the mixotrophic and organotrophic growth of *N. hamburgensis* (Kirstein et al. 1986; Laanbroek, Bodelier, and Gerards 1994; Starkenburg, Arp, and Bottomley 2008b; Spieck and Bock 2005). The AOB
*N. europaea* is capable of using fructose and/or pyruvate in the presence of NH₃ for growth (Clark and Schmidt 1966; Hommes, Sayavedra-Soto, and Arp 2003). Clark’s group (1966) found that mixed cultures of *N. europaea* with added sodium pyruvate experienced a shorter lag phase and higher cell densities (Clark and Schmidt 1966). Uptake and incorporation of carbon was measured using C¹⁴-labeled pyruvate. *N. europaea* has also shown incorporation of fructose in the presence of NH₃ and was demonstrated using radiolabeled fructose into *N. europaea* cells (Hommes, Sayavedra-Soto, and Arp 2003). Since AOB and NOB are surrounded by other microbes and a wide range of organic compounds in non-pure culture environments, the results of Clarks *et al.* (1966) and Hommes *et al.* (2003) suggest that AOB bacteria, including the less metabolically versatile *N. europaea*, are adaptive and take advantage of select carbon compounds when available, although no alternative C sources have been shown to support growth without NH₃.

### 1.3 Co-Culture interactions

In nature, it is not uncommon to find AOB and NOB living in close association. These organisms typically form biofilms with a wide variety of other microorganisms (Wagner *et al.* 1996), with NOB congregated often next to active NO₂⁻-producing AOB. This has been shown in metagenomic studies performed in a variety of environments including engineered systems. In studies focused on the interactions and responses to stimuli of AOB and NOB, researchers typically use continuous-flow cultures. Nitrifiers have been studied in single, co-culture and mixed culture scenarios (Laanbroek and Gerards 1993; Sayavedra-Soto *et al.* 2015; Clark and Schmidt 1966). Axenic cultures of *N. europaea* have been studied extensively in continuous-flow bioreactors (Skinner and Walker 1961) as well as in co-culture with *N. winogradskyi* (Pérez *et al.* 2014; Laanbroek and Gerards 1993). In those studies, *N. europaea* was found to contribute
~80% of total co-culture cell density, or in a 5:1 ratio of cell density. Furthermore, N. europaea and N. hamburgensis have been studied in co-culture with replete and limiting amounts of O\textsubscript{2} (Laanbroek, Bodelier, and Gerards 1994). Despite considerable research on the effects of low O\textsubscript{2} on nitrifiers in co-culture, a full transcriptomic approach has not been taken with N. europaea and N. hamburgensis. Learning more about how these organisms interact and respond to prolonged O\textsubscript{2} limitation may prove beneficial to WWT efficiency and greenhouse gas emission reductions.

### 1.4 Effects of oxygen limitation on AOB and NOB

Ammonia and nitrite oxidation is dependent on O\textsubscript{2} for both N. europaea and N. hamburgensis respectively, and as a consequence when O\textsubscript{2} is limiting, the two organisms must compete for O\textsubscript{2}. There are reports showing that N. europaea has a higher affinity for O\textsubscript{2} compared to N. hamburgensis and other NOB (Laanbroek, Bodelier, and Gerards 1994). This places NOB such as N. hamburgensis at a potential disadvantage when growing in co-culture with an AOB partner. In most nitrification scenarios when O\textsubscript{2} is limiting, NO\textsubscript{2} often accumulates (Bodelier et al. 1996). The authors investigated the affinity for O\textsubscript{2} between lake sediment (O\textsubscript{2}-limited) and oxic soil profiles (O\textsubscript{2} replete) and it was determined that lake sediment AOB population had a lower affinity relative to the AOB in oxic soil samples (Bodelier et al. 1996). This result suggests that certain AOB communities are capable of adapting to low O\textsubscript{2} concentrations. This “adaptation” could be valid, however, the community diversity between the two habitats is likely different and thus cannot be applied to all AOB (Giri et al. 2005).

Additionally, Laanbroek’s study (1994) examined the effects of limited O\textsubscript{2} on co-cultures of N. europaea and N. hamburgensis. Initially, N. europaea outcompeted N. hamburgensis for O\textsubscript{2} as the accumulation of NO\textsubscript{2} was observed when aeration was completely and abruptly removed
from cultures (Laanbroek, Bodelier, and Gerards 1994). When O\textsubscript{2} levels were gradually decreased from 80\% to below 10\% air saturation, NO\textsubscript{2} did not accumulate and the \( K_s \) for O\textsubscript{2} of \textit{N. hamburgensis} decreased nearly 11-fold (5.2 \( \mu \)M h\textsuperscript{-1}) and became slightly less than the \( K_s \) of \textit{N. europaea} (6.9 \( \mu \)M h\textsuperscript{-1}). Because \textit{N. hamburgensis} cell density was higher than that of \textit{N. europaea}, the NOB became a better competitor for O\textsubscript{2} and NH\textsubscript{3} began to accumulate, a result not typically found in co-culture studies. The evidence for the sub-oxic adaption of \textit{N. hamburgensis} to better compete for O\textsubscript{2} is thought to be related to either the increased number of genes encoding respiratory terminal cytochrome oxidases, (Starkenburg et al. 2008) or a unique, alternative cytochrome oxidase expressed exclusively under chemoorganotrophic growth (Kirstein et al. 1986). To date, there is no direct evidence for either mechanism, induced under sub-optimal O\textsubscript{2} conditions. Co-cultures in Laanbroek’s study (1994) contained peptone, pyruvate and a variety of carbon compounds derived from yeast extract. Because \textit{N. hamburgensis} apparently possesses the ability to utilize alternative electron donors, the ability to adapt and outcompete \textit{N. europaea} may have been directly influenced by organic carbon utilization (Racz, Datta, and Goel 2010). The same results may not be observed under standard culturing techniques or relevant in typical lithotrophic environments where these organisms are commonly found. The study of how nitrifying microorganisms alter gene expression in O\textsubscript{2}-limited environments will help to the better understanding of their contribution to the balance of the N cycle and impact on climate change.

1.5 Research objectives

1.5.1 To establish under comparable conditions steady-state single culture and co-culture bioreactors of \textit{N. europaea} and \textit{N. hamburgensis}. 

1.5.2 To compare growth characteristics of *N. europaea* and *N. hamburgensis* in single culture and in co-culture bioreactors under both O$_2$-replete and O$_2$- limited conditions.

1.5.3 To analyze and compare the transcriptome of *N. europaea* and *N. hamburgensis* in O$_2$- replete and O$_2$- limiting from single culture bioreactors.

1.6 Research queries

1.6.1 Are *N. europaea* and *N. hamburgensis* capable of adapting to an O$_2$-limited environment? (i.e. changes in cell density, substrate consumption)

1.6.2 What are the gene expression changes of *N. europaea* / *N. hamburgensis* made in response to O$_2$ limitation? (i.e. transcript levels of key genes that would suggest adaptation)

1.6.3 Are there any physiological responses of *N. europaea* / *N. hamburgensis* under O$_2$ limitation?

1.6.4 Do *N. europaea* and *N. hamburgensis* share commonalities in response to low O$_2$ levels?

1.7 Hypotheses

1.7.1 *N. europaea* and *N. hamburgensis* display reduced capacity for Nitrification (i.e. lower growth yield and substrate consumption).

1.7.2 *N. europaea* and *N. hamburgensis* gene expression changes in response to O$_2$ limitation.

1.7.3 *N. europaea* and *N. hamburgensis* display lower levels of carbon and other assimilatory gene transcripts.
2 Chapter 2:

Experimental: Transcriptome profiling of *Nitrosomonas europaea* and *Nitrobacter hamburgensis* with replete and limited oxygen supply.

2.1 Summary

*Nitrosomonas europaea* and *Nitrobacter hamburgensis* were grown separately and in co-culture under replete and limiting-O\(_2\) levels. Under the conditions of this study, *N. hamburgensis* did not maintain activity in co-culture with *N. europaea* with 60 mM NH\(_4^+\) under O\(_2\) limitation. The cell density in these bioreactors decreased and NO\(_2^-\) accumulated. *N. europaea* and *N. hamburgensis* in single culture bioreactors achieved a steady state under non-limiting and limiting O\(_2\) conditions. The concentrations of NH\(_3\), NO\(_2^-\), NO\(_3^-\) and the cell density (OD\(_{600}\)) were measured daily to assure that the cultures were indeed at steady state in the bioreactors. Cells from individual bioreactors under the two O\(_2\) concentrations were harvested and total RNA was extracted. From these total RNA preparations, mRNA was enriched and concentrated for mRNA-Seq analysis.

The transcriptome of each organism under non-limiting and limiting O\(_2\) conditions was analyzed and compared using CLC-Bio workbench. Compared to replete O\(_2\) growth, the transcriptome of *N. europaea* growing under limited O\(_2\) showed that 917 genes (~35% of total protein encoding genes) changed significantly. Transcript levels for genes encoding terminal cytochromes and stress-related chaperonin proteins were present in higher levels under O\(_2\)-limited-growth. Transcripts encoding AMO, carbon fixation proteins and sulfur metabolism were lower under O\(_2\)-limited growth.
Compared to replete O\textsubscript{2} growth, the transcriptome of N. hamburgensis under limited O\textsubscript{2} showed that 711 of 4716 genes (~15\% of total candidate protein encoding genes) changed significantly. Transcript levels for genes encoding dissimilatory NO\textsubscript{2}– reduction (nirK), lipopolysaccharide biosynthesis, type-IV pilus / conjugation, and Iron (Fe) acquisition were present in significantly higher levels under O\textsubscript{2} limited growth. Transcripts encoding NO\textsubscript{2}– oxidation (nxr), chemotaxis and flagella synthesis, carbon fixation and terminal cytochrome proteins were significantly lower under O\textsubscript{2}-limited growth.

