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Partitioning of Nitrogen and Carbon in Cultures of the Marine Diatom *Thalassiosira fluviatilis* Supplied with Nitrate, Ammonium, or Urea

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Abstract

Small or negligible differences in growth rates, average cell size, yields in cell numbers and total cell volumes were found in cultures of *Thalassiosira fluviatilis* enriched with nitrate, ammonium, or urea. Intracellular pools of unassimilated nitrate, nitrite, and ammonium were found in nutrient-rich conditions, but urea was not accumulated internally. Nitrogen assimilation into organic combination rather than nitrogen nutrient uptake was a critical rate-limiting step in nitrogen utilization. The free amino acid pool, protein, lipid-associated nitrogen, pigments, and total cell nitrogen were all highest in young or mature phase cells and decreased with age in senescent cells, whereas chitan, lipid, carbohydrate, and total cellular carbon all continued to increase during senescence. Dissolved organic nitrogen compounds accumulated in the medium only during senescence. C:N and lipid:protein were sensitive indicators of nitrogen depletion and age in *T. fluviatilis*.

Introduction

Whipple in 1894 first noted the importance of nitrogen as a key limiting factor in the growth of diatoms both in nature and in the laboratory (quoted in Chu, 1943). Nitrogen is also known to play a critical role in extablishing patterns of plant metabolism (Bassham, 1971; Kanazawa *et al.*, 1972). The work presented below examines the partitioning of nitrogen by diatom cells when they are presented with nitrate, ammonium, or urea, all of which might be available to phytoplankton in the natural marine environment. Eppley and Rogers (1970) have shown the need for studies of this type.

Materials and Methods

The euryhaline centric diatom *Thalassiosira fluviatilis* Hustedt, Guillard's strain, was kindly supplied by Dr. J.S. Craigie, Atlantic Regional Laboratory, N.R.C., Canada. Axenic cultures were maintained in a modified Guillard f/2 medium buffered with ferric citrate-citric acid (see Conover, 1974). 500 µM N was supplied as $NaNO_3$, NH_4HCO_3, or urea as desired; 500 µM $NaHCO_3$ was also added to the media containing nitrate or urea. Ammonium bicarbonate and urea were added aseptically after autoclaving the media. Stock and experimental cultures were tested for bacterial contamination using the above media enriched with 0.1% peptone and 0.025% yeast extract (Eppley *et al.*, 1967).

The experiments were carried out in 20-l Pyrex carboys initially containing 15 l of medium. Cultures were aerated with sterilized ammonium-free air. Cultures were inoculated aseptically by means of a large syringe. Experiments were conducted in a constant temperature room at 18°C and irradiated at 0.055 ly min^{-1} by two vertical arrays of Sylvania daylight fluorescent lamps. Cultures were maintained on a 16:8h light:dark cycle.

In each of 3 separate experiments, 3 carboys were enriched with nitrate, ammonium or urea, respectively. Each experiment included appropriate initial measurements, and two samplings. This provided 3 sets of initial data, and 2 sets of data each for young, mature, and senescent cultures. All samples were taken in triplicate, but only two were routinely measured unless they were not in close agreement. Separate samples

were collected for Coulter Counter and chitan analyses (see below). Cells were collected by gentle vacuum filtration through Whatman GF/C glass-fiber filters or prebaked Selas Flotronics 0.8 μ silver filters, depending on the ensuing analysis. The filtrate was used for the analysis of extracellular products. Estimates of analytical variations are presented in Conover (1974); only a small portion of measured variation could be attributed to analytical error.

Nitrate, nitrite, and ammonium were analysed in the filtrates of the nitrate-grown cultures, urea and ammonium in the urea-grown cultures, and ammonium and nitrite in the ammonium-grown cultures. Dissolved organic nitrogen was measured in all filtrates. Nitrates and nitrites were analysed according to Strickland and Parsons (1968), ammonium following Johnston (1966), and urea by the urease procedure of McCarthy (1970) except that the resulting ammonium was analysed using the Johnston method.

Nitrogen from dissolved organic compounds was determined after mineralization with H_2SO_4 and H_2O_2 (Yuen and Pollard, 1954). If the filtrate was from the ammonium-grown culture, the alkaline sample (pH 8 to 9) was evaporated to dryness to drive off the ammonium before the mineralization procedure was started. Samples were cooled and neutralized with NaOH solution to the phenolphthalein end-point before bringing them to volume. An aliquot of neutralized sample was reacted with an equal volume of freshly prepared ninhydrin-hydrindantin reagent (Moore and Stein, 1954). After colour development, the samples were centrifuged and an aliquot of this material was suitably diluted with 50% ethanol, and the absorbance at 570 nm was measured with a spectrophotometer. Dissolved organic nitrogen in the urea medium was obtained by subtracting the nutrient urea determined by urea analysis from the total dissolved organic nitrogen. The dissolved organic nitrogen analyses were started immediately on fresh samples while all other filtrate samples were frozen for subsequent analysis.

Material for intracellular nutrient nitrogen measurements was retained on the GF/C filters. The filter and cells were treated with 2 ml of 6% trichloroacetic acid solution and immediately ground with a motor-driven teflon pestle in a tissue grinder. This extract was rinsed through a second filter with measured volumes of filtered sea water of known nitrogen nutrient concentration. These samples were stored at -20°C. After thawing, samples were neutralized with 4N NaOH and analysed.

Soluble free amino acids were immediately extracted from GF/C filtered cells with hot 80% ethanol, after adding norleucine as an internal standard. The material was subjected to at least three hot 80% ethanol extractions. The combined extracts were filtered, and evaporated to dryness in a rotary evaporator before being redissolved in hot distilled water, and passed slowly through a small column of Dowex 50 resin in the H+ form to absorb the amino acids. The resin was rinsed with distilled water, and the amino acids were eluted with 1 M NH_4OH and evaporated to dryness prior to analysis on a JEOL 5AH amino acid analyzer. Lithium buffers were used to improve the resolution of the non-protein amino acids (Bensen *et al.*, 1967).

Proteins were measured by modifying the Lowry method described by Price (1965). Cells were collected on GF/C filters, ground in 6% trichloroacetic acid, placed in a boiling water bath for 30 min, and then capped and frozen for later analysis. A set of standards containing bovine serum albumin was prepared simultaneously and treated in the same manner. After centrifuging the thawed samples, the supernatants were discarded. The sedimented materials were resuspended in Price's reagent C and heated at 55°C for 30 min. The samples were again centrifuged, aliquots were reacted with the final reagent, and the absorbance of the solutions was read at 750 nm with a spectrophotometer.

Pigments were extracted immediately from cells retained on GF/C filters following Strickland and Parsons (1968). The filter, $MgCO_3$, and cells were ground in 90% acetone and allowed to extract in a refrigerator for approximately 24 h. Chlorophylls *a* and *c* were measured following Wasley *et al.* (1970). Carotenoids were estimated using the equation of Strickland and Parsons (1968).

Cells collected on GF/C filters for carbohydrate analysis were digested in 0.5N H_2SO_4 in a boiling water bath for about 1 h, and then frozen. A set of calibration standards received the same treatment. Thawed samples and standards were centrifuged, and an aliquot was analyzed by the phenol-H_2SO_4 method of Dubois *et al.* (1956). Results were calculated as glucose equivalents.

