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Dissolved free amino acid cycling in an estuarine outflow plume

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ABSTRACT: Microbial cycling of dissolved organic C and N is a major pathway in marine ecosystems, and dissolved free amino acids (DFAAs) are significant components of this cycling. Measurements from 4 cruises (February, June, August 1985, and April 1986) in the outflow plume of Chesapeake Bay (USA) show that DFAAs cycle rapidly, with turnover times averaging 0.5 to 1 h in spring and summer and ca 3 h in winter, as measured by brief (10 min) tritiated tracer uptake and respiration experiments under extremely clean conditions. DFAA uptake and release rates, calculated from turnover and concentration measurements of four of the most abundant amino acids (glu, ser, gly, ala) as the ship followed a surface drogue, were closely coupled and varied over the day, often tending to be highest near noon and lowest at night, suggesting a connection to primary production. Rates also tended to decline as the relatively biologically-rich plume water mixed with less active coastal seawater. Winter rates were much lower than those in spring and summer. Despite concentrations in the low nM range, the release and uptake rates of these 4 amino acids represented a significant fraction (4 to 18 %) of the primary production rate, and incorporation of DFAA-C was 15 to 64 % of estimated bacterial secondary production. N uptake from these 4 DFAAs represented about 44 to 131 % of the calculated bacterial N demand, suggesting that the bacteria in this system probably regenerate inorganic N. One set of measurements in the Hudson plume in summer showed even faster DFAA cycling, with turnover times averaging < 7 min. Rapid turnover indicates that the relative extent of molecular (slow) and eddy (fast) diffusion of DFAAs between sources (various plankton) and sinks (mostly bacteria) may be a major rate-controlling factor at scales on the order of mm. The rapid turnover in both plumes suggests that DFAAs are not advected more than a few km, and tend to cycle within a small volume of water.

INTRODUCTION

Heterotrophic microorganisms play quantitatively significant roles in material and energy transfer in marine food webs, with up to 60 % of total primary production passing through the bacteria (Fuhrman & Azam 1980, 1982, Williams 1981, Azam et al. 1983). A major class of dissolved compounds that help mediate this transfer to marine bacteria is the dissolved free amino acids (DFAAs). Amino acids are among the most abundant monomers in all organisms, and can act as building blocks and energy sources for bacterial metabolism and growth. They contain C and N, and thus contribute to the cycling of both of these major elements. Several previous studies have indicated the importance of DFFAs in carbon and nitrogen cycling of lakes and coastal marine waters (e.g. Crawford et al. 1974, Williams et al. 1976, Mopper & Lindroth 1982, Jørgensen et al. 1983, Carlucci et al. 1984, Fuhrman & Ferguson 1986, Fuhrman 1987, Jørgensen 1987).

This research was performed as part of the MECCAS (Microbial Exchanges and Couplings in Coastal Atlantic Systems) project, a multidisciplinary effort to study microbial food webs in the plume of the Chesapeake Bay, USA. The plume system was chosen because such systems, unlike other open waters, are somewhat coherent and have well-defined boundaries that can be measured by salinity (cf. Boicourt et al. 1987, Malone & Ducklow 1990). The plume acts as a sort of natural 'macrocosm,' and the extent of mixing with surrounding waters can be calculated from salinity data. Thus, in the plume system, one can follow a group of interacting planktonic microorganisms over time, and know how much they are being mixed with organisms from surrounding water bodies. This allowed comparison of microbial activities with minimal concern over changes from advection and mixing. In this report, I show that DFAAs in the plume have low concentrations, apparently resulting from close coupling between release and uptake. These DFAAs cycle very rapidly and represent a major pathway of C and N flow in the system.

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MATERIALS AND METHODS

All work was done on 4 cruises of the MECCAS project during February, June, and August–September 1985, and April 1986, in the outflow plume of Chesapeake Bay (Boicourt et al. 1987, Malone & Ducklow 1990). Except when noted (2 stations in April when the ship was anchored within the plume), experiments were done as the ship followed a submerged surface drogue (ca 1 m deep, 1 m wide, cruciform) in an attempt to stay within a parcel of plume water. Each drogue experiment started with the drogue near the mouth of the estuary in water of low salinity (ca 27 to 28 ‰), and ended when the salinity exceeded 30 ‰ or weather or other conditions demanded it (see Malone & Ducklow 1990 for typical drogue tracks). Sample times were usually 05:00, 09:00, 12:00, 16:00, and 22:00 h. Experimental protocol essentially followed that of Fuhrman (1987), and precautions to avoid DFAA contamination (e.g. from fingerprints) were strictly adhered to.

