Phytoplankton Uptake and Excretion of Assimilated Nitrate in a Small Canadian Shield Lake

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Received for publication 2 December 1977

Nitrate uptake in the epilimnetic waters of a small eutrophic Canadian Shield lake was studied by using a \(^{15}\)N method during summer stratification. Concurrent with inhibition of primary production, 3-(3,4-dichlorophenyl)-1,1-dimethyleurea inhibited NO\(_3^-\) assimilation. Nitrate up to 1 mg of N/liter did not affect the rate of primary production during 3 h of incubation. The NO\(_3^-\) fertilizer added to the lake weekly was consumed through algal assimilation in about 3 days. Excretion of the photoassimilated NO\(_3^-\) as dissolved organic nitrogen represented a significant portion of the nutrient incorporated by the cells. Only 40% of the NO\(_3^-\)\(^{15}\)N which disappeared could be accounted for in the particulate fraction. Although the rest was presumably excreted, only 15% of the \(^{15}\)N label was accounted for as cationic dissolved organic nitrogen by isotope assays. These excreted organic forms were predominantly serine and glycine in the dissolved free amino acid fraction. Bacteria as well as algae might be expected to contribute to and modify the extracellular nitrogen pool.

Lake 227 is one of the 46 lakes of the Experimental Lakes Area in northwestern Ontario (15) first selected for long-term eutrophication studies. The lake measures 5 hectares in surface area, 10 m at maximum depth, and has had its phosphorus and nitrogen budgets controlled. Before artificial nutrient loading, Lake 227 was oligotrophic by all standards. From 1969 to 1974 inclusive, the lake was enriched with nitrogen as NO\(_3^-\) and phosphorus as PO\(_4^{3-}\) (6.29 g of N/m\(^2\) per year and 0.48 g of P/m\(^2\) per year) during the ice-free periods (24, 26). The immediate effect can be described as experimental eutrophication, and, to date, Lake 227 remains eutrophic. The phytoplankton standing crop was near the theoretical maximum (26).

Ambient NO\(_3^-\) and PO\(_4^{3-}\) concentrations in Lake 227 remained low during summer stratification despite the artificial supply of these compounds, presumably due to phytoplankton consumption (26). Before the summer of 1975, when dissolved NO\(_3^-\) was distributed over the lake surface once a week, epilimnetic NO\(_3^-\) concentration was reduced to barely detectable levels (ca. 10 \(\mu\)g of N per liter), and NO\(_3^-\) was not found below 5 m 2 days after its addition (unpublished data). It was therefore essential to assess the contribution of NO\(_3^-\) uptake to the release and cycling of nitrogen in this eutrophic system. Nitrate uptake in the epilimnetic waters, which received nearly all of the fertilizers, had been observed to be much higher than that in the hypolimnion (unpublished data). The direct measurement of inorganic nitrogen uptake by \(^{15}\)N methodology was used in our investigation without precognition of extracellular excretion of assimilated NO\(_3^-\). The kinetic approach had been introduced by Dugdale and Goering (5) who, assuming that excretion of the assimilated nitrogen was insignificant, performed experiments of a few hours of duration.

The dependence of the rate of NO\(_3^-\) uptake on substrate concentration was studied in 1975 to characterize the algal population in Lake 227 with respect to NO\(_3^-\) assimilation. From these kinetic data the turnover time of NO\(_3^-\) (the time required for microbial uptake of the amount of NO\(_3^-\) equivalent to that supplied) was estimated. In addition, excretion of the photoassimilated NO\(_3^-\) as dissolved organic nitrogen (DON) was established and its significance was assessed. The extracellular DON was partially identified as dissolved free amino acids (DFAA) and combined amino acids.

MATERIALS AND METHODS

In the studies reported here, care was taken to clean all the apparatus and glassware by washing with dilute HCl and thorough rinsing with distilled-deionized water before use.