Cells for total carbon-hydrogen-nitrogen analysis were collected on pre-baked silver filters, and dried and stored at room temperature (ca. 21°C) over P_2O_5. Prior to analysis, the filters were fumed with HCl overnight, dried at about 40°C *in vacuo* to drive off any remaining HCl and water, and stored again *in vacuo*

over P_2O_5. Analysis was made using an F & M CHN Analyzer which had been standardized with cyclohexanone-2:4 dinitrophenyl-hydrazene and commercial chitin. Samples were combusted at the maximum temperatures recommended by the manufacturer, as tests showed that this procedure gave the best C and N recovery characteristics.

A modification of the total CHN (carbon, hydrogen and nitrogen) procedure was used for total lipid determination. A second set of samples was collected on silver filters and extracted with a 2:1 mixture of methanol and chloroform (Bligh and Dyer, 1959, water omitted) for about 2 min while the filter remained in the filter holder. A second extraction with methanol:chloroform (1:1) was performed and repeated if any pigments remained, before a final ether rinse was administered. The filters were then stored, fumed, and analysed as described for the total CHN analysis. The carbon, hydrogen, and nitrogen values determined on the extracted filters were subtracted from the total carbon, hydrogen, and nitrogen values for total lipids which, in turn, were further corrected by subtracting the pigment values.

Chitan samples were obtained following the procedure of McLachlan *et al.* (1965). Volumes of whole culture were stirred momentarily in a Waring Blender to shear off the chitan fibers. Cells were sedimented by centrifugation and the supernatant containing the chitan was collected and frozen. After thawing, the chitan was collected on silver filters and washed with successive rinses of distilled water, methanol, chloroform, and ether. Filters were then processed as described for the total CHN samples.

Thalassiosira fluviatilis occurred as single cells in this culture regime. Growth of the cultures was monitored by frequent counts for total numbers and cell volume distribution using a Model Z_B Coulter Counter with a Channelizer. Aliquots of each culture were suspended in newly filtered sea water and counted immediately in triplicate.

In so far as possible, all counts and experimental samplings were started at the same time of each day to minimize complications from diurnal changes in constituents, especially those arising from any possible synchrony of the cultures.

All inocula were found to be axenic with the test media employed. No major bacterial contamination was encountered during the experimental periods, although tests showed some minor contamination in the older populations.

Results

Cell Numbers and Volumes

Growth curves for all *Thalassiosira fluviatilis* cultures were of the common sigmoidal form for both cell numbers and total cell volumes. With the exception of one nitrate-grown culture, the growth curves for each nitrogen source were reasonably good replicates. The maximum rate of cell division occurred between Days 4 and 7, and the maximum rate of increase in total cell volume was found between Days 4 and 8. Growth measured by total cell volume lagged slightly behind that for cell numbers. Cultures matured in the same time period irrespective of the nitrogen source.

In the subsequent discussion the state of culture development will be referred to as "young", "mature", and "senescent", depending on whether the cultures were undergoing rapid cell division, slower cell division as maximum cell counts were approached, or registering little net change following the attainment of maximum cell numbers or total cell volumes. Although these phases in culture development have been given the dimension of culture age, it is recognized that they were induced by changes in the nutritional, chemical and physical environment of the cells. The onset of senescence coincided with the depletion of nutrient nitrogen, although other nutritional and environmental factors may have contributed as well.

Different yields in cell numbers and total cell volumes were observed with the three nitrogen sources. The results are summarized in Table 1. All available data for mature and senescent cultures (Day 8 and days subsequent) were subjected to separate Kruskal-Wallis one-way analyses of variance by ranks (Siegel,

Table 1. *Thalassiosira fluviatilis*. Average cell numbers and total cell volumes of fully developed cultures grown on 3 nitrogen sources. Each value was obtained by pooling data for Day 8 from all 3 cultures with 4 sets of data for the 2 senescent cultures taken between Days 9 and 20

Nitrogen source	Mean cell nos. l^{-1}	% > smallest	Mean total cell volume (μ^3 l^{-1})	% > smallest
Nitrate	219×10^6	5.8	263×10^9	smallest
Ammonium	208×10^6	smallest	308×10^9	17.3
Urea	251×10^6	20.8	345×10^9	31.3

1956) to determine whether observed differences were statistically significant. Differences in cell numbers were significant at the 1% level and differences in total cell volumes were significant at the 5% level.

Average cell sizes were also compared in relation to culture age and nitrogen source (Table 2). Minimum average cell sizes were found either in initial measurements or during culture maturation. Maximum average cell size was achieved during later senescence. Ammonium-grown cells were on the average 10% larger than nitrate- or urea-grown cells.

Table 2. *Thalassiosira fluviatilis*. Average sizes of cells grown with nitrate, ammonium, or urea

Nitrogen source	Mean minimum cell size (μ^3)	Mean maximum cell size (μ^3)	% maximum > minimum	Average cell size, all data (μ^3)
Nitrate	987	1448	47	1193
Ammonium	1095	1577	44	1321
Urea	932	1493	60	1209

Distribution of Nitrogen

Tables 3, 4 and 5 summarize the distribution of the nitrogen in each experiment as it was partitioned between the several extra- and intra-cellular pools. The major features of the nitrogen distribution patterns are summarized in Fig. 1.

The nitrogen contained in the intracellular fractions was computed on a µM nitrogen basis and summed. This value was then compared with the total µM nitrogen determined in the CHN analysis. In all but four cases, total CHN nitrogen was greater than that computed from the summed components, and this difference is shown as "unaccounted for" in the intracellular data included in Tables 3-5 and Fig. 1. In these four cases, all fairly young ammonium- or nitrate-grown cultures, the sum of the components was greater than that determined from total CHN analysis by 7 to 38%.

The total nitrogen value from the CHN analysis or from the summed components, whichever was larger, was added to the total nitrogen value obtained by summing the nitrogen contained in the several components in the medium. If the sum of all these components was less then 92% of the initial total value, the difference was designated an "unaccounted for" portion in the medium. In two cases, indicated in Tables 4 and 5, the totals were about 20% greater than the initial values, indicating undetermined errors in the estimations.

As the precision of nitrogen recovery is not ideal in this study, the data must be viewed as first approximations. The overall results do, however, reflect the complexities of the interrelationships and permit a description of the partition of nitrogen as influenced by nitrogen source and culture age.

