



Oxidation kinetics and inverse isotope effect of marine nitrite-oxidizing isolates

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ABSTRACT: Nitrification, the step-wise oxidation of ammonium to nitrite and nitrate, is important in the marine environment because it produces nitrate, the most abundant marine dissolved inorganic nitrogen (DIN) component and N-source for phytoplankton and microbes. This study focused on the second step of nitrification, which is carried out by a distinct group of organisms, nitrite-oxidizing bacteria (NOB). The growth of NOB is characterized by nitrite oxidation kinetics, which we investigated for 4 pure cultures of marine NOB (*Nitrospina watsonii* 347, *Nitrospira* sp. Ecomares 2.1, *Nitrococcus mobilis* 231, and *Nitrobacter* sp. 311). We further compared the kinetics to those of non-marine species because substrate concentrations in marine environments are comparatively low, which likely influences kinetics and highlights the importance of this study. We also determined the isotope effect during nitrite oxidation of a pure culture of *Nitrospina* (*Nitrospina watsonii* 347) belonging to one of the most abundant marine NOB genera, and for a *Nitrospira* strain (*Nitrospira* sp. Ecomares 2.1). The enzyme kinetics of nitrite oxidation, described by Michaelis-Menten kinetics, of 4 marine genera are rather narrow and fall in the low end of half-saturation constant (K_m) values reported so far, which span over 3 orders of magnitude between 9 and $>1000 \mu\text{M NO}_2^-$. *Nitrospina* has the lowest K_m ($19 \mu\text{M NO}_2^-$), followed by *Nitrobacter* ($28 \mu\text{M NO}_2^-$), *Nitrospira* ($54 \mu\text{M NO}_2^-$), and *Nitrococcus* ($120 \mu\text{M NO}_2^-$). The isotope effects during nitrite oxidation by *Nitrospina watsonii* 347 and *Nitrospira* sp. Ecomares 2.1 were 9.7 ± 0.8 and $10.2 \pm 0.9\%$, respectively. This confirms the inverse isotope effect of NOB described in other studies; however, it is at the lower end of reported isotope effects. We speculate that differences in isotope effects reflect distinct nitrite oxidoreductase (NXR) enzyme orientations.

KEY WORDS: Nitrification · Nitrite-oxidizing bacteria · Isotope effect · Enzyme kinetics · Marine environment

INTRODUCTION

In the global ocean, nitrate is the dominant dissolved inorganic nitrogen (DIN) component. Nitrate sinks include assimilation (mainly but not exclusively by phytoplankton) and respiratory processes, like dissimilatory nitrate reduction to ammonium (DNRA) and denitrification (e.g. Kraft et al. 2014). Only N_2

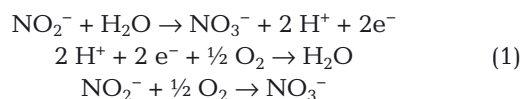
production (via denitrification or anammox) permanently removes nitrogen from the system. Nitrate is synthesized via nitrification, the step-wise oxidation of ammonia to nitrite and nitrate. This study focuses on the second step, which is carried out by chemolithoautotrophic nitrite-oxidizing bacteria (NOB). Marine nitrite concentrations are generally low; however, there are regions where nitrite does accumulate

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to micromolar concentrations. On the one hand, this occurs in the 'primary nitrite maximum' (PNM) at the base of the euphotic zone due to decoupling of ammonia and nitrite oxidation or phytoplankton release of nitrite (Olson 1981, Dore & Karl 1996, Lomas & Lipschultz 2006). On the other hand, nitrite also occurs in the 'secondary nitrite maximum' (SNM), which is found in oxygen-depleted deeper water bodies, where nitrite probably stems from nitrate reduction to nitrite and ammonia oxidation (Lam et al. 2011).

Important oxygen minimum zones (OMZs) with significant nitrite accumulation are located in the Arabian Sea, the eastern tropical North Pacific, and the eastern tropical South Pacific (cf. overview in Wright et al. 2012). Even though OMZs make up <0.1% of the total ocean volume (Codispoti et al. 2001), they are responsible for 30 to 50% of the global marine nitrogen loss (Gruber & Sarmiento 1997), because high rates of nitrification and DNRA fuel nitrogen release via anammox and/or denitrification (e.g. Lam & Kuypers 2011, Kalvelage et al. 2013). Although nitrification is an aerobic process, it is also a key process in OMZs, as some nitrifiers are also active under oxygen depleted conditions (Bristow et al. 2016).

In this manuscript, we focused on marine NOB pure cultures, which gain energy from the chemical conversion of nitrite to nitrate and use CO₂ as a carbon source (Watson et al. 1989, Fiencke et al. 2005):



Four NOB phyla are known: *Nitrospirae*, *Nitrospinae*, *Proteobacteria*, and *Chloroflexi*. The genus *Nitrospira* (Watson et al. 1986) belongs to the phylum *Nitrospirae* (Ehrich et al. 1995), *Nitrospina* (Watson & Waterbury 1971) is phylogenetically affiliated with *Nitrospinae* (Lücker et al. 2013, Spieck et al. 2014), just like the *Candidatus Nitromaritima* (recently identified based on metagenomic data; Ngugi et al. 2016). *Nitrobacter* (Winogradsky 1892, Woese et al. 1984), *Nitrococcus* (Watson & Waterbury 1971, Woese et al. 1985), and *Nitrotoga* (Alawi et al. 2007) belong to the *Alpha*-, *Gamma*- or *Betaproteobacteria*, and *Nitrolancea* (Sorokin et al. 2012) is a member of the phylum *Chloroflexi*.

NOB are phylogenetically heterogeneous (Teske et al. 1994), and cover a wide range of preferences regarding environmental conditions such as temperature or substrate concentration (De Boer et al. 1991). In the marine realm, *Nitrobacter*, *Nitrococcus*, *Nitro-*

spina, *Nitrospira*, and *Candidatus Nitromaritima* have been found. Additionally, *Nitrotoga* has been detected in a marine recirculation aquaculture system (Keuter et al. 2017). However, few strains were isolated, and there is limited knowledge on their overall distribution and abundance (Watson & Waterbury 1971, Watson et al. 1986, Ward 2011, Ngugi et al. 2016). Established data for marine NOB are scarce and mainly available for *Nitrobacter* (Ward & Carlucci 1985), including *Nitrobacter* sp. 355 (Buchwald & Casciotti 2010) isolated from Black Sea surface water, and for *Nitrospira marina* from the Gulf of Maine (Watson et al. 1986).

