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Seasonal succession and taxon-specific bacterial grazing rates of heterotrophic nanoflagellates in Lake Constance

Ernst-Josef Cleven*, Thomas Weisse**

Limnological Institute, University of Konstanz, PO Box 5560, 78457 Konstanz, Germany

ABSTRACT: We investigated the taxonomic composition of the heterotrophic nanoflagellate (HNF) assemblage and its taxon-specific bacterial grazing rates in Lake Constance (Germany) over the course of 1 yr. Bacterial grazing rates were measured using natural fluorescently labelled bacteria (FLB) and compared to bacterial production estimated by the uptake of ^{14}C-leucine incorporation. Glutaraldehyde-fixed, DAPI-stained flagellates were counted using epifluorescence microscopy. Based on annual averages, small species such as *Spumella* sp. (2 to 6 µm) were the most numerous HNF and the dominant bacterivores. Larger flagellates such as *Kathablepharis* sp. contributed significantly to total HNF biomass, in particular during spring, but were relatively unimportant as bacterial grazers. The HNF community structure changed during the transition from the phytoplankton spring bloom to the clearwater phase, with small flagellates such as heterokonts, kinetoplastids and choanoflagellates becoming increasingly abundant. The flagellate community composition was more diverse during summer and autumn than in spring. Per capita ingestion rates ranged from 0 to 31 bacteria HNF^{-1} h^{-1} and changed seasonally up to 10-fold within a given taxon. Mixotrophic species contributed little to total bacterivory. We provide evidence that the relative significance of bacterial ingestion by a given flagellate taxon may change seasonally. Based upon our experimental results, we discuss potential shortcomings inherent in the FLB technique.

KEY WORDS: Heterotrophic nanoflagellates (HNF) · Seasonal succession · FLB · Grazing rate · Negative binomial distribution · Lake Constance

INTRODUCTION

The great significance of minute (2 to 20 µm) heterotrophic nanoflagellates (HNF) as consumers, producers, and nutrient remineralizers in pelagic food webs has been demonstrated in numerous studies over the past 2 decades (e.g. Caron et al. 1988, Sanders et al. 1989, Weisse 1991). As in the case of their primary prey, heterotrophic bacteria, knowledge on the taxonomic composition of HNF in natural freshwater systems is still in its infancy (Laybourn-Parry 1994, Weisse & Müller 1998). This is because, due to the sparsity of characteristic HNF features, identification based upon morphological traits is difficult in fixed material using epifluorescence microscopy. Molecular probes that allow unequivocal identification of individual HNF species are available for a few species only (Lim et al. 1993, Rice et al. 1997), and the majority of HNF taxa, therefore, remains unidentified in most ecological investigations (Lim et al. 1999). There is, however, evidence accumulating that the HNF assemblage in lakes is composed of a variety of coexisting taxa (Salbrechter & Arndt 1994, Weisse & Müller 1998). Similar to planktonic algae and ciliates, HNF seem to show a seasonal succession in temperate lakes (Laybourn-Parry 1994). Niche-partitioning has already been demonstrated for

Present addresses:
*Zoological Institute, Department of General Ecology and Limnology, University of Köln, 50923 Köln, Germany. E-mail: ernst.cleven@uni-koeln.de
**Institute for Limnology of the Austrian Academy of Sciences, Mondseestr. 9, 5310 Mondsee, Austria

some bacterivorous flagellate species, both in the laboratory (e.g. Fenchel 1982, Rothhaupt 1996) and *in situ* (Sanders 1991, Weisse 1997).

We investigated the taxonomic composition and the bacterial consumption by natural HNF in Lake Constance over the course of 1 yr. Bacterivory in this lake has been studied in much detail using various approaches such as a dilution technique (Weisse 1990, 1991), uptake of radiolabelled bacteria (Jürgens & Güde 1991, Simon et al. 1998), uptake of cultured fluorescently labelled bacteria (FLB, Jürgens & Güde 1991), and mass-balanced carbon-flow modelling (Straile 1995, 1998). In contrast to those previous investigations, we used natural FLB to assess, as much as possible, taxon-specific bacterivory. In most cases, bacterial grazers were identified to the family or genus level. We also report species-specific rates for those HNF which could be identified unequivocally in the fixed samples. The goals of this study were (1) to test if all small (~2 to 10 µm) HNF act primarily as bacterivores with similar ingestion rates (null hypothesis), i.e. if they can be lumped together in 1 functional guild, and (2) to assess seasonal changes in taxon-specific bacterial uptake rates. The former assumption has been used in initial studies on bacterial grazing by flagellates (e.g. Weisse 1990) and is inherent in recent attempts to model the carbon flow in Lake Constance and elsewhere (Wylie & Currie 1991, Gaedke & Straile 1994, Straile 1998). Our alternative hypothesis was that significant taxon-specific differences exist among the small HNF with respect to their bacterial consumption. Larger-sized flagellates (>10 µm) tend to feed on prey bigger than most natural bacteria (e.g. Sherr & Sherr 1991, 1994). Details of ciliate bacterivory which was studied in parallel to the present investigation using a similar approach will be presented elsewhere (Cleven & Weisse unpubl. data).

We found it difficult to obtain statistically reliable results from routine application of the FLB method. Thus, we caution against drawing firm conclusions based upon a comparison of FLB uptake rates to independent measurements of bacterial production.

MATERIALS AND METHODS

Investigation area and sampling. Samples were taken from the routine sampling station located at the maximal water depth (147 m) of the Überlinger See, the northwestern basin of Lake Constance, Germany, where all previous investigations of the Limnological Institute Konstanz have been conducted since the mid-1970s. Lake Constance is a 532 km^2 large, deep (max. depth 252 m; mean depth 100 m) pre-alpine lake. This study was carried out during the period of its re-oligotrophication (Gaedke 1998, Häse et al. 1998 and references therein) when the lake was meso-eutrophic. The investigation period lasted from April through October 1991. Samples were taken in weekly to biweekly intervals. Additionally, a late winter sample was taken in February 1992. Seasonal phases were identified according to Müller et al (1991).

Samples for FLB uptake measurements were taken at 3 m water depth using a 2 l water bottle made of acrylic plastic. Subsamples of 50 ml each were immediately fixed with ice-cold glutaraldehyde, buffered with 0.1 M cacodylic acid (Sigma Chemical) to give a final concentratation of 2% (vol/vol).

Ambient parameters. Water temperature (±0.1°C) at 3 m depth was recorded using a mercury thermometer. Chlorophyll *a* concentration was determined spectrophotometrically and computed according to Jeffrey & Humphrey (1975). Primary production was calculated as ^{14}C incorporation according to Eq. (12) of Tilzer & Beese (1988). Phytoplankton species composition was analyzed in Lugol-fixed samples after settling according to Utermöhl (1958).

Determination of microoganisms and bacterial production. Abundances of bacteria and heterotrophic nanoflagellates were determined from Na-cacodylate (0.1 M) buffered, glutaraldehyde (2% final conc.)-fixed samples. Microorganisms were counted at 1250× magnification from subsamples of 3 to 10 ml volume using epifluorescence microscopy (Nikon Labophot II) and DAPI staining according to Porter & Feig (1980). A blue and a blue-violet excitation filter set was used to distinguish non-pigmented from pigmented flagellates through the autofluorescence of the latter. The HNF cells were identified according to the literature (Sleigh 1973, Hänel 1979, Patterson & Larsen 1991) and previous experience obtained in our laboratory (Weisse et al. 1995). In particular, identification was facilitated by including observations of live material and comparison with cultured HNF species isolated from the same sampling station (D. B. Springmann, Limnological Institute Konstanz, unpubl. data). Identification of the latter included scanning electron microscopy (Weisse et al. 1995) and molecular techniques (Bruchmüller 1998).

