Dear Editor,

We found all comments are constructive. However some questions regarding methods were raised due to our under-descriptions in old version. We also modified slightly the model structure for both low and high nutrient cases in order to examine the processes of remineralization, DON release and ammonium uptake by microbes (< 0.7 um). As replied below, our method was simply the integration of the first two time points (trapezoid method) and unique solution can be obtained. According to comments below, we applied ODE and present the ODE-derived results in tables for comparison. The differences in rate or rate constants were caused by length for time integration. However, we need to emphasize this paper is not a model paper. The constructed box model was based on our questions just to project these full time courses of oft-measured pools for validation. Addition to the model extrapolation, we ran sensitivity test for these rate numbers to convince readers the reliability of matrix-derived values. The manuscript had been reorganized and the part of methodology was thoroughly revised.

On behalf of all the authors

Sincerely,

Shuh Ji Kao

Reviewer #2, comment #1

Min Nina Xu and co-workers present an original experimental design to quantify multiple nitrogen transformation processes (rates of ammonium, nitrite and nitrate uptake, ammonia oxidation; nitrite oxidation; nitrite excretion; DON release; and potentially remineralization) by adding a single ^{15}N-labelled ammonium substrate into a single incubation system. No inhibitors were used and special attention was given to minimize the alteration of the system by adding a limited amount of tracer. Examples of field measurements are presented and different calculation methods are discussed. The article is written in a clear and understandable manner and fits well with the scope of Biogeosciences (BG). The study is worthy of publication but the authors need to address a number of comments to improve their manuscript (ms).

Author response:

Thanks.

Reviewer #2, comment #2

I have a concern about the method used to solve the rate law equations. Here the mass balance differential equations for determining the N-transformation rates are not integrated, neither

analytically or numerically. This is rather unusual and in opposition with standard methods acclaimed for the treatment of chemical reaction kinetics. Such an approach, using rates instead of the generated profile of concentrations versus time, presents serious drawbacks, namely regarding the uncertainty on the estimated parameters (rates or rate constants). Unfortunately this point is not addressed in the ms. The authors should therefore convince the reader that their method is at least as good as conventional integration methods in terms of accuracy and precision, and this requires an uncertainty assessment (see specific comments).

Author response:

To avoid confusion, we change dC/dt into $\Delta C/\Delta t$.
The rate constants were determined by using the measurements at time zero and the first time point after that. The matrix equations were not constructed for calculating derivatives but to integrate the differential equation between the first two time points, and then to estimate the "instant flux" (F or k* $\bar{C}$, if time for incubation is short enough). Note that the use of "instant" here is just to make it distinguishable from the longer time incubation (or > two time points). The method we used was a second-order Runge-Kutta method, more specifically, the improved Euler method, to carry out the integration numerically. In our case, we inverted the solutions to solve for the fluxes or rate constants that would give us the correct answers at the first time point. Because the fluxes and rate constants are determined entirely from the data at time zero and the first time point, our method is equivalent to integrating the functions (trapezoid method).
After having the "instant rate" for the first time interval, we constructed a box model (equation-based input-output box model) to predict (i.e., extrapolation) the full time courses for all nitrogen pools. In previous version, the model structures and the numbers of equations and unknowns for the two cases were different (see below). However, we did not described clearly in old version. More details will be given regarding the derivation procedure. The number of equation equals the number of unknown. Thus, no uncertainty exists for the matrix solution. However, reviewer is correct for the uncertainty induced by limited data points for derivation. The major uncertainty will be sourced from analytical uncertainties and sample heterogeneity. However, in all our cases, these extrapolations agreed well with consecutive observations, suggesting a good performance of our estimator for rate or rate constant with good measurements. In this version, we stated explicitly that researchers can applied our approach by using more observational data (enlarged trapezoid) to get an average rate for longer duration if ignorable community change can be assured (see example blow for low nutrient case).
According to reviewers' comments, we modified the model slightly (see revised model structures below for comment #4) and described the two cases together in Methods.

