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Provisional chapter

Stable Isotope Methods for the Study of the Nitrogen Cycle

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Additional information is available at the end of the chapter

1. Introduction

Nitrogen, a limiting element for biological productivity, plays a key role in regulating the biogeochemical processes in the ocean. In today's ocean, all of the major reactions in the N cycle are mediated by assimilatory or dissimilatory functions of marine organisms. Because marine organisms preferentially incorporate lighter stable isotope of nitrogen ^{14}N instead of ^{15}N, each major metabolic reaction in the N cycle involves irreversible kinetic fractionation of nitrogen.

The isotopic composition of a pool of nitrogen can be used to identify the relative importance of sources that are isotopically distinct, or processes that add or remove nitrogen with a characteristic pattern of isotopic discrimination. The strongest isotopic fractionations are associated with dissimilatory processes that mediate the transfer of nitrogen from one inorganic pool to another [1]. In contrast, processes such as primary production, which move nitrogen directly into and through the food web are associated with comparatively weak isotopic fractionations [1]. The extent of nitrogen isotope fractionation also depends upon the kinetics of individual metabolic reactions, concentration of products and reactants, environmental conditions (e.g., oxygen concentrations) and the microbial species involved.

The crucial challenge in using nitrogen isotope methods is the complexity of the marine nitrogen cycle and the potential influence of multiple processes on the isotopic Composition of several biologically active pools of nitrogen. Successful use of nitrogen isotopes in resolving N cycle fluxes and processes requires an understanding of the general distribution of nitrogen isotopes in marine systems, the nature of isotopic fractionation, and a careful consideration of the processes at work in the system of interest. In this chapter I will introduce the reader to the two different approaches of nitrogen isotope analysis:

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natural abundance or tracer methods (^{15}N-labeling). The natural abundance of the stable isotope of nitrogen can provide critical insights into processes acting on a variety of scales with minimal alteration and manipulation of the system being studied. The isotopic signal collected in this type of approach is an integrated value that incorporates large spatial and temporal variation of the relevant processes. Conversely, isotope tracer methods involve short-term incubations of small, isolated samples of water under in situ or simulated in situ conditions. Tracer methods are primarily used for rate experiments that complement larger-scale natural abundance studies.

I begin the chapter with basic definitions of isotopic fractionation and analytical considerations of the sample measurements. I follow with an overview of the processes of nitrogen sources and sinks. I conclude with a discussion of how isotopic data can contribute to the current debate on the balance of N-inputs and losses in the nitrogen cycle.

2. Fundamentals

2.1. Isotopes and calculation of their ratio

Isotopes are atoms of an element that share the same number of protons but a different number of neutrons. In the scientific nomenclature, isotopes are specified in the form ${}^{m}_{n}E$, where "m" indicates the mass number (the sum of protons and neutrons in the nucleus) and "n" refers to the atomic number of an element "E". There are more than 10 nitrogen isotopes known. Most of these isotopes are radioactive and highly unstable with longest half-life time for ${}^{13}_{7}N$ of 10 minutes. The only two stable nitrogen isotopes are ${}^{14}_{7}N$ and ${}^{15}_{7}N$, which have seven protons each and seven or eight neutrons in their nucleus, respectively. ^{15}N is the less frequent stable isotope, constituting of 0.365% of the global nitrogen pool [2]. Consequently, it is more practical to measure the difference or ratio of two isotopes instead of the absolute quantity of each.

Isotopic compositions are expressed in terms of "delta" (δ) values which are given in parts per thousand or per mil (‰). Nitrogen isotope ratios, for example, are expressed as the ‰-difference to atmospheric N_2, which has a constant ^{14}N/^{15}N of 272 ± 0.3 [3]. The δ^{15}N-value in the sample is then calculated by the following equation (1).

$$\delta^{15}N(vs.air) = \left({}^{(15}N\,/^{14}\,N)_{sample} \Big/ {}^{(15}N\,/^{14}\,N)_{air} - 1 \right) \times 1000 \quad (1)$$

The δ-values do not represent absolute isotope abundances but rather the ‰-difference to a widely used reference standard, such as VSMOW (Vienna Standard Mean Ocean Water). The δ-value is then calculated from equation (2), by measuring the isotope ratios (R) for the sample and the reference standard:

$$\delta^{15}N = \left(R_{sample} / R_{standard} - 1 \right) \times 1000 \quad (2)$$

where R_{sample} and $R_{standard}$ represent the isotope ratio $\delta^{15}N_{(vs.air)}$ in the sample and in the standard respectively, calculated using equation (1). By convention, R is the ratio of the less abundant isotope over the most abundant isotope (i.e. $^{15}N/^{14}N$ for nitrogen).

As previously discussed, the $\delta^{15}N$ value changes under the influence of chemical and physical processes. If the process is complete the resulting $\delta^{15}N$ value in the product is equal to the value of the reagent. Only if the reaction is incomplete the fractionation of isotopes happens, which means that the $\delta^{15}N$ values in the product and substrate differ.

2.2. Isotope fractionation effect

There are two different fractionation processes, both of which will be discussed here: equilibrium and kinetic fractionation processes.

Equilibrium fractionation processes are reversible processes. They are mainly driven by changes in the internal energy of a molecule, i.e. vibrations of the atoms within a molecule with respect to each other and rotations around the molecular axes. The equilibrium fractionation factor $\alpha_{eq.}$ is related to the equilibrium constant K as shown in equation (3), where n is the number of exchanged atoms.

$$\alpha_{eq.} = k^{1/n} \quad (3)$$

During equilibrium reactions, the heavier isotope preferentially accumulates in the compounds with a higher number of bonds. During phase changes, the ratio of heavy to light isotopes in the molecules also changes. For example, as water vapor condenses in rain clouds (a process typically viewed as an equilibrium process), the heavier water isotopes (^{18}O and ^{2}H) become enriched in the liquid phase while the lighter isotopes (^{16}O and ^{1}H) remain in the vapor phase. In addition, the equilibrium isotopic effect decreases as the system temperature increases.

Kinetic fractionation processes are also associated with incomplete processes like evaporation, dissociation reactions, biologically mediated reactions and diffusion [4]. Kinetic isotope fractionation reflects the difference in the bond strengths or motilities of the isotopic species. The degree of isotopic fractionation associated with a reaction is commonly expressed with α, which is the ratio of rate constants for molecules containing the different isotopes:

$$\alpha = {}^{14}k / {}^{15}k \quad (4)$$

where ^{14}k and ^{15}k are the rate constants for molecules containing the light and heavy isotopes, respectively. Most biological reactions discriminate against the heavier isotope, yielding

$1.00<\alpha<1.03$. This makes it convenient to define an isotopic enrichment factor (ε) that highlights more clearly the range of variation:

$$\varepsilon = (\alpha - 1) \times 1000 \tag{5}$$

An alternative method for describing isotopic enrichment is the Rayleigh relationship. This is an exponential relationship that describes the partitioning of isotopes between two reservoirs as one reservoir decreases in size. The equation can be used if the following conditions are met: 1) material is continuously removed from a mixed system; 2) the fractionation accompanying the removal process at any instance is described by the fractionation factor α, and 3) α does not change during the process. Under these conditions, the evolution of the isotopic composition in the residual (reactant) material is described by:

$$R = R_0 f^{(\alpha-1)}, \tag{6}$$

where R is the isotopic ratio of the product, R_0 is the initial ratio of the reactant, f is the fraction of the substrate pool remaining and α is the kinetic fractionation factor.

2.3. Measurements of $\delta^{15}N$

Stable isotope measurements are becoming a routine tool in studies of marine ecosystems due to the increasing availability of mass spectrometers. Biological oceanographers usually using continuous flow systems for ^{15}N measurements that integrate a preparatory system (e.g., an elemental analyzer) with a mass spectrometer [5]. The mass spectrometer typically measures the isotopic composition of N_2 generated by combustion and carried in a stream of He gas to an open split interface that introduces a small fraction of the gas stream into the ion source for measurement. This analytical approach requires little or no manipulation of organic samples, though care must be taken to avoid contamination with exogenous nitrogen, particularly when tracer-level ^{15}N experiments are being carried out in the vicinity. Since N_2 is typically the analyte, minimizing atmospheric contamination in the preparatory system is also quite important. Alternatively, N_2O gas may be used for isotope measurements, which practically eliminates the potential contamination from the atmosphere and gives additional information of oxygen isotope composition of nitrogen compound (e.g nitrate or nitrite). In this case, nitrogen compounds must be chemically converted into N_2O prior the isotope analysis [6-11]. Special care has to be taken due to the potential exchange of oxygen isotopes between nitrate and water leading to deviation of initial value (e.g. [10]).

Tracer-level ^{15}N experiments used for rate measurements typically add about 10% of the ambient source concentration of ^{15}N-compound. The initial planning of tracer studies requires consideration of several practical issues. First of all, one has to determine the length of incubation, which is connected to turnover rate of the relevant pools and the detection limits for the analytical methods. The turnover rate (pool size^{-1}×uptake rate) has to be estimated for

both source and product pools to ensure that tracer can be detected in the product pool, while dilution of the source pool is either insignificant or small [12, 13]. Increasing incubation time will increase the amount of tracer in the product but it will reduce the potential of isotope dilution affecting the source pool. Given a best-guess turnover rate for the product pool, one can estimate the relative trade-offs between incubation time and changes in isotopic labeling of the source and product pools at a given enrichment level (7).

$$\text{Turnover rate} \times \text{incubation time} \times \text{source enrichment} = \text{change in enrichment, e.g., } 0.1\ d^{-1} \times 0.2\ d^{-1} \times 10\% = 0.2\%. \quad (7)$$

Generally the shortest incubation possible would resolve this dichotomy for biogeochemical processes such as nitrification or denitrification. However, complications may arise for uptake studies since short incubations primarily represent the transport (movement across the cell membrane) of N-compounds, whereas longer incubations primarily represent assimilation into organic compounds such as amino acids or proteins.

