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Sources and sinks of dissolved free amino acids and protein in a large and deep mesotrophic lake

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Abstract

We studied the microbial cycling of dissolved free amino acids (DFAAs) and protein in mesotrophic Lake Constance, Germany, by examining their release by phytoplankton and various heterotrophic organisms and incorporation by heterotrophic bacterioplankton. Release processes of both substrate classes, measured by an isotope dilution approach, comprised, as an annual mean, 15% of primary production and as much as 64% during the clear-water phase. DFAAs accounted for ~70% of total release during the spring bloom, in the early phase predominantly as photosynthetic extracellular products of rapidly growing algae and toward the end as a result of copepod grazing. Thereafter, during the clear-water phase, when daphnids were most abundant, release was dominated by protein. At this time, and again in late summer, lysis of grazing-damaged and senescent algae, including as well the hydrolytic activity of attached bacteria was one of the most important sources of protein. Rotifers, protozoans, and release processes in the fraction <1 μm were minor sources of DFAA + protein. Concentrations of dissolved combined amino acids (DCAAs) and protein ranged between 750–1,900 and 1–280 nM, respectively, and peaked during phytoplankton blooms in spring and summer. As an annual mean, concentrations of labile protein constituted 8% of DCAAs, and the ratio of DFAAs to DCAAs was 0.16. About 50% of the DCAAs occurred in the molecular weight fraction between DFAA and 3 kDa and 30% in that >30 kDa. Concentrations of DCAAs >3 kDa were closely correlated to chlorophyll *a*, suggesting their phytoplankton origin and thus a ready availability. Protein was the preferred bacterial substrate. As an annual mean, its incorporation supported 45% of bacterial biomass production, compared with 13% by DFAAs. During winter and spring, when DFAA concentrations were highest, DFAA incorporation constituted up to 40% of bacterial production. Annually, the sum of DFAA + protein supported 58% and 80% of the bacterial C and N demand, respectively, indicating that they were the most important bacterial C and N sources.

In pelagic ecosystems, the pool of dissolved organic carbon (DOC) mainly originates from phyto- and zooplankton, and its excretion can account for <10% to >70% of primary production (PP), depending on the trophic state of the system and the physiological state of the phytoplankton (Sundh 1992*a*,*b*; Münster 1993). Heterotrophic bacteria are the most important sink of DOC, consuming up to 80% of the pelagic PP (e.g., Azam et al. 1983; Simon et al. 1998). DOC mainly consists of recalcitrant material, and only 10%–30% so far has been chemically characterized (Benner et al. 1992; Münster 1993). It has been suggested that high-molecular-weight (HMW) DOC is more bioreactive and less diagenetically altered than low-molecular-weight (LMW) DOC (Amon and Benner 1996).

The labile, bioavailable fraction only accounts for <40% of the bulk DOC and mainly consists of amino acids and carbohydrates (Münster 1991, 1993; Tranvik and Jørgensen 1995; Weiss and Simon 1999). Much is known about the turnover of dissolved free amino acids (DFAAs) and their significance for the growth of heterotrophic bacteria (e.g., Jørgensen 1987; Simon and Rosenstock 1992). Several studies found that DFAAs and ammonium are the primary N sources for bacteria (Keil and Kirchman 1991; Jørgensen et al. 1993; Middelboe et al. 1995*a*), whereas others showed that dissolved combined amino acids (DCAAs) are of similar or even higher significance for the growth of bacterioplankton (Coffin 1989; Rosenstock and Simon 1993; Kroer et al. 1994). The DCAA pool is chemically complex and is composed of various types of combined amino acids, including oligo- and polypeptides, protein, and amino acids bound to humic substances (Keil and Kirchman 1993; Hubberten et al. 1994). Even though labile protein accounts for <10% of DCAAs, it can support 20%–65% of the bacterial N demand (Keil and Kirchman 1999).

Dissolved amino acids can be released as photosynthate or by autolysis of senescent algae (Gardner et al. 1987; Sundh 1992*a*,*b*). Grazing activities of zooplankton also lead to the release of amino acids (Fuhrman 1987; Nagata and Kirchman 1991). Possible modes of grazer-associated amino acid release include sloppy feeding, excretion, egestion, and dissolution of fecal material. Further sources of amino acids are the solubilization of organic particles such as lake snow (Grossart and Simon 1998) and virus-induced lysis of algae

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and bacteria (Fuhrman 1992). Specific release processes of DCAAs and protein have been little studied (Nagata and Kirchman 1991; Sundh 1992*a*,*b*). In order to better understand the turnover of amino acids, one of the most important classes of labile dissolved organic matter (DOM), it is important to examine sources and release processes of DFAAs, protein, and DCAAs.

We assessed sources and sinks of DFAAs and protein, applying an isotope dilution approach that used ^{14}C algal protein and a mixture of ^{3}H DFAAs and measured incorporation of these substrates by heterotrophic bacteria and release processes by different plankton fractions. A further aim of this study was to examine the cycling of different molecular weight (MW) fractions of amino acids within the microbial loop and their role as bacterial substrates.

Materials and methods

Lake Constance (Bodensee) is a mesotrophic and warm-monomictic prealpine lake in central Europe with an area of 571 km^2 and maximum and mean depths of 254 and 100 m, respectively. Plankton dynamics are largely dominated by autochthonous processes and have been studied extensively during the last decade in this lake (e.g., Gaedke 1998; Simon et al. 1998; Straile and Geller 1998). The study was carried out between March 1993 and October 1994 at the center of Lake Überlingen, the northwestern fjordlike arm of the lake, with maximum and mean depths of 147 and 90 m, respectively. Samples were collected with a 10-liter van Dorn bottle at 3, 10, 15, and 50 m, withdrawn into acid-rinsed 2-liter polyethylene bottles, and brought to the lab within 2 h. Samples for amino acid concentrations were filtered through 0.2-μm Gelman Acrodiscs (Tuffrin-polysulfon) and kept frozen at -20°C in combusted 20-ml glass vials until analysis. Additional parameters—e.g., temperature, PP, and biomass of ciliates, rotifers, daphnids, and copepods—were measured simultaneously at the same sampling site. They were available from the database of the Special Collaborative Program ''Cycling of Matter in Lake Constance.'' Primary production was measured in situ by the ^{14}C bicarbonate method as described in Tilzer and Beese (1988). Incubations lasted 4 h around noon.

Analysis of dissolved amino acids—Concentrations of DFAAs were measured by high-pressure liquid chromatography (HPLC) according to the method of Lindroth and Mopper (1979) after precolumn derivatization with orthophthaldialdehyde, as described in detail in Rosenstock and Simon (1993). The mean coefficient of variation (CV, standard deviation) of the analyses was <5%.

