Comparison of physiology and genome-wide expression in two *Nitrosomonas* spp. under batch cultivation

By

Mohammad Ghashghavi

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In

Microbiology and Biotechnology

Department of Biological Sciences

University of Alberta

© Mohammad Ghashghavi, 2014

Abstract:

Ammonia oxidizing bacteria (AOB) play a central role in the nitrogen cycle by oxidizing ammonia to nitrite. *Nitrosomonas europaea* ATCC 19718 has been the single most studied AOB that has contributed to our understanding of chemolithotrophic ammonia oxidation. As a closely related species, *Nitrosomonas eutropha* C91 has also been extensively studied. Both of these bacteria are involved in wastewater treatment systems and play a crucial part in major losses of ammonium-based fertilizers globally. Although comparative genome analysis studies have been done before, change in genome-wide expression between closely related organisms are scarce. In this study, we compared these two organisms through physiology and transcriptomic experiments during exponential and early stationary growth phase. We found that under batch cultivation, *N. europaea* produces more N_2O while *N. eutropha* consumes more nitrite. From transcriptomic analysis, we also found that there are selections of motility genes that are highly expressed in *N. eutropha* during early stationary growth phase and such observation was completely absent in *N. europaea*. Lastly, principle homologous genes that have been well studied had different patterns of expression in these strains. This study not only gives us a better understanding regarding physiology and genome-wide expression of these two AOB, it also opens a wide array of opportunities to further our knowledge in understanding other closely related species with regards to their evolution, physiology and niche preference.

Acknowledgements:

I would like to thank Dr. Lisa Stein for her full support and supervision throughout my graduate career. I'm very grateful for all the feedback during my Masters and throughout the writing of this thesis. I would also like to thank Albert Rosana in aiding with the laboratory work of this thesis. I would like to thank Kerim Kits as well for his continuous and constructive comments and support throughout my graduate career. Lastly, I would like to thank everyone in Dr. Stein's lab and within the Department of Biological Sciences for their support.

Furthermore, I would like to thank each and every professor at the Department of Biological Sciences that have helped throughout my career. I would also like to thank the University of Alberta for accepting me and providing me with the funds, through Teaching Assistantship, needed to complete my postgraduate degree.

Table of Contents

CHAPTER 1 INTRODUCTION **1**
1.1 THE EVOLUTION OF THE GLOBAL NITROGEN CYCLE 1
1.2 THE HISTORY OF NITROGEN CYCLE EVOLUTION DURING THE ANOXIC ERA 3
1.3 THE LINK BETWEEN METHANE AND AMMONIA OXIDATION 6
1.4 TAXONOMY OF AMMONIA OXIDIZING BACTERIA 8
1.5 COMPLETE GENOME SEQUENCES OF AOB AND THEIR PROPERTIES 12
1.6 *NITROSOMONAS EUROPAEA/NITROSOMONAS MOBILIS LINEAG*E 14
1.7 GENERAL ISOLATION, ENRICHMENT, AND MAINTENANCE PROCEDURES 18
1.8 ECOLOGY AND APPLICATION 21
1.9 THE BEGINNING OF NITRIFICATION FOCUSED RESEARCH 24
1.10 *NITROSOMONAS EUROPAEA* AND ITS GENOME ANALYSIS 25
1.11 PRINCIPAL NITRIFICATION/DENITRIFICATION ENZYMES IN N. EUROPAEA 27
1.12 *NITROSOMONAS EUTROPHA* AND ITS GENOME ANALYSIS 32
1.13 MY THESIS PROJECT 37

CHAPTER 2 MATERIALS AND METHODS **39**
2.1 CULTURE CARE AND PHYSIOLOGICAL MEASUREMENTS: 39
2.2 RNA EXTRACTION: 40
2.3 rRNA REMOVAL AND RNA PURIFICATION: 42
2.4 RNA ANALYSIS: 43

CHAPTER 3 RESULTS **45**
3.1 COMPARISON OF PHYSIOLOGY 45
3.2 GENE EXPRESSION IN *N. EUROPAEA* 50
3.2.1 Metabolism 50
3.2.2 Enzymes 53
3.2.3 Cellular processes 55
3.2.4 Membrane proteins 56
3.2.5 Remaining categories 57
3.3 GENE EXPRESSION IN N. EUTROPHA 58
3.3.1 Metabolism 59
3.3.2 Enzymes 65
3.3.3 Cellular processes 69
3.3.4 Environmental Information Processing 70
3.3.5 Bacterial Motility 71
3.3.6 Remaining categories 72
3.4 GENE EXPRESSION COMPARISON BETWEEN N. EUROPAEA AND N. EUTROPHA 73
3.4.1 Metabolism 75
3.4.2 Enzymes 77
3.4.3 Cellular processes 77
3.4.4 Environmental information processing 78
3.4.5 Bacterial motility 79
3.4.6 Membrane proteins, Iron-binding proteins and Human diseases 80

CHAPTER 4 DISCUSSION **83**
4.1 SIMILARITIES AND DIFFERENCES IN GROWTH 83
4.2 SIMILARITIES AND DIFFERENCES IN NITRITE PRODUCTION AND O_2 CONSUMPTION 86
4.3 SIMILARITIES AND DIFFERENCES IN N_2O PRODUCTION 90
4.4 GENOME-WIDE EXPRESSION 92

4.5 BACTERIAL MOTILITY 95
4.6 EXPRESSION OF PRINCIPAL GENES 97

CHAPTER 5 CONCLUSION AND FUTURE PERSPECTIVES........101

BIBLIOGRAPHY........ ERROR! BOOKMARK NOT DEFINED.

APPENDIX........121

List of Tables

TABLE 1: LIST OF ALL THE GENES INVOLVED IN METABOLISM IN *N. EUROPAEA*........ 52
TABLE 2: LIST OF ALL THE GENES INVOLVED IN ENZYME CATEGORY IN *N. EUROPAEA*........ 54
TABLE 3: LIST OF ALL THE GENES INVOLVED IN CELLULAR PROCESSES CATEGORY IN *N. EUROPAEA* 56
TABLE 4: REMAINING CATEGORIES INCLUDING MEMBRANE PROTEINS IN *N. EUROPAEA*........ 57
TABLE 5: LIST OF ALL THE GENES INVOLVED IN METABOLISM IN *N. EUTROPHA* 61
TABLE 6: LIST OF ALL THE GENES INVOLVED IN ENZYME CATEGORY IN *N. EUTROPHA* 65
TABLE 7: LIST OF ALL THE GENES INVOLVED IN CELLULAR PROCESSES CATEGORY IN *N. EUTROPHA*........ 69
TABLE 8: LIST OF ALL THE GENES INVOLVED IN ENVIRONMENTAL INFORMATION PROCESSING CATEGORY IN *N. EUTROPHA*........ 71
TABLE 9: UP-REGULATED MOTILITY GENES IN *N. EUTROPHA*........ 72
TABLE 10: REMAINING CATEGORIES IN *N. EUTROPHA*........ 73
TABLE 11: OVERVIEW OF TRANSCRIPTOME ANALYSIS AND GENOME PROPERTIES........ 74
TABLE 12: REGULATION OF PRINCIPLE HOMOLOGOUS GENES IN *N. EUROPAEA* AND *N. EUTROPHA* 76
TABLE 13: LIST OF ALL HOMOLOGOUS GENES IN *N. EUROPAEA* AND *N. EUTROPHA*........ 78

List of Figures

FIGURE 1 2
FIGURE 2 11
FIGURE 3: GROWTH CURVE OF *N. EUROPAEA* AND *N. EUTROPHA* 46
FIGURE 4: O_2 CONSUMPTION AND NO_2^- PRODUCTION 47
FIGURE 5: NITRITE PRODUCTION IN *N. EUROPAEA* AND *N. EUTROPHA*........ 48
FIGURE 6: NITROUS OXIDE PRODUCTION IN *NITROSOMONAS SPP.* 49
FIGURE 7: TOTAL NUMBER OF GENES REGULATED IN *N. EUROPAEA* AND *N. EUTROPHA*........ 82

FIGURE A- 1: GROWTH CURVE OF *N. EUROPAEA* AND *N. EUTROPHA* 121
FIGURE A- 2: NITROUS OXIDE PRODUCTION IN *NITROSOMONAS SPP.*........ 121
FIGURE A- 3: O_2 CONSUMPTION AND NO_2^- PRODUCTION 122
FIGURE A- 4: NITRITE PRODUCTION IN *N. EUROPAEA* AND *N. EUTROPHA.*........ 122
FIGURE A- 5: SAMPLE QPCR RESULTS OF STANDARDS AND SAMPLES........ 124

List of Symbols

AMO	Ammonia monooxygenase
AmoA	Ammonia monooxygenase Subunit A
ANAOB	Anaerobic Ammonia Oxidizing Bacteria (annamox)
AOB	Ammonia Oxidizing Bacteria
cycA	Cytochrome C554
cycB	Cytochrome c_M552
copCD	Copper Resistance genes
cytL	cytrochrome P460
cytS	cytochrome c'-beta
HAO	Hydroxylamine dehydrogenase
HCOs	Aldehydes
HURM	Hydroxylamine/Hydrazine-ubiquinone-redox-module
ITS	Intergenic Transcribed Spacer
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	Liter
M	Molar
MBSU	Molecular biology services unit
mM	millimolar
mL	milliliter
MOB	Methane Oxidizing Bacteria
NCBI	Natural Center for Biotechnology Information
NH_4^+	Ammonium
NH_2OH	Hydroxylamine
nir	Nitrite Reductase
NO	Nitric oxide
NO_2	Nitrogen dioxide
N_2O	Nitrous oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	Nitrite Oxidizing Bacteria
nor	Nitric oxide reductase
NOS	Nitrous Oxide reductase
NrfA	Pentaheme nitrite reductase
PCR	Polymerase Chain Reaction
PMF	Proton Motive Force
pMMO	Particulate Methane monooxygenase
PmoA	Particulate monooxyenase Subunit A
RNS	Reactive Nitrogen Species
rRNA	Ribosomal Ribonucleic Acid
RuBisCo	Ribulose-1,5-bisphosphate carboxylase
tRNA	Transfer Ribonucleic Acid
TCA cycle	The citric acid cycle
µl	Microliter

This thesis is an original work by Mohammad Ghashghavi. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Comparison of physiology and genome-wide expression in two *Nitrosomonas* spp. under batch cultivation", Sept 21st, 2014.

This thesis is an original work by Mohammad Ghashghavi. No part of this thesis has been previously published.

Chapter 1
Introduction

1.1 The evolution of the global Nitrogen cycle

Nitrogen flux through the global biogeochemical nitrogen cycle has been altered dramatically in the past few decades (Galloway & Cowling, 2002). This mostly is a result of anthropogenic processes, such as production of ammonia-based fertilizers via the Haber-Bosch process, agriculture of nitrogen-fixing crops, and the combustion of fossil fuels that ultimately lead to the release of nitrogen oxides (Nevison & Holland, 1997; Galloway & Cowling, 2002). Bacteria are the major facilitators of the extant global biogeochemical nitrogen cycle through nitrogen fixation, nitrification, and denitrification. In 2005, a recent discovery has added a new process, anaerobic ammonia oxidation that has its own contribution to the global nitrogen cycle (Dalsgaard *et al.*, 2005; Jetten *et al.*, 2005).

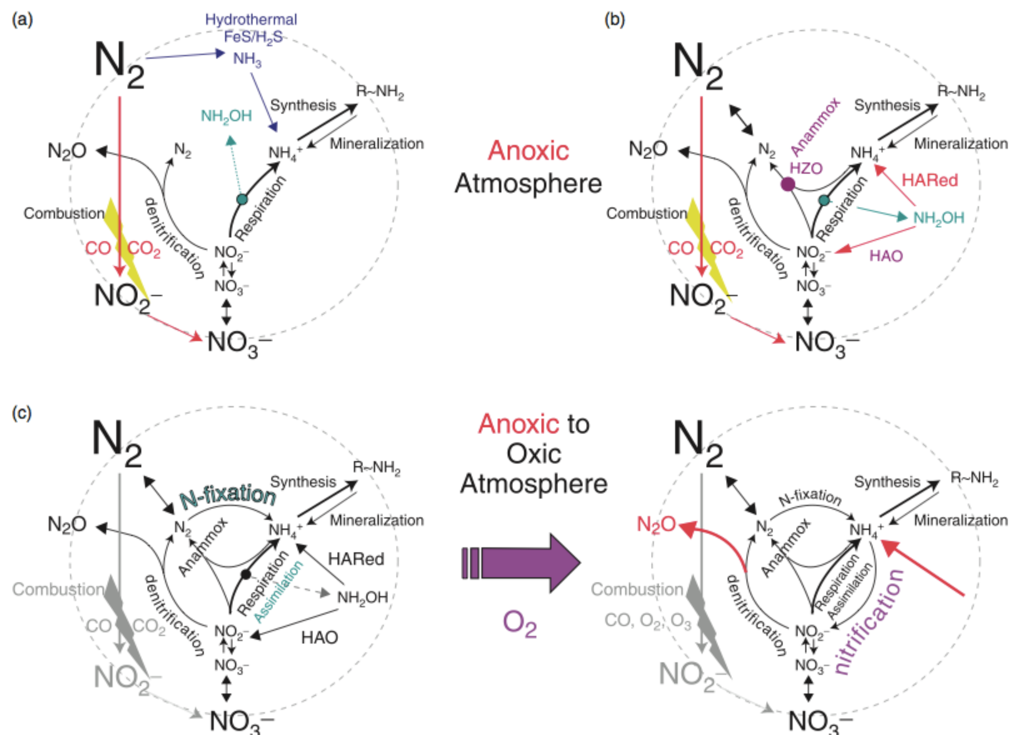


Figure 1: Figure taken from Klotz and Stein, published in 2008 without modification. "Key steps in the evolution of the nitrogen cycle. (a) Very early anaerobic nitrogen metabolism, in which NO_2^- and NO_3^- are produced by combustion and NH_3 is mainly generated from N_2 at hydrothermal vent sites: Incomplete respiratory denitrification (MGD-NarGH/NapAB; cd1-NirS) and respiratory nitrite ammonification [pentaheme-NrfAH (NrfABCD)]. (b) Early anaerobic nitrogen metabolism: To avoid NH_2OH poisoning, invention of hydroxylamine dehydrogenation (HAO/HZO) and reduction (prismane protein), which replenished nitrite and ammonia pools. Resulting emergence of functional HURM and hydrazine hydrolase constitute ANNAMOX process and thus complete recycling of fixed nitrogen. (c) Late anaerobic and early aerobic nitrogen metabolism: Emergence of assimilatory ammonification (siroheme cytochrome c NIR) and N_2 fixation to satisfy increased ammonia demand. Emergence of heme-copper and copper redox centers and subsequent diversification of anaerobic and aerobic respiration (Cu-NirK, HCOs, NOR), complete denitrification (Cu–NOS) as well as aerobic methane- and ammonia oxidation (pMMO/AMO) and thus closure of the biotic nitrogen cycle. Elevated ammonia input from anthropogenic sources leads to elevated nitrifier denitrification in today's nitrogen cycle." (Klotz *et al.*, 2008)

In nitrogen fixation, the breakage of the triple bond in dinitrogen to yield ammonia is conducted using various enzymes and cofactors (Postgate, 1970). Ammonia then continues through sequential oxidation to yield nitrite in the first step of bacterial nitrification, predominantly by ammonia-oxidizing bacteria (AOB) and archaea. Nitrite-oxidizing bacteria (NOB) then complete the whole process by oxidizing nitrite to nitrate in the second step of nitrification (Prosser, 1989). Recently, Thaumarchaea have been shown to be capable of such process in soils, estuarine and marine environments via a different mechanism than that of AOB (Konneke *et al*., 2005; Francis *et al*., 2007, Arp *et al.*, 2007). However, the ammonia oxidizing archaea are not the focus of the present study.

The global nitrogen cycle continues with denitrification, which involves the reduction of nitrate and nitrite as substrates that completes the cycle returning the fixed nitrogen back to its initial gaseous state (Zumft, 1997; Brandes *et al*., 2007). This process is facilitated by either anaerobic respiration in anoxic

environments or by reductive detoxification of nitrite to nitrous oxide in aerobic environments (Lipschultz *et al*., 1981). Anaerobic ammonia oxidation performed by anammox bacteria (ANAOB) couples the oxidation of ammonia to the reduction of nitrite to produce N_2 in anoxic ecosystems (Jetten *et al.*, 2009). Other contributors to the global nitrogen cycles are fungi or plants that release ammonia during degradation of organic matter, as well as assimilatory and respiratory reduction of nitrate or nitrite to ammonia (ammonification). Abiotic processes, such as production of ammonia from N_2, also have their part in modifying the nitrogen cycle (Brandes *et al.*, 1998; Wachtershauser, 2007).

1.2 The history of Nitrogen cycle evolution during the anoxic era

The biggest leap in understanding the evolution of the nitrogen cycle was the recent availability of genomes from ecotypically different AOB as well as that of ANAOB (Chain *et al.,* 2003). The combination of these genomic inventories with the existing literature on the genetics and physiology of AOB and ANAOB has resulted in the identification of individual catabolic modules (Chain *et al*., 2003, Klotz *et al*., 2006, Stein *et al*., 2007, J.M. Norton *et al*., unpublished data, Strous *et al*., 2006). These include the definitive catabolic core to extract and recycle electrons from ammonia, a discovery that has sparked efforts to reformulate the evolutionary history of the nitrogen cycle. Based on both geochemical and oceanographic data, a variety of different speculations on the emergence of both biotic and abiotic components of the biogeochemical nitrogen cycle have risen (Mancinelli & McKay, 1988; Falkowski, 1997; Brandes *et al*.,

1998; Navarro -Gonzalez *et al*., 2001; Raymond *et al.*, 2004; Canfield *et al.*, 2006; Wachtershauser, 2007 and references therein). Some authors have interpreted these data as evidence for a late emergence of denitrification, and others have proposed that N_2 fixation was a very early evolutionary development urgently needed due to rapid depletion of the primordial fixed nitrogen pool (Falkowski, 1997; Navarro-Gonzalez *et al*., 2001). Although key trace metals for nitrogenase function, such as iron and molybdenum, were likely abundant in the Archaean era, early evolution and selection of this function were likely prohibitive due to encoding gene complexity and high cost of the N_2 fixation process (Anbar & Knoll, 2002; Canfield *et al*., 2006).

If it weren't for sufficient recycling of N_2 in the atmosphere by denitrification or anammox, ongoing N_2 fixation even at a lower pace than in modern times would have led to a remarkable decrease in N_2 (Falkowski, 1997; Navarro-Gonzalez *et al*., 2001). An additional argument against an early evolution of the N_2 fixation inventory comes from the fact that eukaryotes lack any N_2-fixing organelles (Klotz and Stein, 2008). These findings have led authors to believe that after independent lineages of cellular life came to stabilize and before abiotic activities could have any control over nitrogen flux, denitrification emerged, earlier and before N_2 fixation (McKay & Navarro-Gonzalez, 2001). When looking at the modern nitrogen cycle, denitrification is widely believed as the main physiological pathway in which the N_2 pool in the atmosphere is replenished (Capone *et al*., 2006). However, the point that is overlooked is that not all

enzymes that function in this current model were likely around during the anoxic Archaean and reducing Proteozoic eras.

Metal cofactors required for the activity of denitrifying enzymes were not widely available. Enzymes with Class B transition metals (e.g. zinc, copper, and cadmium) were likely not functioning in the anoxic Archaean era, whereas enzymes with nickel (e.g. hydrogenase), iron (e.g. sulfur-iron and cytochrome C proteins) and molybdenum (e.g. formate dehydrogenase and nitrate reductase) likely were (Klotz and Stein, 2008). Enzymes with Class B transition metals would possibly become even scarcer in the Proterozoic era where metals like copper would be locked into bio-unavailable sulfidic minerals due to the nature of the oceans. As a result, copper would not be available to serve as a redox cofactor in catalysis (Lewis & Landing, 1992; Canfield, 1998; Anbar & Knoll, 2002; Poulton *et al*., 2004). Hence, present day nitrous oxide reductase (NOS) which is a copper enzyme found in all current canonical denitrifiers that produce dinitrogen would either be preceded by a functional non-copper NOS, evolved from a non-copper NOS, or was a *de novo* invention of the oxic era (Klotz, NSF Microbial Genome Sequencing Program workshop 2007). Authors mostly support the latter hypothesis since a sufficient NO_3^- pool would be available for early denitrifiers supporting anaerobic respiration (Capone & Knapp, 2007).

Current NrfA (pentaheme cytochrome c nitrite reductase) reduces nitric oxide and hydroxylamine to ammonia without the release of Reactive Nitrogen Species (RNS) intermediates (Simon, 2002 and references therein). This may have led to the leak of catalytic intermediates such as hydroxylamine and

because hydroxylamine is a potent mutagen, it would serve as a pressure to evolve a hydroxylamine-scavenging complex that could result in the evolution of the anammox process (Pino *et al*., 2006). This is why currently, two different proteins (ammonia-forming hydroxylamine reductase and hydroxylamine/hydrazine dehydrogenase) have been found in extant strict and facultative AOB and ANAOB (Hooper *et a*l., 2005). Hydroxylamine/hydrazine dehydrogenase is called HAO in AOB and it's an octaheme cytochrome c dehydrogenase (Hooper *et al*., 2005, Schalk *et al*., 2000). Electron flow from hydroxylamine to ubiquinol in AOB follows two different paths. First is a cyclic path to provide reductant to ammonia monooxygenase and second, a linear path for the generation of proton motive force (PMF) (Arp *et al*., 2007). Although the exact evolutionary history of HAO is still unknown, sequence and phylogenetic analysis have linked it to the pentaheme nitrite reductase, NrfA (Bergmann *et al*., 2005).

1.3 The link between methane and ammonia oxidation

The processes involved in the global nitrogen cycle have always been regarded *per se* as different processes that are performed by different organisms that have evolved through different lineages in their evolutionary histories (Strous *et al*., 2006; Kartal *et al*., 2007). By mostly focusing on AMO, many have speculated on the evolution of nitrification (Teske *et al*., 1994; Holmes *et al.,* 1995; Klotz & Norton, 1998; Purkhold *et al*., 2000; Norton *et al*., 2002). It was found that AMO is homologous to particulate methane monooxygenase (pMMO),

a copper enzyme like AMO (Hanson & Hanson, 1996; Murrell & Holmes, 1996). pMMO is responsible for the analogous function of oxidizing methane to methanol in methane oxidizing bacteria (MOB). Although the first published hypothesis on the evolutionary relatedness of AMO and pMMO turned out to be based on a PmoA of a contaminating MOB in the culture, later comparisons of available AmoA and PmoA sequences and enzyme properties did support the hypothesis, that indeed the two are homologous enzymes (Lontoh *et al*., 2000; Purkhold *et al*., 2000; Norton *et al*., 2002).

Further analysis of an MOB genome (*Methylococcus capsulatus* Bath) revealed residence of HAO in this methanotroph resulting in the important finding that HAO is a central player in ammonia oxidation in both AOB and MOB (Bergmann *et al*., 2005; Ward *et al*., 2004). While chemoorganotrophic methanotrophy and chemolithotrophic ammonia-catabolism are evolutionarily linked via common descent of the nitrification module, they possess totally different catabolic lifestyles. This is a demonstration of prokaryotic diversity created by the modular evolution of catabolism (Spirin *et al*., 2006 and references therein).

Aerobic ammonia oxidation would have become a catabolically efficient process only after a large enough pool of reduced inorganic nitrogen could sustain it (Capone *et al*., 2006). It also would likely coincide or quickly succeed the evolution of copper-containing enzymes. These enzymes however would have required Earth's oceans to be oxygenated to release the necessary bio-available copper (Zumft, 1997; Brandes *et al*., 2007). AMO and oxygen-reducing

terminal HCOs at the beginning and the end of the electron transport chain are the most vital copper-containing enzymes (Nakamura & Go, 2005). AMO and pMMO are homologous enzymes that many authors believe are a *de novo* emergence of their ancestor, a promiscuous ammonia-methane monooxygenase (Arp *et al.*, 2007 and references therein). Once these two enzymes were in place to extend the present catabolic units, AMO (to extend the hydroxylamine/hydrazine-ubiquinone-redox-module (HURM)) and pMMO (to extend methanol catabolism) could be involved in niche-dependent adaptation towards higher affinity for their respective substrate in gamma-proteobacterial AOB or MOB. Since AOB have a strict dependence on oxygen and are incapable of catabolizing an alternative natural energy source, this suggests that gamma-AOB and beta-AOB may have evolved by genome economization and reductive evolution while adapting to specific environmental niches. This in turn would create specialized inventories for each AOB ecotype as reported from the genomes of the marine, sewage, and soil AOB (Arp *et al*., 2007; Stein *et al*., 2007).

1.4 Taxonomy of Ammonia Oxidizing Bacteria

As stated earlier, oxidation of ammonia to nitrite and its subsequent oxidation to nitrate are performed by two functional groups via the process of nitrification. Ammonia oxidizers and nitrite oxidizers both contribute to the global nitrogen cycle, which has led to their initial grouping within the *Nitrobacteraceae* family with subdivisions based solely on their source of energy, ammonia or

nitrite (Buchanan, 1917 and 1918). Watson (1971) revised the *Nitrobacteraceae* family in 1971 and as a result, autotrophic AOB were divided into three described genera (*Nitrosomonas, Nitrosococcus* and *Nitrosospira*) and autotrophic nitrite-oxidizing bacteria were also divided into three described genera (*Nitrobacter, Nitrococcus* and *Nitrospina*). Using 16S rRNA gene-based phylogeny, ammonia and nitrite oxidizing bacteria have been shown to be phylogentically distinct with AOB falling under beta- and gamma-proteobacteria (Woese *et al.,* 1984a, 1985). The majority of known AOB belong to the Betaproteobacteria, where they form the *Nitrosomonadaceae* (Watson 1971; Watson *et al.* 1971; Harms *et al.*, 1976; Head *et al.*, 1993).

Early classification of genera and species within the *Nitrosomonadaceae* was solely based on a limited number of morphological and physiological characteristics. These include cell size and shape, presence and organization of intracellular membranes, motility, urease activity, ammonia affinity and sensitivity, and salt requirement and tolerance (Koops *et al.*, 2006). Classification methods have since become much more advanced using gene sequences for both the16S rRNA and *amoA*, which encodes for the alpha subunit of AMO catalyzing the initial oxidation of ammonia to hydroxylamine (Koops *et al.*, 2006). These analyses along with other gene sequences obtained by amplification of nucleic acids extracted from a wide range of environments have created seven well-supported lineages within the genus *Nitrosomonas* (Stephen *et al*., 1996, Purkhold *et al.*, 2000).

The phylogenetic position of AOB was initially determined on the basis of

16S rRNA cataloguing, and subsequently refined using near full-length, PCR-amplified 16S rRNA gene sequences, however, the validity of the relationships inferred from these analyses has been questioned (Woese *et al.*, 1984b, 1985; Fox and Stackebrandt 1988). Although comparative analysis of genes encoding different components of AMO and analysis of intergenic transcribed spacer (ITS) sequences confirmed the relationships, there has been some incongruence between 16S rRNA-derived trees and ITS derived trees (Purkhold *et al.*, 2000; Aakra *et al.*, 2001a, b; Calvo *et al.*, 2005). This could be attributed to the high sequence variation of ITS and difficulties in reliable alignment of such variable sequences, but in broad terms, these analyses have shown that cultivated AOB fall within three main lineages (Koops *et al.*, 2006).

Two distinct lineages represent the genera *Nitrosomonas* and *Nitrosospira* and fall within the Betaprotebacteria while the other that forms the genus *Nitrosococcus* falls within the *Chromatiaceae,* in the Gammaproteobacteria. There are three clusters within the *Nitrosomonas* lineage defined by Stephen *et al*. (1996) and Kowalchuk *et al*. (1997) through comparative analysis of 16S rRNA gene sequences from cultured lithoautotrophic AOB. Further sequences from 16S rRNA and *amoA* led to the recognition of seven lineages (clusters 5, 6a, 6b, 7, 8, *N. cryotolerans*, and cluster 9/Nm143 lineage) within the genus *Nitrosomonas.* There are nine validly named species within these lineages such as *N. communis, N. europaea,* and *N. eutropha* (Fig. 2).

