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Bacterial utilization of dissolved free amino acids, dissolved combined amino acids and ammonium in the Delaware Bay estuary: effects of carbon and nitrogen limitation

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ABSTRACT: The contribution of dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA) and ammonium to the C and N requirements of bacterioplankton was examined in batch cultures enriched with various sources of C and N. DFAA sustained up to 14 and 34% of the C and N requirements, respectively, during exponential growth. DCAA constituted less than 10% of the C demand in all cultures during exponential growth and up to 24% of the N demand in N-limited cultures. In C-limited cultures DFAA, DCAA, and NH_4^+ constituted 37 to 62%, 4 to 10% and 27 to 59% of total N uptake (DFAA + DCAA + NH_4^+) during exponential growth. In nitrogen-limited cultures the corresponding values were 78, 14, and 8% of total N uptake. During the stationary phase the importance of DCAA as a C and N source increased as DFAA and NH_4^+ concentrations decreased. In addition to allowing us to examine the contribution of various compounds to supporting bacterial production, our data on uptake and concentrations suggest that bacteria release free and combined amino acids, especially during the stationary phase.

KEY WORDS: DCAA · DFAA · Ammonium · Bacterial utilization · C- and N-limitation

INTRODUCTION

Bacterial extracellular hydrolysis of polymeric organic compounds and subsequent utilization of the produced mono- and oligomers have been studied intensively during recent years (e.g. Hoppe et al. 1988, Billen 1991). Despite much work on dissolved combined amino acids (DCAA) in particular, the importance of these compounds in supporting bacterial C and N demand is not clearly resolved. Several batch culture studies with natural assemblages of bacteria have found dissolved free amino acids (DFAA) and NH_4^+ to be the primary N sources for bacteria (Keil & Kirchman 1991, 1993, Jørgensen et al. 1993). In these studies utilization of DCAA generally constituted <10% of C and N uptake by bacteria. Other studies, however, found that DCAA may contribute substantially to bacterial growth (Coffin 1989, Tupas & Koike 1990, Simon & Rosenstock 1992, Rosenstock & Simon 1993, Jørgensen et al. 1994, Kroer et al. 1994).

In Lake Constance (W Europe), Simon & Rosenstock (1992) and Rosenstock & Simon (1993) observed that DCAA utilization may explain >100% of bacterial production during periods of low *in situ* concentrations of DFAA. In support of these results, Keil & Kirchman (1993) found a negative correlation between DFAA concentration and the importance of proteins for bacterial growth in coastal waters and suggested that protein utilization was partially controlled by the concentration of DFAA. A similar correlation between DFAA and DCAA concentrations was observed in a cross-

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system study by Kroer et al. (1994), who argued that differences in the DCAA:DFAA ratio may determine their relative significance for bacterial growth. Moreover, Keil & Kirchman (1991) suggested that DCAA constituted an increasingly important substrate for bacteria, as DFAA were depleted from the cultures. Thus, it appears that different C and N sources are utilized in an order of priority by a bacterial assemblage, and that DCAA does not contribute significantly to bacterial growth as long as DFAA or NH_4^+ along with a simple C source are available.

In this study we examined the relative importance of DFAA, DCAA and NH_4^+ for bacterial C and N demand during periods of N and C limitation of bacterial growth in the Delaware Bay estuary (Atlantic coast, USA).

MATERIALS AND METHODS

Experimental design. Two bacterial batch culture experiments were carried out on October 4 (Expt 1) and 8 (Expt 2), 1993, with water samples collected just before high tide outside the surf zone at Lewes Beach, Delaware Bay. Batch cultures were prepared with 80% 0.2 µm-filtered and 20% 0.8 µm-filtered water; 142 mm polycarbonate filters (Nuclepore) and gravity filtration were used. Each experiment consisted of 3 cultures enriched with inorganic or organic compounds and 1 control culture without additions. In Expt 1 the compounds were added immediately before incubation and the cultures received 10 µM NH_4^+, 5 µM glucose, or both. Expt 2 was started as 1 culture until 21 h of incubation after which it was divided into 4 subcultures and amended with 10 µM NH_4^+, 5 µM glucose, or an amino acid mixture containing 400 nM of each of the 12 most common amino acids found in the water samples (5 µM total). Incubations were in 5 l polypropylene bottles in the dark and at *in situ* temperature.

Bacterial abundance. Bacterial abundance was estimated by using acridine orange epifluorescent microscopy (Hobbie et al. 1977), and the bacterial specific growth rate (μ) was calculated from changes in abundance.

Dissolved free amino acids (DFAA). Concentration of individual amino acids was measured in 0.2 µm-filtered subsamples (Acrodisc, Gelman Sciences, MI, USA) by high-performance liquid chromatography (HPLC) after derivatization with *o*-phthaldialdehyde (OPA) according to Lindroth & Mopper (1979), modified according to Jørgensen et al. (1993). Analytical precision was about 5%.

The turnover of amino acids was measured by incubation of subsamples with a mixture of 15 ^{3}H-labelled amino acids (Amersham) with an average specific activity of 51 Ci mmol^{-1}. Twenty-five µCi was added to 20 ml triplicate subsamples (plus 1 control killed with 2% formaldehyde) which were incubated for 20 to 60 min. Incubations were stopped with formaldehyde (2% final concentration) and samples were filtered through 0.2 µm cellulose nitrate filters (Sartorius) and rinsed 4 times with Milli-Q water. The filters were then dissolved for 30 min in ethyl acetate followed by the addition of scintillation cocktail and radioassay by liquid scintillation counting. Respiration of ^{3}H-labelled amino acids during incubation was measured by distillation of the 0.2 µm filtrates (duplicates + killed control). The filtrate was heated to 80°C and distilled under vacuum, and ^3H_2O in 3 to 5 ml distillate was radioassayed. Bacterial respiration was measured once during the exponential (20 to 21 h) and once during the stationary phase (50 to 60 h) in all cultures.

Dissolved combined amino acids (DCAA). To measure concentrations of DCAA, 750 µl sample was freeze dried and hydrolyzed in the vapor phase under N atmosphere for 20 min at 150°C in a microwave oven (N. O. G. Jørgensen unpubl.). Samples were then redissolved in a borate buffer at pH > 10 to obtain optimal OPA reaction and quantified by use of HPLC. Milli-Q water treated as samples were used as blanks; these always contained <10% of sample concentration.