Finally, one has to consider the degree of replication and treatment of the initial, or time zero, concentrations and enrichment of both the product and source pools. To account for small, natural (%) level changes in these pools, studies either assume that particles have constant natural abundance values, or they assess the natural abundance periodically during the study. Alternatively, tracer can be added to the incubation bottle, which is then deployed and then immediately filtered at an initial time point for comparison with the final values. One then needs to decide how to interpret the initial time point, as there is often a substantial time between tracer addition and filtration (often 30 min). During this interval, the population may experience abnormal conditions (e.g., high light exposure or rush uptake due to nutrient perturbation) prior to filtration. It was also shown recently by Mohr *et al.* that measurements of N_2-fixation rates by introducing a tracer in bubble form can lead to significant underestimation of the process [14]. The equilibration, i.e. the isotopic exchange between the $^{15}N_2$ gas bubble and the surrounding water is controlled primarily by diffusive processes. The major variables that influence the rate of isotopic exchange include the surface area to volume ratio of the bubble, the characteristics of the organic coating on the bubble surface [15], and temperature and the rate of renewal of the water-bubble interface [16]. To avoid this problem, the authors recommend adding an aliquot of $^{15}N_2$-enriched water to incubators used in rate measurements.

Samples of oceanographic interest often pose additional analytical challenges simply because they are difficult to obtain in large quantities and most mass spectrometry systems require fine-tuning and careful attention to leaks in order to process samples containing less than a few micromoles of nitrogen. In practice, open ocean samples including suspended particles, small zooplankton, and sinking organic matter are often available only in μmole or sub-μmole quantities. These low concentrations create a strong incentive to minimize the mass requirements of the analytical systems used for marine samples, and to develop methods for correcting for the influence of any analytical blank, which will disproportionately affect small samples. Even with substantial care to reduce this source of contamination, a nitrogen blank on the order of 0.05–0.15 μmol N is typical for systems in current use [17]. For analysis of the

smallest samples, the influence of this blank can be reduced provided that an appropriate range of mass and isotopic standards is run, allowing estimation of the size and $\delta^{15}N$ of the blank.

Operationally, isotope ratio mass spectrometers measure the difference in ^{15}N abundance between a sample and a reference gas calibrated to atmospheric N_2. This reference gas calibration may be carried out directly by comparison to atmospheric values [18], or indirectly using any of a number of organic and inorganic secondary standards (e.g., nitrate and ammonium salts, acetanilide, glutamic acid) available from NIST or IAEA. For precise system calibration and correction of blank effects, it is also important to analyze a size series of a working standard that is chemically similar to the samples of interest.

Correcting for an analytical blank is a multi-step process involving separate regression analyses to evaluate the size of the blank and the dependence of $\delta^{15}N$ values on size. This information can then be used to remove the isotopic influence of the blank. Any real sample can be treated as a mixture of sample material and contaminants of various origins. If the blank contribution is constant in composition and magnitude across analyses, then a simple mass balance can be constructed for the mixture analyzed:

$$M_{mix}\delta^{15}N_{mix} = M_{sample}\delta^{15}N_{sample} + M_{blank}\delta^{15}N_{blank}, \quad (8)$$

where M is the size of an individual pool (sample, blank, or the mixture). In general, the nitrogen analytical blank is small enough that blank corrections are minor for samples containing more than 1.5–2 μmol of nitrogen.

3. Sampling for $\delta^{15}N$ measurements

Modern water column nitrogen isotope analysis is used on broad variety of samples as particles and dissolved nitrogen species, e.g. nitrate, ammonia or N_2 and N_2O. For each of these methods, special care must be taken during sample collection and preparation.

3.1. Particles

The simplest and most common method to remove particles from incubation water is by filtration. During the filtration special care should be taken to minimize the pressure difference to avoid strong cell rupture [18]. Several studies showed that Teflon and aluminium oxide filters capture up to 60% higher amount of particulate organic nitrogen (PON) than the GF/F filters [19, 20]. GF/F filters have been also found to retain only ~50% of bacteria [21]. In addition to incomplete retention of particles, glass-fiber filters adsorb dissolved organics on the high surface area of the filter [22]. For ^{15}N studies, although mass retention of DON is not a major issue, retention of the highly labeled incubation water on the filter has the potential to affect the isotopic ratio of the retained particulates so that wetting the filter prior to filtration and rinsing afterwards with filtered seawater is a common practice. Filtered seawater is used to prevent osmotic shock of the living particles. Regardless of the type of filter, the particle load

needs to be preserved and converted into the gaseous form prior to mass spectroscopic analysis. $\delta^{15}N$ of particles can be analysed directly via combustion (conversion into N_2) or via persulfate oxidation (conversion into NO_3^-) [23].

3.2. Nitrate and nitrite

The most abundant dissolved nitrogen specie in the ocean is nitrate (NO_3^-) followed by nitrite (NO_2^-). In order to perform the isotopic analysis both have to be converted into forms amenable to enter the mass spectrometer. The methods differ by level of reduction, with conversion from NO_3^- to NO_2^-, NO, N_2 or complete reduction to ammonium.

Although NO_2^- concentrations are usually very small compared to NO_3^-, there are cases when both should be analyzed without an interference of $\delta^{15}N$ signals of one another. To do this one has to separate NO_3^- and NO_2^- in the sample by excluding one of them from the sample. Olson [24] used sulfamic acid, while Yakushiji and Kanda [25] took advantage of the reaction of sulfanilic acid with NO_2^-. Rather than forming the final azo dye, the diazonium ion can be destroyed by heating which effectively destroys the initial NO_2^- present in the sample. Two new techniques remove nitrite by reaction with sodium azide in acetic acid buffer to produce N_2O [7] or via reaction with ascorbic acid to produce NO which is then removed bubbling [11].

Nitrate conversion to ammonia commonly uses DeVarda's alloy approach [26]combined with moderate heating. However, this method can add a significant blank that varies with the batch, and combustion of the alloy failed to reduce the blank while also reducing the reduction efficiency [27]. Tanaka and Saino [28] replaced Devarda's alloy with an aluminum reagent that reduced the blank and permitted analysis of nitrate at low concentrations.

Biological reduction of NO_3^- to NO_2^- or gaseous forms has been used by several investigators. Risgaard-Petersen et al. [29] and [30] used cultures of denitrifying bacteria to respire NO_3^- and NO_2^- toN_2 which was then stored for analysis by mass spectrometry. Sigman et al [8] changed this method by using mutant strain of a denitrifying bacteria that only produced N_2O to avoid the problem with contamination of N_2 from the air. The chemical alternative using sodium azide buffer to produce N_2O from NO_2^- was developed by McIlvin and Altabet [7] for natural abundance measurements in seawater samples. It was further improved for samples with low salinities [6] and can be used to analyze nitrate as well as nitrite in the samples.

3.3. Ammonium

Ammonium is not necessarily dominant by mass, but it is often predominant with respect to flux. NH_4^+ is produced primarily by the breakdown and remineralization of organic forms of nitrogen and consumed by autotrophic assimilation and re-incorporation into organic molecules, chemosynthetic nitrification to NO_2^-, and anammox oxidation by NO_2^- to N_2. For isotopic analysis ammonia has to be extracted quantitatively from the solution and converted to suitable gaseous species (e.g. N_2).

Initial approaches to isolating ammonium for isotopic analysis by steam distillation were derived from the soil literature. In all methods involving NH_4^+, great care is required to

minimize ambient contamination (e.g., cleaning compounds such as Windex), from sample/reaction containers and from reagents.

Distillation of NH_4^+ under alkaline conditions with subsequent conversion to dinitrogen gas has been used by several groups but is time consuming and has problems with cross-contamination and fractionation [31-34]. In the diffusion method, ammonia passes through a membrane into an acidic medium and is trapped as an NH_4^+ salt [34-37]. This approach, however, is also time-intensive and not very reliable at low concentrations [34]. For freshwater samples Hg precipitation and cation exchange are reported to show very good results for isotope analysis [32, 38]. An organic reaction with NH_3 producing indophenol permits organic or solid-phase extraction works well for ^{15}N-labeled samples [32] but cannot be used for samples with $[NH_4^+] < 5\mu M$ [38]. Zhang and Altabet [39] developed a robust method for sea water samples at low concentrations (0.5 μM or lower), which involves oxidation of ammonia into NO_2^- and subsequent reduction to N_2O.

3.4. N_2O and N_2

In addition to the previously discussed methods where production of N_2 or N_2O from other forms of nitrogen is an analytical step, there are related methods for analysis of the gases where the gas itself is the form of interest. N_2O can be formed during nitrification and denitrification processes, while N_2 is the end product of denitrification and anammox. Isotope paring technique (IPT) for analysis of N_2 requires analysis of all three isotopic variants of N_2 ($^{28}N_2$, $^{29}N_2$, $^{30}N_2$) [40]. Either a quadrupole or magnetic sector mass spectrometer can be used for the isotope pairing analysis, but the quadrupole can also simultaneously determine the N_2/Ar ratio. Extraction of N_2 from water samples, generally stored in a gas-tight container sealed with a septum (e.g., Exetainer), involves partitioning of the gas into a suitable headspace such as helium. The gas is allowed to equilibrate and the headspace is sampled for introduction of the gas phase into the mass spectrometer [41].

Rather than extraction into a headspace, several investigators have employed membrane inlet mass spectrometers (MIMS) to directly sample N_2 for denitrification and nitrogen fixation studies [30, 42]. MIMS can also be used directly to measure denitrification by monitoring changes in the N_2/Ar ratio over time since the Ar composition reflects changes in temperature and other physical factors while change in the N_2 concentration reflects the balance between nitrogen fixation and denitrification [43]. An advantage of combining MIMS with the isotopes is the ability to separate the two contributions to the net flux. MIMS also obviates the need for the generally slow, separate extraction step. The membrane inlet configuration varies but always features a gas-permeable membrane that separates the mass spectrometer from the solution [44, 45]. A probe inlet [44]permits analysis of depth zonation of rate processes in sediments but has only been employed for N_2/Ar measurements.

