Differential Responses of the Catalytic Efficiency of Ammonia and Nitrite Oxidation to Changes in Temperature

Anne E. Taylor*† and Brett L. Mellbye‡

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Steffen Kolb,
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Man-Young Jung,
Jeju National University, South Korea

*Correspondence:
Anne E. Taylor
Anne.Taylor@oregonstate.edu
†These authors have contributed equally to this work

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INTRODUCTION

Anthropogenic activities have approximately doubled inorganic nitrogen (N) inputs to the global biosphere (Steffen et al., 2015), drastically altering nutrient cycling as evidenced by major changes in N utilization (Butterbach-Bahl et al., 2013; Liang et al., 2016) and carbon (C) sequestration (Davidson and Janssens, 2006). These changes have harmful effects on human and environmental health and severe economic impacts, including eutrophication of waterways (Shelton et al., 2018),
significant changes in the sources and sinks of N oxide gas pollutants (Ni and Groffman, 2018), and the loss of biodiversity (Stevens, 2019). Nutrient cycling is microbially mediated by microorganisms that mineralize organic N to ammonium/ammonia (NH$_4^+$/NH$_3$), oxidize NH$_3$ to nitrite (NO$_2^-$) then nitrate (NO$_3^-$), and denitrify to N oxide and dinitrogen gases. Nitrification, the aerobic sequential oxidation of NH$_3$ to NO$_2^-$ and then to NO$_3^-$, is an important intermediate in the N cycle and contributes to N availability for plants, N oxide gas emissions, and NO$_2^-$ and NO$_3^-$ leaching that leads to eutrophication (Galloway et al., 2008; Ward, 2011).

While environmental change predictions and climate models have focused on plant effects and C sequestration, the evaluation of environmental changes on microbial physiology has lagged behind or simply been treated as a "black box" (Shade et al., 2009; McGuire and Treseder, 2010; Treseder et al., 2012; Bennett et al., 2019). Nitrifying microorganisms are an important group that controls the size and composition of inorganic N pools available to plants (Ward, 2011). There is a critical need to understand the physiological changes that N cycling microbes make in response to climate change, and whether their responses can sustain environmental functionality and resilience. Nitrification is carried out by diverse microorganisms, including bacteria and archaea. NH$_3$-oxidizing bacteria and archaea (AOB and AOA, respectively) generally carry out NH$_3$ oxidation in partnership with NO$_2^-$-oxidizing bacteria (NOB; Daims et al., 2011; Hidetoshi et al., 2011; Sayavedra-Soto and Arp, 2011; Starkenburg et al., 2011). There are several groups of NOB, but in this study we focused on two groups that are the most abundant in soils: the genus Nitrobacter and the genus Nitrospira. A subset of lineage II Nitrospira that has the capability to completely oxidize NH$_3$ to NO$_3^-$ (comammox) has not been included in this study (Ward, 2011; Daims et al., 2015; van Kessel et al., 2015).

Rates of biological enzymatic functions are temperature sensitive and there is evidence of differential responses of soil processes to temperature (Hobbs et al., 2013; Blagodatskaya et al., 2016; Taylor et al., 2017, 2019). Enzyme catalytic rates generally increase with temperature until an optimum is reached and, at higher temperatures, the reaction rate decreases due to thermal inactivation (Laidler and Peterman, 1979). Previous work by our research group and others suggests that AOB and AOA in soils respond differently to temperature (Pierre et al., 2017; Taylor et al., 2017; Duan et al., 2018), and it has recently shown that rates of activity and substrate affinity of soil NO$_2^-$ oxidation are also temperature-sensitive (Taylor et al., 2019; Duan et al., 2020). Increasing temperature can also increase reaction rates which are associated with increased production of N oxide, nitric oxide (NO), and nitrous oxide (N$_2$O) gases (Law et al., 2012; Schreiber et al., 2012; Bonnett et al., 2013; Mellbye et al., 2016). However, increasing temperature can also result in lower affinity for the substrate, which may offset higher reaction rates (Laidler and Peterman, 1979; Snider et al., 2000; Davidson and Janssens, 2006; Blagodatskaya et al., 2016). Different responses to temperature by associated NH$_3$ oxidizers and NO$_2^-$ oxidizers may affect nitrification coupling and potential NO$_2^-$ accumulation (Schaefer and Hollibaugh, 2017; Taylor et al., 2019; Duan et al., 2020; Venterea et al., 2020), but significant biochemical data, particularly $V_{\text{max(app)}}$ and $K_m(\text{app})$, to model the response of nitrifying microorganisms to temperature are lacking.

Most physiological data on nitrifying bacteria and archaea have been generated under optimal growth conditions. For example, Suzuki et al. (1974) showed the response of Nitrosomonas europaea $K_m(\text{app})$ to changes in pH, but did not investigate temperature, and NH$_3$ affinity has been measured in the AOBs Nitrosococcus oceaneus and Nitrosospira briensis, but only at optimum temperature (Ward, 1987; Bollmann et al., 2005). The NO$_2^-$ oxidation kinetics of strains of Nitrobacter and Nitrospira have been determined only at each strain’s optimal growth temperature, pH, and dO$_2$ (Nowka et al., 2015; Ushiki et al., 2017). Exceptions include a study measuring whole-cell kinetics of Nitrosomonas sp. 4W30 at three temperatures, which showed significantly greater affinity for NH$_3$ at lower temperatures (Jones and Morita, 1985), and the evaluation of the AOA “Ca. N. oleophilus” MY3 kinetic response where the apparent $K_m(\text{app})$ increased ~2-fold from 25 to 35°C (Jung et al., 2022).

Previous work demonstrated that the rates of nitrification changed with temperature (Kim et al., 2006; Schaefer and Hollibaugh, 2017; Taylor et al., 2017, 2019; Duan et al., 2018); however, it is unclear if changes in rates in response to temperature results from a shift in community structure or changes in microbial physiology (Blagodatskaya et al., 2016; Taylor et al., 2017; Duan et al., 2020). We hypothesized that individual nitrifiers respond differently to temperature, and that these responses are reflected in changes in substrate affinity and reaction rate. We chose pure culture representative members of AOA, AOB, Nitrobacter NOB, and Nitrospira NOB for evaluation and investigated changes in NH$_3$ and NO$_2^-$ affinity and oxidation rate over a large range of physiologically relevant temperatures. Finally, we applied Michaelis–Menten (MM) and Haldane (H) kinetic models, based on observations, to changes in nitrification rates observed in whole soils over a range of temperatures.