2.2 Materials and Methods

2.2.1 Cultivation, bioreactor operation and maintenance

*Nitrosomonas europaea* (ATCC 19718) and *Nitrobacter hamburgensis* (X14) were grown at 30\° C in 1 and 2 L continuous flow bioreactors (Applikon Biotechnology). Growth medium for co-culture and *N. europaea* single cultures contained 60 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.75 mM MgSO\textsubscript{4}, 0.1 mM CaCl\textsubscript{2} and trace minerals (10 µM FeCl\textsubscript{3}, 1.0 µM CuSO\textsubscript{4}, 0.6 µM Na\textsubscript{2}MoO\textsubscript{4}O\textsubscript{4}, 1.59 µM MnCl\textsubscript{2}, 0.6 µM CoCl\textsubscript{2}, 0.096 µM ZnCl\textsubscript{2}). A phosphate buffer (0.52 mM NaH\textsubscript{2}PO\textsubscript{4}.H\textsubscript{2}O, 3.5 mM KH\textsubscript{2}PO\textsubscript{4}) and carbonate (0.28 mM NaCO\textsubscript{3}) were mixed, adjusting pH to 7.0 with HCl, and sterilized. To prevent precipitation the buffer mix was let stand to cool to room temperature before use, at which point, 6 ml/L were added aseptically to the medium in the bioreactors just prior inoculation. The same media recipe was used for growing *N. hamburgensis* single cultures but with 60 mM NaNO\textsubscript{2} instead of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. In co-culture, *N. hamburgensis* was dependent on the oxidation of NH\textsubscript{3} to NO\textsubscript{2}– by *N. europaea*. The *N. europaea* bioreactors were maintained at pH =7.0 ± 0.1 by dispensing sterile 10\% Na\textsubscript{2}CO\textsubscript{3} into the medium as necessary. The *N. hamburgensis* bioreactors were adjusted to pH= 7.0 ± 0.1 if necessary only at the start of the culture. In rare cases it was necessary to readjust the pH by adding sterile 1.0 M HCl dropwise.
Bioreactors were inoculated with 2.0 % exponential phase batch cultures and remained in “batch mode” until < 10 mM NH₃ (or NO₂⁻ accordingly) remained in the growth medium. Once this point was reached, the bioreactors were set to continuous media feed mode at a flow rate of 10 ml/h for 1L vessels and 20 ml/l for 2 L vessels controlled by Thermoscientific peristaltic pumps. Flow rates for each vessel correspond to a dilution rate of .010 and corresponds to a steady state doubling time of ~70 hours for each organism as calculated:

\[
doubling\ time = \frac{\ln2}{\text{dilution rate } (0.01)}
\]

Air (0.2-µm filtered) was supplemented to the bioreactors during batch mode and O₂-replete steady states. During O₂-replete conditions, cultures were supplied with constant aeration at 40 – 100 ml/min. and stirring to 400 rpm for N. europaea and 600 rpm for N. hamburgensis. O₂-limited steady states were established differently for each organism. The air input for N. europaea bioreactors was suspended completely, while N. hamburgensis bioreactors required an air input of 20 ml/min. into the headspace, instead of bubbling it into the medium. To promote gas exchange in the low O₂ treatment the stirring was increased to 800 rpm. Cultures were periodically checked for contamination on Luria-Bertani agar plates plating ~100 µl of culture and incubated at 30˚C for 4 days.

2.2.2 Cell density and substrate/product determinations

Cell density was determined by measuring the optical density at 600 nm (OD₆₀₀) using a Beckman spectrophotometer. Colorimetric assays were used to determine the concentrations of NH₄⁺, NO₂⁻ and NO₃⁻ as described (Hood-Nowotny et al. 2010).
2.2.3 RNA extraction and purification

Total RNA was extracted as described in Pérez et al. (2014) with some modification. A total of three biological replicates were collected on alternate days for each organism under the two O$_2$ treatments and at steady state. To extract sufficient mRNA for analyses, 40 ml of *N. europaea* cell culture, or 80 ml for *N. hamburgensis* cell culture, were necessary. The cells were harvested by centrifugation at 12400 R.C.F. (Beckman centrifuge model J2-21), resuspended in 1 ml, lysed by sonication using a mini-probe, (Heatsystems Ultrasonic Processor XL), and total RNA was extracted using an RNeasy mini kit following the directions of the manufacturer (Qiagen, Germantown, MD). To deplete the RNA samples of rRNA, a MICROBExpress-bacteria RNA Purification Kit was used following the directions of the manufacturer (Ambion/Life Technologies, NY). The quality of depleted RNA was examined using 6000 Nano Lab-Chip Kit and an Agilent Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). The Illumina-targeted RNA Expression Kit with at least 200 ng of concentrated mRNA was used to synthetize the mRNA-Seq libraries. The libraries were sequenced (100mer paired-end) on a HiSeq 2000 Sequencer (Illumina, San Diego, CA) at the Center for Genome Research and Biocomputing Core Laboratories (CGRB) at Oregon State University.

2.2.4 Transcriptome data analyses

The transcriptome of *N. europaea* and *N. hamburgensis* under O$_2$ replete and O$_2$ limited conditions were comparatively analyzed using CLC Bio Workbench (CLC bio, Prismet, DK). Differential Gene Expression (DGE) between O$_2$ replete and O$_2$ limited steady state samples was analyzed and expressed as a difference in fold change. Significant DGE was defined by a $p$-value threshold $\leq 0.05$. Default values of the software were used and normalized to reads per kilobase per million (RPKM) (Robinson and Smyth 2008; Robinson, McCarthy, and Smyth 2010). Two
DGE comparisons were analyzed: *N. europaea* replete vs. limited O₂ and *N. hamburgensis* replete vs. limited O₂.

### 2.3 Results

#### 2.3.1 Establishment of steady state cultures under O₂ limitation

To establish *N. europaea* and *N. hamburgensis* co-culture bioreactors, full aeration (100 ml/min), stirring (400 rpm), and a flow rate of 10 ml/min. were used. In these conditions, the co-culture of *N. europaea* and *N. hamburgensis* achieved a steady state at cell density of 0.35 OD₆₀₀ consuming ~98% of the growth substrates (NH₃ and NO₂⁻ respectively). In an attempt to establish an O₂-limited environment, the air input was removed and the stirring was maintained. We observed that the activity of both organisms, as expected, decreased over a 20-day period reducing the cell density to 0.075 OD₆₀₀ and the NH₄⁺ and NO₂⁻ concentrations increased to 25-30 mM (Fig. 2). Although the NH₄⁺ concentration tended to stabilize, the NO₂⁻ concentration kept increasing with time, suggesting that *N. hamburgensis* was starved for O₂ and being washed out from the bioreactor. In an attempt to recover *N. hamburgensis*, the air flow was reestablished to the culture at 10 ml/min. This caused the NH₄⁺ concentration to decrease to 10 mM indicating that the cells of *N. europaea* were recovering, however the NO₂⁻ concentration continued to increase suggesting that the cells of *N. hamburgensis* were still inactive. In a further attempt to recover *N. hamburgensis*, the air flow was increased to 20 ml/min. This change resulted in 98% consumption of NH₄⁺ by *N. europaea*, but NO₂⁻ concentration at this point exceeded 55 mM suggesting *N. hamburgensis* was washed out completely from the bioreactor. In the conditions tested, *N. europaea* outcompeted *N. hamburgensis* for O₂ when in limiting supply and in 60 mM NH₄⁺. This can be attributed to a lower $K_s$ for O₂ at 80% air saturation (8.6 µM vs. 57.5 µM respectively), a behavior described by Laanbroek, Bodelier, and Gerards (1994).
Fig 1: Bioreactor schematic.

We proceeded to analyze *N. hamburgensis* and *N. europaea* in single culture in an O$_2$-limited steady state. For *N. europaea*, approximately 98% of the growth substrate was consumed by aeration at 40 ml/min. and stirring at 400 rpm while *N. hamburgensis* required aeration at

Fig. 2: Growth of *N. europaea* and *N. hamburgensis* in 1L bioreactor under O$_2$-replete and O$_2$-limited non-steady states. *N. hamburgensis* washed out as a result of O$_2$ limitation.
100 ml/h and stirring at 600 rpm to consume an equivalent amount of substrate. These bioreactors presented NH$_4^+$ concentrations of less than 2 mM for *N. europaea* and NO$_2^-$ concentrations of less than 1 mM for *N. hamburgensis*. Cultures that persisted with these parameters were considered at steady state, O$_2$ replete and N-substrate limited. The establishment of a *N. europaea* bioreactor that was O$_2$-limited at a steady state was achieved by completely removing the air input and with moderate stirring (400 rpm), however for *N. hamburgensis*, suspending aeration was not conducive to produce a steady state culture and in agreement to the observations in the co-culture bioreactors. A steady state *N. hamburgensis* O$_2$-limited bioreactor required at least 20 ml/min air flow, but into the headspace of the culture and increased stirring to 800 rpm. The O$_2$-limited bioreactors were controlled to consume approximately 60% of the growth substrates i.e. leaving NH$_4^+$ at least 25 mM for *N. europaea* and NO$_2^-$ at 25 mM for *N. hamburgensis* (Figs. 3 and 4).