Nitrite oxidation is catalysed by nitrite oxidoreductase (NXR) enzymes, which shuttle 2 electrons per reaction into the respiratory chain. NXR belongs to a type II DMSO reductase-like family of molybdopterin-binding enzymes (Lücker et al. 2010, Lücker et al. 2013). NXR in different genera of NOB varies in its molecular mass, orientation, and classes of cytochromes that are used in the electron transport chain. NXR is a membrane-bound enzyme associated with the cytoplasmic membrane, and consists of 3 subunits: the catalytical NxrA, the electron-channelling NxrB, and a putative NxrC as membrane anchor (Sundermeyer-Klinger et al. 1984, Lücker et al. 2010). The substrate-binding NxrA subunit faces the periplasmic space in *Nitrospina* (Spieck & Bock 2005, Lücker et al. 2013) and *Nitrospira* (Spieck et al. 1998, Lücker et al. 2010), but is oriented towards the cytoplasm in *Nitrobacter*, *Nitrococcus*, and *Nitrolancea* (Spieck et al. 1996, Sorokin et al. 2012).

The activity of NXR can be described based on Michaelis-Menten kinetics, i.e. the half-saturation constant (K_m) and the maximum nitrite oxidation activity (V_{max}). Based on these parameters and the idea of *K*- and *r*-selection (MacArthur & Wilson 1967, Andrews & Harris 1986), Schramm et al. (1999) classified *Nitrospira* as a *K*-strategist and *Nitrobacter* as a *r*-strategist. *K*-strategy originally meant selection for competitive ability in crowded populations, and *r*-strategy referred to selection for high population growth in uncrowded populations (MacArthur & Wilson 1967). *K*-strategists among microbes have high substrate affinities at low substrate concentrations, and *r*-strategists have high maximum specific growth and substrate utilization rates at high substrate concentrations (Andrews & Harris 1986). So far, the kinetics of nitrite oxidation have not been investigated for marine NOB pure cultures. Recent investigations of natural assemblages in OMZs focused on O₂ dependence during nitrite oxidation and found a very high O₂ affinity, which supports the relevance of

nitrification even in the core of OMZs (Bristow et al. 2016, Peng et al. 2016).

Another technique is based on natural abundances of stable nitrite isotopes to shed light on the origin and fate of nitrite, which have been applied, for example, in the PNM in the central California Current and in a European river (Santoro et al. 2013, Jacob et al. 2016). Nitrite oxidation is coupled to a rare inverse isotope effect, so that the nitrite pool is subsequently depleted in ^{15}N during nitrite oxidation (Casciotti 2009). However, this isotope effect has only been investigated in *Nitrococcus mobilis* 231, *Nitrobacter* sp. 355, and *Nitrospira marina*. It is known that the isotope effect of a specific reaction can vary depending on enzyme properties and genetic diversity (Casciotti et al. 2003), but so far, the database has been too sparse to assess the variability of the isotope effect of nitrite oxidation and its link to enzyme variability and Michaelis-Menten kinetics.

In this study, we investigated 4 marine species (*Nitrospira* sp. Ecomares 2.1, *Nitrospina watsonii* 347, *Nitrobacter* sp. 311, and *Nitrococcus mobilis* 231) for their nitrite oxidation kinetics and 2 species (*Nitrospira* sp. Ecomares 2.1, *Nitrospina watsonii* 347) for their specific kinetic isotope effects, and compared those to non-marine species. We hypothesized that the environmental conditions in the marine realm (e.g. low substrate concentration in comparison to terrestrial habitats) should be reflected in enzyme kinetics, and aimed to investigate a potential link between enzyme kinetics and the isotope effects of nitrite oxidation.

MATERIALS AND METHODS

Bacterial strains and cultivation

Nitrospira sp. Ecomares 2.1 was isolated from a moving-bed filter of an aquaculture system in Büsum, Germany, and has optimal growth conditions in 28 to 30°C seawater (70%) (Keuter 2011). To date, *Nitrospina* has been found exclusively in marine habitats, and *Nitrospina watsonii* 347 was originally sampled from 100 m depth in the Black Sea (Spieck et al. 2014). Its temperature optimum is 28°C in 70% seawater (Watson & Waterbury 1971). *Nitrospira* and *Nitrospina* have substrate optima from 0.5 to 3 mM (Keuter et al. 2011, Spieck et al. 2014). *Nitrobacter* sp. 311 was isolated from surface waters of the tropical Eastern Atlantic Ocean near the west coast of central Africa (J. Waterbury pers. comm. in Starkenburg et al. 2008). *Nitrococcus mobilis* 231 was iso-

lated from surface waters of the South Pacific Ocean near the Galapagos Archipelago. So far, it is the only isolate of this genus, and has to date been found exclusively in marine habitats. Optimal growth conditions are 25 to 30°C in 70 to 100% seawater (Watson & Waterbury 1971).

Chemical analyses

For nitrite oxidation kinetics experiments, nitrite and nitrate concentrations of *Nitrospira*, *Nitrospina*, *Nitrobacter*, and *Nitrococcus* cultures were analysed by high-performance liquid chromatography (HPLC) coupled to an ion pair chromatograph (LiChrospher RP-18 column; Merck) (Meincke et al. 1992), and UV detection in an automated system (LaChrom Elite HPLC system; VWR). Cell protein concentration was determined based on the bicinchoninic acid method (Smith et al. 1985) after cell lysis in 0.15 M NaOH and incubation at 90°C for 30 min.

For nitrite isotope fractionation experiments, nitrite concentrations of *Nitrospina watsonii* 347 incubations were analysed photometrically using standard colorimetric techniques (Grasshoff et al. 2009). Nitrite and nitrate concentrations of *Nitrospira* sp. Ecomares 2.1 incubations were measured with an HPLC system (Jasco) (Meincke et al. 1992). Both methods were in good agreement.