Water was sampled from within 1 m of the surface in cleaned 30 l Niskin bottles, and 50 ml subsamples were measured quickly into duplicate sterile, pre-rinsed polyethylene Whirlpak bags. A trace amount (total ca 0.2 nM , measured by HPLC) of an equimolar mixture of [^{3}H] glutamic acid, serine, glycine, and alanine (all >20 Ci mmol^{-1}; mixture dried and reconstituted in distilled water to eliminate background tritiated water; New England Nuclear) was added to duplicate bags and 10 ml were removed by acid-washed pipette tip for a 'time-zero' filtration through pre-rinsed 0.45 µm pore-size Millipore (type HA, mixed ester) filters in a 10-place stainless-steel and teflon filter unit (Hoefer Scientific). Preliminary tests showed that the 0.45 µm pore-size mixed ester filters collected as much or more radioactivity as did 0.2 µm pore-size polycarbonate Nuclepore filters, yet filtered more rapidly. In February, serine was not used, so there were only 3 labeled amino acids. Filtrates were collected in combusted glass scintillation vials and capped with acid-washed plastic caps. After removal of the vials containing the filtrates, the filters were rinsed 5 times with ca 1 ml 0.45 µm filtered seawater, and then twice more around the rims after the filter towers were removed. For incubation, the bags were quickly taken to an on-deck flowing surface-seawater incubator that was covered with a screen to yield 90 % surface illumination. At 9 min after the initial addition of the isotope (ca 8 min after placement in the incubator), the bags were quickly taken back into the lab for the final duplicate filtrations (10 min total incubation time) as above. All times were recorded to within 10 s, and the total time between Niskin bottle closing and final filtration was $<$ 15 min.

The filter unit was cleaned between experiments with 10 % nitric or phosphoric acid and rinsed copiously with HPLC-grade water (Burdick and Jackson; Muskegon, MI, USA). Each new filter was rinsed many times with such water and its filtrate was checked as a 'blank' by HPLC for DFAAs. Samples with excessively high blanks ($>$ a few nM) were not used. The shipboard concentration measurements (described below) were found to be absolutely necessary for determining the extent of potential contamination (very high) and the cleaning steps necessary to reduce contamination to acceptable levels.

Radioactivity was analyzed from the filter, filtrate, and lyophilized filtrate by liquid scintillation (Ecoscint scintillation fluid, National Diagnostics), with quench corrected by the external standard technique. The difference in counts between the filtrates and lyophilized filtrates was considered to be tritiated water generated by respiration of the tritiated amino acids.

DFAA concentrations in samples, stock solutions, and blanks were measured by high pressure liquid chromatography (HPLC), using an automated modification of the pre-column *o*-phthaldialdehyde derivatization technique of Mopper & Lindroth (1982) as adapted by Fuhrman (1987). Automation was necessary to allow processing at sea of the large number of samples generated by these experiment (>400 per 3 wk cruise, including frequent blanks and standards). The automated system consisted of a Waters Associates model 710B autoinjector, Waters model 420 system controller, and 2 Waters model 6000 pumps. The chromatographic column (4.6 x 100 mm) was commercially packed with Microsorb C-18 bonded silica (Rainin Instruments, Woburn MA, USA). The fluorescence detector was a Kratos model FS970, and integrator a Spectra Physics model 4270. A low-volume mixing chamber was put in line between the autoinjector and column, and was constructed from a precolumn (dimensions 2 cm x 2 mm; Upchurch) containing a 19.5 mm long piece of 1/16 inch (1.6 mm) stainless steel tubing that had been dented in several places with a wire-cutter; the dents cause turbulent flow to facilitate mixing. The needle wash system of the autosampler was disabled (wash frit removed and fluid reservoir empty), as it was found to introduce contamination and was not necessary as long as no high-DFAA (≥ 0.2 µM total) samples or standards were run.

