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### Quantification of multiple simultaneously occurring nitrogen flows in the euphotic ocean

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Abstract. The general features of the N cycle in the sunlit region of the ocean are well known, but methodological difficulties have previously confounded simultaneous quantification of transformation rates among the many different forms of N, e.g., ammonium  $(NH_4^+)$ , nitrite  $(NO_2^-)$ , nitrate  $(NO_3^-)$ , and particulate/dissolved organic nitrogen (PN/DON). However, recent advances in analytical methodology have made it possible to employ a convenient isotope labeling technique to quantify in situ fluxes among oft-measured nitrogen species within the euphotic zone. Addition of a single <sup>15</sup>N-labeled  $NH_4^+$  tracer and monitoring of the changes in the concentrations and isotopic compositions of the total dissolved nitrogen (TDN), PN,  $NH_4^+$ ,  $NO_2^-$ , and  $NO_3^-$  pools allowed us to quantify the <sup>15</sup>N and <sup>14</sup>N fluxes simultaneously. Constraints expressing the balance of <sup>15</sup>N and <sup>14</sup>N fluxes between the different N pools were expressed in the form of simultaneous equations, the unique solution of which via matrix inversion yielded the relevant N fluxes, including rates of  $NH_4^+$ ,  $NO_2^-$ , and  $NO_3^-$  uptake; ammonia oxidation; nitrite oxidation; DON release; and  $NH_4^+$  uptake by bacteria. The matrix inversion methodology that we used was designed specifically to analyze the results of incubations under simulated in situ conditions in the euphotic zone. By taking into consideration simultaneous fluxes among multiple N pools, we minimized potential artifacts caused by non-targeted processes in traditional source-product methods. The proposed isotope matrix method facilitates post hoc analysis of data from ondeck incubation experiments and can be used to probe effects of environmental factors (e.g., pH, temperature, and light) on multiple processes under controlled conditions.

### 1 Introduction

Nitrogen (N), which is an essential element for all organisms, regulates productivity in the surface waters of many parts of the ocean (Falkowski, 1997; Zehr and Kudela, 2011; Casciotti, 2016). As a limiting nutrient in the euphotic zone, nitrogen rapidly interconverts among five major N compartments: particulate organic nitrogen (PN), dissolved organic nitrogen (DON), ammonium (NH<sub>4</sub><sup>+</sup>), nitrite (NO<sub>2</sub><sup>-</sup>), and nitrate  $(NO_3^-)$  (Fig. 1). Studies of the rates of transformation of N in the marine N cycle have had a major impact on our current understanding of the coupling of autotrophic and heterotrophic processes involving carbon and nitrogen as well as the efficiency of the biological pump (Dugdale and Goering, 1967; Caperon et al., 1979; Harrison et al., 1992; Bronk and Glibert, 1994; Dore and Karl, 1996; Laws et al., 2000; Yool et al., 2007). Such information has also facilitated evaluation of ecosystem functions. However, those studies have typically involved inventory and isotope tracer methods that quantified the rates of only one or a few fluxes (Ward, 2008, 2011; Lipschultz, 2008, and references therein). The dynamic nature and complexity of the N cycle make simultaneous resolution of the rates of more than a few of the important fluxes a challenging task.

The inventory method (monitoring the change in substrate and/or product concentrations over time) has often been used to determine the uptake rates of ammonium, nitrite, nitrate, and urea (McCarthy and Eppley, 1972; Harvey and Caperon, 1976; Harrison and Davis, 1977; Howard et al., 2007) and to examine the occurrence and rate of nitrification (Wada and



Figure 1. Model schemes with the most fundamental nitrogen transformation processes in (a) low- and (b) high-nutrient aquatic environments. Arrows represent the transfer flux/rate from the reactant to product pool. The structure and inter-exchanges in the high-nutrient case (b) are the same as in (a), except that  $NO_x^-$  is divided into  $NO_2^-$  and  $NO_3^-$ .

Hatton, 1971; Pakulski et al., 1995; Ward, 2011). However, failure to account for other processes may bias the results. For example, the concentration of ammonium is controlled simultaneously by removal via phytoplankton uptake (PN as the product), nitrification (nitrite/nitrate as the product), and bacterial metabolism (operationally defined DON as product) and by additions via remineralization from heterotrophic bacterial metabolism, zooplankton excretion, and viral lysis. Similarly, the products of nitrification ( $NO_x^-$ ) may be simultaneously consumed by phytoplankton.

The <sup>15</sup>N-labeled tracer technique has been widely used as an assay for specific nitrogen processes since the emergence of isotope ratio mass spectrometry (IRMS). For example, the addition of <sup>15</sup>N-labeled nitrate has been applied to estimate new production (Dugdale and Goering, 1967; Chen, 2005; Painter et al., 2014). Likewise, by incubating water to which  ${}^{15}NH_4^+$  has been added, the nitrification rate ( ${}^{15}NO_3^$ as product; e.g., Newell et al., 2013; Hsiao et al., 2014; Peng et al., 2016) and ammonium uptake rate (<sup>15</sup>N<sub>PN</sub> as product; e.g., Dugdale and Goering, 1967; Dugdale and Wilkerson, 1986; Bronk et al., 1994, 2014) can be measured via incubations in the dark and light, respectively. However, the interpretation of isotope labeling experiments is confounded by the same problems as the inventory method, i.e., multiple processes that occur simultaneously impact the concentrations of substrates and products in the incubation bottle. In fact, those transformations among pools have significant implications for biogeochemical cycles. For instance, Yool et al. (2007) synthesized available global data and concluded that the fractional contribution of nitrate derived from nitrification to nitrate uptake can be as high as 19-33 % in the euphotic zone. However, integration of the relevant rates over a light-dark cycle has been confounded by the fact that nitrate uptake rates have typically been determined during the photoperiod, whereas nitrification rates have been measured under dark conditions (e.g., Grundle et al., 2013). Nitrate uptake may occur in the dark, but not necessarily at the same rate as in the light (Laws and Wong, 1978), and nitrification is inhibited by light (Dore and Karl, 1996). To integrate rates over the light–dark cycle, 24 h incubations have been used to compensate for the diel cycle of light-sensitive processes (Beman et al., 2012). Yet, interpretation of the results of 24 h incubations may be confounded by artifacts due to transfers of <sup>15</sup>N and <sup>14</sup>N among pools. A new method is needed to overcome these problems.

Marchant et al. (2016) have reviewed recent methodological advances using <sup>15</sup>N-labeling substrates combined with nanoSIMS, FISH, or HISH in marine N-cycle studies. These methods provide qualitative information about N transfers at the cellular and molecular level but do not quantify rates at the community level. Elskens et al. (2005) conducted a comprehensive review of oft-used models for rate derivation and concluded that oversimplified models may lead to biased results if their underlying assumptions are violated. However, overly complex models risk misinterpreting random noise as relevant processes. To address this concern, De Brauwere et al. (2005) proposed a model selection procedure. More recently, Pfister et al. (2016) applied an isotope tracer technique and mass conservation model to explore nitrogen flows among dissolved nitrogen pools  $(NH_4^+, NO_2^-, and NO_3^-)$  in tidal pools and found that benthic macrobiota played an important role in regulating remineralization rates. They also found that dilution effects significantly biased the results obtained with source-product models. For the euphotic zone, where competing processes co-occur, an innovative and convenient method is needed to determine the rates of multiple N fluxes from the results of simulated in situ incubations.

In this study, we propose an "isotope matrix method". To avoid perturbations, the concentration of the tracer was limited to <10 or 20% of the substrate concentration, as suggested by previous researchers (Raimbault and Garcia, 2008; Middelburg and Nieuwenhuize, 2000; Painter et al., 2014). One single tracer, <sup>15</sup>NH<sub>4</sub><sup>+</sup>, was added to an incubation bottle to trace the <sup>15</sup>N flow among the nitrogen pools under simulated in situ conditions. Almost all the most fundamental processes in the N cycle can be quantified with this

newly proposed method. To demonstrate the applicability of the method, we conducted incubation experiments with lownutrient water from the western North Pacific and with highnutrient coastal water off the southeastern China coast. As a result of recent advances in the analytical methods for measuring the concentrations and isotopic compositions of various nitrogen species, we were able to use this isotope matrix method to quantify the in situ fluxes of N in the euphotic zone.

#### 2 Isotope matrix method

### 2.1 Framework of the interconnections among nitrogen pools

Figure 1 shows the transformations of N among  $NH_4^+$ ,  $NO_2^-$ ,  $NO_3^-$ , PN, and DON in an aerobic euphotic zone. The PN was operationally defined as the particulate organic nitrogen trapped on a GF/F filter (>0.7 µm). Dissolved inorganic nitrogen (DIN) and DON were equated to the inorganic and organic nitrogen, respectively, in the dissolved fraction that passed through a polycarbonate membrane with a 0.22 µm pore size. Because DON includes the N in numerous dissolved organic N compounds, including unidentified organics, urea, amino acids, amines, and amides, it represents the "bulk" DON and was calculated by subtracting the concentrations of  $NH_4^+$ ,  $NO_2^-$ , and  $NO_3^-$  (DIN) from the total dissolved N (TDN).

