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Estimation of dissolved organic nitrogen release by micrograzers in natural planktonic assemblages

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Abstract: We propose a new approach which combines ^{15}N tracer experiments with the dilution method to evaluate the role of micrograzers on dissolved organic nitrogen (DON) release in coastal planktonic assemblages. To observe possible $DO^{15}N$ release by micrograzers, planktonic assemblages (<94 μm) were incubated with $^{15}NH_4^+$ for 2 or 3 d, before short time (6 or 12 h) dilution experiments. In all dilution experiments from March to November, concentrations of $DO^{15}N$ increased in undiluted seawater, while concentrations decreased gradually with dilution. Based on a simple model calculation, the importance of micrograzers in $DO^{15}N$ release was confirmed statistically in all cases. Release and consumption of $DO^{15}N$ were well coupled, i.e., 58 to 103% of released $DO^{15}N$ were consumed by bacteria. On the average, release rates of $DO^{15}N$ were comparable to 59% of the NH_4^+ regeneration rates obtained in same period, suggesting that DON release by micrograzers is important in the nitrogen cycle in coastal ecosystems.

Key words: DON, micrograzers, the dilution method, ^{15}N tracer, natural assemblage

Introduction

Dissolved organic nitrogen (DON) is the largest pool of organic nitrogen in seawater (Sharp 1983) and some observations suggest that DON is potentially an important nitrogen source for bacteria and phytoplankton (Jackson & Williams 1985; Tupas & Koike 1991; Bronk & Glibert 1993a). However, in natural waters due to a high background concentration of DON and to the existence of consumption processes, it is difficult to evaluate DON release by changes in DON concentration. Recently, reliable methods enabling the determination of the ^{15}N content of DON have been developed and because of their high sensitivity, they have revealed information on DON release and/or uptake in natural planktonic communities (Axler & Reuter 1986; Bronk & Gilbert 1991, 1993a, b; Slawyk & Raimbault 1995).

Micrograzers are ubiquitous in marine environments and their importance in marine food webs has been well recognized (Azam et al. 1983). Laboratory studies have indicated that micrograzers release the organic nitrogen of their prey as DON during egestion and excretion (Andersson et al. 1985; Nagata & Kirchman 1991) and their importance in dissolved organic matter (DOM) fluxes in marine environments has been discussed theoretically (Jumars et al. 1989). However, the contribution of micrograzers to total DON release in a natural planktonic community has never been evaluated, because the organisms they prey upon (i.e. phytoplankton) are also known to release DON (Myklestad et al. 1989; Biddanda & Benner 1997).

In this paper, in order to estimate DON release by micrograzers, we applied the ^{15}N tracer technique with the dilution method (Landry & Hassett 1982) for coastal planktonic assemblages. From the observed patterns of changing $DO^{15}N$ concentrations in a series of dilution experiments, we have endeavored to identify and evaluate the role of organisms which contribute to $DO^{15}N$ release. Based on a simple model calculation, we were able to prove the important role of micrograzers in $DO^{15}N$ release and the rapid consumption of that $DO^{15}N$ by the bacterial population.

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Materials and Methods

Dilution experiments

Surface seawater was sampled in a 20-liter carboys at a station (water depth, 13 meters; 43°01'N, 144°52'E) in Akkeshi Bay, eastern coast of Hokkaido, Japan. Seawater was filtered with a 94-μm-mesh net and poured into a 20-liter carboy. After the addition of $^{15}NH_4^+$ (99.0 atom % ^{15}N) to a concentration of about 0.5 μM, seawater was pre-incubated for 2 or 3 d on a 15 : 9 L/D cycle starting at the middle of the light period in order to label particulate organic matter (POM) with ^{15}N. All incubation temperatures were maintained by running seawater which was 2 to 3°C higher than the ambient temperature.

A portion of this pre-incubated seawater was filtered through a GF/F filter and this filtered seawater was referred as 0% seawater. $^{14}NH_4^+$ (final conc. ca. 8 μmol l^{-1}) and PO_4^{3-} (final conc. ca. 1 μmol l^{-1}) were added to the remaining aliquot of pre-incubated unfiltered seawater, and also to 0% seawater. This unfiltered seawater was mixed with 0% seawater in the ratios 1.0 (100% unfiltered seawater), 0.75 (75%), 0.5 (50%), and 0.25 (25%). Diluted seawater at each dilution ratio (0.25 to 1) was poured into 1-liter polycarbonate bottles. Duplicate bottles were used for each dilution. Samples were incubated in the dark for 12 h (except for August, 6 h), with gentle mixing (0.5- to 2-h interval) to minimize the sedimentation of particles.

At time zero of the dilution experiment, unfiltered seawater (100%) was filtered through a GF/F filter (47 mm). A portion of this filtrate and 0% seawater were further filtered through a 0.2-μm Nuclepore filter (47 mm). These filtrates were frozen for later analysis of nutrients, DON concentration, and the nitrogen isotopic ratios of NH_4^+ and DON. The GF/F filter with POM was frozen until analysis of its carbon and nitrogen contents and nitrogen isotopic ratio. For chlorophyll-*a* (Chl-*a*) analysis, a subsample was filtered through a GF/F filter (25 mm) and the filter was stored frozen. For a bacterial count, a subsample (10 ml) was fixed with buffered formalin (final conc. 1%) and stored at 4°C. Values for POM at time zero for each dilution (0.75, 0.5 and 0.25) were calculated from the analyzed value for 100% seawater. The concentrations of, and ^{15}N atom% for DON at time zero for each dilution were also calculated from the values for 100% and 0% seawater.

