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Role of predation in controlling bacterial and heterotrophic nanoflagellate standing stocks in the coastal Adriatic Sea: seasonal patterns

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ABSTRACT: The role of bacteria and heterotrophic nanoflagellates (HNF) in the microbial food web was studied over 1992/93 in Kaštela Bay (Adriatic Sea). The average production of bacteria (12. 3 µg C d^{-1}) and HNF (4.8 µg C d^{-1}) suggests that these organisms fix a significant amount of organic carbon. Small HNF (<8 µm) were the most important bacterial grazers, accounting, on average, for 80% of the total grazing on bacteria, whereas abundance and production of HNF were controlled by ciliate grazing. Bacterivorous protozoa stimulated bacterial growth and contributed to an enhanced turnover of bacterial biomass. About 20% of annual bacterial production was channelled through the microbial loop. However, bacterial carbon flux through the microbial loop showed marked seasonal oscillations with considerably higher values recorded during the warmer part of the year (June to November). Thus, in that period the microbial loop could be an important link between primary production and higher trophic levels. Grazing rates obtained by an RLB (radioactive-labelled bacteria) method were regularly higher than values obtained by the size fractionation method.

KEY WORDS: Bacteria · Carbon flux · Ciliates · Heterotrophic nanoflagellates · Microbial loop · Predation

INTRODUCTION

In the microbial loop planktonic bacteria are able to use dissolved organic substrates from the aquatic environment, incorporating them in their own biomass. In turn, bacterial biomass may be removed from the aquatic system by several mechanisms among which bacterivory by phagotrophic protozoa has been proved to be one of the most important (Fenchel 1982b, Sherr et al 1989). Flagellates and ciliates are the most important bacterivorous protozoa in most aquatic environments (McManus & Fuhrman 1988, Bloem & Bär-Gilissen 1989). The contribution of bacteria to the total planktonic biomass of most aquatic communities is highly significant (Sieburth 1976, Ducklow et al. 1986). Thus, quantifying the grazing rate on bacteria has been perceived as essential for modeling biomass transfer from the bacterial level to higher trophic levels. Numerous studies dealing with predation rate on bacteria by protozoa have been carried out establishing significant variations in predation rate (Riemann 1985, Sanders et al. 1989, Wikner et al. 1990). In these studies bacterial biomass grazed by protozoa represented between 5 and 430% of the bacterial production. Most of these studies have examined the importance of predation at a particular time of year, reporting the spatial differences in bacterivore activity as well as their short-term (day-to-day) changes rather than seasonal changes in bacterivory (Pace et al. 1990, Marrase et al. 1992). However, there are important differences in the environmental conditions in marine systems during the year. With regard to seasonal fluctuations of temperature as a primary mechanism in controlling activity and composition of the natural microbial community (Pomeroy & Wiebe 1988) it is likely that changes in predation rates are also significant on a seasonal basis. Therefore, the ecological importance of the microbial loop as a pathway of carbon to higher trophic levels can not be completely understood if it is evaluated at a particular time of year or in general terms such as annual means.

The aim of this study was to determine seasonal fluctuations of bacterial carbon flux through the microbial loop in a coastal marine ecosystem. The role of heterotrophic nanoflagellates (HNF) in channeling bacterial secondary production toward the higher trophic levels was particularly studied. Up to now such questions as: 'What organisms are the main consumers of bacteria?' 'What are their grazing rates?' 'Are bacteria a carbon source capable of supporting consumer populations?' 'Do consumers control bacterial population by grazing?' have hardly been addressed in the Adriatic Sea, and this study is the first attempt at answering these questions.

Several techniques have been used to estimate predation rates on bacteria (see McManus & Fuhrman 1988, Nygaard & Hessen 1990), which has caused difficulties in comparing results. In our study different techniques were compared in an effort to evaluate methodological bias.