The turnover of DCAA was estimated by use of the fluorogenic substrate analog L-leucine-4-methyl-coumarinylamide hydrochloride (Leu-MCA), according to Hoppe et al. (1988, 1993). Leu-MCA is hydrolyzed by the bacterial extracellular enzyme leucine aminopeptidase, and is believed to mimic natural peptides (Hoppe et al. 1988). The turnover of Leu-MCA was used as an estimate of the turnover rate for all proteins. Triplicate 1 ml subsamples were incubated with 500 nM Leu-MCA (final concentration) for 3 to 5 h, and Leu-MCA turnover rate was calculated from the increase in fluorescence as the non-fluorescent Leu-MCA was hydrolyzed to the highly fluorescent 7-amino-4-methyl-coumarin (AMC). Background fluorescence at time zero was measured for each triplicate sample. A Spectrovision FD-100 fluorometer was used for fluorescence measurements. The increase in fluorescence was linear with time for the incubation times used. The duration of the Leu-MCA incubations was determined by the appearance of a significant fluorescence signal. Total DCAA hydrolysis was estimated from concurrent measurements of Leu-MCA turnover rate and from the decrease in DCAA concentrations.

Ammonium. The ammonium concentration was measured with an Alpkem autoanalyzer using standard procedures.

RESULTS

Bacterial abundance and growth rate

In Expt 1 bacterial abundance increased exponentially during the first 30 h, then remained fairly constant until 55 to 70 h when the abundance started to decrease (Fig. 1A). The addition of glucose increased the maximum bacterial abundance from 2.5×10^6 cells ml^{-1} (control) to about 4.5×10^6 cells ml^{-1} Bacterial growth rate (Fig. 1B) increased during the first 30 h to maximum rates of 0.08 to 0.11 h^{-1} in cultures with glucose additions and 0.05 to 0.07 h^{-1} in cultures without added glucose.

In Expt 2 the control culture had a lower maximum bacterial abundance (1.7×10^6 cells ml^{-1} after 45 h; Fig. 1C) and lower maximum growth rate (0.04 h^{-1}; Fig. 1D). Addition of ammonium, glucose and DFAA increased the maximum bacterial abundance and bacterial growth rate compared to the control. In NH_4^+ (+NH_4^+) and DFAA (+DFAA) amended treatments, however, bacterial abundance started to decrease before a constant level was reached (Fig. 1C).

Dissolved free amino acids (DFAA)

The initial DFAA concentration in the experiments ranged from 300 to 700 nM; we found no elevation of DFAA concentration due to filtration and preparation of the cultures. Concentrations of DFAA are naturally very high in the Delaware Bay (Coffin 1989, Keil & Kirchman 1993). In both experiments DFAA con-

