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NOTE

A modified ^{15}N tracer method and new calculation for estimating release of dissolved organic nitrogen by freshwater planktonic algae

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ABSTRACT: To estimate the release of DON (dissolved organic nitrogen) by freshwater planktonic algae, we modified the Bronk & Glibert method used for marine planktonic algae, by adding NaCl (3.5%) to the freshwater samples and using ion retardation resin columns to separate DON from inorganic nitrogen in the solutions. Clear separation and high recoveries of amino acids indicate that the ion retardation resin column method can be applied to freshwater samples. We derived equations for the calculation of DON release and net DIN (dissolved inorganic nitrogen) assimilation which enable the estimation of the TNR (total nitrogen release) and NNA (net nitrogen assimilation). Further, we derived equations to estimate RANR (recently assimilated nitrogen release; release of DON produced from DIN assimilated during ^{15}N incubation), and PANR (previously assimilated nitrogen release; release of DON produced before the incubation). Using the modified method and equations, we examined time courses of DON release from the cyanobacterium *Microcystis novacekii*. Both TNR and NNA increased with time during ^{15}N incubation, with more than 91% of the TNR consisting of RANR.

KEY WORDS: Ammonium · Nitrogen uptake · Dissolved organic nitrogen release · Freshwater planktonic algae · *Microcystis novacekii* · Tracer method

Release of DOM (dissolved organic matter) by planktonic algae is one of the main sources of DOM in aquatic environments, along with loss of cell contents during predation by zooplankton and cell lysis by viruses (Jumars et al. 1989, Bronk & Glibert 1993b, Søndergaard et al. 1995, Weinbauer & Höfle 1998). Released DOM is readily assimilated by heterotrophic bacteria (Frogg 1983, Kirchman et al. 1991, Norrman et al. 1995), and therefore plays a key role in the microbial loop and flow of matter in aquatic environments (Azam et al. 1993).

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DOM release from planktonic algae has been examined using dissolved organic carbon (DOC) or dissolved organic nitrogen (DON). DOC release has been studied mostly using ^{14}C tracers (Fogg et al. 1965, Watt 1966, Nalewajko & Schindler 1976, Lancelot 1979, Mague et al. 1980, Giordano et al. 1994) and DON release using ^{15}N tracers (Axler & Reuter 1986, Bronk & Glibert 1991, 1993b, 1994, Glibert & Bronk 1994). DON release in freshwater environments (Axler & Reuter 1986) has only been peripherally examined compared to marine environments.

In most of the previous studies on DOM release, extracellular DIM (dissolved inorganic matter) has been used as the tracer source (e.g. Fogg et al. 1965, Watt 1966). Release of total DOM has been estimated by the calculation analogous to that for DIM uptake (Dugdale & Goering 1967) when stable isotopes are used as tracers. During tracer incubations, extracellular DIM is incorporated into cells and synthesized to intracellular organic matter. A portion of this intracellular organic matter is then released from cells. However, not all the released DOM originates from DOM produced from extracellular DIM during the tracer incubations, as some of the released DOM may originate from organic matter synthesized before the addition of the tracer (Giordano et al. 1994). Therefore, the amount of DOM released may be underestimated due to this mixing, making it difficult to precisely estimate RANR (recently assimilated nitrogen release, i.e. release of DON produced from nitrogen assimilated during ^{15}N tracer incubation; for terminology see Table 1).

Bronk & Glibert (1991) assumed that intracellular low molecular weight (LMW) organic nitrogen was the source of the released DON. Based on this assumption, they calculated the release of LMW DON and total

Table 1. List of abbreviations

Abbreviation	Description	Unit
DOM	Dissolved organic matter	µg-at. N or C
POM	Particulate organic matter	µg-at. N or C
DIM	Dissolved inorganic matter	µg-at. N or C
PON	Particulate organic nitrogen	µg-at. N
DON	Dissolved organic nitrogen	µg-at. N
DIN	Dissolved inorganic nitrogen	µg-at. N
PON_t	PON at time t	µg-at. N
PON_{t_1}	PON at time $t_1 = t + \delta t$	µg-at. N
DON_t	DON at time t	µg-at. N
DON_{t_1}	DON at time $t_1 = t + \delta t$	µg-at. N
A_{DIN}	^{15}N atom% of DIN	%
A_{PON_t}	^{15}N atom% of PON at time t	%
A_{PONt_1}	^{15}N atom% of PON at time $t_1 = t + \delta t$	%
A_{DON_t}	^{15}N atom% of DON at time t	%
A_{DONt_1}	^{15}N atom% of DON at time $t_1 = t + \delta t$	%
TNR	Total nitrogen release (total nitrogen released during δt; RANR + PANR)	µg-at. N
RANR	Recently assimilated nitrogen release (nitrogen assimilated and released during δt)	µg-at. N
PANR	Previously assimilated nitrogen release (nitrogen assimilated before time t and released during δt)	µg-at. N
GNA	Gross nitrogen assimilation	µg-at. N
NNA	Net nitrogen assimilation (GNA – TNR)	µg-at. N