MATERIAL AND METHODS

Study area and sampling procedure. Samples were collected monthly from April 1992 to March 1993 at a station located in the enclosed, shallow basin Kaštela Bay, mid Adriatic Sea (43° 31' N, 16° 22' E). The bay, with a surface area of 61 km^2 and an average depth of 23 m, is subject to considerable land influence. Bacterial production in this area is typically 20 to 30% of primary production (Krstulović 1989). The average surface temperature during the study period was 16.8°C, and ranged from 9.6°C in March to 24.6°C in August. Sampling was performed between 08:30 and 09:30 h from 0, 10, 20 and 35 m depths. All samples employed for cell counts were preserved with formalin (final conc. 2%), and were processed in the laboratory within 3 h after collecting. Temperature was measured *in situ* before each sampling. Mean values integrated for the entire water column were used as input data for the analysis of all parameters.

Bacterial and flagellate counts. Enumeration of bacteria and heterotrophic nanoflagellates (HNF) were made by epifluorescence microscopy using the standard AODC technique (Hobbie et al. 1977) for bacteria, and proflavine staining technique (Haas 1982) for HNF. For biovolume estimates, length and width of bacterial and HNF cells were measured with an eyepiece graticula (New Porton G12; Graticules, Ltd, UK). Biovolume was converted to carbon biomass assuming 0.121 pg C μm^{-3} (Watson et al. 1977) for bacteria, and 0.22 pg C μm^{-3} (Børsheim & Bratbak 1987) for HNF.

Bacterial and flagellate production. Bacterial cell production was measured with the ^{3}H-thymidine incorporation technique (Fuhrman & Azam 1980, 1982). (Methyl-^{3}H)-thymidine was added in 10 ml samples at a final concentration of 10 nM (specific activity 86 Ci $mmol^{-1}$; Amersham Ltd, UK). Triplicate samples and a formalin-killed adsorption control (final conc. 0.5%) were incubated at *in situ* temperature in the dark for 1 h. The incubations were stopped with formalin (final conc. 0.5%). To each 10 ml sample and control an equal volume of ice-cold 10% (wt/vol) TCA was added and mixtures were kept on ice for 15 min. The TCA-insoluble fraction was collected by filtering the sample through a 25 mm 0.2 µm pore size cellulose nitrate filter. The filters were rinsed 5 times with 1 ml of ice-cold 5% (wt/vol) TCA. The filters were dried, placed in scintillation vials, dissolved in 10 ml Filtercount™ (Packard scintillation cocktail) and counted after 24 h storage in a scintillation counter (Packard Tricarb 2500 TR). The conversion factors to estimate bacterial cell production were calculated from bacterial cell numbers and ^{3}H-thymidine incorporations in the <1 µm size fractions (Riemann et al. 1987) as: $CF = (N_2 - N_1)/{}^3H_{inc}$, where N_1 and N_2 are the numbers at the beginning and at the end of the experiment; $^3H_{inc}$ is the integrated ^{3}H-thymidine incorporation rate during the experiment.

Cell production of HNF was estimated using a filtration/inoculation method (Sherr et al. 1984). Production rate was expressed as the increase in flagellate number in grazer-free samples (filtered through a 1 µm polycarbonate filter) after incubation at *in situ* temperature.

Grazing experiments. The consumption of bacterioplankton by predators was estimated by 2 different methods. The first method used was the size fractionation technique (Wright & Coffin 1984, Rassoulzadegan & Sheldon 1986). The size fractions used were: <1 µm (bacteria), <8 µm (bacteria and HNF), and <100 µm (bacteria, HNF and ciliates). The <100 and <8 µm subsamples were filtered by gravity through a 100 µm plankton net and 8 µm polycarbonate filters, respectively. The <1 µm subsamples were filtered through 1 µm polycarbonate filters with a vacuum <2 kPa. Size fractions were incubated for 24 h *in situ*. Grazing on bacteria was calculated with 2 different formulas:

(1) Grazing on bacteria was estimated from difference in bacterial growth between ungrazed (<1 µm) and grazed (<8 and <100 µm) samples (Kuuppo-Leinikki & Kuosa 1990) as:

$$G\ (\text{bact. ml}^{-1}\ \text{h}^{-1}) = bb\ (\text{ungrazed}) - bb\ (\text{grazed}),$$

where $bb = N_0 e^{kt}$; $k = (\ln N_t - \ln N_0)/t$; N_0, N_t are the bacterial numbers (ml^{-1}) at the beginning and at the end of incubation; t is time of incubation (h). Accordingly, grazing by <100 µm protozoa (mostly ciliates) on HNF was estimated from the difference in flagellate growth between ungrazed (<8 µm) and grazed (<100 µm) samples.