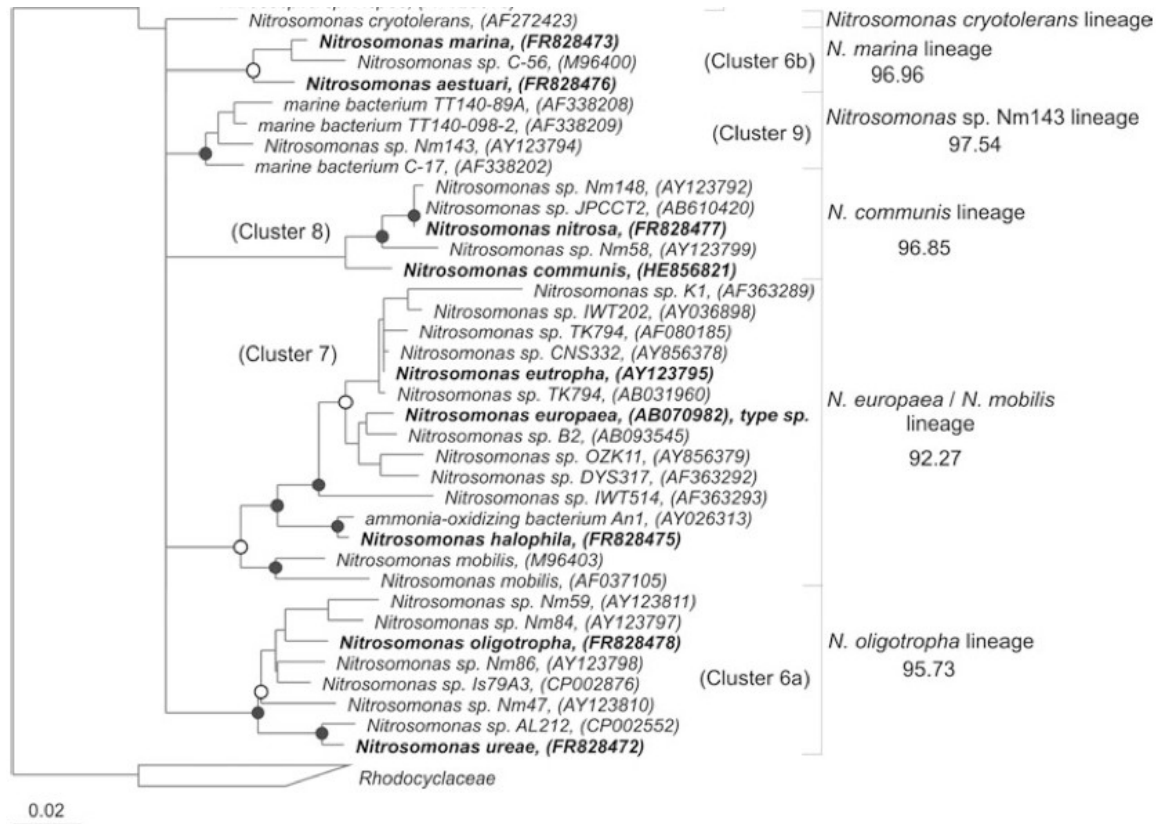


Figure 2: Figure taken from Prosser and colleagues with minor modification. Figure is partially shown. ‘‘Consensus phylogenetic tree of the family Nitrosomonadaceae based on the 16S rRNA gene. Final topology is based on a mix of reconstructions including maximum likelihood (RAxML, Stamatakis 2006), maximum parsimony (ARB, Ludwig 2004), and neighbor joining with the Jukes-Cantor correction. Initial trees were calculated using sequences longer than 1,300 nucleotides. Shorter sequences were added to the consensus tree using the ARB maximum parsimony tool. The sequence dataset and alignment were obtained from the All-Species Living Tree Project (LTP) database (Yarza et al. 2010; http://www.arb-silva.de/projects/living-tree) for validly published species (bold characters) and Silva SSU r114 database (http://www.arb-silva.de/search) for other sequences. Representative sequences from closely related taxa were used as out-groups. In addition, a bacterial homologues site filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Unstable topologies are shown as multifurcations. Filled and empty round nodes indicate neighbor-joining bootstrap values (100 re-samplings) above 90 % and 70 %, respectively. Minimum 16S rRNA sequence similarity among members of each cluster is depicted. Scale bar indicates estimated sequence divergence.” (Prosser *et al*, 2014)

1.5 Complete genome sequences of AOB and their properties

As of this writing, there are five published papers containing closed genome sequences of AOB within the *Nitrosomonadaceae*: *Nitrosomonas europaea* ATCC 19718 (Chain *et al*., 2003), *Nitrosomonas eutropha* C91 (Stein *et al*., 2007)*, Nitrosomonas sp.* AL212 (Suwa *et al.,* 2011), *Nitrosomonas* sp. Is79 (Bollman *et al.,* 2012), and *Nitrosospira multiformis* ATCC 25196 (Norton *et al*., 2008). As this field moves forward and the cost of genome sequencing declines, more isolates will be fully sequenced and publicly released in the near future. Of 5 closed AOB genome sequences, all contain a single circular chromosome and three contain plasmids of variable sizes. Additionally, all contain one complete ribosomal RNA operon and a full complement of tRNA genes. Multiple copies of the key genes involved in oxidation of ammonia are present in all of the *Nitrosomonadaceae* genomes but absent in marine-dwelling gammaproteobacterial *nitrosococci* (Klotz *et al.,* 2006). There is a high degree of recombinogenic activity among the AOB genomes as evidenced by the presence of pseudogenes, numerous IS elements, large tandem repeats in the *N. europaea* ATCC 19716 genome, and a ca. 117 kb genomic island flanked by tRNA genes and phage-related integrase in the *N. eutropha* C91 genome (Chain *et al*., 2003).

As stated earlier, all five *Nitrosomonadaceae* genome sequences maintain multiple copies of operons encoding the central components of the ammonia oxidation pathway such as: ammonia monooxygenase, hydroxylamine dehydrogenase, and two cytochromes (c554 and $_{CM}$552) that are involved in

production of PMF and continuation of ammonia oxidation by relaying electrons from hydroxylamine oxidation to the quinone pool (Sayavedra-Soto and Arp, 2011). Lithotrophic growth of AOB on ammonium is facilitated by *haoAB, cycA,* and *cycB* genes encoding HAO and cytochrome c554 and c_M552 that form the hydroxylamine-ubiquinone redox module (HURM) (Klotz and Stein, 2008). All except for *Nitrosomonas* sp. Is79 genome, although variable, encode for copper resistance genes (*copCD*) immediately following the *amoCABED* operons (Arp *et al.,* 2007). Three copies of the HURM operon is also present in all five genomes, although *N. europaea* ATCC 19716 and *N. eutropha* C91 lack the *cycB* gene in one of these operon copies (Arp *et al.,* 2007).

All *Nitrosomonadaceae* genomes contain sections of the nitrifier denitrification and nitrosative stress tolerance inventory. These include copper-containing nitrite reductase (*nirK* with or without associated genes), cytochrome c-dependent nitric oxide reductase (*norCBQD* and *norSY-senC-orf1*), cytrochrome P460 (*cytL*), and cytochrome c'-beta (*cytS*). However, some of these genes are either missing or have not yet been identified in the oligotrophic strains. The copper-containing *nirK* present in all of the genomes is responsible in assisting hydroxylamine oxidation by *N. europaea* and in the nitrifier denitrification pathway for reduction of nitrite to nitric oxide (Cantera and Stein, 2007a). Lateral gene transfer from several origins has resulted in the acquisition of *nirK* and *norCBQD* into AOB genomes (Cantera and Stein, 2007a; Garbeva *et al.*, 2007).

All of the AOB that have been examined so far use the Calvin cycle for fixation of carbon dioxide. A single variety of Type I RuBisCO has been found in three of the five genomes with *N. eutropha* C91 being the only one that encodes the full suite of genes for carboxysome biosynthesis. Another essential nutrient for AOB is iron as they rely on several cytochromes and the iron-rich HAO enzyme for ammonia chemolithotrophy. *N. europaea* ATCC 19718 and *N. eutropha* C91 have 90 and 28 genes, respectively, with putative involvement in iron uptake. *N. europaea* is the only one of the sequenced *Nitrosomonadacea* that encodes a vast diversity of putatitve iron siderophore uptake genes (Chain *et al.*, 2003).

1.6 *Nitrosomonas europaea/Nitrosomonas mobilis Lineage*

Nitrosomonous europaea ATCC 19718 has been the single most studied organism that has contributed to our understanding of AOB and the process of chemolithotrophic ammonia oxidation (Whittaker *et al*., 2000; Arp and Stein, 2003; Sayavedra-Soto and Arp, 2011). After the cultivation of more strains, other studies on other AOB have revealed properties that are obligate and universally shared amongst them, while some properties are different. For instance, ammonia is used as the energy-deriving substrate in all AOB while obligate autotrophy and the requirement of O_2 by ammonia monooxygenase is different (Arp *et al.*, 2012; Beaumont *et al.*, 2002; Berube *et al.*, 2007). Initially, most of the focus to determine niche preference was put on phenotypes such as tolerance to

toxins and fluctuating ammonium and oxygen concentrations. Recently, studies have shifted their focus and put it more on NO_x production by either ammonia oxidation or nitrifier denitrification pathways (Cruz-Ramos *et al.*, 2012; Hendrich *et al.*, 2002; Lipshultz *et al.*, 1998). To better understand niche preference, some basic phenotypic properties of the *Nitrosomonas europaea/Nitrosomonas mobilis* lineage within the *Nitrosomonadaceae* family will be discussed.

In this lineage, there are isolates with adaptations to relatively high concentrations of ammonium and salinity. *N. europaea* and *N. eutropha*, amongst others, both fall under this lineage. Studies have shown that in environments such as wastewater treatment plants and heavily fertilized soils where the concentration of ammonium is high this lineage of AOB phylotypes usually dominates (Mclain *et al.*, 2005; Levy-Booth *et al.*, 2014). By looking at the regulation of ammonia oxidation in *N. europaea*, studies have shown that ammonia is a transcriptional activator of *amoA* and it enhances ammonia-oxidizing activity in whole cells by raising the AMO holoenzyme expression (Hyman 1995; Stein *et al.,* 1997). Additionally when ammonium substrate is absent, AMO is inactivated by nitrite though nitrite can enhance the recovery of *N. europaea* following starvation from ammonium (Stein and Arp, 1998; Laanbroek and Gerards, 1993). In hypoxic to anoxic conditions where oxygen availability becomes extremely scarce to non-existent, studies have revealed that in *N. europaea* and *N. eutropha* NO_2 can be used as an alternative oxidant for AMO while using nitrite as an electron acceptor (Schmidt *et al.*, 2001; Schmidt *et al*., 2004)

Other pathways that are extensively studied in *N. europaea* include the nitrous oxide-producing pathways. Two specific pathways lead to N_2O production: first is the oxidation of hydroxylamine and second is the reduction of nitrite and nitric oxide (Wrage *et al.*, 2001). The former is produced as a result of active ammonia oxidation and the latter takes place during nitrifier denitrification, which is stimulated under low O_2 tension. Both of these processes leading to N_2O production can take place simultaneously (Stein, 2010). Under conditions of high ammonium and oxygen concentrations, ammonia oxidation is the main driver of N_2O production. On the other hand when nitrite concentrations are high, the NsrR transcriptional repressor releases the promoter of the nitrite reductase operon (*ncgABC-nirK*) and allows its expression in *N. europaea* (Klotz and Stein, 2008; Beaumont *et al.*, 2004).

Furthermore, it was previously shown that strains of *N. europaea* that have been mutagenized to stop the expression of NirK show much higher levels of N_2O production under high oxygen compared to hypoxic environments (Beaumont *et al.*, 2002; Beaumont *et al.*, 2005). These results suggested that in *N. europaea*, complete conversion of hydroxylamine to nitrite is dependent on nitrite reduction by NirK under fully aerobic conditions. In hypoxia, nitrifier denitrification could be involved in the important role of gathering electrons from the cytochrome pool as re-oxidation by the terminal oxidase slows down (Stein, 2010). This can have a potential to create a bottleneck in electron flow. A previous study on *N. eutropha* also looked at effects of NO_2 on gene and protein expression under oxic and anoxic conditions (Kartal *et al.*, 2012). They found that

regardless of the conditions (whether oxic or anoxic), exposure to NO_2 led to increase in proteins involved in energy conservation, including AmoCAB. Furthermore, exposure to NO_2 during anoxia resulted in increased proteins and transcrpts reflective of an energy-deprived state (Kartal *et al.*, 2012). While NorCB was not detected in the proteome, NorY (nitric oxide reductase) was expressed under both oxic-plus-NO_2 and anoxic-plus-NO_2 conditions (Kartal *et al.*, 2012).

In a recent study done by Kozlowski and *et al*. (2014), they revised the function of NirK and NorB in N_2O-production pathways in *N. europaea* under atmospheric and lower O_2 tension. In that study, the authors used wildtype *N. europaea* along with *NirK* mutant, *NorB* mutant and both *NirK* and *NorB* mutant strains to see which is the principle gene involved in nitrous oxide production. Through anoxic resting-cell assay and instantaneous nitrite reduction experiments, the authors illustrated the important role of NorB in N_2O production under both atmospheric and reduced oxygen tension. While NorB was proven to be the sole nitric oxide reductase for nitrifier denitrification, they also concluded that an alternative nitrite reductase to NirK is present and active. This was an important study in expanding our knowledge in enzymology and understanding the exact role of specific enzymes or the possibility of alternate fully functioning enzymes in other ammonia oxidizing bacteria.

1.7 General Isolation, Enrichment, and Maintenance procedures

Members of the *Nitrosomonadaceae* can fairly easily be enriched from environments where they have relatively high abundance. This is routinely achieved in selective inorganic media, which can also be used in pure culture isolation (Koops *et al.*, 2006). Although media composition may vary, all contain ammonium chloride or sulfate, mineral salts, and buffers of different strength. It is required for all media to be buffered since ammonia oxidation to nitrite produces acid equivalents and the optimal growth pH of all cultivated AOB is either at neutral or moderately alkaline conditions (Schmidt *et al.*, 2001). The majority of AOB cannot grow below pH 7 and exhibit minimal growth in pH 6.5 in batch cultures. When it comes to isolating a pure culture however, there are some hurdles to overcome.

Firstly, autotrophic ammonia oxidizers have much lower specific growth rates compared to many of the heterotrophs present in environmental samples that can use organic contaminants in solid media, glassware, volatile compounds entering liquid medium, and by-products of ammonia oxidizers (Prosser *et al.*, 2004). Secondly, growth yield of most AOB are fairly low and highly inhibited by acidity and nitrite toxicity. With both of these difficulties combined, growth of readily visible colonies on solid medium and dominance over heterotrophs in liquid enrichment cultures rarely occur. As a result, re-streaking of isolated colonies and inoculation of liquid media or continued subculture in liquid medium has proven to be difficult in fully eliminating the heterotrophs. However, using these methods has resulted in isolation of pure cultures. Using neutral red (which

stains acid-producing ammonia oxidizer colonies red) and antibiotics to eliminate heterotrophic growth can facilitate these isolation methods. Furthermore, gelatin, silica gel or purified agar can be used to lower contamination of media with organic carbon (Prosser *et al.*, 2004).

Usually in liquid culture, pure cultures are obtained through dilution of heterotrophs to extinction (e.g., Gibbs 1920; Lewis and Pramer 1958; Watson *et al*. 1971). This method can be successful only in cases where the concentration of AOB is higher than the heterotrophs and it involves successive dilution to a level that eliminates heterotrophs followed by inoculation into many tubes of liquid medium. This method was used by Frankland and Frankland in 1890, which led to the isolation of the first ammonia oxidizer. Liquid medium being contaminated with organic carbon can be reduced through oven-heating of glassware and growth sealed vessels with sufficient headspace to avoid oxygen limitation (Aakara *et al.*, 1999). Nowadays enrichment and isolation are accompanied by molecular analysis of source environments, to identify target phylotypes, and enrichment cultures. Naturally this approach is also not full proof, as it will be limited by our current knowledge in detecting organisms outside the range of current primers sets (Prosser *et al.*, 2004).

Growth conditions through modification of salt concentration, pH and incubation temperatures have been modified to closely reflect the environments that the isolate was taken from. However traditionally selective media contain higher ammonium concentrations than most natural environments, which wouldn't be successful in selecting for strains that prefer lower concentrations of

ammonium (MacFarlane and Herbert, 1984; Watson, 1971; Bollmann and Laanbroek, 2001; Bollmann *et al.*, 2011). Therefore, reductions in initial ammonium concentrations and continuous and semi-continuous cultures have been used instead to select for those strains. These methods will also aid in selecting for organisms with much slower growth rates. AOB in many environments are attached to particulate material and their ecophysiological characteristics are influenced by biofilm growth to such an extent that initially, most ammonia oxidizer enrichment media contained biofilms attached to insoluble carbonate (Armstrong and Prosser, 1988; Powell and Prosser, 1992; Allison and Prosser, 1993). Cells would be mostly attached such that dispersed growth was considered impossible. Systems that enable enrichment and isolation of cultures on surfaces or as aggregates may result in isolating strains that have more environmental relevance and increase the diversity of currently cultivated strains.

Initially, AOB were considered to be strict autotrophs. Nowadays, with the advancement of science we know that some ammonia oxidizers have potential for mixotrophic and heterotrophic growth as well (Prosser *et al.*, 2004 and references therein). Our present enrichment methods, however, mostly allow for the isolation of chemolithoautotrophs. These methods can be altered by addition of organic carbon in the media that may lead to the increase in selection of mixotrophic AOB (Ding *et al.*, 2013). However, alterations aren't without consequences and addition of organic carbon could make it more difficult to get rid of highly competitive non-ammonia oxidizing heterotrophs. Maintaining AOB

can be easily achieved through continued subculture. This process does have its downside being that strains that are selected can be the ones that are more specifically adapted to the cultivation conditions and therefore are less environmentally relevant (Prosser *et al.*, 2004). Other disadvantages include the possibility of further contamination by heterotrophs or other isolated strains if multiple strains are maintained simultaneously. AOB can also be frozen at -80C in glycerol and resuscitated later on. Resuscitation however can often take up to 2 months, though recent developments are prolonging freezer storage periods with shorter resuscitation times (Heylen *et al.*, 2012).

1.8 Ecology and Application

Nitrosomonads and nitrosospiras were both isolated from soil and considered to be the main players in driving ammonia oxidation in soil before the discovery of thaumarchaeal *amoA* genes (Leininger *et al.*, 2006). Compared to nitrosospiras, nitrosomonads are much more readily isolated from soil, which could suggest their greater fitness in these AOB communities. However, nearly all soils have greater abundances of gene sequences related to nitrosospiras than to nitrosomonads. *Nitrosomonas* cluster 5 has not yet been cultivated from soil along with no reported evidence of gammaproteobacterial AOB present in this environment. Using molecular techniques, *Nitrosomonadaceae* can be detected in virtually all soils with the exception of more acidic soils, which are currently believed to be dominated by thaumarchaea as the main drivers of nitrification (Martens-Habbena *et al.*, 2009; Lehtovirta *et al.* 2011). Generally, it is

difficult to distinguish patterns between phylogeny and soil environmental characteristics in different phylotypes.

In wastewater treatment systems where the removal of nitrogen is a critical process, ammonia oxidation by ammonia oxidizers and their close physical and metabolic association with nitrite oxidizers is crucial to its reliable operation (Mobarry *et al.*, 1996; Schramm *et al.*, 1996; Okabe *et al.*, 1999; Graham *et al.*, 2007). In these systems, nitrite produced by AOB can be used as an electron acceptor by anaerobic ammonia-oxidizing planctomycetes (anammox bacteria) leading to removal of nitrogen as dinitrogen gas (Kuenen, 2008). Nitrite can also be used as an electron donor for nitrite oxidizers forming nitrate that may be removed through classical denitrification. AOB are commonly considered to be strictly chemolithoautotrophic and their much slower growth rate is the rate-limiting step of nitrification (Verhagen and Laanbroek, 1991; van Nielmetal, 1993; Laanbroek and Gerards, 1993). This poor growth consequently results in AOB being a poor competitor for oxygen and ammonia when compared to other co-occurring heterotrophs. Out of all the strains identified that belong to the *Nitrosomonadacaea*, *N. eutropha* appears to be the most commonly encountered isolate in municipal wastewater treatment plants.

Major losses of ammonium-based fertilizers are as a result of nitrification. In some agricultural systems, as little as 30% of fertilizers are used by crops and the remainder is used as an energy source to nourish the bacterial communities (Prosser *et al.*, 2004 and references therein). This in turn results in the production of other harmful greenhouse gases such as nitrous oxide. Over fertilizing the soil

also leads to natural leaching of nitrate that ends up polluting groundwater, while denitrification converts it to gaseous forms, dinitrogen or nitrous oxide. Recently, the estimated global nitrogen fertilizer input to soil was at 100-150 Tg y^{-1} (Gruber and Galloway, 2008; Schlesinger, 2009). As the use of such fertilizers increase to aid global expansion, so does the economic and environmental significance of ammonia oxidizers. Strategies to remedy the current fertilizer loss include better informed management of fertilizer addition, introduction of ammonia oxidation inhibitors, development of crops that produce natural inhibitors, and persistent nitrification through long term fertilization that results in acidic soils with increased toxic metals (Prosser *et al.*, 2004).

The nitrogen cycle in aquatic environments is also heavily influenced by AOB. Nitrogen budgets are closely related between soil and freshwater with most of the nitrogen in freshwater systems coming from run-off from land (Gruber and Galloway, 2008). In 2009, it was estimated that of the approximate 295 Tg N y^{-1} input of fixed nitrogen to terrestrial environments from both natural and human driven activities, about 110 Tg N y^{-1} is lost to the atmosphere solely through denitrification and 50% of this budget is accounted for by freshwater denitrification systems (Schlesinger, 2009). In marine settings, AOB also play an important role in oxidizing the reduced nitrogen entering the oceans across the planet with the bulk of the nitrogen entering from rivers and atmospheric deposition being in the form of nitrate. All of the 140 Tg N y^{-1} that enters the ocean from nitrogen fixation must first be mineralized and oxidized by ammonia

oxidizers before it can be removed by denitrification, which approximately balances marine nitrogen input (Gruber and Galloway, 2008).

1.9 The beginning of nitrification focused research

In 1892, Sergei Winogradsky studied the process of nitrification in AOB and NOB for the very first time. His work showed that in AOB catabolism, ammonia monooxygenase (AMO) is responsible for the aerobic oxidation of ammonia to hydroxylamine, followed by dehydrogenation of hydroxylamine to nitrite via hydroxylamine dehydrogenase (HAO). This process is proposed to transfer the four extracted electrons to the ubiquinone pool via two interactive cytochromes, c554 and c_M552. Currently, HAO is the best-studied functional component of this process, followed by cytochrome c554 (Sayavedra-Soto *et al.*, 1994). This is due to HAO's highly soluble nature. AMO on the other hand, which is a multimeric transmembrane copper-enzyme, has yet to be functionally isolated, and its structure determined. In order to oxidize ammonia, AMO activation requires the electrons obtained from ubiquinone (Sayavedra-Soto *et al.*, 1998).

Although new molecular techniques using sequence availability of individual genes encoding HAO and AMO has led to surge of research in AOB distribution and abundance, aspects of the molecular biology and biochemistry of these organisms besides carbon assimilation and use of ammonia as an energy source are still vastly unknown. Particularly, almost nothing is known about the regulation of gene expression required for nitrifier denitrification. Astonishing

really given the fact that nitrifier denitrification competes for electrons with primary bioenergetic processes, produces reactive nitrogen species and could compete with NOB for catabolism of substrate (Klotz and Stein, 2008). While AOB and NOB interconnect the pools of fixed and gaseous nitrogen by oxidizing ammonia to nitrate collaboratively and independently of denitrification, ANAOB combine the two into a single process (ammonia oxidation and nitrite reduction leading to dinitrogen production) (Strous *et al.*, 1999).

1.10 *Nitrosomonas europaea* and its genome analysis

As mentioned earlier, in 2003 Chain and colleagues published the complete genome sequence of *N. europaea. N. europaea*'s genome consists of 2,812,094 bp in a single circular chromosome. Through GC skew analysis, it was found that the genome is divided into two unequal replichores with genes being distributed evenly around the genome (Chain *et al.*, 2003). While 47% of the genes are transcribed from one strand, 53% is transcribed from the complementary strand equaling a total of 2,460 protein-encoding genes. Genes necessary for the main physiological function of *N. europaea* such as catabolism of ammonia, energy and reductant generation, biosynthesis and CO_2 and NH_3 assimilation were identified. In contrast, genes for catabolism of organic compounds are limited. While some of the enzymes to breakdown macromolecules to monomers and building blocks are coded by the genome, the full pathways for further degradation are not present. This is probably due to the secreting nature of *N. europaea* to get rid of its organic waste rather than to

recycle it. It also appears that it has a limited ability to transport organic molecules from the environment into the cell (Chain *et al.*, 2003).

Ammonia has been identified as the sole source of energy for this bacterium, making it an obligate chemolithoautotroph and as such, the genome provides no evidence for other lithotrophic capabilities. For instance, the bacterium could also grow with H_2 as the source of energy and reductant had it possessed the genes for a hydrogenase enzyme. Instead, necessary genes for the utilization of H_2, CO, Fe or other inorganic sources of energy were not identified. Nonetheless, complete pathways for a few compounds can be identified in the gene profile such as catabolism of fructose and mannose, and one study showed growth via ammonia oxidation for energy generation and fructose catabolism for carbon assimilation (Hommes *et al.*, 2003). An unexpected finding that came out of the genome analysis was the insight into the strategy of *N. europaea* in accumulating Fe from the environment. Given the number of cytochromes they produce, these bacteria have a great need for iron. Naturally, they would encode receptors for Fe-siderophores as a result. However, the sheer number of genes devoted to different classes of receptors was surprising (Chain *et al.*, 2003).

What's even more surprising, however, is that *N. europaea* almost completely lacks the genes responsible for the biosynthetic pathways for siderophores with the exception of a citrate transporter, which utilizes a product and intermediate of the TCA cycle (Chain *et al.*, 2003 and references therein). This would suggest that *N. europaea* relies on other bacteria in the environment

to produce siderophores, so that it can harvest it using its arsenal of receptors. It is likely that this bacterium pursues Fe in its environment more as a survival strategy than as a co-dependency on other bacteria. Other interesting features of the genome include the multitude of insertion sequences. The variety and the number of elements together suggest that this genome is prone to loss or accession of genetic elements. Comparing *N. europaea*'s genome to many other proteobacteria, it is much smaller in size and appears to have lost many of its possible ancient phototrophic gene modules (Chain *et al.*, 2003 and references therein). Perhaps, this bacterium is continuing to downsize its genome since a minimum genome set is advantageous for lithoautotrophy.

1.11 Principal nitrification/denitrification enzymes in N. europaea

Ammonia oxidizers can utilize molecular oxygen in both formation of hydroxylamine from ammonia and as the terminal acceptor of electrons from the respiratory chain (Wood, 1986). Homologues of genes that encode for the specific enzymes required for nitrite (NIR) and nitric oxide reduction (NOR) are present in *N. europaea*. AOB normally produce NO and in some cases N_2 during ammonia oxidation (Dore and Karl, 1996; Jiang and Bakken, 1999). Enzymes possessing nitrite-reducing activity have been isolated from *N. europaea* suggesting an alternative mode of respiration through the use of NO_2^- (Abeliovich and Vonshak, 1992; Schmidt and Bock, 1997). Other possibilities hint towards a protective role of nitrite reductases in AOB since NO_2^- has a toxic effect during

growth (Yu *et al.*, 2010). Furthermore, HAO has been shown, in vitro, to be able to produce NO and N_2O during the oxidation of NH_2OH (Arciero *et al.*, 1991a; Hommes *et al.*, 1996; Igarashi *et al.*, 1997).

In *N. europaea*, an open reading frame with homology to genes encoding copper-type NirK enzymes was found (Beaumont *et al.*, 2002). This ORF is 930bp in length and translates into a polypeptide of 309 amino acid residues. Through alignment analysis, it was found that NirK found in *N. europaea* is significantly shorter than other Cu-type Nir proteins (Beaumont *et al.*, 2002 and references therein). Furthermore, the analysis of the N terminus of this NirK with the SignalP algorithm predicted that a periplasmic target is present, hence suggesting that this protein resides in the periplasm. Once the *nirK* deficient mutants were obtained by means of conjugation, they were grown along with wild-type *N. europaea* ATCC 19178 in both liquid and solid medium (Schmidt *et al.*, 2004). Using a hydroxylamine assay, nitrite reductase activity was observed to be only present in the periplasmic protein extracts from the wild-type cells but not from the NirK-deficient cells. This activity was resumed in NirK-deficient cells that were complemented by an insertion of a broad-host-range factor that contained a functioning *N. europaea nirK* (Beaumont *et al.*, 2002).

Using time-point experiments, authors showed also that the rate of N_2O production was approximately four times greater than that of the wild-type cells. Furthermore, NH_3-dependent oxygen uptake rates were not significantly different between the two strains indicating that both wildtype and NirK-deficient cells had comparable respiratory potentials. Time-dependent increase in turbidity of

aerobic batch cultures was used to analyze the growth characteristics of both strains. Results indicated a similar rate of growth, with NirK-deficient cells reaching close to 90% of the maximal biomass concentration of the wild-type cells (Beaumont *et al.*, 2002).

As stated earlier, a possible role of NirK was its involvement in tolerance to toxic effects of nitrite to cells of *N. europaea* (Beaumont *et al.*, 2005). This was tested through series of growth experiments in batch cultures where increasing amounts of NO_2^- were added at the start of culturing. Although they found that both wild-type and NirK-deficient strains' growth rate and final biomass was affected, the effects were much more pronounced in the mutant strains (Beaumont *et al.*, 2005). This was more profound at higher concentrations when, while wildtype strains could still grow, the mutant cells were incapable of growing. These results together suggest that the function of NirK is most probably periplasmic and it does not involve lowering the amount of extracellular nitrite to which cells are exposed.

In denitrification, which is an anaerobic form of respiration, nitrate is reduced to dinitrogen via nitrite, nitric oxide and nitrous oxide (Poth *et al.*, 1985; Levy-Booth *et al.*, 2014). Nitrate reductase, nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase are the enzymes involved in this stepwise reduction process (Wrage *et al.*, 2001). The expression of these genes mostly occurs in heterotrophic denitrifying bacteria where oxygen concentrations are extremely low, and one or more of the required denitrification substrates are present (NO_3^-, NO_2^-, or NO) (Zumft *et al.*, 1997). While *N. europaea*'s nitrification

pathway involving AMO and HAO in oxidation of ammonia to nitrite via hydroxylamine is clearly characterized, the physiological relevance of its putative denitrification pathway is still largely unknown (Beaumont *et al.*, 2004). As mentioned, the nitrite and nitric oxide reducing enzymes can function to either lower the toxic effects of NO_2^-, to aid in hydroxylamine oxidation to nitrite, or to serve as an alternative terminal electron accepter when oxygen levels are low. *N. europaea* also contains two Nor homologues which could function to maintain a low NO concentration produced by Nir as it does in heterotrophic denitrifying bacteria (Stein, 2010).

There haven't been many papers that focus on the genome wide transcription regulation in AOB. Only in 2009, Beyer and colleagues looked into mRNA transcripts coding for key metabolic functions in *N. europaea*. They looked at the transcripts in both aerobic as well as anaerobic growth conditions either through nitrification or chemoorganotrophic anaerobic pyruvate-dependent denitrification. They mainly focused on *amoA, hao, rh1, nirK, norB, nsc, ace, idhA, ppc, gltA, odhA, coxA,* carbon dioxide fixation (*cbbL*), gluconeogenesis (*ppsA*), cell growth (*ftsZ*), and oxidative stress (*sodB*) (Beyer *et al.*, 2009). What they found was as expected. Genes correlated with aerobic ammonia oxidation, nitrite reduction and growth rates were upregulated during aerobic growth conditions. On the other hand, during anaerobic growth conditions, mRNA concentrations of *amoA, hao, rh1, coxA, cbbL, ftsZ,* and *sodB* were significantly lowered (Beyer *et al.*, 2009).

In another study, authors looked into a quantitative proteomic analysis of *N. europaea* and comparing the results between growing- and energy-starved cells (Pellitteri-Hahn *et al.*, 2011). A total of 876 proteins were identified which represented 24% of the total predicted proteome. What they found was proteins that were associated with nucleic acid replication and functions closely linked to it collectively comprised the largest group of highly abundant proteins in growing cells (Pellitteri-Hahn *et al.*, 2011). In energy-starved cells, the proteome shifted away from biosynthesis and toward survival functions such as, cell envelope modification, protein protection-degradation, detoxification and implementation of alternative energy generation mechanism (Wei *et al*., 2006; Vajrala *et al.*, 2011; Cho *et al.*, 2006).