Concentrations of DCAAs were analyzed as DFAAs after acid hydrolysis according to the method of Parsons et al. (1984). Five hundred microliters of each sample were hydrolyzed with 6 N HCl in sealed glass ampoules under nitrogen at 110°C for 20 h. Prior to hydrolysis α-amino butyric acid was added as internal standard (160 nM final concentration). After hydrolysis, samples were dried and diluted 1 : 8 with Milli-Q water for HPLC analysis. Concentrations of DCAAs were calculated as total dissolved amino acids (TDAAs) − DFAA. The mean CV of the analyses was <10%.

MW fractionation—The pool of DCAAs was separated into the MW fractions <3, 3–30, and >30 kDa by ultrafiltration in centrifugation tubes (Centricon 3,000 and 30,000, Amicon). In some samples, the <500-Da fraction (MPS-1, Amicon) was also separated. All tubes and filter membranes were cleaned several times with Milli-Q water, 0.1 M NaOH (Centricon), and 2 M NaCl (MPS-1) prior to ultrafiltration. The centrifugation tubes were reused several times and cleaned again by the same procedure. Possible contaminations were taken into account by the analysis of one blank with Milli-Q water per each batch of samples, which was subtracted from each sample. One milliliter of sample was withdrawn into each tube and centrifuged for 30 min at 1,700 × g (MPS-1 and Centricon 30,000) and for 60 min at 3,000 × g (Centricon 3000) in a Labofuge GL (Heraeus Sepatech). Concentrations of DFAAs and DCAAs in the filtrates were determined by HPLC (see above). Concentrations of the different MW fractions—e.g., the 3–30 kDa fraction—were calculated as 30 − 3 kDa. All samples were analyzed in duplicate, which usually agreed within 5% (Centricon) and 10% (MPS-1). Tests with DFAAs, tetra-alanine (MW 302), and hexa-tyrosine (MW 997) showed that these compounds passed the 3-kDa membrane to 93%–96%.

Bacterial biomass production—Bacterial biomass production (BP) was determined by incorporation of ^{14}C leucine (Leu) according to the methods of Kirchman et al. (1985) and Simon and Azam (1989). Six milliliters of sample were incubated with ^{14}C Leu (30 nM final concentration, specific activity 312 mCi mmol^{-1}, Amersham) in triplicate and with a Formalin-killed control at in situ temperature in the dark. Incubations were stopped after 1 h and filtered through 0.45 μm nitrocellulose filters (Sartorius), and macromolecules were extracted with 5% ice-cold trichloroacetic acid for 5 min. Thereafter, the filters were radioassayed by liquid scintillation counting. BP (μg C L^{-1} h^{-1}) was calculated according to the method of Simon and Rosenstock (1992), under the assumption of a 2.4-fold intracellular isotope dilution of Leu and a partitioning of Leu in the protein fraction of 86% of the total macromolecular fraction. To convert bacterial C into N production, we assumed a C : N ratio of bacteria of 4 (Lee and Fuhrman 1987).

Incorporation of amino acids and protein—Incorporation of DFAAs and protein into the bacterial biomass was determined by measuring the turnover rates of a mixture of 15 ^{3}H DFAAs (mean specific activity 31.3 Ci mmol^{-1}, Amersham) and a ^{14}C algal protein (specific activity 44 mCi mmol^{-1}, Amersham). This custom-made protein, extracted from *Anacystis nidulans* (Keith et al. 1974), also constituted 40% of oligo-peptides of <3 kDa and served as a model substrate for labile proteins of a wide range in MW and typical under natural conditions. For calculation of the specific activity, the protein concentration in the stock solution was determined by the bicinchoninic acid method (Keil and Kirchman 1993). The radiolabel was added in triplicate to 6-ml subsamples and a Formalin-killed control not exceed-

ing one tenth of the in situ concentration: 0.8–1.7 nM final concentration for ^{3}H DFAAs and 0.7–3 nM for ^{14}C protein. Incubations were further processed as described for BP. The CV of the triplicate measurements was <10%. Bacterial turnover rates of DFAAs and protein ($T_{DFAA,protein}$) were calculated from the incorporated radioactivity over the added radioactivity and the turnover times as the inverse of the turnover rates. Incorporation rates of DFAAs and protein into bacterial biomass ($I_{DFAA,protein}$) were calculated as

$$I_{DFAA,protein}\ (\mu g\ C\ L^{-1}\ h^{-1}) = \text{Concentration}_{DFAA,protein} \times T_{DFAA,protein}.$$

The CV was usually <8%.

The concentration of labile protein was calculated from $K_t + S_n$, derived from incorporation kinetics according to the method of Wright and Hobbie (1965). K_t is the half-saturation constant, and S_n is the in situ concentration of bioavailable protein. Therefore, incorporation of ^{14}C protein was measured at four concentrations (1, 20, 50, and 100 nM final concentration). Incubation and further processing was done as described for measurements of BP, and S_n was calculated as 50% of $K_t + S_n$ (Billen 1991; Keil and Kirchman 1993). R^2 of the regression to calculate $K_t + S_n$ was >0.93. Even though we did not test the validity of the protein uptake kinetics, data from Keil and Kirchman (1993) indicate that uptake kinetics of various types of defined labile proteins and extracted protein from algae are similar.

Release of DFAAs and protein—Release of protein and DFAAs was examined by an isotope dilution approach in three different plankton size fractions: <1 μm (bacteria, viruses, DOM), 1–140 μm (microplankton), and >140 μm (mesoplankton). To obtain the <1 μm and the microplankton size fractions, 200 ml of water from 3-m depth were filtered by gravity through a 1-μm Nuclepore filter and a 140-μm gauze, respectively. Mesoplankton-related activities were examined in unfiltered samples and by subtracting the activities of the smaller size fractions. Each sample was divided into two 100-ml aliquots. One was taken for measuring the time course of amino acid concentrations (see above) and the other to monitor the radioactivity in the dissolved fraction. Therefore, a mixture of 15 ^{3}H amino acids and ^{14}C protein was added to the latter at 1 and 2 nM final concentrations, respectively. The samples were incubated for 4 h at in situ light conditions (50 μE m^{-2} s^{-1} at 3 m depth), but one experiment was also incubated in the dark. Subsamples were taken at the beginning (t_0), after 1.5 h (t_1), and after 4 h (t_2) for amino acid analyses (see above) and for measuring the extracellular specific activity of ^{3}H DFAAs and ^{14}C protein. Therefore, 6 ml were filtered through 0.45-μm nitrocellulose filters, and 1 ml of the filtrate was radioassayed after adding 4 ml of scintillation cocktail. Measurements of the specific activity of the ^{3}H amino acids and ^{14}C protein in 0.2-μm filtered controls over the incubation period showed that abiotic adsorption of radiolabeled amino acids and protein was <5%. This finding ensured that dynamics of amino acids and proteins were only due to biological uptake and release processes and that this method, originally developed for inorganic nitrogen, yielded reliable results. To correct the release of radiolabeled protein for respiratory production of $^{14}CO_2$, we measured its respiration in four experiments and found that it was <10% of the total amount of labeled protein. The same assumption was made for ^3H_2O produced from tritiated DFAAs. Additional uptake kinetics were performed at each sampling point, to determine the protein concentration, as described earlier. Release rates of DFAAs and protein were determined according to the method of Glibert et al. (1982). This method allows one to determine the release of labile DOC compounds in short-time experiments independent from uptake processes:

$$\text{Release rate} = \frac{\ln(R_0/R_{1,2})}{\ln(C_0/C_{1,2}) \times t_{1,2}} \times (C_0 - C_{1,2}).$$

