Plant Physiol. (1986) 82, 708–712
0032-0889/86/82/0708/05/$01.00/0

Nitrate and Ammonium Induced Photosynthetic Suppression in N-Limited *Selenastrum minutum*[1]

II. EFFECTS OF NO_3^- AND NH_4^+ ADDITION ON CO_2 EFFLUX IN THE LIGHT

Received for publication May 6, 1986 and in revised form June 22, 1986

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ABSTRACT

The effects of nitrate and ammonium addition on net and gross photosynthesis, CO_2 efflux and the dissolved inorganic carbon compensation point of nitrogen-limited *Selenastrum minutum* Naeg. Collins (Chlorophyta) were studied. Cultures pulsed with nitrate or ammonium exhibited a marked decrease in both net and gross photosynthetic carbon fixation. During this period of suppression the specific activity of exogenous dissolved inorganic carbon decreased rapidly in comparison to control cells indicating an increase in the rate of CO_2 efflux in the light. The nitrate and ammmonium induced rates of CO_2 efflux were 31.0 and 33.8 micromoles CO_2 per milligram chlorophyll per hour, respectively, and represented 49 and 48% of the rate of gross photosynthesis. Nitrate addition to cells at dissolved inorganic carbon compensation point caused an increase in compensation point while ammonium had no effect. In the presence of the tricarboxylic acid cycle inhibitor fluoroacetate, the nitrate-induced change in compensation point was greatly reduced suggesting the source of this CO_2 was the tricarboxylic acid cycle. These results are consistent with the mechanism of N-induced photosynthetic suppression outlined by Elrifi and Turpin (1986 Plant Physiol 81: 273–279).

In many cases, nitrogen addition to N-limited microalgae or natural phytoplankton assemblages results in a transient suppression of photosynthetic carbon fixation (3–11, 17–19). Experiments with the N-limited green alga *Selenastrum minutum* Naeg. Collins (Chlorophyta) have led to the development of a model which may explain the mechanism of N-induced photosynthetic suppression (5). This model suggests that N addition to N-limited *S. minutum* results in rapid N assimilation and an increased demand for α-ketoglutarate. In order to maintain the integrity of the tricarboxylic acid cycle during the period of α-ketoglutarate drain, an increase in activity of the tricarboxylic acid cycle would be expected. This would be supported by increased phosphoenolpyruvate carboxylase and pyruvate kinase activity. One source of carbon for these reactions was suggested to be the Calvin cycle. This drain of Calvin cycle intermediates would contribute to a decrease in the rate of RuBP[2] regeneration thus limiting photosynthetic carbon fixation during periods of N resupply (5).

This model presents several testable hypotheses. One prediction is that N resupply to NO_3^--limited *Selenastrum minutum* should result in an increase in tricarboxylic acid cycle activity and consequently an increase in the rate of CO_2 efflux in the light. This hypothesis can be tested in several ways. The first is by measuring CO_2 efflux in the light as reflected by a decrease in the specific radioactivity ($^{14}DIC/^{12}DIC$) of the medium surrounding the cells. The second is to measure changes in the Γ brought about by the addition of exogenous nitrogen sources.

In this report we present evidence that NO_3^- or NH_4^+ addition to NO_3^--limited *S. minutum* results in a major increase in the rate of CO_2 efflux in the light. This observation is consistent with the mechanism of N-induced photosynthetic suppression proposed by Elrifi and Turpin (5).

MATERIALS AND METHODS

Chemostat Culture. *Selenastrum minutum* Naeg. Collins was grown axenically in NO_3^--limited 1.4 L chemostats at 20°C at a growth rate of 0.3 d^{-1}. Inflow medium was a substantially modified Hughes medium (5) enriched to 1 mM $NaNO_3$ and 200 μM K_2HPO_4. This ensured that NO_3^- was the limiting nutrient. The photon flux density at the culture face was 165 $\mu E \cdot m^{-2} \cdot s^{-1}$. Cultures were buffered at pH 7.0 with 50 mM Hepes and bubbled rapidly (10 $L \cdot min^{-1}$) with filter sterilized air. These conditions resulted in steady state cell densities of 1.72 $\mu g\ Chl \cdot ml^{-1}$.