**MATERIALS AND METHODS**

**Nitrifying Strains and Growth Conditions**

Nine nitrifying microorganisms were used in this study and cultivated as previously described (Table 1). We utilized the AOA Nitrososphaera viennensis (Tournier et al., 2011; Taylor et al., 2015), and the AOB *N. europaea* and *Nitrosospira multiformis* (Ensign et al., 1993; Chain et al., 2003; Norton et al., 2008; Taylor et al., 2013). We worked with a total of six NOB strains. *Nitrobacter hamburgensis*, *Nitrobacter winogradskyi*, *Nitrobacter vulgaris*, and *Nitrospira moscoviensis* were grown under the conditions previously used in our laboratories (Bock et al., 1983, 1990; Ehrich et al., 1995; Starkenburg et al., 2006, 2008; Koch et al., 2015; Mellbye et al., 2017a,b), and *Nitrosospira japonica* NJ1 and *Nitrosira* ND1 were cultivated as previously described (Ushiki et al., 2017). Minimal media for all cultures were at pH 8. Cultures were grown at their optimal growth temperatures (30°C for all cultures except for *N. viennensis*, which was grown...
at 42°C and \textit{N. moscoviensis} which was grown at 37°C, Table 1) to maximize growth yields and for the ease of comparison with other studies. Cultures were monitored at regular intervals to check cell density by optical density at 600 nm (OD\textsubscript{600}) and NO\textsubscript{2}\textsuperscript{−} concentrations by the Griess assay.

**Determination of Kinetic Parameters**

Cultures were grown to early stationary phase and then harvested by centrifugation (10,000 \times g, 15 min), washed with their respective substrate-free growth media to remove trace concentrations of NH\textsubscript{3} and NO\textsubscript{2}\textsuperscript{−}, and resuspended in ~15-mL volumes of substrate-free growth media (pH 8) to yield a concentrated cell suspension. The cell density of the concentrated suspension was measured by OD\textsubscript{600}, and the protein content was measured with a Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, United States) to determine the volume of cells to be added to the incubation vials, yielding an average protein concentration of 2.6 ± 2.9 µg/µl for the NOB and 6.5 ± 1.7 µg/µl for NH\textsubscript{3} oxidizers. For each experimental temperature, vials were prepared in triplicate with 5 mL portions of their respective growth media (pH 8) supplemented with a range of substrate concentrations (NH\textsubscript{4}\textsuperscript{+} for the AOA and AOB and NO\textsubscript{2}\textsuperscript{−} for the NOB). The vials were closed with gray butyl stoppers and preincubated at the experimental temperatures. Experiments were initiated with the addition of aliquots of concentrated culture. Vials were sampled immediately and then at ~30 min intervals to obtain NO\textsubscript{2}\textsuperscript{−} concentration at five time points over 2 h, with a final time point obtained at ~4 h after the initiation of the experiment. Rates of NH\textsubscript{3} oxidation by the AOA and AOB were determined by the rate of NO\textsubscript{2}\textsuperscript{−} accumulation in the incubation vials, and NO\textsubscript{2}\textsuperscript{−} oxidation rates by its disappearance. The MM kinetic coefficients of substrate utilization at each temperature were determined by fitting the NO\textsubscript{2}\textsuperscript{−} or NH\textsubscript{3} oxidation rate (\(v\)) for a given substrate concentration (\(S\)) to the equation

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

(1)

to find the maximum reaction rate (\(V_{\text{max}}\)) and the substrate concentration that yields half of this maximum rate (\(K_m\)) using SigmaPlot (Systat Software, San Jose, CA, United States). Because we are working with whole cells, the calculated \(V_{\text{max}}\) and \(K_m\) should be considered to be the apparent \(V_{\text{max}}\) (\(V_{\text{max(app)}}\)) and apparent \(K_m\) (\(K_m(app)\)). Note that we calculated \(V_{\text{max(app)}}\) as a relative \(k_{\text{cat}}\) based on the maximum reaction rate per mg protein of whole cells (Snider et al., 2000). The effects of H inhibition on substrate utilization were determined by fitting the NO\textsubscript{2}\textsuperscript{−} or NH\textsubscript{3} oxidation rate for a given substrate concentration to the equation

\[ v = \frac{V_{\text{max}} \times S}{(K_m + S + S^2/K_a)} \]  

(2)

to find the effects of substrate inhibition on \(V_{\text{max(app)}}\) and \(K_m(app)\), as well as to find the substrate concentration that inhibited \(V_{\text{max(app)}}\) by 50% (\(K_i\)). Specific affinity for substrate (\(a^2_s\)) and the affinity constant (\(K_A\)) were determined as described by Button (1998). Temperature-dependent distribution of NH\textsubscript{3}/NH\textsubscript{4}\textsuperscript{+} was calculated as described by Groeneweg et al. (1994).

**Soil Incubations to Characterize NH\textsubscript{3} Oxidation Response**

Agricultural soils were collected from the western Oregon Coastal Plain in Tillamook County and from the eastern Oregon Columbia River basin in Morrow County (Waggoner et al., 2021). The western Oregon Coastal Plain soil is identified as a Quillamook series silty loam soil. It receives a mean annual precipitation of 2,159 mm and has an isomesic temperature regime (temperatures averaging 6.5°C in winter and 14°C in summer) with a mean annual temperature of 10°C and has a pH of 5.8. The soil was cropped with a silage corn (\textit{Zea mays} L.) and rye grass (\textit{Lolium} spp.) rotation. The eastern Oregon Columbia River basin soil receives a mean annual precipitation of 178 mm and has a mesic temperature regime (temperatures averaging 2°C in winter to 22°C in summer) with a mean annual temperature of 12°C and has a pH of 8.5. It is identified as a Sagheer series sandy loam soil (see text footnote 1). It is cropped under a silage corn (\textit{Z. mays} L.) and triticale (\textit{Triticosecale}) rotation. Three field replicates were collected at each site, each of which was created using a composite of 3–5 samples from the surface 0–20 cm that

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**Table 1** | Ammonia (NH\textsubscript{3}) and nitrite (NO\textsubscript{2}−) oxidizer strains for this study were grown under optimal growth conditions and temperatures.

| Genus         | Species          | AOA/AOB/NOB\textsuperscript{b} | Strain designation | Growth temperature (°C) |
|---------------|------------------|---------------------------------|--------------------|------------------------|
| Nitrosospheara| viennensis       | AOA                             | EN76               | 42                     |
| Nitrosospira  | multiformis      | AOB                             | ATCC 25196(T)      | 30                     |
| Nitrosomonas  | europaea         | AOB                             | ATCC 19718         | 30                     |
| Nitrobacter   | winogradskyi     | NOB                             | NB-255             | 30                     |
| Nitrobacter   | hamburgensis     | NOB                             | X14                | 30                     |
| Nitrobacter   | vulgaris         | NOB                             | AB\textsubscript{1} | 30                     |
| Nitrosira     | moscoviensis     | NOB                             | M1                 | 37                     |
| Nitrosira     | japonica         | NOB                             | NJ1                | 30                     |
| Nitrosira     | sp.              | NOB                             | ND1                | 30                     |

\textsuperscript{a}Bock et al. (1983, 1990), Ehrich et al. (1995), Chain et al. (2003), Starkenburg et al. (2006, 2008), Norton et al. (2008), Touma et al. (2011), Koch et al. (2015), Melbye et al. (2017b), and Ushiki et al. (2017). \textsuperscript{b}NH\textsubscript{3}-oxidizing archaeaen, bacterium, or NO\textsubscript{2}−-oxidizing bacterium.
were mixed in the field, and then brought to the laboratory where they were sieved at 2.36 mm and stored at 4°C until use.