Activity measurements and calculation of nitrite oxidation kinetics

Nitrite oxidation kinetics were calculated based on activity measurements performed after Nowka et al. (2015). *Nitrospira* sp. Ecomares 2.1, *Nitrospina watsonii* 347, *Nitrobacter* sp. 311, and *Nitrococcus mobilis* 231 were grown in biological replicates (see Table 2) in 1 l flasks, each with 500 ml marine NOB-medium at 28°C (modified Spieck & Lipski 2011, after Watson & Waterbury 1971). Nitrite-oxidation dependent oxygen consumption was analysed in a microrespiration system (Unisense AS), which was constructed with a 1 channel oxygen sensor amplifier (OXY-Meter), a Clark-type oxygen microsensor (OX-MR, polarized for at least 48 h before use), a stirring system with glass-coated magnets, 2 ml glass chambers with glass stoppers, a rack for 8 chambers, and the data acquisition software MicOx 3.0. All incubations were stirred at 200 rpm in a recirculated water bath in thermostat-regulated rooms. Twelve to 48 h after complete nitrite consumption (early stationary

phase), sub-samples were transferred to 2 ml glass chambers, which were sealed with glass stoppers. The measurements started with nitrite concentrations of up to 150 μM for *Nitrospira* sp. Ecomares 2.1, 230 μM for *Nitrospina watsonii* 347, 472 μM for *Nitrobacter* sp. 311, and 1250 μM for *Nitrococcus mobilis* 231 (cf. Nowka et al. 2015) (see Fig. 1).

Oxygen consumption rate profiles based on a minimum of 5 different concentrations and at least triplicate incubations of each NOB strain were used to calculate specific oxidation kinetics. Nitrite consumption was calculated from oxygen consumption based on a ratio of 2:1. These consumption data were used to calculate oxidation kinetics based on Michaelis-Menten kinetics:

$$V = \left(\frac{V_{\max} \times [S]}{K_m + [S]} \right) \quad (2)$$

where V is the activity, V_{\max} is the maximum specific activity ($\mu\text{mol mg protein}^{-1} \text{h}^{-1}$), K_m is the half-saturation constant for nitrite oxidation (μM), and $[S]$ is the nitrite concentration in μM . Note that we did not work with purified enzyme, but as growth can be discounted due to the short-term incubations of only a few hours, the use of the terms K_m and V_{\max} is justified.

Setup for nitrite fractionation experiments

The batch experiments of *Nitrospina watsonii* 347 and *Nitrospira* sp. Ecomares 2.1 represent closed systems during a relatively short time, where any nitrite loss is due to nitrite oxidation. Sterile control flasks were incubated in duplicate to exclude abiotic nitrite turnover.

Both NOB strains were cultured in batch incubations of marine NOB-medium with varying amounts of nitrite (modified Spieck & Lipski 2011, after Watson & Waterbury 1971) in the dark at 28°C without stirring. Flasks (250 ml) with 100 ml fresh growth medium were inoculated with 10 ml active stock culture. The medium was then amended with nitrite of a known isotope value ($\delta^{15}\text{N} = -27.5\text{‰}$).

We performed 6 *Nitrospira* sp. Ecomares 2.1 and 7 *Nitrospina watsonii* 347 experiments, which had nitrite concentration between ~ 100 and ~ 1600 μM (Table 1), i.e. within the substrate optima for the respective species (Keuter et al. 2011, Spieck et al. 2014). Samples were taken daily until nitrite was consumed. At each sampling time, 6 ml of culture

Table 1. Overview of nitrite oxidation experiments for the calculation of isotope effects (ϵ). Results of fractionation experiments: isotope effects and standard deviations

Incubation ID	Bacteria species	Initial nitrite concentration ($\mu\text{mol l}^{-1}$)	Number of samples used for ϵ calculation	Isotope effect (‰)	Standard deviation (‰)
1	<i>Nitrospira</i> sp.	921	5	9.0	0.2
2	Ecomares 2.1	831	5	10.0	0.3
3		849	5	10.8	0.2
4		564	4	11.5	0.3
5		563	5	9.9	0.2
6		560	4	9.8	0.2
7	<i>Nitrospina</i>	1624	5	8.2	0.7
8	<i>watsonii</i> 347	1613	5	10.5	0.7
9		1533	5	10.4	0.2
10		1600	5	9.1	1.9
11		123	3	9.7	1.7
12		114	3	10.1	2.3
13		600	11	9.7	1.8

were removed with a sterile pipette and centrifuged (20 min at $16\,000 \times g$). The supernatant was analysed for nutrient and isotope composition. Isotope effects of *Nitrospira* sp. Ecomares 2.1 and *Nitrospina watsonii* 347 were based on 3 to 11 technical replicates per nitrite concentration that showed a linear decrease in substrate concentration over time (Table 1).

Isotope analyses

Nitrite isotopes were analysed using the denitrifier method (Sigman et al. 2001, Casciotti et al. 2002, Böhlke et al. 2007). In brief, media samples were injected into a *Stenotrophomonas nitrireducens* bacteria suspension. These bacteria selectively reduce nitrite to N_2O gas, which was then analysed on a Gas Bench II coupled to a Delta V isotope ratio mass spectrometer (Thermo Fisher Scientific). The sample volume was always adjusted to a final N_2O gas amount of 5 nmol.

Isotope values are reported using the delta notation:

$$\delta^{15}\text{N} [\text{‰ vs. std}] = \left(\frac{\left(\frac{^{15}\text{N}}{^{14}\text{N}} \right)_{\text{sample}}}{\left(\frac{^{15}\text{N}}{^{14}\text{N}} \right)_{\text{std}}} - 1 \right) \times 1000 \quad (3)$$

where the standard for nitrogen is atmospheric N_2 .

All samples were analysed in replicate and against in-house potassium nitrite and sodium nitrite standards with known $\delta^{15}\text{N}$ values of -81.5 and -27.5‰ , determined independently via elementaranalysator-

isotope ratio mass spectrometry analysis. The standard deviation of standards and replicates was $<0.3\%$ ($n > 3$).

Calculation of isotope effects

The kinetic isotope effect, ϵ , can be calculated based on the Rayleigh closed-system equation (Mariotti et al. 1981, see Eq. 4), with samples of exponential growth phase only. Recently, some inconsistency has emerged in reporting isotope effects, with some authors reporting inverse fractionation with a negative sign. However, we refer to Mariotti et al. (1981) as one of the fundamental works on isotope effects, and decided to use their original notation. Hence, positive isotope effects indicate inverse fractionation:

$$\epsilon = \frac{\delta^{15}\text{N}_{\text{substrate}} - \delta^{15}\text{N}_{\text{initial}}}{\ln(f)} \quad (4)$$

where f is the remaining fraction of substrate at the time of sampling, and $\delta^{15}\text{N}_{\text{initial}}$ and $\delta^{15}\text{N}_{\text{substrate}}$ represent the initial isotope value of nitrite and the isotope value at the time of sampling, respectively.