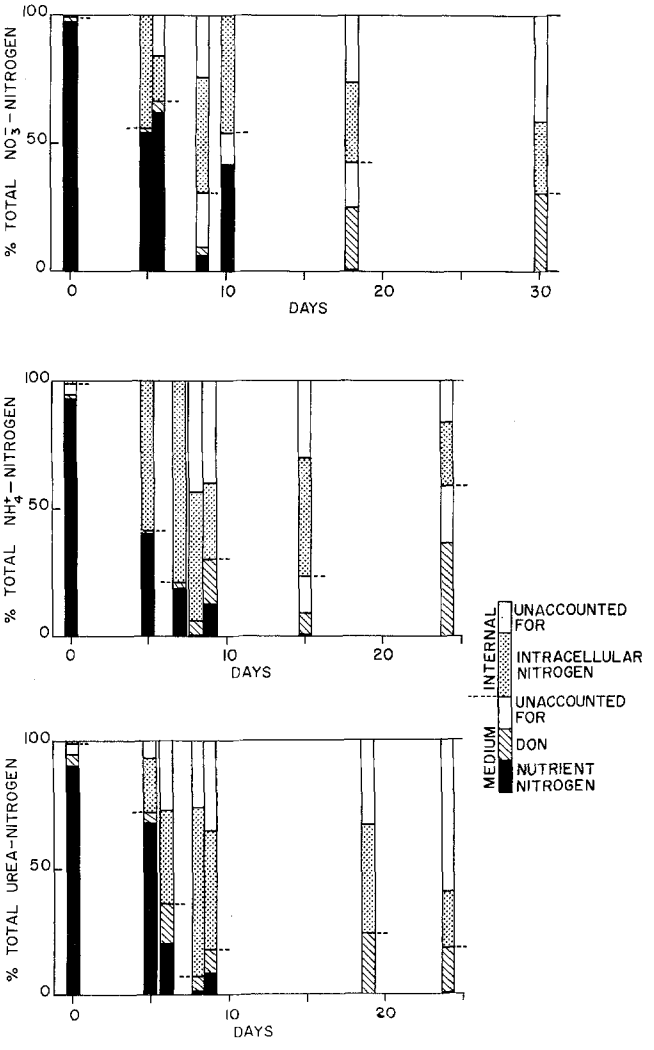


Fig. 1. Partitioning of total available nitrogen in medium and within cells of nitrate-, ammonium-, and urea-grown cultures of *Thalassiosira fluviatilis*. Total available nitrogen, 500 µM per culture, is represented as 100%. Each histogram represents 1 sample with exception of initial data which are averages of 3 samples. See text for calculation methods. DON: dissolved organic nitrogen

Table 3. *Thalassiosira fluviatilis*. Partitioning of nitrogen in cultures supplied with 500 µM nitrate-N. Total µM cell N was computed using either total CHN-N or total N, summed fractions, whichever was greater. Distribution of nitrogen amongst intracellular fractions is computed on percentage basis for comparison with similar data for ammonium and urea in Tables 4 and 5, respectively. See text for calculation procedure for "unaccounted for" portions. Values for cell numbers per liter and total cell volumes per liter are also included. DON: dissolved organic nitrogen

Nitrogen	Days						
	0	5	5.5	8.5	10	18	30
Extracellular							
Nutrient N (µM)	488	273	312	29	207	45	6
DON (µM N)	9	7	23	20	0	126	155
Unaccounted for N (µM)	0	0	0	106	67	87	0
Intracellular							
Total cell N (µM)	3.5	237	164	329	210	273	340
Total CHN-N (pM/cell)		1.83	1.27	1.87	0.75	1.55	1.27
Total N, summed fractions (pM/cell)		2.72	0.68	1.21	0.84	0.85	0.51
Percentage distribution of intracellular nitrogen computed as fractions of total CHN-N or of summed fractions, whichever is greater							
Nitrate		18.2	8.9	1.3	37.5	0	0
Nitrite		0.1	0.2	0.2	0.4	0.3	0.1
Ammonium		1.0	4.4	0	13.6	0	0
Amino acids		3.6	1.1	2.6	3.4	1.6	0.7
Protein		55.7	15.8	45.9	13.3	27.0	13.0
Chitan		4.6	8.3	13.5	13.9	25.8	24.1
Pigment		0.8	0.9	1.0	1.0	0.1	0.1
N extracted with lipid		16.0	13.7	0	17.0	0	2.2
Unaccounted for N		T[a]	46.7	35.5	T[a]	45.2	59.8
Population measurements							
No. of cells x 10^6 l^{-1}		87	129	176	250	176	268
Total cell volume x 10^9 μ^3 l^{-1}		103	123	226	263	257	410

[a] T: Total CHN-N less than total summed fractions.

Extracellular Nitrogen

Nutrient Nitrogen. External nutrient nitrogen was nearly all consumed by the time cultures entered the mature phase. Considerable enrichments (up to six times initial values) of nitrite were found in the medium in young and mature-phase nitrate-grown cultures. This phenomenon was first observed by Orr (1926) with marine diatoms, and has been reported several times since. It is due to nitrite formed by nitrate reductase within the cells accumulating and leaking out into the medium more rapidly than it can be reduced to ammonium internally. Small amounts of ammonium accumulated in the medium of some of the younger nitrate- or urea-grown cultures; this was almost certainly the result of leakage from the internal pool of metabolically produced ammonium although it could, in the case of the urea-grown culture, be interpreted as evidence for an extracellular urea-splitting enzyme.

Dissolved Organic Nitrogen. Little dissolved organic nitrogen was found in the young cultures. These products tended to accumulate with age, and apparently were not used as a nitrogen source to regenerate the cultures.

Table 4. *Thalassiosira fluviatilis*. Partitioning of nitrogen in cultures supplied with 500 µM ammonium-N. Total µM cell N was computed using either total CHN-N or total N, summed fractions, whichever was greater. Distribution of nitrogen amongst intracellular fractions is computed on percentage basis for comparison with similar data for nitrate and urea in Tables 3 and 5, respectively. See text for calculation procedure for "unaccounted for" portions. Values for cell numbers per liter and total cell volumes per liter are also included

Nitrogen	Days						
	0	5[a]	7	8	9	15	24
Extracellular							
Nutrient N (µM)	471	201	95	24	63	29	4
DON (µM N)	3	2	13	32	88	41	185
Unaccounted for N (µM)	24	0	0	0	0	75	110
Intracellular							
Total cell N (µM)	3	367	422	469	355	416	210
Total CHN-N (pM/cell)		1.93	1.35	2.12	1.83	2.02	1.11
Total N, summed fractions (pM/cell)		3.53	2.05	1.14	0.72	1.24	0.69
Percentage distribution of intracellular nitrogen computed as fractions of total CHN-N or of summed fractions, whichever is greater							
Ammonium		66.4	66.0	3.3	0	13.2	0.1
Amino acids		3.2	2.1	2.1	4.0	1.3	2.3
Protein		23.8	18.4	29.1	21.4	19.1	10.5
Chitan		2.1	4.0	8.5	12.8	14.3	48.8
Pigment		0.6	0.6	1.1	0.9	0.4	0.1
N extracted with lipid		3.9	8.8	10.0	0	13.4	0
Unaccounted for N		T[b]	T[b]	46.0	60.9	38.4	38.3
Population measurements							
No. of cells x 10^6 l^{-1}		104	206	221	194	206	189
Total cell volume x 10^9 μ^3 l^{-1}		129	199	315	310	298	344

[a] Combined totals of extracellular and intracellular nitrogen ca. 20% greater than initial total.

[b] T: Total CHN-N less than total summed fractions.

Intracellular Nitrogen

Intracellular nitrogen data is presented in Tables 3-5. Figs. 2, 3 and 4 present the relative pool sizes and probable pathways of nitrogen metabolism for representative samples of young, mature, and senescent-phase cultures supplied with the three sources of nutrient nitrogen.

Nutrient Nitrogen. Young nitrate- and ammonium-grown cells accumulated large internal concentrations of their respective nitrogen nutrients. The maximum internal nitrate pool measured represented a concentration factor of 1560. The maximum ammonium concentration factor determined was 13753 and, in this case, it represented more than 50% of all the nitrogen in the culture.

Free Amino Acids. The size of the free amino acid pool was markedly reduced with age as the pool sizes in senescent cells ranged from 1/4 to 1/10 the size of the pools found in the youngest cells examined.