![N. europaea Bioreactor](image)

**Fig. 3:** Typical growth of *N. europaea* in 1 L bioreactor. Steady state under O$_2$-replete was established by aeration with filtered air. O$_2$-limited growth was possible by interrupting aeration into the reactor by day 16 while maintaining stirring.
O₂ consumption kinetics and theoretical O₂ concentrations for O₂ replete and O₂-limited bioreactors were calculated using the Michaelis-Menten Kinetics equation:

\[ v = \frac{V_{\text{max}}[S]}{K_s + [S]} \]

When bioreactors of either *N. europaea* or *N. hamburgensis* were grown under O₂ replete conditions, ~98 to 99% of NH₃ and NO₂⁻ was consumed thus cultures were considered to have saturating, non-limiting amounts of O₂ (Table 1). When O₂-limited and bioreactors were in steady state, they consumed approximately 50% of the available substrate (Table 2). Using the consumption rate of NH₃ and NO₂⁻ for each culture, the known ratio of substrate to O₂ consumption required for NH₃ and NO₂⁻ oxidation (1.5:1 and 0.5:1 respectively), the dilution rate of bioreactors (0.01)

![N. hamburgensis Bioreactor](image)

Fig. 4: Typical growth of *N. hamburgensis* in a 2 L bioreactor. Steady state under O₂-replete was established by aeration with filtered air. O₂-limited growth was established by aeration into the headspace of reactor on day 21 and increasing stirring to promote gas diffusion.

and the reported \( K_s \) of O₂ for each organism, the theoretical concentration of O₂ and the rate of O₂ consumption was calculated. Based on the equation above, *N. europaea* consumed
approximately 0.467 µmols ml\(^{-1}\) h\(^{-1}\) O\(_2\) under O\(_2\)-limited conditions vs. 0.145 µmols ml\(^{-1}\) h\(^{-1}\) O\(_2\) consumed by \textit{N. hamburgensis}. The concentration of O\(_2\) in O\(_2\)-limited bioreactors was 9.25µM O\(_2\) and 54.8µM O\(_2\) for \textit{N. europaea} and \textit{N. hamburgensis} respectively. The behavior of a typical bioreactor of \textit{N. europaea} and \textit{N. hamburgensis} and their transition to a steady state elicited by O\(_2\) limitation is depicted in Figs. 3 and 4. During the start of a bioreactor under replete O\(_2\) conditions, \textit{N. europaea} grew to a cell density of 0.16 OD\(_{600}\) and consumed approximately 59 mM (or 98%) of the available NH\(_4^+\) accumulating ~57 mM NO\(_2^-\) in about 6 days post inoculation (Fig. 3). The apparent discrepancy in stoichiometry of the conversion of NH\(_4^+\) to NO\(_2^-\) is in accordance to previous observations (Pérez \textit{et al.} 2014) and is likely due to the production of N\(_2\)O gas as reported by our laboratory and others (Kool \textit{et al.} 2011; Ahn, Kwan, and Chandran 2011). At day 6, fresh medium was fed to establish a steady state culture as described in the Materials and Methods section. Post inoculation, it usually took a 7 to 10-day period to observe a steady state culture (Fig. 3). Cells were collected and total RNA was extracted on alternate consecutive days. Typically, by day 16, the air input could be interrupted to limit the O\(_2\) supply. Stirring was manipulated to achieve a \textit{N. europaea} cell density of about 0.07 OD\(_{600}\) over a 6-day period. In this condition, the NH\(_3\) concentration increased to ~30 mM and the NO\(_2^-\) concentration decreased to ~27 mM (Fig. 3, Table 1), again with a slight discrepancy in the stoichiometry most likely due to the generation of NO\(^-\) and N\(_2\)O. The experiments for the O\(_2\) replete and O\(_2\)-limited cultures in steady state were maintained for 10 and 13 days respectively. RNA samples for each condition were during these times. A volume of 40 ml typically yielded 15-60 µg of total RNA and yielded ~500 ng for mRNA-Seq.

The single culture bioreactors of \textit{N. hamburgensis} in O\(_2\)-replete conditions grew to 0.078 OD\(_{600}\) and consumed 99% of available NO\(_2^-\) approximately 11 days (Fig. 4). The lag phase for
Nitrobacter cultures was on average 6 days longer than that of *N. europaea*, with a fresh media feed being initiated 11 days post inoculation compared to 6 days for *N. europaea*. An O$_2$ replete steady state was typically observed between days 13 and 19 where NO$_2^-$ remained < 1 mM and NO$_3^-$ concentrations were approximately 58 mM (Fig. 4, Table 1). The discrepancy in N-stoichiometry can again be explained by the production NO$_x$ gases (Ahn, Kwan, and Chandran 2011). O$_2$ was made the limiting growth factor on day 21 by altering the method of aeration and air flow injected into the head space of the bioreactor was reduced to 20 ml/min with stirring at 800 rpm to increase gas diffusion. The OD$_{600}$ decreased to 0.045, while NO$_2^-$ increased to ~32 mM, NO$_3^-$ decreased to ~28 mM and persisted (Table 2). A new O$_2$-limited steady state was achieved 5 days post aeration modifications and steady state lasted 6 days during which RNA was extracted (Fig. 4). Total RNA was extracted during the O$_2$ replete and limited steady states as described above. Using identical techniques for total RNA extraction as described for *N. europaea* resulted in < 10 µg total RNA, an insufficient yield for downstream applications. RNA yields were increased by increasing the duration of sonication to 30 sec on/off for a total of 2 min as well as doubling the extract volume (80 ml) resulting in approximately 20 µg total RNA and ~500 ng for mRNA-Seq. Under O$_2$-limited steady state, *Nitrobacter* cell morphology was noticeably altered. Cell pellets from this condition formed clumps and were more difficult to re-suspend for RNA extraction. The color of the cell pellet was also a darker brown color compared to pellets extracted from O$_2$-replete conditions. These observations were unique to *N. hamburgensis* cultures and were caused by O$_2$ limitation during growth.
Table 1: O₂ replete bioreactors mass balance

<table>
<thead>
<tr>
<th>Nitrogen Conc.</th>
<th>N. europaea</th>
<th>N. hamburgensis</th>
<th>Co-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄⁺ in feed⁠</td>
<td>60</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>NO₂⁻ in feed⁠</td>
<td>0</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>NH₄⁺ in effluent⁠</td>
<td>0-1</td>
<td>0</td>
<td>0-1</td>
</tr>
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<td>NO₂⁻ in effluent⁠</td>
<td>57-60</td>
<td>0-1</td>
<td>0-1</td>
</tr>
<tr>
<td>NO₃⁻ in effluent⁠</td>
<td>0</td>
<td>55-59</td>
<td>52-60</td>
</tr>
<tr>
<td>Cell density⁠</td>
<td>0.15-0.17</td>
<td>0.072-0.08</td>
<td>0.32-0.37</td>
</tr>
</tbody>
</table>

⁠a Nitrogen concentration (mM)

⁠b Cell Density (OD₆₀₀)

Table 2: O₂-limited bioreactors mass balance

<table>
<thead>
<tr>
<th>Nitrogen Conc.</th>
<th>N. europaea</th>
<th>N. hamburgensis</th>
<th>Co-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄⁺ in feed⁠</td>
<td>60</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>NO₂⁻ in feed⁠</td>
<td>0</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>NH₄⁺ in effluent⁠</td>
<td>24-35</td>
<td>0</td>
<td>1-38</td>
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<td>NO₂⁻ in effluent⁠</td>
<td>28-32</td>
<td>28-33</td>
<td>29-60</td>
</tr>
<tr>
<td>NO₃⁻ in effluent⁠</td>
<td>0</td>
<td>26-32</td>
<td>2-30</td>
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<tr>
<td>Cell density⁠</td>
<td>0.059-0.062</td>
<td>0.043-0.05</td>
<td>0.06-0.10</td>
</tr>
</tbody>
</table>

⁠a Nitrogen concentration (mM)

⁠b Cell density (OD₆₀₀)
2.3.1.1 *Nitrosomonas europaea* transcriptome analysis

The transcriptome of *N. europaea* were analyzed using CLC Bio Workbench. The DGE between O$_2$ replete and O$_2$ limited steady state samples were expressed as a difference in fold change. Significant DGE was defined by a *p*-value threshold ≤ 0.05. A total of 917 of the 2,640 protein-encoding genes in *N. europaea* were found to be differential expressed and significantly different between treatments (~35% of total genome) (Table 3). Genes related to energy production, carbon assimilation, motility and stress response were heavily impacted by the limitation of available O$_2$. The largest increase in fold change was observed in a hypothetical protein hypothesized to be an integral component of the cell membrane (138.60-fold increase in expression). The largest decrease in fold change was observed in the *cbbOSQ* gene cluster (21.50, 10.90, 9.00) (Table 3), two of which code for the carbon fixation proteins of RuBisCO.