Reviewer #2, comment #3

The authors are not the first to propose a mass balance approach to derive multiple N-transformation rates. As far as I know, such an approach was used and discussed at least in three previous publications. 1. Elskens et al., Global Biogeochemical Cycles, vol. 19, gb4028, doi:10.1029/2004gb002332, GBC-2005 2. De Brauwere et al. Chemometrics and Intelligent Laboratory Systems 76, 163– 173, CILS-2005 3. Pfister et al., Biogeosciences, 13, 3519–3531,

BGS-2016. In the GBC approach, the rate law equations are analytically integrated while in the BGS, the differential equations are solved numerically using an ODE function. Currently the use of the ODE function for solving ordinary differential equations is easy to implement (see https://cran.rproject.org/web/packages/deSolve/deSolve.pdf) and the generated profile of concentrations versus time can be fitted using least squares methods (see GBC, CILS and BGS papers). It would be appropriate to address these points in the introduction, and throughout the discussion, the authors should argue why their simplified approach can be an asset when compared to the aforementioned papers.

Author response:

Following this comment, we changed our statement about "mass balance". The statement is now "This is a convenient method specifically for euphotic zone to quantitatively and simultaneously resolve complicated nitrogen transformation rates, albeit with some uncertainties.". Above mentioned models had been referred in revision.

The rate derived from ODE is a mean of integration over time that requires a concentration time course (three points at least) for iteration and integration, thereby differs from our "instant rate" determined by two time points as replied above. Although our method is simple mathematically, we do integration. We agree with reviewer that ODE may have advantages with the support of longer time course, however, our two-point matrix solution also gave good performance for extrapolation (see figures for comment #5 by Reviewer #1).

Nevertheless, we applied ODE and made a comparison for fixed r_{NH4+} condition (see the example below for high nutrient case with 80% sPAR). The rate values obtained by matrix and ODE were consistent. The difference in rate, if any, was caused by the duration for integration, i.e., shorter time (two points for the first ~2 hours) for ours and longer time (5 points for ~15 hours) for ODE. Since time series monitoring in prolonged on-deck incubation is inconvenient and inappropriate due to rapid nutrient turnover and microbial community change. Thus, we select two time point for integration. The model was constructed to reduce the potential bias in traditional source-product method caused by ^{15}N flows among boxes. Our aim is to provide a less biased and convenient (in term of on-deck implementation and post-hoc data processing) measure for multiple transformation rates (more specifically, the "instant rate" researchers are eager to know). As indicated by Elskens et al. (2005), over complex models can misinterpret part of the random noise as relevant processes. These boxes, i.e. PN, nitrate, nitrite and ammonium, were the most often measured species and these exchanges we applied among pools were the most fundamental processes.

Table 2a. Results of high nutrient case under 80% PAR.

Rate ($k^* \bar{C}$) nmol L^{-1} h^{-1}	The percentage of r_{NH4+} decrease in 15 h					
	0		1%	10%	20%	50%
	ODE*	Isotope Matrix	Isotope Matrix	Isotope Matrix	Isotope Matrix	Isotope Matrix
NH_4^+ uptake (F1)	361	397	397	399	401	408
Remineralization (F2)	0	0	21	211	424	1043
NO_2^- uptake (F3)	28	29	29	29	29	29
NH_4^+ oxidation (F4)	1.1	0.4	0.4	0.4	0.4	0.4
NO_3^- uptake (F5)	189	149	149	149	149	149
NO_2^- oxidation (F6)	1.1	0	0	0	0	0
DON release (F7)	0	0	0	0	0	0
Bacteria uptake NH_4^+ (F8)	268	282	303	490	701	1314

*Ordinary Differential Equation

Reviewer #2, comment #4

Also I'm not convinced that adding a single ^{15}N-labelled ammonium tracer into the incubation system allows an accurate determination of the ammonium, nitrite and nitrate uptake rates. According to me the kinetic reactions corresponding to the matrix expressions (Eqns 16-17) with the labelling of a single ammonium substrate is underidentified. Under this condition, the ^{15}N-labelling of PN proceeds via the uptake of ammonium and/or via nitrification and the subsequent uptakes of nitrite and nitrate. These processes are thus not independent, and may result in a multimodal optimization problem, i.e., multiple solutions providing similar responses. The authors should address this point, especially because little information is available in the ms regarding the method used to solve Eqns. (16-17).