RESULTS

Nitrite oxidation kinetics

The key parameters of nitrite oxidation kinetics are V_{max} and K_m . They were assessed based on nitrite-dependent oxygen consumption. In nitrite-depleted, early-stationary-phase cultures, oxygen consumption was initially small, and increased within a few minutes after substrate addition. The O_2 consumption rates depended on the added nitrite concentration and followed Michaelis-Menten kinetics (Fig. 1, Table 2). The V_{max} values of the investigated species revealed a clear differentiation of specific activities:

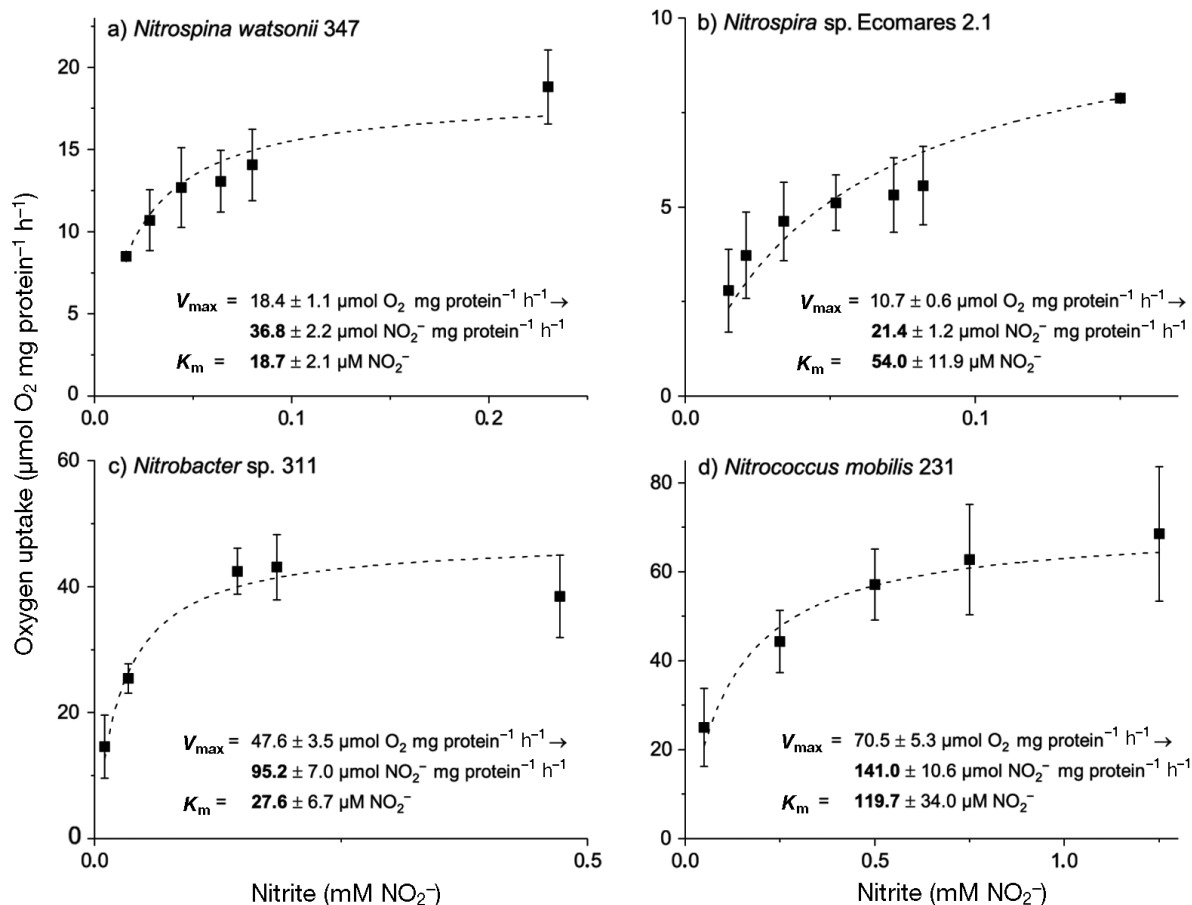


Fig. 1. Nitrite oxidation kinetics of *Nitrospina watsonii* 347, *Nitrospira* sp. Ecomares 2.1, *Nitrobacter* sp. 311, and *Nitrococcus mobilis* 231. Michaelis-Menten plots of oxygen uptake versus nitrite concentration of early-stationary-phase cells at 28°C. Mean values and standard deviations resulted from 2 to 4 biological replicates (cf. Table 2). The kinetic parameters were calculated by non-linear fitting of Michaelis-Menten equation to the data

Nitrococcus mobilis 231 had the highest maximum oxidation activities ($141 \pm 11 \mu\text{mol NO}_2^- \text{ mg protein}^{-1} \text{ h}^{-1}$), followed by *Nitrobacter* sp. 311 ($95 \pm 7 \mu\text{mol NO}_2^- \text{ mg protein}^{-1} \text{ h}^{-1}$). In contrast, the maximum specific activities in *Nitrospina watsonii* 347 and *Nitrospira* sp. Ecomares 2.1 were much lower: 37 ± 2 and $21 \pm 1 \mu\text{mol NO}_2^- \text{ mg protein}^{-1} \text{ h}^{-1}$, respectively. *Nitrospina watsonii* 347, *Nitrobacter* sp. 311, and *Nitrospira* sp. Ecomares 2.1 had relatively low K_m values (19 ± 2 , 28 ± 7 , and $54 \pm 12 \mu\text{M NO}_2^-$, respectively), whereas *Nitrococcus mobilis* 231 had a comparatively high value ($120 \pm 34 \mu\text{M NO}_2^-$; Fig. 1, Table 2).