At the end of the experiment, identical sampling procedures as were carried out for 100% seawater at time zero were followed for each incubation bottle. All GF/F filters were pre-combusted at 450°C for 3 h and all bottles were acid washed and rinsed thoroughly with distilled water prior to use. When GF/F filters were used, filtration was done at <50 mm Hg to minimize the disruption of cells.

Chemical, isotopic, Chl-*a* and bacterial analyses

NH_4^+ and PO_4^{3-} concentrations were determined with a Technicon autoanalyzer (Strickland & Parsons 1972). DON concentrations were measured by the wet oxidation method of Solórzano & Sharp (1980).

To prepare the sample for nitrogen isotopic measurements of NH_4^+, we applied the conventional steam distillation method of Bremner & Keeney (1965). NH_4^+ from a 15-ml sample with a 1.5-μmol NH_4^+ spike as the carrier, was distilled and recovered in 5 ml of 0.01 N H_2SO_4. The recovered NH_4^+ was concentrated to a final volume of <1 ml by a rotary evaporater and the concentrate was absorbed to a GF/F filter (25 mm) and dried at 100°C for ^{15}N analysis.

Samples to be used for the determination of the nitrogen isotopic ratio of DON were prepared by the method described by Slawyk & Raimbault (1995) with modifications after Hasegawa et al. (2000). In short, the dissolved inorganic nitrogen in each seawater sample was reduced to NH_4^+ and removed under alkaline conditions (pH 9.5) using vacuum evaporation (50°C). After removal, DON was digested to NO_3^- and then was reduced to NH_4^+. This NH_4^+ was recovered into H_2SO_4 soaked in GF/F filter (25 mm).

The nitrogen isotopic ratio of DON, particulate organic nitrogen (PON), and NH_4^+, and the organic carbon and nitrogen contents of POM were analyzed using a continuous flow mass spectrometer (Tracermass, Europa Scientific) equipped with a CN analyzer (Roboprep–CN, Europa Scientific) (Kanda et al.1998). POM on the GF/F filter was acidified with HCl vapor to remove carbonate before analysis. Sodium L-glutamate monohydrate (0.366 atom% ^{15}N) and glycine (1.66 to 4.51 atom% ^{15}N) were used as the standard samples. Standard deviation for atom% of L-glutamate monohydrate (1 μmol N) is smaller than 0.0012 (n=6). Chlorophyll-*a* was determined by the fluorometric method of Strickland & Parsons (1972) as modified by Suzuki & Ishimaru (1990), using a Turner Designs fluorometer. Bacteria were counted by epifluorescence microscopy after DAPI staining (Porter & Feig 1980).

The dilution method and evaluation of contribution to DO^{15}N release

In the dilution method, we assume that the growth of phytoplankton is constant with enough nutrients (N and P) available and that ingestion by micrograzers is proportional to the frequency of encounter between micrograzers and phytoplankton. The biomass of phytoplankton at time t (h) is expressed as follows,

$$P_t = P_0 e^{(\mu - Xg)t}, \quad (1)$$

where P_t and P_0 are the biomass of phytoplankton at times t and 0, respectively. X is the dilution factor. The growth rate of phytoplankton (μ) and the grazing coefficient: (g) were estimated by dilution theory (Landry & Hassett 1982).

In this study, we pre-labeled phytoplankton with $^{15}NH_4^+$ for 2 to 3 d. If micrograzers release a part of their prey-nitrogen as DO^{15}N, adequate ^{15}N labeling of the phytoplankton will increase the sensitivity of the detection of this ef-

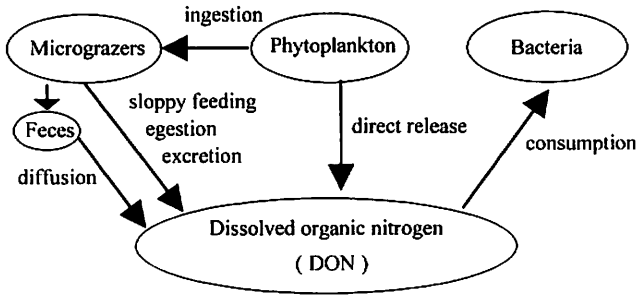

Fig. 1. Schematic representation of the organic nitrogen flux in the upper water column.

fect. On the other hand, if $DO^{15}N$ is directly released by phytoplankton, the source can be considered to be low molecular weight organic compounds in the cells (Bronk & Gilbert 1991). Kanda & Hattori (1988) showed that NH_4^+ that is taken up by phytoplankton starts to be incorporated into higher molecular weight compounds within 30 min over a wide range of species and growth phases. This suggests that during our pre-incubation period (2 or 3 d), most of the ^{15}N would be incorporated into high molecular weight compounds. Furthermore, due to the addition of excess $^{14}NH_4^+$ at the beginning of the experiment, most of the low molecular weight compounds within phytoplankton cells probably consists of cold nitrogen. Thus, the effect of direct $DO^{15}N$ release by phytoplankton is minimized in our experiment.