Several AOB, including *N. europaea*, possess a divergent monocistronic copy of *amoC* (*amoC3*) of previously unknown function (Sayavedra-Soto *et al.*, 1998). It was suggested that *amoC3* could have a possible functional role as part of the sigmaE stress response regulon during extended ammonia starvation in *N. europaea*, thus indicating its importance during the exit of cells from starvation (Berube and Stahl, 2012). The authors utilized global transcription analysis to look at the specific function of *amoC3*. They found that general stress response is required for an efficient exit from starvation and that the divergent AmoC3 subunit participates in this specific response. The encoded proteins during starvation were found to be involved in the cytoplasmic and membrane general stress responses and most often functioned in the degradation or repair of damaged or miss-folded proteins (Berube and Stahl, 2012).

They also illustrated that in contrast to heterotrophic bacteria, it seems that *N. europaea* delays repair mechanisms until an energy source is replenished (Berube and Stahl, 2012). While the mechanistic functions of either AmoC subunits is not fully understood, *N. europaea* seemed to have evolved an auxiliary AmoC subunit that confers greater enzyme stability to effect a physiological advantage under stressful conditions. A similar prediction can be made of the homologous particulate methane monooxygenase subunit (Holmes *et al.*, 1995). Additionally, *amoCAB* operons also were significantly up-regulated during recovery compared to exponential-phase cells. The *amoE* and *amoD* genes are conserved in all AOB, and homologous genes are also found in closely related methane oxidizing bacteria (Murrell *et al.*, 1996; Bodelier and Laanbroek, 2004; El sheikh *et al.*, 2008). Their presence in high transcripts also suggests a possible role in the recovery of *N. europaea* from starvation (Berube and Stahl, 2012).

1.12 *Nitrosomonas eutropha* and its genome analysis

Up until now, all characterized aerobic AOB are phylogenetically affiliated with the *Nitrosomonas* and *Nitrosospira* genera of the Betaproteobacteria as well as the *Nitrosococcus* genus of the Gammaproteobacteria. Few of these *Nitrosomonas* isolates can oxidize ammonia both aerobically and anaerobically. This includes *N. eutropha* C91, which was isolated by Watson and *et al* (1971) from a sewage disposal plant in Chicago, IL, USA. As a member of the ammonia-oxidizing bacteria, *N. eutropha* is an autotrophic nitrifying bacterium capable of

catalyzing the oxidation of ammonia to meet its energy requirement for growth (Stein *et al.*, 2007).

N. eutropha is a Gram-negative Betaproteobacterium and it is stated in the literature to be closely related to *N. europaea* (Schmidt *et al.*, 2009; Stein *et al.*, 2007; Zart *et al.*, 2000). It is pleomorphic and is mostly found in short chains in eutrophic environments such as municipal and industrial sewage disposal systems (Stein *et al.*, 2007). The name '*eutropha*' entails a high tolerance to much elevated ammonia concentrations. Although literature often suggests a high similarity between these two bacteria (*N. europaea* and *N. eutropha*), there are some notable differences. For instance, *N. eutropha* possesses carboxysomes unlike *N. europaea* (Kartal *et al.*, 2012). These along with other findings suggest that significant differences in metabolic capability could be present in these two closely related species. In a study done in 2007, researchers were able to sequence the complete genome of *N. eutropha* C91 and compared it to *N. europaea* in a genome wide comparison (Stein *et al.*, 2007).

Similar to *N. europaea*, *N. eutropha* C91's genome is composed of a single circular chromosome of 2,661,057 bp in length (Beaumont *et al.*, 2002; Stein *et al.*, 2007). It also contains two large plasmids, both of which that contains an identical 11,414 bp transposon flanked by short inverted repeats. Similar to *N. europaea*, it also contains several families of insertion sequence elements, repeated up to 22 times within the chromosome (Stein *et al.*, 2007). Furthermore, it harbors two and three copies of the gene clusters that encode functional AMO

and hydroxylamine oxidoreductase and associated cytochromes respectively. Since bacteria that experience difference in O_2 levels tend to maintain flexible respiratory chains by encoding multiple classes of proton-pumping haem-coper oxidases, *N. eutropha* is no exception in containing these gene clusters (Stein *et al.*, 2007).

As stated earlier, aerobic AOB have been shown to reduce nitrite to NO and N_2O in a process known as nitrifier denitrification (Wrage *et al.*, 2001), and the necessary gene clusters are present in *N. eutropha*. These include *nirK* and *norCBQD,* and *norSY-senC-orf1*, which have been previously discussed in this review in *N. europaea* and pose the same function in *N. eutropha*. Much like *N. europaea*, *N. eutropha* also possesses ribulose bisphosphate carboxylase/oxygenase (RuBisCO) to fix carbon dioxide via the Calvin-Benson-Bassham cycle. Both of these strains also contain heavy metal and multidrug resistance gene clusters such as mercury and copper resistance. Although, *N. europaea* does not produce its own siderophores for iron requirement and instead, its genome encodes about 90 genes for iron acquisition. However, *N. eutropha*'s genome contains a six-gene cluster that resembles the aerobactin biosynthesis operon, and two genes in this cluster are orthologous to aerobactin synthases of *N. oceani* (Stein *et al.*, 2007 and references therein). Siderophore biosynthesis by *N. eutropha* is still to be investigated.

Since the two bacteria, *N. europaea* and *N. eutropha*, share a 97% identity in their 16S rRNA genes, they occupy a tightly clustered lineage based on 16S rRNA phylogenetic tree (Stein *et al.*, 2007 and references therein). However,

looking at the genomic organization along with its content, hypotheses arise as to why *N. eutropha*-like species are better adapted to eutrophic ecosystems. Having many heavy metal and multidrug transport and two additional gene clusters for respiratory HCOs differentiates *N. eutropha* from the rest of aerobic AOB and anammox genomes sequence to date (Stein *et al.*, 2007 and references therein). Significant amount of horizontal gene transfer could be responsible for such unique qualities for survival and fitness in each of these specialized bacteria.

In a study done in 2012 by Kartal *et al.*, effects of nitrogen dioxide on gene and protein expression in *N. eutropha* C91 under oxic and anoxic conditions were investigated. A continuous culture was maintained in a chemostat fed with ammonium under oxic, oxic-plus-NO_2, and anoxic-plus-NO_2 culture conditions and later moved into batch cultivation for biomass harvesting and mRNA and protein analysis. This was a long-term physiological representation in adaptations to NO_2 and anoxia plus NO_2 rather than short-term stress responses. What they found was that NO_2 cannot act as a sole oxidant for anaerobic growth or a vital co-oxidant with O_2 in *N. eutropha* C91. This conclusion was obtained from the fact that no biomass was gained during anoxic-plus- NO_2 culturing conditions and the rate of ammonia oxidation to nitrite under oxic-plus- NO_2 atmosphere was much lower. However, the argument still remains that a possible short-term NO_2 exposure could stimulate ammonia-oxidizing activity very briefly (Kartal *et al.*, 2012).

Looking at the transcriptome and proteome results, elevated levels of transcript and presence of protein for aerobactin synthase (siderophore) and

cytochrome c peroxidase along with higher expression of metal homeostasis genes were observed in anoxic-plus- NO_2 incubations of *N. eutropha* C91 (Kartal *et al.*, 2012). This would in turn indicate some sort of overlap with typical oxidative or heavy metal stress responses. As mentioned previously, *amoC* gene has been implicated to aid in the recovery of *N. europaea* ATCC 19718 from starvation as a general stress response. In batch cultures, there was an increase in AmoCAB and other redox-active proteins along with diminished biosynthetic and nutrient storage activities. This could explain a possible short-term enhancement of ammonia oxidation rates, as more energy would be diverted to more essential metabolism instead of nutrient storage (Kartal *et al.*, 2012 and references therein).

Interestingly, there was no detectable change in protein or transcript levels of *nirK* or any significant change in expression of nitric oxide reductase (NorCB) proteins or *norBQD* under any condition. They did observe an expression of the nitric oxide reductase (NorY) in both oxic and anoxic conditions indicating that it could be of more importance than NorB for NO_x metabolism by *N. eutropha* C91. Some of the previously observed changes in similar experiment in *N. europaea* grown in short-term batch cultures (significant increase in expression of *nirK, norB*, and *ncyA* in presence of ammonia, nitrite and NO_2) were not observed in this study (Kartal *et al.*, 2012). These prior measurements of change in expression could reflect the change observed in their environmental conditions in such short term.

1.13 My thesis project

As discussed earlier, *N. europaea* and *N. eutropha* are two closely related species yet they are capable of occupying different environments. Major questions remain unanswered by the current literature: How do these organisms regulate their genome wide expression in order to occupy a certain niche and to be able to adapt and compete in that environment? Although comparative genome analysis studies have been done before and mentioned in this review, transcriptomic studies comparing closely related organisms in controlled experimental conditions are scarce. We already know that having certain homologous gene inventory does not always translate to the same exact physiology, gene function, or gene regulation in related species.

Therefore, by comparing their physiology along with their global transcription profiles from mid- to late-log of growth in batch culture, we were able to directly compare responses of *N. europaea* and *N. eutropha* and better understand how these two species compare to each other at a genome wide transcriptional level. The central hypothesis of this thesis was that these two closely related bacteria would have differential physiology and genome-wide responses to the similar environmentally relevant stress of approaching stationary phase. The following chapters examine how each of these bacteria differs in both physiology and regulation of their gene expression, translating these observations to fitness, competition and adaptation in their environment.

N. europaea ATCC 19718 was one of the strains used because this organism plays a central role in the availability of nitrogen to plants and limiting their CO_2 fixation (Suzuki *et al.*, 1974; Chain *et al.*, 2003). It is also extremely

important in the treatment of industrial and sewage waste as already mentioned. *N. europaea* is also the model organism in the field of ammonia oxidation and since it has its genome closed and fully annotated, it would make it a great candidate for a transcriptomic study of this kind. The other stain used in this study was *N. eutropha*. Although closely related to *N. europaea*, this strain is found in strongly eutrophic environments with high tolerance to elevated ammonia concentrations. Its genome is also closed and fully annotated. Besides environmental relevance, *N. eutropha* also has been used in health industries as having possible skin benefits acting as a cleanser, deodorant, anti-inflammatory and immune booster by feeding on the ammonia in our sweat and converting it to nitrite and nitric oxide.

The two time points that were chosen for this study were mid-log and late-log (early stationary) phase. During logarithmic phase, growth is not limited, and doubling will continue at a constant rate in consecutive time period. In late log or early stationary, a factor will be limiting growth and as such growth rate slows down. This slow down in growth eventually equals the rate of cell death resulting in a plateau in growth curves. Therefore, the two time points chosen in this experiment are to reveal the different responses of cells to their environment as they are growing exponentially and when they enter stationary phase and how those responses differ between these two species.

Chapter 2
Materials and Methods

2.1 Culture care and physiological measurements:

Nitrosomonas europaea strain ATCC 19718 and *Nitrosomonas eutropha* strain C-91 were grown (in replicates, n = 6) in a mineral medium containing per liter: 5mM $(NH_4)_2SO4$, 10mM NaCl, 0.4mM KH_2PO_4, 0.2mM $MgSO_4.7H_2O$, 1.0mM $CaCl_2.2H_2O$, 1.0mM KCl, 0.02% phenol red, 1ml Trace solution (which contains per liter Mili-Q water: 11.5mM NA_2-EDTA, 10.0mM $FeCl_2.4H_2O$, 0.50mM $MnCl_2.2H_2O$, 0.10mM $NiCl_2.6H_2O$, 0.10mM $CoCl_2.6H_2O$, 0.10mM $CuCl_2.2H_2O$, 0.50mM $ZnCl_2$, 0.10mM $Na_2MoO_4.2H_2O$, and 1.0mM H_3BO_3), 15mM Hepes buffer all dissolved in 1000ml total Milli-Q water. The pH of the media was adjusted using 1M or 10M NaOH to ca. pH 7.6-7.8. 100mL of media was poured aseptically into 250mL Wheaton bottles with septum caps. Bottles were then inoculated by 1mL of culture at ca.10^9 cells/mL. Cultures were incubated at 30°C while shaken on a rotary platform at 100rpm. During growth, the pH was maintained at approximately 7.7 using 5% sodium bicarbonate that was added daily after the first 48h of growth using sterile syringes.

Replicated experiments (n = 6) were also done using media containing per litre: 25mM $(NH_4)_2SO4$, 10mM NaCl, 0.4mM KH_2PO_4, 0.2mM $MgSO_4.7H_2O$, 1.0mM $CaCl_2.2H_2O$, 1.0mM KCl, 0.02% phenol red, 1ml Trace solution (which contains per liter Mili-Q water: 11.5mM NA_2-EDTA, 10.0mM $FeCl_2.4H_2O$, 0.50mM $MnCl_2.2H_2O$, 0.10mM $NiCl_2.6H_2O$, 0.10mM $CoCl_2.6H_2O$, 0.10mM $CuCl_2.2H_2O$, 0.50mM $ZnCl_2$, 0.10mM $Na_2MoO_4.2H_2O$, and 1.0mM H_3BO_3), 30mM Hepes buffer all dissolved in 1000ml total Milli-Q water.

Every 24h, cells were extracted from their respective bottles and counted using a hemocytometer under phase contrast light microscopy. During the same period, O_2 and N_2O levels were analyzed using gas chromatography (thermal conductivity detector; Hayesep D column). 200μl of gas from the headspace was injected with the GC at an attenuation of 4 to analyze O_2 levels and the same volume was injected with the GC at an attenuation of -2 to analyze N_2O levels. Specific gas concentrations were calculated using standard curves using pure gases (Sigma-Aldrich). Nitrite production measurements were done via a nitrite assay. In this assay, 1mL of N-1-Naphthylethylenediamine dihydrochloride (NNEQ) (75mL of 10M HCl was added to 425mL of Nanopure water in 500mL Whatman bottle, then 100mg NNEQ was added and the bottle was covered with foil to avoid light) is added to a test-tube containing 1mL of sulfanilamide solution (75mL of 10M HCl was added to 425mL of Nanopure water in a 500mL Whatman bottle, and finally 5g of sulfanilamide was mixed into the bottle) plus 2.5μL of sample. After letting the test-tubes settle for about 10-15min, the specific concentration of nitrite was measured using a spectrophotometer at Abs540 and calculated through standard curves using a dilution series of $NaNO_2$ as a substrate. Microsoft Excel was used to further analyze the data and create figures and tables.

2.2 RNA extraction:

Based on previous growth curves, the two time points to collect cells during mid-log and early stationary was chosen to be at 36h and 72h, respectively. During extraction, 12 bottles (6 biological replicates with each

having an experimental replicate) were set up for analysis. Physiology measurements were done on the first set of the experimental replicates while the second set was not disturbed to minimize any change in temperature. This was done in order to minimize experimental errors and the first experimental set acted as a control to extract cells from the seond set at their correct growth stage (mid-log and late-log). Within each bottle the concentrations of cells at mid-log and late-log was roughly 10^7cells/mL and 10^8cells/mL, respectively. Each bottle was poured into a 250mL Nalgene bottle and 17mL of ice-cold phenol stop solution was added to each bottle and left on ice until other bottles were treated. Nalgene bottles were then centrifuged at 10,000 rpm for 30min at 4 degrees Celsius. A total of six Nalgene bottles were used for each of the biological replicates within each condition (e.g. for one of RNA samples sent for mid-log, RNA was extracted from a total of 6 Nalgene bottles with each being a biological replicate). Once the supernatant was discarded, the samples were collected by re-suspending the pellet in 2mL of Tris EDTA buffer. To each sample, 330µL of Tissue and Cell lysis solution (prepared by adding 25µL of 20mg/mL Proteinase K to 1mL of T&C solution) was added and vortexed for 5 seconds. Each sample were then incubated at 65°C for 20min, vortexing every 5min for 5 seconds and checked for clearing of suspension.

Samples were cooled down on ice for 5min and 175µl of MasterPure™ Complete protein precipitation reagent was added. Samples were then vortexed vigorously for 10 seconds and incubated on ice for 5min before it was transferred into new 1.5mL microfuge tubes. Samples were then centrifuged for 10min at

4°C, ≥ 13000 x g and 500μL of the supernatant were added into a new 1.5mL microfuge tube containing equal volume of ice-cold isopropanol (RNA grade) and the tubes were inverted 30-40 times until nucleic acid precipitated. After it was pelleted at 4°C, ≥ 13000 x g for 10min, the supernatant was carefully pipetted-out and the pellet was rinsed with 1.0mL 75% ethanol (RNA grade). The pellet was dislodged from the bottom and spun at 4°C, ≥ 13000 x g for 4 min. Once the ethanol was fully removed and the pellet was left to dry, it was re-suspended in 50μL of nuclease-free water.

In order to get rid of the DNA in the sample, 1/10th volume 10X DNase I buffer and 1/20th rDNase I enzyme were added and pipette mixed. Samples were incubated at 37°C for 15min and another shot of 1/20th volume rDNAse I enzyme was added. The reaction was stopped with 1/20th volume 50mM EDTA and RNA was stabilized with 1μL Superase-In. 1.5μL of sample was quantified via Nanodrop spectrophotometer for a quick quality and quantity check of the nucleic acid and the rest was stored at -80°C until further analysis. Quantitative PCR using both degenerate 16S rRNA and *Nitorosomonas europaea* amoA sequences was performed to check for any genomic DNA contamination in the samples (see Appendix). Samples were treated with DNAse I and rDNAse multiple times until all genomic DNA present in the sample was eliminated.

2.3 rRNA removal and RNA purification:

Before any further analysis, RNA quality and concentration in each sample was checked through Agilent Bioanalyzer 2100 and Qubit Fluorometer

respectively (using kits available at MBSU). A total of 9 samples were processed further after satisfying both quality and quantity standards set by Hiseq illumina sequencing at Oregon state for RNA analysis. Four biological replicates in *N. europaea* (two biological replicates for mid-log and 2 biological replicates for early stationary) and five biological replicates in *N. eutropha* (3 biological replicates for mid-log and 2 biological replicates for early stationary) were further analyzed. In order to get rid of any ribosomal RNA, the Ribo-Zero™ Magnetic Kit (Bacteria) by epicentre was used. The protocol was followed as stated by the manufacturer's instructions without any modifications. Finally, RNA Clean & Concentrator™-5 and RNA Clean & Concentrator™-25 by Zymo Research was used to purify the RNA samples. The protocols were again carried out as stated by the manufacturer's instructions without any modification. Finally, samples were sent for library construction and stranded sequencing by illumina Hiseq 2500 at Oregon State University.

2.4 RNA analysis:

The raw reads from the illumina Hiseq sequencing were sent back and uploaded into the CLC Genomics Workbench version 7.0.4 (Latest version as of this writing) for RNA analysis. Once the files were uploaded for each sample, the raw reads were trimmed by using Trim Sequences functionality under NGS core tools without changing the parameters. Once all the raw reads were trimmed, by using RNA-Seq Analysis under RNA-Seq Analysis toolbox, the raw reads were mapped to their corresponding annotated genome (NC_004757.1 and

NC_008344.1) that had been uploaded from NCBI and RAST. Once mapped, Set Up Experiment under Transciptomics Analysis tool was used to calculate the RPKM (reads per kilobase per million) of the genes within each experimental condition. By using the Normalize tool under Transformation and Normalization toolbox, the results were normalized and the quality of the reads were checked through Box Plot, Hierarchical Clustering of Samples and Principal Component Analysis under Quality Control toolbox. Finally, On Proportions under Statistical Analysis were used to only look at statistically significant changes in expression from mid-log to stationary phase between the biological and experimental replicates of one bacterium based on Bonferroni corrected statistics under Baggerly's test. Along each step of the process, the parameters set by CLC was kept constant to ensure reproducibility. Lastly, Computational Biology at ORNL (http://genome.ornl.gov/), National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) were used to analyze and discuss the results from the transciptomics data.

Chapter 3
Results

3.1 Comparison of Physiology

The growth curve for *N. europaea* and *N. eutropha* followed a nearly identical trend (Fig. 3). Both cultures reached mid-log after 36 h post inoculation with *N. eutropha* having a maximum doubling time at $7.9h^{-1}$ compared to *N. europaea's* $7.7h^{-1}$ doubling time. Starting concentration for both were at 10^6/ml and they grow up to 10^8/ml after 72h where they reach early stationary phase. As expected, oxygen consumption followed a similar trend as well between these two bacteria (Fig. 4). Starting from 23% oxygen in the headspace, after 48h nearly half of the available oxygen was consumed with oxygen levels reaching hypoxia at ca. 120h. In fact by early stationary phase, only 5% oxygen was present in the gas headspace. The same growth experiments were done with 50mM ammonia and the growth rate showed the same pattern in both *N. europaea* and *N. eutropha*, indicating that the approach to stationary phase was not due to substrate limitation (see Appendix, Fig. A-1).

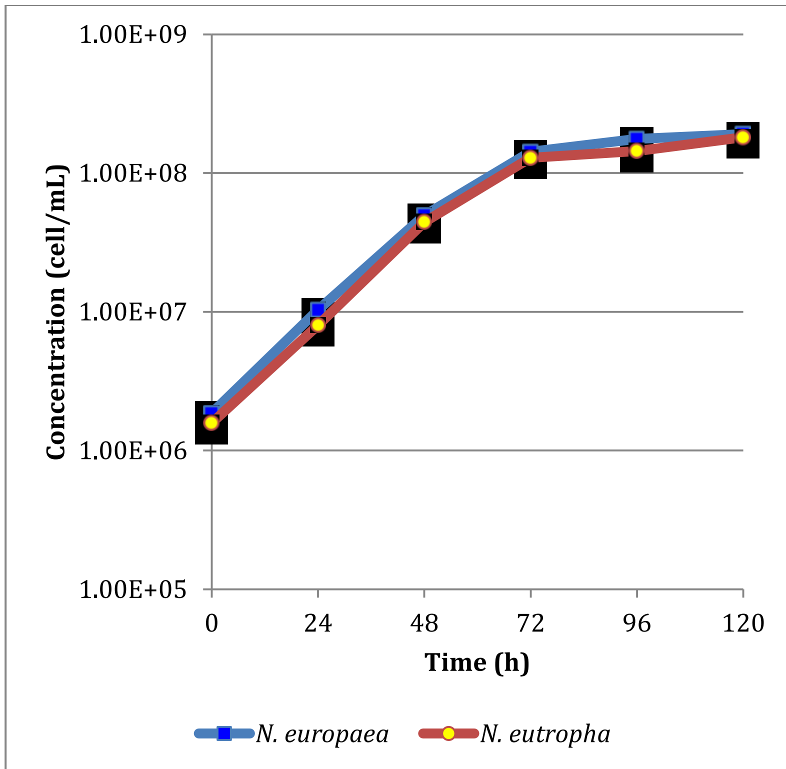
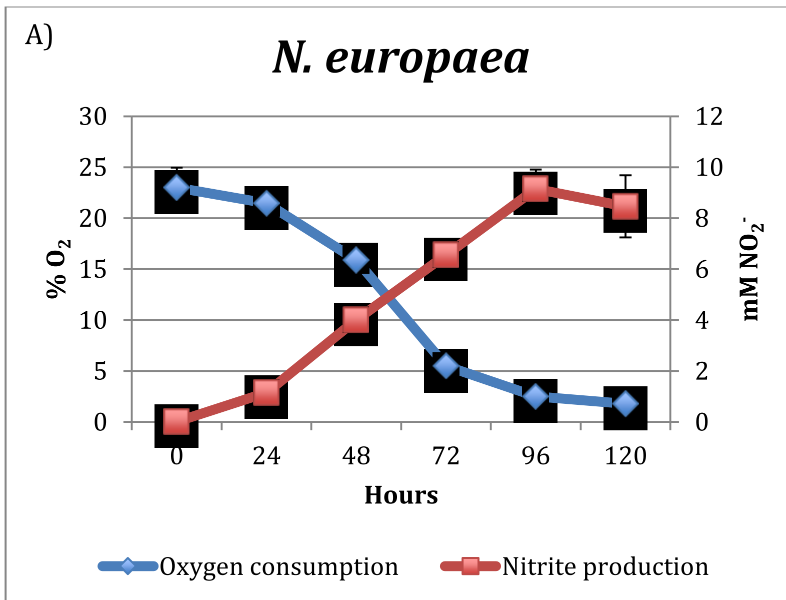


Figure 3: Growth curve of *N. europaea* and *N. eutropha*. Cells were grown in H.K. media in batch culture at 30°C incubation. The blue line represents *N. europaea* (n = 6) and the red line represents *N. eutropha* (n = 6). Data are presented as means with ± SEs.



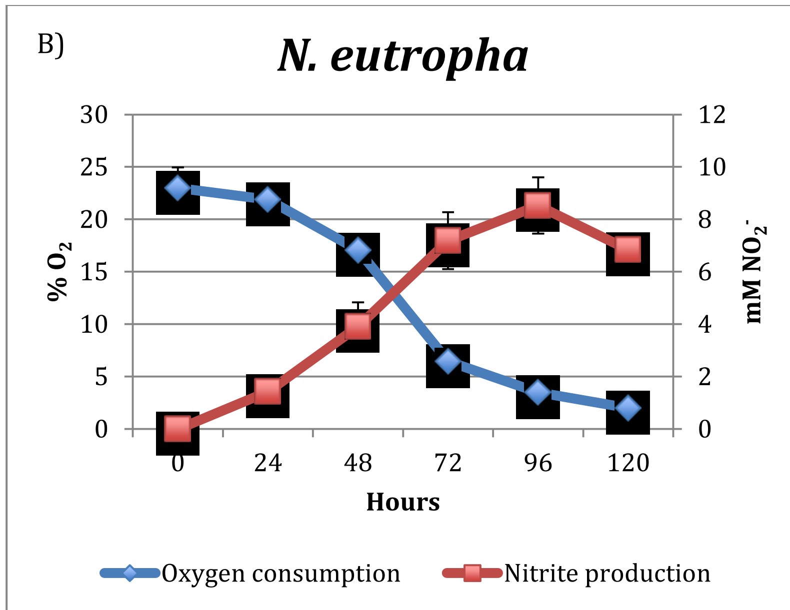


Figure 4: O_2 consumption and NO_2^- production. O_2 consumption were measured starting at 23% in the headspace and compared to nitrite production in (A) *N. europaea* (n = 6) and (B) *N. eutropha* (n = 6). Cells were grown in H.K. media in batch culture at 30°C incubation. All data are presented as means ± SEs.

The similarities between the two bacteria continue as we look at the graph for nitrite production and oxygen consumption (Fig. 4 & 5). Both start producing nitrite as early as 24h after inoculation with *N. eutropha* having a faster maximum rate at 6.05 mM/h^{-1} compared to *N. europaea*'s slower 7.4 mM/h^{-1} during mid-log. Furthermore, they both nearly converted all the available 10mM ammonia to nitrite at 96 h where oxygen levels were less than 5% gas headspace concentration. An interesting point to consider is that in *N. eutropha*, nitrite levels showed a statistically significant (based on t-test) drop from 96h to 120h falling from an average of 9.15mM nitrite to 8.45mM. A similar pattern of nitrite production was also observed when *N. europaea* and *N. eutropha* were grown in 50mM ammonia with no statistically significant difference in total nitrite produced,

again indicating a limitation on growth other than ammonium availability since the pH was controlled at circum-neutral throughout growth (see Appendix, Fig. A-4).

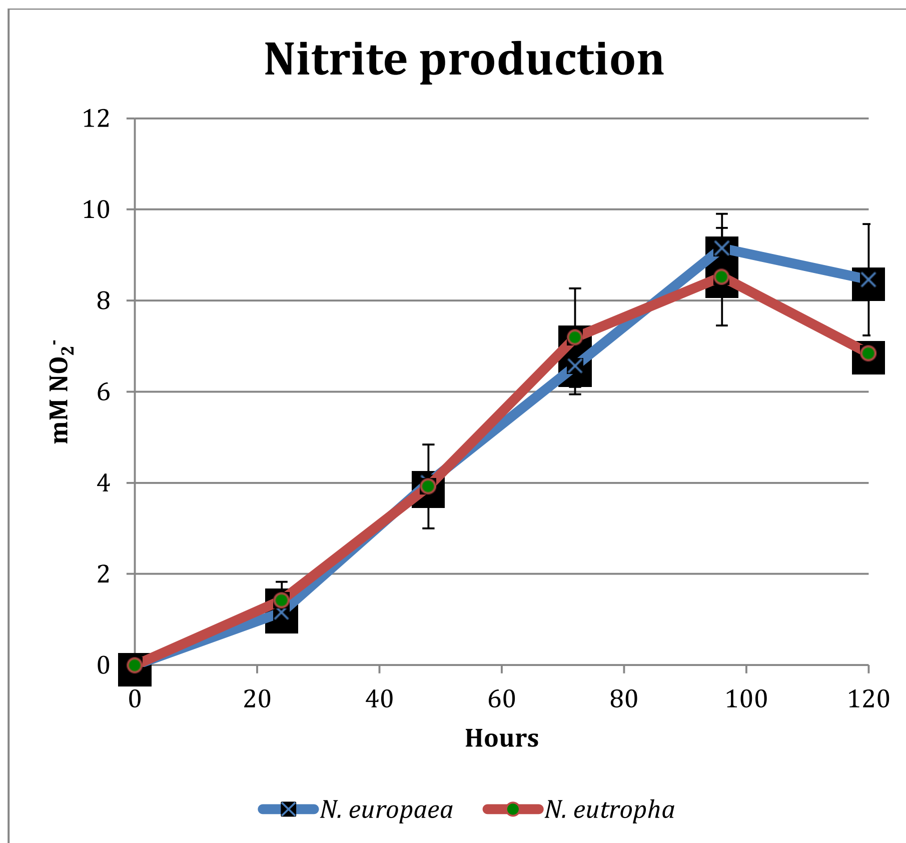


Figure 5: Nitrite production in *N. europaea* and *N. eutropha*. Cells were grown in H.K. media in batch culture at 30°C incubation. Nitrite production was measured using chemical assays and a spectrophotometer. The red line represents *N. eutropha* (n = 6) and the blue line represents *N. europaea* (n = 6). Data are presented as means ± SEs.

Finally, the last physiological measurement and the most dissimilar pattern was observed in nitrous oxide production between the two bacteria (Fig. 6). In the case of *N. europaea*, N_2O measurement was not detectable until 48h of growth, by which an average of 9.16μM N_2O was produced. This production continued to rise until an average of 18.06μM N_2O was observed after 96h of growth followed by a slight decrease after 120h. In the case of *N. eutropha* however, this pattern was significantly different. In fact, no N_2O production was detected until after 72h

of growth. Even at 72h, only an average of 4.31μM N_2O was produced which was much lower than the 16.01μM in *N. europaea* at the same time point. The N_2O production in *N. eutropha* continued in more of a linear fashion with still only an average of 4.52 μM N_2O produced after 120 h post inoculation. During growth experiments where higher initial concentration of ammonium was added to the media (50mM), N_2O production in *N. europaea* and *N. eutropha* followed the same pattern (See Appendix, Fig. A-2). Thus, nitrite consumption at stationary phase, and N_2O production showed the greatest differences in physiological characteristics between the two strains.

