Journal of
Plankton Research

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J. Plankton Res. (2019) 00(00): 1–4. doi:10.1093/plankt/fbz034

BRIEF COMMUNICATION

Quantifying the overestimation of planktonic N_2 fixation due to contamination of $^{15}N_2$ gas stocks

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Received October 24, 2018; editorial decision 3, 2019; accepted June 24, 2019

Corresponding editor: Pia Moisander

The $^{15}N_2$-tracer assay [Montoya *et al.* (1996) A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. *Appl. Environ. Microbiol.*, **62**, 986–993.] is the most used method for measuring biological N_2 fixation in terrestrial and aquatic environments. The reliability of this technique depends on the purity of the commercial $^{15}N_2$ gas stocks used. However, Dabundo *et al.* [(2014) The contamination of commercial $^{15}N_2$ gas stocks with ^{15}N-labeled nitrate and 142 ammonium and consequences for nitrogen fixation measurements. *PLoS One*, **9**, e110335.] reported the contamination of some of these stocks with labile ^{15}N-labeled compounds (ammonium, nitrate and/or nitrite). Considering that the tracer assay relies on the conversion of isotopically labeled $^{15}N_2$ into organic nitrogen, this contamination may have led to overestimated N_2 fixation rates. We conducted laboratory and field experiments in order to (i) test the susceptibility of ^{15}N contaminants to assimilation by non-diazotroph organisms and (ii) determine the potential overestimation of the N_2 fixation rates estimated in the field. Our findings indicate that the contaminant ^{15}N-compounds are assimilated by non-diazotrophs organisms, leading to an overestimation of N_2 fixation rates in the field up to 16-fold under hydrographic conditions of winter mixing.

KEYWORDS: marine N_2 fixation; $^{15}N_2$ contamination; nitrogen isotopes; diazotrophs

Biological N_2 fixation is an important source of nitrogen into the ocean (Gruber, 2008), and the $^{15}N_2$-tracer addition technique (Montoya *et al.*, 1996) is the most extended method for measuring it in both terrestrial and aquatic environments. The $^{15}N_2$-tracer assay is based on the assimilation and transformation of isotopically

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labeled $^{15}N_2$ into organic nitrogen in incubations of natural planktonic communities. The accuracy of this approach is based on the assumption that the ^{15}N enrichment of particulate matter can only be due to biological reduction of N_2. Nevertheless, Dabundo *et al.* (2014) reported the presence of substantial concentrations of ^{15}N-contaminants ($^{15}NH_4^+$, $^{15}NO_3^-$, $^{15}NO_2^-$) in some commercial $^{15}N_2$ gas stocks supplied by Sigma-Aldrich, which may be assimilated by microorganisms. Their findings imply that the contaminant ^{15}N-compounds could lead to significant overestimations in some of the N_2 fixation rates reported in the literature. However, the magnitude of the overestimation of N_2 fixation measured in the field when using contaminated $^{15}N_2$ remains unknown.

In the framework of the NICANOR project, biological N_2 fixation was quantified between 2014 and 2015 in a station located in productive waters of the temperate upwelling region off NW Iberian Peninsula (off Ría de A Coruña, 43.42°N–8.44°W; depth = 80 m) (see Supplementary data S1 and Moreira-Coello *et al.*, 2017). Most of N_2 fixation rates measured throughout 2014 reached values comparable to the higher rates described for (sub) tropical oligotrophic regions (up to 82 nmol N L^{-1} d^{-1}) (Luo *et al.*, 2012). The concern that this could be due to the use of a $^{15}N_2$ gas stock substantially contaminated with ^{15}N-labeled ammonium, nitrate and nitrite motivated further laboratory and field experiments. The main goals of this study were (i) to test the susceptibility of ^{15}N-contaminants to assimilation by non-diazotroph organisms and (ii) to determine the magnitude of potential overestimation of the biological N_2 fixation rates estimated in the field.