To examine gene expression levels relative to their biological functions, the genes were organized clusters of orthologous groups (COGs) for *N. europaea* (Fig. 5). Of the 18 functional groups, *N. europaea*, under O$_2$ limitation, presented the mRNA levels of 381 genes at higher levels while the mRNA levels of 488 genes were at lower levels. Genes related to lipid metabolism, intracellular trafficking and secretion, cell motility, signal transduction, transcription and ribosomal structure and translation were at higher mRNA levels under low O$_2$ concentrations. Genes related to secondary metabolites, inorganic ion transport, coenzyme metabolism, nucleotide synthesis, amino acid transport, carbohydrate transport, energy production, post translational modification, cell envelope biogenesis, defense mechanisms, cell division and DNA replication were all at lower levels.
Transcripts of genes involved in energy production were altered as a result of O₂ limitation. For example, the mRNA levels of the 3 subunits of AMO (amoA, amoB and amoC) were in lower amounts when O₂ was limiting (1.75, 1.42, 3.86) (Table 3). In contrast, the transcript levels of HAO were not affected. The mRNA levels of cytochromes involved in electron transport following NH₃ oxidation (c-554 and c-552) did not present different levels with the exception of genes for the terminal electron accepting complex: cytochrome c-oxidase. Transcript levels of the gene cluster (coxA, coxA2, coxB1, coxB2 and coxC) were higher (1.57, 4.98, 9.66, 2.75, 1.74) under O₂ limitation (Table 3). Cytochrome c-oxidase assembly gene (ctaG) was also at higher level (1.76) (Table 3). Interestingly, the transcript levels for both ATP
synthase and NADPH dehydrogenase were not significantly different between treatments despite being downstream of the genes for the terminal cytochrome complex. The transcript level of *aniA (nirK)*, encoding a dissimilatory NO₂⁻ reductase, was more than 4-fold lower (Table 3). The mRNA of *nor* (the gene for nitric oxide reductase responsible for N₂O production) was not significantly different under O₂ limitation. Nitrogen assimilatory genes also did not change transcript levels as a result of O₂ limitation in *N. europaea*.

### 2.3.1.1.2 Carbon fixation

*N. europaea* showed a large decrease in the number transcripts associated with carbon fixation under limited O₂ (Table 3). The *cbb* gene cluster consisting of *cbbO, cbbQ, cbbS* and *cbbR* was differentially expressed. As noted above, the mRNA of the genes *cbbO* (VWA domain-containing protein), *cbbQ* (ATPase) and *cbbS* (ribulose bisphosphate carboxylase) were at considerably lower levels (21.51, 9.00, 10.90). Interestingly, the mRNA for *cbbR* (the gene for a transcriptional regulator of the cluster) was at higher levels (4.03) (Table 3). Genes involved in the glycolytic pathway did not show a significant difference in mRNA levels under limited O₂.

### 2.3.1.1.3 Motility, chemotaxis and cell membrane

Genes associated with chemotaxis and motility were differentially expressed under O₂ limitation (Table 3). The mRNAs of the two-component regulatory genes responsible for conferring a twitching motility *pilI* and *pilH* were at higher levels under O₂ limitation (Table 3). Twitching motility protein PilT transcripts were increased more than 2-fold. The mRNA of genes for flagella biosynthesis protein FliS, motor switch protein FliG, and additional biosynthesis protein FliR, were at lower levels (2.67, 2.39, 1.60) (Table 3). The *che* gene cluster consisting of (*cheWYZR*) was mostly unaffected by the change in O₂ concentration with the exception of the
gene the response regulator protein CheY which showed a 2.09 fold mRNA increase (Table 3).

Transcripts for the *mur/mra/fts* gene cluster involved in peptidoglycan biosynthesis (NE0982-NE0993) were lower under O$_2$ limitation (Table 3).

### 2.3.1.1.4 Stress response

Many genes involved in stress response were at higher level during O$_2$-limited steady state. The mRNA of genes for cold shock proteins (NE1312, NE1731), and heat shock protein Hsp2 (NE2074) were all at higher level (4.66, 3.63, 1.56) (Table 3). Transcript levels of universal stress genes coding for chaperone proteins also increased between 1.64 and 1.90-fold (Table 3). The mRNA of the gene for superoxide dismutase (*sodB*), which encodes for a protein responsible for detoxifying free radicals generated by high levels of O$_2$, was nearly 4-fold lower when O$_2$ was limiting (Table 3). Genes for ribosomal proteins were differentially expressed under O$_2$ limitation, favoring increased transcript numbers (Table 3).

Table 3: *N. europaea* statistically significant changes in transcript levels for genes encoding electron transport and transformation, C fixation and metabolism, reductant consumption, stress response-related genes, motility and chemotaxis and sulfur metabolism-related proteins.

<table>
<thead>
<tr>
<th>Gene number</th>
<th>Gene name</th>
<th>Role</th>
<th>Fold change$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy transformation and e$^-$ transport</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE0943 – NE0945, NE1411, NE2062-NE20664, NE0962, NE2044, NE0924, NE0683, NE0684, NE1013, NE1016, NE1017</td>
<td><em>amoABC</em></td>
<td>Ammonia oxidation</td>
<td>-1.42 to -3.86</td>
</tr>
<tr>
<td>NE0809 – 0811</td>
<td><em>hao2, hao1, hao3, coxA2, coxB1, coxC, coxA, coxB2</em></td>
<td>Hydroxylamine oxidation</td>
<td>1.00</td>
</tr>
<tr>
<td>NE0809 – 0811</td>
<td><em>cytB</em></td>
<td>Cytochrome bc1 (Ubiquinol-Cyt, Cyt B)</td>
<td>2.02 to 2.79</td>
</tr>
<tr>
<td>NE0809 – 0811</td>
<td><em>ctaG</em></td>
<td>Cytochrome assembly</td>
<td>1.74</td>
</tr>
</tbody>
</table>
### Reductant consumption

<table>
<thead>
<tr>
<th>Gene Cluster</th>
<th>Gene(s)</th>
<th>Function</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE0924</td>
<td>aniA(nirK)</td>
<td>Nitrite reductase and electron transport</td>
<td>-4.54</td>
</tr>
<tr>
<td>NE2003 – NE2006</td>
<td>norBDQ</td>
<td>Nitric oxide reductase</td>
<td>1.00</td>
</tr>
</tbody>
</table>

### Carbon fixation and metabolism

<table>
<thead>
<tr>
<th>Gene Cluster</th>
<th>Gene(s)</th>
<th>Function</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE1918-1920</td>
<td>cbbOQS</td>
<td>RuBisCO gene cluster</td>
<td>-9.00 to -21.51</td>
</tr>
<tr>
<td>NE1922</td>
<td>cbbR</td>
<td>LysR transcriptional regulator</td>
<td>4.03</td>
</tr>
<tr>
<td>NE1921</td>
<td>rbcL</td>
<td>RuBisCO LC</td>
<td>-4.33</td>
</tr>
<tr>
<td>NE1926</td>
<td>cynT</td>
<td>Carbonic anhydrase</td>
<td>-1.52</td>
</tr>
</tbody>
</table>

### Stress Response

<table>
<thead>
<tr>
<th>Gene Cluster</th>
<th>Gene(s)</th>
<th>Function</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE0870</td>
<td>sodB</td>
<td>Superoxide dismutase</td>
<td>-3.10</td>
</tr>
<tr>
<td>NE1312, NE2074, NE009</td>
<td>cpsD1, cspD2, Hsp2</td>
<td>Temperature stress response; chaperonins, etc.</td>
<td>1.56 to 4.66</td>
</tr>
<tr>
<td>NE1028</td>
<td>uspA</td>
<td>Universal stress protein</td>
<td>1.90</td>
</tr>
<tr>
<td>NE2047 – NE2050</td>
<td>rplIAK</td>
<td>50s ribosomal proteins</td>
<td>-1.46 to -2.42</td>
</tr>
<tr>
<td>NE1292, NE1293, NE1466, NE1484, NE1644, NE2054, NE2055, NE2143, NE2340</td>
<td>rpmAGF, rpiUM, rpsGLT</td>
<td>30s, 50s ribosomal proteins</td>
<td>2.09 to 5.22</td>
</tr>
</tbody>
</table>

### Sulfur Metabolism

<table>
<thead>
<tr>
<th>Gene Cluster</th>
<th>Gene(s)</th>
<th>Function</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE0576 – 0578, NE05282</td>
<td>cysA, cysW, cysU, Sbp1</td>
<td>Sulfate import and metabolism</td>
<td>-2.21 to -2.73</td>
</tr>
</tbody>
</table>

### Cell membrane

<table>
<thead>
<tr>
<th>Gene Cluster</th>
<th>Gene(s)</th>
<th>Function</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE2022, NE0797</td>
<td>exoT, exoD</td>
<td>Exopolysaccharide synthesis</td>
<td>1.67 to 2.15</td>
</tr>
<tr>
<td>NE0982 – NE0993</td>
<td>mur, mra, fts</td>
<td>Peptidoglycan synthesis</td>
<td>-1.64 to -4.93</td>
</tr>
<tr>
<td>NE2296-NE2300</td>
<td>BioDCHFB</td>
<td>Biotin synthesis</td>
<td>-1.62 to -2.45</td>
</tr>
<tr>
<td>NE2218</td>
<td>N/A</td>
<td>Hypothetical membrane protein</td>
<td>168.68</td>
</tr>
</tbody>
</table>