In preparation for the automated measurements, two 4 ml autosampler vials containing conical glass low-volume inserts were filled with ca 30 µl of derivatization solution (made from 25 mg *o*-phthaldialdehyde + 25 µl beta mercaptoethanol + 500 µl methanol + 500 µl Sodium borate buffer, pH 13) and placed in the autosampler, next to vials containing blanks, samples or standards. All blanks, samples, and standards had been cleanly filtered and preserved with 10 % HPLC-grade methanol in pre-combusted glass autosampler

vials (note: standards in distilled water rapidly lost DFAAs when kept in combusted vials, probably due to adsorption onto the activated glass surface, but this was not a problem with seawater). Tests showed that the samples preserved in 10% methanol had the same DFAA concentrations as unpreserved samples, but could be safely left at room temperature in the autosampler for at least several hours, or refrigerated for a few days.

The automated derivatization proceeds (unattended) as follows: With zero initial flow from the pumps, the controller instructs the autosampler to withdraw 150 µl of sample, then 3 µl of derivatization solution, then another 150 µl of sample, and finally another 3 µl of derivatization solution. This produces a 4-layer 'sandwich' of sample and solution within the tubing of the autosampler. Then the flow starts, with a flow rate linearly increasing from 0 to 1 ml min^{-1} over 1 min, and this pushes the sample through the mixing chamber and into the column. The rest of the separation proceeds the same as a manual injection (e.g. Fuhrman & Bell 1985), and uses a gradient from 20 to 80% methanol over ca 20 min (gradients were optimized for individual columns). The gradient was specialized for separating the labeled amino acids, and was not ideal for measurement of DFAAs eluting after methionine. At the end of each automated run it was essential to have zero flow for at least 2 min (possibly more) to allow the system pressure to fall to zero; otherwise residual flow interferes with the next injection. The time between injections was 35 min, and up to 8 samples were run automatically at a time.

DFAA release and uptake rates were calculated from the 'instantaneous' turnover rates of tracers and DFAA concentrations as they changed over time. All calculations included corrections for respiration of tracer The equations are:

$$\text{Turnover rate} = (1/\text{turnover time}) = (\ln(\text{lyoph}_{tzero}/\text{lyoph}_t)/t$$

$$\text{Uptake rate} = \text{concentration} \times \text{turnover rate}$$

$$\text{Release rate} = \text{uptake rate} + [(\text{concn}_{t2} - \text{concn}_{t1})/(t2 - t1)]$$

Where lyoph_{tzero} and lyoph_t = dpm ml^{-1} in initial and final lyophilized filtrates from uptake experiments (i.e. 10 min apart); t1 and t2 = consecutive sampling times from the plume (e.g. at 05:00 and 09:00 h).

RESULTS

The studied amino acids had total cumulative concentrations averaging 6.5 to 23 nM over the 4 cruises, with lowest average values in February (Table 1). The C:N ratio (by atoms) of these amino acids in plume water was 3 in February, June, and August, and was 3.5 in April. The molar ratios of the 4 amino acids averaged 1.75:2.13:2.02:1 (glu:ser:gly:ala) and ranged (0.66 to 3.9):(1 to 4.7):(0.83 to 2.9):1. There was no clear seasonal pattern to these ratios, but glutamic acid and serine tended to be higher proportions of the total in April compared to other months (also reflected in the C:N ratios). The percentage of DFAA uptake that was respired averaged 46% in February, 47% in June, 37% in August, and 35% in April.

Turnover rates of the 4 DFAAs were rapid, with turnover times averaging 0.5 to 1 h in summer and < 3 h in winter (Table 1). In a set of 5 similar measurements made over 1 d in the outflow plume of the Hudson River, USA, in September 1985, the average turnover time was a remarkably fast 6.8 min (Table 2).

