NITRATE REDUCTION TO AMMONIUM IN *NAUTILIA PROFUNDICOLA* VIA A PROPOSED REVERSE HURM PATHWAY

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master in Marine Studies

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"It takes a village to raise a child."-Anonymous

The proverb was true of my journey as a graduate student. The entirety of this project was reliant on the guidance and equipment usage of many faculty and staff. I could not have completed this degree without the cooperation and generosity of these people to whom I am grateful and must acknowledge.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Nautilia profundicola is an epsilonproteobacterium that inhabits hydrothermal vents on the dorsal epithelium of *Alvinella pompejana* or as a free-living bacterium. *N. profundicola* was isolated with polysulfide as the electron acceptor for respiratory growth, but is also able to utilize nitrate as an electron acceptor for growth. Interestingly, the *N. profundicola* genome lacks homologs of known enzymes used in nitrite reduction. Therefore, the pathway of nitrate reduction is likely to be novel. Genome annotation suggested that the reverse hydroxylamine ubiquinone redox module (rHURM) may be used for anaerobic cellular respiration in *N. profundicola* when nitrate is the terminal electron acceptor. The proposed rHURM pathway includes rHaoA and hcp/har which catalyze the reduction of nitrite to hydroxylamine, as an intermediate, and hydroxylamine to ammonium, respectively. In this study, cell growth was monitored in batch cultures under varied concentrations of sulfide, nitrate, formate, and H_2. Optimal concentrations were used for culturing cells in continuous culture and in batch culture for substrate addition assays. The four nitrogen compounds in the proposed pathway were measured in the substrate addition assays—nitrate, nitrite, hydroxylamine, and ammonium. Hydroxylamine was present in small concentrations during nitrate reduction to ammonium. When given as a substrate, hydroxylamine was directly and entirely reduced to ammonium (up to 5 mM). An anaerobic continuous culture apparatus for *N. profundicola* was developed to grow cells in steady state with different electron acceptors to eliminate growth rate variations that have affected prior gene expression measurements. The results

presented here are consistent with hydroxylamine as a free intermediate in nitrate reduction to ammonium in *N. profundicola*. Additional experiments including in vitro assays of enzymatic activity and gene disruption will be required to fully address the role of the proposed rHURM pathway in nitrate reduction.

PREFACE

The comparison of deeply-branching hydrothermal vent bacteria like *Nautilia* to recent-branching host-associated bacteria like *Campylobacter* gives insight into the evolution of metabolic pathways and pathogenicity. As will be detailed below, the epsilonproteobacterium *Nautilia profundicola* and members of the *Campylobacteriales* are predicted on the basis of genome annotation to possess a novel pathway for nitrate reduction. For host-associated *Campylobacteriales*, nitrate reduction may provide an alternative route for the disposal of electrons in the absence of oxygen for energy conservation. In anoxic conditions, this alternate metabolism may allow bacteria to persist, bolstering their pathogenicity by increasing their adaptability. The motivation to determine specific substrate requirements for *N. profundicola* was to obtained consistent growth rates and yields on nitrate. The ability to precisely control *N. profundicola* growth will enable us to better define the importance of the nitrate reduction pathway to *N. profundicola* and confirming the sequence of reactions. Furthermore, controlled growth will allow for more precise measurements of substrate-dependent gene expression that will help define the genetic components required for this pathway in *N. profundicola* and, by extension, other *Epsilonproteobacteria*.

Nitrate and nitrite can be reduced for respiratory and/or assimilatory purposes by bacteria. The first steps for all of these pathways are the reduction of nitrate to nitrite by nitrate reductase and the subsequent reduction of nitrite by nitrite reductase. Classical nitrite reductases are NirB and NrfA used in nitrite ammonification to reduce

nitrite to ammonium and NirS and NirK used in denitrification to reduce nitrite to nitric oxide (Rodionov et al. 2005; Stolz & Basu 2002). Interestingly, *N. profundicola*, a deeply branching member of the *Epsilonproteobacteria*, consumes nitrate and produces ammonium, but the genome does not contain any genes that encode homologs of the classical nitrite reductases described above (Campbell et al. 2009). Instead, the genome contains a gene whose product is homologous to hydroxylamine oxidoreductase (*hao*). Hydroxylamine is converted to nitrite by Hao as part of the hydroxylamine:ubiquinone redox module (HURM) in ammonium oxidizing bacteria (Klotz & Stein 2008; Jetten et al. 2009). Additionally, genes encoding a nitrate reductase (*napA*), a cytochrome c (*cycB*) and genes for a hybrid cluster protein (*hcp*) that has shown hydroxylamine reductase activity in *Rhodobacter capsulatus* (Cabello et al. 2004) were identified in the *N. profundicola* genome. From this combination of genes, it is feasible that the *N. profundicola* NapA reduces nitrate to nitrite, Hao reduces nitrite to hydroxylamine, and Har reduces hydroxylamine to ammonium for energy conservation and/or assimilation of nitrogen into biomass. While this pathway is feasible based on *N. profundicola*'s genome, it is only a prediction. Further support for this predicted reaction sequence is the fact that all reactions of the novel pathway are thermodynamically favorable (Table 1). That is, all steps may conceivably be coupled to the conservation of energy in biologically useful forms: proton motive force and/or ATP.

Electron accepting reaction	E^0 (V)	ΔG^0 (kJ mol^{-1})
$2H^+ + NO_3^- + 2e^- \rightarrow NO_2^- + H_2O$	0.873	-169
$5H^+ + NO_2^- + 4e^- \rightarrow NH_2OH + H_2O$	0.270	-104
$3H^+ + NH_2OH + 2e^- \rightarrow NH_4^+ + H_2O$	1.780	-344

Table 1. Standard reduction potentials and standard Gibbs free energy values of half-reactions for electron acceptors.

The presence of these genes and the favorable thermodynamics of reduction via a hydroxylamine intermediate led to the proposal of the pathway termed reverse-HURM (rHURM) in *N. profundicola* and other *Epsilonproteobacteria*. Three other *Epsilonproteobacteria—Campylobacter concisus, Campylobacter curvus,* and *Campylobacter fetus*—encode the aforementioned genes (Hanson et al. 2013; Kern & Simon 2009).

At the time this project started, nitrate dependent growth of *N. profundicola*, the stoichiometric transformation of nitrate to ammonium in cultures, and the assimilation of nitrate-derived ammonium into *N. profundicola* had been demonstrated. However, the role of hydroxylamine in nitrate reduction and global patterns of nitrate-dependent gene regulation in *N. profundicola* had not been clearly established. Therefore, the present study sought to determine 1) optimal growth conditions for *N. profundicola* on nitrate as the electron acceptor 2) the feasibility of hydroxylamine as an intermediate in nitrate reduction to ammonium in *N. profundicola* and 3) an anaerobic continuous culturing system for *N. profundicola* that would allow collection of RNA from cells growing at a steady rate with different electron acceptors.

Chapter 1

INTRODUCTION

Characteristics of *Epsilonproteobacteria*

The class of *Epsilonproteobacteria* is composed of well-known host-associated species isolated from clinical systems as well as free-living species isolated from environmental systems (Campbell et al. 2006). *Campylobacter* and *Helicobacter* are two genera of host-associated pathogenic bacteria found in the digestive tract of humans (Park 2002). *Wolinella succinogenes* is a non-pathogenic, host-associated bacterium found in cow intestines (Baar et al. 2003). *Campylobacter*, *Helicobacter*, and *Wolinella* genera are classified in the order *Campylobacteriales*, tend to be heterotrophic, and all contain virulence factors in their genomes. Homologs for pathogenicity found in these bacteria include hemolysin-related genes, adhesion factors, pili-generating proteins, invasins, antigenicity factors, and certain resistance genes and proteases (Baar et al. 2003). The ecological niche for these bacteria is the oral-fecal tract of animal hosts, characterized by high concentrations of organic substrates, microaerophillic conditions, and low salinity compared to seawater. In contrast, *Nautilia* and *Caminibacter* are genera belonging to the *Nautiliales* order. These bacteria are often autotrophic, and are found in sulfidic habitats like hydrothermal vent systems (Miroshnichenko 2004; Campbell et al. 2001; Campbell et al. 2006). Virulence genes are not abundant in the genomes of these bacteria and the ecological niches tend to be comparatively lower in organic substrates, higher in inorganic substrates, anaerobic, and high in salinity.

While the niches inhabited by physiologically diverse *Epsilonproteobacteria* vary, the class is related through shared 16S rRNA sequence and phylogenetic clustered bacteria seem to share ecotypes, environmental conditions, and metabolisms (Campbell et al. 2006). In Figure 1, animal host-associated clades found in clinical systems are shown in black: *Helicobacter* and *Campylobacter*. All other sequences can be placed into four phylogenetic clusters: *Nautiliales*, *Sulfurospirililium*, *Arcobacter*, and "environmental" retrieved from marine or terrestrial systems indicated by blue and green coloring, respectively. *Sulfurospirllum* and *Arcobacter* sequences from marine, terrestrial, and some clinical samples, branch deeper in the *Campylobacteriales*, showing an earlier evolutionary divergence than the *Helicobacter* and *Campylobacter* clusters that are dominated by clinical sequences. In addition, the deepest branching *Epsilonproteobacteria* belonging to the *Nautiliales* order thrive at hydrothermal vents where conditions are anoxic and sulfidic.

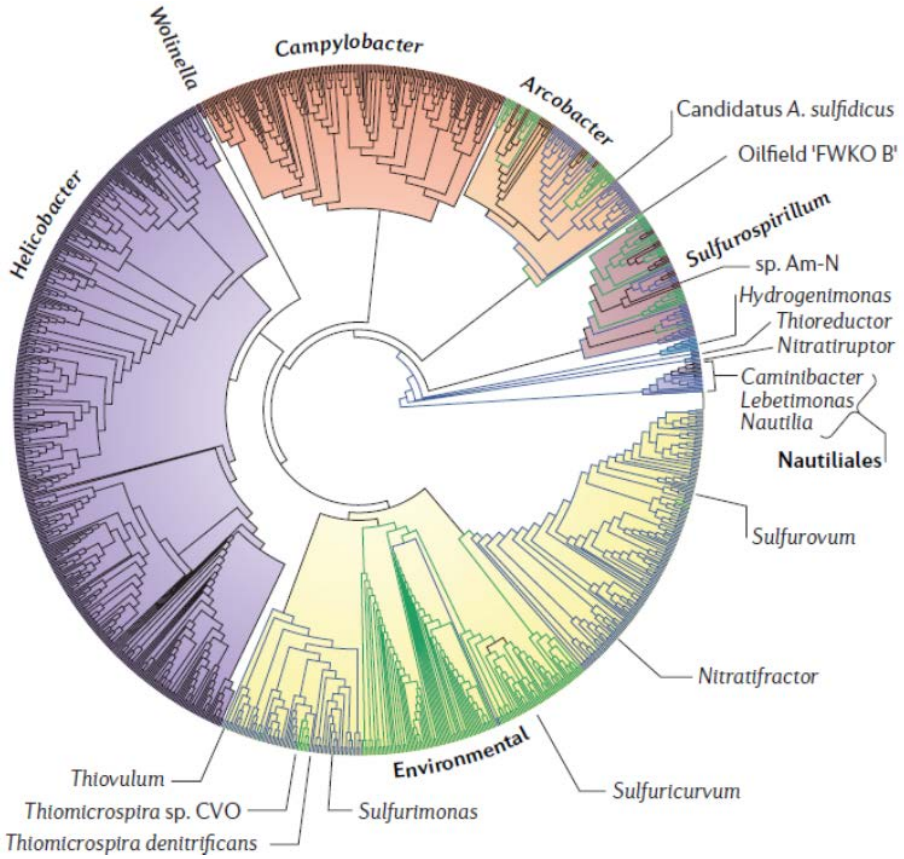

Figure 1. The diversity within the *Epsilonproteobacteria* class (Campbell, et al. 2006). The black brackets represent clinical sytems, the green brackets represent terrestrial systems, and blue brackets represent marine systems. The major groups identified are printed in bold.

Physical properties of hydrothermal vents

Hydrothermal vents are geothermal fissures in the seafloor that allow hot, nutrient rich fluid to flow from the earth's crust. Most hydrothermal vents are part of a submarine system along interfaces of tectonic plates. Seawater at a temperature of 2-4°C seeps into the permeable crust and is heated by underlying magma. The hot water becomes modified by reduced, inorganic compounds from surrounding crustal rock and gaseous compounds from magmatic fluid. Shifting tectonic plates create cracks, allowing hydrothermal fluid to plume into the deep ocean (Stein & Fisher 2003; Le Bris et al. 2006). The hydrothermal fluid reenters the seawater through vents at temperatures of 300-400°C (Koschinsky et al. 2008) and provides substrates for

cellular respiration (Haase et al. 2007). Chemolithoautotrophic bacteria use inorganic substrates to fuel their metabolism and produce organic substrates. At hydrothermal vent communities chemolithoautotrophs are the primary producers which provide organic substrates for higher trophic levels.

Epsilonproteobacteria at hydrothermal vents

Phylogenetic survey studies of 16S rRNA genes have shown that *Epsilonproteobacteria* are numerically dominant key players in the cycling of nutrients such as carbon, nitrogen, and sulfur at hydrothermal vents (Corre 2001; Campbell et al. 2001; Longnecker 2001; Voordeckers et al. 2008). Figure 2 shows the breadth of species that have been isolated from the hydrothermal vents. Even though the *Epsilon* class of *Proteobacteria* is very diverse, many members within the class use hydrogen as an electron donor (Gross et al. 1998; Smith et al. 2008). A feature of most *Epsilonproteobacteria* is their ability to use more than one electron acceptor (Campbell et al. 2001; Takai et al. 2005), including nitrate, oxygen, and various forms of sulfur (Fig. 2). Metabolic diversity is a key mechanism for survival at hydrothermal vents (Grzymski et al. 2008). The dominance of *Epsilonproteobacteria* may be explained by this adaptability in fluctuating substrate conditions.