Bacterial biovolume was measured by a semi-automatic image-analysis system (VIDS IV, AI Tectron) and calculated according to Kuuppo-Leinikki (1990). Biovolume was converted to carbon units using the allometric equation provided by Simon & Azam (1989).

Bacterial biomass production (BP) was determined by incorporation of ^{14}C-leucine (Leu) according to Kirchman et al. (1985) and Simon & Azam (1989). Production estimates were performed on the same day as the grazing experiments using a subsample from the

same water. Ten millilitres of sample were incubated with ^{14}C-leucine (30 nM final conc., specific activity 312 mCi mmol^{-1}, Amersham) in triplicate and a formalin-killed control (2% final conc.) at *in situ* temperature in the dark. Incubations were stopped after 1 h, 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 Simon & Rosenstock (1992), assuming 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. The mean coefficient of variation of the triplicate measurements was <10%.

Mean biovolume of preserved HNF was determined by the same image-analysis system used for bacteria. The C content of HNF was then calculated by multiplying the biovolume with a conversion factor of 220 fg C µm^{-3} (Børsheim & Bratbak 1987). According to measurements conducted in our laboratory in parallel to this study (D. B. Springmann, Limnological Institute Konstanz, unpubl. data), we assumed that the cellular biovolume of the different taxa changed seasonally (Table 1).

Preparation of fluorescently labelled bacteria. Three to 4 d prior to an experiment, ca 15 l of lake water were collected from 3 m water depth and filtered successively through 100 and 10 µm-mesh gauze and 1.0-polycarbonate membrane filters (Nuclepore). The <1 µm-filtered water was filtered onto a 0.2 µm membrane filter, and the retentate was stained with 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF, Sigma Chemical) following Sherr et al. (1987). After staining, the cells were concentrated by filtration on a 0.2 µm Nuclepore membrane, washed with 0.05 M phosphate-buffered saline, and resuspended in 0.02 M tetrasodium pyrophosphate solution by sonification. The suspended, labelled bacteria were then portioned and deep-frozen at –20°C. The concentration of the FLB suspension was checked by epifluorescence microscopy under blue light excitation prior to each grazing experiment.

***In situ* grazing experiments.** Experiments were conducted in a 2 l container made of acrylic plastic. The container was incubated at 3 m depth at the sampling station. The natural bacterial abundance was estimated microscopically on the day preceding an experiment. The concentration of the FLB used in the experiments was adjusted to 20 to 30% of the respective bacterial abundance. The actual abundance of natural bacteria on the day of the experiment was later determined in another subsample taken parallel the experimental samples. The field experiment started by adding FLB to the containers. To ensure even distribution of the tracer particles, the water in the containers was carefully mixed by means of a perforated disc. Subsamples of 50 ml each were taken 0, 20 and 30 min after the beginning of the experiments and immediately fixed with ice-cold glutaraldehyde (final conc. of 2%), buffered with 0.1 M cacodylic acid (Sigma Chemical). All experiments were conducted without replicates and carried out between 09:00 and 10:00 h.

Microscopic analyses and statistical data evaluation. An aliquot of the fixed samples (2 to 10 ml) was filtered onto a polycarbonate filter (Nuclepore, pore size 1 µm), stained with DAPI (Porter & Feig 1980) and inspected by epifluorescence microscopy using a Nikon Labophot II microscope. A total of at least 100 HNF were examined for ingested FLBs under a combination of UV excitation (for the flagellates) and blue light excitation (detection of ingested FLB). Results were presented only when a minimum of 30 specimens per taxon was counted.

A frequency distribution of the ingested FLB was calculated for each taxon and each subsample (number of HNF with x = 1, 2, 3, 4,...n FLB HNF^{-1}), and the mean per grazer ingestion of FLB and the variance of the mean was then estimated. The variance-to-mean ratio indicated a 'contagious', i. e. aggregated or clumped, distribution of the data (χ^2 test, Pearson & Hartley 1966, Sachs 1984). The negative binomial distribution (NBD) is an approximate model for a relationship if the variance is greater than the mean, and can also be applied to the corresponding pattern of dispersion, that is, the contagious distribution of the observed FLB ingestion. The NBD is defined by 2 parameters (Bliss & Fisher 1953): the arithmetic mean m and the positive

Table 1. Mean biovolume (µm^3) of major taxa of heterotrophic nanoflagellates (HNF) in Lake Constance for 5 periods during 1991 and 1992 ('Ovalis': undetermined small HNF species; *Spumella*: heterokont flagellates of the genus *Spumella*; Kinetopl.: Kinetoplastida; *Kathabl.*: *Kathablepharis* sp.; Choanofl.: Choanoflagellida; 'Others': all other HNF combined; data provided by D. B. Springmann unpubl.)

Period (1991, 1992)	HNF taxon					
	'Ovalis'	*Spumella*	Kinetopl.	*Kathabl.*	Choanofl.	'Others'
9 Apr–7 May	11.3	12.8	22.1	61.1	50.2	73.3
14 May–26 Jun	8.3	9.1	9.1	43.6	22.6	62.2
2 Jul–10 Sep	9.8	10.1	11.7	58.7	33.2	69.0
24 Sep–22 Oct	9.1	10.8	9.6	49.5	27.4	105.1
4 Feb	9.2	12.9	91.0	50.4	31.4	72.6

exponent k. The probability of x = 1, 2, 3, 4,...n FLB HNF^{-1} was calculated according to

$$p(x) = [1 + (m/k)]^{-k} \cdot (k + x - 1)!/x!(k-1)! \cdot (m/k + m)^x \quad (1)$$

The parameters m and k were estimated from the frequency distribution; k was estimated according to the maximum likelihood method (Bliss & Fisher 1953). Although the subsample frequency distribution did not follow a normal distribution, we calculated least-square linear regressions between the FLB uptake by the flagellates and the experimental time following the central-limit theorem (e.g. Sachs 1984, W. Nagl pers. comm.). The central-limit theorem states that the means of a large number of random samples from a given population will be approximately normally distributed. The overall mean, m, then approaches the true population mean μ, and the variance s of the sample equals the population variance σ^2.

Based upon the comparison between the expected (NBD model) and the observed (sample) frequencies, we decided upon removal or retention of potential outliers. Those may have a significant effect on the slope of the regression line and, thus, the calculated ingestion rate. If the probability of the occurrence of a potential outlier was high enough to be encountered in 1 ml of sample volume, the data point was included in the calculation of the regression. These calculations considered protists which did not ingest any FLB in the course of the experiments. The different variances of the mean individual ingestion, as well as the variable abundance of a single taxon in the 3 subsamples of each experiment (0, 20 and 30 min), both influence the slope as well as the y-intercept of the regression and, therefore, the calculated ingestion rate. This impact was considered by the 'weight' statement within the general linear module (GLM-procedure) of the SAS statistical program. The 95% confidence limits for the slope were determined by multiplying the standard error of the regression coefficient by a Student's coefficient for $\alpha = 0.05$ with $n - 2$ degrees of freedom (Prepas 1984, Sachs 1984). Because of the few data points available for the calculation of the regression, the 95% confidence limits were generally large.

Clearance rates (C, ml^{-1}) of the flagellates were calculated by dividing the estimated ingestion rate by the mean natural bacterial abundance assessed on the day of the experiments. In order to compare bacterial losses due to HNF grazing to bacterial production, the bacterial ingestion rates were converted into units of carbon using our measured bacterial biovolume and the allometric equation provided by Simon & Azam (1989). The result was compared to bacterial production estimated at the sampling location in parallel to the present study (Simon et al. 1998, Simon unpubl. data).