R is the extracellular specific activity (Ci mmol^{-1}), and C is the concentration of DFAAs or protein at time t_0, t_1, or t_2. Release rates were calculated as means of the time intervals $t_0 - t_1$ and $t_0 - t_2$. The means of these two time intervals varied from 5% to 54% in the 22 experiments, but in 75% they agreed within 19%. We calculated individual release rates for the microplankton fraction as <140 minus <1 μm and for the mesoplankton fraction as unfiltered minus <140 μm. The bacterial assimilation efficiency of TDAAs was calculated as incorporation of DFAA + protein into bacterial biomass (see above) over total uptake of DFAA + protein, which was determined from the decrease in concentrations in the <1-μm fraction over time.

Amino acid release by various plankton organisms—To relate release processes to the phytoplankton and ciliates occurring in the size fraction 1–140 μm and to rotifers, we determined the release rates of these groups of organisms separately. Therefore, the abundance of ciliates and rotifers was raised by a factor of 2–8 (20 in two experiments) in the size fractions <140 μm, by use of model organisms isolated from Lake Constance. As a model organism for ciliates, we selected *Balanion planctonicum*, which is highly abundant in Lake Constance and constitutes up to 40% of the ciliate biomass (Müller et al. 1991), and *Keratella cochlearis* as a model rotifer of up to 30% of total rotifer biomass in the second half of the year (Pauli 1990). Ciliates were added at four experiments from April to June 1994 and rotifers at two experiments in June and July 1994. These additions enriched the ciliate samples by up to 100% of DCAAs and the rotifer samples by up to 60% of DFAAs, respectively. These enrichments, however, had no effect on calculation of the release rates because they were included in the C_0 values. The enrichment factor for ciliates and rotifers was calculated as the abundance in the enriched <140 μm fraction over the natural abundance of a mixed sample (0–8 m) from Lake Constance from the same day. Ciliate and rotifer numbers were determined in 50-ml Lugol-fixed subsamples with the Utermöhl method (Müller et al. 1991). Release rates under in situ conditions were recalculated by dividing the release rates of DFAAs and protein in the experiments by the enrichment factors.

We found a close correlation ($r = 0.97$, $P < 0.05$) between the production rates of ciliates (P_{Cil}) and corresponding in situ release rates of the sum of DFAA + protein (R_{Cil}). Therefore, we calculated amino acid release rates due to cil-

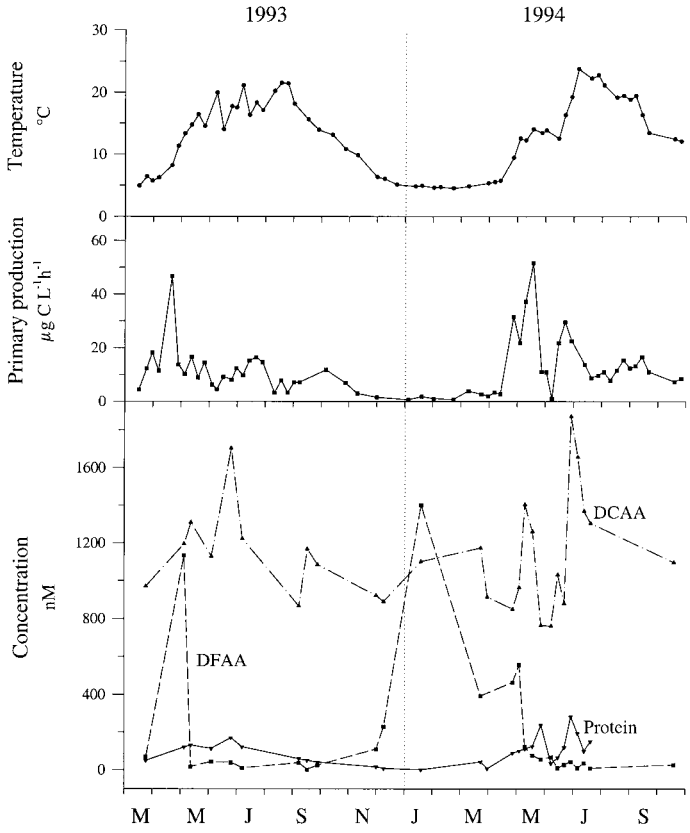

Fig. 1. Temperature, primary production, and concentrations of DCAAs, DFAAs, and protein at 3 m depth in Lake Constance between March 1993 and October 1994.

iates from their production rates in all experiments without ciliate addition as $R_{Cil} = 0.72 \times P_{Cil} + 0.05$. The same assumption was made for rotifers, to obtain release rates of DFAA + protein (R_{Rot}) from their biomass production (P_{Rot}) as $R_{Rot} = 0.05 \times P_{Rot} - 0.01$. The biomass production of ciliates and rotifers in Lake Constance was calculated from production : biomass ratios according to the method of Straile (1998). Phytoplankton-derived amino acid release (R_{Phyto}) was calculated from the different release rates for DFAA + protein as $R_{Phyto} = R_{1-140\mu m} - R_{Cil} - R_{Rot}$.

Results

In the 2 yr studied, pronounced differences in plankton dynamics occurred because of different thermal regimes of Lake Constance. In 1994, warming of the lake started 1 month later than in 1993 but developed in two distinct short phases (Fig. 1). Accordingly, the maximum of PP during the phytoplankton spring bloom in 1994 occurred in mid-May, compared with mid-April in 1993. A second algal bloom developed in 1994 in late June, whereas in 1993 primary production fluctuated fairly little in summer and fall.

On the basis of physical, chemical, and biological parameters, we differentiated the year into five typical seasonal phases (Geller et al. 1991): (1) the spring bloom from April to May, characterized by high phytoplankton biomass and high abundances of copepods and ciliates; (2) the clear-water phase at the end of May and early June, with a massive grazing of algae by daphnids; (3) summer (July, August), when diatoms dominated the phytoplankton; (4) fall (September, October), with again elevated PP rates and zooplankton abundances; and (5) winter, with generally low plankton biomass.