### Chemotaxis and Motility

<table>
<thead>
<tr>
<th>Gene Cluster</th>
<th>Gene(s)</th>
<th>Function</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE0285, NE2174, NE2431</td>
<td>pil, pilT</td>
<td>Twitching motility</td>
<td>1.67 to 2.60</td>
</tr>
<tr>
<td>NE1006 – NE1008</td>
<td>pilH</td>
<td>Chemotaxis response regulators</td>
<td>1.48 to 3.35</td>
</tr>
<tr>
<td>NE1596, NE2084, NE0457</td>
<td>fliS, fliG, fliR</td>
<td>Flagella biosynthesis</td>
<td>-1.60 to -2.67</td>
</tr>
</tbody>
</table>

*aFold change is the difference in mRNA transcripts between the control (replete O₂) and the treatment (O₂-limited) (p ≤ 0.05). A value of 1.00 indicates no statistically significant change in at least one of the listed genes.*
2.3.1.2 Nitrobacter hamburgensis transcriptome analysis

The *N. hamburgensis* transcriptome was analyzed in the same manner as *N. europaea* with the same set of parameters. A total of 711 of the 4,716 genes were found to be differential expressed and significantly different between treatments (~15% of total genome) (Fig. 6). Genes related to energy production, carbon assimilation, polysaccharide synthesis and sulfur metabolism were heavily impacted by the limitation of available O\(_2\) (Table 4). The largest increase in fold change was observed in the *vir* gene cluster responsible for conjugation and type-IV pilus construction (1.94 to 33.60) (Table 4). The largest decrease in fold change was observed in the *fixK* a protein involved in microaerobic metabolism of closely related organism *Bradyrhizobium japonicum* (Table 4).

**Fig. 6:** *N. hamburgensis* genes grouped as clusters of orthologous groups (COG) under O\(_2\)-limited steady state growth in continuous flow bioreactor.
The clusters of orthologous groups (COGs) for *N. hamburgensis* were created to represent gene expression levels relative to major biological functions (Fig. 6). Of the 18 orthologous groups, *N. hamburgensis* expressed 232 genes at higher levels while 182 genes were expressed at lower levels (Fig. 6). Many of the *N. hamburgensis* genes are hypothetical or without a classified proposed role, thus are not assigned to a functional group that can be quantified. Genes related to lipid, coenzyme, nucleotide and amino acid metabolism, as well as energy production, intracellular trafficking, cell envelope biogenesis, defense mechanisms, cell division, DNA replication, transcription and ribosomal biogenesis were at higher mRNA levels under low O₂ concentrations. Genes related to inorganic transport, carbohydrate metabolism, post-translational modification, cell motility, and signal transduction pathways were expressed at lower levels (Fig. 6).

2.3.1.2.1 Energy transformation and electron transport

Under low O₂ conditions, *N. hamburgensis* showed differential expression of genes involved in nitrogen metabolism and energy production. The transcripts for nitrite oxidoreductase (*nxrABDG*) were slightly at lower levels from 1.51 to 1.84-fold (Table 4). The gene for dissimilatory nitrite reductase (*nirK*) responsible for NO production, showed higher transcript levels (1.73-fold) and the nitrite/nitrate transporter protein Nrt (Nham_3444) showed a decrease in transcript levels (1.44) under O₂ limitation (Table 4). Genes involved in nitrogen assimilatory pathways were not significantly different between treatments. Similar to the transcriptome of *N. europaea*, transcripts encoding for NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome C reductase (complex IV) and ATP synthase (complex V), all involved in energy transformation and electron transport, were not altered under O₂-limited growth. *N. hamburgensis* contains several genes encoding b-type cytochromes (*b₅₆₁*),
an important electron transfer component in aerobic carboxidotrophic bacteria and whose activity in *N. hamburgensis* is thought to be linked to the low O$_2$ conditions (Kirstein et al. 1986). Of the eight type-*b* cytochromes present in the genome, only two showed a significant increase in transcript numbers (Nham_2570 and Nham_3599) and only changed about 2 fold (Table 4). The remaining type-*b* cytochromes did not show a significant difference between treatments and two showed lower levels of transcripts (1.63 and 3.38).

### 2.3.1.2.2 Carbon fixation

*N. hamburgensis* contains 3 copies of RuBisCO throughout its genome and plasmids. Only the copy of type I RuBisCO on plasmid pPB13 (Nham_4332 and Nham_4333) was significantly different and only slightly at lower transcript levels (1.72, 1.62) (Table 4). Interestingly, carboxysome shell proteins, including a carboxysome specific carbonic anhydrase (CA), were at higher transcript level (1.41, 1.61) (Table 4). Upstream of RuBisCO is a set of P-II regulatory protein genes *glnB* and *glnK*. These genes are involved in regulating nitrogen assimilation in most bacteria and were found to have higher transcript levels (2.99, 2.86-fold) under O$_2$ limitation (Table 4).

### 2.3.1.2.3 Motility, chemotaxis and cell membrane

In O$_2$-limited cultures, *N. hamburgensis* increased transcript levels of a gene cluster responsible for the production of polysaccharides containing glycosyl transferases, hydrolases, and homopolysaccharide components (e.g. Nham_1049 to Nham_1064) (Table 4). Transcripts for a chemotaxis regulator gene *cheY* were negatively impacted by O$_2$ limitation and were lower 2.51-fold (Table 4). Genes involved in the main pathway dedicated to peptidoglycan biosynthesis
were not significantly different between treatments for *N. hamburgensis* as opposed to *N. europaea* (Table 3, Table 4).

### 2.3.1.2.4 Conjugation

Several genes involved in type-IV pilus synthesis were at higher level in response to O$_2$ limitation (Table 4). Conjugal transfer protein (*vir*) transcripts that comprise the type-IV secretion system in *N. hamburgensis* were at higher levels ranged from 1.93 to 33.60 under low O$_2$ concentration (Table 4). Interestingly, the Type II and type III secretory pathways did not show differential expression.

### 2.3.1.2.5 Iron metabolism

The transcript levels of genes involved in Iron (Fe) acquisition and O$_2$ reduction increased under O$_2$ limitation (Table 4). There was a 6.79 fold increase in a Fe$^{2+}$-Oxygenase protein PKHD-type hydroxylase (Nham_3637) adjacent to a siderophore receptor (Nham_3678) which was 10.29-fold higher (Table 4). An additional siderophore receptor (Nham_3719) was also found to have higher transcript levels (6.55) (Table 4). Bacterioferritin (*bfr*), an iron storage protein, was expressed 7.63-fold higher as were transcripts of a non-heme iron protein used to reversibly bind O$_2$ for transport and storage (Nham_1456) (Table 4). Genes *fixK1* and *fixK2* were found to be negatively impacted by O$_2$-limitation; *fixK2* presenting the largest decrease in fold change for the entire transcriptome (7.48 and 63.90 respectively) (Table 4). Interestingly, an autoinducer synthase gene (Nham_4466) flanked by hypothetical proteins on plasmid PB113 was 24.94 fold higher under O$_2$ limitation (Table 4).
Table 4: *N. hamburgensis* statistically significant changes in transcript levels of genes encoding electron transport and transformation, C fixation and metabolism, reductant consumption, motility, cell membrane and sulfur metabolism-related proteins.