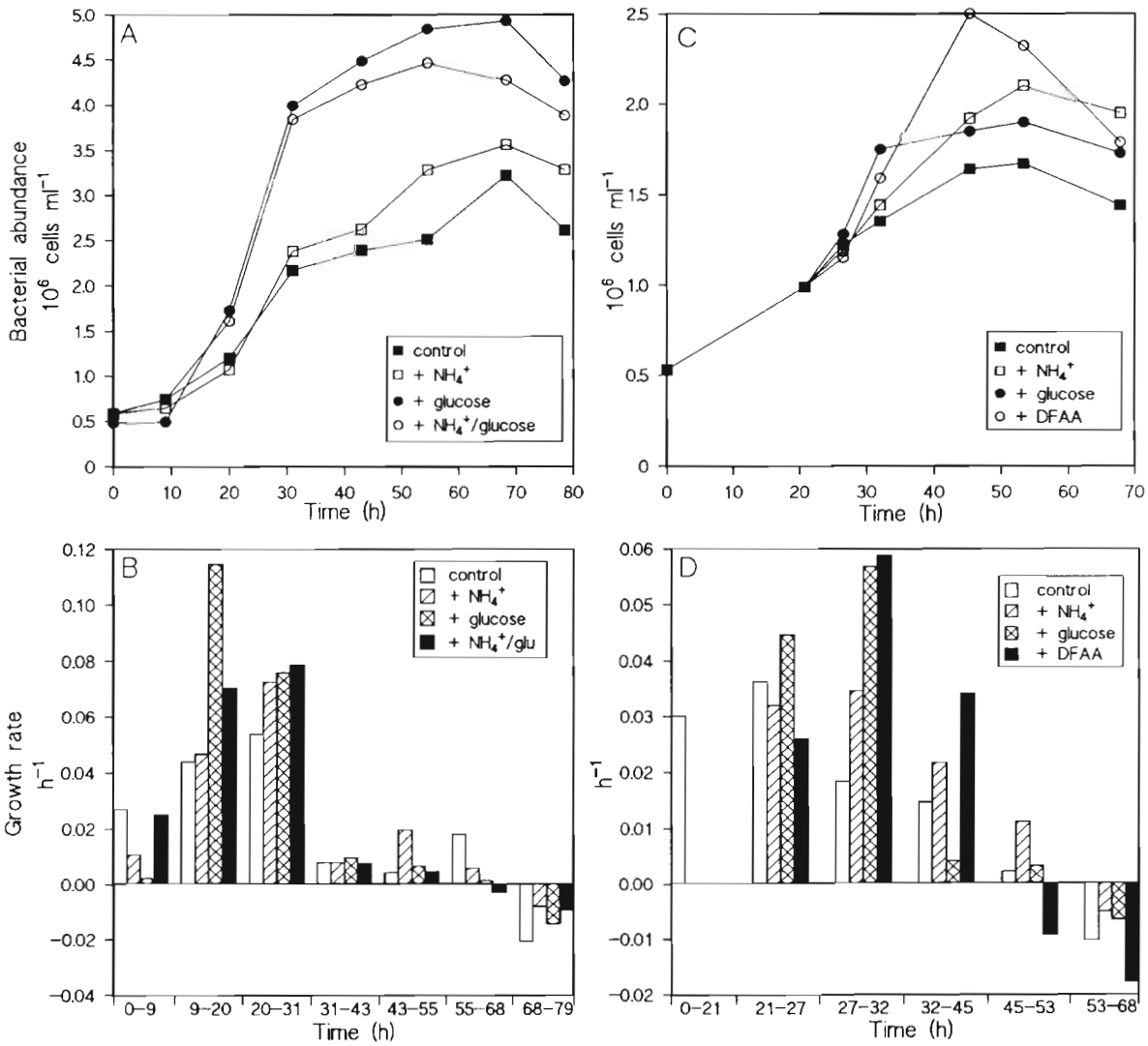

Fig. 1. Bacterial abundance and growth rate in (A, B) Expt 1 and (C, D) Expt 2

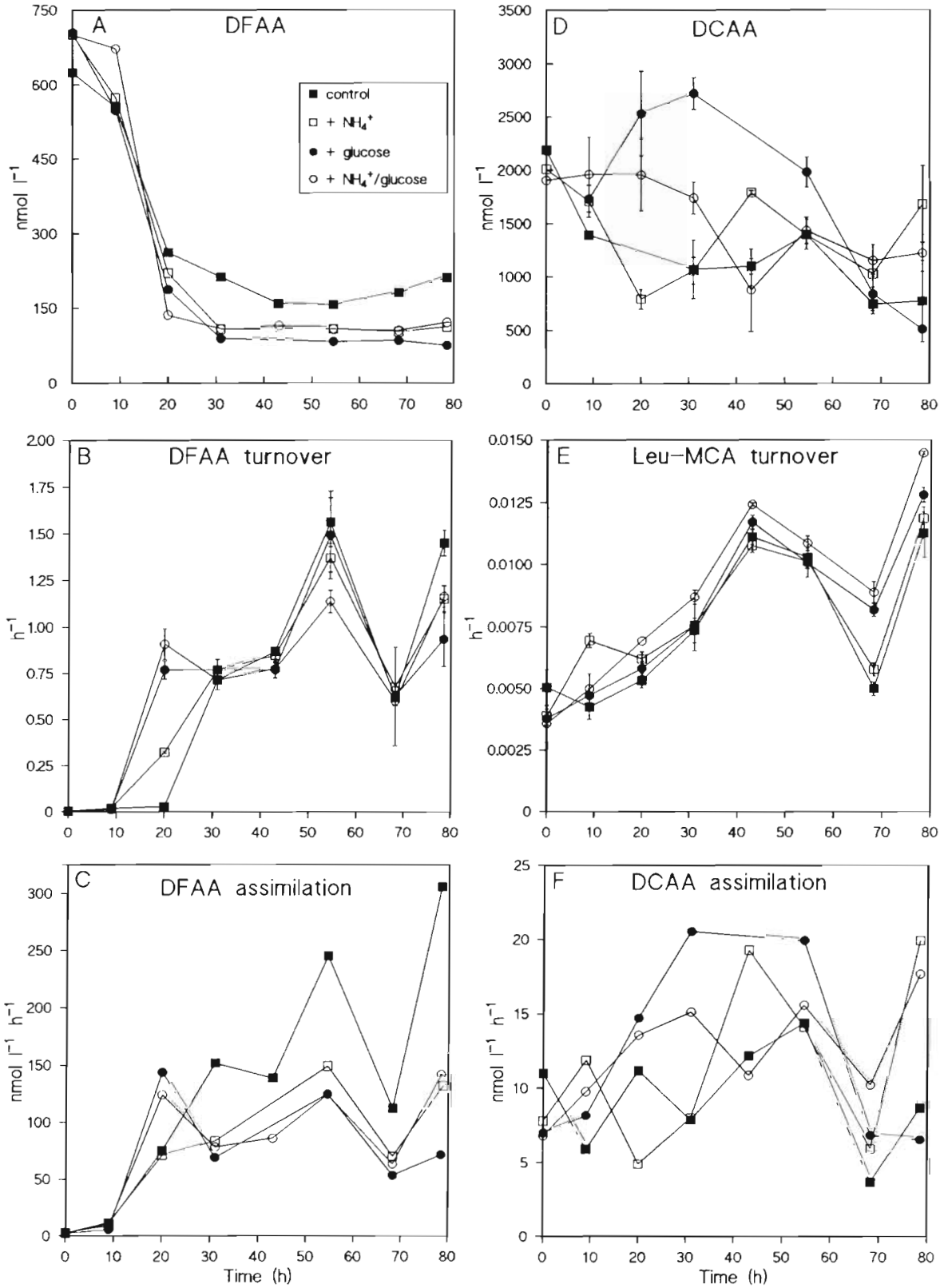

Fig. 2. Expt 1. (A) Concentration, (B) turnover and (C) assimilation rate of dissolved free amino acids; (D) concentration of dissolved combined amino acids (DCAA), (E) Leu-MCA turnover and (F) estimated DCAA assimilation rate

centration decreased during the first 20 to 30 h to about 100 to 200 nM (Figs. 2A & 3A), and remained constant throughout the rest of the incubation. The DFAA added in Expt 2 was utilized 32 h after addition.

Dissolved combined amino acids (DCAA)

The initial DCAA concentration in Expt 1 was approximately 2000 nM and constituted 75% of the total pool of amino acids (Fig. 2D). In the control and $+NH_4^+$ cultures, DCAA concentrations were reduced to about 1000 nM during the first 31 h. In glucose-enriched cultures DCAA remained constant or increased at the beginning of the incubation, followed by a decrease after 31 h (Fig. 2D).

The *in situ* DCAA concentration had decreased from 2000 to 1400 nM between the 2 sampling dates and constituted 81% of total amino acids in Expt 2 (Fig. 3D). After decreasing to 1200 nM during the first 21 h, DCAA concentration tended to increase in all cultures in this experiment. After 32 h a decrease in DCAA concentration was observed in all cultures (Fig. 3D).

DFAA assimilation and incorporation

Respiration of ^{3}H-amino acids did not vary significantly between cultures or during the incubation in any of the experiments; the averages were 43.5% ± 5.8% (±SD, n = 8) and 50.1% ± 7.5% (n = 5) of the assimilated ^{3}H-amino acid mixture in Expts 1 and 2, respectively. In the following discussion DFAA turnover and assimilation rates are based on total assimilation (i.e. incorporation + respiration) of ^{3}H-labelled DFAA.

DFAA turnover increased in both experiments concomitant with increased bacterial abundance and peaked at 1.1 to 1.5 h^{-1} in Expt 1 and 0.7 to 1.0 h^{-1} in Expt 2 after about 50 h (Figs. 2B & 3B). DFAA assimilation followed the turnover with maximum assimilation rates of 542 nmol l^{-1} h^{-1} in +DFAA and 100 to 300 nmol l^{-1} h^{-1} in the other cultures (Figs. 2C & 3C).