Seventeen amino acids and amides were found in this organism: alanine, β-alanine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, glycine,

Table 5. *Thalassiosira fluviatilis*. Partitioning of nitrogen in cultures supplied with 500 µM urea-N. Total µM cell N was computed using either total CHN-N or total N, summed fractions, whichever was greater. Distribution of nitrogen amongst intracellular fractions is computed on percentage basis for comparison with similar data for nitrate and ammonium in Tables 3 and 4, respectively. See text for calculation procedure for "unaccounted for" portions. Values for cell numbers per liter and total cell volumes per liter are also included

Nitrogen	Days						
	0	5	6	8	8.5	19	24[a]
Extracellular							
Nutrient N (µM)	453	339	104	5	42	0.4	0.5
DON (µM N)	23	22	81	32	38	121	92
Unaccounted for N (µM)	22	0	0	23	0	0	0
Intracellular							
Total cell N (µM)	3.5	150	322	399	441	375	434
Total CHN-N (pM/cell)		2.05	1.72	1.65	2.49	1.37	1.81
Total N, summed fractions (pM/cell)		1.52	0.96	1.15	1.45	0.78	0.49
Percentage distribution of intracellular nitrogen computed as fractions of total CHN-N or of summed fractions, whichever is greater							
Urea		0.9	0	0	0	0.2	1.1
Ammonium		2.5	0.02	3.8	0	5.5	0
Amino acids		6.7	5.7	3.6	2.0	2.1	1.0
Protein		38.9	26.1	25.1	30.3	30.9	8.3
Chitan		7.2	7.1	13.4	7.5	15.6	16.2
Pigment		0.8	1.0	1.0	0.8	0.2	0.2
N extracted with lipid		17.3	16.3	22.7	17.7	2.3	0.1
Unaccounted for N		25.7	43.8	30.3	41.7	43.2	73.0
Population measurements							
No. of cells x 10^6 l^{-1}		73	187	242	177	274	240
Total cell volume x 10^9 μ^3 l^{-1}		80	174	340	235	370	353

[a] Combined totals of extracellular and intracellular nitrogen ca. 20% greater than initial total.

isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine. Only 6 of these were major components, i.e., each frequently accounting for 5% or more of the total free amino acid pool. These were alanine, asparagine, aspartic acid, glutamine, glutamic acid, and proline. Aspartic acid and glutamine-glutamic acid represented approximately the same proportion of the total pool regardless of the pool size, the culture age, or the nitrogen source. Alanine remained a constant proportion of the total pool as the cultures aged, but there may have been a slight alanine enrichment in ammonium-grown cultures and a little impoverishment in the nitrate-grown cultures. Very young cultures had large proline pools, regardless of the nitrogen source. Asparagine enrichment was characteristic of both young ammonium- and urea-grown cultures, while nitrate-grown cultures had detectable amounts of asparagine on only one occasion.

Protein. The youngest cells analyzed contained the largest amounts of protein, and the oldest cells contained the smallest amounts with the exception of a single value for the mature phase of one nitrate-grown culture.

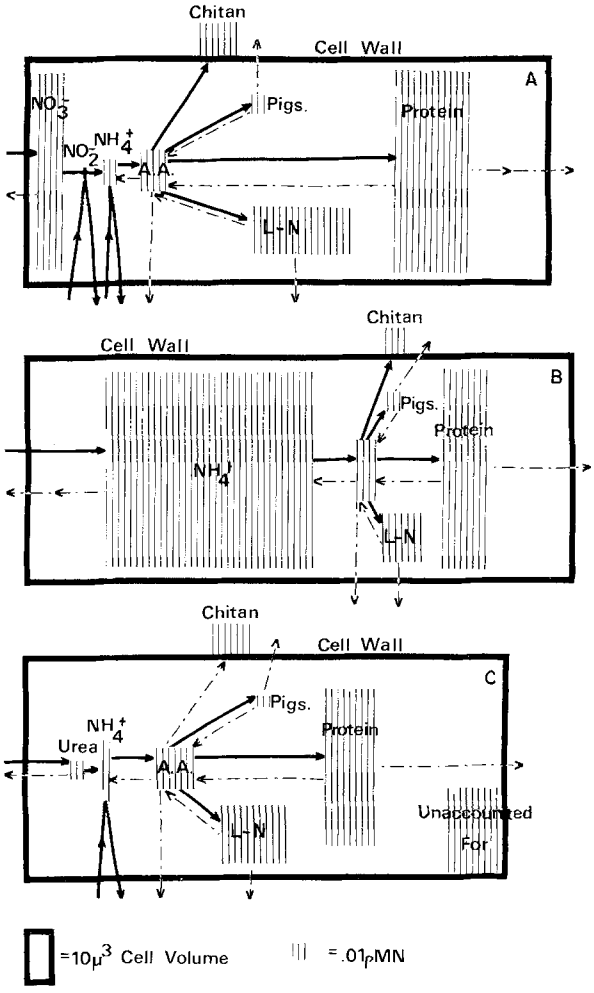


Fig. 2. *Thalassiosira fluviatilis*. Relative size of internal nitrogen pools of cells supplied with nitrate, ammonium, or urea. Average cell volume is represented by areas enclosed by solid lines. Solid arrows indicate pathways known with reasonable certainty, dashed lines possible metabolic pathways. A.A.: amino acids; Pigs.: pigments; L-N: nitrogen extracted with lipid fraction. Representative young-phase cells. (A) Nitrate-grown culture, Day 5; (B) ammonium-grown culture, Day 5; (C) urea-grown culture, Day 5

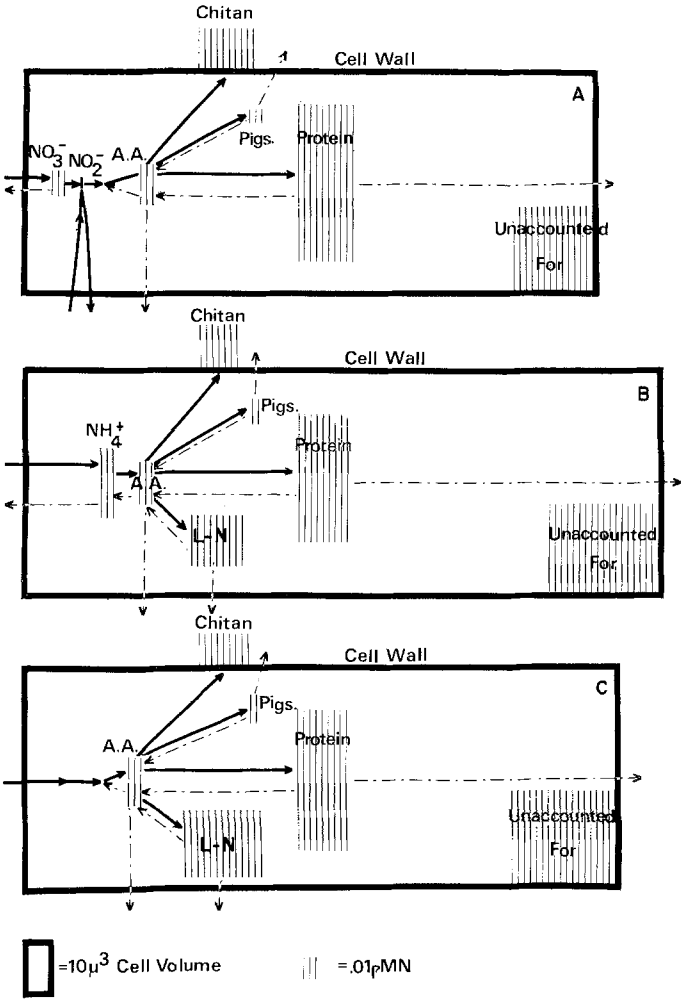


Fig. 3. *Thalassiosira fluviatilis*. Relative size of internal nitrogen pools of cells supplied with nitrate, ammonium, or urea. Representative mature-phase cells. (A) Nitrate-grown culture, Day 8.5; (B) ammonium-grown culture, Day 8; (C) urea-grown culture, Day 8.5. For further explanation see legend to Fig. 2

Chitan. Chitan accumulated in all cultures as the population aged, and this appeared to be a continuous process, rather than one which reached a plateau and then ceased at some point in the aging process.

