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Reduced isotope fractionation by denitrification under conditions relevant to the ocean

K. Kritee [a,*], Daniel M. Sigman [a], Julie Granger [b], Bess B. Ward [a], Amal Jayakumar [a], Curtis Deutsch [c]

[a] Department of Geosciences, Princeton University, Guyot Hall, Washington Road, Princeton, NJ 08544, United States
[b] Department of Marine Sciences, University of Connecticut, 1080 Schennecossett Road, Groton, CT 06340, United States
[c] Department of Atmospheric and Oceanic Science, University of California – Los Angeles, 7125 Math Science Building, 405 Hilgard Ave., Los Angeles, CA 90095, United States

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Abstract

Experiments with two well-studied denitrifiers and one recently isolated marine suboxic zone denitrifier show that the cellular-level denitrification N isotope effect ($^{15}\varepsilon$) is typically lower than the canonical value of ~25‰ under many conditions prevalent in the ocean. Across all three strains, $^{15}\varepsilon$ is 10–15‰ at cellular nitrate reduction rates that are more representative of the environment than the very high rates under which we and previous investigators measure $^{15}\varepsilon$ to be 20–30‰. A sharp decrease in $^{15}\varepsilon$ is also observed in individual nitrate drawdown assays as the extracellular nitrate concentrations approach 2–35 μM and nitrate uptake becomes the rate-limiting step. On an apparently strain-specific basis, lower values of $^{15}\varepsilon$ are observed under diverse conditions common in the natural environment: less reduced carbon sources, small inputs of oxygen, nutrient availability, agitation, and age of starter culture (i.e., initiation of assays with cells that had recently depleted a large previous nitrate amendment or were more recently in the exponential growth ("bloom") phase). A conserved oxygen-to-nitrogen isotope relationship across the experiments for all three denitrifiers ($^{18}\varepsilon/^{15}\varepsilon = 0.93 \pm 0.06$ (1SD)) supports the interpretation that fractionation is imparted solely by the internal respiratory nitrate reductase, with the amplitude of $^{15}\varepsilon$ varying with the proportional importance of cellular nitrate efflux relative to uptake. Aspects of the $^{15}\varepsilon$ variation are unexpected; nevertheless, the occurrence of lower $^{15}\varepsilon$ is robust. It is uncertain if our lower $^{15}\varepsilon$ estimates apply to oceanic water column denitrification because field studies have generally yielded $^{15}\varepsilon_{wc}$ between 20–30‰, more similar to previous culture estimates and our estimates at high cell specific nitrate reduction rates. If denitrification in the ocean's major suboxic zones does have an $^{15}\varepsilon$ of ~10–15‰, it would remove an apparent imbalance between global ocean N inputs and outputs previously suggested by fixed N isotope budgeting.

1. INTRODUCTION

Biologically available (or fixed) nitrogen (N) is a major phytoplankton nutrient that limits photosynthesis in large expanses of the ocean, with implications for fertility of aquatic ecosystems, global ocean carbon storage, and total emissions of the greenhouse gas nitrous oxide. The major input of oceanic fixed N is N fixation in surface waters. Most oceanic N loss is due to denitrification by heterotrophic nitrate respiration to N_2 and by autotrophic anammox in low oxygen environments in the water column and in sediment pore waters (Gruber and Galloway, 2008); both of these processes require the reduction of nitrate. Canonical microbial denitrification refers to the process of respiratory nitrate reduction (also called dissimilatory nitrate reduction) accompanying the oxidation of an electron

* Corresponding author. Address: Environmental Defense Fund, 257 Park Avenue South, NY 10010, United States. Tel.: +1 732 277 8134.
E-mail address: kriteek@gmail.com (K. Kritee).

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donor (e.g., organic matter) that ultimately produces molecular N (N_2) through a series of less oxidized intermediate N oxide products [i.e., nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O)]. Respiratory nitrate reduction is performed primarily by heterotrophic bacteria, although denitrifiers are now known in all main microbial phylogenetic groups, including some bacterial autotrophs and archaeal denitrifiers (Cabello et al., 2004), foraminifera (Pina-Ochoa et al., 2010) and fungi (Morozkina and Kurakov, 2007). There is strong regulation of the expression of genes encoding the enzymes responsible for heterotrophic denitrification (hereafter, denitrification unless specified otherwise) and the transporter(s) involved in uptake and efflux of denitrification substrates and intermediate products. Low oxygen tension and the availability of an N oxide are common triggers of enzyme and transporter synthesis (Zumft, 1997).

Experiments with pure cultures of bacterial denitrifiers have shown that the nitrate reduction can occur 2–3% (i.e., 20–30‰) more slowly for ^{15}N-than for ^{14}N-bearing nitrate molecules (Table 1). As in multi-step microbial transformations of other elements (Canfield, 2001; Brunner and Bernasconi, 2005; Kritee et al., 2009), the "cellular level" stable isotopic discrimination of nitrogen and oxygen associated with denitrification – the isotope discrimination that applies to the disappearance of nitrate from the external medium – is the combined result of fractionation at all of the steps associated with uptake and reduction of nitrate by a denitrifying cell up to and including the first irreversible step in cellular nitrate reduction. Heterotrophic denitrifiers do not re-oxidize the nitrite produced from nitrate (Wunderlich et al., 2012), although such nitrite re-oxidation can occur on an environmental scale (Anderson et al., 1982). Thus, when the nitrate transport is reversible, nitrate reduction is the first irreversible step of both cellular-level nitrate assimilation and denitrification and thus the last step that affects the cellular-level isotopic discrimination (Granger et al., 2008). If transport is irreversible (e.g., at very low extracellular nitrate concentrations ($[NO_3^-]_{external}$)), however, nitrate uptake is the only step contributing to the cellular-level isotopic discrimination. Below, isotope ratios for all experiments are reported in delta notation, in units of per mil (‰), relative to N_2 in air for $\delta^{15}N$ [$= ((R_{sample}/R_{air}) - 1) * 1000‰$, where $R = {}^{15}N/{}^{14}N$] and relative to Vienna Standard Mean Ocean Water (SMOW) for $\delta^{18}O$ [$= ((R_{sample}/R_{SMOW}) - 1) * 1000‰$, where $R = {}^{18}O/{}^{16}O$]. The denitrification N isotope effect, $^{15}\varepsilon$, is defined as $[(^{14}k/^{15}k) - 1] * 1000‰$, where ^{14}k and ^{15}k refer to the reduction rates of nitrate bearing ^{14}N and ^{15}N, respectively.

The present mechanistic understanding of fractionation during heterotrophic denitrification is guided by a large number of studies on algal nitrate assimilation and a smaller number of studies on heterotrophic respiratory denitrification. Altogether, these studies make a case for an "efflux model" such that that cellular-level N isotope effect during nitrate assimilation and denitrification originates largely from isotope fractionation during enzymatic nitrate reduction inside the cell. The expression of this fractionation outside the cell in the ambient medium depends on the rate of efflux of intracellular nitrate (relative to the rate of uptake or reduction), which transfers ^{14}N-depleted (or ^{15}N-enriched) internal nitrate back into the medium. Supporting observations from algal studies include: (1) high measured isotope effects for purified algal nitrate reductases (up to 30‰; (Ledgard et al., 1985; Schmidt and Medina, 1991; Karsh et al., 2012)) that are similar to the highest cellular-level isotope effects observed in both nitrate assimilators (Wada and Hattori, 1976) and denitrifiers (Wellman et al., 1968; Delwiche and Steyn, 1970); (2) elevation of the cellular-level isotope effect by inhibition of nitrate reductase activity with tungstate, which increases the ratio of nitrate efflux to reduction (Shearer et al., 1991); (3) elevation of $\delta^{15}N$ (and $\delta^{18}O$) of the large internal nitrate pool of algae relative to external nitrate, indicating that nitrate undergoes strong isotopic fractionation inside the cell (Granger et al., 2004; Needoba et al., 2004); (4) an inverse correlation between internal nitrate ^{15}N enrichment and the cellular-level isotope effect, indicating that expression of the isotope fractionation in the external medium is greater when efflux is more important (Needoba et al., 2004); (5) the same ratio

Table 1
Summary of measured $^{15}\varepsilon$ for denitrification in laboratory cultures and in environmental samples.

Experimental system	$^{15}\varepsilon$ (‰)	Reference	Notes on experimental conditions and methodology
Pseudomonas stutzeri	20–30	Wellman et al. (1968)	Complex organic medium, single batch culture, fractionation factor lower during exponential phase
Paracoccus denitrificans	13–20	Delwiche and Steyn (1970)	Glucose, no Rayleigh plots, instantaneous fractionation factor calculated for 5 resting batch cultures
Paracoccus denitrificans	28.6 ± 1.9	Barford et al. (1999)	Chemostat, acetate, starting nitrate 30 mM, CSNR of 50–250 $* 10^{-12}$ μmol cell^{-1} min^{-1}
Pseudomonas stutzeri	5.4–19.7	Granger et al. (2008)	Batch, starting nitrate 85–513 μM, complex organic medium, CSNR calculation not possible
Pseudomonas aureofaciens	16.9–23	Granger et al. (2008)	Batch, starting nitrate 95–315 μM, complex organic medium, CSNR calculation not possible
Paracoccus denitrificans	17.6–24.8	Granger et al. (2008)	Batch, starting nitrate 160–310 μM, complex organic medium, CSNR calculation not possible
Marine denitrifier	14–21	Miyake and Wada (1971)	Experimental conditions and methodology details not available, isotope analysis of product N_2
Freshwater denitrifier	1.9–12.3	Wada et al. (1975)	Experimental conditions and methodology details not available, isotope analysis of product N_2

of increase in $\delta^{18}O$ and $\delta^{15}N$ in internal nitrate and medium nitrate during nitrate assimilation, suggesting that nitrate isotope fractionation is driven by a single intracellular reaction (Granger et al., 2004); and (6) a similar increase in the ratio of $\delta^{18}O$-to-$\delta^{15}N$ (i.e., ~1.0) of nitrate during *in vitro* reduction by purified assimilatory nitrate reductase enzyme (Karsh et al., 2012) which matches the observed ratio of intracellular and medium $\delta^{15}N$ and $\delta^{18}O$ increase during *in vivo* nitrate assimilation. A similar mechanism for denitrifiers is encouraged by the following observations: (1) the $\delta^{18}O$-to-$\delta^{15}N$ ratio of change during denitrification is similar to that by nitrate assimilators (Granger et al., 2008; this study); and (2) the cellular-level isotope fractionation is lost when nitrate reduction by the cell approaches uptake limitation (e.g., when $[NO_3^-]_{external}$ approaches the half-saturation constant for the nitrate transporter of denitrifiers), indicating that transport itself is not a major driver of isotope fractionation (Granger et al., 2008; this study).