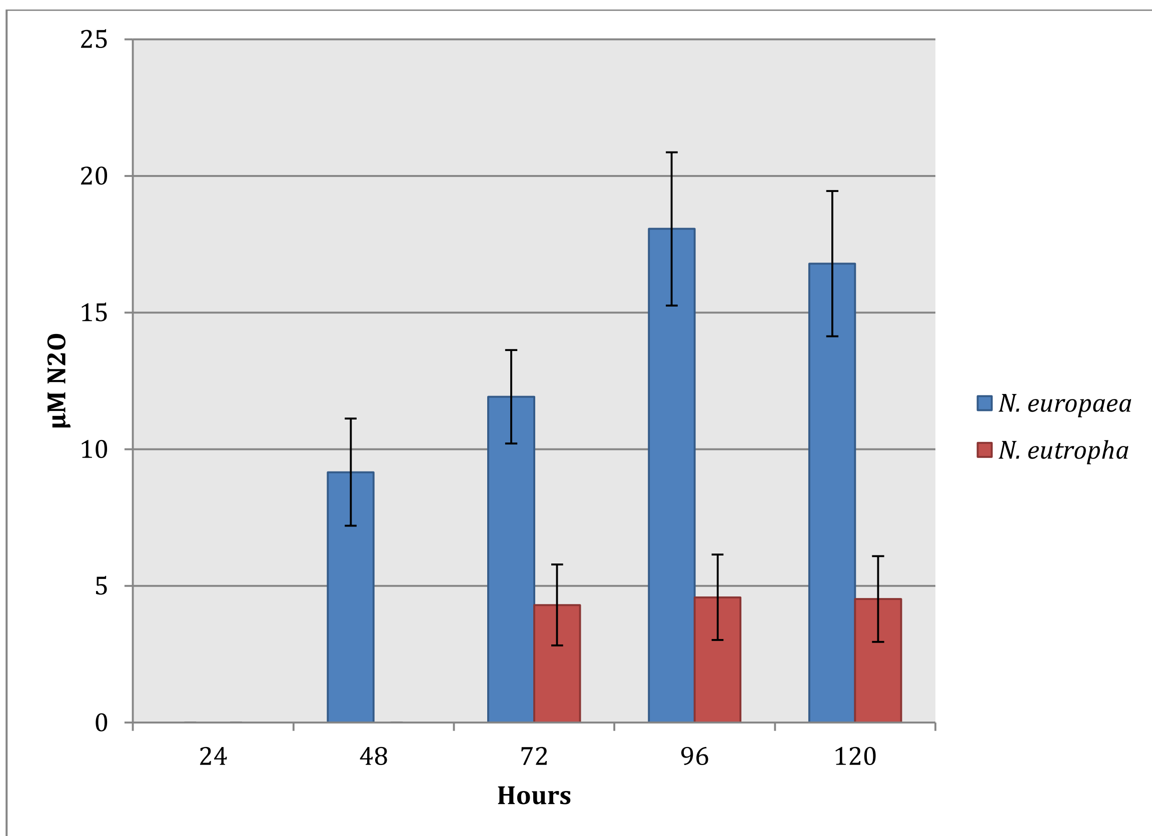


Figure 6: Nitrous oxide production in *Nitrosomonas spp.*. Cells were grown in H.K. media in batch culture at 30°C incubation. N_2O measurments from *N. europaea* (n = 6) and *N. eutropha* (n = 6). 200 μL injections from the headspace were injected into the GC. Note: No detectable N_2O were measured at 24h for *N. europaea* (blue bar) and at 24h and 48h in *N. eutropha* (red bar). Data are presented as means ± SEs.

3.2 Gene expression in *N. europaea*

When looking at the genome wide expression levels in replicated experiments of *N. europaea* in early stationary phase compared to mid-log, a total of 212 genes were analyzed to have had significantly different levels of transcripts. 120 of these genes had lower number of transcripts present in early stationary while the rest (93 genes) had a higher number of transcripts. These included both genes of unknown and known functions ranging from 34-fold increase in transcript level at the top of the list, all the way to 12-fold decrease at the bottom. In fact, the most up-regulated and down-regulated genes code for hypothetical proteins of yet unknown functions. Out of the 212 genes analyzed, there were 153 genes grouped under metabolism, enzymes, iron-binding proteins, cellular processes, environmental information processing, membrane proteins and human diseases (Fig. 7). However, only genes with equal to or higher than 2-fold change in their expression were considered significant and were used in the following results and discussion.

3.2.1 Metabolism

There were a total of four genes involved in carbon metabolism that were significantly different between mid- and late-log (Table 1). These genes code for 2-oxoglutarate dehydrogenase (E1 component), citrate synthase, dihydrolipoamide succinyltransferase, and ribulose bisphosphate carboxylase (large chain). All the genes involved in this sub-category were down-regulated. For instance, the gene coding for ribulose bisphosphate carboxylase large chain was down-regulated by ~2-fold, going from ~4002 RPKM in mid-log to only

~1814 RPKM in early stationary phase. There were a total of three genes involved in amino acid metabolism that were regulated (Table 1). Interestingly, all of these genes were down-regulated. They were present in fairly similiar numbers of reads in both mid-log and early stationary phase. When looking at carbohydrate metabolism, there was a total of seven genes that were regulated, most of which overlap with the ones involved in carbon metabolism mentioned above. Besides the gene coding for inositol-1 monophosphate, all of the genes in this sub-category were down-regulated, which follows the pattern seen in carbon and amino acid metabolism.

Eight more regulated genes were grouped under energy metabolism (Table 1). Interestingly, with the exception of the genes coding for RuBisCO and ferredoxin reductase, the rest of the genes were all up-regulated. This group included genes coding for ammonia monooxygenase (A-subunits), cytochrome c oxidase, ferredoxin reductase, ATP synthases, ribulose bisphosphate carboxylase (large chain), etc. This group contained most of the genes in *N. europaea*'s transcriptome that were present in high RPKM during both mid-log and early stationary growth phase. Furthermore, it also included one of the most highly down-regulated genes coding for ferredoxin reductase, going from ~3435 RPKM in mid-log down to only ~326 RPKM in early stationary phase.

A small number of regulated genes (seven in total) fell within the rest of the categories that are cofactors and vitamins, glycan biosynthesis, lipid, oxocarboxylic acid, and nitrogen metabolism (Table 1). Due to the small number of genes divided between the other sub-categories, no real pattern of expression

can be deduced. It is interesting to note that gene expression levels for these particular categories in *N. europaea* were not vastly different when entering stationary phase.

Table 1: List of all the genes involved in metabolism in *N. europaea*. Only the genes with ≥ 2-fold change were included to be significant. Note that some genes are repeated as they are involved in more than one process within metabolism. Fold change is normalized and the RPKM values are average of all replicates (n = 2 for Mid-Log, n = 2 for Late-Log). Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4).Groupings based on K.E.G.G..

Proteins involved in Metabolism in *N. europaea*	Fold change	ML-RPKM	LL-RPKM	Gene ID
Carbon metabolism				
2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	-2.19	158.77	72.31	NE2375
Citrate synthase (EC 2.3.3.1)	-2.02	121.71	60.28	NE2373
Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (EC 2.3.1.61)	-2.02	126.36	62.52	NE0360
Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	-2.2	4002.41	1814.84	NE1921
carbohydrate metabolism				
2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	-2.19	158.77	72.31	NE2375
Citrate synthase (EC 2.3.3.1)	-2.02	121.71	60.28	NE2373
Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (EC 2.3.1.61)	-2.02	126.36	62.52	NE0360
Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	-2.2	4002.41	1814.84	NE1921
NAD-dependent epimerase/dehydratase	-2.42	290.88	119.78	NE0679
Inositol-1-monophosphatase (EC 3.1.3.25)	2.02	83.32	168.6	NE0781
UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158)	-2.29	130.84	57.038	NE0993
Amino acid metabolism				
2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	-2.19	158.77	72.31	NE2375
Anthranilate synthase, aminase component (EC 4.1.3.27)	-2.72	150.96	55.6	NE2150
UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10)	-2.16	119.19	55.11	NE0987
Energy metabolism				
Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	-2.2	4002.41	1814.84	NE1921
ATP synthase A chain (EC 3.6.3.14)	2.09	56.7	118.83	NE0200
ATP synthase C chain (EC 3.6.3.14)	2.78	66.63	185.84	NE201
Cytochrome c oxidase polypeptide II (EC 1.9.3.1)	2.12	446.12	946.69	NE0684
Ferredoxin reductase	-10.51	3435.81	326.85	NE1771
Ammonia monooxygenase A-subunit (EC 1.14.13.25)	2.07	5370.13	11130.86	NE2062

Ammonia monooxygenase A-subunit (EC 1.14.13.25)	2.22	5408.39	12013.81	NE2063
Ubiquinol-cytochrome C reductase iron-sulfur subunit (EC 1.10.2.2)	2.35	63.84	150.06	NE0809
Cofactors and vitamins metabolism				
Biotin synthase (EC 2.8.1.6)	2.01	124.92	250.89	NE2300
Glycan biosynthesis metabolism				
Lipid carrier: UDP-N-acetylgalactosaminyltransferase (EC 2.4.1.-)	2.22	197.05	439.4	NE0991
UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158)	-2.29	130.84	57.038	NE0993
UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10)	-2.16	119.19	55.11	NE0987
Lipid metabolism				
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	-2.64	258.59	98.01	NE1548
Putative cyclooxygenase	2.57	358.67	922.61	NE1240
Oxocarboxylic acid metabolism				
Citrate synthase (EC 2.3.3.1)	-2.02	121.71	60.28	NE2373
Nitrogen metabolism				
Ammonia monooxygenase A-subunit (EC 1.14.13.25)	2.07	5370.13	11130.86	NE2062
Ammonia monooxygenase A-subunit (EC 1.14.13.25)_2	2.22	5408.39	12013.81	NE2063
Cytochrome c oxidase polypeptide II (EC 1.9.3.1)	2.12	446.12	946.69	NE0684

3.2.2 Enzymes

Eight of the regulated genes involved in coding for enzymes were grouped as oxidoreductases, constructing the biggest sub-group within this category (Table 2). Exactly half of the genes were down-regulated and the other half were up-regulated in early stationary phase. This includes the gene coding for ferredoxin reductase that was already mentioned. Most of the genes involved had a high number of reads present in both mid-log and early stationary phase with a few exceptions such as the ones coding for ubiquinol-cytochrome C reductases. There were 10 more genes grouped under transferases, ligases, isomerases and lyases (Table 2). All of the genes coding for lyases, ligases and isomerases were down-regulated. These included a gene coding for cyanophycin synthase that

showed a nearly 9-fold decrease going from ~1557 RPKM in mid-log to only ~173 RPKM in early stationary phase. There were four genes coding for transferases, half of which were up-regulated in stationary phase. These included the genes coding for biotin synthase and UDP-N-acetylgalactosaminyltransferase.

Table 2: List of all the genes involved in enzyme category in *N. europaea.* Only the genes with ≥ 2-fold change are included to be significant. Note that some genes are repeated as they are involved in more than one process. Fold change is normalized and the RPKM values are average of all replicates (n = 2 for Mid-Log, n = 2 for Late-Log). Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4).Groupings based on K.E.G.G.

Enzymes in *N. europaea*	Fold Change	ML-RPKM	LL-RPKM	Gene ID
Transferases				
2-oxoglutarate dehydrogenase E1 component (EC 1.2.4.2)	-2.19	158.77	72.31	NE2375
Biotin synthase (EC 2.8.1.6)	2.01	124.92	250.89	NE2300
Lipid carrier: UDP-N-acetylgalactosaminyltransferase (EC 2.4.1.-)	2.22	197.05	439.4	NE0991
Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex (EC 2.3.1.61)	-2.02	126.36	62.52	NE0360
Ligases				
Cyanophycin synthase (EC 6.3.2.29)	-8.99	1557.63	173.07	NE0923
Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	-2.44	203.48	83.11	NE1125
UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10)	-2.16	119.19	55.11	NE0987
Isomerases				
NAD-dependent epimerase/dehydratase	-2.42	290.88	119.78	NE0679
Oxidoreductases				
Ammonia monooxygenase A-subunit (EC 1.14.13.25)	2.07	5370.13	11130.86	NE2062
Ammonia monooxygenase A-subunit (EC 1.14.13.25)	2.22	5408.39	12013.81	NE2063
Cytochrome c oxidase polypeptide II (EC 1.9.3.1)	2.12	446.12	946.69	NE0684
Ferredoxin reductase	-10.51	3435.81	326.85	NE1771
Putative cyclooxygenase	2.57	358.67	922.61	NE1240
Putative tyrosinase	2.00	718.44	1430.19	NE1241

Thiol peroxidase, Bcp-type (EC 1.11.1.15)	-2.12	705.06	331.73	NE2465
Ubiquinol-cytochrome C reductase iron-sulfur subunit (EC 1.10.2.2)	2.35	63.84	150.06	NE0809
UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158)	-2.29	130.84	57.038	NE0993
Butyryl-CoA dehydrogenase (EC 1.3.99.2)	-2.64	258.59	98.01	NE1548
Lyases				
Anthranilate synthase, aminase component (EC 4.1.3.27)	-2.72	150.96	55.6	NE2150
Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	-2.2	4002.41	1814.84	NE1921

3.2.3 Cellular processes

There were a total of eight regulated genes involved in transcription and translation (Table 3). A very interesting point here is that all of the genes involved in translation were up-regulated in stationary phase. In contrast, three out of the possible five genes in transcription were down-regulated with a much larger difference in fold expression between mid-log and stationary phase. Some of the genes such as the ones coding for translation elongation factors and iron-sulfur cluster regulator IscR were present in a high number of reads while others, such as the ones coding for ferric uptake regulation protein and iron-sulfur cluster regulator SufR were present in a much smaller number of reads in both mid-log and early stationary phase. Four other regulated genes that complete the cellular processes category fell within cell division and DNA repair and transport sub-categories (Table 3). Under cell division, it can be noted that all of the genes coding for cell division proteins were down-regulated in stationary phase. In fact the only gene in this sub-category that was up-regulated coded for chaperone protein DnaK. A single gene grouped under DNA repair and transport was up-regulated in stationary phase (Table 3).

Table 3: List of all the genes involved in cellular processes category in *N. europaea.* Only the genes with ≥ 2-fold change are included to be significant. Fold change is normalized and the RPKM values are average of all replicates (n = 2 for Mid-Log, n = 2 for Late-Log). Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4). Groupings based on K.E.G.G..

Cellular processes proteins in *N. europaea*	Fold Change	ML-RPKM	LL-RPKM	Gene ID
Cell divison				
Cell division protein FtsI [Peptidoglycan synthetase] (EC 2.4.1.129)	-2.49	153.63	61.65	NE0985
Cell division protein FtsL	-4.35	183.3	42.16	NE0984
Cell division protein MraZ	-2.11	206.39	97.41	NE0982
Chaperone protein DnaK	2.03	204.6	417.18	NE1949
Translation				
Translation elongation factor G	2.49	217.59	542.1	N/A
Translation elongation factor Tu	2.39	283.46	677.51	N/A
Translation initiation factor	4.21	46.23	194.94	NE0957
Transcription				
DNA-directed RNA polymerase specialized sigma subunit	-8.98	244.99	27.26	NE2046
Ferric uptake regulation protein FUR	-8.33	29.49	3.53	NE0616
FIG000325: clustered with transcription termination protein NusA	3.44	37.34	128.52	NE0760
Iron-sulfur cluster regulator IscR	2.23	373.93	834.59	N/A
Iron-sulfur cluster regulator SufR	-4.67	153.31	32.8	N/A
DNA repair and transport				
Putative DNA transport competence protein	2.08	79.52	165.6	NE0940

N/A corresponds to gene ID not being available on the K.E.G.G. website.

3.2.4 Membrane proteins

Continuing on with the results from *N. europaea*'s transcriptome data, eight regulated genes were grouped as coding for membrane proteins (Table 4). These included genes coding for efflux pumps, bipolymer transporters, ABC-transporters and generic transporters. Minus a single gene coding for outer membrane lipoprotein under efflux pump, all of the genes were down-regulated in

stationary phase. In fact, the gene with the largest fold change in its expression coded for a putative membrane protein believed to be a membrane transporter.

3.2.5 Remaining categories

Only five more regulated genes populate the environmental information processing, iron-binding proteins and human diseases categories. These genes were present in a fairly small number of RPKM during both mid-log and early stationary growth phase (Table 4).

Table 4: Remaining categories including membrane proteins in *N. europaea.* Only the genes with ≥ 2-fold change are included to be significant. Fold change is normalized and the RPKM values are average of all replicates (n = 2 for Mid-Log, n = 2 for Late-Log). Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4). Groupings based on K.E.G.G..

Membrane proteins in *N. europaea*	Fold Change	ML-RPKM	LL-RPKM	Gene ID
Efflux pump				
Cobalt-zinc-cadmium resistance protein CzcA; Cation efflux system protein CusA	-7.97	116.35	14.59	NE1638
Outer membrane lipoprotein omp16 precursor	2.1	167.82	353.21	NE0036
Probable Co/Zn/Cd efflux system membrane fusion protein	-4.63	77.18	16.64	NE0375
Transporter				
Ferric siderophore transport system, periplasmic binding protein TonB	-4.2	110.88	26.37	NE0321
MotA/TolQ/ExbB proton channel family protein	-4.76	281.32	59.02	NE0215
Putative membrane protein	-8.07	1206.06	149.3	NE0802
Bipolymer transporters				
Biopolymer transport protein ExbD/TolR	-2.82	276.46	97.79	NE0216
ABC-transporter				
ABC-transporter ATP binding	-3.6	72.99	20.24	NE1404
Environmental Information Processing				
Signal transduction				
RND efflux system, membrane fusion protein CmeA	-2.03	146.5	72.04	NE1112
two-component system regulatory protein_1	-3.24	54.2	16.68	NE0017

Ubiquinol-cytochrome C reductase iron-sulfur subunit (EC 1.10.2.2)	2.35	63.84	150.06	NE0809
Iron-binding proteins				
Ferritin				
Iron binding protein IscA for iron-sulfur cluster assembly	2.27	115.49	262.26	N/A
Human diseases				
Drug resistance				
RND efflux system, membrane fusion protein CmeA	-2.03	146.5	72.04	NE1112

N/A corresponds to gene ID not being available on the K.E.G.G. website.

3.3 Gene expression in N. eutropha

In the case of *N. eutropha*, when looking at the number of statistically significant differences in genes expressed in mid- versus stationary phase of growth, 455 genes were analyzed . Out of the 455 genes, nearly half of them (221 genes) were down-regulated while the rest were up-regulated during stationary phase. As expected, the list is populated with genes of both known and unknown functions. While the gene coding for a putative membrane protein was at the bottom of the list for being the most down-regulated, the gene coding for flagellar transcriptional activator FlhD was at the top going from ~40 RPKM present in mid-log to ~2560 RPKM in early stationary phase, an almost 60-fold increase. 400 genes with known functions out of the original 455 genes could be analyzed and grouped under Metabolism, Enzymes, Cellular processes, Environmental information processing, Membrane proteins, Human diseases, Bacterial motility and Iron-binding proteins (Fig. 7). Similar to *N. europaea*'s trancriptome, only genes with equal to or higher than a 2-fold change in their expression between mid-log and stationary phase of growth were considered significant.

3.3.1 Metabolism

There was a total of 13 regulated genes coding for proteins involved in carbon metabolism (Table 5). With the exception of the genes coding for pyruvate kinase and serine hydroxymethyltransferase, all of the genes involved in carbon metabolism were surprisingly up-regulated in stationary phase. These included genes coding for carboxysome shell protein (CsoS1), Ribulose bisphosphate carboxylase (small and large chain), ribose-phsophate pyrophosphokinase and RuBisCO activation protein.

Genes involved in nitrogen metabolism were present in high number of reads in both mid-log and early stationary phase (Table 5). A total of six genes were grouped in this category. With the exception of the ones coding for hydroxylamine dehydrogenase precursor and ammonia monooxygense C-subunit, all of them were up-regulated in early stationary phase. A gene coding for a multicopper oxidase of unknown function was the most highly up-regulated gene in this sub-category, going from having ~440 RPKM during mid-log to 3093 RPKM in early stationary phase.

12 regulated genes were grouped into the carbohydrate category, with only three genes being down-regulated and the rest being up-regulated in stationary phase (Table 5). Most of the genes were present in fairly small number of RPKM in both mid-log and early stationary phase. Genes coding for ribulose bisphosphate carboxylase large and small chain have high number of reads whereas genes coding for 2-isopropylmalate synthase, GDP-mannose 4,6-dehydratase and UDP-N-acetylenolpyruvoylglucosamine reductase were amongst some of the lowest transcribed genes in both growth phases. The gene

coding for UDP-N-acetylenolpyruvoylglucosamine reductase had the highest change in its fold expression, however, it was present in much smaller total number of RPKM.

Continuing on with *N. eutropha*'s transcriptome data, there was a total of 28 regulated genes coding for proteins involved in amino acid metabolism with 22 of the genes being up-regulated in stationary phase (Table 5). Most genes were present in similar numbers of RPKM in both mid-log and early stationary phase with the exception of the gene coding for glutathione synthetase. This gene was down-regulated by ~3-fold going from ~4232 RPKM in mid-log to ~1362 RPKM in early stationary phase. It is interesting to note that genes in this sub-category form the second largest group under metabolism when we take a look at *N. eutropha*'s transcriptome data.

Energy metabolism was the largest sub-category under metabolism with total of 33 regulated genes (Table 5). A total of 11 genes were down-regulated while the rest were up-regulated during stationary phase. It also included some of the mostly highly transcribed genes in both mid-log and early stationary phase in addition to a sizable fold-change in expression from mid-log to stationary phase. For instance, genes coding for cytochrome c class I, cytochrome c class IC, and multicopper oxidase_9 were amongst the most highly up-regulated genes in early stationary phase with the fold change of 12.48, 19.67 and 7.01 respectively. In contrast, genes coding for cytochrome c-type biogenesis protein (CcmE), cytochrome O ubiquinol oxidase subunit II and ammonia monooxygenase C-

subunit were amongst the most down-regulated genes with all showing around 6-fold decrease in early stationary phase.

There were 11 more regulated genes that grouped under cofactors and vitamins with about half being down-regulated and the rest being up-regulated in stationary phase (Table 5). The gene coding for quinolate synthetase had the highest change in expression in this sub-category. There were four more sub-categories that include the remaining regulated genes under metabolism. Most of the genes in these sub-categories were up-regulated during stationary phase. In fact, there were only three genes that were down-regulated; two involved in glycan biosynthesis and one that involved in nucleotide metabolism (Table 5). It is interesting that genes for lipid, nucleotide, and trepnoids and polyketides were all upregulated in stationary phase.

Table 5: List of all the genes involved in metabolism in *N. eutropha*. Only the genes with ≥ 2-fold change were included to be significant. Note that some genes are repeated as they are involved in more than one process within metabolism. Fold change is normalized and the RPKM values are average of all replicates (n = 3 for Mid-Log, n = 2 for Late-Log). Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4). Groupings based on K.E.G.G..

Metabolism in *N. eutropha*	**Fold change**	**ML-RPKM**	**LL-RPKM**	**Gene ID**
Carbon				
Acetyl-coenzyme A carboxyl transferase beta chain (EC 6.4.1.2)	2.32	82.34	191.12	Neut1154
Aminomethyltransferase (EC 2.1.2.10)	2.55	115.55	295.19	Neut1955
carboxysome shell protein CsoS1	3.1	193.22	599.55	Neut0806
carboxysome shell protein CsoS1	3.39	825.16	2805.1	Neut0807
D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	2.54	56.96	144.99	Neut1571
Phosphoglycerate kinase (EC 2.7.2.3)	2.31	94.3	218.41	Neut1579
Phosphoribulokinase (EC 2.7.1.19)	2.25	103.3	232.95	Neut0772
Pyruvate kinase (EC 2.7.1.40)	-2.01	342.01	170.62	Neut0979
Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)	2.95	136.4	402.91	Neut1140

Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	2.5	877.89	2197.97	Neut0804
Ribulose bisphosphate carboxylase small chain (EC 4.1.1.39)	2.11	270.54	571.3	Neut0805
Rubisco activation protein CbbO	5.83	6.51	38.03	N/A
Serine hydroxymethyltransferase (EC 2.1.2.1)	-2.66	345.2	129.47	Neut1552
Nitrogen				
Copper-containing nitrite reductase (EC 1.7.2.1)	4.02	554.19	2230.44	Neut1403
Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	3.75	519.49	1952.37	N/A
Hydroxylamine oxidoreductase precursor (EC 1.7.3.4)	3.24	172.85	561.38	Neut1672
Hydroxylamine oxidoreductase precursor (EC 1.7.3.4)	-2.22	492.29	221.57	Neut1793
Multicopper oxidase	7.01	440.67	3093.03	Neut1406
Ammonia monooxygenase C-subunit (EC 1.14.13.25)	-6.82	7289.75	1067.34	Neut2078
Carbohydrate				
2-hydroxy-3-oxopropionate reductase (EC 1.1.1.60)	-3.14	272.48	86.67	Neut1881
2-isopropylmalate synthase (EC 2.3.3.13)	3.35	67.02	224.85	Neut1244
Acetyl-coenzyme A carboxyl transferase beta chain (EC 6.4.1.2)	2.32	82.34	191.12	Neut1154
GDP-mannose 4,6-dehydratase (EC 4.2.1.47)	3.67	19.2	70.59	Neut0156
Phosphoglycerate kinase (EC 2.7.2.3)	2.31	94.3	218.41	Neut1579
Pyruvate kinase (EC 2.7.1.40)	-2.01	342.01	170.62	Neut0979
Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)	2.95	136.4	402.91	Neut1140
Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	2.5	877.89	2197.97	Neut0804
Ribulose bisphosphate carboxylase small chain (EC 4.1.1.39)	2.11	270.54	571.3	Neut0805
Serine hydroxymethyltransferase (EC 2.1.2.1)	-2.66	345.2	129.47	Neut1552
UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158)	5.5	10.13	55.74	Neut0244
UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7)	-2.25	126.57	56.21	Neut1556
Amino acid				
2-isopropylmalate synthase (EC 2.3.3.13)	3.35	67.02	224.85	Neut1244
3-isopropylmalate dehydratase large subunit (EC 4.2.1.33)	-2	358.63	178.93	Neut1144
5-methyltetrahydropteroyltriglutamate (EC 2.1.1.14)	2.45	42.589	104.75	Neut1555
Acetylglutamate kinase (EC 2.7.2.8)	2.55	35.38	90.29	Neut2384
Adenylosuccinate synthetase (EC 6.3.4.4)	2.15	46.48	100.16	Neut0967
Aminomethyltransferase (EC 2.1.2.10)	2.55	115.55	295.19	Neut1955
Anthranilate phosphoribosyltransferase (EC 2.4.2.18)	2.32	72.91	169.61	Neut0134
Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4)	2.42	56.34	136.72	Neut0901
Aspartokinase (EC 2.7.2.4)	2.03	159.19	324.26	Neut2084
ATP phosphoribosyltransferase regulatory subunit (EC 2.4.2.17)	2.28	46.39	105.78	Neut1205
Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	2.46	47.75	117.63	Neut0455
Carbamoyl-phosphate synthase small chain (EC 6.3.5.5)	2.69	64.05	172.85	Neut0454
CDP-diacylglycerol-serine O-phosphatidyltransferase (EC 2.7.8.8)	2.94	100.98	297.57	Neut1258

Chorismate mutase I (EC 5.4.99.5)	3.01	20.4	61.63	Neut1570
D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	2.54	56.96	144.99	Neut1571
GDP-mannose 4,6-dehydratase (EC 4.2.1.47)	3.67	19.2	70.59	Neut0156
Glutathione synthetase (EC 6.3.2.3)	-3.1	4232.63	1362.46	Neut0953
Glycine dehydrogenase (EC 1.4.4.2)	2.44	68.57	167.82	Neut1952
Glycine dehydrogenase (EC 1.4.4.2)	3.04	30.76	93.62	Neut1953
Histidinol dehydrogenase (EC 1.1.1.23)	2.09	76.14	159.44	Neut1206
Imidazole glycerol phosphate synthase cyclase subunit (EC 4.1.3.-)	2.08	57.91	120.48	Neut1907
O-acetylhomoserine sulfhydrylase (EC 2.5.1.49)	-2.19	102.03	46.51	Neut0424
Pantoate--beta-alanine ligase (EC 6.3.2.1)	3.14	17.55	55.22	Neut2292
Phosphoribosyl-ATP pyrophosphatase (EC 3.6.1.31)	-2.58	293.18	113.62	Neut1911
Serine hydroxymethyltransferase (EC 2.1.2.1)	-2.66	345.2	129.47	NEUT1552
UDP-N-acetylmuramate--alanine ligase (EC 6.3.2.8)	3.01	25.18	75.71	Neut0245
UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10)	4.11	12.34	50.76	Neut0250
Urea carboxylase (EC 6.3.4.6)	-2.7	72.55	26.83	Neut2475
Energy				
ATP synthase A chain (EC 3.6.3.14)	3.01	143.96	433.32	Neut0278
ATP synthase B chain (EC 3.6.3.14)	2.18	174.2	380.84	N/A
ATP synthase beta chain (EC 3.6.3.14)	2.25	356.84	804.3	N/A
ATP synthase C chain (EC 3.6.3.14)	2.99	162.16	486.45	N/A
ATP synthase delta chain (EC 3.6.3.14)	3.08	307.56	947.92	N/A
ATP synthase epsilon chain (EC 3.6.3.14)	2.8	212.2	595.59	N/A
ATP synthase gamma chain (EC 3.6.3.14)	2.09	326.68	685.15	N/A
Copper-containing nitrite reductase (EC 1.7.2.1)	4.02	554.19	2230.44	Neut1403
Cytochrome c family protein	-2.93	169.47	57.65	N/A
Cytochrome c family protein	-2.12	707.24	332.68	N/A
Cytochrome c heme lyase subunit CcmF	-4.4	1072.6	243.7	N/A
Cytochrome c oxidase (B(O/a)3-type) chain I (EC 1.9.3.1)	2.05	135.2	278.08	Neut1875
Cytochrome c oxidase (B(O/a)3-type) chain II (EC 1.9.3.1)	2.05	87.24	179.67	Neut1874
Cytochrome c oxidase polypeptide III (EC 1.9.3.1)	3.75	519.49	1952.37	N/A
Cytochrome c, class I	12.48	145.79	1819.79	N/A
Cytochrome c, class IC	19.67	54.06	1064	Neut1652
Cytochrome c-type biogenesis protein CcmE	-6.18	1177.12	190.39	N/A
Cytochrome c-type biogenesis protein (EC 1.8.1.8)	-2.28	132.65	57.98	N/A
Cytochrome O ubiquinol oxidase subunit I (EC 1.10.3.-)	-5.32	247.54	46.48	N/A
Cytochrome O ubiquinol oxidase subunit II (EC 1.10.3.-)	-6.94	271.82	39.13	N/A
Cytochrome O ubiquinol oxidase subunit III (EC 1.10.3.-)	-3.85	135.6	35.2	N/A
Cytochrome oxidase biogenesis protein	4.83	489.79	2367.87	N/A
D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	2.54	56.96	144.99	Neut1571
Multicopper oxidase	7.01	440.67	3093.03	Neut1406