DON, and assessed the importance of direct release of DON by phytoplankton in seawater samples containing phytoplankton and bacteria. The assumption enables the direct measurement of the release of DON by phytoplankton in seawater samples. However, it is necessary to measure RANR to examine the intracellular nitrogen pool of nitrogen release from phytoplankton in more detail. If RANR by phytoplankton is large, the intracellular nitrogen pool of dissolved LMW DON is probably small or saturated. Therefore, RANR may be useful as an indicator of the status of nitrogen nutrition in planktonic algae.

In this study, we have modified the calculation method for DIN uptake (Dugdale & Goering 1967, Bronk & Glibert 1991, Bronk et al. 1994). By considering the mass balance among DIN, intracellular DON and extracellular DON and using this modified calculation method, we can estimate the total nitrogen release (TNR) along with the RANR and the nitrogen assimilation. As to the experimental procedure, we modified Bronk & Glibert's (1991) experimental method to allow analysis of freshwater phytoplankton samples and using ^{15}N we measured the DON release by the cyanobacterium *Microcystis novacekii* (Kom.) Comp. during the ^{15}N incubation.

Materials and methods. To measure DON release, we determined ^{15}N atom% and N contents of DON and algal particles after incubation with ^{15}N. First, we added ^{15}N tracers $^{15}NH_4Cl$ (99.6 atom%) or $Na^{15}NO_3$ (99.0 atom%) to algal suspensions in 100 ml glass bottles (final 90 µg-at. N l^{-1}) and incubated for a given time as described below. After ^{15}N incubation, we added formalin (final 0.2%) to the suspension to stop the incubation at a set time, and then immediately filtered the samples with glass fiber filters (Whatman GF/C, 47 mm ∅) by gravity filtration to separate filtrates and particulate matter. Sodium chloride was added to 9 ml of the filtrates (3.5%) to increase the salinity. The NaCl-added filtrates were then loaded on ion retardation resin columns (BioRad #142-7834; AG 11 A8, 50 to 100 mesh) to separate DON from DIN. Elution of DON was carried out with deionized water (Bronk & Glibert 1991). After elution, pH of the eluted DON solutions was adjusted to 7.0. The solutions were then concentrated to <2 ml by evaporation. The concentrated eluates were impregnated onto precombusted (420°C) glass fiber filters (Whatman GF/C, 47 mm ∅) and the filters were dried. Small amounts of the eluate were repeatedly impregnated and dried to avoid any loss of the solution from the filters. ^{15}N atom% and N contents of DON on the filters were measured by a quadrupole mass spectrometer (Anelva TE-150) fitted with a combustion furnace (Watanabe & Miyazaki 1996).

The filters of the particulate matter were washed with deionized water 3 times to remove any adhered traces of the isotope on the filter and filtered material and then dried. ^{15}N atom% and N contents of particulate material were determined with the mass spectrometer (Anelva TE-150) as above.

Separation capability of the ion retardation resin column was examined using phenylalanine and ammonium or nitrate. Nine ml of 3.5% NaCl solution containing phenylalanine (0.27 µg-at. N) and NH_4Cl or

$NaNO_3$ (0.27 µg-at. N) was loaded on the ion retardation resin column. Phenylalanine and inorganic nitrogen were eluted with deionized water. Phenylalanine was monitored at 270 nm with a Shimadzu UV-200 spectrophotometer. Concentrations of ammonium and nitrate were monitored by the methods of Sagi (1966) and Mullin & Riley (1955), respectively.