Author response:

Reviewer is correct about the optimization problem. We did not make clear and correct descriptions in our old version. The problem no longer exists after our modification.
In previous version for the simple case (low nutrient open ocean, include NO_2^- into NO_x^- as one pool), we set nitrite release from the PN pool along with r_{PN} (F5 in panel (a) of Figure r1 below). We found this not reasonable since nitrite release occurs during intra-cell nitrate reduction. Meanwhile, this flux should be minor relative to other fluxes. In this version, we modified the model structure, of which the nitrite release was included as an internal cycle inside the NO_x^- pool, which can be precisely measured by bacteria method. On the other hand, the remineralization input of NH_4^+ (F2) was connected to the DON pool instead of PN to more realistically reflect the dilution effect. As mentioned in our manuscript, once the isotopic composition of ammonium at the end-point can be measured accurately, no assumption or sensitivity test for r_{NH4+} is needed. Currently, we manipulated r_{NH4+} values to examine the effect of

remineralization. Via our extrapolation process, the effect of remineralization was evaluated.

According to other reviewer's suggestion, we discussed the missing nitrogen for both high and low nutrient cases (see the example of F5 and F6 in panel (b) of Figure r1 below for low nutrient case), which had been pointed out yet unresolved in previous study by Laws (1985). Here in this version, F5 in low nutrient case was the DON release from PN following the isotope ratio of PN and F6 was defined as ammonium uptake by microbes that passed through the GF/F filter (0.7 μm).

The modified model structure is shown below in (b) accompanied with the old one in (a) for comparison. According to this modification, unique solution can be obtained by matrix (6 unknowns and 6 independent equations).

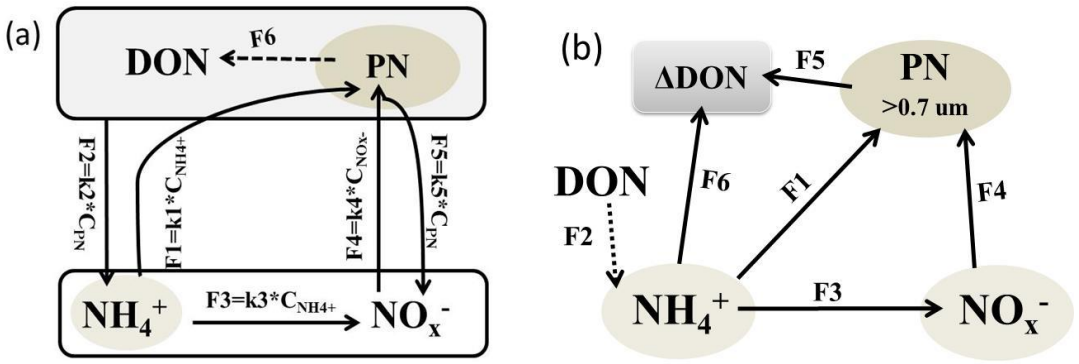

Figure r1. The old (a) and revised (b) model structures for low nutrient case.

Equations for the low nutrient case:

$$\frac{\Delta\left[{}^{15}NH_4^+\right]}{\Delta T} = \overline{F_2}\times 0.00366 - \overline{F_1}\times\overline{r_{NH_4^+}} - \overline{F_3}\times\overline{r_{NH_4^+}} - \overline{F_6}\times\overline{r_{NH_4^+}}$$

$$\frac{\Delta\left[{}^{15}NO_x^-\right]}{\Delta T} = \overline{F_3}\times\overline{r_{NH_4^+}} - \overline{F_4}\times\overline{r_{NO_x^-}}$$

$$\frac{\Delta\left[{}^{15}PN\right]}{\Delta T} = \overline{F_1}\times\overline{r_{NH_4^+}} + \overline{F_4}\times\overline{r_{NO_x^-}} - \overline{F_5}\times\overline{r_{PN}}$$

$$\frac{\Delta\left[{}^{14}NH_4^+\right]}{\Delta T} = \overline{F_2}\times(1-0.00366) - \overline{F_1}\times(1-\overline{r_{NH_4^+}}) - \overline{F_3}\times(1-\overline{r_{NH_4^+}}) - \overline{F_6}\times(1-\overline{r_{NH_4^+}})$$

$$\frac{\Delta\left[{}^{14}NO_x^-\right]}{\Delta T} = \overline{F_3}\times(1-\overline{r_{NH_4^+}}) - \overline{F_4}\times(1-\overline{r_{NO_x^-}})$$

$$\frac{\Delta\left[{}^{14}PN\right]}{\Delta T} = \overline{F_1}\times(1-\overline{r_{NH_4^+}}) + \overline{F_4}\times(1-\overline{r_{NO_x^-}}) - \overline{F_5}\times(1-\overline{r_{PN}})$$

For the high nutrient complex case (NO_2^- and NO_3^- in separable pools), we indeed encountered equifinality problem in old version since we have 6 independent equations and 7 unknowns.

