Reviewer Comments on Plouviez et al. 2025

General comments

Plouviez et al. take on a significant problem within the biogeosciences – namely, that the N_2O budget is not closed, and one of the main hurdles in closing the budget is accurately accounting for all sources and sinks of N_2O since it can be produced / consumed by multiple biotic and abiotic pathways. A powerful tool in this space is measuring "Site Preference" (SP), which quantifies the relative 'preference' of ^{15}N for the central ('alpha') or outer ('beta') site in the asymmetrical, linear N_2O molecule. Therefore, many groups have been working to systematically measure the SP of all known sources and sinks of N_2O in an effort to close the N_2O budget, as well as identify sources of N_2O that may be mitigated to prevent greenhouse gas emissions.

Plouviez et al. measure the N_2O SP of eukaryotic and bacterial photosynthesizers, which have been shown to produce N_2O outside of the metabolic pathways that N_2O production has been typically attributed to (denitrification and nitrification, either by bacteria or fungi). Measuring this value is particularly important for understanding N_2O cycling in the oceans, since denitrifiers, nitrifiers, and algae all coexist in complex microbial assemblages – therefore, finding potentially unique SP signatures for algae may help disentangle complex marine N_2O cycling. They state that they describe a new method for the accurate laser-based analysis of N_2O isotopes, which enables them to conduct novel SP measurements of algal N_2O. They find significantly different SP signatures for the eukaryotic algae (*C. reinhardtii* and *C. vulgaris*) compared to the cyanobacteria (*M. aeruginosa*).

I have two main comments regarding this manuscript. The first is about the technical aspects of the measurement. The second is about the interpretation of the isotopic signatures.

For the first point, I cannot independently evaluate the quality of the data presented because it lacks key outputs that would enable independent calculation of this. I assume that authors are measuring the major isotopologues $^{14}N^{14}N^{16}O$ (446), $^{14}N^{15}N^{16}O$ (456), $^{15}N^{14}N^{16}O$ (546) and $^{14}N^{14}N^{18}O$ (448) and not the rarer clumped species (i.e. $^{14}N^{15}N^{18}O$), though this is never explicitly stated. SP is calculated as the relative difference between the ^{15}N isotopologues (SP = δ456 – δ546) and the bulk nitrogen isotopic composition is the average of the alpha and beta sites ($\delta^{15}N_{bulk}$ = (δ456 – δ546)/2; see Kanterova et al. 2022 *GCA*, for example, for calculations) – therefore, understanding issues of sample bracketing, variations among samples, and etc. can be masked by reporting SP only. This is because variations in SP can be driving by variations in one isotopologue alone, since SP simply describes a relative difference in 456 and 546. In addition, as noted in Griffith 2018 *GMT*, several commercial manufacturers offer optical analyzers based on laser or FTIR spectroscopy that report results in various ways – as an isotopologue mole fraction and/or total mole fractions and/or in 'traditional' isotope delta values. Plouviez et al. do not report the equations used to convert from raw, instrument measurements to final delta values. They also do not report δ456 and δ546 (also denoted as δ^{15}N-alpha and δ^{15}N-beta), nor do they show that the calculated $\delta^{15}N_{bulk}$ from these values match the measured $\delta^{15}N_{bulk}$ values. They do report some necessary data in reporting "a new method" – i.e. Figure 1 and 5 – but, again. they do not report their full dataset and only their final calculated values. For

example, Table 1 gives the averaged isotopic measurements across all replicates for each species, but the individual measurements behind each average are not in the main text or supplement. Therefore, it is difficult to independently evaluate the quality of their data. I would encourage the authors to publish a more complete dataset, as well as equations involved in converting from raw, instrument measurements to final, reported delta values. This could be amended to the existing supplemental.

