Dynamics of atmospheric combined inorganic nitrogen utilization in the coastal waters off North Carolina

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ABSTRACT: Phytoplankton in nitrogen-depleted coastal Atlantic waters off North Carolina, USA, had a positive response to nitrogen added as rain (DIN: NO_3^- and NH_4^+) or directly as NO_3^- or NH_4^+. Increases in primary production, photopigments, and cellular protein concentrations were observed when nitrogen limitation was alleviated. NO_3^- concentrations decreased faster than those of NH_4^+ in 670 l mesocosm experiments, performed in October 1993 and March and April 1994. Stable nitrogen isotope measurements ($\delta^{15}N$) of particulate N typically showed similar responses to the nitrogen additions. The $\delta^{15}N$ decreased as the different DIN sources, having $\delta^{15}N$ values near 0‰, were incorporated into cell biomass. The smallest changes (about 1‰) occurred in the $\Delta^{15}N$ ($\delta^{15}N_{initial} - \delta^{15}N_{final}$) from nitrate additions. A greater shift of about 2‰ was observed with added DIN from rain, even though $\delta^{15}N$ of total DIN was similar. Ammonium additions resulted in the largest difference from the control, about 6 to 7‰. This fractionation is indicative of isotopic fractionation during enzymatic incorporation and active transport of ammonium into the cells. In parallel incubations, ^{14}C-bicarbonate was added along with rain in addition to all N additions and controls. Subcellular ^{14}C-labeled fractions from these samples showed a short-term response to nitrogen additions and included an increase in the low molecular weight fraction after the first light incubation (from dawn to dusk). Carbon was allocated into protein after a 24 h period that encompassed the night incubation.

KEY WORDS: Atmospheric deposition · $\delta^{15}N$ · Nitrogen cycling · Phytoplankton · Subcellular ^{14}C-incorporation

INTRODUCTION

Nitrogen (N) is the primary limiting nutrient in many marine environments, and therefore exerts an important control on primary production (Ryther & Dunstan 1971, Eppley & Peterson 1979). Nitrogen is assimilated and incorporated into proteins, some of which mediate photosynthetic assimilation of CO_2. When N is available for uptake, chlorophyll a (chl a) is synthesized, more CO_2 is fixed and more particulate and dissolved N are accumulated at different trophic levels.

The degree to which phytoplankton growth can be N limited is temporally and spatially variable. Shifts in N availability can determine patterns of primary production depending on the response of phytoplankton to N availability (McCarthy & Goldman 1979). Coastal and estuarine environments receive N inputs from terrestrial and anthropogenic sources. Oceanic waters have also been shown to be N limited (Dugdale 1967), although other elements such as iron can also enhance primary production (Martin et al. 1994). Open ocean waters have less exposure to terrestrial sources and no direct contact with benthic N regeneration. Therefore, allochthonous sources of 'new' nitrogen are of fundamental importance to primary production in this environment.

New nitrogen sources supporting primary production and phytoplankton growth include (1) cyanobac-

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terial and eubacterial N_2 fixation; (2) vertical mixing (i.e. upward flux) and upwelling of nitrate in coastal regions; (3) possible groundwater intrusions in shelf regions; and (4) atmospheric deposition which can supply nutrients directly into the open ocean. Regenerated N is considered an internal source (Eppley & Peterson 1979), a product of metabolic activities. Regenerated N inputs include ammonium remineralization by zooplankton and bacteria, which can result in a significant influx of available N within the euphotic zone. A second component in coastal environments is the flux of ammonium (NH_4^+) or nitrate (NO_3^-) to the water column from sediments.

In coastal areas adjacent to agricultural land, runoff of fertilizer and animal waste has been shown to create a significant impact on N loading. In urban areas, discharges from sewage treatment plants and industry have resulted in the eutrophication of almost all major Eastern US estuaries (Nixon 1986). While steps have been taken to decrease N loading from these 2 sources, combustion of fossil fuels and volatilization of ammonia from agricultural practices has remained unregulated, resulting in a steady increase of N to the atmosphere and coastal watersheds (Briblecombe & Stedman 1982, Fisher & Oppenheimer 1991).

Important characteristics of atmospheric deposition (AD) include the episodic nature of rainfall events and storms, as well as spatial patchiness in coastal and open oceans (Michaels et al. 1993). The magnitude and frequency of AD depend on the spatial and temporal characteristics of the atmospheric patterns and processes at any given location. For example, rainfall events vary from short-lived intense storms in a small area (e.g. summer thunderstorms) to large frontal systems that have regional impacts (e.g. hurricanes). Atmospheric deposition of N is of quantitative importance, with a load of 20 to 100 mmol N m^{-2} yr^{-1} (Duce et al. 1991). Atmospherically derived dissolved inorganic nitrogen (DIN: NO_3^-, NH_4^+) contributes 25 to 50% of the total N loading to estuaries, coastal and open ocean environments. Even remote regions are particularly influenced by the N present in rain events (Paerl 1993). Remote regions may find much greater need for atmospheric N because of isolation from other sources.

Nitrogen concentrations in rain collected on the North Carolina coast varied from 0.7 to 144 μM for NO_3^- and 0.5 to 164 μM for NH_4^+ (Paerl & Fogel 1994). Nitrogen loading from atmospheric deposition may be crucial in the transfer of this nutrient to nitrogen-limited ecosystems. Nitrogen-depleted coastal and offshore waters tend to respond quickly to the addition of N via rain events. Primary production and chl a concentrations increased within 24 h in experiments where individual nitrogen sources and rain water were added to nitrogen-depleted, oligotrophic North Car-

olina Atlantic coastal waters (Paerl et al. 1990, Willey & Paerl 1993). Nitrogen deposition can also stimulate longer-term effects in new production and may be a factor for initiating nuisance algal bloom formation (Paerl 1993).

Usually, different sources of N added to an environment exhibit distinct isotopic signatures or $\delta^{15}N$ values. In order to trace the different N sources in an ecosystem, stable isotope tracers at the natural abundance level have been examined (Fogel & Cifuentes 1993). The range in $\delta^{15}N$ values of various DIN sources can be diagnostic when studying the $\delta^{15}N$ of particulate N assemblages consisting of phytoplankton, zooplankton, and bacteria (Paerl & Fogel 1994). Particulate N (PN) $\delta^{15}N$ values in relatively pristine coastal waters of North America range from +4.5 to +6.5‰, whereas values of +7 to +10‰ have been measured in more polluted coastal areas. Regenerated ammonium should have $\delta^{15}N$ values slightly greater than those of PN (Cifuentes et al. 1989, Paerl & Fogel 1994). Riverine nitrate and ammonium inputs to coastal waters of North Carolina ranged between +4 and +16‰ (Showers et al. 1990).

In studies of $\delta^{15}N$ in DIN from rainfall, isotopic values of the ammonium and nitrate are considerably lower than either regenerated or riverine sources (Heaton 1986). In North Carolina, $\delta^{15}N$ of ammonium, nitrate and DON in rain were measured from different rain events throughout the year. The $\delta^{15}N$ values of the ammonium averaged –3.13‰ (range –12.5 to +3.6‰), whereas the nitrate and dissolved organic nitrogen averaged +1‰ (–2.0 to +4.7‰). In North Carolina, the N species contained in atmospheric deposition most likely originate from long-distance transport of combustion byproducts (NO_x), DON and/or local agricultural emissions (NH_3/NH_4^+) (Paerl & Fogel 1994).

The influence of AD sources of N to phytoplankton growth was studied by Paerl & Fogel (1994) in micro- and mesocosm experiments using N-depleted coastal ocean water. Stable N isotopes were used to identify the uptake of N from incoming rain; isotope fractionations during uptake proved minimal. However, isotope fractionations can occur during every enzymatic step involved in the uptake and assimilation of nitrate and ammonium and hence are dependent on concentrations of DIN and relative rates of uptake. Because of these metabolic complexities in isotopic labeling, $\delta^{15}N$ values of PN from coastal and open ocean environments may be difficult to interpret as to the dominant species of N incorporated or to the predominant source of the N. Isotopic fractionation of nitrate is usually small and concentration independent (Montoya et al. 1990, Fogel & Cifuentes 1993), whereas ammonium fractionation is larger and concentration dependent (Cifuentes et al. 1989, Hoch et al. 1992, 1994).