We used two different models to analyze our data: a lownutrient model to represent the open ocean and a highnutrient model to represent estuarine and coastal environments (Fig. 1a and b). In the high-nutrient model,  $NH_4^+$ ,  $NO_2^-$ , and  $NO_3^-$  were assumed to co-exist. The rationale for the two model structures is as follows.

The consumption of reactive inorganic nitrogen  $(NH_{4}^{+})$ ,  $NO_2^-$ , and  $NO_3^-$ ) is dominated by photosynthetic uptake by phytoplankton ( $F_1$  and  $F_4$  in Fig. 1a;  $F_1$ ,  $F_3$ , and  $F_5$  in Fig. 1b). Heterotrophic bacteria may also play an important role in  $NH_4^+$  assimilation (Laws, 1985; Middelburg and Nieuwenhuize, 2000; Veuger et al., 2004). We took heterotrophic bacterial assimilation of NH<sup>+</sup><sub>4</sub> into account as well ( $F_6$  in Fig. 1a and  $F_8$  in Fig. 1b) to explore its importance. Though  $NO_2^-$  may be released during  $NO_3^-$  uptake (Lomas and Lipschultz, 2006), little  $NO_2^-$  production from  $NO_3^-$  was detected by Santoro et al. (2013). Nitrate assimilation may be inhibited in aerobic water, especially in estuaries and coastal seas where the  $NH_{4}^{+}$  concentration is high, and in the absence of nitrate uptake, there is no release of nitrite. Thus, nitrite release was ignored in our model. Due to DIN assimilation by phytoplankton, the PN pool may increase, but DON may be released during assimilation ( $F_5$  in Fig. 1a and  $F_7$  in Fig. 1b) as noted by Bronk et al. (1994), Bronk and Ward (2000), and Varela et al. (2005). The size of the  $NH_4^+$  pool is increased by remineralization ( $F_2$  in both Fig. 1a and b) and decreased by nitrification. The latter consists of two basic steps: ammonium oxidation by archaea/bacteria (AOA/AOB) to nitrite ( $F_4$  in Fig. 1b) and nitrite oxidation to nitrate by nitrite-oxidizing bacteria (NOB) ( $F_6$  in Fig. 1b). Although recent studies have revealed a single microorganism that can completely oxidize NH<sub>4</sub><sup>+</sup> to NO<sub>3</sub><sup>-</sup> (comammox) (Daims et al., 2015; van Kessel et al., 2015), the importance of comammox in the marine environment remains unclear.

Specific mechanisms or processes such as grazing and viral lysis may alter the concentrations of  $NH_4^+$ , nitrite, and DON. However, the scope of this study is to determine the nitrogen fluxes among the often-measured and operationally defined nitrogen pools. The organisms that mediate the relevant fluxes are not specifically included in the model. Thus, the results of specific processes such as grazing and viral lysis have been incorporated into the paradigm depicted in Fig. 1.

## 2.2 Analytical methods to determine the amounts of <sup>15</sup>N / <sup>14</sup>N in various pools

To trace the <sup>15</sup>N movement among pools, our isotope matrix method couples the <sup>15</sup>N-labeling and inventory methods by considering changes in both concentrations and isotopic compositions. Analytical methods to determine the concentrations and isotopic compositions of both high and low levels of inorganic/organic nitrogen are in most cases well established and have been reported elsewhere. We determined all the relevant concentrations and isotopic compositions with the exception of the isotopic composition of  $NH_4^+$ .

Concentrations of NH<sub>4</sub><sup>+</sup> higher than 0.5  $\mu$ M were measured manually by using the colorimetric phenol hypochlorite technique (Koroleff, 1983). Nanomolar NH<sub>4</sub><sup>+</sup> concentrations were measured by using the fluorometric *o*-phthaldialdehyde (OPA) method (Zhu et al., 2013). Concentrations of NO<sub>2</sub><sup>-</sup> and of NO<sub>x</sub><sup>-</sup> (NO<sub>2</sub><sup>-</sup> + NO<sub>3</sub><sup>-</sup>) were determined with the chemiluminescence method following the protocol of Braman and Hendrix (1989). The detection limits of NO<sub>2</sub><sup>-</sup> and NO<sub>x</sub><sup>-</sup> were both ~ 10 nmol L<sup>-1</sup>, and the corresponding relative precision was better than 5 % within the range of concentrations that we measured. By using persulfate as an oxidizing reagent, we oxidized TDN and PN separately to nitrate (Knapp et al., 2005) and then measured the nitrate by using the analytical method for NO<sub>x</sub><sup>-</sup> described above.

We determined the  $\delta^{15}$ N of NO<sub>2</sub><sup>-</sup> with the azide method by following the detailed procedures in McIlvin and Altabet (2005). The  $\delta^{15}$ N of NO<sub>x</sub><sup>-</sup> was determined by using a distinct strain of bacteria that lacked N<sub>2</sub>O reductase activity to quantitatively convert NO<sub>x</sub><sup>-</sup> to nitrous oxide (N<sub>2</sub>O), which we then analyzed by IRMS (denitrifier method; Sigman et al., 2001; Casciotti et al., 2002). The isotopic composition of NO<sub>3</sub><sup>-</sup> was determined from isotope mass balance (NO<sub>x</sub><sup>-</sup> - NO<sub>2</sub><sup>-</sup>) or measured by the denitrifier method after eliminating preexisting NO<sub>2</sub><sup>-</sup> with sulfamic acid (Granger and Sigman, 2009). To determine the  $\delta^{15}$ N of TDN and PN, both species were first converted to NO<sub>3</sub><sup>-</sup> with the denitrifier method, and then the  $\delta^{15}$ N of the NO<sub>3</sub><sup>-</sup> was determined as described above. The detection limit of  $\delta^{15}$ N<sub>PN</sub> can be reduced to the nanomolar level (absolute amount of nitrogen), which is significantly lower than the detection limit using high-temperature combustion with an elemental analyzer connected to an IRMS.

The most popular way to determine the N isotopic composition of  $NH_4^+$  is the "diffusion method", which involves conversion of dissolved  $NH_4^+$  to  $NH_3$  gas by raising the sample pH to above 9 with magnesium oxide (MgO) and subsequently trapping the gas quantitatively as (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> on a glass fiber (GF) filter; the isotope ratios of the  $^{15}N$  /  $^{14}N$ are then measured using an elemental analyzer coupled with an IRMS (Holmes et al., 1998; Hannon and Böhlke, 2008). Alternatively, after removing the preexisting  $NO_2^-$  from the seawater samples using sulfamic acid, NH<sub>4</sub><sup>+</sup> is first quantitatively oxidized to NO<sub>2</sub><sup>-</sup> by hypobromite (BrO<sup>-</sup>) at pH  $\sim$  12 (BrO<sup>-</sup> oxidation method), and the protocol of McIlvin and Altabet (2005) is then used to reduce the  $NO_2^-$  to  $N_2O$ (Zhang et al., 2007). Unfortunately, neither of these methods has been established in our lab yet. The isotope matrix method requires the isotopic composition of  $NH_4^+$  as well, but this requirement can be circumvented by making certain assumptions, as illustrated in our case studies below.

We estimated the amount of <sup>14</sup>N and <sup>15</sup>N atoms in every individual pool for which we knew the concentration and  $\delta^{15}N$  ( $\delta^{15}N\%_{c} = [(R_{sample} - R_{atm N_2}) / R_{atm N_2}] \times 1000$ ). By assuming the <sup>15</sup>N content of standard atmospheric nitrogen to be 0.365% (Coplen et al., 1992), we calculated  $R_{sample}$ (<sup>15</sup>N / <sup>14</sup>N). By defining  $r_{sample}$  as <sup>15</sup>N / (<sup>14</sup>N+<sup>15</sup>N), we directly derived the <sup>15</sup>N and <sup>14</sup>N concentrations of all forms of N, with the exception of NH<sub>4</sub><sup>+</sup> and DON. The *r* value of the NH<sub>4</sub><sup>+</sup> was assumed to equal either its initial value or an arbitrarily chosen fraction thereof, and the <sup>15</sup>N and <sup>14</sup>N content of the NH<sub>4</sub><sup>+</sup> was then determined.

#### 2.3 Formation of matrix equations

In this isotope matrix method, we added a limited amount of  ${}^{15}\text{NH}_4^+$  into incubation bottles at the very beginning and then monitored the changes in  ${}^{15}\text{N}$  and  ${}^{14}\text{N}$  in the measured pools every few hours. We assumed isotopic mass balance at every time point in the incubation bottle. In other words, the sum of the variations in the total N,  ${}^{15}\text{N}$ , and  ${}^{14}\text{N}$  concentrations was zero for any time interval. The fluxes of  ${}^{15}\text{N}$  and  ${}^{14}\text{N}$ were therefore equal to the total flux multiplied by  $r_{\text{substrate}}$ and  $(1-r_{\text{substrate}})$ , respectively. Although we did not consider isotope fractionation, it could have been introduced into the equations by dividing the  ${}^{14}\text{N}$  flux by the ratio of the specific rate constants of  ${}^{14}\text{N}$  and  ${}^{15}\text{N}$  to obtain the flux of  ${}^{15}\text{N}$ .

