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 ocean have been recognized, but quantitative information about multiple transformation rates among nitrogen pools, i.e., ammonium ($\text{NH}_4^+$), nitrite ($\text{NO}_2^-$), nitrate ($\text{NO}_3^-$) and particulate/dissolved organic nitrogen (PN/DON), are insufficient due to methodological difficulties. Recent advances in analytical methods for isotopic composition of oft-measured nitrogen species allowed us to establish a convenient isotope labelling method to quantify in situ dynamic nitrogen flows for euphotic water. By adding a single $^{15}$N-labelled $\text{NH}_4^+$ tracer, we monitored the changes in concentration and isotopic composition of the total dissolved nitrogen (TDN), PN, $\text{NH}_4^+$, $\text{NO}_2^-$, and $\text{NO}_3^-$ pools to trace the $^{15}$N and $^{14}$N flows. Based on mass and isotope conservations of every individual pool as well as the whole system, we formulated matrix equations with unique solution to simultaneously derive multiple nitrogen transformation rates, such as rates of $\text{NH}_4^+$, $\text{NO}_2^-$, and $\text{NO}_3^-$ uptake; ammonia oxidation; nitrite oxidation; DON release and $\text{NH}_4^+$ uptake by bacteria. This isotope matrix method was designed specifically for euphotic water column incubation under simulated in situ condition. With consideration of multi-flows among pools, we minimized potential biases caused by non-targeted processes in traditional source-product method. The proposed isotope matrix method is convenient in terms of on-deck incubation and post-hoc data analysis and is feasible to probe effects of environmental factors (e.g., pH, temperature and light) on multiple processes under manipulated conditions.
Keywords

Ammonium oxidation, isotope, new production, nitrification, regenerated production
1. Introduction

Nitrogen (N), which is an essential element in organisms’ metabolic processes, 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$_4^+$), nitrite (NO$_2^-$), and nitrate (NO$_3^-$) (Fig. 1). Quantitative information on transformation rates in the marine N-cycle may advance our understanding of the coupling of autotrophic and heterotrophic processes involving carbon and nitrogen as well as the efficiency of the biological pump. Such information would also facilitate evaluation of ecosystem functions. However, the dynamic nature and complexity of the reactions involving nitrogen make it a difficult task to resolve the rates of multiple simultaneous nitrogen transformations. Inventory and isotope tracer methods have often been used to measure rate of specific process in previous studies (Ward, 2008, 2011; Lipschultz, 2008 and references therein).

The inventory method (monitoring the change of substrate and/or product concentrations over time) was often 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 Hatton, 1971; Pakulski et al., 1995; Ward, 2011). However, unwanted processes may bias the result. For example, the substrate (ammonium) pool
is controlled simultaneously by consumptions via phytoplankton (PN as the product), nitrifier (nitrite/nitrate as the product) and bacteria (operationally defined DON as product) and by additions via remineralization from heterotrophic bacterial metabolism, zooplankton excretion, and viral lysis. Similarly, the product (NO₃⁻) pool of nitrification is consumed contemporarily by phytoplankton during incubation.

The $^{15}$N-labeled tracer technique has been widely used as a direct measure for specific nitrogen processes since the emergence of isotope ratio mass spectrometry (IRMS). For example, the addition of $^{15}$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 $^{15}$NH₄⁺ has been added, nitrification rate ($^{15}$NO₃⁻ as product; e.g. Newell et al., 2013; Hsiao et al., 2014; Peng et al., 2016) and ammonium uptake rate ($^{15}$NPN as product; e.g. Dugdale and Goering, 1967; Dugdale and Wilkerson, 1986; Bronk et al., 1994, 2014) can be measured, respectively, with dark and light incubation. However, isotope labelling encounters similar bias problem in the inventory method, i.e., multiple processes occur simultaneously involving either source or product terms in the incubation bottle. In fact, these transformations among pools have significant implications in biogeochemical cycle. For instance, Yool et al. (2007) synthesized available global data and indicated that the fractional contribution of nitrate derived from nitrification to nitrate uptake can be as high as 19–33% in the euphotic zone. However, nitrate uptake rates were determined under light conditions, and nitrification was determined under dark conditions (e.g. Grundle et al., 2013),
which are not comparable in terms of their effects on these processes. To overcome this problem, 24-h incubations have been used to compensate for the diel cycle of light-sensitive processes (Beman et al., 2012). Yet, 24-h incubations may cause calculation artifacts due to the interference from significant transfers of $^{15}$N and $^{14}$N among pools. A new method is needed to reconcile the above-mentioned biases and the uncomparable parallel incubations.

Marchant et al. (2016) have reviewed recent method advances in marine N-cycle studies using $^{15}$N-labeling substrates combined with nanoSIMS, FISH, or HISH. These methods provide qualitative information for N transfers at cellular and molecular level but no quantitative rates at community level. A comprehensive review of oft-used models for rate derivation was conducted by Elskens et al. (2005), who concluded that oversimplified models would risk bias when their underlying assumptions are violated; nevertheless, overly complex models could misinterpret part of the random noise as relevant processes. Therefore, a model selection procedure was subsequently proposed (De Brauwere et al., 2005). More recently, Pfister et al. (2016) applied isotope tracer technique and mass conservation model onto tidal ponds study to explore nitrogen flows among dissolved nitrogen pools ($\text{NH}_4^+$, $\text{NO}_2^-$ and $\text{NO}_3^-$) and found that benthic macrobiota plays important role in regulating remineralization flow. They also proved that the dilution effect significantly biased the results obtained by source-product models. Nevertheless, for the euphotic zone where competing processes co-occur, an
Innovative and convenient method for on-deck incubation to measure in situ multiple N flows is needful.

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, $^{15}$NH$_4^+$, was added into incubation bottle to trace the $^{15}$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 for low-nutrient water in the western North Pacific and for high-nutrient coastal water off southeastern China coast. Thank for recent advances in these analytical methods for concentration and isotopic composition of various nitrogen species, this isotope matrix method becomes applicable to quantify in situ dynamic nitrogen flows for euphotic water.

2. Isotope matrix method

2.1 Framework of the inter-connections among nitrogen pools

In the oxygenated and well-lit euphotic zone, the transformations of N among NH$_4^+$, NO$_2^-$, NO$_3^-$, PN, and DON are shown in Fig. 1. The PN is operationally defined as the particulate organic nitrogen trapped on a GF/F filter (> 0.7μm). Dissolved inorganic nitrogen (DIN) and DON are the inorganic and organic nitrogen, respectively, in the dissolved fraction that passes through a polycarbonate membrane with a 0.22 μm
pore size. Since DON includes the N in numerous dissolved organic N compounds, including unidentified organics, urea, amino acids, amines, and amides, DON represents the “bulk” DON and is calculated by subtracting the concentrations of NH$_4^+$, NO$_2^-$, and NO$_3^-$ (DIN) from the total dissolved N (TDN).

We consider two different types of schemes in our method: low nitrogen and high nitrogen (Fig. 1a and 1b). The low nutrient scheme is for the open ocean. The high nutrient scheme is for estuary and coastal environments where three dissolved inorganic nitrogen species co-exist. Below, we describe the rationale of model structures for the two cases.

