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Interactions of ammonium, nitrate, and D- and L-amino acids in the nitrogen assimilation of two species of estuarine benthic diatoms*

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ABSTRACT: Assimilation of D- and L-amino acids by axenic cultures of *Navicula salinarum* and *Amphiprora* cf. *paludosa* was measured by High Performance Liquid Chromatography (HPLC). Ammonium ions at concentrations of 5 to 100 µmol l^{-1} effectively suppressed the utilization of concentrations of 100 µmol l^{-1} of nitrate, but did not eliminate the uptake of amino acids in concentrations of 0.05 to 0.8 µmol l^{-1}. *N. salinarum* assimilated low concentrations of several amino acids parallel with high concentrations of ammonium, whereas *A.* cf. *paludosa* depleted the concentration of aspartic and glutamic acid more rapidly than that of ammonium. Nitrogen-deprived cultures of both species assimilated spikes of amino acids very rapidly: half of the concentration added was assimilated in 0.5 h. The assimilation of 6 D-amino acids was compared with that of the corresponding L-forms; the effect of the isomeric form on the uptake rate differed widely among the amino acids. The possible application of these uptake experiments to interstitial water is discussed.

INTRODUCTION

Marine algae are able to assimilate nitrogen from a variety of sources, e.g. ammonia, nitrate and organic nitrogen. Ammonia and nitrate are commonly regarded as the main source of nitrogen for phytoplankton, whereas the contribution of other sources, e.g. urea, has been measured only in a limited number of cases (McCarthy 1981, Kaufman et al. 1983, Kristiansen 1983, Price et al. 1985). Scarcely any data compare the use made of amino nitrogen and inorganic nitrogenous nutrients by natural assemblages of phytoplankton (Butler et al. 1979, Syrett 1981). Organic sources of nitrogen may be more important for microphytes inhabiting estuarine and marine sediments, than for the phytoplankton. Dissolved free amino acids have been found in concentrations as high as 10 µmol l^{-1} in interstitial waters (Jørgensen et al. 1980) and, moreover, the ability of numerous species of benthic diatoms to use amino acids as a nitrogen source or as a substrate for heterotrophic growth has been demonstrated in axenic cultures (Hellebust & Lewin 1977, Admiraal & Peletier 1979, Saks & Kahn 1979, Admiraal et al. 1984, 1986, Flynn & Syrett 1986). All the amino acids used in these experiments were in the 'natural' L-form, whereas it can be speculated that D-amino acids are relatively important in the benthos.

The simultaneous utilization of several inorganic and organic nitrogen sources by marine algae raises problems of metabolic regulation. An aspect relatively well studied for phytoplankton is the suppression of nitrate uptake by concentrations of ammonia of ca 1 µmol l^{-1} (cf. Syrett 1981). Maestrini et al. (1982, 1986) demonstrated that oyster-pond plankton and benthic microphytes showed ammonium thresholds for simultaneous uptake of ammonium and nitrate at concentrations of 7 to 40 µmol l^{-1} of ammonium. Furthermore, Liu & Hellebust (1974) showed that amino acids suppressed the nitrate reductase in the neritic *Cyclotella cryptica*. Recently, Flynn & Wright (1986) found that high con-

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centrations of L-arginine and ammonia are utilized simultaneously by the semi-planktonic, semi-benthic species *Phaeodactylum tricornutum*. These observations indicate that neritic and benthic micro-algae are highly versatile in assimilating sources of nitrogen.

Our research aimed to ascertain whether or not nitrate, ammonia and amino acids are simultaneously utilized by truly benthic diatom species. Experiments were performed with D- and L-amino acids in view of the possibility that both forms are abundant in intertidal sediments. Two estuarine test species were used: *Navicula salinarum*, which was abundant on an organically polluted mudflat, and *Amphiprora* cf. *paludosa*, which was isolated from a sandflat. The interaction of the 3 nitrogen sources was studied in long-term culture experiments and in short-term uptake experiments.