Isotope analyses and isotope effects

The initial nitrite concentrations are summarized in Table 1; initial nitrite $\delta^{15}\text{N}$ was -27.5% . During common lag phases, substrate consumption and isotope changes in each assay were small, and thereafter, concentrations and isotope values of nitrite changed rapidly. Nitrite was stoichiometrically converted to nitrate (not shown in plots). The isotope values of nitrite decreased during nitrite oxidation in all experiments. At the end of the experiment, $\delta^{15}\text{N-NO}_2^-$ values of up to -50% for *Nitrospina watsonii* 347 and -65% for *Nitrospira* sp. Ecomares 2.1 were reached. Isotope effects were calculated based on Eq. (4). In *Nitrospina watsonii* 347 cultures, isotope effects were 8.2 to 10.5%. The replicate regression lines had standard deviations of $<2.3\%$ and were not significantly different from each other (2-tailed t -test, $p > 0.05$). We thus calculated a mean isotope effect, ϵ , of $9.7 \pm 0.8\%$ (Fig. 2a, Table 1). In *Nitrospira* sp. Ecomares 2.1 cultures, ϵ ranged from 9.0 to 11.5%. The standard deviation of each regression was smaller than 0.3%, and replicates were statistically similar (2-tailed t -test, $p > 0.05$). Therefore, we calculated a mean isotope effect of $10.2 \pm 0.9\%$ (Fig. 2b, Table 1).

Table 2. Results of nitrite oxidation experiments: mean (\pm SD) maximum specific activities (V_{max}) and half-saturation constants (K_m) of 4 marine nitrite oxidizing bacteria (NOB). Number of biological replicates in parentheses

Bacteria species	$V_{\text{max}} \pm \text{SD}$ ($\mu\text{mol NO}_2^- \text{ mg protein}^{-1} \text{ h}^{-1}$)	$K_m \pm \text{SD}$ ($\mu\text{M NO}_2^-$)
<i>Nitrospina watsonii</i> 347 (2)	36.8 ± 2.2	18.7 ± 2.1
<i>Nitrospira</i> sp. Ecomares 2.1 (4)	21.4 ± 1.2	54.0 ± 11.9
<i>Nitrobacter</i> sp. 311 (3)	95.2 ± 7.0	27.6 ± 6.7
<i>Nitrococcus mobilis</i> 231 (3)	141.0 ± 10.6	119.7 ± 34.0

Overall, we found that the ϵ of *Nitrospira* sp. Ecomares 2.1 was not significantly different from that determined in *Nitrospina watsonii* 347 ($p > 0.05$).

DISCUSSION

Nitrite oxidation kinetics

So far, investigations on nitrite oxidation kinetics have mainly been based on non-marine pure cultures, enrichment cultures, or natural assembles (Prosser 1989, Both et al. 1992, Laanbroek et al. 1994, Schramm et al. 1999, Blackburne et al. 2007, Nowka et al. 2015). The aim of this study was to investigate the substrate affinity of marine NOB to link it to environmental conditions and nitrite oxidation kinetics, which thus far has been difficult because nitrite affinity of marine NOB had not yet been investigated in pure cultures. To close this gap, we investigated the nitrite oxidation kinetics of 4 marine NOB genera, including NOB with NXR oriented towards the cytoplasm and periplasm, respectively, and compared them with non-marine NOB strains (Fig. 3). The investigated marine K_m values varied from 19 to $120 \mu\text{M NO}_2^-$ (Fig. 1, Table 2), which is rather narrow and at the low end of K_m values reported so far in comparison to all reported kinetics today that span over 2 orders of magnitude between 9 and $>1000 \mu\text{M NO}_2^-$ (Fig. 3) (Nowka et al. 2015 and references therein). In nature, NOB compete for nitrite, and this competition should be reflected in inter- and intra-specific niche differentiation concerning nitrite oxidation kinetics (Schramm et al. 1999, Kim & Kim 2006, Nogueira & Melo 2006, Blackburne et al. 2007). In the light of relatively similar K_m values, these niche differentiations seem to be less pronounced among marine NOB, probably because the availability of nitrite as a substrate is normally low (Wada & Hatton 1971). K_m values of all the marine NOB we investigated seem to represent an adaptation to these low nitrite concentrations.

Using whole cells from isolates to calculate oxidation kinetics is a well-established method, and thus our calculated K_m values are comparable to previous studies with data from pure cultures (Both et al. 1992, Laanbroek et al. 1994). Furthermore, the results are an important guideline for environmental studies. Even though substrate affinities in the presented dataset are $>20 \mu\text{M NO}_2^-$, our kinetic experiments revealed ongoing nitrite oxidation at low substrate concentrations (i.e. $16 \mu\text{M}$ for *Nitrospina watsonii* 347, $15 \mu\text{M}$ for *Nitrospira* sp. Ecomares 2.1, $10 \mu\text{M}$ for