NAD-dependent epimerase/dehydratase family protein	3.55	21.41	76.2	Neut0922
Ammonia monooxygenase C-subunit (EC 1.14.13.25)	-6.82	7289.75	1067.34	Neut2078
Phosphoglycerate kinase (EC 2.7.2.3)	2.31	94.3	218.41	Neut1579
Phosphoribulokinase (EC 2.7.1.19)	2.25	103.3	232.95	Neut0772
Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	2.5	877.89	2197.97	Neut0804
Ribulose bisphosphate carboxylase small chain (EC 4.1.1.39)	2.11	270.54	571.3	Neut0805
Rubisco activation protein CbbO	5.83	6.51	38.03	N/A
Serine hydroxymethyltransferase (EC 2.1.2.1)	-2.66	345.2	129.47	NEUT1552
Xylulose-5-phosphate phosphoketolase (EC 4.1.2.9)	-2	169.61	84.67	Neut2087
Cofactors and Vitamins				
1-deoxy-D-xylulose 5-phosphate synthase (EC 2.2.1.7)	2	77.68	155.8	Neut1501
2-amino-4-hydroxy-6- hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)	2.71	27.33	74.15	Neut2295
3,4-dihydroxy-2-butanone 4-phosphate synthase (EC 4.1.99.12)	-2.77	272.07	97.96	Neut2516
Aminomethyltransferase (EC 2.1.2.10)	2.55	115.55	295.19	Neut1955
Dihydrofolate synthase (EC 6.3.2.12)	2.3	95.66	220.22	Neut1155
Dihydropteroate synthase (EC 2.5.1.15)	-2.6	252.06	95.66	Neut0999
Pantoate--beta-alanine ligase (EC 6.3.2.1)	3.14	17.55	55.22	Neut2292
Quinolinate synthetase (EC 2.5.1.72)	-3.69	786.89	212.87	Neut2299
Radical SAM domain protein	-2.07	171.64	82.62	Neut0292
Riboflavin synthase eubacterial/eukaryotic (EC 2.5.1.9)	-2.35	101.69	43.15	Neut2517
Serine hydroxymethyltransferase (EC 2.1.2.1)	-2.66	345.2	129.47	Neut1552
Lipid				
Acetyl-coenzyme A carboxyl transferase beta chain (EC 6.4.1.2)	2.32	82.34	191.12	Neut1154
CDP-diacylglycerol--serine O-phosphatidyltransferase (EC 2.7.8.8)	2.94	100.98	297.57	Neut1258
Phosphate:acyl-ACP acyltransferase PlsX	4.46	48.25	215.62	Neut0471
Phosphatidylserine decarboxylase (EC 4.1.1.65)	2.08	147.78	307.94	Neut1259
Glycan Biosynthesis				
D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	-2.02	110.49	54.65	Neut0223
UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158)	5.5	10.13	55.74	Neut0244
UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7)	-2.25	126.57	56.21	Neut1556
UDP-N-acetylglucosamine--N-acetylmuramyl-pyrophosphoryl-undecaprenol (EC 2.4.1.227)	2.99	20.04	60.12	Neut0246
UDP-N-acetylmuramate--alanine ligase (EC 6.3.2.8)	3.01	25.18	75.71	Neut0245
UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10)	4.11	12.34	50.76	Neut0250
Nucleotide				
5-nucleotidase SurE (EC 3.1.3.5)	2.06	91.56	188.7	Neut1831
Adenine phosphoribosyltransferase (EC 2.4.2.7)	2.91	35.56	103.85	Neut0262
Adenylosuccinate synthetase (EC 6.3.4.4)	2.15	46.48	100.16	Neut0967

Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	2.46	47.75	117.63	Neut0455
Carbamoyl-phosphate synthase small chain (EC 6.3.5.5)	2.69	64.05	172.85	Neut0454
Pyruvate kinase (EC 2.7.1.40)	-2.01	342.01	170.62	Neut0979
Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)	2.95	136.4	402.91	Neut1140
Trepnoids and polyketides				
1-deoxy-D-xylulose 5-phosphate synthase (EC 2.2.1.7)	2	77.68	155.8	Neut1501
Acetyl-coenzyme A carboxyl transferase beta chain (EC 6.4.1.2)	2.32	82.34	191.12	Neut1154

N/A corresponds to gene ID not being available on the K.E.G.G. website.

3.3.2 Enzymes

There were a total of 104 regulated genes coding for enzymes (Table 6). Most of the genes code for oxidases and transferases within the enzyme category. The rest of the genes grouped under hydrolases, ligases, lyases, isomerases and proteases. While it is difficult to assess a pattern in some of the sub-categories, most genes coding for hydrolases were down-regulated during stationary phase, while most of the genes coding for oxidoreductases, transferases, ligases, and lyases were up-regulated. In fact, genes coding for proteases were the only ones in that sub-category where all the genes followed the same pattern of down-regulation going into early stationary phase (Table 6). Lastly, genes that code for oxidoreductases and ligases were amongst some of the most highly expressed genes in the enzyme category (Table 6).

Table 6: List of all the genes involved in enzyme category in *N. eutropha.* Only the genes with ≥ 2-fold change are included to be significant. Fold change is normalized and the RPKM values are average of all replicates (n = 3 for Mid-Log, n = 2 for Late-Log). Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4).Groupings based on K.E.G.G..

Enzymes in *N. eutropha*	Fold Change	ML-RPKM	LL-RPKM	Gene ID
Hydrolases				
3,4-dihydroxy-2-butanone 4-phosphate synthase	-2.77	272.07	97.96	Neut2516

(EC 4.1.99.12)				
5-nucleotidase SurE (EC 3.1.3.5)	2.06	91.56	188.7	Neut1831
Amidohydrolase	-5.25	100.71	19.17	Neut1692
Carboxyl-terminal protease (EC 3.4.21.102)	-2.44	392.27	160.58	Neut0917
Chemotaxis response regulator protein-glutamate methylesterase CheB (EC 3.1.1.61)	5.63	22.63	127.51	Neut1173
D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	-2.02	110.49	54.65	Neut0223
Exodeoxyribonuclease VII small subunit (EC 3.1.11.6)	-3.95	65.35	16.52	Neut1510
Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3)	-3.67	290.68	79.04	Neut0084
Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3)	-7.03	144.57	20.53	Neut0084
Lipoprotein signal peptidase (EC 3.4.23.36)	3.06	88.42	270.86	Neut1438
Peptide deformylase (EC 3.5.1.88)	-3.14	255.54	81.14	Neut0392
Phosphoribosyl-ATP pyrophosphatase (EC 3.6.1.31)	-2.58	293.18	113.62	Neut1911
Probable protease htpX homolog (EC 3.4.24.-)	-4.05	549.98	135.6	Neut1535
Ribonuclease III (EC 3.1.26.3)	2.42	125.78	305.46	Neut1778
Ribonuclease P protein component (EC 3.1.26.5)	-3.05	1216.15	397.88	Neut2152
Xaa-Pro aminopeptidase (EC 3.4.11.9)	-2.92	170.81	58.47	Neut2095
Transferase				
1-deoxy-D-xylulose 5-phosphate synthase (EC 2.2.1.7)	2	77.68	155.8	Neut1501
2-amino-4-hydroxy-6- hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)	2.71	27.33	74.15	Neut2295
2-isopropylmalate synthase (EC 2.3.3.13)	3.35	67.02	224.85	Neut1244
5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (EC 2.1.1.14)	2.45	42.589	104.75	Neut1555
Acetylglutamate kinase (EC 2.7.2.8)	2.55	35.38	90.29	Neut2384
Adenine phosphoribosyltransferase (EC 2.4.2.7)	2.91	35.56	103.85	Neut0262
Aminomethyltransferase (EC 2.1.2.10)	2.55	115.55	295.19	Neut1955
Anthranilate phosphoribosyltransferase (EC 2.4.2.18)	2.32	72.91	169.61	Neut0134
Aspartokinase (EC 2.7.2.4)	2.03	159.19	324.26	Neut2084
ATP phosphoribosyltransferase regulatory subunit (EC 2.4.2.17)	2.28	46.39	105.78	Neut1205
Biosynthetic Aromatic amino acid aminotransferase beta (EC 2.6.1.57)	-3.47	248.39	71.49	N/A
CDP-diacylglycerol-serine O-phosphatidyltransferase (EC 2.7.8.8)	2.94	100.98	297.57	Neut1258
Chemotaxis protein methyltransferase CheR (EC 2.1.1.80)	3.41	28.39	97.02	Neut1171
Dihydropteroate synthase (EC 2.5.1.15)	-2.63	252.06	95.66	Neut0999
Glycosyl transferase	3.01	55.17	166.42	Neut0041
Imidazole glycerol phosphate synthase cyclase subunit (EC 4.1.3.-)	2.08	57.91	120.48	Neut1907
O-acetylhomoserine sulfhydrylase (EC 2.5.1.49)	-2.19	102.03	46.51	Neut0424
Phosphate:acyl-ACP acyltransferase PlsX	4.46	48.25	215.62	Neut0471
Phosphoglycerate kinase (EC 2.7.2.3)	2.31	94.3	218.41	Neut1579
Phosphoribulokinase (EC 2.7.1.19)	2.25	103.3	232.95	Neut0772

Protein-L-isoaspartate O-methyltransferase (EC 2.1.1.77)	2.06	131.94	272.48	Neut0197
Protein-N(5)-glutamine methyltransferase PrmC, methylates polypeptide chain release factors RF1 and RF2	-3.47	965.28	277.86	Neut0828
Pyruvate kinase (EC 2.7.1.40)	-2.00	342.01	170.62	Neut0979
Quinolinate synthetase (EC 2.5.1.72)	-3.69	786.89	212.87	Neut2299
Riboflavin synthase eubacterial/eukaryotic (EC 2.5.1.9)	-2.35	101.69	43.15	Neut2517
Ribonuclease PH (EC 2.7.7.56)	2.43	65.29	159.19	Neut0308
Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)	2.95	136.409	402.91	Neut1140
Serine hydroxymethyltransferase (EC 2.1.2.1)	-2.66	345.2	129.47	Neut1552
UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7)	-2.25	126.57	56.21	Neut1556
UDP-N-acetylglucosamine--N-acetylmuramyl-pyrophosphoryl-undecaprenol (EC 2.4.1.227)	2.99	20.04	60.12	Neut0246
Oxidoreductase				
2-hydroxy-3-oxopropionate reductase (EC 1.1.1.60)	-3.14	272.48	86.67	Neut1881
Copper-containing nitrite reductase (EC 1.7.2.1)	4.02	554.19	2230.44	Neut1403
D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	2.54	56.96	144.99	Neut1571
Glycine dehydrogenase (EC 1.4.4.2)	2.44	68.57	167.82	Neut1952
Glycine dehydrogenase (EC 1.4.4.2)	3.04	30.76	93.62	Neut1953
Histidinol dehydrogenase (EC 1.1.1.23)	2.09	76.14	159.44	Neut1206
Hydroxylamine oxidoreductase precursor (EC 1.7.3.4)	3.24	172.85	561.38	Neut1672
Hydroxylamine oxidoreductase precursor (EC 1.7.3.4)	-2.22	492.29	221.57	Neut1793
Multicopper oxidase	7.01	440.67	3093.03	Neut1406
NAD-dependent epimerase/dehydratase family protein	3.55	21.41	76.2	Neut0922
NADH dehydrogenase, subunit 5	7.29	38.55	281.14	N/A
NADH ubiquinone oxidoreductase chain A (EC 1.6.5.3)	2.74	79.71	219.08	N/A
NADH-ubiquinone oxidoreductase chain C (EC 1.6.5.3)	3.18	201.99	644.23	N/A
NADH-ubiquinone oxidoreductase chain D (EC 1.6.5.3)	2.55	119.99	306.32	N/A
NADH-ubiquinone oxidoreductase chain E (EC 1.6.5.3)	3.04	197.37	601.62	N/A
NADH-ubiquinone oxidoreductase chain F (EC 1.6.5.3)	3.45	102.28	353.78	N/A
NADH-ubiquinone oxidoreductase chain G (EC 1.6.5.3)	5.1	134.44	686.49	N/A
NADH-ubiquinone oxidoreductase chain H (EC 1.6.5.3)	3.38	80.36	271.8	N/A
NADH-ubiquinone oxidoreductase chain I (EC 1.6.5.3)	4.97	58.87	292.74	N/A
NADH-ubiquinone oxidoreductase chain J (EC 1.6.5.3)	5.67	57.59	326.68	N/A
NADH-ubiquinone oxidoreductase chain K (EC 1.6.5.3)	4.86	40.26	195.96	N/A
NADH-ubiquinone oxidoreductase chain L (EC 1.6.5.3)	4.73	69.46	329.21	N/A
NADH-ubiquinone oxidoreductase chain M (EC 1.6.5.3)	3.1	74.54	231.61	N/A
NADH-ubiquinone oxidoreductase chain N (EC 1.6.5.3)	2.44	82.675	201.99	N/A
Peptide methionine sulfoxide reductase MsrA (EC 1.8.4.11)	-5.81	270.86	46.57	Neut0419
PqqC-like protein	3.14	626.71	1970.02	Neut1553
UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158)	5.5	10.13	55.74	Neut0244

Ligases				
Acetyl-coenzyme A carboxyl transferase beta chain (EC 6.4.1.2)	2.32	82.34	191.12	Neut1154
Adenylosuccinate synthetase (EC 6.3.4.4)	2.15	46.48	100.16	Neut0967
Asparagine synthetase (EC 6.3.5.4)	2.42	56.34	136.72	Neut0901
Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	2.46	47.75	117.63	Neut0455
Carbamoyl-phosphate synthase small chain (EC 6.3.5.5)	2.69	64.05	172.85	Neut0454
Cyanophycin synthase (EC 6.3.2.29)	-5.55	227.56	40.96	Neut1401
Cyanophycin synthase (EC 6.3.2.30)	-2.61	119.85	45.75	Neut1402
Dihydrofolate synthase (EC 6.3.2.12)	2.3	95.66	220.22	Neut1155
Glutathione synthetase (EC 6.3.2.3)	-3.1	4232.63	1362.46	Neut0953
Histidyl-tRNA synthetase (EC 6.1.1.21)	3.01	61.78	186.38	Neut2167
Pantoate--beta-alanine ligase (EC 6.3.2.1)	3.14	17.55	55.22	Neut2292
UDP-N-acetylmuramate--alanine ligase (EC 6.3.2.8)	3	25.18	75.71	Neut0245
UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10)	4.11	12.34	50.76	Neut0250
Urea carboxylase (EC 6.3.4.6)	-2.7	72.55	26.83	Neut2475
Lyases				
3,4-dihydroxy-2-butanone 4-phosphate synthase (EC 4.1.99.12)	-2.77	272.07	97.96	Neut2516
3-isopropylmalate dehydratase large subunit (EC 4.2.1.33)	-2	358.63	178.93	Neut1144
Chorismate mutase I (EC 5.4.99.5)	3.01	20.4	61.63	Neut1570
GDP-mannose 4,6-dehydratase (EC 4.2.1.47)	3.67	19.2	70.59	Neut0156
Orotidine 5'-phosphate decarboxylase (EC 4.1.1.23)	4.45	38.5	171.64	Neut0402
Phosphatidylserine decarboxylase (EC 4.1.1.65)	2.08	147.78	307.94	Neut1259
Radical SAM domain protein	-2.07	171.64	82.62	Neut0292
Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	2.5	877.89	2197.97	Neut0804
Ribulose bisphosphate carboxylase small chain (EC 4.1.1.39)	2.11	270.54	571.3	Neut0805
Xylulose-5-phosphate phosphoketolase (EC 4.1.2.9)	-2	169.61	84.67	Neut2087
Isomerase				
FIG010773: NAD-dependent epimerase/dehydratase	3.37	100.54	338.87	N/A
FKBP-type peptidyl-prolyl cis-trans isomerase	2.17	131.71	286.89	Neut0192
Proteas				
ATP-dependent Clp protease adaptor protein ClpS	-3.17	264.708	83.31	Neut2047
ATP-dependent Clp protease ATP-binding subunit ClpA	-2.7	344.61	127.35	Neut2048
ATP-dependent Clp protease ATP-binding subunit ClpX	-3.16	1133.95	358.63	Neut0202
ATP-dependent protease La (EC 3.4.21.53) Type 1	-2.11	374.97	177.06	Neut0201
ATP-dependent protease La (EC 3.4.21.53) Type 2	-2.56	146.05	57.01	Neut0970

N/A corresponds to gene ID not being available on the K.E.G.G. website.

3.3.3 Cellular processes

In this category, there was a total of 32 regulated genes grouped into sub-categories that consist of DNA binding and DNA directed proteins, translation, transcription, cell division, DNA repair, and mitochondrial biogensesis (Table 7). In the DNA binding and DNA directed category, no real pattern in expression could be observed, however more than half of the genes were down-regulated in stationary phase. All of the genes under translation were up-regulated while most of the genes under transcription were down-regulated (Table 7). Moreover, most of the genes involved in cell division were down-regulated including the ones coding for BolA, FtsH and FtsJ while the rest were up-regulated in stationary phase.

Two genes coding for CspD cold shock protein and GroES heat shock protein were both up-regulated in stationary phase (Table 7). The gene coding for CspD has a nearly 4-fold increase in its expression going from ~2636 RPKM in mid-log to ~10495 RPKM in early stationary phase and the gene coding for GroES has a ~15-fold increase going from only 195 RPKM in mid-log to ~3048 RPKM in early stationary phase. Most of the genes involved in mitochondrial biogenesis were down-regulated in stationary phase. Lastly, there were only two regulated genes grouped under the DNA repair subcategory (Table 7).

Table 7: List of all the genes involved in cellular processes category in *N. eutropha.* Only the genes with ≥ 2-fold change are included to be significant. Fold change is normalized and the RPKM values are average of all replicates (n = 3 for Mid-Log, n = 2 for Late-Log). Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4). Groupings based on K.E.G.G..

Cellular processes in *N. eutropha*	Fold chang	ML-Means	LL-Means	Gene

	e			
DNA-binding and DNA-directed polypeptides				
DNA-binding protein	-7.7	27.87	3.61	N/A
DNA-binding protein Fis	4.81	18.48	88.93	N/A
DNA-directed RNA polymerase alpha subunit (EC 2.7.7.6)	4.2	361.04	1519.1	N/A
DNA-directed RNA polymerase alpha subunit (EC 2.7.7.6)	-5.76	1519.13	263.33	N/A
RNA polymerase sigma factor RpoH	-4.25	620.47	145.79	N/A
Cell divison				
Cell division protein BolA	-7.01	79.33	11.3	Neut1391
Cell division protein FtsH (EC 3.4.24.-)	-4.89	2562.26	523.09	N/A
Cell division protein FtsJ	-4.39	288.63	65.72	N/A
DNA repair				
Primosomal replication protein N	4.03	204.44	825.16	Neut0117
Mitochondrial biogenesis				
Chaperone protein DnaK	-5.79	1439.49	248.39	Neut0412
Heat shock protein 60 family co-chaperone GroES	15.55	195.96	3048.45	Neut0207
Heat shock protein GrpE	-6.46	462.42	71.57	Neut0411
Primosomal replication protein N	4.03	204.44	825.16	Neut0117

N/A corresponds to gene ID not being available on the K.E.G.G. website.

3.3.4 Environmental Information Processing

There was a total of nine regulated genes in this category grouped under stress response, signal transduction and membrane transport and cellular processes (Table 8). Genes involved in the latter two sub-categories were all up-regulated during stationary phase. Interesting points are the genes involved in stress response. All of the genes coding for putative stress proteins were down-regulated, with the exception of the gene coding for NnrS. It is remarkable to see a ~20-fold decrease in expression levels when we look at the gene coding for general stress protein going from ~13666 RPKM in mid-log to only ~657 RPKM in early stationary phase. This is in contrast to the up-regulation of the stress proteins CspD and GroES during stationary phase as mentioned above.

Table 8: List of all the genes involved in environmental information processing category in *N. eutropha*. Only the genes with ≥ 2-fold change are included to be significant. Fold change is normalized and the RPKM values are average of all replicates (n = 3 for Mid-Log, n = 2 for Late-Log). Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4). Groupings based on K.E.G.G..

Environmental Information processing in *N. eutropha*	Fold change	ML-Means	LL-Means	Gene
Stress response				
General stress protein	-20.78	13666.64	657.59	N/A
NnrS protein involved in response to NO_2	3.66	59.73	219.03	Neut0425
Universal stress protein	-2.35	166.33	70.75	N/A
Signal transduction				
Chemotaxis protein methyltransferase CheR (EC 2.1.1.80)	3.41	28.39	97.02	Neut1171
Chemotaxis response regulator protein-glutamate methylesterase CheB (EC 3.1.1.61)	5.63	22.63	127.51	Neut1173
Methyl-accepting chemotaxis protein I (serine chemoreceptor protein)_1	5.15	19.95	102.84	Neut1161
Positive regulator of CheA protein activity (CheW)	4.12	19.55	80.67	Neut1168
Membrane transport and cellular processes				
Chemotaxis protein methyltransferase CheR (EC 2.1.1.80)	3.41	28.39	97.02	Neut1171
Chemotaxis response regulator protein-glutamate methylesterase CheB (EC 3.1.1.61)	5.63	22.63	127.51	Neut1173

N/A corresponds to gene ID not being available on the K.E.G.G. website.

3.3.5 Bacterial Motility

Within this category, there is only one sub-category that encompassed all of the regulated genes, which is the flagellar system (Table 9). Out of the 15 genes responsible for bacterial motility, only one gene coding for flagellar motor rotation protein (MotB) was down-regulated. The rest of the genes were up-regulated and some showed a huge fold difference when expression levels were compared between mid-log and early stationary phase. For instance, genes coding for flagellar transcriptional activator FlhC and FlhD were both highly up-regulated in early stationary phase by ~41-fold and ~60-fold respectively.

Table 9: Up-regulated motility genes in *N. eutropha*. Table contains all of the motility genes, being up-regulated over 2-fold in early stationary phase compared to mid logarithmic growth in *N. europaea*. Fold change is normalized and the RPKM values are average of all replicates (n = 3 for Mid-Log, n = 2 for Late-Log). Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4). Groupings based on K.E.G.G..

Protein name	Gene ID	Fold Change	M.L.-RPKM	L.L.-RPKM
Flagellar basal-body rod modification protein FlgD	Neut0337	6.62	10.09	66.91
Flagellar basal-body rod protein FlgC	Neut0336	10.87	2.7	29.43
Flagellar biosynthesis protein FliL	Neut2055	6.33	6.94	43.99
Flagellar hook protein FlgE		3.49	30.39	106.14
Flagellar hook-basal body complex protein FliE	Neut0748	3.05	21.73	66.31
Flagellar M-ring protein FliF	Neut0745	5.54	8.75	48.61
Flagellar motor rotation protein MotB	Neut0756	-3.15	83.15	26.39
Flagellar motor switch protein FliG	Neut0744	2.28	45.08	102.8
Flagellar motor switch protein FliN	Neut2057	8.45	6.612	55.92
Flagellar sensor histidine kinase FleS	Neut0746	13.26	8.03	106.65
Flagellar transcriptional activator FlhC	Neut0730	41.72	27.17	1,133.95
Flagellar transcriptional activator FlhD	Neut0729	59.64	42.95	2,562.25

3.3.6 Remaining categories

Only 12 regulated genes were grouped under three separate categories: Membrane protein, Human diseases and Iron-binding proteins (Table 10). Membrane proteins included efflux pumps, transporters, and Type I secretion proteins. Due to the fact that each of these sub-categories had a very small number of genes involved, a clear pattern of expression could not be determined. In contrast, all of the six genes grouped under human disease that were involved in drug resistance were down-regulated in stationary phase (Table 10). Lastly,

only a single gene coding for bacterioferritin was grouped under iron-binding proteins going from ~439 RPKM in mid-log to ~1046 RPKM in early stationary phase.

Table 10: Remaining categories in *N. eutropha*. Table contains three remaining categories including membrane protein, Human diseases and Iron-binding proteins. Only the genes with ≥ 2-fold change are included to be significant. Fold change is normalized and the RPKM values are average of all replicates (n = 3 for Mid-Log, n = 2 for Late-Log). Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4). Groupings based on K.E.G.G..

Membrane protein	Fold change	M.L.-RPKM	L.L.-RPKM	Gene ID
Transporter				
Membrane protein involved in the export of O-antigen, teichoic acid lipoteichoic acids	12.71	10.68	135.93	N/A
Human diseases				
Drug resistance				
Arsenic resistance protein ArsH (1)	-7.98	46.57	5.83	Neut0668
Arsenic resistance protein ArsH (2)	-4.25	47.8	11.22	Neut2263
Arsenical resistance operon repressor (1)	-8.82	60.55	6.86	N/A
Arsenical resistance operon repressor (2)	-8.44	54.59	6.46	N/A
Iron-binding proteins				
Ferritin				
bacterioferritin possibly associated with carboxysome	2.38	439.26	1046.89	Neut1195

N/A corresponds to gene ID not being available on the K.E.G.G. website.

3.4 Gene expression comparison between N. europaea and N. eutropha

The overview of genome characteristics and our transcriptome analysis of *N. europaea* and *N. eutropha* are shown in Table 11. When comparing the genome analysis between the two bacteria, we found that there were more than twice the number of genes in *N. eutropha* that were significantly up- or down-regulated during early stationary phase compared to *N. europaea* (455 genes in *N. eutropha* compared to 212 genes in *N. europaea*). Comparing the genome

wide transcription levels, while more than half of the genes in *N. europaea* had fewer reads in early stationary phase compared to mid-log, more than half of the genes in *N. eutropha* had significantly higher RPKM values in stationary compared to mid-log.

Table 11: Overview of transcriptome analysis and genome properties. This table contains general information regarding genome-wide expression regulation in *N. europaea* and *N. eutropha* from our analysis. It also contains general characteristics of each genome including the number of genes involved in certain protein category based on COGS functional groups (http://genome.ornl.gov/microbial/neur/final/fun.html).

Genome properties	*N. europaea*	*N. eutropha*
Number of chromosomes	1	1
Number of basepairs in chromosome	2,812,094	2,661,057
GC content (%)	50.7	48.49
Coding density (%)	88.4	85.6
Number of predicted protein coding genes	2,460	2,443
Number of predicted protein with putative function	1.863	2,001
Number of unknown proteins unique to each bacterium	312	442
Total average counted fragments (from M.L. and L.L.)	81,546,248	102,398,566
% of total map to genes	43.29	42.09
% of total map to intergenic	56.7	57.91
Total number of genes expressed in any conditions	2872	2892
Number of genes in protein category (Based on COGs)		
Energy production and conversion	178	187
Transport	187	152
DNA replication	131	149
Transcription	123	102
Translation	173	168
Signal transduction mechanism	115	102

Amino acid metabolism	208	206
Carbohydrate metabolism	117	131
Nucleotide metabolism	61	61
Coenzyme metabolism	171	152
Lipid metabolism	57	59
Secondary metabolites	82	94
Cellular processes	743	720
General functions	146	284
Cell motility	92	94

3.4.1 Metabolism

In total, there were 145 regulated genes in *N. eutropha* involved in metabolism, which was more than three times of the number of regulated genes in *N. europaea* (only 44 genes) (Fig. 7). In both bacteria, the sub-group with the most number of regulated genes was energy metabolism. However, when we take a look at each sub-category, we can see that the division of genes between the sub-categories does not follow the same pattern between the two bacteria (Fig. 7). In any case, there were only two homologous genes in metabolism that between the two bacteria that had higher than 2-fold change in their expression, though the patterns were opposite (Table 12).

The gene coding for rubilose bisphosphate carboxylase (large subunit) was down-regulated in *N. europaea* while it was up-regulated in *N. eutropha* (Table 13). The same held true for the gene coding for UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6- diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10). Lastly in *N. eutropha*, there were arrays of genes involved in

trepnoids and polyketides metabolism. This sub-category was not regulated in *N. europaea*'s trancriptome data while in *N. europaea*, genes involved in oxocarboxylic acid metabolism were not regulated in *N. eutropha*'s transcriptome (Fig. 7).

Table 12: Regulation of principle homologous genes in *N. europaea* and *N. eutropha*. This table compares some of the principle and well-studied homologous genes between *N. europaea* and *N. eutropha*. The asterisk (*) next to the genes means they do not show a statistically significant difference in their fold expression and are present only to for the sake of comparison between the two species. M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from all replicates using CLC Genomics Workbench (version 7.0.4).

Nitrosomonas eutropha	**Gene ID**	**Fold change**	**M.L.(RPKM)**	**L.L.(RPKM)**
Ribulose bisphosphate carboxylase large chain	Neut0804	2.5	877.89	2,197.97
Ribulose bisphosphate carboxylase small chain	Neut0805	2.11	270.54	571.30
Carboxysome shell protein	Neut0807	3.39	825.16	2,808.10
**Rubisco activation protein CbbO	N/A	5.83	6.51	38.03
Copper-containing nitrite reductase	Neut1403	4.02	554.19	2,230.44
*Ammonia monooxygenase A-subunit (1)	Neut2077	(-1.00)	(18,593.19)	(18,593.19)
*Ammonia monooxygenase A-subunit (2)	Neut2318	(-1.00)	(18,614.67)	(18,614.67)
*Ammonia monooxygenase B-subunit (1)	Neut2317	(-1.00)	(14,733.83)	(14,733.83)
*Ammonia monooxygenase B-subunit (2)	Neut2076	(1.41)	(28,803.74)	(40,686.53)
Ammonia monooxygenase C-subunit (1)	Neut2078	-6.82	7,289.75	1,067.34
*Ammonia monooxygenase C-subunit (2)	Neut2319	(-1.06)	(40,686.53)	(38,241.86)
*Ammonia monooxygenase C-subunit (3)	Neut1529	(-1.33)	(38,241.86)	(28,803.74)
Nitrosomonas europaea	**Gene ID**	**Fold change**	**M.L.(RPKM)**	**L.L.(RPKM)**
Ribulose bisphosphate carboxylase large chain	NE1921	-2.2	4,002.41	1,814.84
Ribulose bisphosphate carboxylase small chain	NE1920	-1.92	6,253.9	3,256.49
**Rubisco activation protein CbbO	N/A	(-1.55)	(123.17)	(49.53)
*Copper-containing nitrite reductase	NE0924	(1.03)	(2,904.75)	(2,816.22)
Ammonia monooxygenase A-subunit (1)	NE2062	2.07	5,370.13	11,130.86
Ammonia monooxygenase A-subunit (2)	NE2063	2.22	5,408.39	12,013.81
*Ammonia monooxygenase B-subunit (1)	NE2062	(-1.17)	(17,625.90)	(15,116.99)
*Ammonia monooxygenase B-subunit (2)	NE0943	(-1.00)	(16,421.72)	(16,421.72)
*Ammonia monooxygenase C-subunit (1)	NE2064	(-1.00)	(46,581.43)	(46,581.43)
*Ammonia monooxygenase C-subunit (2)	NE0945	(-1.04)	(2,645.67)	(2,550.69)
*Ammonia monooxygenase C-subunit (3)	NE1411	(-1.00)	(26,456.53)	(26,456.53)

N/A corresponds to gene ID not being available on the K.E.G.G. website.

