NITROGEN AND OXYGEN ISOTOPE EFFECTS OF NITRITE OXIDATION IN VARIOUS SPECIES OF NITRITE-OXIDIZING BACTERIA

by

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ABSTRACT

Bioavailable nitrogen is a limiting nutrient in many parts of the ocean. Nitrite oxidation produces biologically available nitrate, which in turn can help regulate primary production and the absorption of carbon dioxide from the atmosphere. Despite the importance, the global mass and nitrogen isotope budgets are poorly constrained. This thesis uses dual isotope tracers of nitrogen and oxygen in nitrite to investigate bacterial nitrite oxidation isotope systematics which may be used to improve future oceanic models requiring isotope effect data. Nitrogen and oxygen isotope effects of nitrite oxidation in continuous culture from *Nitrococcus mobilis, Nitrospira marina* and *Nitrobacter winogradskyi* were recorded. Nitrogen isotope effect values ranged from -31‰ to -10‰, whereas oxygen isotope values ranged from -12‰ to 1‰. Low oxidation rates seen in *N. marina* and *N. winogradksyi* were associated with increased inverse nitrogen isotopic fractionation. Variable oxidation rates observed here may be due to differences in NXR enzyme active sites and altered demands for oxygen. Nitrogen and oxygen nitrite isotope effects for *Nitrospina gracilis* in batch culture are also reported ($^{15}\varepsilon_{NXR}$ of -11‰ and $^{18}\varepsilon_{NXR}$ of -2‰). *N. gracilis* is of particular importance to the study of nitrogen cycling in oxygen deficient zones due to its prevalence and flexible metabolic pathways of nitrite oxidation and nitrate reduction. This thesis contributes to the field of oceanic isotope research by helping to disentangle individual isotope effects for nitrite oxidation in various species of nitrite oxidizing bacteria and shows how oxidation rate can affect isotopic fractionation.

LIST OF ABBREVIATIONS AND SYMBOLS USED

$CaCl_2$	Calcium Chloride
CO_2	Carbon Dioxide
$CoCl_2$	Cobalt Chloride
H_2O	Water
HCl	Hydrochloric Acid
K_2CO_3	Potassium Carbonate
K_2HPO_4	Dipotassium Phosphate
$MgSO_4$	Magnesium Sulfate
$MnCl_2$	Manganese (II) Chloride
N	Nitrogen
Na_2MoO_4	Sodium Molybdate
NaN_3	Sodium Azide
$NaNO_2$	Sodium Nitrite
NaOH	Sodium Hydroxide
NH_3	Ammonia
NO_2^-	Nitrite
NO_3^-	Nitrate
O	Oxygen
$ZnSO_4$	Zinc Sulfate
ETNP	Eastern Tropical North Pacific
IRMS	Isotope-ratio Mass Spectrometer
NOB	Nitrite Oxidizing Bacteria
NXR	Nitrite Oxidoreductase Enzyme
ODZ	Oxygen Deficient Zones
PSU	Practical Salinity Unit
UV	Ultraviolet
VSMOW	Vienna Standard Mean Ocean Water
$^{15}\varepsilon$	Nitrogen Kinetic Isotope Effect
$^{15}\varepsilon_{NXR}$	Nitrogen Kinetic Isotope Effect of nitrite oxidoreductase

$^{18}\varepsilon$	Oxygen Kinetic Isotope Effect
f	Fraction of unreacted substrate
µL	Micromole per litre
µM	Micromoles per litre
mL	Millilitre
M	Moles per litre
nM	Nanomoles per litre
nmol	Nanomole
$\delta^{15}N$	Nitrogen Isotopic Ratio
$\delta^{15}N_{NO2}$	Nitrogen Isotopic Ratio of nitrite
$\delta^{15}N_{NO3}$	Nitrogen Isotopic Ratio of nitrate
$\delta^{18}O$	Oxygen Isotopic Ratio
$\delta^{18}O_{H2O}$	Oxygen Isotopic Ratio of water
$\delta^{18}O_{NO2}$	Oxygen Isotopic Ratio of nitrite
$\delta^{18}O_{NO3}$	Oxygen Isotopic Ratio of nitrate

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CHAPTER 1 INTRODUCTION

1.1 IMPLICATIONS FOR GLOBAL NITROGEN BUDGET

Fixed nitrogen (N) is a limiting nutrient in many parts of the ocean. Deep water sources of nitrate (NO_3^-) can partially regulate the rate of primary production and carbon export from the surface oceans (Eppley and Peterson 1979). Nitrogen cycling has been studied for many decades but due in part to difficulties in measuring bacterial processes at depth there are many uncertainties with regards to global N budgets (Wang et al. 2019; Fowler et al. 2015; Gruber and Galloway 2008; Deutsch et al. 2004). Global balances of nitrate are currently poorly constrained, and we lack sufficient data to accurately model N cycling processes. Currently, some studies show the rate of N loss is predicted to outweigh the rate of N input (Wang et al. 2019; Fowler et al. 2015; Deutsch et al. 2004), whereas others show a balanced budget (Gruber and Galloway 2008). Due to the importance of the nitrate inventory regarding primary production and climate change, we need to work towards a better understanding of the processes that control the production and consumption of nitrate.

The main sources of fixed N include biological N fixation and agricultural run-off at the surface ocean. Sinks include various microbial processes occurring in anoxic areas of the water column, also known as oxygen deficient zones (ODZs), and sedimentary denitrification and burial (Thamdrup and Dalsgaard, 2002; Codispoti et al., 2001). Microbial processes operating in low oxygen regions such as ammonium oxidation and denitrification result in the production of N_2 and a loss of fixed N. Nitrification and nitrogen fixation have also recently been shown to occur in areas of low oxygen (Beman et al., 2013; Buchwald and Casciotti, 2013; Füssel et al., 2012). The contribution of each

process to nitrite and nitrate production is still unclear. Difficulty in resolving the N budget as well as areas of the ocean where production and loss occur close together has led to the use of dual isotope tracers of N and oxygen (O) to model oxidation in the ocean (Sigman et al. 2005).

1.2 Dual N and O Isotopic Tracers

The ratio of stable isotopes ^{15}N to ^{14}N ($\delta^{15}N = [(^{15}N/^{14}N)NO_2 \div (^{15}N/^{14}N)N_2 - 1] \times 1000$) and ^{18}O to ^{16}O ($\delta^{18}O = [(^{18}O/^{16}O)NO_2 \div (^{18}O/^{16}O)VSMOW - 1] \times 1000$) of nitrite in comparison to a reference gas in a collected sample can be measured on an isotope-ratio mass spectrometer (IRMS). In most scenarios, bacteria will preferentially take up the lighter isotope, leaving behind a heavier product. This partitioning of heavy to light ratios during a metabolic reaction is defined as a kinetic isotope effect ($^{15}\varepsilon$ and $^{18}\varepsilon$). $\delta^{15}N$ and $\delta^{18}O$ values can be measured at different depths and plotted to create an isotope profile. Isotope profiles can only be used to accurately interpret metabolic processes if isotope fractionation factors for each individual process are known. For example, N fixation as a source of N can be occurring in the surface ocean, whereas denitrification as a sink of N can be occurring in the sediments below. Various processes that produce and consume N can occur simultaneously. In parts of the ocean where denitrification and N fixation are both occurring, the $\delta^{15}N$ enrichment from denitrification can mask N fixation (Sigman et al. 2005). If additional processes are also occurring, it can be difficult to separate out the fractionation factor specific to denitrification and N fixation. Sigman et al. (2005) introduced a tracer to disentangle denitrification from N fixation. This tracer $\Delta(15,18) = (\delta^{15}N_{NO3} - \delta^{15}N_{NO3\ deep\ water}) - {}^{15}\varepsilon/{}^{18}\varepsilon * (\delta^{18}O_{NO3} - \delta^{18}O_{NO3\ deep\ water})$ can be useful because O isotopes will be affected differently than N isotopes from denitrification and N fixation.

Deep water is found at a depth below the thermocline and above the seabed. $\delta^{15}N_{NO3\ deep\ water}$ is approximately 5‰ relative to atmospheric N_2 (Sigman et al. 2000). $\delta^{18}O_{NO3\ deep\ water}$ is consistent among regions with differing oxygen concentrations and only varies ± 1‰ (Casciotti et al. 2002). These values serve as a baseline to determine changes occurring to $\delta^{15}N_{NO3}$ and $\delta^{18}O_{NO3}$ due to processes going on in other areas of the water column.

If only denitrification is occurring, the $\delta^{15}N$ will be enriched as the associated isotope effect is within a predicted range of 12-30‰ (Kritee et al. 2012; Granger et al. 2008; Voss et al. 2001; Wada et al. 1975). In areas of the water column where this is the case, the slope of $\delta^{15}N_{NO3}$ vs $\delta^{18}O_{NO3}$ will be equal to one (Sigman et al. 2005; Granger et al. 2004). If the isotope values do not fall on this slope, there are two possibilities to explain the local isotope anomaly. The first is remineralization of newly fixed N being added to the region. The second could be nitrite re-oxidation (cycling between nitrite and nitrate) in suboxic regions (Sigman et al. 2005). Nitrite oxidation is a unique process with an inverse isotope effect where nitrite oxidizing bacteria (NOB) preferentially take up heavier isotopes, leaving a lighter nitrite substrate pool (Cascioitti 2009). It is important to use both nitrate and nitrite isotopic measurements to differentiate between nitrite oxidation and N fixation, as a deviation from the slope of one could be due to either process.

1.3 Use of Isotope Profiling in Oceanic Modelling

Natural abundance stable isotope profiles of nitrite and nitrate have been used in modelling studies to try and determine what metabolic processes are occurring (Buchwald et al. 2018; Granger and Wankel 2016; Buchwald et al. 2015; Casciotti et al. 2013). Models can also use isotope profiles to calculate an *in-situ* isotope effect,

assuming there is enough data to predict the processes in play. For example, a model constructed by Casciotti et al. (2013) used nitrate and nitrite isotopic measurements from the Peruvian ODZ to try and better understand the relative rates of nitrate oxidation and reduction. However, despite using wide ranges of fractionation factors and $\delta^{15}N$, the values could not simulate isotope effects that matched published values for NOB (Buchwald and Casciotti 2010; Casciotti 2009).

Many models based on isotope profiles cannot simulate the observed large difference between light $\delta^{15}N_{NO2}$ and heavy $\delta^{15}N_{NO3}$. One possible explanation for this could be enzyme level interconversion (Buchwald et al. 2018; Kemeny et al. 2016). Under normal aerobic conditions, the nitrite oxidoreductase (NXR) protein catalyzes the nitrite oxidation reaction by converting nitrite to nitrate. NXR has also been shown to be reversible (Sundermeyer-Klinger et al. 1984). This reversible enzymatic reaction under anaerobic conditions could promote isotopic equilibrium and help explain the observed isotopic patterns in addition to the presence of nitrite oxidation. Additionally, previous models have not allowed the simulated isotope effect to change based on recorded oxidation rates. It was assumed that the rate would not change the isotope systematics. However, *Chapter 2* of this thesis discusses the impact oxidation rate can have on isotope effects, and how lighter $^{15}\varepsilon$ values required for models to produce values that match recorded isotope profile can be reconciled with results from pure culture experiments.