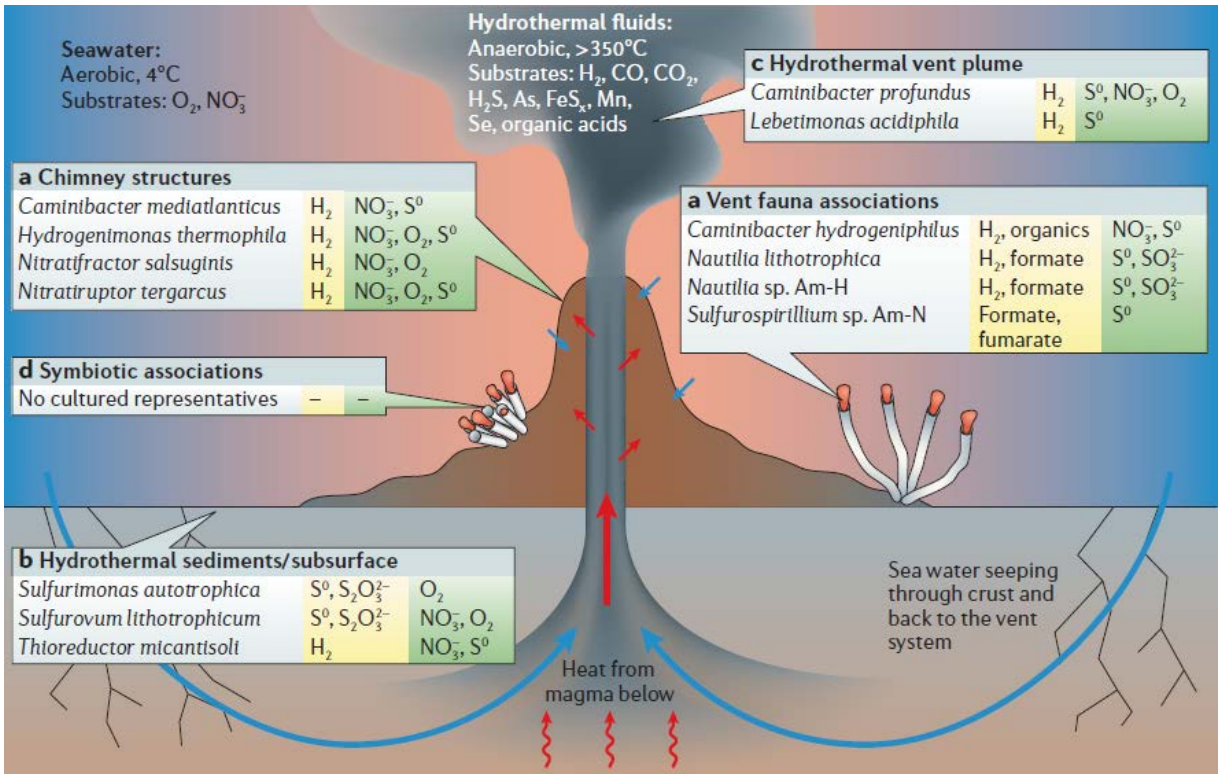

Figure 2. Hydrothermal vent *Epsilonproteobacteria* and electron donors/acceptors they use (Taken from Campbell, et al 2006).

N. profundicola as a model system

In 1999, the first hydrothermal vent Epsilonproteobacterium was isolated from 13°N along the East Pacific Rise (Campbell et al. 2001). Strain AmH was isolated from the dorsal epithelium of *Alvinella pomejana*, but was also found as a free-living bacterium in mats on vent surfaces(Campbell et al. 2001). *A. pompejana* is a polycheate worm that lives in the transition zone of hydrothermal fluid and seawater. Bacteria associated with *A. pompejana* are considered moderate thermophiles in comparison to other *Epsilonproteobacteria* found in the vent plume (Grzymski et al. 2008).

AmH belongs to the deepest branching order of *Epsilonproteobacteria*, the *Nautiliales* (Campbell et al. 2001). Smith et al. (2008) named the characterized AmH

strain *Nautilia profundicola*, meaning an inhabitant of the abyss. *N. profudicola* has slight-curved rod morphology with dimensions of 0.4 by 0.3µm. The optimal temperature for growth is 45°C. The bacterium grows on minimal medium with the addition of ammonium, elemental sulfur, and hydrogen sulfide or nitrate and hydrogen sulfide and a 20% H_2:80%CO_2 gas mixture or a 20%N_2:80%CO_2 gas mixture with the addition of formate.

The genome of *N. profundicola* was sequenced and annotated (Campbell et al. 2009). The single circular chromosome was 1.7 mb, which is short in comparison with other free-living *Epsilonproteobacteria*, and lacked duplicated gene clusters which explains the why genome is so compact. Annotation revealed two putative fumarate reductase/succinate dehydrogenase (Fdr/Sdh) gene complexes present in other chemolithoautotrophs using the reductive tricarboxylic acid (rTCA) cycle. CO_2 obtained directly from the environment or from the oxidation of formate ($COOH^-$) is used as a source of carbon.

The presence of multiple encoded hydrogenase homologs and one presumptive formate dehydrogenase agrees with the physiologically determination that the oxidation of hydrogen or formate is used as a source of electrons for the electron transport chain (Fig. 3).

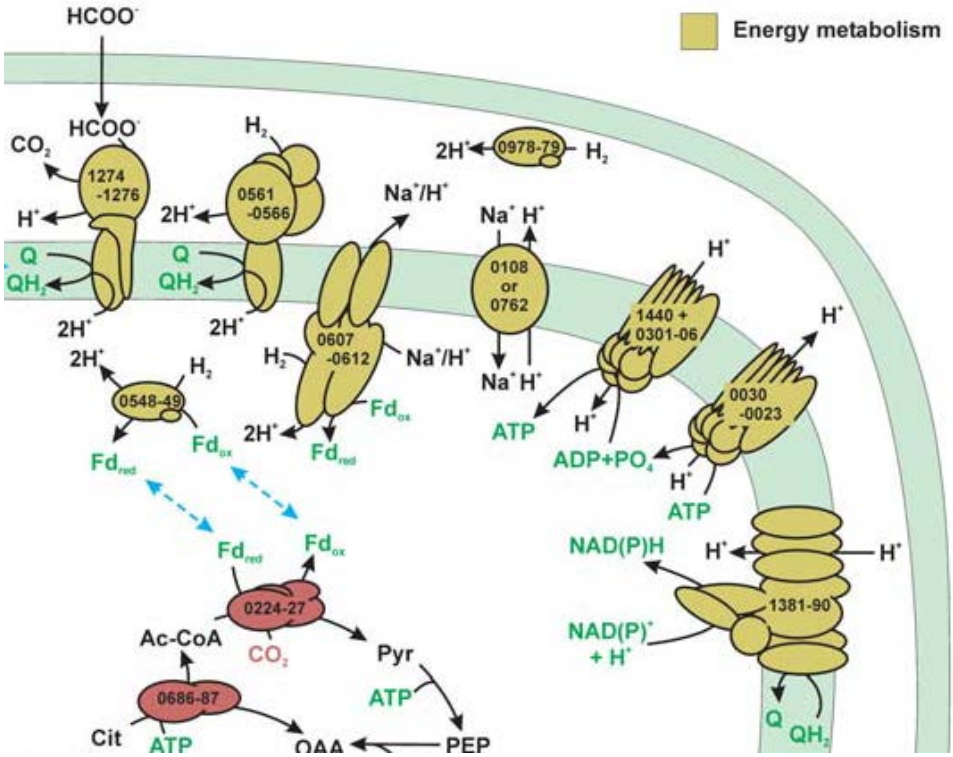

Figure 3. The use of hydrogen or formate as an electron donor (Campbell, et al. 2009).

N. profundicola uses hydrogen or formate as the electron donor, both of which are common electron donors for nitrate reducing *Epsilonproteobacteria* (Campbell et al. 2006). Hydrogen gas donates two electrons to the quinol pool and produces two hydrogen ions while formate is split by formate dehydrogenase, donating one electron and producing one hydrogen ion (Takai et al. 2005; Campbell et al. 2009). Even though the products differ in quantity, the energetics per molecule of hydrogen/formate calculated at standard conditions from the Nernst equation is the same (Table 2). Therefore, hydrogen and formate are thermodynamically equivalent as electron donors.

Coupled Reaction	ΔG^0 (kJ mol^{-1})	per H_2 or CH_2O_2
H_2 Oxidation		
$4H_2 + NO_3^- + 2H^+ \rightarrow NH_4^+ + 3\ H_2O$	-937	-234
$3H_2 + NO_2^- + 2H^+ \rightarrow NH_4^+ + 2\ H_2O$	-688	-229
$H_2 + S_{3\text{-}8}^{2-} \rightarrow H^+ + S_{2\text{-}7}^{2-} + HS^-$	-28	-28
Formate Oxidation		
$4CH_2O_2 + NO_3^- + 2H^+ \rightarrow 4CO_2 + NH_4^+ + 3\ H_2O$	-946	-237
$3CH_2O_2 + NO_2^- + 2H^+ \rightarrow 3CO_2 + NH_4^+ + 2\ H_2O$	-695	-232
$CH_2O_2 + S_{3\text{-}8}^{2-} \rightarrow CO_2 + S_{2\text{-}7}^{2-} + H^+ + HS^-$	-32	-32

Table 2. Thermodynamics of the coupled reaction for respiration in *N. profundicola* (Hanson et al., 2013)

Additionally, nitrate reduction is more advantageous than polysulfide reduction under standard conditions. Nitrate reduction yields 7 to 8 times the amount of energy yield per mole of electron donor than polysulfide reduction: ~ -230kJ mol^{-1} from nitrate reduction compared to ~ -30kJ mol^{-1} from polysulfide reduction (Table 2).

N. profundicola contains genes encoding a membrane-bound polysulfide reductase (*psr*) suggesting that polysulfide can be used as an electron acceptor in the electron transport chain which corroborates the isolation and growth of AmH on this substrate. Two sulfur species are utilized by *N. profundicola*: hydrogen sulfide and polysulfide. Elemental sulfur commonly occurs as an eight-membered ring structure and has low solubility in water; however, when hydrogen sulfide is present, the ring structure can be reductively cleaved, producing a linear polysulfide (Steudel & Eckert 2003; Kamyshny et al. 2007). Polysulfide outside the cell enters the periplasm as a substrate for cellular respiration or continues into the cytoplasm for assimilation (Klimmek et al. 1999). The reduction of polysulfide is a process that pumps hydrogen ions into the periplasm and increases the proton gradient that allows ATP synthesis. Hydrogen sulfide ions can be obtained directly from the environment or produced in the cytoplasm from polysulfide (Fig. 4). Sulfur is potentially assimilated via a NAD(P)H polysulfide oxidoreductase (GAmH_0923) that reduces polysulfide to

produce sulfide. The genome encodes two potential sulfide assimilation genes for production of cysteine and methionine, O- acetyl-serine sulfhydrylase (GAmH_1543) and O-acetyl-L- homoserine sulfhydrylase (GAmH_0859) (Hanson et al. 2013).

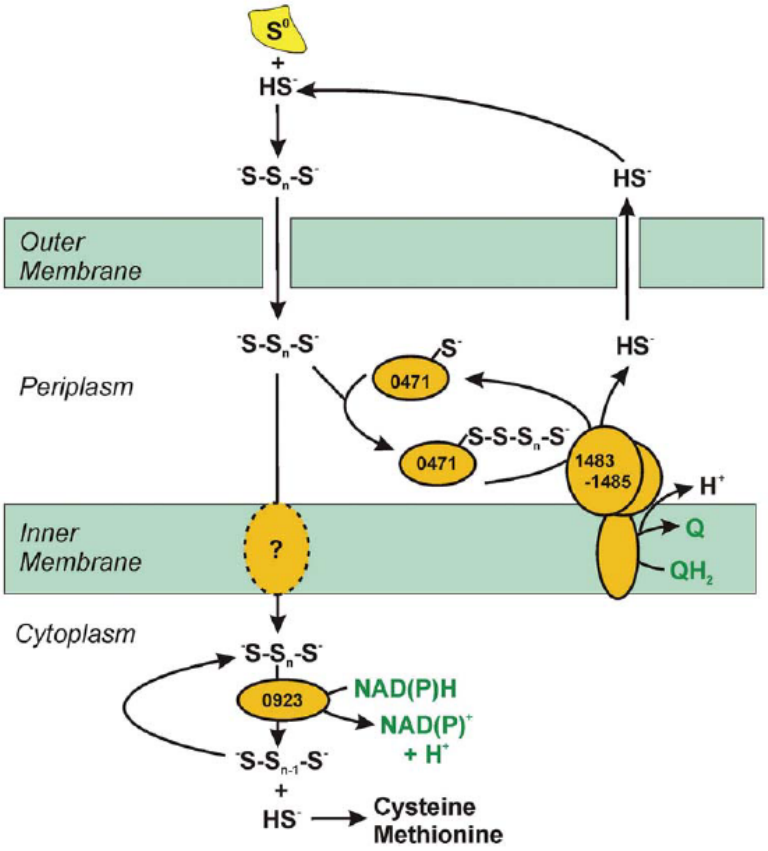

Figure 4. Sulfur metabolism in *N. profundicola* (Taken from Campbell, et al. 2009).

Nitrogen is assimilated via two AmtB transporters (GAmH_0198 and GAmH_0380) that make ammonium available in the cytoplasm, in addition to NADPH- glutamate synthase (GAmH_1535/ GAmH_1024) and glutamine synthetase (GAmH_0480), which incorporate nitrogen into the biomass. While dissimilatory periplasmic nitrate reductase (*napAGHBFD*, GAmH_0533-0527) was encoded, canonical nitrite reduction genes were absent despite the physiological determination that *N. profundicola* consumes nitrate and produces ammonium. This quandary led to further genome analysis and the proposal of the novel rHURM pathway.

Proposed rHURM pathway

Denitrification and nitrate ammonification are nitrate reduction pathways which use a nitrate reductase followed by a nitrite reductase to produce either nitrous oxide or ammonium, respectively (Cabello et al. 2004; Kern & Simon 2009). Most bacteria use a reductase such as Nir or Nrf for the reduction of nitrite to ammonium. Dissimilatory nitrate reduction to ammonium (DNRA) is a microbial process that uses Nir or Nrf in the reduction of nitrate to ammonium to oxidize organic carbon for energy conservation. Two genera that employ DNRA are *Aeromonas* and *Vibrio* (Bonin 1996; Takeuchi 2006). Other chemolithoautotrophic bacteria reduce nitrate to ammonium to generate proton motive force for respiration, not for dissimilation, and use Nrf, or reduce nitrate to ammonium to assimilate ammonia into biomass and use Nir (Smith et al. 2007; Stolz & Basu 2002). *W. succinogenes* and *Sulfurospirillum deleyianum* are two chemolithoautotrophic species that couple nitrate reduction to sulfide oxidation for respiration (Strohm et al. 2007). There is also some evidence to support the utilization of nitrite reduction as a nitrosative stress response in order to rid the cell of excess nitrite, which can interrupt other functional pathways (Kern et al. 2011).