Sampling. Composite water samples were collected between 0800 and 1000 h from a depth of 1.25 to 1.75 m at the center station on Lake 227 by using an opaque 3-liter Van Dorn bottle. These were stored in the dark.
Nitrified uptake measurements. The "light-starved" (dark-incubated) bulk samples were dispensed into 125-ml Pyrex reagent bottles, some of which were modified to provide lightproof conditions. Each replicate was then treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or PO₄³⁻ according to the particular experimental design after receiving a portion of K¹⁵NO₃ (95.0 atom %, International Nuclear & Chemical Corp.). Controls contained, in addition, 20 mg of HgCl₂ per liter. Incubation was carried out in an illuminated water-bath incubator (7, 25) at 20°C ± 1°C and 6,285 J of illumination per m² per min. After incubation, particulate nitrogen was retained on grade 984H Reeve Angel glass-fiber filters (which had been preignited at 500°C for 20 h) by vacuum filtration (125 mm Hg) and then determined as N₂ by dry combustion (20). The ¹⁵N abundance of each particulate nitrogen sample was determined by emission spectrometry on a Statron NOI-5 N-15 analyzer by the procedure developed by Flett (R. J. Flett, Ph.D. thesis, University of Manitoba, Winnipeg, 1976). Enrichment in ¹⁵N of the particulate fraction over that of background was taken to measure NO₃⁻ incorporation. Colorimetric determinations of NO₃⁻ and NO₂⁻ were also performed on the incubated water samples according to the method of Sawicki and Scaringelli (21), and that of NH₃ was performed by the method of Stainton et al. (28). To study the effect of DCMU on the kinetics of NO₃⁻ uptake, primary production was measured simultaneously by the ¹⁴C technique (29), but acidification and bubbling (27), instead of filtration, were employed to determine carbon uptake.

Examination for extracellular products of nitrate assimilation. The problem of possible phytoplankton excretion of NO₃⁻ assimilation products was investigated by the following experimental design to obtain unequivocal evidence for the existence of such excretion, to identify the chemical forms of these excretion products, and to estimate the net excretion rate under experimental conditions. The procedures employed are summarized in Fig. 1 and described in detail below. At the time of the experiment, the phytoplankton in Lake 227 was predominantly made up of green algae (Spongiosisium and Scenedesmus) and small Chrysophycean species. The test experiment consisted of a 4-liter episodic water sample in an open cylindrical Pyrex jar. The sample was constantly mixed by means of a Teflon-coated stirring magnet driven by a motor-stirrer at a moderate rate and kept illuminated by two Sylvania Gro-Lux lamps installed 15 cm directly above. The fluorescent light source was enriched in the 435- and 650-nm regions, which together made up 35% of the total photo output. Its intensity was not measured but was lower (6,285 J/m² per min) than that used in the NO₃⁻ uptake experiments. At zero time, the sample was enriched with 100 µg of K²¹⁵NO₃-N (95.0 atom %) per liter. During the 4-h incubation period at 22°C, the water temperature never rose more than 3°C, i.e., between 22 and 25°C. Control experiments also set up in parallel consisted of K²¹⁵NO₃-enriched or DCMU- or HgCl₂-treated water samples similarly illuminated and a K²¹⁵NO₃-enriched sample incubated in darkness. At the termination of incubation, subsamples were withdrawn for ¹⁵NO₃⁻ uptake measurements in the manner described above before the entire contents of each jar were continually fed to a Heraeus-Christ Junior 15000 centrifuge (ca. 100 ml/min) to remove the large particulate matter at about 12,000 × g. All samples were centrifuged within 0.5 h of the end of the incubation time, and those awaiting centrifugation were kept under diffused light conditions to minimize continued photosynthetic activity. After centrifugation, 2.5 liters of each sample of the supernatant was consecutively filtered on preignited Whatman GF/C filters and membrane filters (Millipore Corp. HA, 0.45 µm). The filtration pressure was maintained at 125 mm of Hg.

Samples to be analyzed for amino acids and peptides, the suspected organic forms of excreted nitrogen, were first prepared from the filtrates by concentration on a Brinkman Rotovap at 55°C to 1/100 of their original volumes. Fractions of the concentrates were used for peptide detection (13), protein estimation (17), and cation-exchange chromatography to isolate DON.