Analysis of $^{15}N_2O$ during nitrification and denitrification rate studies has become increasingly popular. Punshon and Moore [46] further developed the method to extract $^{15}N_2O$ produced from either $^{15}NH_4^+$ or $^{15}NO_3^-$ by natural samples. Using purge-trap gas chromatography with a quadrupole mass spectrometer, they were able to determine production rates for N_2O from nitrification. The origin of N_2O (denitrification or nitrification) can be determined using

isotopic signature of oxygen isotope. $\delta^{18}O$ isotope signature in N_2O molecule is a product of mixing from dissolved O_2 and H_2O and any fractionation process associated with its production [47]. For instance, production of N_2O via hydroxylamine oxidation (nitrification) will only include oxygen atoms from dissolved O_2, while via denitrification it will have $\delta^{18}O$ isotope of NO_2^- or NO_3^-, originating from both dissolved O_2 and H_2O [47, 48].

The site preference isotopic signature (isotopomers) is also used to identify pathways of N_2O production. In the molecule of N_2O the ^{15}N can be at a central (α) or outer (β) position. The intermolecular distribution of ^{15}N is expressed as site preference (SP) (eq.9)[49].

$$SP=\delta^{15}N^{\alpha}- \delta^{15}N^{\beta} \quad (9)$$

In contrast to $\delta^{18}O$ signature, SP is not dependent on the substrate's isotopic composition but reflects the microbial production mechanism [48]. For example, production of N_2O via hydroxylamine oxidation results in an SP of 33‰, while via denitrification the SP is 0‰ [50]. Good summery on isotopomers for soil studies is presented in the book of Environmental Isotope Geochemistry [51] showing the variable range of SP values for nitrification. In general, isotopomers are valuable tool for distinguishing between nitrification and denitrification processes of N_2O production.

4. Main processes of nitrogen cycle and their influence on isotope balance

Most nitrogen in marine environments is present in five forms: N_2, a quite stable molecule that requires specialized enzymatic systems to break and use it; NO_3^-, the most oxidized form of nitrogen and the dominant form of N within oxic environments; NH_4^+, the most reduced natural form of N and the dominant biologically available form found in anoxic environments; particulate nitrogen, predominant within sediments and primarily in the form of organic N, and dissolved organic N (DON), which is a complex mixture of compounds with a wide range of compositions [52-55]. Nitrate, nitrite, ammonium, and organic nitrogen are typically grouped together as "fixed N" in discussions about the availability of nitrogen. Although each form has a different level of reactivity, a complex web of reactions links these different compounds. The most important processes discussed here are: N_2 fixation, nitrification, assimilation and N-loss (conversion of fixed N to N_2). As it was mentioned already earlier, all biologically mediated processes lead to nitrogen isotope fractionation. For example, denitrification converts NO_3^- to N_2, which has the net effect of depleting the ocean's supply of combined nitrogen and doing so with strong discrimination against ^{15}N. This results in an increase in the $\delta^{15}N$ of the major pool of oceanic combined nitrogen (NO_3^-) concomitant with a reduction in its overall size. In contrast, N_2-fixation adds combined nitrogen with a low $\delta^{15}N$ to the ocean, counteracting both the mass and isotopic effects of denitrification (figure 1). In the subsequent parts of this chapter I will discuss all these processes in detail and their influence on the nitrogen isotope budget in the ocean.

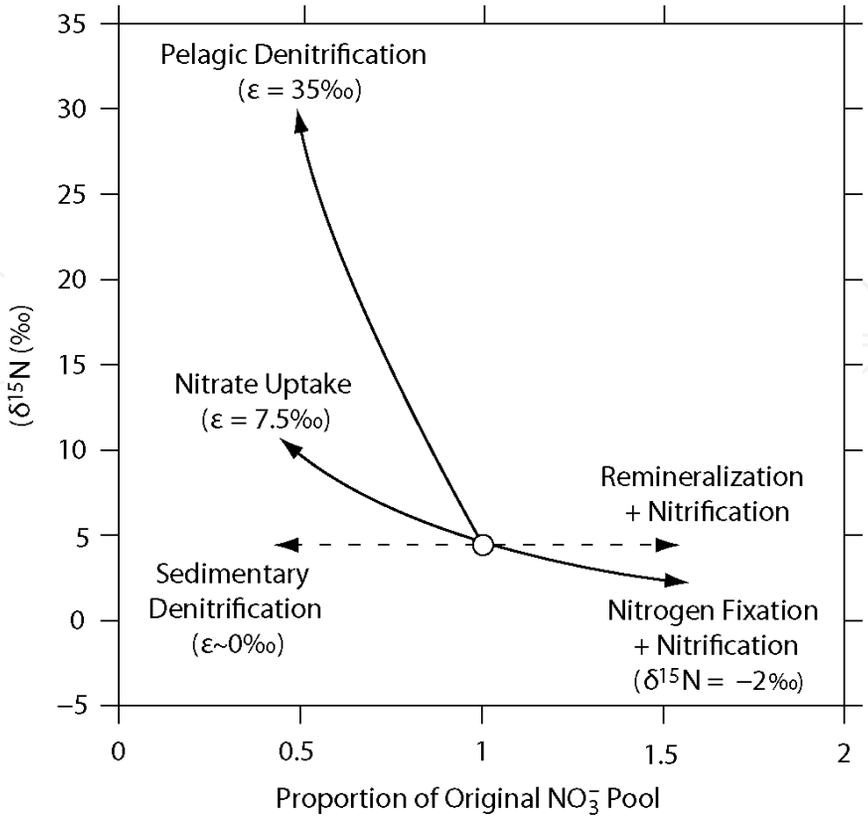


Figure 1. The impact of different processes on the $\delta^{15}N$ of oceanic NO_3^- [56]. Axes show deviation of $\delta^{15}N$ signal from oceanic average and loss/input of nitrogen due to different processes.

4.1. Nitrogen fixation in the ocean

Microbial N_2 fixation in the ocean inputs "new" N into the surface waters by reducing N_2 gas into two molecules of ammonia. The reaction is logically consumes energy and is catalyzed by nitrogenase enzyme complex, which is extremely oxygen sensitive (req. 10).

$$N_2 + 8\,H^+ + 16\,ATP + 8\,e^- \rightarrow 2\,NH_3 + H_2 + 16\,ADP + 16\,P_i \quad (10)$$

The nitrogenase proteins are highly similar among diazotrophs, and the well-conserved *nif*H gene is commonly used for phylogenetic and ecological studies [57]. In the open ocean N_2 fixation research has focused most intensively on the cyanobacterium, *Trichodesmium* [58, 59], which often occur as aggregates (often referred to as colonies) visible to the naked eye ('sea sawdust'). It can also occur, however, as individual filaments. In early reports N_2 fixation was observed in the pelagic zone and associated with the cyanobacterial epiphytes (*Dichothrix fucicola*) of the brown macroalga, *Sargassum* [60] and cyanobacterial endosymbionts of certain oceanic diatoms [61, 62]. *Trichodesmium* has a unique physiology that employs spatial and temporal segregation and increased oxygen consumption to allow it to simultaneously fix N_2 and CO_2 (and thus evolve O_2)[63].

Evidence of N_2 fixation began to first accumulate in the late 1970s with several pioneering N isotope studies examining the natural abundance of the ratio of $\delta^{15}N$ in the western Pacific Ocean [59, 64]. The average isotopic signature of nitrate in the deep ocean lay about 5‰ [27, 65, 66]. Nitrogen fixation is the process by which N_2 is incorporated from the air into the cell. As the $\delta^{15}N_{air}$ is set to 0‰, the kinetic isotope effect of the process is small (-2‰ to +2‰). Surface particulate and dissolved inorganic N pools in the Kuroshio often had a ^{15}N-depleted isotopic signature, indicative of a N_2 fixation source [67]. Using *nif*H gene studies, N_2-fixing was found also in the picoplankton, as well as in heterotrophic bacteria from the guts of copepods [68]. Heterotrophic N_2 fixation does not consume CO_2 during the process as diazotrophs do but instead produces it. However, the mechanism of heterotrophic N_2 fixation is not yet understood and it is still unclear and the potential contribution of these organisms to marine N_2 fixation is uncertain.

4.1.1. Controlling factors for N_2 fixation and its distribution

Numerous factors, physical, chemical and biotic, can affect the extent of N_2 fixation in an ecosystem [69-72]. Many factors that affect nitrogenase activity co-vary, such as light, temperature, oxygen and turbulence, making it difficult to determine the relative impact of each. Species-level responses to these variables also differ. The heterocystous diazotrophs, for instance, are typically restricted to special marine environments. Metabolically active populations of *Trichodesmium* have been observed at 18.3 °C [54], but activity was low, and substantial growth is typically not seen until water temperature exceeds 20 °C [73]. In the cooler Baltic waters *Nodularia* spp. and *Aphanizomenon* spp. Are observed, which are restricted to 16–22 °C and 25–28 °C, respectively [74]. In warmer waters of the tropics and subtropics, heterocystous diazotrophs (such as *Richelia intracellularis*) are only encountered as symbionts of diatoms like *Hemiaulus* spp., and *Rhizosolenia* spp. [75].

For photoautrophic diazotrophs, nitrogenase activity is intimately linked to photosynthesis [76]. Thus, light is an obvious and important factor potentially regulating or constraining this process. Carpenter [73] has summarized much of the early marine work largely relating to *Trichodesmium* spp. with respect to their relationship to light. Whereas many non-heterocystous cyanobacteria fix nitrogen during the night, and thereby uncouple N_2 fixation from photosynthesis, *Trichodesmium* fixes nitrogen exclusively during the light period and shows a strong diel pattern of activity with maxima during midday [59, 63, 69, 77]. Natural populations of *Trichodesmium*, often found in the upper layers of the euphotic zone, appear to be adapted to high light with a relatively shallow compensation depth (typically 100–200 mmol quanta m^{-2} s^{-1}) for photosynthesis [78].