N. profundicola lacks all known nitrite reductase homologs but does convert nitrite to ammonium (Campbell et al. 2009). Ammonium, obtained from the environment or produced through a nitrate reduction pathway, is used for biosynthesis of nitrogenous compounds in *N. profundicola* but cannot be utilized for respiration (Smith et al., 2008). While the substrate and product is known, the specific nitrite reduction enzyme and the chemical intermediates have not previously been described.

The hydroxylamine ubiquinone redox module (HURM) describes a group of genes utilized by bacteria for aerobic hydroxylamine oxidation (Bergmann et al. 2005). Ammonia monooxygenase (AMO) uses electrons from ubiquinol to split oxygen which then attack ammonia to form hydroxylamine (NH_2OH), water, and ubiquinone (oxidized form):

$$NH_3 + O_2 + \text{Ubiquinol } (2e^-) \leftrightarrow NH_2OH + H_2O + \text{Ubiquinone}$$

Hydroxylamine oxidoreductase (HAO) further oxidizes hydroxylamine to form nitrite and return the electron carrier to the reduced form, ubiquinol (Klotz & Stein 2008; Klotz et al. 2008):

$$NH_2OH + 2\ \text{Ubiquinone} + H_2O \leftrightarrow NO_2^- + 2\ \text{Ubiquinol } (4e^-)$$

If ammonium can be oxidized to nitrite via a hydroxylamine intermediate, could the opposite reaction be possible via "reverse HURM" enzymes?

The annotation of the *N. profundicola* genome showed genes similar to that of the HURM. These genes include a homolog for hydroxylamine oxidoreductase (*haoA* GAmH_1248) and a cytochrome c-like protein in the NapC family (*cycB* GAmH_0536). HAO in *N. profundicola* is proposed to work in reverse. The $[HaoA]_3$ complex has shown reverse activity, catalyzing the reduction of nitrate to hydroxylamine when reductant is available to donate electrons for the reaction (Kostera et al. 2008). In addition, two ammonium transporters (*amtB*-1GAmH_ 0198 and *amtB-2* GAmH_0380), an NAD(P)H-dependent hydroxylamine reductase/hybrid cluster protein (*har/hcp* GAmH_1044), and an NAD(P)H regenerating iron-sulfur cluster (GAmH_1302) are present as well (Campbell et al. 2009; Kern et al. 2007; Klotz et al. 2008).

As shown in Figure 5, the proposed pathway begins with the reduction of nitrite via reverse HAO and cytochrome c protein allowing the transfer of electrons from the ubiquinol pool to form hydroxylamine. Hydroxylamine is transferred into the cytoplasm by one of two ammonium transporters where Har/Hcp reduces the hydroxylamine to ammonium in the cytoplasm using NAD(P)H as the reductant (Campbell et al. 2009). Ammonium is assimilated via glutamine synthetase and glutamine:2-oxoglutarate aminotransferase or is transported out of the cell. Hanson et al., 2013 showed that rHURM genes were uprregulated when grown on nitrate and while nitrate is utilized for both energy conservation and nitrogen, the production of excess exogenous ammonium and the lack of repression seen in rHURM genes when given both nitrate and ammonium suggests that nitrate is used primarily for energy conservation. The purpose of the current study was to improve cell growth on nitrate medium, obtain chemical support for hydroxylamine as an intermediate in the proposed pathway, and grow cells continuously in steady state at a constant growth rate.

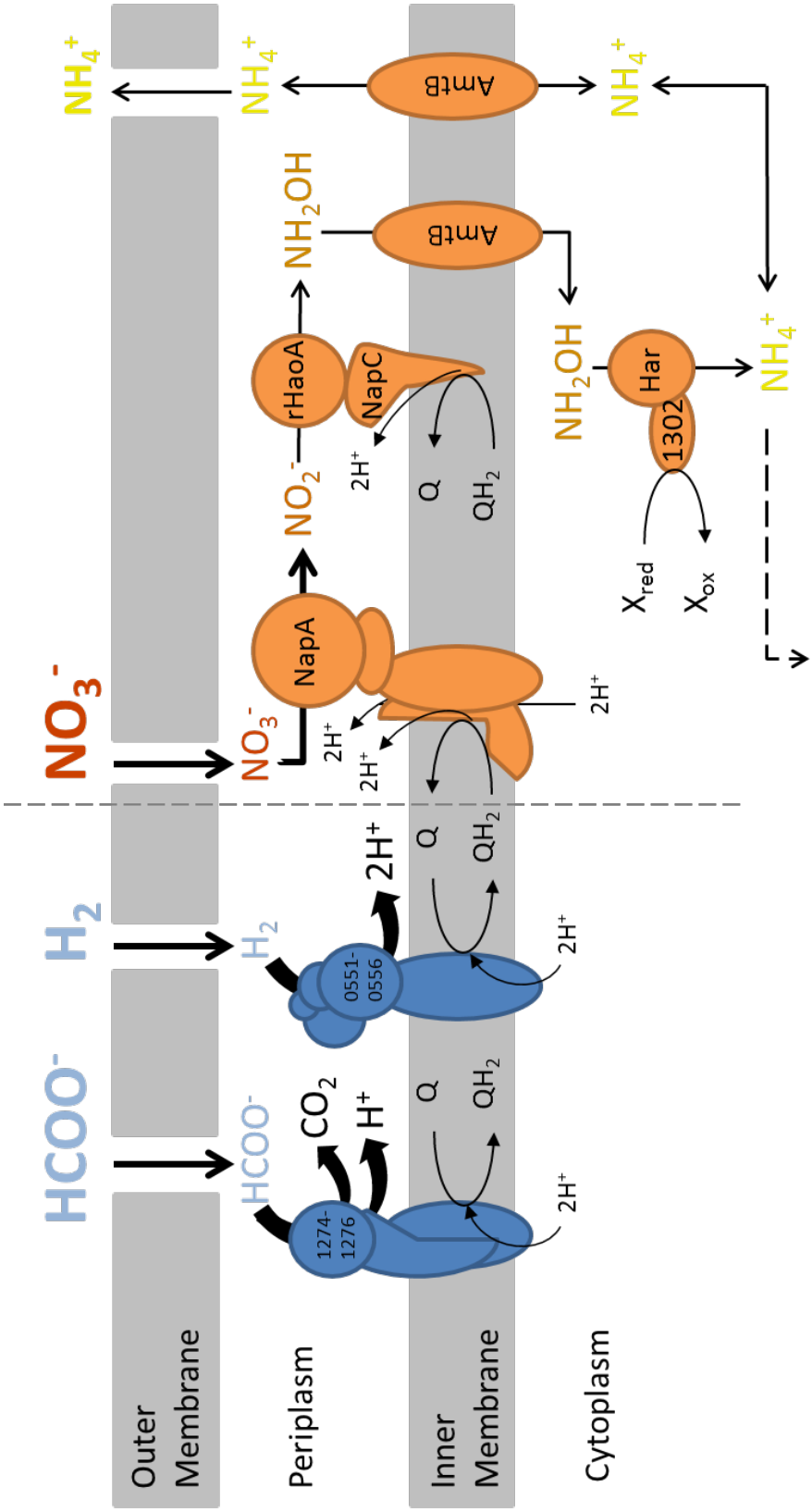

Figure 5. Nitrogen metabolism *N. profundicola*: Nitrate reduction to ammonium for cellular respiration and assimilation via the proposed rHURM pathway.

Chapter 2

METHODS

Growth and Optimization Experiments

Medium composition and preparation

Growth curves were used to track cell yield and specific growth rate. *N. profundicola* was grown on anoxic minimal autotrophic medium composed of 200 g NaCl, 30 g $MgCl_2 \cdot 6\ H_2O$, 1.5 g $CaCl_2 \cdot 2\ H_2O$, and 5 g KCl per liter. Nanopure water was used to prepare all medium and solutions. The medium was autoclaved for 20 min at 15 PSI, 121°C and immediately placed under an 80% N_2: 20% CO_2 headspace while cooling on ice for 15 minutes. One ml per L of the following sterile, anoxic solutions was added to the basal medium: 1.47 M KH_2PO_4, selenite-tungstate solution (4 g NaOH, 3.025 mg $NaSeO_3$, and 8 mg $Na_2WO_4 \cdot 2\ H_2O$ per liter), and trace elements solution (2.1 g $FeSO_4 \cdot 7H_2O$ dissolved in 12 ml of 7.69 M HCl, 30 mg H_3BO_3, 100 mg $MnCl_2 \cdot 4H_2O$, 190 mg $CoCl_2 \cdot 6H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, 2 mg $CuCl_2 \cdot 2H_2O$, 144 mg $ZnSO_4 \cdot 7H_2O$, and 36 mg $NaMoO_4 \cdot 2H_2O$ per liter). For small batch cultures, the final solution was dispensed into 18 x 150 mm Balch tubes or serum bottles made of borosilicate glass under 80% N_2: 20% CO_2 gas and sealed with a butyl rubber stopper and aluminum crimp cap. For large batch cultures (500 ml), the final solution was stored under 80% N_2: 20% CO_2 gas in a 1 L Kimax GL 45 media bottle with a GL 45 PTFE faced silicone septa (Pyrex™) and a GL 45 screw thread open top cap (Corning™ Pyrex™). If medium was not used within 8 hours of preparation, the bottle was stored in a vinyl anaerobic chamber (Coy). The medium additions tested

for growth optimization were sulfide as a reductant and S-source (0.016 mM – 2 mM), nitrate as an electron acceptor (5 mM – 20 mM), $\%H_2$ (5 – 80%) and formate (2.5 mM – 60 mM) as electron donors, and 25 mM 2-(N-morpholino) ethanesulfonic acid (MES) for pH control.

Culture preparation

The basal medium was amended with sodium sulfide, sodium nitrate and an electron donor (80% H_2 or 80% H_2+formate) 15 minutes prior to inoculation. Cells were grown from a glycerol freezer stock stored at –80°C in 10 ml culture medium and subsequently transferred into 10 ml fresh medium at least once more before each experiment to ensure cultures were viable and robust. *N. profundicola* was grown in 10 ml culture medium volume for sample collection. Each inoculation is a 1:10 ratio of cells to fresh medium. Samples were taken at initial inoculation and increments during log phase up to 24 hours.

Direct Cell Counts

Cell samples were diluted in 1X phosphate buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 800 ml water, pH 7.4), fixed with 2% paraformaldehyde and stained with 20 ng/ml 4',6-diamidino-2-phenylindole (DAPI) (Porter & Feig 1980). Samples were filtered onto 0.2 µm pore size black polycarbonate filters and mounted onto microscopes slides with 10 µl of oil and a coverslip. Samples not analyzed immediately were stored at -20°C until time of analysis. An Olympus Provis AX70 microscope using Image Pro Plus, Media Cybernetics, identified DAPI stained cells with a filter set of excitation of 360 +/- 40

and emission of 460 ± 50. Images were captured with a SPOT RT monochrome camera from Diagnostics, Inc. and cell density was calculated in the Microbe Counter program using the following equation (Cottrell & Kirchman 2003).

Substrate Conversion Assays

Preparation of cells

For each resting cell assay, a 10% glycerol freezer stock was transferred to fresh medium at a 1:10 dilution and subsequently transferred twice (final transfer into 500 ml batch culture). Cultures used in the assays were grown on 80%H_2: 20%CO_2 + 20 mM formate. Cells were harvested in stationary phase (8 hours post-inoculation) and centrifuged at 10,000 x *g* in a Sorvall RC-5B refrigerated super speed centrifuge for 10 minutes. The supernatant was discarded and the cells were washed twice with ~100 ml nitrate and sulfide free basal medium and pelleted. Cells were eluted with nitrate and sulfide free basal medium to a final concentration around $4x10^8$ cells/ml. Cell suspension was transferred to Balch tubes in the anaerobic chamber and stoppered. The headspace in each tube was exchange for 1 minute with 20% CO_2: 80% H_2, pressurized to 100 kPa, and incubated for 2 hours at 45°C at 250 rpm in the shaker incubator. Test substrate was added and samples were taken every 5 or 10 minutes for 40-60 minutes. Aliquots of spiked cell suspension were filtered through a 0.2 µm nylon filter and the concentrations of nitrate, nitrite, ammonium, and/or hydroxylamine were measured in the filtrate as described below.

Substrate quantification

Samples for nitrate and nitrite analysis were kept frozen at -20°C until analysis. Nitrate and nitrite concentrations were quantified on a Shimadzu Scientific class VP HPLC system equipped with a SPD-10AV Ultra Violet Absorbance detector measuring at 210 nm using a Prevail Organic Acids 5 μm column eluted with 25 mM potassium phosphate buffer at pH 2.5. Detection limits were ~50 μM for nitrate and 200 μM for nitrite.

Ammonium samples were prepared immediately using the reagent diethyl ethoxymethylene malonate (DEEMM) to derivatize ammonium into an aminoenone for HPLC quantification (Gómez-Alonso et al. 2007). Standards were prepared from ammonium chloride in a range of 0.125 mM to 5 mM. Ammonium samples and standards were quantified with a Shimadzu Scientific VP HPLC system equipped with a Shimadzu Scientific SPD-M10A VP Diode Array detector using an Prevail C18 5 μm column eluted with buffer A: 25 mM acetate, pH 6.8, and buffer B: 80:20 acetonitrile:methanol (Table 3). The detection limit was 122 μM for ammonium.

Time (min)	0.0	10.00	15.30	16.80	32.50	36.50	39.00	41.00	42.30	42.40	45.00
Eluent A (%)	90	90	83	83	80	28	18	0	0	90	90
Eluent B (%)	10	10.0	17.0	17.0	20.0	72.0	82.0	100.0	100.0	10.0	10

Table 3. Eluent gradient used for HPLC determination of DEEMM derivatized ammonium concentrations.

Hydroxylamine was quantified through a colorimetric assay. Samples were prepared immediately through the assay previously described (Frear and Burrell, 1955) and measured on a diode array spectrophotometer (Hewlett Packard 8452A) and using OLIS 4.8.42 SpectralWorks software. Hydroxylamine hydrochloride standards ranging from 0.1 mM to 2 mM were prepared with the same method. The detection limit was 4 μM. Samples were scanned for absorbance between 200 and 800 nm and

an average of 10 scans per sample were collected for each sample at the maximum absorbance peak for hydroxylamine at 706 nm.

A control of medium without cells was tested. One tube of nitrate and sulfide free medium was warmed at 45°C at 250rpm for 1 hour. Hydroxylamine was added at a final concentration of 1 mM and samples were taken every 5 minutes from 0-45 minutes.