The consumption of reactive inorganic nitrogen (NH$_4^+$, NO$_2^-$, and NO$_3^-$) is dominated by photosynthetic uptake by phytoplankton (F1 and F4 in Fig. 1a; F1, F3, and F5 in Fig. 1b). Heterotrophic bacteria may also be important actors for NH$_4^+$ assimilation (Laws, 1985), and was confirmed by studies later on (e.g. Middelburg and Nieuwenhuize, 2000; Veuger et al., 2004). We took it into account as well (F6 in Fig. 1a and F8 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 (Santoro et al., 2013), especially in high NH$_4^+$ estuary and coastal sea, nitrate assimilation may be inhibited in oxygenated water and, subsequently, so is the nitrite release. Thus, the 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 (F5 in Fig. 1a and F7 in Fig. 1b) as indicated by previous studies (Bronk et
al., 1994; Bronk and Ward, 2000; Varela et al., 2005). On the other hand, remineralization may refuel the NH$_4^+$ pool (F2 in both Fig. 1a and 1b). Meanwhile, ammonium pool is reduced by nitrification process, which consists of two basic steps: the ammonia oxidation by archaea/bacteria (AOA/AOB) to nitrite (F4 in Fig. 1b) and the nitrite oxidation to nitrate by nitrite-oxidizing bacteria (NOB) (F6 in Fig. 1b).

Although recent studies have revealed a single microorganism that may completely oxidize NH$_4^+$ to NO$_3^-$ (comammox) (Daims et al., 2015; van Kessel et al., 2015), its importance 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 flows and exchanges among the often measured and operationally defined nitrogen pools. In this context, grazers and viruses belong to the operationally defined PN and DON pools, respectively. Thus, the resulance of specific process such as grazing and viral lysis has been incorporated in the paradigm depicted in Figure 1.

**2.2 Analytical methods to determine the amounts of $^{15}$N/$^{14}$N in various pools**

To trace the $^{15}$N movement among pools, our isotope matrix method couples the $^{15}$N-labelling and inventory methods through considering both concentration and isotopic composition changes. 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 of the
mentioned concentrations and isotopic compositions except the isotopic composition of NH$_4^+$.

Concentrations of NH$_4^+$ higher than 0.5 $\mu$M were measured manually by using the colorimetric phenol hypochlorite technique (Koroleff, 1983). Nanomolar NH$_4^+$ concentrations were measured by using the fluorometric o-phthaldialdehyde (OPA) method (Zhu et al., 2013). Concentrations of NO$_2^-$ and NO$_x^-$ (NO$_2^-$ + NO$_3^-$) were determined with the chemiluminescence method following the protocol of Braman and Hendrix (1989). The detection limits of NO$_2^-$ and NO$_x^-$ were both $\sim$ 10 nmol L$^{-1}$, 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$_x^-$ described above.

We determined the $\delta^{15}$N of NO$_2^-$ with the azide method by following the detailed procedures in McIlvin and Altabet (2005). The $\delta^{15}$N of NO$_x^-$ was determined by using a distinct strain of bacteria that lacked N$_2$O reductase activity to quantitatively convert NO$_x^-$ to nitrous oxide (N$_2$O), which we then analyzed by IRMS (denitrifier method; Sigman et al., 2001; Casciotti et al., 2002). The isotopic composition of NO$_3^-$ was determined from isotope mass balance (NO$_x^-$ minus NO$_2^-$) or measured by the denitrifier method after eliminating preexisting NO$_2^-$ with sulfamic acid (Granger and Sigman, 2009). To determine the $\delta^{15}$N of TDN and PN, both species were first converted to NO$_3^-$ with the denitrifier method, and then the $\delta^{15}$N of the NO$_3^-$ was
determined as described above. The detection limit of $\delta^{15}N_{\text{PN}}$ can be reduced to nanomole level (absolute amount of nitrogen), which is significantly lower than that by using high temperature combustion with an elemental analyzer connected to 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$_4$)$_2$SO$_4$ on a glass fiber (GF) filter; the isotope ratios of the $^{15}$N/$^{14}$N are then measured using a coupled elemental analyzer 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$_4^+$ is first quantitatively oxidized to NO$_2^-$ by hypobromite (BrO$^-$) at pH ~12 (BrO$^-$ oxidation method), and the protocol of McIlvin and Altabet (2005) is then used to reduce the NO$_2^-$ to N$_2$O (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 $^{14}$N and $^{15}$N atoms in every individual pool for which we knew the concentration and $\delta^{15}$N ($\delta^{15}N$ % = $[(R_{\text{sample}} - R_{\text{atmN}_2})/R_{\text{atmN}_2}] \times 1000$). By assuming the $^{15}$N content of standard atmospheric nitrogen to be 0.365%, we calculated $R_{\text{sample}}$ ($^{15}$N/$^{14}$N). By defining $r_{\text{sample}}$ as $^{15}$N/$(^{14}$N+$^{15}$N), we directly derived the $^{15}$N and
14N concentrations of all forms of N, with the exception of NH₄⁺ and DON. The r value of the NH₄⁺ was assumed to equal either its initial value or an arbitrarily chosen fraction thereof, and the ¹⁵N and ¹⁴N content of the NH₄⁺ was then determined.

2.3 Formation of matrix equations

In this isotope matrix method, we added limited amount of ¹⁵NH₄⁺ into incubation bottles at the very beginning and then monitored the changes of ¹⁵N and ¹⁴N in the measured pools every a few hours. We assumed isotopic mass balance at every time point in the incubation bottle. In other words, the sums of the variations in the total N, ¹⁵N, and ¹⁴N concentrations were zero for any time interval. We assumed no fractionation between ¹⁵N and ¹⁴N for all the transfer reactions among the pools. The fluxes of ¹⁵N and ¹⁴N were therefore equal to the total flux multiplied, respectively, by r_{substrate} and (1 – r_{substrate}). Note that we did not consider isotope fractionation, though it could easily be introduced into the equations if necessary, i.e., dividing ¹⁴N flux by α (the ratio of specific rate constant of ¹⁴N to ¹⁵N), and the flux of ¹⁵N is obtained. Below, we illustrated equations for the two model cases.

According to mass balance, the net changes of the ¹⁵N (or ¹⁴N) concentration of individual N pool in certain time interval are determined by the inflow and outflow of ¹⁵N (or ¹⁴N) (see Fig. 1 and Eqs. below). In the low-nitrogen case, the changes of the ¹⁵N concentrations of the NH₄⁺, NO₃⁻, and PN pools were expressed by Eq. 1, 2 and 3, respectively. Similarly, the temporal dependence of ¹⁴N-NH₄⁺, ¹⁴N-NO₃⁻, and ¹⁴N-PN were expressed by Eq. 4, 5 and 6, respectively. The mean rate of change in nitrogen
pool, i.e. the left side of the equation, was determined from the data at time zero and the first time point. For example, when sampling time interval is short, $\Delta[^{14}\text{NH}_4^+] / \Delta t$ at the first time point was approximately $([^{14}\text{NH}_4^+]_{t1} - [^{14}\text{NH}_4^+]_{t0})/(t1-t0)$ where the subscripts indicate the times at which the concentrations were measured. The $r$ value applied in the equation for substrate was the average of the $r$ values at time zero and the first time point after that for measured pool.