Although our experiment was designed to minimize the effect of $DO^{15}N$ release by phytoplankton, the relative contributions to $DO^{15}N$ release by the micrograzers and phytoplankton were tested statistically. Statistical protocols basically followed those described by Andersen et al. (1991) for the estimation of N and P sources contributing to phytoplankton growth.

The flow of organic nitrogen is schematically shown in Fig. 1. We identify phytoplankton as the main source of released $DO^{15}N$ because most of $^{15}NH_4^+$ were taken up by phytoplankton (Hasegawa, unpublished data). We assume two main pathways which contribute to $DO^{15}N$ release in a community of small organisms (<94 μm). One is the direct $DO^{15}N$ release by phytoplankton (Myklestad et al. 1989; Biddanda & Benner 1997) and the other is the $DO^{15}N$ release that is mediated by micrograzers (Andersson et al. 1985; Nagata & Kirchman 1991).

Another assumption we made is that the released $DO^{15}N$ was consumed only by bacteria. Some studies report that phytoplankton use DON but other than for urea, DON utilization by phytoplankton is generally considered to be negligible (Antia et al. 1991). Furthermore, we added an excess of NH_4^+ at the beginning of the dilution experiment. From these facts, $DO^{15}N$ consumption by phytoplankton will be of only minor importance in our experiment.

The rate of $DO^{15}N$ concentration change, $\Delta DO^{15}N$, can be described as follows,

$$\Delta DO^{15}N=\Delta DO^{15}N_{micro}+\Delta DO^{15}N_{phyto}-\Delta DO^{15}N_{bact} \quad (2)$$

where $\Delta DO^{15}N_{micro}$ and $\Delta DO^{15}N_{phyto}$ are the release rates of $DO^{15}N$ by micrograzers and phytoplankton, respectively, and $\Delta DO^{15}N_{bact}$ is the consumption rate of $DO^{15}N$ by bacteria.

Possible mechanisms for $DO^{15}N$ release mediated by micrograzers (e.g. protozoa, copepod nauplii) include sloppy feeding, egestion, excretion and diffusion from feces. Although quantitative evaluations of each process have not been possible, we assume that $DO^{15}N$ release by micrograzers is approximately proportional to their ingestion rate, because all mechanisms mentioned above are functions of ingestion rate.

In the dilution method, the biomass of micrograzers and phytoplankton changes proportionally to X. Thus the frequency of encounters between micrograzers and phytoplankton, that is, the ingestion rate of the micrograzer population, is proportional to X^2. $\Delta DO^{15}N_{micro}$ can be described as follows,

$$\Delta DO^{15}N_{micro}=a\times X^2, \quad (3)$$

where a is the $DO^{15}N$ release rate (N nmol l^{-1} h^{-1}) mediated by the micrograzer population in undiluted seawater.

If phytoplankton release $DO^{15}N$ directly, the release rate will be proportional to the amount of source $DO^{15}N$ within the phytoplankton community. Thus, $\Delta DO^{15}N_{phyto}$ can be described as follows,

$$\Delta DO^{15}N_{phyto}=b\times X, \quad (4)$$

where b is the $DO^{15}N$ release rate (N nmol l^{-1} h^{-1}) by phytoplankton in undiluted seawater.

$DO^{15}N$ consumption by bacteria is probably related to their cell numbers. In our experiments, a significant proportion of the bacteria passed through the GF/F filter and entered into 0% seawater. These bacteria accounted for from 6.3 to 36% of the total cell number and these values differed in each experiment. Thus we used the average cell number of bacteria for the incubation period substituted for X. Average cell number was estimated from cell numbers at the beginning and end of incubation and from the assumption that bacteria grew exponentially.

$$\Delta DO^{15}N_{bact}=c\times B, \quad (5)$$

where c is the $DO^{15}N$ consumption rate (N nmol l^{-1} h^{-1}) by bacteria in undiluted seawater. B is the average bacterial cell number for the incubation period expressed as a ratio against the maximal average bacterial cell number (i.e. cell numbers in 100% seawater) for the same experimental series.

Substituting Eqs 3, 4, 5 into Eq. 2, we can relate $\Delta DO^{15}N$ to X and B with the coefficients a, b, and c which reflect the contribution to $DO^{15}N$ flux by micrograzers, phytoplankton and bacteria, respectively.

$$\Delta DO^{15}N=aX^2+bX-cB. \quad (6)$$

Table 1. Duration of pre-incubation (days), concentrations of chlorophyll *a* (Chl *a*: $\mu g\ l^{-1}$), particulate organic nitrogen (PON: $\mu mol\ l^{-1}$), and particulate organic carbon (POC: $\mu mol\ l^{-1}$), and the C/N ratio ($\mu mol/\mu mol$) and PON/Chl *a* ratio ($\mu mol/\mu g$) of particulate organic matter before and after the pre-incubation.