1.4 Nitrification and Nitrite-Oxidizing Batch Culture Experiments

Nitrite oxidation is the second step of nitrification, with the first being ammonia oxidation. Ammonia oxidation is done by ammonia-oxidizing bacteria and archaea. Nitrite oxidation is carried out by NOBs. In nitrite oxidation, an oxygen atom from water

is incorporated into nitrite. This process generates nitrate and contributes significantly to resuppling the surface ocean with biologically available N (Yool et al. 2007). Various oceanic and fresh-water species of NOB exist. Certain species of NOB such as *Nitrospina* and *Nitrococcus* have been found in high numbers in ODZs (Beman et al. 2013; Füssel et al. 2012). *Nitrospina* is considered a prominent NOB and can also be found in the open ocean and sediments in addition to ODZs (Beman et al. 2013; Santoro et al. 2010; Mincer et al. 2007).

Under aerobic chemolithoautotrophic growth conditions, NOB will oxidize readily available nitrite by using oxygen as a terminal electron acceptor and inorganic CO_2 as a carbon source. In these species, the enzyme NXR is responsible for the oxidation reaction. However, certain NOB, such as *N. mobilis*, have flexible metabolic pathways and may also undergo the reverse reaction (Füssel et al. 2017; Koch et al. 2015). Under anaerobic conditions, nitrate is used as a terminal electron acceptor and organics such as acetate or formate are used as a carbon source (Füssel et al. 2017; Koch et al. 2015). There is no evidence of any enzymes capable of nitrate reduction in the *N. mobilis* genome, suggesting that NXR may also be responsible for the reduction process (Lücker et al. 2010; Sundermeyer-Klinger et al. 1984).

In addition to models, pure culture studies have also been used to investigate nitrite oxidation in NOB. Previous batch culture experiments and incubation studies have recorded isotope effects for nitrite oxidation, denitrification and ammonia oxidation in various NOB species (Buchwald et al. 2010; Casciotti et al. 2010; Lehmann et al. 2004). These isotope effect values are important for modelling purposes to get a better understanding of N cycling. A culture study by Füssel et al. (2017) with *Nitrococcus*

mobilis helped determine the NOB species' ability to reduce nitrate to nitrite under anaerobic conditions. Although these types of studies are useful, batch culture experiments represent a closed system environment. Cells in a batch culture are not seeing a steady rate of supply of nutrients. Instead, they are given a large concentration of initial substrate and consume the nutrient until there is none left. At this point, cells are no longer growing exponentially and waste products begin to build up. Because of this, cells cannot grow at a steady state. It is also not possible to accurately control their cellular growth rate. To overcome these challenges, continuous culture experiments in a chemostat can be done. In a continuous culture, media and key substrates are provided at the same rate as media, cells and waste products are removed. The incoming substrate concentration is not changing and it is more representative of an open system where it is more likely that limiting nutrients are supplied at a constant rate and then instantly consumed. Steady state growth of cells can be achieved because cells must grow at the same rate as they are removed. For this reason, the growth of cells is much slower in a chemostat and can be more accurately controlled.

1.5 Factors Influencing Nitrite Oxidation Rate

The bacterial growth rate (exponential rate constant) in a chemostat should be equivalent to the dilution rate because cells need to match their growth rate to the rate of the media being removed. The dilution rate is setting the bacterial turn-over rate. Chemostats can be used to set growth rates and determine nutrient uptake rates based on steady state equations of growth. The nitrite oxidation rate in NOB is the rate at which the bacterial cell consumes nitrite. We initially thought that the faster the growth rate, the more nitrite should be consumed to match energy demands. However, oxidation rate may

not be linearly linked to growth rate. Previous studies conducted by Laanbroek et al. (1994) and Both et al. (1992) show that growth rate had no predictable relationship with oxidation rate. Instead, the oxidation rate and associated isotope effects may be influenced by how a specific species of bacteria grows. *Chapter 2* of this thesis also discusses the relationship seen between oxidation and growth rate, and the effect of these factors on isotope fractionation.

Nitrococcus, Nitrospira, Nitrospina and *Nitrobacter* species all express NXR proteins, which catalyze the nitrite oxidation process. The active site of NXR in *Nitrococcus* and *Nitrobacter* is found in the cytoplasm, whereas the active site in *Nitrospira* and *Nitrospina* is found in the periplasm, which is more energetically favourable (Lücker et al. 2010; Spieck and Bock 2005). Additionally, *Nitrococcus/Nitrobacter* have multiple copies of NXR-A (the substrate binding subunit) clustered away from oxidative genes. *Nitrospira* and *Nitrospina* on the other hand have multiple NXR-A copies clustered with oxidative genes but have no oxidative stress genes, thus making these species more adapted to low nitrite low oxygen environments (Lücker et al. 2010; Sundermeyer-Klinger et al. 1984). Differences in metabolism found in NOB impact how the cells grow and how they oxidize nitrite. Species that oxidize nitrite slower in continuous culture are more representative of rates seen in the ocean.

1.6 Objectives and Thesis Structure

The main objective of this work is to better understand the isotope effects associated with nitrite oxidation by NOB. Establishing accurate isotope effects for nitrite oxidation is required to improve upon current models that may be based on batch culture experiments or insufficient data. This thesis will focus on providing isotope effect data

for a variety of NOB species grown in continuous culture: *Nitrococcus mobilis, Nitrospira marina, Nitrobacter winogradskyi*. Additionally, isotope effect data is provided for *Nitrospina gracilis* grown in batch culture. The controls on oxidation rate and growth rate in NOB will be explored, as well as how this can impact expressed isotope effects. This thesis also aims to seek explanations for why oceanic models require isotope effect values for nitrite oxidation that do not necessarily reconcile with previous pure culture experiments or recorded values from ODZs.

This thesis is split into two data chapters written in manuscript format, followed by a conclusion chapter. *Chapter 2* will provide a discussion around nitrogen and oxygen isotope effects in continuous cultures of *Nitrospira marina, Nitrococcus mobilis* and *Nitrobacter winogradskyi.* The impact of growth rate and nitrite oxidation rate on isotope effects will also be discussed. *Chapter 2* is written as a paper in preparation with co-author Dr. Carolyn Buchwald. We are planning to submit to the Limnology and Oceanography journal. I was responsible for carrying out experiments, recording and analyzing data and for writing the first draft of the text. Dr. Buchwald supervised the project, devised the study design and contributed to editing as well. *Chapter 3* will present nitrite isotope effect data from *Nitrospina gracilis* in batch culture. Details on the isotope effects associated with oxygen atom exchange will also be provided. *Chapter 3* is also written in manuscript format; however it will not be submitted for publication as more work is required. In this chapter, I was responsible for carrying out experiments, recording and analyzing data and for writing the first draft of the text. Dr. Buchwald supervised the project, devised the study design and contributed to editing. *Chapter 4* summarizes conclusions from this thesis.

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CHAPTER 2 - THE RELATIONSHIP BETWEEN NITRITE OXIDATION RATE AND ^{15}N AND ^{18}O ISOTOPE EFFECTS IN THREE NITRITE-OXIDIZING BACTERIA GROWN IN CONTINUOUS CULTURE

2.1 INTRODUCTION

Biologically available nitrogen (N) is fundamental for life and is a limiting nutrient in many parts of the ocean. As a result, fixed N in the forms of nitrate (NO_3^-), nitrite (NO_2^-), and ammonia (NH_3) can play a large role in regulating the rate of primary production and carbon export from the surface oceans (Eppley and Peterson 1979). Despite the importance, there are still many unanswered questions about the microbes and their role in the cycling of these nutrients. Microbes are largely responsible for production and consumption of fixed N, yet the relative contributions of processes controlling oxidation and reduction of nitrate remain uncertain. This is part of the reason why the global N budget is currently poorly constrained.

Nitrification is a key pathway in the nitrogen cycle. In a two-step process, ammonia is converted to nitrate, with nitrite-oxidizing bacteria (NOB) performing the latter step by converting nitrite to nitrate. This occurs in the aerobic ocean in the upper water column and is responsible for generating the majority of the biologically available nitrate, which can then be reduced by microorganisms in primarily anoxic regions of the ocean. The dominant reductive processes occurring in oxygen deficient zones (ODZs) via microbial activities are anaerobic ammonium oxidation (conversion of nitrite and ammonium to N_2 gas) and denitrification (reduction of nitrate to N_2 gas). However, it has been observed that steps of the nitrification pathway also occur in anoxic regions of the ocean (Beman et al. 2013; Buchwald and Casciotti 2013; Füssel et al. 2012). Certain species of NOB such as *Nitrospina* and *Nitrococcus* have been found in high numbers in

ODZs, possibly due to their flexible metabolic pathways (Füssel et al. 2017; Beman et al. 2013; Füssel et al. 2012). NOBs have been shown to reduce nitrate to nitrite in anaerobic conditions if necessary (Füssel et al. 2017). To help clarify the extent of which processes are occurring, dual isotope tracers of N and oxygen (O) have been used (Buchwald et al. 2015; Casciotti et al. 2013; Casciotti 2009; Sigman et al. 2005).

The ratio of stable isotopes ^{15}N to ^{14}N ($\delta^{15}N = [(^{15}N/^{14}N)NO_2 \div (^{15}N/^{14}N)N_2 - 1] \times 1000$) or ^{18}O to ^{16}O ($\delta^{18}O = [(^{18}O/^{16}O)NO_2 \div (^{18}O/^{16}O)VSMOW - 1] \times 1000$) in comparison to a reference gas in a collected sample can be measured on an isotope-ratio mass spectrometer (IRMS). Most metabolic reactions incorporate the lighter isotope compound at faster reaction rates because it expends less cellular energy. The difference in reaction rate of light versus heavy causes isotopic fractionation. Nitrite oxidation is a unique process with an inverse isotope effect (Cascioitti 2009) where NOB preferentially take up heavier isotopes, leaving a lighter nitrite substrate pool. The ratio between the reaction rates of heavy and light isotopes during chemical processes can be described by a kinetic isotope effect ($^{15}\varepsilon$ and $^{18}\varepsilon$). Previous batch culture experiments and nitrogen cycling models have produced $^{15}\varepsilon$ and $^{18}\varepsilon$ values which can be used to model processes occurring in ODZs (Buchwald and Casciotti 2010; Wankel et al. 2007; Casciotti et al. 2007; McIlvin and Altabet 2005; Sigman et al. 2005; Casciotti et al. 2002). However, they have measured a wide range of possible isotope effect values.

A previous batch culture study with *N. mobilis*, *N. marina* and *Nitrobacter* reported $^{15}\varepsilon$ and $^{18}\varepsilon$ of nitrite oxidation for each of the species (Buchwald and Casciotti 2010). Batch cultures were given a starting concentration of 50 µM nitrite, which was continuously taken up and converted to nitrate aerobically. *N. mobilis* and *N.*

winogradskyi completely consumed the nitrite in 10-48 hrs, whereas *N. marina* took two weeks. The $^{15}\varepsilon_{NXR}$ and $^{18}\varepsilon_{NXR}$ ranged from -22.0 to -7.8‰ and -1.0 to -10.2‰ respectively, with the least amount of fractionation occurring from *N. marina*. Within this study, there was a difference of 14‰ between isotope effects produced in different species.

In addition to pure culture experiments, many studies have used natural abundance stable isotope profiles of nitrite and nitrate to try and model what nitrogen cycle processes are occurring in various areas of the ocean (Table 2.1). In some cases, models can suggest what isotope effect values should be based on $\delta^{15}N$ and $\delta^{18}O$ data and oxidation and reduction rates. Often we see isotope effects that are different in pure culture experiments than what is predicted from models based on isotope profiles. This may be because the observed isotope effect in the environment is a result of a combination of metabolic processes occurring and there is not enough published isotope effect data to accurately account for this when modelling. Those values are not necessarily true bacterial isotope effects and it is difficult to differentiate to what extent each process contributes to the observed isotope effect.

Table 2.1: Comparison of nitrite oxidation kinetic isotope effect values ($^{15}\varepsilon_{NXR}$ and $^{18}\varepsilon_{NXR}$) from batch culture and modelling studies. Error is shown as ± 1 standard deviation.