DCAA turnover and hydrolysis

In both experiments Leu-MCA turnover were 2 orders of magnitude lower than DFAA turnover and ranged from 0.003 h^{-1} to 0.015 h^{-1} in Expts 1 and 2 (Figs. 2E & 3E). DCAA hydrolysis was 1 order of magnitude lower than DFAA assimilation and ranged from 4 to 23 nmol l^{-1} h^{-1} (Figs. 2F & 3F).

Ammonium concentration

NH_4^+ concentration decreased by about 2 µM in control, glucose (+glucose) and ammonium ($+NH_4^+$) amended cultures (Fig. 4A), while NH_4^+ concentration in cultures with the addition of both ammonium and glucose ($+NH_4^+$/glucose) decreased by 7.3 µM over 43 h and then increased by 2.5 µM (Fig. 4A). At the onset of Expt 2, the *in situ* NH_4^+ concentration had decreased to <0.3 µM, and decreased further to about the detection limit of the method (ca 0.10 µM) in control and +glucose incubations (Fig. 4B). In the $+NH_4^+$ treatment the concentration decreased slightly after the ammonium addition, while enrichment with DFAA resulted in an increase of 4.03 µM within 25 h after addition.

C and N budget

Bacterial C and N budgets were calculated separately for the exponential (0 to 20–30 h) and stationary (20–30 to 45–55 h) phases. We did not analyze the last period when bacterial abundance decreased. Contributions of DFAA and DCAA to bacterial C and N demand were calculated both from turnover of ^{3}H-DFAA and Leu-MCA and from changes in concentrations of DFAA and DCAA over time. C and N content of DFAA and DCAA were based upon the C and N content of individual amino acids.

In both experiments there was a substantial discrepancy between C and N incorporation estimated from ^{3}H-DFAA uptake and from decreases in DFAA concentration. Apart from the first 20 h of Expt 1, during which DFAA assimilation rates agreed with rates of decrease in DFAA concentrations (Fig. 2A & C), uptake rates of DFAA-N and DFAA-C exceeded the corresponding decreases in DFAA concentration by 200 to 400% during the exponential phase (Tables 1 to 4). Differences between the 2 estimates of DFAA uptake increased even more during the stationary phase, where DFAA concentrations remained relatively constant despite a progressive increase in ^{3}H-DFAA uptake.

In Expt 1 net DFAA incorporation based on decreases in DFAA concentrations constituted 8 to 14% of bacterial C demand and 18 to 30% of their N demand during exponential growth; the greatest contribution of DFAA was observed in cultures without glucose additions (Tables 1 & 2). In Expt 2 the decrease in DFAA concentrations could account for 14% of the C demand and 34% of the N demand during the first 21 h (Tables 3 & 4). Except for the +DFAA culture the contribution of DFAA to C and N demand decreased during the stationary phase in Expt 2.

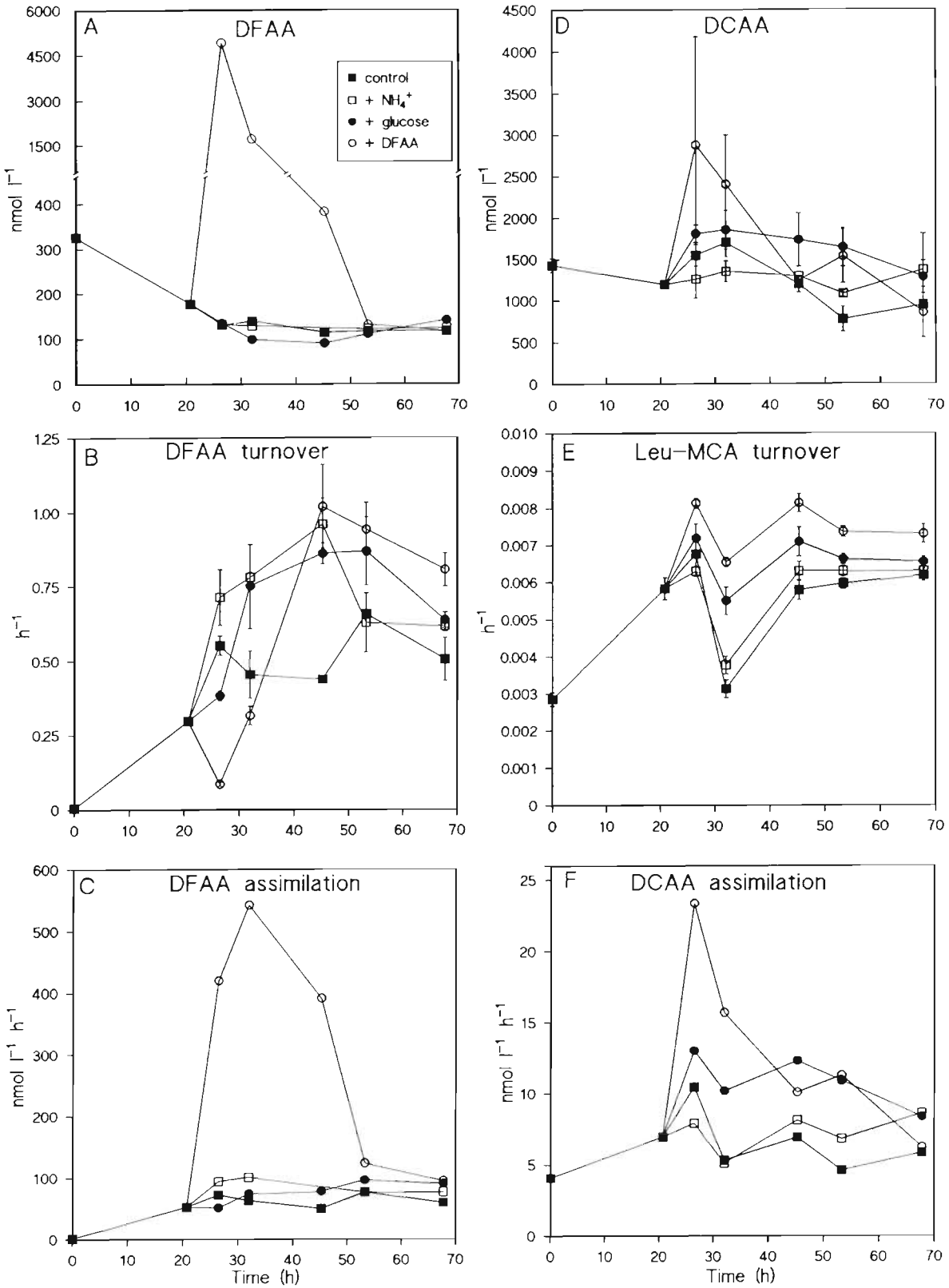

Fig. 3. Expt 2. (A) Concentration, (B) turnover and (C) assimilation rate of dissolved free amino acids; (D) concentration of dissolved combined amino acids (DCAA), (E) Leu-MCA turnover and (F) estimated DCAA assimilation rate