Pigments. Chlorophylls *a* and *c* contain nitrogen, and so are included in Figs. 2-4; for convenience, carotenoids will be considered in this section as well. On a per cell basis, maxima for chlorophyll *a*, chlorophyll *c*, and the carotenoids were found in either the young or the mature phases, and minima were found in the oldest cultures. For example, the younger nitrate-grown cells had 26 times the chlorophyll *a*, 15.4 times the chlorophyll *c*, and 3.7 times the carotenoids of the oldest cells. This pattern of changing pigment concentrations was consistent for cells grown on all three nitrogen sources. The only significant differences in carotenoid:chlorophyll *a* and chlorophyll *c*:chlorophyll *a* ratios were found in senescent cultures (Fig.5).

Lipid-Associated Nitrogen. Maximum values per cell of nitrogen associated with the lipid fraction could be found any time

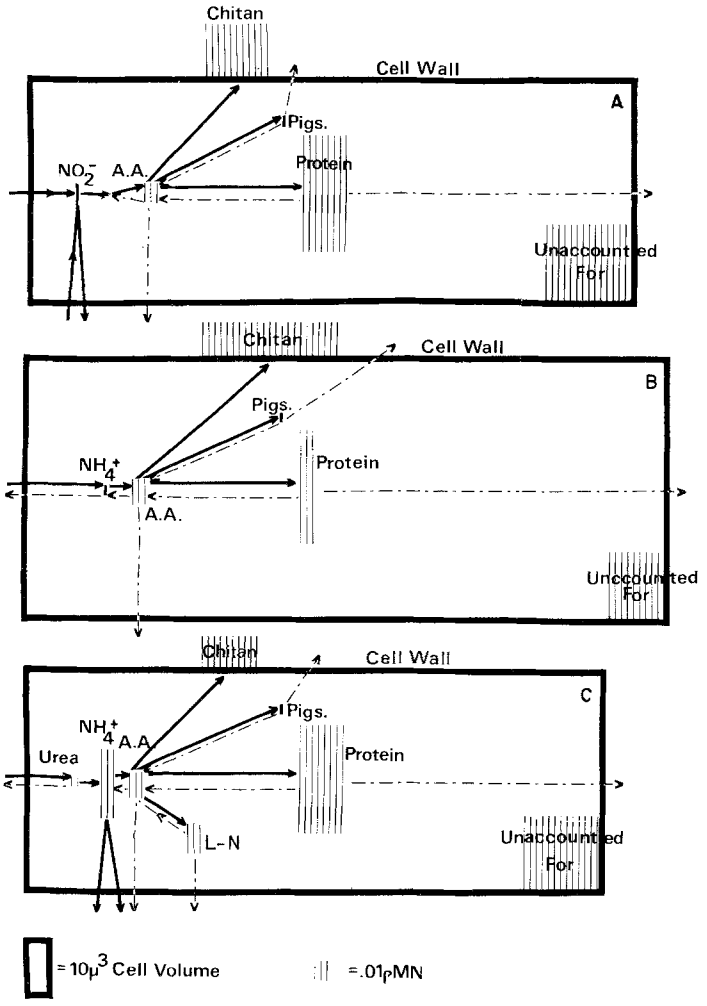


Fig. 4. *Thalassiosira fluviatilis*. Relative size of internal nitrogen pools of cells supplied with nitrate, ammonium, or urea. Representative senescent-phase cells. (A) Nitrate-grown culture, Day 18; (B) ammonium-grown culture, Day 24; (C) urea-grown culture, Day 19. For further explanation see legend to Fig. 2

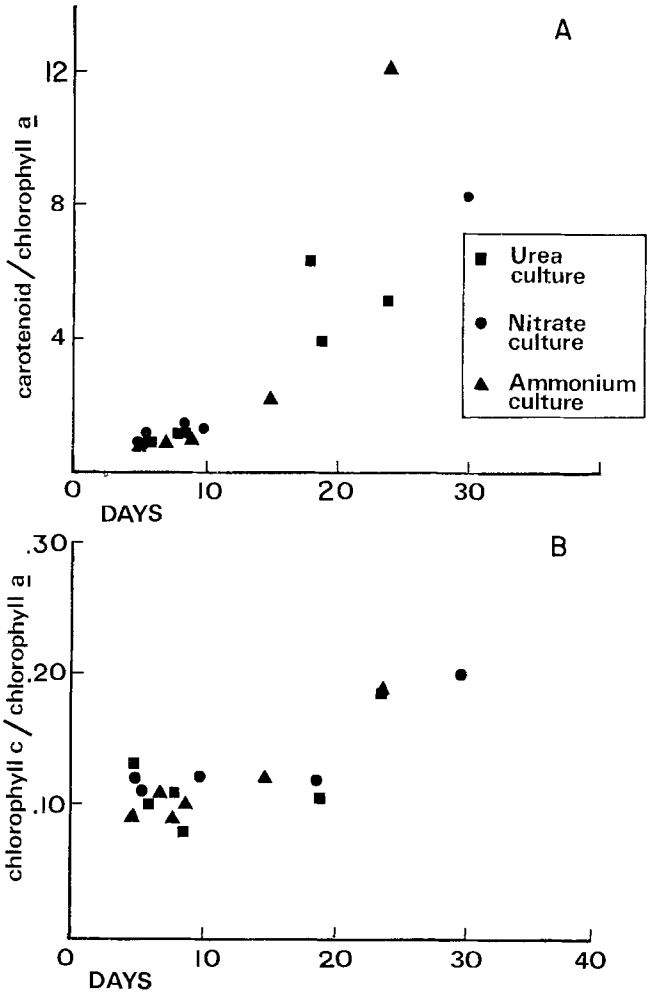


Fig. 5. *Thalassiosira fluviatilis*. Changes in carotenoid:chlorophyll *a* and chlorophyll *c*:chlorophyll *a* ratios with time (age) in nitrate-, ammonium-, and urea-grown cultures. All experimental data are included

from the young phase to early senescence. The oldest cells always had the least nitrogen associated with the lipid fraction. There were four cases where there was no apparent nitrogen in the lipid extract. Because this nitrogen fraction was determined by difference calculation, this absence of nitrogen was considered to be an artifact rather than a true estimate.

Total Cellular Nitrogen. Using data from CHN analysis, maximum nitrogen concentrations were found in mature phase cells. Minimum values were approximately half as much, and were found in the senescent cells. It should be recalled that in four cases the total CHN nitrogen value was less than that determined from the sum of the components.