(2) Grazing on bacteria in <8 and <100 µm fractions was calculated from the difference between bacterial cell production measured with the ^{3}H-thymidine technique and the observed numbers of bacteria (Kuuppo-Leinikki & Kuosa 1990) as:

$$G\ (\text{bact. ml}^{-1}\ \text{h}^{-1}) = \sum (N_{2est} - N_{2obs})/n,$$

where $N_{2est} = N_{1obs} + P_1$; N_{1obs}, N_{2obs} are the bacterial numbers (ml^{-1}) at times t_1 and t_2; P_1 is bacterial cell production at time t_1 using the conversion factor determined in each experiment; n is number of samplings.

The second method used to determine grazing rate on bacteria was a modification of the methods described by Riemann (1985), Nygaard & Hessen (1990) and Pace et al. (1990) based on the radioactive-labelled bacteria (RLB) technique. Natural seawater samples were filtered through 1 µm polycarbonate filters to remove all predators and labelled with 10 nM (methyl-^{3}H)-thymidine (86 Ci mmol^{-1}; Amersham Ltd) for about 10 h at *in situ* temperature. To 100 ml natural seawater samples (containing the natural density of bacterial predators) an equal volume of the labelled natural bacterioplankton, at natural concentrations, was added, and incubations were continued *in situ* for 30 min. The incubations were stopped with formalin (final conc. 0.5%). Size distribution of incorporated radioactivity was determined by filtering 10 ml subsamples immediately after fixation through 0.2, 1 and 8 µm polycarbonate filters. To obtain net activity in predator fractions the background values (zero-time incubations) caused by bacteria retained on 1 and 8 µm filters were subtracted from these values. Triplicate samples and 1 zero-time incubation were made from each water sample. The filters were dissolved in scintillation cocktail (Filter-count™, Packard) and radioassayed by liquid scintillation counting.

The predator community clearance rate (CCR) in ml h^{-1} by predators from 1 l was calculated as consumed radioactivity by the formula:

$$\text{CCR (ml h}^{-1}\ \text{l}^{-1}) = A_P/(A_B\, t),$$

where A_P is radioactivity in predators (DPM l^{-1}) corrected for radioactivity absorbed by control; A_B is radioactivity in bacterioplankton (DPM ml^{-1}); and t is incubation time (h).

RESULTS AND DISCUSSION

Bacterial and HNF counts

Seasonal fluctuations of bacterial and HNF counts are shown in Fig. 1A. Bacterial abundance ranged from 0.40 × 10^6 to 2.06 × 10^6 cells ml^{-1} with a mean value of 1.31 × 10^6 cells ml^{-1} [CV (coefficient of variation) = 43%]. The values for HNF varied from 0.18 × 10^3 to 3.45 × 10^3 cells ml^{-1}, with mean of 1.59 × 10^3 cells ml^{-1} (CV = 71%). High bacterial abundance from May to September was followed by a marked decrease in October/November. Another bacterial peak was observed in winter (December/January). On the other hand, HNF abundance was significantly higher during the warmer part of the year (July to November) in comparison to the colder winter-spring period. HNF abundance was more variable than bacterial abundance across the sampling period, as typically found in field studies (Wright et al. 1987, Vaqué & Pace 1992). The HNF peak reached in August could be a response to the bacterial summer peak, but there was no response to the bacterial winter peak. Therefore, correlation between bacterial and HNF abundance was not established across the year. Although it has been demonstrated that bacterial abundances are positively correlated with HNF abundances across a wide range of marine and freshwater environments (Berninger et al. 1991, Sanders et al. 1992), this has not always been noted in field data (Wright et al. 1987, Gasol & Vaqué 1993).