In previous version, we applied linear programming (Excel solver) to obtain the optimal solution for 7 unknowns. The non-linear GRG (Generalized Reduced Gradient Algorithm) was selected. The target function is the root mean square error for all six equations. When the value of target function reaches minimum the optimal solution was provided. After obtaining the optimal solution, we simulate time courses by using the constructed Stella model. Time course extrapolation provided by Stella were surprisingly good, thus, we overlooked the multimodel

optimization problem pointed out by reviewer.

The old and revised model are shown below in (a) and (b), respectively, for high nutrient case. Similar to the simple case, in this version we removed F7, nitrite excretion (see panel (a) below in Figure r2). In high NH_4^+ estuary and coastal sea, nitrate assimilation may be inhibited in oxygenated water and subsequently, the nitrite release. Thus, the ignorance of nitrite release from PN (F7 in lower panel (a)) should be acceptable. In old version, equations for PN pool were not applied independently. In order to discuss the missing ammonium, we now introduced PN into equation set. Thereby, the number of total parameters is eight. With eight independent equations a unique parameter combination can be obtained (see equations below). During the revision, we compared with ODE-derived results (see reply to comment #3 above, new Table 2a). We also examined the sensitivity of parameters in accordance with the target function (see reply below to the last specific comment) and found all rates converged to unique solutions.

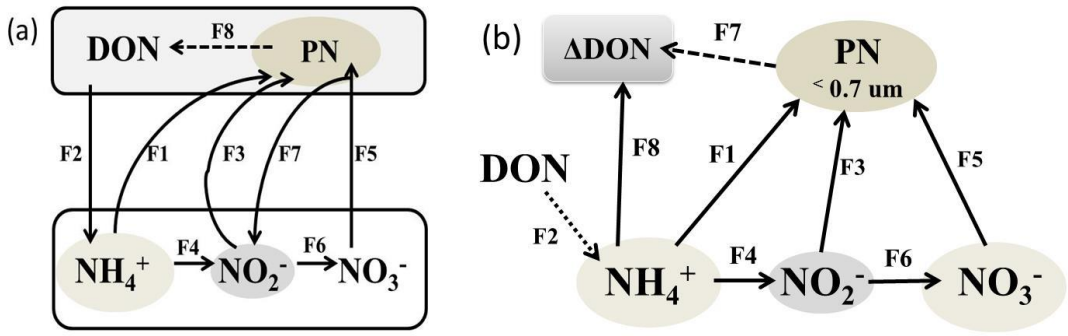

Figure r2. The old (a) and revised (b) model structures for high nutrient case.

Equations for the high nutrient case:

$$\frac{\Delta\left[{}^{15}NH_4^+\right]}{\Delta T} = \overline{F_2}\times 0.00366 - \overline{F_1}\times\overline{r_{NH_4^+}} - \overline{F_4}\times\overline{r_{NH_4^+}} - \overline{F_8}\times\overline{r_{NH_4^+}}$$

$$\frac{\Delta\left[{}^{15}NO_2^-\right]}{\Delta T} = \overline{F_4}\times\overline{r_{NH_4^+}} - \overline{F_3}\times\overline{r_{NO_2^-}} - \overline{F_6}\times\overline{r_{NO_2^-}}$$

$$\frac{\Delta\left[{}^{15}NO_3^-\right]}{\Delta T} = \overline{F_6}\times\overline{r_{NO_2^-}} - \overline{F_5}\times\overline{r_{NO_3^-}}$$

$$\frac{\Delta\left[{}^{15}PN\right]}{\Delta T} = \overline{F_1}\times\overline{r_{NH_4^+}} + \overline{F_3}\times\overline{r_{NO_2^-}} + \overline{F_5}\times\overline{r_{NO_3^-}} - \overline{F_7}\times\overline{r_{PN}}$$

$$\frac{\Delta\left[{}^{14}NH_4^+\right]}{\Delta T} = \overline{F_2}\times(1-0.00366) - \overline{F_1}\times(1-\overline{r_{NH_4^+}}) - \overline{F_4}\times(1-\overline{r_{NH_4^+}}) - \overline{F_8}\times(1-\overline{r_{NH_4^+}})$$