To examine the recovery of amino acids, 9 ml of amino acid mixture solution containing 3.5% NaCl and 3.2 µg-at. N of each amino acid component (valine, glycine, phenylalanine, isoleucine, serine, aspartic acid and glutamic acid) was loaded on the ion retardation resin column. Nitrogen contents of eluted fractions were measured by mass spectrometry (Anelva TE-150).

DON release by *Microcystis novacekii* (Kom.) Comp. was examined. *M. novacekii* was supplied by Dr M. Watanabe of Tsukuba Botanical Garden, National Science Museum (Tsukuba Algal Collection TAC 19). This alga was axenically grown in a 2 l flask containing modified WC medium (Guillard & Lorenzen 1972, Watanabe & Miyazaki 1996) at 25°C under an irradiance of 150 µmol photons m^{-2} s^{-1} supplied by cool-white fluorescent lamps (14:10 h LD). The culture medium was continuously aerated (16 ml s^{-1}) and stirred with a magnetic bar.

After 10 d growth, 70 ml of the cultivated algal suspension was dispensed into 100 ml glass bottles and $^{15}NH_4Cl$ (99.6 atom%) was added to the bottles (90 µg-at. N l^{-1} (Miyazaki et al. 1985). This concentrationn of ammonium has been shown not to inhibit growth of *Microcystis novacekii* (Watanabe & Miyazaki 1996). The algal suspensions in the glass bottles were incubated in duplicate for 3, 6 or 9 h at 25°C under an irradiance of 150 µmol photons m^{-2} s^{-1}. After incubation, samples were fixed with formalin (0.2%) (Watanabe & Miyazaki 1996), and processed on a ion retardation resin column as described above.

Equations for the calculation of DON release and DIN assimilation are based on the mass balance of nitrogen among DIN, DON and PON (particulate organic nitrogen). Abbreviations used in the text are summarized in Table 1. A schematic diagram of DIN assimilation and DON release is given in Fig. 1.

When DON release and assimilation occur simultaneously, during δt (time difference between t and $t_1 = t + \delta t$), mass balance of total nitrogen ($^{14}N+^{15}N$) is described as

$$TNR + PON_{t1} = PON_t + GNA \quad (1)$$

where TNR is the total DON released during δt. PON_{t1} and PON_t are PON at t_1 and t, respectively. GNA (gross nitrogen assimilation) is gross (released + unreleased) nitrogen assimilated during δt.

Mass balance of ^{15}N is described by

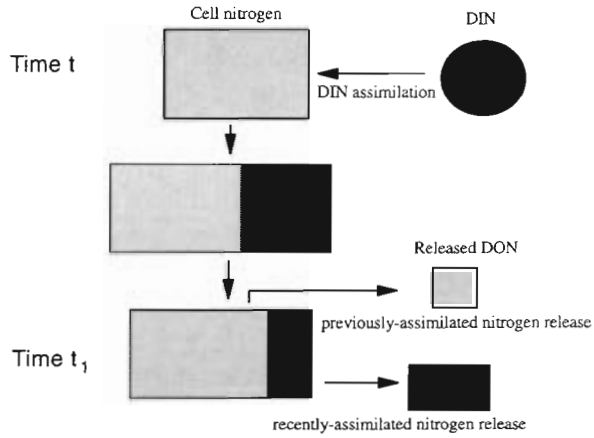

Fig. 1. Schematic diagram of DIN assimilation and DON release

$$A_{PON_t} \cdot PON_t + A_{DIN} \cdot GNA = A_{PON_{t1}} \cdot PON_{t1} + A_{DON_{t1}} \cdot DON_{t1} - A_{DON_t} \cdot DON_t \quad (2)$$

where A_{PON_t} and $A_{DON_{t1}}$ are ^{15}N atom% of PON at time t and t_1, respectively. A_{DIN} is ^{15}N atom% of DIN. DON_{t1} and DON_t are DON at time t_1 and t, respectively. $A_{DON_{t1}}$ and A_{DON_t} are ^{15}N atom% of DON at t_1 and t, respectively.