In order to understand the exact nature and timing of N uptake by phytoplankton in response to AD, a set of time-course experiments in 670 l mesocosms was conducted. Advantages of mesocosms include the following: (1) isolation of a mass of water, thereby minimizing complexities resulting from tidal forces or sedimentary fluxes, and (2) manipulations of the water mass are easier, where several N additions in the form of rain, nitrate and ammonium can be performed. Here, we document the differential uptake of nitrate versus ammonium nitrogen by coastal phytoplankton.

METHODS

Mesocosms. Experiments were conducted in 670 l outdoor mesocosms filled with Bogue Sound (North Carolina) water, pumped in from the dock of the Institute of Marine Sciences into a holding tank prior to mesocosm filling, then distributed into the different mesocosms. These experiments were performed in October 1993 and March and April 1994 from water pumped from Bogue Sound at different intervals throughout the year. Bogue Sound is a tidal sound, which on an incoming tide supplies full-salinity (32 to 35 ppt), N-depleted coastal water. Mesocosms consisted of laminated fiberglass cylinders that were filled with water from a depth of 2 m, pumped with a Pacer centrifugal pump from the incoming tide, usually from 6 to 12 h before nutrient additions were made. Once contained, the water was bubbled continuously with air to provide mixing and aeration. The mesocosms were surrounded by flowing Bogue Sound water to ensure natural ambient temperatures. In all experiments, initial NH_4^+ and NO_3^- concentrations were below 1 µM.

In each experiment, 2 or 3 mesocosms were maintained as controls, i.e. no additions were made. Nutrient additions for the different treatments included 4 to 8 % rain (v/v), nitrate, and ammonium additions (10 µM, the weighed amount was previously dissolved into 2 l distilled water). Specific additions to each experiment are shown in Table 1. These experiments were performed in October 1993 (Expt 1), end of March (Expt 2) and April (Expt 3) 1994. During these times the availability of nitrogen was generally low.

The rain used for mesocosm additions was collected in acid washed and rinsed polyethylene tubs, screened through 100 µm mesh to remove large particles, filtered through a GF/F filter, then placed in acid washed 10 l cubitainers and kept frozen until it was thawed for the additions (Paerl & Fogel 1994). Before addition, rainwater nutrient (NH_4^+ and NO_3^-) concentrations were determined, and 2 l subsamples were saved in

Table 1. Final mesocosm concentrations from nitrogen additions to mescosm experiments, in the form of rain and direct N additions

| | Final DIN (µM) | | |
	Expt 1	Expt 2	Expt 3
Control			
Nitrate	1	0.3	0.3
Ammonium	<0.1	0.2	1
Additions			
Rain	4%	R1	
Nitrate	2	2.1	
Ammonium	0.4	0.6	
Rain	8%	R2	$8\% + NH_4^+$
Nitrate	4	1.6	0.25
Ammonium	0.8	0.4	11
Nitrate	10	14	0.4
Ammonium	14	10	–

acid washed polypropylene bottles for isotope analysis of the dissolved N components.

Analytical methods. (1) *In situ* incubations were used for primary production measurement by $NaH^{14}CO_3$ incorporation. Both morning and afternoon incubations were conducted in light and dark bottles, which were suspended in the corresponding mesocosm. 10 µCi of $NaH^{14}CO_3$ (58 µCi µmol^{-1} specific activity) were added to 125 ml Pyrex bottles and incubated for at least 4 h. Following the incubation, the entire volume was filtered on 25 mm GF/F filters, placed in a 7 ml polypropylene tube, then 250 µl of 2 N sulfuric acid was added to the filter in a vial, and vented overnight under a hood to volatilize the unincorporated $H^{14}CO_3^-$. Finally, 5 ml of Universol Liquid Scintillation Cocktail was added. Tubes were left in the dark overnight and then counted on a Beckman scintillation counter.

(2) Dissolved inorganic carbon (DIC) was collected by completely filling a 20 ml glass liquid scintillation vial and keeping it at 4°C until analyses. DIC was measured as CO_2 by injecting 250 µl of the sample into 50 % phosphoric acid, under a stream of argon flowing into a Beckman Model 850 infrared CO_2 detector (Paerl 1987).

(3) Chl a was determined following filtration through a 25 mm GF/F filter unless indicated otherwise. Chl a was extracted by a modification of the 90 % acetone: DMSO extraction method of Shoaf & Lium (1976). The extract was measured by fluorometry using a Turner Design Model 110 fluorometer, calibrated against chl a (Sigma Chemical Company, St. Louis, MO). Chl a and carotenoid accessory pigments were also extracted in 90 % acetone and analyzed by HPLC photodiode array

spectrophotometry (Wright et al. 1991, Van Heukelem et al. 1992).

(4) Material for protein analysis was collected by filtration through 25 mm GF/F filters, extracted with 0.2 N sodium hydroxide and reacted with Coomasie blue (Bradford 1976). Absorbance was measured with a UV160U Shimadzu spectrophotometer at 595 nm. Bovine serum albumin was used as the standard.

(5) Nutrient analyses were made on samples filtered using combusted 47 mm GF/F filters, then analyzed for nitrite and nitrate, ammonium and orthophosphate using high sensitivity colorimetric techniques (Solórzano 1969, Strickland & Parsons 1972).

(6) Nitrate reductase activity was determined by the method of Lowe & Evans (1964).

Stable isotope analysis. For stable N isotope analyses, particulate material from each treatment (40 to 80 l) was filtered through a 1.0 µm Nuclepore 142 mm membrane by positive pressure filtration with nitrogen using a maximum pressure of 10 psi (703 g cm^{-2}). Particles were gently scraped from the membrane and stored at −20°C until analysis. Then the sample was thawed and concentrated by centrifugation at 17 000 rpm (26 000 × g) for 20 min in acid-washed and rinsed polycarbonate tubes. Once the whole sample was concentrated into 1 tube, it was freeze-dried overnight and put into a 60°C oven to ensure dryness. Filtered (GF/F) and scraped particles showed very similar δ^{15}N values when compared directly.

Dried samples were weighed and loaded into a pre-combusted (500°C for 1 h) quartz tube with 1.5 g copper and 2.0 g copper oxide, evacuated and sealed under vacuum. The tube was combusted with the sample at 900°C for 1 h and cooled at a controlled rate. Nitrogen was separated cryogenically from CO$_2$ and water, then analyzed in a double focusing isotope ratio mass spectrometer (modified by Nuclide Corp, State College, PA). CO$_2$ was collected after water was separated from the gas with a dry ice/methanol slurry. The CO$_2$ samples were analyzed for δ^{13}C in a Finnigan MAT 252 isotope ratio mass spectrometer (Bremen). The isotope ratio (δ^{15}N and δ^{13}C) is defined as:

$$\delta^{15}N = \frac{(^{15}N/^{14}N \text{ sample})}{(^{15}N/^{14}N \text{ standard})} - 1 \times 1000$$

For δ^{13}C, isotopic ratios of ^{13}C/^{12}C are used in the calculations. Standard deviation of these samples and standards (N$_2$ and CO$_2$) was ±0.2‰.

In certain experiments, particulate N for isotope analysis was sampled by filtration on combusted 47 mm GF/F filters. A volume of 700 to 1000 ml of mesocosm water yielded 50 to 100 µg N. The glass fiber filters were minced with scissors and added to 5 g of CuO reagent and 3 g Cu metal. After evacuation, samples were processed as above.

For dissolved ammonium analysis from the rain additions, ammonium was adsorbed onto Zeolite (W-85, Union Carbide), filtered onto a pre-combusted 47 mm GF/F and dried for 48 h. The Zeolite with adsorbed NH$_4^+$ was placed into a quartz tube, evacuated and combusted as above (Velinsky et al. 1989). For nitrate and any dissolved organic nitrogen, the rainwater used was ammonium free (post Zeolite sample), then it was freeze-dried in a CentriVap concentrator (Labconco). The resulting material was then put in a quartz tube and processed as above.