\[
\begin{align*}
\frac{\Delta[^{15}\text{NH}_4^+]}{\Delta T} &= F_2 \times 0.00366 - F_1 \times r_{^{14}\text{NH}_4^+} - F_3 \times r_{^{14}\text{NH}_4^+} - F_6 \times r_{^{14}\text{NH}_4^+} \\
\frac{\Delta[^{15}\text{NO}_x^-]}{\Delta T} &= F_3 \times r_{^{14}\text{NH}_4^+} - F_4 \times r_{^{14}\text{NO}_x^-} \\
\frac{\Delta^{15}\text{PN}}{\Delta T} &= F_1 \times r_{^{14}\text{NH}_4^+} + F_4 \times r_{^{14}\text{NO}_x^-} - F_5 \times r_{^{14}\text{PN}} \\
\frac{\Delta[^{14}\text{NH}_4^+]}{\Delta T} &= F_2 \times (1 - 0.00366) - F_1 \times (1 - r_{^{14}\text{NH}_4^+}) - F_3 \times (1 - r_{^{14}\text{NH}_4^+}) - F_6 \times (1 - r_{^{14}\text{NH}_4^+}) \\
\frac{\Delta[^{14}\text{NO}_x^-]}{\Delta T} &= F_3 \times (1 - r_{^{14}\text{NH}_4^+}) - F_4 \times (1 - r_{^{14}\text{NO}_x^-}) \\
\frac{\Delta^{14}\text{PN}}{\Delta T} &= F_1 \times (1 - r_{^{14}\text{NH}_4^+}) + F_4 \times (1 - r_{^{14}\text{NO}_x^-}) - F_5 \times (1 - r_{^{14}\text{PN}})
\end{align*}
\]

In this study, we conducted a time series monitoring for over 24 hours, however, we took the first two time points for the rate calculation since such rate derivations might be more close to the instant rates in the original environments. Note: researchers may apply this method onto longer time interval, however, rates may vary as a result
of substrate consumption and/or community change, shorter-term incubation is suggested (see below).

Since the total number of equations and unknowns are equal, a unique solution therefore can be obtained via matrix solution for the low nutrient model.

In high nutrient cases, similarly, equations (Eqs. 7-14) can be constructed by using transformations among NH$_4^+$, NO$_2^-$, NO$_3^-$ and PN (Fig. 1b).

\[
\Delta^{15}[NH_4^+] \Delta T = \overline{F_2} \times 0.00366 - \overline{F_1} \times \overline{r_{NH_4^+}} - \overline{F_4} \times \overline{r_{NH_4^+}} - \overline{F_6} \times \overline{r_{NH_4^+}} \tag{7}
\]

\[
\Delta^{15}[NO_2^-] \Delta T = \overline{F_4} \times \overline{r_{NH_4^+}} - \overline{F_3} \times \overline{r_{NO_2^-}} - \overline{F_6} \times \overline{r_{NO_2^-}} \tag{8}
\]

\[
\Delta^{15}[NO_3^-] \Delta T = \overline{F_6} \times \overline{r_{NO_2^-}} - \overline{F_5} \times \overline{r_{NO_3^-}} \tag{9}
\]

\[
\Delta^{15}[PN] \Delta T = \overline{F_1} \times \overline{r_{NH_4^+}} + \overline{F_3} \times \overline{r_{NO_2^-}} + \overline{F_5} \times \overline{r_{NO_3^-}} - \overline{F_7} \times \overline{r_{PN}} \tag{10}
\]

\[
\Delta^{14}[NH_4^+] \Delta T = \overline{F_2} \times (1-0.00366) - \overline{F_1} \times (1-\overline{r_{NH_4^+}}) - \overline{F_4} \times (1-\overline{r_{NH_4^+}}) - \overline{F_6} \times (1-\overline{r_{NH_4^+}}) \tag{11}
\]

\[
\Delta^{14}[NO_2^-] \Delta T = \overline{F_4} \times (1-\overline{r_{NH_4^+}}) - \overline{F_3} \times (1-\overline{r_{NO_2^-}}) - \overline{F_6} \times (1-\overline{r_{NO_2^-}}) \tag{12}
\]

\[
\Delta^{14}[NO_3^-] \Delta T = \overline{F_6} \times (1-\overline{r_{NO_2^-}}) - \overline{F_5} \times (1-\overline{r_{NO_3^-}}) \tag{13}
\]

\[
\Delta^{14}[PN] \Delta T = \overline{F_1} \times (1-\overline{r_{NH_4^+}}) + \overline{F_3} \times (1-\overline{r_{NO_2^-}}) + \overline{F_5} \times (1-\overline{r_{NO_3^-}}) - \overline{F_7} \times (1-\overline{r_{PN}}) \tag{14}
\]
Again, a unique solution can be obtained since the numbers of equations and unknowns are equal.

In the above matrix equations, $r_{\text{NH}_4^+}$, which we did not measure in this study, is necessary for the solution. Here we set various degrees of remineralization to test the effect of isotope dilution (NH$_4^+$ addition) in our experimental cases. We reduced $r_{\text{NH}_4^+}$ values at a constant reduction rate and the total reduction of $r_{\text{NH}_4^+}$ was 0%, 1%, 10%, 20% and 50% for the full time span of incubation ($r_{\text{NH}_4^+}$ of remineralization is assumed to be 0.00366). The F2 coupled with given $r_{\text{NH}_4^+}$ values allowed us to resolve rates under different remineralization conditions, and the derived F2 was introduced into STELLA model for extrapolations (see below). We compared the observed and remineralization-associated simulations to reveal the effect of remineralization on rate measure for time series incubations.

### 2.4 Validation by STELLA

As the aforementioned, the “instant rate” at the original condition is what researchers pursue. Note that the use of “instant” here is just to make it distinguishable from the longer time incubation or more than two time points. To evaluate the applicability of matrix-derived instant rate, here we applied STELLA 9.1.4 software (Isee systems, Inc.) to construct box models that were consistent with the scenarios depicted in Figure 1. The constructed STELLA model contained two modules (Figs. S1 and S2), one for $^{15}$N and the other for $^{14}$N. The connection between these two modules was through the $^{15}$N atom % ($r_N$), which was a measured parameter in the incubation
experiment. The model started to run with these measured initial values for nitrogen pools at time zero and to project continuous changes of corresponding nitrogen pools. Since the rate numbers based on the first two time points may not guarantee a good performance for the full time course due to system variation, i.e., changes in substrate concentration and microorganism community, we took this model practice (extrapolation) as a validation.

In this study, we assumed the first order reaction for both cases, thus, the initial rate constant “k” can be derived via dividing matrix-derived F by $\bar{C}$ (the average substrate concentration of the first two time points). After setting the initial concentrations of $^{15}$N and $^{14}$N to every pool, the model ran for 24 h according to matrix-derived short-term k values. As depicted in Figure 1, all these monitored N pools are regulated by F, which is concentration dependent according to our assumption (Figs. S1 and S2). The output includes the time courses of the $^{15}$N and $^{14}$N concentrations and the $^{15}$N atom % ($r_N$) of each N species. Through this comparison, we could observe the course evolution of the isotopic composition in various N pools.