According to mass balance, the net changes in the  ${}^{15}N$  (or  ${}^{14}N$ ) concentration of an individual N pool in a time in-

terval are determined by the inflow and outflow of <sup>15</sup>N (or <sup>14</sup>N) (see Fig. 1 and Eqs. 1–14 below). In the low-nitrogen case, the changes in the <sup>15</sup>N concentrations of the NH<sub>4</sub><sup>+</sup>, NO<sub>x</sub><sup>-</sup>, and PN pools were expressed by Eqs. (1), (2), and (3), respectively. Similarly, the temporal dependence of <sup>14</sup>N-NH<sub>4</sub><sup>+</sup>, <sup>14</sup>N-NO<sub>x</sub><sup>-</sup>, and <sup>14</sup>N-PN were expressed by Eqs. (4), (5), and (6), respectively. The mean rate of change in the nitrogen pool, i.e., the left side of each equation, was determined from the data at time zero ( $t_0$ ) and the first time point ( $t_1$ ). For example, when the sampling time interval was short,  $\Delta [^{14}NH_4^+]_t 1 - [^{14}NH_4^+]_{t_0}]/(t_1 - t_0)$ , where the subscripts indicate the times at which the concentrations were measured. The *r* value in each equation was the average of the *r* values for the pool at time zero and the first time point.

$$\frac{\Delta \left[ {}^{15}\mathrm{NH}_{4}^{+} \right]}{\Delta T} = \overline{F_{2}} \times 0.00366 - \overline{F_{1}} \times \overline{r_{\mathrm{NH}_{4}^{+}}}$$

$$- \overline{F_{3}} \times \overline{r_{\mathrm{NH}^{+}}} - \overline{F_{6}} \times \overline{r_{\mathrm{NH}^{+}}}$$

$$(1)$$

$$\frac{\Delta \begin{bmatrix} {}^{15}\text{NO}_x^- \end{bmatrix}}{\Delta T} = \overline{F_3} \times \overline{r_{\text{NH}_4^+}} - \overline{F_4} \times \overline{r_{\text{NO}_x^-}}$$
(2)

$$\frac{\Delta [^{13}\text{PN}]}{\Delta T} = \overline{F_1} \times \overline{r_{\text{NH}_4^+}} + \overline{F_4} \times \overline{r_{\text{NO}_x^-}} - \overline{F_5} \times \overline{r_{\text{PN}}}$$
(3)

$$\frac{\Delta \left[ {}^{14}\text{NH}_4^+ \right]}{\Delta T} = \overline{F_2} \times (1 - 0.00366) - \overline{F_1} \times (1 - \overline{r_{\text{NH}_4^+}}) \qquad (4)$$
$$- \overline{F_3} \times (1 - \overline{r_{\text{NH}_4^+}}) - \overline{F_6} \times (1 - \overline{r_{\text{NH}_4^+}})$$

$$\frac{\Delta \left[ {}^{14}\text{NO}_x^- \right]}{\Delta T} = \overline{F_3} \times (1 - \overline{r_{\text{NH}_4^+}}) - \overline{F_4} \times (1 - \overline{r_{\text{NO}_x^-}})$$
(5)

$$\frac{\Delta \left[ {}^{14}\text{PN} \right]}{\Delta T} = \overline{F_1} \times (1 - \overline{r_{\text{NH}_4^+}}) + \overline{F_4} \times (1 - \overline{r_{\text{NO}_x^-}}) \qquad (6)$$
$$- \overline{F_5} \times (1 - \overline{r_{\text{PN}}})$$

The time series in this study lasted for 24 h. However, we used only the first two time points for the rate calculations because we felt those rates would be closest to the instantaneous in situ rates of the original samples. Although the isotope matrix method may be applied to longer time intervals, rates may vary as a result of substrate consumption and/or community change. Relatively short-term incubations are therefore advisable (see below).

Because the total number of equations and unknowns is equal, a unique solution can be obtained via matrix inversion for the low-nutrient model.

In high-nutrient cases, analogous equations (Eqs. 7–14) can be constructed to describe the fluxes between  $NH_4^+$ ,  $NO_2^-$ ,  $NO_3^-$ , and PN (Fig. 1b).

$$\frac{\Delta \begin{bmatrix} ^{15}\text{NH}_{4}^{+} \end{bmatrix}}{\Delta T} = \overline{F_2} \times 0.00366 - \overline{F_1} \times \overline{r_{\text{NH}_{4}^{+}}}$$

$$- \overline{F_4} \times \overline{r_{\text{NH}_{4}^{+}}} - \overline{F_8} \times \overline{r_{\text{NH}_{4}^{+}}}$$
(7)

$$\frac{\Delta \left[ {}^{15}\text{NO}_2^- \right]}{\Delta T} = \overline{F_4} \times \overline{r_{\text{NH}_4^+}} - \overline{F_3} \times \overline{r_{\text{NO}_2^-}} - \overline{F_6} \times \overline{r_{\text{NO}_2^-}}$$
(8)

$$\frac{\Delta \left[ {}^{15}\text{NO}_3^- \right]}{\Delta T} = \overline{F_6} \times \overline{r_{\text{NO}_2^-}} - \overline{F_5} \times \overline{r_{\text{NO}_3^-}}$$
(9)

$$\frac{\Delta \left[ {}^{15}\text{PN} \right]}{\Delta T} = \overline{F_1} \times \overline{r_{\text{NH}_4^+}} + \overline{F_3} \times \overline{r_{\text{NO}_2^-}} + \overline{F_5} \times \overline{r_{\text{NO}_2^-}} - \overline{F_7} \times \overline{r_{\text{PN}}}$$
(10)

$$\frac{\Delta \left[ {}^{14}\mathrm{NH}_{4}^{+} \right]}{\Delta T} = \overline{F_{2}} \times (1 - 0.00366) - \overline{F_{1}} \times (1 - \overline{r_{\mathrm{NH}_{4}^{+}}}) \quad (11)$$
$$- \overline{F_{4}} \times (1 - \overline{r_{\mathrm{NH}_{4}^{+}}}) - \overline{F_{8}} \times (1 - \overline{r_{\mathrm{NH}_{4}^{+}}})$$

$$\frac{\Delta \left[ {}^{14}\text{NO}_2^- \right]}{\Delta T} = \overline{F_4} \times (1 - \overline{r_{\text{NH}_4^+}}) - \overline{F_3} \times (1 - \overline{r_{\text{NO}_2^-}})$$
(12)

$$-F_6 \times (1 - r_{NO_2^-})$$

$$\frac{^{14}NO_3^-}{} = \overline{F_6} \times (1 - \overline{F_{NO_2^-}}) - \overline{F_5} \times (1 - \overline{F_{NO_2^-}})$$

$$\frac{\Delta \begin{bmatrix} 1^{4} \mathrm{NO}_{3}^{-} \end{bmatrix}}{\Delta T} = \overline{F_{6}} \times (1 - \overline{r_{\mathrm{NO}_{2}^{-}}}) - \overline{F_{5}} \times (1 - \overline{r_{\mathrm{NO}_{3}^{-}}})$$
(13)

$$\frac{\Delta [^{1}PN]}{\Delta T} = \overline{F_1} \times (1 - \overline{r_{\text{NH}_4^+}}) + \overline{F_3} \times (1 - \overline{r_{\text{NO}_2^-}})$$
(14)

$$+ \overline{F_5} \times (1 - \overline{r_{\text{NO}_3^-}}) - \overline{F_7} \times (1 - \overline{r_{\text{PN}}})$$

A unique solution can again be obtained via matrix inversion because the number of equations and unknowns are equal.

In the above matrix equations, the value of  $r_{\rm NH^+}$ , which we did not measure in this study, was needed to obtain a solution. To address this issue, we assumed various degrees of remineralization to test the effect of isotope dilution (NH<sub>4</sub><sup>+</sup> addition) on our calculated fluxes. We reduced  $r_{\mathrm{NH}^+_4}$  values of the 24 h incubation. The  $r_{NH_4^+}$  for remineralization ( $F_2$ ) was assumed to be constant (0.00366) and equal constant rates that led to total reductions of  $r_{\text{NH}^+_4}$  by 0, 1, 10, 20, or 50 % by the end. The value of  $F_2$  coupled with the assumed  $r_{\rm NH^+}$  values allowed us to resolve rates under different remineralization scenarios, and the derived  $F_2$  was introduced into a STELLA model for extrapolation purposes (see below). We compared the observed and remineralization-associated simulations to elucidate the effect of remineralization on the calculated rates for the time series incubations.