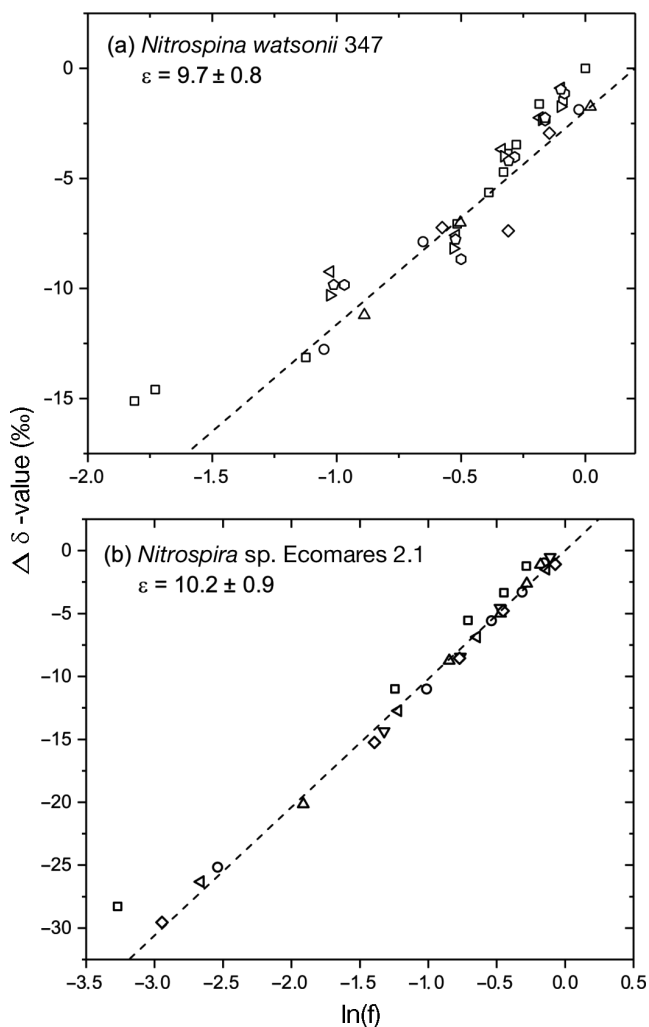


Fig. 2. Rayleigh-plot for (a) 7 *Nitrospina watsonii* 347 experiments and (b) 6 *Nitrospira* sp. Ecomares 2.1 experiments. Decrease of $\delta^{15}\text{N-NO}_2^-$ relative to the initial $\delta^{15}\text{N-NO}_2^-$ is plotted as a function of $\ln(\text{NO}_2^-/\text{NO}_2^-_{\text{initial}})$ during nitrite oxidation (cf. Granger et al. 2004 and our Eq. 4). Every experiment has its own symbols. Standard deviation of a sample is $<0.3\%$ and smaller than the symbol. The average of all regressions defines the dashed line and the corresponding isotope effect (ϵ) with an average ϵ of $9.7 \pm 0.8\%$ in the case of *Nitrospina watsonii* 347 (a), and an average ϵ of $10.2 \pm 0.9\%$ in the case of *Nitrospira* sp. Ecomares 2.1 (b)

Nitrobacter sp. 311, and $50 \mu\text{M}$ for *Nitrococcus mobilis* 231 (Fig. 1). We assume, though, that environmental conditions are probably more complex due to coupled processes and different bacterial assemblages. Nevertheless, the results obtained should be seen as one vital component for the determination of NOB kinetics.

The K_m value of *Nitrospira* sp. Ecomares 2.1 ($54 \mu\text{M NO}_2^-$) is high compared to other *Nitrospira* species. The lowest K_m values in this genus were measured in

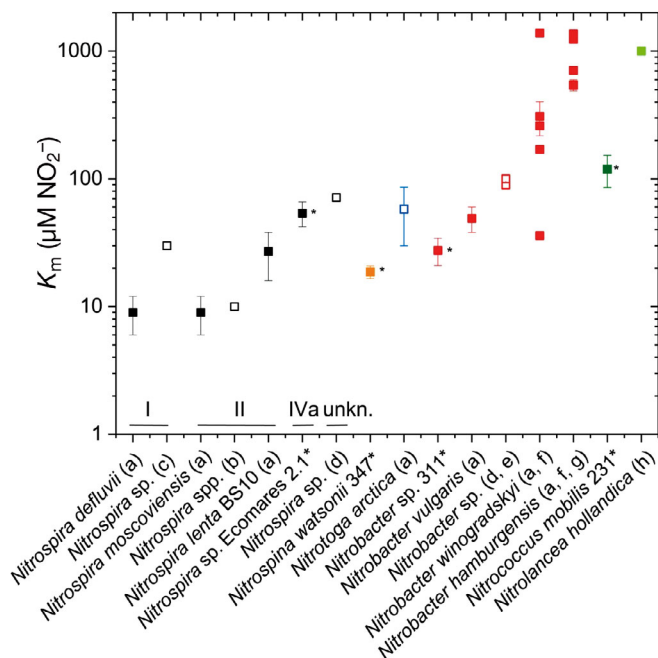


Fig. 3. Nitrite affinities of *Nitrospira* (black), *Nitrospira* (orange), *Nitrotoga* (blue), *Nitrobacter* (red), *Nitrococcus* (dark green), and *Nitrolancea* (light green). *Nitrospira* lineages are indicated as I, II, IVa or unknown above the species name (modified after Nowka et al. 2015). Marine strains from this study are indicated with an asterisk (*). Filled symbols: pure cultures; open symbols: enrichment cultures. Further half-saturation constant (K_m) values are from references (a) Nowka et al. (2015), (b) Schramm et al. (1999), (c) Maixner et al. (2006), (d) Blackburne et al. (2007), (e) Vadivelu et al. (2006), (f) Both et al. (1992), (g) Laanbroek et al. (1994), and (h) Sorokin et al. (2012)

Nitrospira moscoviensis (isolate from heating systems; $9 \mu\text{M NO}_2^-$) and *Nitrospira defluvii* (activated sludge; $9 \mu\text{M NO}_2^-$; Nowka et al. 2015, our Figs. 1 & 3). One possible explanation for the relatively high K_m values of *Nitrospira* sp. Ecomares 2.1 may be its origin; this strain was isolated from a moving-bed reactor of a fish farm, where high nitrite concentrations are common (Keuter et al. 2011).

All the other species we investigated were isolated from natural marine environments. *Nitrospira watsonii* 347 isolated from the Black Sea had the lowest K_m value of $19 \mu\text{M NO}_2^-$. Interestingly, *Nitrobacter* sp. 311 had the second smallest K_m value of our investigated marine species, and of all *Nitrobacter* species investigated to date (Fig. 3). Isolates from soils, like *Nitrobacter hamburgensis* and *Nitrobacter winogradskyi*, have significantly higher K_m values of up to $1370 \mu\text{M}$ (Both et al. 1992, Laanbroek et al. 1994). Within the marine NOB, *Nitrococcus mobilis* 231 has the highest K_m value of $120 \mu\text{M NO}_2^-$, which is in the range reported for *Nitrobacter*. High K_m values of *Ni-*

trobacter and *Nitrococcus* indicate low substrate affinity, which could be due to low affinities of the nitrite transporter proteins that shuttle nitrite across the cytoplasmic membrane (Nowka et al. 2015).

Overall, our results are somewhat ambiguous. We expected K_m values to fall in the lower range, indicating high substrate affinities, because marine nitrite concentrations are usually low. This is, however, not entirely the case — while K_m values indeed generally are at the lower end of those reported so far, other non-marine NOB species within the genus of *Nitrospira* exhibit even higher substrate affinities and lower K_m values (Nowka et al. 2015). We speculate that the relatively narrow range of marine kinetics represents an adaptation to constant low substrate concentrations in the ocean in comparison to much more diverse conditions in terrestrial habitats.

Inverse kinetic isotope effects during nitrite oxidation

In accordance with previous studies (Casciotti 2009, Buchwald & Casciotti 2010, our Fig. 4) we found that the isotope effect of nitrite oxidation is inverse, so that the nitrite pool is successively depleted in ^{15}N . This inverse effect is because in a transition state complex with NXR and water molecules, $^{15}\text{NO}_2^-$ is preferentially oxidized to nitrate, whereas the complex with $^{14}\text{NO}_2^-$ is more likely to decompose before further oxidation (Friedman et al. 1986).