Two resin columns, modified from 10-cm³ glass syringes and packed with Bio-Rad 50W-X12 (100 to 200 mesh, H form), were employed for each sample. Before the sample (concentrated filtrate) was applied to the first column, its pH was adjusted to about 2.0 with 0.1 HCl. After adsorption, the sample was washed with 40 ml of 0.1 N HCl, and the washing was applied to the second column. Each of the two columns was then washed with 40 ml of deionized water. Cations thus bound were then eluted with 1 M NH₄OH; a 25-ml portion of eluate was collected from each column and then pooled. Each pool sample was estimated for Lowry protein and divided into appropriate fractions according to protein content. These fractions were reduced to about 1-ml samples by rotary evaporation at 36°C on a Brinkmann Evapo-Mix before amino acid analyses and ¹⁵N determination were performed.

The efficacy of the chromatographic procedure was tested by the separate application of known quantities of L-tyrosine, bovine serum albumin, calf-thymus deoxyribonucleic acid, and yeast ribonucleic acid (Sigma Chemical Co.) to the cation-exchange columns as described above. In each case, the test amino acid, protein, and nucleic acids were bound to the resin. Furthermore, recovery of these standards was close to 100% when the first 25 ml of eluate from each column was collected and assayed by absorption measurement at 260 or 280 nm.

To remove the ammonia contained in the column eluates, these were first evaporated to dryness at 110°C and stored until amino acid analysis. The amino acid composition of acid-hydrolyzed and unhydrolyzed duplicate samples was determined by a Beckman 121 analyzer according to the method of Dexter and Dronzek (4).

Fractions from the column eluates allocated for N isotope ratio determination were loaded onto preignited glass-fiber filters in a manner similar to that for the examination of glass-fiber filtrates. Traces of ammonia on the desiccated filters left from the column...
elution procedure were removed when the filters were heated and evacuated in the sample preparation unit before $^{15}$N analysis. The amount of amino acids and DON and their $^{15}$N content were computed by knowing the original water filtrate volume represented by the sample analyzed.

**RESULTS**

**Nitrate uptake kinetics.** At an enrichment of 50 $\mu$g of NO$_3^-$-N per liter, uptake was linear with time during 8 h of incubation on 23 August 1975. No lag period was observed. Nitrate uptake rates in the light with 1 and 10 $\mu$M DCMU and in the dark were in the ratio of 8:2:1. The fractional uptake rate of NO$_3^-$, $V_{NO_3^-}$, was 0.005 $\mu$g of N/(microgram of total N) per h, or simply 0.005 h$^{-1}$, under light saturation. Dark uptake of NO$_3^-$ was small compared with that in the light.

By using enrichment levels up to 500 $\mu$g of NO$_3^-$-N per liter on 5 September 1975, the light data displayed the Michaelis-Menten hyperbolic relationship between the rate of uptake and
substrate concentration (Fig. 2) and yielded the half-saturation transport constant, $K_t$, and the maximum fractional uptake rate, $V_{\text{max}}$, by the Lineweaver-Burk, or double-reciprocal, plot (Fig. 3). These kinetic constants were determined to be about 73.6 $\mu$g of N per liter (or 5.2 $\mu$M) and 0.006 h$^{-1}$, respectively. Although not shown here, Hofstee, or Eadie, plot ($V_{\text{NO}_3^-}$ versus $V_{\text{NO}_3^-}/[\text{NO}_3^-]$) and Hanes plot ([NO$_3^-$] / $V_{\text{NO}_3^-}$ versus [NO$_3^-$]) of the same rectangular hyperbola also gave similar values of the kinetic constants. No Michaelis-Menten kinetic function was readily discernible from data obtained with the dark-incubated samples because of the low uptake rates. Therefore, these results were not amenable to linear analysis. Addition of 10 and 50 $\mu$g of P/liter seemed to enhance and inhibit NO$_3^-$ uptake, respectively (Fig. 2). However, unpublished data from studies of several lakes from the Experimental Lakes Area have shown phosphorus uptake with as high as 150-$\mu$g of P per liter enrichment level for 24 h without any apparent toxic effect due to overloading (F. P. Healey, personal communication).