Thus, the basic "efflux model" framework for understanding the controls on $^{15}\varepsilon$, centered around the ratio of rate of nitrate efflux to uptake (which is indirectly related to the ratio of reduction to uptake as well), is likely to be appropriate for the bacterial denitrification isotope effect. However, physico-chemical environmental parameters that control the rates of nitrate uptake, efflux and reduction are not clear. As a result, this framework has not, in itself, yielded a predictive understanding of the environmental controls on $^{15}\varepsilon$.

The cellular level N isotope effect of denitrification is central to many applications of the N isotope systematics, in the ocean and in other environments. For example, the isotope-based constraint on the global marine fixed N budget depends strongly on the value of $^{15}\varepsilon$. Here, we examine variations in $^{15}\varepsilon$ through experiments with three pure cultures of denitrifying bacteria under changing internal physiological conditions and external environmental parameters; discuss the implications of our results for the value of $^{15}\varepsilon$ in the suboxic zones of the ocean water column; and briefly consider the potential implications of our findings for the global ocean's fixed N budget.

2. METHODS

2.1. Strains

We studied two facultative denitrifiers, *Pseudomonas chlororaphis* ATCC 43928 (Christensen and Tiedje, 1988) and *Paracoccus denitrificans* ATCC 19367 (Kelly et al., 2006) under freshwater growth conditions and *Marinobacter* sp. strain 23-5 under seawater growth conditions *P. denitrificans* strain ATCC 19367 was previously used by Granger et al. (2008) for examining $^{15}\varepsilon$. Further, 16S rRNA sequence comparison suggests that it is extremely closely related to the *P. denitrificans* type strain ATCC 17741 used by other groups (e.g., Barford et al., 1999) because the 16S sequences of two strains are 100% similar without any gap or mismatch. *Marinobacter* sp. strain 23-5 was isolated from the Arabian Sea oxygen deficient zone (ODZ) and has high identity at both 16S rRNA and *nirS* gene level with *Marinobacter aquaeolei*, representing a *nirS* clade found in the Arabian Sea and Eastern Tropical South Pacific ODZs (data not shown). This strain, a rod shaped bacterium 3–4 μm in length whose motility appears to be mediated by polar flagella, is the first ODZ strain to be investigated for $^{15}\varepsilon$.

2.2. Denitrification assays

2.2.1. Preparation of starter cultures

The inoculum for preparing the starter culture came from 3 to 5 days old solid medium plates streaked with glycerol stocks of the respective cultures. In turn, the starter cultures that were used for inoculating the assay reactors were grown at room temperature on a rotary shaker in rich liquid medium containing 2 mM potassium nitrate in either tryptic soy broth (for freshwater conditions) or yeast extract and bactopeptone-based medium (for seawater conditions) for 2–11 days (Table 2). These starter cultures were harvested after all added nitrate was consumed, but the time period spent in stationary phase (without the presence of nitrate) varied from 1 to 10 days. Inocula for the actual assay were prepared by centrifuging starter cultures to obtain pellets and re-suspending the pellets in the assay medium by gentle pipetting (see Section 2.2.3).

2.2.2. Assay (re-suspension) medium composition

We explored the effect of multiple factors on the extent of stable isotope fractionation during denitrification (Tables 2 and 3): the type of carbon (C) source (acetate, glucose, or complex organics as bactopeptone and casein extracts), the concentration of dissolved O_2 in the growth medium ($[O_2]$), and the starting nitrate concentrations in the assay medium ($[NO_3^-]_{external}$). For specific cultures, we also investigated the effect of the growth phase of the inoculum, the presence/absence of mechanical stirring in the assay reactors, and the timing of nitrate supply with respect to the timing of supply of electron donor and other nutrients (Table 2).

The composition of the freshwater assay media for *P. denitrificans* and *P. chlororaphis* were similar to the medium used earlier (Granger et al., 2008), with 2 g/L of either acetate, glucose or complex organic matter (bactopeptone and casein extract) as C sources. As opposed to acetate and glucose, which are both single C sources with well defined chemical structure and biochemical properties, bactopeptone and casein extract are composed of multiple poorly characterized organic compounds. The seawater assay medium for *Marinobacter* sp. consisted of filtered seawater amended with *f*/2 vitamins and AQUIL trace elements, and yeast extract and bactopeptone as C sources (Granger et al., 2008). For a given C source, the reactors assays were also subjected to two kinds of treatments with respect to dissolved O_2 (0 or 4 μM $[O_2]$) by purging the reactors with ultra pure N_2 gas stream with or without O_2. Starting $[NO_3^-]_{external}$ among assays was varied between 15 and 800 μM. All assay media included 1 mM ammonium to serve as a nutritional source of fixed nitrogen.

2.2.3. Reactor assembly and assay conditions

Denitrification assays were conducted in 1 L glass reactors that allowed for periodic sub-sampling from the

Table 2
Conditions varied for denitrifiers during assays to measure $^{15}\varepsilon$.

Conditions	*P. denitrificans*	*P. chlororaphis*	*Marinobacter* sp.
Medium	Freshwater	Freshwater	Seawater
Complex organic C source	Yes	Yes	Yes
Acetate as C source	Yes	Yes	No growth
Glucose as C source	Yes	Yes	No growth
Dissolved O_2 concentration	0 vs. 4 μM	0 vs. 4 μM	0 vs. 4 μM
Mechanical stirring	Yes	Yes	Yes ("standard" assays) vs. No
Age of starter culture (days)	⩾4	⩾4 ("standard" assays) vs. <2–3	4
Nutrient re-amendment	Only for non-standard assays	No	No

Table 3
Parameters affecting with $^{15}\varepsilon$ of denitrification.

Parameter	Condition simulated by its decrease absence
Cell specific nitrate reduction	Absence of optimum growth conditions
Nitrate concentration	Low nitrate concentration in the ocean
(Presence of O_2)	Margins of oxygen minimum zones
Presence of mixing	Stagnation of water masses
Nutrient re-amendments	Organic matter limitation
Energy availability (reducing power of C source)	Organic matter limitation
Age of denitrifying cells (time spent in stationary phase)	Denitrifier bloom

reactor. As described previously, 500–600 ml of autoclaved medium components (except as noted below) were assembled aerobically in a pre-sterilized 1 L reactor, and the assay medium was bubbled with N_2 for 45 min to purge any dissolved O_2 (Kritee et al., 2008). To initiate the assays, cell pellets from starter cultures were re-suspended in the assay medium and were also bubbled with N_2 for 45 min. Bubbled anaerobic inocula (~50 ml) thus prepared were amended with the desired amounts of filter-sterilized nitrate and C source and were anaerobically pumped into the main reactors to start the assays. Assay cultures were bubbled continually with ultra pure N_2 (or 4 μM [O_2] in N_2) under constant stirring. During the assays, $[NO_3^-]_{external}$ was monitored closely, and 5–10 samples were collected periodically as nitrate was consumed over typically a 3–9 h period.

2.2.4. Species-specific denitrification assays

2.2.4.1. Nitrate re-amendments for P. denitrificans. An intermediate nitrite pool did not accumulate from nitrate reduction in *P. denitrificans*, which allowed us to investigate the effect of re-amending nitrate with or without all other nutrients including reduced carbon source, ammonium, vitamins and trace metals upon depletion of the original amendment of nitrate. The experiments with nitrate amendment alone simulate organic matter limitation (Table 3). In the other two strains, it took too long to complete one cycle of reduction of both nitrate and nitrite, so the effect of similar re-amendments could not be investigated. To discern the difference between first (initial) and second amendment of nitrate, "standard" assay conditions for *P. denitrificans* are intended to identify the first cycle of nitrate reduction in the assay reactors, irrespective of the age of the starter culture (Table 2).

2.2.4.2. Impact of inoculum age for P. chlororaphis. Unlike the *P. denitrificans* experiments, our first set of *P. chlororaphis* experiments indicated that $^{15}\varepsilon$ was sensitive to the number of days for which the starter culture was allowed to grow. This led us to undertake an explicit test of the effect of the age (i.e., growth phase) of the inoculum on $^{15}\varepsilon$ during denitrification by *P. chlororaphis*. In this case, "standard" assay conditions for the *P. chlororaphis* strain correspond to use of starter cultures grown for 4–11 days, the first cycle of nitrate reduction in the presence of mechanical stirring. These 'standard' assays were compared with assays where starter cultures were grown for only 2–3 days (and were hence closer to "bloom" phase, Table 3). For *P. chlororaphis*, rapid re-amendment of nitrate was not investigated because of the presence of residual nitrite, and unstirred conditions were also not investigated (Table 2).

2.2.4.3. Impact of mechanical stirring for Marinobacter sp.. For *Marinobacter* sp. strain 23-5, the "standard" assay conditions correspond to use of exactly 4 day old starter cultures and the first cycle of nitrate reduction in the assay reactors, in the presence of mechanical stirring. We incidentally discovered that the complete absence of mechanical stirring decreases $^{15}\varepsilon$ during denitrification, and follow-up experiments were performed to confirm this finding. It is possible that these experiments are relevant to conditions in the ODZs, the waters in which may not be continuously turbulent (Table 3). Starter culture age was kept constant at 4 days (~2 days after complete nitrate reduction), and greater culture ages were not explored (Table 2).

2.3. Sample processing

Assay sub-samples were passed through two 0.2 μm pore-size PTFE syringe filters and were then treated with sulfamic acid to remove nitrite (NO_2^-) and neutralized with NaOH (Granger and Sigman, 2009) before being frozen for

nitrate isotopic analysis within 1–7 days. Viable cell counts (colony forming units (CFU)/ml) were made by dilution plating on tryptic soy agar-containing solid medium (Kritee et al., 2007).