3.4.2 Enzymes

The differences between the two transcriptomes continues with the enzyme category. While there was a total of 162 regulated genes in *N. eutropha*, less than one third of that number of genes in the enzyme category were regulated in *N. europaea* (only 53 genes) (Fig. 7). While genes coding for oxidoreductases made the biggest sub-category in *N. europaea*, in *N. eutropha* transferases held the largest number of regulated genes. There was one new gene in this category that is homologous between the two bacteria and it codes for cyanophacin synthase (Table 13). This gene was highly down-regulated in both bacteria during stationary phase although it was present in different number of RPKM between the two growth phases of the two bacteria. Lastly, there were a few genes coding for hydrolases and proteases in the *N. eutropha* transriptome and these sub-categories were not regulated in the *N. europaea* trancriptome.

3.4.3 Cellular processes

A total of 49 genes involved in cellular processes were regulated in *N. eutropha* compared to 23 genes in *N. europaea* (Fig. 7). While there was a wide array of genes involved in DNA-binding and DNA-directed polypeptides, such a sub-category was missing in the regulated genes of *N. europaea*. However in both bacteria, all genes involved in translation were up-regulated in stationary phase while most genes in transcription were down-regulated. There were two new genes in this category that are homologous between the two bacteria and they code for FtsL (a cell division protein) and DnaK (a chaperone protein) (Table 13). They followed theopposite pattern of expression in these two bacteria. While

the one coding for FtsL was up-regulated in *N. eutropha*, it was down-regulated in *N. europaea* and vice versa for the gene coding for DnaK.

Table 13: List of all homologous genes in *N. europaea* and *N. eutropha*. This table compares all the homologous genes between *N. europaea* and *N. eutropha* under each category. Only the genes with ≥ 2-fold change are included to be significant. Fold change is normalized and the RPKM values are average of all replicates. Gene ID based on ORNL website (http://genome.ornl.gov/). M.L. and L.L. correspond to mid-log and late-log respectively. RPKM (Reads Per Kilobase Per Million) was calculated from reads mapped to each gene on CLC Genomics Workbench (version 7.0.4). Groupings based on K.E.G.G..

Metabolism	**Fold Change**	**ML-RPKM**	**LL-RPKM**	**Gene ID**
N. europaea				
Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	-2.2	4002.41	1814.84	NE1921
UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10)	-2.16	119.19	55.11	NE0987
N. eutropha				
Ribulose bisphosphate carboxylase large chain (EC 4.1.1.39)	2.5	877.89	2197.97	Neut0804
UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10)	4.11	12.34	50.76	Neut0250
Enzymes	**Fold Change**	**ML-RPKM**	**LL-RPKM**	**Gene ID**
N. europaea				
Cyanophycin synthase (EC 6.3.2.29)	-8.99	1557.63	173.07	NE0923
N. eutropha				
Cyanophycin synthase (EC 6.3.2.29)	-5.55	227.56	40.96	Neut1401
Cellular processes	**Fold Change**	**ML-RPKM**	**LL-RPKM**	**Gene ID**
N. europaea				
Cell division protein FtsL	-4.35	183.3	42.16	NE0984
Chaperone protein DnaK	2.03	204.6	417.18	NE1949
N. europaea				
Cell division protein FtsL	3.37	44.85	151.51	N/A
Chaperone protein DnaK	-5.79	1439.49	248.39	Neut0412

N/A corresponds to gene ID not being available on the K.E.G.G. website.

3.4.4 Environmental information processing

There were nine regulated genes involved in stress, signal transduction and membrane transport and cellular processes in *N. eutropha*. In comparison, only three genes were grouped under this category in *N. europaea*, all of which

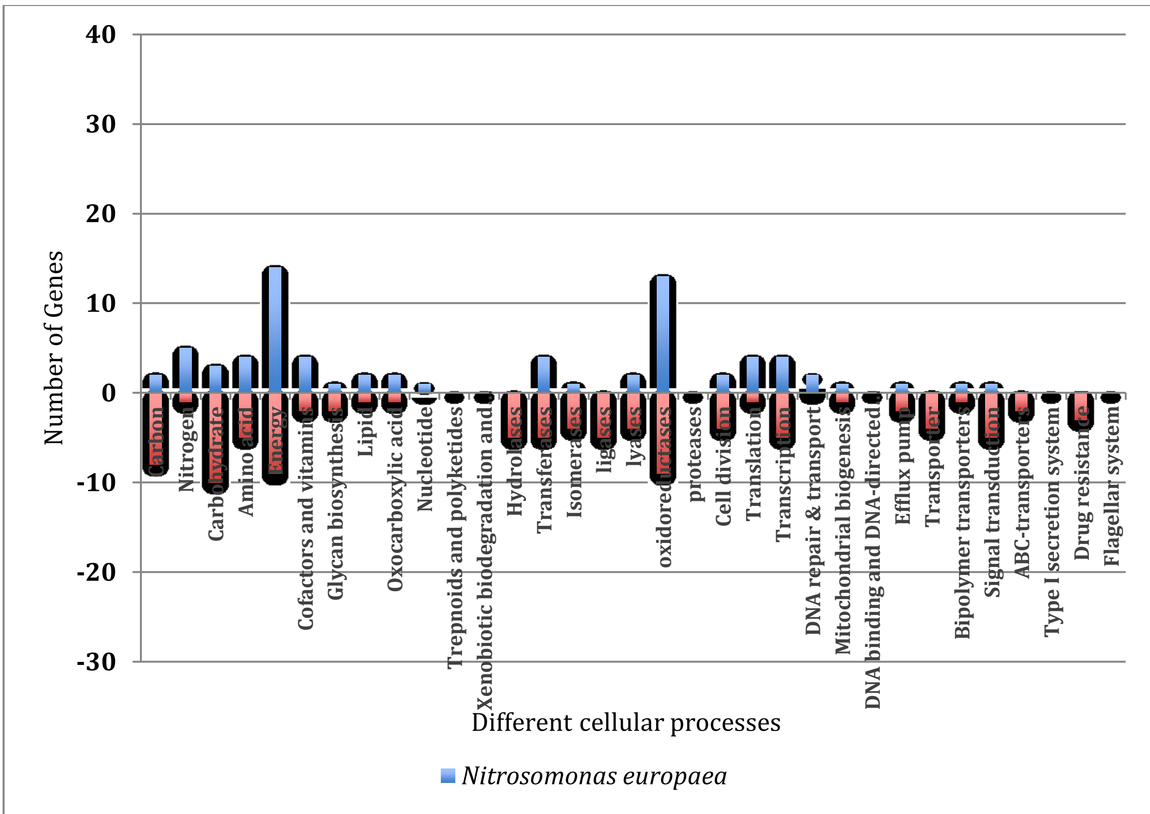
that were involved in signal transduction (Fig. 7). While there were no homologous genes within this category, the biggest difference was in the array of genes under stress in *N. eutropha*. The only gene in *N. europaea* responding to stress coded for DnaK and it was involved in cell division. While this gene was highly up-regulated, genes coding for stress proteins in *N. eutropha* were down-regulated some by a massive 20-fold (in the case of General stress protein). However, the gene in *N. eutropha* coding for NnrS protein involved in response to NO_2 is up-regulated as well as genes coding for cold-shock CspD and general co-chaperone GroES proteins. Such a regulation in the *N. europaea* transcriptome was completely missing. Lastly, while all of the genes in *N. eutropha* involved in signal transduction were up-regulated in stationary phase, such a pattern could not be deduced from *N. europaea*'s transcriptome.

3.4.5 Bacterial motility

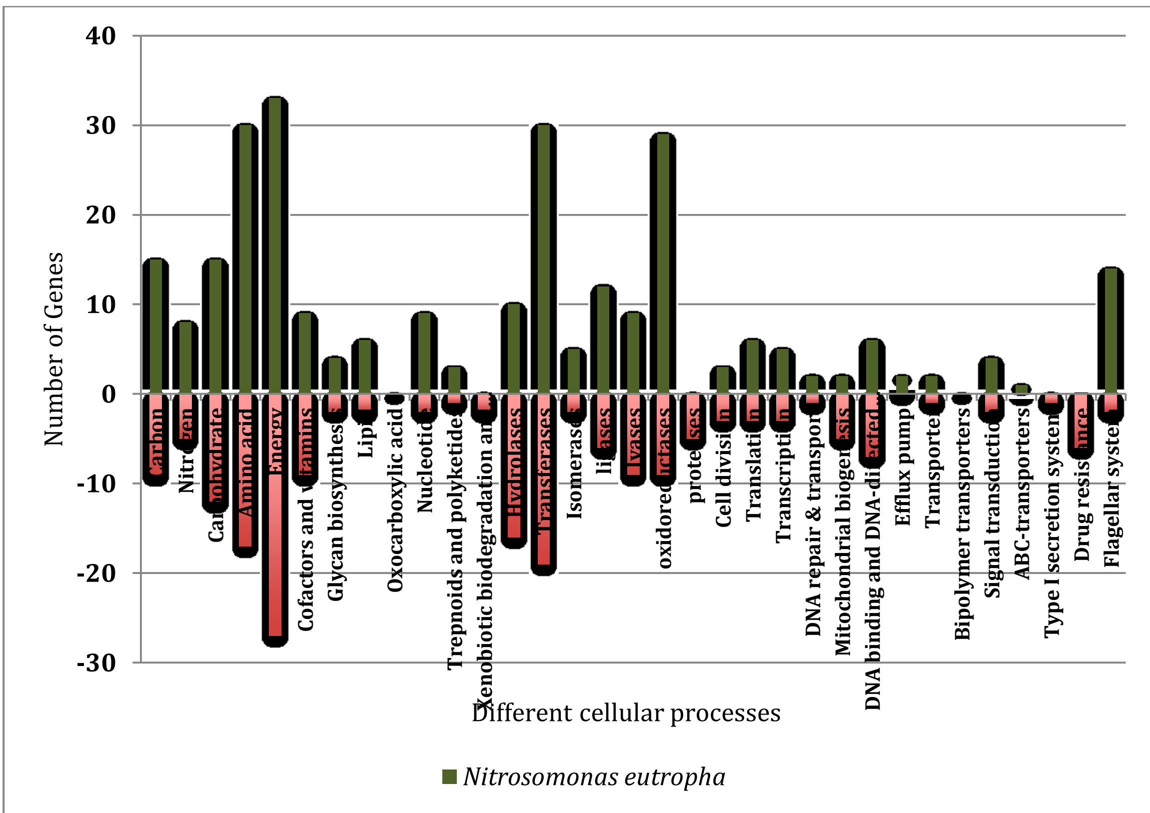
This is a category where one of the biggest differences between the two transcriptome data exists. Looking at *N. eutropha*'s data, there was a total of 15 regulated genes in this category with all being involved in the flagellar system (Fig. 7). With the exception of one gene, all were highly up-regulated in stationary phase, including a whopping 60-fold increase in the gene transcripts coding for FlhD. However, when we look at *N. europaea*'s transcriptome, such a regulation in motility was completely absent as this bacteria reaches early stationary phase. This is the first time where a whole category based on the KEGG website was only present in one of the transcriptome data sets.

3.4.6 Membrane proteins, Iron-binding proteins and Human diseases

The rest of the regulated genes fell within these three categories (Fig. 7). Due to the small number of genes in each category, they were not compared or considered significant in this study. Finally, some of the principal genes involved in the nitrogen cycle that are well characterized and well studied were not regulated in either transciptome. This included the *norCBQD* and the norSY-senC-orf1 operons coding for nitric oxide reductases. However, copper-containing nitrite reductase was highly up-regulated in *N. eutropha* as the cells entered early stationary phase. The nirK gene was not regulated in the *N. europaea* transcriptome, although the RPKM values were high for this gene in both mid-log and early stationary phase indicating that it was highly expressed in both conditions.



40
30
20
10
0
-10
-20
-30
Number of Genes
Nitrogen
Cofactors and vitamins
Glycan biosynthesis
Oxocarboxylic acid
Nucleotide
Trepnoids and polyketides
Xenobiotic biodegradation and
Hydrolases
Transferases
Isomerases
ligases
lyases
proteases
Cell division
Translation
Transcription
DNA repair & transport
Mitochondrial biogenesis
DNA binding and DNA-directed
Efflux pump
Transporter
Bipolymer transporters
Signal transduction
ABC-transporters
Type I secretion system
Drug resistance
Flagellar system
Different cellular processes
Nitrosomonas europaea



40
30
20
10
0
-10
-20
-30
Number of Genes
Glycan biosynthesis
Oxocarboxylic acid
Trepnoids and polyketides
Xenobiotic biodegradation an
Isomerase
Cell divisi
Translati
Transcripti
DNA repair & transpor
Efflux pump
Transporte
Bipolymer transporters
Signal transductio
ABC-transporters
Type I secretion system
Different cellular processes
Nitrosomonas eutropha

Figure 7: Total number of genes regulated in *N. europaea* and *N. eutropha*. This is a general representation of total number of statistically significant genes (based on Bonferroni corrected statistics under Baggerly's test) in *N. europaea* (blue bars) and *N. eutropha* (green bars). The positive numbers represent total number of up-regulated genes while the negative numbers (red bars in each figure) represents total number of down-regulated genes in early stationary, compared to mid logarithmic growth phase within each category. The x-axis represents all the different cellular processes that the genes are involved in based on K.E.G.G. (http://www.genome.jp/kegg/).

Chapter 4
Discussion

4.1 Similarities and differences in Growth

As previously discussed, *N. europaea* is a Gram-negative obligate chemolithoautotroph that derives all of its energy by oxidizing ammonia to nitrite (Whittaker *et al.*, 2000). This bacterium is found in places like sewage, freshwater, soil and other places where the environment contains high levels of nitrogen compounds (Vlaeminck *et al.*, 2011). Since it needs to consume a fairly large amount of ammonia to have enough energy to divide, the growth rate is not as robust and takes several days (Utaker *et al.*, 1995). This is apparent in this study when we take a look at its growth curve (Fig. 3). As mentioned in the results, it takes roughly 72h post inoculation for this bacterium to reach early stationary phase due to limiting factors in batch culture (Fig. 3). *N. europaea* shows a doubling time of $7.7h^{-1}$ during mid-log, which is fairly rapid for an ammonia-oxidizer. This number is an overall average of biological and experimental replicates (n = 6). This bacterium may achieve this fast growth rate in batch culture as all nutrients necessary for growth were available, environmental stresses such as temperature and pH variations were reduced, and competition for substrates with other organism were nonexistant since it is in pure culture.

During logarithmic growth, there are two key enzymes that are necessary for obtaining energy in order for the cells to divide. Ammonia monooxygenase (AMO) and hydroxylamine dehydrogenase (HAO) are involved in the first part of what is now known as the rate-limiting step of ammonia oxidation, which is the

oxidation of ammonia to nitrite. AMO is a trans-membrane copper protein that catalyzes the oxidation of ammonium to hydroxylamine, during which it uses two electrons directly from the quinone pool (Whittaker *et al.*, 2000). Although the exact stoichiometry varies in different AOB species (usually 1.5 molecules of oxygen is needed to oxidize 1 molecule of ammonia), AMO requires one oxygen molecule for this reaction (Arp *et al.*, 2012; Beaumont *et al.*, 2002; Berube *et al.*, 2007). Once this step is completed, HAO helps convert hydroxylamine to nitrite. This trimeric multiheme c-type enzyme then produces four electrons (Arciero and Hooper, 1993; Arciero *et al.*, 1991a). This reaction occurs in the periplasm and the four electrons are channeled through cytochrome c_{554} to a membrane-bound cytochrome c_{M552} (Arciero *et al.*, 1991a). Out of the four electrons, two are used by AMO again for further oxidation of ammonia while the other two are used to generate a proton motive force and to reduce nicotinamide adenine dinucleotide phosphate (NAD(P)) through reverse electron transport (Hommes *et al.*, 1996).

Therefore, as our culture reaches stationary phase, growth rate slows down dramatically with the culture staying at the concentration of 10^8 cells/mL for the next several days. There could be many reasons behind this slow down. One particular reason could be due to oxygen availability. These cultures are growing in wheaton bottles that have septum caps with a finite concentration of oxygen and nutrients available. Therefore, as both nutrients and oxygen are consumed for energy production and growth, less and less become available which can cause the bacteria to enter stationary phase. Nutrient limitation was disregarded as a possibility since the experiments done with 50 mM ammonia followed the

same growth pattern (see Appendix, Fig. A-1). In those experiments, cells were also only capable of producing around 8mM nitrite before they reached stationary phase. Although pH and temperature were kept constant, there were slight variations between each physiological measurement. These along with possible oxygen limitations could be responsible for cells entering stationary phase.

Similar to *N. europaea*, *N. eutropha* is a Gram-negative bacterium that belongs to the Betaprotebacteria (Stein *et al.*, 2007). It is an aerobic chemolithoautotroph as well and just like *N. europaea*, it generates energy by oxidizing ammonia to nitrite (Stein *et al.*, 2007). As mentioned before, it is mostly found in eutrophic environments such as municipal and industrial sewage disposal systems where not only it has high tolerance for elevated concentrations of ammonia, it thrives on it. In this study however, this bacterium was grown in the same environmental conditions as *N. europaea* with the media containing only 10mM ammonia (Fig. 3). One would expect that on top of the stresses mentioned above, lower relative ammonia concentrations could also be a limiting factor for their growth. Ammonia limitation was further examined when *N. eutropha* was grown in 50 mM where it reached stationary phase after almost the same period with similar final nitrite concentration of around 8mM and cell titre at 10^8 cells/mL (see Appendix, Fig. A-1). Therefore, other possibilities than ammonia limitation (O_2, pH, temperature, carbon, etc.) are likely causing this bacterium to enter stationary phase.

Based on the growth curve obtained from our replicates (n = 6) in this study, *N. eutropha* exhibited a similar maximum doubling time at mid-log,

doubling every 7.9h^{-1} (Fig. 3). This is really interesting as it demonstrates *N. eutropha*'s wide adaptability and gives us a possible insight to its fitness in nature. *N. eutropha* also reached early stationary phase around the same time as *N. europaea* at 72h therefore, limiting factors other than ammonia limitation likely caused both organisms to enter stationary phase simultaneously.

4.2 Similarities and differences in nitrite production and O_2 consumption

Since the growth rates between the two bacteria were highly similar, one would expect nitrite production to be highly similar as well. Although this may seem to be the case at first glance, there were some notable differences (Fig. 4). Both bacteria produced nitrite as early as 24h after inoculation. However, in accordance to the growth rate data, *N. eutropha* showed faster nitrite production with nitrite concentrations doubling every 6.05h^{-1} during mid-log compared to 7.4h^{-1} in *N. europaea* (Fig. 5). These are an average from six biological replicates.

At around 60h post inoculation, about half of the oxygen available in the headspace was consumed while about half of the total ammonia was oxidized to nitrite by both bacteria (Fig. 4). Past 72h where cells were at early stationary phase, there was still an increase in nitrite production until 96h post inoculation. However, this increase was not experimentally significant in *N. eutropha* since the error bars at 72h and 96h overlap with one another. This, however, is not the case in *N. europaea* where a significant increase in nitrite production did occur from 72h to 96h producing an average of 6.5mM and 9.1mM nitrite, respectively.

This increase in nitrite production occurred with a slight increase in total cell number of *N. europaea* as well. This is interesting considering that at 72h, the amount of oxygen available in the headspace was at nearly 5%. So although growth rate had dramatically slowed down, *N. europaea* managed to still oxidize ammonia using the remaining ≤ 5% oxygen to produce slightly more nitrite while growing marginally as well (Fig. 4). Cells grown in 50mM nitrite concentration showed a similar pattern in nitrite production as well (See Appendix, Fig. A-4).

In contrast, we see a significant decrease in total nitrite concentration in the media from 96h to 120h in *N. eutropha* (Fig. 4). This occurred while there was a slight increase in the number of cells between the two time points as well (Fig. 3). There are several possibilities to describe this observation. One could be based on chemistry alone and how reactive NO_x intermediates are lost due to their reaction with other intermediates or molecules in the media (Beaumont *et al.*, 2002). The other could be due to nitrite being reduced enzymatically via nitrite reductase (Beaumont *et al.*, 2004). If we take a quick glance at *N. eutropha*'s transcriptome data, we can see a 4-fold increase in expression of the gene coding for the copper-containing nitrite reductase (NirK) in early stationary phase (Table 5). At the same time we can also see a 3.6-fold increase in expression of gene coding for *NnrS*, which is a haeme- and copper-containing membrane protein along with *NnrR* involved in regulating the expression of nitrite reducing genes such as nitrite and nitric oxide reductases. These observations along with the physiology data suggest that *N. eutropha* could be reducing nitrite as a hypoxic metabolism.

As mentioned earlier, NO and N_2O production via the reduction of NO_2^- observed in *N. europaea* cells suggests that they may be capable of denitrification as well. In 2004, Beaumont and collegues focused on the activity of NirK and its importance in protection against NO_2^- and possibly 'true' denitrification involvement during oxygen limited growth in this bacterium. They found that NsrR, was encoded in *N. europaea* as a novel NO_2^- sensing transcription repressor. NsrR plays a crucial role in NirK expression as a response to concentrations of NO_2^- and the pH (Van Spanning *et al.*, 1995). Howeever, in the present study, the lack of regulation of nirK, norB, or *norY* genes in *N. europaea* suggest that this pathway was not significantly induced during early stationary phase of *N. europaea.* As for *N. eutropha,* this bacterium has the ability to use NO_2^- as an alternate oxidant for ammonia oxidation under extreme hypoxia to anoxia (Schmidt and Bock, 2001; Kartal *et al.*, 2012). Hence, in the present experiment, *N. eutropha* could be employing denitrifying inventory to survive as oxygen level is going below 5%, while *N. europaea* is not.

Difference in transcriptional responses to relatively high nitrite concentrations between *N. europaea* and *N. eutropha* is an observation that was also shown in a study done by Cua and Stein in 2011. The authors found a lack of increased *nirK* mRNA levels in *N. eutropha* after being exposed to nitrite. Since *ncgABC-nirK* operon, promoter-proximal *NsrR*-binding motif, and *NsrR* repressor share high sequence indentity between *N. europaea* and *N. eutropha* (Cantera & Stein, 2007a; Stein *et al.*, 2007), it seems that expression of NirK enzyme may play diffierent roles in these two closely related spp. (Schmidt *et al.*,

2004; Beaumont *et al.*, 2005; Cantera & Stein, 2007b). Similar to the findings of this study, Cua and Stein (2011) also found that norB expression were unchanged in *N. europaea* and *N. eutropha* despite previous studies suggesting its induction during anaerobic metabolism (Beyers *et al.*, 2009) or growth in the presence of $NaNO_2$ (Yu & Chandran, 2010).

In another study, the authors illustrated that NO and NO_2 could be the driving forces of ammonia oxidation in *N. eutropha* during anoxia (Schmidt *et al.*, 2001). While more than 1ppm of of trace gases NO (NO_x) or NO_2 have been shown to have bactericidal effects on many chemoorganotrophic organisms (Mancinelli and McKay, 1983), *N. eutropha* was not detrimentally affected by NO_2 concentrations as high as 50 ppm (Schmidt *et al.*, 2001). However, NO concentrations above 10 ppm led to a decrease in anaerobic ammonia oxidation in this bacterium. They also found that NO and NO_2 could have important regulatory effect on aerobic nitrification (Schmidt *et al.*, 2001).

A previous study on *N. eutropha* also looked at effects of NO_2 on gene and protein expression under oxic and anoxic conditions (Kartal *et al.*, 2012). They found that regardless of the conditions (whether oxic or anoxic), exposure to NO_2 led to increase in proteins involved in energy conservation, including AmoCAB. Furthermore, exposure to NO_2 during anoxia resulted in increased proteins and transcrpts reflective of an energy-deprived state (Kartal *et al.*, 2012). While NorCB was not detected in the proteome, NorY (nitric oxide reductase) was expressed under both oxic-plus-NO_2 and anoxic-plus-NO_2 conditions (Kartal *et al.*, 2012).

4.3 Similarities and differences in N_2O production

Nitrous oxide is a usual product of denitrification where nitrate is reduced to nitrite, nitric oxide, nitrous oxide and eventually dinitrogen in a stepwise reduction process (Lipschultz *et al.*, 1981). These reactions include enzymes called nitrate reductase, nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase respectively (Lipschultz *et al.*, 1981; Choi *et al.* 2006). Some organisms do not have the full denitrification gene inventory and are therefore only capable of doing part of this reduction process (Wrage *et al.*, 2001; Prosser, 1989; Klotz and Stein, 2008). It has been argued for many years that there are two possible ways for N_2O to be produced in AOB. One is through the chemical auto-oxidation of hydroxylamine during nitrification and the other occurs during nitrifier denitrification as already talked about in the introduction (Kozlowski *et al.*, 2014). The argument here is that as oxygen becomes a limiting factor, more N_2O will be produced via the latter process. In these two bacteria, their gene inventory would enable the bacteria to to produce nitrous oxide via both of these pathways (Chain *et al.*, 2003; Stein *et al.*, 2007).

Perhaps the biggest difference and inconsistency between the two physiological data sets lies in the production of N_2O. In the case of *N. europaea*, the cells were able to produce detectable N_2O 48h post inoculation (Fig. 6). The production continued to increase until 96h, after which a slight decrease was observed at 120h. More nitrous oxide was produced as oxygen levels decreased. While none of the nitrifier denitrification gene inventories were up-regulated in *N. europaea*, they were highly expressed during both mid- and late-logarithmic growth phases (some are included in Table 12). In fact, they were present in

extremely high RPKM in both growth phases. One possible explanation for this observation is that from mid-log to early stationary phase, expression of genes coding for Nir or Nor was not regulated in *N. europaea*, although the proteins could be present and active (Wei *et al.*, 2006).

However in the case of *N. eutropha*, we see a much lower and much later production of N_2O (Fig. 6). No detectable N_2O was observed until 72h post inoculation and even then, the total concentrations produced were much lower than in *N. europaea* (around 4.5μM). This is very interesting considering that both of these organisms have the necessary genes to produce nitrous oxide and as already discussed, we saw in our *N. eutropha*'s transcriptome that *nirK* was highly up-regulated in early stationary phase compared to mid-log (Table 5). One possible explanation is that in *N. eutropha*, oxygen limitation along with the presence of one or more denitrification substrates (NO_3^-, NO_2^-, or NO) triggered the expression of denitrifying genes (Zumft *et al.*, 1997). This would explain why no N_2O was observed until 72h after inoculation when oxygen levels were nearly at 5% in the headspace. Interestingly, in experiments where *N. eutropha* was grown at 50 mM ammonia, the same exact pattern of N_2O production was observed (see Appendix, Fig A-2). Therefore, one possible explanation for the difference here is that while N_2O production can be occurring throughout exponential growth in *N. europaea*, certain conditions need to trigger such activity in *N. eutropha* to produce N_2O. This makes sense considering the involvement of NO and NO_2 in hypoxic and anoxic respiration by *N. eutropha*, but not in N. europaea, as discussed above.

The observations of differences between expressions of denitrifying genes are extremely important with regards to metagenomic studies. Using sequences of specific genes, scientists can produce a profile of bacterial diversity in an environmental sample. These studies show us a greater picture of the bacterial communities involved in certain environments that have been vastly missed by our cultivation-based methods (Hugenholz *et al.*, 1998). However, they also tend make the assumption that the species of bacteria that are present in high numbers are main drivers of the physiology observed in these environments. Specifically in the case of N_2O, there have been metagenomic studies where the most prevalent bacteria were considered the main drivers of N_2O production (Bartossek *et al.*, 2012; Orellana *et al.*, 2014). As the results from this study entails, this is not necessarily always the case as one strain (*N. europaea*) produced higher concentrations of N_2O, whereas the other strain (*N. eutropha*) did not. Further specific physiological and gene/protein expression studies from cultivated AOB species would give us a better understanding of each strain's involvement in a specific physiological process.

4.4 Genome-wide expression

Most of the differences between the two *Nitrosomonas* spp. observed in this study lie within their genome-wide expression (Fig. 7). As mentioned already in the introduction, these two *Nitrosomonas spp.* have circular chromosomes (that are very similar in size) and are closely related based on 16S phylogenetic trees (Chain *et al*., 2003; Beaumont *et al.*, 2002; Stein *et al.*, 2007) (Table 11).

However, when it comes to gene expression in mid-log versus early stationary phase, it is clear that *N. eutropha* had more than triple the number of genes with statistically significant changes in their fold expression compared to *N. europaea* (Fig. 7). These results come from a total of nine biological replicates with each having an experimental replicate to avoid any experimental bias in this study. One possible explanation for this observation could be that a higher number of regulated genes translates to a more stringent control over gene expression and possibly better and quicker adaptability of *N. eutropha* compared to *N. europaea*. This would make *N. eutropha* capable of having a faster and more appropriate response to environmental stresses than *N. europaea* (Cua and Stein, 2011; Schmidt and Bock, 2001; Wei *et al.*, 2006).

In a study done by Pellitteri-Hahn and colleagues (2011), they found that proteins associated with nucleic acid replication collectively comprised the largest group of highly abundant proteins in growing cells of *N. europaea*. In energy-starved cells, the proteome shifted away from biosynthesis and toward survival functions such as cell envelope modification, protein protection-degradation, detoxification and implementation of alternative energy generation mechanism (Pellitteri-Hahn *et al.*, 2011). In this study, genes coding for proteins involved in energy, carbohydrate and amino acid metabolism were amongst the highest regulated genes, being both up- or down-regulated in late logarithmic growth phase (Table 5).

Another explanation for why more genes have statistically significant difference in their fold expression in *N. eutropha* could be that since it is growing

in much lower concentrations of ammonia than what it is used to, the cells are experiencing more stress than usual as discussed earlier. On the other hand, it is probably more economical to have fewer genes regulated, hence the focus on catabolism instead of anabolism. This could be why *N. europaea* seems to be more lenient in its gene regulation in early stationary phase while it shows similar physiological characteristics to *N. eutropha*. Some of the highly expressed genes in *N. europaea* had no statistically significant change in their RPKM value from mid-log to early stationary phase (Table 12). Therefore, it seems that the change in environmental conditions in our batch cultivation did not trigger a change in gene expression in this strain. This was also illustrated in another study where in contrast to heterotrophic bacteria, *N. europaea* seemed to delay repair mechanisms when stressed until an energy source was replenished (Berube and Stahl, 2012).