2.5 Study sites and incubation experiments

Incubation experiments were conducted in two environments with very different nutrient levels. The low nutrient case 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 spring 2015. The site for the high nutrient case is in the Wuyuanwan Bay (WYW) (24.5° N, 118.2° E) in the southern coast of China.
The water samples at WNP station were collected using a 24-bottle rosette sampler. The sampling depth was 25 m with moderate light intensity (12% the surface water irradiance). Two pre-washed 10-L polycarbonate carboys (Nalgene, USA) were used for the incubation. A total of 1.5 mL of 200 μM 15N-labelled NH₄Cl tracers containing 98 atom% 15N (Sigma-Aldrich, USA) was injected into each incubation bottle separately to achieve a final concentration of 30 nM. Incubation was carried out immediately with a constant simulated light intensity (35 μmol E m⁻² s⁻¹) in a thermostatic incubator (GXZ-250A, Ningbo) at in situ temperature.

The WYW station is an inner bay with a regular semidiurnal tide. As a coastal bay, Wuyuanwan suffers from anthropogenic influences that result in high nutrient concentrations analogous to other coastal zones in China. However, the bay water is still well ventilated and constantly saturated with dissolved oxygen due to tidally induced water exchange. It is an ideal research site to study the dynamic transformation processes of the coastal nitrogen cycle.

The WYW samples were taken on 19 January, 2014 from water depth of 0.3 m and 2.3 m, respectively, with a light intensity of 80 % and 2% of the surface water irradiance. Duplicate water samples were collected for each depth by using submersible pump into pre-washed 10-L polycarbonate bottles (Nalgene, USA). 15N-labeled NH₄Cl (98 atom % 15N, Sigma-Aldrich, USA) was added to the incubation bottles with final concentration of 1μM (~4 % of the ambient concentration). The incubations were carried out immediately in the field. Neutral density-screen that allows 80% and 2%
light penetration was applied, respectively, for incubation bottles of shallow and deep samples. The temperature was maintained at ~13.7 °C by continuously pumped seawater throughflow.

Sample of the first time point (t0) was taken immediately after tracer addition. Subsequent samples were taken at approximately 2–4 h interval for DIN and PN analyses. An aliquot of 200 mL was filtered through a 47-mm polycarbonate membrane with a 0.22 μm pore size (Millipore, USA), and 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), under a pressure of <100 mm Hg. The GF/F filters were freeze-dried and stored in a desiccator for PN concentration and isotopes.

3. Results

3.1 Ambient condition and initial concentrations

The water temperature and salinity of the WNP low nutrient case from 25m was 18.4°C and 34.8, respectively. The dissolved oxygen (DO) was 7.3 mg L⁻¹. The concentration of NH₄⁺, NO₃⁻ and phosphate was 113 ± 5 nmol L⁻¹, 521 ± 18 nmol L⁻¹ and 74 ± 2 nmol L⁻¹, respectively. The N/P ratio was lower than 16, indicating the system is N limited.

The water temperature and salinity of the WYW whole water column for high nutrient case was 13.5 ± 0.1°C and 29.5 ± 0.1, respectively. The DO saturation ranged 135-140%. The concentrations of nitrogenous species were relatively high with
inorganic nutrient concentrations of 30.9 ± 0.7 μmol L⁻¹ for NO₃⁻, 22.3 ± 4.3 μmol L⁻¹ for NH₄⁺, 5.4 ± 0.2 μmol L⁻¹ for NO₂⁻, and 9.3 ± 0.7 μmol L⁻¹ for PN. Phosphate was 1.5 ± 0.1 μmol L⁻¹.

3.2 Time-courses of incubations

3.2.1 Low nutrient case

The observed variation patterns for the bulk NH₄⁺, NO₃⁻, PN, and TDN concentrations and δ¹⁵N of NO₃⁻ and PN during incubation are shown in Figure 2. Concentrations of NH₄⁺ and NO₃⁻ decreased rapidly from 143 ± 5 to 48 ± 5 nM and 521 ± 18 to 127 ± 11 nM, respectively (Figs. 2a and 2b). In contrast, 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). Opposite to the trend of NO₃⁻ concentration, δ¹⁵N-NO₃⁻ increased from 8.9 ± 0.2 to 171 ± 2 ‰ (Fig. 2e). In addition, δ¹⁵N-PN exhibited great changes, increasing from 46.8 ± 0.2 to 6950 ± 314 ‰ (Fig. 2f).

3.2.2 High nutrient cases

The time-series of observational parameters for samples from depths of 80% and 2% sPAR exhibited similar variation trends during incubation (Fig. 3). During the course of incubation, NH₄⁺ decreased significantly and continuously from 26.6 ± 0.1 (initial concentration) to 17.4 ± 0.1 μmol L⁻¹ with a mean reduction rate of 0.63 μmol L⁻¹ h⁻¹ for 80% sPAR sample (Fig. 3a). Compared with that of 80% sPAR, NH₄⁺ of 2% sPAR sample decreased slower from 24.6 ± 0.1 (initial concentration) to 18.2 ± 1.0 μmol L⁻¹
with a mean reduction rate of 0.47 μmol L⁻¹ h⁻¹ (Fig. 3a). NO₃⁻ in 80% and 2% sPAR decreased from 30.1 ± 0.1 to 28.3 ± 0.1 μmol L⁻¹ and from 31.1 ± 0.1 to 29.7 ± 0.1 μmol L⁻¹, respectively (Fig. 3c). Overall, the nitrate reduction rates were much lower than that of NH₄⁺. Compared to nitrate, NO₂⁻ displayed even slower declining trends yet with significantly higher rate for 80% sPAR sample relative to that of 2% sPAR sample (Fig. 3b). Similar to the low nutrient case, PN increased steadily from 8.8 ± 0.1 to 17.7 ± 0.9 μmol L⁻¹ with a mean rate of 0.61 μmol L⁻¹ h⁻¹ for 80% sPAR sample and from 9.9 ± 0.1 to 16.0 ± 2.0 μmol L⁻¹ with a mean rate of 0.44 μmol L⁻¹ h⁻¹ in 2% sPAR (Fig. 3d). The increase rates in PN concentration were very close to the decrease rates of NH₄⁺ indicating ammonium was the major nitrogen source for growth. The TDN concentration decreased from 78.7 ± 1.6 to 68.4 ± 0.1 μmol L⁻¹ and form 72.8 ± 2.5 to 67.1 ± 0.8 μmol L⁻¹ for 80% and 2% sPAR samples, respectively (Fig. 3e). The δ¹⁵N-NO₂⁻ increased from −9.0 ± 0.1 to 12.1 ± 0.1 ‰ and −8.8 ± 0.1 to 23.3 ± 0.6 ‰ in 80% and 2% sPAR incubation, respectively (Fig. 3g); Since nitrate pool was relatively large, the values of δ¹⁵N-NO₃⁻ ranged from 6.8 to 10.1 ‰ with no significant trend over time (Fig. 3h). In addition, δ¹⁵N-PN increased from 14.8 ± 0.3 to 3078 ± 180 ‰ and from 15.0 ± 0.5 to 2738 ± 66 ‰ for 80% and 2% sPAR sample, respectively (Fig. 3i). These significant changes in both concentration and isotopic compositions of nitrogen pools over time suggested that nitrogen transformed significantly among pools and the labelled ¹⁵N in NH₄⁺ flowed through the system except nitrate.

