

## Use of $^{15}\text{N}$ to measure dissolved organic nitrogen release by marine phytoplankton (reply to comment by Bronk and Ward)

In their comment Bronk and Ward attempt to clarify the questions addressed by Slawyk et al. (1998); i.e., (1) what is the general significance of dissolved organic nitrogen (DON) release measured with  $^{15}\text{N}$  tracer, (2) are extremely high DON release rates (up to 86%) reported by Bronk and Ward reasonable, and thus (3) do traditional  $^{15}\text{N}$  techniques underestimate new production by up to 74% (Bronk et al. 1994)? We fully agree with Bronk and Ward that “though the amount of  $^{15}\text{N}$  that ultimately passes into the DON pool may be small, it can translate into large DON release rate relative to the rate of gross nitrogen uptake,” but only so long as one does not mix DON release with dissolved inorganic nitrogen (DIN) loss as DON.

At the end of a  $^{15}\text{N}$ -incubation experiment  $^{15}\text{N}$  is in three pools: DIN, particulate organic nitrogen (PON), and extracellular DON (Fig. 1). Since trichloroacetic acid (TCA) can only be used to trace incorporation of  $^{15}\text{N}$ -labeled DIN into PON (Bronk and Glibert 1991), the intracellular organic nitrogen (ON) is methodologically divisible into two parts: a TCA soluble fraction ( $\text{ON}_{\text{TCA}_s}$ ) and a TCA insoluble fraction ( $\text{ON}_{\text{TCA}_{\text{ins}}}$ ). Transfers of  $^{15}\text{N}$ -labeled nitrogen between N pools (sensu Sheppard 1962) depicted in Fig. 1 by  $\rho_1$  to  $\rho_5$  are given by Eqs. 1–5 (Fig. 1). Measurements of DON release, i.e., the transport of nitrogen from intracellular ON to extracellular DON ( $\rho_3$ ), are based on the assumption that  $\text{ON}_{\text{TCA}_s}$  is the source pool and DON is the target pool (Bronk and Glibert 1991, 1993). The rate of this transport is given by Eq. 3 and actually corresponds to  $\text{ON}_{\text{TCA}_s}$  release. Bronk (1999) referred to the latter rate as intracellular pool (IP) DON release. If, however,  $^{15}\text{N}$ -labeled ON is also released as  $\text{ON}_{\text{TCA}_{\text{ins}}}$  ( $\rho_4$ ),  $\text{ON}_{\text{TCA}_s}$  release can be determined only if the  $^{15}\text{N}$  enrichment (R) of  $\text{ON}_{\text{TCA}_s}$  in the extracellular DON pool is measured. Since the intracellular ON source is not exactly known and only the  $^{15}\text{N}$  enrichment of  $\text{ON}_{\text{TCA}_s}$  has been measured, actual available data on DON release may only represent rough estimates of the total amount of DON released ( $\rho_5$ ).

A further problem with the calculation of DON release based on an intracellular ON pool may arise from the fact that it probably does not respect a basic principle of tracer methodology, i.e., constancy of  $^{15}\text{N}$  enrichment in the source pool. It is to be expected that this enrichment increases in some fashion with incubation time.

To get around these latter difficulties, Bronk et al. (1994) introduced a new protocol for the calculation of DON release, assuming that extracellular DIN is the source pool of DON release. Bronk (1999) called this release extracellular pool (EP) DON release and calculated it as the difference between gross uptake and net uptake. However, this procedure included a mathematical correction of the atom percentage  $^{15}\text{N}$  enrichment of PON (after some estimate of DON release) through a process of iteration (Bronk pers. comm.). Note that Bronk and Ward could obtain EP DON release directly from their Eq. 2 presented in the comment by omit-

ting the term  $(\text{PN} \times \text{PN at } \%xs)$  in the numerator. Slawyk et al. (1998) quantified the identical rate in terms of DIN loss ( $\rho_2$ ) with Eq. 2 (Fig. 1) without needing to calculate first the gross and net DIN uptake. They named the rate of this DON release  $\rho_{\text{DIN}}^{\text{loss}}$  since it equals the rate of  $\text{DI}^{15}\text{N}$  originally transported into the cells (PON) and then present (lost) outside the cells as  $\text{DO}^{15}\text{N}$  (cf. Flynn and Berry 1999).

There is a fundamental difference between the two methods used by Bronk and Ward to calculate DON release (intracellular ON source pool method vs. extracellular DIN source pool method) with regard to the significance of the resulting rates. Although rates of DON release obtained from the two latter methods reflect the same process, they are not the same. Choosing one or the other method is not, as Bronk (1999) claimed, simply a question of convenience (less experimental labor) or robustness of results.