MATERIALS AND METHODS

Culture technique and analysis. Two benthic diatoms *Navicula salinarum* Grunow and *Amphiprora* cf. *paludosa* W. Smith, were isolated and grown axenically in synthetic seawater medium and were incubated at 14 °C under 14 h illumination per day at 100 µE m^{-2} s^{-1} (for details see Admiraal et al. 1984). L-amino acids (Sigma) and D-amino acids (Fluka) were added to culture media in concentrations of ca 1 µmol l^{-1} and their concentrations were measured by High Performance Liquid Chromatography (HPLC) of *o*-phthaldialdehyde derivates, following Lindroth & Mopper (1979). Samples of culture media were stored frozen for a few weeks before analysis. The L-amino acids used were: aspartic acid (asp), glutamic acid (glu), glycine (gly), histidine (his), arginine (arg), serine (ser), alanine (ala), valine (val) and leucine (leu). The following D-amino acids were tested: asp, arg, ser, ala, val and leu.

Diatom cells and media were separated by gentle filtration on glass fibre filters (Whatman GF/C). In contrast to the observations made by Fuhrman & Bell (1985) this treatment did not lead to leakage of cellular amino acids (cf. Admiraal et al. 1986).

Nitrate was measured by automated analysis, according to the method described by Strickland & Parsons (1968). Ammonia forms a weakly fluorescent derivative with *o*-phthaldialdehyde and this was measured by HPLC (Jørgensen et al. 1981). Protein was measured according to Lowry et al. (1951).

Experimental procedures. Two kinds of experiments were carried out: long-term culture experiments (of 14 d) and short-term uptake experiments (of 8 h). In the growth experiments low concentrations of nitrate-sufficient cells were inoculated into media to which 8 or 9 amino acids had been added, each in a concentration of ca 1 µmol l^{-1}.

However, most of the nitrogen was obtained from other sources: one culture flask contained ca 100 µmol l^{-1} of ammonia, another ca 100 µmol l^{-1} of nitrate, and the third contained nitrate plus ammonia. Concentrations of ammonia and nitrate used in the media were the same as those commonly observed in interstitial water (cf. Riaux-Gobin 1985). Cell concentrations (counted in microscope counting chambers) and concentrations of nitrogen in the medium were measured, until the cultures reached the stationary phase. Samples from the cultures were taken each day at the same time (1000 h) to avoid the potential effects of the light-dark cycle.

For the uptake experiments, diatom cells were grown in 2 conditions. Nitrate-sufficient cells were harvested from dilute cultures that initially contained 675 µmol l^{-1} of nitrate. Hence, these cells were saturated with nitrate. Nitrogen-deprived cells were obtained by growing cultures in media that initially contained 30 µmol l^{-1} of nitrate. This small nitrogen source was exhausted after ca 2 d; the nitrogen-limitation of the diatoms was evident from their decreased pigmentation. Nitrate-sufficient and nitrogen-starved cells were concentrated aseptically and dispersed in test media containing L- or D-amino acids. The uptake of the amino acids during incubations in the light was measured by HPLC. Control experiments were carried out without adding amino acid and with amino acids in the original culture medium (instead of being added fresh).

RESULTS

Long-term experiments

The presence of concentrations of ammonia higher than ca 5 µmol l^{-1} effectively suppressed the utilization of nitrate by *Navicula salinarum* (Fig. 1A) and *Amphiprora* cf. *paludosa* (Fig. 2A). L-amino acids were utilized by *N. salinarum* generally parallel with ammonia, even though their combined concentration was an order of magnitude lower than that of ammonia. In the presence of 100 µmol l^{-1} of nitrate the L-amino acids were assimilated more rapidly than the nitrate (Fig. 1). In *A.* cf. *paludosa* the available pool of amino acids, e.g. aspartic acid was more rapidly depleted than that of ammonium even though the concentration of the individual amino acids was only 1 % of the ammonium concentration.