Phytoplankton primary production was not affected by NO$_3^-$ enrichment up to 1 mg of N per liter (Table 1). Inhibition of primary production by DCMU was incomplete; at 1 $\mu$M, DCMU inhibited photosynthesis by 96% but still permitted CO$_2$ fixation at a rate which was consistently two times higher than that of dark-incubated samples. Since an identical inhibition effect was noted in NO$_3^-$ uptake experiments where 1 $\mu$M DCMU was included (Fig. 4), it suggested that NO$_3^-$ uptake is directly depend-
weight extracellular nitrogen was likely to be expected as a reasonable product to account for the observed NO$_3^-$ uptake discrepancies. The increase of $^{15}$N label in the DON isolated from filtrates of incubated water samples by cation-exchange chromatography and the concurrent uptake of $^{15}$NO$_3^-$ are presented in Table 2. Increase in total particulate nitrogen after incubation was estimated to be less than 10%. Isotope analysis of total particulate nitrogen and DON demonstrated the cycling of NO$_3^-$-N into DON. If the net DON production originated from the particulate fraction alone, about 20 to 45% of this DON in the light-incubated samples was primarily due to phytoplankton excretion. In the DCMU-treated and dark-incubated samples, 13 and 34%, respectively, of the DON released was due to phytoplankton and bacteria. It was noted that DCMU inhibited only 50% of NO$_3^-$ uptake in the DCMU experiment (20 July 1976, Table 2). A variation in the efficiency of inhibition by DCMU was therefore possible when different samples were used. DCMU appeared to inhibit DON production as well as NO$_3^-$ uptake. The net rate of fractional DON production was at least one-tenth as fast as that of fractional NO$_3^-$ uptake; the rate of heterotrophic utilization of DON was unknown. As previously stated, nitrogen balances measured by colorimetric analysis and by particulate $^{15}$N determination showed a discrepancy of about 60% in terms of the total nitrogen applied. Of this, only about one quarter (15% of total) could be accounted for as DON by the isotope assay.

Table 3 presents results from amino acid analyses of the isolated DON performed with and without acid hydrolysis. Analytical results obtained with acid treatment represented total amino acid content including both combined amino acids (peptides) and DFAA, whereas those from untreated samples represented DFAA only. Scrutiny of Table 3 discloses a

![Graph 1](http://aem.asm.org)  
**FIG. 4.** Effect of DCMU on the kinetics of NO$_3^-$ uptake in Lake 227 epilimnetic waters (7 September 1975). Incubation was carried out for 3 h under the following conditions: I, light saturation; II, with 1 $\mu$M DCMU; III, in the dark. Maximum primary production was also estimated in the experiment by a $^{14}$C method. See also Fig. 1.

![Graph 2](http://aem.asm.org)  
**FIG. 5.** Correlation between $^{15}$N tracer and colorimetric determination of NO$_3^-$ uptake in Lake 227 epilimnetic waters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$P_{\text{max}}$ (pg C/h) with added NO$_3^-$ at (ng of N/liter):</th>
<th>0</th>
<th>50</th>
<th>150</th>
<th>300</th>
<th>500</th>
<th>1,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Light-saturated</td>
<td>9.56</td>
<td>2.95</td>
<td>ND</td>
<td>ND</td>
<td>2.98</td>
<td>2.89</td>
<td>2.73</td>
</tr>
<tr>
<td>II. With 1 $\mu$M DCMU</td>
<td>ND</td>
<td>0.11</td>
<td>0.12</td>
<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>III. Dark-incubated</td>
<td>0.06</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.06</td>
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$^a$ NO$_3^-$ enrichment up to 1,000 ng of N per liter in Lake 227 epilimnetic waters (7 September 1975). Water samples were incubated for 3 h. See also Fig. 4.

$^b$ ND, Not determined.
convenient partition of the data into two groups, namely, A and B versus C and D (controls). Each group has common DFAA and peptide concentrations that are significantly different from those of the other. A comparison of the two groups showed two prominent features. First, light-incubated samples enriched with either $^{15}$NO$_3$ or $^{14}$NO$_3$ were about 150 nM higher in total DFAA concentration than control samples treated with DCMU or HgCl$_2$. Treatment with HgCl$_2$ did not promote cell lysis because there was no significant increase in total DFAA or combined amino acids over background levels. Second, most individual DFAA concentrations were low (usually <50 nM) and similar in all samples except for serine and glycine. The peptides contained more glutamate, glycine, and alanine than the other amino acid residues. However, serine concentration was clearly higher in A and B than in the controls; whereas glutamate and alanine contents in the peptides of A and B were higher.