<table>
<thead>
<tr>
<th>Gene number</th>
<th>Gene name</th>
<th>Role</th>
<th>Fold change$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy transformation and e$^-$ transport</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nham_3445 - 3449, 2961</td>
<td><em>nxrABDG</em></td>
<td>Nitrite/nitrate oxidation</td>
<td>-1.47 to -2.18</td>
</tr>
<tr>
<td>Nham_3444</td>
<td><em>nrt</em></td>
<td>NO$_2^-$ / NO$_3^-$ transporter</td>
<td>-1.44</td>
</tr>
<tr>
<td>Nham_4177, 3458, 0256</td>
<td><em>Caa3, cyt</em></td>
<td>Terminal electron acceptors, electron transport</td>
<td>-1.54 to -7.89</td>
</tr>
<tr>
<td>Nham_2570 - 3599</td>
<td><em>cytB</em></td>
<td>High affinity, alternative cytochrome B561</td>
<td>1.50 to 2.17</td>
</tr>
<tr>
<td><strong>Reductant composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nham_3281</td>
<td><em>nirK</em></td>
<td>Nitrite reductase</td>
<td>1.73</td>
</tr>
<tr>
<td><strong>Carbon fixation and metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nham_4324 , 4330(p)</td>
<td><em>glnB, glnK</em></td>
<td>Nitrogen/carbon regulatory proteins</td>
<td>2.99, 2.86</td>
</tr>
<tr>
<td>Nham_4332, 4334</td>
<td><em>rbcl1, rbcl2</em></td>
<td>Ribulose-1,5-bisphosphate Carboxylase/oxygenase large subunit</td>
<td>-1.69, -1.72</td>
</tr>
<tr>
<td>Nham_4334, 4339</td>
<td><em>csoS2</em></td>
<td>Carboxysome Shell Proteins (plasmid)</td>
<td>1.41, 1.49</td>
</tr>
<tr>
<td>Nham_4335</td>
<td><em>csoS3</em></td>
<td>Carbonic anhydrase (carboxysome)</td>
<td>1.61</td>
</tr>
<tr>
<td><strong>Motility and chemotaxis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nham_3373 - 3375</td>
<td><em>cheW,</em></td>
<td>Chemotaxis response regulator protein</td>
<td>-1.43 to -2.51</td>
</tr>
<tr>
<td>Nham_1382 - 1384</td>
<td><em>flgA, flgG, flgF</em></td>
<td>Flagella biosynthesis</td>
<td>-1.61 to -2.36</td>
</tr>
<tr>
<td><strong>Conjugation / Type IV pilus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nham_4445,4446, 4467, 4562, 4566, 4567, 4569</td>
<td><em>virB4, virB3, virB5, virB11</em></td>
<td>Conjugal transfer proteins and pilus formation</td>
<td>1.80 to 33.60</td>
</tr>
<tr>
<td><strong>Low oxygen regulator proteins/stress proteins/Fe acquisition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nham_4375</td>
<td><em>fixK1</em></td>
<td>Transcriptional regulator protein</td>
<td>-7.48</td>
</tr>
<tr>
<td>Nham_1951</td>
<td><em>fixK2</em></td>
<td>Transcriptional regulator protein</td>
<td>-63.90</td>
</tr>
<tr>
<td>Nham_4466</td>
<td><em>N/A</em></td>
<td>Autoinducer synthase protein</td>
<td>24.94</td>
</tr>
<tr>
<td>Nham_1456</td>
<td><em>N/A</em></td>
<td>Hemerythrin protein</td>
<td>25.85</td>
</tr>
<tr>
<td>Nham_1458</td>
<td><em>N/A</em></td>
<td>Universal stress protein</td>
<td>4.01</td>
</tr>
<tr>
<td>Nham_2904</td>
<td><em>bfr</em></td>
<td>Bacterioferritin</td>
<td>7.63</td>
</tr>
<tr>
<td>Nham_3637</td>
<td><em>ybiX</em></td>
<td>PKHD-type hydroxylase</td>
<td>6.79</td>
</tr>
<tr>
<td>Nham_3638</td>
<td>Siderophore receptor</td>
<td>10.29</td>
<td></td>
</tr>
</tbody>
</table>
Nham_3719
**Sulfur metabolism**
Nham_3557 - cysW123
(3564, 3558 - 3561)
Siderophore receptor
6.55
Nham_1049 -
(1064)
**Cell membrane**
Nham_1270 - mur, mra
(1282)
LPS biosynthesis
1.62 to 2.58
Peptidoglycan biosynthesis cluster
1.00

*Fold change is the difference in mRNA transcripts between the control (replete O\textsubscript{2}) and the treatment (O\textsubscript{2}-limited) (p ≤ 0.05). A value of 1.00 indicates no statistically significant change in at least one of the listed genes.

### 2.4 Discussion

The purpose of this study was to further contribute to the understanding of the effects of low O\textsubscript{2} levels to the process of nitrification. In this study, the responses of *N. europaea* and *N. hamburgensis* in co-culture and single culture bioreactors to O\textsubscript{2} limitation were characterized. The first evidence that the organisms were limited for O\textsubscript{2} was that the cultures were not metabolizing all of the supplied growth substrate (60 mM NH\textsubscript{4}\textsuperscript{+} or NO\textsubscript{2}\textsuperscript{-}). The same bioreactors under O\textsubscript{2}-replete conditions normally consumed ~98-99% of the growth substrate.

Typically, when O\textsubscript{2} was being gradually decreased to achieve the desired ~50% substrate consumption, the cultures of *N. europaea* and *N. hamburgensis* showed a near linear decrease in cell density, paired with an increase of growth substrate. After approximately 5 days, both *N. europaea* and *N. hamburgensis* bioreactors stabilized, i.e. the cells began to grow at the same rate, but at a lower cell density and limited now by the lower concentration of O\textsubscript{2}. The concentration of the growth substrate (NH\textsubscript{3} or NO\textsubscript{2}\textsuperscript{-} accordingly) was maintained at steady levels (~50%). Although the supply of O\textsubscript{2} was the main driver in producing differential gene expression from replete and O\textsubscript{2}-limited states, potential effects of excess substrate cannot be fully ignored.
We observed incomplete nitrification in our experiments when O₂ was limiting, but we also cannot ignore that the conditions tested do not represent what is observed in engineered environments due to the presence of other microorganisms and nutrient content (Abeliovich 1987).

A first hurdle in this study was to determine the O₂ concentration when limited in the bioreactors. In preliminary attempts O₂ probes displayed very low concentrations in the medium of O₂-limited bioreactors, still the cells were utilizing the growth substrate but at lower cell densities. This suggested that the cells had sufficient O₂ to consume 50% of the substrate, but left little dissolved O₂ and was therefore undetectable by a probe. Therefore, we used our measurements of substrate consumed and the known $K_s$ for the organisms to determine the O₂ concentration. For the substrate consumed in each bioreactor, one can assume that the O₂ concentration is close to the reported $K_s$ for each organism. The O₂ concentration and consumption rate in O₂-limited bioreactors of *N. europaea* and *N. hamburgensis* was calculated roughly equal to the $K_s$ of each organism as reported by Laanbroek, Bodelier, and Gerards (1994). Based on the amount of O₂ required for NO₂⁻ oxidation and the relative $K_s$ for O₂ of *N. hamburgensis*, the O₂ concentration in the bioreactor was approximately 6 times higher (54.8 µM) than that of *N. europaea* (9.25 µM). It is not surprising that when NH₄⁺ is abundant in O₂-limited co-culture bioreactors, *N. europaea*’s higher affinity for O₂ can outcompete *N. hamburgensis* causing washout of O₂-starved *N. hamburgensis* cells.

It is interesting to note that the growth rate of *N. europaea* in this study was the same under both O₂ levels. Still, the O₂ limitation in the steady state bioreactors resulted in ~35% of the genome being differentially expressed compared to growth with replete O₂. Other studies have described nitrifying bacteria, such as *N. europaea*, as capable of adapting to low O₂
concentrations (Laanbroek and Gerards 1993; Laanbroek, Bodelier, and Gerards 1994). Studies in other laboratories have focused on the response of specific genes and more molecular evidence for how cells adapt to low O_2 environments was needed. The transcriptomic analyses of *N. europaea* and *N. hamburgensis* in this study provides new information about the response to O_2 limitation during nitrification and hints at potential compensation tactics to grow in O_2-limited environments on a global transcriptome scale.

The transcript levels of genes related to nitrogen metabolism and energy production were different in the response of *N. europaea* to O_2-limited growth states. The enzyme AMO (encoded by *amoA, amoB* and *amoC* in two identical gene loci in the chromosome) uses O_2 for the oxidation of NH_3. The AMO transcript levels were at lower levels in O_2-limited bioreactors (Table 1). There was NH_4^+ available in the medium, but there was not sufficient O_2 to use all the NH_4^+. *N. europaea* may be conserving energy by producing fewer transcripts and potentially less protein dedicated to NH_3 oxidation. The genes involved in subsequent steps of NH_3 oxidation, particularly *hao* (three identical gene copies NE0962, NE2044 and NE2339) were not significantly different between replete and O_2-limited treatments. *N. europaea* can retain high levels of HAO protein and activity under NH_4^+ starvation (Nejidat, Shmuely, and Abeliovich 1997) and it would be consistent with no changes in the *hao* mRNA levels. The effects of low dissolved O_2 on the levels for the HAO mRNA have not been characterized until our study. The stability of HAO transcripts observed, may be linked to a similar mechanism related to low intracellular levels of NH_4^+ as described by (Nejidat, Shmuely, and Abeliovich 1997). There is also evidence that when NH_4^+-starved cells are incubated in NH_4^+-rich medium, the levels of the *hao* mRNA did not change as dramatically as the *amo* transcripts (Wei et al. 2006; Sayavedra-Soto et al. 1996). According to previous studies, *N. europaea* was found to express higher
mRNA levels of AMO and HAO in the presence of limited O\textsubscript{2} in batch cultures (Yu and Chandran 2010). The authors concluded the response was based on a specific mechanism to cope with low O\textsubscript{2} where \textit{N. europaea} becomes more efficient at utilizing nitrogen (Yu and Chandran 2010). Our results contradict this finding, as \textit{amo} transcripts were in lower amounts when O\textsubscript{2} was limited. With NH\textsubscript{4}\textsuperscript{+} in excess, it may be in the best interest of \textit{N. europaea} to reduce the amount of AMO in an attempt to save energy and divert resources to more vital components of cell maintenance. Related to NH\textsubscript{4}\textsuperscript{+} oxidation are genes for the components comprising electron transport and energy production. The subsequent steps of NH\textsubscript{4}\textsuperscript{+} oxidation releases free electrons used for oxidative phosphorylation and ATP and NADH production that drives cellular processes and growth. Surprisingly, most transcript levels for components of this pathway were not significantly different under O\textsubscript{2} limitation, with the exception of those for the terminal electron acceptor cytochrome c oxidase whose transcript level saw an increase (Table 3). Perhaps by increasing the number of mRNA for the terminal cytochromes, \textit{N. europaea} is attempting to scavenge O\textsubscript{2}. This observation may explain why \textit{N. europaea} can maintain its growth rate at lower cell densities during O\textsubscript{2} limitation.