RESULTS

Ambient parameters

Experiments commenced in mid-April 1991 when the water column was stratified and the spring phytoplankton maximum began. Water temperature ranged from 7 to 10°C, and the chlorophyll *a* (chl *a*) concentration increased rapidly from 7.5 to 47 mg m^{-3} during this period. Water transparency (Secchi-disc readings) declined from 5.4 to 1.7 m within a few days. The phytoplankton biomass was dominated by the small cryptophyte *Rhodomonas minuta* (Cryptophyta) during the first chlorophyll peak (47 mg m^{-3}: 16 April) and by *R. lens* during the second (19 mg m^{-3}: 30 April) and third (9.5 mg m^{-3}: 22 May) peaks. Co-occurring diatoms and chlorophytes were mainly small *Stephanodiscus* spp. and microalgae of the *Chlorella* type.

Water temperature varied between 13 and 18°C during the clearwater phase (28 May to 16 July) following the spring peak. Seasonal phases identified in this investigation were defined according to Müller et al. (1991). Chl *a* concentration ranged from 1.3 to 5.7 mg m^{-3} during the clearwater phase, corresponding to Secchi depths of up to 14.7 m. Cryptophytes still dominated the phytoplankton biomass, but the small *Rhodomonas* species were replaced by larger algae of the genus *Cryptomonas* (mainly *C. ovata*, *C. marsonii* and *C. rostratiformis*).

Water temperature peaked at 21.5°C in late summer, while chl *a* never exceeded 10.7 mg m^{-3} during this seasonal phase. The summer phytoplankton community was dominated by the pennate diatom *Fragilaria crotonensis*. Water temperature varied between 12 and 18°C during autumn. Chl *a* concentration ranged from 1.8 to 5.4 mg m^{-3}, corresponding to Secchi depths of up to 11.4 m. Water transparency and chl *a* measured in winter were similar to the respective values recorded during the clearwater phase.

Phytoplankton primary production (PP) varied between 1 mg C m^{-3} h^{-1} (winter), 5 mg C m^{-3} h^{-1} (clearwater phase) and >40 mg C m^{-3} h^{-1} (spring bloom). The annual primary production in Lake Constance amounted to 280 g C m^{-2} in 1991 (Häse et al. 1998, Simon et al. 1998), similar to the values measured in the 1980s (Tilzer & Beese 1988), but considerably higher than those recorded in more recent years at reduced phosphorus levels (Häse et al. 1998).

Bacterial abundance, biomass and production

Bacterial abundance ranged from late-winter minimum of 0.8×10^6 cells ml^{-1} (recorded on 4 February) to a maximum of 7.7×10^6 cells ml^{-1} measured on 28 May

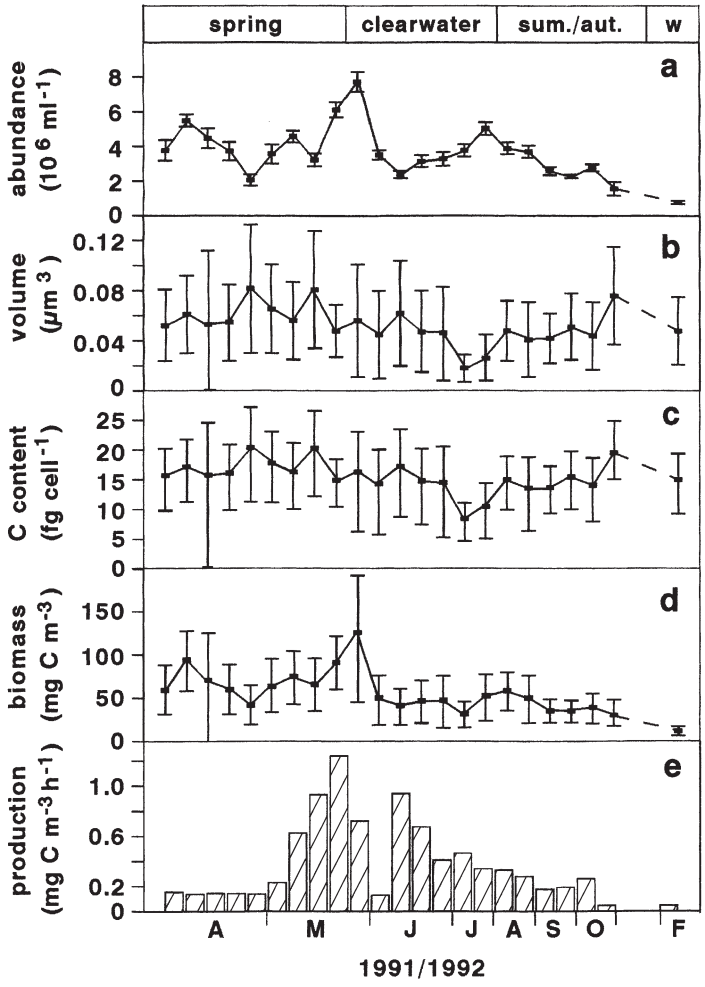

Fig. 1. Seasonal succession of (a) bacterial abundance, (b) cell volume, (c) cellular carbon content, (d) community biomass and (e) bacterial production in Lake Constance. Abscissa is distorted to show the changes in spring more clearly. Error bars denote counting variabilities (a), standard deviations (SD) of mean values (b), calculated upper and lower C contents (c) based on the standard deviations of (b), and upper as well as lower biomasses (d) calculated from the limits of (a) and (c). w: winter season

(Fig. 1a). Bacterial abundance fluctuated widely during the spring bloom and towards the beginning of the clearwater phase. Seasonal variation of the mean bacterial biovolume was less pronounced (0.052 ± 0.015 µm^3, n = 23; Fig. 1b). The bacterial biovolume decreased during the clearwater phase from 0.061 (±0.012) to 0.043 (±0.016) µm^3. This trend was, however, statistically insignificant (*t*-test). The annual mean bacterial biovolume corresponded to a cellular carbon content of 15.4 (±2.7 fg; Fig. 1c). Total bacterial biomass (Fig. 1d) followed the seasonal dynamics of the bacterial abundance. The annual mean bacterial biomass was 55.5 (±24.7) mg C m^{-3}. Bacterial biomass was positively correlated with temperature during the phytoplankton spring bloom (Spearman's rank correlation; $p < 0.05$; $r_s^2 = 0.635$) and negatively correlated with PP during the clearwater phase ($p < 0.05$; $r_s^2 = 0.563$). Bacterial biomass appeared unrelated to chl *a*.

Bacterial production (BP) increased at the end of the phytoplankton spring bloom, reaching its maximum of 1.2 mg C m^{-3} h^{-1} during the early clearwater phase (Fig. 1e). With 1 exception, BP remained relatively high, although bacterial biomass decreased during the clearwater phase. Averaged over the year, BP was uncorrelated to environmental parameters (temperature, chl *a*, PP). The annual bacterial production (0 to 20 m) amounted to 29.5 g C m^{-2} in 1991, corresponding to 10.5% of PP (Simon et al. 1998).

Cell numbers, biomass and taxonomic composition of heterotrophic nanoflagellates (HNF)

Major flagellate taxa such as kinetoplastids, choanoflagellates and heterokonts ('chrysomonads') could be unequivocally identified under the epifluorescence microscope. Some other flagellates, possibly colourless cryptomonads and euglenids, which we could not clearly assign to a certain taxon were combined as 'others'. Within the kinetoplastids the genera *Bodo* and *Rhynchomonas* could be identified. Their abundances were, however, low throughout the study period. Different representatives of filter-feeding choanoflagellates could be differentiated but could not be identified to the species level. These cells occurred as both solitary and colonial forms. The latter were, in most cases, attached to particles or pennate diatoms of the genera *Fragillaria* and *Asterionella*, in particular during late summer and autumn when abundances of these algae peaked (C. Milanesi pers. comm.).