In *Navicula salinarum* the 8 amino acids tested were taken up in roughly the same way, whereas *Amphiprora* cf. *paludosa* was very selective (Fig. 3), assimilating aspartic acid and glutamic acid in a very early stage

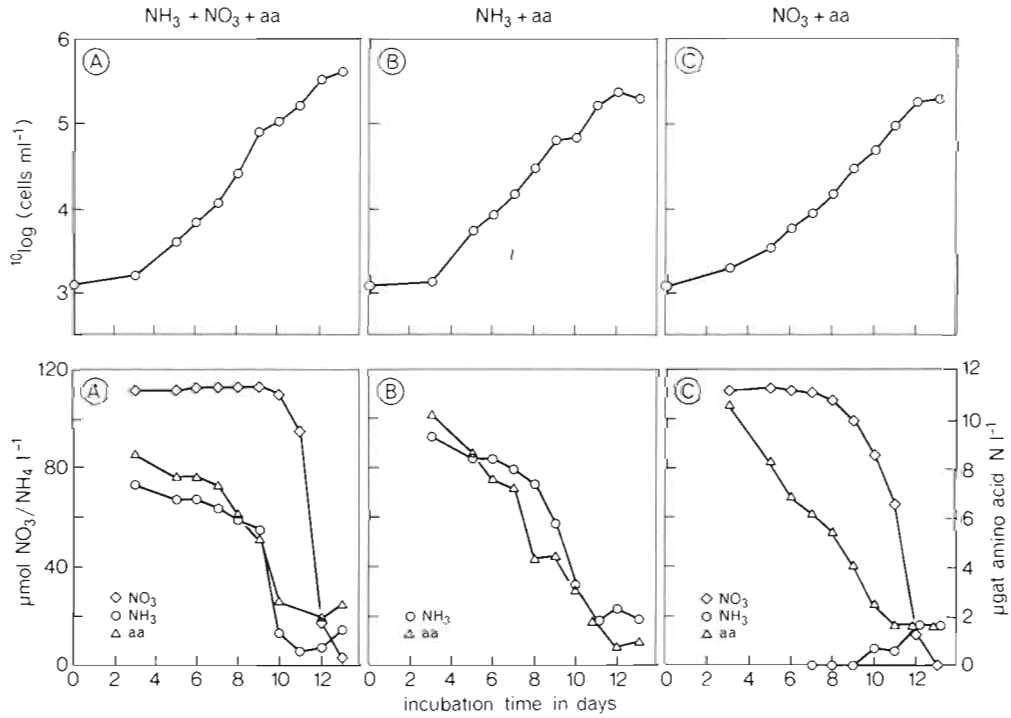

Fig. 1. *Navicula salinarum*. Development of concentrations of cells (upper plots) and nitrogen compounds (lower plots) in culture media supplied with a mixture of 8 amino acids (aa, totals given on right-hand scale) and with ammonia (B), or nitrate (C), or ammonia plus nitrate (A); concentrations on left-hand scale. Note the difference in scale