Concentration and MW distribution of dissolved amino acids—Concentrations of DFAAs, DCAAs, and protein ranged from 2 to 1,400, 750 to 1,900, and 1 to 280 nM, respectively. DFAAs exhibited maxima in winter until the spring bloom, whereas DCAAs and protein peaked during the phytoplankton blooms in spring and summer (Fig. 1). As an annual mean, ratios of DFAA : DCAA were 0.16, but during the growing season ratios were usually <0.05 and in winter were sometimes as high as 1.3. Protein accounted for 1%–31% of DCAAs, with highest and lowest fractions during the clear-water phase and winter. The means of these periods varied from 1% to 18% (Table 1). From May until fall, ratios of protein : DCAA were four times higher than that of DFAA : DCAA. The LMW fraction of DCAAs (<3 kDa) dominated the TDAA pool, composing 50% of it (Table 2). The highest proportions occurred during the spring bloom and during the fall. The fraction >30 kDa constituted ~30% of TDAAs, with the highest proportions during the spring bloom and summer. DFAAs and the intermediate fraction (3–30 kDa) constituted ~10% of TDAAs as an annual

Table 1. Mean values ± standard deviation for various seasonal phases of labile protein as percentage of DCAAs, turnover times, and incorporation rates of protein and DFAAs (percentage of bacterial production [BP]), and the bacterial assimilation efficiency of protein (incorporation/total consumption in %) in 1993 and 1994 at 3 m depth in Lake Constance. ND, not determined.

Seasonal phases	Protein concentration	Turnover		Incorporation		Assimilation efficiency
		Protein	DFAA	Protein	DFAA	Protein
1993/1994	% DCAA	(h)	(h)	% BP	% BP	(%)
Spring	9 ± 1	14 ± 8	107 ± 120	60 ± 31	26 ± 11	33
Clear-water	18 ± 13	22 ± 14	72 ± 63	35 ± 12	10 ± 6	26
Summer	11 ± 3	8 ± 4	46 ± 69	47 ± 41	2 ± 1	64 ± 6
Fall	5 ± 1	8 ± 1	21 ± 18	36 ± 8	7 ± 4	77
Winter	1 ± 1	73 ± 62	2,764 ± 3,025	13 ± 9	13 ± 11	ND
Mean	8 ± 7	24 ± 37	564 ± 1,673	45 ± 39	13 ± 17	57 ± 17

Table 2. Mean concentrations ± standard deviation of different MW fractions of dissolved amino acids and the ratio of each fraction to TDAAs at 3 m depth in Lake Constance from March 1993 to October 1994.

MW fraction (kD)	Concentration (nM)	MW fraction (% TDAA)	*n*
DFAA	186 ± 344	11 ± 15	26
DCAA			
<3	631 ± 185	50 ± 14	26
3–30	120 ± 94	11 ± 8	26
>30	363 ± 209	28 ± 13	26
DCAA			
<0.5	450/586	27/33	2
0.5–3	299/387	18/22	2

mean, each. Although the intermediate fraction always constituted low proportions, DFAAs constituted high proportions during late winter and the early spring bloom, when absolute concentrations were also high. In two experiments in June and September 1993, using a cutoff of 500 Da, amino acids <500 Da, including DFAAs, accounted for ~40% of TDAAs.

The vertical distribution of different MW fractions of DCAAs varied according to plankton dynamics in Lake Constance. In March, when chlorophyll *a* was still low, the bulk of DCAAs in the upper 15 m constituted 36% (range 25%–58%) of HMW compounds >3 kDa (Fig. 2A). In June and July, when Chl *a* concentrations were enhanced in the euphotic zone, HMW-DCAA constituted 56% (range 45%–70%) of the total (Fig. 2B) and were closely correlated to Chl *a* ($r = 0.80$, $P < 0.004$). At 50 m depth, the pool of DCAAs was dominated by LMW-DCAA, which constituted 62%. The close and highly significant correlation of HMW-DCAA with bulk DCAAs ($r = 0.80$, $P < 0.001$; Fig. 2C) indicated that below the intercept with the x-axis of 327 nM, only LMW-DCAA would be available. Similar correlations with DCAAs >30 kDa and protein indicated that these fractions become 0 below DCAA concentrations of 471 and 678 nM, respectively.

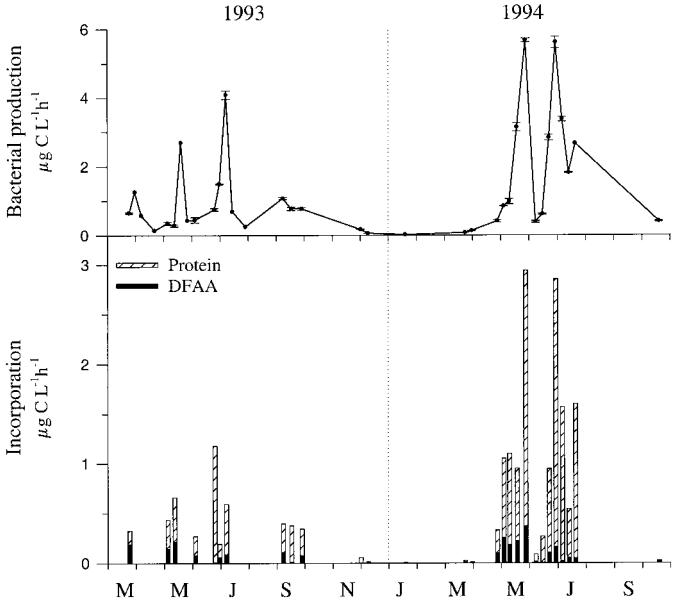

Fig. 3. Bacterial production and incorporation rates of protein and DFAAs at 3 m depth between March 1993 and October 1994.

Turnover and bacterial incorporation of DFAAs and protein—Bacterial production covaried with PP and reached maxima during the late spring bloom in May and the summer bloom in July with >2.8 μg C L^{-1} h^{-1} (Figs. 3 and 4). During the clear-water phase in June and the winter period, BP decreased to minima <0.5 μg C L^{-1} h^{-1}. BP and PP were positively correlated ($r = 0.56$, $P < 0.005$). This cor-