Within the heterokont genus *Spumella*, 2 as yet undescribed species, a small one of 2 to 6 µm in length (fixed cells) and a larger one of >6 µm, were present throughout the year. Both species were isolated into culture and identified by molecular techniques (denaturing gradient gel electrophoresis [DGGE], ssu rDNA sequencing: Bruchmüller 1998). The species could be distinguished based on sequence variation, but both belong to the genus *Spumella* (I. Bruchmüller et al. unpubl. data). Another heterotrophic flagellate of a similar size to the small *Spumella* species, but with an ovally shaped cell body, remained unidentified. Due to its characteristic shape, we refer to this taxon as 'ovalis' in the following. This heterokont flagellate deviated from *Spumella* in the more peripheral location of

the nucleus and a 'speckled' appearance of the cell plasma. The species identity of the large (10 to 15 µm in length) flagellate of the genus *Kathablepharis* known from Lake Constance has not been determined (Weisse 1997, Weisse & Müller 1998).

Cell numbers of the major HNF taxa varied seasonally (Fig. 2). Maximum cell numbers of the smallest flagellates, *Spumella* sp. (2 to 6 µm) and 'ovalis', as well as of *Kathablepharis* sp., were close to 1200 cells ml^{-1} and were recorded during spring. The other taxa reached considerably lower abundances and peaked during different times of the year. Choanoflagellates occurred almost exclusively during summer. Cell numbers of all HNF taxa were low during the clearwater phase. Mixotrophic species such as *Dinobryon* spp. occurred at low numbers (<1 ml^{-1}) and were unimportant as bacterial grazers during this study. Corresponding to changes in cell numbers, the biomass of each flagellate taxon varied seasonally (Fig. 2). Because of its high individual cell volume (Table 1), the biomass of the *Kathablepharis* sp. was up to 18 mg C m^{-3} during spring. The biomass of the smaller flagellates, *Spumella* sp. (2 to 6 µm) and 'ovalis', which were of equal abundance at that time, did not exceed 4 mg C m^{-3}.

The relative contribution of the various taxa to total HNF abundance and biomass is shown in Fig. 3. The smallest flagellates dominated the HNF community in particular during the clearwater phase, when the small *Spumella* sp. reached 83% of the total biomass. *Kathablepharis* sp. contributed >50% to the total HNF biomass during the second half of the spring bloom. The biomass fraction of the larger *Spumella* sp. ranged from 0.6 to 14.7%. With the onset of the clearwater phase, the HNF community became more diverse owing to the appearance of choanoflagellates and kinetoplastids. The kinetoplastids were similar to *Rhynchomonas* sp. or *Bodo saltans*, but could not be identified unequivocally. Larger kinetoplastids of the *B. designis*/

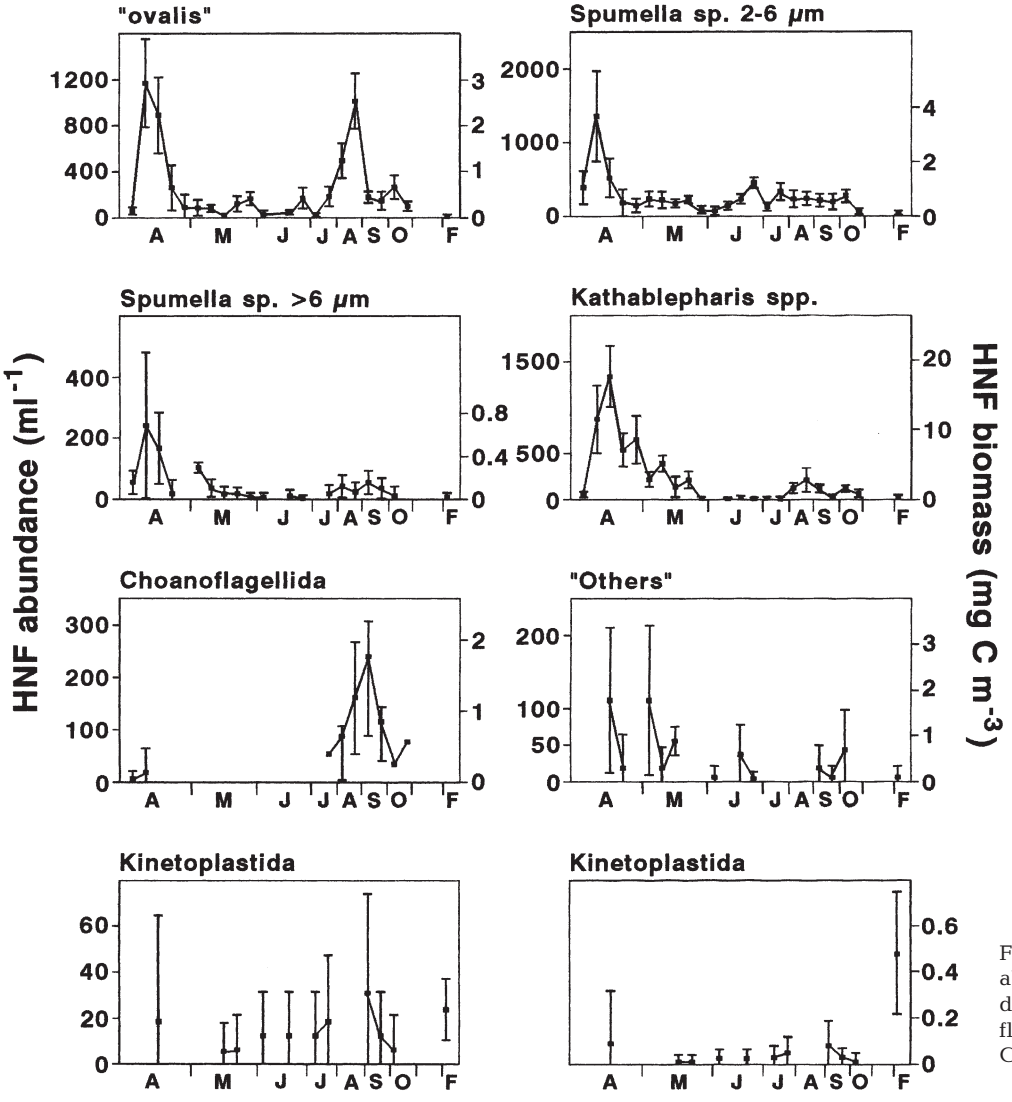

Fig. 2. Seasonal succession of abundance and biomass of different heterotrophic nanoflagellate (HNF) taxa in Lake Constance. Error bars denote counting variabilities