TNR is expressed as

$$TNR = DON_{t1} - DON_t \quad (3)$$

From Eqs. (1), (2) & (3), GNA and TNR are calculated according to

$$GNA = \frac{A_{DON_t} - A_{PON_{t1}}}{A_{DON_t} - A_{DIN}} \cdot PON_{t1} - \frac{A_{DON_{t1}} - A_{DON_t}}{A_{DON_t} - A_{DIN}} \cdot DON_{t1} + \frac{A_{PON_t} - A_{DON_t}}{A_{DON_t} - A_{DIN}} \cdot PON_t \quad (4)$$

and

$$TNR = \frac{A_{DIN} - A_{PON_{t1}}}{A_{DON_t} - A_{DIN}} \cdot PON_{t1} - \frac{A_{DON_{t1}} - A_{DON_t}}{A_{DON_t} - A_{DIN}} \cdot DON_{t1} + \frac{A_{PON_t} - A_{DIN}}{A_{DON_t} - A_{DIN}} \cdot PON_t \quad (5)$$

We define NNA (net nitrogen assimilation) as

$$NNA = GNA - TNR \quad (6)$$

TNR is composed of RANR (recently assimilated nitrogen release; release of DON produced from DIN assimilated during δt) and PANR (previously assimilated nitrogen release; nitrogen assimilated before time t and released during δt). Thus,

$$TNR = RANR + PANR \quad (7)$$

Mass balance of ^{15}N present in TNR is described by

$$A_{DIN} \cdot RANR + A_{PON_t} \cdot PANR = A_{DON_{t1}} \cdot DON_{t1} - A_{DON_t} \cdot (DON_{t1} - TNR) \quad (8)$$

From Eqs. (7) & (8), RANR is calculated.

$$RANR = TNR\frac{A_{DON_t} - A_{PON_t}}{A_{DIN} - A_{PON_t}}\,TNR + \frac{A_{DON_{t1}} - A_{DON_t}}{A_{DIN} - A_{PON_t}} \cdot DON_{t1} \quad (9)$$

Results. Use of the ion retardation resin column allowed separation of phenylalanine from ammonium and from nitrate in the freshwater samples containing 3.5% NaCl (Fig. 2). The concentration maximum of phenylalanine appeared at 20 to 25 ml eluates, whereas the maxima of ammonium and nitrate appeared at 65 to 70 ml. Recovery of phenylalanine in the first 40 ml eluate was 92.4% when a mixture of phenylalanine and ammonium was loaded on the column (Fig. 2A). Ammonium recovery in the first 40 ml eluate was 18.0% in the phenylalanine/ammonium mixture. Ammonium

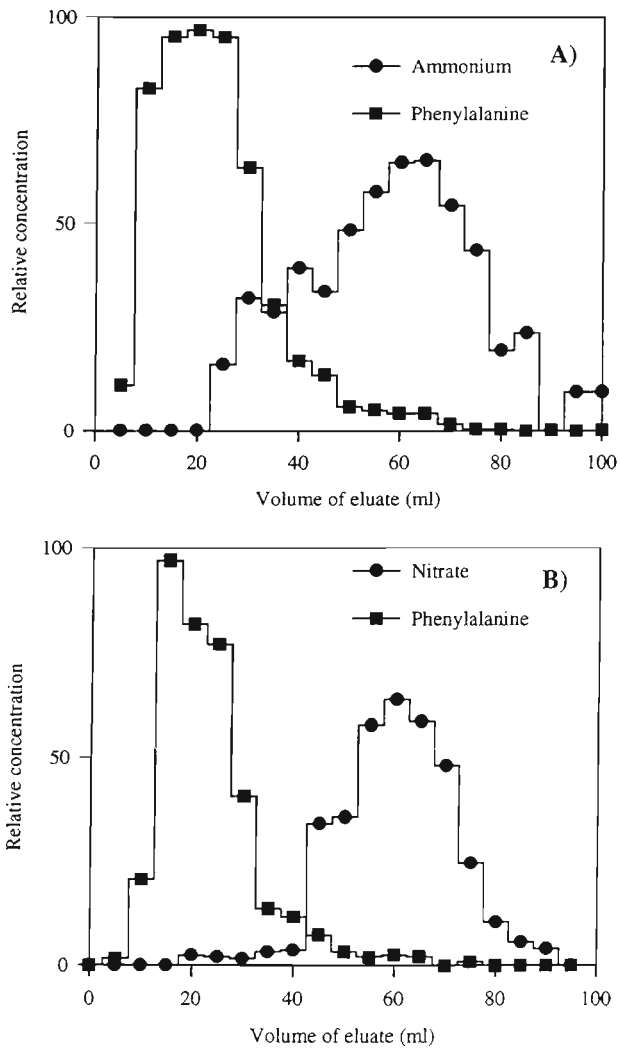

Fig. 2. Changes in concentration of (A) NH_4^+ and phenylalanine and (B) NO_3^- and phenylalanine in eluates from the ion retardation column