DIC Measurement. DIC concentration was determined by GC. Aliquots (100 μl) were removed from the experimental cuvette and injected into a modified gas chromatograph (Shimadzu GC 8A) similar in design to that described by Birmingham and Colman (1).

DIC Compensation Point (Γ) Measurement. For Γ determinations cells were harvested by centrifugation and resuspended in N-free low DIC medium, buffered at pH 8.0 with 50 mM Hepes (low DIC buffer), at a final density of approximately 4.0 $\mu g\ Chl \cdot ml^{-1}$. This suspension was placed in a water-jacketed (20°C) experimental cuvette (20). This cuvette was equipped with several serum stoppered sampling ports and was bubbled with CO_2 free air (400 $ml \cdot min^{-1}$) to maintain air levels of O_2. At this bubbling rate and pH no detectable DIC loss from the medium occurred over the duration of the experiment (data not shown). Upon attaining the Γ the effects of NO_3^- or NH_4^+ addition in the presence or absence of various inhibitors were observed.

Specific Radioactivity ($^{14}DIC/^{12}DIC$) Measurement. Cells were harvested and treated as previously outlined for compensation point experiments. Upon reaching compensation point $NaH^{12}CO_3$ and $NaH^{14}CO_3$ (Atomic Energy Commission of Canada) were added to bring the DIC concentration to 150 μM and specific radioactivity to 2.4 $\mu Ci \cdot \mu mol^{-1}$. Samples (750 μl) were withdrawn over a period of 40 min and injected into N_2 purged,

[1] Supported by the School of Graduate Studies and Research of Queen's University and the Natural Sciences and Engineering Research Council of Canada.

[2] Abbreviations: RuBP, ribulose 1,5-bisphosphate; DIC, dissolved inorganic carbon; GOGAT, glutamine 2-oxoglutarate aminotransferase (EC 2.6.1.53); P_g, gross photosynthesis; Γ, DIC compensation point; R, respiration; FA, fluoroacetate; Aza, azaserine.

sealed vials and frozen immediately in liquid N_2. Samples were later thawed and aliquots taken from the vials for analysis of DIC as previously described. Acid stable radiolabel was determined by adding 50 μl aliquots from the sealed vials to 300 μl of kill solution (80% aqueous ethanol, 5% HCOOH). Samples were evaporated to dryness and resuspended in 1.0 ml distilled H_2O and 2.0 ml Scintiverse I (Fisher Scientific). Total radiolabel was determined by adding 50 μl aliquots from the sealed vials to 1.0 ml of distilled H_2O alkalized with 1 drop of saturated KOH. Two ml of Scintiverse I were added and radiolabel quantitated by liquid scintillation counting. The specific radioactivity of DIC was determined as $(dpm_{total} - dpm_{acid\ stable}) \cdot DIC^{-1}$. The effect of NO_3^- or NH_4^+ addition was observed by raising the $NaNO_3$ or NH_4Cl concentrations to 5 mM at the time of DIC addition.

Gross Carbon Fixation at Compensation Point (Γ). The rate of gross carbon fixation at the Γ was measured by short-term incorporation of ^{14}C into acid stable products. Cultures were brought to the Γ in the presence or absence of added NO_3^- or NH_4^+ as previously described. $NaH^{14}CO_3$ was added to approximately 175 $\mu Ci \cdot \mu mol^{-1}$ and 0.9 ml samples were withdrawn at frequent time intervals and placed into 5.0 ml scintillation vials containing 500 μl of kill solution. No measurable changes in DIC levels resulted from $NaH^{14}CO_3$ addition. Quantification of radiolabel was as previously described.

Metabolic Inhibitors. Aza, an inhibitor of GOGAT, was added to the experimental cuvette at a concentration of 5 mM. Experiments were initiated following a 60 min preincubation. To partially inhibit the tricarboxylic acid cycle cells were preincubated in the light for 60 min with 50 mM fluoroacetate.