Using a method previously adapted by DeAngelis et al. (2010), DNA extraction was performed using phenol:chloroform:isopropyl alcohol (25:24:1), followed by precipitation in PEG6000 solution [30% (w/v) polyethylene glycol 6000 in 1.6 M NaCl] (DeAngelis et al., 2010). Each soil sample was extracted two times per replicate, with three replicates. Extracts from each replicate were pooled. Quantitative polymerase chain reaction (qPCR) was then used to determine gene copy numbers of AOA amoA, AOB amoA, and NOB nxrA and nxrB using reagents, primers, and conditions as described in Supplementary Table 1. Standards for qPCR were prepared from N. viennensis (AOA amoA), N. multiformis (AOB amoA), N. winogradskii (nxrA), and N. moscowiensis (nxrB).

Soil incubations were performed to evaluate the contributions of AOA and AOB to soil nitrification when NH4+ was supplied as NH4Cl. Addition of 1-octyne (4 μM) allows differentiation of AOA and AOB through inactivation of the AOB NH3 monoxygenase (AMO), and additions of acetylene (10 μM) were used to prevent all NH4+-mediated nitrification of both AOA and AOB, serving as a negative control (Taylor et al., 2013, 2015). To evaluate the effect of temperature on NH3 oxidation, incubations were established at a range representative of temperatures during the growing season at each sampling site, plus one temperature 10°C higher than normal temperatures to evaluate the effect of temperature stress. The incubation temperatures for Coastal Plain soils were 10, 20, and 30°C, while Columbia Basin soils were incubated at 10, 20, 30, and 40°C. Prior to experimentation, three field replicates of each soil were leached to remove background NO3− to increase colorimetric assay sensitivity. Briefly, soil aliquots were placed in a funnel lined with a paper filter and covered with crushed ice, then held at room temperature while the ice melted and percolated through the soil. This leaching method allows the soil to retain most of its structure. Soils were then allowed to partially air dry to ~30% gravimetric moisture content for the Coastal Plain and 5% for the Columbia Basin. Leached soils (2.5 g) were measured into 125-ml Wheaton bottles and preincubated at room temperature (23°C) for 12–24 h before experimentation.

To initiate the incubations, soils received 5 μmol NH4Cl g−1 soil in dH2O to bring the soil moisture content slightly above the field capacity for each soil (72% for the Coastal Plain and 22% for the Columbia Basin). A control that received dH2O, but no NH4+ source, was included for each treatment. Each bottle was capped using a lid with an n-butyl septum and alkylene treatments imposed as described above. Alkylene stocks were prepared and added in gaseous forms as previously described to the +octyne and -acetylene treatments (Giguere et al., 2015). The soils were then incubated in the dark for 24 h at 10, 20, 30, or 40°C.

At the end of the incubations, the accumulations of NO2− and NO3− + NO−3 in soils were determined as previously described (Miranda et al., 2001; Hood-Nowotny et al., 2010). The difference in NO2− or NO−2 + NO3− compared to incubations that included acetylene was considered to represent the rate of nitrification performed by the nitrifiers, or total AOA and AOB activity. The difference between +octyne treatments and +acetylene treatments represents only AOA activity. AOB activity was then calculated as the difference between total nitrification activity and AOA-only activity. NOB was considered to be equal to the total rate of soil nitrification minus any accumulated NO2−.

**Modeling the Potential Soil Response of AOA, AOB, and NOB Cultures**

**Coastal Plain Soil**

To determine whether MM kinetics and H (substrate-induced inhibition) parameters could be used to predict the rates of NH3 and NO2− oxidation activity in soil incubations, certain assumptions were made to apply the rates from cultures (μmol substrate mg protein−1 h−1) to soils in which population size was determined by qPCR of AOM and NO2−-oxidoreductase (NXR) genes (Supplementary Table 1), and the rates were expressed as μmol substrate g soil−1 h−1. Because the qPCR primers did not discriminate between the strains of AOB or NOB, when evaluating the potential soil activity of each of the cultures we assumed that each strain made up the entire population. It was also assumed that all gene copy numbers determined through qPCR came from active cells, and this number was divided by the number of gene copies per genome as determined by the Basic Local Alignment Search Tool (BLAST; Supplementary Table 2). From Urukawa et al. (2011), the relationship between cell volume and cell protein content of *Nitrosopumilus maritimus* has been determined, and we extrapolated that information to estimate the protein content per cell of the cultures used in this study (Supplementary Table 3); and in turn estimate the amount of cell protein in each gram of soil (see Supplementary Material for further explanation on the conversion of μmol substrate g soil−1 h−1 to μmol substrate g soil−1 h−1).

It was also necessary to make assumptions about starting substrate concentrations in the soil. The Coastal Plain soil without NH4+ addition had 16 μmol g soil−1 KCl extractable NH3 + NH4+ and a soil water content of 72%, and the assumption was made that all extractable NH4+ would be in soil solution, yielding a concentration of 0.22 μM NH3 + NH4+. In the incubations with NH4+ addition (5 μmol g soil−1), it was assumed that all NH4+ would be in the soil solution yielding a concentration of 6 mM NH3 + NH. To evaluate how the rates of MM or H kinetics of NOB cultures would respond under soil conditions, it was necessary to estimate possible concentrations of NO2− in the soil. Our confident limit of detection of NO2− in soil is ≥2 μM NO2−. There was no detectable NO2− in Coastal Plain soils prior to, or during incubations, suggesting that the rate of soil NOB activity was equal to the rate of soil NH3 oxidation; but does not rule out the possibility that trace NO2− accumulated during active soil nitrification. For the Coastal Plain soil without NH4+ addition, there was 0.22 μM extractable NH3 + NH4+ and, if completely oxidized to NO2−, would yield 0.22 μM NO2−; this value was used as the substrate concentration. For Coastal Plain soils with NH4+ addition, we assumed a NO2− concentration just below our level of detection (2 μM NO2−).

The above assumptions were used with the kinetic parameters determined in this study (Supplementary Table 5) for each...
of the microbial strains to estimate the potential rate of activity in the soil using MM Eq. (1) and H Eq. (2) kinetics at each soil incubation temperature. The average value of population size, cell protein content, gene copy number, \( V_{\text{max(app)}} \), \( K_{m(app)} \), and \( K_{i(app)} \) were used in the calculations as there were an excessive number of potential combinations (26 or 64 combinations at each temperature) of these values if both extremes of the standard deviation (SD) were evaluated. We assumed no inhibition of NH\(_3\) oxidation in the incubations without NH\(_4^+\) addition. Additionally, \( K \) kinetics were not determined for NOB in the treatments without NH\(_4^+\) addition where NO\(_2^-\) was assumed to be 0.22 \( \mu \text{M} \) and several orders of magnitude lower than \( K_i \) determined in this study.