Table 2. Recoveries of amino acids, calculated as (nitrogen collected in the first 40 ml eluates)/(amino acid nitrogen loaded on the ion retardation column) × 100 (%). Recoveries were measured in duplicate

Amino acid	% recovery ± SD
Valine	94.4 ± 0.1
Glycine	86.5 ± 0.3
Alanine	94.0 ± 1.6
Phenylalanine	96.8 ± 0.4
Isoleucine	91.1 ± 0.4
Serine	78.2 ± 2.8
Leucine	90.9 ± 0.6
Aspartic acid	97.8 ± 0.4
Glutamic acid	76.5 ± 1.5
Average	89.6 ± 0.9

in this fraction was removed during concentration and drying. In the mixture of phenylalanine and nitrate, phenylalanine recovery in the first 40 ml eluate was 89.9% and nitrate recovery in the eluate was 5.4%.

When a mixture of amino acids was loaded on the column, the recoveries of amino acids in the first 40 ml eluate were more than 76.5% (Table 2). The highest recovery was 97.8 ± 0.4% for aspartic acid. The average recovery was 89.6 4 ± 0.9%. The recoveries in this study are similar to or higher than those in the study of Bronk & Glibert (1991), who determined amino acid concentrations with HPLC. This loss may lead to some underestimation of the DON release. However, the relative extent of any underestimation is similar among samples. The ion retardation resin column method is therefore applicable to freshwater samples by adding NaCl, if we deal with relative releases.

TNR, RANR and NNA by *Microcystis novacekii* increased with time (see Fig. 3, as an example). TNR showed a linear increase; at 3 h TNR was 0.07 µg-at. N µg-at. N^{-1} and at 9 h TNR was 0.14 µg-at. N µg-at. N^{-1}. RANR increased similarly, although RANR was marginally lower. This shows that almost all the TNR was due to RANR and that little nitrogen which had been assimilated before the incubation was released. NNA increased linearly for 3 h, although after 3 h it gradually levelled off. NNA at 3 h was 0.27 µg-at. N µg-at. N^{-1}. Twenty-six percent of GNA was released at 9 h.

Discussion. Separation of amino acids from inorganic nitrogen (ammonium or nitrate) (Fig. 2) and the percentage recoveries of the amino acids (Table 2) suggest that the ion retardation resin can be applied to freshwater samples with added NaCl (3.5%). However, when NaCl was not added to the freshwater samples, peaks of amino acids and inorganic nitrogen did not appear during the first elution of 100 ml. Thus, we could not use samples without the added NaCl. We examined only separation of amino acids as an example of DON from ammonium or nitrate, as carried out

by Bronk & Glibert (1991). It might, however, be necessary to further examine whether the ion retardation resin works similarly with DON of matter other than amino acids, such as oligopeptides in freshwater samples.

We added ^{15}N tracer at a final concentration of 90 µg-at. N l^{-1}. This concentration is higher than *in situ* concentrations of inorganic nitrogen (Dugdale & Goering 1967). This higher concentration was partly because the detection limit of the mass spectrometer (Anelva TE-150) was <1 µg-at. N for nitrogen and because we tried to measure release and uptake of nitrogen at a saturated level of nitrogen concentration. If a more sensitive mass spectrometer was used, concentrations of added tracers could be lowered to *in situ* levels.

The ion retardation resin column separated phenylalanine from nitrate more readily than from ammonium (Fig. 2). Similar results for marine samples were obtained by Bronk & Glibert (1991). Percentage recoveries of amino acids in this study were similar to those obtained by Bronk & Glibert (1991).

Cellular nitrogen-specific net nitrogen assimilation (NNA/cell-N) at 3 h (0.27 ± 0.02 µg-at. N µg-at. N^{-1}) by *Microcystis novacekii* was similar to inorganic nitrogen uptake by marine planktonic algae (Dugdale & Goering 1967) and similar to the inorganic nitrogen uptake value of 0.26 ± 0.02 µg-at. N µg-at. N^{-1} found for *M. novacekii* grown under nitrogen limitation (Watanabe & Miyazaki 1996). The percentage of TNR to NNA was 34 % at 9 h incubation (cf. Fig. 2). In northern Chesapeake Bay, the ratio of LMW DO^{15}N release to the ammonium uptake varied from 32 to 42 % (Bronk & Glibert 1991). This indicates that the TNR and NNA values obtained in this study are in the same range as DON release and nitrogen uptake in the literature.