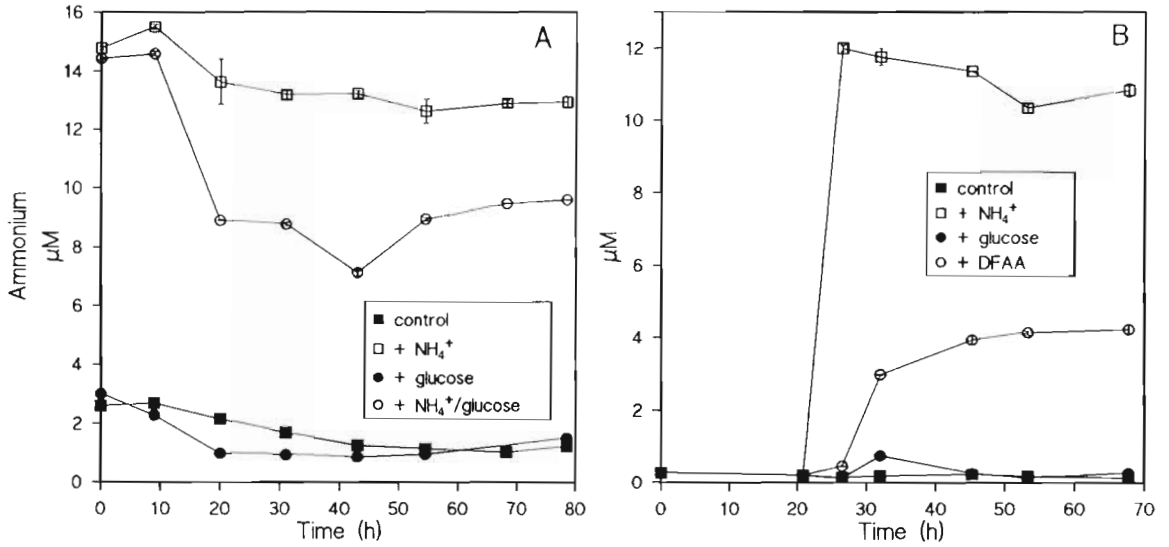

Fig. 4. Ammonium concentration in (A) Expt 1 and (B) Expt 2

Like DFAA uptake, we observed quite large differences between the 2 concurrent measurements of DCAA hydrolysis (Tables 1 to 4). Generally, estimates of DCAA hydrolysis based on decreases in DCAA concentration were larger than those obtained by Leu-MCA hydrolysis. DCAA release, i.e. increases in DCAA concentration, was observed during part of the incubation in all cultures.

Based on the tracer approach, DCAA sustained 5 to 8% of the C demand and 10 to 16% of the N demand during the exponential phase in Expt 1 (Tables 1 & 2). During the stationary phase the DCAA contribution increased to 23–43% and 46–85% of the C and N demand, respectively, with increasing importance in cultures without an ammonium addition. In Expt 2 DCAA sustained 10% and 24% of C and N demand, respectively, during the first 21 h (Tables 3 & 4). During the stationary phase the importance of DCAA as an N source increased to 30% of N demand in the glucose-enriched culture, while it decreased to 13–17% in the other cultures.

Based on turnover of ^{3}H-DFAA and Leu-MCA during exponential growth DFAA, DCAA and ammonium accounted for 37 to 62%, 4 to 10% and 27 to

Table 1 Expt 1. Net uptake of C by incorporation of DFAA and DCAA compared to estimated bacterial net C demand during exponential and stationary phases

	Net C demand[a] (µg C l^{-1})	Net DFAA incorporation µg C l^{-1} [b]	% of demand	µg C l^{-1} [c]	% of demand	Net DCAA incorporation µg C l^{-1} [d]	% of demand	µg C l^{-1} [e]	% of demand
0 to 31 h									
Control	104	37.8	36	10.5	10	7.9	7.6	34.5	33
+NH_4^+	115	29.6	26	15.6	14	6.8	5.9	28.9	26
+glucose	201	43.9	22	15.9	7.9	10.7	5.3	−26.5	−13
+NH_4^+/glucose	191	41.6	22	15.3	8.0	9.5	5.0	2.90	1.5
31 to 55 h									
Control	17.9	73.2	410	1.44	8.0	7.63	43	−14.4	−81
+NH_4^+	47.3	51.6	110	0.01	0.02	10.7	23	−17.4	−37
+glucose	44.6	40.8	92	0.33	0.70	12.5	28	21.9	49
+NH_4^+/glucose	32.6	45.3	140	0.03	0.10	8.31	26	12.6	39

[a]Assuming a cell volume of 0.05 µm^3 (t = 0 h), 0.10 µm^3 (t = 9 h) and 0.15 µm^3 (t > 9 h) (Jørgensen et al. 1993, M. Middelboe unpubl. data) and 0.35 pg C µm^{-3} (Bjørnsen 1986)
[b]Estimated from the turnover rate of ^{3}H-labelled DFAA and DFAA concentrations
[c]Estimated from changes in DFAA concentrations, and 44% respiration (see text)
[d]Estimated from the turnover rate of Leu-MCA and DCAA concentrations, and 44% respiration
[e]Estimated from changes in DCAA concentrations, and 44% respiration

Table 2. Expt 1 Bacterial N uptake by incorporation of DFAA, DCAA and NH_4^+, compared to estimated bacterial N demand during exponential and stationary phases