Solving Eq. 2 (Fig. 1) for  $R_{\text{DON}}$  and substituting this in Eq. 3 (Fig. 1) yields:

$$\rho_{\text{ON}_{\text{TCA}_s}} = \rho_{\text{DIN}}^{\text{loss}}(R_{\text{DIN}}/R_{\text{ON}_{\text{TCA}_s}}), \quad (7)$$

or, using Bronk's (1999) terminology,

$$\text{IP DON release} = \text{EP DON release}(R_{\text{DIN}}/R_{\text{ON}}). \quad (8)$$

These equations show that for a given DIN loss rate (EP DON release rate),  $\text{ON}_{\text{TCA}_s}$  release (IP DON release) is proportional to the ratio  $R_{\text{DIN}}/R_{\text{ON}_{\text{TCA}_s}}$  ( $R_{\text{DIN}}/R_{\text{ON}}$ ). In other words, the more  $^{15}\text{N}$ -labeled substrate ( $\text{DI}^{15}\text{N}$ ) is diluted by initial unenriched cell nitrogen in the course of its metabolism, the higher is the rate of  $\text{ON}_{\text{TCA}_s}$  release (IP DON release) in order to meet the DIN loss (EP DON release). Only when  $R_{\text{ON}_{\text{TCA}_s}} = R_{\text{DIN}}$  ( $R_{\text{ON}} = R_{\text{DIN}}$ ) does  $\rho_{\text{ON}_{\text{TCA}_s}} = \rho_{\text{DIN}}^{\text{loss}}$  (IP DON release = EP DON release). Further,  $\rho_{\text{ON}} < \rho_{\text{DIN}}^{\text{loss}}$ , as found by Bronk and Ward when repeating Slawyk et al.'s (1998) calculations, is theoretically impossible because it would require that  $R_{\text{DIN}}/R_{\text{ON}} < 1$ .

If isotopic equilibrium is reached between the extracellular DIN pool and the intracellular  $\text{ON}_{\text{TCA}_s}$  pool, this latter should be very small, having a very short turnover time. Results from a time course nitrate uptake experiment, however, show that the  $\text{ON}_{\text{TCA}_s}$  pool is rather large (15–35% of total PON) and that the  $R_{\text{NO}_3}/R_{\text{ON}_{\text{TCA}_s}}$  ratio decreases with incubation time from 23.8 to 7.9 (Fig. 2). Note that the decreasing trend of the latter ratio resulted from the increase of  $R_{\text{ON}_{\text{TCA}_s}}$  since  $^{15}\text{N}$ -isotope dilution of  $R_{\text{NO}_3}$  was zero. This ratio may be overestimated due to isotope dilution of  $\text{O}^{15}\text{N}_{\text{TCA}_s}$  by unenriched  $\text{ON}_{\text{TCA}_s}$  from cells not taking up nitrate. However, even a dilution factor of 2, although unlikely given the average PON/Chl *a* ratio of 1.12, would result in  $R_{\text{NO}_3}/R_{\text{ON}_{\text{TCA}_s}}$  ratios  $\gg 1$ . Therefore, according to Eqs. 7 and 8, release of  $\text{ON}_{\text{TCA}_s}$  (IP DON release) would be  $\gg \rho_{\text{NO}_3}^{\text{loss}}$  (EP DON release). This is the reason why we argued that extremely high DIN loss (EP DON release) rates (50–90% of gross DIN uptake) would produce negative changes in PON concentration of cells taking up DIN (Slawyk et al. 1998), es-