There are several potential artifacts that should be considered in DON release studies (Bronk & Glibert 1991). We used glass fiber filters (Whatman GF/C) for filtration. Pore size (approximately 1 µm) of this filter may be larger than certain bacteria. If bacteria were present in samples, we cannot estimate the exact content of DON. In this case, glass fiber filters with a smaller pore size are necessary. We used glass fiber filters instead of nucleopore filters (Bronk & Glibert 1991), because our calculation method is based on the mass balance of DIN, DON and PON and it is necessary to determine accurately the nitrogen content of each fraction of the same sample. To minimize the release of matter from cells during the filtration process, we used gravity filtration instead of vacuum filtration. Kirchman et al. (1989), however, reported that release of dissolved free amines increases even when gravity filtration is used. In the present study, unlabeled nitrogen, which had been incorporated into the cell contents before the ^{15}N incubation, was not released, because the TNR was

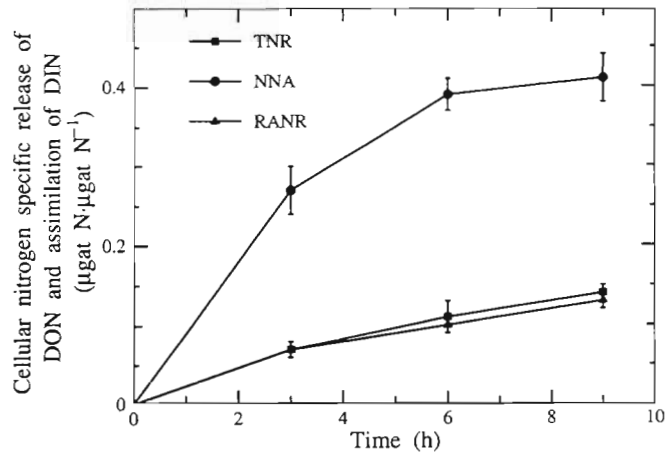

Fig. 3. *Microcystis novacekii*. Changes in NNA, TNR, RNR of batch-cultured cyanobacteria during a 9 h time course experiment

not significantly different from the RANR (Fig. 3). Only a low amount of DON was released by *Microcystis novacekii* grown in nitrogen-limited conditions (Nagao & Miyazaki unpubl.). Therefore, DON release might not be significantly influenced by gravity filtration. As TNR/cell-N was almost the same as NNR/cell-N (Fig. 3), other experimental procedures such as formalin fixation did not appear to have affected the release data, although Shimura et al. (1978) suggested that formalin fixation might affect releases of photosynthetic products by the marine cyanobacterium *Trichodesmium thiebautii*. There is also a possibility that phytoplankton take up DON during incubation and release experiments. Bronk & Glibert (1993a) suggested that newly produced DON is assimilated into phytoplankton. Collos et al. (1992) also observed DON uptake by the diatom *Synedra planctonica* in the dark. If phytoplankton take up DON, then total nitrogen release might be underestimated.

In *Microcystis novacekii*, total nitrogen release (TNR/cell-N) increased with increasing net nitrogen assimilation NNR(NNA/cell-N) (Fig. 3). This relationship between TNR/cell-N and NNA/cell-N is similar to that found in previous studies (e.g. Bronk & Glibert 1991, 1993b). In *M. novacekii*, almost all TNR/cell-N was composed of recently-assimilated nitrogen release (RANR/cell-N). This indicates that the bulk of nitrogen was released during the ^{15}N incubation and then suggests that the intracellular nitrogen pool was small or saturated. Giordano et al. (1994) suggested that DOC released from the green alga *Dunaliella salina* is composed mainly of recently fixed photosynthate. It seems that *M. novacekii* exhibits similar DON release characteristics to *D. salina*.

There may be differences in the release patterns of DON among functionally and ecologically distinct

phytoplankton. DON release by *Microcystis novacekii* originated from recently assimilated nitrogen during the 9 h incubation period in this study. However, in *Scenedesmus quadricauda*, released DON originates from nitrogen assimilated before the ^{15}N incubation (Nagao & Miyazaki unpubl.). Using the method described here, we can examine any differences in release patterns between freshwater planktonic algae.

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