To calculate how much nitrate is produced via N_2-fixation, Redfield stoichiometry can be used [79]. With the given $NO_{3\,exp}^-$ and the measured concentration of the actual dissolved inorganic nitrogen (DIN = NO_3^- + NO_2^- + NH_4^+), the amount of NO_3^-, which is removed by denitrification ($NO_{3\,def}^-$), can be calculated as follows:

$$NO_{3\,def}^- = NO_{3\,exp}^- - DIN \tag{11}$$

An alternative stoichiometric method involves the quasi-conservative tracer N* [80, 81]. Gruber and Sarmiento used high quality data from JGOFS and GEOSECS databases to develop a general relationship between fixed inorganic nitrogen (DIN) and phosphate (PO_4^{3-}) for the world's oceans:

$$N^* = DIN - 16\left[PO_4^{3-}\right] + 2.90 \quad (12)$$

The value of 2.90 is the deviation from the amount of DIN predicted by the Redfield stoichiometry (N:P =16) and the world-ocean N:P regression relationship. Negative values of N* are interpreted to show the net *denitrification* whereas positive values show the net *nitrogen fixation*.

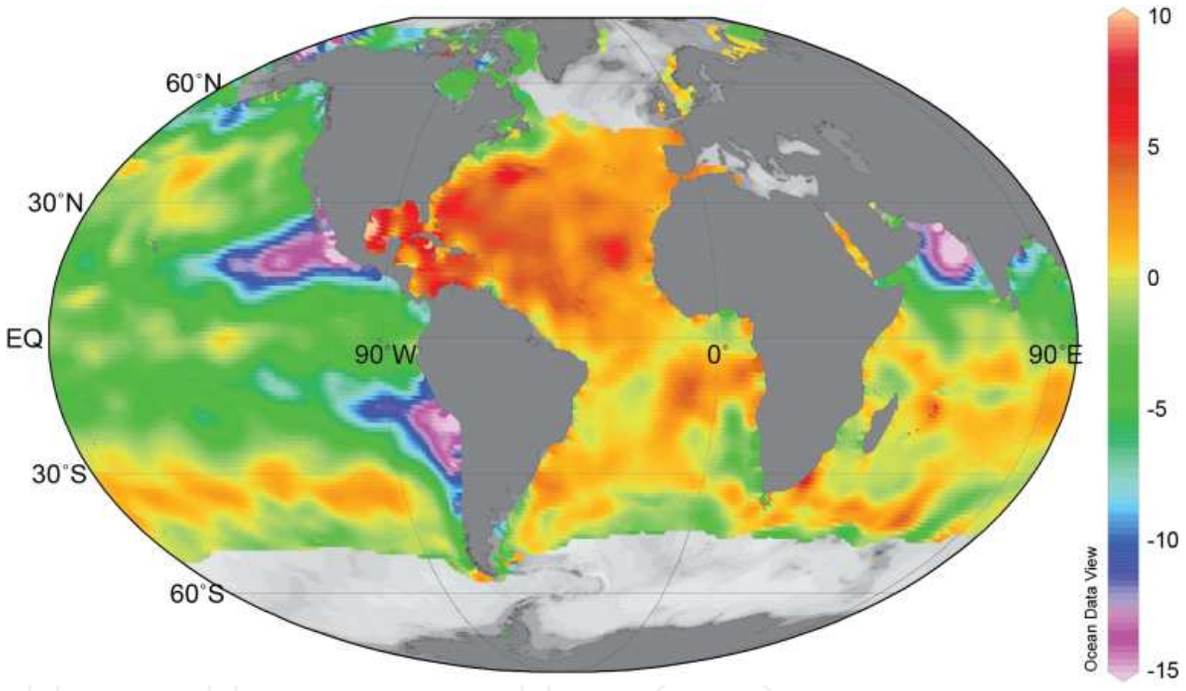


Figure 2. N*= [NO_4^{3-}] – 16*[PO_4^{3-}] + 2.9 on the 26.5 sigma theta surface (World Ocean Atlas 2005). Note the large negative N* values in the Tropical Pacific and Arabian Sea and positive N* values in the Tropical Atlantic Ocean.

Strong positive N* values have been observed in the tropical and subtropical North Atlantic (fig.2) and are proposed to be a result of the input into these areas of diazotrophic (i.e. nitrogen fixer) biomass with a higher N:P content than that typical of eukaryotic phytoplankton of the upper ocean [80-82]. The observation of relatively high concentrations of DON in surface waters of regions of the tropical oceans have also been attributed to nitrogen fixation [83-86]. At the Hawaiian Ocean Time (HOT) series station, pools of DON increased during a period in which microbial nitrogen fixation also became more prominent [87, 88].

Iron limitation may be important for cyanobacterial N_2 fixation as Fe is a cofactor in photosynthesis and nitrogenase, leading to is ~5 times higher Fe requirement for diazotro-

phy than that for ammonia assimilation [77]. Even though the concentration of dissolved Fe (dFe) is low in many of the environments where N_2 fixation occurs [89-92], a number of studies have speculated on diazotroph response to mineral dust Fe fertilization [93, 94]. A 1999 Saharan dust event coincided with increases in dissolved Fe concentrations on the west Florida shelf and a 100-fold increase in *Trichodesmium* biomass [95]. N_2 fixation rates were not measured, but DON concentrations doubled, presumably due to exudation by N_2 fixers [95]. A Saharan dust addition experiment to surface water samples collected along a west African cruise transect (35°W–17°W), found a minimal increase in CO_2 fixation and a large stimulation in N_2 fixation, suggesting that diazotrophs were co-limited by both P and Fe [96]. It should be noted however, the analysis of aerosol dust shows that while providing Fe, the dust also supplies P and combined N [96-98]. Work on *Trichodesmium* spp. culture has identified mechanisms to deal with P limitation. For instance, under P stress conditions it uptakes a greater amount of DIP and dissolved organic P (DOP), decreasing the requirement for cellular P.

N_2 fixation is an energetically costly process in comparison to ammonia assimilation, thus it is logical to assume that presence of high concentrations of fixed N play a role of an inhibitor. Indeed, under mM concentrations of ammonia (concentrations essentially never observed in the real surface ocean), N_2 fixation is inhibited in most diazotrophs [99]. However, mM concentrations of NO_3^- stopped N_2 fixation in some strains [100] but have little to no effect in others [101]. The higher energy requirement for the nitrate reduction compared to 'direct' assimilation of ammonia might explain these differences. Addition of ammonia to natural samples of *Trichodesmium* spp. can shut down nitrogenase activity [69]. However, pure culture studies have shown mixed results, with some cultures indicating no reduction of their activity [102] and others showing a reduction in activity under short-term additions of fixed N sources [103]. Interestingly, indication for N_2 fixation has been found also in the nitrate-rich upwelling areas off Chile and Peru [104, 105], indicating that N_2 fixation may not be as strongly regulated by fixed N as previously presumed.

4.1.2. Nitrogen fixation in sediments

Within the marine realm, N_2 fixation in sediments has been mostly recognized in pelagic environments or in benthic microbial mats, sea grass sediments, and coral reef systems [106]. A recent study by Bertics et al. [107] revealed that N_2-fixation, coupled to sulfate reduction, is stimulated by bio-irrigation in organic-rich coastal sediments. The authors suggested that N_2-fixation has been largely overlooked in sediments, and that its occurrence might be significant, contradicting the general belief that sediments are areas of net nitrogen loss through denitrification and anaerobic ammonium oxidation [108, 109].

4.2. Nitrification

Nitrification process oxidizes NH_4^+ into NO_2^- and further to NO_3^-. Ammonium rarely occurs at high concentrations in the surface ocean as it is rapidly recycled between heterotrophic and N_2 fixing organisms and many heterotrophic and photosynthetic plankton species. N_2 fixing

organisms play a role of ammonia source as they excrete NH_4^+ directly or release organic N that is microbialy degraded to NH_4^+, while plankton utilize ammonia as a N source for building up its biomass. Similarly, accumulation of NO_2^- is rarely observed in oxygenated habitats, although NO_2^- is an essential intermediate in several oxidation and reduction processes in the N cycle.

Nitrification is carried out through a combination of two microbial processes: ammonia oxidation to NO_2^- and nitrite oxidation to NO_3^-. The first step of the process is enabled by nitrifying organisms, ammonium-oxidizing bacteria and archaea (AOB and AOA), while the second step is carried out by nitrite-oxidizing bacteria (NOB). No known organism can carry out both reactions. The overall reaction of NH_4^+ oxidation (eq. 13) for AOB shows that the process consumes molecular oxygen and produces NO_2^- and hydrogen ions. Molecular oxygen is required in the first step of the reaction, which is catalyzed by a monooxygenase (NH_3 monooxygenase, AMO). Oxygen is also consumed by the terminal oxidase as a result of electron transport generating adenosine-5′-triphophate (ATP) for cellular metabolism. The immediate product of AMO is hydroxylamine, which is further oxidized by hydroxylamine oxidoreductase (HAO) to NO_2^-. In contrast, AOA apparently do not possess the hydroxylamine reductase gene, so the pathway of ammonia oxidation in these organisms must be quite different.

$$NH_4^+ + O_2 + H^+ + 2\,e^- \xrightarrow{AMO} NH_2OH + H_2O \xrightarrow{HAO} NO_2^- + 5\,H^+ + 4\,e^- \quad (13)$$

Depending on the conditions, NO, N_2O and even N_2 have been reported as secondary products in autotrophic NH_4^+ oxidation by both marine and terrestrial strains [110, 111]. Although N_2O and NO can be produced in vitro by HAO from hydroxylamine [112], the reduction of NO_2^- appears to be the dominant pathway in all cells [113-115]. One of the first studies of kinetic isotopic effect of nitrification in pure cultures of *Nitrosomonas europaea* was made by the Mariotti group [116], where a fractionation factor of 34.7 ± 2.5‰ was determined for conversion of NH_4^+ into NO_2^-. The later work of Casciotti [117] identified that the fractionation factor for different *Nitrosomonas* cultures of ammonia-oxidizing bacteria was linked to amino acid sequences. The isotopic effect in these cultures ranged from 14.2‰ to 38.2‰.