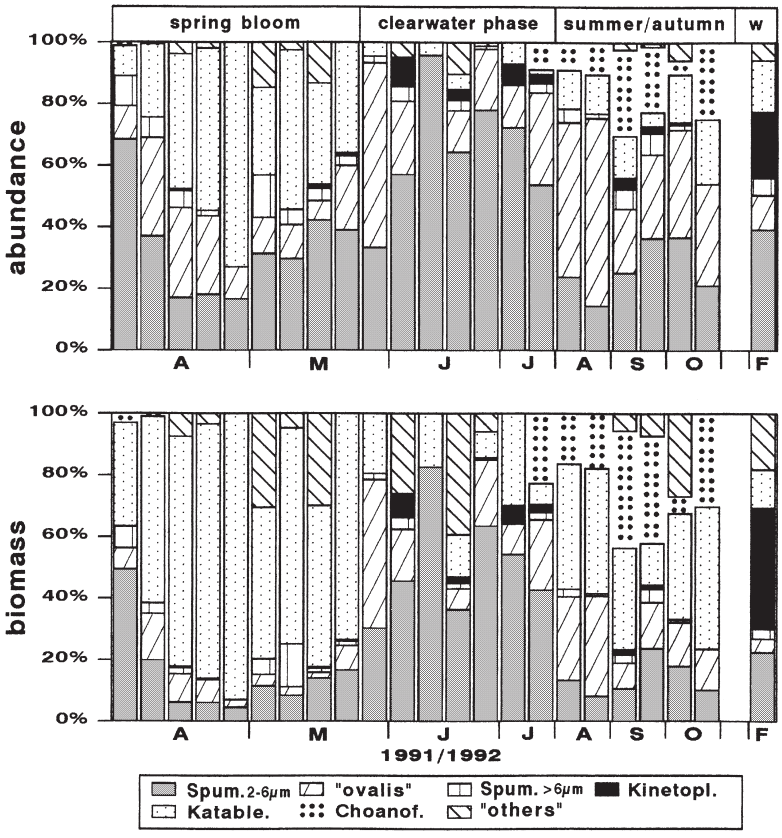

Fig. 3. Seasonal succession of HNF community composition (relative units) in terms of abundance and biomass. Full taxon names are given in Fig. 2. w: winter

B. curvifilus type were prominent during midwinter. The collective HNF category 'others' contained relatively large (6 to 20 µm) species. Accordingly, their mean biomass reached 8.7% (max. 39.2%), compared to their mean abundance of only 2.9% (max. 14.7%).

The community biomass of HNF equalled 8.3 (±8.3)% of the bacterial biomass averaged over the investigation period. We tested for correlations between our dominant HNF taxa and their potential food items, i.e. bacteria and phytoplankton. While correlations were insignificant during most of the season, HNF and their foods were significantly correlated in spring. During the spring phytoplankton bloom, the biomass of *Spumella* sp. (2 to 6 µm) correlated positively with bacterial biomass ($p < 0.05$; $r_s^2 = 0.540$; $n = 9$), while *Kathablepharis* sp. were significantly related to chl *a* ($p < 0.05$; $r_s^2 = 0.567$; $n = 9$).

Bacterial ingestion rates

We observed large species-specific and seasonal differences in the ingestion rates of major HNF taxa (Fig. 4). Ingestion rates of *Spumella* sp. (2 to 6 µm, Fig. 4a), the flagellate type 'ovalis' (Fig. 4b), and of *Kathablepharis* sp. (Fig. 4c) were generally low (0 to 2 bacteria HNF^{-1} h^{-1}) during the phytoplankton spring peak. Higher values (2 to 6 bacteria HNF^{-1} h^{-1}) were measured at the transition to the clearwater phase when both bacterial biomass and production were higher than in spring (cf. Fig. 1). Bacterial ingestion of *Spumella* sp. (2 to 6 µm) was positively correlated to bacterial biomass during this period ($p < 0.01$; $r_s^2 = 0.894$; $n = 7$), while the effect of temperature was insignificant. Per capita ingestion rates of choanoflagellates ranged from 0 to 11 bacteria HNF^{-1} h^{-1}, peaking in late summer (Fig. 4d). For the larger *Spumella* sp., grazing rates could be calculated on 3 occasions, 2 in May (6 and 12 bacteria HNF^{-1} h^{-1}) and 1 in September (31 bacteria HNF^{-1} h^{-1}). The latter was the highest HNF per capita ingestion recorded during this study. During the rest of the year, cell numbers of this flagellate were too low to measure uptake of FLB in at least 30 specimens. For the same reason, bacterial uptake by kinetoplastids could be quantified in 2 experiments only (September and February), when their ingestion rates were low (2 and 3 bacteria HNF^{-1} h^{-1}, respectively). The undetermined flagellates ('others') occurred in numbers too low to estimate their ingestion rates with any statistical reliability. The per capita ingestion rate averaged over all HNF taxa including the unidentified species ranged from <0.1 (19 April) to 7.3 bacteria (24 September) HNF^{-1} h^{-1}; Fig. 4e). The reported seasonal trends of the species-specific HNF grazing rates were statistically insignificant due to generally large confidence intervals of the means (Fig. 5).

Clearance rates of *Spumella* sp. (2 to 6 µm), the flagellate type 'ovalis', *Kathablepharis* sp., and of the Choanoflagellida, ranged from 0 to 4.9 nl $cell^{-1}$ h^{-1}. The highest rates were measured during autumn. For the larger *Spumella* sp. (>6 µm), the clearance rates were 2.0 nl $cell^{-1}$ h^{-1} in May and 13.6 nl $cell^{-1}$ h^{-1} in September.

We compared the taxon-specific bacterial ingestion rates reported above (Fig. 4) to the bacterial production estimates shown in Fig. 1e. The highest relative bacterial ingestion (33% of bacterial production) was reached by the small *Spumella* sp. in early April, when this species was highly abundant (1.4×10^3 ind. ml^{-1}; Fig. 6a). During the rest of the year, *Spumella* sp. (2 to 6 µm) cropped <10% of the bacterial production. The

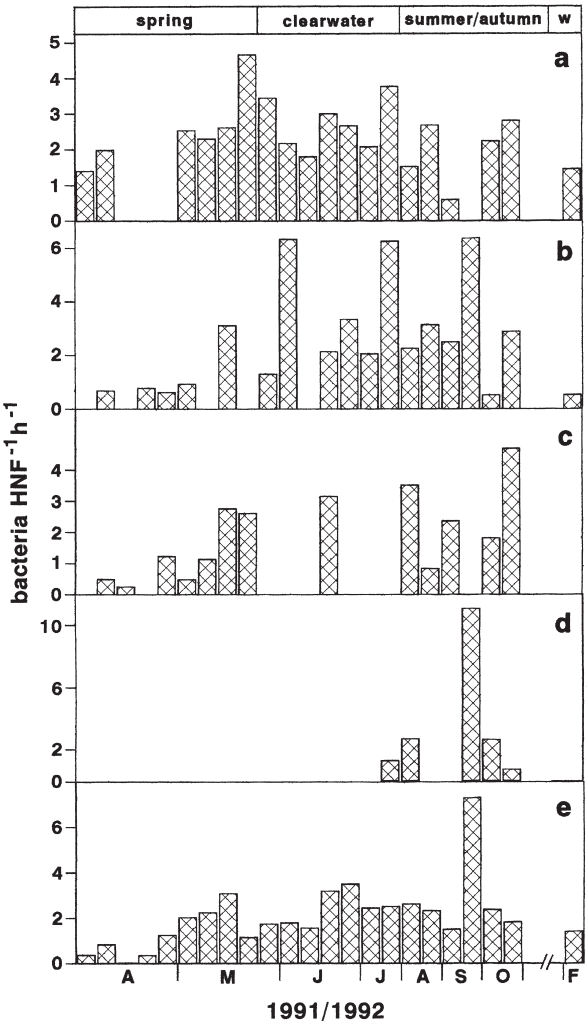

Fig. 4. Mean grazing rates of (a) *Spumella* sp. (2 to 6 µm), (b) the flagellate 'ovalis', (c) *Kathablepharis* sp., (d) Choanoflagellida and (e) averaged over all flagellate taxa including the unidentified species. w: winter

undetermined flagellate 'ovalis' contributed significantly to total bacterial grazing loss in spring and summer/autumn, when up to 15.5% of the bacterial production were ingested (Fig. 6b). A similar pattern was obvious for *Kathablepharis* sp. (Fig. 6c). Choanoflagellates removed 10% of bacterial production on 1 occasion in late September (Fig. 6d). The combined grazing loss of all major HNF taxa (the small *Spumella* sp., 'ovalis', *Kathablepharis* sp., and choanoflagellates, ranged from 0.5 (11 June) to 48.3% (16 April) (Fig. 6e).