The transcript for \textit{nirK} (nitrite reductase, NE2003) encoding the enzyme responsible for the conversion of NO\textsubscript{2}\textsuperscript{-} to NO gas, was 4.54 fold lower under O\textsubscript{2} limitations, suggesting NOx gas production may not be increased during steady state, O\textsubscript{2}-limited growth. Previous studies have demonstrated \textit{N. europaea} produces more NO and N\textsubscript{2}O during transitional periods from high to low dissolved O\textsubscript{2} and vice versa (Perez-Garcia et al. 2014). Our observation of decreased transcript levels of \textit{nirK} during O\textsubscript{2}-limited steady state seems to agree with this interpretation. Future studies measuring NOx emissions during steady state and O\textsubscript{2} transitions coupled to
enzyme activity, molecular analyses and transcriptomics may provide more evidence for the phenomenon of NO\textsubscript{x} gas production as a result of low O\textsubscript{2}.

Elevated NO\textsubscript{2}\textsuperscript{−} concentration has also been shown to result in higher gene expression of \textit{nirK} and \textit{norB}, the genes that code for the enzymes responsible for producing NO and N\textsubscript{2}O gases, a putative cell response to reduce the negative effects of NO\textsubscript{2} to the cells (Yu and Chandran 2010). Under O\textsubscript{2} limitation, the concentration of NO\textsubscript{2}\textsuperscript{−} was ~50% lower when compared to O\textsubscript{2} replete bioreactors, thus if one assumes \textit{nirK} and \textit{norB} expression may be regulated in part by NO\textsubscript{2}\textsuperscript{−} concentrations, lower transcript levels of each protein should be observed. Our results are in agreement with this interpretation, for \textit{nirK} mRNA levels were lower, but the \textit{norB} transcript levels were not different (Table 3). The control of \textit{nor} expression remains enigmatic and without physiology data one cannot speculate further on the production of either gas. Inorganic carbon limitation has previously shown to have a direct effect on transcript level of \textit{nirK} and NO\textsubscript{x} gas emissions (Jiang et al. 2015). Clearly, more research is needed to understand AOB production of NO\textsubscript{x} gas.

\textit{N. europaea} bioreactors under O\textsubscript{2} limitation resulted in lower transcript levels of carbon fixation genes (Table 3). Ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO) is used to fix CO\textsubscript{2} and provide cells with carbon for biosynthesis. Bioreactors were supplied with HCO\textsubscript{3}− in the main media reservoir as well as additional CO\textsubscript{3}− as method to control pH. CO\textsubscript{3}− is in equilibrium with HCO\textsubscript{3}−, which then is converted to CO\textsubscript{2} by carbonic anhydrase (CA) in the cytoplasm of actively growing cells. The transcript levels for CA were lower in O\textsubscript{2}-limited treatments (Table 3) suggesting \textit{N. europaea} requires less carbon during O\textsubscript{2} limitation. The process of C fixation is expensive, requiring both ATP and NADH and is only required for actively growing cells. RuBisCO activity is negatively impacted by high levels of O\textsubscript{2}, a condition
not experienced by cells growing under limited O$_2$ (Nisbet and Nisbet 2008). While the growth rate under O$_2$ limitation was the same as when O$_2$ was non-limiting, albeit at a lower cell density, one possibility is that RuBisCO is more stable due to the lack of oxygenase activity and other stresses brought on by replete levels of O$_2$ (Mellbye et al. 2016 manuscript submitted for publication).

An increase in transcripts for genes related to stress response was observed in the N. europaea transcriptome under O$_2$-limited growth (Table 3). Among these, chaperone and temperature related stress protein transcripts were in higher numbers (Table 3). It is widely accepted that hsp and cps genes are not solely controlled by changes in temperature and are commonly associated with a global stress response by several organisms (Parsell and Lindquist 1993). Furthermore, many hsp encode proteins that are known to function as molecular chaperones that prevent aggregation and promote the proper refolding of denatured proteins that may be present in cells experiencing limited O$_2$ (Parsell and Lindquist 1993). N. europaea COG’s showed a large increase in the number of transcripts related to ribosomal proteins and translational machinery (Fig. 5, Fig. 7). A closer examination revealed several mRNA’s for ribosomal proteins to be expressed at higher levels under O$_2$ limitation (Table 3). Ribosomal proteins are required for building a functional ribosomes as well as extraribosomal functions including gene expression (Pechmann, Willmund, and Frydman 2013). The increase in associated ribosomal proteins may be due to stress caused by sub-optimal O$_2$, as elevated levels have been observed in other prokaryotes experiencing limited resources (Graumann et al. 1996).

The transcripts for genes responsible for twitching motility were at higher levels under O$_2$ limitations while genes related to flagellum synthesis were in lower numbers. Pérez et al. (2014) found that when N. europaea was grown in bioreactors with high levels of NO$_2^-$, flagellum
synthesis was increased and it was postulated this was a mechanism for movement away from high levels of NO\textsubscript{2}. It would be expected to see a shift towards enhanced motility rather than immobilization under O\textsubscript{2} limitation as \textit{N. europaea} may want to seek a new environment with more available O\textsubscript{2}, similar to what was observed when grown in high NO\textsubscript{2}. In contrast, the higher transcript levels of \textit{pilI}, \textit{pilT} and the chemotaxis response regulator \textit{pilH} suggest \textit{N. europaea} may transition to biofilm growth under O\textsubscript{2} limitation (O’Toole and Kolter 1998). Biofilms are generally the most common method of growth in natural environments (Wagner et al. 1996). Twitching motility may also be preferred under conditions where energy is scarce as less ATP is required for motility (Mattick 2002). Furthermore, a gene coding for an unidentified membrane protein (NE2218) was at levels nearly 169-fold higher under O\textsubscript{2} limitation. While the annotation and function of this gene is not known, the dramatic change in its level may play a vital role and further experimentation is required to characterize its significance. Genes involved in the major pathway for peptidoglycan synthesis were fewer, and may be involved in a strategy for \textit{N. europaea} to overcome low dissolved O\textsubscript{2}. It has been proposed that bacteria may reduce their peptidoglycan content to deal with inhibitions of cell wall biosynthesis and thus increase chances of survival during stressful conditions (Vollmer, Blanot, and Pedro 2008).

The \textit{N. hamburgensis} transcriptome revealed that \textasciitilde{}15\% of the total protein encoding genes were altered in response to an O\textsubscript{2}-limited steady state. Similar to \textit{N. europaea}, single-cultures of \textit{N. hamburgensis} maintained identical growth rates when under replete and limited O\textsubscript{2} while the transcriptome data revealed key differences. \textit{N. hamburgensis} COG’s showed higher transcript numbers when compared \textit{N. europaea} (Fig. 5, Fig. 6). Indications of \textit{b-type} cytochrome synthesis, enhanced cell membrane biogenesis and increased Fe acquisition indicate
*N. hamburgensis* has different strategies than *N. europaea* for growth under low dissolved O$_2$ (Table 4, Fig. 7).

Fewer NXR transcripts were present in *N. hamburgensis* cultures under limited O$_2$ conditions, suggesting a reduced capacity for energy transformation and overall nitrogen metabolism (Table 4). With growth rates similar to that of cells under O$_2$-replete conditions, *N. hamburgensis* consumed less NO$_2^-$ while maintaining stable growth rates, compensating for the O$_2$ limitation. A possible explanation for this response is that *N. hamburgensis* synthesizes alternative high affinity *b*-type cytochromes capable of utilizing lower concentrations of O$_2$ (Kirstein et al. 1986). While our study showed increased transcripts coding *b*-type cytochromes (Table 4), it is not clear if they are active or beneficial. Furthermore, it has been proposed that *N. hamburgensis* is capable of re-routing electrons away from terminal cytochromes and ATP production towards NADH generation that may result in enhanced carbon fixation and PHB synthesis (Arp, Chain, and Klotz 2007). According to a putative pathway, when *N. hamburgensis* experiences O$_2$ limitation, *nirK* expression increases NO formation which inhibits terminal cytochromes thus shifting electron flow towards the generation of NADH that enhances carbon fixation and PHB synthesis used as a method for storing energy (Arp, Chain, and Klotz 2007). We found fewer transcripts for the terminal electron acceptor cytochrome c-oxidase as well as slightly higher levels of *nirK* mRNA (Table 4). However, genes involved in PHB biosynthesis were not in significantly different mRNA levels under O$_2$ limitation and RuBisCO, responsible for C fixation, had fewer transcripts. It is possible that this pathway requires lower concentrations of O$_2$ than were implemented in our study.

Phenotypic differences were observed when harvesting *N. hamburgensis* cells from O$_2$-limited bioreactors for subsequent RNA purification. The cells tended to form a dark-colored
clump that was not as easily disrupted compared to O$_2$-replete cells, which had a pale color and were readily re-suspended. Potentially related to this observation is the fold change increase of transcripts for genes related to lipopolysaccharide synthesis (Table 4). LPS composition is linked to adhesion and may be the causal agent for the phenotypic observation of difficult re-suspension of cells during RNA extractions (Burks et al. 2003). Additionally, flagella biosynthesis genes were lower under O$_2$ limitation and further hint that *N. hamburgensis* is not attempting to migrate from its low O$_2$ environment. Contrary to what was observed in *N. europaea*, *N. hamburgensis* did not alter gene expression of peptidoglycan biosynthesis suggesting the use of a slightly different strategy when it comes to altering membrane composition and adhesion properties.