Table 1. Summary of concentration and rate information, Chesapeake Plume, all cruises. Data are from glu, ser (except February), gly, and ala only. DFAA release rates averaged within 5% of uptake rates on all cruises. Number of samples indicated were each analyzed in duplicate

	DFAA concentration (nmol C l^{-1})	(nmol N l^{-1})	Turnover time (min)	Uptake (µg C l^{-1} h^{-1})
February				
Mean (n = 16)	19.6	6.53	162	0.086
Range	12.6–25.5	4.2–8.5	86–300	0.036–0.140
June				
Mean (n = 42)	33.1	10.9	60	0.69
Range	8.7–76.9	2.9–21.7	13–126	0.035–4.0
August				
Mean (n = 25)	38.2	12.9	46.9	0.594
Range	11.8–98.9	3.6–34.3	24–250	0.105–2.12
April				
Mean (n = 48)	80	22.9	63.8	0.902
Range	15.4–242	5–73.5	23–353	0.113–2.89

Table 2. Hudson River plume DFAA results, including data only for glu, ser, gly, and ala; 4 September 1985

Time (h)	DFAA concentration (nmol C l^{-1})	(nmol N l^{-1})	Uptake (μg C l^{-1} h^{-1})	Turnover time (min)
06:18	40.6	11.4	3.74	7.9
09:40	29.4	9.0	2.45	8.6
13:05	7.0	1.85	0.74	6.5
16:30	57.5	15.5	7.89	5.3
22:30	26.9	6.0	3.41	5.6

On all cruises, the average release rates were essentially identical to average uptake rates, despite short-term variations (e.g. see Fig. 1). This indicates that the changes in ambient concentrations were extremely small compared to the potential changes one might expect from the rapid turnover.

There was a great deal of variability in the DFAA flux rates from sample to sample and drogue to drogue (Fig. 1), so it is difficult to make firm conclusions about the differences between cruises (Table 1). The most dramatic difference, ca 10-fold, was between February and the other cruises. While it must be kept in mind that only 3 amino acids were followed in February (no serine, unlike the other cruises), this would only be expected to lead to a difference of about a factor of 1.5 because serine contributed about 30% to the average cumulative concentration of the 4 amino acids studied.

In most of the samples from June and several from other months, the total flux of amino acids (both release and uptake) followed a diel pattern, with highest val-