Inhibitors for specific enzymes suggested in the BRENDA database (http://www.brenda-enzymes.info/) were used in an attempt to disrupt specific enzymatic reactions and increase the concentration of a specific product. Inhibitors were added 15 minutes prior to addition of substrate. Methionine Sulfoximine (MSX) at 100 µM was used to inhibit the GS/GOGAT system to block ammonium assimilation. Sodium thiocynate at 100 µM and *o*-phenanthroline at 1 mM were used as potential inhibitors of Har/Hcp to block the reduction of hydroxylamine. *N. profundicola* was grown in batch culture in the presence and absence of each inhibitor to test the ability of each inhibitor to prevent nitrate dependent growth *of N. profundicola*.

Anaerobic Continuous Culture

Chemostat setup

A detailed schematic of the anaerobic continuous culture system developed here is provided in Figure 6. A No. 3 tap was used to thread holes into GL 45 caps. The medium bottle cap had 2 threaded holes and the culture cap had 4 threaded holes, all fitted with female luer bulkheads ¼-28 UNF thread to 1/16" hose barbs. The female luer bulkheads were connected to male luers with lock rings to 1/16" hose barbs on the exterior of the cap assembly. FDA-Compliant Viton tubing with an inner

diameter of 1/16” and an outer diameter of 1/8” was chosen for its impermeability to gas and was connected to the fitted barbs as shown in the schematic (Fig. 6). Gauge 24 copper wire was used to secure the tubing to barb connections. A Gilson Minipuls® 3 peristaltic pump with a 4-channel pump head (only 2 channels needed) was used to control the inflow of medium to the culture and outflow of culture to the spent medium container. The GL 45 caps were fitted with silicone rubber sealing O-ring (Pyrex®) to maintain anoxic conditions in the medium bottle and the culture vessel. A Fisher Scientific™ Isotemp™ Digital Stirring Hotplate was used for temperature control and culture agitation. A Pyrex® glass container filled with DI water was used to stabilize temperature at 42°C.

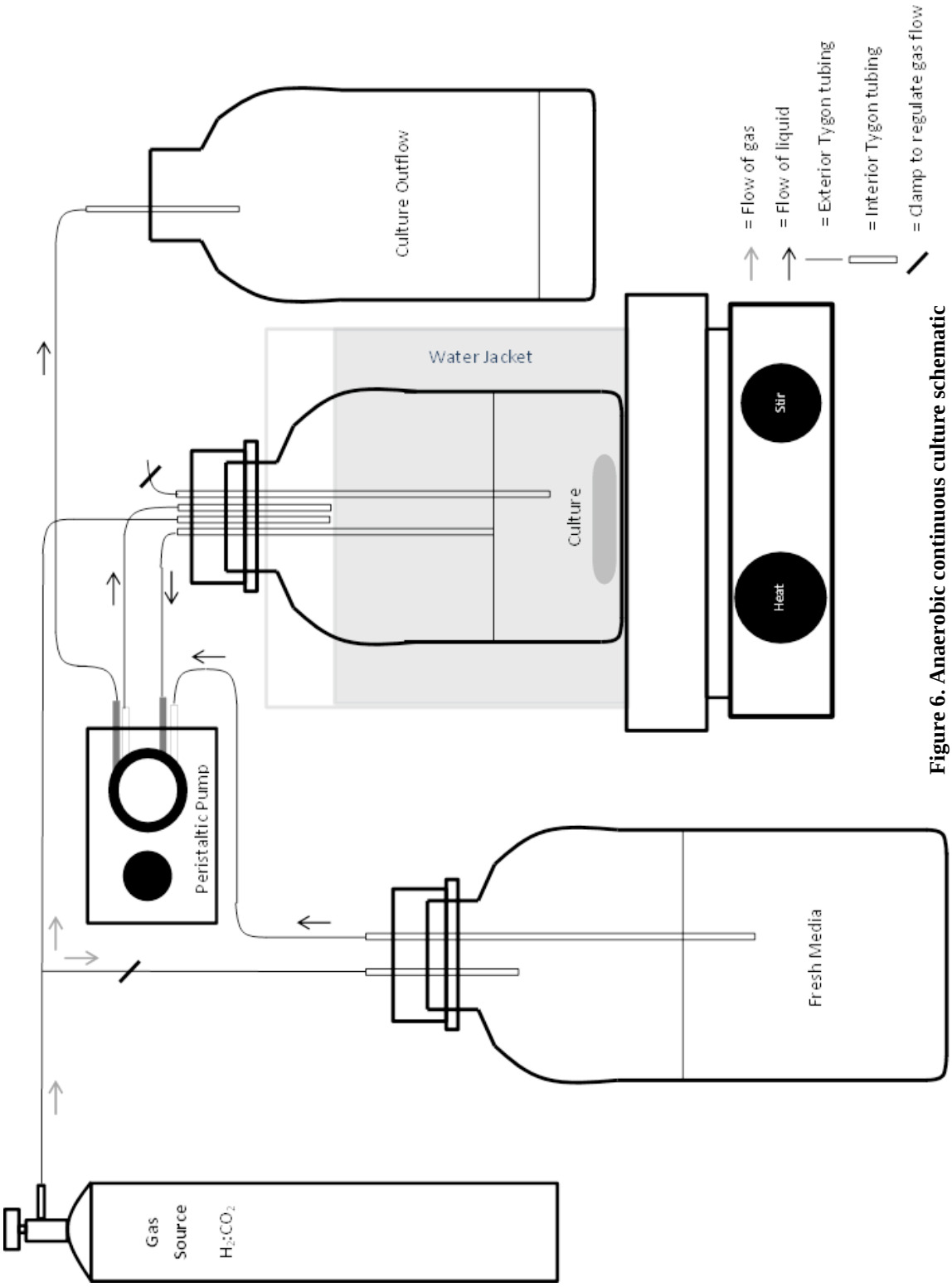

Figure 6. Anaerobic continuous culture schematic

Culture preparation

The assembled apparatus was autoclaved prior to culturing. Medium was prepared as described above in 500ml batches. A 1 ml freezer stock was used to inoculate a 10 ml batch culture that was allowed to grow for 2 hours. The culture was used to inoculate 60 ml of fresh medium. After 2 hours, the culture was transferred to the 100ml Kimax GL 45 media bottle in a anaerobic chamber (Coy) and the vessel was sealed with a GL 45 PTFE faced silicone septa (Pyrex™) and a GL 45 screw thread open top cap (Corning™ Pyrex™). Empty bottles were used as proxies for flushing the tubing and cap apparatus with 80% N_2: 20% CO_2 for 10 minutes at 5 PSI and then the outflow and sampling port were clamped to restrict gas flow. Nitrate (20 mM) was added to the medium inflow bottle through the septum and the inflow cap was placed loosely on the bottle. The culture vessel cap was placed loosely on the 100 ml culture bottle and both the medium inflow and culture vessel bottles were allowed to gas under 80% H_2: 20% CO_2 for 5 minutes. Sulfide (0.5 mM) was added to the medium inflow bottle and the caps to both bottles were tightened. After 15 minutes, the flow rate was started at 20 ml/hr. Optical density (OD) was measured over time at 600nm with an Eppendorf® BioPhotometer to monitor growth rate.

Chapter 3

RESULTS

Growth medium optimization

To improve the growth of *N. profundicola* on nitrate, components of the defined medium were tested for optimum concentration. Cell yields taken in stationary phase represent the amount of substrates that were converted into biomass. Specific growth rates were taken in log phase and represent the exponential increase of biomass over time. For sulfide and nitrate experiments, biological duplicate samples were tested. For formate and H_2, biological triplicates samples were tested and standard deviation are shown in Figures 9 and 10. Generally, cell yield and specific growth rate as functions of substrate concentration in batch culture can be described by Monod kinetics where cell yield and growth rate increase with substrate concentration until the cells reach their physiological maximum (K_{max}) and cannot increase cell yield or growth rate in the presence of increased substrate concentration.

Optimized Sulfide Concentration

Prior work had shown that sulfide could be used in place of polysulfide as a S-source for *N. profundicola*. Therefore, I investigated whether there was an optimal concentration of sulfide. In order to determine the physiological requirement for sulfide as a sulfur source, sulfide was not added during medium preparation; rather it was added to each tube 15 minutes prior to inoculation. Sulfide concentration affected both cell yield and growth rate in a similar way. Both increased as a function of

sulfide concentration up to 0.4 mM sulfide (Fig. 7). Cell yield ($5.46x10^8$ cells ml^{-1}) and growth rate (0.15 h^{-1}) at 0.4 mM did not maintain at the K_{max} for sulfide concentration greater than 0.4 mM. Instead, 2 mM sulfide caused a decrease in both cell yield ($9.43x10^7$ cells ml^{-1}) and growth rate (0.05 h^{-1}), indicating that sulfide was necessary to fulfill physiological sulfur requirements but was also detrimental to cells at 2 mM. Based on these results, medium was amended to 0.5 mM sulfide for all subsequent experiments.

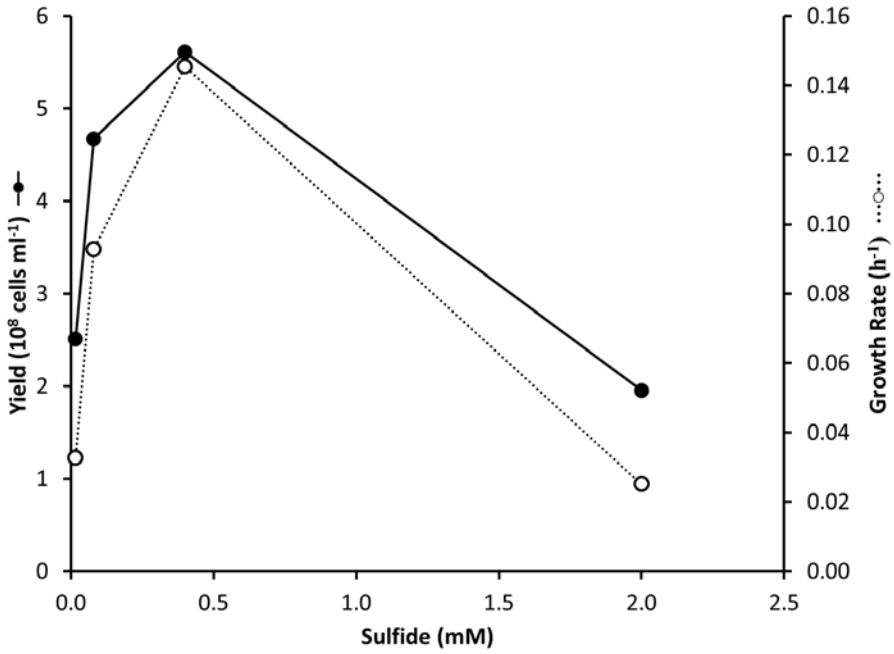

Figure 7. Effect of sulfide concentration on stationary phase cell yield (—●—) and log phase growth rate (···○··).

Optimized Nitrate Concentration

N. profundicola could utilize nitrate as the sole electron acceptor and nitrogen source. Nitrate was positively associated with cell yield but not growth rate. Cell yield increased from 5 mM nitrate to 10 mM nitrate and maintained at the observed yield ($4.39x10^8$ cells ml^{-1}), showing that cell yield is a function of nitrate concentration

when nitrate is <10 mM (Fig. 8). Growth rate was independent of nitrate concentration in the range of concentrations tested. The lowest concentration tested, 5 mM, was enough to satisfy the nitrate requirement for biomass production in exponential phase and increased concentrations of nitrate did not further stimulate biomass production. In this experiment, K_{max} is reached at 10 mM; however, because there was no adverse effect of the higher nitrate concentration, 20 mM nitrate was used in subsequent experiments. This was to prevent nitrogen starvation in conditions conducive to higher growth rate as seen in proceeding substrate optimization conditions which led to higher growth rate and yield.

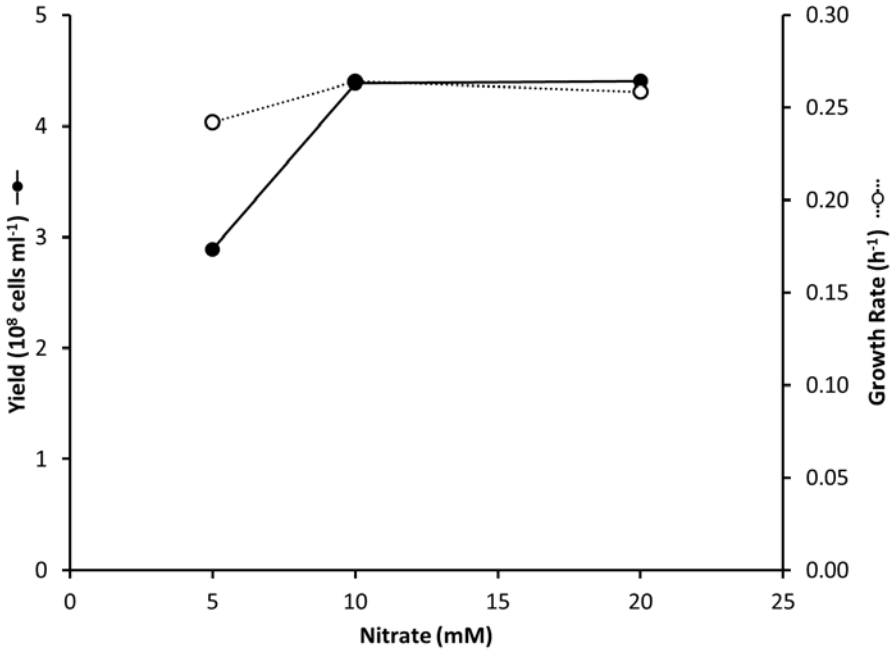

Figure 8. Effect of nitrate concentration on stationary phase cell yield (—●—) and log phase growth rate (···○···).

Optimized Formate Concentration

Formate was given as an electron donor but also could have been utilized as a carbon source. Formate as the sole electron donor was not sufficient for growth when

nitrate was given as the electron acceptor with 80% N_2: 20% CO_2 in the headspace; therefore, the cells were grown on 80% H_2: 20% CO_2 with the addition of formate at varying concentrations. Both cell yield and growth rate increased as a function of formate concentration up to 20 mM. Cell yield decreased with formate concentrations greater than 20 mM. Growth rate decreased slightly with formate concentrations greater than 20 mM but did not follow the steep decline with concentration seen in cell yields (Fig. 9). This suggests that concentrations of formate above 20 mM do not deter growth in log phase but have a negative effect on cells post-log phase.