*B. japonicum* and *N. hamburgensis* share a 98.1% homology of the 16s ribosomal DNA and are phylogenetically related (Bagg and Neilands 1987). It is known that *B. japonicum* adapts to different environmental O$_2$ concentrations by triggering expression of multiple high-affinity terminal cytochrome oxidases and that are similar to those in the *N. hamburgensis* genome (Nellen-Anthamatten et al. 1998). These genes may also play a role in the previously observed ability of the organism to increase its affinity for O$_2$ as well as trigger the expression of additional genes when severely limited for O$_2$ (Laanbroek, Bodelier, and Gerards 1994). Many micro-aerobes, including *B. japonicum*, have been shown to express alternate high-affinity cytochrome *cbb*$_3$ oxidases and show activity at < 10 nM concentrations of O$_2$ (Zufferey et al. 1996). The concentration of O$_2$ is the main effector of the *fixNOQP* regulon in *B. japonicum*, a gene cluster that is not present in the genome of *N. hamburgensis*. FixK is an effector, responsible for activating genes involved in microaerobic respiration and denitrification which include the synthesis of the high affinity *cbb*$_3$-type terminal oxidases and denitrification genes in *B. japonicum*, and thus potentially *N. hamburgensis* (Bedmar et al. 2005). The cascade of genes
activated by FixK also promotes transcription of additional fix and nif genes which are important in N₂ fixation in B. japonicum (Mesa et al. 2008a). The transcripts for two putative nifU-like genes in N. hamburgensis (Nham_0016, Nham_1656) were not significantly different between treatments. Because fixK transcripts were observed to be much lower in N. hamburgensis under low dissolved O₂, in our experiment the O₂ concentration may not have been low enough to trigger a response (Mesa et al. 2008b). In vivo activity of FixK has been reported to only be achieved by anoxic conditions where nitrate respiration dominates (Philip, Batut, and Boistard 1990). FixK may also work as a negative regulator of genes involved in micro-aerobic growth and requires further examination in N. hamburgensis (Batut et al. 1989).

Under limiting amounts of O₂, N. hamburgensis increased the number of transcripts for proteins involved in Type-IV pilus construction. A typical stress response for prokaryotes is to initiate genetic exchange, a task that may be accomplished by the conjugatory pili in N. hamburgensis; genes which mRNAs were at dramatically higher levels under O₂-limited growth (Table 4). One strategy for N. hamburgensis may be to initiate conjugation during stress conditions such as O₂ limitation and is supported by the increase in transcripts for genes related to DNA repair and recombination (Fig. 6). Further support for plasmid conjugation between cells was the higher levels of transcripts for a putative quorum sensing (QS) autoinducer synthase (Nham_4466) which was nearly 25-fold higher under low dissolved O₂. It has been shown that N. winogradsky, another member of the Nitrobacter genus, is capable of producing N-acyl-homoserine lactones (acyl-HSL) autoinducers in a cell-density, growth-phase-dependent manner (Mellbye, Bottomley, and Sayavedra-Soto 2015). The role of acyl-HSLs in Nitrobacter spp. are currently unknown, although our evidence suggests they may play a role in coordinating a response under O₂ limitation in N. hamburgensis and potentially other Nitrobacter spp.
Interestingly, the mRNA of genes involved in Fe acquisition were at higher levels in response to O$_2$ limitation (Table 4, Fig. 7), a response similar to what has been observed in *N. europaea* in response to growth in co-culture with *N. winogradskyi* (Pérez 2014). Fe plays the vital role of a co-factor in several enzymes including cytochromes. PKHD-type hydroxylase is a member of the 2-oxoglutarate (2OG) oxygenase super family of proteins and had transcript levels nearly 7-fold higher under O$_2$-limited conditions (Table 4). Fe$^{2+}$-dependent di-oxygenases are widespread in bacteria and higher organisms and catalyze a multitude of reactions, typically the oxidation of organic molecules using O$_2$ (Falnes, Johansen, and Seeberg 2002). In *E.coli*, protein AlkB, an Fe$^{2+}$ 2OG oxygenase, is responsible for mediating non-specific, Fe-dependent DNA repair (Falnes, Johansen, and Seeberg 2002). If PKHD is crucial and *N. hamburgensis* is seeking to repair DNA caused by stress due to low O$_2$, increased transcript numbers of this gene may be a logical response. Also linked to Fe, two siderophore receptors showed a large increase in mRNA concentration in response to low dissolved O$_2$ (Table 4). Siderophores are high-affinity Fe binding molecules that are secreted from the cell in an attempt to scavenge environmental Fe (Bagg and Neilands 1987). Due to the increase in transcripts for both siderophore receptors, *N. hamburgensis* may be attempting to acquire more Fe while experiencing O$_2$ limitation. Furthermore, mRNA coding for bacterioferritin (*bfr*), an iron storage protein, was more than 7-fold higher between treatments. Assembled into a hollow, roughly spherical 24-mer containing an heme Fe-binding center, bacterioferritins are thought to not only protect the oxidation state of Fe (Fe$^{2+}$) but also act as a general storage system that protects against Fe toxicity (Carrondo 2003). Transcripts of a non-heme iron protein (hemerythrin) used to reversibly bind O$_2$ for transport and storage was found to be highly expressed (Table 4) (Carrondo 2003). It is postulated that hemerythrin proteins serve to distribute O$_2$ efficiently throughout the cell and
supply it to important enzymes such as the methane monooxygenase of *Methylococcus capsulatus* (Chen et al. 2012). It has also been proposed that the O₂-binding domain acts as an O₂ sensor for chemotaxis in *Desulfovibrio vulgaris* and potentially other microbes (Xiong et al. 2000). *N. hamburgensis* could be using this heme containing protein to efficiently capture and partition O₂ concentrations in areas central to energy production in an attempt to compensate for the low intracellular levels. *N. hamburgensis* siderophore production may also be linked to the demand for Fe required for ferritin and hemerythrin proteins that play a role in managing the response to low O₂ environments.

In summary, *N. europaea* and *N. hamburgensis* are able to compensate to low O₂ environments. The overall response to low O₂ by *N. europaea* appears to be a general reduction of activity based on the observation of lower transcript levels of genes in nitrogen metabolism and carbon assimilation. Genes important in membrane composition and energy metabolism are expressed at higher levels under O₂ limitation and suggest *N. europaea* is attempting to scavenge what O₂ is available in the environment. *N. hamburgensis* displayed a different compensation strategy to low O₂, one that focused on biofilm formation, exchange of genetic material, and the acquisition of Fe related to internal O₂ homeostasis. Transcriptome analyses suggests quorum sensing may be central to the regulation of several genes involved in low O₂ compensation, but these results need to be confirmed experimentally. Most studies with *N. hamburgensis* under low O₂ were done in the presence of organic carbon, an attribute that was not part of our study. Due to its diverse, mixotrophic capabilities, it is hard to compare similar studies where O₂ was limiting and an adaption to low O₂ was observed.

*N. europaea* and *N. hamburgensis* appear to utilize different strategies to combat low dissolved O₂ in pure culture. A shared response of both organisms was lower transcript levels of
amo and nxr, used to catalyze the first step on N-oxidation in each species respectively. The N. europaea transcriptome under O₂ limitation revealed an increase in transcripts of the main subunits of the terminal electron accepting cytochrome complex, suggesting a compensatory response to scavenge environmental O₂ while the N. hamburgensis transcriptome revealed biofilm formation, quorum sensing, exchange of genetic material, and Fe acquisition may be part of a primary response to O₂ limitation.

A continuation of this study would be to focus on the transition period from replete O₂ to steady state O₂ limitation. This transitional period may show more of a transcriptional response as many cells may be eliciting a starvation response to low O₂ levels. Other studies have showed NOₓ gasses are produced in larger quantities when N. europaea was entering and exiting O₂ limited states (Chandran et al. 2011). Considering the role of NO as a signaling molecule in many biological systems, NOₓ gas production as a physiological signal may be an important area of future research.
Fig. 7: Model for the overall responses of *N. europaea* and *N. hamburgensis* to low O$_2$ environments. *N. europaea* (top) and *N. hamburgensis* (bottom) cells are depicted as spheres. The large exterior triangle represents the shift in O$_2$ concentration: at the left of the illustration are O$_2$-replete conditions; the right is O$_2$-limited conditions. Internal triangles represent level of gene expression of COG’s or specific cellular processes: as the triangles narrow, gene expression is lower. In *N. europaea*, O$_2$ limited cultures increased expression of stress response genes, reduced expression of energy metabolism, cell membrane, cell motility and carbon fixation genes and reduced NO$_2^-$ concentration, resulting in a lower cell density. In *N. hamburgensis*, O$_2$ limited cultures increased expression of cell envelope, iron metabolism, conjugation genes, reduced expression of energy metabolism, cell motility and carbon fixation genes and reduced NO$_3^-$ concentrations, resulting in a lower cell density.
3. References


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