Reference	Bacteria vs. Model	Average $^{15}\varepsilon_{NXR}$ (‰)	Average $^{18}\varepsilon_{NXR}$ (‰)
Buchwald and Casciotti 2010	*N. mobilis* pure culture	-20.2 ± 2.8	-8.2 ± 2.5
Buchwald and Casciotti 2010	*N. marina* pure culture	-9.1 ± 1.8	-1.3 ± 0.4
Buchwald et al. 2018	Model fit based on pore water concentration and isotope profiles in North Atlantic	-15	-3
Granger and Wankel 2016	Model fit based on isotope profiles in groundwater	-35 to 0	-7 to -3
Buchwald et al. 2015	Model fit based on isotope profiles in Costa Rica ODZ	-30	-3
Casciotti et al. 2013	Model fit based on isotope profiles in Peru ODZ	-15	-3

There is currently a large variation in predicted isotope effect values for nitrite oxidation (over 20‰) from both models and previous batch culture experiments (Table 2.1). This suggests that there are various isotope effect values for oxidation based on the bacterial species and their environmental conditions. It is possible that batch culture experiments are not truly indicative of the open ocean environment, as steady state growth can never be achieved. Bacteria will typically grow faster in batch culture as opposed to an ocean environment. Cells in a batch cultures may be given high concentrations of substrate, which will be consumed until it is depleted. If true isotope

effect variability does exist, the best way to test for factors that influence isotope effects is with continuous culturing in chemostats.

Chemostats can be used to grow bacterial cells in continuous culture to reach steady state, which is the condition where all variables for cellular growth (rate of nutrient supply, consumption, cell concentration etc.) remain constant. Nutrient media is supplied to a well-mixed vessel at a setrate. To achieve steady state, the outflow volume rate must equal the inflow rate (Madigan et al. 2014). Temperature, pH, dissolved oxygen, gas concentrations and other parameters can be tightly controlled in the vessel. Chemostats can be used to set growth rates and determine nutrient uptake rates based on steady state equations of growth. At steady state, growth rate is equivalent to dilution rate since there is no change in cell concentration over time. As the dilution rate changes, cells must increase or decrease their rate of growth in tandem or risk being washed out of the vessel. A change in dilution rate should cause a change in oxidation or consumption rates of key substrates.

In the present study we wanted to explore the effects of oxidation rate on nitrite oxidation isotope effects in three different NOBs: *Nitrococcus mobilis*, *Nitrospira marina,* and *Nitrobacter winogradskyi*. This study will help inform previous studies and N cycling models that have interpreted $\delta^{15}N$ and $\delta^{18}O$ data. We need to re-evaluate how future models are created to allow for changing oxidation rates to impact the predicted isotope effects. It is important that models are accurately representing N cycling in ODZs and how a changing climate could impact these areas in the future. ODZs are expanding due to climate change, altering rates of N transformations (Gilly et al. 2013). Additionally, the rate of loss of N has varied considerably based on the changing

dissolved oxygen levels (Deutsch et al. 2011). By improving existing models of N cycling, we can gain a better understanding of where N loss is occurring in parts of the ocean, what processes are occurring, and how they might continue to change in the future.

2.2 METHODS

2.2.1 Continuous Culture Incubations

Nitrococcus mobilis, Nitrospira marina and Nitrobacter winogradskyi strains were used in these experiments. *N. mobilis* was isolated off the Galapagos Archipelago (Watson and Waterbury 1971) and *N. marina* and *N. winogradskyi* were isolated from the Gulf of Maine (Watson et al. 1986; F. Valois, personal communication). *N. mobilis* was grown in artificial seawater-based media. Artificial seawater was made by adding the following salts to filtered water: 0.47 µmol/L NaCl, 0.01 µmol/L KCL, 0.01 µmol/L $CaCl_2$,*$2H_2O$, 0.025 µmol/L $MgCl_2$*$6H_2O$, 0.028 µmol/L $MgSO_4$*$7H_2O$ and 2.2 mmol/L HCO_3^-. Artificial seawater was diluted with filtered water to 75% salinity of natural seawater. The following salts were then added: 400 µmol/L $MgSO_4$, 30 µmol/L $CaCl_2$, 5 µmol/L K_2HPO_4, 2.3 µmol/L Geigy iron, 0.1 µmol/L Na_2MoO_4, 0.25 µmol/L $MnCl_2$, 0.002 µmol/L $CoCl_2$ and 0.08 µmol/L $ZnSO_4$. *N. marina* was grown in natural seawater-based media. Natural seawater was diluted with filtered water to a strength of 75% salinity of seawater. The water was then amended with the same salts as the artificial seawater media. Both natural and artificial seawater-based media was adjusted to pH 6.8 with HCl before autoclaving. *N. winogradskyi* was grown in freshwater media: 400 µmol/L $MgSO_4$, 30 µmol/L $CaCl_2$, 0.5 µmol/L K_2HPO_4, 2.3 µmol/L Geigy iron, 0.1 µmol/L Na_2MoO_4, 0.25 µmol/L $MnCl_2$, 0.002 µmol/L $CoCl_2$ and 0.08 µmol/L ZnSO4.

Freshwater media was adjusted to pH 7.4 with K_2CO_3 before autoclaving. Sterilized 1M $NaNO_2$ was added to all media after autoclaving to a final concentration of 10mM in batch culture.

All cells were grown chemolithoautotrophically in 100mL batch culture and transferred while in log growth phase to continuous culture in the Eppendorf BioFlo 120. The culture volume was 2L. All cultures were well aerated, stirred constantly, and kept at a temperature of 22 degrees Celsius through the use of a water-cooling system and electric heat blankets. Cultures were provided with nitrite-free media when initially transferred to continuous culture until all measurable nitrite from the batch culture transfer was oxidized. This took between 3-5 days on average, depending on initial nitrite concentrations. Media containing 50 μM nitrite was provided for the remainder of the continuous culture experiments for all species.

Cultures were considered to be at steady state when the outflow nitrite concentration remained constant (within 1-3μM) for three consecutive days. Media inflow and outflow pumps were calibrated to within one tenth of a mL. The time needed to achieve steady state varied by the rate at which cells were washed out (Table 2.2). Once steady state was reached, the dilution rate was decreased twice to achieve two additional growth conditions based on nitrite concentrations. Experiments were performed in duplicate (*N. mobilis* E1 and E2, *N. marina* E1 and E2, and *N. winogradskyi* E1 and E2).

Table 2.2: Summary of dilution rate used to achieve three steady states of growth in multiple species of NOBs grown in continuous culture.

Species	Growth Media	Dilution Rate (hr^{-1}) Experiment 1	Dilution Rate (hr^{-1}) Experiment 2
Nitrococcus mobilis	Artificial Seawater	0.036	0.045
		0.024	0.030
		0.018	0.018
Nitrospira marina	Natural Seawater	0.030	0.030
		0.015	0.015
		0.006	0.006
Nitrobacter winogradskyi	Fresh Water	0.030	0.030
		0.021	0.021
		0.012	0.012

2.2.2 Nitrite Concentration

Nitrite concentrations were measured in duplicate immediately after sampling using the Griess-Ilosvay method (Strickland and Parsons 1972). Standard deviation from duplicate nitrite concentration analysis was calculated. The standard deviation represents the amount of variation of the duplicate concentration values from the mean. Samples were diluted if necessary when concentrations were above 25 μM, then analyzed at 543nm using a UV spectrophotometer. Nitrite standards ranging from 0 μM to 25 μM were run at the beginning and used to calculate the concentration in each sample. The limit of quantitation using this method is 0.5 μM, with a limit of detection of 0.02 μM (Strickland and Parsons 1972). The range over which nitrite could be quantified using this assay is 0.5-25 μM. The average standard deviation of duplicate samples was 0.05 μM.

2.2.3 Cell Concentration

1mL samples were collected for cell counts. Cells were preserved with 1% paraformaldehyde and put in the dark for 20 minutes. Samples were then flash frozen and kept at -80 °C until being thawed for analysis. When thawed, 500 µL of cell sample were put through a 35µm filter. Cells were then stained with 5 µL of 100x dilution of SYBR green stock and incubated in the dark for ten minutes. Cells were then counted using a flow cytometer (C6 Accuri; BD). Three samples were collected in duplicate for each steady state. Standard deviation was calculated for the mean of all values. Relative standard error was calculated for log transformed values. The relative error in log(cell concentration) was calculated from log(e)*(standard deviation of cell concentration ÷ cell concentration).

2.2.4 Nitrite Oxidation Rate Analysis

Nitrite oxidation rates were calculated from the difference between nitrite concentrations of the inflow and outflow samples, multiplied by the dilution rate. Oxidation rates were then divided by the cell number to get a nitrite oxidation rate per cell. Propagated error of the cellular oxidation rate values results from error in nitrite concentration measurements as well as error in cell concentration measurements. The error in nitrite concentration is the standard deviations of duplicate analyses of nitrite concentration and the error from cell count measurements is the standard deviations of duplicate analyses for cell count measurements. The error in the subtraction of nitrite concentrations was calculated from the square root of the sum of squares of the error in

the measured value. The error in the division was calculated from the square root of the sum of squares of the error divided by the measured value.

2.2.5 Nitrite Isotope Measurements

The $\delta^{15}N$ and $\delta^{18}O$ of each sample was analyzed by converting nitrite to N_2O using sodium azide (NaN_3) and then measured on an IRMS (McIlvin and Altabet 2005). 30 mL of sample was immediately preserved with 0.6 mL of 6M NaOH to bring the same to a pH of 13.1 to limit abiotic exchange of oxygen at room temperature. Sample volume was calculated based on the volume needed to produce 20nmol or 10nmol of N_2O, depending on the nitrite concentration, and added to 20mL glass headspace vials. If sample salinity was below the value of seawater, combusted sodium chloride was added to the vial until PSU 35 was reached. Samples were diluted with a 1M pH 13.1 sodium chloride if needed to achieve a final volume of 15mL and were crimped shut with gray butyl septa and aluminum seals. Nitrite was then reduced with the addition of 0.8 mL purged 2M NaN_3 in 45% acetic acid. The solution reacted with samples at 30 degrees Celsius for 1 hour before being neutralized with the addition of 0.5 mL 10M NaOH.

Nitrite isotope standards MAA1_NEW, SHA1 and SHA2 were used to calibrate with determined $\delta^{15}N$ of -59.18‰, 17.51‰, and 118.88‰ and $\delta^{18}O$ of 19.72‰, 35.90‰, and 46.14‰ respectively (S. Haas unpubl.). These standards were calibrated from pre-existing WILIS10, WILIS11 and WILIS20 (Buchwald et al. 2016). Bracketing standards (between 5nmol-20nmol) were run in parallel at the beginning, middle and end of the run to correct for variation in sample size (Casciotti et al. 2007). Samples and standards were analyzed in duplicate on the Delta V IRMS (Thermo Scientific) coupled to a PreCon and

ConFlo III. The PreCon serves to trap focus nitrous oxide and remove water and carbon dioxide and the ConFlo III introduces the reference gas into the IRMS.

Isotope effects were calculated from $^{15}\varepsilon = (\delta^{15}N_{NO2\ Outflow} - \delta^{15}N_{NO2\ Inflow}) \div f$ or $^{18}\varepsilon = (\delta^{18}O_{NO2\ Outflow} - \delta^{18}O_{NO2\ Inflow}) \div f$. Values needed to calculate the isotope effect were fraction reacted (f), and the difference between δ values of the inflow and outflow samples. The error in f can be calculated from standard deviations of duplicate analyses for nitrite concentration. Propagated error of the isotope effect values results from the error in f, as well as from standard deviations of duplicate analyses for $\delta^{15}N$ and $\delta^{18}O$. The error in a result from an addition and/or subtraction was calculated from the square root of the sum of squares of the error in the measured value. The error in a result from a division and/or multiplication was calculated from the square root of the sum of squares of the error divided by the measured value.