Nitrate Analysis. One ml culture samples were filtered through Whatman 934-AH glass fiber filters. Nitrate content of the filtrate was determined using the method of Strickland and Parsons (12) modified for flow-through sample injection.

Photosynthetic Kinetics. Aliquots of chemostat cultures were centrifuged and resuspended in low DIC medium in the experimental cuvette (20). Photosynthetic kinetics with respect to DIC were measured by monitoring O_2 evolution with a Clarke-type electrode (Yellow Springs Instruments) (20).

Other Measurements. Chl and cell numbers were measured as previously described (4).

RESULTS

Photosynthetic Kinetics. Cells harvested from the chemostat vessels exhibited half-saturation constants for photosynthesis with respect to [DIC] ($K_{1/2}^{DIC} \pm$ SE) of 98 ± 22 μM. The maximum carbon saturated rate of net photosynthetic O_2 evolution was 298 ± 37 $\mu mol\ O_2 \cdot mg^{-1}\ Chl \cdot h^{-1}$ (data not shown).

Effect of NO_3^- and NH_4^+ on Γ. In measuring the response of the Γ to N addition, a great deal of day to day variability was observed. Table I represents the mean and SE as determined from all measurements, whereas Figures 1 and 2 illustrate representative experiments. Cells resuspended in low DIC medium reduced [DIC] to an average compensation point of 29.7 ± 2.3 μM ($\bar{x} \pm$ SE; Table I). Following NO_3^- addition the level of DIC in the medium increased nearly 4-fold until a new compensation point was reached (111.7 ± 16.9 μM; Table I). The initial net rate of DIC efflux following NO_3^- addition was 39.2 ± 5.0 $\mu mol \cdot mg^{-1}\ Chl \cdot h^{-1}$ (Table II). During this time NO_3^- uptake was constant at a rate of 107.1 $\mu mol\ NO_3^- \cdot mg^{-1}\ Chl \cdot h^{-1}$ (Fig. 1).

Nitrate addition following partial inhibition of the tricarboxylic acid cycle with fluoroacetate resulted in a lower rate of net CO_2 efflux in response to NO_3^- addition (9.8 ± 1.7 $\mu mol \cdot mg^{-1}\ Chl \cdot h^{-1}$; Fig. 2) and the establishment of an intermediate compensation point (59.8 ± 13.8 μM; Table I). Inhibition of NO_3^- assimilation by the GOGAT inhibitor Aza alleviated any effect of NO_3^- on the Γ (Table I; Fig. 2). No increase in the Γ was seen when NH_4^+ was added to NO_3^--limited cultures and no change was observed when NO_3^- was added to NO_3^--sufficient cultures (data not shown).

Table I. *Effects of Nitrate and Metabolic Inhibitors on the DIC Compensation Point of NO_3^--Limited S. minutum*

Condition	Final Γ	Change from Control
	μM	%
Control	29.7 ± 2.3	
$+NO_3^-$	111.7 ± 16.9	+276
$+NO_3^-$ + FA[a]	59.8 ± 13.8	+101
$+NO_3^-$ + Aza[b]	30	0

[a] 50 mM fluoroacetate (about 46% inhibition of dark respiration). [b] 5 mM Aza.

Table II. *Estimates of the Rate of CO_2 Efflux from NO_3^--Limited S. minutum in the Light*

	CO_2 Efflux in the Light		
	S.A. experiments[a]	^{14}C-Fixation at Γ[b]	Net CO_2 efflux[c]
	$\mu mol\ CO_2 \cdot mg^{-1}\ Chl \cdot h^{-1} \pm$ SE		
Control	6.4 ± 2.4	5.3 ± 0.9	0.0
$+NO_3^-$	37.4 ± 6.9	56.8 ± 14.7	39.2 ± 5.0
$+NH_4^+$	40.2 ± 3.5	5.9 ± 1.0	0.0
NO_3^--induced change in CO_2 efflux[d]	31.0	51.5	39.2
NH_4^+-induced change in CO_2 efflux[d]	33.8	0.6	0.0

[a] Calculated as the rate of $^{12}CO_2$ release required to give the initial rate of decrease in exogenous specific activity. [b] Calculated from the rate of gross $^{14}CO_2$ fixation occurring at the Γ assuming that $P_g = R$. [c] The initial net rate of DIC efflux upon pulsing with N at the Γ. [d] The rate of CO_2 efflux in the presence of NO_3^- or NH_4^+ less the control rate.