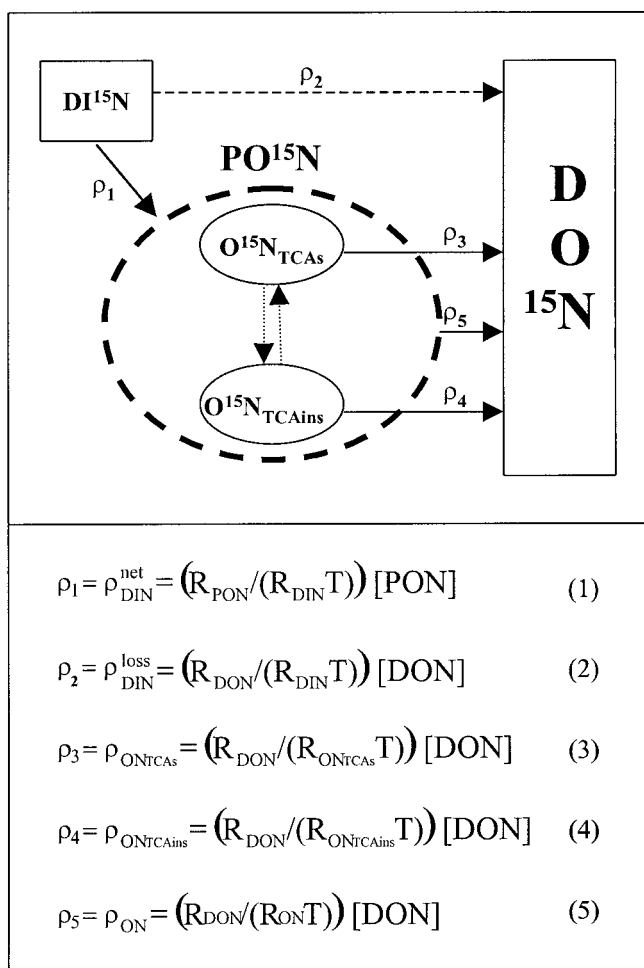


Fig. 1. Possible pathways of  $^{15}N$  following addition of  $^{15}N$ -labeled dissolved inorganic nitrogen ( $DI^{15}N$ ) to a sample of seawater contained in an incubation bottle and equations to calculate N transport between dissolved and particulate N pools. Depicted are DIN net uptake by the particulate organic nitrogen (PON) pool ( $\rho_1$ , sensu Dugdale and Wilkerson 1986), DIN loss as dissolved organic nitrogen (DON) to the extracellular DON pool ( $\rho_2$ , Slawyk et al. 1998; Flynn and Berry 1999; equivalent to [EP] DON release in Bronk 1999), active trichloroacetic acid (TCA) soluble cellular organic nitrogen ( $ON_{TCAAs}$ ) release ( $\rho_3$ , equivalent to DON release in Bronk and Glibert 1991 and to IP DON release in Bronk 1999), active TCA insoluble ON ( $ON_{TCAins}$ ) release ( $\rho_4$ , not measured), and passive  $ON_{TCAAs}$  and  $ON_{TCAins}$  release during zooplankton grazing and/or cell lysis ( $\rho_5$ , not measurable with the  $^{15}N$  tracer). Note that for  $\rho_2$  passage of  $^{15}N$ -labeled N through the PON pool was ignored. Gross DIN uptake ( $\rho_{DIN}^{gross}$ ) equals  $\rho_1 + \rho_2$  (Slawyk et al. 1998) but not necessarily  $\rho_1 + \rho_3$ ,  $\rho_1 + \rho_4$  or  $\rho_1 + \rho_5$ . Dotted arrows represent exchange between intracellular ON pools (protein synthesis and breakdown). EP and IP stand for extracellular and intracellular pool, respectively. In equations  $R$  is  $^{15}N$  atom percentage excess enrichment,  $T$  is time, and [PON] and [DON] are final concentrations (in  $\mu M$ );  $\rho$  has units of  $\mu M \text{ time}^{-1}$ .

pecially when large ON cell fractions such as  $ON_{TCAins}$  with relatively low  $^{15}N$  abundance ( $R_{ON_{TCAins}} < R_{ON_{TCAAs}} < R_{DIN}$ ) are released to meet these high DIN loss rates.

On the other hand, it should be emphasized that low DIN loss (EP DON release) rates do not rule out the possibility of high (IP) DON release rates. Bearing this in mind, Raim-

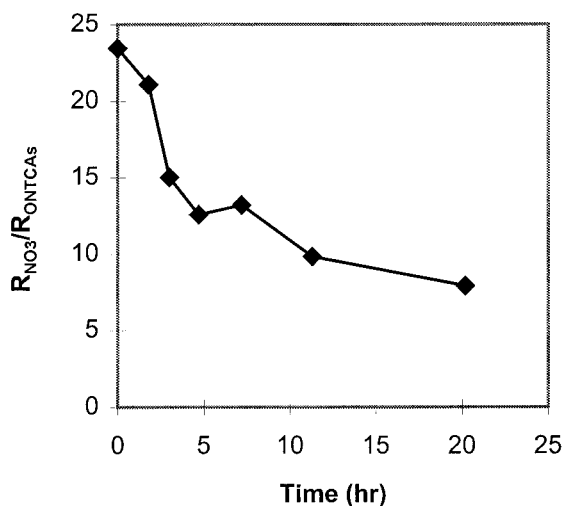


Fig. 2. Time course of the ratio atom percentage enrichment of extracellular nitrate/atom percentage enrichment of TCA soluble intracellular organic nitrogen ( $R_{NO_3}/R_{ON_{TCAAs}}$ ) from a nitrate uptake experiment conducted in subsurface waters (50% light depth) off North Brittany during summer.  $R_{NO_3} = 8.7\%$ . Initial concentrations of nitrate and chlorophyll  $a$  were  $1.53 \mu M$  and  $1 \mu g L^{-1}$ , respectively. The final PON concentration was  $1.32 \mu M$ . The slope of a linear regression of PON ( $\mu M$ ) vs. Chl  $a$  ( $\mu g L^{-1}$ ) was 1.12 ( $r = 0.72$ ,  $n = 15$ ).