**DISCUSSION**

Nitrates uptake in the epilimnetic waters of Lake 227 during summer stratification was largely phytoplanktonic. Uptake was reduced when water samples were treated with DCMU,
an inhibitor of photosystem II and noncyclic photophosphorylation, which parallels the effect of dark incubation. In general, photosynthetic assimilatory reduction of NO$_3^-$ by algae is operative through the supply of reducing power indirectly from photosynthates (carbohydrates) and adenosine 5'-triphosphate from photophosphorylation (11). In blue-green algae (cyanobacteria), however, the photosynthetic reduction of NO$_3^-$ may be direct and independent of reducing power and adenosine 5'-triphosphate through a ferredoxin-NO$_3^-$-reducing system (1). The small NO$_3^-$ uptake in the dark probably represented the endogenous uptake by phytoplankton as well as uptake by bacteria.

The $K_I$ value of 73.6 µg of N per liter (5.2 µM) for NO$_3^-$ uptake by phytoplankton in Lake 227 is high compared with values (generally, ca. 1 µM) reported for natural populations and cultures of marine algal species (6, 18). The $K_I$ value falls in the upper part of the range (about 0.1 to 10 µM) determined for marine algae (6) and probably reflects the uptake characteristics of the dominant algal species belonging to the genera *Scenedesmus, Oscillatoria*, and *Aphanizomenon* (8) that have adapted to the eutrophic conditions in the epilimnion of Lake 227. This relatively high $K_I$ suggests that the NO$_3^-$ uptake rate was limited by the low ambient NO$_3^-$ concentration (<10 µg of N per liter), which prevailed in the lake during the summer months despite the addition of fertilizer NO$_3^-$ before 1975. The fertilizers added to the lake had contained adequate nitrogen to support primary production. In 1975, when the experiments were carried out, the fertilizer N:P weight ratio was reduced from 14 to 5, and there was a limitation of primary production. However, this nitrogen-deficient fertilization favored the development of N$_2$-fixing blue-green algae (*Aphanizomenon gracile*), thus lessenning the nitrogen deficiency in the lake (23). Ambient NO$_3^-$ concentration in the surface waters was still largely controlled by phytoplankton during summer stratification. It was estimated by isotope tracer technique that at the maximum uptake rate, the turnover of NO$_3^-$-N (uptake of NO$_3^-$ equal to that provided) could be as long as 5 days if possible exchange with the particulate fraction is neglected.

Low-molecular-weight nitrogen compounds in the form of DFAA and combined amino acids were detected as part of the extracellular DON produced by phytoplankton assimilation of NO$_3^-$ and CO$_2$ in Lake 227 surface waters. These are probably metabolic intermediates and thus are type I extracellular products according to the classification of Pogg (9). Since their appearance depends on metabolic activity, the presence of consumers, and other factors such as species composition, the rate of their release may change even during relatively short experiments.

Uptake of NO$_3^-$ and its subsequent excretion as DON was inhibited by DCMU, suggesting that these processes were light dependent. Hence, phytoplankton played a more important role than bacterioplankton in the contribution to the extracellular DON pool, although bacteria might significantly modify DON composition and quantity by heterotrophic uptake. This is not the first report on phytoplankton release of DFAA. The phenomenon has been observed by Schell in marine phytoplankton populations enriched with $^{15}$NO$_3^-$ (22). He found that at least 10% of the $^{15}$N was released as DON within 48 h but that plant processes were not differentiated from those of bacteria. However, our $^{15}$N tracer experiments provided very strong evidence for such photosynthetic extracellular production in Lake 227 phytoplankton. To date, the relation between assimilation of inorganic nitrogen and excretion of organic nitrogen has scarcely been examined in freshwater phytoplankton, although the release of peptides in *Anabaena* cultures was suggested to be linked with dinitrogen fixation (30). The possibility that this observation might have been an artifact resulting from cellular fragility and decomposition was ruled out by the use of biostatic controls. Comparison of our primary production data with those recorded during the peak season did not suggest that the extracellular release was stress induced. Although the frequency of DON measurements was limited, the discrepancy between disappearance of NO$_3^-$ and $^{15}$N uptake was consistent. It remains unknown whether amino acids were solubilized from colloidal forms when the pH of the concentrated filtrate was lowered to 2.0 for cation-exchange chromatography. Assuming that such a reaction in fact occurred, and that the colloids in both experiments and controls released the same proportion of amino acids on acidification, the observed results would still be meaningful.