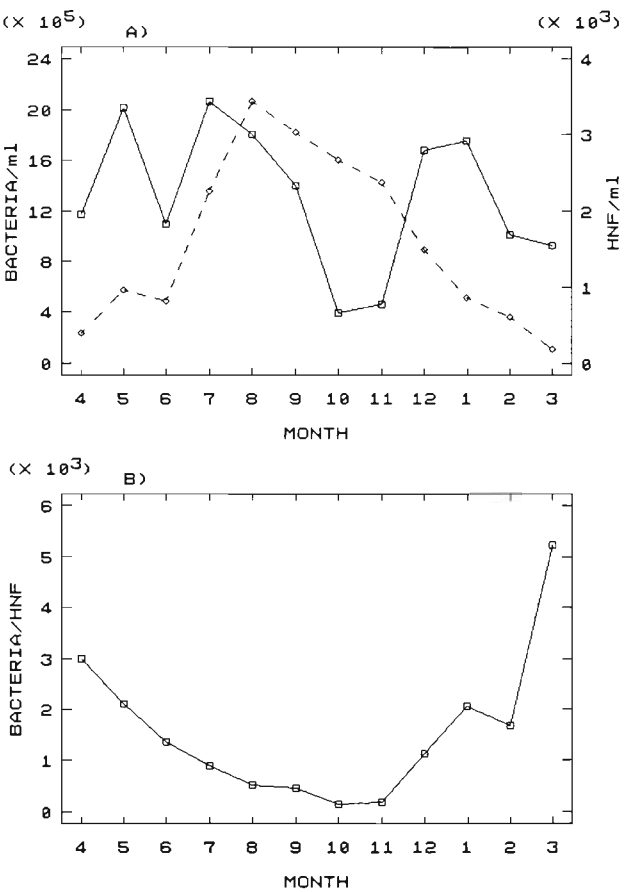

Fig. 1. (A) Seasonal fluctuations of bacterial (solid line) and HNF (dashed line) abundance. (B) Bacteria:HNF ratio

An average bacterial carbon biomass was 15.11 µg C l^{-1} (range 4.54 to 23.71 µg C l^{-1}), assuming a mean cell volume of 0.095 µm^3 (4092 cells were measured). Average HNF carbon biomass was 6.93 µg C l^{-1} (range 0.77 to 15.05 µg C l^{-1}), assuming a mean cell volume of 19.83 µm^3 (2860 cells were measured).

The ratio between bacterial and HNF abundance ranged between 148 and 5225 with a mean of 1568 (Fig. 1B). Expressed as bacteria:HNF carbon biomass ratio the mean value was 4.1 (ranged from 0.4 to 13.8). The minimum ratio was obtained in October and November when the HNF biomass was twice the bacterial biomass. This was because in that period an HNF abundance maximum corresponded with a bacterial abundance minimum. Higher values of the ratio were observed during the colder part of the year (January to May) with the maximum value in March. The average abundance ratio of 1568 is in accordance with 3 orders of magnitude as a general difference between bacterial and HNF abundance (Fenchel 1986, Sanders et al. 1992).

Bacterial and HNF production

Conversion factors to transform thymidine uptake values into bacterial cell production calculated from the <1 µm size fraction were used in this study instead of literature values. The conversion factors obtained in this study ranged from 0.48×10^{18} to 3.31×10^{18} cells mol^{-1}, with mean value of 1.70×10^{18} cells mol^{-1} (CV = 33.5%; n = 96), which is in accordance with factors reported for coastal seas (Fuhrman & Azam 1982, Riemann et al. 1987).