2.3 RESULTS

2.3.1 The Effect of Dilution Rate on Cellular Oxidation Rate

All NOB converted nitrite to nitrate using oxygen as a terminal electron acceptor. During these chemostat experiments, the dilution rate was adjusted several times to change the rate at which the bacteria were receiving nitrite from the inflow media. A change in dilution rate did not cause a change in cell concentration for *N. mobilis* and *N. marina* throughout the course of the experiments (Figure 2.1). *N. winogradksyi* cell concentrations did not significantly change between dilution rates of 0.03/hr and 0.021/hr or between rates of 0.021/hr and 0.012/hr. However, there was a significantly higher cell concentration at a dilution rate of 0.012/hr compared to a dilution rate of 0.03/hr. ($p=0.02$) based on results from a t-Test where $p<0.05$ is significant.

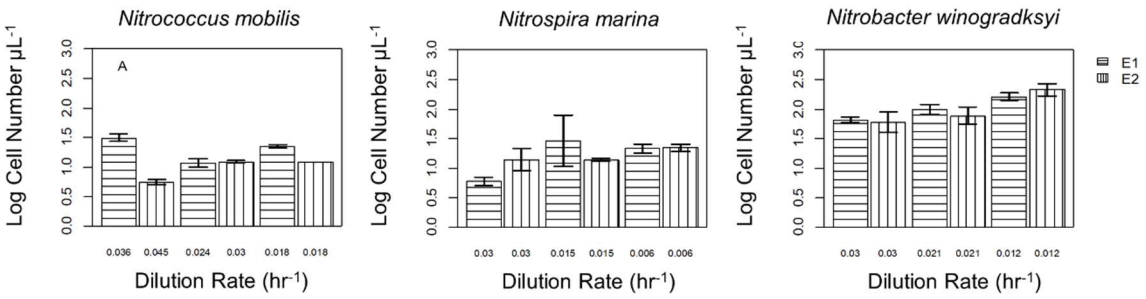

Figure 2.1: Number of bacteria determined by flow cytometry in steady state cultures of *N. mobilis* E1 (A) and E2 (D), *N. marina* E1 (B) and E2 (E) and *N. winogradsky* E1 (C) and E2 (F) at three dilution rates. Error bars shown for the log transformed cell concentration values represent the relative error of cell concentrations, calculated from the standard deviation of replicate cell count measurements.If error bars are not visible, they fall within the boundaries of the symbol.

The cellular nitrite oxidation rate of the bacteria was affected by changes in dilution rate. The cellular nitrite oxidation rate is the concentration of nitrite oxidized to nitrate over a period of time, per cell. Propagated error in cellular nitrite oxidation values was determined from the standard deviation or nitrite concentrations as well as standard deviation from cell concentration, as described in the methods. At the highest dilution rate, *N. marina* cells oxidized nitrite more slowly compared to when cells were subjected to a slower dilution rate (Figure 2.2). The oxidation rates of *N. mobilis* ranged from $2.8 \times 10^{-8} \pm 1.2 \times 10^{-9}$ to $1.1 \times 10^{-7} \pm 5.1 \times 10^{-9}$ µM/cell hr^{-1}, whereas oxidation rates of *N. marina* and *N. winogradskyi* were slower and ranged from $1.2 \times 10^{-9} \pm 3.9 \times 10^{-10}$ to $1.6 \times 10^{-8} \pm 2.6 \times 10^{-9}$ µM/cell hr^{-1} and $1.4 \times 10^{-9} \pm 5.5 \times 10^{-10}$ to $6.6 \times 10^{-9} \pm 1.2 \times 10^{-9}$ µM/cell hr^{-1} respectively. The *N. mobilis* E2 experiment had a larger range of dilution rates compared to E1 to ensure that the bacteria experienced both high and low concentrations of available nitrite.

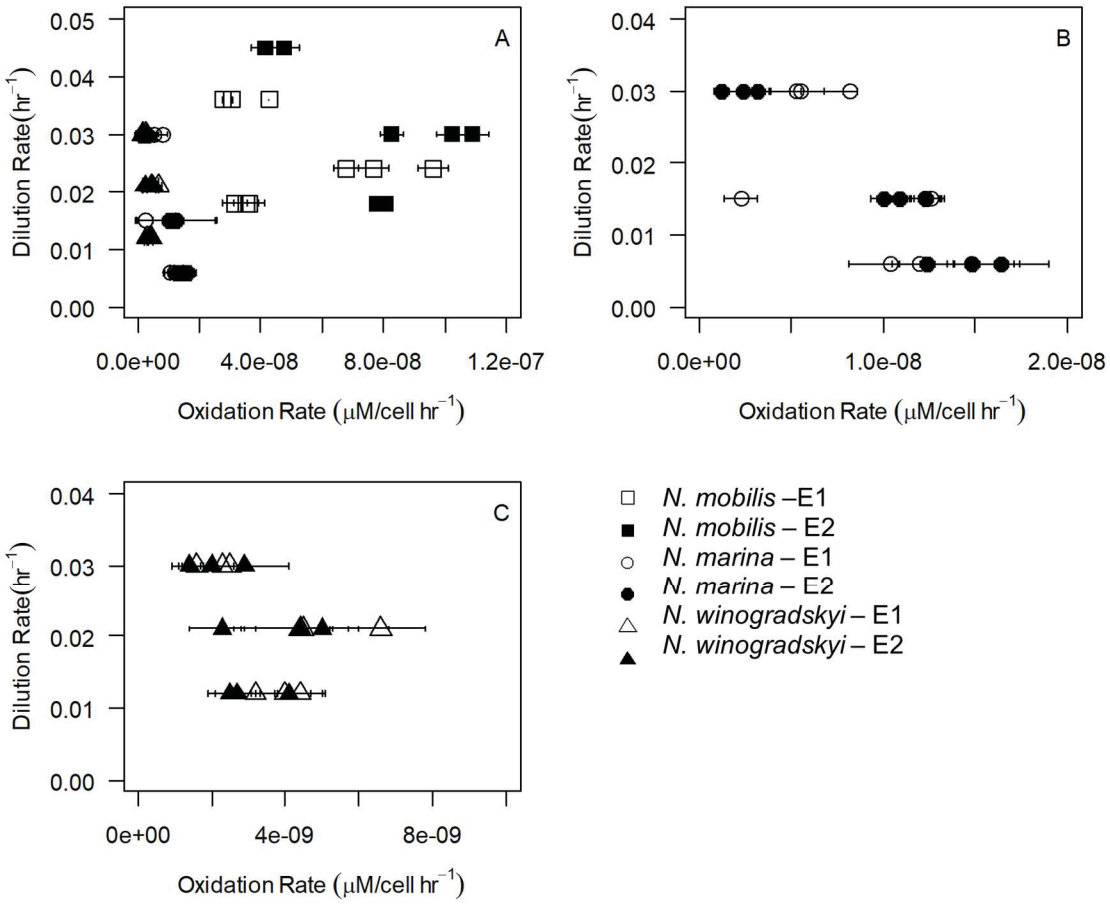

Figure 2.2: The cellular nitrite oxidation rate of *N. mobilis*, *N. marina* and *N. winogradskyi* (A) in duplicate at various dilution rates. Each dilution rate had 3 cell count measurements in duplicate and associated oxidation rates. The nitrite oxidation rate of *N. marina* may be faster at slower growth rates (B) and *N. winogradskyi* nitrite oxidation rate had no relationship to growth rate (C). Error bars represent propagated error from standard deviations in replicate nitrite concentration measurements and cell count measurements. If error bars are not visible, they fall within the boundaries of the symbol.

The fraction of initial nitrite reacted (f) changed based on differing dilution rates. Once steady state was reached at the maximum dilution rate, all species of bacteria consumed only a small percentage of the total inflow nitrite concentration (Figure 2.3). Conversely, when the dilution rate was very low, all cultures were able to consistently

consume a high percentage of the inflow nitrite, leaving a very low concentration of nitrite in the outflow. There was always 1µM or more of nitrite in the outflow to allow for accurate detection. It was difficult to achieve an intermediate consumption rate where only half of the nitrite was consumed. Altering the dilution rate to control for nitrite consumption may not have been sensitive enough.

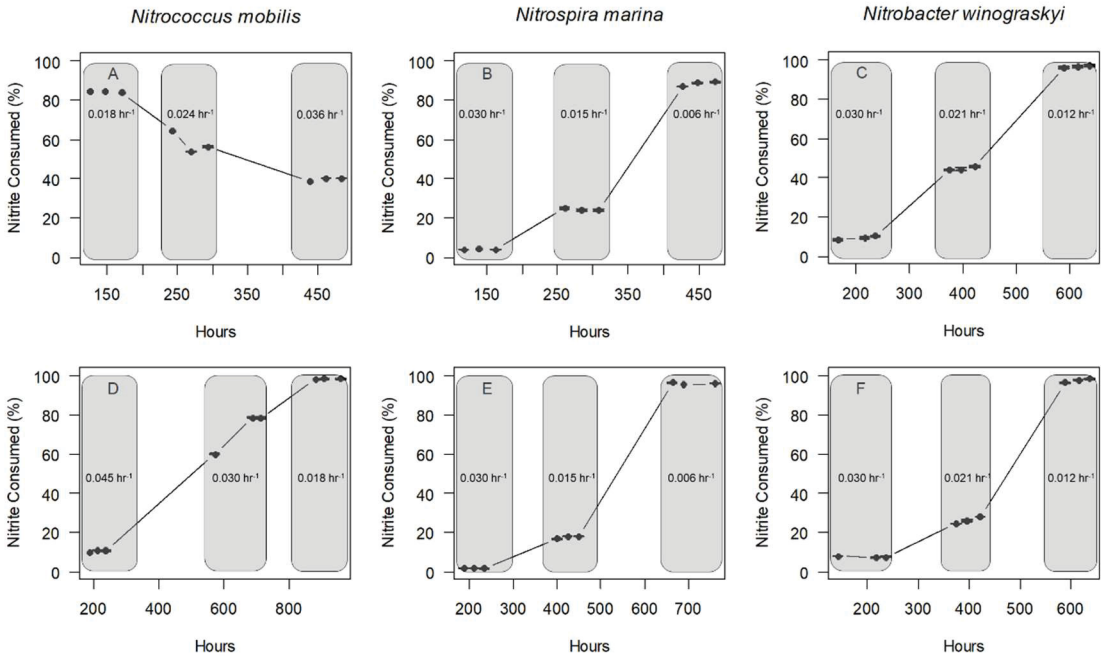

Figure 2.3: Percent of inflow nitrite consumed of *N. mobilis* E1 (A) and E2 (D), *N. marina* E1 (B) and E2 (E) and *N. winogradskyi* E1 (C) and E2 (F) at three dilution rates. Each dot represents the fraction of nitrite reacted at various time points. Dilution rate was a parameter that changed over the course of the experiment (hours on the x-axis). The dilution rate values are indicated in the shaded regions, above the time period where dilution rate stayed at the indicated value and cells were in a steady state. Error bars show propagated error from duplicate nitrite concentration analyses of inflow and outflow samples. If error bars are not visible, they fall within the boundaries of the symbol.