DISCUSSION

This is one of the first studies investigating bacterial grazing by different HNF taxa. Our approach is similar to that taken by Šimek et al. (1997) in a eutrophic freshwater reservoir. These authors also used FLB to estimate bacterial uptake rates and compared the latter to bacterial production measured via radiolabelled thymidine incorporation. In contrast to our investigation, the study by Šimek and co-workers was restricted to a study period of 5 wk in late summer. Before comparing our taxon-specific grazing results to literature data, we will first consider the taxonomic composition of the HNF assemblage and shortcomings inherent in the FLB approach used.

HNF community composition and seasonal succession

The results obtained in this study confirmed and complemented earlier findings from Lake Constance and similar environments. The community composition of the HNF, their biomass and seasonal succession were found to be similar to the results obtained by D. B. Springmann (quoted in Weisse et al. 1995, Weisse & Müller 1998) at the same sampling location during the previous year. These authors reported that small heterokont flagellates, mainly *Spumella* spp., contributed, on the annual average, 57% to the total HNF biomass in 1990, followed by *Kathablepharis* sp. and other morphologically cryptomonad-like flagellates (24%), choanoflagellates (14%), and kinetoplastids (5%). Similar to the present study, choanoflagellates were restricted to late summer and autumn (Weisse et al. 1995).

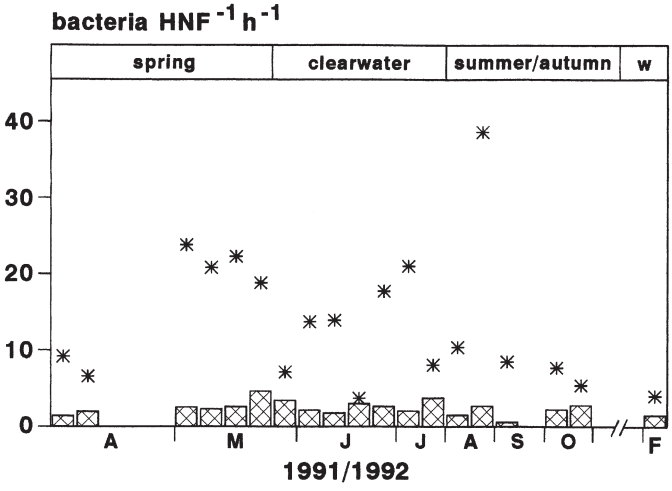

Fig. 5. *Spumella* sp. Mean grazing rates (bars) and upper 95% confidence limits (✳). Latter were derived from the slopes of the respective least-squares linear regressions of fluorescenly labelled bacteria (FLB) uptake vs time (see 'Materials and methods' for further explanation). w: winter

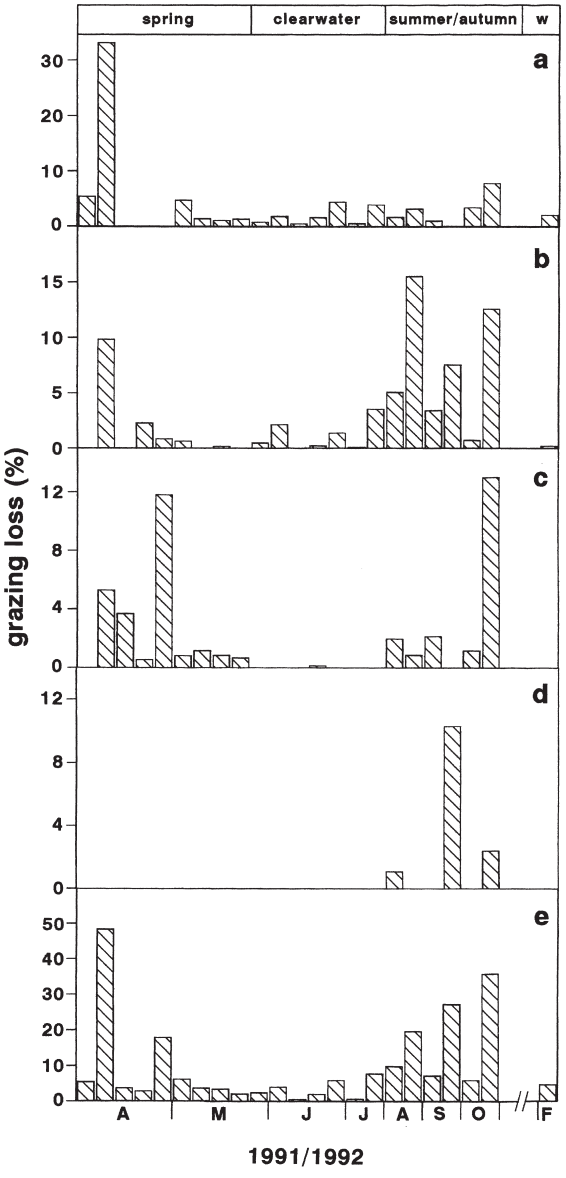

Fig. 6. Mean loss of bacterial production due to grazing by (a) *Spumella* sp., (b) the flagellate 'ovalis', (c) *Kathablepharis* sp., (d) Choanoflagellida, and (e) all identified flagellate taxa combined. w: winter

The contributions of the different taxa to total HNF numbers and biomass in Lake Constance are similar to those reported for other temperate freshwater environments of comparable lake trophy (summarized by Weisse & Müller 1998, see also Carrias et al. 1998). Such comparisons should, however, consider that conversion of abundances into units of biomass and carbon content is still controversial (e.g. Choi & Stoecker 1989, Menden-Deuer & Lessard 2000 and references therein). Similar cell numbers and seasonal succession of the 2 conspicuous taxa, the small *Spumella* sp. and the large *Kathablepharis* sp., were also found at the sampling site in 1992/1993 (Weisse 1997). The latter author pointed to the fact that production rates of these 2 dominant HNF taxa were seasonally displaced in Lake Constance. The omnivorous *Kathablepharis* sp. peaked in relation to the phytoplankton maxima in spring and, to a lesser extent, in late summer/autumn. Its seasonal dynamics were primarily controlled by water temperature. In agreement with this conclusion, we found lower FLB uptake rates of *Kathablepharis* sp. during spring than during the clearwater phase. It should be investigated if *Kathablepharis* sp. reduces bacterial ingestion when phytoplankton food is abundant. For the primary bacterivore *Spumella* sp. (2 to 6 µm), temperature and bacterial food supply were of comparable significance and together explained 84% of the observed seasonal variation in the lake (Weisse 1997). Overall, top-down control via predation by ciliates, rotifers and crustaceans is, however, more important in governing the population dynamics of HNF in Lake Constance than is bottom-up control by bacterial food supply (Weisse 1991). During most of the year, HNF numbers are controlled within the microbial food web, with oligotrich and prostomatid ciliates being the primary flagellate grazers (Müller et al. 1991, Weisse 1991, Weisse & Müller 1998). It is only during the relatively short period of the clearwater phase (May to June) that *Daphnia* spp. strongly reduce the HNF population size (Gaedke & Straile 1998).

These findings from Lake Constance are in agreement with a statistical analysis based upon sampling of 16 Quebec lakes and including literature data (Gasol et al. 1995). Gasol and co-workers concluded that the abundance of HNF is primarily controlled by resources (bottom-up) among lakes differing greatly in resource supply. Within lakes, predator control (top-down) was, on the annual average, of similar importance as resource control. The relative significance of bottom-up and top-down control changed seasonally: resources were more important in spring and predation in summer (Gasol et al. 1995).