For the second point, I would: 1) Encourage the authors to comment more on the potential mechanism behind the large difference in SP values between the eukaryotic vs. bacterial algal strains; and 2) Have some clarifying questions regarding controls in their experimental systems. As the authors are likely aware of, in both eukaryotic and bacterial algae, it is thought that there are primarily two sources of N_2O: flavodiiron proteins (FLV) and cytochrome p450s (CYP55). FLVs are used in pseudo-cyclic electron flow for Photosystem I (PSI) photoprotection, where electrons are put onto O_2 instead of being used to generate NADPH. It has been shown that NO can be reduced instead of O_2, generating N_2O in the process (Burlacot et al 2020 *PNAS*). CYP55s are a broad class of enzymes involved in multiple metabolic pathways, including pigment biosynthesis and lipid metabolism – i.e., reactions not involved in the light reactions of photosynthesis. Hence, as noted by the authors, it has been shown in *C. reinhardtii* that FLV produce N_2O in the light, while CYP55 produces N_2O in the dark (Burlacot et al. 2020 *PNAS*). Due to similarities between the species, *C. vulgaris* should use a similar pathway, as noted by the authors. Prior work by some of the authors (Fabisik et al. 2023 *Biogeosciences*) performed a BLASTP search on *M. aeruginosa* and found hits for FLV and CYP55, suggesting that similar pathways exist in this strain as well.

Plouviez et al. perform all cell suspensions in the dark – this should isolate the CYP55 signal. The SP signals from *C. reinhardtii* and *C. vulgaris* are similar to that of the fungal nitric oxide reductase (Figure 2), which also belongs to the CYP55 family. However, the SP signals from *M. aeruginosa* are quite different and better match the bacterial nitric oxide reductase (Figure 2). One interpretation of their results is that CYP55 from eukaryotic and bacterial algae are quite different, and that is reflected in their N_2O SP values – this appears to be the primary interpretation that the authors make, though they do not attribute it to the enzyme explicitly. Alternatively, in *M. aeruginosa*, since they note that the pathway has not been fully 'elucidated,' non-CYP55 sources of N_2O may be possible. Potentially relevant, an enzyme called flavohemoglobin protein (FHP) has recently been measured for N_2O SP (Wang et al. 2024 *PNAS*). FHP is similar to FLV as they both have flavins as a co-factor – diflavins like FLV have two, while FHP has a flavin and heme cofactor. Measured N_2O SP values in Wang et al. 2024 *PNAS* of FHP from *P. aeruginosa*, *A. baumannii* and *S. aureus* are similar to those measured from *M. aeruginosa* in this paper (roughly 0 to 15‰ in Wang et al., depending on strain, compared to 2±7‰ for *M. aeruginosa* in this paper). The authors should also explicitly note if *M. aueruginosa* has a nitric oxide reductase (NOR) or not, since that would aid in interpretation of this unique signal. In addition, it may be potentially relevant that the standard deviation of their reported SP values from *M. aeruginosa* is much larger than that of *C. reinhardtii* and *C. vulgaris*, though they do not report the non-averaged data nor the 456 / 546 data, so this is difficult to interpret. That may also help better interpret why the SP values of *M. aeruginosa* are so different.

For this point, did the authors repeat their experiments in the light? Given the established light-dependent nature of N_2O production in *C. reinhardtii*, if FLV does have a different N_2O SP, one would expect to see a shift in the N_2O SP of *C. reinhardtii*.

In addition, other enzymes besides NOR, FLV, and CYP55 can produce N_2O. Currently, nitric oxide reductases (NOR), P450nor, cytochrome P460, cytochrome p450 (CYP55), cytochrome c554, flavodiiron proteins (FLVs) and flavohemoglobin proteins (FHPs) have been shown to produce N_2O as a direct product of an enzymatic reaction (see Ferousi et al. 2020 *Chem Rev*, Kuypers et al 2018 *Nat Rev Microbiol* and Poole & Hughes 2000 *Mol Microbiol* for review). Did the authors attempt to check if their wild-type (WT) strains have genes encoding any of these potential enzymatic sources? This check can be doing through searches like BLASTP, qPCR, RNAseq, or other similar techniques for working on non-genetically tractable strains (i.e. strains where making clean deletions of a certain gene are difficult).