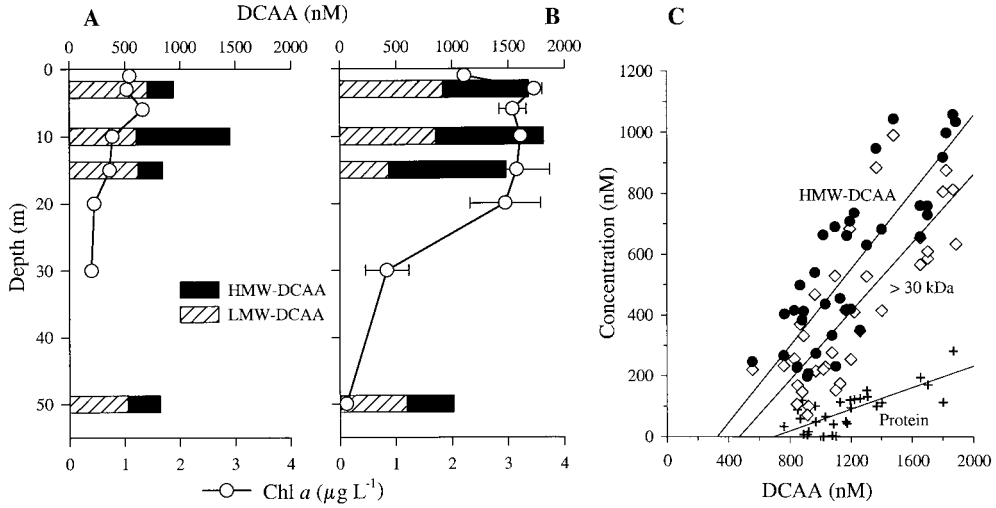

Fig. 2. Depth profile (3, 10, 15, and 50 m) of LMW (<3 kDa) and HMW (>3 kDa) DCAAs and Chl *a* in (A) March and (B) June. (C) Linear regressions between concentrations of DCAAs and protein ($y = 0.18 \times -122$, $r = 0.7$, $P < 0.001$), DCAAs and HMW DCAAs ($y = 0.63 \times -206$, $r = 0.8$, $P < 0.001$), and DCAAs and the fraction >30 kDa ($y = 0.56 \times -264$, $r = 0.8$, $P < 0.001$).

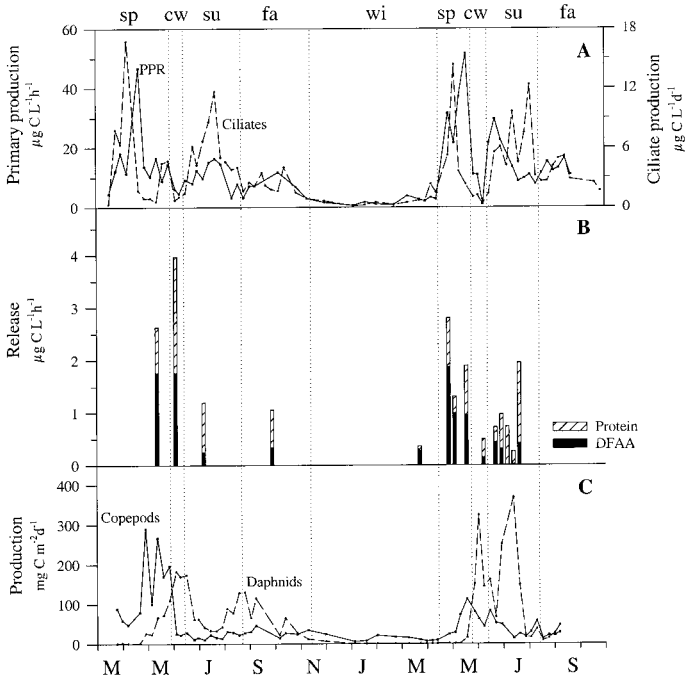

Fig. 4. (A) Primary production and production of ciliates and (C) of copepods and daphnids. (B) Release rates of protein and DFAAs. All data presented are from 3 m depth, except the production of copepods and daphnids, which are integrated values for the upper 50 m. Seasonal phases are separated by dotted lines (sp, spring; cw, clear-water; su, summer; fa, fall; wi, winter).

relation further improved under the assumption of a lag of BP of 1 week ($r = 0.71$, $P < 0.005$).

Bacterial production was fuelled by varying fractions of TDAAs during different seasonal phases (Fig. 3). DFAAs were relatively more important as a bacterial substrate from January until May, ranging from 30% to 60% of the total incorporation of DFAA + protein. We have no indication that individual DFAAs were preferentially utilized, because in the incubations of 1-μm filtrates in which we measured bacterial consumption of DFAAs and DCAAs, the mol% distribution remained constant. Incorporation of protein dominated from June to November, with a mean of 85% of total incorporation. Maximum incorporation rates coincided with or shortly preceded peaks of BP. In spring, ~85% of the bacterial production were supported by incorporation of DFAA + protein but during the other seasonal phases much less (Table 1). As an overall mean, protein accounted for 45% and DFAA for 13% of BP. Hence, ~40% of BP was supported by organic carbon other than dissolved amino acids. Since amino acids are relatively enriched in N, compared with bacteria, the N demand of heterotrophic bacteria was, on average, supported to ~80% by the incorporation of DFAAs and protein. Assimilation efficiencies of protein increased during the growing season from 26% to >64% and were 57% as an annual mean (Table 1). The turnover of protein was always faster than that of DFAAs and ranged from <8 to >73 h (Table 1). During the growing season, turnover times of protein were usually <22 h and were shortest during summer and fall. Turnover times of DFAAs were >21 h and increased to >100 d in winter.

Release of dissolved amino acids—Release of DFAA + protein ranged from 0.4 to 4 μg C L^{-1} h^{-1}, with the highest rates during the spring bloom and the clear-water phase in 1993, reaching 64% of PP (Fig. 4B). The annual mean was 15% of PP. During the clear-water phase in 1994, however, only low release rates were recorded. These differences were mainly due to the different times of the experiments in both years. In 1993, abundance and production of daphnids peaked at the time of the experiment, whereas in 1994 the daphnid production had already declined (Fig. 4C). From January until May, total release of amino acids was dominated by DFAAs to 71% ± 11%, whereas later on protein dominated by 67% ± 14%. High release rates of DFAAs during spring coincided with enhanced concentrations and incorporation rates of DFAA (Figs. 1, 3). As an overall mean, protein release was 17% higher than that of DFAAs.

Amino acid release by the mesoplankton size fraction, predominantly as DFAAs, was highest in spring and during the clear-water phase (Fig. 5A). Copepods and daphnids apparently mediated the release in this fraction to a great extent, since their production peaked in late spring and the clear-water phase (Fig. 4). At the end of April 1994, however, when the production of copepods and daphnids was low, other sources of amino acids in the mesoplankton fraction must have contributed to the release. A second maximum of the daphnid production in summer did not result in enhanced release of amino acids. Release of amino acids by the microplankton fraction was mainly due to phytoplankton and was also dominated by DFAAs in spring and by protein later on during the season (Fig. 5F). Even though we calculated the release of amino acids by phytoplankton and did not measure it directly, we found a close correlation between release rates and PP ($r = 0.86$, $P < 0.0001$), ignoring two dates in the clear-water phase and in late summer. One experiment in the latter season, with simultaneous light and dark incubations, showed that release of DFAA + protein in the dark constituted 55% of the total release. At these times, amino acids obviously were not released by actively growing but rather by lysing phytoplankton. Ciliates, on average, supported 10% of the total release with highest values in spring and summer (Fig. 5E). Rotifers were a negligible source for amino acids (Fig. 5D). From summer until winter, phytoplankton contributed ~60% to the total release and, on average, 44%. During the same period, ciliates contributed ~20% to the amino acid release, which was the same proportion as that of the mesozooplankton (Fig. 5E). Normalizing the release to biomass production revealed that the phytoplankton released only <10% of their biomass net production as TDAAs, but that of ciliates was >100%. In the picoplankton fraction, substantial release of amino acids only occurred during spring and the clear-water phase, not matching enhanced rates of BP (Fig. 5C). In one experiment with <0.2-μm filtered water, we observed increasing concentrations of DFAAs and protein over time, presumably originating from the pool of TDAAs. Hence, in the fraction <1 μm, the activity of extracellular enzymes might have fuelled the pools of DFAAs and protein.