Date	Duration		Chl *a*	PON	POC	C/N	PON/Chl *a*
12 March	3	before	12	4.8	49	10	0.40
		after	13	5.7	63	11	0.44
28 May	3	before	2.1	3.0	23	7.8	1.5
		after	3.9	3.3	31	9.2	0.85
2 July	3	before	3.8	4.2	31	7.4	1.1
		after	5.5	4.1	38	9.4	0.72
27 Aug.	3	before	3.6	3.0	67	22	0.85
		after	3.5	3.8	75	20	1.1
12 Nov.	2	before	2.6	2.6	31	12	1.0
		after	3.9	3.1	36	12	0.78

Based on the above rationalization, we estimated the coefficients, *a*, *b* and *c* with a multiple linear regression (Andersen et al. 1991). To avoid collinearity between X^2 and X, both sides of Eq. 6 were divided by X.

$$\Delta DO^{15}N/X = aX + b - cB/X. \quad (7)$$

The regression analysis used X and $1/X$ as the independent variables and $\Delta DO^{15}N/X$ as the dependent variable.

Results

Change in particulate matter during pre-incubation

Seasonal changes in Chl-*a* concentrations (values before pre-incubation) varied by a factor of six, but those for PON and particulate organic carbon (POC) were about two- and threefold, respectively (Table 1). Concentrations of Chl-*a*, PON and POC increased during the pre-incubation period with few exceptions (Table 1). C/N ratios of particulate matter after pre-incubation varied from 9.2 to 20, and only in the August experiment, did they decrease. Low values (0.44 to 1.1) for PON/Chl-*a* ($\mu mol/\mu g$) at the end of the pre-incubation period indicated that PON consisted mainly of phytoplankton (McCarthy & Nevins 1986).

The growth rates of phytoplankton and grazing coefficients

At the end of incubation, concentrations of NH_4^+ and PO_4^{3-} were comparable to 77 and 95% of initial values, respectively, even in the August experiment, indicating that growth of phytoplankton was not limited by these nutrients. Except for May and July, apparent growth rates, determined by the change in Chl-*a* concentration, increased linearly with decreases in the dilution factor (Fig 2). For these cases, estimated grazing coefficients ranged from 0.009 to $0.052\ h^{-1}$. When we use changes in PON concentrations instead of Chl-*a* for the calculation, the apparent growth rates increased linearly with decreases in the dilution factor for all experiments except March and August (Fig 3). Calculated grazing coefficients varied over the range of 0.021 to $0.037\ h^{-1}$. A significant grazing coefficient was obtained in all dilution experiments for at least one of the methods of estimation (Chl-*a* and PON).

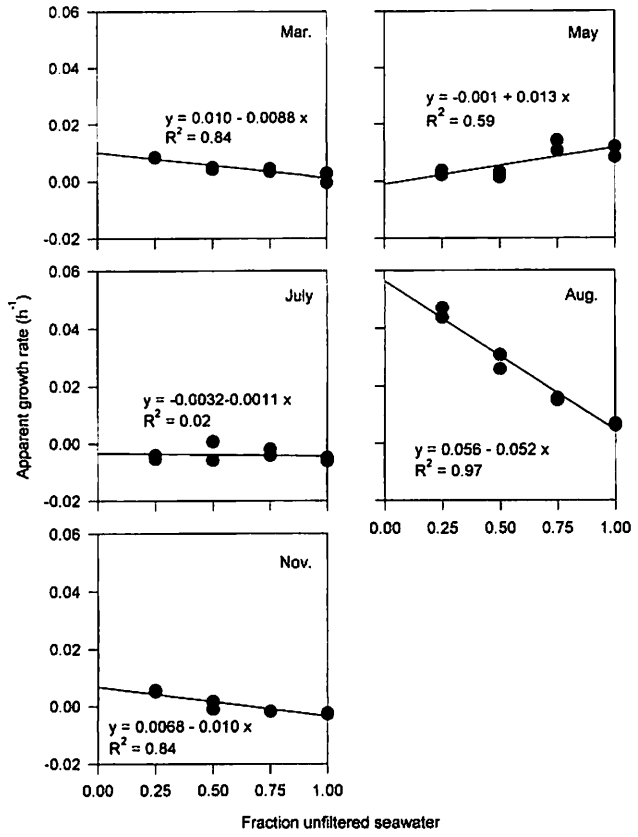

Fig. 2. Apparent growth rates determined by the change in Chl-*a* concentrations as a function of the dilution factor in the experiments. The straight lines are estimated by least squares fits after Landry & Hassett (1982).