In our experiments, we found isotope effects of $9.7 \pm 0.8\text{‰}$ for *Nitrospira watsonii* 347 (Fig. 2a) and $10.2 \pm 0.8\text{‰}$ for *Nitrospira* sp. Ecomares 2.1 (Fig. 2b, Table 1). This is in line with the previously determined isotope effect of nitrite oxidation by *Nitrospira marina* ($9.1 \pm 1.8\text{‰}$; Buchwald & Casciotti 2010), but clearly lower than the considerably varying isotope effects of the marine species *Nitrococcus mobilis* ($12.8 \pm 1.5\text{‰}$; Casciotti 2009 to $20.2 \pm 2.8\text{‰}$; Buchwald & Casciotti 2010) or *Nitrobacter* sp. 355 ($20.6 \pm 3.2\text{‰}$; Buchwald & Casciotti 2010). We point out that we investigated *Nitrobacter* sp. 311 regarding nitrite oxidation kinetics, whereas Buchwald & Casciotti (2010) investigated the isotope effect of *Nitrobacter* sp. 355 in their study.

Interestingly, it seems that the differences in isotope effects can be linked to variability in K_m values. *Nitrobacter* and *Nitrococcus* exhibit more inverse isotope effects and generally have high K_m values and thus low substrate affinities, whereas *Nitrospira* and *Nitrospira*, with slightly less inverse isotope effects, have high substrate affinities. We speculate

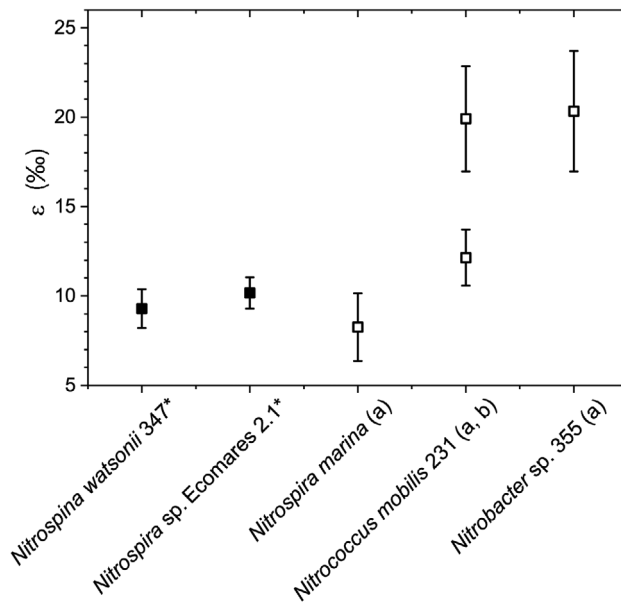


Fig. 4. Overview of isotope effects (ϵ) during nitrite oxidation of this study (*) and others: references (a) Buchwald & Casciotti (2010) and (b) Casciotti (2009)

that these high affinities might be reflected in a more efficient turnover of nitrite bearing the light ^{14}N species, possibly due to a better stabilization of the transition state. This would then result in a lower bulk isotope effect. The contrary might thus apply for *Nitrobacter* and *Nitrococcus* with significantly lower substrate affinities: If the transition state decomposed more often and the higher stability with ^{15}N was more pronounced, these would promote a more pronounced isotope effect.

Beside substrate affinity, the orientation of NXR enzymes and thus energy efficiency differ. *Nitrospira* and *Nitrospira* with NXRs oriented towards the periplasm are more competitive at lower nitrite concentrations than *Nitrobacter* and *Nitrococcus* with cytoplasmically oriented NXRs (Sorokin et al. 2012). The higher efficiency of periplasmic NXR conserves more energy per oxidized nitrite molecule. The surrounding water provides the additional oxygen atom for the oxidation of nitrite, and 2 protons are released in the periplasmic space of NOB with periplasmic NXR. Additionally, during O_2 reduction to water, 2 protons are consumed in the cytoplasm. Therefore, the activity of periplasmic NXR directly contributes to the proton motive force. However, in NOB with cytoplasmic NXR, protons are released and consumed on the same side of the membrane, and thus no contribution to the proton motive force occurs and little energy is obtained during nitrite oxidation (Lücker et al. 2010). The orientation of NXR therefore distin-

guishes between a more economical pathway (NXR towards periplasm, e.g. *Nitrospina* and *Nitrospira*), and a less energy efficient pathway (NXR towards cytoplasm, e.g. *Nitrobacter* and *Nitrococcus*).

Another reason for the range in isotope effects may be the orientation of NXR with regards to nitrite transport, as suggested previously by Buchwald & Casciotti (2010). In cytoplasmically oriented NXR (*Nitrobacter* and *Nitrococcus*), transporters shuttle nitrite into and nitrate out of the cell (Spieck et al. 1996, Starkenburg et al. 2006, Sorokin et al. 2012) to avoid accumulation of nitrate in the cell and potential unfavourable conditions. This transport can potentially be a bottleneck for oxidation, depending on the substrate affinity and activity of the transporter (Daims et al. 2016). In periplasmically oriented NXR (*Nitrospira* and *Nitrospina*) neither nitrite nor nitrate need to be transported through the cytoplasmic membrane, which hinders active enrichment and may be reflected in lower nitrite availability, so that a more efficient turnover of nitrite is an advantage.

Moreover, the 2 groups of NXRs are not only separated by their subcellular orientation, but also by their evolutionary development based on their phylogenetic affiliation within the type II DMSO reductase family of molybdopterin cofactor-binding enzymes and subcellular location (e.g. Lücker et al. 2013). *Nitrospira* and *Nitrospina* lack intracytoplasmic membranes and NXRs oxidize nitrite on the periplasmic side of the membrane (Lücker et al. 2013). NXRs of these NOB belong to a distinct phylogenetic lineage within the type II enzymes of the DMSO reductase family (Lücker et al. 2010, Lücker et al. 2013), which cluster together with NxrAs of anammox bacteria. This NXR type clusters together with the anammox organism '*Candidatus* K. stuttgartiensis' (Lücker et al. 2010).