#### 2.4 Validation by STELLA

The initial rates are of particular interest because they are presumably most similar to the in situ rates at the time the sample was collected. The initial rate is here distinguished from rates derived from incubations that extended beyond time point  $t_1$ . To evaluate the applicability of the matrixderived initial rate, we used STELLA 9.1.4 software (Isee systems, Inc.) to construct box models that were consistent with the scenarios depicted in Fig. 1. The constructed STELLA model contained two modules (Figs. S1 and S2 in the Supplement), one for <sup>15</sup>N and the other for <sup>14</sup>N. These two modules were connected through the  $^{15}N$  atom % (rN), which was a parameter measured in the incubation experiment. A model run was initialized with the measured values of the nitrogen pools at time zero, and the model then projected the values of those pools as a continuous function of time. Because the rates based on the first two time points might not accurately represent the behavior of the system throughout the full time course due, for example, to changes in substrate concentrations and the composition of the microbial community, this extrapolation using the initial rates amounted to a test of the hypothesis that the rates did not change.

We assumed first-order reaction kinetics in both the lownutrient and high-nutrient cases. The initial rate constant "k" could therefore be derived by dividing the matrix-derived flux F by  $\overline{C}$ , the average substrate concentration during the first two time points. After the concentrations of <sup>15</sup>N and <sup>14</sup>N were initialized in every pool, the model ran for 24 h using the matrix-derived short-term k values. As depicted in Fig. 1, all the monitored N pools were regulated by F, which was assumed to be concentration dependent (Figs. S1 and S2). The output of the model included the time courses of the <sup>15</sup>N and <sup>14</sup>N concentrations and the <sup>15</sup>N atom % (rN) of each N species. Through this analysis, we could observe the temporal evolution of the isotopic composition of the various N pools.

#### 2.5Study sites and incubation experiments

The incubation experiments were conducted in two environments with very different nutrient levels. The low-nutrient study was conducted on-deck of the R/V Dongfanghong 2 on a cruise to the western North Pacific (WNP) (33.3° N, 145.9° E) in the spring of 2015. The site of the high-nutrient study was Wuyuanwan Bay (WYW) (24.5° N, 118.2° E) in the southern coast of China.

The water samples at the WNP station were collected using a 24-bottle rosette sampler. The sampling depth was 25 m, at which the light intensity was 12 % of the surface irradiance. Two pre-washed 10L polycarbonate carboys (Nalgene, USA) were used for the incubation. A total of 1.5 mL of 200 µM <sup>15</sup>N-labeled NH<sub>4</sub>Cl tracer containing 98 atom % <sup>15</sup>N (Sigma-Aldrich, USA) was injected into each incubation bottle separately to achieve a final concentration of 30 nM. The incubation was carried out immediately with a constant simulated light intensity of 35  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup> in a thermostatic incubator (GXZ-250A, Ningbo) at the in situ temperature.

The WYW station was located in the inner bay, where the tide was semidiurnal. Wuyuanwan, a coastal bay, suffers from the same anthropogenic influences that cause eutrophication in other coastal areas of China. However, the bay water is well ventilated and constantly saturated with dissolved oxygen due to tidally induced water exchange. It is an ideal



**Figure 2.** The observational data in the low-nutrient case for (a)  $[NH_4^+]$ , (b)  $[NO_x^-]$ , (c) [PN], (d) [TDN], (e)  $\delta^{15}N$ -NO<sub>x</sub><sup>-</sup>, and (f)  $\delta^{15}N$ -PN. The regular and inverse open triangles represent the paralleled samples and the analytical errors are shown.

site to study the dynamic transformations that characterize the coastal nitrogen cycle.

The WYW samples were taken on 19 January 2014 from water depths of 0.3 and 2.3 m, where the light intensities were 80 and 2%, respectively, of the surface water irradiance. Duplicate water samples were collected from each depth by using submersible pump to fill pre-washed 10L polycarbonate bottles (Nalgene, USA). <sup>15</sup>N-labeled NH<sub>4</sub>Cl (98 atom % <sup>15</sup>N, Sigma-Aldrich, USA) was added to the incubation bottles to a final concentration of 1  $\mu$ M (~4% of the ambient concentration). The incubations were carried out immediately in the field. Neutral density screens that allowed 80 and 2% light penetration were used to simulate the light intensities at 0.3 and 2.3 m, respectively. The temperature was maintained at ~ 13.7 °C by continuously pumping seawater through the incubators.

The sample at the first time point ( $t_0$ ) was taken immediately after tracer addition. Subsequent samples were taken at approximately 2–4 h intervals for DIN and PN analyses. An aliquot of 200 mL was filtered through a 47 mm polycarbonate membrane filter with a 0.22 µm pore size (Millipore, USA). The filtrates were frozen at -20 °C for chemical analyses in the lab. Particulate matter was collected by filtering 500 mL seawater through pre-combusted (450 °C for 4 h) 25 mm GF/F filters (Whatman, GE Healthcare, USA) at a pressure of < 100 mm Hg. The GF/F filters were freeze-dried and stored in a desiccator prior to analysis of PN concentrations and <sup>15</sup>N atom %.

#### **3** Results

#### 3.1 Ambient conditions and initial concentrations

The water temperature and salinity at a depth of 25 m in the WNP were 18.4 °C and 34.8, respectively. The dissolved oxygen (DO) was 7.3 mg L<sup>-1</sup>. The concentrations of NH<sub>4</sub><sup>+</sup>, NO<sub>x</sub><sup>-</sup>, and phosphate were  $113 \pm 5$ ,  $521 \pm 18$ , and  $74 \pm 2$  nmol L<sup>-1</sup>, respectively.

The water temperature and salinity throughout the water column of the WYW were  $13.5 \pm 0.1$  °C and  $29.5 \pm 0.1$ , respectively. The DO saturation fell in the range 135-140 %. The concentrations of nitrogenous species were relatively high. The inorganic nutrient concentrations were  $30.9 \pm 0.7$  for NO<sub>3</sub><sup>-</sup>, 22.3 ± 4.3 for NH<sub>4</sub><sup>+</sup>,  $5.4 \pm 0.2$  for NO<sub>2</sub><sup>-</sup>, and  $1.5 \pm 0.1 \,\mu$ mol L<sup>-1</sup> for phosphate. The PN concentration was  $9.3 \pm 0.7 \,\mu$ mol L<sup>-1</sup>.

#### **3.2** Time courses of incubations

#### 3.2.1 Low-nutrient case in the WNP

The observed patterns of change in the bulk NH<sub>4</sub><sup>+</sup>, NO<sub>x</sub><sup>-</sup>, PN, and TDN concentrations and the  $\delta^{15}$ N of NO<sub>x</sub><sup>-</sup> and PN during the incubation are shown in Fig. 2. Concentrations of NH<sub>4</sub><sup>+</sup> and NO<sub>x</sub><sup>-</sup> decreased rapidly from 143 ± 5 to 48 ± 5 nM and 521 ± 18 to 127 ± 11 nM, respectively (Fig. 2a and b). In contrast, the PN concentration increased from 437 ± 9 to 667 ± 14 nM (Fig. 2c), and the TDN concentration remained stable, with an average of 6511 ± 209 nM (Fig. 2d). In contrast to the trend of NO<sub>x</sub><sup>-</sup> concentration,  $\delta^{15}$ N-NO<sub>x</sub><sup>-</sup> increased from 8.9 ± 0.2 to 171 ± 2% (Fig. 2e). In addition,  $\delta^{15}$ N-



**Figure 3.** The observational data in the high-nutrient case for (a)  $[NH_4^+]$ , (b)  $[NO_2^-]$ , (c)  $[NO_3^-]$ , (d) [PN], (e) [TDN], (f) [PN+TDN], (g)  $\delta^{15}N-NO_2^-$ , (h)  $\delta^{15}N-NO_3^-$ , and (i)  $\delta^{15}N-PN$ . The light and dark red diamonds represent the paralleled samples in 80 % sPAR case and the black regular and inverse open triangles represent the paralleled samples in 2 % sPAR case. Bars represent analytical errors.

PN exhibited great changes, increasing from  $46.8 \pm 0.2$  to  $6950 \pm 314 \%$  (Fig. 2f).