N_2O is produced by AOB through two separate pathways: hydroxylamine decomposition and nitrite reduction, so-called "nitrifier denitrification" [114]. The isotopic compositions ($\delta^{15}N_{bulk}$, $\delta^{18}O$, $\delta^{15}N_{\alpha}$, $\delta^{15}N_{\beta}$, and site preference = $\delta^{15}N_{\alpha} - \delta^{15}N_{\beta}$) of the produced N_2O can provide insight into the mechanisms of N_2O production under different conditions [48, 50, 118]. For example, N_2O production through nitrifier denitrification has low $\delta^{15}N_{bulk}$ and low site preferences relative to that produced by hydroxylamine decomposition [119]. As discussed earlier, the N_2O site preference (SP) is determined by the enzymatic mechanism [49, 120]. The site preference of N_2O produced during nitrification is +30‰ to +38‰, while N_2O production via denitrification and nitrifier-denitrification has a SP of -10‰ to +5‰ [48, 118, 121, 122]. The large difference between the SP values of these two mechanisms of N_2O production provides a good tool with which to distinguish their contributions.

The biochemistry of the NO_2^- oxidation is simpler than that of NH_4^+ oxidation because it is only involves a transfer of two electrons without intermediates (eq. 14). The additional oxygen atom in NO_3^- is derived from water, and the molecular oxygen that is involved in the net reaction results from electron transport involving cytochrome oxidase.

$$NO_2^- + H_2O \xrightarrow{\text{nitrite oxydoreductase}} NO_3^- + 2H^+ + 2e^- + 0.5\,O_2 \xrightarrow{\text{cytochrome oxidase}} NO_3^- + H_2O \quad (14)$$

A recent study of isotopic fractionation during nitrite oxidation to nitrate [1] showed a unique negative isotopic effect of -12.8‰. Because of the inverse isotope effect, when nitrite oxidation is taking place, the $\delta^{15}N_{NO2}$ (and $\delta^{18}O_{NO2}$) values are expected to be lower than the NO_2^- initially produced by ammonia oxidation or nitrate reduction. In most parts of the ocean, however, NO_2^- does not accumulate and the isotope effects of different processes complicate the interpretation. In the euphotic zone NO_2^- is either oxidized to NO_3^- or assimilated into particulate nitrogen (PN) and the heavy isotope can be preferentially shunted in one direction vs. the other [119].

4.2.1. Controlling factors of nitrification and its distribution

Nitrifying bacteria are traditionally considered to be obligate aerobes; they require molecular oxygen for reactions in the N oxidation pathways and for respiration. A significant positive correlation between apparent oxygen utilization (AOU) and N_2O accumulation is often observed in marine systems [123-125]. The relationship implies that nitrification is responsible for N_2O accumulation in oxic waters where it is released as a byproduct. The relationship breaks down at very low oxygen concentrations (~6 mM, [126]), where N_2O is consumed by denitrification.

There is abundant evidence from culture studies that both AOB and NOB are photosensitive. Several studies of nitrification rates in surface seawaters from various geographical regions show profiles that are consistent with light inhibition of both NH_4^+ and NO_2^- oxidation [127-129]. The highest nitrification rates occur in a region near the bottom of the euphotic zone. It is in this interval where nitrifying bacteria can compete with phytoplankton for NH_4^+, as the rates of nutrient assimilation are reduced due to light limitation. A sharp peak of nitrification rate is often observed at water column depth where the light intensity is 5–10% of surface light intensity [118, 127-129].

In both deep and shallow sediments, nitrification can be one of the main sinks for oxygen [130, 131]. In continental shelf sediments, nitrification and denitrification are often closely linked. Coupled nitrification/denitrification is invoked to explain the observation that the rate of N_2 flux out of sediments can greatly exceed the diffusive flux of NO_3^- into the sediments [132]. Ammonium is produced during aerobic and anaerobic remineralization of organic matter. It is then oxidized to NO_3^- and subsequently reduced to N_2. Anaerobic oxidation of NH_4^+ can be a factor supporting the imbalance between supply and consumption of NO_3^-. Nitrification can supply up to 100% of the NO_3^-, which is later consumed by denitrification [133, 134].

Although oxygen and NH_4^+ conditions likely differ between planktonic and sediment environments, there is no clear evidence from clone libraries that water column and sediment nitrifying communities are significantly different in composition and regulation.

To measure nitrification rates, isotope dilution can be used. In this method, the isotope dilution of the NO_2^- pool (in the case of NH_4^+ oxidation) or the NO_3^- pool (in the case of NO_2^- oxidation) is monitored over time [135]. Alternatively, in the ^{15}N method $^{15}NH_4^+$ or $^{15}NO_2^-$ is added to a sample and then the appearance of ^{15}N label in the NO_2^- or NO_3^- pools is measured over time [24]. One disadvantage of the method is that the increase in NH_4^+ or NO_2^- concentrations caused by the tracer addition can result in enhanced NH_4^+ or NO_2^- oxidation rates [129]. When small additions of ^{15}N are used, the isotope dilution of the substrate pool must be monitored over time.

4.3. Nitrogen assimilation

Nitrification and nitrogen assimilation are usually found at similar depths in the ocean as they compete for the same reactants (NH_4^+). At the same time, nitrification, producing NO_2^- and NO_3^-, provides assimilation with additional reactants. Nitrogen assimilation is the process of incorporation of reactive nitrogen species (NO_3^-, NO_2^- and NH_4^+) into the bacterial cell. Ammonium, which is produced in the photic zone by heterotrophic processes, is assimilated immediately by phytoplankton and heterotrophic bacteria before it can be nitrified. Ammonium is often the dominant form of dissolved N assimilated in a variety of marine and estuarine systems. This form of N is energetically efficient for cells to use, because it is already reduced and is a common cellular transient in N metabolism, requiring little additional energy for assimilation. Despite the low NH_4^+ concentrations in oceanic systems, uptake and regeneration of NH_4^+ are tightly coupled and result in rapid turnover times [136].

Nitrate concentrations in the surface ocean are usually maintained at low levels as a) phytoplankton assimilate NO_3^- rapidly and b) nitrate can be supplied by mixing or diffusion from the deep NO_3^- reservoir only at the regions with strong upwelling. The assimilation of NO_3^- is more energetically demanding than NH_4^+, because it requires a synthesis of NO_3^- and NO_2^- reductases, associated active transport systems, and the turnover of cellular ATP and nicotinamide adenine dinucleotide phosphate (NADPH) [136]. In order to assimilate NO_3^- for growth, phytoplankton must first possess the genetic capacity to synthesize the necessary enzymes and transport systems- a capacity which not all phytoplankton have. Further, the supply of NO_3^- is limited by nitrification and vertical mixing.

Since nitrite reductase (NiR) is required for NO_3^- assimilation, organisms that assimilate NO_3^- can by default assimilate NO_2^-. Consequently, some organisms that cannot use NO_3^- can still use NO_2^- (e.g., some clones of *Prochlorococcus*; [137]). It has been suggested that this rather unique N physiology has arisen from the evolutionary loss of genes, which are necessary for the assimilation of NO_3^- [138, 139]. The resultant evolution of many ecotypes specifically adapted for unique environments where NO_2^- production can be high.

Nitrogen isotope fractionation can take place at different stages of the processes. Wada and Hattori [140] first argued that N isotope fractionation by phytoplankton occurs during *nitrate*

reduction, while Montoya and McCarthy [141] ultimately favored fractionation associated with *nitrate transport* into the cell. The N isotope data presented in Granger at al. [142] suggest that an isotope effect is driven solely by the reductase, and not by uptake at the cell surface and that this effect results in a fractionation factor of 5 – 10‰. Ammonium assimilation is shown to have higher fractionation factor of +14 – +27‰ [143, 144].

In most parts of the ocean, however, assimilation and nitrification occur concurrently, which additionally complicates the isotope analysis. In general, nitrite oxidation, as discussed above, transfers NO_2^- with an elevated $\delta^{15}N$ to the NO_3^- pool, while nitrite assimilation transfers the residual NO_2^- with a lower ^{15}N:^{14}N ratio into the PN pool. If the fractionation factor of nitrite assimilation is 1‰ [144] and fractionation factor of nitrite oxidation is -15‰ [1], then the $\delta^{15}N_{NO2}$ at steady state will be lower than the source of NO_2^-, unless nitrite assimilation is >95% of the NO_2^- sink. This has the opposite effect of the ammonia oxidation/assimilation branching where ammonia oxidation transfers low ^{15}N:^{14}N material into the NO_2^- and NO_3^- pools and higher ^{15}N:^{14}N material into the PN pool [119].

4.3.1. Factors controlling nitrogen assimilation and its distribution

Most nitrogen assimilation takes place in the surface waters of the ocean. The most important factors controlling assimilation are the presence of oxygen for respiration and/or light for photosynthesis and enzymes responsible for metabolic processes. Most of the enzymes involved in the uptake and assimilation of N are bound to energy sources and thus are affected by light, presence of oxygen, and the supply of enzyme co-factors and metabolic substrates. For example, uptake and reduction of NO_3^-, NO_2^-, and urea have been linked to the light supply in phytoplankton due to the necessity for ATP and NADPH from photo-phosphorylation. While uptake and reduction of these compounds is thought to proceed at maximum rates only in the light under nutrient replete conditions, active uptake of these compounds may occur even in the dark [145].

The accumulation of intracellular product pools, on the other hand, can result in the inhibition of uptake and assimilation. The posttranslational modification of enzymes can regulate uptake and assimilation by modulating the number of active sites available for catalysation of specific reactions. Studies examining the transcriptional activation of enzymes involved in the process have begun to demonstrate clear functional relationships between genomes and ecological capabilities. For example, the presence of NH_4^+ represses protein expression (i.e., enzymes) involved in the assimilation of alternative N sources (e.g., NO_3^- and N_2) in some organisms [146]. Overall, most factors controlling assimilation are directly connected with enzymes, which require substrate compounds or energy and oxygen supply for their metabolic activity.

4.4. Nitrogen loss (denitrification and anammox) in the ocean

The term "nitrogen loss" is commonly used for the processes in which fixed nitrogen (nitrate, nitrite or ammonia) is converted into non-bioavailable N_2 in the absence of oxygen. This includes denitrification process, in which combined nitrogen is reduced to gaseous end products, as well as newly discovered process of anammox (anaerobic ammonium oxidation),

which converts nitrite and ammonia into N_2 [147]. Another important process of reduction is DNRA (dissimilatory nitrate reduction to ammonia) will be also discussed in this section as it is an important source of ammonia in the ocean. Even though the reduction of nitrate to ammonia is not a N-loss as such, but it is also taking place in suboxic waters, and the ammonia produced can diffuses up to the anoxic waters and may well be oxidized to N_2 by anammox bacteria [148].