The rate of gross carbon fixation at the Γ was linear over the time it was measured (Fig. 3). At compensation points in the absence of added NO_3^- and NH_4^+, the rates of gross carbon fixation were low ($\bar{x}$ = 5.3 ± 0.9 $\mu mol\ CO_2 \cdot mg^{-1}\ Chl \cdot h^{-1}$; Table II). Following NO_3^- addition and the establishment of a higher compensation point, gross carbon fixation had increased ($\bar{x}$ = 56.8 ± 14.7 $\mu mol\ CO_2 \cdot mg^{-1}\ Chl \cdot h^{-1}$; Table II). Intermediate rates of gross carbon fixation were observed at the intermediate compensation points obtained following NO_3^- addition to fluoroacetate treated cultures (17.3 $\mu mol\ CO_2 \cdot mg^{-1}\ Chl \cdot h^{-1}$).

Effects of N on Photosynthesis and CO_2 Efflux. Addition of 150 μM HCO_3^- to control cells at compensation point resulted in initial rates of gross C fixation of 124.1 $\mu mol \cdot mg^{-1}\ Chl \cdot h^{-1}$ (Fig. 4A). The initial net rate of C fixation was 120.0 $\mu mol \cdot mg^{-1}\ Chl \cdot h^{-1}$ (Fig. 4B). Over the duration of the experiment there was little change in the specific activity of exogenous DIC (Fig. 4C). Cells pulsed with NO_3^-, however, exhibited lower initial rates of gross C fixation (62.8 $\mu mol \cdot mg^{-1}\ Chl \cdot h^{-1}$; Fig. 4A) and greatly reduced rates of net C fixation (5.9 $\mu mol \cdot mg^{-1}\ Chl \cdot h^{-1}$; Fig. 4B). Over the duration of the experiment there was a major (52.4%) decrease in the specific activity of the exogenous DIC pool (Fig. 4C). Addition of NH_4^+ to NO_3^--limited cells yielded a similar response (Table II).

DISCUSSION

Consistent with our previous results the addition of NO_3^- or NH_4^+ to N-limited *S. minutum* resulted in a suppression in photosynthetic C-fixation (Fig. 4, A and B). Recently, we have