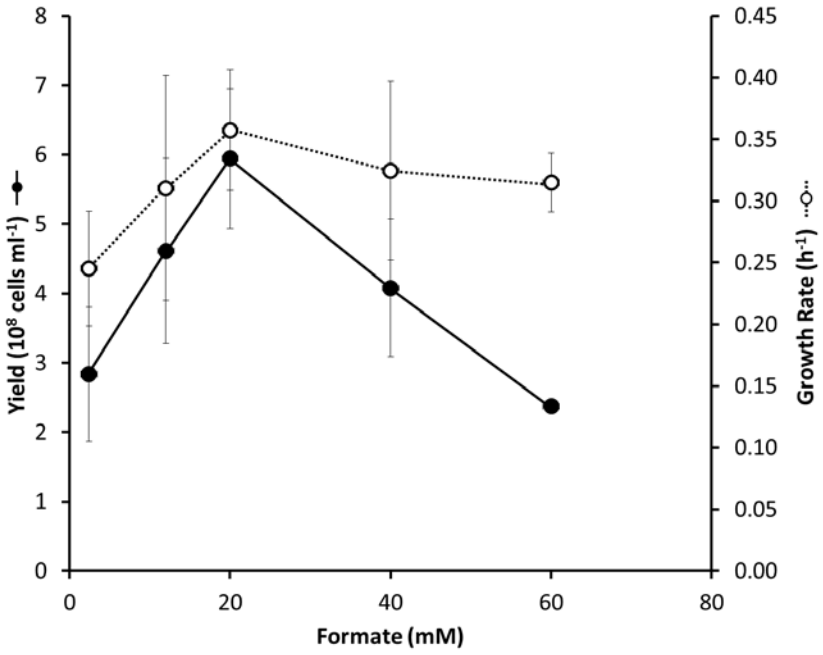

Figure 9. Effect of formate concentration in the presence of 80%H_2 on stationary phase cell yield (—●—) and log phase growth rate (···○···).

MES Buffer Addition

Given the speculation that pH might be a factor in formate toxicity, the addition of a strong buffering agent was investigated. Therefore, the pH of *N. profundicola* cultures was monitored and was found to increase from an initial pH of 6

to 8 within 24 hours. To minimize the increase in pH during growth, MES buffer (2-(N-morpholino) ethanesulfonic acid) with a pH of 6 was added to medium during preparation at 25mM final concentration. The addition of MES did not completely stabilize the pH during growth; however, but pH was checked at random at end point growth and the observed pH in high yield cultures was from 6.8-7.1.

Optimized H_2 Concentration

Hydrogen was either given as the sole electron donor at a concentration of 80% H_2 or in varying concentrations with 20 mM formate. Both cell yield and growth rate increased from 5% to 40% H_2 and maintained the observed K_{max} (~$1x10^9$ cells ml^{-1}, 0.32 h^{-1}) at 80% H_2 in the presence of formate (Fig. 10). There was no adverse effect of increasing H_2; however, H_2 as the sole electron donor at 80% had a slightly lower growth rate and cell yield than 80% H_2 and 20 mM formate.

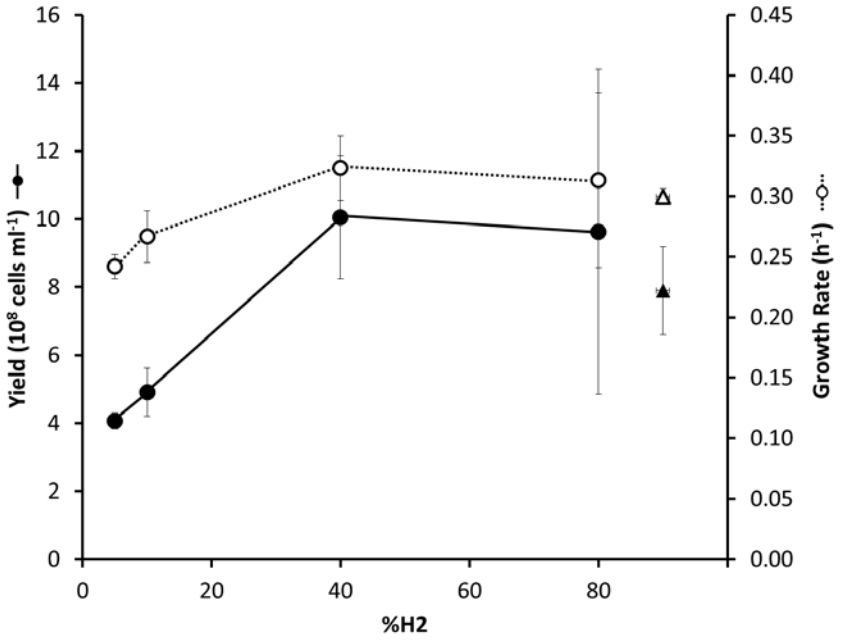

Figure 10. Effect of %H_2 concentration in the presence of 20 mM formate on stationary phase cell yield (—●—) and log phase growth rate (···○···). Growth on H_2 as the sole electron donor is shown for comparison of stationary phase cell yield (Δ) and log phase growth rate (▲) at 80% H_2.

Substrate conversion assays

Hanson et al., 2013 showed transcriptional data suggesting increased expression of rHURM genes in the presence of nitrate. Substrate conversion assays were designed to provide a direct link between those results and the reduction of nitrate to ammonium in *N. profundicola*. Resting cell suspensions of *N. profundicola* were grown under the optimal conditions outlined above and provided with nitrate, nitrite, or hydroxylamine as the electron acceptor and 80% H_2 and 20 mM formate as the electron donors. The concentrations of nitrate, nitrite, ammonium and hydroxylamine were followed over time.

Conversion of NO_3^- to NO_2^- and NH_4^+

In replicated resting cell assays, cells with added nitrate produced ammonium at a rate up to $1x10^{-3}$ μM min^{-1} $(10^8$ cells$)^{-1}$ while cells with no addition do not produce ammonium. At high (50 mM) initial nitrate concentrations, similar ammonium production rates were observed: 110 and 116 μM/min. In one resting cell assay, nitrite was produced at a rate of 126 μM/min (Fig. 11A), but was not detected in the other replicate (Fig. 11B). Nitrate consumption was far greater in one replicate than in the other—445 μM/min and 59 μM/min in Fig. 11A and 11B, respectively. Total consumption of nitrate in both replicates was only a fraction of the nitrate provided to cells during these resting cell assays. Hydroxylamine was not detected at 706 nm using a standard colorimetric assay in these experiments, suggesting that if it was present, it was lower than the detection limit for the assay.

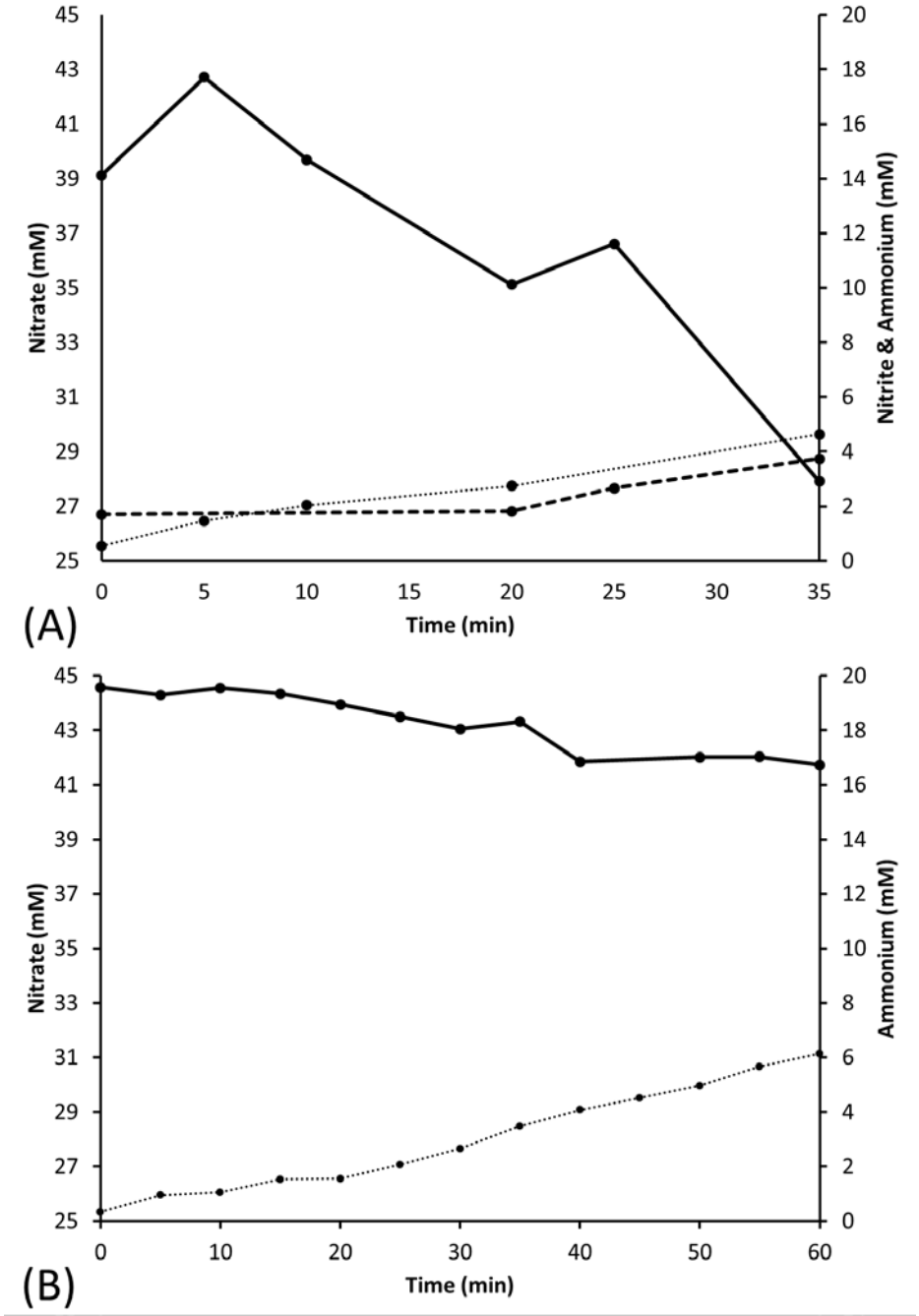

Figure 11. Substrate conversion of nitrate (—●—) to nitrite (– ●– –) and ammonium (⋯●⋯) during resting cell assays. The concentration of nitrate was greater than the production of ammonium. In effect, an appropriate range of for nitrate consumption was used and ammonium was placed on a secondary y-axis.

In addition to ammonium, an unknown compound was detected in the DEEMM assay with a retention time of 7 minutes (U7). This compound was detected as a DEEMM derivative, which strongly suggests it contains a reactive amino functional group. U7 was only detected when nitrate was supplied to resting cells and appeared to increase in concentration with time (Fig. 12 A). Additionally, it was noted that the color of the assays for hydroxylamine were substantially different when cells and nitrate were present. This led to the observation that a substance absorbing at 438 nm in the hydroxylamine assay accumulated in a nitrate dependent fashion (Fig. 12 B). Standards of glutamine, glutamate, nitrite, hydroxylamine, and ammonium were tested for comparison to the U7 peak and substance measured at 438. The observed compound did not co-elute corresponded to these standards.

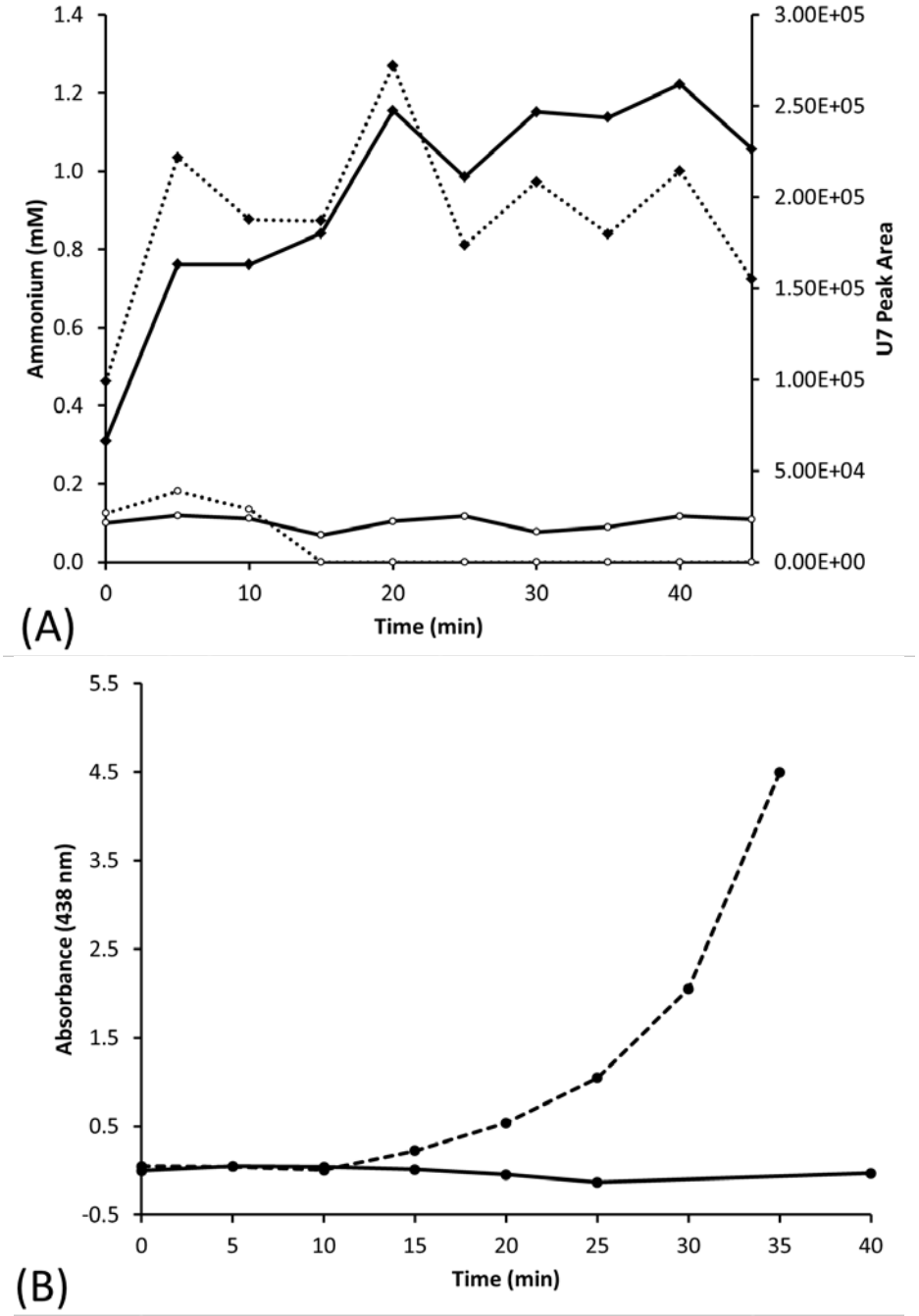

Figure 12. (A) Concentration of NH_4^+ with NO_3^-(—♦—), NH_4^+ without NO_3^- (—○—) and Peak U7with added NO_3^- (···♦···) and U7 without added NO_3^- (··○··). (B) Production of a nitrate-dependent substance at 438nm with added NO_3^- (– ●– –) and without added NO_3^- (—●—).