Finally, regarding experimental controls, it is established that N_2O can be produced abiotically (i.e. 'chemodenitrification' Stanton et al. 2018 *Geobiology*), and this process is strongly pH-dependent, where acidic pHs produce nitric oxide radicals that can then be further reduced to N2O (i.e. Su et al 2019 *ES&T*). Did the authors control or check pH of their growth media? Though the media composition is given, there is no indication that the pH of the system was checked prior to incubation, or what the target pH of their media is. In addition, did the authors perform any no-cell controls, where the media was incubated with no cells? I may have missed this, but it does not appear that the authors did this. In addition, N_2O can be formed readily from NO radicals, which makes it important to control for all sources of NO radicals, particularly in wild-type (WT) strains. Both bacteria and eukaryotes can create NO through a diverse set of nitric oxide synthases (Forstermann & Sessa 2011 *Eur Heart J*), and these NO radicals can spontaneously react to form N_2O in the absence of oxygen. Did the authors check for these potential NO sources in their strains?

Overall, Plouviez et al. tackle an important problem in the biogeosciences – constraining the N_2O SP of eukaryotic and bacterial photosynthesizers, which produce N_2O outside of the metabolic pathways that N_2O production has been typically attributed to (denitrification and nitrification, either by bacteria or fungi). Their work offers an important starting point for further, more detailed physiological work that will enable this measurement to be used to disentangle complex microbial communities of denitifiers, nitrifiers, and photoysnthesizers, helping the community close the N_2O budget and disentangle complex marine N2O cycling.

Specific questions

The authors fine extremely depleted $\delta^{15}N_{bulk}$ values of about –100‰. This is outside of the range of their standards, and also of N_2O in air (ranged from ~9 to 6‰ over the past 300 years; Park et al 2012 Nat Geosci). Did they use a very depleted source of nitrite? The $\delta^{15}N$ of the nitrite supplied should be included.

In Table 1, what does "F" mean in the footnotes? At first I thought it meant fraction consumed, but one value of F is 1200.

For Table 3, what are the $\delta^{15}N$-alpha and $\delta^{15}N$-beta values for the standards used?

For Figure 1, what are the δ^{15}N-alpha and δ^{15}N-beta values, not just the SP values? In addition, just to clarify, only USGS52 was measured over time, and not USGS51 as well? Related to this, I am slightly confused because Figure 5 shows only USGS51 and not USGS52, and Figure 6 suggests that both reference gases were measured regularly.

For Figure 2a, Wang et al. 2024 *PNAS* offers a more recent compilation of N_2O SP measurements than Denk et al. 2017.

For Figure 2b, the authors are comparing their data vs. that from published denitfier data. In the text (line 113) and in the figure legend, which experimental denitrifer data are the authors comparing their data to? (In addition, the plot should specific 'bacterial denitrifiers' instead of just 'denitrifiers'). Multiple groups have measured bacterial denitrifiers and there is a larger range of values than they show in their figure. For example, see Wang et al. 2024 *PNAS* or Toyoda et al 2017 *Mass Spectrom Rev* for recent compilations. In addition, unless the authors are using nitrite with the exact same δ^{15}N as that study, one would not expect the δ^{15}N-N_2O and δ^{18}O-N_2O to be the same. Instead, the relative fractionation (i.e. $^{15}\varepsilon$ or $^{18}\varepsilon$) are comparable, not the bulk values. Therefore, the epsilons should be calculated and plotted instead.

For Figure 5, this is something where showing the full suite of data (δ^{15}N-alpha, δ^{15}N-beta, SP, δ^{15}N-N_2O and δ^{18}O-N_2O) would be helpful. The legend says that these are all measurements of USGS51, which should have a SP value of –1.67 at their target of 1000 ppb (1 ppm). However, at that pressure, the SP measured is between –25 and –30‰. Since this is showing "measurement bias," am I to understand that the SP value being measured is between –26.67 and –31.67‰? In addition, what happened to the 5/10/2023 run? The authors do not talk about it in the figure legend or text. Was data from that run discarded? In addition, it would be helpful to show the "experimentally determined, linear correction function" (Line 276) to show how they correct for variation in cell pressure, and how that consistent or not consistent that correction was for all experiments.

Technical corrections

There's a little floating "1)" in the upper left corner for Figure 3. Is this supposed to be there? And, the "2" in H2O and CO2 in the figure are not subscripted.