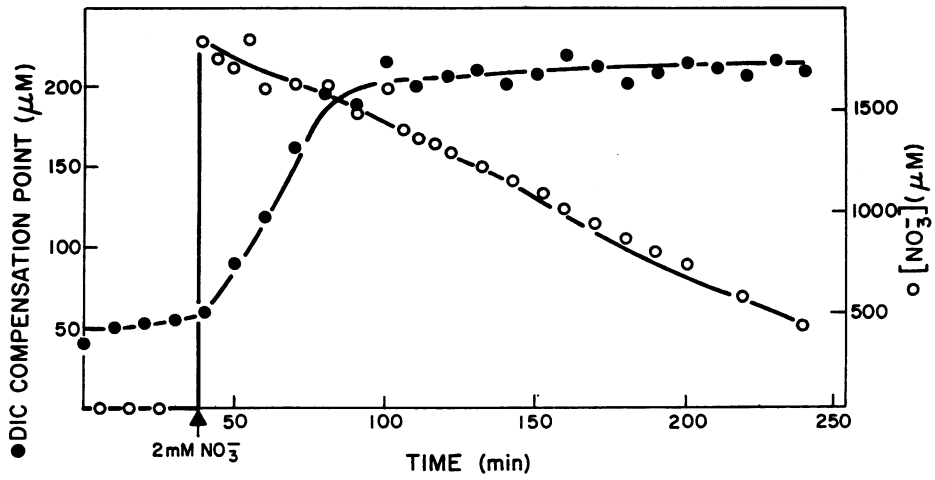

FIG. 1. A representative experiment showing the effect of NO_3^- addition (2 mM) on the Γ of NO_3^--limited chemostat grown cells of *S. minutum*; DIC concentration (●); NO_3^- concentration (O). Addition of 2 mM NO_3^- (t = 40 min) to cells at compensation point caused a net increase of culture DIC at a rate of 53.0 μmol·mg^{-1} Chl·h^{-1}. A new DIC compensation point of 220 μM was eventually reached. Over the duration of the experiment NO_3^- uptake was linear at a rate of 107 μmol·mg^{-1} Chl·h^{-1}.

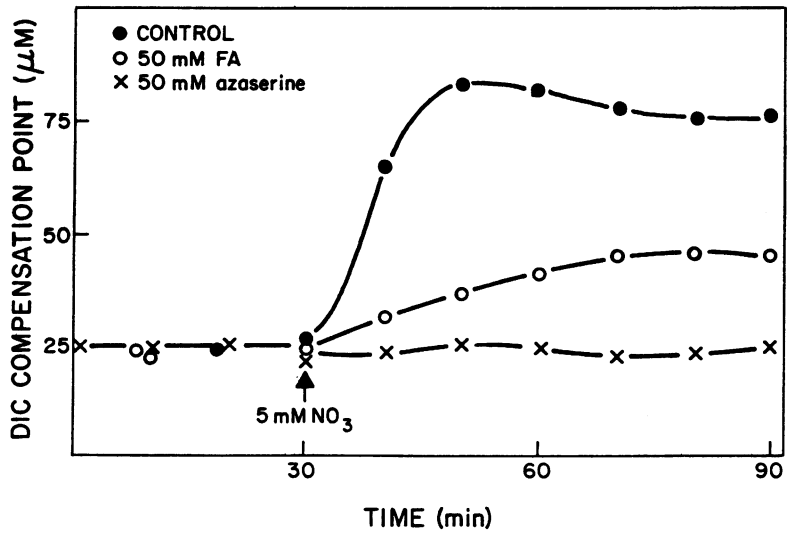

FIG. 2. The effect of metabolic inhibitors on the NO_3^- induced increase in Γ in NO_3^--limited chemostat grown cells of *S. minutum*. The initial compensation point was 25 μM while the addition of NO_3^- (5 mM, t = 30 min) resulted in a final Γ of 80 μM (●). Nitrate addition to cells treated with 50 mM fluoroacetate (O) resulted in a final Γ of 49.8 μM. Nitrate addition to cells treated with 5 mM Aza exhibited no change in DIC compensation point (×).

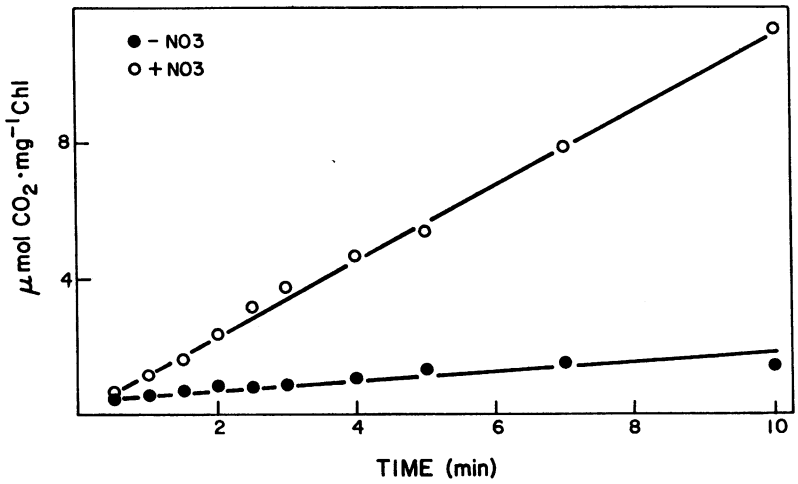

FIG. 3. An example of $^{14}CO_2$ incorporation at the DIC compensation point as a function of time in a control culture with a compensation point of 8.3 μM DIC (●); and a nitrate-pulsed culture with a compensation point of 288 μM DIC (O).