Conversion of NH_2OH to NH_4^+

The ability of *N. profundicola* to convert hydroxylamine to ammonium was first tested in high cell density incubations (~10^{10} cells ml^{-1}). The result (Fig. 13) was that no hydroxylamine was detected, even in initial time point samples taken immediately after the addition of substrate to resting cells. The amount of ammonium recovered was the same amount of hydroxylamine added. A cell free control experiment demonstrated that hydroxylamine was not consumed in the absence of cells. Together, the observations suggested that the rate of hydroxylamine conversion by *N. profundicola* was very fast. Therefore, the dependence of hydroxylamine conversion rate with cell concentration was examined.

The conversion of hydroxylamine to ammonium was stoichiometric and independent of cell concentration (Fig. 14). The rate of conversion was controlled by cell concentration. Since the hydroxylamine concentration was 5 mM, the estimated time of conversion by $1.97x10^8$ cells ml^{-1} with a rate of ~100 μM min^{-1} was about 40 minutes. With a cell concentration of ~$2x10^8$ cells ml^{-1}, 5 mM hydroxylamine was converted to ammonium over 45 min (Fig. 15). The rate of hydroxylamine consumption was equal to the rate of ammonium production (28.6 μM min^{-1} (10^8 cells)$^{-1}$).

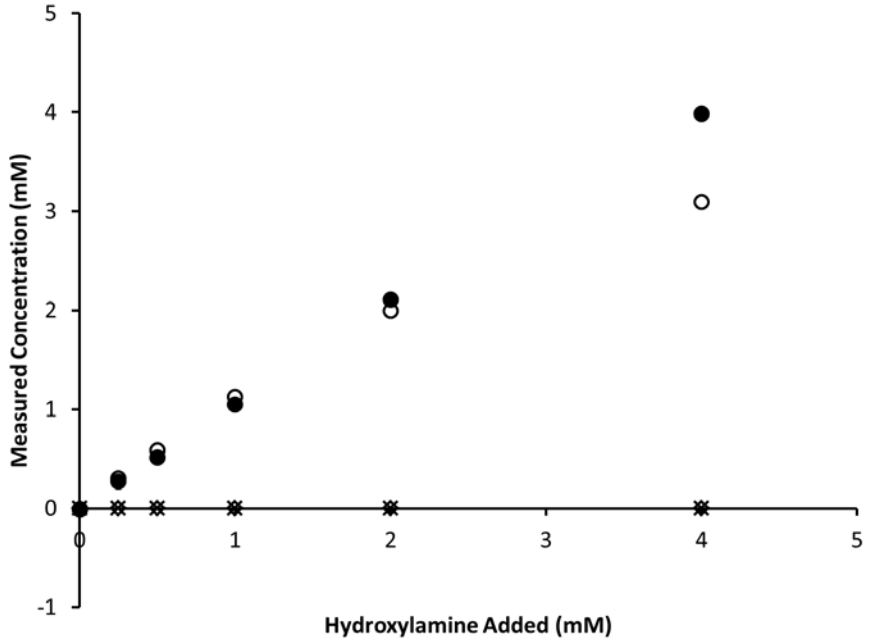

Figure 13. Initial concentration (×) and final concentration (◊) of hydroxylamine with initial concentration (●) and final concentration (○) of ammonium converted in ~10^{10} cells ml^{-1} resting cells.

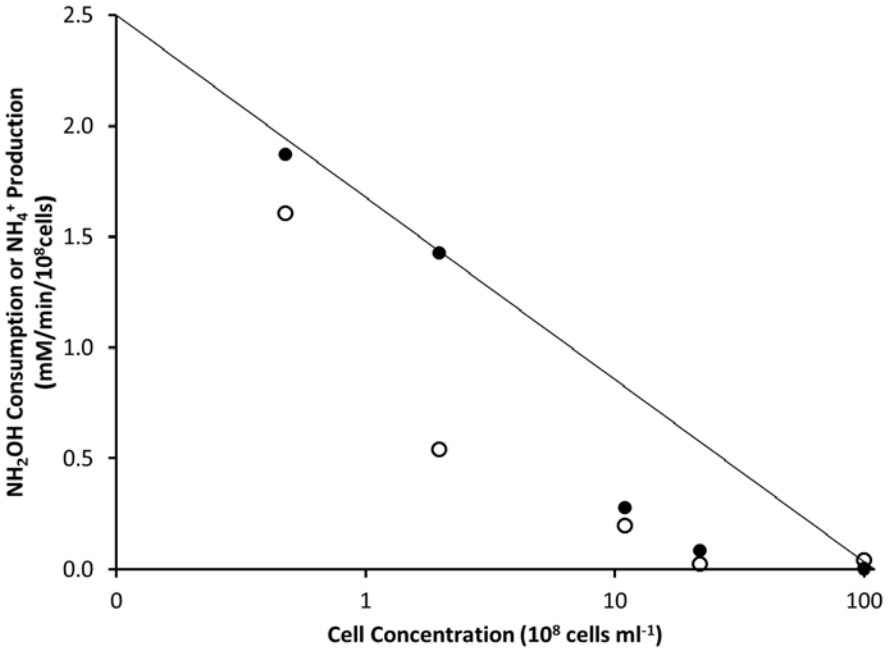

Figure 14. Effect of cell concentration on the rates of ammonium production (●) and hydroxylamine consumption (○). One to one line (——).

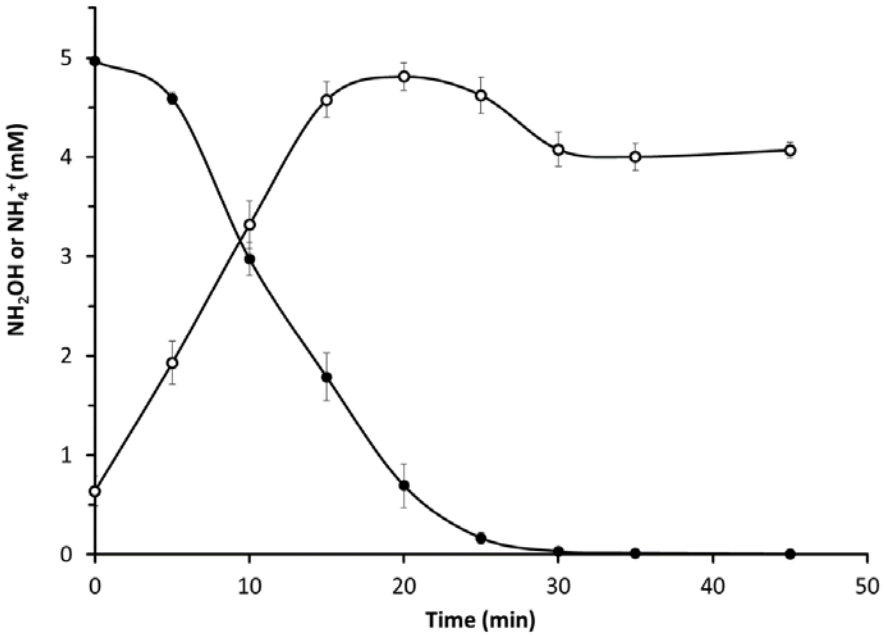

Figure 15. Conversion of NH_2OH (—●—) to NH_4^+ (—○—).

Attempts to inhibit rHURM pathway enzymes

Inhibitors were added to resting cells given 4 mM of substrate in attempt to block the rHURM pathway at defined places in the hopes of observing the accumulation of substrates upstream of the inhibited enzyme. Concentrations of nitrate, nitrite, ammonium and hydroxylamine were measured over time. The effectiveness of each inhibitor was further assessed by examining growth inhibition—with and without the inhibitor. In the absence of inhibitors, hydroxylamine given as the electron acceptor yielded the fastest rate of ammonium production whereas nitrate yielded the slowest rate (Fig. 16).

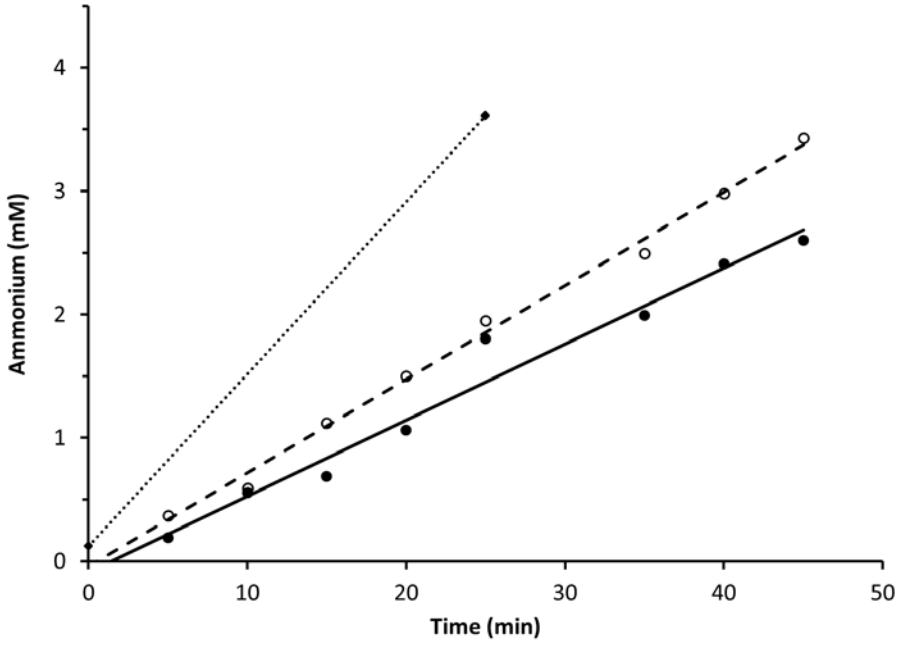

Figure 16. Substrate conversion of nitrate (——), nitrite (– – –), and hydroxylamine (·····) to ammonium during resting cell assays without any inhibitors.

To restrict the assimilation of ammonium into biomass, methionine sulfoximine (MSX) was used to inhibit glutamine synthetase, the major route of ammonium incorporation in most bacteria. When nitrate was given as the substrate with MSX as an inhibitor, ammonium production was 3.04 μM min^{-1} (10^8 cells)$^{-1}$—a

43% increase due to the exogenous disposal of ammonium. However, no hydroxylamine was observed when the downstream enzyme for ammonium assimilation was restricted.

In an attempt to directly restrict the reduction of hydroxylamine, sodium thiocynate was used as an inhibitor (Nicholas 1986). Sodium thiocynate did not change the rate of ammonium production, nor did it inhibit growth. Another inhibitor for hydroxylamine reductase was *o*-phenanthroline (Cabello et al. 2004; Roussos & Nason 1960). When nitrate was given as the substrate with *o*-phenanthroline as an inhibitor, ammonium production was 0.28 μM min^{-1} $(10^8$ cells$)^{-1}$—an 87% decrease. The decrease in ammonium production seemed promising but there was a deficit in the mass balance of nitrogen (Fig. 17). The highest concentration of hydroxylamine was 49 μM at 0 minutes. By 10 minutes, hydroxylamine was no longer detected for the duration of the incubation. At 50 minutes when nitrate is completely consumed, 1.3 mM nitrogen was missing from the nitrogen balance, when the maximum hydroxylamine measured was included in the products.

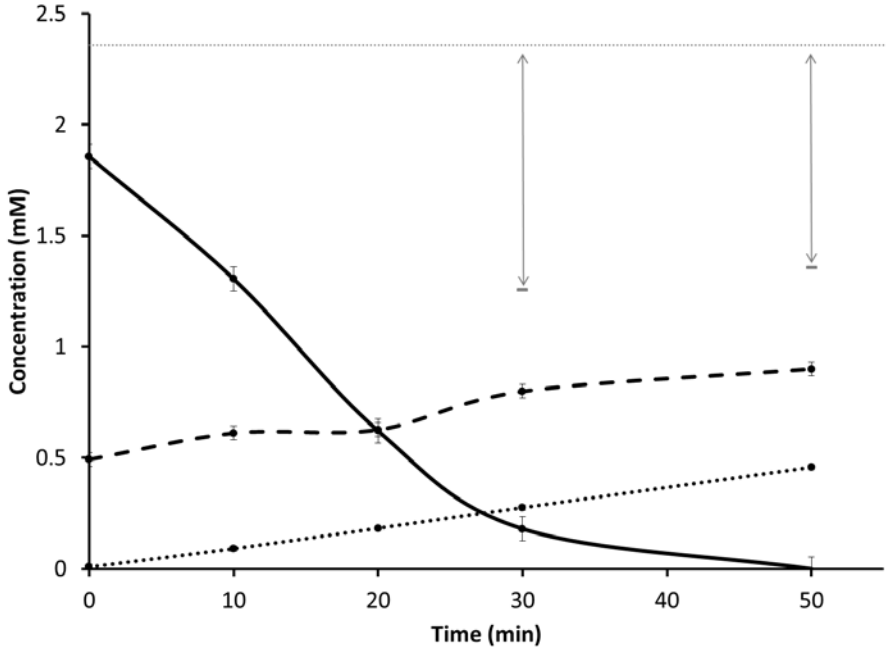

Figure 17. Conversion of nitrate (—●—) to nitrite (– –● –) and ammonium (⋯●⋯) during resting cell assays in the presence of phenanthroline. Arrows indicate the difference in total nitrogen at 30 and 50 minutes compared to the total initial nitrogen (⋯).

Anaerobic Continuous Culture

The continuous culture was designed as to provide better control over previous rHURM transcript abundances measured in batch culture. In a continuous culture system, growth rate can be controlled and sustained for RNA analysis more readily than in batch culture. An anaerobic continuous culture apparatus was built, assembled, and tested with *N. profundicola* cultures. The anaerobic system maintained a positive pressure at ~5 PSI of 80% H_2: 20% CO_2 gas. The water bath made of a glass dish filled with water set atop the heated stir plate was sufficient to keep the temperature steady at 42-45°C for days. The culture agitation was limited by the outflow tube and the angle of the vortex from the spinning stir bar. The maximum rate of stirring was 400 rpm in a culture with a volume of 80 ml.