Contribution to $DO^{15}N$ metabolism

Changing rates of $DO^{15}N$ concentration for each dilution

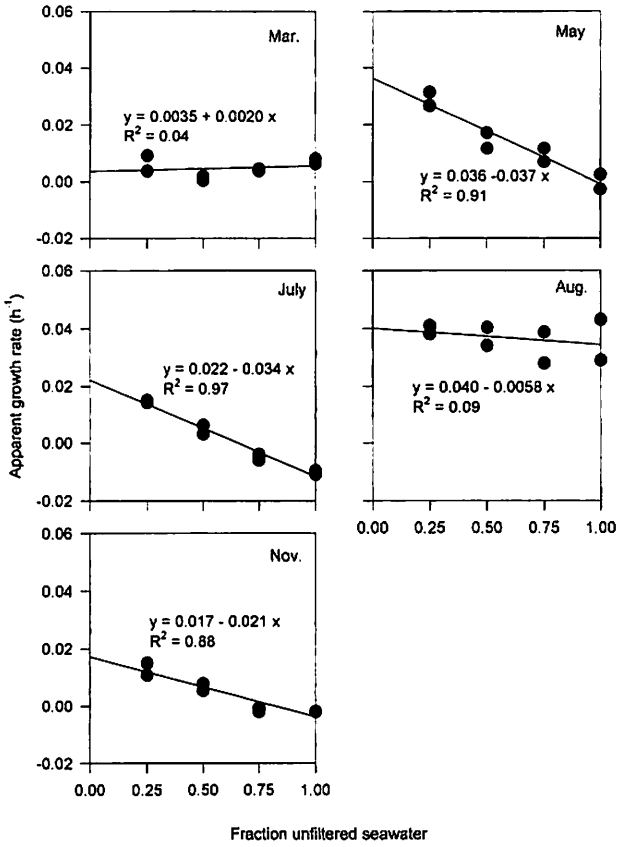

Fig. 3. Apparent growth rates determined by the change in PON concentrations as a function of the dilution factor in the experiments. The straight lines are estimated by least squares fits after Landry & Hassett (1982).

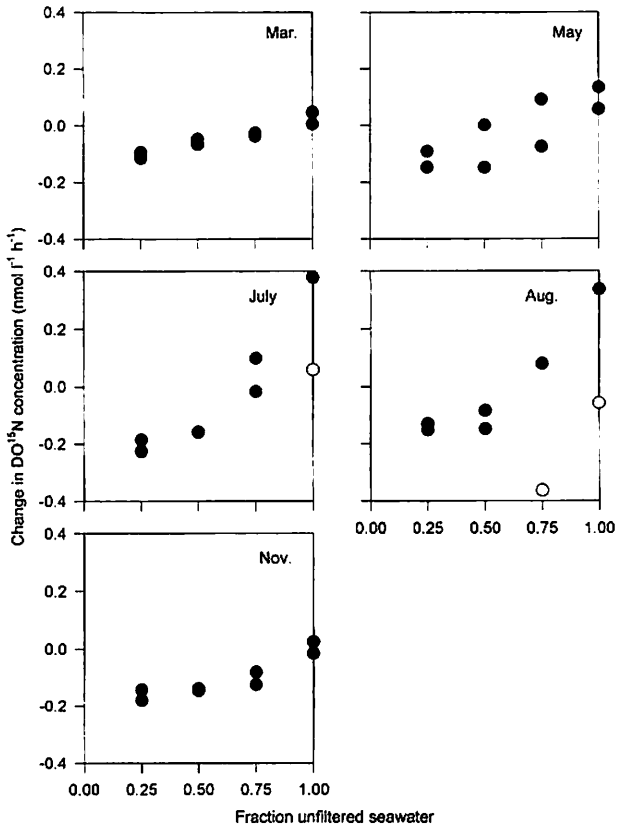

Fig. 4. Changing rates of $DO^{15}N$ concentration in the dilution experiments. Open symbols on July and August were not included in the calculation (see text).

experiment are shown in Fig. 4. In undiluted seawater, the $DO^{15}N$ concentration always increased and changing rates were in the range of 0.005 to 0.22 nmol l^{-1} h^{-1} (Fig. 4). These positive values indicate that the release of $DO^{15}N$ generally exceeded the consumption of $DO^{15}N$ in the undiluted seawater, irrespective of the season. With increasing dilution, consumption of $DO^{15}N$ gradually began to exceed release and net changes in $DO^{15}N$ concentration reached minimum values of −0.10 to −0.21 nmol l^{-1} h^{-1} at a dilution factor of 0.25 (Fig. 4). This suggests that dilution led to decreases in the release rate of $DO^{15}N$ rather than in the consumption rate of $DO^{15}N$.

Contribution to $DO^{15}N$ metabolism by each biological component was estimated with Eq. 7 by a forward stepwise multiple regression (F=3.6 to enter the regression). Coefficients entered into the final regression are listed in Table 2. In all cases, the coefficient for release that was retained was the coefficient for micrograzers (a), suggesting an important role for micrograzers in $DO^{15}N$ release. Percentage ratio of bacterial $DO^{15}N$ consumption to rate of release by micrograzers ranged from 58 to 103%. These values imply close coupling between DON release and consumption in the planktonic assemblages observed.

Discussion

Laboratory experiments have demonstrated that micro-

Table 2. Coefficients of $DO^{15}N$ metabolism (±SD) for grazing (a), phytoplankton (b) and bacteria (c) obtained from Equation 7.