4.4.1. Canonical denitrification

Canonical denitrification is a heterotrophic process in which nitrogen oxides serve as the terminal electron acceptor for organic carbon metabolism [149]. The nitrogen oxides are reduced mainly to molecular nitrogen and some nitrous oxide may be formed as a side product. The sequential reduction is enabled by four well-studied enzyme systems: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase [150] (eq. 15).

$$NO_3^- \xrightarrow{\text{nitrate reductase}} NO_2^- \xrightarrow{\text{nitrite reductase}} NO \xrightarrow{\text{nitric oxide reductase}} N_2O \xrightarrow{\text{nitrous oxide reductase}} N_2 \quad (15)$$

The intermediate NO_2^- is known to escape the cell and is frequently found in denitrifying environments; likewise, N_2O also can accumulate externally [151]. This leads to local maxima of both intermediates, which tend to occur near the boundaries of the suboxic zone. Another intermediate, NO, is actually a free radical and, thus, very reactive and highly toxic to most bacteria including denitrifiers [152]. In order to minimize the accumulation of NO within the cell, nitrite reductase and nitric oxide reductase are controlled interdependently at both the transcriptional and enzyme activity levels [152, 153].

4.4.2. Anammox

Anammox bacteria are chemoautotrophic bacteria that fix CO_2 using NO_2^- as the electron donor [154] and are thought to be strict anaerobes. Recent publication on the tolerance of anammox bacteria to oxygen levels, however, showed that they can perform a significant activity even under ~20 μmol/l O_2 concentrations [155]. All anammox species found have evolved a membrane-bound intra-cytoplasmic compartment called the anammoxasome. The anammoxasome membrane is made up of high-density lipids, called ladderanes because of their ladder-like structure, which are thought to be specific to anammox bacteria. The proposed mechanism of anammox involves a hydrazine hydrolase, which catalyzes the combination of hydroxyl ammine and ammonium to form hydrazine. The hydrazine-oxidizing enzyme subsequently oxidized it to N_2 [156, 157]. The ladderane-lipid membrane is thought to act as a barrier to diffusion thus isolating the toxic intermediates of the anammox reaction within the anammoxasome. Interestingly, for every 1 mol of N_2 produced, 0.3 mol of NO_3^- is also produced alongside from NO_2^-. This reaction is believed to be important for replenishing electrons for the acetyl-CoA carbon fixation pathway [158]. At the same time, these bacteria can perform dissimilatory NO_3^- reduction to NH_4^+ (DNRA), which is subsequently combined with NO_2^- to produce N_2, thus mimicking denitrification [159].

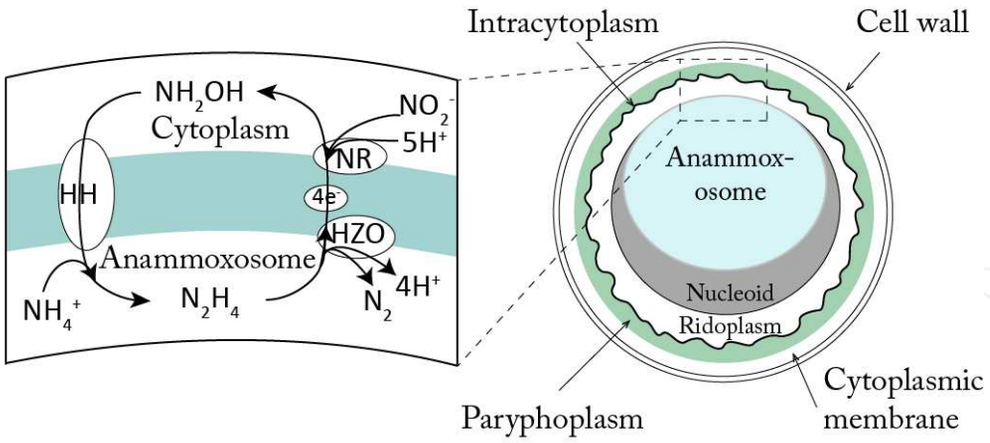


Figure 3. Morphology of the anammox cell and proposed model for the anammox process. HH, hydrazine (N_2H_4) hydrolase; HZO, hydrazine oxidizing enzyme; NR, nitrite reducing enzyme [160].

Anammox bacteria have been identified in many naturally occurring anoxic environments including marine, freshwater, and estuarine sediments [161, 162], oxygen minimum zones [163, 164], soils [165-167], wetlands [168], and anoxic tropical freshwater lakes [169]. These bacteria are not only widespread but may also be very active (e.g. ~67% fixed N-loss [162]). Anammox organisms may live in low abundance but are capable of significant growth and metabolism when a shift in environmental parameters permits anammox communities to grow [170]. The anammox bacteria are slow growing, with a doubling time of ~11 days, even under optimal growth conditions in bioreactors [171]. The state-of-the art method to identify anammox is currently based on isotopic labeling of NH_4^+ or NO_x^-. It relies on the fact that N_2 produced by anammox combines 1:1 atoms from NH_4^+ and NO_2^- [172]. The selective formation of $^{15}N^{14}N$ ($^{29}N_2$) rather than $^{15}N^{15}N$ ($^{30}N_2$) is a clear evidence of anammox activity[162].

4.4.3. Dissimilatory nitrate reduction to ammonium or DNRA

The dissimilatory reduction of nitrate to ammonium is a bacteria-mediated heterotrophic process occurring in anaerobic conditions. Nitrate is first reduced to nitrite and then to NH_4^+. Bock et al. [173] also showed that *N. europaea* and *N. eutropha* were able to nitrify and denitrify at the same time when grown under oxygen limitation ([O_2] ~ 0.2 - 0.4 μmol/l). Under these conditions, oxygen and NO_2^- served simultaneously as electron acceptors and both N_2 and N_2O were produced, whereas under anaerobic conditions N_2 was the predominant end product. This type of nitrification–denitrification pathway may help to explain why ammonium oxidizers remain active in nearly suboxic environments and enhanced N_2O production is observed [174, 175].

The reaction has been reported for sediments underlying anoxic waters [176, 177] and sediments with substantial free sulfide, possibly due to sulfide inhibition of nitrification and denitrification [42]. Suboxic NO_3^- reduction to NH_4^+ appears to occur in environments where there is excess available carbon, such as estuarine sediments [178]. Perhaps more important are the NO_3^-fermenting organisms such as *Thioploca* and *Thiomargarita*, that are found in

sediments underlying the major suboxic-denitrifying water columns of the Arabian sea, eastern Tropical Pacific and Namibia [179-181]. These organisms are able to couple the reduction of NO_3^- to NH_4^+ with the oxidation of reduced sulfur compounds. Both *Thioploca* and *Thiomargarita* are able to concentrate NO_3^- at up to 500 mM levels in large vacuoles within their cells for subsequent sulfide oxidation [180, 181].

4.4.4. Controlling factors for N-loss processes and its distribution

The controlling factor for all N-loss processes is molecular oxygen. When the concentration of the denitrification intermediate NO_2^- is plotted against dissolved oxygen, we see that NO_2^- does not appear in the water columns of the Eastern Tropical Pacific until oxygen concentrations are reduced below about 2 μM (see figure 4 [182, 183]).

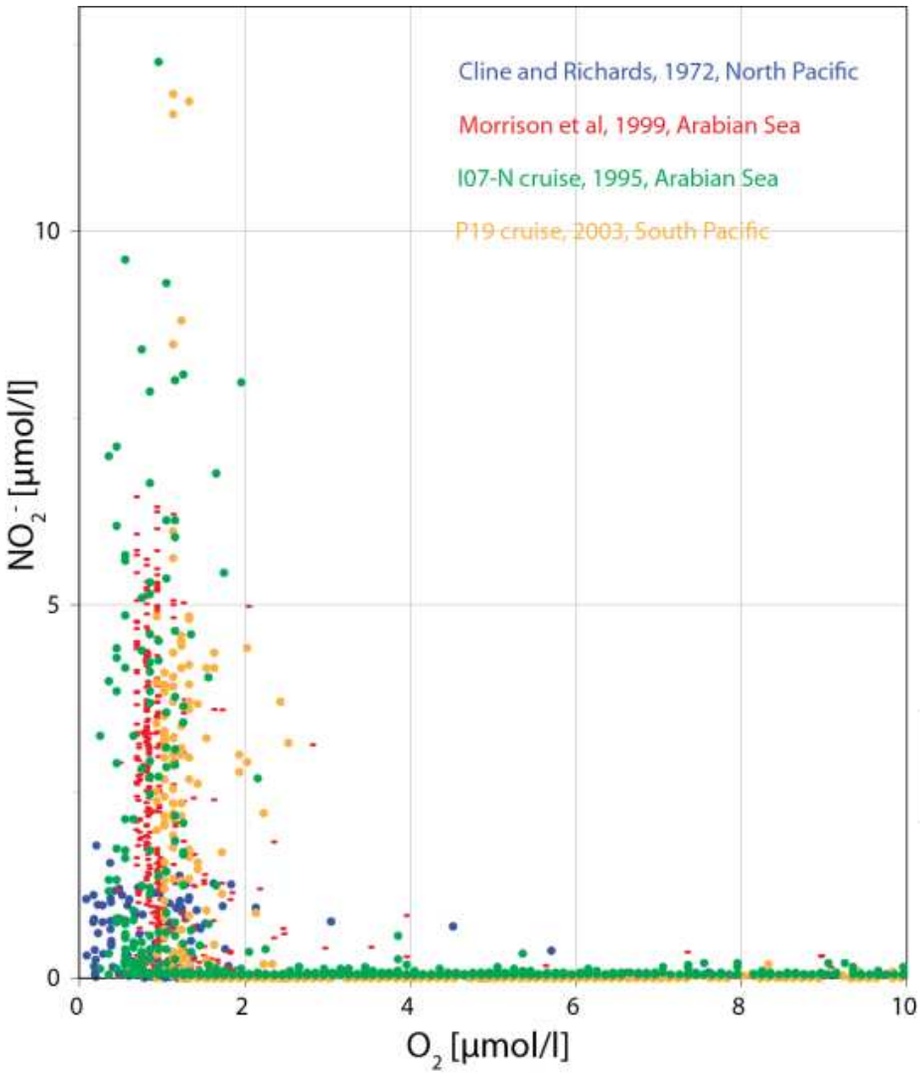


Figure 4. Dissolved oxygen versus nitrite for OMZs. Presented data are taken from publically available cruises I07-N, P19 (http://woce.nodc.noaa.gov/woce_v3/wocedata_2/bathymetry/default.htm) and publications of Cline & Richards [182] and Morisson [183].