**Columbia Basin Soil**

The assumptions made on the population size were the same as for the Coastal Plain soil detailed above. The Columbia Basin soil without NH\(_4^+\) addition had 0.01 \( \mu \text{mol g}^{-1} \) KCl extractable NH\(_3\) + NH\(_4^+\) and a soil water content of 22\%, and the assumption was made that all extractable NH\(_4^+\) would be in the soil solution yielding a concentration of 0.05 \( \mu \text{M} \) NH\(_3\) + NH\(_4^+\). In the incubations with NH\(_4^+\) addition (5 \( \mu \text{mol g}^{-1}\)), it was assumed that all NH\(_4^+\) would be in the soil solution yielding a concentration of 22 mM NH\(_3\) + NH. There was no detectable NO\(_2^-\) in the Columbia Basin soil prior to incubation or in the incubations without NH\(_4^+\) addition, suggesting that the rate of soil NOB activity was equal to the total rate of soil nitrification. In the Columbia Basin soil incubations without NH\(_4^+\) addition, we assumed a NO\(_2^-\) concentration of 0.05 \( \mu \text{M} \) (all extractable soil NH\(_4^+\) oxidized to NO\(_2^-\)). However, in the Columbia Basin soils with NH\(_4^+\) addition, NO\(_2^-\) accumulated during incubations to concentrations of 2, 700, 1,900, and 400 \( \mu \text{M} \) at 10, 20, 30, and 40\ºC, respectively; and these values were used in the calculation of MM and H kinetic rates. In the treatments with NH\(_4^+\) addition in which NO\(_2^-\) accumulated, the rate of NO\(_2^-\) oxidation was the NH\(_3\) oxidation rate minus the rate of accumulated NO\(_2^-\).

**Statistical Analysis**

Analysis of variance (ANOVA) using the Holm–Sidak method in SigmaPlot (Systat Software, San Jose, CA, United States) was used to evaluate if there were significant differences in kinetic parameters across temperatures. Regression analyses in SigmaPlot compared the MM and H kinetic models with the rates of NH\(_3\) and NO\(_2^-\) oxidation measured in Coastal Plain and Columbia Basin soils.

**Bioinformatic Analyses**

The National Center for Biotechnology Information\(^3\), Kyoto Encyclopedia of Genes and Genomes\(^3\), and BLAST\(^4\) were used to identify AmoA and NxrA homologs in nitrifiers and other bacteria (Supplementary Datasets 1, 2; Boratyn et al., 2013; Kanchisa et al., 2017). MUSCLE was used for multiple alignment of amino acid sequences (Edgar, 2004), and phylogenetic analyses were conducted using MEGA11 (Kumar et al., 2016). The evolutionary history of AmoA and NxrA was inferred using the Maximum Likelihood method, based on the JTT matrix model (Jones et al., 1992). Phylogenetic trees were visualized using FigTree 1.4.4\(^5\).

**RESULTS**

**Homology of AmoA and NxrA in Nitrifying Bacteria and Archaea**

Enzyme kinetics are notably different between AOB and AOA, and between *Nitrobacter* NOB and *Nitrospira* NOB; perhaps due to different niche specialization and temperature optima (Martens-Habbena et al., 2009; Verhamme et al., 2011; Prosser and Nicol, 2012; Pester et al., 2014; Gruber-Dorninger et al., 2015; Nowka et al., 2015; Kits et al., 2017; Ushiki et al., 2017). We chose to analyze the oxidation rate and affinity of nine nitrifying microorganisms isolated from soils and wastewater systems, and we focused on microorganisms that could accumulate the biomass necessary for our experiments (Table 1). For ammonia oxidizers (AOA and AOB), we selected the AOA *N. viennensis* and the AOB *N. multiformis* and *N. europaea*. The evolutionary history of the putative substrate-binding subunits of AMO (putative substrate-binding subunit, AmoA) is shown in Figure 1A. For NOB, *N. winogradskyi*, *N. hamburgensis*, *N. vulgaris*, *N. moscoviensis*, *N. japonica*, and *N. ND1* were selected. The evolutionary history of the putative substrate-binding subunits of NXR (putative substrate-binding subunit, NxrA) is shown in Figure 1B.

**Nitrifier Kinetics Change With Temperature**

The oxidation rates of nitrifiers and their affinity in pure culture incubations were investigated. Both MM and H kinetic models were used to determine \( V_{\text{max(app)}} \), \( K_{m(app)} \), and catalytic efficiency (\( V_{\text{max(app)}}/K_{m(app)} \)) from pure culture data (Supplementary Table 5). We have presented these data showing changes in \( V_{\text{max(app)}} \), \( K_{m(app)} \), and catalytic efficiency in Figure 2. As expected, \( V_{\text{max(app)}} \) increased with temperature in all microorganisms we investigated; however, there were significant differences in how enzyme affinity (\( K_{m(app)} \)) changed with temperature between the different groups of nitrifiers.

Bacterial and archaeal NH\(_3\) oxidizers had significantly different changes in enzyme affinity with temperature. For the AOA representative, *N. viennensis*, \( V_{\text{max(app)}} \) increased \(~100\text{-fold}\) from 10 to 42\ºC, with maximum rates of 10.3 \( \mu \text{mol substrate mg protein}^{-1} \text{h}^{-1} \). *N. viennensis* had no significant change in \( K_{m(app)} \) with temperature, with values ranging from 6.1 to 20.3 \( \mu \text{M} \) NH\(_3\) + NH\(_4^+\), suggesting that temperature generally does not significantly affect enzyme affinity but does

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\(^{3}\)http://www.ncbi.nlm.nih.gov/\(^{4}\)http://www.genome.jp/kegg/\(^{5}\)http://tree.bio.ed.ac.uk/software/figtree/
increase catalytic efficiency (Figure 2A and Supplementary Tables 5, 6 compares the affinity of the NH$_3$ oxidizers for total NH$_3$ + NH$_4^+$ vs. NH$_3$). There was evidence of temperature-independent H substrate-induced inhibition of N. viennensis at 0.3–2.4 mM NH$_3$ + NH$_4^+$ (Supplementary Table 5). In contrast, the K$_m$(app) of AOB significantly increased with temperature from 10 to 37°C, suggesting that increasing temperature decreases bacterial AMO affinity; potentially explaining previous observations of different temperature optima between AOA and AOB (Figure 2B). In addition, while K$_m$(app) of NH$_3$ oxidation for AOB increased 10- to 12.7-fold with temperature (from 73.7 to 938.6 μM NH$_3$ + NH$_4^+$), the V$_{max}$(app) of AOB also increased 10- to 17-fold (from 51.5 to 128.7 μmol NH$_3$ + NH$_4^+$ mg protein$^{-1}$ h$^{-1}$), resulting in increased catalytic efficiency. In both N. viennensis and N. multiformis, V$_{max}$(app) decreased when temperature increased past their temperature optima resulting in decreased catalytic efficiency (Figure 2C).