$$\frac{\Delta\left[{}^{14}NO_2^-\right]}{\Delta T} = \overline{F_4}\times(1-\overline{r_{NH_4^+}}) - \overline{F_3}\times(1-\overline{r_{NO_2^-}}) - \overline{F_6}\times(1-\overline{r_{NO_2^-}})$$

$$\frac{\Delta\left[{}^{14}NO_3^-\right]}{\Delta T} = \overline{F_6}\times(1-\overline{r_{NO_2^-}}) - \overline{F_5}\times(1-\overline{r_{NO_3^-}})$$

$$\frac{\Delta\left[{}^{14}PN\right]}{\Delta T} = \overline{F_1}\times(1-\overline{r_{NH_4^+}}) + \overline{F_3}\times(1-\overline{r_{NO_2^-}}) + \overline{F_5}\times(1-\overline{r_{NO_3^-}}) - \overline{F_7}\times(1-\overline{r_{PN}})$$

Our approach differs from that in Pfister et al. (2016), in which the ratio of ammonium uptake to nitrate uptake was fixed by trial and error. According to comments below and from other reviewers, we also presented additional case and discussed the light effect.

Specific comments

Line 47 – p3: What is meant by the inventory method?
Author response – We followed B. B. Ward (2011). We made a more clear description. The sentence is now "The inventory method (monitoring substrate and/or product change over time) is often used …".

Line 100 – p6: The term validation is not appropriate since the Stella model is based on the reaction kinetics outlined in Fig.1, and thereby submitted to the same underlying hypotheses than Eqns (16-17). At best we can say that the matrix solutions are consistent with a model run generating concentration versus time curves through back calculation.
Author response – We partly agree with reviewer. This question was raised due to the under-descriptions of our method. For both cases, the "instant rate" for the first time interval was obtained and then served as prescribed values in Stella box model to predict the time course and to compare with consecutive observations. Since the rate is concentration dependent (first order reaction), the rate constant derived from the first time interval would not guarantee a good performance for the full time course due to decline of substrate and contemporary community change. The extrapolation is a kind of validation.
In the ocean, the rate we are eager to know is the *in situ* rate (or the instant rate at the time of sampling) before microbial community changes. Thus, short-term incubation was suggested in our previous version. Stand on this point, "validation" is a proper term.

Lines 280/281/397/399/417. Please pay attention to the number of significant decimals when reporting data (e.g. 22.3± 4.3 μM or 5376.4 nM).
Author response – Carefully checked and corrected.

Line 348/354: How did the authors define 'undetectable' or 'below detection limit' in their ms?
Author response – We change to "below the detection limit".

Line 420 – p23: In Fig.4 a nonlinear behavior for the concentration versus time doesn't demonstrate that the rate laws follow first order.
Author response – Reviewer is correct. Now an assumption of first order reaction was made explicitly instead of by the judgement from apparent non-linear behavior.

Line 438 – p23: What is meant by 'this positive offset was compensated for by organic nitrogen utilization'.
Author response – We admit the old sentence was confusing. The sentence is now "Since both ammonium and NO_x^- fitted well within 12 hours, the extra non-fitted PN at the time point of 12 hours in observation likely indicated an additional nitrogen source, such as organic nitrogen, was utilized by phytoplankton when inorganic nitrogen reached threshold levels (Sunda and Hardison, 2007)." In fact, our flow cytometry data (see panel below) showed clearly the cell abundance of pico-eukaryotes increased within the first 24 hours and then decreased rapidly, very likely due to nutrient limitation. By contrast, the *Synechococcus* grew continuously even when ammonium and nitrate was around the limiting level. *Synechococcus* may thus uptake DON or recycled nitrogen

for growth. Such result is not only supportive of the importance of short-term incubation also indicative of rate might change rapidly due to community change.

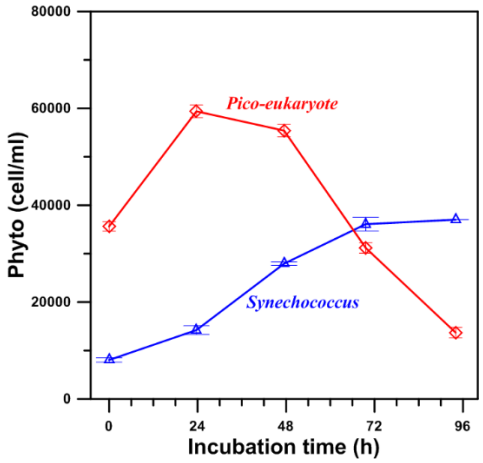

Figure r3. The variations of cell abundances of *Pico-eukaryote* and *Synechococcus* determined by flow-cytometry.