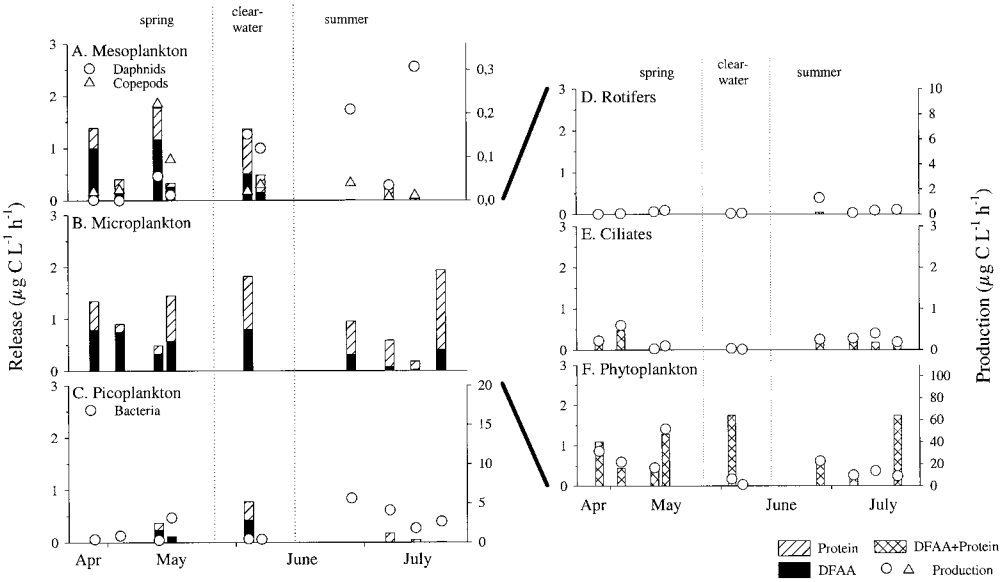

Fig. 5. Release rates of protein and DFAAs by (A) mesoplankton (>140 μm), (B) microplankton (1–140 μm), and (C) picoplankton (<1 μm) at 3 m depth. In the microplankton fraction, release rates of protein + DFAA are further separated into proportions of (D) rotifers, (E) ciliates, and (F) phytoplankton. Circles and triangles indicate production rates of the respective organisms.

Discussion

Our main results showed that phytoplankton-related processes were most important in releasing DFAAs and protein but that zooplankton, in particular during the spring bloom and clear-water phase, was also a major source of these substrates. During winter and the spring bloom, DFAAs were much more important as release products than later on in the season, when dissolved proteins dominated. These release patterns were also reflected by the in situ concentrations of both substrate classes. In winter and during the spring bloom, DFAAs exhibited substantially higher concentrations than protein, whereas, thereafter, protein concentrations were relatively higher. DCAAs were dominated by oligopeptides <3 kDa, but the molecular size fraction of >30 kDa also contributed high proportions. Despite these differences, protein was always the major substrate source for bacterioplankton growth, even though during winter and the spring bloom the relative fraction of DFAAs was enhanced.

Isotope dilution approaches, so far, have been applied in plankton communities to assess release of inorganic and organic nitrogen (e.g., Glibert et al. 1982; Bronk et al. 1994) and DFAAs (Fuhrman 1987; Rosenstock and Simon 1993) but not to other compounds and to simultaneously measure release processes of two different organic substrates. This approach is attractive because release processes can be determined independently of concentration dynamics of the released products. We used a dual label approach with radiotracers to examine the release of DFAAs and protein. Their concentrations were determined in one subsample and the radioactivity in a parallel under the assumption that release in the two subsamples was similar. Even though we routinely used only one subsample for each parameter, we found in various other experiments, using duplicates, similar concentration dynamics of DFAAs and protein over time (B. Rosenstock and M. Simon unpubl. data), suggesting that release of DFAAs and protein in the two subsamples was also similar. A crucial point of this method is the evaluation of protein concentrations, which were determined as 50% of $K_t + S_n$ from concentration kinetics (Wright and Hobbie 1965) according to the method of Billen (1991). Keil and Kirchman (1993) found that S_n accounted for 65%–100% of the total term and assumed that the protein concentration (S_n) equaled 100% of $K_t + S_n$. With our more conservative value, we think that we did not underestimate protein concentrations, because incorporation of protein constituted high proportions of BP, up to 90% in spring. Applying this kinetic approach, protein concentrations are only based on incorporation rates, not accounting for respiration, and thus underestimate in situ concentrations of dissolved protein. When we take the assimilation efficiencies into account (Table 1), corrected protein concentrations are 1.3–3.8 and, on average, are 1.8 times higher. Weiss and Simon (1999) determined that concentrations of labile amino acids are a function of TDAAs, approaching 0 at 720 nM TDAAs. If we also determine this relationship according to Fig. 2C, but based on TDAAs, the corrected concentration of labile protein + DFAA reaches zero at 726 nM. This value is similar to that of the regression analysis of Weiss and Simon (1999), justifying the use of 50% of $K_t + S_n$ to calculate protein concentrations in Lake Constance. Because we routinely determined protein concentrations on the basis of assimilation into the bacterial biomass, these values were used for the uptake and release calculations. To ensure that measured processes reflect in situ conditions as precisely as possible, we used a model protein that consisted of different MW size classes. Forty percent were <3 kDa and 60% were composed of the higher MW proteinaceous fraction. We think that using a peptide mixture

of different size-classes instead of a single protein mimics much more closely in situ rates of release and uptake of dissolved amino acids.

The microplankton fraction, 1–140 μm, was always a major source of DFAA and protein, in particular during summer. This size fraction included phytoplankton, protozoans, and rotifers. Rotifers were never an important source of dissolved amino acids. With respect to protozoans, heterotrophic nanoflagellates (HNFs) presumably were only of minor importance as an amino acid source. Previous studies showed that these organisms release <23% of their ingested nitrogen as amino acids (Andersson et al. 1985; Nagata and Kirchman 1991). A mass-balanced carbon flow analysis revealed that, in Lake Constance, HNFs contributed to total grazing activities substantially less than ciliates but were the dominant consumers of bacteria (Gaedke and Straile 1994; Simon et al. 1998). Ciliates, however, were the most important phytoplankton grazers and are of great importance for the recycling of organic matter. On the basis of experiments with *B. planktonicum,* a typical and dominant ciliate in Lake Constance (Müller et al. 1991), we estimated that ciliates contributed 18% on average but up to 50% of the amino acid release in the microplankton fraction. *B. planktonicum* largely dominates the ciliate community in Lake Constance during the spring bloom and is one of the two dominant members in summer. If the release rates we determined were not applicable to situations when this model organism did not dominate the ciliate community, relative rates by ciliates might have been biased. However, this could only have been the case when the relative release by ciliates was high, such as in early July, but would have only little effect at other times. As an annual mean, they contributed 20% to the zooplankton-mediated amino acid release. The high release rates normalized to biomass production we found, which are consistent with another study (Taylor et al. 1985), indicate that ciliates have a low transfer efficiency and contribute to total release relatively more than other zooplankton. Also, Strom et al. (1997) found evidence that marine ciliates were important in releasing DOM, even exceeding phytoplankton- and copepod-mediated release.