As we saw in Table 11 showing the genome properties of these two bacteria, the numbers of genes involved in each cellular process between the two species are highly similar. Looking at our transcriptome results, the number of genes involved in different sub-categories within each category differed greatly between the two species (Table 1 compared to Table 5 & Table 2 compared to Table 6). This provides an overall view of what aspect of cellular processes, such as metabolism or enzymes, are more regulated in early stationary phase. One such a big difference is in amino acid metabolism, where *N. eutropha* showed a much larger number of genes involved in this sub-category when compared to *N. europaea*. Another difference is in drug resistance, however, it is known that

having many heavy metal and multidrug resistance genes differentiates *N. eutropha* from the rest of aerobic AOB and anammox genomes sequenced to date (Stein *et al.*, 2007 and references therein). Our results follow previous findings and we can see that the numbers of expressed genes involved in drug resistance in *N. eutropha* are more than double that in *N. europaea* (Fig. 7).

One should keep in mind that lower numbers of gene transcripts present in early stationary phase does not necessarily mean that there was a decrease in gene expression. Other secondary processes or environmental stresses can cause the half-life of a transcript to decrease dramatically when cells are under stress (Berube *et al.*, 2012; Beyers *et al.*, 2009; Hirota *et al.*, 2006). Since these transcriptome data are a snapshot of the cellular processes taking place during each growth phase, it is difficult to hold 'down-regulation' as the only reason responsible for a gene to be present in fewer transcripts.

4.5 Bacterial Motility

The second biggest difference between the two species besides total number of genes being expressed is in their expression of an array of motility genes (Fig. 7 & Table 9). As we already know, both of these *Nitrosomonas spp*. are able to swim using lash-like appendages called flagella (Hooper, 1969; Bardy *et al.*, 2003). In some species, it is not only used for locomotion, but also as a sensory organelle sensing the alterations in temperature or chemicals surrounding the cell (Wang *et al.*, 2005). Flagella consist of different proteins that make a long hollow tube with a sharp bend outside the outer membrane (the

hook) and these protein components are added at the flagellar tip during assembly (Lefebvre, 2001). Proteins forming the *Mot* complex (the motor) propel the bacterium by rotating the hook. This rotary engine, powered by proton motive force, is made up of proteins that are located at the flagellum's anchor point and can operate at 200 to 1000 rpm when the flagellar filaments are attached to it. There are a couple of switch proteins responsible for the instantaneous change in direction of the rotation to propel the bacterium in different directions (Bardy *et al.*, 2003).

In *N. eutropha*, genes coding for chemotaxis protein (CheR and CheB), regulator of CheA protein (CheW), flagellar basal-body rod protein (FlgD and FlgC), flagellar biosynthesis protein (FliL), flagellar hook protein (FlgE), flagellar hook-basal body complex protein (FliE), flagellar M-ring protein (FliF), flagellar motor switch protein (FliG and FliN), flagellar sensor histidine kinase (FleS), and flagellar transcriptional activator (FlhC and FlhD) were all highly up-regulated in early stationary phase (Table 9). Surprisingly, no such expression could be seen in *N. europaea*. This data suggests that when nutrients along with available oxygen become limited, cells of *N. eutropha* express some of its motility genes. There could be several reasons behind this modification. If such expression translates to motility, this could potentially help the bacterium to have higher and better adaptability by being able to scavenge for nutrients. This is further supported by the up-regulation of genes involved in chemotaxis as well, which is possibly triggered by low concentration of nutrients in early stationary phase (Wang *et al.*, 2005). Another explanation for this regulation could be that the cells

are trying to swim away from the build up of nitrite in their surroundings, which as discussed earlier, has been shown to be toxic at high concentrations. Regardless of the exact reasoning for this difference in expression, such an observation defines one of the most distinctive characteristics of *N. eutropha* compared to *N. europaea* that can be further examined in future studies to understand whether it is related to any physiological advantages.

4.6 Expression of principal genes

In our transcriptomic data, we observed that some well-studied and principal genes in these strains showed a different pattern of regulation in their expression (Table 12). For instance, the genes involved in carbon metabolism highly differed in their expression between *N. europaea* and *N. eutropha*. Ribulose-1,5-bisphosphate carboxylase/oxygenase, which is commonly abbreviated as RuBisCO is an enzyme involved in the first step of carbon fixation (Feller *et al.*, 2008). It was already mentioned in the introduction that both of these *Nitrosomonas spp.* fix carbon via Calvin-Benson-Bassham cycle (Stein *et al.*, 2007). This enzyme is fairly inefficient in its process and it is generally considered as the rate-limiting step of this cycle (Klotz, 2008). However, what is remarkable is the difference in its expression between the two bacteria. In *N. europaea*, a high number of reads were present (~4000 RPKM) in mid-log and this number decreased by 2-fold in early stationary phase. Therefore, all of the necessary RuBisCO for carbon fixation were present early in logarithmic growth phase and naturally the number declined as limiting factors caused cells to enter

stationary phase. In contrast in *N. eutropha*, we saw an increase in this gene's expression in early stationary phase. The same pattern also exists for the genes coding for carboxysome shell proteins. These proteins form a microcompartments within the bacteria that will contain the necessary enzymes, such as RuBisCO, for carbon fixation (Kartal *et al.* 2012). These compartments will help concentrate carbon dioxide to overcome the inefficiency of RuBisCO's active site that can otherwise be occupied more easily by O_2. Kartal and colleagues (2012) made a similar observation in their proteomic study regarding carboxysome shell protein (CsoS2) being up-regulated as cells were experiencing anoxia. So while in *N. europaea* the genes necessary for carbon fixation are expressed in high numbers during mid-log, they are up-regulated in early stationary phase in *N. eutropha.* Presumably, in early stationary phase, RuBisCO could be functioning more efficiently in the carboxysome given the fact that the concentration of external oxygen is decreased drastically.

Ammonia monooxygenase has been talked about in great detail in this study. We already know its properties and exact function with regards to ammonia oxidation. It consists of three A-, B- and C-subunits (Sayavedra-Soto *et al.*, 1998). As previously discussed, some AOB possess a divergent copy of *amoC* called *amoC3,* which has been suggested to be involved in ammonia starvation (Berube and Stahl, 2012). In our transcriptome data, *amoC* is present in a high number of reads in *N. eutropha* and its expression is highly decreased in early stationary phase (Table 12). This gene's expression is unchanged in *N. europaea*'s transcriptome (Table 12). In contrast, both *amoA1* and *amoA2* are

present in much higher number of reads in early stationary phase in *N. europaea* (Table 12). Other studies, such as the one done by Sayavedra-Soto and colleagues have shown that different subunits of ammonia monooxygenase do not always follow the same pattern of expression between different *Nitrosomonas spp.* (1994). So it seems that while some of the AMO subunits are down-regulated, others are up-regulated suggesting that different copies of the same homologous enzyme subunits could be involved in more than just one process in different species (Berube *et al.*, 2007).

Another principal gene involved in ammonia oxidation is the one coding for copper-containing nitrite reductase (Cantera and Stein, 2007a). As discussed previously when we looked at the physiology of these two species, *nirK* was highly up-regulated in *N. eutropha* as cells enter stationary phase. Although there was no fold difference in its expression in *N. europaea*, it was present in extremely high number of reads in both mid-log and early stationary phase (Table 12). Therefore, in *N. europaea*, this gene is always expressed regardless of the growth state the cells are in while it is up-regulated in *N. eutropha*. Other principle genes that are unchanged in their fold expression follow the findings of previous literature. Beaumont and colleagues found that NorB was not up-regulated during N_2O production or be involved in protection of cells against nitrite toxicity in *N. europaea* (Beaumont *et al.*, 2005). The same finding can be said from this study regarding NorB in both *N. europaea* and *N. eutropha* as the cells enter stationary phase.

Further research needs to be done to fully understand the specific regulatory mechanisms employed by *N. europaea* to jointly counter substrate starvation and stress. The same can be said about the findings of this study regarding regulation of dentrification genes in *N. europaea*. There seems to be no specific trigger during mid- or late-logarithmic growth to cause a change in this already highly expressed gene, even though the NsrR regulator is deactivated under certain conditions of high nitrite load in *N. europaea*, causing induction of nirK and its associated genes (Beaumont *et al.*, 2004). Looking at the transcriptome and proteome results of a previous study (Kartal *et al.* 2012), they also observed no detectable change in protein or transcript levels of *nirK* or any significant change in expression of nitric oxide reductase (NorCB) proteins or *norBQD* under any conditions. However, this same study did show expression of the NorY nitric oxide reductase, suggesting again a specific trigger for nitric oxide reductase under some conditions.

Chapter 5
Conclusion and future perspectives

This is the first study in the field of ammonia oxidation reserach where we have compared whole-genome expression levels along with their physiology in two closely related *Nitrosomonas spp*. under batch cultivation conditions. Studies that have been done before, in certain environmental conditions, mostly examined specific genes (Beaumont *et al.*, 2004; Berube *et al.*, 2007; Beyers *et al.*, 2009; Hirota *et al.*, 2006). While they further our understanding of specific gene functionality in different species, this study gives us a more general understanding of differences and similarities in genome-wide regulation in closely related ammonia oxidizers. This is extremely useful in understanding both physiology and reasons for niche preference of different cultivated strains. Since this study was done in batch culture, it answers more environmentally relevant questions. In batch culture after a period of feast, bacteria eventually reach a point of famine where they are completely starved of nutrients and oxygen. This type of study more closely resembles their environmental habitat since many environmental factors change simultaneously (such as temperature, pH, ammonia concentration, etc.). The central hypothesis of this thesis, as mentioned in the introduction, was that these two closely related bacteria would have differential genome-wide responses grown in similar conditions.

The results from the physiological experiments along with transcriptome data supported our hypothesis. There are few main conclusions that can be made from the results of this project. First: *N. europaea* produces more N_2O

while *N. eutropha* consumes more nitrite as cells enter stationary phase. Second: Higher numbers of genes are regulated in *N. eutropha* compared to *N. europaea* in the same phase. Third: There are selections of motility genes that are highly up-regulated in *N. eutropha* during early stationary growth phase and such regulation is completely absent in *N. europaea*. Fourth: principle genes that have been well studied have different patterns of regulation in these two closely related strains. However, the majority of the principle genes were not differentially regulated in either strain.

While significant, each of these points can be taken further to understand more about these two well studied organism. Firstly, it would be interesting to see whether such a difference in total number of genes regulated would follow the same pattern when cells are grown in continuous culture. This would give us a broader understanding about these species' 'imminent' versus 'prolonged' responses to environmental changes. Moreover, transcriptomic data from the 50mM ammonia concentration experiments can help us look at *N. eutropha*'s gene regulation when grown in media that is closer to its optimal growth conditions and to see how it relates to the results from this study. Furthermore, it could give us a broader view into how *N. europaea* adapts to higher ammonia concentrations and what gene inventories are responsible for such adaptation. It would be fascinating to do further motility experiments to see whether this huge difference in genes responsible for motility is environmentally beneficial to the cells. Lastly, transcriptomic studies give us one aspect of the cellular mechanism and following these studies with proteomics and metabolomics would give us a

more complete picture with regards to understanding the physiology of AOB on multiple levels.

Bibliography

Aakra A., Utaˆker J.B., Nes I.F. (2001a) Comparative phylogeny of the ammonia monooxygenase subunit A and 16S rRNA genes of ammonia-oxidizing bacteria. FEMS Microbiol Lett 205:237–242.

Abeliovich, A., & Vonshak, A. (1992). Anaerobic metabolism of *Nitrosomonas europaea*. *Archives of Microbiology, 158*(4), 267-270.

Anbar A.D. and Knoll A.H. (2002) Proterozoic ocean chemistry and evolution: a bioinorganic bridge? Science 297: 1137–1142.

Arciero D.M., Pierce B.S., Hendrich M.P. and Hooper A.B. (2002) Nitrosocyanin, a red cupredoxin-like protein from *Nitrosomonas europaea*. Biochemistry 41: 1703–1709.

Arciero, D. M., and A. B. Hooper. (1993). Hydroxylamine oxidorectase is a multimer of an octa-heme subunit. *J. Biol. Chem.* 268:14645–14654.

Arciero, D., C. Balny, and A. B. Hooper. (1991a). Spectroscopic and rapid kinetic studies of reduction of cytochrome *c*554 by hydroxylamine reductase from *Nitrosomonas europaea*. *J. Biol. Chem.*269:11878–11886.

Arp, D. J., P. S. G. Chain, and M. G. Klotz. 2007. The impact of genome analyses on our understanding of ammonia-oxidizing bacteria. *Ann. Rev.Microbiol.* 61:21–58.

Arp, D. J., Sayavedra-Soto, L. A., & Hommes, N. G. (2002). Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*. *Archives of Microbiology, 178*(4), 250-255.

Bardy S.L., Ng S.Y., Jarrell K.F., (2003). "Prokaryotic motility structures". *Microbiology (Reading, Engl.)* 149 (Pt 2): 295–304. doi:10.1099/mic.0.25948-0. PMID 12624192.

Bartossek R., Spang A., Weidler G., Lanzen A. and Schleper C. (2012). Metagenomic analysis of ammonia-oxidizing archaea affiliated with the soil group. doi: 10.3389/fmicb.2012.00208.

Beaumont, H. J. E., Hommes, N. G., Sayavedra-Soto, L. A., Arp, D. J., Arciero, D. M., Hooper, A. B., et al. (2002). Nitrite reductase of *Nitrosomonas europaea* is not essential for production

of gaseous nitrogen oxides and confers tolerance to nitrite. *Journal of Bacteriology, 184*(9), 2557-2560. Retrieved from SCOPUS database.

Beaumont, H. J. E., Lens, S. I., Reijnders, W. N. M., Westerhoff, H. V., & Van Spanning, R. J. M. (2004). Expression of nitrite reductase in *Nitrosomonas europaea* involves NsrR, a novel nitrite-sensitive transcription repressor. *Molecular Microbiology, 54*(1), 148-158. Retrieved from SCOPUS database.

Beaumont, H. J. E., Lens, S. I., Westerhoff, H. V., & Van Spanning, R. J. M. (2005). Novel nirK cluster genes in *Nitrosomonas europaea* are required for NirK-dependent tolerance to nitrite. *Journal of Bacteriology, 187*(19), 6849-6851. Retrieved from SCOPUS database.

Beaumont, H. J. E., Van Schooten, B., Lens, S. I., Westerhoff, H. V., & Van Spanning, R. J. M. (2004). *Nitrosomonas europaea* expresses a nitric oxide reductase during nitrification. *Journal of Bacteriology, 186*(13), 4417-4421. Retrieved from SCOPUS database.

Bergmann, D. J., A. B. Hooper, and M. G. Klotz. 2005. Structure and sequence conservation of genes in the *hao* cluster of autotrophic ammonia- oxidizing bacteria: evidence for their evolutionary history. *Appl. Environ. Microbiol.* 71:5371–5382.

Bergmann D.J., Zahn J.A., Hooper A.B. and DiSpirito A.A. (1998) Cytochrome P460 genes from the methanotroph Methylococcus capsulatus Bath. J Bacteriol 180: 6440–6445.

Berube, P. M., S. C. Proll, and D. A. Stahl. (2007). Genome-wide transcriptional analysis following the recovery of *Nitrosomonas europaea* from ammonia starvation, abstr. H-105. Abstr. 107th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC.

Berube, P. M., R. Samudrala, and D. A. Stahl. (2007). Transcription of all *amoC* copies is associated with recovery of *Nitrosomonas europaea* from ammonia starvation. *J. Bacteriol.* 189:3935–3944.

Berube, P. M., & Stahl, D. A. (2012). The divergent AmoC3 subunit of ammonia monooxygenase functions as part of a stress response system in *Nitrosomonas europaea*. *Journal of Bacteriology, 194*(13), 3448-3456. doi:10.1128/JB.00133-12; 10.1128/JB.00133-12.

Beyer, S., Gilch, S., Meyer, O., & Schmidt, I. (2009). Transcription of genes coding for metabolic key functions in *Nitrosomonas europaea* during aerobic and anaerobic growth. *Journal of Molecular Microbiology and Biotechnology, 16*(3-4), 187-197. Retrieved from SCOPUS database.

Bodelier P.L.E. and Laanbroek H.J. (2004) Nitrogen as a regulatory factor of methane oxidation in soils and sediments. FEMS Microbiol Ecol 47: 265–277.

Braker, G., and J. M. Tiedje. (2003). Nitric oxide reductase (*norB*) genes from pure cultures and environmental samples. *Appl. Environ. Microbiol.* 69:3476–3483.

Brandes J.A., Boctor N.Z., Cody G.D., Cooper B.A., Hazen R.M. and Yoder H.S. (1998) Abiotic nitrogen reduction on the early Earth. Nature 395: 365–367.

Brandes J.A., Devol A.H. and Deutsch C. (2007) New developments in the marine nitrogen cycle. Chem Rev 107: 577–589.

Buchanan R.E. (1917) Studies on the nomenclature and classification of the bacteria. III. The families of the Eubacteriales. J Bacteriol 2:347–350.

Buchanan R.E. (1918) Studies in the nomenclature and classification of 1188 the bacteria. VI. Subdivisions and genera of the Spirillaceae and Nitrobacteriaceae. J Bacteriol 3:175–181.

Cabello P., Pino C., Olmo-Mira M.F., Castillo F., Roldan M.D. and Moreno-Vivian C. (2004) Hydroxylamine Assimilation by *Rhodobacter capsulatus* E1F1: requirement of the hcp gene hybrid cluster protein) located in the nitrate assimilation nas gene region for hydroxylamine reduction. J Biol Chem 279: 45485–45494.

Calvo´ L., Cortey M., Garc´ıa-Mar´ın J.L., Garcia-Gil L.J. (2005) Polygenic analysis of ammonia-oxidizing bacteria using 16S rDNA, amoA, and amoB genes. Int 1195 Microbiol 8:103–110.

Canfield DE (1998) A new model for Proterozoic ocean chemistry. Nature 396: 450–453.

Canfield D, Rosing M & Bjerrum C (2006) Early anaerobic metabolisms. Philos Trans Roy Soc B: Biol Sci 361: 1819–1836.

Cantera J.J.L. and Stein L.Y. (2007a) Role of nitrite reductase in the ammonia-oxidizing pathway of *Nitrosomonas europaea*. Arch Microbiol 188: 349–354.

Cantera J.J.L. and Stein L.Y. (2007b) Molecular diversity of nitrite reductase genes (nirK) in nitrifying bacteria. Environment Microbiol 9: 765–776.

Casciotti, K. L., and B. B.Ward. (2005) Phylogenetic analysis of nitric oxide reductase gene homologues from aerobic ammonia-oxidizing bacteria. *FEMS Microbiol. Ecol.* 52:197–205.

Capone D.G. (2000) The marine microbial nitrogen cycle. Microbial Ecology of the Oceans (Kirchman DL, ed), pp. 455–494. Wiley-Liss, New York, NY.

Capone D.G. and Knapp A.N. (2007) Oceanography: a marine nitrogen cycle fix? Nature 445: 159.

Capone DG, Popa R, Flood B & Nealson KH (2006) Geochemistry: follow the nitrogen. Science 312: 708–709.

Chain, P., Lamerdin, J., Larimer, F., Regala, W., Lao, V., Land, M., et al. (2003). Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. *Journal of Bacteriology, 185*(9), 2759-2773.

Cho, C. M., Yan, T., Liu, X., Wu, L., Zhou, J., & Stein, L. Y. (2006). Transcriptome of a *Nitrosomonas europaea* mutant with a disrupted nitrite reductase gene (nirK). *Applied and Environmental Microbiology, 72*(6), 4450-4454. doi:10.1128/AEM.02958-05

Choi, P. S., Z. Naal, C. Moore, E. Casado-Rivera, H. D. Abruna, J. D. Helmann, and J. P. Shapleigh. (2006) Assessing the impact of denitrifier- produced nitric oxide on other bacteria. *Appl. Environ. Microbiol.* 72:2200–2205.

Colliver B.B. and Stephenson T. (2000) Production of nitrogen oxide and dinitrogen oxide by autotrophic nitrifiers. Biotechnol Adv 18: 219–232.

Cruz-Ramos H., Crack J., Wu G., Hughes M.N., Scott C., Thomson A.J., Green J. and Poole R.K. (2002) NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. EMBO J 21: 3235–3244.

Cua, L. S., & Stein, L. Y. (2011) Effects of nitrite on ammonia- oxidizing activity and gene regulation in three ammonia- oxidizing bacteria. *FEMS Microbiology Letters, 319*(2), 169-175.

Dalsgaard T., Thamdrup B. and Canfield D.E. (2005) Anaerobic ammonium oxidation (anammox) in the marine environment. Res Microbiol 156: 457–464.

de la Torre, J. R., C. B. Walker, A. E. Ingalls, M. Könneke, D. A. Stahl. 2008. Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environ. Microbiol.* 10:810–818.

Ding, S., Zheng, P., Lu, H., Chen, J., Mahmood, Q., & Abbas, G. (2013). Ecological characteristics of anaerobic ammonia oxidizing bacteria. *Applied Microbiology and Biotechnology, 97*(5), 1841-1849.

Dore J.E. and Karl D.M. (1996) Nitrification in the euphotic zone as a source for nitrite, nitrate, and nitrous oxide at Station ALOHA. Limnol Oceanogr 4: 1619–1628.

El Sheikh, A. F., and M. G. Klotz. (2008) Ammonia- dependent differential regulation of the gene cluster that encodes ammonia monooxygenase in *Nitrosococcus oceani* ATCC 19707. *Environ. Microbiol.* 10:3026–3035.

Elmore B.O., Bergmann D.J., Klotz M.G. and Hooper A.B. (2007) Cytochromes P460 and c0-beta; A new family of high-spin cytochromes c. FEBS Lett 581: 911–916.

Ensign, S. A., M. R. Hyman, and D. J. Arp. (1993) In vitro activation of ammonia monooxygenase from *Nitrosomonas europaea* by copper. *J. Bacteriol.* 175:1971–1980.

Falkowski, P. G. 1997. Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. *Nature* 387:272.

Feller U., Anders I., Mae T (2008). "Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated". *J. Exp. Bot.* **59** (7): 1615–24. doi:10.1093/jxb/erm242. PMID 17975207

Fox G.E. and Stackebrandt E. (1988) The application of 16s rrna cataloguing and 5s rrna sequencing in bacterial systematics. Method Microbiol 19:405–458.

Francis C.A., Beman J.M. and Kuypers M.M.M. (2007) New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. The ISME J 1:19–27.

Galloway J.N. and Cowling E.B. (2002) Reactive nitrogen and the world: 200 years of change. Ambio 31: 64–71.

Garbeva, P., Baggs, E. M., & Prosser, J. I. (2007). Phylogeny of nitrite reductase (nirK) and nitric oxide reductase (norB) genes from *nitrosospira* species isolated from soil. *FEMS Microbiology Letters, 266*(1), 83-89. doi:10.1111/j.1574-6968.2006.00517.x

Garcia-Horsman J.A., Barquera B., Rumbley J., Ma J. and Gennis R.B. (1994) The superfamily of heme-copper respiratory oxidases. J Bacteriol 176: 5587–5600.

Gibbs W.M. (1920) The isolation and study of nitrifying bacteria. Soil Sci 8:427–481.

Gunsalus R.P. and Wang H. (2000) The nrfA and nirB nitrite reductase operons in Esc*herichia coli* are expressed differently in response to nitrate than to nitrite. J Bacteriol 182: 5813–5822.

Hanson R.S. and Hanson T.E. (1996) Methanotrophic bacteria. Microbiol Rev 60: 439–471.

He, J., Hu, H., and Zhang, L. (2012). Current insights into the autotrophic thaumarchaeal ammonia oxidation in acidic soils. *Soil Biology and Biochemistry, 55*, 146-154. Retrieved from SCOPUS database.

Harms H., Koops H.P., Wehrmann H. (1976) An ammonia-oxidizing bacterium, *Nitrosovibrio tenuis* nov. gen. nov. sp. Arch Microbiol 108:105–111.

Head I.M., Hiorns W.D., Embley T.M., McCarthy A.J., Saunders J.R. (1993) The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J Gen Microbiol 139:1147–1153

Hendrich, M. P., A. K. Upadhyay, J. Riga, D. M. Arciero, and A. B. Hooper. (2002) Spectroscopic characterization of the NO adduct of hydroxylamine oxidoreductase. *Biochemistry* 41:4603–4611.

Hirota, R., A. Kuroda, T. Ikeda, N. Takiguchi, H. Ohtake, and J. Kato. (2006) Transcriptional analysis of the multicopy *hao* gene coding for hydroxylamine oxidoreductase in *Nitrosomonas* sp. strain ENI-11. *Biosci. Biotechnol. Biochem.* 70:1875–1881.

Hirota, R., A. Yamagata, J. Kato, A. Kuroda, T. Ikeda, N. Takiguchi, and H. Ohtake. (2000) Physical map location of the multicopy genes coding for ammonia monooxygenase and hydroxylamine oxidoreductase in the ammonia-oxidizing bacterium *Nitrosomonas* sp. ENI-11. *J. Bacteriol.* 182:825–828.

Holmes A.J., Costello A., Lidstrom M.E. and Murrell J.C. (1995) Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol Lett 132: 203–208.

Hommes, N. G., L. A. Sayavedra-Soto, and D. J. Arp. (1996) Mutagenesis of hydroxylamine oxidoreductase in *Nitrosomonas europaea* by transformation and recombination. *J. Bacteriol.* 178:3710–3714.

Hommes, N. G., L. A. Sayavedra-Soto, and D. J. Arp. (1998) Mutagenesis and expression of amo, which codes for ammonia monooxygenase in *Nitrosomonas europaea*. *J. Bacteriol.* 180:3353–3359.

Hommes N.G., Sayavedra-Soto L.A., Arp D.J. (2003) Chemolithoorganotrophic growth of Nitrosomonas europaea on fructose. J Bacteriol;185(23):6809-14.

Hooper, A. B. (1968) A nitrite-reducing enzyme from *Nitrosomonas europaea*. *Biochim. Biophys. Acta* 162:49–65.

Hooper, A. B. (1969) Biochemical basis of obligate autotrophy in *Nitrosomonas europaea*. *J. Bacteriol.* 97:776–779.

Hooper AB, Arciero DM, Bergmann D & Hendrich MP (2005) The oxidation of ammonia as an energy source in bacteria in respiration. Respiration in Archaea and Bacteria: Diversity of Procaryotic Respiratory Systems, Vol. 2. (Zannoni Davide, ed), pp. 121–147. Springer, Dordrecht, the Netherlands.

Houghton J, Ding Y, Griggs D, Noguer M & van der Linden PJ (2001) Climate Change 2001: The Scientific Basis. Cambridge University Press, Cambridge, UK.

Hugenholz, P.; Goebel B.M.; Pace N.R. (1998). "Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity". *J. Bacteriol* **180** (18): 4765–74. PMC 107498. PMID 9733676.

Hyman MR, Arp DJ (1995) Effects of ammonia on the de novo synthesis of polypeptides in cells of *Nitrosomonas europaea* denied ammonia as an energy source. J Bacteriol 177:4974–4979.

Igarashi, N., Moriyama, H., Fujiwara, T., Fukumori, Y., & Tanaka, N. (1997). The 2.8 Å structure of hydroxylamine oxidoreductase from a nitrifying chemoautotrophic bacterium, *Nitrosomonas europaea*. *Nature Structural & Molecular Biology, 4*(4), 276-284.

Itoh, Y., Sakagami, K., Uchino, Y., Boonmak, C., Oriyama, T., Tojo, F., *et al*. (2013). Isolation and characterization of a thermotolerant ammonia-oxidizing bacterium *Nitrosomonas* sp. JPCCT2 from a thermal power station. *Microbes and Environments, 28*(4), 432-435. Retrieved from SCOPUS database.

Jetten, M. S. M., L. v. Niftrik, M. Strous, B. Kartal, J.T. Keltjens, and H. J. M. Op den Camp. (2009) Biochemistry and molecular biology of anammox bacteria. *Crit. Rev. Biochem. Mol. Biol.* 44:65–84.

Jiang, Q.Q., and L. R. Bakken. (1999) Nitrous oxide production and methane oxidation by different ammonia-oxidizing bacteria. *Appl. Environ. Microbiol.* 65:2679–2684.

Kartal, B., Wessels, H. J., van der Biezen, E., Francoijs, K. J., Jetten, M. S., Klotz, M. G., et al. (2012). Effects of nitrogen dioxide and anoxia on global gene and protein expression in long-term continuous cultures of *Nitrosomonas eutropha* C91. *Applied and Environmental Microbiology, 78*(14), 4788-4794. doi:10.1128/AEM.00668-12; 10.1128/AEM.00668-12.

Klotz, M. G. (2008) Evolution of the nitrogen cycle, an ~omics perspective: evolution of the marine nitrogen cycle through time I, p. PP42A-02. Abstr. Fall meeting of the American Geophysical Union, San Francisco, CA.

Klotz, M. G., and J. M. Norton. (1998) Multiple copies of ammonia monooxygenase (*amo*) operons have evolved under biased AT/GC mutational pressure in ammonia-oxidizing autotrophic bacteria. *FEMS Microbiol. Lett.* 168:303–311.

Klotz, M. G., and Stein, L. Y. (2008). Nitrifier genomics and evolution of the nitrogen cycle. *FEMS Microbiology Letters, 278*(2), 146-156. Retrieved from SCOPUS database.

Koops H.P., Purkhold U., Pommerening-Rö̈ser A., Timmermann G., Wagner M. (2006) The lithotrophic ammonia-oxidizing bacteria. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The prokaryotes: a handbook on the biology of bacteria, vol 5, Proteobacteria: alpha and beta subclass. Springer, New York, pp 778–811.

Kowalchuk G.A., Stephen J.R., De Boer W., Prosser J.I., Embley T.M., Woldendorp J.W. (1997) Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl Environ Microbiol 63:1489–1497.

Kozlowski J.A., Price J., Stein L.Y. (2014). Revision of N_2O-Producing Pathways in the Ammonia-Oxidizing Bacterium *Nitrosomonas europaea* ATCC 19718. *Appl. Environ. Microbiol.* 80(16):4930-5.

Laanbroek HJ, Gerards S (1993) Competition for limiting amounts of oxygen between Nitrosomonas europaea and Nitrobacter winogradskyi grown in mixed continuous cultures. Arch Microbiol 159:453–459.