Subcellular ^{14}C-incorporation. Cells were separated into major biochemical compounds from 2 different size fractions; particulate material retained on a pre-combusted GF/F filter and an 8 µm Nuclepore filter. Water from the mesocosm was dispensed into two 1 l acid-washed polycarbonate bottles per treatment and 30 µCi of NaH^{14}CO$_3$ were added. The containers were incubated in situ, and left floating in the mesocosms. Cells from one of the bottles were harvested after the light period (ca 12 h), and the other one before dawn (ca 18 h). In both cases, 500 ml were filtered through a 25 mm GF/F filter and the other 500 ml were filtered through an 8 µm Nuclepore filter. This procedure was repeated every afternoon and morning for 3 d. Subcellular fractionation employs sequential extractions in which low molecular weight compounds, lipids, nucleic acids, carbohydrates, and protein components are separated from one another. We followed the procedures of Roberts et al. (1963) as modified by Cuhel (1993).

Experiments. In the first experiment (Expt 1), duplicate mesocosms were monitored for the uptake of both NO$_3^-$ and NH$_4^+$ at approximately 2 to 5 h time intervals during a 30 h period. Because NO$_3^-$ and NH$_4^+$ concentrations did not decrease to ambient levels, a second experiment was conducted in which we followed all parameters for 76 h in duplicate mesocosms (Expt 2). Third, we tested the reproducibility of an experiment by running triplicate mesocosms for the control and rainwater additions, and followed them for 3 d (Expt 3). Finally, in order to examine the partitioning of fixed carbon as a function of added N, we measured ^{14}C-bicarbonate uptake into different subcellular biochemical fractions in parallel to stable N isotope incorporation.

RESULTS

Expt 1

Nutrients

Ammonium. In October 1993, ammonium concentrations in Bogue Sound were around 0.1 µM (Fig. 1A). Over a 31 h period, concentrations fluctuated in the
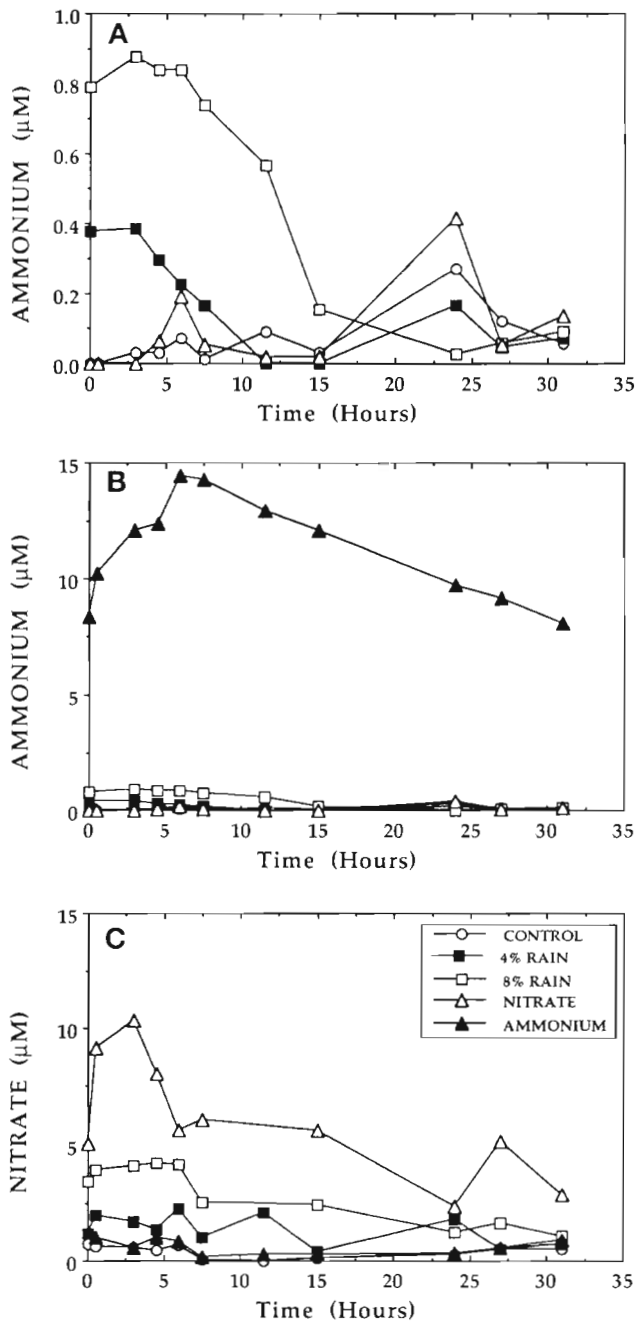
Fig. 1. Expt 1. Nutrient concentration change (µM). (A) Ammonium concentration in control, rain and nitrate additions; (B) ammonium concentration in the ammonium addition; (C) nitrate concentration change in all treatments

mesocosm, but never exceeded 0.25 µM. In response to the 4 and 8% rain additions (0.4 and 0.8 µM ammonium respectively), ammonium was stripped completely from the water after 11 h and 24 h, respectively. When higher initial concentrations were administered (14 µM, Fig. 1B), ammonium steadily declined throughout the experiment, reaching a final concentra-

tion of 8 µM after 30 h. With the addition of the nitrate, ammonium followed the same response as was observed in the control.

Nitrate. Nitrate concentrations in the control were 1 µM and remained so throughout the 30 h (Fig. 1C). In the 4 and 8% rain additions, nitrate concentrations started at 2.5 and 5 µM respectively and declined to half that during the first 7 h. In the case of the nitrate addition (9 µM), a rapid decrease in the concentration was observed after 7 h to a level of 5 µM. In the ammonium additions, the nitrate concentrations were 1 µM and remained at that concentration throughout the experimental period.

Phosphorus. Phosphate concentrations in ambient waters were between 0.2 and 0.25 µM. With added rain, there was an increase to 0.45 µM. At the end all mesocosm treatments, $HPO_4^=$ decreased to approximately 0.1 µM.

Biological parameters

Photopigments. Initial chl *a* concentrations were around 5 µg l^{-1}, and there was no increase in the concentration until 24 h after the start of the experiment (Fig. 2A). After 30 h, concentrations in the control reached 12 µg l^{-1}, while 4% rain, nitrate and ammonium treatments increased to around 20 µg l^{-1}. The 8% rain addition yielded the highest chl *a* concentration (27 µg l^{-1}).

Fucoxanthin is an accessory pigment found mainly in diatoms (Jørgensen 1977). Initial concentrations of this accessory pigment ranged between 1.5 and 2.5 µg l^{-1} (Fig. 2B). Concentrations remained within that range until the start of the next light period the second day. After 24 h, values rose to between 3 and 4.5 µg l^{-1}. At the end of the experiment, the control exhibited the lowest concentration, followed by the increasing concentrations in 4% rain, nitrate, 8% rain and ammonium treatments.

Primary productivity. Primary productivity exhibited a pattern similar to the fucoxanthin and chl *a* concentrations. After 24 h, the control exhibited the lowest rate, 22 mgC m^{-3} h^{-1}, and increasing values were observed with the 4 and 8% rain, nitrate and ammonium treatments (Table 2). In ammonium additions the enhancement factor with respect to the control was 2.1, whereas rain additions showed a factor of 1.3, and nitrate addition 1.8 (Table 2).