It is obvious that more advanced techniques such as oligonucleotide probe identification (Lim et al. 1993, 1999, Rice et al. 1997) and electron microscopy (e.g. Clay & Kugrens 1999) are needed to improve the taxonomic resolution of the natural HNF assemblage. Our study benefitted from the additional information provided by live observations and comparison with cultured HNF isolates obtained from Lake Constance. With epifluorescence microscopy alone, and when using fixed material in particular, unequivocal identification of species and even genera remains the exception rather than the rule.

Potential shortcomings of the FLB approach

There is at present no 'perfect' method available to measure bacterial grazing loss rates, and the use of 'hybrid' techniques has been recommended to obtain independent and complementing estimates (Landry 1994, Weisse 1999). The primary advantage of the FLB approach is that it allows estimation of HNF taxon-specific grazing rates. Benefits and disadvantages of the FLB technique have been extensively discussed in the literature (McManus & Okubo 1991, Sherr & Sherr 1993, Landry 1994, Vaqué et al. 1994) and shall not be repeated here in detail. We want, however, to draw the attention of the reader to some often neglected problems inherent in the FLB technique.

It remains an open question if the large percentage of non-bacterial feeders observed in this study (Table 2, Fig. 7) and comparable investigations (Bratvold et al. 2000 and references therein) reflects specimens that did not feed during the period of incubation or others that do permanently select against FLB. Both possibilites may include experimental artifacts. The former may be caused by the handling of the natural HNF communities during the experiments. To minimize the handling effect, we reduced subsampling to 3 occasions (0, 20, 30 min) during each experiment. The physiological conditions of the grazers such as their nutritional status (Hatzis et al. 1993, Jürgens & DeMott 1995) or the timing of their cell division and mouth-part formation may also lead to temporary non-feeding (Bratvold et al. 2000). Prolongation of the experimental period does not overcome this problem, because incubation times in excess of digestion time yield unrealistic results (e.g. Dolan & Šimek 1998, Bratvold et al. 2000). Bacterial digestion time of small HNF species has been reported to range from <30 min to 1.5 h (Sherr et al. 1988, Gonzalez et al. 1990b, 1993). It should, however, be noted that Berninger et al. (1992) reported a linear increase of the FLB uptake by some mixotrophic protists and HNF for as long as 2 h. Some bacterivores may, therefore, commence feeding only during a longer incubation period.

Table 2. Comparison of observed and expected fluorescently labelled bacteria (FLB) distribution according to 3 different models. Values are results for *Spumella* sp. 20 min after the beginning of the feeding experiment of 16 July 1991. Pos.-bin: positive binomial; Neg.-bin: negative binomial distribution

FLB ind.$^{-1}$	Number of individuals			
	Expected			Observed
	Pos.-bin.	Poisson	Neg.-bin.	
0	20.6	20.9	26.3	26
1	14.3	13.6	7.5	9
2	4.2	4.4	3.2	2
3	0.7	1.0	1.5	2
4	<0.1	0.2	0.7	0
5	<0.01	0.1	0.4	0
6	<0.001	<0.01	0.2	0
7	<0.0001	<0.001	0.1	1

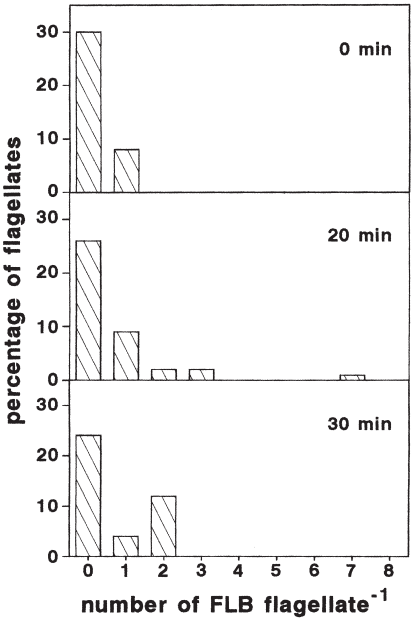

Fig. 7. Typical frequency distribution of the food vacuole content (fluorescently labelled bacteria, FLB) of small *Spumella* sp. *y*-axis shows the percentage of *Spumella* sp. cells with 0, 1, 2... FLB ingested; total of 100 HNF had been inspected. Experiment was conducted on 16 July 1991

Bacterial grazing measured as protists' food vacuole content of FLB after a certain time of incubation may deviate from the normal distribution. In our investigation the bacterial ingestion could be fitted best to a negative binomial distribution (Table 2) rather than to a Poisson distribution. Note that the bias originating from incorrectly assuming a Poisson distribution would be relatively small. Calculating ingestion rates from uptake values presented in Fig. 7 based upon a Poisson distribution would yield an ingestion rate of 10.0 bacteria HNF^{-1} h^{-1}, compared to 9.8 bacteria HNF^{-1} h^{-1} derived from assuming a negative binomial distribution.

Our results confirm earlier findings of McManus & Okubo (1991) and Bratvold et al. (2000). The latter authors reported that both a truncated Poisson distribution (Poisson distribution with extra zeros) and the negative biomial distribution adequately described the uptake of FLB by cultured flagellates, while the Poisson distribution and the binomial distribution were inadequate models. Bratvold et al. pointed out that the fit of FLB uptake data to a negative binomial model suggests subpopulations with a range of grazing rates extending to zero. The truncated Poisson model, in

contrast, suggests only 2 categories of grazers—non-feeders and a feeding group with a single grazing rate. In natural flagellate populations, the former seems to be the biologically more realistic assumption.

Ingestion rates are commonly derived from the slope of a least-squares linear regression of HNF bacterial uptake versus time (Sherr & Sherr 1993, Šimek et al. 1997, this study). Considering that the FLB uptake deviates from the normal distribution, this approach is problematic. The central limit theorem may allow for the calculation of a linear regression assuming that the mean value derived from a series of grazing experiments does not deviate from the normal distribution. The standard error involved in this calculation is, however, large, as the regression is based upon a few data pairs and, thus, highly sensitive to potential outliers. Since any microscopic measurement of bacterial ingestion is laborious, it is impossible in routine studies to process a large number of replicates to increase the statistical significance of the results obtained. The recent application of flow cytometry to measuring uptake and dissappearance rates of FLB (Vazquez-Dominguez et al. 1999, Bratvold et al. 2000) appears to be a promising approach to overcoming the significance problem.

We assume that our bacterial ingestion rates are conservative estimates: (1) the FLB had been rendered non-motile by the heat-inactivation step in the staining process, and bacterivores appear to feed at a higher rate on motile compared to non-motile bacteria (Monger & Landry 1992, Gonzalez et al. 1993); (2) the DAPI concentration of 0.01 mg ml^{-1} used in this study (according to Porter & Feig 1980) may lead to underestimates of bacterial and HNF cell numbers (Hoff 1993, Sherr et al. 1993); (3) the natural bacteria used for FLB preparation had been prefiltered through a 1.0 µm filter. We, therefore, cannot rule out that the FLB were biased towards smaller-sized cells, which are less liable to be grazed by HNF (e.g. Chrzanowski & Šimek 1990, Gonzalez et al. 1990a). However, in an earlier investigation, 77% of the free-living bacteria from Lake Constance were smaller than 1.0 µm on an annual average (Simon 1987). The average biovolume that we measured and its seasonal variation was virtually identical to the results obtained by Simon with different methods (i.e., glutaraldehyde fixation and scanning electron micrographs); (4) as discussed above, our short incubation times may have underestimated feeding of some bacterivores.