proposed that this suppression is due in part to the competing demands for carbon skeletons between the processes of N assimilation and CO_2 fixation (5). We suggested that sudden N resupply to NO_3^--limited cultures of *S. minutum* resulted in increased tricarboxylic acid cycle activity associated with the supply of carbon skeletons for N assimilation. These increased demands on tricarboxylic acid cycle intermediates would require anaplerotic reactions to maintain cycle activity. One source of carbon for these reactions was suggested to be Calvin cycle generated triose-P, the depletion of which would decrease RuBP regeneration thereby limiting carbon fixation. This hypothesis was consistent with the increases in dark carbon fixation and respiration observed in response to N addition as well as the observed effects on photosynthetic O_2 evolution (5).

A testable prediction of this model is that the N-induced increase in tricarboxylic acid cycle activity should result in an increase in CO_2 efflux in the light as a result of increased carbon flow through isocitrate dehydrogenase, pyruvate dehydrogenase

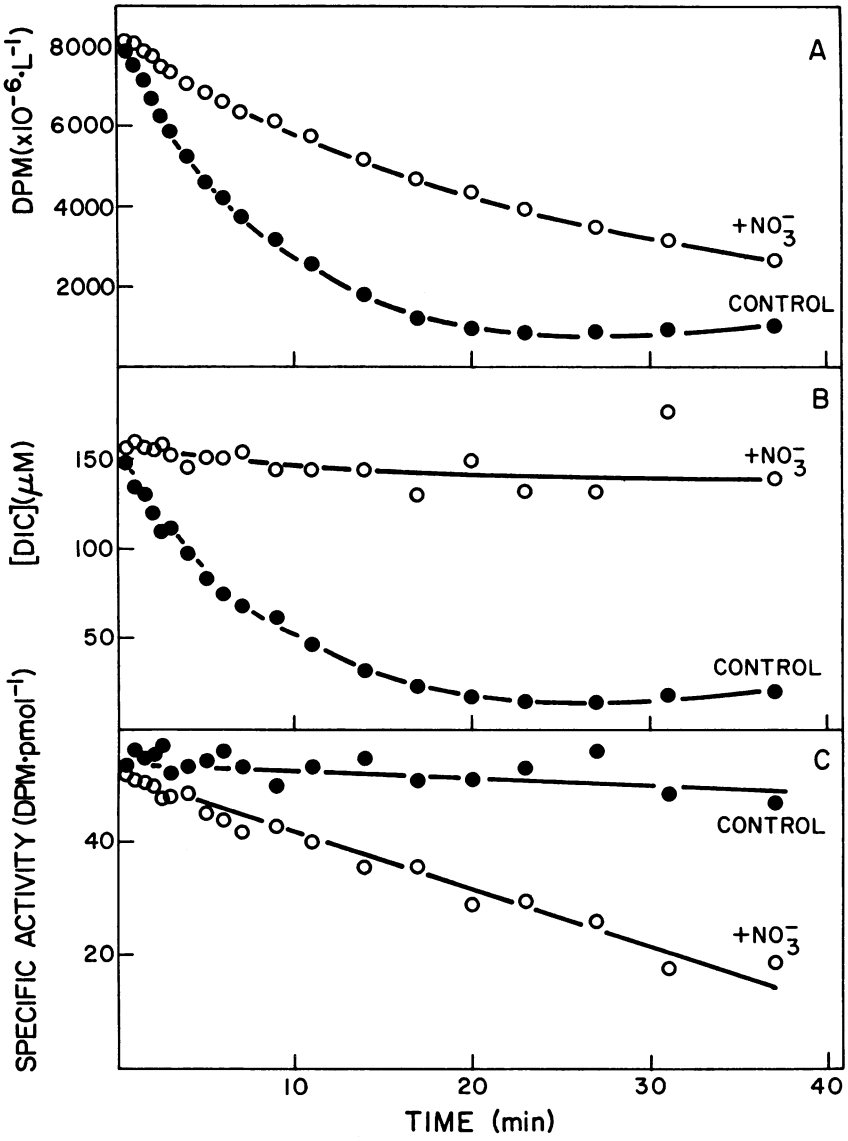

FIG. 4. Effects of NO_3^- addition on gross, and net photosynthesis and the specific activity of exogenous DIC. Nitrate-limited, chemostat grown cells of *S. minutum* at a density of 7.1 μg Chl·ml^{-1} were placed in a culture cuvette and brought to 150 μM $NaHCO_3$ in the absence (●) or presence of 5 mM NO_3^- (○). A, Decrease in ^{14}C in the external media; B, decrease in exogenous DIC; C, resulting changes in specific activity.

complex and α-ketoglutarate dehydrogenase complex. Earlier work by Syrett (13–16) showed that addition of NO_3^- to N-starved cells of *Chlorella* resulted in an increase in both CO_2 production and O_2 consumption in the dark. If our model is to account for the process of photosynthetic suppression, a similar response must occur in the light. One method of evaluating CO_2 efflux in the light is to follow the change in the specific activity of exogenous DIC using the technique of Birmingham *et al.