### 3.3 Solutions of the matrix equation and STELLA extrapolation
3.3.1 Low nutrient case

The matrix-derived rate constants (ki) and rates (Fi) are shown in Table 1(A) and 1(B), respectively. Under no remineralization condition (i.e. \( r_{NH4+} \) decreased 0% within 24 hours), the NO\(_x\) uptake (\( k4 = 0.059 \) h\(^{-1}\); \( F4 = 27.2 \) nmol L\(^{-1}\) h\(^{-1}\)) was the highest among all in terms of flux, followed by NH\(_4^+\) uptake (\( k1 = 0.038 \) h\(^{-1}\); \( F1 = 4.9 \) nmol L\(^{-1}\)) and DON release (\( k5 = 0.024 \) h\(^{-1}\); \( F5 = 11.5 \) nmol L\(^{-1}\) h\(^{-1}\)). NH\(_4^+\) uptake by bacteria (\( k6 = 0.007 \) h\(^{-1}\); \( F6 = 1.0 \) nmol L\(^{-1}\) h\(^{-1}\)) was much lower than that by phytoplankton. The rate constant for nitrification (\( k3 = 0.0005 \) h\(^{-1}\)) was the lowest among all fluxes (\( F3 = 0.07 \) nmol L\(^{-1}\) h\(^{-1}\)).

By introducing initial \( ^{15}N \) and \( ^{14}N \) concentrations of NH\(_4^+\), NO\(_x\)^{−}, PN and DON and the calculated rate constants (k1 to k6) into STELLA (Fig. S1), we obtained full time courses for all parameters (Fig. 4). Generally, the model outputs fitted well with the measured values except the last time point for PN associated \( ^{15}N \) concentration, \( \delta^{15}N \), and \( r_N \) (Figs. 4 c, k and o). In general, the rates of the first time interval can well predict the following up observations, demonstrating a good predictive performance by using the matrix method instant rate. Since both substrates, e.g., ammonium and NO\(_x\)^{−}, fitted well within 24 hours, the extra non-fitted PN in observation after the time point of 12 hours likely indicated the participation 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 these test runs of regeneration with $r_{NH4^+}$ reduction by a total amount of 1 %, 10 %, 20 % and 50 %, we found that the NH$_4^+$ 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). More specifically, greater NH$_4^+$ regeneration resulted in larger differences between these three PN-associated values ($^{15}$N-PN, $^{15}$N-PN, and $r_{PN}$) and the STELLA-projected data (Figs. 4 c, k and o). Meanwhile, the dilution effect was more significant after 12 hours of incubation. On the other hand, the effect of $r_{NH4^+}$ on NO$_x^-$-associated parameters was trivial (Figs. 4 b, 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 remineralization rate is high and incubation prolongs. Besides remineralization, offsets along the time course might possibly be induced by the community change as incubation prolongs.

### 3.3.2 High nutrient cases

The results of 80% sPAR and 2% sPAR under the assumption of fixed $r_{NH4^+}$ are shown in Table 2(A) and 2(B), respectively. For the high light sample (80 % sPAR), the NH$_4^+$ uptake by phytoplankton (F1, 397 nmol L$^{-1}$ h$^{-1}$) and by bacteria (F8, 282 nmol L$^{-1}$ h$^{-1}$) were much higher than the other rates and were followed by the NO$_3^-$ uptake rate (F5, 149 nmol L$^{-1}$ h$^{-1}$). The NO$_2^-$ uptake (F3) rate was 29 nmol L$^{-1}$ h$^{-1}$, much lower than that of NH$_4^+$ and NO$_3^-$ uptake. The ammonia oxidation rate (F4) was 0.4 nmol L$^{-1}$ h$^{-1}$, and the nitrite oxidation rate (F6) was zero (Table 2A). Since this incubation was...
conducted in winter with low temperature and under 80% sPAR light conditions, low rates of ammonium and nitrite oxidation were reasonable because both nitrifiers and NOB are sensitive to light (e.g. Olson, 1981a, 1981b; Horrigan et al., 1981; Ward, 2005; Merbt et al., 2012; Smith et al., 2014). The DON release rate by phytoplankton (F7) was zero in this case.

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

By introducing initial concentrations and calculated rate constants (k1–k8) into the STELLA model (Fig. S2), we obtained successive variations of $^{15}\text{N}$ and $^{14}\text{N}$ concentrations and $r_N$ of $\text{NH}_4^+$, $\text{NO}_2^-$, $\text{NO}_3^-$, PN and DON over time (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 5B). Since ammonium uptake was the dominant process, the alteration of the PN pool was more significant in comparison with the other pools (Figs. 5 d, n and s). We found again, as F2 increased, F1 and F8 increased to maintain a constant reduction of the measured $\text{NH}_4^+$ concentration (Table 2). Similar to low nutrient case, as regeneration increased the projected course of $^{15}\text{N}$-PN deviated more from observation and the turning point also appeared earlier, resulting in a larger curvature of r-PN and $\delta^{15}\text{N}$-PN (Fig. 5d and 5s). This model exercise confirmed the
influence of the isotope dilution effect; however, the effect is insignificant in the early stage of incubation.

4. Discussion

4.1 Method comparisons

4.1.1 Model structure and rate derivation

The most widespread $^{15}$N model was proposed by Dugdale and Goering (1967), who assumed the isotopic and mass balances in the particulate fraction, resulting in the commonly used formula for nitrogenous nutrient uptake. Dugdale and Wilkerson (1986) modified their rate equations further and highlighted the importance of short-term incubation. Although short-term incubation was requested, Collos (1987) demonstrated that the formula based on the concentration of particles at the end of the experiment, rather than at the beginning, is more reliable when more than one N source are simultaneously incorporated by the phytoplankton population. That is, the equation by Collos (1987) corrected the bias caused by unlabeled multiple N utilization.

Different from the above mentioned equations, Blackburn (1979) and Caperon et al. (1979) proposed $^{15}$N isotope dilution models based on the substrate rather than product. By measuring the isotope values and concentrations of the substrate, e.g. NH$_4^+$, and then both NH$_4^+$ consumptions (DON and/or PN as product) and regeneration rate can be obtained. Glibert et al. (1982) further modified the isotope dilution method and calculated the uptake rate into PN fraction by substituting the exponential average $\Gamma_{NH4^+}$.
at the beginning and at the end of incubation to correct the isotope dilution existing in
the model of Dugdale and Goering (1967). Besides method improvements, imbalance
was often observed between the substrate reduction and the increase in particulate
phase in field studies. Laws (1985) introduced a new model with consideration of the
imbalance and calculated the “net uptake rate” (into PN). Later on, Bronk and Glibert
(1991) revised Law’s model on the basis of the model proposed by Glibert et al. (1982)
to calculate the “gross uptake rate” (substrate incorporation into PON plus DON).
Overall speaking, none of the above models considered mass balance at whole system
scale. Although rates were obtained via analytical solutions, the bias potential due to
multiple flows was not completely solved.