2.3.2 The Effect of Oxidation Rate on Isotope Fractionation

The chosen range of dilution rates allowed us to assess how the isotope effect changed based on changing oxidation rates. *N. mobilis* average $^{15}\varepsilon_{NXR}$ ranged from -16.9‰ to -9.7‰ and $^{18}\varepsilon_{NXR}$ ranged from -5.4‰ to 1.1‰ (Figure 2.4). There was little change in isotopic composition based on oxidation rate or dilution rate changes in *N. mobilis*. Additionally, there was also no change in isotope effects when the dilution rate in the *N. marina* experiments was very low and oxidation rates were faster. In these conditions, the average $^{15}\varepsilon_{NXR}$ ranged from -15.7‰ to -12.8‰. However, when the oxidation rates of *N. marina* were slowest, more fractionation was occurring. The average $^{15}\varepsilon_{NXR}$ at this steady state was found to be -27.2‰ ± 1.9‰. The average $^{15}\varepsilon_{NXR}$ ranged from -31.2‰ to -18.7‰ and the average $^{18}\varepsilon_{NXR}$ ranged from -7.2‰ to 0.2‰ from *N. winogradskyi.*

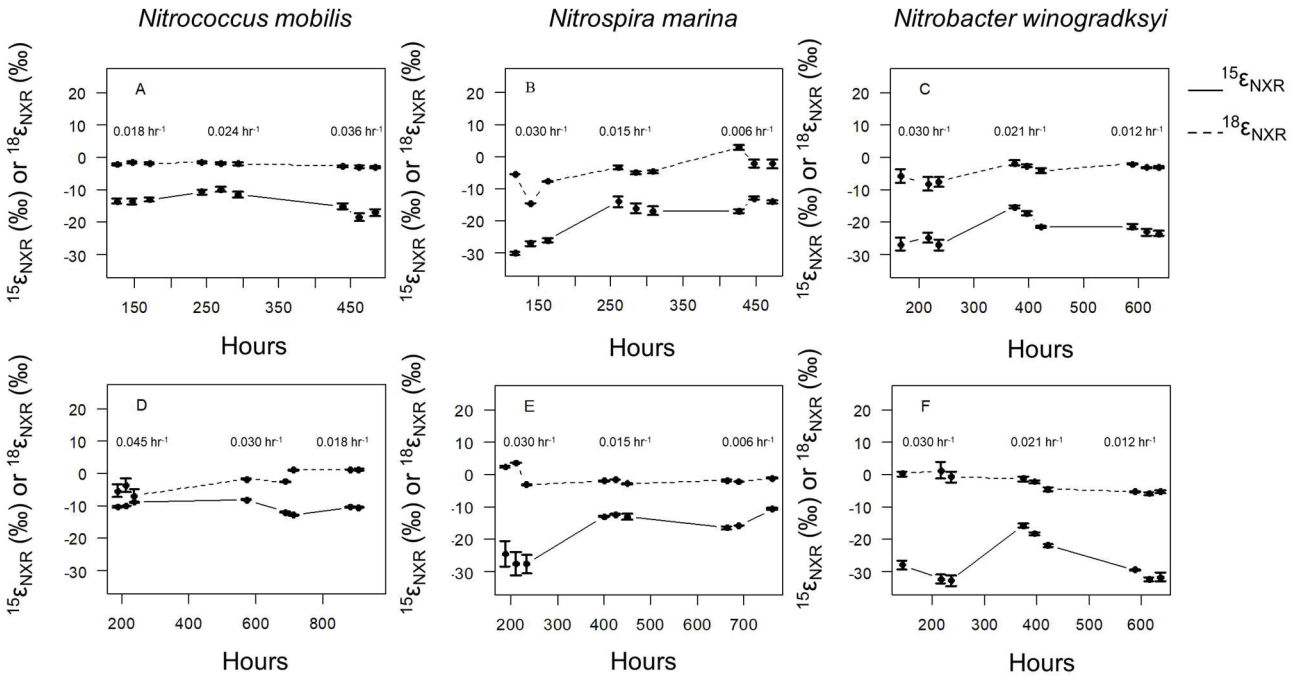

Figure 2.4: $^{15}\varepsilon_{NXR}$ and $^{18}\varepsilon_{NXR}$ of *N. mobilis* E1 (A) and E2 (D), *N. marina* E1 (B) and E2 (E) and *N. winogradskyi* E1 (C) and E2 (F) at three dilution rates. Dilution rates are shown for each steady state. Error bars represent propagation of error from standard deviations of duplicate nitrite concentration and duplicate $\delta^{15}N$ and $\delta^{18}O$ measurements. If error bars are not visible, they fall within the boundaries of the symbol.

2.4 Discussion

2.4.1 The Effect of Dilution Rate on Oxidation Rate

The chemostat pumped media into the vessel at a rate that was equal to the rate at which the cells and media were pumped out of the vessel. A large change between the concentration of nitrite in the inflow media and the nitrite concentration measured in the media pumped out indicated that the bacteria in the chemostat vessel were consuming a large percentage of the nitrite provided to them. We were aiming to achieve high, low and intermediate percentages of nitrite consumption. Initially, it was expected that there would be a linear change in nitrite consumption as the dilution rate was changed. However, it was difficult to achieve an intermediate level of consumption because the

nitrite consumption and the dilution rate did not change as expected. Small, controlled changes in dilution rate resulted in larger changes in oxidation rate and nitrite consumption. In addition to this, we were expecting to see the highest oxidation rate in the experiments with the highest dilution rates. As the bacteria grow faster, it was thought that this would necessitate faster oxidation for energy. Despite this, we saw the opposite effect where the oxidation rates were slowest during a time of fast cell growth. This may be attributed to a change in cell size; however, due to the shape and size of the bacteria, it was difficult to assess if the cells were changing throughout the experiment and this should be looked into further.

The lack of relationship between oxidation rate and growth rate has been shown before in previous kinetics studies with *Nitrosomonas europaea*, *Nitrobacter hamburgensis* and *Nitrobacter winogradskyi* (Laanbroek et al. 1994; Both et al. 1992). Laanbroek et al. (1994) found that dilution rate had no significant effect on the maximum nitrite oxidation rate for *N. europaea* or *N. hamburgensis*. Both et al. (1992) found that although the maximum nitrite oxidation rate per cell of *N. hamburgensis* decreased as growth rate increased, the actual oxidation rate in the vessel was independent of growth rate, whereas the oxidation rate of *N. winograskyi* increased with increasing growth rate. This might indicate that the relationship between growth rate and nitrite oxidation rate is bacteria dependent. The critical dilution rate is the highest rate possible where steady state growth can be maintained. This threshold differs for individual species of bacteria. At this critical rate, it may be possible that oxidation rate and growth rate uncouple.

2.4.2 Isotope Effects Change Based on Oxidation Rate

No previous studies or N cycling models have probed the relationship between isotope effects and cellular oxidation rate in NOB. In this study there was no observed relationship between $^{18}\varepsilon_{NXR}$ and oxidation rates in any of the bacterial species (Figure 2.5). Additionally, no relationship between *N. mobilis* $^{15}\varepsilon_{NXR}$ and oxidation rates was observed. The *N. mobilis* cells in each steady state had faster oxidation rates than what was observed in *N. marina* or *N. winogradksyi.* Both the *N. marina* and *N. winogradskyi* cells had slower oxidation rates compared to *N. mobilis,* and larger inverse isotope effects were observed. The slowest oxidation rates in *N. marina* correlated with a larger inverse $^{15}\varepsilon_{NXR}$ value (-27‰) compared to what has been recorded in previous published values in batch culture for this species (-9‰) (Buchwald and Cascoitti 2010). As the oxidation rates got faster in *N. marina,* there was less fractionation observed. Overall, the $^{15}\varepsilon_{NXR}$ and oxidation rates from all species combined fit a logarithmic trend with an R^2 value of 0.65. Regardless of species, it can be suggested that if cells have a slower nitrite oxidation rate, such as what was seen in *N. marina* and *N. winogradskyi,* there can be more isotopic fractionation associated with nitrite oxidation. If cells are growing at a faster oxidation rate, similar to what was seen in *N. mobilis,* there may be less fractionation occurring. If we could have forced *N. mobilis* to have a slower oxidation rate, we could potentially have seen a more inverse fractionation as well.

Bacterial cultures typically grow much faster in pure culture than in the ocean environment. The slowest oxidation rates found in the chemostat vessel were still faster than those found in the ocean (Table 2.3). However, the slowest oxidation rate of *N. marina* where more fractionation was occurring is the closest value to average oxidation

rates predicted in ODZs (709 nM day^{-1}). The average isotope effect value of -27‰ to -30‰ found in this study in bacteria oxidizing slowly may be more representative of the environmental bacterial isotope effect.

Table 2.3: Estimated oxidation rates from field sampling or pure culture experiments in comparison with results from this study. *N. marina* oxidation rates were closest to those found in ODZs. Oxidation rates were not able to be normalized per cell due to the uncertainties in finding a reliable NOB cell count in ODZs.

Reference	Species or Location	Average or Estimated Oxidation Rate (nanomolar/day)
Beman et al. 2013	ETNP ODZ	50 to 213
Füssel et al. 2012	Namibian ODZ	14 to 372
Clark et al. 2008	North Atlantic	13 to 30
Buchwald et al. 2010	*N. mobilis* in batch culture	120 000
This Study	*N. mobilis* in continuous culture	5870 to 22 671
This Study	*N. marina* in continuous culture	710 to 7679
This Study	*N. winogadskyi* in continuous culture	3346 to 15 017

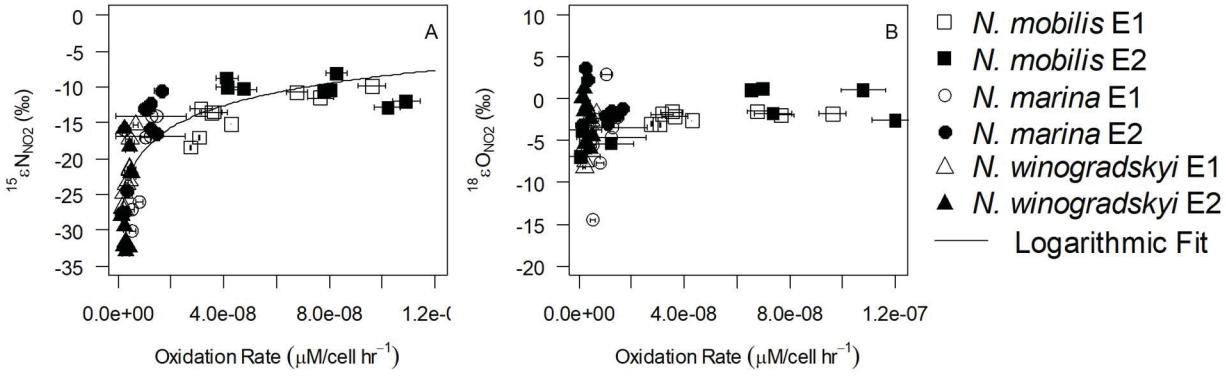

Figure 2.5: The $^{15}\varepsilon_{NXR}$ of *N. mobilis*, *N. marina* and *N. winogradskyi*, (A) and the $^{18}\varepsilon_{NXR}$ of each species in duplicate (B) produced at various calculated nitrite oxidation rates. The slower oxidation rates seen with *N. marina* corresponded to greater inverse fractionation occurring. Error bars show propagated error of oxidation rate values as described in the methods. If error bars are not visible, they fall within the boundaries of the symbol.