Distribution of Non-Nitrogenous Components

Intracellular Components

Several analyses of non-nitrogenous components were made which contribute to the understanding of the physiology of *Thalassiosira fluviatilis* relative to nitro-

gen source and age. In addition, the comparison between total CHN carbon and the sum of the carbon contained in the several components (Tables 6-8) provides a check on the nitrogen budget. Certain assumptions were made in selecting conversion factors in these carbon computations. Amino acid nitrogen data were converted to carbon using a molecular C:N ratio of 4.887 derived from the free amino acid distribution data. Data of Cheucas and Riley (1969) were used to estimate a similar ratio for protein of 3.442. Carbohydrates were computed as glucose (weight of carbohydrate x 0.4/12.01), and the carotenoids as 85% carbon by weight (weight of carotenoid x 0.85/12.01), assuming 1 µSPU (Specific Pigment Unit, see Strickland and Parsons, 1968) = 1 µg. The agreement between the CHN data and the summed components is much better with the carbon data than with the nitrogen data (Tables 3-5), suggesting that a certain degree of confidence in the various measurements is justified.

Lipids. Maximum lipid accumulations were usually found in mature to late senescent phase cultures.

Carbohydrates. Generally the minimum carbohydrate levels (as pM C) were found in the youngest cells, while maximum amounts were observed in the oldest ones regardless of the nitrogen source supplied to the cells. It was concluded that actively growing cells contained a minimum of carbohydrate reserves.

Total Carbon. Maximum total intracellular carbon was found in senescent cells. The minimum amount of carbon per cell was always found in the second young-phase sample except in the case of the mature phase of the one atypical nitrate-grown culture. Urea-grown cultures averaged the most, and nitrate-grown cultures the least, total carbon per cell.

The percentage distribution of moles of carbon amongst the several measured fractions is also presented in Tables 6-8. The percentage of the total intracellular carbon found in amino acids, proteins, and plant pigments declined with age. Chitan carbon remained as a relatively constant proportion of the total carbon, and increased only in late senescence except in the urea-grown culture. Lipids accounted for less of the total carbon in the youngest cells than in succeeding stages. Carbon in the lipid fraction accounted for the largest proportion of total cell carbon in all but two (both young-phase cultures) of the 18 samples measured. Carbohydrate carbon was a elatively constant proportion in all phases of culture development and averaged approximately 20% of the total carbon. Urea-grown cells tended to put more carbon into the lipid fraction, otherwise there were no major differences in relative size of the various fractions attributable to nitrogen source.

Table 6. *Thalassiosira fluviatilis.* Partitioning of intracellular carbon in cells grown on nitrate. Values for culture age, total carbon from CHN analysis, and total carbon computed from summed fractions are presented. Percentage distribution of carbon amongst fractions is also included. See Table 3 for cell numbers and total cell volumes

Carbon	Days					
	5	5.5	8.5	10	18	30
Total CHN-C (pM/cell)	12.72	11.15	26.16	6.90	29.17	9.64
Total C, summed fractions (pM/cell)	15.71	12.34	24.38	5.53	21.71	7.35
Percent distribution of carbon computed as fractions of total CHN-C or of summed fractions, whichever is greater						
Amino acids	3.0	0.6	0.9	2.1	0.4	0.5
Protein	33.2	5.6	11.3	5.6	4.9	5.9
Chitan	6.4	6.8	7.7	13.6	10.9	25.3
Lipid	35.1	58.0	51.9	32.2	40.3	27.9
Carbohydrate	18.7	26.4	19.1	23.0	17.2	15.7
Chlorophyll *a*	1.7	1.1	0.8	1.4	0.1	0.1
Chlorophyll *c*	0.2	0.1	0.1	0.2	trace	trace
Carotenoids	1.8	1.4	1.4	2.1	0.6	1.0
Unaccounted for	0	0	6.8	19.9	25.6	23.7

Ratios

Molecular C:N. Using CHN analysis data, the molecular C:N ratio per cell was found to increase with culture age (Fig. 6). There was no significant difference due to nitrogen source, so a linear regression of C:N ratio on age was fitted to all the data, except the Day 30 value for the atypical nitrate-grown culture. The regression coefficient was significant at $P < 0.001$. A nonlinear function might provide a better mathematical description, since intracellular carbon tended to increase with cell age, while total intracellular nitrogen tended to decrease.

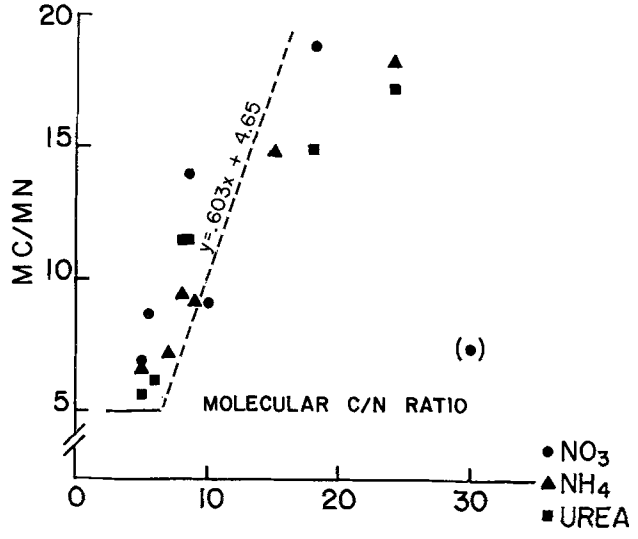
Carbohydrate:Protein. This ratio was calculated using carbohydrate and protein values determined by direct analysis, and thus differs slightly from that calculated by Myklestad and Haug (1972) and Haug *et al.* (1973) who mathematically converted t o t a l cell nitrogen measurements to protein.

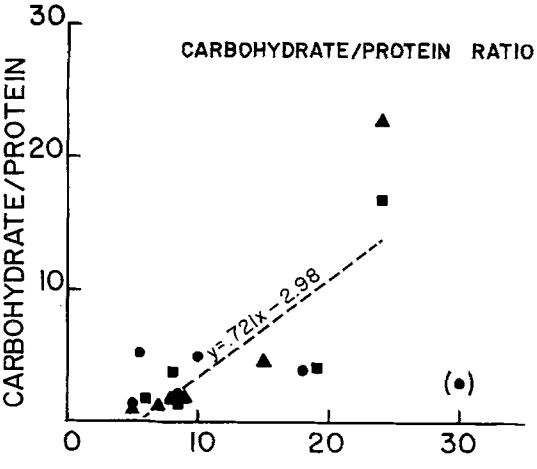
Table 7. *Thalassiosira fluviatilis*. Partitioning of intracellular carbon in cells grown on ammonium. Values for culture age, total carbon from CHN analysis, and total carbon computed from summed fractions are presented. Percentage distribution of carbon amongst fractions is also included. See Table 4 for cell numbers and total cell volumes

Carbon	Days					
	5	7	8	9	15	24
Total CHN-C (pM/cell)	12.51	9.89	20.32	16.83	30.02	20.32
Total C, summed fractions (pM/cell)	9.62	9.96	15.89	13.12	29.92	23.18
Percent distribution of carbon computed as fractions of total CHN-C or of summed fractions, whichever is greater						
Amino acids	4.4	2.1	1.1	2.2	0.4	0.5
Protein	23.1	13.1	10.4	8.0	4.4	1.7
Chitan	4.8	6.7	7.0	11.2	7.7	18.7
Lipid	16.8	49.4	32.7	36.2	68.1	44.8
Carbohydrate	23.5	25.3	23.4	17.6	17.7	33.3
Chlorophyll *a*	2.0	1.6	1.4	1.2	0.4	0.1
Chlorophyll *c*	0.2	0.2	0.2	0.1	trace	trace
Carotenoids	2.2	1.7	2.0	1.5	1.0	0.9
Unaccounted for	23.1	0	21.8	22.0	0.3	0