Date	a (nmol l^{-1} h^{-1})	b (nmol l^{-1} h^{-1})	c (nmol l^{-1} h^{-1})	r^2 (n)
12 March	0.46±0.074	—[a]	0.41±0.049	0.88(8)
28 May	0.41±0.11	—	0.29±0.056	0.77(8)
2 July	0.83±0.068	—	0.48±0.027	0.98(7)
27 Aug.	0.77±0.099	—	0.48±0.047	0.95(6)
12 Nov.	0.50±0.093	—	0.52±0.056	0.93(8)

[a] Not entered into the final regression.

grazers can release a significant amount of their prey's organic matter as DOM (Nagata & Kirchman 1991; Strom et al. 1997). Because grazers and prey organisms that were used in these laboratory experiments are limited to a few species, extrapolation of these data into field situations must be done with caution. However, in natural communities, DOM release by micrograzers has never been evaluated, since it is difficult to separate micrograzers from phytoplankton, which are the other potential organisms involved in DON release. Even if separation could be achieved, it would result in a break in the relationship between prey and predator, meaning that it would not reflect natural conditions.

Bronk & Gilbert (1991, 1993b) used the molecular weight of $DO^{15}N$ as an indicator for the release mechanisms. High molecular weight compounds ($>10^4$ Daltons) are mainly released by micrograzers and a dominance of low molecular weight $DO^{15}N$ means that direct release by phytoplankton is the main source. However, as micrograzers also release low molecular weight compounds (e.g. free amino acids), evaluation of the contributions though each mechanism is confused (Andersson et al. 1985; Nagata & Kirchman 1991).

To evaluate DON release by micrograzers in natural planktonic assemblages, we attempted a new approach. In our method, to make $DO^{15}N$ release by micrograzers more resolvable and to minimize the effect of direct $DO^{15}N$ release by phytoplankton, we combined pre-incubation with ^{15}N and excess cold NH_4^+ addition before the dilution experiments. As a result of these procedures, stepwise multiple regression revealed the important role of micrograzers on DON release in all cases (Table 2). Applying stepwise multiple regression analysis to our data, entering b into the final regression was rejected. However, this dose not mean that phytoplankton have no contribution towards $DO^{15}N$ release.

The dilution method was originally developed to estimate the grazing impact of micrograzers on the growth of phytoplankton under an excess of N and P nutrients (Landry & Hassett 1982). In this method, whereas the biomass of each organism is diluted in proportion to the dilution factor X, ingestion by the micrograzer population decreases proportionally to X^2. Accordingly, the balance between the source of $DO^{15}N$ (ingestion by micrograzers: X^2) and the $DO^{15}N$ sink (bacteria: X) was lost with the dilution, as observed in Fig. 4. Thus, we were able to evaluate release by micrograzers and consumption by bacteria from the net change in $DO^{15}N$ concentrations throughout the dilution gradient (Table 2).

It is not clear why we failed to detect significant grazing effects on the growth of phytoplankton through changes in Chl-*a* concentrations (May and July) or in PON concentrations (March and August). We added excess $^{14}NH_4^+$ to minimize the effect of direct $DO^{15}N$ release by phytoplankton (see above). However, due to $^{14}NH_4^+$ uptake by phytoplankton, longer incubation times should result in higher ^{15}N isotope dilution in phytoplanktonic nitrogen. This would reduce the sensitivity for evaluating $DO^{15}N$ release due to grazing. We therefore minimized the incubation time (6 to 12 h) and incubated under dark conditions when micrograzers actively feed (Litaker et al. 1988; Wheeler et al. 1989). In the previous application of the dilution method, the incubation period was over 24 h (Landry & Hassett 1982). Paranjape (1987) reported that he failed to obtain significant values for the grazing coefficient with a 24-h incubation, but succeeded after a further 24 h of incubation. This result suggests that our 6- to 12-h incubation time might not be long enough to estimate μ and g from the change in Chl-*a* and/or PON concentrations. Because we detected grazing on phytoplankton with at least one of the two evaluative methods (Chl-*a* and PON) that were consistent with the dilution theory, we judged that ingestion by grazers was proportional to X^2.

We assumed that ingestion by micrograzers is proportional to the square of the dilution factor (X^2) after Landry & Hassett (1982). Gallegos (1989) showed a saturation effect for ingestion and recommended nonlinear regression to estimate the grazing coefficient. If the above consideration is true for our experiment, the contribution of micrograzers to $DO^{15}N$ release would be indistinguishable from that due to release by phytoplankton, because the specific grazing rate would be almost constant toward the undiluted seawater and ingestion by micrograzers would start to be proportional to X. Bacteria are also known to release DON through the decomposition of PON and this is thought to be due mostly to bacteria attached to particles (Smith et al. 1992). If this was the case, we may overestimate $DO^{15}N$ release by phytoplankton because these attached bacteria should be retained on the GF/F filter and their concentrations would vary in direct proportion to the dilution factor X (i.e. same as for phytoplankton). In spite of these biases, which underestimate the contribution to DON release by micrograzers or overestimate that by phytoplankton, the importance of micrograzers to DON release was confirmed statistically in all cases. This implies that nitrogen, incorporated into phytoplankton and synthesized into higher molecular weight compounds, is not easily released without grazing effects.