2.4.3 Metabolic Differences between NOB

There are several key differences between NOB species. *Nitrococcus, Nitrospira* and *Nitrobacter* species all express nitrite oxidoreductase (NXR) proteins. These NOB convert nitrite to nitrate via the membrane bound NXR. However, the active site of NXR in *Nitrococcus* and *Nitrobacter* is found in the cytoplasm, whereas the active site in *Nitrospira* is found in the periplasm, which is more energetically favourable (Lücker et al. 2010; Spieck and Bock 2005). Additionally, *Nitrococcus* and *Nitrobacter* have multiple copies of NXR-A (the substrate binding subunit) clustered away from oxidative genes. *Nitrospira* on the other hand has multiple NXR-A copies clustered with oxidative genes but has no oxidative stress genes, thus making it more adapted to low nitrite low oxygen environments (Lücker et al. 2010; Sundermeyer-Klinger et al. 1984). It is clear that there are several metabolic differences between *Nitrococcus/Nitrobacter* and

Nitrospira that could be causing variability seen in oxidation rates across these three species.

The *N. mobilis* bacteria never oxidized nitrite at as slow of a rate as the *N. marina* or *N. winogradskyi* which may be why there was no impact on the *N. mobilis* $^{15}\varepsilon_{NXR}$ (Figure 2.5). Additionally, when *N. marina* cells oxidized slowly, they were growing at a critical rate, meaning if the dilution rate were further increased the bacteria would not have survived. *N. mobilis* may not have been at this critical growth rate in the chemostat. *N. winogradskyi* oxidized nitrite at consistently low oxidation rates, similar to the slowest oxidation rates seen from *N. marina*. In this case, the $^{15}\varepsilon_{NXR}$ averaged between -32‰ and -18‰ for the entire experiment, unlike what was seen in *N. mobilis* or *N. marina*.

2.4.4 NXR Reversibility Possible Mechanism for Relationship between Isotope Effect and Oxidation Rate

The reversibility of the NXR protein may help explain the relationship between isotope effect and oxidation rate. The process of nitrite oxidation occurs with an inverse isotope effect (Casciotti 2009). NOB preferentially incorporate heavier isotopes into their metabolic processes, leaving lighter isotopes in the substrate pool. True enzyme level inverse isotope effects are rare in biology, but could be explained in NOB partially by transition state theory. When undergoing nitrite oxidation, the N atom is more strongly bonded to the transition state molecule than to the substrate, which could lead to an inverse isotope effect for nitrite oxidation (Casciotti 2009). However, the isotope effect is likely a combination of the kinetic isotope effect for the forward reaction and an equilibrium isotope effect between nitrite and nitrate (Kemeny et al. 2016; Wunderlich et

al. 2013). Under anaerobic conditions, *Nitrococcus* can convert nitrate to nitrite despite having no nitrate reductase genes (Füssel et al. 2012) and partially purified NXR from *Nitrobacter* has also shown reversibility (Yamanka and Fukumori 1988). The forward reaction of nitrite oxidation has an inverse isotope effect, whereas the reverse reaction of nitrate reduction has a 'normal' isotope effect. The reverse reaction has been shown to occur, although minimally, in addition to the dominating forward reaction in aerobic conditions (Casciotti 2009). A changing oxidation rate may impact the ratio of forward to reverse reactions, and expression of the equilibrium isotope effect. In slower reactions, there is more time for isotope equilibration to occur between nitrite and nitrate, increasing the proportions of light N in the substrate pool. In this case, there would be more backwards reaction expressed, potentially causing the more negative inverse isotope effects seen in *N. marina* and *N. winogradskyi* at slow oxidation rates. As oxidation rates increase, there is less time for equilibration and less reverse reaction could be expressed, leading to a more 'normal' isotope effect.

2.4.5 Implications on Past Modelling in ODZs

Previous N cycling models have difficulties reconciling observed isotope profiles with results published from pure culture studies. For example, a model constructed by Casciotti et al. (2013) investigated the mechanism of N cycling in the Peru ODZ by using oxygen isotope exchange data between nitrate and water along with nitrite oxidation rates to be able to accurately fit their collected nitrate $\delta^{18}O$ data from the ETSP. However, with the lack of nitrite $\delta^{18}O$ data it was difficult to constrain the rate of nitrate produced by nitrite oxidation. Large ranges of reaction rate constants run through the Casciotti model were unsuccessful at simulating the extremely low $\delta^{15}N_{NO2}$ that could be fit to models

using published $^{15}\varepsilon_{NXR}$ values for bacterial nitrite oxidation between -8 to -22‰ (Buchwald and Casciotti 2010; Casciotti 2009). Another model constructed by Buchwald et al. (2015) used natural abundance stable isotope profiles of the secondary nitrite maximum of the ETNP off Costa Rica. Their model predicted NXR fractionation factors much larger than previously measured in cultures, corresponding to a $^{15}\varepsilon_{NXR}$ of -30‰. These values are much more similar to what we observed in *N. marina* (-27‰) and *N. winogradskyi* (-32‰) when oxidation rates were slow and closer to the magnitude observed in the ocean. Models based on isotope profiles often cannot simulate the large difference between light $\delta^{15}N_{NO2}$ and heavy $\delta^{15}N_{NO3}$ of up to 60‰ (Buchwald et al. 2018; Buchwald et al. 2015). A larger isotope effect for nitrite oxidation may help to reconcile this difference.

2.4.6 Conclusion

The overall purpose of our work is to better understand how bacteria collectively express isotope effects in the ocean. Pure batch culture experiments can help to isolate which bacteria are responsible for which process; however, there is no way to achieve steady state, or to assess other factors such as oxidation rate that could be affecting isotope effects. A more ideal way to determine true isotope effects is by using chemostats, as we have done here. We have shown that there is a relationship between oxidation rate and isotope effects, as well as growth rate and oxidation rate. However, further work needs to go into characterising NXR in various species of NOB to better understand if the reversibility is impacting the variability in the measured isotope effects. It is also possible that the amount of nitrite or substrate surrounding the bacterial cells is important, in addition to the oxidation rates. Regardless, it is clear that the rate at which

bacteria take up nitrite is important, and it would be helpful to know if oxidation rate always slows at critical growth rate, as we saw with *N. marina* and *N. winogradskyi*, or if that is based on how bacteria grow in aerobic conditions. Ultimately, further insight will help us improve existing models of ODZs and other areas of the ocean where oxidation occurs, and can help us predict how they may change in the future.

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CHAPTER 3 NITRITE ISOTOPE SYSTEMATICS AND OXYGEN EXCHANGE IN *NITROSPINA GRACILIS* AEROBIC BATCH CULTURE

3.1 INTRODUCTION

Deep water sources of nitrate (NO_3^-) can partially regulate the rate of primary production and carbon export from the surface oceans (Eppley and Peterson 1979). Despite the importance, there are still many uncertainties with regards to global N budgets and the roles that microbially mediated metabolic processes have in controlling the N cycle (Wang et al. 2019; Gruber and Galloway 2008). Nitrification is a two-step process in which ammonia is converted to nitrate. Nitrite-oxidizing bacteria (NOB) perform the second step by converting nitrite to nitrate. The contribution of this process to overall nitrite and nitrate production in the ocean is still unclear. Difficulty in resolving the N budget has led to the use of dual isotope tracers of N and oxygen (O) to model oxidation in the ocean (Buchwald et al. 2015; Sigman et al. 2005). The ratio of stable isotopes ^{15}N to ^{14}N ($\delta^{15}N = [(^{15}N/^{14}N)NO_2 \div (^{15}N/^{14}N)N_2 - 1] \times 1000$) or ^{18}O to ^{16}O ($\delta^{18}O = [(^{18}O/^{16}O)NO_2 \div (^{18}O/^{16}O)VSMOW - 1] \times 1000$) in comparison to a reference gas in a collected sample can be measured on an isotope-ratio mass spectrometer (IRMS). The partitioning of heavy to light ratios during a metabolic reaction is defined as a kinetic isotope effect ($^{15}\varepsilon$ and $^{18}\varepsilon$). Nitrite oxidation is a unique process with an inverse isotope effect (Casciotti 2009) where NOB preferentially take up heavier isotopes, leaving a lighter nitrite substrate pool.

Previous batch culture experiments and nitrogen cycling models have either measured or predicted $^{15}\varepsilon$ and $^{18}\varepsilon$ values respectively, which can be used to model processes occurring in oxygen deficient zones (ODZs) (Buchwald and Casciotti 2010; Wankel et al. 2007; Casciotti et al. 2007; McIlvin and Altabet 2005; Sigman et al. 2005;

Casciotti et al. 2002). These are areas of the ocean dominated by anaerobic processes. It has also been shown that nitrite oxidation occurs in these areas and NOB can be found in large numbers (Füssel et al. 2012). Oceanic models can use $\delta^{15}N$ and $\delta^{18}O$ data and oxidation and reduction rates to predict what isotope effect values should be. However, often there is a difference found between values produced from models and values measured in pure bacterial culture experiments. Many models have trouble reconciling these pure culture results and observed isotope profiles and have predicted a wide range of possible nitrite isotope effect values (0 to -60‰). For example, a model constructed by Buchwald et al. (2015) used stable isotope profiles of the ETNP off Costa Rica. Their model predicted NXR fractionation factors significantly larger than previously measured in cultures, corresponding to a $^{15}\varepsilon_{NXR}$ of -30‰.

Pure culture experiments of NOB can also provide isotope effect data for nitrite oxidation and nitrate reduction. A previous batch culture study with *N. mobilis*, *N. marina* and *Nitrobacter sp.* reported $^{15}\varepsilon$ and $^{18}\varepsilon$ of nitrite oxidation for each of the species (Buchwald and Casciotti 2010). Within this study, there was a difference of 14‰ between isotope effects produced in different species. This suggests that kinetic isotope effects might differ based on different growth conditions among species. Buchwald and Casciotti (2010) experiments were also conducted with ^{18}O-labelled water to follow the $\delta^{18}O$ of nitrite and nitrate during nitrite oxidation to better understand the balance of the deep ocean nitrate budget. Using their data, the estimated range of $\delta^{18}O$ of nitrate produced was estimated to be -8.3‰ to -0.7‰.

The Buchwald (2010) study did not provide any data for the *Nitrospina gracilis* species. *N. gracilis* is one of the most prominent NOB found in marine environments and

has been detected in open ocean and ODZs (Beman et al. 2013; Füssel et al. 2012; Santoro et al. 2010; Mincer et al. 2007). *N. gracilis* is a gram-negative bacteria isolated from the surface water that grows chemoautotrophically with nitrite as an energy source and CO_2 as a carbon source (Watson and Waterbury 1971). The species can grow adequately in low oxygen environments by transporting NXR complexes to the periplasm which is more energy efficient (Lücker et al. 2013). Additionally, *N. gracilis* encodes a *cbb*$_3$-type oxidase which has high O_2 affinity to help respiration (Cosseau and Batut 2004). *Nitrospina* made up 1.3% of total microbial communities in the upper ODZ in the Namibian upwelling (Füssel et al. 2012). Despite this, there has been little genetic data available and the metabolic potential of this species is still not well understood. Nitrite oxidation is the only known microbial pathway leading to the production of nitrate. Since nitrate accounts for up to 88% of fixed nitrogen (Gruber 2008), understanding the flexible metabolic pathways of NOB is crucial to better understand nitrate budgets in the deep ocean.

This study presents nitrite isotope effect values associated with nitrite oxidation in *N. gracilis*. The original intent of this study was also to measure the oxygen atom exchange between water and nitrite to help better understand what controls the $\delta^{18}O$ values of nitrate produced by NOB. Although this was not accomplished, the $^{15}\varepsilon_{NO2}$ and $^{18}\varepsilon_{NO2}$ of nitrite oxidation in *N. gracilis* is still valuable information that can be added to the known nitrite isotope effects for use in future models.