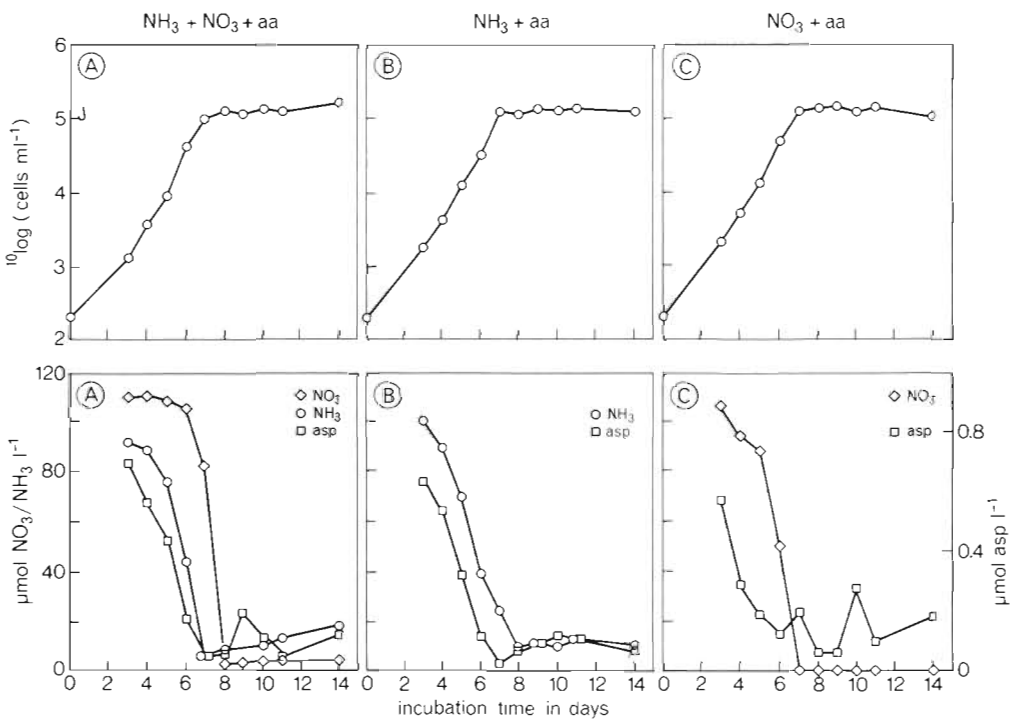

Fig. 2. *Amphiprora* cf. *paludosa*. As Fig. 1. Results for amino acids are given for aspartic acid only (for other amino acids see Fig. 3)

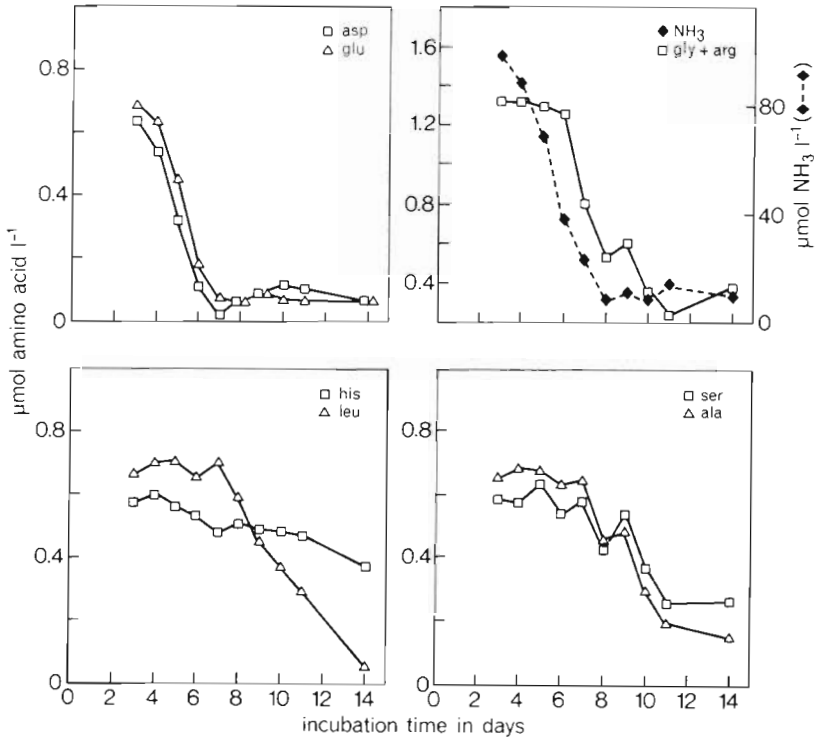

Fig. 3. *Amphiprora* cf. *paludosa*. Depletion of ammonia and 8 L-amino acids in cultures with these nitrogen sources (same experiment as in Fig. 2)

of growth, and serine, alanine, histidine and leucine in the stationary growth stage only.

Both diatom species seemed to release some ammonia and some amino acids at the end of the exponential growth or when growth stopped. This effect, attributed to the stress effect brought about by the changing nutrient conditions, had been observed earlier in planktonic diatoms (cf. Poulet & Martin-Jézéquel 1983, Admiraal et al. 1986).

Short-term experiments

D- and L-amino acids were taken up rapidly by *Navicula salinarum* not only when the cells were nitrogen-starved, but also when they had adapted to high concentrations of nitrate (Fig. 4). The decrease in the concentrations of amino acid in the media was exponential, consistent with the fact that half-saturation concentrations for uptake in diatoms are generally higher than the concentrations tested here (cf. Hellebust & Lewin 1977). The rate of amino acid decrease in the media was calculated from the data in Fig. 4 and from similar data on other amino acids and the other diatom species.

Table 1 summarizes the specific rates of decrease in dissolved amino acids, normalized to cell suspensions containing 1 mg protein l^{-1}; the actual concentration of protein varied between 3.5 and 5.8 mg l^{-1}.