Protein. Initial protein concentrations were around 60 µg l^{-1}, increasing after dark to a range between 60 and 150 µg l^{-1}. The control mesocosm remained at the initial value, but increasing concentrations were observed in 4% rain, 8% rain, ammonium and nitrate additions. After 24 h, at the beginning of the second
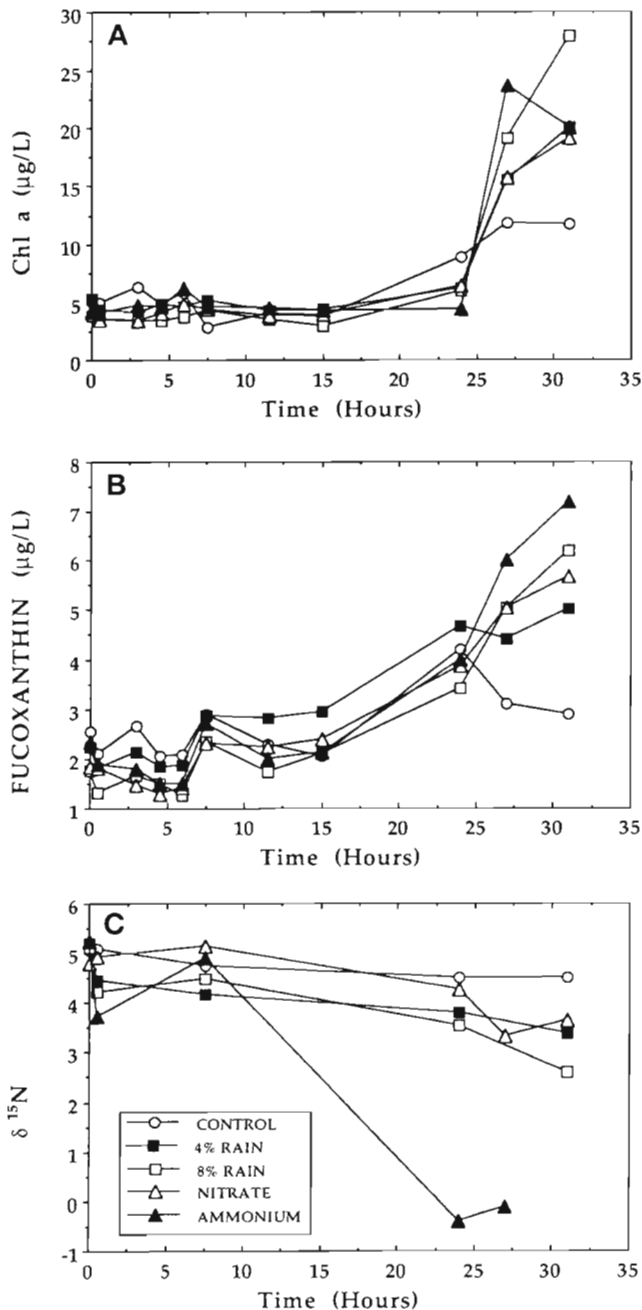
Fig. 2. Expt 1 (A) Chl *a* (µg l^{-1}) concentration change in all treatments; (B) fucoxanthin (µg l^{-1}); (C) changes in δ^{15}N values in all treatments

Table 2. Primary production (PP) measurements performed throughout the mesocosm experiments. The data shown here represent the production at around 24 h. Stimulation by additions is indicated by the enhancement factor (EF)

| | Primary production (mgC m^{-3} h^{-1}) | | | | | |
| | Expt 1 | | Expt 2 | | Expt 3 | |
	PP	EF	PP	EF	PP	EF
Control	22	1.0	18	1.0	15	1.0
Additions						
Rain 4%	29	1.3				
R1			26	1.4		
R2			21	1.2		
Rain 8%	30	1.4			47	3.1
Rain 8% + NH$_4^+$					52	3.5
Nitrate	40	1.8	25	1.4		
Ammonium	46	2.1	36	2.0		

Stable isotopic compositions

The δ^{15}N of particulate material >1.0 µm varied in response to treatments (Fig. 2C). The initial δ^{15}N values for the particulate material and the initial values for the additions are shown in Table 3. Immediately after nutrient or rain additions, the initial δ^{15}N values ranged between +5 and +3.5‰. Changes in the control from start to end were very small, Δ^{15}N = 0.75, (Table 4). Δ^{15}N is defined as δ^{15}N$_{initial}$ − δ^{15}N$_{final}$. The increasing differences in PN δ^{15}N values mainly occurred as a function of added N. The largest difference was observed in response to the ammonium addition, where a continuous decline in the δ^{15}N was observed throughout the experiment (Δ^{15}N = 7.82, Table 4). The δ^{15}N values were more negative than at

Table 3. δ^{15}N initial values for the nitrogen additions in the different mesocosm experiments

| | | δ^{15}N (‰) | |
	Expt 1	Expt 2	Expt 3
	δ^{15}N-PN		
Control	5.20	4.68	9.33
Additions	δ^{15}N-DIN		
Rain	4%	Rain 1	
Nitrate		+1.0	
Ammonium		+1.64	
Rain	8%	Rain 2	8% + NH$_4^+$
Nitrate	−1.14	+1.94	−0.91
Ammonium	−0.92	+3.64	+1.27
Nitrate	0.61		
Ammonium	−0.68		−0.68

light period, protein concentrations in the control had increased to 180 µg l^{-1}; in the rain treatments protein levels were 260 µg l^{-1}, while the ammonium treatment had 300 µg l^{-1}. The highest protein increase occurred in response to the nitrate treatment. The pattern of protein synthesis response was similar to that observed for chl *a* and primary productivity.

Table 4. Isotope shifts in particulate N ($\Delta^{15}N$) as a function of treatment in mesocosm experiments. $\Delta^{15}N = {}^{15}N_{initial} - {}^{15}N_{final}$. Maximum values are reported when $\delta^{15}N$ increased at the end of the experiment

| | Isotope fractionation ($\Delta^{15}N$) | | |
	Expt 1	Expt 2	Expt 3
Control	0.75	0.21	0.07
Additions			
Rain 4%	1.81		
R1		1.40	
R2		0.98	
Rain 8%	2.52		
Rain 8% + NH_4^+			6.16
Nitrate	1.16	1.87	
Ammonium	7.82	5.48	

Table 5. Isotope shifts in particulate C ($\Delta^{13}C$) relative to the control in mesocosm experiments. These differences were calculated from values measured at the end of each experiment. $\Delta^{13}C = {}^{13}C_{treatment} - {}^{13}C_{control}$

| | Isotope fractionation ($\Delta^{13}C$) | | |
	Expt 1	Expt 2	Expt 3
Rain 4%	+1.48		
R1		+1.37	
R2		+1.26	
Rain 8%	+0.93		
Rain 8% + NH_4^+			R1 +2.03
			R2 +1.85
Nitrate	+1.14	+1.93	
Ammonium	+1.99	+1.90	

the start of the time course and similar to $\delta^{15}N$ of the added ammonium.

Stable isotopes of carbon in the particulate material of the different treatments were heavier than the control by an average 1.2‰ (Table 5). The nitrate addition was similar to that of the rain, however ammonium additions had a $\Delta^{13}C$ of almost 2‰.

Expt 2

Nutrients

Ammonium. During March 1994, ammonium concentrations in Bogue Sound were around 0.2 µM (Fig. 3A). Concentrations fluctuated during the 73 h experiment, reaching a value of 0.6 µM. Both 8.5% rain additions had a final ammonium concentration of 0.7 µM. The uptake of ammonium was observed within
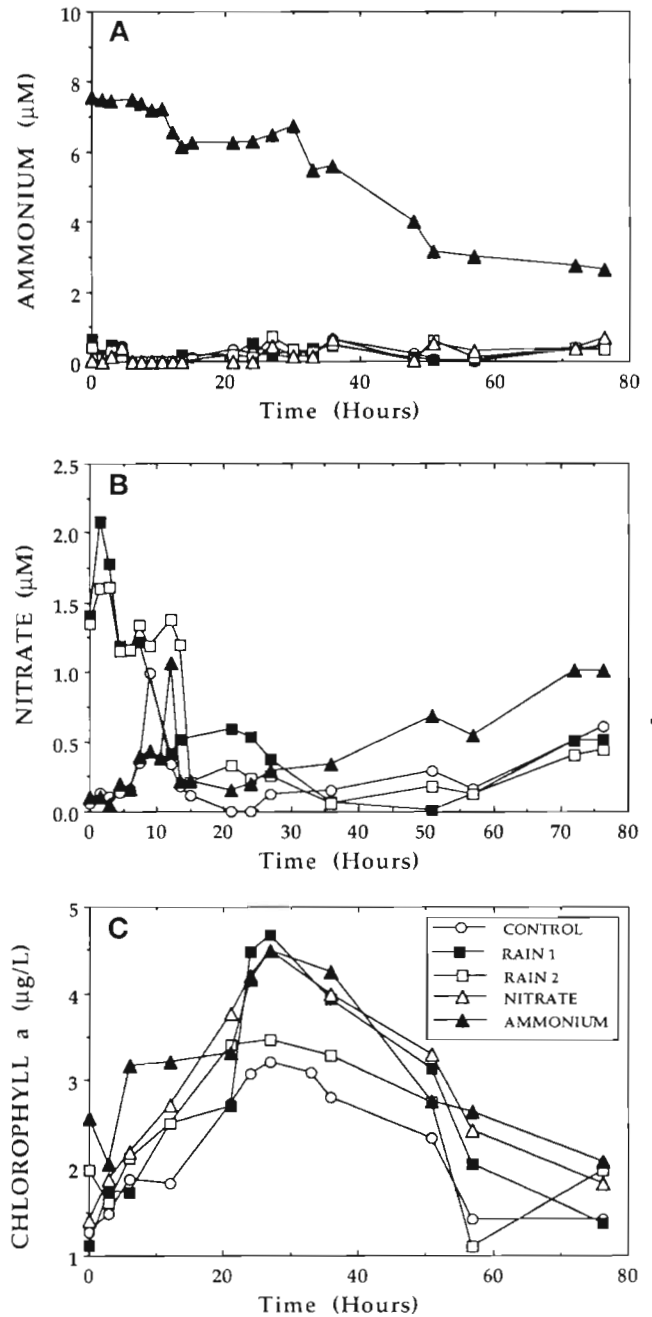