Grazing control of bacterial production in Lake Constance

Based upon our conservative estimates, only 9.7 (±12.4)% of bacterial production were removed by the small HNF on an annual average. This seems to contradict earlier findings from Lake Constance which, based upon 3 independent approaches and different years of study, concluded that HNF were responsible for 50 to 60% of the bacterial loss rates in Lake Constance (summarized by Weisse & Müller 1998). It is in agreement with this discrepancy that Vaqué et al. (1994) concluded from a literature review that methods that measure grazing rates by the uptake of fluorescent particles yield significantly lower bacterial loss rates than methods which determine community grazing rates (by dilution, inhibition, or filtration). Irrespective of the large uncertainty involved in any comparison of bacterial production and grazing loss rates derived from FLB ingestion, it appears obvious that grazers other than the small HNF contribute significantly to the total bacterial mortality in Lake Constance.

While we considered the bacterial uptake by small potentially mixotrophic species such as *Ochromonas* spp., the contribution of larger (>10 µm) mixotrophic species may have been underestimated in our study. The relative significance of *Dinobryon* spp. as bacterial grazers in Lake Constance increased in the years following our study period. In 1991, during our investigation, mixotrophic species contributed approximately 25% to the total summer phytoplankton biomass. In 1992, 1993 and 1996 their share increased to 40 to 50% during summer (Gaedke 1998). Colonial *Dinobryon* spp. may have exerted a pronounced grazing pressure on bacteria during their blooms (Henrichs 1997 cited in Gaedke 1998, Simon et al. 1998). The mass occurrence of mixotrophic species was, however, restricted to a relatively short period during summer (Gaedke 1998). We conclude, therefore, that larger mixotrophic species contributed little to the total annual bacterivory during our investigation. Similarly, bacterivorous ciliates, which were investigated parallel to this study (Cleven & Weisse unpubl. data), removed, on average, <5% of the bacterial production. This finding is in agreement with size-fractionated grazing measurements of ^{14}C-leucine labelled bacteria conducted in Lake Constance during 1994 (Simon et al. 1998). On the seasonal (May to September) average, the 1 to 10 µm size fraction, which comprised most of the HNF, accounted for 69% of the total bacterial grazing loss. The size fraction 10 to 50 µm contributed 20%, and organisms >50 µm contributed 11% to total bacterivory in the lake. High proportions of grazing by the >50 µm fraction were associated with late-summer peaks of colonial *Dinobryon* spp. (Simon et al. 1998). Earlier during the season, grazing in the large size fraction was primarily due to rotifers and daphnids. *Daphnia* spp. may contribute as much as 90% to total bacterial grazing at the height of their population peak

during the clearwater phase (Güde 1988), when they control the entire planktonic food web structure in Lake Constance by their grazing activity (Gaedke & Straile 1998).

Another important factor of bacterial mortality is phage-induced cell lysis (Bergh et al. 1989, Proctor & Fuhrman 1990). In Lake Constance, viral infection accounted for <2 to 24% of total bacterial mortality for the period April to August (Hennes & Simon 1995, Simon et al. 1998). In conclusion, grazing is, on an annual average, the dominant loss factor of bacterial production in Lake Constance, and small HNF species seem to be the single most important group among the bacterivores.

The significance of protistan grazing for inducing morphometric and taxonomic shifts in the bacterial community (e.g. Šimek & Chrzanowski 1992, Jürgens & Güde 1994, Šimek et al. 1999) has not yet been studied in Lake Constance.

Taxon-specific bacterial ingestion rates

In order to compare the taxon-specific ingestion and clearance rates we measured to literature data (Table 3), we assumed that the FLB and the alternate techniques were internally consistent. That is, we ignored problems originating from different experimental protocols including fixation and counting and assumed that the bias involved in the calculation of bacterial uptake rates levelled out if many samples were analyzed over a longer period of time. We further ignored the fact that the results reported in Table 3 were obtained at varying temperatures, ranging from 5 to 30°C.

With all these restrictions in mind, our data fall within the range, but toward the lower end, of previously published data. Overall, taxon-specific clearance rates seem to vary less than per capita ingestion rates. It seems obvious from the large variability of the data reported in Table 3 that we need improved techniques to measure species-specific bacterial grazing rates. We also need to learn more about the variablity of ingestion rates within a given flagellate population (Weisse & Kirchhoff 1997, Boenigk & Arndt 2000).

Irrespective of the uncertainty involved in current estimates of taxon-specific bacterial uptake rates, our study confirmed that per capita ingestion of small HNF ranges from 0 to 50 bacteria HNF^{-1} h^{-1}. A part of the large variability reported in literature appears to originate from seasonal differences. We found that bacterial uptake rates by a given flagellate taxon may vary seasonally by a factor of up to 10. In the obligate bacterivorous species *Spumella* sp. (2 to 6 µm) ingestion rates were, similar to its previously measured growth rates (Weisse 1997), positively correlated to bacterial biomass when the latter was relatively high. In contrast to its growth rates, temperature did not significantly impact bacterial ingestion rates of *Spumella* sp. It remains to be investigated to what extent taxonomic

Table 3. Ingestion and clearance rates of heterotrophic nanoflagellates (HNF) obtained by various methods. RLB: radiolabelled bacteria; FLB: fluorescently labelled bacteria; Chrysom: Chrysomonadida = Heterokonta, e.g. *Spumella* sp. (other taxon abbreviations as in Table 1)

Method	Ingestion rate (bacteria $cell^{-1}$ h^{-1})	Clearance rate (nl $cell^{-1}$ h^{-1})	Taxon	Source
FLB	10–15		*Spumella*	Jürgens & Güde (1991)
FLB	10–14	1.2–1.9	*Spumella*	Hwang & Heath (1997)
FLB	13–37	1.9–6.5	Choanofl.	Hwang & Heath (1997)
FLB	21		*Spumella*-like	Šimek et al. (1997)
FLB	36		Bodonids	Šimek et al. (1997)
FLB	53		Choanofl.	Šimek et al. (1997)
FLB	0–5	0–1.9	*Spumella* (2–6 µm)	This study
FLB	0–6	0–2.8	'Ovalis'	This study
FLB	6–31	1.8–13.6	*Spumella* >6 µm	This study
FLB	0–5	0.1–3.0	*Kathabl.*	This study
FLB	0–11	0.5–4.9	Choanofl.	This study
Minicells	13–73		Choanofl.	Vaque & Pace (1992)
Live observation	14–38	0.06–24	*Spumella*	Holen & Boraas (1991)
Microspheres	3–23	0.4–3.0	*Spumella*	Sanders et al. (1989)
Microspheres	8–42	0.9–5.5	Choanofl.	Sanders et al. (1989)
Microspheres	0.1–0.7		Chrysom.	Pace & Bailiff (1987)
Microspheres	1.6–27		*Monas*-like	Carrias et al. (1996)
Microspheres	1.7–33.6		Choanfl.	Carrias et al. (1996)
RLB/FLB		11	Choanfl.	Pace et al. (1990)
Cultured bacteria	27	2.0	Choanofl.	Fenchel (1982)

and physiological changes in the bacterial community affect species-specific HNF grazing rates.

Among the small HNF, the ubiquitous genus *Spumella* appears to be of pivotal importance. *Spumella* flagellates have been found in many contrasting freshwater environments (summarized by Bruchmüller 1998). Our results obtained for the small and the larger *Spumella* species suggest that significant species-specific differences exist within this genus. Other HNF such as *Kathablepharis* and choanoflagellates are either only facultative bacterivores (the former) or quantitatively important bacterivores at certain periods of the annual cycle (the latter).

In conclusion, in spite of the statistical uncertainty inherent in the FLB method, the assumption that all HNF act similarly as primary bacterivores appears incorrect. Seasonal variation of the bacterial ingestion rates within individual taxa seems to be more pronounced than assumed until recently. We have only begun to become aware of large inter- and intraspecific differences among the heterotrophic nanoflagellates.

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