#### 3.2.2 High-nutrient case in the WYW

The time series of observational parameters for samples from depths of 80 and 2% of surface PAR (sPAR) exhibited similar trends during the incubation (Fig. 3). During the course of the incubation, NH<sub>4</sub><sup>+</sup> decreased significantly and continuously from  $26.6 \pm 0.1$  (initial concentration) to  $17.4 \pm 0.1 \,\mu\text{mol}\,\text{L}^{-1}$ . The mean reduction rate was  $0.63 \,\mu\text{mol}\,\text{L}^{-1}\,\text{h}^{-1}$  for the 80% sPAR sample (Fig. 3a). The  $NH_4^+$  concentration of 2% sPAR sample decreased more slowly from  $24.6 \pm 0.1$  (initial concentration) to  $18.2\pm1.0\,\mu\text{mol}\,L^{-1}$  with a mean reduction rate of 0.47  $\mu mol \, L^{-1} \, h^{-1}$  (Fig. 3a).  $NO_3^-$  in 80 and 2 % sPAR samples decreased from  $30.1 \pm 0.1$  to  $28.3 \pm 0.1 \,\mu\text{mol}\,\text{L}^{-1}$ and from  $31.1 \pm 0.1$  to  $29.7 \pm 0.1 \,\mu\text{mol}\,\text{L}^{-1}$ , respectively (Fig. 3c). Overall, the nitrate reduction rates were much lower than the  $NH_4^+$  reduction rates. Compared to nitrate,  $NO_2^-$  displayed even slower rates of decline, yet the rate was significantly higher at 80 % sPAR than at 2 % sPAR (Fig. 3b). Similar to the low-nutrient case, PN increased steadily

from  $8.8 \pm 0.1$  to  $17.7 \pm 0.9 \,\mu\text{mol L}^{-1}$ , with a mean rate of  $0.61 \,\mu\text{mol L}^{-1} \,\text{h}^{-1}$  at  $80 \,\%$  sPAR and from  $9.9 \pm 0.1$  to  $16.0 \pm 2.0 \,\mu\text{mol L}^{-1}$  with a mean rate of  $0.44 \,\mu\text{mol L}^{-1} \,\text{h}^{-1}$ at  $2 \,\%$  sPAR (Fig. 3d). The rates of increase in the PN concentration were very close to the rates of decrease in NH<sup>4</sup><sub>4</sub>, the indication being that ammonium was the major nitrogen source for growth. The TDN concentration decreased from  $78.7 \pm 1.6$  to  $68.4 \pm 0.1 \,\mu\text{mol L}^{-1}$  and from  $72.8 \pm 2.5$ to  $67.1 \pm 0.8 \,\mu\text{mol L}^{-1}$  at 80 and  $2 \,\%$  sPAR, respectively (Fig. 3e).

The  $\delta^{15}$ N-NO<sub>2</sub><sup>-</sup> increased from  $-9.0 \pm 0.1$  to  $12.1 \pm 0.1 \%_0$  and from  $-8.8 \pm 0.1$  to  $23.3 \pm 0.6 \%_0$  at 80 and 2 % sPAR, respectively (Fig. 3g). Because the nitrate pool was relatively large, the values of  $\delta^{15}$ N-NO<sub>3</sub><sup>-</sup> ranged from 6.8 to  $10.1 \%_0$  with no significant trend over time (Fig. 3h). In addition,  $\delta^{15}$ N-PN increased from  $14.8 \pm 0.3$  to  $3078 \pm 180 \%_0$  and from  $15.0 \pm 0.5$  to  $2738 \pm 66 \%_0$  at 80 and 2 % sPAR, respectively (Fig. 3i). These significant changes in both the concentration and isotopic composition of the nitrogen pools over time suggests that there were significant movements of nitrogen among pools and that the labeled <sup>15</sup>N in the NH<sub>4</sub><sup>+</sup> moved throughout the system, with the exception of nitrate.

### 3.3 Solutions of the matrix equation and STELLA extrapolation

#### 3.3.1 Low-nutrient case

The matrix-derived rate constants  $(k_i)$  and rates  $(F_i)$ are shown in Table 1a and b, respectively. Under the no-remineralization condition (i.e.,  $r_{\rm NH_4^+}$  decreased 0% within 24 h), NO<sub>x</sub><sup>-</sup> uptake  $(k_4 = 0.059 \, {\rm h}^{-1};$  $F_4 = 27.2 \, {\rm nmol} \, {\rm L}^{-1} \, {\rm h}^{-1}$ ) was the highest among all forms of inorganic nitrogen in terms of flux, followed by NH<sub>4</sub><sup>+</sup> uptake  $(k_1 = 0.038 \, {\rm h}^{-1}; F_1 = 4.9 \, {\rm nmol} \, {\rm L}^{-1} \, {\rm h}^{-1})$  and DON release  $(k_5 = 0.024 \, {\rm h}^{-1}; F_5 = 11.5 \, {\rm nmol} \, {\rm L}^{-1} \, {\rm h}^{-1})$ . NH<sub>4</sub><sup>+</sup> uptake by bacteria  $(k_6 = 0.007 \, {\rm h}^{-1}; F_6 = 1.0 \, {\rm nmol} \, {\rm L}^{-1} \, {\rm h}^{-1})$  was much lower than that by phytoplankton. The rate constant for nitrification  $(k_3 = 0.0005 \, {\rm h}^{-1})$  was the lowest among all fluxes  $(F_3 = 0.07 \, {\rm nmol} \, {\rm L}^{-1} \, {\rm h}^{-1})$ .

By introducing the initial <sup>15</sup>N and <sup>14</sup>N concentrations of  $NH_4^+$ ,  $NO_x^-$ , PN, and DON and the calculated rate constants  $(k_1 \text{ to } k_6)$  into STELLA (Fig. S1 in the Supplement), we obtained a full time courses for all parameters (Fig. 4). Generally, the model outputs fitted well with the measured values, except for the last time point for PN, the associated <sup>15</sup>N concentration,  $\delta^{15}$ N, and rN (Fig. 4c, k and o). The fact that the rates during the first time interval generally predicted the subsequent observations rather well demonstrated a good predictive performance with the matrix method initial rate. Because the concentrations of both ammonium and  $NO_r^-$  were described well during the 24 h experiment, the extra PN that was not well described in observations after 12h likely reflected the influence of an additional nitrogen source, i.e., dissolved organic nitrogen that was utilized by phytoplankton (see discussion below) when inorganic nitrogen reached threshold levels (Sunda and Ransom, 2007).

In the test runs with  $r_{\rm NH^+_4}$  reduction by a total of 1, 10, 20, and 50 %, we found that the NH<sub>4</sub><sup>+</sup> consumption rates ( $k_1$ and  $k_6$  increased as the regeneration ( $k_2$ ) increased (Table 1). As indicated in previous studies, such regeneration-induced isotope dilution indeed altered the original results (Table 1 and Fig. 4). Specifically, greater NH<sub>4</sub><sup>+</sup> regeneration resulted in larger differences between the three PN-associated values ( $^{15}$ N-PN,  $\delta^{15}$ N-PN, and  $r_{PN}$ ) and the STELLA-projected data (Fig. 4c, k, and o). The dilution effect was more significant after 12 h of incubation. In contrast, the effect of  $r_{\rm NH^+}$ on parameters associated with  $NO_x^-$  was trivial (Fig. 4b, f, j, n and r). The comparison between the simulation and observation suggested that  $NH_4^+$  regeneration needs to be considered for PN (i.e., uptake) when the remineralization rate is high and the incubation is longer than 12 h. Besides remineralization, discrepancies along the time course might possibly be caused by changes in the composition of the microbial community as the incubation continues.

#### 3.3.2 High-nutrient cases

The results at 80 and 2 % sPAR on the assumption of a fixed  $r_{\rm NH^+}$  are shown in Table 2a and b, respectively. For the 80 % sPAR sample, the NH<sub>4</sub><sup>+</sup> uptake by phytoplankton ( $F_1$ , 397 nmol  $L^{-1} h^{-1}$ ) and by bacteria (*F*<sub>8</sub>, 282 nmol  $L^{-1} h^{-1}$ ) were much higher than the other rates and were followed by the NO<sub>3</sub><sup>-</sup> uptake rate ( $F_5$ , 149 nmol L<sup>-1</sup> h<sup>-1</sup>). The NO<sub>2</sub><sup>-</sup> uptake  $(F_3)$  rate was 29 nmol L<sup>-1</sup> h<sup>-1</sup>, much lower than that of  $NH_4^+$  and  $NO_3^-$ . The ammonia oxidation rate ( $F_4$ ) was 0.4 nmol L<sup>-1</sup> h<sup>-1</sup>, and the nitrite oxidation rate ( $F_6$ ) was zero (Table 2a). Because this incubation was conducted in winter with low temperature and at 80 % sPAR, low rates of ammonium and nitrite oxidation were reasonable because both nitrifiers and NOB are sensitive to light (e.g., Olson, 1981a, b; Horrigan et al., 1981; Ward, 2005; Merbt et al., 2012; Smith et al., 2014). The DON release rate by phytoplankton  $(F_7)$ was zero in this case.

In comparison, all the rates at 2% sPAR showed a very similar pattern (Table 2b). The only difference was that all the uptake rates were lower at 2% sPAR, except for ammonia oxidation, which was higher in the low light.

By introducing initial concentrations and calculated rate constants ( $k_1$ – $k_8$ ) into the STELLA model (Fig. S2), we obtained a time series of <sup>15</sup>N and <sup>14</sup>N concentrations and the rN values for NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, PN, and DON (Fig. 5). In general, the modeled and measured values remained consistent throughout the 15 h incubation, demonstrating the capability of the isotope matrix method.