In order to investigate the potential assimilation of ^{15}N-contaminants by non-diazotrophic organisms, the marine green alga *Tetraselmis suecica* was cultured in f/2 growth media (f/32 for nitrate) equilibrated with $^{15}N_2$ from different commercial suppliers: Sigma-Aldrich lot MBBB0968V and Cambridge Isotope lot I-19168A. The Sigma lot showed substantial contamination with ^{15}N-labeled ammonium, nitrate and nitrite (Dabundo *et al.*, 2014), whereas the composition of the specific Cambridge lot used was unknown. However, other analyzed stocks of the same commercial supplier showed negligible contamination (Dabundo *et al.*, 2014). Three 250-mL Erlenmeyer flasks were filled with 200 mL of growth medium equilibrated during 60 h with $^{15}N_2$ from the Sigma stock, three with medium equilibrated with $^{15}N_2$ from the Cambridge stock and finally another three with unamended medium (control). The nine flasks were inoculated with a stock culture of *T. suecica* (28 μg L^{-1} of initial concentration of chlorophyll *a* in each flask) and incubated during 4 days. For comparing treatments we used the $\delta^{15}N$ parameter, which expresses the $^{15}N/^{14}N$ isotopic ratio in a sample in relation to the standard value in the atmospheric N_2. The treatment with the Sigma stock resulted in statistically significant (Kruskal–Wallis, $P < 0.001$) enrichments in ^{15}N abundance of the particulate organic nitrogen ($\delta^{15}N_{PN} \sim 1.5‰$), in relation to the treatment with the Cambridge stock and the control ($\delta^{15}N_{PN}$ ranging from -0.90 ± 0.01 to $-1.4 \pm 0.3‰$), both during the exponential growth (Day 2) and stationary phase under nitrate-depleted conditions (Day 4). Sigma cultures were ^{15}N enriched because the microalgae assimilated the inorganic ^{15}N compounds present in the contaminated lecture bottle. No statistically significant (Kruskal–Wallis, $P > 0.05$) differences were observed between the control and the cultures equilibrated with $^{15}N_2$ from the Cambridge stock (Fig. 1). If we compute the N_2 fixation rate in the cultures of *T. suecica* equilibrated with contaminated $^{15}N_2$ from Sigma assuming that this microalga is a N_2-fixer, the observed difference in $\delta^{15}N$ and ^{15}N atom% between control and Sigma treatments would result in an apparent N_2 fixation rate ranging from 0.6 to 1.1 nmol N L^{-1} d^{-1}, after 4 and 2 days of incubation, respectively. This value of N_2 fixation is an artifact since *T. suecica* is not a diazotroph. Therefore, in a system where N_2 fixation is *per se* relatively low, the effect of using contaminated $^{15}N_2$ may be significant. More details on this experiment are available in Supplementary data S2.

In December 2014, we measured biological N_2 fixation rates in the field at surface waters and at 70-m depth by the $^{15}N_2$-uptake technique (Montoya *et al.*, 1996) using concomitantly two $^{15}N_2$ lecture bottles, the contaminated

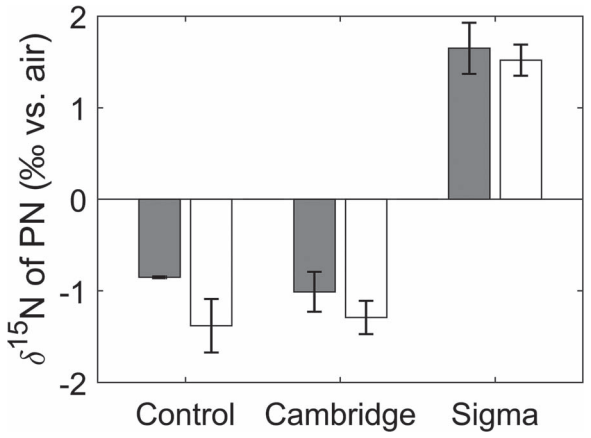

Fig. 1. ^{15}N content of particulate organic nitrogen versus the reference value in atmospheric N^2 ($\delta^{15}N_{PN}$, ‰ vs. air) in *T. suecica* cultures equilibrated with $^{15}N_2$ gas from Cambridge Isotope and Sigma-Aldrich commercial stocks sampled 2 days (exponential growth, gray bars) and 4 days (stationary phase under nitrate-depleted conditions, white bars) after inoculation. Error bars represent standard deviation (n=3).