2.4. Nitrate concentration and isotopic analyses

The combined $[NO_3^-]_{external}$ and $[NO_2^-]_{external}$ before the addition of sulfamic acid or $[NO_3^-]_{external}$ after addition of sulfamic acid in the growth media were measured by conversion to NO followed by chemiluminescence detection with a Teledyne NO analyzer (Braman and Hendrix, 1989). The N and O isotopic composition of NO_3^- remaining in the reactors was determined using the denitrifier method (Sigman et al., 2001; Casciotti et al., 2002).

We also calculated the O-to-N isotope effect ratio ($\Delta\delta^{18}O/\Delta\delta^{15}N$) in remaining nitrate, where Δ represents the difference between the isotopic composition ($\delta^{18}O$ or $\delta^{15}N$) at a given point minus the isotopic composition of the medium at the beginning of the assay. The N isotope effect of a given process, ${}^{15}\varepsilon_x$, is defined as $[({}^{14}k/{}^{15}k) - 1] * 1000‰$, where ${}^{14}k$ and ${}^{15}k$ refer to the intrinsic rates of processing of nitrate bearing ^{14}N and ^{15}N, respectively; x refers to the specific process, be it nitrate reduction by respiratory nitrate reductase (NaR), nitrate uptake (uptake), or nitrate efflux (efflux). When ${}^{15}\varepsilon$ lacks any subscript, it refers to the cellular level isotope effect of denitrification, that is, the isotope effect that applies to the disappearance of nitrate from the external medium. The N isotope fractionation factor for nitrate reduction ($\alpha_{15/14} = R_{reactant}/R_{product}$; "product" refers to instantaneous product) was calculated from the corresponding slope of the Rayleigh plot [$\ln(R_{reactant}/R_{reactant\ at\ time\ zero})$ vs. $\ln(f)$] and converted to the isotope effect, ${}^{15}\varepsilon$ (where f is the fraction of nitrate remaining in the reactor and ${}^{15}\varepsilon = (\alpha_{15/14} - 1) * 1000‰$). The linear regressions and errors associated with fractionation factors were determined using the York method (York, 1966) in which each data point is weighted according to its uncertainty in both X and Y.

3. RESULTS

During all assays, viable cell number and the nitrate reduction rate remained constant (e.g., Fig. EA1, Electronic Annex), and as the experiments progressed, the reactant nitrate remaining in the reactor increased in d15N and d180 and followed Rayleigh fractionation (e.g., Fig. EA2, Electronic Annex). The range of values for cellular-level isotope effect for denitrification (${}^{15}\varepsilon$) observed for the assays with the three heterotrophic bacterial denitrifiers was quite large (Fig 1a). ${}^{15}\varepsilon$ for *P. denitrificans* ranged between 9.3‰ and 22.3‰ and for *P. chlororaphis* between 11‰ and 29.5‰. *Marinobacter* sp. strain 23-5 also showed a smaller but substantial range for ${}^{15}\varepsilon$, 14.8–22.8‰.

For all three species, a common pattern is that the ${}^{15}\varepsilon$ of denitrification is smaller when the rate at which a single cell reduces nitrate (the "cell specific nitrate reduction" (CSNR) rate) is low (see also Electronic Annex Section EA1.1). This pattern is especially clear when only the results of assays done under "standard" conditions (i.e., assays inoculated with starter cultures $\geqslant$4 days old and undergoing the initial round of nitrate reduction under mechanically stirred conditions) for each of the three species are examined (Table 2 and Fig. 1b). Lower CSNR rates tended to correspond with a less reduced carbon (C) source and higher $[O_2]$, though not necessarily among all three strains and/or under all conditions. *P. denitrificans* clearly showed slower CSNR and correspondingly lower ${}^{15}\varepsilon$ when the assay medium consisted of glucose or acetate as C sources as compared to medium with complex organic constituents. *P. chlororaphis*, though only assayed with glucose and acetate as C sources, showed a broader range of both CSNR ($1–1000 * 10^{-12}$ compared to $0.2–17 * 10^{-12}$ μmol cell^{-1} min^{-1}) and ${}^{15}\varepsilon$ (11–29.5‰ compared to 9.3–22.3‰) than *P. denitrificans*. Whether complex organic substrates would yield even higher CSNR could not be determined because addition of organic constituents increased the propensity of *P. chlororaphis* cells to flocculate, precluding the enumeration of viable cells (and thus the determination of CSNR rate). Assays of *Marinobacter* sp. were conducted only with complex organic constituents because the strain did not grow in any investigated defined medium containing glucose, acetate, or lactate as sole carbon sources.

The presence of oxygen resulted in slower CSNR in *P. denitrificans* experiments, and correspondingly lower isotope effects (Fig. 1b). For *P. chlororaphis,* the presence of oxygen tended to correspond to slower CSNR as compared to corresponding no-oxygen treatment on that particular day, but it was not systematically associated with a lower ${}^{15}\varepsilon$ among days of experiments. In the case of the *Marinobacter* strain, higher dissolved oxygen did not necessarily yield lower CNSR (Fig. 1b). The incidence of lower ${}^{15}\varepsilon$ was nevertheless associated with lower CNSR rate for this marine denitrifier, with a sharp decline in ${}^{15}\varepsilon$ between CSNR rates of 13 and $10 * 10^{-12}$ μmol cell^{-1} min^{-1}, although the factors underlying the variation in CSNR are not evident.

Interestingly, when *P. denitrificans* cells were assayed for ${}^{15}\varepsilon$ during a second cycle of nitrate reduction following a re-amendment of nitrate to the assay medium, the isotope effect and CSNR appeared dependent on whether other non-nitrate nutrients were re-amended as well. A re-amendment of nutrients and vitamins along with nitrate to spent assay medium tended keep the CSNR and ${}^{15}\varepsilon$ similar to the values for the first "standard" cycle, whereas re-amendment of only nitrate resulted in a smaller isotope effect and a slower CSNR (Fig. 2a). The association of the decrease in ${}^{15}\varepsilon$ associated with slower CSNR is qualitatively consistent with the other findings, but the resulting relationship between CSNR and ${}^{15}\varepsilon$ is different from that seen in the initial nitrate amendment assays (Fig. 2a).

While variation of C source did not affect ${}^{15}\varepsilon$ for *P. chlororaphis* assays with the starter cultures in their late stationary phase (i.e., under "standard" assay conditions, Fig. 1b), ${}^{15}\varepsilon$ did show sensitivity to carbon source as seen for *P. denitrificans* when assays were done with younger starter cultures: lower (${}^{15}\varepsilon \leqslant 15‰$) occurred with acetate, a carbon source more reduced than glucose (~24‰) (Fig 2b). Another condition that resulted in lower ${}^{15}\varepsilon$ in the *Marinobacter* sp. assays was the absence of mechanical stirring in the assay reactors (Fig. 2c).

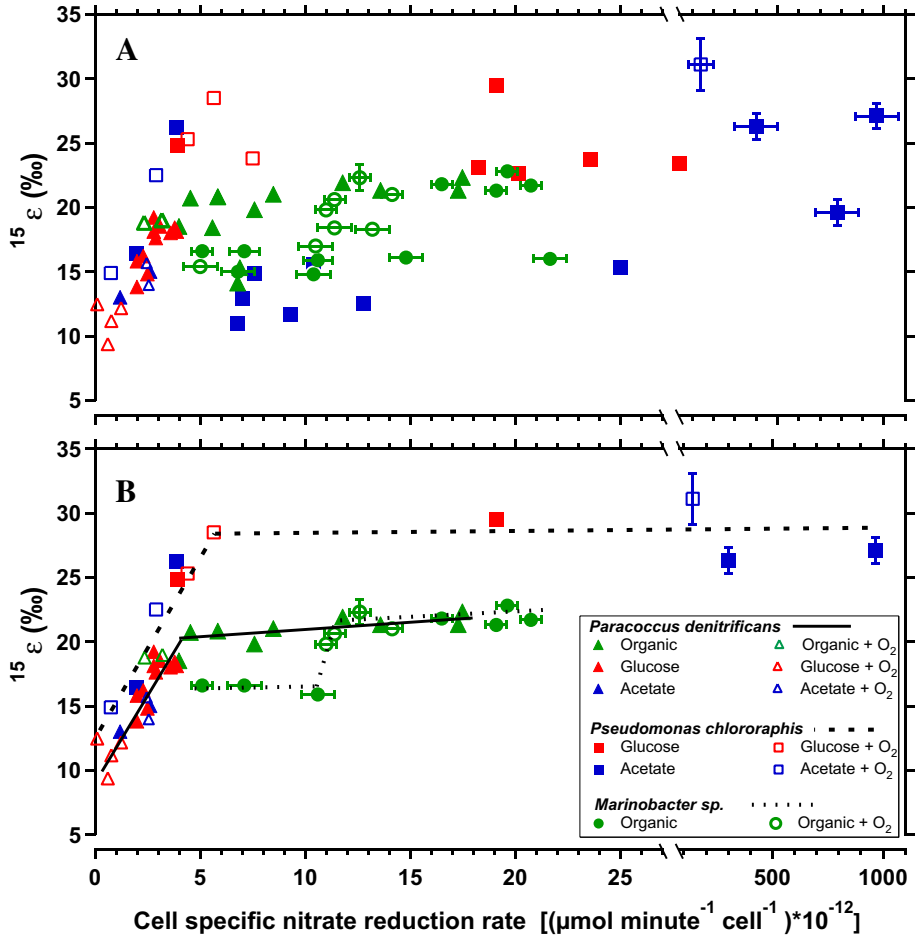

Fig. 1. N isotope effect ($^{15}\varepsilon$) vs. cell specific nitrate reduction (CSNR) rate during denitrification by *P. denitrificans*, *P. chlororaphis* and *Marinobacter* sp. 23-5 under all investigated conditions (A) and "standard" assay conditions for each strain (B). Where shown, error bars are equal to ±1SD. For most cases, 1SD is $<\pm 0.2‰$ for ε and $<\pm 0.2 * 10^{-12}$ µmol cell^{-1} min^{-1} for CSNR rate, both smaller than the symbol size.

For *P. chlororaphis* (Fig. 2b) and *Marinobacter* sp. (Fig. 2c), initial [NO_3^-] did not yield systematic differences in $^{15}\varepsilon$. Since all the assays of *P. denitrificans* with the highest initial [NO_3^-] were also conducted with organic constituents in assay (re-suspension) media that corresponded to the highest CSNR rates for *P. denitrificans*, it is not clear if initial [NO_3^-] affected $^{15}\varepsilon$ in this strain (Fig. 2a).