Another indication for the denitrification process is consumption of N_2O within the water column, where oxygen concentrations are reduced levels less than c. 5 μM [65]. In oceanic systems the relative importance of heterotrophic denitrification, compared to autotrophic anammox has been debated and believed to depend on the amount of organic matter (OM) available [184, 185]. However, in most of the cases N-loss processes co-exist and it is rather a question which one is playing the first fiddle [186, 187].

About less than half of the current marine denitrification of the global ocean is thought to occur in the three main pelagic Oxygen Minimum Zones (OMZs): Eastern Tropical North Pacific (ETNP), the Eastern Tropical South Pacific (ETSP) and the Northern Arabian Sea (Fig. 4). These zones occur in intermediate waters (~150–1000 m) in locations where the ventilation rate is insufficient to meet the oxygen demand. To calculate nitrogen loss in the water column, the amount of substrate that is consumed in N-loss processes or the amount of N_2 produced should be determined. Similar to estimations of the N-fixation, the Redfield stoichiometry is used to calculate N-deficit or N*. Negative values of N* are interpreted to show the net *N-loss* whereas positive values show the net *nitrogen fixation* (see above).

Devol et al. [188] used N_2:Ar ratios in the Arabian Sea OMZ to determine the amount of N_2 produced during denitrification. The amount of N_2 excess in the OMZ was computed from the increase in the N_2:Ar ratio over that present in the source waters. Measurements of N_2 excess predicted a larger nitrogen anomaly than that estimated by nitrate deficit. The discrepancy is said to be due to incorrect assumptions of the Redfield stoichiometry [189]. Inputs of new nitrogen through N-fixation, N_2 contributions from sedimentary denitrification along continental margins, the anammox reaction, or metal catalyzed denitrification reactions all lead to a shift of the N:P ratio from the Redfield stoichiometry.

Due to N-loss processes, i.e. denitrification and anammox, the $\delta^{15}N$ values in the water column increase to 15‰ or more. For the eastern tropical North Pacific OMZ Brandes et al. [190] calculated the fractionation factors between 22‰ (Arabian Sea closed system model) and 30‰ (eastern tropical North Pacific open-system model). Several authors [190-193] have observed high nitrogen isotopic values and nitrate deficits in the Pacific and Arabian Sea OMZ's and estimated fractionation factor, which all cluster around a value of +25‰. The fractionation factor of N-loss processes in the sediments was shown to be significantly lower (~3 ‰) [194]. Thus, in the waters with strong upwelling, the water column signal can be altered via mixing with water originating from the sediments.

4.4.5. Sedimentary denitrification

Another location where oxygen is typically depleted and denitrification takes place is in marine sediments, especially the continental margin and hemipelagic sediments. Respiratory processes within the sediments act as sinks of oxygen, while overlying water deliver nitrate to the sediment. In most continental shelf sediments, oxygen penetrates to less than 1 cm below the sediment–water interface and even in most deep-sea sediments oxygen penetration is restricted to the approximately upper 10 cm. Thus, ample environments exist for N-loss in marine sediments. Due to the fact that marine sediments are usually rich with organic matter, denitrification and DNRA are the prevailing mechanisms in the sediments [176].

5. Modern ocean nitrogen budget

The discussion of N-budget is usually based on balance or imbalance of nitrogen sources and sinks. Nitrogen sources in this case are N_2-fixation, atmospheric wet and dry deposition, and riverine input. Nitrogen sinks include denitrification and anammox in the water column, sedimentary denitrification, and sedimentation of organic matter. Nitrification and DNRA processes "recycle" nitrogen from ammonia into nitrate and vice versa. Although the primary sources and sinks are relatively well known, the quantification of their fluxes is associated with considerable uncertainty [81, 195-199] (see figure 5). The major problem in calculating global budgets of N sources and sinks is extrapolating measured fluxes from a limited number of oceanic regions to the global ocean.

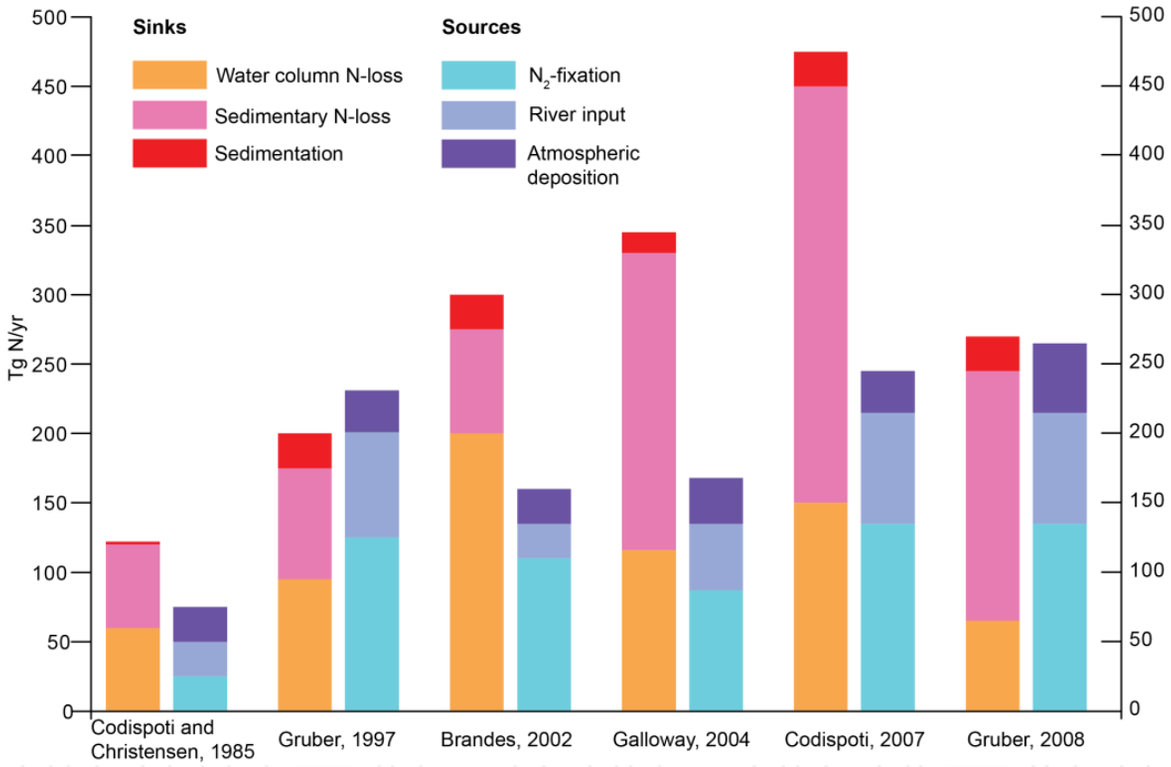


Figure 5. Nitrogen budget estimations in the literature.

Additional complications in calculating N budgets are caused by interplay between and potential coexistence of N cycle processes at the same depth. A schematic water column profile with corresponding processes over O_2, NO_2^-, and N_2O gradients is presented in the figure 6 (based on water column profiles in the South Pacific ocean [65]). In the surface layer, nitrogen assimilation and rapid recycling is taking place, producing nitrite where oxygen and bioavailable N are plentiful enough. Ammonia oxidation (first step of nitrification) lead to efflux of N_2O [200] in the surface waters. Ammonium release and its oxidation in the well-oxygenated waters lead to high production of NO_2^- (primary nitrite maximum). At oxygen concentration ~ 5 μmol/l, denitrification process become more preferential producing to "secondary nitrite

maximum" in the OMZ. This NO_2^- can also be a source for anammox process at the top of OMZ, while DNRA process can provide a source of ammonia [148]. N_2O consumption in the water column provides a strong evidence for denitrification producing N_2. At the borders of OMZ nitrification and denitrification processes may coexist [196], leading to production of N_2O at these water depths.