Similar to the low-nutrient case, we evaluated the effect of regeneration (see Table 2 and Fig. 5a and b). Because ammonium uptake was the dominant process, changes in the PN pool were more significant in comparison with the other pools (Fig. 5d, n, and s). We found again that as  $F_2$  increased,  $F_1$  and  $F_8$  increased to maintain a constant reduction of the measured NH<sup>+</sup><sub>4</sub> concentration (Table 2). Similar to the low-nutrient case, as regeneration increased, the projected course of <sup>15</sup>N-PN deviated more from observations, and the turning point also appeared earlier, resulting in a larger curvature of *r*-PN and  $\delta^{15}$ N-PN (Fig. 5d and s). This modeling exercise confirmed the influence of the isotope dilution effect. However, the effect was insignificant in the early part of the incubation.

#### 4 Discussion

#### 4.1 Method comparisons

#### 4.1.1 Model structure and rate derivation

The most widespread <sup>15</sup>N model was proposed by Dugdale and Goering (1967), who assumed isotopic and mass balances in the particulate fraction, the result being the commonly used equation for nitrogenous nutrient uptake. Dug-



**Figure 4.** The observed and STELLA-derived values in the low-nutrient case for (**a**)  $[{}^{15}NH_{4}^{+}]$ , (**b**)  $[{}^{15}NO_{x}^{-}]$ , (**c**)  $[{}^{15}N-PN]$ , (**d**)  $[{}^{15}N-DON]$ , (**e**)  $[{}^{14}NH_{4}^{+}]$ , (**f**)  $[{}^{14}NO_{x}^{-}]$ , (**g**)  $[{}^{14}N-PN]$ , (**h**)  $[{}^{14}N-DON]$ , (**i**)  $r_{NH_{4}^{+}}$ , (**j**)  $r_{NO_{x}^{-}}$ , (**k**)  $r_{PN}$ , (**l**)  $r_{DON}$ , (**m**)  $\delta^{15}N-NH_{4}^{+}$ , (**n**)  $\delta^{15}N-NO_{x}^{-}$ , (**o**)  $\delta^{15}N-PN$ , (**p**)  $\delta^{15}N-DON$ , (**q**)  $[NH_{4}^{+}]$ , (**r**)  $[NO_{x}^{-}]$ , (**s**) [PN], and (**t**) [DON]. The black regular and inverse open triangles represent the paralleled observed values; the black, green, blue, magenta, and pink solid lines represent the STELLA model simulations when  $r_{NH_{4}^{+}}$  decreases 0, 1, 10, 20, and 50 % in 24 h, respectively. The dashed lines in (**b**), (**f**), (**j**), (**n**), and (**r**) were generated from nonlinear least-squares curve fitting by MATLAB following Santoro et al. (2010).

**Table 1.** The isotope matrix results for (**a**) the specific rates and (**b**) average rates of N processes in the low-nutrient case during the first interval under different  $r_{\rm NH_4^+}$  variation conditions. In addition, all N transformation rates via ODE following Pfister et al. (2016) on the assumption of no remineralization were estimated for comparison. Note:  $r_{\rm NH_4^+}$  variation was manipulated artificially by decreasing  $r_{\rm NH_4^+}$  values at a constant reduction rate and the total reduction of  $r_{\rm NH_4^+}$  was 0, 1, 10, 20, and 50 % of the full time span (24 h) of incubation.

(a)									
The percentage of $r_{\rm NH_4^+}$ decrease in 24 h									
	(	)	1 %	10 %	20%	50 %			
Rate constant $(k)$ h <sup>-1</sup>	ODE		Isotope matrix						
$NH_4^+$ uptake $(k_1)$	0.040	0.038	0.038	0.038	0.038	0.039			
Remineralization $(k_2)$	0	0	0.00001	0.0001	0.0002	0.001			
$NH_4^+$ oxidation ( $k_3$ )	0.0004	0.0005	0.0005	0.0005	0.0005	0.0005			
$NO_x^{-}$ uptake $(k_4)$	0.060	0.059	0.059	0.059	0.059	0.059			
DON release $(k_5)$	0.017	0.024	0.024	0.024	0.024	0.024			
Bacteria uptake $NH_4^+$ ( $k_6$ )	0.005	0.007	0.008	0.011	0.015	0.028			
(b)									
	The percentage of $r_{\mathrm{NH}_4^+}$ decrease in 24 h								
	(	)	1 %	10 %	20 %	50 %			
Rate $(k \times \overline{C})$ nmol L <sup>-1</sup> h <sup>-1</sup>	ODE		Isc	otope matr					
$NH_4^+$ uptake $(F_1)$	3.8	4.9	4.9	4.9	5.0	5.1			
Remineralization $(F_2)$	0.0	0.0	0.1	0.6	1.2	3.0			
$NH_4^+$ oxidation (F <sub>3</sub> )	0.04	0.07	0.1	0.1	0.1	0.7			
$NO_x^{\frac{1}{2}}$ uptake (F <sub>4</sub> )	19.3	27.2	27.2	27.2	27.2	27.2			
DON release $(F_5)$	9.6	11.5	11.5	11.6	11.6	11.8			
Bacteria uptake $NH_4^+$ ( $F_6$ )	0.5	1.0	1.0	1.5	2.0	3.7			

dale and Wilkerson (1986) modified their rate equations further and highlighted the importance of short-term incubations. Collos (1987) demonstrated that an equation based on the concentration of particles at the end of the experiment, rather than at the beginning, was more reliable when more than one N source was simultaneously incorporated by the phytoplankton. That is, the equation by Collos (1987) corrected for the bias caused by use of unlabeled multiple N sources.

Unlike the abovementioned equations, Blackburn (1979) and Caperon et al. (1979) proposed <sup>15</sup>N isotope dilution models based on the substrate rather than the product. By measuring the isotope values and concentrations of the substrate (e.g.,  $NH_4^+$ ), both  $NH_4^+$  consumption (DON and/or PN as product) and regeneration rates can be obtained. Glibert et al. (1982) further modified the isotope dilution method and calculated the uptake rate into the PN fraction by substituting the exponential average of  $r_{NH_4^+}$  at the beginning and at the end of an incubation to correct for the isotope dilution in the model of Dugdale and Goering (1967). Despite the methodological improvements, imbalance was often observed between the substrate reduction and the increase in the particulate phase in field studies. Laws (1985) introduced

a new model that considered the imbalance and calculated the "net uptake rate" (into PN). Later on, Bronk and Glibert (1991) revised Laws' model on the basis of the model proposed by Glibert et al. (1982) to calculate the "gross uptake rate" (substrate incorporation into particulate organic nitrogen plus DON). None of the above models considered the mass balance at the whole system scale. Although rates were obtained via analytical solutions, the bias potential due to multiple fluxes was not completely resolved.

To address this problem, Elskens et al. (2002) formulated a new model that takes into account multiple co-occurring N fluxes in a natural system. The model contains 3n + 1 equations and an equal number of flux rates, where n is the number of labeled N substrates. The rates in their model were estimated using a weighted least-squares technique. Elskens et al. (2005) subsequently created a process-oriented model (PROM) that accounted for as many N processes as needed to quantify how specific underlying assumptions affected the behavior of the estimates of all the abovementioned models. The authors concluded that uncertainties may increase as the incubation is prolonged and that oversimplified models may risk bias when their underlying assumptions are violated. The most recent attempt to resolve simultaneous N processes was **Table 2.** The isotope matrix results for the rates of N processes in the high-nutrient case at the depth of (a) 80% sPAR and (b) 2% sPAR under different  $r_{\rm NH_4^+}$  variation conditions. In addition, all N transformation rates via ODE following Pfister et al. (2016) on the assumption of no remineralization were estimated for comparison. Note:  $r_{\rm NH_4^+}$  variation was manipulated artificially by decreasing  $r_{\rm NH_4^+}$  values at a constant reduction rate and the total reduction of  $r_{\rm NH_4^+}$  was 0, 1, 10, 20, and 50% of the full time span (15 h) of incubation.