With respect to the decreasing [NO_3^-] during individual assays, for all three species, when [NO_3^-]$_{external}$ approached 35 µM or less, δ^{15}N for the nitrate remaining in the medium began to fall below the values expected from a constant $^{15}\varepsilon$ (Fig. 3).

In spite of large variations in $^{15}\varepsilon$ and CSNR rate in our study, the O-to-N isotope effect ratio ($\Delta\delta$18O/$\Delta\delta$15 N in remaining nitrate) did not deviate systematically from ~0.9 and the ratio of $^{18}\varepsilon/^{15}\varepsilon$ was 0.93 ± 0.06 (1SD) (Fig. 4). Similarly, in individual assays, the plateau in δ^{15}N as [NO_3^-] fell below 35 µM (Fig. 3) also applied to δ^{18}O.

4. DISCUSSION

Our results from 80 assays (33 for *P. denitrificans,* 27 for *P. chlororaphis*, 21 for *Marinobacter* sp. strain 23-5) represent the largest integrated set of culture estimates reported for $^{15}\varepsilon$ so far. The amplitude of $^{15}\varepsilon$ for each experiment was based on plotting at least five data points in Rayleigh space (e.g., Fig. EA2). We note that several of the earliest denitrifier studies sampled only the product (N_2) (Wellman et al., 1968), and/or the calculation of $^{15}\varepsilon$ was based on only two samples of the reactant (NO_3^-) (Delwiche and Steyn, 1970) (Table 1). There is some previous culture evidence for $^{15}\varepsilon$ significantly lower than 20–30‰ (Miyake and Wada, 1971; Wada et al., 1975) and for changes in $^{15}\varepsilon$ values for a single species with growth phase (Wellman et al., 1968) or other physiological variables (Wada and Hattori, 1978), although experimental conditions were not specified rigorously. We found larger ranges of $^{15}\varepsilon$ for *P. chlororaphis* (9.3–22.3‰) and *P. denitrificans* (11–31‰) than previously reported (see Table 1 and (Barford et al., 1999; Granger et al., 2008) and references therein). *Marinobacter* sp. strain 23-5, the first ODZ strain to be investigated for its denitrification isotope effect, also showed a substantial range for $^{15}\varepsilon$ (14.8–22.8 ‰).

Below, we examine in detail the conditions (Tables 2 and 3) that systematically and directly lead to $^{15}\varepsilon$ values lower

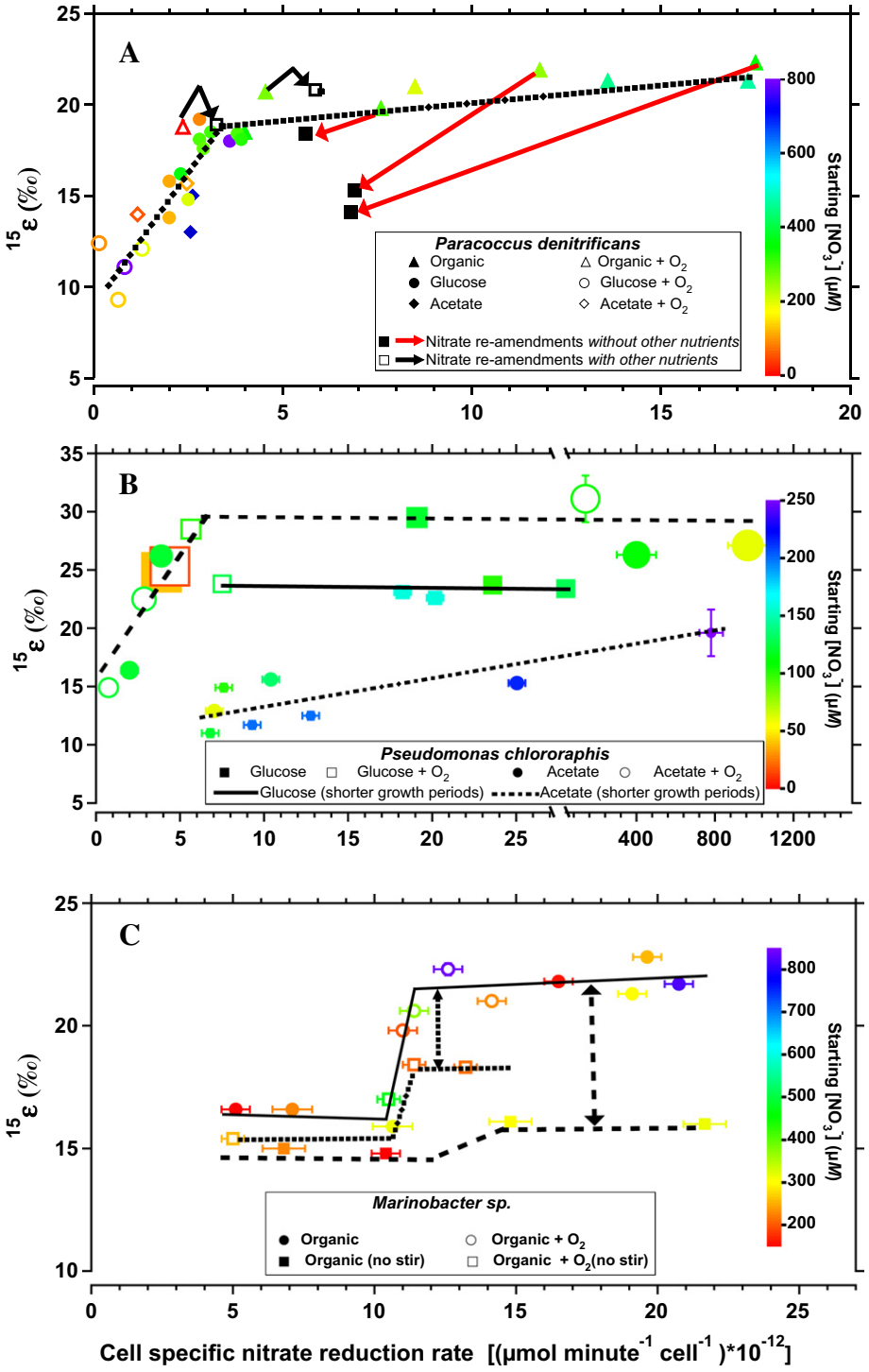

Fig. 2. N isotope effect ($^{15}\varepsilon$) vs. cell specific nitrate reduction (CSNR) rate during denitrification by *P. denitrificans* as a function of the starting $[NO_3^-]_{external}$ and in nitrate re-amendments to the assay medium (A); by *P. chlororaphis* as a function of the starting $[NO_3^-]_{external}$ in the assay medium. (B, symbol sizes vary with the length of time for which bacterial inoculum was grown before being harvested for the assay); by *Marinobater* sp. Libra as a function of the starting $[NO_3^-]_{external}$ and mixing conditions in the assay medium. (C, all experiments were with inoculum grown for 4 days).

than the canonical value of 25‰. In the light of "efflux model", we infer the effect of CSNR rate and of other examined culture conditions on the relative rates of nitrate uptake, efflux and reduction.

4.1. Efflux model for respiratory nitrate reduction

A simplified formulation of nitrate reduction (Shearer et al., 1991) illustrates that enzymatic bond breakage is

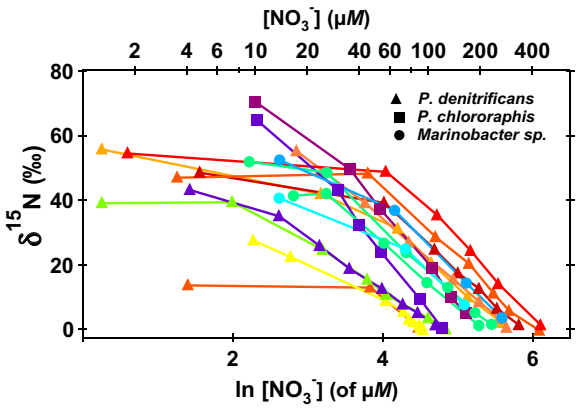

Fig. 3. Rayleigh plots of the change in the N isotopic composition of nitrate ($\delta^{15}N$) as a function of the natural logarithm of $[NO_3^-]_{external}$ to highlight the asymptotic behavior of nitrate N isotopic fractionation at lower $[NO_3^-]_{external}$.

the first unidirectional step in the reaction sequence, associated with reaction rate constant k_5 in the reaction sequence below:

$$NO_3^-{}_{out} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} NO_3^-{}_{in} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} NO_3^- \cdot NaR \xrightarrow{k_5} NO_2^- \cdot NaR \qquad (1)$$

where k_1 and k_2 are the rate constants for nitrate uptake into the cell and the efflux from the cell, respectively; NaR is the intracellular respiratory nitrate reductase; and k_3, and k_4 are rate constants for the enzyme-substrate binding and enzyme-substrate dissociation, respectively. Fractionation of the N and O isotopes of nitrate occurs at the enzymatic step because the rate of N–O bond breakage is slower for the heavier isotopologues of nitrate (i.e., ${}^{15}k_5 < {}^{14}k_5$ and ${}^{18}k_5 < {}^{16}k_5$). Fractionation during the uptake and efflux steps is taken to be minimal because of the lack of bond breakage, an assumption to which we will return below.

It is assumed that the binding and unbinding of substrate at the enzyme involve minimal isotope fractionation (Bryan, Shearer, Skeeters and Kohi, 1983). If so, considering the overall enzymatic mechanism, the isotope fractionation factor observed for the enzymatic reaction, α_{NaR}, is a function of the intrinsic isotope fractionation factor associated with the irreversible, isotopically-sensitive step (N–O bond rupture) $\alpha_5 = {}^{14}k_5/{}^{15}k_5$ and the extent to which this enzymatic step is rate-limiting (O'Leary, 1980):

$$\alpha_{NaR} = \frac{\alpha_5 + \frac{k_5}{k_4}}{\frac{k_5}{k_4}} \qquad (2)$$

The rate limitation of the enzymatic step is measured as the ratio k_5/k_4 and is termed the "commitment to catalysis." As the commitment to catalysis increases, the enzyme level isotope effect is expected to decline, as the binding of nitrate begins to become the first irreversible step; it is the subsequent reduction step that imparts the isotope fractionation. The isotope fractionation factor manifest at the cellular-level, $\alpha_{cellular\text{-}level}$, then depends on fractionation imposed by enzymatic catalysis, α_{NaR}, and upon the rates of the uptake and efflux, as has been demonstrated for N isotopes during nitrate assimilation (Shearer et al., 1991) and for C isotopes during CO_2 fixation in plants (Farquhar et al., 1982) and in algae (Francois et al., 1993):

$${}^{15}\varepsilon_{cellular\text{-}level} = E/U * ({}^{15}\varepsilon_{NaR}) \qquad (3)$$

where E/U f is the relative proportion of nitrate efflux to gross uptake E/U.