Fig. 3. Expt 2. Nutrient concentration change (µM). (A) Ammonium in all treatments; (B) nitrate concentration in all treatments; (C) chl a (µg l^{-1})

5 h, after which ammonium regeneration was observed during the night. This regenerated nitrogen was reincorporated during the following day, and this pattern was repeated several times during the course of the experiment. Rain 2 (8% addition), which was initially higher in nitrate, and the nitrate addition had similar ammonium concentration changes throughout. In the ammonium addition (9 µM), a decrease in the concen-

tration was observed after 7 h (Fig. 3A). There was a steady decrease in the concentration to 5 µM at 20 h (at dawn), then an increase of 1 µM during the day, returning to the 5 µM concentration at dusk. After the second day of the experiment, a decrease in concentration was observed, reaching 3 µM at the end of the experiment.

Nitrate. Ambient concentration of nitrate in Bogue Sound was ~0.3 µM (Fig. 3B). Nitrate concentrations in mesocosms following the rain additions were 2.1 µM for Rain 1 and 1.6 µM for Rain 2 additions. Nitrate in the rain additions decreased after 3 h and declined to 1.2 µM after 13 h. Concentrations decreased in both rain-amended mesocosms, and approached detection limits. In both rain-amended incubations, nitrate increased slightly at the end of the experiment to ~0.5 µM. In the ammonium addition, nitrate concentrations showed a slight increase from 0.25 µM to 0.75 µM at the end of the experiment. In the 10 µM nitrate addition, the decrease in nitrate concentration started at 10 h and declined to 5 µM at 76 h (data not shown).

Biological parameters

Photopigments. Initial concentrations of chl a (Fig. 3C) were ~1 µg l^{-1}, increasing to 3 µg l^{-1} after 24 h. Highest concentrations, ~4.5 µg l^{-1}, were observed in Rain 1 and ammonium-treated incubations; chl a concentrations in the other treatments remained similar. Concentrations of chl a in all mesocosms decreased at 55 h, leveling off at ~1.5 µg l^{-1} at 78 h. Parallel stimulation in fucoxanthin concentrations were observed. Initial fucoxanthin concentrations were around 1 µg l^{-1}, reaching a maximum ~3.5 µg l^{-1} in the Rain 1 (4% addition) and ammonium additions. Fucoxanthin concentrations were as high as chl a concentrations during this mesocosm incubation.

Primary productivity. Immediately after N additions, primary productivity in all treatments was ~20 to 25 mgC m^{-3} h^{-1}. By 24 h, primary productivity increased in the NH_4^+-amended mesocosms by 2-fold, but less in other treatments (Table 2). By the second day, greater differences in productivity could be detected. The control exhibited the lowest rate, followed by Rain 2, NH_4^+, Rain 1 and NO_3^-. The highest enhancement factor with respect to the control was in the ammonium addition with a value of 2.0 (Table 2).

Protein. Initial protein concentrations (<20 µg l^{-1}) appeared uniform throughout the first day, then increased only after 18 h. Further increases in protein concentrations were measured during the second light period; after the second night's incubation maximum protein concentrations of ~160 µg l^{-1} were reached. Nitrogen enrichment from all sources resulted in over-
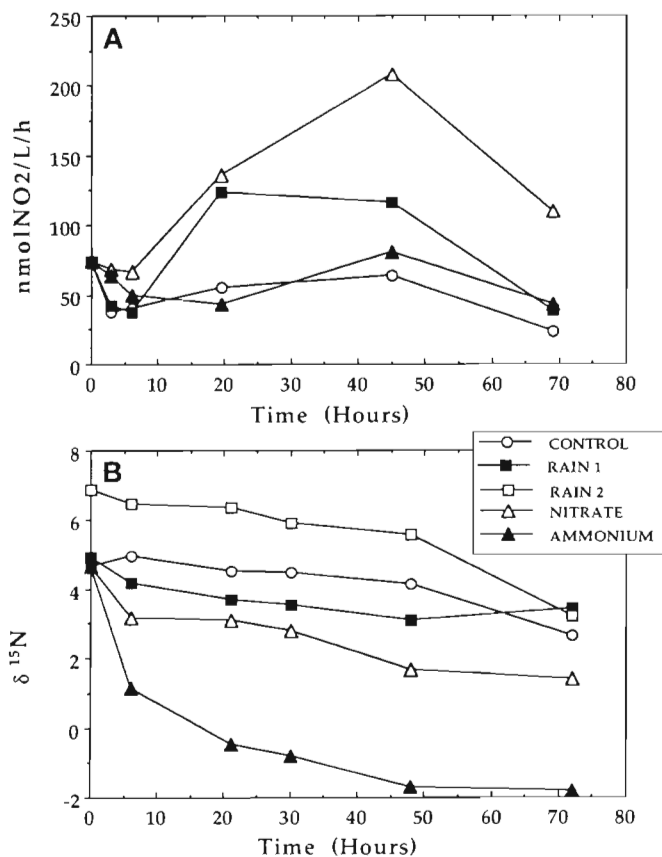

Fig. 4. Expt 2. Nitrate reductase activity (nmol l^{-1} h^{-1}); (B) changes in $\delta^{15}N$ (‰) values in all treatments

all greater concentration of protein when compared to controls.

Nitrate reductase activity. The rate of nitrate reductase activity (NRA) increased only after 10 h in mesocosms amended with nitrate and rain (Fig. 4A). Background NRA was around 50 nmol NO_2^- l^{-1} h^{-1}. In the rain-treated mesocosms, NRA reached a maximum of 120 nmol NO_2^- l^{-1} h^{-1}, whereas in the nitrate addition, activity was 210 nmol NO_2^- l^{-1} h^{-1} at 45 h. A decrease in activity was measured near the end of this experiment, in both the rain and the nitrate additions. In the control and the ammonium amended mesocosms, NRA increased after 45 h to 60 and 80 nmol NO_2^- l^{-1} h^{-1} respectively. NRA activity is enough to account for the observed nitrate disappearance.

Stable isotopic compositions

The $\delta^{15}N$ of particulate material declined in direct response to individual treatments (Fig. 4B). At the time of the additions, initial $\delta^{15}N$ values varied from +5 to +7. $\delta^{15}N$ values of the additions are presented in Table 3. Changes in nitrogen isotopic compositions in

the control throughout the experiment were small, ca 1‰ (Fig. 4B). Shifts in the $\delta^{15}N$ values in Rain 1-amended mesocosm were also near 1‰, but $\delta^{15}N$ values in the Rain 2 additions decreased by 3.5‰ at the end of the experiment. In mesocosms having direct N additions, $\delta^{15}N$ decreased by 2.5‰ with added NO_3^-, and similar to our first experiment, the greatest change in $\delta^{15}N$ was measured in NH_4^+-amended treatments ($\Delta^{15}N$ 5.48, Table 4). The control had the lowest $\Delta^{15}N$ of 0.21, then increasing values were measured in PN from Rain 2, Rain 1, NO_3^- and NH_4^+ (Table 4).

The $\delta^{13}C$ values of the particulate material (size >1 µm) from the rain-amended treatments were +1.3‰ heavier than the control (Table 5). Both nitrate and ammonium additions had increased $\delta^{13}C$ values by almost 2‰ relative to controls.

Expt 3

Nutrients

Ammonium. Ambient ammonium concentrations in Bogue Sound were around ~1 µM, decreasing to below the detection limits very quickly (Fig. 5A). Concentrations increased slightly after ~36 h, reaching 2 µM at 48 h, then decreased to initial concentrations after 70 h. Rain + 10 µM ammonium additions showed a rapid decrease; after 12 h only 50% of the ammonium added was left. Ammonium decreased until reaching background concentrations at 60 h. Rain 1 was added before dawn, whereas in Rain 2 rain was added during mid-morning.