	Net N demand[a] (µg N l^{-1})	DFAA incorporation µg N l^{-1} [b]	% of demand	µg N l^{-1} [c]	% of demand	DCAA incorporation µg N l^{-1} [d]	% of demand	µg N l^{-1} [e]	% of demand	NH_4^+ incorporation µg N l^{-1} [f]	% of demand
0 to 31 h											
Control	30.3	28.2	93	6.4	21	4.7	16	23.0	76	12.7	42
+NH_4^+	33.5	22.7	68	10.1	30	4.2	13	17.3	52	22.4	67
+glucose	58.7	32.6	56	10.8	18	6.5	11	−8.43	−14	28.8	49
+NH_4^+/glucose	55.8	48.8	87	10.1	18	5.7	10	4.71	8.4	79.1	140
31 to 55 h											
Control	5.21	66.1	130	1.23	24	4.43	85	−3.66	−71	7.56	150
+NH_4^+	13.8	45.5	330	−0.13	−0.90	6.3	46	−4.25	−31	7.70	56
+glucose	13.0	35.8	280	0.14	1.1	7.6	58	12.2	94	−0.28	−2.2
+NH_4^+/glucose	9.49	44.9	470	0.01	0.12	4.6	49	5.02	53	−2.38	−25

[a]Estimated from bacterial C demand (Table 1) assuming a molar C/N ratio of 4 (Lee & Fuhrman 1987)
[b]Estimated from the turnover rate of ^{3}H-labelled DFAA and DFAA concentrations
[c]Estimated from changes in DFAA concentrations
[d]Estimated from the turnover rate of Leu-MCA and DCAA concentrations
[e]Estimated from changes in DCAA concentrations
[f]Estimated from changes in NH_4^+ concentrations

Table 3. Expt 2. Net uptake of C by incorporation of DFAA and DCAA compared to estimated bacterial net C demand during exponential and stationary phases

	Net C demand[a] (µg C l^{-1})	Net DFAA incorporation µg C l^{-1} [b]	% of demand	µg C l^{-1} [c]	% of demand	Net DCAA incorporation µg C l^{-1} [d]	% of demand	µg C l^{-1} [e]	% of demand
0 to 21 h									
Control	25.4	10.6	42	3.56	14	2.50	10	5.63	22
21 to 45 h									
Control	51.5	31.1	60	1.12	2.2	4.0	7.8	−1.65	−3.2
+NH_4^+	66.2	44.0	67	0.44	0.7	3.9	5.9	−5.74	−8.7
+glucose	62.5	34.7	56	1.53	2.4	7.0	11	−39.9	−64
+DFAA	96.5	315	330	142	150	10.0	10	−10.6	−11

[a]Assuming a cell volume of 0.05 µm^3 (t = 0 h), 0.10 µm^3 (t = 9 h) and 0.15 µm^3 (t > 9 h) (Jørgensen et al. 1993, M. Middelboe unpubl. data) and 0.35 pg C µm^{-3} (Bjørnsen 1986)
[b]Estimated from the turnover rate of ^{3}H-labelled DFAA and DFAA concentrations
[c]Estimated from changes in DFAA concentrations, and 50% respiration (see text)
[d]Estimated from the turnover rate of Leu-MCA and DCAA concentrations, and 50% respiration
[e]Estimated from changes in DCAA concentrations, and 50% respiration

59% of total N uptake (DFAA + DCAA + NH_4^+), respectively, in Expt 1. The importance of NH_4^+ increased at the expense of DFAA in NH_4^+-enriched cultures. In Expt 2 the corresponding values were 78, 14 and 8% of total N uptake, respectively, during the initial 21 h.

Fig. 5 presents an overview of the contributions of DFAA, DCAA and NH_4^+ to bacterial N demand during different stages of the incubations. In both experiments there appeared to be a shift in the main bacterial DON (dissolved organic N) source from DFAA during the first 20 h towards a dominance of DCAA at the end of the incubations. Especially in glucose-enriched cultures not supplied with NH_4^+, DCAA constituted an important N source for the bacteria during the stationary phase. The DFAA-enriched culture deviated from this general pattern since DFAA in this culture constituted the single most important N source.

In Expt 1, NH_4^+ was the dominant N source in all the enriched cultures during the first 20 h. NH_4^+ then decreased in importance and eventually was regenerated during the last period. In contrast, the contribu-

Table 4. Expt 2. Bacterial N uptake by incorporation of DFAA, DCAA and NH_4^+, compared to estimated bacterial N demand during exponential and stationary phases

	Net N demand[a] (µg N l^{-1})	DFAA incorporation µg N l^{-1} [b]	% of demand	µg N l^{-1} [c]	% of demand	DCAA incorporation µg N l^{-1} [d]	% of demand	µg N l^{-1} [e]	% of demand	NH_4^+ incorporation µg N l^{-1} [f]	% of demand
0 to 21 h											
Control	7.40	9.80	130	2.51	34	1.80	24	5.87	79	0.98	13
21 to 55 h											
Control	15.0	27.7	190	0.93	6.2	2.5	17	0.10	0.7	−0.42	−2.8
+NH_4^+	19.3	39.6	210	0.37	1.9	2.5	13	−1.3	−6.7	8.82	46
+glucose	18.2	31.9	180	1.26	6.9	5.5	30	−19.7	−110	−0.84	−4.6
+DFAA	28.2	241	860	91.5	320	4.6	16	−13.1	−47	−55.2	−200

[a]Estimated from bacterial C demand (Table 3) assuming a molar C/N ratio of 4 (Lee & Fuhrman 1987)
[b]Estimated from the turnover rate of ^{3}H-labelled DFAA and DFAA concentrations
[c]Estimated from changes in DFAA concentrations
[d]Estimated from the turnover rate of Leu-MCA and DCAA concentrations
[e]Estimated from changes in DCAA concentrations
[f]Estimated from changes in NH_4^+ concentrations

tion of NH_4^+ in Expt 2 increased at the end of the incubation in all but the DFAA-enriched culture.

DISCUSSION

Differences in bacterial responses to nutrient additions strongly suggested that the carrying capacity (i.e. maximum bacterial abundance) in the 2 experiments was limited by different nutrients. In Expt 1 the bacteria were C limited, since the addition of only glucose caused an increase in bacterial abundance relative to the control (Fig. 1A). The 360 µg C l^{-1} added as glucose could explain the entire increase in bacterial C demand, if we assume an overall growth yield of 27% (Table 1).