When ingestion by micrograzers decreases proportionally to X^2, one might expect that lower levels of ingestion by grazers would reduce the amount of DON release through improvements their assimilation efficiencies. If this was the case, the pattern of $DO^{15}N$ release by micrograzers with the dilution gradient might approach the cubic or higher powers of dilution factors. In such a situation, considerations of the assimilation efficiency with lower and higher levels of ingestion by micrograzers would be necessary in order to better analyze $DO^{15}N$ release.

$DO^{15}N$ concentration and composition might affect bacterial $DO^{15}N$ availability. $DO^{15}N$ concentration at the end of experiment was as much as 65% of that at the beginning even in the most decreased case (November experiment at a

Table 3. Average ^{15}N atom% for PON during the incubation period and rates for the release and consumption of DON (nmol h^{-1}) in the surface seawaters of Akkeshi Bay.

	12 March	28 May	2 July	27 Aug.	12 Nov.
^{15}N atom% in PON[a]	5.99	6.88	5.66	6.52	3.47
release[b]	7.7	6.0	15	12	15
uptake[b]	6.9	4.3	8.5	7.4	15

[a] Calculated by the assumption that ^{15}N atom% in PON changed exponentially.
[b] Calculated by the assumption that ^{15}N atom% of released and consumed DON were the same as that of PON.

dilution factor of 0.25), and we considered the effect of $DO^{15}N$ concentration on its availability to bacteria to be minimal. The refractory nature of some of the components of $DO^{15}N$ might restrict $DO^{15}N$ availability. However, as the $DO^{15}N$ in our experiment was freshly released into seawater (maximum 3-d old), we judged the major proportion of $DO^{15}N$ to be labile. Since $DO^{15}N$ concentration increased in the undiluted seawater, $DO^{15}N$ was obviously released within the incubation period for all the experiments (Fig. 4). These facts allow us to discount possible problems associated with $DO^{15}N$ availability.

If we assume that the ^{15}N atom% of DON released is the same as that for PON (Table 3), we can calculate total DON release rates due to micrograzers from the $DO^{15}N$ release rate (*a*: Table 2) and the ^{15}N atom% for DON (Dugdale & Goering 1967). These rates ranged from 6.0 to 15 nmol l^{-1} h^{-1}. The total DON consumption rates by bacteria ranged from 4.3 to 15 nmol l^{-1} h^{-1} (Table 3). In the same period in Akkeshi Bay, the ammonium regeneration rates estimated by the ^{15}N isotope dilution method (Blackburn 1979; Caperon et al. 1979) varied from 5.7 to 34 nmol l^{-1} h^{-1} (Hasegawa, unpublished data). On average, DON release rates accounted for 59% of NH_4^+ regeneration rates, implying that DON release by micrograzers is an important pathway in the nitrogen cycle in coastal marine environments.

Acknowledgments

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Literature Cited

Andersen, T., A. K. L. Schartau & E. Paasche 1991. Quantifying external and internal nitrogen and phosphorus pools, as well as nitrogen and phosphorus supplied through remineralization, in coastal marine plankton by means of a dilution technique. *Mar. Ecol. Prog. Ser.* **69**: 67–80.

Andersson, A., C. Lee, F. Azam & Å. Hagström 1985. Releaes of aminoacids and inorganic nutrients by heterotrophic marine microflagellates. *Mar. Ecol. Prog. Ser.* **23**: 99–106.

Antia, N. J., P. J. Harrison & L. Oliveira 1991. The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. *Phycologia* **31**: 1–89.

Axler, R. P. & J. E. Reuter 1986. A simple method for estimating the ^{15}N content of dissolved organic matter ($DO^{15}N$) in N-cycling studies. *Can. J. Fish. Aquat. Sci.* **43**: 130–133.

Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil & F. Thingstad 1983. The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.* **10**: 257–263.

Biddanda, B. & R. Benner 1997. Carbon, nitrogen, and carbohydrate fluxes during the production of particulate and dissolved organic matter by marine phytoplankton. *Limnol. Oceanogr.* **42**: 506–518.

Blackburn, T. H. 1979. Method for measuring rates of NH_4^+ turnover in anoxic marine sediments, using a ^{15}N-NH_4^+ dilution technique. *Appl. Environ. Microbiol.* **37**: 760–765.

Bremner, J. M. & D. R. Keeney 1965. Steam distillation methods for determination of ammonium, nitrate and nitrite. *Anal. Chim. Acta* **32**: 485–495.

Bronk, D. A. & P. M. Gilbert 1991. A ^{15}N tracer method for the measurement of dissolved organic nitrogen release by phytoplankton. *Mar. Ecol. Prog. Ser.* **77**: 171–182.

Bronk, D. A. & P. M. Gilbert 1993a. Application of a ^{15}N tracer method to the study of dissolved organic nitrogen uptake during spring and summer in Chesapeake Bay. *Mar. Biol.* **115**: 501–508.

Bronk, D. A. & P. M. Gilbert 1993b. Contrasting patterns of dissolved organic nitrogen release by two size fractions of estuarine plankton during a period of rapid NH_4^+ consumption and NO_2^- production. *Mar. Ecol. Prog. Ser.* **96**: 291–299.