The absence of fractionation at lower $[NO_3^-]$, when transport becomes the rate-determining step in denitrification (Fig. 4), argues that the isotope effect of nitrate uptake among denitrifiers is trivial (Granger et al., 2008). The conserved O-to-N isotope relationship of ~0.9 across the experiments is consistent with the interpretation that fractionation of the N and O isotopes of nitrate is imparted by a single process, namely internal enzymatic reduction by the respiratory NaR (Granger et al., 2004, 2008; Karsh et al., 2012; this study). This implies that the variations in the amplitude of ${}^{15}\varepsilon$ are largely driven by the proportional

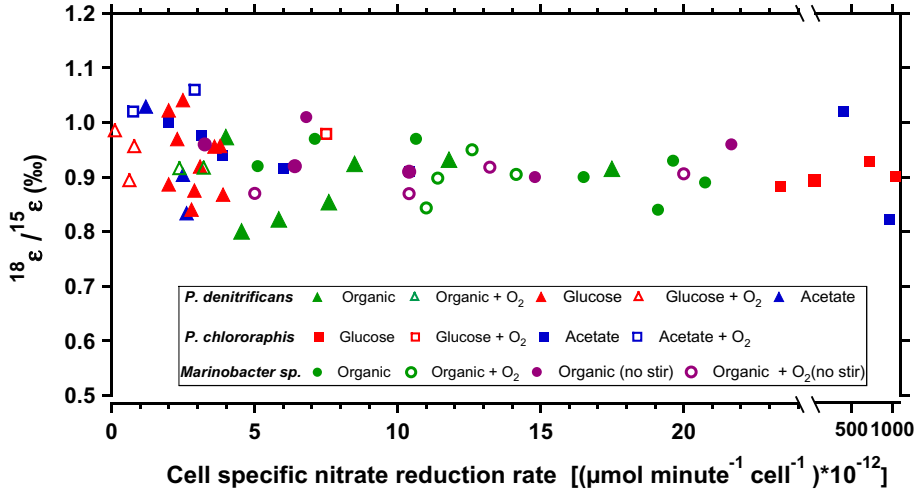

Fig. 4. The ratio of ${}^{18}\varepsilon$ to ${}^{15}\varepsilon$ vs. CSNR rate during denitrification by *P. denitrificans*, *P. chlororaphis* and *Marinobacter* sp. 23-5 under all examined assay conditions.

importance of cellular nitrate efflux relative to uptake. Thus, the efflux-to-uptake ratio is the focus of our discussion below.

4.2. Cell specific nitrate reduction (CSNR)

A salient trend in our results is that $^{15}\varepsilon$ tended to decrease with CSNR rate (Fig. 1). Slower CSNR resulted partly from distinct assay conditions, including a less reduced carbon source and oxygenation. In some cases, however, decreases in CSNR rates do not explicitly appear to be the result of purposeful manipulations in the assay conditions. Regardless, the results argue for a lowering of the isotope effect associated with a decrease in the CSNR rate. These results were not anticipated: in studies of microbial sulfate reduction (Sim et al., 2011) and methanogenesis (Penning et al., 2005), lower cell specific substrate reduction/consumption rates were associated with higher, not lower, sulfur (S) and carbon (C) isotope fractionation (see Section EA1.2 in Electronic Annex for detailed comparison of S and N isotope studies).

In considering the basic steady state relationship between efflux and reduction rates (uptake = efflux + reduction), it is clear that a decrease in CSNR indicates a slower rate of nitrate reduction, which, in isolation (i.e., with uptake rate remaining constant), would tend to raise nitrate efflux and thus increase $^{15}\varepsilon$. Given this decline in absolute nitrate reduction rate, the efflux model *requires* that the rate of nitrate uptake must decrease even more than the rate of reduction, so as to yield a net reduction in the ratio of nitrate efflux to uptake. In other words, nitrate uptake relative to nitrate reduction must be maximized at elevated CSNR rates, resulting in an increase in the relative rate of nitrate efflux (Fig. 5). Thus, high efflux resulting from rapid nitrate uptake causes the intracellular nitrate reductase isotope effect ($^{15}\varepsilon_{NaR}$) to be nearly fully expressed in the medium (Fig. 5a). Previous studies with denitrifying bacteria have most often observed $^{15}\varepsilon$ to be 20–30‰ ((Barford et al., 1999; Granger et al., 2008) and references therein), which is the range seen in our experiments when CSNR rates are high (Fig. 1). We suggest that this range for $^{15}\varepsilon$ applies under optimal conditions for cellular energy generation by respiratory nitrate reduction, which laboratory studies typically seek to provide. Conversely, when CSNR rate is lower and more typical of environmental rates (Fig. 5b), a smaller proportion of nitrate uptake leads to efflux and the efflux/uptake ratio is smaller. Consequently, $^{15}\varepsilon_{NaR}$ is poorly expressed outside the cell, and thus $^{15}\varepsilon$ is lower.

4.2.1. Energy dependence of nitrate uptake, efflux and reduction

We propose that the relationship between CSNR rate and $^{15}\varepsilon$ is the result of a high sensitivity of nitrate uptake to the energy generated by respiratory nitrate reduction. A question that arises is whether this proposition is consistent with other information on the relative dependencies of nitrate uptake, reduction, and efflux on the energy obtained from respiratory nitrate reduction. We do not call upon efflux to be directly controlled by CSNR; we take it to be due

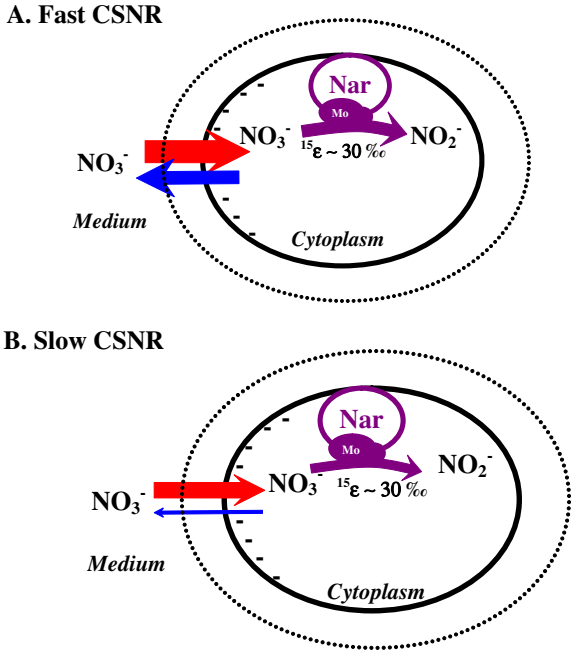

Fig. 5. Model depicting relative rates (arrow thickness) and proposed relative dependence of nitrate uptake, efflux and reduction on energy derived from denitrification (color). Warmer colors represent higher relative dependence on energy obtained from respiratory nitrate reduction such that nitrate uptake (red) requires more energy as compared to nitrate reduction (purple). At faster CSNR (a), uptake is much higher than reduction rate and thus the ratio of rates of efflux to uptake is higher than at slower CSNR (b). Higher efflux leads to higher expression of enzymatic isotope effect outside the cell. Efflux (blue) is not affected by CSNR, being dictated by intracellular [NO_3^-] and other constraints on its passive diffusion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to passive diffusion, driven by the intracellular [NO_3^-] and the favorable electrochemical gradient (pK_a of HNO_3 is −1.3 and cytoplasmic membrane potential is ~180 mV and negative inside). Rather, we focus on the uptake and reduction of nitrate, which are directly susceptible to cellular energetics. Nitrate reduction is obviously energy-dependent, as energy is expended in the biosynthesis of the enzyme nitrate reductase (NaR), the provision of cofactors based on molybdenum (the transport of which is energy intensive (Berks et al., 1995; Zumft, 1997)), and the supply of cellular reductants (e.g., NADH). Nitrate transport into the cell is necessarily also an energy dependent process as well, as the nitrate anion must be moved against the strong proton chemical gradient. Indeed, in the absence of active nitrate transport, passive diffusion out of the cell limits intracellular [NO_3^-] to 0.1% of its external concentration (Goddard et al., 2008). Additionally, nitrate uptake is tightly coupled with intracellular nitrate reduction (Rowe et al., 1994) because maximum catalysis by nitrate reductase requires saturating intracellular [NO_3^-] in the range of 300–3800 μM (Zumft, 1997). Hence, nitrate uptake requires the energy and electrochemical gradients derived from denitrification (Boogerd et al., 1983; Incharoensakdi and Laloknam, 2005; Kucera, 2005; Goddard et al., 2008)

and is in turn critical for nitrate reduction (Rowe et al., 1994) and hence energy generation by this respiratory process. Nitrate uptake is proposed to occur via either nitrate/H^+ symporters and/or nitrate/nitrite antiporters; and the energy allotted to nitrate transport is implicit in nitrite production in the case of nitrate/nitrite antiporters (Rowe et al., 1994; Sharma et al., 2006) or in the form of the proton gradient in the case of nitrate/H^+ symporters (Wood et al., 2002; Kucera, 2005; Goddard et al., 2008).

The efflux model implies that the rate of nitrate transport is more dependent on the energy generated by respiratory nitrate reduction than is nitrate reduction, making the former more sensitive to variation in CSNR. Thus, it follows that during faster CSNR, cells maintain a high intracellular [NO_3^-] by maximizing nitrate uptake, the high rate of which is afforded by the energy generated from respiratory nitrate reduction. At slower CSNR, the energy allotted to nitrate transport is diminished relative to total cellular energetic demands, such that intra-cellular [NO_3^-] is reduced. We infer that at low CSNR rates, the uptake rate is lowered to more closely match the nitrate reduction rate, such that uptake processes do not re-consume a disproportionately large fraction of the energy produced by nitrate reduction.