The apparatus was suitable for anaerobic cell growth as a constant rate was maintained over a period of 4 hours. In one attempt (Fig. 18A), optical density ranged from 0.15-0.17 (3.73-4.39 x 10^8 cells ml^{-1}) before dropping off to 1.21 x 10^8 cells ml^{-1} after 2.5 hours. In the initial 30 min, the average growth rate 0.02 hr^{-1} is slightly positive at the average medium flow rate of 17.2 ml hr^{-1}. After 30 min, the average medium flow rate was increased to an average of 23.5 ml hr^{-1} and the result was a decline of cell population. In a subsequent attempt (Fig. 18B), the medium flow rate was kept constant at 20 ml hr^{-1}. While optical density was lower than in the previous experiment, it sustained at an optical density of 0.112 or about 2.55 x 10^8 cells ml^{-1} for 4 hours. This suggests that an appropriate dilution rate for *N. profundicola* in continuous culture is around 0.25 hr^{-1}.

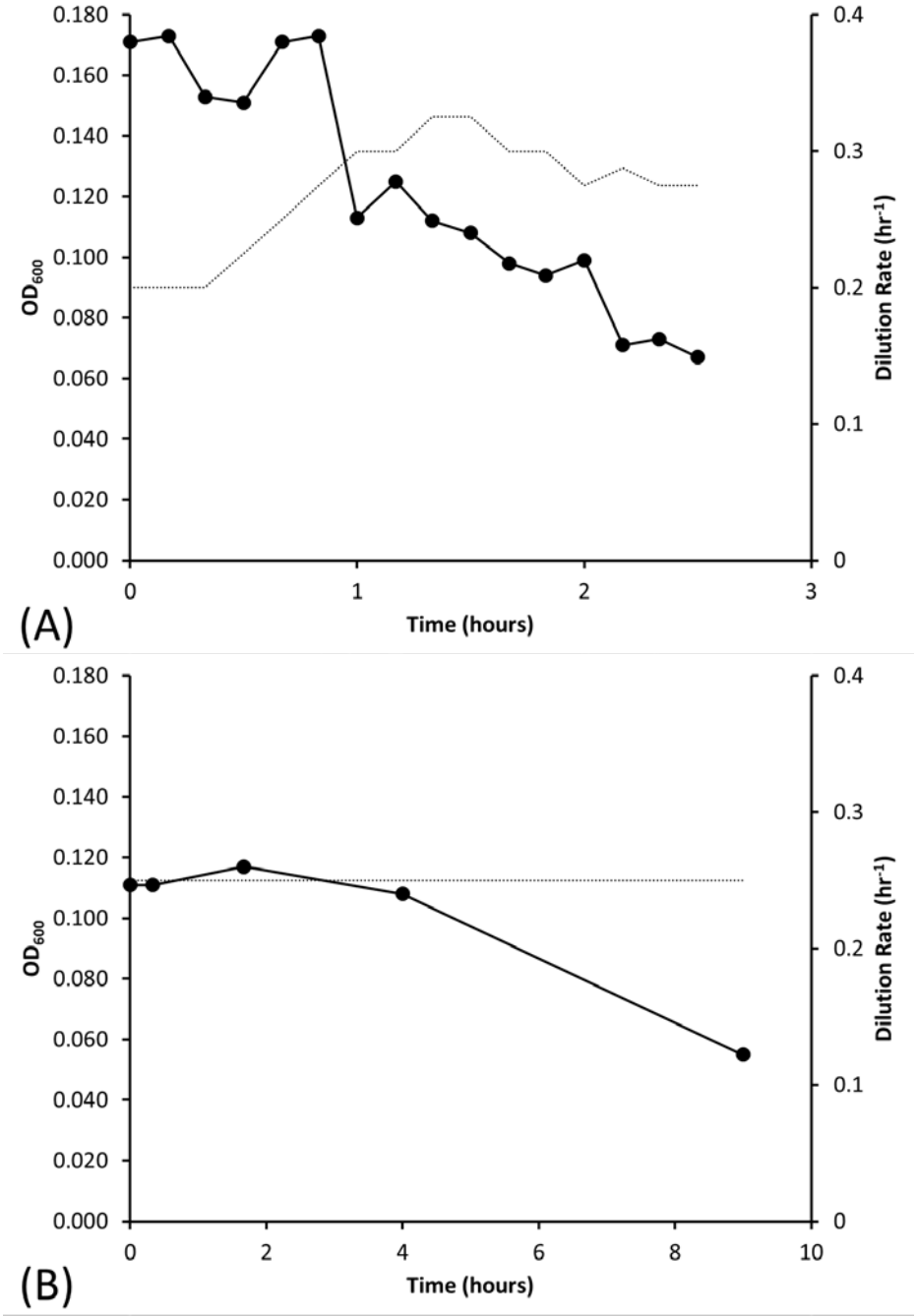

Figure 18. Optical Density of cells (OD_{600}) on the left (—●—) and dilution rate on the right (·····) over time in an anaerobic continuous culture. (A) is an initial experiment to find the correct dilution rate. (B) is a subsequent experiment with a constant dilution rate at 0.25 hr^{-1}.

Chapter 4

DISCUSSION

Effects of various substrate concentrations during growth on nitrate

Optimal concentrations of substrates for *N. profundicola* were tested for growth on nitrate. The electron acceptor nitrate and electron donor H_2 showed no adverse effect on growth with increasing concentration. The K_{max} for both these substrates was observed in the growth plots over time. In contrast, sulfide and formate had peak concentrations at which growth was least inhibited by substrate deficiency but also least repressed by adverse effects of higher substrate concentration.

Sulfide as the sulfur source and reductant

Sulfide is a strong reductant which is why historically it has been used in anaerobic cultures to remove oxygen. However, excess sulfide is problematic because of its redox potential. *N. profundicola* was highly sensitive to sulfide (2 mM) during growth on nitrate. In comparison, wild-type *Chlorobaculum tepidum*, a green sulfur phototrophic bacterium, is tolerant to concentrations of sulfide up to 8 mM. Sulfide: quinone oxidoreductases (SQRs) in *C. tepidum* enable use of sulfide an electron donor while simultaneously disposing of the toxic substrate through oxidation. However, a mutant strain lacking functional SQR's (CT0117::TnOGm, CT1087::TnEm) showed decreased protein yield and sulfide sensitivity above 2 mM (Chan et al. 2009). Similar to *N. profundicola* grown on nitrate, the *C. tepidum* mutant strain cannot utilize sulfide as an electron donor in energy conservation; therefore, the physiological need for

sulfide is lower, and without the respective mechanisms that diminish the pool of excess sulfide, sensitivity to sulfide is higher.

Sulfide sensitivity could be due to the abundance of enzymes containing transition metals which are integral to transferring electrons as part of the electron transport chain. Such enzymes include membrane bound cytochromes responsible for direct interaction with the quinone pool. The interconversion of iron oxidation state from reduced (Fe^{2+}) to oxidized (Fe^{3+}) or vice versa in the heme center of some cytochromes allows these enzymes to oxidize or reduce substrates (Hill et al. 1984; Pietri et al. 2011). Sulfide is a highly reductive anion that readily complexes with transition metals, like iron. So when sulfide complexes with the iron in membrane bound cytochromes, the electron transfer function is lost and the enzyme can no longer oxidize or reduce substrates.

Looking at nitrate reduction in *N. profundicola*, both NapA and rHAO rely on a such a protein for electron transfer and therefore function of these enzymes rely on the cytochromes to provide electrons from the quinone pool in order to reduce nitrate and nitrite. At 2 mM sulfide, growth was inhibited; so at this concentration, it would seem that sulfide may be complexed with iron in cytochromes, thereby reducing growth by restricting electron transfer concomitant with proton motive force and the generation of ATP. Interestingly, free sulfide concentrations in *A. pompejana* tubes where *N. profundicola* was first isolated were <50 µM (Luther et al. 2001) indicating that *N. profundicola* is used to low levels of sulfide *in situ*. Higher concentrations of total sulfide were found but the remaining pool was iron sulfide.

When considering the optimum sulfide concentrations under different electron acceptors, it seemed that *N. profundicola* was less sensitive to sulfide when growing

on polysulfide than when growing on nitrate, 2.5 mM and 0.5 mM sulfide, respectively. *N. profundicola* cannot utilize sulfide or elemental sulfur as the sole electron acceptor; rather, both are required for the synthesis of polysulfide. It is possible there may be an unknown enzyme working to convert sulfide in polysulfide grown cells. However, an alternative explanation is that sulfide, both provided in the medium and produced by polysulfide reduction, reacts with excess elemental sulfur S_8^o ring in the medium through nucleophilic cleavage (Boyd & Druschel 2013; Steudel 2003), thereby producing polysulfide and reducing the concentration of free hydrogen sulfide. Sulfide serves a dual purpose during growth on polysulfide, a precursor to the electron acceptor and to sulfur containing amino acids cysteine and methionine. When nitrate is provided as the electron acceptor, the purpose of sulfide is solely for assimilation. Under these conditions, the pool of free sulfide is dependent on the added hydrogen sulfide concentration and the rate of incorporation into biomass and therefore, nitrate grown cells have a lower optimal sulfide concentration. While the level of toxicity between 0.4 mM to 2 mM sulfide in *N. profundicola* was not explored during this study, the elucidation of sulfide sensitivity would be an interesting complementary experiment to the physiological studies described in this project.

Nitrate as an electron acceptor and nitrogen source

Previous work showed that *N. profundicola* preferred nitrate over polysulfide as an electron acceptor (Hanson et al. 2013). It was also shown that ammonium is produced in excess of the physiological nitrogen requirement (~400 µM) suggesting that nitrate reduction to ammonium is employed primarily for energy conservation. In

accordance with these findings, growth on nitrate increased with concentration up to 10 mM most likely due the increased amount of electron acceptor available for energy conservation. Nitrate >10 mM did not adversely affect growth, suggesting that *N. profundicola* is not sensitive to excess nitrate. However, nitrite and hydroxylamine, the proposed intermediates of nitrate reduction in the rHURM pathway, may adversely affect growth. *N. profundicola* likely avoids toxicity from these pathway intermediates by regulating rHURM enzymes. Data in this study showed that nitrate reduction is the rate-limiting step and each successive reduction step is slightly faster in order to dispose of toxins immediately upon production (Fig. 16).

Considerations of pH during growth and the effect of formate addition

pH increased as a function of growth in all cultures grown on nitrate, independent of the electron donor provided. At least two mechanisms may be responsible for the shift from slightly acidic to slightly basic pH. One mechanism is the consumption and removal of hydrogen ions from the aqueous medium. Another mechanism is the consumption of CO_2 during autotrophic growth which affects CO_2 as a buffering system. Carbon was provided to cells through CO_2, bicarbonate, and formate when it was included as an electron donor. These carbon sources also support a bicarbonate buffering system within the aqueous medium. *N. profundicola* uses CO_2 as a substrate for the rTCA cycle and the removal of CO_2 from the medium reduces buffering capacity against pH changes. To counterbalance the increase in pH, MES was added to cultures and the extra buffering capacity led to higher growth yields. However, after log phase growth and a brief to nonexistent stationary phase, cell yields decreased and pH was observed to be basic in these cultures. While growth on

nitrate supports more robust growth than polysulfide, the rapid consumption of substrates leads to increased pH and conditions which are suboptimal for growth and subsistence.

Similarly, the addition of formate in cultures may have an additive affect. When formate is provided to cultures as an electron donor, it potentially serves other purposes for growth. After oxidation to CO_2, formate is available as a carbon source but also as an added source of bicarbonate buffering. Growth of *N. profundicola* decreased with formate concentrations >20mM given in addition to 80% H_2 as electron donors. While formate consumption was observed in cultures grown on both formate and H_2, 20% CO_2 was always provided to cells in the headspace of culture tubes; so it is unclear whether the presence of formate was beneficial as an additional carbon source and buffering agent or if formate was utilized primarily as an additional electron donor. Perhaps the consumption of formate at higher concentrations led to a greater imbalance in the buffering capacity of the system. In this study, the consumption of formate over time was not followed with respect to pH; however, the elucidation of whether or not the consumption of formate affects pH could explain why higher concentration of formate adversely affected growth in *N. profundicola*.

Comparison of H_2 and formate as electron donors

Smith et al. (2008) showed that *N. profundicola* was can utilize either H_2 or formate independently as the sole electron donor for growth on polysulfide. Based on the comparable calculated free energies for H_2 and formate regardless of the electron acceptor, it was expected that either electron donor could be utilized for growth. Furthermore, formate was delivered as a liquid in the medium and is more readily

available to cells in liquid medium than H_2 which is provided as a gas in the headspace. However, growth on nitrate only supported the utilization of H_2 or H_2 and formate, but not formate alone. Formate and 80% H_2 had a slightly higher yield than 80% H_2 alone. Under dual electron donor growth, formate is consumed so formate must provide some benefit for growth. Still, this does not explain why formate cannot support growth of *N. profundicola* as the sole electron donor with nitrate as the electron acceptor.

Annotation revealed a gene homolog for formate dehydrogenase (FDH) in *N. profundicola* (Campbell et al. 2009). Physiological data from Smith et al. (2008) showed formate could be used as a sole electron donor which suggests that the *N. profundicola* FDH was involved primarily in electron transfer for energy conservation during growth on polysulfide. Perhaps the nature of the electron acceptor dictates the role that FDH plays during growth. Interestingly, nitrate represses FDH in *E. coli* (*fdhF*) (Wang & Gunsalus 2003). During anaerobic growth, repression was gradual with increasing nitrate concentration but as little as 1mM nitrate was enough to repress *fdhF* expression by 30%. The concept of regulatory nitrate control on *fdhF* expression in *E. coli* is comparable to the inability of *N. profundicola* to utilize formate as an electron donor causing growth inhibition in the presence of 20 mM nitrate, twice the reported concentration required for complete repression of *fdhF*. A study of FDH expression in *N. profundicola* may elucidate the link between formate utilization and nitrate repression.

Improved growth conditions with nitrate as the electron acceptor

Previous to this study, *N. proufundicola* was routinely grown on polysulfide. Here, the optimal concentrations of substrates for growth on nitrate as the electron acceptor were explored. Overall, cell yield of cultures grown on 20 mM nitrate, 0.5 mM sulfide, and 80% H_2: 20% CO_2 increased by 2 fold over the reported cell yield on polysulfide, 4-5x10^8 cells ml^{-1} to 10^9 cells ml^{-1}, respectively. Specific growth rate doubled from 0.15 (h^{-1}) to around 0.3(h^{-1}) and doubling time decreased 2-3 fold from the previously reported 6 hours (Smith et al. 2008; Hanson et al. 2013). As predicted by the calculated free energies, nitrate was the preferred electron acceptor for rapid and robust *N. profundicola* growth (Fig. 19).