Nitrate. Initial nitrate concentration was 0.3 µM and increased to 0.6 µM after 24 h in both the control and rain additions. Concentrations in the control then decreased to initial values, while the rain additions decreased to 0.4 µM. The rain added contained virtually no nitrate.

Phosphorus. Initial phosphate concentrations were <0.1 µM and increased to ~0.3 µM after 36 h.

Biological parameters

Photopigments. Initial chl *a* concentrations were ~1 µg l^{-1} (Fig. 5B), with similar concentrations also observed for fucoxanthin (Fig. 5C). Both pigments showed increased concentrations after 24 h. Chl *a* concentrations in the control remained the same throughout the experiment; fucoxanthin showed a similar pattern, but increased slightly after 60 h. Treatments receiving rain showed an increase in chl *a* as well as fucoxanthin. Rain 2 (mid-morning addition) chl *a* reached 2.5 µg l^{-1} whereas fucoxanthin was ~4 µg l^{-1}.
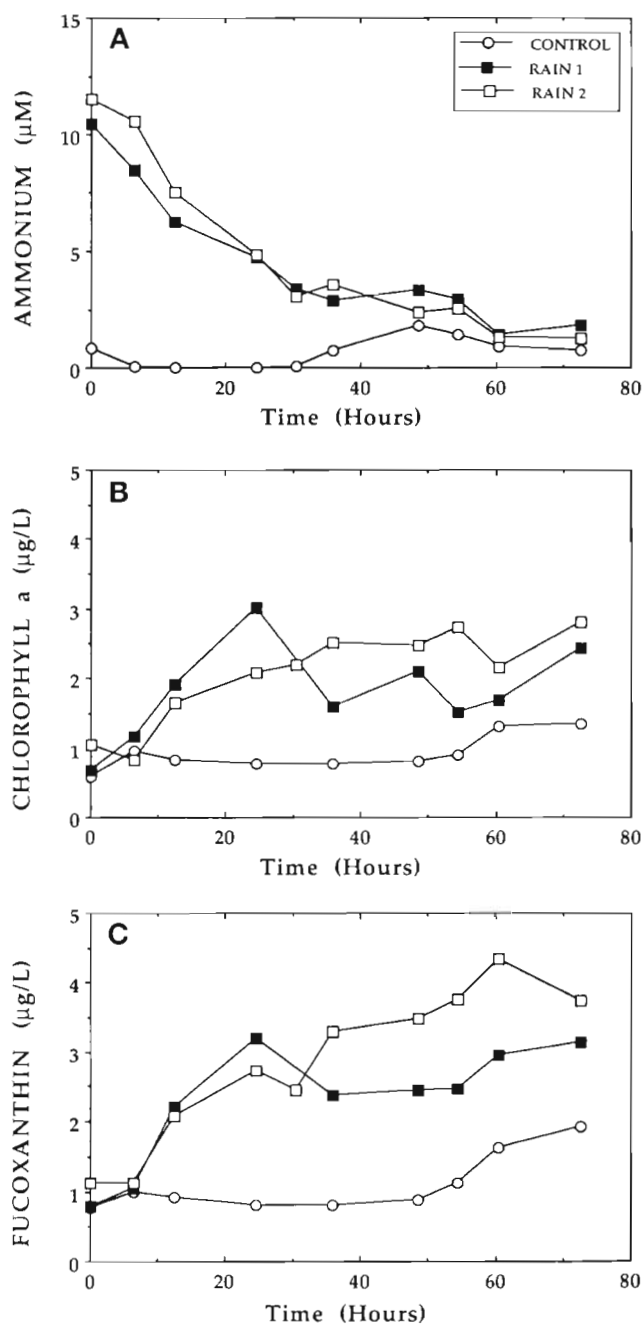

Fig. 5. Expt 3. Nutrient concentration change (µM) in all treatments. (A) Ammonium; (B) chl *a* (µg l^{-1}); (C) fucoxanthin (µg l^{-1})

Rain 1 (pre-dawn addition) concentrations were 2 and 2.5 µg l^{-1} respectively. Chl *a* in the 8 µm size fraction had the same trends as observed in whole water (GF/F fraction). The control had the lowest values and Rain 2 had the highest: chl *a* in this fraction (>8 µm) represented at least 75% of the total chl *a*.

Primary productivity. Primary productivity values initially were ~5 mgC m^{-3} h^{-1}, then increased to

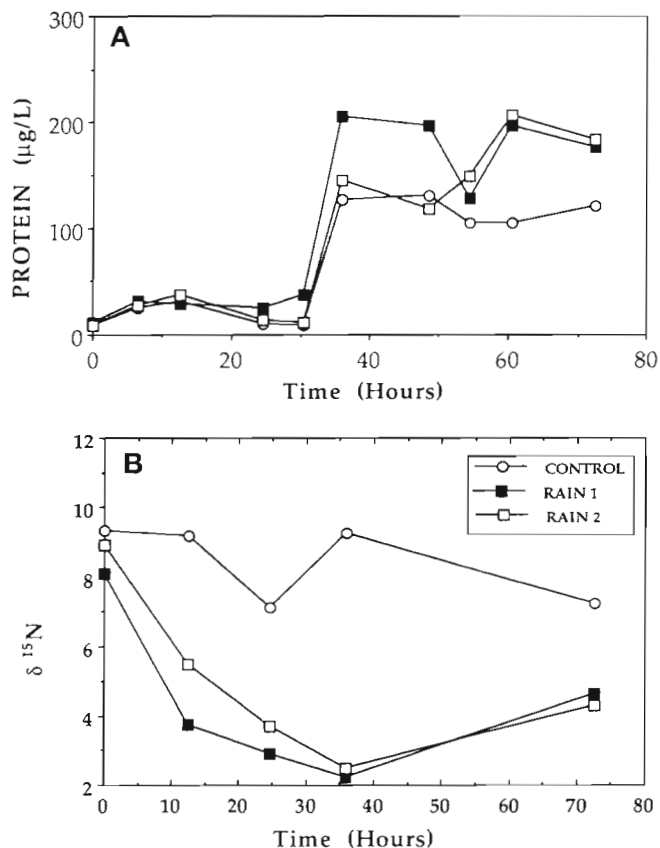Fig. 6. Expt 3. (A) Protein (μg l^{-1}); (B) changes in δ^{15}N (‰) values in all treatments

15–20 mgC m^{-3} h^{-1} in controls during the first day (Table 2). After 24 h, the N-amended treatments showed a further increase, reaching ~50 mgC m^{-3} h^{-1}, over 3-fold greater than controls. Production values decreased to initial values after 55 h. Enhancement factor with respect to the control reached a value of 3 in both rain additions (Table 2).

Protein. Initial protein concentrations were around 30 μg l^{-1}, increasing after 24 h to 150 μg l^{-1} in the control and R1 and to 200 μg l^{-1} in R2 (Fig. 6A).

Stable isotopic compositions

The control exhibited a slight decrease in the δ^{15}N values (initial values are shown in Table 3), but the value remained around 9‰ (Fig. 6B). The rain additions showed a 4‰ decrease after the first day, decreasing to lower values after the second day. The δ^{15}N values increased at the end of the experiment, at the same time when the NH$_4^+$ levels were depleted. In this experiment, Δ^{15}N (δ^{15}N$_{initial}$ – δ^{15}N$_{final}$) in the control was 0.07 and in the rain addition was 6.16 (Table 4). Stable isotopes of carbon in the particulate material

were heavier than the control. Table 5 shows the difference at the end of the experiment. Both the rain and the ammonium amended mesocosms had δ^{13}C values almost 2‰ more positive than controls. These Δ^{13}C values are similar to those found in mesocosms with added nitrogen rather than those found in mesocosms with only rain.

Subcellular fractionation

GF/F size fraction. Subcellular biochemical fractionations from the rain additions showed protein synthesis stimulation (almost double), when compared to the control (Table 6). After the first light period, an increase in the low molecular weight (LMW) fraction, which includes components having fast turnover rates such as amino acids (Morris 1981), was observed in Rain 1 (early morning pre-dawn addition). After the first night period there was an increase in protein, most likely stimulated by the N addition (Table 6). During the second day there was another increase in the LMW component which reflected N. The LMW values in the additions were clearly higher than the control. There was a further increase in protein after the second night. At this time, the carbon fixed was highest in the N additions (Table 6). During the third day, an increase was observed with respect to the control and after the night the control as well as the treatments increased, which shows that protein synthesis occurred at night. The LMW fraction showed an increase until the third day, when the ammonium had been consumed.