3.2 METHODS

3.2.1 Culture Maintenance

Nitrospina gracilis was originally isolated from surface waters of the Atlantic and cultures were provided by John Waterbury and Frederica Valois (Watson and Waterbury 1971). Cultures were grown in natural seawater media. Natural seawater was diluted with filtered water to achieve 75% salinity of seawater. The following salts were then added: 400 µmol/L $MgSO_4$, 30 µmol/L $CaCl_2$, 5 µmol/L K_2HPO_4, 2.3 µmol/L Geigy iron, 0.1 µmol/L Na_2MoO_4, 0.25 µmol/L $MnCl_2$, 0.002 µmol/L $CoCl_2$ and 0.08 µmol/L $ZnSO_4$. Sterilized 1M $NaNO_2$ was added after autoclaving to a final concentration of 2 mM in batch culture. Cultures were kept in 500 mL flasks in a dark 22 degrees Celsius incubator.

3.2.2 *Nitrospina gracilis* Pure Culture Nitrite Oxidation Experiments

Media required for the nitrite oxidation experiments was prepared as above with the addition of ^{18}O labelled water with a $\delta^{18}O_{H20}$ of 5000‰ vs. VSMOW. Between 0 to 10 mL of labelled water was added to media flasks before autoclaving to achieve $\delta^{18}O_{H20}$ values that should have been -1.39‰ (Flask A and Flask B), +48.25‰ (Flask C and Flask D) and +98.25‰ (Flask E and Flask F). However, due to an error, the flasks had actual $\delta^{18}O_{H20}$ values of -4.2‰ (Flask A and Flask B), -2.8‰ (Flask C and Flask D) and -1.3‰ (Flask E and Flask F). After autoclaving, media was adjusted to pH 8.2. Prior to the initiation of the experiment, sterile 1M $NaNO_2$ was added to the media for a final concentration of 60µM.

Pure cultures of *N. gracilis* were grown chemolithoautotrophically in 500 mL flasks and harvested while in log growth phase by centrifugation at 4000rpm for 30 minutes. Cell pellets were resuspended in 1mL of natural seawater media and inoculated into the prepared media. Duplicate flasks were inoculated. Flasks were immediately subsampled for nutrient and isotopic analysis. Samples were taken again every 8-12 hours afterwards until all nitrite was converted to nitrate. All samples were filtered.

3.2.3 Concentration Analysis and Isotope Measurements

Nitrite concentrations were measured immediately after sampling using the Griess-Ilosvay method (Strickland and Parsons 1972). Further details on this method can be found in *Chapter 2*.

The $\delta^{15}N_{NO2}$ and $\delta^{18}O_{NO2}$ of each sample with 1µM or more of nitrite was analyzed by converting nitrite to N_2O using sodium azide (NaN_3) and then measured on an isotope ratio mass spectrometer (McIlvin and Altabet 2005). Further details on this method can be found in *Chapter 2*. The $\delta^{18}O_{H2O}$ samples were analyzed using a Picarro L2130-I CRDS analyzer with a vaporization module A0211 coupled to an HTC-xt Leap Pal Technologies autosampler (Walker et al. 2015).

3.3 RESULTS

3.3.1 Nitrite Oxidation

Nitrospina gracilis converted all the nitrite to nitrate in each experiment. Starting nitrite was consumed within 55-60 hours (Figure 3.1). The $\delta^{15}N_{NO2}$ of each culture after adding cells was 4.3 ± 0.08‰ and decreased over time depending on minor changes in

initial cell densities and how quickly each culture was consuming nitrite (Figure 3.1). The starting $\delta^{18}O_{NO2}$ was 3.5 ± 0.05‰ and decreased slightly over time.

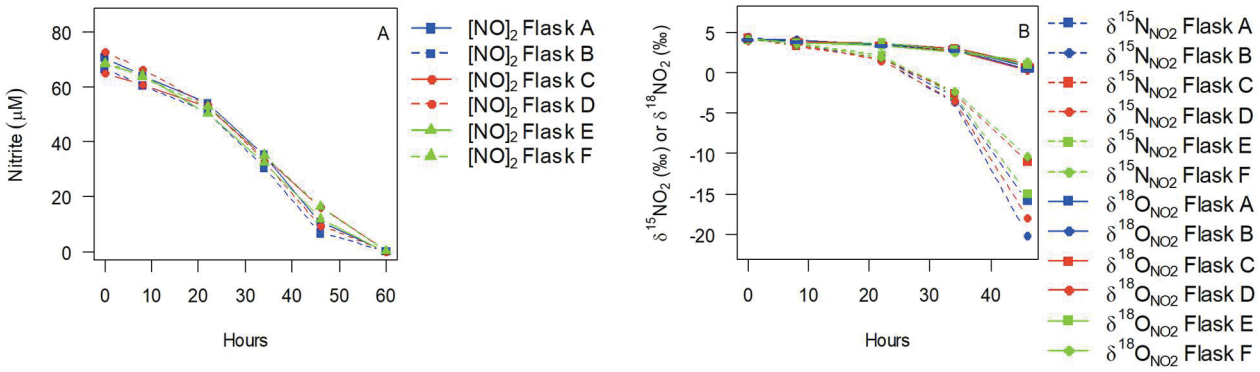

Figure 3.1: Nitrite concentrations (μM) during the *N. gracilis* incubation experiment (A). The $\delta^{15}N_{NO2}$ started at a value of +4.3 ‰ while the $\delta^{18}O_{NO2}$ started at +3.5‰ (B). Each set of duplicate flasks had a $\delta^{18}O_{H2O}$ of -4.2‰ (Flasks A and B), -2.8‰ (Flasks C and D), and -1.3‰ (Flasks E and F).

3.3.2 Nitrite Kinetic Isotope Effects

Kinetic isotope effects of nitrite ($^{15}\varepsilon_{NO2}$ and $^{18}\varepsilon_{NO2}$) can be calculated from the slope of the $\delta^{15}N_{NO2}$ or $\delta^{18}O_{NO2}$ vs $-\ln(f)$ plots (Figure 3.2). The fraction of nitrite remaining (f) = $[NO_2^-]/[NO_2^-]_{initial}$. A linear relationship was found between $-\ln(f)$ and $\delta^{15}N_{NO2}$ or $\delta^{18}O_{NO2}$, which is expected to occur with closed system Rayleigh fractionation (Scott et al. 2004). The average $^{15}\varepsilon_{NXR}$ calculated from all experiments is -10.8 ± 0.3‰ and the average $^{18}\varepsilon_{NO2}$ was found to be -1.8 ± 0.2‰ (Table 3.1). The R^2 value for each line in the $^{15}\varepsilon_{NO2}$ Rayleigh plot was above 0.99. The R^2 value for each line in the $^{18}\varepsilon_{NXR}$ Rayleigh plot differed slightly from 0.99 and ranged from 0.98 to 0.99.

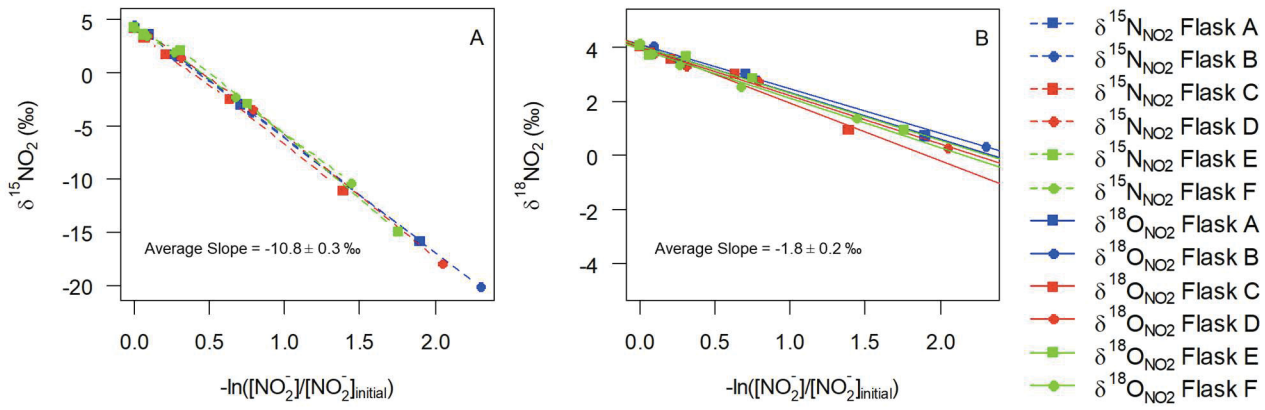

Figure 3.2: Rayleigh plots for $^{15}\varepsilon_{NXR2}$ (A) and $^{18}\varepsilon_{NXR}$ (B) of nitrite oxidation in *Nitrospina gracilis*. Each set of duplicate flasks had a $\delta^{18}O_{H2O}$ of -4.2‰ (Flasks A and B), -2.8‰ (Flasks C and D), and -1.3‰ (Flasks E and F). Average slope error is represented by standard deviation between each calculated slope.

Table 3.1: Calculated $^{15}\varepsilon_{NXR}$ and $^{18}\varepsilon_{NXR}$ in *Nitrospina gracilis* batch culture with a starting nitrite of 60 µM. Error represents ± 1 standard deviation.

Experiment	$\delta^{18}O_{H20}$ of Media	$^{15}\varepsilon_{NXR}$ (‰)	Average $^{18}\varepsilon_{NXR}$ (‰)	$^{18}\varepsilon_{NXR2}$ (‰)	Average $^{18}\varepsilon_{NXR}$ (‰)
Flask A	-4.2‰	-10.7 ± 0.1	-10.7 ± 0.03	-1.7 ± 0.08	-1.7 ± 0.07
Flask B	-4.2‰	-10.7 ± 0.05		-1.6 ± 0.07	
Flask C	-2.8‰	-10.9 ± 0.2	-10.9 ± 0.02	-2.1 ± 0.2	-2.0 ± 0.3
Flask D	-2.8‰	-10.9 ± 0.3		-1.8 ± 0.08	
Flask E	-1.3‰	-11.0 ± 0.5	-10.6 ± 0.6	-1.7 ± 0.1	-1.8 ± 0.05
Flask F	-1.3‰	-10.2 ± 0.1		-1.8 ± 0.2	
Average			**-10.8 ± 0.3**		**-1.8 ± 0.2**

3.4 DISCUSSION

3.4.1 *Nitrospina gracilis* Metabolism and Cell Structure

N. gracilis are long, slender Gram-negative rods that grow to be between 0.3-0.4 µM wide and 2.7-6.5 µM long (Watson and Waterbury 1971). This species is an obligate autotroph and must fix CO_2 via the rTCA cycle (Berg 2011). All energy necessary for growth comes from the oxidation of nitrite. The *nirA* gene for ferredoxin-nitrite reductase and *nirC* for a nitrite transporter were found in the *N. gracilis* genome (Lücker et al. 2013). Nitrite oxidation is catalyzed by the nitrite oxidoreductase (NXR) enzyme attached to the cytoplasmic membrane. Two operons encode the three subunits of *nxrABC*. Both operons are very similar with an identity of 95% (Lücker et al. 2013). The *N. gracilis* genome also encodes two copies of NirK, however the function is still unclear (Lücker et al. 2013). Unlike other NOB such as *Nitrobacter* and *Nitrococcus*, the *Nitrospina* cells have the NXR active site in the periplasm (Lücker et al. 2013; Lücker et al. 2010; Spiek et al. 1998). Periplasmic-oriented NXR are more energy efficient because the enzymatic activity can contribute to proton motive force (Lücker et al. 2013). NOB with periplasmic NXR placement can survive on lower nitrite concentrations than NOB with cytoplasmic NXR (Sorokin et al. 2012). *N. marina* also shares a periplasmic-oriented NXR and has also been found to survive in lower oxygen environments (Lücker et al. 2010). Despite being an aerobe, *Nitrospina* encodes a *cbb3*- type terminal oxidase that has a high oxygen affinity (Cosseau and Batut 2004). This may also help in low oxygen environments and increase survival in ODZs.