To solve multiple co-occurring N processes, Elskens et al. (2002) formulated a
new model, containing 3n+1 equations (n stands for the number of labelled N
substrate) and 3n+1 flux rates, by taking multiple co-occurring N fluxes in natural
system into account. Approximate rates in their model were resolved by a weighted
least squares technique. Additionally, Elskens et al. (2005) created a process-oriented
model (PROM) accounting for as many N processes as needed to quantify how
specific underlying assumptions affect the estimation behavior of all above-mentioned
models. The authors concluded uncertainties may increase as the incubation prolongs
and oversimplified models may risk bias when their underlying assumptions are
violated. The most recent attempt to resolve simultaneous N processes was conducted
by Pfister et al. (2016) who applied parallel incubations ($^{15}$N labelled NH$_4^+$ and NO$_3^-$)
in tidepools to measure multiple flows among benthic, ammonium, nitrite and nitrate pools. In their experiment, six differential equations (with seven unknowns) were constructed basing on mass and isotope balances and solved by using the ODE function of R language. Since benthic algae were not measured due to difficulty in sampling and spatial heterogeneity in biomass, the whole system scale mass balance cannot be reached; thus, the flux of DON release cannot be obtained.

Compared with methods or models mentioned above, the advantages of isotope matrix method include (1) the potential biases caused by multi-flows were considered under the circumstance of mass balance at system scale; (2) one tracer addition for multiple in situ flows (parallel incubations, i.e., dark and light or $^{15}$NH$_4^+$ and $^{15}$NO$_3^-$, were not needed); (3) simple post-hoc data processing and unique solution can be obtained via matrix derivation; (4) no extra laboratory work is demanded (see below).

4.1.2 Rate comparisons

Following Pfister et al. (2016), we estimated all N transformation rates via ODE for the three cases on the assumption that there is no remineralization for comparison (see Table 1–3). In general, the rate values obtained by the matrix and ODE were consistent. The rate difference, if any, was caused by the duration for integration, i.e., shorter time (the first two time points for calculation) for isotope matrix method and longer time (4 or 5 points for the entire incubation) for ODE. In Pfister et al. (2016), 3 monitoring points within 5 hours were implemented for ODE. Unfortunately, such intensive sampling for on-deck incubation is not practical; however, we still strongly
recommend the short-term incubation for water column study as previously suggested. With proper duration, two time points for integration may be more convenient and realistic for instant rate measure.

Below, we present a comparison with conventional source-product rate measurements (Collos, 1987) of ammonium oxidation and uptake (Table 3). The matrix-derived $\text{NH}_4^+$ uptake rates for all experimental cases were consistent with those (difference < 8%) from the traditional source-product method when the final PN concentration was applied for calculation. The deviations were larger (13–21%) when the initial PN was applied, which was supported by the conclusion of previous studies that estimate involving the final PN concentration more reliable. Obviously, deviation could be higher when the phytoplankton growth rate was higher.

On the other hand, the end-products of ammonium oxidation or nitrification are consumed by phytoplankton continuously, particularly in euphotic layer full of photosynthetic autotrophs. In many cases, nitrate uptake occurs in both light and dark conditions (e.g. Dugdale and Goering, 1967; Lipschultz, 2002; Mulholland and Lomas, 2008). The significant consumption of end-products ($\text{NO}_x^-$ and $\text{NO}_2^-$) apparently violate the underlying assumption of source-product rate calculation. Therefore, the $\text{NH}_4^+$ oxidation/nitrification rate could not be obtained, such as all cases in our study since phytoplankton consumption resulted in a net reduction of $\text{NO}_x^-$ (Figs. 2b, 3b and 3c) (see Table 3).
In most cases, the final isotopic composition rather than final concentration of NO\textsubscript{\textgreek{x}} was measured; as such, researchers may not be aware of the greater $^{15}\text{NO}_{\text{x}}$ outflow than inflow. For dark incubation, researchers may also assume insignificant NO\textsubscript{\textgreek{x}} consumption. However, the “net decrease in end-product” is almost unavoidable when incubation is conducted under simulated *in situ* light condition for ammonium oxidation. To overcome this consumption effect induced by the first-order reaction, Santoro et al. (2010, 2013) took NO\textsubscript{\textgreek{x}} removal into account and formulated a new equation, a function of nitrification rate (F) and NO\textsubscript{\textgreek{x}} uptake rate (k). Following 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 $^{15}\text{N-NO}_{\text{x}}$/$^{14}\text{N-NO}_{\text{x}}$ measurements) to be 0.05 nmol L$^{-1}$ h$^{-1}$ (Table 3), which was (~30%) lower than the matrix-derived rate (0.07 nmol L$^{-1}$ h$^{-1}$). By contrast, their nitrate uptake rate (k = 0.010 h$^{-1}$) was only one-sixth of that (0.059 h$^{-1}$) derived from the matrix method, although a comparable nitrification rate was obtained when the consumption term was taken into account.

Surprisingly, when we introduced the two parameters by using the method of Santoro et al. (2010) into STELLA to generate the time courses of parameters, we found simulations of $\delta^{15}\text{NO}_{\text{x}}$ and r\textsubscript{NO\textsubscript{\textgreek{x}}} agreed well with that of isotope matrix method (Figs. 4j and 4n), yet, much slower decreasing trends were found for $^{15}\text{NO}_{\text{x}}$, $^{14}\text{NO}_{\text{x}}$, and NO\textsubscript{\textgreek{x}} (Figs. 4 b, f and r). Finally, we realized that the formula produced by Santoro et al. (2010) is constrained only by the ratio changes rather than the individual
concentration changes in $^{15}\text{NO}_3^-$ and $^{14}\text{NO}_3^-$. Thus, the nonlinear curve-fitting method by Matlab may only provide a correct simulation for the ratio change. This implies that the nitrate uptake rate derived from the non-linear curve-fitting method in Matlab should be validated also by the final concentration of nitrate, as was done by Santoro et al. (2013).

In summary, (1) an accurate measurement of concentration time series is vital for all kinds of transformation rate estimate including the isotope matrix method and (2) the isotope matrix method overcame various biases that traditional methods might encounter.

4.2 Implications for nitrogen biogeochemical processes

Through the isotope matrix method, biogeochemical implications were obtained from various aspects.

4.2.1 Remineralization, regeneration and community succession

The matrix solution fit well with the model runs with variable r-$\text{NH}_4^+$ in early stage, suggesting that dilution effect was negligible during the early incubation period at least in our case. Dilution effect could be significant when remineralization is intensive and incubation prolongs. Pfister et al. (2016) found macrofauna (mussel) play an important role in remineralization. While zooplankton in the water column of our sampled cases was not abundant, it might be a reason for low remineralization rates in our short-term incubation.
In the WNP low nutrient case, after 24-hour incubation the levels of nitrate and ammonium approached the concentration threshold for phytoplankton utilization (e.g., <30–40 nM NH$_4^+$ for *Emiliania huxleyi*; Sunda and Ransom, 2007). In Figure 4, STELLA projection fitted well with PN parameters only for the first 12 hours. In this case, in fact, we have observed phytoplankton succession. Our flow cytometry data (shown in authors reply for Reviewer #2) demonstrated that the cell number of living eukaryotes (4 times higher than *Synechococcus*) increased in the first 24 hours and started to drop rapidly after 24 hours. On the contrary, the growth of *Synechococcus* continued throughout 94 hours under constantly low nitrogen nutrient situation. Such phenomenon suggested that phytoplankton community competed for nitrogen source and a major community shift started at around 24 hours. After the time point of 12 hours, observed parameters associated with PN was higher than the projection by STELLA. The most intriguing phenomenon among PN associated parameters was the additional $^{15}$N, which should not come from $^{15}$NH$_4^+$, in PN. The most likely nitrogen source candidate with enriched $^{15}$N to support *Synechococcus* growth was the nitrogen released from dead eukaryotes, which contained freshly consumed $^{15}$N tracer, rather than the ambient DON. More studies are needed to explore nutrient thresholds for different phytoplankton species. Nevertheless, our results suggested that short-term incubation is crucial for nitrogen uptake studies in oligotrophic ocean.