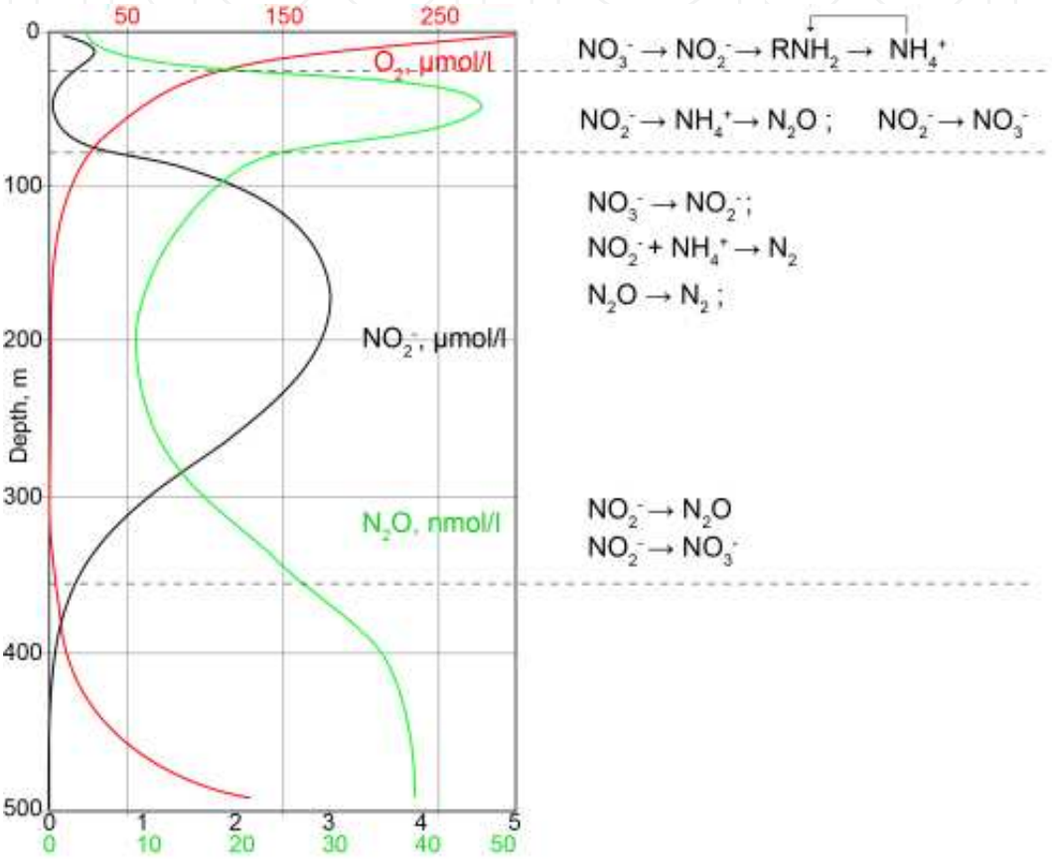


Figure 6. Schematic water column profile with corresponding processes over O_2, NO_2^-, and N_2O gradients.

Nitrogen isotope ratios in the water column and in sedimentary records provide additional constraints for nitrogen budget calculation. Average $\delta^{15}N$ of fixed nitrogen in the deep ocean is close to 5‰. The sedimentary records show that this global average increased during deglaciation periods, when stronger denitrification was taking place. However, the global average $\delta^{15}N$ range for the last 30.000 years was in the range 4-6‰ [201], which suggests that N sources and sinks should be balanced, at least the millennial time scale. In this case, the processes increasing $\delta^{15}N$ (assimilation, N-loss) has to be balanced with those, which are decreasing it (N_2-fixation).

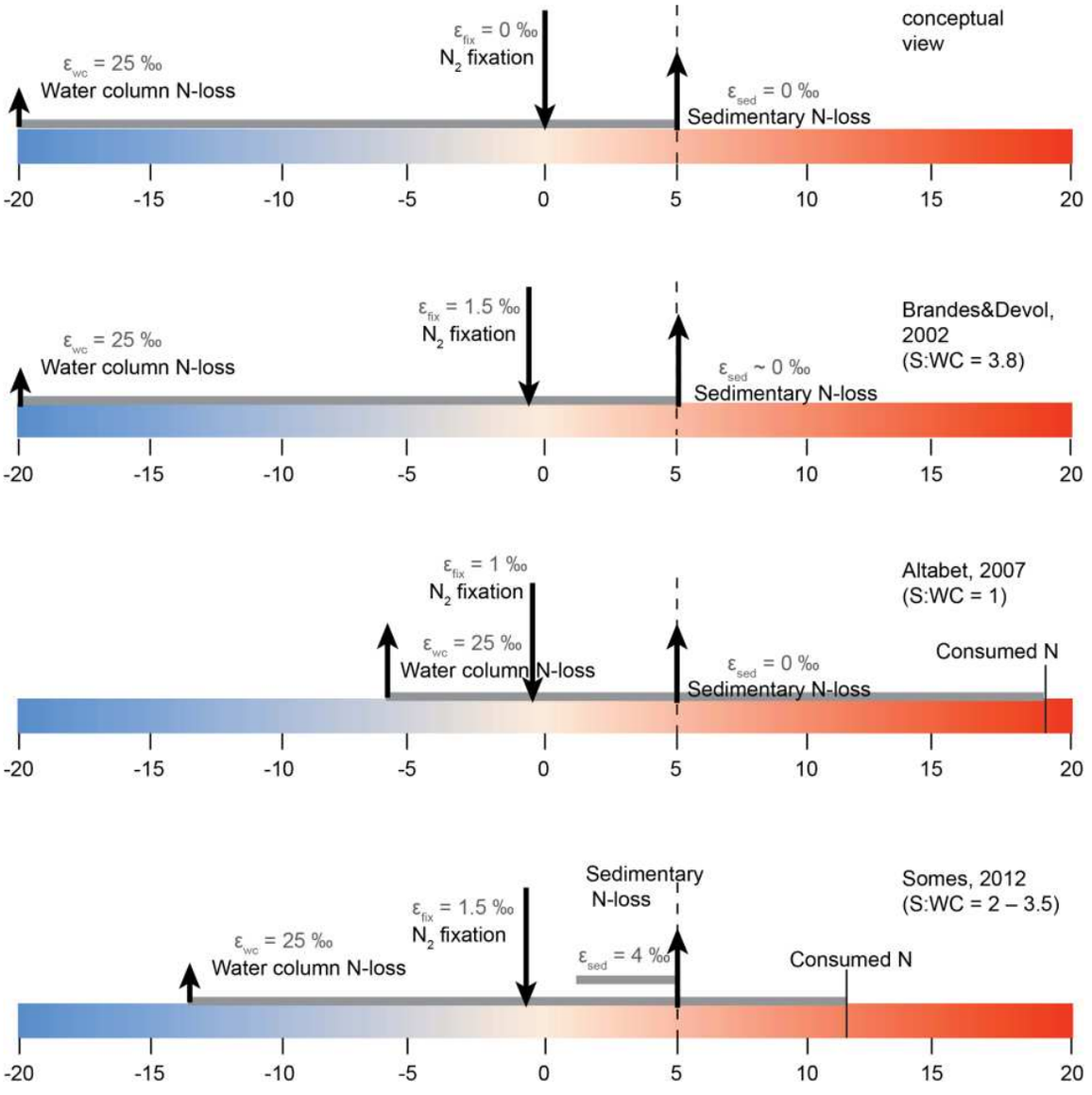


Figure 7. Nitrogen isotope range and processes influencing on the ^{15}N budget. Dashed line at 5‰ represent an oceanic average value [197, 202, 203].

The conceptual model of the nitrogen isotope budget is initially based on two processes: N_2-fixation and denitrification. Sedimentary denitrification was believed not to change the isotopic signature (ε_{sed}) of nitrogen due to N-limitation in the sediments, while water column denitrification (ε_{wc}) has a fractionation factor of ε between 25 and 35 ‰. The N_2- fixation fractionation factor ε_{fix} is close to 0‰ as it incorporates nitrogen from the atmosphere. An early attempt to construct a one-dimensional model was undertaken by Brandes and Devol [197], where they set fractionation factor to ε_{fix} and ε_{sed} to 1.5‰; and ε_{wc} to 25‰. On the figure 8 scale of nitrogen isotope is illustrated. In the Brandes and Devol model, sedimentary N-loss does not change the ^{15}N budget substantially (ε_{sed} in the range of 0 to 1.5‰). Thus, a large amount of sedimentary denitrification is necessary to balance water column denitrification and bring overall isotopic values of the losses into the range of sources, leading to the value of Sediment

to Water column denitrification (W:WC) ratio of 3.8. The authors noticed that with fluxes estimations of total N-loss of 400 Tg N/yr and fixation of 100 Tg N/yr, "the combined budget would be out of balance by over -200 Tg N/yr" [197]. This implies that fixation rates should be 2–3 times higher than 100 Tg N/yr, which was an accepted value at that time. Recent observations that N_2- fixation is indeed underestimated by up to two fold support these calculations.

More recent calculation of the nitrogen isotope budget in year 2007 included so-called "consumed-N" into the model, which represents nitrogen utilization in the euphotic zone [202]. During N utilization, nitrogen fractionation takes place, shifting the "initial" value of the ambient NO_3^- from +5‰ to 17 ‰. Water column N-loss leads to production of N_2 gas with $\delta^{15}N$ values 25‰ lighter than the "initial". This process shifts the nitrogen isotope budget significantly and even leads to balance of water column and sedimentary denitrification (S:CW =1). A recent model of the pre-industrial N-budget includes a sedimentary fractionation factor ε_{sed} of 4‰ and ε_{fix} of 1.5‰ [203]. The value of "consumed-N" was set to +12‰, taking into account the fractionation factor of utilization of 7‰. In this model, the S:CW denitrification ratio varies from 2 to 3.5. Current budget estimates can, however, differ from these prior estimates due to an increase of riverine and atmospheric input of nitrogen into the ocean [198].

6. Conclusions

Of the two approaches whereby nitrogen isotope measurements are used to study marine N cycle processes, stable isotope natural abundance measurements have the great advantage of providing information on integrated processes with little or no manipulation of the system. In contrast tracer-based rate measurements are better used for rate measurements. The natural abundance and tracer approaches are strongly complementary, providing insight into the nitrogen cycle on very different spatial and temporal scales. The magnitude of processes that have distinctive isotopic fractionation patterns can potentially be quantified if the fractionations are known accurately. Thus, there is a significant ongoing need for careful measurement of isotopic fractionation patterns under controlled laboratory conditions as well as under field conditions, which in the best case should be combined. In order to interpret isotopic distributions and trends and to construct meaningful isotopic budgets, the openness of the system (ventilation rates) and the nature of N-exchanges should be known.

The growing set of $\delta^{15}N$ measurements makes it increasingly feasible to incorporate isotopic constraints into ecosystem and biogeochemical models of nitrogen in the ocean. A robust isotope budget for the ocean will require extension of these sampling efforts. Time-series measurements from a variety of locations will also greatly enhance the utility of nitrogen isotope measurements, particularly in quantifying the flux of nitrogen through the biota during blooms and other transient events. Such transient events are effectively natural tracer experiments created by isotopic fractionation during phytoplankton consumption of inorganic nitrogen and could provide an ecosystem-level analog to the short-term tracer experiments typically carried out in small volumes aboard ship. Complementing nitrogen stable isotope measurements with focused rate measurements may ultimately provide the best strategy for studying the dynamics of the nitrogen cycle across a variety of temporal and spatial scales.

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