Table 8. *Thalassiosira fluviatilis*. Partitioning of intracellular carbon in cells grown on urea. Values for culture age, total carbon from CHN analysis, and total carbon computed from summed fractions are presented. Percentage distribution of carbon amongst fractions is also included. See Table 5 for cell numbers and total cell volumes

Carbon	Days					
	5	6	8	8.5	19	24
Total CHN-C (pM/cell)	12.22	10.67	18.99	28.71	20.49	31.68
Total C, summed fractions (pM/cell)	11.30	9.44	21.45	26.17	20.01	29.71
Percent distribution of carbon computed as fractions of total CHN-C or of summed fractions, whichever is greater						
Urea	0.1	0	0	0	trace	trace
Amino acids	5.5	4.4	1.3	0.8	0.7	0.3
Protein	22.4	14.5	6.6	9.0	7.1	1.6
Chitan	9.6	9.1	8.2	5.2	8.4	7.5
Lipid	24.0	35.0	59.8	63.2	55.7	60.5
Carbohydrate	27.4	21.4	21.7	10.6	24.7	23.1
Chlorophyll *a*	1.5	1.9	0.9	0.9	0.2	0.1
Chlorophyll *c*	0.2	0.2	0.1	0.1	trace	trace
Carotenoids	1.7	1.9	1.3	1.3	0.9	0.6
Unaccounted for	7.6	11.5	0	8.9	2.3	6.2





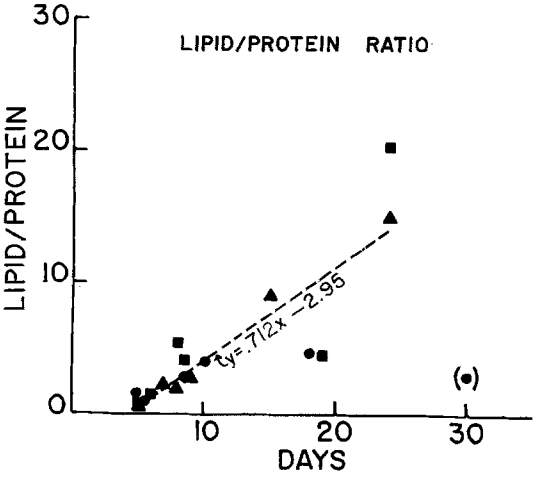


Fig. 6. *Thalassiosira fluviatilis*. Changes in cell C:N (molecular basis), cell carbohydrate: protein, and cell lipid:protein with time (age) in nitrate-, ammonium-, and urea-grown cultures. All experimental data are included. Linear regressions for all data through Day 24 are indicated. The atypical Day-30 nitrate-grown culture values (in parentheses) were omitted from regression calculations

The carbohydrate:protein ratio increased with age and no significant difference due to nitrogen source was observed. The correlation coefficient for a linear regression fitted to these data, again omitting the Day 30 nitrate-grown value, was significant at $P < 0.001$. The distribution of data points shown in Fig. 6 suggests caution in using this relationship as a predictive tool. A nonlinear mathematical description might be better for this data as well as the C:N data, since carbohydrate tended to increase and protein to decrease with age in *Thalassiosira fluviatilis*.

Lipid:protein. To compute this ratio, the weight of lipid was estimated as pM lipid C per cell x 12 x 1.2, and then divided by pg protein per cell. Results are shown in Fig. 6. The ratio increased with age, and was independent of nitrogen source. The linear regression of lipid:protein on age (30-day nitrate-grown culture value omitted) had a correlation coefficient significant at $P < 0.001$, although this data, too, may be nonlinear. Because lipid constitutes the largest carbon fraction and shows large variations with age, this ratio might be a useful indicator of culture age and nitrogen depletion in *Thalassiosira fluviatilis*.

Discussion

Thalassiosira fluviatilis was able to use all three nitrogen sources. No single nitrogen source conferred any advantage in terms of culture development (growth rate). Similar results were noted by Grant *et al.* (1967). Caperon and Meyer (1972a) found equal maximum specific growth rates for a variety of phytoplankton organisms with ammonium and nitrate, and Paasche (1971) found either equal or different growth rates in *Dunaliella tertiolecta* cultures supplied with nitrate or ammonium, depending on the light regime. In the present study with *T. fluviatilis*, there were small but significant differences in yields of cell numbers and total cell volumes per unit of culture volume with the three nitrogen sources. Average ammonium-grown cells were larger than urea- or nitrate-grown cells, although culture age was a much more important factor affecting cell size than nitrogen source.

Some possible explanations can be advanced for the "unaccounted for" nitrogen fractions. Extra-cellular ammonium-N may have been lost to the atmosphere since the pH of the cultures was greater than 8, but three instances of apparent loss of nitrate from the media cannot be accounted for. The discrepancy between total intracellular CHN-N and N in the summed fractions when the former is larger was due in part to the measurement of only the free amino acid component of the "soluble" or "peptidal" nitrogen pool (see Synge, 1968). Data of Hattori (1958) and Lui and Roels (1972) suggest that 5 to 15% of the total cell nitrogen could have been lost in this unsampled fraction. When the total summed N fractions were greater than total CHN-N, perhaps the large pools of intracellular nutrient nitrogen were not adequately measured by CHN analysis or were overestimated by the chemical tests. In spite of imperfectly balanced nitrogen and carbon budgets, considerable insight on nitrogen and carbon metabolism was obtained from these data.

There was one significant difference in the method of utilizing the three nitrogen nutrients by *Thalassiosira fluviatilis:* nitrate and ammonium were accumulated in large internal pools, but little urea was stored intracellularly.

Uptake and metabolism of the various nitrogen nutrients are known to be independent acts in at least some instances. Heimer and Filner (1970, 1971) clearly separated nitrate uptake from the first step in nitrate reduction; McCarthy (1972) was able to demonstrate that urea uptake was independent of subsequent metabolism. Ammonium uptake and assimilation are known to be very rapid, a matter of seconds in *Escherichia coli* (Schutt, 1972) and 1 to 2 min in *Chlorella* species (Hiller, 1970; Kanazawa *et al.*, 1972), so it is more difficult to separate the two processes.

The pathways and enzymes of internal nutrient nitrogen utilization are relatively well known. Nitrate reduction is catalyzed by nitrate and nitrite reductases, and urea by urease (present in the only diatom tested to date; see Leftly and Syrett, 1973), or urea amidolyase. Thus nitrogen, no matter what its original form, is metabolically assimilated as ammonium with the possible exception of certain exogenously-supplied amino acids.

The size of the internal nutrient pools in *Thalassiosira fluviatilis* may provide information on limiting steps in nutrient nitrogen reduction and assimilation. The internal nitrite pool was much smaller than either the nitrate or the ammonium pools. While this could be due at least in part to leakage from the cells, and/or an estimation error due to the action of trichloroacetic acid on nitrite (Beevers and Hageman, 1969), it is consistent with the observations of

Hewitt *et al.* (1968) and Weissman (1972) that, when both systems are fully functional, the reduction of nitrite is more rapid than that of nitrate. Internal urea, when present at all, was found in lower concentrations than ammonium. An ammonium pool was found with all nutrient nitrogen sources and suggests that ammonium assimilation into organic compounds was a critical rate-limiting step in internal nitrogen metabolism. Lui and Roels (1972) found internal ammonium pools in *Biddulphia aurita* of variable size depending on the nitrogen source, as in this study. Eppley and Rogers (1970) also found internal ammonium pools in *Ditylum brightwellii,* but observed that the pool size did not vary with the nitrogen source. Ammonium in high concentrations is often toxic to plants including phytoplankton (Zgurovskaya and Kustenko, 1968), but these internal pools are apparently sufficiently segregated or otherwise rendered harmless, so that the *T. fluviatilis* cultures grew quite successfully. Internal pools were exhausted as the cultures aged and the external nutrient supply was depleted.