### 4.2.2 Evaluate the contribution of nitrification to new production
Nitrification in the sunlit ocean drew not much attention until recent decades after the widespread ammonia oxidizing archaea (AOA) discovery in the perspective of molecular evidence (Francis et al., 2005; Santoro et al., 2010, 2013; Smith et al., 2014) and rate measurements based on isotope (Ward, 2011; Santoro et al., 2010; Grundle et al., 2013; Smith et al., 2014). As mentioned in Introduction, the conventional “new” production has been overestimated 19–33% on a global scale due to the “regenerated” nitrate via nitrification process. However, a more realistic evaluation for the fractional contribution of nitrification to NO$_3^-$ uptake can only be achieved when the incubation is conducted in the same bottle under in situ light conditions instead of parallel incubations in 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 ocean. In all our experimental cases, the contributions of nitrification to new production were < 1% (Table 4). The relatively low contribution was probably due to the light inhibition on nitrifiers for the WNP case and because of the low temperature in the sampling season.

Nevertheless, light effect in our case studies is significant. Light suppresses nitrification (Ward, 2005; Merbt et al., 2012; Peng et al., 2016). NH$_4^+$ oxidation rate in 80% sPAR reduced by 36% relative to that in 2% sPAR. Results agreed with current knowledge although some recent evidences showed that some taxa of marine AOA hold genetic capabilities to reduce oxidative stress and to repair ultraviolet damage (Luo et al., 2014; Santoro et al., 2015). More study cases are needed in the future to
explore vertical distributions of the relative contribution of nitrification to new production in euphotic zone.

4.2.3 Nutrient preference

Phytoplankton takes different nitrogenous species as nutrients for growth. McCarthy et al. (1977) introduced a relative preference index (PRI) to assess the relative utilization of a specific N form, and when RPI value >1 indicates a preference for the specific substrate over the other N forms. As shown in Table 4, in the NO$_3^-$ prevailed low nutrient case, the PRI (NO$_3^-$) was very close but slightly higher to PRI (NH$_4^+$), which was probably due to the phytoplankton community structure as mentioned above. This result was in line with the result in the Sargasso Sea (Fawcett et al., 2011). While in the high NH$_4^+$ bay, the PRI (NH$_4^+$) > 1 > PRI (NO$_3^-$) > PRI (NO$_2^-$), suggesting that phytoplankton preferred NH$_4^+$ over NO$_3^-$ and NO$_2^-$, similar to the result in Chesapeake Bay (McCarthy et al., 1977).

4.2.4 Quantify various ammonium consumption pathways

In the upper ocean, NH$_4^+$ cycles rapidly due to various microorganisms’s metabolic pathways competing for ammonium. Ammonium may serve as nitrogen source for phytoplankton assimilation, and as energy source for ammonia oxidizing organisms (AOM). Moreover, many studies have shown that bacteria also play a part in NH$_4^+$ utilization (Middelburg and Nieuwenhuize, 2000; Veuger et al., 2004). Our result in the low nutrient case showed that phytoplankton was the main NH$_4^+$ consumer (82%
of the total NH$_4^+$ consumption), bacteria accounted for another ~17% and AOM utilized the rest 1%. While in the eutrophic WYW bay, phytoplankton and bacteria each consumed ~50% of the total NH$_4^+$ (Table 4).

5. Conclusion

This isotope matrix method was designed specifically for euphotic water column incubation under simulated in situ condition. By considering multi-flows among pools on the assumption of 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 concentration and isotopic composition of nitrogen species, the isotope matrix method presents a promising avenue for the study of rates of nitrogen processes with 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 the interactive N processes in one single incubation bottle.

Acknowledgement

We sincerely thank Wenbin Zou and Tao Huang at the State Key Laboratory of Marine Environmental Science (Xiamen University, China) for their valuable help with the water sampling and the on-board trace NH$_4^+$ concentration analysis during the 2015 NWP cruise. Yu-ting Shih from the Department of Geology at National Taiwan University in Taiwan is thanked for his help on ODE application. This research was
funded by the National Natural Science Foundation of China (NSFC U1305233, 2014CB953702, 91328207, 2015CB954003).

References


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36 / 52


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\textsubscript{NH4+} variation conditions. And all N transformation rates via ODE following Pfister et al. (2016) on the assumption of no remineralization were estimated for comparison. Note r\textsubscript{NH4+} variation was manipulated artificially by decreasing r\textsubscript{NH4+} values at a constant reduction rate and the total reduction of rNH4+ was 0%, 1%, 10%, 20% and 50% of the full time span (24 h) of incubation.

### (A)

<table>
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<tr>
<th>Rate constant ((k)) (h^{-1})</th>
<th>The percentage of r\textsubscript{NH4+} decrease in 24 h</th>
<th>(0)</th>
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<th>10%</th>
<th>20%</th>
<th>50%</th>
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### (B)

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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 $r_{NH4^+}$ variation conditions. And all N transformation rates via ODE following Pfister et al. (2016) on the assumption of no remineralization were estimated for comparison. Note: $r_{NH4^+}$ variation was manipulated artificially by decreasing $r_{NH4^+}$ values at a constant reduction rate and the total reduction of $r_{NH4^+}$ was 0%, 1%, 10%, 20% and 50% of the full time span (15 h) of incubation.

(A)

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<th>Rate ($k^* C$) nmol L$^{-1}$ h$^{-1}$</th>
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<tr>
<td>NO$_3^-$ uptake (F5)</td>
<td>190</td>
<td>149</td>
<td>149</td>
</tr>
<tr>
<td>NO$_2^-$ oxidation (F6)</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DON release (F7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria uptake NH$_4^+$ (F8)</td>
<td>268</td>
<td>282</td>
<td>303</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>Rate ($k^* C$) nmol L$^{-1}$ h$^{-1}$</th>
<th>The percentage of $r_{NH4^+}$ decrease in 15 h</th>
<th>ODE</th>
<th>Isotope Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4^+$ uptake (F1)</td>
<td>228</td>
<td>208</td>
<td>208</td>
</tr>
<tr>
<td>Remineralization (F2)</td>
<td>0</td>
<td>0</td>
<td>18.1</td>
</tr>
<tr>
<td>NO$_2^-$ uptake (F3)</td>
<td>7.3</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>NH$_4^+$ oxidation (F4)</td>
<td>1.1</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>NO$_3^-$ uptake (F5)</td>
<td>106</td>
<td>72</td>
<td>72</td>
</tr>
<tr>
<td>NO$_2^-$ oxidation (F6)</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DON release (F7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria uptake NH$_4^+$ (F8)</td>
<td>202</td>
<td>265</td>
<td>283</td>
</tr>
</tbody>
</table>
Table 3. Comparison of the NH$_4^+$/ NO$_x^-$ uptake and NH$_4^+$ oxidation/nitrification rates derived from different methods.