* (2). If exogenous DIC is enriched with ^{14}C, the fixation of both species of carbon (^{14}C and ^{12}C) will be in direct proportion to their relative abundance. In the short term, however, any CO_2 efflux from the cells will be primarily unlabeled $^{12}CO_2$. Consequently, $^{12}CO_2$ efflux in the light can be detected as a decrease in the specific activity of exogenous DIC over time. The relative constancy of the specific activity in control cells (Fig. 4C) indicates low rates of CO_2 efflux in the light (6.4 ± 2.4 μmol CO_2·mg^{-1} Chl·h^{-1}; Table II). This is in general agreement with the rates reported for green algae by Birmingham *et al.* (2). In the present study both NO_3^- and NH_4^+ addition decreased net and gross C-fixation. Over the same period there was a major decrease in the specific activity of DIC. The rates of CO_2 efflux required to produce the observed changes in specific activity were 37.4 ± 6.9 and 40.2 ± 3.5 μmol CO_2·mg^{-1} Chl·h^{-1} upon NO_3^- and NH_4^+ addition, respectively. These fluxes represent approximately a 6-fold increase in the rate of CO_2 efflux in the light in response to NO_3^- or NH_4^+ (Table II). During the period of NO_3^- or NH_4^+ induced photosynthetic suppression observed in these experiments (Fig. 4), these rates were equivalent to 49 and 48% of gross photosynthesis, respectively.

Another indication of the magnitude of CO_2 efflux in the light is the response of the Γ to NO_3^- or NH_4^+ addition. Compensation point is achieved when the rate of gross carbon fixation equals the rate of CO_2 release. If NO_3^- or NH_4^+ addition were to enhance CO_2 efflux in the light, NO_3^- or NH_4^+ addition should result in an increase in the compensation point. Our results for NO_3^- addition are consistent with this prediction (Fig. 1). The initial net rate of CO_2 efflux in reponse to NO_3^- addition was 39.2 ± 5.0 μmol CO_2·mg^{-1} Chl·h^{-1} (Table II). Cells treated with 50 mM fluoroacetate and subsequently pulsed with 5 mM NO_3^- exhibited a lower initial net rate of CO_2 efflux and a correspondingly lower Γ (Table I; Fig. 2). The inhibitory effect of fluoroacetate on this increase in compensation point was taken as evidence that a major source of this CO_2 was from the tricarboxylic acid cycle. The alleviation of the NO_3^--induced increase in compensation point by the GOGAT inhibitor, Aza, demonstrated that this increase in CO_2 efflux was dependent upon the process of N

assimilation (Fig. 2). These observations are in agreement with our model of N-induced photosynthetic suppression in N-limited microalgae.