There were strikingly different kinetic responses of NO$_2^-$ oxidation to temperature between Nitrobacter and Nitrospira and also between the different lineages (Figures 2D–I and Supplementary Table 5). In Nitrobacter, both V$_{max}$(app) and K$_m$(app) increased significantly as temperature increased from 20 to 37°C (Figures 2D,E). While V$_{max}$(app) increased from ~2- to 2.6-fold (from 33.6 to 102.4 μmol NO$_2^-$ mg protein$^{-1}$ h$^{-1}$), fold increases in K$_m$(app) were approximately two times that of V$_{max}$(app) (3.8–6.9-fold) and ranged from 416.5 to 1662 μM NO$_2^-$ as temperature increased; resulting in significantly decreased catalytic efficiency (Figure 2F and Supplementary Table 5).

There were interesting differences among the three Nitrospira strains tested. The lineage II Nitrosira, N. moscoviensis (growth temperature 37°C), and N. japonica NJ1 (growth temperature 30°C), behaved similarly, except for notable differences in temperature optima that resulted in maximum V$_{max}$(app) at 37°C or 42°C for N. moscoviensis and N. japonica NJ1, respectively, (Figure 2G). There were also indications of H inhibition of N. moscoviensis that became most pronounced at 10°C (Supplementary Table 5). V$_{max}$(app) increased from 1.5- to 4.1-fold, to 19.9 to 35.4 μmol NO$_2^-$ mg protein$^{-1}$ h$^{-1}$ for lineage II Nitrospira while K$_m$(app) varied between 6.6 and 36.8 μM NO$_2^-$ with little relationship to temperature (Supplementary Table 5). Except for a ~4-fold increase in catalytic efficiency for N. moscoviensis at 20°C, there was little difference in catalytic efficiency over the temperature ranges tested for the two evaluated lineage II Nitrospira (Figure 2I). The lineage I Nitrosira strain, Ns. ND1, had a 3.4-fold increase in V$_{max}$(app) up to 24.5 μmol NO$_2^-$ mg protein$^{-1}$ h$^{-1}$, and little change in K$_m$(app) (13.9 to 50.4 μM NO$_2^-$), resulting in small changes in catalytic efficiency from 10 to 30°C. However, from 30 to 42°C, V$_{max}$(app) stopped increasing and eventually decreased, and K$_m$(app) drastically increased to 505.9 μM,
and catalytic efficiency significantly decreased (Figure 2I and Supplementary Table 5).

Comparison of the Nitrification Temperature Response Predicted by Michaelis–Menten and Haldane Kinetic Models to Soil Nitrification Activity

After determining kinetic values for NH$_3$ and NO$_2^-$ oxidation, we applied the MM and H kinetic models to the changes in nitrification rates observed in short-term incubations of whole soils with or without NH$_4^+$ addition and incubated at temperatures from 10 to 40°C (Figures 3, 4). We reasoned that this approach would yield insight into how well pure culture microorganisms used to make the model resemble naturally occurring nitrifying microorganisms. Agricultural soils from two climatic regions of Oregon that demonstrate different nitrification properties were used in this study. AOA outnumbered AOB in both soils (Supplementary Table 4). There was more *Nitrobacter* than *Nitrospira* NOB in Coastal Plain soils; however, there was no significant difference in these two populations in the Columbia Basin soil. Nitrification in Coastal Plain soils was coupled and NO$_2^-$ did not accumulate, while NO$_2^-$ accumulated in Columbia Basin soils despite having lower overall rates of NH$_3$ oxidation (Figures 3, 4). H models include substrate inhibition and are expected to be important in AOA and *Nitrospira* NOB that prefer low concentrations of NH$_3$ or NO$_2^-$, respectively.

Model Predictions Compared to Nitrification in Coastal Plain Soils

Soil AOA had activity in Coastal Plain soils without NH$_4^+$ addition, and the NH$_3$ oxidation rate predicted by the MM model was a good fit for soil AOA activity ($R^2 = 0.815$, Supplementary Table 6).

![Figure 2](https://example.com/figure2.png) **Figure 2** | Response of ammonia (NH$_3$) and nitrite (NO$_2^-$) oxidizers to temperature. Michaelis–Menten (MM) kinetic parameters for NH$_3$-oxidizing bacteria and archaea (AOA, AOB; A–C), *Nitrobacter* NO$_2^-$-oxidizing bacteria (NOB; D–F), and *Nitrospira* NOB (G–I). Cultures were grown at their optimal growth temperature, and $V_{\text{max(app)}}$ and $K_{\text{m(app)}}$ were determined at the indicated temperatures during short incubations. The response of NH$_3$ oxidizers is expressed in terms of total NH$_3$+NH$_4^+$; see Supplementary Table 6 for the response to NH$_3$. Catalytic efficiency was calculated as $V_{\text{max}}/K_{\text{m}}$. Error bars represent the standard deviation (SD) of the average of three replicates.
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FIGURE 3 | Comparison of the potential response of AOA, AOB, and NOB cultures evaluated with the response of soil nitrifiers in short-term (24 h) incubations of Coastal Plain soil without no NH$_4^+$ addition (A–D), and with NH$_4^+$ addition (5 µmol/g soil, E–H). The soil nitrifier response measured during the incubations is indicated by the bars corresponding to the left y-axis, and the response modeled using the kinetics of nitrifier cultures determined in this study (Supplementary Table 5) is indicated by the symbols corresponding to the right y-axis. Individual nitrifier names are preceded by either MM or Haldane (H) to indicate that MM or H parameters, respectively, were used to calculate the potential rates of activity.

$\rho = 0.01$, Supplementary Table 7, and Figure 3A). Unlike AOA, no activity of soil AOB was measured in the absence of NH$_4^+$ addition in Coastal Plain soils, and the AOB MM models also predicted no contribution to NH$_3$ oxidation by soil AOB (Figure 3B). In the absence of NH$_4^+$ addition, the MM model did not predict any significant response by
N. japonica NJ1 or Nitrospira ND1; however, the rate response of N. moscoviensis determined by the kinetic parameters of the MM model matched the data at 10 and 20°C, but not 30°C (Figure 3C). The rate response of N. vulgaris determined by the kinetic parameters of the MM model could potentially contribute to all the NO\(_2^-\) oxidation observed in this soil (R\(^2\) = 0.641, p = 0.01), but the MM models for the remaining NOB do not predict any contribution to NO\(_2^-\) oxidation (Figure 3D).