In Expt 2 the control culture contained low concentrations of both C and N available for bacterial utilization. The glucose addition caused a large increase in growth rate (Fig. 1D) but an increase of only 11 µg C l^{-1} in biomass (Table 3), thus indicating that the lack of an easily available N source determined the carrying capacity in these glucose-amended cultures. Similarly, NH_4^+ addition caused a small increase in the maximum bacterial abundance, and cell production apparently became C limited in these cultures (Fig. 1C). Although DFAA was expected to fulfill the C and N demands of the bacteria, the addition of 260 µg C l^{-1} as DFAA resulted in a biomass increase of only 45 µg C l^{-1} (Table 3). Possibly, flagellate grazing or viral lysis reduced bacterial biomass before the carrying capacity was reached in this culture (Fig. 1C). These results indicated that cell production in Expt 2 may have been limited by an easily available N source and that DCAA were not able to sustain the bacterial N requirements during periods of limiting NH_4^+ and DFAA concentrations.

The evaluation of DFAA and DCAA as C and N sources for bacterial growth in the 2 experiments was complicated by the large discrepancies among the different ways of estimating their utilization by bacteria. Generally, estimates of DFAA assimilation based on ^{3}H-DFAA uptake exceeded decreases in DFAA concentrations, and, especially during the stationary phase, exceeded the cumulative C demand (Expt 1) and N demand of the bacteria (Expt 1 & 2) (Tables 1 to 4). Although previously noticed (Jørgensen et al. 1993, Rosenstock & Simon 1993) this phenomenon has not been examined closely. Rosenstock & Simon (1993) found a good agreement between the decrease in DFAA concentration and the concurrent incorporation of DFAA estimated from net incorporation of ^{3}H-DFAA in batch cultures, and the authors used this correspondence as an argument for a negligible DFAA respiration. However, assuming 40 to 80% respiration of ^{3}H-DFAA, which has been observed several times (e.g. Suttle et al. 1991, Jørgensen et al. 1994, this study), DFAA uptake estimated by Rosenstock & Simon (1993) would also exceed the decrease in DFAA concentration by more than 100%. In a similar study, Jørgensen et al. (1993) found that the estimated DFAA uptake (not including respired DFAA) exceeded the decrease in DFAA concentration by a factor of 12 to 36 during the first 26 h of incubation. The authors suggested that DCAA was the main source of incorporated DFAA, but they did not observe a corresponding decrease in DCAA concentrations. DCAA hydrolysis and uptake of released DFAA may provide part of the

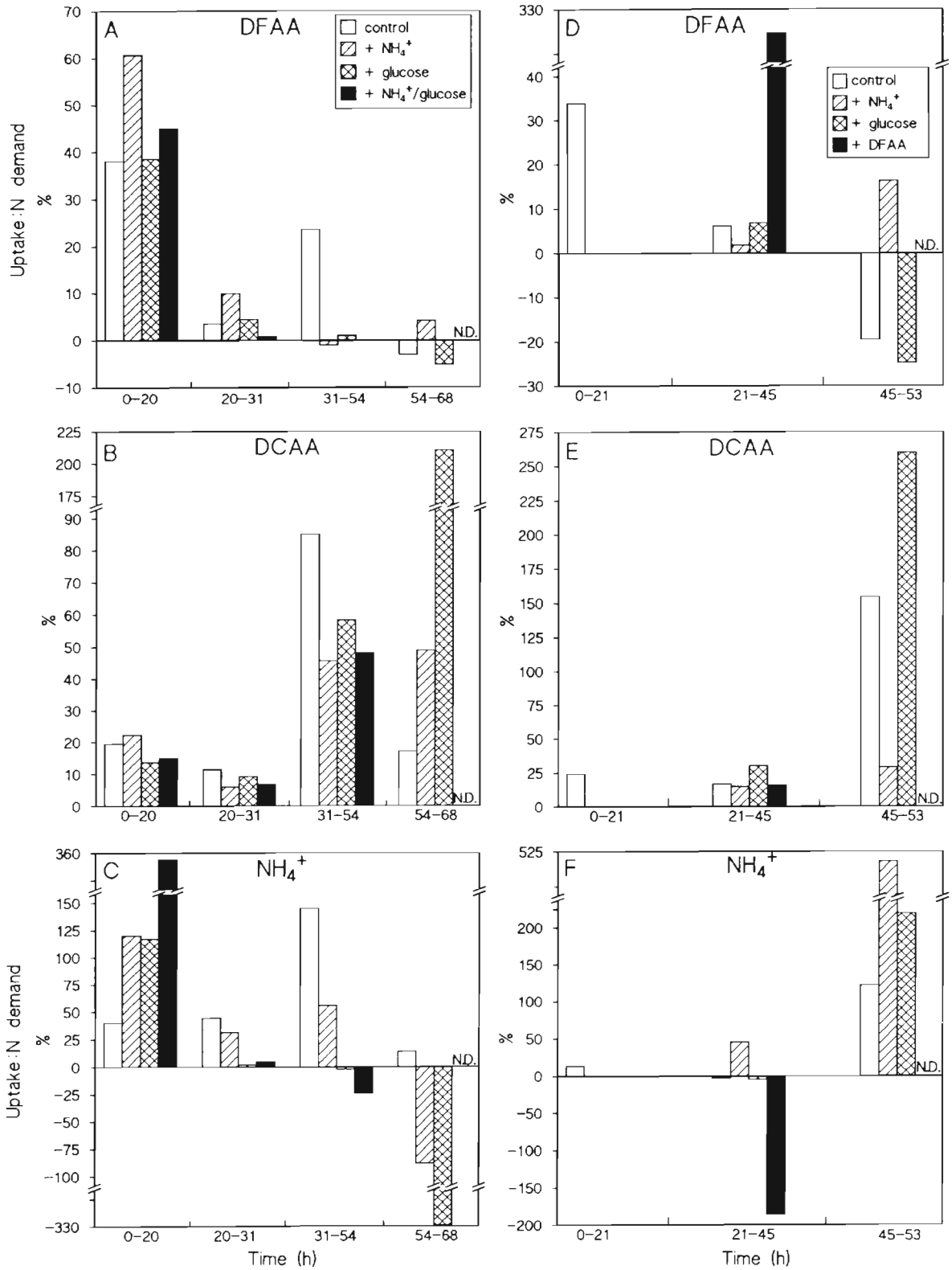

Fig. 5. Uptake:demand ratio of N supported by DFAA, DCAA and NH_4^+, respectively, in (A to C) Expt 1 and (D to F) Expt 2. Values are based on Leu-MCA turnover and changes in DFAA and ammonium concentration

explanation for the observed disparity (Tables 1 to 4; Jørgensen et al. 1993), assuming that products from DCAA hydrolysis actually enter the bulk pool of DFAA prior to assimilation.