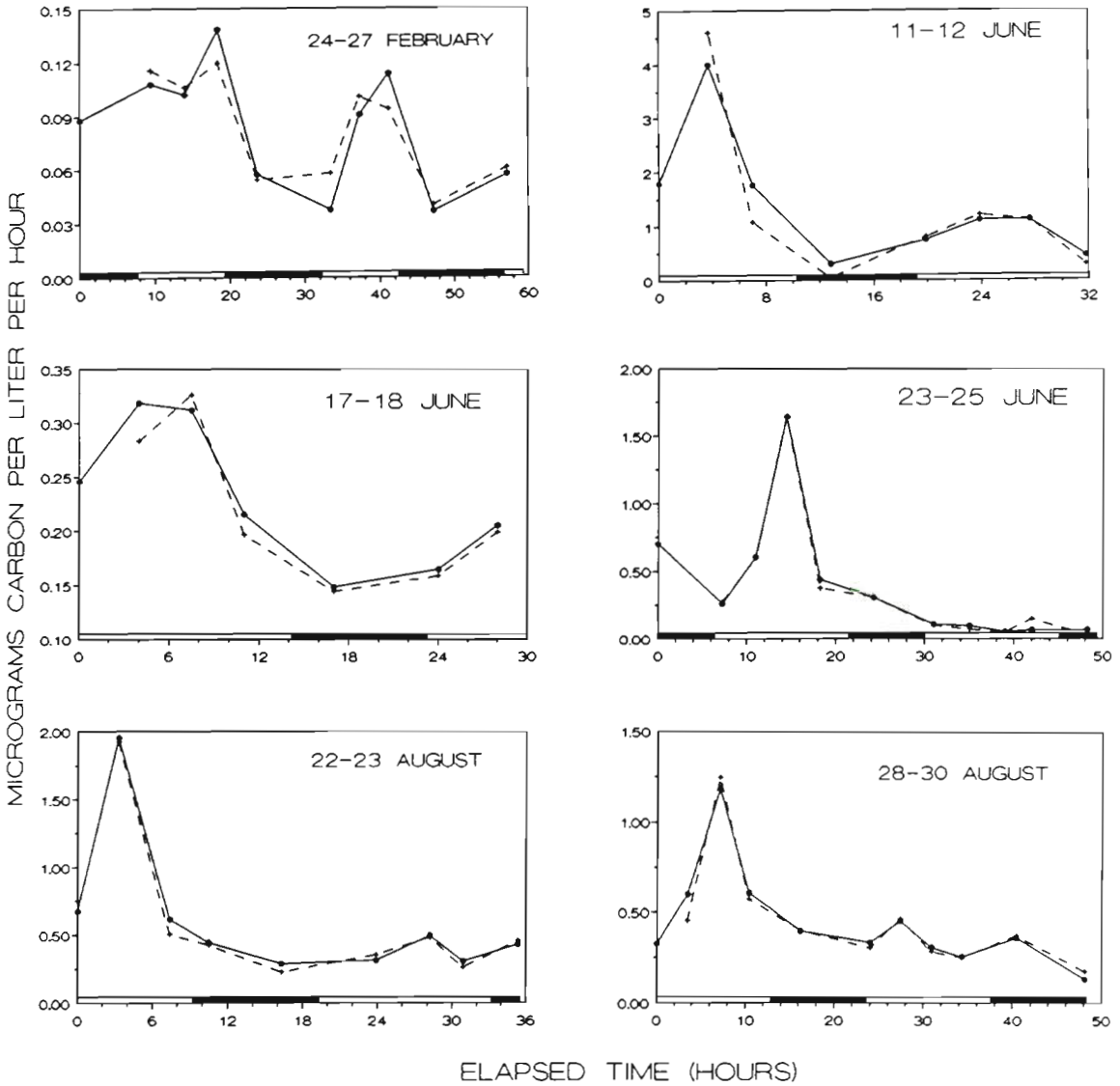

Fig. 1. Time courses of DFAA uptake (•) and release (+) for the dates indicated. Dark and light bars along the x-axis indicate periods of darkness and daylight

Table 3. Calculation of DFAA average uptake in relation to average bacterial productivity (BP) and primary productivity (PP) measurements (productivities from Malone & Ducklow 1990)

	DFAA uptake ($\mu g\ C\ l^{-1}\ h^{-1}$)	BP ($\mu g\ C\ l^{-1}\ h^{-1}$)	DFAA/BP C only[a]	DFAA/BP N only[b]	PP ($\mu g\ C\ l^{-1}\ h^{-1}$)	DFAA/PP C only
February	0.086	0.167	0.280	0.806	1.250	0.069
June	0.690	1.708	0.214	0.642	16.583	0.042
August	0.594	2.083	0.151	0.444	16.167	0.037
April	0.902	0.917	0.638	1.313	4.917	0.183

[a] Estimate of DFAA contribution to bacterial C biomass production, calculated from incorporation of DFAA after respiration loss, not total uptake as is used elsewhere
[b] Assumes C:N ratio of 4 for bacteria

ues near noon and lowest near midnight (Fig. 1). Even when the pattern was not smooth, there was a tendency for the higher values to occur mid-day and lowest during darkness or morning. It was also observed that the DFAA flux tended to decline as the plume water mixed with less rich coastal seawater (Fig. 1), as might be expected. The superimposition of these factors often led to a pronounced rate peak near noon of the first day, and a smaller or no peak on the next day (Fig. 1). Note that even in summer when primary production was high (Table 3), the magnitude of the diel change was sometimes small.

Background data on temperature, salinity, and bacterial abundance (from Malone & Ducklow 1990) are as follows: The salinity of the plume samples always averaged between 27.2 and 28‰, and temperatures averaged 4.6°C in February, 11.4°C in April, 21.5°C in June, and 26.1°C in August. Bacterial counts by epifluorescence microscopy of acridine-orange stained cells were 0.7×10^9 cells $l^{-1} \pm 32\%$ (mean ± coeff. of variation) in February, 4.6 ± 31% in April, 5.6 ± 27% in June, and 3.6 ± 16% in August.