While the relevant studies have not yet occurred to test if nitrate transport is indeed more dependent on energy generated by respiratory nitrate reduction and hence is more sensitive to variation in CSNR than is nitrate reduction, our proposition does have support. The difference in the half saturation constants (K_m) for nitrate uptake and nitrate reduction specifically for *P. denitrificans* (5 μM (Parsonage et al., 1985) vs. 300 μM (Craske and Ferguson, 1986)) and in general the known range for denitrifiers (2–18 μM (Murray et al., 1989) vs. 300–3800 μM (Zumft, 1997)) indicate that the accumulation of intracellular nitrate is a strategy for enhancing CSNR rates. Indeed, it is a common metabolic strategy to regulate uptake and concentrate substrate intracellularly, rather than expending cellular energy on enzyme biosynthesis and function, especially in field conditions (Button, 1985). However, such a substrate accumulation strategy is likely to be tightly modulated by the energetic yield and favorability of nitrate respiration. This explains why the nitrate uptake step in denitrification is tightly regulated by environmental conditions (e.g., [NO_3^-] and [O_2]) (Berks et al., 1995; Zumft, 1997; Moir and Wood, 2001). One demonstrated biochemical mechanism for the influence of CSNR on nitrate uptake rate lies in the dependence of nitrate uptake on the redox state of the quinone pool within the respiratory chain (Alefounder et al., 1983; Denis et al., 1990; Moir and Wood, 2001; Sohaskey, 2005), with nitrate uptake occurring only when the quinone pool is significantly reduced. A decrease in reduced state of the quinine pool (e.g., due to a decrease in the reducing power of the C substrate and thus CSNR rate) will leave the quinone pool more oxidized (Soballe and Poole, 1999), thereby decreasing nitrate uptake and thus possibly also the ratio of efflux to uptake.

4.2.2. Influence of CSNR rate on $^{15}\varepsilon_{NaR}$

The quantitative framework provided above (Eqs. (1)–(3)) illustrates the possibility that the amplitude of the enzyme level isotope effect ($^{15}\varepsilon_{NaR}$) could also vary, especially given that the enzymatic commitment to catalysis in some enzymes has been shown to change in response to changes in the rate of enzyme-specific activity. Bryan et al. (1983) showed that a slower enzymatic nitrite reduction rate result in a higher enzymatic isotope effect for NaR. By analogy, if the commitment to catalysis of NaR could change, then slower CSNR might be expected to reduce the commitment to catalysis and thus raise $^{15}\varepsilon_{NaR}$ (Eq. (2)), in the opposite sense as required to explain our results (see Section EA1.3 in Electronic Annex for more detailed discussion). Indeed, data for nitrate assimilation argues against modulations of $^{15}\varepsilon_{NaR}$ playing a major role in the expression of the cellular-level isotope effect (Needoba et al., 2004; Karsh et al., 2012). Our interpretation is also consistent with a sequence of catalytic steps followed by NaR enzymes that would not allow for variations in the commitment to catalysis (Campbell, 2001). Given these lines of evidence, modulation of $^{15}\varepsilon_{NaR}$ is probably not the cause of the reduced cellular level isotope effects observed at lower CSNR rates.

4.3. Dependence of $^{15}\varepsilon$ on culture conditions

Denitrification is highly regulated according to environmental conditions. In the transcription of genes involved in the transport and reduction of different N species, denitrifiers use multiple promoters that are regulated by diverse environmental variables; this is interpreted as a means of adjusting gene expression in response to the varying environmental concentrations of O_2, N species, trace metals and reductants (Zumft, 1997). Thus, the relative ratio of nitrate efflux to uptake is expected to vary in response to many physiological variables, and it is not surprising that our assays show that a low CSNR rate is not the only condition that can lower $^{15}\varepsilon$. We have identified a number of assay conditions that can decrease $^{15}\varepsilon$ below the canonical value of 25‰ at even a high CSNR rate (Table 3). In contrast, there is no evidence that, at a low CSNR rate, $^{15}\varepsilon$ can be raised back closer to the canonical value of 25‰. This is consistent with the interpretation that, if energy generation by denitrification is compromised, denitrifiers will reduce the energetically costly process of intracellular nitrate accumulation.

4.3.1. Nitrate concentration and uptake limitation

Starting $[NO_3^-]_{external}$ in the range of 15–800 μM seems to have no effect on $^{15}\varepsilon$ (Fig. 2), which is perhaps not surprising in that the nitrate transport is in all likelihood saturated at these starting $[NO_3^-]_{external}$ [K_m for nitrate uptake is 2–18 μM (Parsonage et al., 1985; Murray et al., 1989)]. Results from experiments with all three species do, however, show that, for assays starting at high concentrations, $^{15}\varepsilon$ decreases when the $[NO_3^-]_{external}$ in the medium falls in the range of 2–35 μM (Fig. 3). At low $[NO_3^-]_{external}$, we suggest that nitrate uptake, a step thought to cause negligible N isotope fractionation (Shearer et al., 1991; Granger et al., 2008), begins to become the rate limiting and first-irreversible step in the overall process of cellular nitrate reduction (Eq. (1)). This pattern aligns with the

observations made for S isotope fractionation by sulfate reducing bacteria. Similar to our results, it has been demonstrated that a suppression of the S isotope effect occurs when sulfate concentrations become limiting (Habicht et al., 2002).

For a denitrifier species with given genetic/biochemical properties associated with nitrate uptake (i.e., given values of enzyme turnover number (k_{cat}) and half saturation constant (K_m)), uptake rate will depend not only on the [NO_3^-] in the medium but also on the total density of nitrate transporters (uptake rate = [transporters] * k_{cat} * [NO_3^-]/(K_m + [NO_3^-])). Previous studies have suggested that $^{15}\varepsilon$ decreases when [NO_3^-] in the medium approaches the K_m of nitrate uptake (Lehmann et al., 2007; Granger et al., 2008). The K_m values for nitrate uptake should be fixed for a given species. However, the relative concentration of transporters and NaR molecules per cell will also have a significant influence on when uptake becomes rate-limiting. The [NO_3^-] at which fractionation is suppressed varies, and the assays with higher starting [NO_3^-] tend to be associated with a suppression in fractionation at [NO_3^-] higher than those with lower starting [NO_3^-] (Fig. 3). This may occur because higher starting [NO_3^-]$_{external}$ induces the production of a higher concentration of nitrate reductase molecules per cell, leading to earlier onset of uptake limitation for the cellular denitrification (see Section EA1.4 in Electronic Annex for more details).

4.3.2. Carbon and nutrient conditions

Nutrient conditions, especially the reducing power of the carbon source, have a significant effect on $^{15}\varepsilon$. Lower $^{15}\varepsilon$ values were obtained during assays done with less thermodynamically favorable carbon sources (glucose vs. acetate) for *P. denitrificans* (Figs. 2a and 1b) and *P. chlororaphis* (Fig. 2b). These results once again suggest that the energy dependence of nitrate uptake is greater than that of nitrate reduction (see Section 4.2.1. above). Consistent with our results, Wunderlich et al. (2012) found that $^{15}\varepsilon$ values for two different denitrifiers were 4–6‰ lower when toluene or benzoate was used as a C source as opposed to acetate. They proposed that this decrease in $^{15}\varepsilon$ was due to the adverse effect of toluene and benzoate on cell membrane fluidity and/or their positive effect on the intracellular concentration of NaR. This would lead to a decrease in the uptake rate or an increase in the ratio of reduction to efflux, both of which cause decrease in the efflux-to-uptake ratio. Unlike our assays where cell concentrations remained constant, Wunderlich et al. (2012) did their study under typical batch conditions where cell concentrations increased during the experiments and the range of the observed CSNR rates were 100–1000 fold more than the rates at which we observe lower $^{15}\varepsilon$ values. Thus, they found no significant effect of the kinetic or thermodynamic favorability of C substrates on the CSNR rates (Wunderlich et al., 2012).

The *P. denitrificans* nitrate re-amendment experiments which utilized complex organic matter as a C source show that reduction of re-amended nitrate (following complete removal of initially added nitrate and nitrite produced during the first cycle) leads to expression of lower $^{15}\varepsilon$ in the medium when the re-amendment does not contain other nutrients (Fig. 2a). To explain these results, we once again propose a nutrient/energy limitation-driven decrease in nitrate uptake, associated with a smaller decrease in the reduction rate, such that the efflux-to-uptake ratio decline. Although complex organic compounds used in our study (bactopeptone and casein extract) are a "rich" mixture of multiple organic sources of C, it is plausible that the favorable carbon sources are used in the first cycle. Depletion of readily consumable (kinetically favorable) and/or more reduced carbon (thermodynamically favorable) source in the medium following the first cycle of nitrate reduction may cause the decrease in both CSNR and $^{15}\varepsilon$ that we observe for assays where reduced carbon and other nutrients were not added along with re-amendment of nitrate. Another possibility is that the assay cultures with re-amendments of nitrate alone invest less toward nitrate transport due to disproportionate energy consumption by other functions of cellular metabolism because they must contend with resource limitation and/or adaptation to new environmental conditions; this lower rate of nitrate transport then translates to a reduced efflux-to-uptake ratio and a lower $^{15}\varepsilon$.

4.3.3. Oxygen

For *P. denitrificans*, an oxygen tolerant denitrifier, the denitrification rate of which is sensitive to dissolved [O_2] (Barford et al., 1999), we find that $^{15}\varepsilon$ was lower when the assay was done in the presence of 4 μM dissolved O_2 rather than in anoxic conditions. These data are also consistent with the previous suggestion (Granger et al., 2008) that O_2 availability may cause nitrate uptake to be down-regulated. One possible mechanism for this effect is that the presence of oxygen oxidizes the quinone pool in the membrane, reducing nitrate uptake (Alefounder et al., 1983; Denis et al., 1990); reduced nitrate uptake would then lead to less intracellular nitrate accumulation, a lower nitrate efflux-to-uptake ratio, and thus a lower $^{15}\varepsilon$.