N. gracilis was unable to grow in a chemostat using conditions employed in the *Chapter 2 Methods* with *Nitrobacter winogradskyi, Nitrococcus mobilis* and *Nitrospira marina*. *N. gracilis* cells were unable to grow fast enough to match even very slow dilution rates. One possible explanation could be due to the shape of the bacteria. These slender rod-shaped cells were known to be fragile when isolated, compared to other NOB at the time (Watson and Waterbury 1971). It is possible that the bubbling and/or stirring necessary to maintain growth in the chemostat disturbed the *N. gracilis* cells too much, or could have sheared them due to their fragile shape. It may be possible to grow them in continuous culture in the future by gently stirring and bubbling with gas periodically, instead of consistently. Additionally, if doing experiments aerobically, it is probably only necessary to bubble with air when the dissolved oxygen levels drop significantly.

3.4.2 ^{15}N and ^{18}O Nitrite Oxidation Kinetic Isotope Effects

In this study, we have recorded both N and O kinetic isotope effects for nitrite oxidation (Table 3.1). An inverse isotope effect is expected (Casciotti 2009) and has been observed previously in several pure NOB culture experiments (Table 3.2). Both the $^{15}\varepsilon_{NXR}$ and $^{18}\varepsilon_{NXR}$ in *N. gracilis* (-11‰ and -2‰ respectively) were identical within uncertainty to the isotope effects reported in batch culture experiments with *N. marina* (-9‰ and -1‰) (Buchwald et al. 2010). These two bacteria both share similar metabolisms with a periplasmic-oriented NXR. Additionally, these values were also comparable to *N.marina* isotope effects (-16‰ to -13‰) when grown in continuous culture at fast oxidation rates (*Chapter 2*).

Table 3.2: Average nitrite oxidation kinetic isotope effects from batch culture experiments and oceanic models, compared with results from this study.

Reference	Bacteria vs. Model	Average $^{15}\varepsilon_{NXR}$ (‰)	Average $^{18}\varepsilon_{NXR}$ (‰)
Buchwald et al. 2018	Model fit based on pore water concentration and isotope profiles in deep sea North Atlantic	-15	-3
Granger and Wankel 2016	Model fit based on isotope profiles in groundwater near Cape Cod	-35 to 0	-7 to -3
Buchwald et al. 2015	Model fit based on isotope profiles in Costa Rica ODZ	-30	-3
Casciotti et al. 2013	Model fit based on isotope profiles in Peru ODZ	-15	-3
Buchwald and Casciotti 2010	*N. mobilis* pure culture	-20	-8
Buchwald and Casciotti 2010	*N. marina* pure culture	-9	-1
This Study	***N. gracilis* pure culture**	**-11**	**-2**

As seen in *Chapter 2*, growth rate can influence nitrogen isotope fractionation in nitrite oxidation. *N. gracilis* oxidized nitrite in steady state batch culture at an approximate rate of 1.8 µM/hr (43 200 nM/day). Comparing this to the fast oxidation rate of *N. marina* in continuous culture at 7679 nM/day or *N. mobilis* at 22 671 nM/day, it is clear that growth in batch cultures is considerably faster, even after significant starting cell dilutions. At even slower oxidation rates, both *Nitrobacter* and *Nitrospira* had larger isotopic fractionation and a more negative $^{15}\varepsilon_{NXR}$ value. It is possible that this might also

be the case with *Nitrospina,* if the oxidation rates were much slower. This can be important as the slow oxidation rates seen in *N. marina* were more similar to average rates recorded or predicted in ODZs (700 nM/day). It can be assumed that *N. gracilis* also grows much more slowly in ODZs than in batch culture and may have a different isotope effect in environmental conditions, when compared to values recorded in pure culture batch experiments. This is why it is important to learn to grow this species in continuous culture to more easily control for oxidation rate to see how the isotope effect changes. Due to the fact that *N. gracilis* is so prevalent in ODZs, it is important to get an accurate idea of the nitrogen fractionation occurring, to improve upon models that rely on isotope profiles. However, growth rate may not be the only factor affecting isotope fractionation.

Although many models suggest that a large negative $^{15}\varepsilon$ isotope effect is needed for nitrite oxidation in ODZs, this study has recorded a $^{15}\varepsilon_{NXR}$ value of -10‰ in *N. gracilis* culture. It is possible that fast nitrite oxidation rates in batch culture could help explain this difference in predicted isotope effects in models and what is observed in culture experiments. Additionally, enzyme level interconversion could also be a factor (Buchwald et al. 2018; Kemeny et al. 2016). Under normal aerobic conditions, the NXR protein catalyzes the nitrite oxidation reaction by converting nitrite to nitrate. NXR has also been shown to be reversible (Sundermeyer-Klinger et al. 1984). In slower reactions, there is more time for isotope equilibration to occur between nitrite and nitrate, increasing the proportions of light N in the substrate pool. This reversible enzymatic reaction under anaerobic conditions could promote isotopic equilibrium and help explain the observed isotopic patterns in addition to the presence of nitrite re-oxidation (Buchwald et al. 2018).

3.4.3 Oxygen Isotope Exchange

Nitrite oxidation requires a series of steps involving the NXR enzyme. First, NXR binds to an oxygen atom from water. This complex then binds to nitrite, to form an enzymatic intermediate or transition state (Friedman et al. 1986). This intermediate can either complete the oxidation process to form nitrate or revert to nitrite. If one of the original nitrite oxygen atoms is removed when transitioning back to nitrite, it would be replaced by one from water. This would result in a change in the $\delta^{18}O$ over time. However, studies show that little exchange exists between nitrite and water (Buchwald et al. 2010; Friedman et al. 1986). This is likely because less energy is required to break the new N-O single bond, compared to dissolving the original N-O double bond (Buchwald et al. 2010). However, oxygen exchange is relevant to the production of nitrate and can impact the $\delta^{18}O_{NO3}$. The kinetic isotope effect of nitrite oxidation ($^{18}\varepsilon_{NO2}$), the kinetic isotope effect for water incorporation ($^{18}\varepsilon_{H2O}$) and the fraction of nitrite oxygen atoms exchanged with water can all affect the $\delta^{18}O$.

In this study, we were unable to obtain $\delta^{18}O_{NO3}$ data, $^{18}\varepsilon_{H2O}$ values or a value for the exchange of oxygen atoms between nitrite and water. Different labelled $\delta^{18}O$ water sources should have been used to make media. However, there was an error made which resulted in very similar starting $\delta^{18}O$ of water across all experiments. We could have calculated the $^{18}\varepsilon_{H2O}$ during nitrite oxidation; however, a value representing the oxygen atom exchange would have needed to be assumed. This experimental setup should ideally be done again to investigate the oxygen atom exchange and $\delta^{18}O_{NO3}$ values associated with *N. gracilis* nitrite oxidation. The $\delta^{18}O_{NO3}$ value is necessary to create an accurate $\delta^{18}O$ budget. Buchwald et al. (2018) used $\delta^{18}O_{NO2}$ from ammonia-oxidizing bacteria, a

$\delta^{18}O_{H2O}$ value of 0, and measured $^{18}\varepsilon_{H2O}$ from their study to determine the expected range of $\delta^{18}O_{NO3}$ is -8.3‰ to -0.7‰. However, without more data from other NOB, large ranges are still possible.

3.4.4 Conclusion

The goal of this work is to provide accurate isotope effects expressed by NOB to improve upon existing ODZ models. This study provided kinetic isotope effects of nitrite oxidation in *N. gracilis* grown in batch culture. Isotope profiles taken from ODZs or other parts of the ocean often represent a combination of different processes and associated isotope effects. With culture experiments, it is easier to disentangle what processes are occurring to get a better sense of how NOB microbial activities contribute to the influx and efflux of nitrogen. Future work should include growing *N. gracilis* in continuous culture to investigate how isotope effects are altered by a changing oxidation rate. Additionally, this experiment should be repeated to record an oxygen exchange value for subsequent modelling.

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CHAPTER 4 CONCLUSION

The goal of this thesis was to use chemostat experiments to get a better understanding of the isotope effects associated with NOB and how these values change based on differing metabolism rates. This thesis provides nitrogen and oxygen isotope effects for nitrite oxidation in various species of NOB (*Nitrococcus mobilis, Nitrospira marina* and *Nitrobacter winogradskyi*) in continuous culture. This is one of the only results from NOB grown in chemostats to date. Average $^{15}\varepsilon_{NXR}$ and $^{18}\varepsilon_{NXR}$ values ranged from -31‰ to -10‰ and -7‰ to 0‰ respectively. This study suggests that oxidation rate and growth rate are not always linearly related and that oxidation rate may have an impact on isotopic fractionation. Oxidation rates in *N. mobilis* were highest and ranged from 6000 to 23000 nM/day. Rates in *N. marina* and *N. winogradskyi* were slower and ranged from 700 to 15000 nM/day. A decrease in oxidation rate was related to an increase in inverse nitrogen isotope fractionation. Isotope effect values of nitrite oxidation in *Nitrospina gracilis* batch culture were also reported. Average $^{15}\varepsilon_{NXR}$ and $^{18}\varepsilon_{NXR}$ values in this species were -10‰ and -2‰ respectively.

This thesis provides much needed isotope effect data from NOB in continuous culture under aerobic conditions. The large negative nitrogen isotope effect values observed in the pure continuous cultures can potentially help to explain why models often predict the need for larger inverse isotope effect values than what is recorded in batch culture. Additionally, this work and subsequent experiments of this type can help inform future models of areas where denitrification occurs simultaneously with nitrogen fixation and nitrite oxidation. Climate change is causing ODZs to grow, altering rates of N transformations and the rate of loss of N. By improving existing models of N cycling, we

can gain a better understanding of what processes are occurring, and how they might continue to change in the future. By potentially allowing nitrite oxidation isotope effect values to change based on known or estimated bacterial oxidation rates, models could become more accurate.

The observed isotope effects from *N. gracilis* ($^{15}\varepsilon_{NXR}$ of -10‰ and $^{18}\varepsilon_{NXR}$ of -2‰) from batch culture are novel results and have not been previously reported. These reported values in *N. gracilis* do not match the values that models require for nitrite oxidation, as often they require larger negative $^{15}\varepsilon_{NXR}$ closer to -30‰; however, this may be due to the slow oxidation rate and/or enzymatic conversion seen in ODZs. This thesis then shows the significance of continuous culture experiments, as opposed to batch culturing, and adds to the growing understanding of NOB isotope systematics.

In the future, investigating factors that affect NOB nitrite oxidation rate should be done. Further characterizing of NXR in various species of NOB is needed to understand how enzyme reversibility may be impacting measured isotope effects. Additional experiments with NOB in anaerobic continuous culture should also be conducted. Since NOB are found in regions of the ocean with little to no oxygen, it is important to get an accurate isotope effect value for nitrite oxidation as well as nitrate reduction to best model these areas.

Nitrospina gracilis is one of the most prominent NOB found in ODZs, so it will also hopefully be possible to grow *N. gracilis* in continuous culture in the future. This might be achievable by intermittently adding air or gas bubbles to the cell media, as opposed to continuously bubbling. The *N. gracilis* cells are fragile and break apart easily under constant perturbation. Additionally, gentle periodic stirring of the vessel contents

could also help. Due to the significance of *N. gracilis* in ODZs, it is imperative to get a better understanding of how this species contributes to the production and loss of nitrate via nitrite re-oxidation and/or anaerobic nitrate reduction.

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