Addition of NH\(_4^+\) to Coastal Plain soils increased the rates of NO\(_2^-\) + NO\(_3^-\) production by soil AOA ∼5–10-fold (Figure 3E). H substrate-induced inhibition of N. viennensis predicted a lower AOA contribution to NH\(_3\) oxidation than that observed in the soil at 10 and 20°C, although the potential response of AOA like N. viennensis exceeded the response observed in the soil at 30°C (R\(^2\) = 0.685, p = 0.006, Figure 3E). In the Coastal Plain soil with NH\(_4^+\) addition, soil AOB contributed to nitrification at all three incubation temperatures, providing NH\(_3\) oxidation rates approximately equal to that of soil AOA at 10 and 20°C, and ∼25% of total NO\(_2^-\) + NO\(_3^-\) production at 30°C; however, neither the MM or H models for N. multiformis or N. europaea were a good fit to the data (Supplementary Table 7 and Figure 3F). The H model predicted no significant effects of substrate inhibition on N. europaea (data not shown) but predicted a reduction in N. multiformis contributions at 20°C. The MM models for N. europaea and N. multiformis showed increasing rates of NO\(_2^-\) + NO\(_3^-\) production as temperature increased from 10 to 30°C, whereas soil AOB rates were the highest at 20°C. The MM models predicted that AOB with kinetic characteristics like those of N. europaea and N. multiformis could contribute much of the NO\(_2^-\) + NO\(_3^-\) production observed at 10 and 20°C, but exceeded soil nitrification rates observed at 30°C. When NH\(_4^+\) was added to increase NH\(_3\)-oxidation activity in this soil, there was no accumulation of NO\(_2^-\) in the Coastal Plain soil above our limit of detection (2 μM NO\(_2^-\)), and under these conditions the MM models using kinetic parameters obtained for Nitrospira NOB in this study did not predict Nitrosira contributions to soil NO\(_2^-\) oxidation (R\(^2\) = 0.09–0.32, Figure 3G). The greatest MM model-predicted response for Nitrosira was N. moscoviensis, but it would only account for ∼3% of the total soil NOB activity at its highest activity rates. In contrast, the MM model for the Nitrobacter NOB N. vulgaris predicted rates of NO\(_2^-\) oxidation that met or exceeded the response observed by soil NOB, but the MM models did not predict that other Nitrobacter could contribute under these conditions with low concentrations of soil NO\(_2^-\) (Figure 3H).

Model Predictions Compared to Columbia Basin Soils

In Columbia Basin soils with no NH\(_4^+\) addition, soil AOA had much higher rates of activity than that predicted by the MM model of N. viennensis (Figure 4A). In the absence of addition of NH\(_4^+\) to soil, AOB had activity at 20, 30, and 40°C, but neither of the MM models using the kinetic parameters from N. multiformis and N. europaea had high enough substrate affinity to predict the oxidation of NH\(_3\) at such low concentrations (Supplementary Table 7 and Figure 4B). NO\(_2^-\) did not accumulate in the Columbia Basin soil in the absence of the addition of NH\(_4^+\); therefore, the rates of soil NOB activity matched that of NH\(_3\) oxidation. In the absence of accumulated NO\(_2^-\), none of the Nitrospira NOB models predicted significant contributions under these low NO\(_2^-\) conditions (Figures 4C,D).

When NH\(_4^+\) was added to the Columbia Basin soil, there was no significant change in the rates of NH\(_3\) oxidation by soil AOA over the incubations without NH\(_4^+\) addition at 10, 20, and 40°C, but the rates at 30°C declined ∼2-fold, suggesting evidence of substrate inhibition (Figure 4E). While the MM model for N. viennensis predicted high rates of AOA NH\(_3\) oxidation in excess of those observed in the Columbia Basin soil, the H model accurately predicted the soil AOA activity (R\(^2\) = 0.918, p = 0.000, Figure 4E). With the addition of NH\(_4^+\) to Columbia Basin soils, there were significant increases in the rates of activity by soil AOB with maximum rates of NH\(_3\) oxidation at 30°C (Figure 4F). Both N. europaea and N. multiformis MM models describe the NH\(_3\) oxidation response to NH\(_4^+\) addition at 10, 20, and 30°C, but overestimated the AOB response at 40°C; resulting in the models that poorly fit the soil AOB activity (Supplementary Table 7). Although the rates of NO\(_2^-\) oxidation by soil NOB increased ∼2-fold in response to the higher rates of NH\(_3\) oxidation (Figures 4G,H), NO\(_2^-\) accumulated in the Columbia Basin soil when NH\(_4^+\) was added (2, 700, 1,900, and 400 μM NO\(_2^-\) at 10, 20, 30, and 40°C, respectively), indicating that soil NOB did not have the capacity to immediately respond to higher rates of NH\(_3\) oxidation. However, the MM models of both Nitrospira and Nitrobacter NOB indicated a positive benefit in predicted rates of NO\(_2^-\) oxidation from the accumulated NO\(_2^-\), and only N. moscoviensis showed substrate-induced inhibition at 30 and 40°C (Figure 4G). The MM model for Nitrospira NOB predicted rates equal to or greater than soil NOB at 20 and 40°C, but could not match the rate of soil NOB at 10 or 30°C. The MM and H models with accumulated NO\(_2^-\) for all three Nitrobacter NOB predicted the capacity to express a higher activity than the observed soil NOB activity but resulted in a significant fit of the soil data (p ≤ 0.004, Supplementary Table 7, and Figure 4H).

**DISCUSSION**

**The Effect of Temperature on Catalytic Efficiency**

The K\(_{\text{m(app)}}\) of all three Nitrospira species increased substantially with temperature at a much greater rate than V\(_{\text{max(app)}}\), resulting in a substantial decrease in catalytic efficiency at higher temperatures. In contrast, both V\(_{\text{max(app)}}\) and K\(_{\text{m(app)}}\) values of the Nitrospira species increased similarly with temperature, on a much smaller scale, and their overall catalytic efficiency generally did not change significantly. This difference between Nitrobacter and Nitrospira makes sense in view of the analysis of the phylogeny of NxrA suggesting
that NO$_2^-$ oxidation has arisen independently multiple times (Lücker et al., 2010; Sorokin et al., 2012), resulting in different biochemical or functional characteristics. For example, the NXR complex is located in the periplasm space of *Nitrospira* sp. and in the cytoplasm space of *Nitrobacter* sp. (Daims et al., 2016), which may contribute to the observed...