Based on a steady state culture study with *P. denitrificans,* Barford et al. (1999) concluded that $^{15}\varepsilon$ was constant at 28.6 ± 1.9‰ over a range in dissolved [O_2] of 0–0.3 μM. These authors found evidence for a lower $^{15}\varepsilon$ of 12.4‰ at dissolved [O_2] of 1.2 μM, but it was disregarded due to the possibility of contamination of their N_2 samples with air. However, their nitrate $\delta^{15}N$ data alone (corresponding to $f = 0.2$ and $f = 0$ in Fig. 4 in Barford et al., 1999), also argue for a lower isotope effect in their experiments with [O_2] of 1.2 μM.

Since many denitrifiers can denitrify in the presence of oxygen concentrations up to 10 μM (Devol, 1978) or higher (Lloyd et al., 1987), our dataset shows that yet another variable could lead to a significant reduction in $^{15}\varepsilon$ under environmental conditions.

4.3.4. Growth phase of denitrifiers

The *P. chlororaphis* experiments suggest that growth phase of the starter cultures could have a significant impact on the cellular level denitrification isotope effect ($^{15}\varepsilon$). The values of $^{15}\varepsilon$ were lower when cells were taken from early stationary phase (age of starter culture = 2–3 days; i.e., 1–2 days after complete nitrate reduction by the starter

culture) rather than later in the stationary phase (age of starter culture $\geqslant$4 days; i.e., $\geqslant$3 days after complete nitrate reduction by the starter culture). Davidson et al. (2009) observed a similar trend in isotope fractionation with growth phase in sulfate reducing bacteria (SRB), with a significantly higher S isotope fractionation during sulfate reduction in stationary/maintenance phase. Consistent with other S isotope studies (see Section EA1.2), the higher S isotope effect in stationary phase was explained by them as the result of a reduction in cell specific sulfate reduction rates leading to a higher ratio of sulfate efflux to reduction (e.g., Sim et al., 2011).

The $^{15}\varepsilon$ obtained from assays with starter culture late in the stationary phase (i.e., "standard" assay conditions for *P. chlororaphis*) appears consistent with the canonical value for $^{15}\varepsilon$ and with the $^{15}\varepsilon$ observed for other strains at high CSNR rates (compare Fig. 1b and the dashed line in Fig. 2b). It is the early stationary phase cells that yield a $^{15}\varepsilon$ that is substantially lower. Thus, these experiments from earlier harvesting of *P. chlororaphis* cells are most in need of explanation. The efflux model for $^{15}\varepsilon$ variation would require a lower efflux-to-uptake ratio in these cultures in the early stationary phase (i.e., cultures closer to a "blooming" phase).

The response of the early stationary phase cultures may be driven by the fact that they have recently been actively dividing, which requires that a higher proportion of their energy be directed to cellular growth and maintenance. This would leave proportionally less energy available for cellular nitrate uptake, leading to a low efflux-to-uptake ratio and thus a low $^{15}\varepsilon$. Other explanations include higher stability of transporters as opposed to nitrate reductase enzymes following the exponential growth phase and decreased cell size during stationary phase. In stationary phase, protein synthesis decreases (Kolter et al., 1993) and turnover of existing proteins increases (Navarro Llorens et al., 2010), raising the possibility of a decrease in the concentration of active nitrate reductase enzymes that is more pronounced than the decrease in the concentration of nitrate transporters. Finally, in stationary phase, reductive division and "dwarfing" of cells cause reduction in cytoplasmic volume (Nystrom, 2004), making cells smaller, with a higher the surface-to-volume ratio and thus potentially more susceptible to cellular efflux.

4.3.5. Turbulence

The difference in $^{15}\varepsilon$ values for *Marinobacter* sp. assays between stirred and unstirred conditions is significant, especially at higher CSNR rates. This suggests that turbulence or mixing can enhance the nitrate efflux to uptake ratio in marine denitrifiers. The relative motion between medium and microbial cells, due to either the inherent motility of the strain or turbulence, may act to reduce the thickness of the diffusion boundary layer around the cells, enhancing the transport rate of molecules both to and away from the cell surface (Jumars et al., 1993; Karp-Boss et al., 1996). Thus, for a strain such as the *Marinobacter* sp. strain 23-5 investigated here, one might suspect that turbulence could increase $[NO_3^-]$ available for uptake at the cell surface, increasing the efflux-to-uptake ratio and thus raising $^{15}\varepsilon$ as observed. However, this hypothesis does not hold up at a quantitative level. An estimation of rates of diffusion, uptake and reduction based on the observed CSNR rates, efflux/uptake ratios, starting $[NO_3^-]_{external}$ and diffusivity of nitrate ions using methodology described by Kritee et al. (2009) shows that a bacterium of the size of *Marinobacter* sp. strain 23-5 cannot be diffusion-limited (see Electronic Annex Section EA1.5).

Hence, we do not as yet have a mechanistic hypothesis to explain the effect of turbulence on $^{15}\varepsilon$ in *Marinobacter* sp. strain 23-5. This total lack of mechanistic understanding deters us from assuming that turbulence/mixing will have the same effect of lowering the isotope effect in the environment. Nevertheless, we note that for all our assays, cultures were vigorously bubbled and thus well-mixed even when there was no mechanical stirring. Thus, it seems likely that even the turbulent environment of denitrifiers in the ocean water column is closer to the unstirred cultures. Thus, if CSNR rate is ever as high as observed for the upper range encountered in our *Marinobacter* sp. strain 23-5 assays (which is unlikely), the unstirred experiments should provide a better prediction of $^{15}\varepsilon$.

4.3.6. Summary

The range in both the magnitude of $^{15}\varepsilon$ (Fig. 1) and the physiological and environmental conditions examined here (Table 2) extend beyond any previous culture studies of the denitrification isotope effect. The experiments have identified diverse conditions under which $^{15}\varepsilon$ is much lower than its canonical value of 25‰. The data in hand encourage explanation of these lower $^{15}\varepsilon$ values in terms of a lowering of the efflux-to-uptake ratio, calling on a more rapid/stronger response of the rate of nitrate uptake as compared to nitrate reduction rate to changing environmental conditions. These explanations are of variable quality: in some cases convincing but pending proof (effect of CSNR rate), and in other cases highly tentative (e.g., effect of growth phase) or even absent (effect of turbulence). Strict proofs can only be obtained by measuring the variation in the rates of uptake, reduction and efflux. Thus, in all cases, more studies are required.

4.4. Implications for open ocean denitrification

In ocean ODZs, denitrification rate varies from ~0.3 to a maximum of 300 nM N/day (Devol et al., 2006; Devol, 2008; Ward et al., 2009; Bulow et al., 2010). Using a minimum denitrifier cell density typical for ODZs (~10^5–10^6 colony forming units (CFU)/ml (Ward et al., 2008, 2009)), the observed maximum *in situ* CSNR rate is lower than $\sim 2 * 10^{-12}$ μmol cell^{-1} min^{-1} even in the coastal regions, and 10–1000 times lower than the CSNR rate typically observed in previous culture studies (Barford et al., 1999; Wunderlich et al., 2012). Thus, our lowest CSNR rates appear most comparable to heterotrophic nitrate reduction in the ocean. These considerations suggest that $^{15}\varepsilon_{wc}$ (the N isotope effect as applicable to the ODZ water column) could be more similar to our mean $^{15}\varepsilon$ of 13.3 ± 2.3‰ ($\pm$1SD ($n = 9$)) for CSNR $< 2 * 10^{-12}$ μmol cell^{-1} min^{-1} (Fig. 1) than to the often-adopted

canonical value of 25‰ (Brandes and Devol, 2002; Deutsch et al., 2004).

The new species-specific results also suggest that other environmental conditions prevalent in the ocean could lower $^{15}\varepsilon_{wc}$ (Table 3). Denitrification in the ocean water column appears to be strongly limited by the availability of labile organic matter (Ward et al., 2008, 2009), such that energy generation by denitrification is typically non-optimal. For *P. denitrificans* (Fig. 1), $^{15}\varepsilon$ was lower when the carbon source was acetate or glucose as opposed to complex and "rich" organic matter, suggesting that use of recalcitrant or thermodynamically unfavorable organic carbon sources, which yield less energy during respiratory nitrate reduction, will lead to lower $^{15}\varepsilon$. This effect can be explained by the same dynamic of down-regulated nitrate uptake rate when denitrification is less energetically profitable. In addition, the examination of the effect of nitrate re-amendments without medium replenishment (Fig. 3) suggest that more recalcitrant C sources and/or non-optimal nutrient conditions, while reducing CSNR rate of denitrifiers, will more sharply down-regulate their nitrate uptake rate, also lowering $^{15}\varepsilon$.

There are also suggestions that denitrification in the ocean is sporadic, with blooms occurring in response to pulsed inputs of organic matter from the overlying euphotic zone (Ward et al., 2008, 2009; Jayakumar et al., 2009). Relevant to this, if highly uncertain, we have found that denitrification assays initiated from more recently grown "younger" *P. chlororaphis* cells that have bloomed only a few days before can also show a lower $^{15}\varepsilon$ than cells harvested further along in stationary phase (Fig. 4).

Assays with *Marinobacter* sp. strain 23-5 (Fig. 5) show that lack of turbulence can decrease $^{15}\varepsilon$. Turbulent kinetic energy dissipation rates are high at the surface and decay rapidly with depth (Karp-Boss et al., 1996). Therefore, we suspect that a lack of turbulent mixing in ODZs where heterotrophic denitrification primarily occurs could be yet another reason to expect lower $^{15}\varepsilon$ in the ocean.

In individual drawdown assays, we consistently observed a reduction in $^{15}\varepsilon$ as $[NO_3^-]_{external}$ fell below ~35 μM (Fig. 2). Evidence for such a decrease in $^{15}\varepsilon$ under low $[NO_3^-]_{external}$ has been observed in the porewater of sediments from the Bering Sea (Lehmann et al., 2007). However, $[NO_3^-]$ of $\leqslant$35 μM in ocean ODZs will not necessarily lower $^{15}\varepsilon$ in these environments. In our experiments, CSNR is typically high, and the rapidly decreasing $[NO_3^-]$ in the assay medium cannot be countered completely by increasing the concentration of nitrate transporter(s) in the cell membrane, leading to uptake limitation and a collapse in $^{15}\varepsilon$. However, if CSNR rate is as low as typically observed in the ODZs, it is likely that the low internal nitrate demand would allow nitrate uptake to maintain a high intracellular $[NO_3^-]$, allowing for the high degree of efflux that yields higher (and non-zero values of) $^{15}\varepsilon$. Moreover, denitrifiers may adjust to their ambient $[NO_3^-]$ by increasing the number of nitrate uptake enzymes embedded in their external membrane. Consistent with this view, several of our *P. chlororaphis* assays started with an initial $[NO_3^-]_{external}$ of 15 or 40 μM and yet the $^{15}\varepsilon$ values were close to 25‰ (Fig. 4) until $[NO_3^-]_{external}$ dropped to 4.3 or 2 μM, respectively.