In contrast, *Nitrobacter*, *Nitrococcus*, and *Nitrolancea* oxidize nitrite on the cytoplasmic side of the membrane, and this type of NXR is closer related to the respiratory nitrate reductase (NAR) of denitrifying bacteria (Lücker et al. 2010, Sorokin et al. 2012). Thus, the differences in isotope effects may reflect the NxrA phylogeny. However, anammox-bacteria oxidize nitrite in the anammoxosome (de Almeida et al. 2015) with an inverse isotope effect of about 31‰ (Brunner et al. 2013), which is much more pronounced than what we found in *Nitrospina* and *Nitrospira*. Indeed, the previously determined isotope effects of *Nitrobacter* and *Nitrococcus* are apparently more pronounced and thus closer to that of anammox bacteria. This is possibly linked to a high reversibility of nitrite oxidation in anammox bacteria

(Brunner et al. 2013), which may presumably also play a role in *Nitrobacter* (Sundermeyer-Klinger et al. 1984, Freitag et al. 1987) and in *Nitrococcus* (Füssel et al. 2017).

Environmental relevance and model application

Our measurements represent the first assessment of kinetics of 4 marine NOB, and we hypothesize that marine NOB tend to be *K*-strategists, probably because of the low substrate availability and relatively little environmental fluctuations in marine habitats. The marine NOB strains we used in our study belong to environmentally relevant genera. *Nitrospina* and *Nitrococcus* in particular are widespread in OMZs, where 30 to 50% of the global oceanic nitrogen loss takes place (Gruber & Sarmiento 1997, Codispoti et al. 2001, Gruber & Galloway 2008). In the Arabian Sea OMZ, *Nitrospina* spp., and a novel lineage of NxrA clustering between anammox and *Nitrospina* sequences were identified (Lüke et al. 2016). *Nitrospina* was further found near Costa Rica in the eastern tropical North Pacific (Buchwald et al. 2015), and *Nitrospina* and *Nitrococcus* make up ~9% of the microbial community in the Namibian OMZ (Füssel et al. 2012). There are numerous studies addressing the ubiquitous occurrence of the genus *Nitrospina*, which make our results to some extent representative for the marine NOB community in at least some sampling sites.

Furthermore, the isotope effects for *Nitrospina watsonii* 347 and *Nitrospira* sp. Ecomares 2.1 that we calculated are significantly different from the previously determined higher isotope effects of *Nitrococcus* and *Nitrobacter*. These differences have important consequences for the interpretation of nitrification and model calculations. Biogeochemical models are often applied in marine systems where nitrite accumulates, such as the SNM of OMZs (Lam et al. 2011, Gaye et al. 2013), and the isotope effects of nitrite generation and removal are important constraints for such models.

Generally, a wide range of isotope effects has been applied in different models: In the OMZ of the eastern tropical North Pacific off Costa Rica, an isotope effect of 30‰ was calculated for nitrite oxidation (Buchwald et al. 2015). In another model study focusing on the eastern tropical South Pacific, measurements of $\delta^{15}\text{N-NO}_2^-$ could not be reproduced using currently published isotope effects—an isotope effect of 32‰ (close to that determined experimentally for anammox bacteria; Brunner et al. 2013) would have been required to reproduce the data (Casciotti

et al. 2013). Contrastingly, in a recent study on isotopic overprinting during combined nitrification–denitrification (Granger & Wankel 2016), the authors used $16.0 \pm 4.5\%$ as a model parameter for nitrite oxidation, and also on the lower end of the applied isotope effects, Gaye et al. (2013) assumed an isotope effect of 13% for nitrite oxidation in the Arabian Sea (cf. Casciotti 2009). A change in the isotope effect of nitrite oxidation, even by only 3% , will alter the computed isotope values of nitrite in ocean models (T. Rixen pers. comm.). The use of such a lower isotope effect may be appropriate because other OMZ regions (like the eastern tropical North Pacific off Costa Rica OMZ, the Namibian OMZ, the Black Sea OMZ, and the Baltic Sea; Labrenz et al. 2007, Fuchsman et al. 2011, Füssel et al. 2012, Spieck et al. 2014, Buchwald et al. 2015) host predominantly *Nitrospina*, for which we find a low isotope effect in this study. This shows that a more accurate assessment of isotope effects and the kinetic parameters that determine these effects is urgently needed to better constrain biogeochemical models.

CONCLUSIONS

We investigated the nitrite oxidation kinetics of 4 marine NOB (*Nitrospina watsonii* 347, *Nitrospira* sp. Ecomares 2.1, *Nitrobacter* sp. 311, and *Nitrococcus mobilis* 231). The range of the K_m values was comparatively narrow, which might reflect a niche specialization towards low substrate concentration and little fluctuation of environmental conditions in marine habitats and places all K_m values of marine NOB in a range similar to clear (terrestrial) K -strategists. For *Nitrospina watsonii* 347 and *Nitrospira* sp. Ecomares 2.1, we also determined the isotope effects of nitrite oxidation, which were 9.7 ± 0.8 and $10.2 \pm 0.9\%$, respectively. This is in line with former investigations of *Nitrospira marina*, but significantly different from *Nitrobacter* sp. 355 and *Nitrococcus mobilis* 231, which both have significantly higher isotope effects.

Based on our data, we speculate that the significant differences between *Nitrospina watsonii* 347, *Nitrospira* sp. Ecomares 2.1, and *Nitrospira marina* on the one side, and *Nitrobacter* sp. 355 and *Nitrococcus mobilis* 231 on the other side, may be due to differences in NXR orientation or phylogenetic relationships because, e.g., anammox bacteria also show a distinctly different (and much more pronounced) isotope effect during nitrite oxidation. Thus, our data sets the basis for an assessment of variances in isotope effects due to phylogeny and enzyme orienta-

tion, which should be pursued in the future with an assessment including non-marine NOB.

Furthermore, our investigation of nitrite oxidation kinetic data and isotope effects will help verify and improve biogeochemical models. Models are often applied in hotspots of nitrogen cycling like OMZs, where especially *Nitrospina* and *Nitrococcus* are widespread. It may be an interesting perspective to link the dominant nitrite oxidizer species to environmentally determined isotope effects and their relatedness to anammox bacteria. Overall, our data provide an overview of potential nitrite oxidation kinetics and enlarge the database for isotope effects. There is a certain mismatch between measured isotope effects and those assumed in models; some modelling approaches require isotope effects significantly above those measured to date, including in this study. This suggests that a re-evaluation of modelling approaches may be required to match the isotope effects because there is no indication for higher isotope effects in NOB from marine OMZs.

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