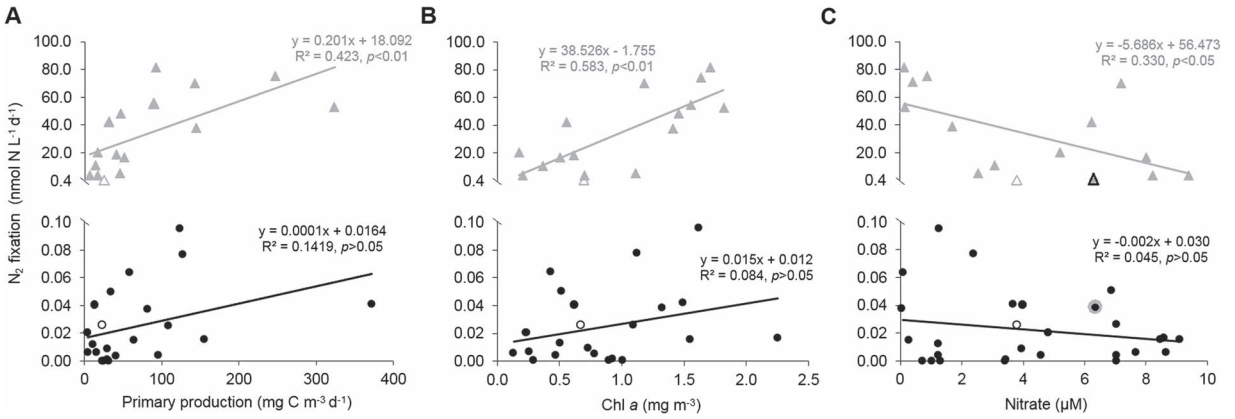

Fig. 2. (**A**) Total primary production, (**B**) chlorophyll *a* concentration and (**C**) nitrate concentration versus volumetric biological N_2 fixation rates obtained in the framework of the NICANOR project (February 2014 to December 2015) by using the contaminated Sigma-Aldrich lot (gray triangles), and the presumably non-contaminated Cambridge Isotope lot (black circles). Samples in which N_2 fixation was measured using both Sigma and Cambridge stocks are represented for December 2014 at 0 m depth by empty triangles and empty circles, respectively, and for December 2014 at 70 m by a gray triangle with black edge and a black circle with gray edge. Regression lines are plotted for each data set. Only samples with chlorophyll *a* < 2.5 mg m^{-3} have been represented in order to improve the data visualization in panels A and B, leaving out a sample with the highest chlorophyll *a* (6.5 mg m^{-3}) and primary production (396.4 mg C m^{-3} d^{-1}) and N_2 fixation of ~72 nmol N L^{-1} d^{-1} obtained using the contaminated Sigma stock.

gas from Sigma (stock MBBB0968V) and the presumably non-contaminated gas from Cambridge (I-19168A), and compared the results to ascertain the potential overestimation of N_2 fixation rates caused by the assimilation of ^{15}N contaminants. This experiment is described in detail in Supplementary data S3. These results were obtained under hydrographic conditions of winter mixing, which was characterized by a deep mixed layer (51 m), low chlorophyll *a* concentration (0.2–0.7 mg m^{-3}) and low primary production (4.8–23.5 mg C m^{-3} d^{-1}) (Fig. S2). The volumetric rates of N_2 fixation obtained were significantly higher, by a factor of 16, when using $^{15}N_2$ from Sigma (0.4 ± 0.1 and 0.6 ± 0.3 nmol N L^{-1} d^{-1}, respectively) than when using the Cambridge stock (0.03 ± 0.01 and 0.04 ± 0.01 nmol N L^{-1} d^{-1}, respectively) (Kruskal–Wallis, $P < 0.05$) (calculations are provided in Supplementary data S4 and S5). The Cambridge gas stock showed undetectable contamination according to our results with *T. suecica* cultures (Fig. 1), and the N_2 fixation rates equal to zero, or below the detection limit, measured in our field experiments during 2015 (Fig. 2). The reliable rates obtained by using the Cambridge stock in the samplings of 2015 and December 2014 did not correlate significantly either with total primary production ($R^2 = 0.142$, $P > 0.05$), chlorophyll *a* ($R^2 = 0.084$, $P > 0.05$) or environmental nitrate concentration ($R^2 = 0.045$, $P > 0.05$). The N_2 fixation measured during 2014 using the contaminated Sigma stock, including the December sampling, ranged from 0.4 to 82 nmol N L^{-1} d^{-1} and correlated positively with both primary production ($R^2 = 0.423$, $P < 0.01$) and chlorophyll *a* ($R^2 = 0.583$, $P < 0.01$), and negatively with nitrate concentration ($R^2 = 0.330$, $P < 0.05$) (Fig. 2). Thus, lower overestimation of N_2 fixation rates occurred in conditions of elevated nitrate concentration. By contrast, higher overestimation of N_2 fixation rates occurred in conditions of high productivity and biomass. The high phytoplankton production and biomass characteristic of conditions such as spring or summer blooms are linked to a high assimilation of nutrients, among which are the contaminants included in the stocks. Hence, the magnitude of overestimation depends on biomass and productivity of the phytoplankton present. In this coastal region, both variables are subject to marked seasonal variability and influenced by wind-driven upwelling, causing large changes in phytoplankton dynamics over short temporal scales (Gilcoto *et al.*, 2017). Thus, the overestimation will be different according to the prevailing hydrographic setting. In the widely studied oligotrophic (sub)tropical oceans, where N_2 fixers predominate, inflated rates may have gone unnoticed due to the high N_2 fixation rates that commonly characterize these systems.