On the whole, our study suggests that, under environmental conditions relevant to the ocean, the denitrification isotope effect could be as low as 10–15‰. Vertical profiles of nitrate $\delta^{15}N$, $[NO_3^-]$ and $[PO_4^{3-}]$ through the core of ocean's ODZs have, however, yielded ε_{wc} estimates of 20–30‰ (Brandes et al., 1998; Altabet et al., 1999; Voss et al., 2001; Sigman et al., 2003). These field studies (Altabet et al., 1999; Voss et al., 2001; Sigman et al., 2003) would seem to contradict our culture-based argument for an oceanic $^{15}\varepsilon_{wc}$ closer to 10–15‰.

The field estimates of ε_{wc} are by in large based on the Rayleigh substrate equation: $\delta^{15}N = \varepsilon_{wc} * \ln(f)$, where f is the fraction of initial nitrate remaining, which applies to a closed system where nitrate is being progressively consumed with a constant isotope effect. A Rayleigh model applicable to closed systems may not be appropriate for the ocean suboxic zones, because of resupply of nitrate to waters undergoing denitrification. However, simple resupply of nitrate during the consumption process, as is included in the context of a steady state model, would not greatly alter most of the field estimates of ε_{wc} that have been generated so far (Brandes et al., 1998; Sigman et al., 2003). More importantly, failing to account for such nitrate resupply tends to underestimate, not overestimate, ε_{wc}. Nevertheless, there are processes that might lead to overestimation of ε_{wc} from the field studies (Deutsch et al., 2010). These processes must be investigated in detail to address whether our culture-based prediction of ε_{wc} ~13‰ is plausible for the ocean suboxic zones.

Finally, we must note that not all field studies have yielded estimates of ε_{wc} between 20‰ and 30‰. Data from eastern tropical South Pacific, interpreted in the context of the Rayleigh model, yield an overall $^{15}\varepsilon$ estimate of 11.4‰, with the data from closer to the continental margin yielding 7.6‰ and the data from further off-shore yielding 16.0‰ (Ryabenko et al., 2012). The authors tentatively explain these internal variations as the result of a variable impact from sedimentary N loss. Sedimentary N loss is known to impart little N isotope fractionation on nitrate in the water column (Brandes and Devol, 1997; Lehmann et al., 2004, 2007) and the relatively low overall $^{15}\varepsilon$ observed by Ryabenko et al. (2012) can at least partly be due to sedimentary N loss. However, this does not rule out the possibility that ε_{wc} (i.e., the $^{15}\varepsilon$ of water column denitrification) alone is 16‰ or lower in this region.

4.5. Global marine N isotope budget

The N isotope based constraint on the global oceanic N budget is one of the most important geochemical applications of N isotope systematics that relies on the amplitude of $^{15}\varepsilon_{wc}$. This constraint derives from the $\delta^{15}N$ of mean ocean nitrate. Biological N fixation introduces newly fixed N with a $\delta^{15}N$ of −2‰ to 0‰ (Wada and Hattori, 1976). The $\delta^{15}N$ of mean ocean nitrate is ~5‰ (Sigman et al., 2000), higher than the $\delta^{15}N$ of newly fixed N because of the isotopic discrimination of denitrifiers (Brandes and Devol, 2002). As opposed to water column denitrification, during benthic denitrification, the cellular-level isotope effect is only minimally expressed in the overlying water

column because nitrate is nearly completely consumed within the sediment pore waters (Brandes and Devol, 1997; Lehmann et al., 2004, 2007). Brandes and Devol (2002), assuming isotope effects for water column (WCD) and benthic denitrification (BD) of 25‰ and 1.5‰, respectively, and a $\delta^{15}N$ of −1‰ for newly fixed N, calculate that a mean ocean nitrate $\delta^{15}N$ of 5‰ requires a BD rate that is much greater than that of WCD (i.e., BD/WCD ~3.6). Assuming a WCD rate of ~75 Tg N/year, the BD rate must be ~270 Tg N/year, yielding a total denitrification rate of 345 Tg N/yr (Brandes and Devol, 2002). If the global ocean rate of N fixation is ~140 Tg N/year (Brandes and Devol, 2002; Deutsch et al., 2007), the N budget is out of balance, with an apparent excess loss of up to 250 Tg N/year (Codispoti et al., 2001; Brandes and Devol, 2002; Codispoti, 2007). This budget is altered quantitatively by moving beyond a one-box model (Deutsch et al., 2004; Altabet, 2007; Sigman et al., 2009), but the basic imbalance appears to persist (Deutsch et al., 2004).

However, an isotope effect of 10–15‰ for water column denitrification would imply a much lower ratio of benthic to water column denitrification. Given a WCD rate of 65 Tg N/year (Deutsch et al., 2007), $^{15}\varepsilon_{wc} = 13‰$ (this study), and otherwise similar assumptions as Brandes and Devol (2002), and using a simple one box model, BD is calculated to be ~105 Tg N/year and the global ocean denitrification rate is ~170 Tg N/year (Fig. 6, solid red[1] line), as opposed to a total N loss rate of 330 Tg N/year when $\varepsilon_{wc} = 25‰$ (Fig. 6; dashed red line). Our calculation here is not intended to suggest that other dynamics, such as the "dilution effect" of Deutsch et al. (2004), do not significantly influence the ocean's N isotope budget. Rather, it shows that downward revision of $^{15}\varepsilon_{wc}$ to 10–15‰ would undercut the previous isotopic rationale for a high rate of benthic and thus total denitrification, removing the apparent imbalance in the ocean N isotope budget.

Previous support for a high benthic denitrification rate derived from a parameterization of local benthic denitrification, the extrapolation of which suggested a global BD rate of 230–285 Tg N/yr (Middelburg et al., 1996). However, with this same parameterization, a global biogeochemical model using a different distribution of organic carbon fluxes yields a BD rate of ~150 Tg N/yr (Sarmiento and Gruber, 2006), ~100 Tg N/yr lower than originally estimated (Middelburg et al., 1996). Moreover, comparison with a new compilation of observed benthic denitrification rates from diverse denitrifying environments suggests that the parameterization itself overestimates local rates by 2–4-fold (Fennel et al., 2009). Thus, if a $^{15}\varepsilon$ of 10–15‰ applied to most water column denitrification, no compelling argument would remain that total denitrification rate is significantly higher than current estimates of the global rate of fixed N input (Gruber, 2004; Deutsch et al., 2007). This highlights the critical importance of understanding the relationship between our culture results and the denitrification occurring in the real ocean water column.

[1] For interpretation of the references to color in Fig. 6, the reader is referred to the web version of this article.

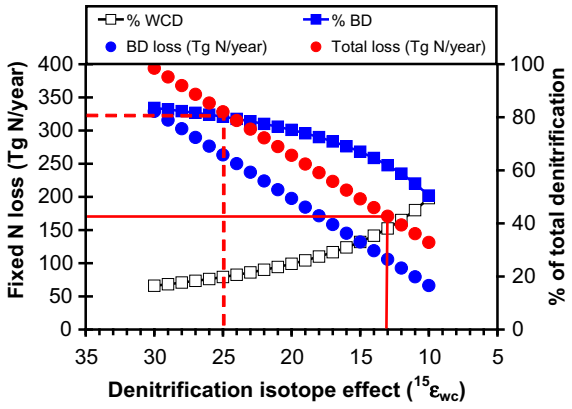

Fig. 6. Percentage contributions of WCD (open square) and BD (closed blue square) to total fixed N loss (closed red circle) as a function of cellular level N isotope effect as applicable to the water column ($^{15}\varepsilon_{wc}$) estimated using a simple one box model given the mean ocean nitrate $\delta^{15}N$ of 5‰, a fixed WCD rate of 65 Tg N/year and otherwise similar assumptions as Brandes and Devol (2002). Net loss due to BD in Tg N/year is also shown as (blue circle) as a function of $^{15}\varepsilon_{wc}$. (For interpretation to colours in this figure the reader is referred to the web version of this paper.)

5. CONCLUSIONS

Based on the previous body of laboratory culture studies (Table 1), we and others have tended to assume a culture-based cellular level denitrification isotope effect ($^{15}\varepsilon$) of 20–30‰. The results of this study show that $^{15}\varepsilon$ varies as a function of environmental conditions and that previous studies may have tended to capture the upper end of the range. The tendency toward high $^{15}\varepsilon$ in previous culture studies may be largely due to the use of optimal growth conditions for cellular energy generation by respiratory nitrate reduction, which in turn supported very high cell-specific nitrate reduction (CSNR) rates. Experiments with three denitrifiers show that $^{15}\varepsilon$ is 10–15‰ at CSNR rates that are more representative of rates measured under environmental conditions. A sharp decrease in $^{15}\varepsilon$ is also observed in individual assays as the nitrate uptake becomes the rate-limiting step in the overall process of cellular nitrate reduction. On an apparently strain-specific basis, a lower $^{15}\varepsilon$ is also observed under diverse other physico-chemical conditions, many of which may better characterize the natural environment than do typical culture conditions. While the explanations for the drivers of variation in $^{15}\varepsilon$ remain unproven, they in general point toward a higher sensitivity and stronger response of nitrate uptake compared to nitrate reduction to environmental conditions, which causes variability in the fraction of intracellular nitrate that is effluxed rather than reduced. In any case, the canonical range of 20–30‰ for $^{15}\varepsilon$ appears too high, and a value of 10–15‰ may be more applicable to the ocean. If $^{15}\varepsilon_{wc}$ were indeed in the range of 10–15‰, an apparent large imbalance in the global ocean N budget previously suggested on the basis of the N isotopes would be resolved (i.e., removed). However, most field estimates of $^{15}\varepsilon_{wc}$ have fallen in the range of 20–30‰, and we must be able to square these findings with our culture results before the latter can be taken to reflect oceanic conditions.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gca.2012.05.020.

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