Cumulative C fixation was highest in the Rain 1 in the GF/F fraction (Fig. 7A), increasing up to 1200 μgC

Table 6. Percent distribution of assimilated ^{14}C-bicarbonate in subcellular fractions on both GF/F and 8 μm Nuclepore filters

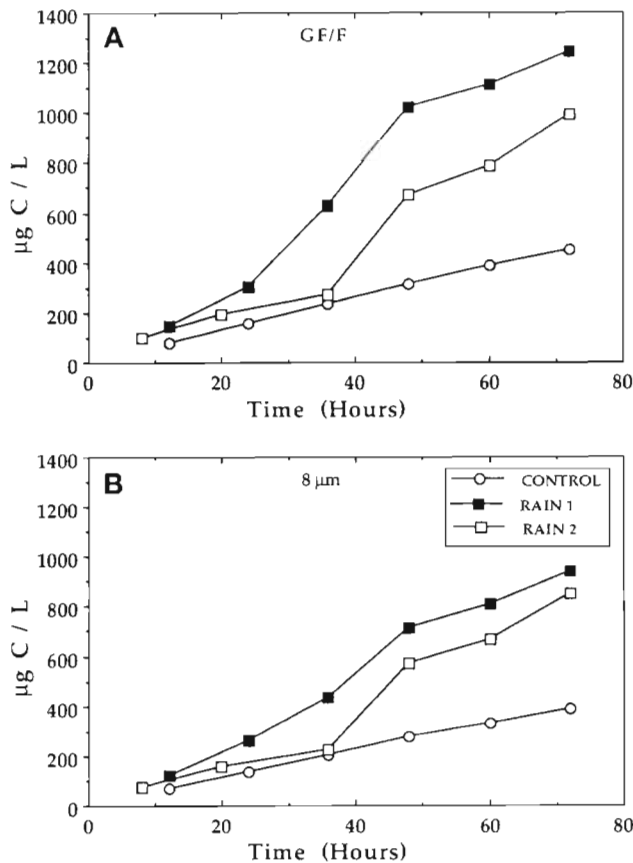| Filter type	Additions	%LMW	%LIPID	%HTCA	%PROT
GF/F					
1st day	Control	46.6	25.5	15.9	12.0
1st day	Rain 1	65.3	14.7	7.1	13.0
1st day	Rain 2	46.3	22.3	12.1	19.3
1st night	Control	45.2	25.6	13.6	15.6
1st night	Rain 1	41.9	19.6	11.5	27.0
1st night	Rain 2	46.4	18.0	10.9	24.7
8 μm					
1st day	Control	38.4	30.1	16.8	14.7
1st day	Rain 1	46.8	22.8	9.8	20.6
1st day	Rain 2	28.7	29.6	14.4	27.3
1st night	Control	41.6	26.6	12.8	19.1
1st night	Rain 1	28.5	28.0	12.5	31.0
1st night	Rain 2	21.7	26.8	13.6	37.8

Fig. 7. Expt 3. Cumulative carbon concentration (μgC l^{-1}) in different size fractions for all treatments; (A) GF/F fraction; (B) 8 μm fraction

DISCUSSION

In mesocosm experiments, as new N from rain or direct inorganic N sources (nitrate and ammonium) was assimilated by phytoplankton, primary production and protein synthesis were stimulated. Typically, increases in photosynthetic pigments occurred within the first and/or second day of incubation. Stimulation of protein synthesis was pronounced during the first and second night period, at which time concentrations of cellular protein were significantly higher than the control. Similar cycles were observed in chemically measured protein, with the control maintaining the lowest values, and increasing concentrations observed in response to rain, nitrate and ammonium additions.

Physiological changes must occur before production and other biotic parameters respond to N additions (e.g. chl a, accessory pigment and protein synthesis). Nutrient-deficient cells are usually low in protein and rich in carbohydrates or lipids for storage; these cells exhibit an increase in the rate of uptake of the deficient nutrient in the darkness, as well as in the light (Healey 1973). At night there is an increase in dark respiration, which has been observed in a broad spectrum of phytoplankton, including diatoms, green algae and cyanobacteria (Healey 1979). Marine phytoplankton exhibit either C_3 or C_4 metabolic pathways (Kerby & Raven 1985). For example, the physiological state of cells influences the overall amount of carbon fixed into cell components by either β-carboxylases (in dinoflagellates) or PEP carboxykinase (in diatoms).

Physiological effects from rainfall N additions were dependent on the relative proportions of NH_4^+ and NO_3^- in the rainwater added in the mesocosm experiments. As there is a large variability in the N composition of rain, the response of phytoplankton to rainfall additions appears to reflect different ammonium and nitrate concentrations. The effects of the individual inorganic N species additions were also evaluated by measuring the uptake dynamics and response time in a N limited environment. Usually, there was a short term positive response to all DIN additions, but nitrate concentrations tended to decrease faster than the ammonium concentrations. In general, the overall response in biomass and primary productivity was greatest in ammonium additions, followed by nitrate and rain.

Seasonal differences were also observed in response to N additions: these differences are reflected in primary production, protein synthesis and changes in $\delta^{15}N$ values. In October, ambient N concentrations were low (Fig. 1), and the response to the additions was considerably more rapid, consistent with responses observed in nutrient-limited populations (Healey 1979). Primary production doubled, and a similar response was observed in protein production.

l^{-1}, and responded faster to the nitrogen addition than the Rain 2 (added during mid-morning). Rain 2 achieved a productivity level of 800 and the control 200 μgC l^{-1}.

8 μm size fraction. During the first day of incubation, LMW was lower in large phytoplankton after 12 h (Table 6), while both lipids and to a lesser extent protein were significantly enhanced relative to total phytoplankton. After the first night period, an increased proportion of carbon was found in the protein fraction, while the LMW fraction decreased.

The diel trend of higher % ^{14}C in the LMW fraction during daylight incubation followed by flux from this pool into protein at night persisted for the 3 d of the experiment (data not shown). The degree of day/night difference became progressively more muted with time, particularly after added N supplies had been assimilated. The response was similar in this size fraction, since it is a subset of the GF/F size fraction. Newly fixed carbon increased rapidly to 1000 μgC l^{-1} in Rain 1, 800 in Rain 2 and 400 in the control (Fig. 7B).

Stable N isotope shifts ($\delta^{15}N$) during N uptake were highest in October and early March (Table 4). As water temperature increased in late March and April, increased production and mineralization of recently fixed organic matter resulted in relatively large diel fluctuations in $\delta^{15}N$, observed both in the control and rain additions (Fig. 6B). According to our results, N concentrations in Bogue Sound were lower in the fall than in the spring. The availability of the N may have influenced the assimilation rates of N observed during the mesocosm experiments.

Distinctions between day and night responses to new N additions were related to photosynthetic and respiratory processes. During the day, CO_2 fixation and synthesis of chl a and accessory pigments (e.g. fucoxanthin) were the dominant processes. During the night, protein synthesis remained important, and an increase in biomass was typically observed after 24 h. Similar responses and time intervals were observed in all experiments. Mitochondrial respiration can support nitrate reduction during photosynthesis, and it stimulates TCA cycle activity to a greater degree than during the ammonium assimilation (Weger 1988). Ammonium was regenerated at night and its increased concentration in the water was measured in experiments where N concentrations had decreased significantly. This diel pattern of uptake and regeneration has been observed previously in algae exhibiting nutrient deficiencies (Healey 1979). Our results were consistent with diel patterns of N uptake during the day and remineralization during the night, which were also observed in the ^{14}C-subcellular fractionation experiments.