The overestimated N_2 fixation rates due to the contamination obtained during 2014 ranged from 310 to 2260 µmol N m^{-2} d^{-1} (0.4–82 nmol N L^{-1} d^{-1}, Fig. 2), reaching similar values to the highest ever reported in (sub)tropical regions (Luo *et al.*, 2012). These rates would imply a depth-integrated diazotrophic biomass between 80 and 600 mg C m^{-2}, representing between 4% and 25% of the total phytoplankton biomass in the station

studied (Teira *et al.*, 2003), which is highly unrealistic (details on these calculations available in Supplementary data S6).

The experiment with *T. suecica* confirmed the uptake of ^{15}N contaminants by non-diazotrophs. Furthermore, the comparison, through field estimates, of the N_2 fixation rates obtained simultaneously with the Sigma and Cambridge stocks allowed to demonstrate that using contaminated $^{15}N_2$ yielded an overestimation of the rates by up to 16-fold. It is expected that the assimilation of dissolved inorganic nitrogen, mainly ammonium and nitrate, is preferred to the energy-costly N_2 fixation process (Stam *et al.*, 1987). Thus, the estimates of N_2 fixation reported in the literature may be distorted depending on the commercial $^{15}N_2$ source used. Past reports obtained using $^{15}N_2$ gas from Sigma should be interpreted with caution. However, the published rates obtained with $^{15}N_2$ from the Cambridge supplier are a priori reliable. In any case, in order to ensure the accuracy of future biological N_2 fixation determinations, it is recommendable to use the commercial stocks presumably free of contaminants according to Dabundo *et al.* (2014) (Cambridge and Campro Scientific) and to conduct control tests such as those herein described prior to use the $^{15}N_2$ lecture bottles acquired.

SUPPLEMENTARY DATA

Supplementary data can be found at *Journal of Plankton Research* online.

ACKNOWLEDGEMENTS

We thank the *Centro de Investigación Mariña* of the University of Vigo (ECIMAT) for the technical support during the experiments as well as the crew of the B/O *Lura* for their help during the sampling at sea. We are also very grateful to Paloma Chouciño, Antonio Fuentes-Lema, Elisa Guerrero-Feijóo, Carlota Rodríguez and Fátima Eiroa for logistic and technical support during the work at sea and in the laboratory.

FUNDING

Galician Government (NICANOR project, EM2013/021) to B.M.-C.; Instituto Español de Oceanografía (RADIALES project; www.seriestemporales-ieo.net); Formación de Profesorado Universitario (FPU) (predoctoral fellowship, FPU13/01674) to V.M.-C.

REFERENCES

Dabundo, R., Lehmann, M. F., Treibergs, L., Tobias, C. R., Altabet, M. A., Moisander, P. H. *et al.* (2014) The contamination of commercial $^{15}N_2$ gas stocks with ^{15}N–Labeled nitrate and ammonium and consequences for nitrogen fixation measurements. *PLoS One*, **9**, e110335.

Gilcoto, M., Largier, J. L., Barton, E. D., Piedracoba, S., Torres, R., Graña, R. *et al.* (2017) Rapid response to coastal upwelling in a semienclosed bay. *Geophys. Res. Lett.*, **44**, 2388–2397.

Gruber, N. (2008) The marine nitrogen cycle: Overview and challenges, in *Nitrogen in the marine environment*, eds D. G. Capone, D. A. Bronk, M. R. Mulholland, E. J. Carpenter, ed Elsevier Inc. (Amsterdam, The Netherlands), 1–50.

Luo, Y.-W., Doney, S. C., Anderson, L. A., Benavides, M., Bode, A., Bonnet, S. *et al.* (2012) Database of diazotrophs in global ocean: abundances, biomass and nitrogen fixation rates. *Earth Syst. Sci. Data Discuss.*, **5**, 47–106.

Montoya, J. P., Voss, M., Kahler, P. and Capone, D. G. (1996) A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. *Appl. Environ. Microbiol.*, **62**, 986–993.

Moreira-Coello, V., Mouriño-Carballido, B., Marañón, E., Fernández-Carrera, A., Bode, A. and Varela, M. M. (2017) Biological N_2 fixation in the upwelling region off NW Iberia: magnitude, relevance, and players. *Front. Mar. Sci.*, **4**, doi:10.3389/fmars.2017.00303.

Stam, H., Stouthamer, A. H. and van Verseveld, H. W. (1987) Hydrogen metabolism and energy costs of nitrogen fixation. *FEMS Microbiol. Rev.*, **46**, 73–92.

Teira, E., Abalde, J., Álvarez-Ossorio, M. T., Bode, A., Cariño, C., Cid, Á. *et al.* (2003) Plankton carbon budget in a coastal wind-driven upwelling station off A Coruña (NW Iberian Peninsula). *Mar. Ecol. Prog. Ser.*, **265**, 31–43.