Bacterial production ranged from 0.37×10^4 to 12.42×10^4 cells ml^{-1} h^{-1}, with a mean value of 4.45×10^4 cells ml^{-1} h^{-1} (CV = 90%). Production of HNF varied between 3.08 and 166.21 cells ml^{-1} h^{-1} with a mean value of 45.88 cells ml^{-1} h^{-1}, and were more variable across the year (CV = 125%) than bacterial production. Both bacterial and HNF production were maximal during summer (July to September) (Fig. 2A). A less pronounced peak of bacterial production also occurred in December. Therefore, a strong seasonal relationship between bacterial and HNF production was established (Fig. 2B). Expressed in carbon biomass the average bacterial and HNF production was 12.28 µg C l^{-1} d^{-1} and 4.80 µg C l^{-1} d^{-1}, respectively. Therefore, on average biomass production of HNF was 39% of bacterial production (this value varied across the year from 11 to 71%). The average biomass-specific productivity, which is useful in comparing the productivity of microorganisms of different sizes, for bacteria was 0.034 µg C µg C^{-1} h^{-1}, or 3.4% h^{-1} (CV = 65%), and for HNF was 0.024 µg C µg C^{-1} h^{-1}, or 2.4% h^{-1} (CV = 83%).

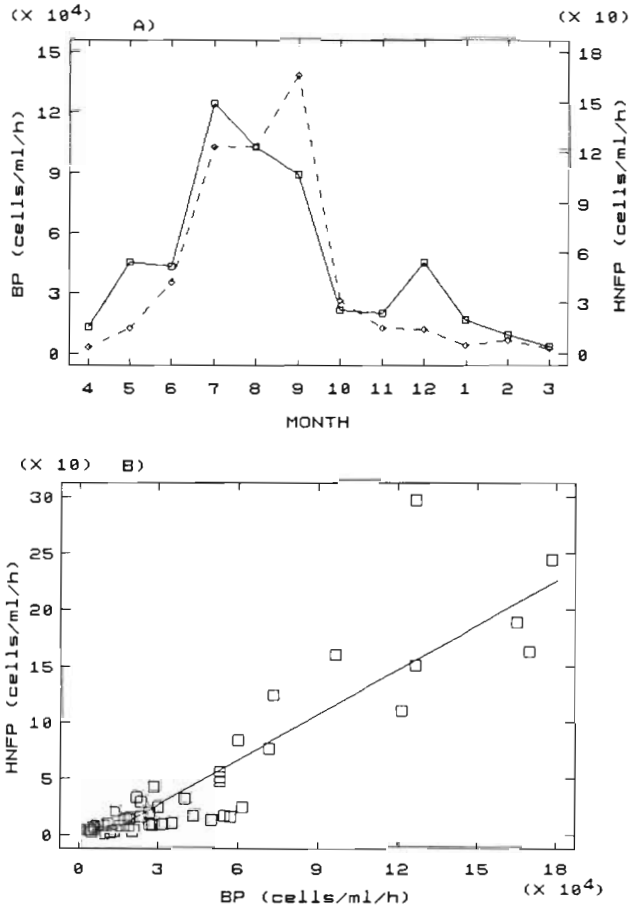

Fig. 2. (A) Seasonal fluctuations of bacterial (solid line) and HNF (dashed line) production. (B) Relationship between bacterial (BP) and HNF (HNFP) production ($y = -13.3 + 0.0013x$; r = 0.90; $p < 0.0001$; n = 48)

Population doubling time ranged from 0.6 to 7.8 d (mean 2.1 d) and from 0.6 to 5.6 d (mean 2.7 d) for bacteria and HNF, respectively (Fig. 3A). Therefore, population doubling times were very similar for both bacteria and HNF, but their seasonal distributions were somewhat different. Bacterial doubling time was very short (about 1 d or less) during the whole warmer part of the year (from June to December). In contrast, population doubling time of HNF significantly increased from October to December. This suggests a strong proportional relationship between bacterial specific growth rate and temperature (Fig. 3B). The coefficient of determination, which measures the degree of association between specific growth rate and temperature, was $R^2 = 0.86$ ($p < 0.0001$; n = 48). That is, 86% of the variance in the growth rate can be explained with temperature, implying that temperature was a primary mechanism in controlling bacterial growth. Since specific growth rate is a function of temperature and food supply (White et al. 1991) it can be concluded that during the study period organic substrate supply was not a

limiting factor for bacterial growth, or that substrate supply was also controlled by temperature. Therefore, the analysis of the relationship between bacterial production (BP, cells ml^{-1} h^{-1}) and bacterial abundance (BAB, cells ml^{-1}) was significantly improved by the inclusion of *in situ* temperature (T, °C) as an additional independent variable:

$$\log BP = -2.14 + 0.88 \log BAB$$
$$(R^2 = 0.25;\ p < 0.0005;\ n = 48)$$

$$\log BP = -7.50 + 1.02 \log BAB + 0.22\ T$$
$$(R^2 = 0.88;\ p < 0.0001;\ n = 48)$$