DISCUSSION

These results show how substances that occur at low nM concentrations can still have a very large flux rate and major contribution to overall food web processes as long as the cycling rate is rapid. An implication of the rapid cycling we observed is tight coupling between DFAA sources and sinks (Fuhrman 1987). Although diel changes in concentrations may seem to contradict this (i.e. by suggesting sources sometimes get ahead of sinks and vice versa), the notable diel cycles often seen are another manifestation of tight coupling, suggesting that a major DFAA source has a strong diel cycle and that the bacteria follow this cycle with only a minimal lag. Such a lag is sometimes visible in the data as changes in release rate slightly preceding those of uptake (Fig. 1), although I cannot rule out the possibility that this is an artifact of the calculation method. The most obvious candidate for the diel-variable source is phytoplankton.

Other authors have investigated diel changes in DFAA uptake. Carlucci et al. (1984) also found daytime highs and night-time lows in marine DFAA uptake rates, and also ascribed these changes to a relationship to primary production. However, Jørgensen et al. (1983) observed different patterns in Danish lakes, with one lake having maxima near 09:00 h and midnight and another showing no obvious pattern. Jørgensen (1987) also found no obvious patterns in lakes. Diel variations of DFAA concentrations have been found in the Baltic Sea by Mopper & Lindroth (1982), who interpreted night-time declines as indicating uptake at rates up to 6 $\mu gAA^{-1}\ h^{-1}$, about 3 to 6 times higher than the rates reported here. However, they did not follow uptake with tracers, nor could they rule out changes due to patchiness or water advection and internal waves. Lack of information on the extent of patchiness is a common problem in biological oceanography, and even following a drogue does not always eliminate questions. For example, in this study, there were several instances with no obvious diel patterns of DFAA release and uptake, including the experiment in the Hudson (Table 2). I cannot say if this was due to a true lack of a pattern among a coherent group of organisms or, alternatively, sampling different patches of water (with different activities) over time.

With the extremely rapid turnover found in the Hudson plume and in many summer Chesapeake samples, the diffusion between DFAA sources (phytoplankton, etc.) and sinks (primarily bacteria) can be an important factor because turnover occurs in about the same time (a few minutes) that it takes a molecule to travel a few millimeters by molecular diffusion (Mitchell et al. 1985). This suggests that enhanced microzones around DFAA sources may be particularly important in these systems. However, turbulence and the accompanying eddy diffusion would speed the mixing rate by orders of magnitude; motility of the sources (passive sinking

or active swimming) must be considered as well (Mitchell et al. 1985). In estuarine plumes, microzones may be enhanced by strong stratification that tends to reduce eddy diffusion (Mitchell et al. 1985). Direct observations of significant centimeter-scale variability in the Chesapeake plume coincident with this study were reported by Mitchell & Fuhrman (1989), indicating that small-scale processes may be very important here.

Comparisons of DFAA flux to primary and bacterial production suggest that these DFAAs make a large contribution to bacterial nutrition, with bacterial incorporation of DFAAs contributing tens of % to bacterial biomass production, and that the flux of these 4 DFAAs is several percent of the total primary production overall (Table 3). Jørgensen (1987) found similarly high percentages in lakes, as did Craven & Carlucci (1989) in a California Borderland basin. Note that it may not be correct to think of the DFAA flux strictly as a % of primary production because all the possible sources are not in situ and some flux may be from import of dissolved organic nitrogen (e.g. from Chesapeake Bay). If this is the case, the imported material would likely be polymers, such as polypeptides, proteins, and complex mixed condensates, because the extremely rapid uptake of DFAAs observed in the plume would preclude significant lateral transport of DFAAs (maximum transport of a few kilometers at typical current velocities). This mechanism might be particularly important in April, when the DFAA concentrations and flux rates seem very high in comparison to the other values.

The estimated DFAA contribution of these 4 DFAAs to bacterial N demand was high, 44 to 131 % (Table 3). Because there are several other possible organic N sources likely taken up by the bacteria, such as other DFAAs, nucleic acid bases, and amino sugars, it would seem that in the course of organic matter uptake, the bacteria as a group are likely to obtain more N than they need for growth. This suggests that in this system, the bacteria as a group probably do not need to take up inorganic N to fulfill their growth demands, so they are probably net regenerators of nitrogen. I use the phrase 'as a group' because I do not know if it is reasonable to treat individual bacteria as 'averages' of the entire bacterial assemblage, and it is possible that some bacteria take up inorganic N while others are regenerating it.