(a)									
	The percentage of $r_{\rm NH_4^+}$ decrease in 15 h								
	0		1%	10 %	20 %	50%			
Rate $(k \times \overline{C})$ nmol L <sup>-1</sup> h <sup>-1</sup>	ODE		Ise	Isotope matrix					
$NH_4^+$ uptake $(F_1)$	360	397	397	399	401	408			
Remineralization $(F_2)$	0	0	21	211	424	1043			
$NO_2^-$ uptake (F <sub>3</sub> )	27	29	29	29	29	29			
$\operatorname{NH}_{4}^{\overline{+}}$ oxidation (F <sub>4</sub> )	1.1	0.4	0.4	0.4	0.4	0.4			
$NO_3^{-}$ uptake (F <sub>5</sub> )	190	149	149	149	149	149			
$NO_2^{-}$ oxidation (F <sub>6</sub> )	1.7	0	0	0	0	0			
DOÑ release $(F_7)$	0	0	0	0	0	0			
Bacteria uptake $NH_4^+$ ( $F_8$ )	268	282	303	490	701	1314			
(b)									
	The percentage of $r_{\rm NH_4^+}$ decrease in 15 h								
	0		1%	10 %	20 %	50%			
Rate $(k \times \overline{C}) \operatorname{nmol} L^{-1} h^{-1}$	ODE		Is	Isotope matrix					
$NH_4^+$ uptake $(F_1)$	228	208	208	209	211	216			
Remineralization $(F_2)$	0	0	18.1	179	361	895			
$NO_2^-$ uptake (F <sub>3</sub> )	7.3	3.1	3.1	3.1	3.1	3.1			
$NH_4^{\ddagger}$ oxidation (F <sub>4</sub> )	1.1	0.7	0.7	0.7	0.7	0.7			
$NO_3^-$ uptake (F <sub>5</sub> )	106	72	72	72	72	72			
$NO_2^{-}$ oxidation (F <sub>6</sub> )	2.0	0	0	0	0	0			
DON release $(F_7)$	0	0	0	0	0	0			
Bacteria uptake $NH_4^+$ ( $F_8$ )	202	265	283	442	623	1152			

conducted by Pfister et al. (2016), who used parallel incubations ( $^{15}$ N labeled NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup>) in tidepools to measure multiple flows among benthic N, ammonium, nitrite, and nitrate. In their experiment, six differential equations were constructed based on mass and isotope balances and solved by using the ODE function of the R language. Because the N content of benthic algae was not measured due to sampling difficulties and spatial heterogeneity of biomass, a mass balance at the whole system scale could not be achieved. Specifically, the rate of DON release could not be determined.

Compared with the methods or models mentioned above, the advantages of the isotope matrix method include (1) the potential biases caused by multiple flows were taken into consideration subject to the constraint that there be a mass balance at the system level; (2) one tracer addition was sufficient to quantify multiple in situ flows; parallel incubations, i.e., light and dark or  ${}^{15}\text{NH}_4^+$  and  ${}^{15}\text{NO}_x^-$ , were not needed; (3) post hoc data processing was simple, and a unique solution can be obtained via matrix inversion; and (4) no extra laboratory work was necessary (see below).

#### 4.1.2 Rate comparisons

In accord with Pfister et al. (2016), we estimated all N transformation rates using ordinary differential equations (ODEs) for the three cases on the assumption that  $r_{\rm NH_4^+}$  was constant (see Tables 1–3). In general, the rates obtained by matrix inversion and integration of the ODEs were consistent. Differences, when apparent, were caused by the duration of the integration. The isotope matrix method was applied to only the first two time points (i.e., time intervals of either 2 or 4 h), whereas the ODEs were integrated for the entire 24 h incubation. In Pfister et al. (2016), ODEs were used to analyze data collected at three time points within a 5 h time interval. Unfortunately, such intensive sampling for on-deck incubations is not practical. However, we strongly recommend short-term incubations for water column studies. Two time points sep-



Figure 5.

arated by a few hours may be more convenient and realistic for instantaneous rate estimates.

Below, we present a comparison between our results and conventional source–product rate measurements (Collos, 1987) of ammonium oxidation and uptake (Table 3). The matrix-derived  $NH_4^+$  uptake rates for all of the experiments were consistent with the rates (difference < 8 %) from the traditional source–product method when the final PN concentration was used in the calculation. The fact that the deviations were larger (13–21 %) when the initial PN was used is consistent with the conclusions of previous studies that estimates involving the final PN concentration are more reliable. The deviation could obviously be higher if the phytoplankton growth rate were higher.

In contrast, the end products of ammonium oxidation or nitrification are consumed by phytoplankton continuously in the euphotic zone. In many cases, nitrate uptake has been shown to occur in both the light and dark (e.g., Dugdale and Goering, 1967; Lipschultz, 2001; Mulholland and Lomas, 2008). The significant consumption of end products  $(NO_x^- \text{ and } NO_2^-)$  violates the assumption that underlies the source–product rate calculation. Therefore, the NH<sub>4</sub><sup>+</sup> oxidation/nitrification rate cannot be determined with a source– product model. Although phytoplankton consumption resulted in a net reduction of  $NO_x^-$  in all of our experiments, we were nevertheless able to determine NH<sub>4</sub><sup>+</sup> oxidation/nitrification rates with the isotope matrix method (Figs. 2b, 3b, and c) (see Table 3).

In most previous studies, the final isotopic composition but not the final concentration of  $NO_x^-$  has been measured. As a result, researchers may not have been aware that the outflow



**Figure 5.** The observed and STELLA-derived values in the high-nutrient case: in the first part 80% sPAR depth and in the second part 2% sPAR depth for (a) [ $^{15}NH_{4}^{+}$ ], (b) [ $^{15}NO_{2}^{-}$ ], (c) [ $^{15}NO_{3}^{-}$ ], (d) [ $^{15}N-PN$ ], (e) [ $^{15}N-DON$ ], (f) [ $^{14}NH_{4}^{+}$ ], (g) [ $^{14}NO_{2}^{-}$ ], (h) [ $^{14}NO_{3}^{-}$ ], (i) [ $^{14}N-PN$ ], (j) [ $^{14}N-DON$ ], (k)  $r_{NH_{4}^{+}}$ , (l)  $r_{NO_{2}^{-}}$ , (m)  $r_{NO_{3}^{-}}$ , (n)  $r_{PN}$ , (o)  $r_{DON}$ , (p)  $\delta^{15}N-NH_{4}^{+}$ , (q)  $\delta^{15}N-NO_{2}^{-}$ , (r)  $\delta^{15}N-NO_{3}^{-}$ , (s)  $\delta^{15}N-PN$ , (t)  $\delta^{15}N-DON$ , (u) [ $NH_{4}^{+}$ ], (v) [ $NO_{2}^{-}$ ], (w) [ $NO_{3}^{-}$ ] (x) [PN], and (y) [DON]. The black regular and inverse open triangles represent the duplicate observational values; the black, green, blue, magenta, and pink solid lines represent the STELLA model simulations of  $r_{NH_{4}^{+}}$  decreases 0, 1, 10, 20, and 50 % in 15 h, respectively.

of <sup>15</sup>NO<sub>x</sub><sup>-</sup> was greater than the inflow. During dark incubations, researchers may also assume insignificant NO<sub>x</sub><sup>-</sup> consumption. However, a "net decrease in end product" is almost unavoidable when an incubation is conducted under simulated in situ light conditions to estimate ammonium oxidation. To address this consumption effect, Santoro et al. (2010, 2013) took NO<sub>x</sub><sup>-</sup> removal into account and formulated a new equation that took account of the nitrification rate (*F*) and NO<sub>x</sub><sup>-</sup> uptake rate (*k*). In accord with Santoro et al. (2010), we calculated the nitrification rate for the low-nutrient case via a nonlinear least-squares curve-fitting routine in MATLAB by using the first three time points of the <sup>15</sup>N-NO<sub>x</sub><sup>-</sup> / <sup>14</sup>N-NO<sub>x</sub><sup>-</sup> are measurements. The calculated rate, 0.05 nmol L<sup>-1</sup> h<sup>-1</sup> (Table 3), was ~ 30% lower than the matrix-derived rate of 0.07 nmol L<sup>-1</sup> h<sup>-1</sup>. In contrast, the nitrate uptake rate constant (k = 0.010 h<sup>-1</sup>) was only one-sixth of the rate constant (0.059 h<sup>-1</sup>) derived from the matrix method, although a comparable nitrification rate was obtained when the consumption term was taken into account.

Process	Case	Depth (m)	Isotope Matrix method (this study)	Rates based on Ref A*	Traditional method Ref B*	Rates followed Ref C*
				(nmol L		
$NH_4^+$ uptake	Low nutrient	25	4.9	3.8	4.6	
Nitrification	Low nutrient	25	0.07	0.04	_	0.05
$NO_x^-$ uptake	Low nutrient	25	27.2	19.3		4.6
$NH_4^+$ uptake	High -80 %sPAR	0.2	397	360	387	
$NH_4^+$ oxidation	High -80 %sPAR	0.2	0.4	1	_	
$NH_4^+$ uptake	High −2 % sPAR	2.3	208	228	192	
$NH_4^+$ oxidation	High −2 % sPAR	2.3	0.7	1	-	

**Table 3.** Comparison of the  $NH_4^+$  /  $NO_x^-$  uptake and  $NH_4^+$  oxidation/nitrification rates derived from different methods.

\* Ref A represents rate calculation by ODE following Pfister et al. (2016). Ref B represents rate calculation following Collos (1987). Ref C represents rate calculation following Santoro et al. (2010).

**Table 4.** The contribution of nitrification derived  $NO_x^-$  to  $NO_x^-$  uptake (%), N preference index, and the proportion of  $NH_4^+$  consumption by phytoplankton, bacteria, and nitrifier to total  $NH_4^+$  consumption in low- and high-nutrient cases.