Line 518 – p27: I guess it is rather an 'accurate measurement of…'
Author response – Changed as suggested.

Line 544 – p 29: 'The uncertainty estimate for this isotope matrix method is not a simple statistical question'. Yet the authors have the means to do so. If they build rate profiles from their concentration measurements, and optimize values for Fi or ki (Eqns 16- 17) using a least squares method, they will get access to the uncertainty on these parameters via the variance-covariance matrix.
Author response – This question was raised due to more equations for unknowns. As replied above, after revision unique solution can be obtained via the matrix method. Thus, this specific uncertainty question does not exist. We mentioned uncertainties in previous version since we also cannot deny uncertainties caused by chemical analyses. Meanwhile, errors along the time course might possibly come from the community change as replied above.
According to this suggestion, we applied a sensitivity test (see Figure r4 below) by using Excel for the low nutrient case under r_{NH4+} constant condition. We set reasonable ranges for parameters and then conducted 10000 times random selection for individual parameters within the given range to generate 10000 sets of parameter combination for RMSE estimate. We can see clearly randomly selected parameters converge toward the unique solution we obtained (red inverse triangle). The RMSE is near zero. Such consistency suggests uncertainties will be sourced from chemical analyses and the heterogeneity of water for incubation rather than method itself.

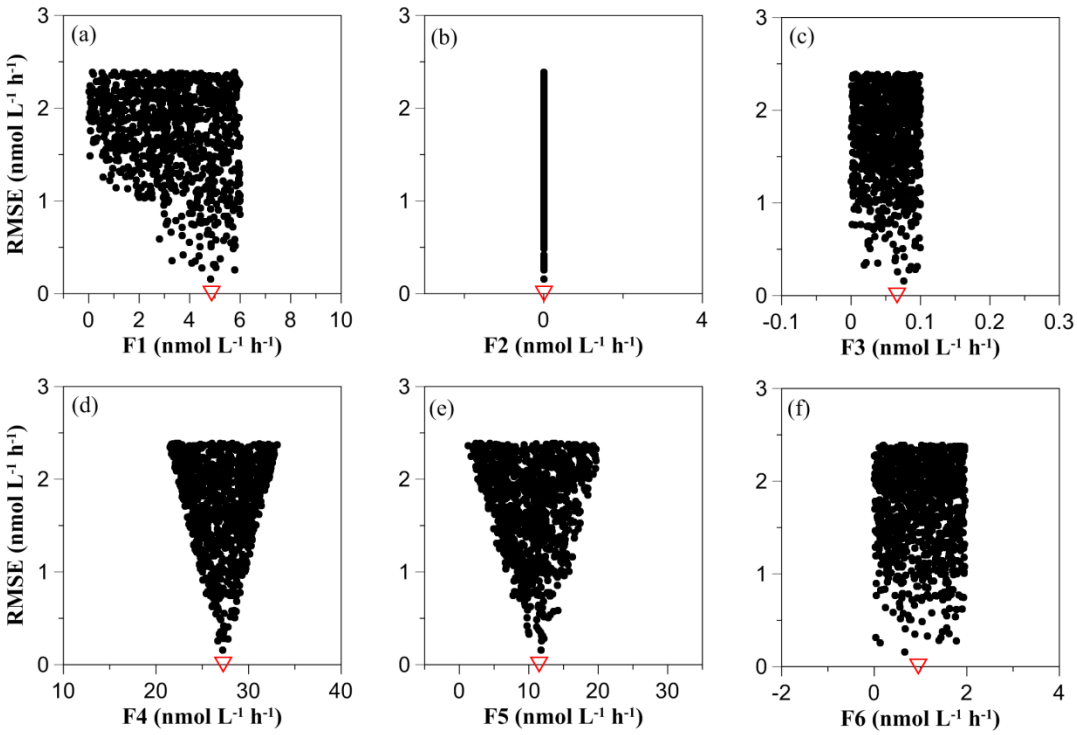

Figure r4. The sensitivity test of parameters. Root mean square error was applied as performance measure. Inverse triangle stands for unique solution from isotope matrix method.