Amino acid release in the microplankton size fraction was dominated in most cases by phytoplankton-related processes. They were not measured directly but are calculated as the residual of the total release in the size fraction 1–140 μm minus that of ciliates and rotifers. The close and highly significant correlation between PP and phytoplankton-mediated release indicates that this calculation yielded valid data. During the spring bloom at high phytoplankton growth rates, release of DFAAs dominated, whereas, later on in the season, at lower growth rates, that of protein was relatively more important, mainly because of the reduced release of DFAAs. Sundh (1992*a*,*b*), examining the biochemical composition and molecular weight fractions of photosynthetic extracellular release in different Swedish lakes of various trophic states, found that amino acid release was dominated by the <1 kDa fraction, whereas protein in most cases was relatively less important. Even though our results are not directly comparable to that of Sundh (1992*b*), they are consistent in the way that amino acid release was dominated by the LMW fraction, including DFAAs. Glycine and serine are the dominant products of photorespiration and are released from growing algal cells (Ogren and Chollet 1982). Therefore, since these two amino acids usually dominate the DFAA pool in Lake Constance (Simon 1998), we assume that, during the spring bloom, release due to photorespiration was an important release mechanism of DFAAs, but other processes presumably contributed as well, mainly leading to the release of protein. We hypothesize that later on, during the clear-water phase and summer, these release processes, mainly by senescent phytoplankton and by algae damaged from grazing and after egestion, became more important. Sundh (1992*b*) also found that in Lakes Erken and Valentunasjön, DFAAs are preferentially released in spring as opposed to more protein during the summer, mainly because of reduced DFAA release rates. Hydrolytic release of amino acids due to microaggregate-associated bacteria in the size fraction 1–140 μm can, in general, be assumed to be of minor importance. In Lake Constance, bacteria attached to microaggregates of 8–150 μm account for <3.5% of total bacterial numbers (T. Brachvogel and M. Simon unpubl. data). At two data points in the clear-water phase and in late summer when algal blooms were breaking down, there was a discrepancy between primary production and the release of amino acids (see Fig. 5F). At these situations, the hydrolytic activity of attached bacteria presumably was important in dissolving senescent algal material (Middelboe et al. 1995*b*).

In the mesoplankton size fraction, >140 μm, release during the spring bloom was dominated by DFAAs, compared with protein during the clear-water phase. During summer, release in this size fraction was of minor importance, presumably because the zooplankton did not consume phytoplankton of this size fraction, such as chain-forming diatoms, *Ceratium hirundinella,* and *Dinobryon* spp., which are abundant during this season (Gaedke 1998). During the early spring bloom, when high release rates of DFAAs were recorded, zooplankton abundance was still low, but the abundance of chain-forming diatoms such as *Asterionella formosa* was high. Therefore, we assume that photosynthetic extracellular release was an important release process at this time (see above). A further potentially important source of dissolved amino acids is bacterial hydrolysis of lake snow–associated particulate combined amino acids. From our experimental design, we cannot distinguish between the different release mechanisms. Middelboe et al. (1995*b*) report an increase of aminopeptidase activities of attached bacteria during the progression of a diatom bloom in a eutrophic lake to up to 75% of the total, implying that bacterially mediated amino acid release was of high significance. In mesotrophic Lake Constance, however, release of amino acids by aggregate-associated bacteria presumably was less important, even though Grossart and Simon (1998) report that aminopeptidase activity on lake snow was substantially enhanced during the clear-water phase and the blooms in spring and fall. These authors, however, further report that lake snow–associated aminopeptidase activity never exceeded 18% of the total. In addition, lake snow aggregates predominantly released DCAAs, whereas in our experiments release of DFAAs prevailed over protein in spring. Because the very fragile lake snow aggregates are destroyed by transferring

water samples from a bottle to other containers if no special care is taken, we assume that our samples did not contain intact lake snow aggregates but rather physically disintegrated ones, if any.

During the late phase of the spring bloom, when copepods dominated the mesozooplankton, DFAAs were still the main release product, but, during the clear water-phase, when daphnids took over, protein was predominantly released. We assume that these different release pattern reflect the different digestion and egestion properties of copepods and cladocerans. The former produce fecal pellets, whereas the latter egest colloidal material, which is recycled rapidly (Simon et al. 1993). Rapid recycling of the colloidal material obviously leads to a preferred release of protein, whereas the packaging of the protein in the fecal pellets may enhance its enzymatic hydrolysis and thus release of DFAAs. Further differences in the feeding-mode of both zooplankton groups may also contribute to these different release patterns, but experimental evidence is lacking. Bochdansky et al. (1995) reported that grazing by copepods enhances the concentration of dissolved enzymes, which may also lead to enhanced release rates of DFAAs. Comparable studies with cladocerans, however, are not available.

In the <1-μm fraction, release rates in general were much lower than those in the other fractions. At two instances, however—in the late spring bloom and in the clear-water phase, when bacterial production rates were low—release in this fraction was substantial. There is evidence that bacteria release DCAAs during growth and in the late stationary phase (Simon and Rosenstock 1992; Rosenstock and Simon 1993; Kroer et al. 1994; Middelboe et al. 1995*a*), but phage-induced mortality could also have caused release of protein (Fuhrman 1992). DFAAs may be the product of the activity of extracellular proteases, which are released by zooplankton activity (Bochdansky et al. 1995). We assume that the mentioned processes caused the release of DFAA and protein in the <1-μm fraction, but we cannot further differentiate among them.

Release of DFAA and protein constituted substantial fractions of PP, indicating that losses into the dissolved phase accompany the transfer of PP to higher trophic levels, thus fuelling the microbial loop (Azam et al. 1983). The only comparable data on release of DFAAs we know of are from Long Island Sound and the Chesapeake Bay, exhibiting similar ranges, 2%–40% and 4%–18% of PP, respectively (Fuhrman 1987). Release of protein in relation to PP has not yet been determined. Total release, including also other organic compounds and predominantly carbohydrates (Sundh 1992*a*,*b*), however, yields even higher rates, highlighting the significance of DOM release for biogeochemical processes in pelagic ecosystems. Kato and Stabel (1984) measured release of extracellular organic carbon in Lake Constance and found highest rates of 69% of PP during the clear-water phase. These numbers are close to the release of DFAAs and protein that we found during this period and suggest that these compounds dominate total release at this time.