**FIGURE 4** Comparison of the potential response of AOA, AOB, and NOB cultures evaluated with the response of soil nitrifiers in short-term (24 h) incubations of Columbia Basin soil with no NH$_4^+$ addition (A–D), and with NH$_4^+$ addition (5 µmol/g soil, E–H). The soil nitrifier response measured during incubations is indicated by the bars corresponding to the left y-axis, and the response modeled using the kinetics of nitrifier cultures determined in this study (Supplementary Table 5) is indicated by the symbols corresponding with the right y-axis. Individual nitrifier names are preceded by MM or H to indicate that MM or H parameters, respectively, were used to calculate the potential rates of activity.
differences in substrate affinity between the groups (Nitrospira $K_m(app) = 9–24$ µM, Nitrobacter $K_m(app) = 49–544$ µM; Nowka et al., 2015; Daims et al., 2016; Ushiki et al., 2017). Additionally, while there was a 50–70% decline in catalytic efficiency with increasing temperature for each of the three Nitrobacter evaluated in this study, there was more variation in the response of Nitrospira; ranging from a ∼50% decline in catalytic efficiency over 10 to 37°C by Ns. ND1, to no change in catalytic efficiency by N. japonica NJ1, and a uniform response in catalytic efficiency across temperature by N. moscoviensis except at 20°C where there was a 4-fold increase. The phylogeny of NxrA indicates that there is more diversity within Nitrospira than in Nitrobacter (Daims 2016 Trends), and all Nitrobacter evaluated in this study have very similar NxrA (92–98% identity) compared to lineages I and II Nitrospira. As for N. japonica and N. moscoviensis, their NxrA have only ∼87% sequence identity and are clearly separated by phylogenetic analysis even though both NOB are in lineage II; the differences in their adaptation to different growth temperatures (30 and 37°C, respectively) may have contributed to their phylogenetic separation and differences in physiological response. N. japonica NJ1 is similarly divergent compared to Nitrospira ND1 (lineage I, 88% identity; Figure 1B).

Although the limited number of NH₃ oxidizer cultures utilized in this study prevents definitive conclusions on the response of catalytic efficiency to temperature change, the results agree with previous studies and are in sharp contrast to that of the NOB. Numerous studies have demonstrated higher rates of NH₃ oxidation in natural environments as temperatures increase (Myers, 1975; Groeneweg et al., 1994; Avrahami et al., 2003; Tourna et al., 2008; Gubry-Rangin et al., 2017; Ouyang et al., 2017; Taylor et al., 2017; Duan et al., 2018, 2020; Mukhtar et al., 2019; Zheng et al., 2020; Bello et al., 2021), but there is a dearth of information on temperature-dependent changes of substrate affinity and catalytic efficiency. In this study, AOB showed 3–7-fold increases in $K_m(app)$ with temperature (decrease in affinity), but greater increases in $V_{max(app)}$ with temperature (8–16-fold), which resulted in 2.5–2.8-fold increases in catalytic efficiency. This agrees with previous results with Nitrosomonas sp. 4W30 that had a 1.9–2.7-fold increase in catalytic efficiency as temperatures increased from 5 to 20°C (Jones and Morita, 1985). In the case of the AOA N. viennensis, there was no significant change in $K_m(app)$ but $V_{max(app)}$ increased nearly two orders of magnitude resulting in a significant increase of catalytic efficiency as temperatures increased. This increase in catalytic efficiency with temperature by N. viennensis was similar to the response of the AOA “Ca. N. oleophilus” MY3 over 25–35°C where there was ∼2-fold increase in catalytic efficiency (Jung et al., 2022).

To date, it is unknown if temperature differentially affects NH₃ and NO₂⁻ oxidation in comammox Nitrospira. Previous evaluation of the kinetic parameters of NH₄⁺ and NO₂⁻ oxidation in comammox cultures of Nitrospira inopinata and “Candidatus Nitrospira krebii” at their optimal growth temperatures (37°C and room temperature, respectively) found that the catalytic efficiency of NH₃ oxidation was one to two orders of magnitude greater than that of NO₂⁻ oxidation (Kits et al., 2017; Sakoula et al., 2020). The different catalytic efficiencies of NH₃ oxidation (19.6) and NO₂⁻ oxidation (0.05) of N. inopinata may have caused the accumulation of NO₂⁻ until all NH₄⁺ was consumed (Daims et al., 2015; Kits et al., 2017). In contrast, NO₂⁻ did not accumulate in “Candidatus Nitrospira krebii” cultures where there were smaller differences between the catalytic efficiency of NH₃ (33–42) and NO₂⁻ oxidation (4–8; van Kessel et al., 2015; Sakoula et al., 2020). Because the activity and catalytic efficiency of NH₃ and NO₂⁻ oxidizers affect N availability and productivity in soil, marine, and surface waters, and engineered systems, future studies are needed to investigate the coupling of nitrification, including comammox in response to temperature.

A Few Observations on the Michaelis–Menten and Haldane Kinetic Parameters of NH₃ and NO₂⁻ Oxidation

Ammonia and NO₂⁻ are poor energy sources to support an autolithothrophic lifestyle; therefore, it makes intuitive sense that the optimal growth temperature ($T_{opt}$) of the NH₃ oxidizers evaluated in this study was at, or near, the temperature where the response of MM kinetics resulted in near peak catalytic efficiency for NH₃ oxidation (Figure 2). In contrast, the growth $T_{opt}$ of the six NOB evaluated in this study did not occur at the temperatures where their catalytic efficiency of NO₂⁻ oxidation was at its best. Under optimal growth conditions, it has been calculated that NOBs spend ∼26.7% of the energy generated from NO₂⁻ oxidation in cellular homeostasis, while those demands rise greater than 3-fold under non-growth conditions (Vadivelu et al., 2006; Ni et al., 2008). Perhaps, NOBs prioritize cell maintenance over growth. Nitrobacter NOBs have multiple energy storage cell inclusions, such as poly-$\beta$-hydroxybutyrate, glycogen, and polyphosphate granules (Watson et al., 1989; Spieck and Bock, 2005), and the energy generated during the oxidation of NO₂⁻ may be used to create or enrich these storage molecules until optimal growth conditions are met. We also observed that the H substrate inhibition of N. viennensis, N. europaea, N. moscoviensis, and N. winogradskiyi, was temperature-sensitive (Supplementary Table 5), which may be another potential explanation for why $T_{opt}$ for growth and the temperature for optimal catalytic efficiency are not aligned, for it has been long known that substrate inhibition can occur during the isolation and culture of nitrifiers (Both et al., 1992; Bartosch et al., 2002; Spieck et al., 2006; Spieck and Lipski, 2011; Tourna et al., 2011). The H inhibition constant $K_i$ was the highest (lowest sensitivity) at the optimal growth temperature (30°C) of N. winogradskiyi and N. vulgaris, suggesting that substrate sensitivity may determine the $T_{opt}$ for growth rather than optimal catalytic efficiency. We also observed that N. moscoviensis, N. japonica strain NJ1, and N. vulgaris all had their highest sensitivity ($K_i < 0.9$ mM) to NO₂⁻ at 10°C, which is interesting because Venterea et al. (2020) reported NO₂⁻ accumulation in soil at temperatures of 5 and 10°C, whereas it was quickly
consumed at 22 and 30°C. Intriguingly, we found that there was a trend for the AOA \textit{N. viennensis} to be the most sensitive to NH$_4^+$ ($K_i$ 0.3–0.5 mM) at its optimal growth temperature, which may help explain why it has been challenging to isolate AOA cultures.