Case	Depth (m)	Nitrification to NO <sub>3</sub> <sup>-</sup> uptake (%)	RPI for NH <sup>+</sup> <sub>4</sub>	RPI for NO <sub>2</sub> <sup>-</sup>	RPI for NO <sub>3</sub>	A/TNH <sup>+</sup> * consumption (%)	B/TNH <sup>+</sup> * consumption (%)	C/TNH <sub>4</sub> <sup>+</sup> * consumption (%)
Low nutrient	25	0.3	0.9	0.6	1.0	82.1	16.8	1.2
High –80 % SPAR High –2 % SPAR	2.3	0.5	1.6	0.6	0.5	58.4 43.9	41.5 56.0	0.1

\* A, B, and C represent NH<sup>4</sup><sub>4</sub> utilized by phytoplankton, bacteria, and nitrifier, respectively. TNH<sup>4</sup><sub>4</sub> consumption represents total NH<sup>4</sup><sub>4</sub> consumption.

Surprisingly, when we introduced the values of *F* and *k* determined with the method of Santoro et al. (2010) into STELLA to generate time courses of variables, we found that the simulated values of  $\delta^{15}NO_x^-$  and  $r_{NO_x^-}$  agreed well with those determined by the isotope matrix method (Fig. 4j and n). However, much slower decreasing trends were found for  ${}^{15}NO_x^-$ ,  ${}^{14}NO_x^-$ , and  $NO_x^-$  (Fig. 4b, f, and r). Finally, we realized that the equation proposed by Santoro et al. (2010) was constrained only by the changes in the ratios rather than by the changes in the individual concentrations of  ${}^{15}NO_x^-$  and  ${}^{14}NO_x^-$ . Thus, nonlinear curve fitting may provide a correct simulation only of the change in the ratio. This conclusion implies that the nitrate uptake rate derived from nonlinear curve fitting should be validated by the final concentration of nitrate, as was done by Santoro et al. (2013).

In summary, (1) accurate measurements of concentrations during a time series are vital for all kinds of transformation rate estimates, including the isotope matrix method, and (2) the isotope matrix method can overcome various biases that impact estimates made with traditional methods.

### 4.2 Implications for nitrogen biogeochemical processes

Results of use of the isotope matrix method suggest several conclusions with respect to biogeochemical processes.

# 4.2.1 Remineralization, regeneration, and community succession

The matrix solution was consistent with the model runs with variable  $rNH_4^+$  at time points of no more than 12 h, the implication being that dilution effects were negligible during the early incubation period, at least in our studies. Dilution effects could be significant when remineralization is intensive and the incubation longer. Pfister et al. (2016) found that macrofauna (mussels) play an important role in remineralization. The fact that zooplankton in our water samples were not abundant might be a reason for the low remineralization rates in our short-term incubations.

In the WNP low-nutrient case, after an incubation of 24 h, the levels of nitrate and ammonium approached the concentration threshold for phytoplankton utilization (e.g., < 30-40 nM NH<sub>4</sub><sup>+</sup> for *Emiliania huxleyi*; Sunda and Ransom, 2007). In Fig. 4, the STELLA projection agreed well with the PN concentrations for only the first 12 h. In this case, we ac-

tually observed phytoplankton succession. Our flow cytometry data (Fig. S3) demonstrated that the number of living eukaryotic cells (4 times higher than Synechococcus) increased in the first 24 h and started to drop rapidly after 24 h. In contrast, the growth of Synechococcus continued after 24 h, even though nitrogen concentrations dropped to a constantly low level. These observations suggest that the phytoplankton community was competing for nitrogen, and a major community shift started at around 24 h. After the time point at 12 h, the observed concentrations of <sup>14</sup>N and <sup>15</sup>N in the PN were higher than those projected by STELLA. The most intriguing phenomenon among PN-associated parameters was the additional <sup>15</sup>N, which could not have come from  ${}^{15}NH_4^+$ . The most likely source of nitrogen with enriched <sup>15</sup>N to support Synechococcus growth was the nitrogen released from dead eukaryotes, which contained freshly consumed <sup>15</sup>N tracer, rather than the ambient DON. More studies are needed to explore nutrient thresholds for different phytoplankton species. Nevertheless, our results suggest that incubations must last no more than a few hours for nitrogen uptake studies in the oligotrophic ocean.

# 4.2.2 Evaluation of the contribution of nitrification to new production

Nitrification in the euphotic zone of the ocean drew little attention until recent years after molecular evidence led to the discovery of the widespread occurrence of ammoniaoxidizing archaea (AOA) (Francis et al., 2005; Santoro et al., 2010, 2013; Smith et al., 2014) and rate measurements based on isotopic studies (Ward, 2011; Santoro et al., 2010; Grundle et al., 2013; Smith et al., 2014). As mentioned in the Introduction, the conventional "new" production may have been overestimated 19-33 % on a global scale due to the nitrate regenerated in the euphotic zone via nitrification. However, a more realistic assessment of the fractional contribution of nitrification to  $NO_3^-$  uptake can only be achieved when incubations are conducted in the same bottle under in situ light conditions instead of parallel incubations in the dark and light. The isotope matrix method is so far the most convenient and suitable method for evaluating the relative importance of co-occurring nitrification and new production in the euphotic zone. In all our experimental studies, the contributions of nitrification to new production were < 1 % (Table 4). This relatively low contribution was probably due to light inhibition of nitrifiers in the WNP and the low water temperature.

Nevertheless, light effects in our studies were significant. Light suppresses nitrification (Ward, 2005; Merbt et al., 2012; Peng et al., 2016). The  $NH_4^+$  oxidation rate at 80 % sPAR was reduced by 36 % relative to the rate at 2 % sPAR. These results are consistent with current knowledge, although some recent evidence has shown that some taxa of marine AOA have the genetic capability to reduce oxidative stress and to repair ultraviolet damage (Luo et al., 2014; Santoro et al., 2015). More case studies are needed in the future to explore the vertical distribution of the relative contribution of nitrification to new production in the euphotic zone.

#### 4.2.3 Nutrient preference

Phytoplankton use a variety of nitrogenous species for growth. McCarthy et al. (1977) introduced the concept of a relative preference index (RPI) to assess the relative use of different forms of N, and an RPI > 1 indicates a preference for the specific substrate over other forms of N. As shown in Table 4, in the low-nutrient case NO<sub>3</sub><sup>-</sup> was preferred. The fact that the RPI for NO<sub>3</sub><sup>-</sup> was slightly higher than the RPI for NH<sub>4</sub><sup>+</sup> was probably due to the phytoplankton community structure, as mentioned above. This result is consistent with studies in the Sargasso Sea (Fawcett et al., 2011). However, in the high-nutrient case, the order of the RPI values was NH<sub>4</sub><sup>+</sup> > 1 > NO<sub>3</sub><sup>-</sup> > NO<sub>2</sub><sup>-</sup>, the suggestion being that phytoplankton preferred NH<sub>4</sub><sup>+</sup> over NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup>, similar to the results of studies in Chesapeake Bay (McCarthy et al., 1977).

# 4.2.4 Quantifying various ammonium consumption pathways

In the upper ocean,  $NH_4^+$  cycles rapidly due to the metabolic pathways of the various microorganisms that compete for ammonium. Ammonium may serve as a nitrogen source for phytoplankton assimilation, and as an energy source for ammonia-oxidizing organisms (AOMs). Moreover, many studies have shown that bacteria also play a part in  $NH_4^+$ utilization (Middelburg and Nieuwenhuize, 2000; Veuger et al., 2004). Our results in the low-nutrient case showed that phytoplankton were the main consumers of  $NH_4^+$  (82 % of the total  $NH_4^+$  consumption). Bacteria accounted for another ~17 %, and AOMs used the remaining 1 %. In the highnutrient study, phytoplankton and bacteria each consumed ~50 % of the total  $NH_4^+$  (Table 4).

#### 5 Conclusions

The isotope matrix method was designed specifically for incubations in the euphotic zone under simulated in situ conditions. By considering multiple flows among pools and requiring mass balance at the whole-system level, we minimized potential biases caused by non-targeted processes in traditional source–product methods. Given the progress in analytical techniques for measuring concentrations and isotopic compositions of nitrogen species, the isotope matrix method is a promising approach for studying of rates of nitrogen fluxes from a system-wide perspective. Furthermore, the matrix method is also appropriate for probing the effects of environmental factors (e.g.,  $CO_2$ , pH, temperature, and light intensity) on interactive N processes in a single incubation bottle. *Data availability.* The data associated with the paper are available from the corresponding author upon request.

### The Supplement related to this article is available online at doi:10.5194/bg-14-1021-2017-supplement.

*Competing interests.* The authors declare that they have no conflict of interest.

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#### M. N. Xu et al.: Quantification of multiple simultaneously occurring nitrogen flows

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