bault et al.'s (1999) assumption that DON release from one or several unknown intracellular pools may represent ~20–100% of new production is not necessarily in conflict with the low DIN loss (EP DON release) rates (~15% of gross DIN uptake) obtained from the same field study (Slawyk et al. 1998). Note that different possible intracellular source pools, ranging from recently produced photosynthate to whole cell fractions, have also been suggested for the release of dissolved organic carbon (DOC) (Karl et al. 1998). The latter authors called attention to exactly the same problem as we did for DON release; i.e., if DOC is released from whole cell fractions (with relatively low specific radioactivity), this release needs to be much larger than that of recently produced DOC (with relatively high specific radioactivity) for an equivalent flux of  $^{14}C$ -DOC (DIC loss in fact).

Bronk and Ward question the DIN/DON extraction method of Slawyk and Raimbault (1995), especially with respect to the efficiency of DON recovery. They found that this method was unable to extract more than 43% of the total DON pool and contend that low DON release rates reported from our laboratory (Slawyk et al. 1998; Raimbault et al. 1999; Diaz and Raimbault 2000; Raimbault et al. 2000) and another laboratory (Hasegawa et al. 2000) are indicative of losses of labile  $^{15}N$ -labeled DON as ammonia during initial DIN removal. Raimbault et al. (2000) checked for this possibility by inspecting samples from 5-m depth at 15 stations in oligotrophic waters of the Mediterranean Sea where the  $^{15}N$ -labeled substrate (ammonium) was exhausted after a 24-h incubation period. In the 300-ml filtrates, to which were added ~3  $\mu mol$  of ammonium carrier, the average  $^{15}N$  atom percentage enrichment of the extracted carrier (2.91  $\mu mol$ , SD = 0.21) was 0.3696 (SD = 0.0044), which corresponds to natural abundance. However, that of the DON pool (1.73  $\mu mol$ , SD =

0.43) was 0.4213 (SD = 0.0371), which corresponds to an average atom percentage excess enrichment of 0.0517. From these results it can be concluded that (1) initial ammonium was, indeed, exhausted, (2) recovery of the carrier (97%) was not higher than expected, (3) DON recovery was not lower than expected, (4) DON release actually occurred, and (5) apparently no  $^{15}\text{N}$ -labeled DON was lost to the ammonium pool during the step of DIN removal when processing samples with Slawyk and Raimbault's (1995) standard method.

On the other hand, any unrecovered  $^{15}\text{N}$ -labeled DIN carried over into the DON pool during the DON extraction procedure would result in an artificial  $^{15}\text{N}$  enrichment of this latter pool and hence in an overestimation of DIN loss (EP DON release) rates. Bronk and Ward (1999) reported 100% DIN removal efficiencies "monitored via wet chemical analysis of the concentrations of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  just prior to UV oxidation." However, this crucial information can only be obtained correctly from 'time zero' experiments using the  $^{15}\text{N}$  tracer and running the experiments under standard experimental conditions (Slawyk and Raimbault 1995) and not from chemical methods even if these latter have low detection limits. Therefore, incomplete DIN removal may explain the very high DIN loss (EP DON release) rates reported (up to >90% of gross DIN uptake, cf. Bronk and Ward 1999) rather than filtration (cell rupture) or environmental (light) stress.

In conclusion, as long as the species and the  $^{15}\text{N}$  enrichment of an intracellular ON source pool for DON release are not exactly known, it is impossible to measure total DON release ( $\rho_5$ ) by phytoplankton (see Fig. 1) with the  $^{15}\text{N}$  tracer. As Bronk and Ward rightly pointed out, the present IP method "requires a host of assumptions." Furthermore, it is more than likely that IP DON release rates (whatever the source) are higher than EP DON release (DIN loss) rates ( $\rho_5 > \rho_4 > \rho_3 > \rho_2$ ), except when the  $^{15}\text{N}$  abundance of the intracellular ON pool equals that of the extracellular DIN pool. But this extreme case needs to be proved. Note that Bronk (1999) found on average that the IP method gave rates up to 3 times higher than those obtained with the EP method ( $\rho_3 = 3 \rho_2$ ).

These problems, however, do not prevent estimating of DIN loss (EP DON release) correctly and hence gross DIN uptake rates (Slawyk et al. 1998). As far as the magnitude of published DIN loss (EP DON release) rates are concerned, Bronk and Ward are now focusing on the lower estimates (10 to ~34% of gross DIN uptake). This latter range is consistent with results from comparisons between DIN disappearance rates obtained from chemical measurements and net uptake rates obtained from classical  $^{15}\text{N}$  experiments (Slawyk et al. 1990; Raimbault et al. 2000).

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