Only a small fraction of the total extracellular material was represented by amino acids and peptides from the photoassimilated carbon in some marine algae (12). Watt reported that glutamate, valine, and serine are minor extracellular products of a freshwater diatom (31, 32). The extracellular DON partially identified as DFAA by automated amino acid analyses in our experiments contained a relatively high abundance of serine and glycine. Serine and glycine have also been found to be the major DFAA in Upper Klamath Lake (Oregon) and Lake Mendota (Wisconsin) surface waters (2, 10). These amino acids are characterized by their low heat of
combustion and therefore are thought to be unsuitable for energy storage (1). The small amount of dissolved free glutamic acid as opposed to the large amount found in the peptides perhaps reflected the high affinity of the indigenous heterotrophic microflora for glutamate (2). Though less obvious, a similar relation was observed in the case of glycine.

The exact nature of extracellular products was not known and forms such as nucleotides and enzymes other than DFAA and peptides might be excreted since, although the latter forms were probably significant products, they did not fully account for the large difference (about 60%) between particulate $^{15}$N uptake and NO$_3^-$ disappearance measured by colorimetric determination. It should be emphasized that neutral or anionic molecules, if excreted, could have escaped detection. Loss of amino acids through combination with colloids was also possible. Finally, if the turnover and exchange rates of certain amino acids were fast, they would not be detectable in natural aquatic environments. In these cases, failure to demonstrate DFAA production does not prove its absence. The present findings indicate that measurement of NO$_3^-$ uptake by phytoplankton as $^{15}$N incorporation alone would likely represent an underestimation. Assuming an underestimation of NO$_3^-$ uptake of 60% by the $^{15}$N method, the turnover time of NO$_3^-$ in Lake 227 epilimnion would be 5 instead of 5 days, which is consistent with the observation mentioned earlier.

The presently available techniques are still unsatisfactory for the elucidation of the mechanism and function of the extracellular products of plankton assimilation. By applying microautoradiography and scanning electron microscopy to study phosphorus cycling in plankton, Paerl and Lean (19) recently showed that the fine filaments extruded from blue-green algae and a colloidal pool of phosphorus were identical entities. The colloidal phosphorus was produced during filtration, which stripped off the filaments without concurrent cell damage. The colloidal phosphorus might also be a fraction not retained by 0.45-$\mu$m filters (16). If this is also true for NO$_3^-$ metabolism, it can explain the movement of nitrogen between the soluble and particulate fractions in lake waters. Paerl and Lean (19) further hypothesized that deposition of phosphorus around the algal capsule served to maintain phosphorus availability by preventing its loss through sedimentation. The excretion of amino acids may be caused by production temporarily exceeding consumption because of uncontrolled synthesis in the absence of mechanisms for enzyme repression (14). The variation in NO$_3^-$ uptake kinetics in relation to species composition as well as environmental factors (e.g., temperature, light intensity, and nutrient availability) that determine NO$_3^-$-N dynamics, including both assimilation and excretion, requires further study. Controlled laboratory experiments with axenic phytoplankton in continuous cultures would be indispensable to such investigations.

ACKNOWLEDGMENTS

This work was supported by a grant from Environment Canada.

We thank E. Tai for technical assistance. A. G. Dwilow kindly performed some of the emission spectrometric analyses. R. Gillespie did the EM work, and B. L. Dronzek provided analyses of the amino acid samples. F. P. Healey, E. J. Fee, and D. W. Schindler criticized the manuscript.

LITERATURE CITED