Phytoplankton fractionate the stable isotopes of N in nitrate and ammonium, and carbon isotopes in CO_2 during assimilation into organic matter (Fogel & Cifuentes 1993). Stable isotope fractionations were observed in response to all N additions. The responses differed as a function of the type of N source provided to the phytoplankton. The most important differences involved the assimilation of nitrate or ammonium. Nitrate concentrations decreased faster than ammonium concentrations in rain and direct additions, and it can be assimilated directly into the cell by active transport in which no isotopic fractionation would occur (Falkowski 1975, Mariotti et al. 1982). Inside the cell, nitrate is usually reduced by nitrate and nitrite reductases to ammonia, which is then incorporated into organic matter. The assimilation and reduction of nitrate requires ATP. During this process, only small fractionations, in the order of 1 to 2‰, were observed during the time course experiments (Table 4). Only when nitrate is supplied in great excess of growth requirements, is the full isotope fractionation (9‰) expressed (Pennock et al. 1996).

Ammonium incorporation occurs chiefly via glutamate dehydrogenase, which is a reversible reaction, or it can occur via glutamine synthetase (GS), a unidirectional reaction requiring energy (ATP) to be active. GS is the primary enzyme for ammonium assimilation in phytoplankton and bacteria, in the aquatic environment studied. During ammonium assimilation in coastal North Carolina by phytoplankton, a consistently greater isotopic fractionation with an average of 7‰ has been measured. The potential isotope fractionation during the uptake of NH_4^+ is even greater (27‰), owing to isotope effects in diffusion and equilibrium isotope effects between NH_3 and NH_4^+ (Hoch et al. 1994, Pennock et al. 1996). The magnitude of the fractionation of NH_4^+ in our mesocosm experiments is indicative of active transport (Kleiner 1985), where most of the NH_4^+ transported into the cell is incorporated into organic matter.

With the rain additions, the mixture of both nitrate and ammonium present in different proportions in each event results in isotopic N fractionations that lie between the values observed in the nitrate additions (1‰) and those from the ammonium additions (7‰). The degree of isotopic fractionation depends on the proportion of nitrate to ammonium added to the mesocosm. If nitrate was the dominant N species, the fractionation measured was smaller than if the ammonium was the dominant component. In Expt 2, for example, Rain 1 had a slightly higher nitrate concentration than Rain 2; as a result, $\Delta^{15}N$ values were 1.4 and 0.98‰ respectively (Table 4).

Carbon isotopes were also measured during the time-course experiments (Table 5), and were consistently heavier with respect to the control in all experiments. Increased productivity was related to increased carbon isotope fractionation. Rain enhancements had a steeper slope than did ammonium enrichments.

The enzyme ribulose 1,5-bisphosphate carboxylase (Rubisco) is involved in the fractionation of carbon during photosynthesis (O'Leary 1988). Active transport and diffusion are also an important part in the fixation of CO_2 into organic matter (Fogel & Cifuentes 1993). In addition to primary fixation of CO_2, there is biochemical evidence for the importance of phosphoenolpyruvate carboxylase (PEPcase) in the carbon fixation at night associated with NH_4^+ assimilation (Elrifi et al. 1988). Guy et al. (1989) observed that when N limitation is relieved by the addition of NH_4^+, photosynthetic carbon fixation declines, and PEPcase is stimulated, followed by more positive $\delta^{13}C$ values. In Expt 3, subcellular fractionation during the night (Table 6) showed an increase in fixed carbon coupled to a 2.5‰ increase in $\delta^{13}C$. Both responses presumably are the result of increased PEPcase activity.

Nitrogen stimulation of phytoplankton is related to carbon fixation, biomass accumulation and cell division (von Caemmerer et al. 1984). The influence of the new N additions on carbon allocation into different subcellular components was addressed in Expt 3. N additions caused a substantial increase in protein synthesis at night and an increase in the low molecular weight material during the day. When N availability becomes limiting for growth (<1 µM), and reaches the initial low N concentrations present in Bogue Sound, cells allocate C very similar to the control. This similarity was observed at the beginning of the third day during this particular mesocosm experiment. Our experiments demonstrate that episodic N alters certain physiological responses on the short term (within 24 h) and results in elevated biomass which enters the biogeochemical N and C cycles.

The biochemical composition of newly formed particulate material from phytoplankton growth was affected by different chemical (nutrient availability) and physical (light, temperature) parameters. Measurement of macromolecular synthesis by phytoplankton permits analysis of specific end products of carbon fixation (e.g. protein, carbohydrate, and lipid) and may provide the means for identifying nutrient deficiency in phytoplankton (Hobson & Pariser 1971, Cuhel & Lean 1987). In the biochemical fractionation from rain+nitrogen additions, stimulation of protein synthesis relative to the control was measured. Overall, protein synthesis occurred predominantly at night. The low molecular weight fraction showed relative increases with N additions until the third day, when the ammonium had been consumed.

The balance between the uptake of the available N and its conversion into protein at night is reflected in an increase in biomass and primary production during the first 2 d. It has been observed that assimilation of added N to N-starved cells induces high assimilation and respiration rates and a decrease of intracellular carbohydrates (Syrett 1956) due to their consumption during protein synthesis (Morris 1981). This pattern was observed in our experiments (Table 6), where an increase of LMW was concomitant with a decrease of carbohydrates. Ammonium assimilation requires ATP synthesis from mitochondrial respiration in the light (Weger 1988). In Weger's study, free amino acid synthesis followed the addition of ammonium which ultimately increased the TCA insoluble material (i.e. protein). Our results from the subcellular fractionation are consistent with an increase in LMW (amino acids) after N addition.

In the 8 µm fraction larger cells, mainly diatoms, were assayed. Allocation into the lipid fraction is greater than the carbohydrate fraction, which is consistent with diatom dominance in this fraction. Protein synthesis also occurred at night, but by the last night's incubation N was already incorporated and therefore not available for assimilation.

Similar patterns of carbon fixation via photosynthesis were observed in both GF/F and 8 µm size fractions (Fig. 7A & B respectively). In both size classes, the total C fixed by the control was about 30 % of C fixed in particulate material in the rain+nitrogen additions. Carbon is readily fixed when N-starved cells have N available. Based on differences in the time of the additions, it seems that having the nutrient available in the early morning (R1, pre-dawn addition; Fig. 7) resulted in a faster assimilation of carbon, when compared to the mid-morning addition (R2; Fig. 7). The difference in carbon fixation in the 8 µm fraction (~900 µgC l^{-1}) from larger cells and the GF/F (~1200 µgC l^{-1}), a combination of larger and smaller cells, provides the amount fixed by the intermediate size fraction (~300 µgC l^{-1}), that are probably important in remineralization processes in this environment.

In summary, when N is added to a N-limited ecosystem there will be primary and secondary responses by the community. What is unclear, however, is the timing and the magnitude of the responses in terms of N additions. When it rains, N is added to a film of surface water, is mixed by waves and wind action, and phytoplankton encounter the N by simple diffusion. If it rained at night, what would the response of phytoplankton be? Our experiments have demonstrated that ammonium added at night could stimulate CO_2 uptake by β-carboxylases, a process distinct from the primary photosynthetic response measured during ^{14}C-bicarbonate uptake. Nitrate additions from rain at night would probably not enter the biomass until the following day. At this time, if the nitrate had not been sufficiently diluted or mixed with ambient waters low in N, it would be taken up rapidly by phytoplankton. In both instances, not until after the first light cycle does this N end up as high-molecular-weight protein that can be utilized for growth and secondary production. Accordingly, it is not until the following second day that phytoplankton will respond to episodic additions of N. By this time, ambient levels of N in the water column should be re-established.

Likewise, stable isotope tracers of this added atmospheric source of N will also lose resolution in terms of dilution and mixing. In order for N and C isotopes to be clearly useful as tracers in a natural ecosystem, the timing of rainfall would need to be in early morning with low wind conditions, and consequently little mixing. Moreover, phytoplankton must be starved for N. Only after 24 to 36 h would it be possible to detect the source of N in the phytoplankton before it is remineralized in the second night cycle, mixed with the standing biomass of particulate N, and lost by dilution.

Questions that still remain are the following: if we relieve N stress by the addition of N from rain or a synthetic source, what will limit the phytoplankton next? Will phosphate become secondarily limiting, and its lack in the water column result in less efficient or rapid uptake of N? If ATP synthesis is shunted towards N uptake, will other biosynthetic reactions be bypassed — such as nucleic acid synthesis and cell division? If ammonium stimulated CO_2 uptake at night, will pools of carbon dioxide in cells be depleted when light fixation becomes important during the day? Further research into the interactions of atmospheric deposition of nitrogen with phosphorus, iron and carbon dioxide will address some of these issues.

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