Concentrations of DCAAs were substantially higher than those of DFAAs, with large seasonal variations. At the declining spring bloom and later on in the growing season, protein constituted a low but consistently higher fraction of TDAAs than DFAAs. Early in the spring bloom, DFAAs concentrations exceeded that of protein, presumably because of the high release rates (see above). As an annual mean, protein constituted 8% of DCAAs, with highest proportions of 31% during the clear-water phase. In the Delaware Bay, Keil and Kirchman (1993) found proportions in the same range, whereas the same authors found substantially higher proportions in the oligotrophic Sargasso Sea (Keil and Kirchman 1999). The TDAA pool was dominated by the LMW (<3 kDa) and the >30 kDa fractions, constituting 50% and 28%, respectively, whereas DFAAs and the intermediate-MW fraction constituted much lower proportions. During the spring bloom, when release rates of DFAAs were high, relative concentrations were enhanced as well. In agreement with our results, Coffin (1989) found that in the Delaware Estuary during phytoplankton blooms, the DCAA pool was dominated to at least 60% by peptides <1 kDa. This high proportion of the LMW fraction suggests that release of higher-MW DCAAs, including bioavailable protein, is channeled predominantly into this fraction. Rapid hydrolysis to LMW peptides and utilization by bacteria presumably were the major sinks of the HMW fraction (>30 kDa) of DCAAs. The HMW fraction was positively correlated to TDAAs and constituted higher proportions with increasing TDAA concentrations, such as during phytoplankton blooms in summer, and becoming 0 below 327 nM DCAAs (Fig. 2C). Such low concentrations occur in Lake Constance at 50 m during winter (Simon 1998). The DCAA pool at this time and at such low concentrations obviously consists only of poorly degradable LMW DCAA. These notions on the MW distribution and bioavailability of DCAAs are consistent with the size-reactivity continuum model proposed by Amon and Benner (1996) for total DOC.

Protein was the preferred amino acid source for bacterioplankton growth, covering at least 35% of the C demand for biomass production during the growing season but as a mean nearly 50%, a 3.5-fold higher fraction than DFAAs. Only during winter were both substrate classes of similar but low significance. DFAAs and protein together covered between 30% of the bacterial C demand in winter and >80% during the spring bloom with an annual mean of 60%, demonstrating their great significance as a bacterial C source. Also previously in Lake Constance, but in other environments as well, it has been shown that protein and DCAAs are relatively more important as bacterial substrates than DFAAs (Coffin 1989; Rosenstock and Simon 1993; Jørgensen et al. 1994; Kroer et al. 1994; Keil and Kirchman 1999). Only Keil and Kirchman (1993, 1999), however, used radiolabeled protein to examine the relative significance of protein versus DFAAs as bacterial substrates. They found that protein was a preferred substrate in the oligotrophic Sargasso Sea (Keil and Kirchman 1999), but DFAAs were preferred in the Delaware Bay (Keil and Kirchman 1993). The relative preference of either of these compounds obviously depends on the availability and thus dominant release process (see above, Kroer et al. 1994; Middelboe et al. 1995*a*). Because incorporation of TDAAs did not cover 100% of the bacterial C demand and because some amino acids are always respired, other C and N sources must have supported bacterioplankton growth as well. Bunte and Simon (1999)

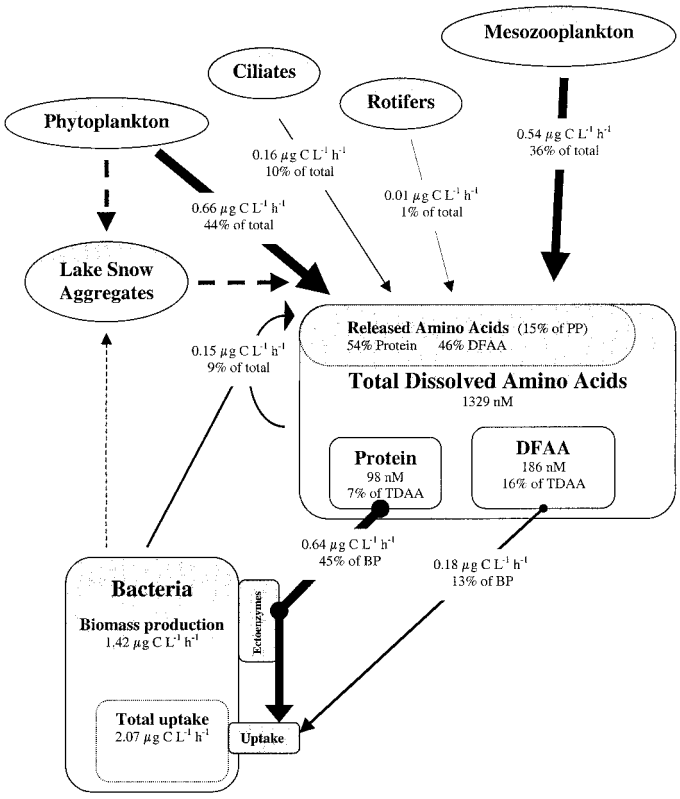

Fig. 6. Fluxes of protein + DFAA from plankton (phytoplankton, ciliates, rotifers, mesozooplankton, and picoplankton) and lake snow aggregates to the pool of TDAAs and bacteria in Lake Constance as measured in this study. Absolute values and relative proportions of the annual means are given. Total uptake of amino acids includes incorporation and respiration.

measured bacterioplankton incorporation of dissolved free monosaccharides in Lake Constance and found that they usually cover 20%–40% of the C demand for BP. Further, Weiss and Simon (1999) studied the bacterial consumption of dissolved combined carbohydrates (DCCHOs) simultaneously with that of TDAAs and found that, at high concentrations of labile amino acids and high bacterial growth rates, amino acids were preferred against DCCHOs. The latter were consumed to higher proportions during nonbloom situations and at low bacterial growth rates. Hence, amino acids and carbohydrates together cover the vast majority, if not totally, the C demand of the heterotrophic bacterioplankton in Lake Constance and presumably in other pelagic ecosystems as well.

This study comprehensively investigated the cycling of DFAAs and protein, the most important labile components of TDAAs in Lake Constance, by examining their release processes as well as their major sink—i.e., bacterial consumption. During most of the growing season, incorporation of protein sustained much higher proportions of BP than DFAAs, constituting 45%. Phytoplankton- and mesozooplankton-mediated release were the major sources of dissolved amino acids, contributing as a mean 44% and 36% to the total (Fig. 6). Other compartments such as ciliates, rotifers, and bacteria were of minor importance. Protein and DFAAs were released to nearly similar proportions, but concentrations of DFAAs, on average, were twice as high as that of protein. Concentrations and release rates of protein corrected for respiration, however, were on average 1.8 times higher and accounted for 14% of PP and, together with DFAAs, 21%.

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