Suprisingly, ammonium addition to cells at Γ failed to induce the predicted increase in the rate of net CO_2 efflux (Table II). The observation that both NO_3^- and NH_4^+ caused CO_2 efflux at high DIC, as determined from the specific activity experiments (Table II), yet NH_4^+ had no effect at compensation point may point to an important difference in carbon partitioning during NH_4^+ assimilation at low and high CO_2 levels. In the specific activity experiments, DIC levels were relatively high (~150 μM) and photosynthesis following N addition was probably limited by RuBP regeneration (5). At the Γ, however, photosynthesis is limited by CO_2 supply. This difference may result in newly assimilated ammonia moving into alanine and aspartate when cells are at compensation point rather than into glutamine and glutamate. If most of the carbon flux was into alanine and aspartate there would be no tricarboxylic acid cycle mediated increase in CO_2 efflux. However, it is still unclear as to why NO_3^- and NH_4^+ addition invoke such different responses. Although we are currently evaluating these discrepancies, our results show that at relatively high levels of DIC both NO_3^- and NH_4^+ addition stimulate CO_2 efflux in the light.

At the Γ gross photosynthesis equals respiration ($P_g = R$). Consequently, an additional estimate of the rate of CO_2 efflux at the Γ can be obtained by measuring the rate of gross carbon fixation. The rate of CO_2 release from control cells at the Γ was calculated to be 5.3 ± 0.9 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$ (Table II). The rate of CO_2 efflux (determined as gross C-fixation) obtained at the high compensation points following NO_3^- addition was much greater (56.8 ± 14.7 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$). The difference between these two rates (51.5 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$) is an indication of the magnitude of NO_3^--induced CO_2 efflux (Table II). The rate of NO_3^--induced CO_2 efflux in the presence of fluoroacetate as determined by this method was substantially reduced (9.8 ± 1.7 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$). This increase in ^{14}C incorporation upon NO_3^- addition to cells at compensation point may at first seem contrary to the observation of N-induced photosynthetic suppression at higher DIC. At compensation point it is CO_2 supply which limits the rate of carbon fixation. Consequently, any increase in compensation point will result in an increased rate of carbon fixation. At high DIC however, the N-induced suppression of photosynthesis is due to RuBP limitation (5). NH_4^+ addition unlike NO_3^-, failed to produce any increase in the rate of CO_2 efflux as determined from these carboxylation rate measurements (Table II). This is consistent with the lack of an NH_4^+ induced increase in compensation point. It also rules out the possibility that NH_4^+ may have caused an increase in CO_2 efflux, with its effect on the compensation point being masked by a simultaneous increase in carbon fixation.

Our measurements of CO_2 efflux from unperturbed cells were low and account for less than 4% of the measured rate of photosynthesis. This is in agreement with observations made by other workers (2). The addition of NO_3^- to NO_3^--limited *S. minutum* resulted in a major increase in this rate. The three independent estimates of the rate of CO_2 efflux resulting from NO_3^- addition were in reasonable agreement. Estimates from specific activity experiments indicated a CO_2 efflux of 31.0 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$, gross photosynthesis at compensation point of 51.5 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$ and net rate of CO_2 efflux at compensation point of 39.2 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$ (Table II). These yielded an average rate of 40.6 ± 6.0 μmol $CO_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$. Given a respiratory quotient of 1 it would be expected that gross O_2 consumption in the light would increase by a similar value upon NO_3^- addition. It is interesting to note that NO_3^- addition in the dark resulted in an increase in the rate of O_2 consumption by a similar magnitude (61.0 μmol $O_2 \cdot mg^{-1}$ Chl $\cdot h^{-1}$) (5).

In summary these results show that NO_3^- addition to N-limited cells resulted in a major increase in the rate of CO_2 efflux in the light. Similarly, NH_4^+ addition at relatively high levels of DIC produced nearly identical results. These observations are consistent with the suggestion that N-induced photosynthetic suppression results in part from competition for carbon skeletons between the processes of N-assimilation and photosynthetic carbon fixation. It also shows that under conditions of transient N resupply to *S. minutum* in the light, dark respiration may be of a similar magnitude to photosynthetic carbon fixation.

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