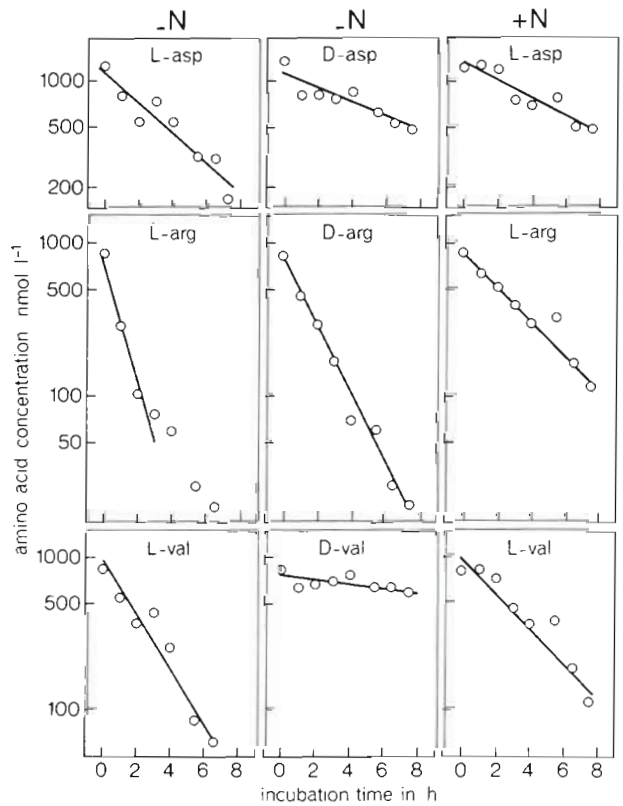

Fig. 4. *Navicula salinarum*. Short-term uptake of L- and D-amino acids in nitrogen-deprived (−N) and nitrate-sufficient (+N) cultures. Results for 3 out of 9 L-amino acids and 3 out of 6 D-amino acids are shown

In nitrogen-starved *Navicula salinarum* cultures 9 L-amino acids disappeared at a more or less uniform rate of 0.064 to 0.232 h^{-1} (mg prot l^{-1}). In contrast, *Amphiprora* cf. *paludosa* was highly active in the uptake of L-aspartic acid, L-glutamic acid and L-arginine, whereas other acids were assimilated only slowly (Table 1).

High concentrations of nitrate suppressed the short-term L-amino acid uptake by *Amphiprora* cf. *paludosa* nearly completely, in contrast to the response of *Navicula salinarum*.

The isomeric form of the amino acid had significant effects on the uptake by the diatoms. The uptake rate of D-aspartic acid and D-arginine acid was ca 60 % that of the corresponding L-forms. For D-alanine, D-serine, D-valine and D-leucine this percentage was 29, 14, 6 and 0, respectively. So leucine, taken up effectively by the 2 diatom species in the L-form, was not assimilated in the D-form during 7 h of exposure (Table 1).

Table 1. Decrease in concentrations of L-amino acids (L-aa) and D-amino acids (D-aa) in media of nitrate-depleted (−N) and nitrate-sufficient cultures (+N, initially containing 675 µmol l^{-1} of nitrate). Decrease calculated as specific rate of decrease (in h^{-1}) from Fig. 4 and other data, and normalized to cultures with 1 mg cell protein l^{-1} −: not tested

Amino acid	−N		+N
	L-aa	D-aa	L-aa
	(h^{-1} [mg prot l^{-1}]$^{-1}$)		(h^{-1} [mg prot l^{-1}]$^{-1}$)
Navicula salinarum			
asp	0.064	0.030	0.031
glu	0.077	–	0.040
gly	0.068	–	0.026
his	0.089	–	0.023
arg	0.232	0.141	0.058
ser	0.093	0.013	0.041
ala	0.175	0.051	0.045
val	0.111	0.007	0.056
leu	0.173	0.000	0.060
Amphiprora cf. *paludosa*			
asp	0.374	0.241	0.005
glu	0.305	–	0.002
gly	0.000	–	0.000
his	0.000	–	0.000
arg	0.440	0.291	0.000
ser	0.007	0.000	0.000
ala	0.009	0.000	0.000
val	0.000	0.000	0.000
leu	0.038	0.000	0.000