Nitrogen assimilation occurs when ammonium combines with internally synthesized carbon skeletons to form amino acids. In *Thalassiosira fluviatilis,* the free amino acid pool sequestered about 7 to 0.7% of the total cellular nitrogen, and 5.7 to 0.3% of the cellular carbon. These values agreed well with those found by other workers (Hattori, 1958; Lui and Roels, 1972). The variation in pool size with age has been noted in several studies with higher plants (Craven *et al.*, 1972; Lu and Smith, 1972) and algae (Daumas, 1968; Berland *et al.*, 1970).

The amino acid composition of the pool was similar to other algae, and indeed to plants in general. Proline appeared to be a temporary nitrogen sink under nitrogen-rich conditions; Citharel (1966) and Daumas (1968) also found large proline fractions in the free amino acid pools of other algae under conditions of nutrient enrichment.

A wide variety of plant materials show decreases in amounts of protein with age, although in many cases the aging process itself may be the result of exhaustion of the nitrogen source. In this study with *Thalassiosira fluviatilis,* the maximum values for protein nitrogen as a fraction of total cell nitrogen ranged from 56% in the nitrate-grown cultures to 29% in the ammonium-grown cultures, while the oldest cultures ranged from 13% in the nitrate-grown to 8% in the urea-grown ones. Even the maximum values measured here are below the average obtained by other workers, but the relatively good agreement in comparisons of summed internal fractions with the total measured carbon permits some confidence to be placed in the protein values. Two factors undoubtedly contributed to these apparently low values. First, the total cell nitrogen value includes internal pools of unmetabolized nutrient nitrogen not normally found in measurements of total cell nitrogen, and second, this species has a chitan component which also adds to the total cell nitrogen value. While nitrate seemed to promote largest protein accumulations in this species, other workers have found maximum protein production with other nitrogen sources, or equal amounts of protein were found irrespective of the nitrogen source. Consequently, each system must be investigated independently.

McLachlan and Craigie (1966) found that chitan continued to be synthesized under conditions of extreme nitrogen deficiency at the expense of pigment and other cytoplasmic components. In the present study, chitan accumulation also occurred even though amino acids, proteins, pigments, and lipid-associated nitrogen all decreased.

That chlorotic plants result from nitrogen deficiency has long been recognized, and Chu (1943) and Harvey (1953) observed this in diatoms. The pigments disappeared at different rates in the aging cells, but pigment ratios were here found to be useful indices of culture age only in extreme senescence.

Significant concentrations of dissolved organic nitrogen per unit of culture volume were not recorded until cultures passed into senescence. To test whether the low observed concentrations of dissolved organic nitrogen in younger cultures were simply a function of small population size, production of dissolved organic nitrogen was also calculated on a per cell basis. Significant "excretion" of dissolved organic nitrogen was characteristic only of senescent cells of *Thalassiosira fluviatilis.* There is no way of knowing whether the dissolved organic nitrogen compounds measured in the medium were products of anabolism or catabolism, excretion, secretion, osmotic leakage, or cell lysis. Increases in dissolved organic nitrogen were not usually accompanied by any marked decrease in cell numbers, suggesting that this accumulation may result from excretion of catabolized nitrogen compounds rather than from cell destruction.

There is also no way of knowing what proportion of the "excreted" material may have been reusable as nitrogen, carbon, or other nutrient sources. Total ni-

trogen per cell and several intracellular nitrogen fractions decreased as cells became more senescent. It was concluded that regeneration of nitrogen from dissolved organic compounds was insufficient to maintain cultures in a healthy state. As with chitan, potential nutrient nitrogen was removed from metabolic recycling by *Thalassiosira fluviatilis* once it entered this fraction.

As nitrogen was depleted and protein and other internal nitrogeneous compounds decreased, new photosynthate accumulated as lipid and carbohydrate in *Thalassiosira fluviatilis*. The lipid fraction was the most important carbon sink. The nutrient regime is known to affect lipid synthesis. Nitrogen deficiency promotes lipid accumulation in some systems (Collyer and Fogg, 1955; Strickland *et al.*, 1969; Werner, 1970), but this is not universal (Antia *et al.*, 1963; Tokuda, 1969). Myklestadt and Haug (1972) found alterations in amounts of lipid with changes in the N:P ratio (not concentration) in the media. Werner (1970) reported a marked increase in lipid and apparent enhancement of lipid synthesis in silica-deficient diatoms. Silica was not measured in this study with *T. fluviatilis*, so it is not known whether silica deficiency could have influenced lipid production in these cultures.

Minimum amounts of carbohydrate per cell coincided with the period of maximum overall synthesis and growth rate. Carbohydrates were less variable than lipids with age as a proportion of the total carbon of *Thalassiosira fluviatilis*.

Physiological events associated with nutrient depletion and culture aging are reflected in the C:N ratio, which increased as nitrogen became limiting and the cells entered senescence. Cells continued to accumulate carbon well into the stationary phase. The changes in internal nitrogen concentration were relatively small compared with those for carbon, hence the change in the ratio was largely due to carbon accumulation. C:N ratios were substantially similar in *Thalassiosira fluviatilis* cells irrespective of nitrogen source, even though amounts of carbon and nitrogen per cell were somewhat different. This contrasts with the situation for another member of this genus, *T. nordenskioldii*, where nitrate-grown cells produced more carbon per unit of nitrogen than did ammonium-grown material (Conover, 1974).

The carbohydrate:protein ratio might be used in conjunction with other data for assessing the age or physiological state of a population, but the relatively small change per unit of time and the scatter of data points suggest that it might be a less useful indicator than the C:N ratio. The lipid:protein ratio showed the best fit as a linear function of age in *Thalassiosira fluviatilis*, but even this relationship can only be interpreted if other information is available.

The results of this study are relevant to studies involving nutrient uptake and its relationship to growth. Droop (1968, 1973), Fuhs (1969) and Caperon and Meyer (1972a, b) have all demonstrated the difficulty of applying the Monod uptake-growth relationship to phytoplankton. They found that growth was not a direct function of nutrient uptake and did not describe a section of a rectangular hyperbola unless an additional factor, the internal cell nutrient concentration, was included in the description. If that is the case, then it needs to be determined whether the additional parameter(s) required can be satisfied by a single measure of total cell nutrient, or whether the factor is the result of a complex set of factors including intracellular pool size, transport, and turnover functions. Hattori (1960) found a linear relationship between total cell nitrogen and consumption (uptake) rates of urea and ammonium which suggests that the single measurement might do in the case of nitrogen, but uptake rates are notoriously variable depending on such factors as light, temperature, culture age, culture history, state of cell division, relative nutrient concentration, internal unassimilated nutrient pool size, etc., so that it would be surprising if a single measurement of total cellular nitrogen could suffice. This study with *Thalassiosira fluviatilis* suggests that assimilation rate rather than uptake rate would be a more meaningful parameter.

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