As an additional explanation we suggest that DFAA are recycled efficiently within the bacterial assemblage. DFAA and DCAA may be released from the bacteria by cell lysis, flagellate grazing and viral infection, and utilized by other bacteria. This hypothesis was supported by the fact that during the first 10 to 20 h, DFAA assimilation was equal to the sum of the decrease in DFAA and the estimated release of DFAA from DCAA hydrolysis, and only at the end of the exponential phase did recycling of DFAA apparently become an increasingly important part of the DFAA flux. During substrate limitation, bacteria thus seem able to sustain a 'regenerated' production based on recycling of bacterial biomass. This may have important consequences for the interpretation of bacterial growth in batch cultures. Changes in cell counts or biomass may seriously underestimate gross production and may not be comparable to incorporation rates of amino acids and thymidine even during short incubations (e.g. 20 h). Consequently, bacterial recycling of organic compounds may bias measurements of conversion factors which relate bacterial thymidine or leucine incorporation to cell production. Our observations may explain why conversion factors measured in batch cultures frequently have been higher than expected, based on theoretical calculations, especially when those factors are calculated from changes in incorporation rates over time (e.g. Kirchman et al. 1982).

We calculated bacterial DCAA utilization from the decrease in DCAA concentrations in order to assess the applicability of Leu-MCA hydrolysis in estimating total DCAA hydrolysis. Such a comparison is, however, not straightforward, since DCAA concentrations increased during part of the experiments. The comparison was limited to periods of decreasing DCAA, and thus only reflected net removal of DCAA during those periods. From a total of 23 periods in the 2 experiments where it was possible to compare the 2 estimates of DCAA hydrolysis, we found that the Leu-MCA based estimate on average constituted 42% ± 37% (±SD, n = 23) of DCAA assimilation based on decreases in DCAA concentration. In the Leu-MCA based estimate we assumed all the measured DCAA to be potential substrates for the aminopeptidase. Since only a part of the measured DCAA may constitute a substrate for bacterial extracellular hydrolysis (Keil & Kirchman 1993), rates of DCAA hydrolysis based on Leu-MCA are probably overestimated. Moreover, since a decrease in DCAA concentration only reflected net removal by the bacteria it represented a minimum estimate of DCAA hydrolysis. Consequently, our results indicate that hydrolysis of Leu-MCA, when used in tracer concentrations, may underestimate actual protein hydrolysis. Although aminopeptidases as detected by Leu-MCA are expected to be important in processing of natural peptides (Hoppe et al. 1988), the model substrate obviously did not fully reflect total protein hydrolysis in the cultures. Part of the decrease in DCAA concentration may, however, be ascribed to uptake of small peptides (<6 amino acids), which can be utilized without exoproteolysis (Payne 1980).

Utilization of DCAA as estimated from Leu-MCA hydrolysis was generally of minor importance during exponential growth in Expt 1 (Tables 1 & 2), and it accounted for at most 25% of the N demand in Expt 2 (Table 4). Based on the decrease in DCAA concentration, DCAA utilization in Expt 1 appeared to be repressed by the glucose addition, since DCAA utilization was delayed in +glucose and $+NH_4^+$/glucose in contrast to control and $+NH_4^+$ cultures (Fig. 2D, Tables 1 & 2). The importance of DCAA as an N source increased during the stationary phase, and DCAA was the dominant N-source for bacteria growing without an NH_4^+ addition in Expt 1 (Table 2, Fig. 5). Similarly, DCAA was the most important N-source in the +glucose culture during the stationary phase in Expt 2 (Table 4, Fig. 5). The results indicate that DCAA may contribute significantly to bacterial C and N demand when easily available C and N compounds are low, consistent with the conclusions of previous studies (Keil & Kirchman 1991, 1993, Simon & Rosenstock 1992, Kroer et al. 1994). Moreover, DCAA concentration measurements demonstrated that DCAA were released from the bacteria, especially during the stationary phase (Figs. 2D & 3D). This DCAA release is consistent with our hypothesis about DFAA release during the stationary phase. In contrast to DFAA, DCAA were not taken up as fast as DFAA and therefore accumulated periodically.

During exponential growth DFAA + DCAA + NH_4^+ sustained 109 to 170% and 71 to 78% of the estimated N-demand in cultures with and without addition of NH_4^+, respectively (Tables 2 & 4). Values <100% may be explained by the utilization of other N sources like NO_3^- as demonstrated by Jørgensen et al. (1993, 1994). Estimates of bacterial N uptake in excess of their estimated demand have previously been observed (Keil & Kirchman 1991, Jørgensen et al. 1994, Kroer et al. 1994). Although this phenomenon in some cases may be partly explained by the likely interference of DFAA recycling, unbalanced N budgets appear to be a general feature of bacterial growth in batch cultures. Kroer et al. (1994) suggested the release of N compounds like methylamines and urea as a possible explanation for the N imbalance since they did not find changes in bacterial C:N ratio during excessive N uptake. More-

over, bacterial nitrification of NH_4^+ may be an important process in batch experiments (Bronk & Glibert 1993, Søndergaard & Middelboe 1995), causing an overestimation of bacterial NH_4^+ uptake. Finally, our comparisons of bacterial nutrient uptake and demand are associated with some uncertainty since estimates of bacterial C and N demand rely on the use of theoretical conversion factors to calculate cell C and N content from cell abundance.

The DFAA source in batch cultures is limited to a small concentration of DFAA present at the time of sampling in contrast to productive natural systems, characterized by a constant input of DFAA from various sources. The rapid utilization of DFAA and ammonium and the shift towards utilization of DCAA at the end of the incubations support the opinion that bacterioplankton growth in natural systems is generally based on a close coupling between supply and uptake of readily available organic and inorganic nutrients, and that polymeric compounds may provide important C and N sources during periods of low input of labile substrates (Coffin et al. 1993, Middelboe & Søndergaard 1993, 1995). Going from productive coastal systems to oligotrophic oceanic waters, the contribution of DCAA to bacterial C demand would probably increase, since bacterial hydrolysis of POC (particulate organic C) and DCAA may provide the main source of DFAA in such low-productive systems (Hoppe et al. 1993).

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