Lawrence J.G. and Hendrickson H. (2005) Genome evolution in bacteria: order beneath chaos. Curr Opin Microbiol 8: 572.

Lefebvre P.A. (2001) "Assembly and Motility of Eukaryotic Cilia and Flagella. Lessons from Chlamydomonas reinhardtii". *Plant Physiol.* **127** (4): 15001507.doi:10.1104/pp.010807. PMC 1540183. PMID 11743094.

Levy-Booth, D. J., Prescott, C. E., & Grayston, S. J. (2014). Microbial functional genes involved in nitrogen fixation, nitrification and denitrification in forest ecosystems. *Soil Biology and Biochemistry, 75*, 11-25. Retrieved from SCOPUS database.

Lewis B.L. and Landing W.M. (1992) The investigation of dissolved and suspended-particulate trace metal fractionation in the Black Sea. Marine Chem 40: 105.

Lewis R.F., Pramer D. (1958) Isolation of *Nitrosomonas* in pure culture. J Bacteriol 1363 76:524–527.

Lin J.T. and Stewart V. (1998) Nitrate assiimilation in bacteria. Adv Microbial Physiol 39: 1–30.

Lipschultz F., Zafiriou O.C., Wofsy S.C., Melroy M.B., Valois F.W. and Watson S.W. (1981) Production of NO and N_2O by soil nitrifying bacteria. Nature 294: 641–643.

Lontoh S., DiSpirito A.A., Krema C.L., Whittaker M.R., Hooper A.B. and Semrau J.D. (2000) Differential inhibition in vivo of ammonia monooxygenase, soluble methane monooxygenase

and membrane-associated methane monooxygenase by phenylacetylene. Environment Microbiol 2: 485–494.

Mancinelli, R. L., and C. P. McKay. (1988) The evolution of nitrogen cycling. *Orig. Life Evol. Biosph.* 18:311–325.

Martens-Habbena, W., P. M. Berube, H. Urakawa, J. R. de la Torre, and D. A. Stahl. (2009) Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* 461:976–979.

McKay C.P. and Navarro-Gonzalez R. (2001) The absence of nitrogen-fixing organelles due to timing of the nitrogen crisis. Symbiosis (Seckbach J, ed), pp. 221–228. Springer, The Netherlands.

McLain J.E.T. and Martens D.A. (2005) Nitrous oxide flux from soil and amino acid mineralization. Soil Biol Biochem 37: 289–299.

McTavish, H., J. A. Fuchs, and A. B. Hooper. (1993a) Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. *J. Bacte- riol.* 175:2436–2444.

McTavish, H., F. LaQuier, D. Aciero, M. Logan, G. Mundfrom, J. A. Fuchs, and A. B. Hooper. (1993b) Multiple copies of genes coding for electron transport proteins in the bacterium *Nitrosomonas europaea*. *J. Bacteriol.* 175:2445–2447.

Monteiro, M., Séneca, J., and Magalhães, C. (2014). The history of aerobic ammonia oxidizers: From the first discoveries to today. *Journal of Microbiology, 52*(7), 537-547. Retrieved from SCOPUS database.

Moreno-Vivian, C., P. Cabello, M. Martinez- Luque, R. Blasco, and F. Castillo. (1999) Prokaryotic nitrate reduction: molecular properties and functional distinction among bacteria nitrate reductases. *J. Bacteriol.* 181:6573–6584.

Murrell J.C. and Holmes A.J. (1996) Molecular biology of particulate methane monooxygenase. Microbial Growth on C1 Compounds (Lidstrom ME & Tabita FR, eds), pp. 133–140. Kluwer Academic Publishers, the Netherlands.

Nakamura K. and Go N. (2005) Function and molecular evolution of multicopper blue proteins. Cell Mol Life Sci 62: 2050–2066.

Navarro-Gonzalez R., McKay C.P. and Mvondo D.N. (2001) A possible nitrogen crisis for Archaean life due to reduced nitrogen fixation by lightning. Nature 412: 61–64.

Norton, J. M., J. J. Alzerreca, Y. Suwa, and M. G. Klotz. (2002) Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. *Arch. Microbiol.* 177:139–149.

Norton, J. M., M. G. Klotz, L.Y. Stein, D. J. Arp, P. J. Bottomley, P. S. G. Chain, L. J. Hauser, M. L. Land, F. W. Larimer, M. W. Shin, and S. R. Starkenburg. (2008) Complete genome sequence of *Nitrosospira multiformis*, an ammonia-oxidizing bacterium from the soil environment. *Appl. Environ. Microbiol.* 74:3559–3572.

Orellana L.H., Rodriguez-R L.M., Higgins S., Chee-Sanford J.C., Sanford R.A., Ritalahti K.M., Loffler F.E., Konstantinidis K.T. (2014). Detecting nitrous oxide reductase (nosZ) genes in soil metagenomes: Method development and implications for the nitrogen cycle. doi:10.1128/mBio.01193-14.

Pellitteri-Hahn, M. C., Halligan, B. D., Scalf, M., Smith, L., and Hickey, W. J. (2011). Quantitative proteomic analysis of the chemolithoautotrophic bacterium *Nitrosomonas europaea*: Comparison of growing- and energy-starved cells. *Journal of Proteomics, 74*(4), 411-419. Retrieved from SCOPUS database.

Pino C., Olmo-Mira F., Cabello P., Martinez-Luque M., Castillo F, Roldan M.D. and Moreno-Vivian C (2006) The assimilatory nitrate reduction system of the phototrophic bacterium *Rhodobacter capsulatus* E1F1. Biochem Soc Trans 34: 127–129.

Postgate J.R. (1970) Biological nitrogen fixation. Nature 226: 25–27.

Poth, M. andFocht, D. D. (1985). ^{15}N kinetic analysis of N_2O production by *nitrosomonas europaea*: An examination of nitrifier denitrification. *Applied and Environmental Microbiology, 49*(5), 1134-1141. Retrieved from SCOPUS database.

Potter, L., H. Angove, D. Richardson, and J. Cole. (2001) Nitrate reduction in the periplasm of Gram-negative bacteria. *Adv. Microb. Physiol.* 45:51–86.

Poulton S.W., Fralick P.W. and Canfield D.E. (2004) The transition to a sulphidic ocean [sim] 1.84 billion years ago. Nature 431: 173.

Prosser J.I. (1989) Autotrophic nitrification in bacteria. Adv Microb Physiol 30: 125–181.

Prosser, J. I., and G. W. Nicol. (2008) Relative contributions of archaea and bacteria to aerobic ammonia oxidation in the environment. *Environ. Microbiol.* 10:2931–2941.

Prosser J.I., Head I. M., Stein, L.Y. (2014). The Nitrosomonadaceae. E. Rosenberg et al. (eds.), The Prokaryotes – Alphaproteobacteria and Betaproteobacteria, DOI 10.1007/978-3-642-30197-1_372.

Purkhold U., Pommerening-Roser A., Juretschko S., Schmid M.C., Koops H.P. and Wagner M. (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Appl Environment Microbiol 66: 5368–5382.

Raymond, J., J. L. Siefert, C. R. Staples, and R. E. Blankenship. (2004) The natural history of nitrogen fixation. *Mol. Biol. Evol.* 21:541–554.

Robertson G.P., Paul E.A. and Haarwood R.R. (2000) Greenhouse gases in intensive agriculture: contributions of individual gases to the radiative forceing of the atmosphere. Science 289: 1922–1925.

Rodrigues M.L., Oliveira T., Matias P.M., Martins I.C., Valente F.M., Pereira I.A. and Archer M. (2006) Crystallization and preliminary structure determination of the membrane-bound complex cytochrome c nitrite reductase from *Desulfovibrio vulgaris* Hildenborough. Acta Crystallogr Sect F 62: 565–568.

Rotthauwe, J. H., K. P. Witzel, and W. Liesack. (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* 63:4704–4712.

Sayavedra-Soto, L. A., N. G. Hommes, and D. J. Arp. (1994) Characterization of the gene encoding hydroxylamine oxidoreductase in *Nitrosomonas europaea*. *J. Bacteriol.* 176:504–510.

Sayavedra-Soto, L. A., N. G. Hommes, J. J. Alzer- reca, D. J. Arp, J. M. Norton, and M. G. Klotz. (1998) Transcription of the *amoC*, *amoA* and *amoB* genes in *Nitrosomonas europaea* and *Nitrosospira* sp. NpAV. *FEMS Microbiol. Lett.* 167:81–88.

Schalk J., de Vries S., Kuenen J.G. and Jetten M.S.M. (2000) Involvement of a novel hydroxylamine oxidoreductase in anaerobic ammonium oxidation. Biochemistry 39: 5405–5412.

Schmidt I., Bock E. and Jetten M.S.M. (2001) Ammonia oxidation by *Nitrosomonas eutropha* with NO_2 as oxidant is not inhibited by acetylene. Microbiology 147: 2247–2253.

Schmidt I., Steenbakkers P.J.M., op den Camp H.J.M., Schmidt K. and Jetten M.S.M. (2004) Physiologic and proteomic evidence for a role of nitric oxide in biofilm formation by *Nitrosomonas europaea* and other ammonia oxidizers. J Bacteriol 186: 2781–2788.

Schmidt, I. (2009). Chemoorganoheterotrophic growth of nitrosomonas europaea and *nitrosomonas eutropha*. *Current Microbiology, 59*(2), 130-138.

Schmidt, I., & Bock, E. (1997) Anaerobic ammonia oxidation with nitrogen dioxide by *Nitrosomonas eutropha*. *Archives of Microbiology, 167*(2-3), 106-111.

Schmidt, I., van Spanning, R. J., & Jetten, M. S. (2004) Denitrification and ammonia oxidation by *Nitrosomonas europaea* wild-type, and NirK- and NorB-deficient mutants. *Microbiology (Reading, England), 150*(Pt 12), 4107-4114. doi:10.1099/mic.0.27382-0

Schmidt, I., Zart, D., & Bock, E. (2001) Gaseous NO_2 as a regulator for ammonia oxidation of *Nitrosomonas eutropha*. *Antonie Van Leeuwenhoek, 79*(3-4), 311-318.

Shailaja M.S., Narvekar P.V., Alagarsamy R. and Naqvi S.W.A. (2006) Nitrogen transformations as inferred from the activities of key enzymes in the Arabian Sea oxygen minimum zone. Deep-Sea Res I 53: 960–970.

Simon J. (2002) Enzymology and bioenergetics of respiratory nitrite ammonification. FEMS Microbiol Rev 26: 285–309.

Spirin V., Gelfand M.S., Mironov A.A. and Mirny L.A. (2006) A metabolic network in the evolutionary context: multiscale structure and modularity. PNAS 103: 8774–8779.

Stein L.Y. and Yung Y.L. (2003) Production, isotopic composition, and atmospheric fate of biologically produced nitrous oxide. Annual Rev Earth Planet Sci 31: 329–356.

Stein, L. Y., Arp, D. J., Berube, P. M., Chain, P. S., Hauser, L., Jetten, M. S., *et al*. (2007) Whole- genome analysis of the ammonia- oxidizing bacterium, *Nitrosomonas eutropha* C91: Implications for niche adaptation. *Environmental Microbiology, 9*(12), 2993-3007.

Stein, L. Y. (2010) Surveying N_2O-producing pathways in bacteria. Retrieved from SCOPUS database.

Stein, L. Y., and D. J. Arp. (1998) Loss of ammonia monooxygenase activity in *Nitrosomonas europaea* upon exposure to nitrite. *Appl. Environ. Microbiol.* 64:4098–4102.

Stein, L. Y., D. J. Arp, and M. R. Hyman. (1997) Regulation of the synthesis and activity of ammonia monooxygenase in *Nitrosomonas europaea* by altering pH to affect NH_3 availability. *Appl. Environ. Micro- biol.* 63:4588–4592.

Stein, L. Y., Sayavedra-Soto, L. A., Hommes, N. G., & Arp, D. J. (2000) Differential regulation of amoA and amoB gene copies in *Nitrosomonas europaea*. *FEMS Microbiology Letters, 192*(2), 163-168. Retrieved from SCOPUS database.

Stephen J.R., McCaig A.E., Smith Z., Prosser J.I., Embley T.M. (1996) Molecular diversity of soil and marine 16S rRNA gene sequences related to b-subgroup ammonia-oxidizing bacteria. Appl Environ Microbiol 62:4147–4154.

Strous M., Pelletier E., Mangenot S. *et al*. (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature 440: 790.

Strous, M., Kuenen, J. G., & Jetten, M. S. (1999) Key physiology of anaerobic ammonium oxidation. *Applied and Environmental Microbiology, 65*(7), 3248-3250.

Suwa Y., Norton J.M., Bollmann A., Klotz M.G., Stein L.Y., Laanbroek H.J. *et al*. (2011) Genome sequence of *Nitrosomonas* sp. strain AL212, an ammonia-oxidizing bacterium sensitive to high levels of ammonia. J Bacteriol 193:5047–5048.

Suzuki, I., Dular, U., & Kwok, S. C. (1974) Ammonia or ammonium ion as substrate for oxidation by *Nitrosomonas europaea* cells and extracts. *Journal of Bacteriology, 120*(1), 556-558.

Tallec, G., Garnier, J., Billen, G., & Gousailles, M. (2006) Nitrous oxide emissions from secondary activated sludge in nitrifying conditions of urban wastewater treatment plants: Effect of oxygenation level. *Water Research, 40*(15), 2972-2980. Retrieved from SCOPUS database.

Upadhyay, A. K., A. B. Hooper, and M. P. Hen- drich. (2006) NO reductase activity of the tetra-heme cytochrome c_{554} of *Nitrosomonas europaea. J. Am. Chem. Soc.* 128:4330–4337.

Utaker, J. B., L. Bakken, Q. Q. Jiang, and I. F. Nes. (1995) Phylogenetic analysis of seven new isolates of ammonia-oxidizing bacteria based on 16S rRNA gene sequences. *Syst. Appl. Microbiol.* 18:549–559.

Van Spanning R.J., De Boer A.P., Reijnders W.N., Spiro S., Westerhoff H.V., Stouthamer A.H. and Van der Oost J. (1995) Nitrite and nitric oxide reduction in *Paracoccus denitrificans* is under the control of NNR, a regulatory protein that belongs to the FNR family of transcriptional activators. FEBS Lett 360: 151–154.

van Teeseling, M. C., Neumann, S., & van Niftrik, L. (2013) The anammoxosome organelle is crucial for the energy metabolism of anaerobic ammonium oxidizing bacteria. *Journal of Molecular Microbiology and Biotechnology, 23*(1-2), 104-117. doi:10.1159/000346547; 10.1159/000346547.

Vajrala N., Sayavedra-Soto L.A., Bottomley P.J., Arp D.J. (2011) Role of a Fur homolog in Iron metabolism in *Nitrosomonas europaea*. DOI: 10.1186/1471-2180-11-37

Vlaeminck, S. E., Hay, A. G., Maignien, L., & Verstraete, W. (2011) In quest of the nitrogen oxidizing prokaryotes of the early earth. *Environmental Microbiology, 13*(2), 283-295.

Wachtershauser G. (2007) On the chemistry and evolution of the pioneer organism (review). Chem Biodiversity 4: 584–602.

Wang, Q.; Suzuki, A.; Mariconda, S.; Porwollik, S.; Harshey, R. M. (2005). "Sensing wetness: A new role for the bacterial flagellum". *The EMBO Journal* 24 (11): 2034–42. doi:10.1038/sj.emboj.7600668. PMC 1142604. PMID 15889148.

Ward N., Larsen O., Sakwa J. *et al*. (2004) Genomic insights into methanotrophy: the complete genome sequence of *Methylococcus capsulatus* (Bath). PLoS Biol 2: e303.

Watson S.W. (1971) Taxonomic considerations of the family Nitrobacteraceae Buchanan. Requests for opinions. Int J Syst Bacteriol 21:254–270.

Watson S.W., Mandel M. (1971) Comparison of the morphology and deoxyribonucleic acid composition of 27 strains of nitrifying bacteria. J Bacteriol 107:563–569.

Wei, X., Yan, T., Hommes, N. G., Liu, X., Wu, L., McAlvin, C., et al. (2006) Transcript profiles of *Nitrosomonas europaea* during growth and upon deprivation of ammonia and carbonate. *FEMS Microbiology Letters, 257*(1), 76-83.

Whittaker, M., D. Bergmann, D. Arciero, and A. B. Hooper. (2000) Electron transfer during the oxidation of ammonia by the chemolithotrophic bacterium *Nitrosomonas europaea*. *Biochim. Biophys. Acta* 1459:346–355.

Winogradsky S. (1892) Contributions a` la morphologie des organismes de la nitrification. Arch Sci Biol 1: 88–37.

Woese C.R., Stackebrandt E., Weisburg W.G., Paster B.J., Madigan M.T., Fowler V.J., Hahn CM, Blanz P, Gupta R, Nealson KH, Fox GE (1984a) The phylogeny of purple bacteria: the alpha subdivision. Syst Appl Microbiol 5:315–326.

Woese C.R., Weisburg W.G., Paster B.J., Hahn C.M., Tanner R.S., Krieg N.R., Koops H.P., Harms H., Stackebrandt E. (1984b) The phylogeny of the purple bacteria: the beta subdivision. Syst Appl Microbiol 5:327–336.

Wood, P. M. (1986) Nitrification as bacterial energy source, p. 39-62. In J. I. Prosser (ed.), Nitrification. Society for General Microbiology, IRL Press, Oxford, United Kingdom.

Wrage, N., Velthof, G. L., Van Beusichem, M. L., & Oenema, O. (2001) Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry, 33*(12-13), 1723-1732. Retrieved from SCOPUS database.

Yu, R. and Chandran, K. (2010) Strategies of *Nitrosomonas europaea* 19718 to counter low dissolved oxygen and high nitrite concentrations. *BMC Microbiology, 10* Retrieved from SCOPUS database.

Yung Y.L. and McElroy M.C. (1979) Fixation of nitrogen in the prebiotic atmosphere. Science 203: 1002–1004.

Zart, D., Schmidt, I., & Bock, E. (2000) Significance of gaseous NO for ammonia oxidation by *Nitrosomonas eutropha*. *Antonie Van Leeuwenhoek, 77*(1), 49-55.

Zehr, J. P., and B. B. Ward. (2002) Nitrogen cycling in the ocean: new perspectives on processes and paradigms. *Appl. Environ. Microbiol.* 68:1015–1024.

Zumft W.G. (1997) Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev 61: 522–616.

Zumft, W. G., and P. M. H. Kroneck. (2006) Respiratory transformation of nitrous oxide (N_2O) to dinitrogen by bacteria and archaea. *Adv. Microb. Physiol.* 52:107–227.

Appendix

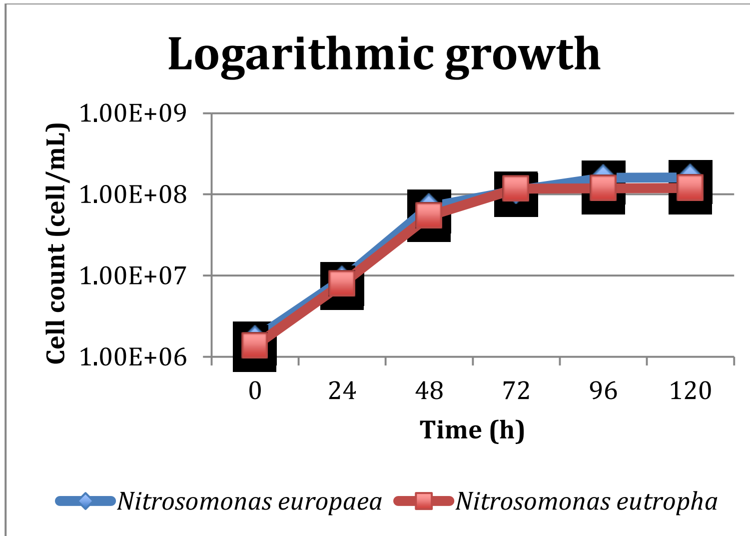


Figure A- 1: Growth curve of *N. europaea* and *N. eutropha*. Cells were grown in 50 mM ammonia H.K. media in batch culture at 30°C incubation. The blue line represents *N. europaea* (n = 6) and the red line represents *N. eutropha* (n = 6). Data are presented as means with ± SEs.

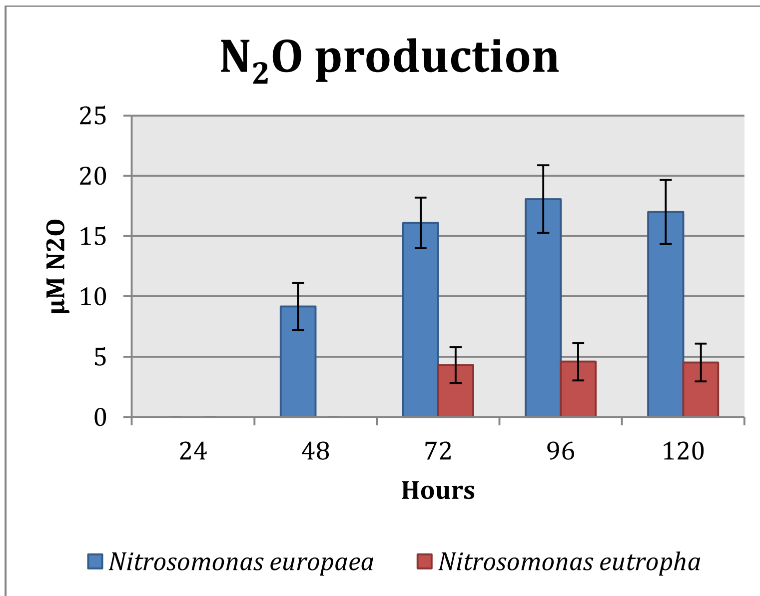


Figure A- 2: Nitrous oxide production in *Nitrosomonas spp.*. Cells were grown in 50 mM ammonia H.K. media in batch culture at 30°C incubation. N_2O measurments from *N. europaea* (n = 6) and *N. eutropha* (n = 6). 200 µL injections from the headspace were injected into the GC. Note: No detectable N_2O were measured at 24h for *N. europaea* (blue bar) and at 24h and 48h in *N. eutropha* (red bar). Data are presented as means ± SEs.

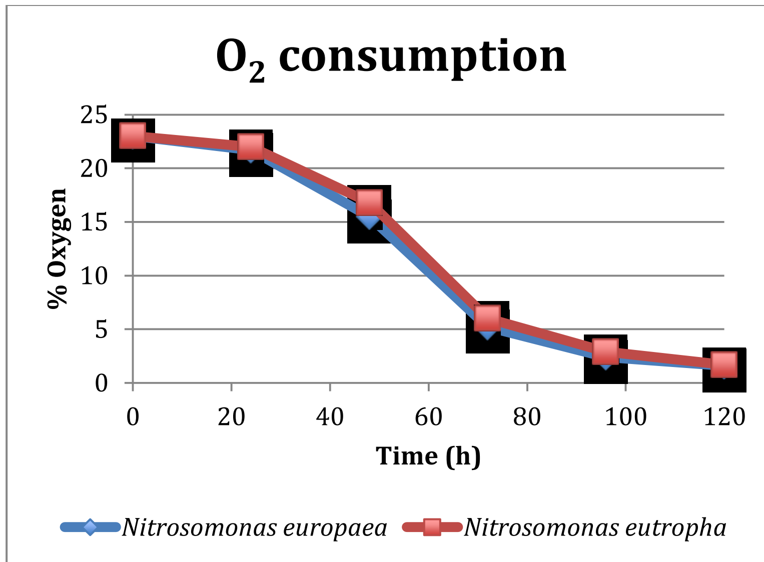


Figure A- 3: O_2 consumption and NO_2^- production. O_2 consumption were measured starting at 23% in the headspace in (A) *N. europaea* (n = 6) and (B) *N. eutropha* (n = 6). Cells were grown in 50 mM ammonia H.K. media in batch culture at 30°C incubation. All data are presented as means ± SEs.

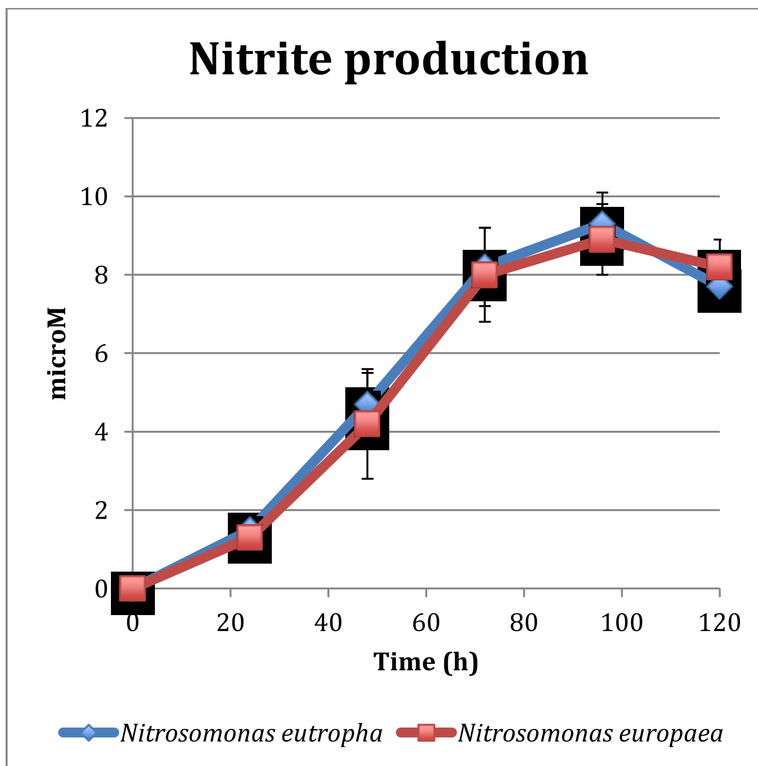


Figure A- 4: Nitrite production in *N. europaea* and *N. eutropha*. Cells were grown in 50 mM ammonia H.K. media in batch culture at 30°C incubation. Nitrite production was measured using chemical assays and a spectrophotometer. The red line represents *N. europaea* (n = 6) and the blue line represents *N. eutropha* (n = 6). Data are presented as means ± SEs.

Primer sequences: List of primers used for qPCR analysis for genomic DNA elimination in our RNA samples.

amoA primers:
Set #1:
Forward: TGG CTG GTT ACC GCG TTA GT
Rerverse: ATC AGC TAC GAC TGG CAG ATG G

Set#2:
Forward: ATC TGC CAG TCG TAG CTG ATG
Reverse: ACG GAC ATA TTC TGG TGT ACC G

Set #3:
Forward: CCT TCT TTG CGG CGT TTG TAT C
Reverse: CTC TTT GCA CGA TAC GTC CTC TT

16S primers:
341F: CCT ACG GGA GGC AGC AG
518R: ATT ACC GCG GCT GGT GG

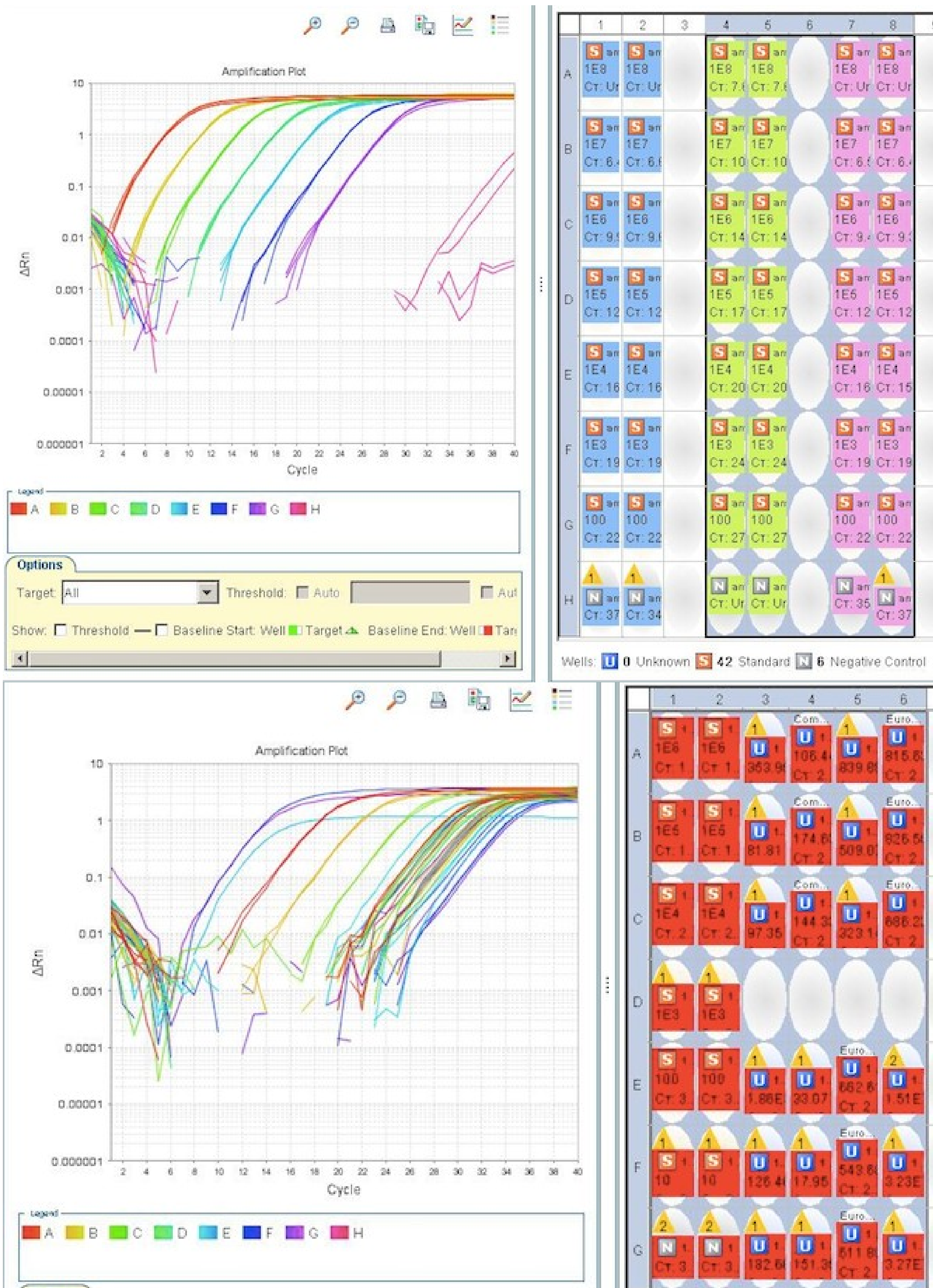


Figure A- 5: Sample qPCR results of standards and samples. Here is a representation of what the qPCR results illustrates regarding the standards (top) and samples (bottom). Samples were retreated with rDNAse I until no amplifications were obsereved after all 40 cycles were completed.