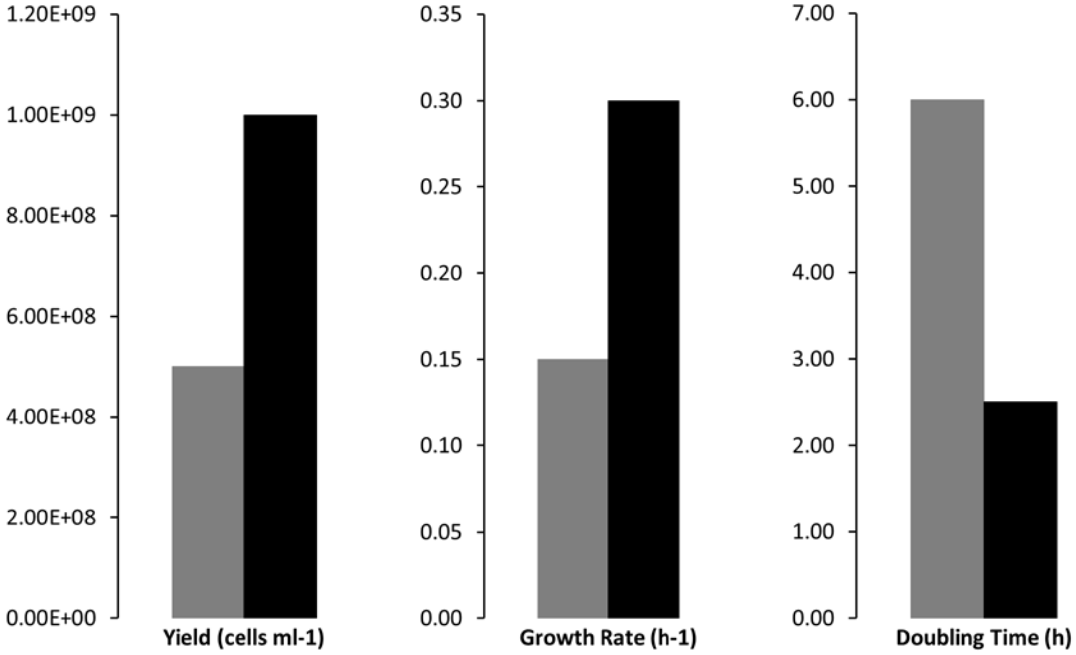

Figure 19. Comparison of growth on nitrate in Hanson et al., 2013 (grey bars) and the present study (black bars).

Linking the nitrate ammonification to the upregulation of rHURM genes

Rapid conversion of hydroxylamine to ammonium

Initially, dense cell suspensions (~10^{10} cells ml^{-1}) were used with a high nitrate concentration (~40 mM) in hopes of inducing fast substrate turnover to measure intermediates in the pathway. While nitrite was detected in one of these assays, it was not consistently detected. In addition, hydroxylamine was not detected in any assay using these cell and nitrate concentrations.

To test the feasibility of hydroxylamine as an intermediate in the rHURM pathway, hydroxylamine was added directly to dense cell suspensions and the resulting conversion of hydroxylamine to ammonium was complete and instantaneous. Hydroxylamine is a strong nucleophile, making it a highly reactive compound. Hydroxylamine causes DNA transition mutations (Brown & Phillips 1965) and inhibits enzymatic function by binding the transition metal in the reaction center (Mei & Yocum 1991). Since hydroxylamine is toxic even at micromolar concentrations, it was surprising that the maximum concentration tested in this study (5 mM) did not render cells inactive but instead led to the conversion of hydroxylamine to ammonium in a stoichiometric fashion even in less dense concentrations of cells (~10^{8} cells ml^{-1}). While the data does not prove that hydroxylamine is produced as an intermediate, it does make the hydroxylamine intermediate feasible because cells can handle the high concentration of mutagenic compound.

Looking at ammonium production from the consumption of each substrate in the pathway, it is clear that hydroxylamine is converted to ammonium rapidly with nitrite conversion to ammonium happening at about half the rate and nitrate

conversion slightly slower than nitrite. Consequently, nitrate reduction is the rate limiting step in the rHURM pathway and produces 2.13 μM min^{-1} $(10^8$ cells$)^{-1}$ of ammonium. This data corroborates the inability to consistently detect nitrite and hydroxylamine based on ammonium production rates in the initial experiments using nitrate as the substrate.

Effect of inhibitors on substrate conversion to ammonium

In attempt to show the production of hydroxylamine as an intermediate, inhibitors were used to restrict key enzymes in hopes that detectable levels of hydroxylamine were produced during nitrate reduction to ammonium in *N. profundicola*. Growth inhibition was used to indicate that essential enzymes needed for growth—like the rHURM pathway enzymes used for energy conservation—were restricted by the inhibitor. Inhibited growth and the increase in ammonium suggests that MSX as an inhibitor seemed to be adequate at restricting GS-GOGAT in *N. profundicola*. Perhaps if the ammonium concentration reached a certain level, feedback inhibition by the end product would result in a detectable pool of intermediary hydroxylamine. However, blocking N assimilation increased the final product of nitrate reduction instead of inhibiting reduction to ammonium as anticipated.

Since downstream regulation of hydroxylamine reduction did not lead to a detectable buildup of the proposed intermediate, direct inhibitors of hydroxylamine reductase were tested. Sodium thiocynate was not a sufficient inhibitor; however, *o*-phenanthroline caused a reduction in ammonium production. Additionally, a

comparatively large pool of nitrite was measured. The highest measured concentration of hydroxylamine (49 μM) was present in the substrate conversion assay on *o*-phenanthroline. This value is 10 fold greater than previously measured concentrations during growth. Still, the inhibitor was not specific to HAR and the reduction of hydroxylamine. Nitrate was completely consumed and nitrite reduction seemed to become the rate limiting step based on exceptionally high accumulation of nitrite. The inability to detect the production of hydroxylamine leaves uncertainty as to whether hydroxylamine is truly an intermediate of the rHURM pathway or simply a byproduct. Nevertheless, the data in the substrate conversion assays support the hydroxylamine as a feasible intermediate.

Production of unknown nitrate dependent substances

In the initial nitrate conversion assay, a nitrate dependent substance was detected in the DEEMM method for quantifying ammonium which we called U7. The unknown substance followed the similar peaks and valleys as ammonium. Another nitrate dependent substance was detected in the colorimetric assay for hydroxylamine at 438 nm. Since both these methods were used to detect nitrogenous compounds, we tested perspective nitrogen containing compounds that may have been produced by nitrate reduction. Glutamine, glutamate, nitrite, hydroxylamine and ammonium were not the U7 peak or the substance at 438 nM in the DEEMM method or in the colorimetric assay. The identities of these compounds are still unknown and there is no direct correlation between the substances. The compounds may share the same identity or they may be independent of each other. Further investigation is required to explore the role, if any, of these substances in nitrate reduction.

N. profundicola growth in anaerobic continuous culture

Previous work had shown increased transcript abundance for key rHURM genes during growth on nitrate in batch culture. The difference in growth rate between nitrate and polysulfide grown cells may have been problematic in measuring the relative abundances of mRNA compared to total cell RNA. In a recent proteomic study in *C. tepidum*, differential protein abundance was observed in early log phase and compared to late log phase (Falkenby et al. 2011). Some protein regulation could be correlated to changes in the pathway of interest, but the majority of variation was not linked to specific metabolic activity. Similarly, batch cultures grown on different electron acceptor, having different growth rates, likely express genes differentially during growth.

In order to normalize the growth rate of cells on each electron acceptor, nitrate and polysulfide, an anaerobic continuous culture was designed to control growth rate and allow for the collection of steady state RNA. Anaerobic continuous culture of *N. profundicola* was achieved for ~4 hrs. Further studies described in the discussion on growth optimization are required to increase the longevity of cultures growing in continuous culture on nitrate. Additionally, the small culture volume (80 ml) was used to cut down on the volume of medium supplied to cells over time, initially thought to be on the scale of days. In effect the cell concentration was highly sensitive to the medium flow rate. The difficulties faced in establishing the continuous culture for *N. profundicola* do not overshadow the accomplishment of setting the ground work for this method. Further trial may allow for steady state growth and comparable total cell RNA, providing refined evidence of rHURM upregulation on nitrate and a clear role of the rHURM pathway and metabolic variation in *N. profundicola*.

Broader considerations of the rHURM pathway

Previous studies showed that *N. profundicola* has mechanisms to utilize both nitrate and polysulfide as electron acceptors (Campbell et al. 2009; Hanson et al. 2013) and has been detected at multiple vent sites along the EPR with varying vent fluid chemistry (Campbell et al. 2001). At 13°N EPR, inside *A. pompejana* tubes, up to 20 µM nitrate and 9.7 µM ammonium have been detected. Ambient sea water has a concentration of 30 µM nitrate and 0.1 µM ammonium (Di Meo-Savoie et al. 2004). Additionally, polysulfides are found at some but not all vent sites along the EPR (Luther, III et al. 2001). In diffuse flow zones where polysulfide and ammonium are plentiful, polysulfide reduction is likely the primary mechanism for energy conservation. However, in mixing zones where there is little or no polysulfide but nitrate is available, the ability to reduce nitrate would allow continuous growth. Calculated standard free energies and batch growth showed that nitrate provides 8x more energy and supports more robust growth than polysulfide; but the fastest growth may not always be the best growth strategy for *N. profundicola*. In addition to rapid growth, nitrate reduction results in increasing pH and the accumulation of ammonium as a waste product. These two negative effects of nitrate reduction in *N. profundicola* may have led to cell death in the continuous culture experiments and suggest that, *in situ*, nitrate reduction is employed for survival when polysulfide is not available and not as the primary mechanism for energy conservation. Regardless of which substrate is preferred *in situ* by *N. profundicola*, the ability to exploit multiple substrates for energy conservation provides adaptability at diffuse flow hydrothermal vents where substrate availability changes.

Similarly, the presence of rHURM genes in host associated Campylobacterales suggests that nitrate reduction could be used for nitrogen assimilation in the absence of sufficient oxygen. One could imagine that in an environment with high nitrate and low oxygen, such as terrestrial runoff or sewage, nitrate reduction may allow survival of these cells until they return to a host and higher concentrations oxygen. Survival of host-associated pathogens outside the host increases pathogenicity. Additionally, microniches within the host may be hypoxic or anoxic such as in tissue lining and require a means of anaerobic respiration for survival. For instance, *Pseudomonas aeruginosa* infects sputum in the lungs of cystic fibrosis patients in which oxygen is limited (Palmer et al. 2007). *P. aeruginosa* can respire aerobically, but can also reduce nitrate for energy conservation in the absence of oxygen and *P. aeruginosa* infections are the leading cause of fatalities in cystic fibrosis patients (Høiby et al. 2005). In general pathogens are metabolically diverse which is why they can adapt to changing conditions throughout host systems. It is important to elucidate pathogenic mechanisms for growth and survival to effectively treat infections. By studying pathways like rHURM that are reliant on inorganic substrates not required by host metabolism, we can target specific enzymes/pathways to inhibit pathogen growth without detriment to the host.

While this study is far from providing that sort of insight, the hydroxylamine conversion to ammonium results, Figure 6 of Hanson, et al. (2013), was an integral part in its publication. The complete reduction of 5 mM hydroxylamine to ammonium coupled with the production of 49 µM hydroxylamine under *o*-phenanthroline inhibition makes hydroxylamine a probable intermediate in the rHURM pathway. Additionally, the growth optimization studies on *N. profundicola* revealed key insights

of sensitivity to sulfide and critical consideration of biochemical interactions affecting growth during nitrate reduction to ammonium. Continuous culture of *N. profundicola* was maintained for 4 hours showing that the system for continuous culture is sufficient for growth and cost thousands of dollars less than a manufactured bioreactor system. Additional hardware for monitoring pH and cell density could be easily added. The decline of cells after 4 hours suggests that the substrate composition needs to be modified during continuous culture. In light of discussion on pH increase during nitrate growth and the 1000 fold lower concentration of nitrate seen by *N. profundicola*, *in situ*, it may be beneficial to decrease the concentration of nitrate provided in continuous culture. Not only could growth rate be controlled by nitrate availability, cell death may also be delayed or lessened.

Future directions for examining the rHURM pathway

To fully address the role of the proposed rHURM pathway in nitrate reduction, additional experiments would be required. Isolation of the rHURM ezymes from *N. profundicola* and subsequent measurement of specific enzymatic activity of substrate conversion will elucidate the function of these genes. The specific activity of rHAO on nitrite should conclusively show whether or not hydroxylamine is the direct product of nitrite reduction by rHAO. It may also be interesting to measure the inhibitory effect of *o*-phenanthroline on rHAO and HCP/HAR *in vitro* to clarify which enzyme is most inhibited, bolstering the results presented here that suggest rHAO may be more sensitive than HCP/HAR to *o*-phenanthroline.

Another crucial experiment would be to disrupt rHURM genes to show whether or not growth is inhibited in mutant strains grown on nitrate. If r*hao* and *hcp/har* are part of the mechanism of nitrate reduction for energy conservation, then their mutants should not grow on nitrate but not be affected during polysulfide growth. Currently, gene knockout experiments require that cells have the ability to grow directly on solid agar medium or have a competent vector that the plasmid can be implanted into with the ability to grow on agar to show the mutation process has worked correctly. Currently, *N. profundicola* cannot grow on agar medium nor does it have a competent system for *N. profundicola* plasmid transplant. Barbara Campbell at Clemson University is working towards growing *N. profundicola* on agar plates.

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Appendix

PERMISSION LETTER

November 21, 2013

This letter is to grant permission to Katie Kalis to utilize the following figures from two of my first author papers in her Master's thesis:

Campbell, B. J., Engel, A. S., Porter, M. L., & Takai, K. (2006). The versatile epsilon-proteobacteria: key players in sulphidic habitats. *Nature reviews. Microbiology*, *4*(6), 458–68. doi:10.1038/nrmicro1414
Figure 1 (listed as Figure 1 in thesis),
Figure in Box 1 (listed at Figure 2 in thesis)

Campbell, B. J., Smith, J. L., Hanson, T. E., Klotz, M. G., Stein, L. Y., Charles, K., … Cary, S. C. (2009). Adaptations to Submarine Hydrothermal Environments Exemplified by the Genome of Nautilia profundicola. *PloS Genetics*, *5*(2), 22–25. doi:10.1371/journal.pgen.1000362
Part of Figure 2 (listed as Figure 3 in thesis),
Figure 3 (listed as Figure 4 in thesis)

If you have any questions, please feel free to contact me. I can be easily reached via email (bcampb7@clemson.edu) or phone (864-656-0559).
Sincerely,

Barbara J. Campbell, PhD

Assistant Professor