DISCUSSION

Antagonism of inorganic and organic nitrogen sources

The few data available so far indicate that benthic micro-algae regulate the uptake of inorganic and organic nitrogen sources in a way somewhat different from that used by the phytoplankton. The data presented confirm the observation made earlier by Maestrini et al. (1982, 1986) that oyster-pond algae, associated with the benthos, assimilate nitrate at considerably higher concentrations of ammonia than do truly planktonic micro-algae. Benthic diatoms, and some neritic forms too, seem to prefer organic nitrogen (e.g. amino acids) to inorganic nitrogen. In the case of nitrate this is illustrated by the response of *Navicula salinarum* in the present study. In accord with the findings of Flynn & Wright (1986) this species also utilized L-amino acids in the presence of very high concentrations of ammonia. Flynn & Wright (1986) also showed that L-arginine in high concentrations of 250 µmol l^{-1} suppressed the ammonium uptake. On the other hand, Shah & Syrett (1982) found that ammonia suppressed the uptake of guanine in the benthic diatom *Amphora coffeaeformis*. However, generally, the state of nitrogen nutrition of the cells is assumed to be the prime regulator of the rate of amino acid assimilation (Hellebust & Lewin 1977).

In summary, these observations may indicate that, despite the antagonistic effects, the order of preference of the benthic or neritic diatoms for the nitrogen sources we studied is: amino acids, ammonium and nitrate. The repression or induction of the assimilation of these 3 nitrogen sources deserves further research, such as the study done by Dortch & Conway (1984) on the interactions of nitrate and ammonia assimilation in phytoplankton.

Uptake of natural concentrations of amino acids

North (1975) was probably the first to attempt to measure amino acid uptake from interstitial water by micro-algae. She found that fluorescamine-positive material (mainly amino acids) diminished rapidly when nitrogen-limited *Platymonas* cells were added. Jørgensen et al. (1980) measured 3.9 to 28.5 µmol l^{-1} of dissolved free amino acids in interstitial water, harvested from a Danish estuary by pressure filtration or centrifugation. We tried to measure the concentrations of amino acids in interstitial waters, harvested by draining in order to avoid squeezing the organisms, and filtered immediately over sterile filters. We found the concentration of amino acid to be at least one order of magnitude lower than Jørgensen et al. (1980) and, more remarkably, the concentrations and composition to be variable. Current techniques of harvesting dissolved free amino acids (cf. Braven et al. 1984, Fuhrman & Bell 1985) and defining their chemical state (cf.

Christensen & Blackburn 1980) seem insufficient to elaborate the initial experiment by North (1975).

Diatoms were shown to assimilate amino acids from their medium at a rate of ca 0.1 h^{-1} (mg prot. l^{-1})$^{-1}$ (Table 1). Since we estimate the concentrations of diatom protein in the top layers of intertidal sediments in the range of 100 to 1000 mg prot. l^{-1}, one may hypothesize that the *in situ* turnover rate for amino acids in interstitial water realized by diatoms could be 0.17 to 1.7 min^{-1} Actually, Christensen & Blackburn (1980) measured a turnover rate of ca 0.2 min^{-1} for ^{14}C-labelled alanine injected into sediment cores. Hence, diatoms present in superficial sediment layers may participate to a significant extent in the turnover of free amino acids in the benthos.

The isomeric composition of dissolved free amino acids in the sediment is largely unknown. Bada & Hoopes (1979) found considerable amounts of D-alanine in sedimenting particular material in the top 500 m water layer of the oceans. Analogously, deposits of estuarine sediments could be enriched in D-amino acids; this prompted us to test the uptake of the 2 isomeric forms. Some of the D-forms were effectively utilized in this study, but there is no proof of actual cellular metabolism using these D-forms. However, Landymore & Antia (1977) found slow growth of *Navicula incerta* on D-tyrosine and D-phenylalanine after a long adaptation period. Jung & Lüttge (1980) found that D-alanine, as well as L-alanine, is taken up by duckweed. However, we found specific differences in the uptake of 6 pairs of D- and L-amino acids. Thus it seems that these differences need further evaluation before they can be used first to characterize the amino acid uptake systems in diatoms and secondly to estimate the relative importance of D-amino acids in the marine environment.

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