There are several procedural issues about this study that deserve brief discussion. With such rapid cycling, it was necessary to perform very fast experiments (< 15 min from sampling to final filtration). Longer incubations, as were common in most previous studies, would lead to underestimates of rates, because the tracer is rapidly depleted. Even with 10 min incubations, there was often significant depletion that necessitated a non-linear equation to calculate uptake rates. Because conditions may change upon sampling (e.g. light cut off), and turnover may be fueled very rapidly by primary production, it was important not to allow the samples to remain in the Niskin bottles or in the ship's lab for more than a few minutes. Ideally, some sort of rapid ultra-clean in situ sampler-incubator-filtering device should be used, but none is currently available.

With the necessity for such rapid experiments, I decided to use mixtures of 4 DFAAs instead of a single DFAA. Logistically it was not possible to perform duplicate determinations routinely with more than one type of tracer (individual or mixture). Mixtures permit wider coverage in each experiment, but there is a question of the accuracy of using the average turnover rates (measured) to calculate uptake rates of mixtures. The 4 DFAAs (glu, ser, gly, ala) were chosen because they represent different classes of amino acids and were found to be consistently high in concentration relative to most of the other amino acids; together they typically make up on the order of 50 % or more of the total DFAAs. Similar results have been found in other aquatic systems by Mopper & Lindroth (1982), Jørgensen et al. (1983), Carlucci et al. (1984), Fuhrman & Bell (1985) and Jørgensen (1987). Another consideration in this study was that these DFAAs could readily and rapidly be measured with the automated HPLC. Regarding the accuracy of calculating uptake with mixed tracers, Williams et al. (1976), Dawson & Gocke (1978), Ferguson & Sunda (1984), Carlucci et al. (1984), Jørgensen (1987), and Craven & Carlucci (1989) all have data on individual amino acid uptake that suggest turnover of mixed tracers should reasonably well approximate the sum of individual rates. Therefore, I believe that the results reported here are basically accurate.

Many of the results were calculated from radioactivity in lyophilized filtrates that was assumed to be from labeled DFAAs that were not taken up. It is possible that some of this radioactivity was actually in very small bacteria, viruses, or metabolites that passed through the 0.45 µm pore size mixed ester filters, and thus the rate estimates may be low. However, we routinely use such filters to prepare filtered seawater to be used as a 'blank' in direct epifluorescence microscopy of bacteria, and we rarely find more than about 1 % of the bacteria pass these filters. Also, the 10 min incubation time probably rules out significant losses of radioactivity into viruses. Released metabolites are probably low because Fuhrman (1987) showed a similar result is obtained if the uptake is monitored as loss of radioactivity from specific DFAAs as measured from peaks eluting from the HPLC or from loss of radioactivity in lyophilized filtrates. Thus I believe any underestimates to be minor.

The way release was calculated in this study is differ-

ent from that of Fuhrman (1987), who measured release during the incubation period within the small container holding the sample. Here, release was measured from change in concentration and uptake rates in the plume itself as a water parcel was followed with a drogue. This was judged to be best because all the naturally-occurring release processes would continue without interference. It is possible that the drogue may not have been following exactly the same water over time, due to meter- or kilometer-scale patchiness and mixing. However, concentrations changed little compared to uptake rates, even from day to day, so the error is probably small. In considering possible alternative ways to measure this release in a system with such rapid turnover and overall activity, I know of no way to contain an uncontaminated parcel of seawater for the necessary times without causing artifacts that are probably much worse than the errors in these measurements.

In summary, although DFAA concentrations were only a few nM, very rapid cycling of these compounds made them highly significant agents of C and N cycling in estuarine plumes. The rapid cycling and frequently-observed diel cycles suggested close coupling between DFAA sources (presumably including phytoplankton) and bacteria. This system has a strong potential to support microzones of enhanced microbial activities.

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