<table>
<thead>
<tr>
<th>Process</th>
<th>Case</th>
<th>Depth (m)</th>
<th>Isotope Matrix method (this study)</th>
<th>Rates based on Ref A* (nmol L$^{-1}$ h$^{-1}$)</th>
<th>Traditional method Ref B* (nmol L$^{-1}$ h$^{-1}$)</th>
<th>Rates followed Ref C* (nmol L$^{-1}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH$_4^+$ uptake</td>
<td>Low nutrient</td>
<td>25</td>
<td>4.9</td>
<td>3.8</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Nitrification</td>
<td>Low nutrient</td>
<td>25</td>
<td>0.07</td>
<td>0.04</td>
<td>−</td>
<td>0.05</td>
</tr>
<tr>
<td>NO$_x^-$ uptake</td>
<td>Low nutrient</td>
<td>25</td>
<td>27.2</td>
<td>19.3</td>
<td>−</td>
<td>4.6</td>
</tr>
<tr>
<td>NH$_4^+$ uptake</td>
<td>High -80% sPAR</td>
<td>0.2</td>
<td>397</td>
<td>360</td>
<td>387</td>
<td></td>
</tr>
<tr>
<td>NH$_4^+$ oxidation</td>
<td>High -80% sPAR</td>
<td>0.2</td>
<td>0.4</td>
<td>1</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NH$_4^+$ uptake</td>
<td>High -2% sPAR</td>
<td>2.3</td>
<td>208</td>
<td>228</td>
<td>192</td>
<td></td>
</tr>
<tr>
<td>NH$_4^+$ oxidation</td>
<td>High -2% sPAR</td>
<td>2.3</td>
<td>0.7</td>
<td>1</td>
<td>−</td>
<td></td>
</tr>
</tbody>
</table>

Ref A* stands of rates calculation by ODE followed Pfister et al. (2016)
Ref B* stands of rates calculation followed Collos (1987)
Ref C* stands of rates calculation followed Santoro et al. (2010)
Table 4. The contribution of nitrification derived NO$_2^-$ to NO$_3^-$ 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.

<table>
<thead>
<tr>
<th>Case</th>
<th>Depth (m)</th>
<th>nitrification to NO$_3^-$ uptake (%)</th>
<th>RPI for NH$_4^+$</th>
<th>RPI for NO$_2^-$</th>
<th>RPI for NO$_3^-$</th>
<th>*A/TNH$_4^+$ consumption (%)</th>
<th>*B/TNH$_4^+$ consumption (%)</th>
<th>*C/TNH$_4^+$ consumption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low nutrient</td>
<td>25</td>
<td>0.3</td>
<td>0.9</td>
<td>1.0</td>
<td>1.0</td>
<td>82.1</td>
<td>16.8</td>
<td>1.2</td>
</tr>
<tr>
<td>High -80% sPAR</td>
<td>0.2</td>
<td>0.3</td>
<td>1.6</td>
<td>0.6</td>
<td>0.5</td>
<td>58.4</td>
<td>41.5</td>
<td>0.1</td>
</tr>
<tr>
<td>High -2% sPAR</td>
<td>2.3</td>
<td>0.9</td>
<td>1.8</td>
<td>0.1</td>
<td>0.5</td>
<td>43.9</td>
<td>56.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*A, *B, *C stands for NH$_4^+$ utilized by phytoplankton, bacteria and nitrifier, respectively. TNH$_4^+$ consumption stands for total NH$_4^+$ consumption.
Figure Captions

**Fig. 1.** Model schemes with the most fundamental nitrogen transformation processes in low- (a) and high- (b) nutrient aquatic environments. Arrows stand for the transfer flux/rate from the reactant to product pool. The structure and inter-exchanges in the high-nutrient case (Fig. 1b) are the same as in (a), except that NO$_x^-$ is divided into NO$_2^-$ and NO$_3^-$.

**Fig. 2.** The observational data in the low-nutrient case for (a) [NH$_4^+$], (b) [NO$_x^-$], (c) [PN], (d) [TDN], (e) δ$_{15}$N-NO$_x^-$, (f) δ$_{15}$N-PN. The regular and inverse open triangles stand for the paralleled samples and the analytical errors are shown.

**Fig. 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) δ$_{15}$N-NO$_2^-$, (h) δ$_{15}$N-NO$_3^-$ and (i) δ$_{15}$N-PN. The light and dark red diamonds stand for the paralleled samples in 80% sPAR case and the black regular and inverse open triangles stand for the paralleled samples in 2% sPAR case. The analytical errors are shown in figures.

**Fig. 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$_{NH4^+}$, (j) r$_{NOx}$, (k) r$_{PN}$, (l) r$_{DON}$, (m) δ$_{15}$N-NH$_4^+$, (n) δ$_{15}$N-NO$_x^-$, (o) δ$_{15}$N-PN, (p) δ$_{15}$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 stand for the STELLA model simulations when r$_{NH4^+}$ 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).

**Fig. 5.** The observed and STELLA-derived values in the high-nutrient case of (A) 80% sPAR depth and (B) 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) δ$_{15}$N-PN, (l) δ$_{15}$N-DON, (m) r$_{NH4^+}$, (n) r$_{NOx}$, (o) r$_{PN}$, (p) r$_{DON}$, (q) [NH$_4^+$], (r) [NO$_x^-$], (s) [PN] and (t) [DON].
[\textsuperscript{14}N-DON], (k) \( r_{\text{NH}_4^+} \), (l) \( r_{\text{NO}_2^-} \), (m) \( r_{\text{NO}_3^-} \), (n) \( r_{\text{PN}} \), (o) \( r_{\text{DON}} \), (p) \( \delta^{15}\text{N-NH}_4^+ \), (q) \( \delta^{15}\text{N-NO}_2^- \), (r) \( \delta^{15}\text{N-NO}_3^- \), (s) \( \delta^{15}\text{N-PN} \), (t) \( \delta^{15}\text{N-DON} \), (u) \( [\text{NH}_4^+] \), (v) \( [\text{NO}_2^-] \), (w) \( [\text{NO}_3^-] \), (x) \( [\text{PN}] \) and (y) \( [\text{DON}] \). 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 \( r_{\text{NH}_4^+} \) decreases 0%, 1%, 10%, 20% and 50% in 15 h, respectively.
Fig. 1

(a) 
\[ \Delta \text{DON} \rightarrow \text{PN} \rightarrow \text{NO}_x^- \]
\[ \text{NH}_4^+ \rightarrow \text{PN} \rightarrow \text{NO}_x^- \]

(b) 
\[ \Delta \text{DON} \rightarrow \text{PN} \rightarrow \text{NO}_x^- \]
\[ \text{NH}_4^+ \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^- \]
Fig. 2

(a) $\text{NH}_4^+$ (mmol L$^{-1}$)

(b) $\text{NO}_x^-$ (mmol L$^{-1}$)

(c) PN (mmol L$^{-1}$)

(d) TDN (mmol L$^{-1}$)

(e) $\delta^{15}\text{NO}_x$ (‰)

(f) $\delta^{15}\text{N-PN}$ (‰)

Incubation time (h)
Fig. 3

(a) NH$_4^+$

(b) NO$_3^-$

(c) NO$_4^-$

(d) PN

(e) TDN

(f) PN + TDN

(g) $\delta^{15}$NO$_2^-$

(h) $\delta^{15}$NO$_3^-$

(i) $\delta^{15}$N-PN

Incubation time (h)
Fig. 4
Fig. 5(A)
Fig. 5(B)