Both \textit{Nitrobacter} and \textit{Nitrospira} have demonstrated the ability to utilize NO$_3^-$ as a terminal electron acceptor in the presence of organic molecules, using NXR in the reverse direction to reduce NO$_3^-$ to NO$_2^-$ (Aleem and Sewell, 1981; Tanaka et al., 1983; Sundermeyerling et al., 1984; Freitag et al., 1987; Meincke et al., 1992; Lücker et al., 2010); and in the case of \textit{Nitrobacter}, NXR possessed a higher affinity for NO$_3^-$ (0.3–0.5 mM NO$_3^-$) than for NO$_2^-$ (0.54–3.6 mM NO$_2^-$). Intriguingly, the highest rates of ATP production were observed in whole-cell incubation of \textit{N. winogradskii} when both oxygen and NO$_2^-$ were provided as electron acceptors, suggesting the potential for NO$_2^-$ oxidation and NO$_3^-$ reduction to occur concurrently (Freitag and Bock, 1990). There are examples of temperature affecting the balance between the forward and reverse direction of enzymes in different ways. The equilibrium in favor of the forward reaction of glucose isomerase increased from 50 to 60°C (Dehkordi et al., 2009) while the affinity of the forward reaction of glycerol-2-phosphate dehydrogenase was higher at 5°C compared to 21°C or 37°C (Ruberto et al., 2019). It is unknown how temperature will affect the balance between the forward and reverse actions of NXR, or if NXR of \textit{Nitrobacter} and \textit{Nitrospira} will respond in different ways.

**Protein Structure or Membrane Differences Between Groups**

Bacterial and archaeal AMOs are integral membrane proteins (Norton et al., 2002; Urakawa et al., 2011), NXRs of \textit{Nitrobacter} are membrane-bound proteins (Sundermeyerling et al., 1984; Meincke et al., 1992), and NXR of \textit{Nitrospira} are soluble proteins anchored to membranes (Mundinger et al., 2019); and it may be worth considering differences in membrane lipid composition between lineages to explain dissimilar temperature responses. This is based on (i) membrane dynamics and fluidity change in response to temperature, and the degree to which they change depends on lipid composition and lipid phase transition temperature (Mansilla et al., 2004), (ii) membrane protein activity and stability can be dependent on lipid composition and dynamics (Hong and Tamm, 2004; Reading et al., 2017; Sanders et al., 2018), (iii) lipid composition of \textit{Nitrobacter} and \textit{Nitrospira} is quite different (Lipski et al., 2001), indicating that their lipid membranes will respond differently to temperature, and (iv) AOA and other Thaumarchaeota have a high proportion of crenarcheol, a core lipid that is unique in structure and potentially function from those of bacteria (Pitcher et al., 2010; Elling et al., 2014). It is well known that the archaeal and bacterial membrane lipid composition is dependent on growth temperature and is often used as a proxy for past temperatures (Elling et al., 2014; De Jonge et al., 2019), and that both bacteria and archaea actively modify lipid membrane composition in response to transient changes in temperature (Balogh et al., 2013; Siliakus et al., 2017). The cyclization of thaumarcheal glycerol dialkyl tetraethers (GDGTs) membrane-spanning lipids is strongly correlated with sea surface temperature (Elling et al., 2014; Qin et al., 2015), and the degree of methylization and cyclization of branched bacterial glycerol dialkyl glycerol tetraethers (GDGTs) that are abundant in terrestrial and aquatic systems varies with the mean annual temperature (De Jonge et al., 2019). While NH$_3$ and NO$_3^-$ oxidizers may have the ability to modify the lipid composition of cellular and periplasmic membranes in response to transient temperature changes, the incubations in this study were likely not long enough for such changes to occur. However, the cultures in this study grown under optimal conditions would differ in lipid composition in ways that could influence lipid dynamics, and may be as important as variation in protein structure in governing the temperature response of individual nitrifying organisms.

**Implications of Changes in Catalytic Efficiency in Natural Environments**

Based on these data, increasing temperature should result in an increase in the catalytic efficiency of NH$_3$ oxidation, while \textit{Nitrospira} NOBs are not predicted to increase catalytic efficiency with temperature, and the catalytic efficiency of \textit{Nitrobacter} NOB decline significantly. This differential change in catalytic efficiency may help explain the accumulation of NO$_2^-$ previously observed in other studies. For example, increasing temperature decoupled NO$_2^-$ oxidation from NH$_3$ oxidation in coastal waters (Heiss and Fulweiler, 2016; Schaefer et al., 2017), and in multiple soil types (Giguere et al., 2017; Taylor et al., 2019; Duan et al., 2020; Waggoner et al., 2021). We have previously observed that the accumulation of NO$_2^-$ was required to stimulate the rates of NO$_2^-$ oxidation to match those of NH$_3$ oxidation in a range of soils (Giguere et al., 2018; Taylor et al., 2019); but accumulated NO$_2^-$ also has the potential to inhibit NOB activity (Park and Bae, 2009; Zheng et al., 2021; Supplementary Table 5). Rates of NO$_2^-$ oxidation were stimulated ~2-fold across all temperatures when NO$_2^-$ accumulated in NH$_4^+$-amended Columbia Basin soil incubations (Figure 3); however, these concentrations were greater than the temperature-dependent $K_i$ of four of the NOB cultures evaluated in this study, and our modeling suggests temperature-dependent H inhibition, particularly of \textit{Nitrospira} NOB.

Changes in enzyme catalytic efficiency with temperature are not specific to nitrification, and previous studies of some enzyme activities linked to processing of organic C, N, and P (cellulbiohydrolase, β-glucosidase and xylanase, phosphatase, leucine-aminopeptidase, and tyrosine-aminopeptidase) in soil have observed changes in catalytic efficiency in response to temperature. Catalytic efficiency was positively correlated with temperature at sites ranging from boreal to tropical forests.
(German et al., 2012) and in long-term N addition plots (Stone et al., 2012). In other studies, catalytic efficiency increased with temperature or did not change depending on the enzyme evaluated (Blagodatskaya et al., 2016; Razavi et al., 2016). Curiously, none of these studies observed pronounced declines in catalytic efficiency as temperature increased as was observed in this study with *Nitrobacter NOB*.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

AT was responsible for experimental design, data analysis, and writing of the manuscript. BM was responsible for execution of experiments, phylogenetic analysis, and writing of the manuscript. Both authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: [https://www.frontiersin.org/articles/10.3389/fmicb.2022.817986/full#supplementary-material](https://www.frontiersin.org/articles/10.3389/fmicb.2022.817986/full#supplementary-material)

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