Caperon, J., D. Schell, J. Hirota & E. Laws 1979. Ammonium excretion rates in Kaneohe Bay, Hawaii, measured by a ^{15}N isotope dilution technique. *Mar. Biol.* **54**: 33–40.

Dugdale, R. C. & J. J. Goering 1967. Uptake of new and regenerated forms of nitrogen in primary productivity. *Limnol. Oceanogr.* **12**: 196–206

Gallegos, C. L. 1989. Microzooplankton grazing on phytoplankton in Rhode River, Maryland: nonlinear feeding kinetics. *Mar. Ecol. Prog. Ser.* **57**: 23–33.

Hasegawa, T., I. Koike & H. Mukai 2000. Dissolved organic nitrogen dynamics in coastal waters and the effect of copepods. *J. Exp. Mar. Biol. Ecol.* **244**: 219–238.

Jackson, G. A. & P. M. Williams 1985. Importance of dissolved organic nitrogen and phosphorus to biological nutrient cycling. *Deep-Sea Res.* **32**: 223–235.

Jumars, P. A., D. L. Penry, J. A. Baross, M. J. Perry & B. W. Frost 1989. Closing the microbial loop: dissolved carbon pathway to heterotrophic bacteria from incomplete ingestion, digestion and

absorption in animals. *Deep-Sea Res.* **36**: 483–495.

Kanda, J. & A. Hattori 1988. Ammonium uptake and synthesis of cellular nitrogenous macromolecules in phytoplankton. *Limnol. Oceanogr.* **33**: 1568–1579.

Kanda, J., K. Sada, I. Koike & K. Yokouchi 1998. Application of an automated carbon-nitrogen analyzer for elemental and isotopic analysis of samples retained on glass-fiber filters. *Intern. J. Environ. Anal. Chem.* **72**: 163–171.

Landry, M. R. & R. P. Hassett 1982. Estimating the grazing impact of marine micro-zooplankton. *Mar. Biol.* **67**: 283–288.

Litaker, W., C. S. Duke, B. E. Kenney & J. Ramus 1988. Diel chl a and phaeopigment cycles in a shallow tidal estuary: potential role of microzooplankton grazing. *Mar. Ecol. Prog. Ser.* **47**: 259–270.

McCarthy, J. J. & J. L. Nevins 1986. Sources of nitrogen for primary production in warm-core rings 79–E and 81–D. *Limnol. Oceanogr.* **31**: 690–700.

Myklestad, S., O. Holm-Hansen, K. M. Varum & B. E. Volcani 1989. Rate of release of extracellular amino acids and carbohydrates from the marine diatom *Chaetoceros affinis*. *J. Plankton Res.* **11**: 763–773.

Nagata, T. & D. C. Kirchman 1991. Release of dissolved free and combined amino acids by bactivorous marine flagellates. *Limnol. Oceanogr.* **36**: 433–443.

Paranjape, M. A. 1987. Grazing by microzooplankton in the eastern Canadian arctic in summer 1983. *Mar. Ecol. Prog. Ser.* **40**: 239–246.

Porter, K. G. & Y. S. Feig 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* **25**: 943–948.

Sharp, J. H. 1983. The distributions of inorganic nitrogen and dissolved and particulate organic nitrogen in the sea, p. 1–35. In *Nitrogen in the Marine Environment* (eds. Carpenter, E. J. & D. G. Capone). Academic Press, New York.

Slawyk, G. & P. Raimbault 1995. Simple procedure for simultaneous recovery of dissolved inorganic and organic nitrogen in ^{15}N-tracer experiments and improving the isotopic mass balance. *Mar. Ecol. Prog. Ser.* **124**: 289–299.

Smith, D. C., M. Simon, A. L. Alldredge & F. Azam 1992. Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. *Nature* **359**: 139–141.

Sólorzano, L. & J. H. Sharp 1980. Determination of total dissolved nitrogen in natural waters. *Limnol. Oceanogr.* **25**: 751–754.

Strickland, J. D. H. & T. R. Parsons 1972. *A Practical Handbook of Seawater Analysis, 2nd ed.* Fish. Res. Bd.Can. Bull.167, 310 pp.

Strom, S. L., R. Benner & M. J. Dagg 1997. Planktonic grazers are a potentially important source of marine dissolved organic carbon. *Limnol. Oceanogr.* **42**: 1364–1374.

Suzuki, R. & T. Ishimaru 1990. An improved method for the determination of phytoplankton chlorophyll using N, N-Dimethylformamide. *J. Oceanogr. Soc. Jpn* **46**: 143–194.

Tupas, L. & I. Koike 1991. Simultaneous uptake and regeneration of ammonium by mixed assemblages of heterotrophic marine bacteria. *Mar. Ecol. Prog. Ser.* **70**: 273–282.

Wheeler, P. A., D. L. Kirchman, M. R. Landry & S. A. Kokkinakis 1989. Diel periodictiy in ammonium uptake and regeneration in the oceanic subarctic Pacific: Implications for interactions in microbial food webs. *Limnol. Oceanogr.* **34**: 1025–1033.