The correlation between specific growth rate of HNF and temperature was also statistically significant (R^2 = 0.21; $p < 0.0005$; n = 48), but temperature explained only a part (21%) of the growth rate variance. A much better proportional relationship (R^2 = 0.46; $p < 0.0001$; n = 48) was established between growth rate of HNF and bacterial production as a source of food for HNF. It suggests that bacteria were dominant prey for HNF and an important factor in controlling HNF growth.

A significant relationship was identified between HNF production (FP, cells ml^{-1} h^{-1}) and HNF abundance (FAB, cells ml^{-1}):

$$\log FP = -4.34 + 1.04 \log FAB$$
$$(R^2 = 0.54;\ p < 0.0001;\ n = 48)$$

A substantial portion of the residual variation from this relationship can be accounted for by bacterial production (BP, cells ml^{-1} h^{-1}):

$$\log FP = -8.91 + 0.33 \log FAB + 0.94 \log BP$$
$$(R^2 = 0.81;\ p < 0.0001;\ n = 48)$$

Finally, temperature accounts for some of the residual variation from this relationship:

$$\log FP = -7.59 + 0.14 \log FAB + 0.77 \log BP + 0.11\ T$$
$$(R^2 = 0.84;\ p < 0.0001;\ n = 48)$$

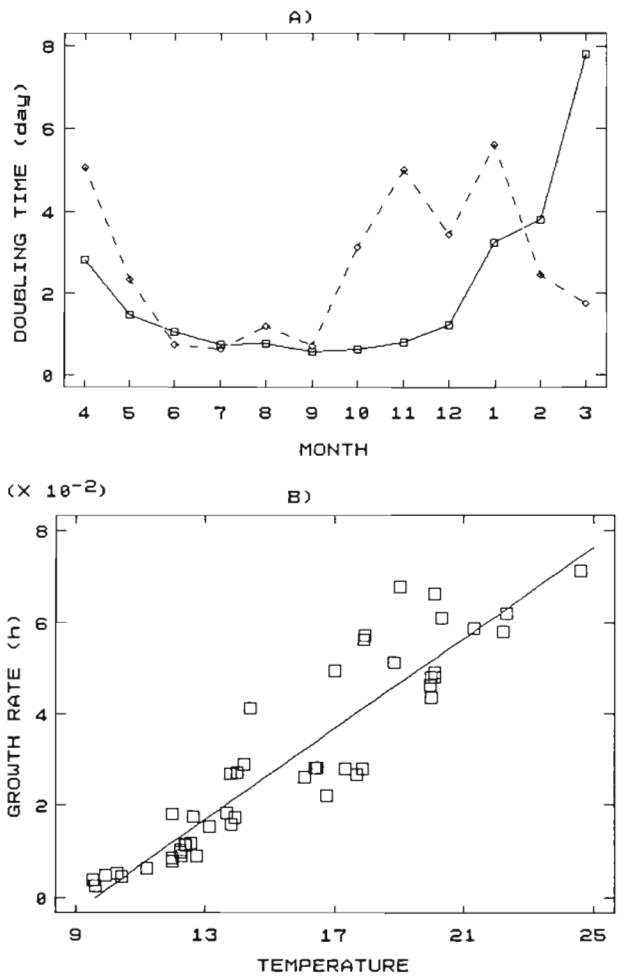

Fig. 3. (A) Seasonal fluctuations of bacterial (solid line) and HNF (dashed line) population doubling time. (B) Specific growth rate of bacteria as a function of temperature ($y = -0.048 + 0.005x$; r = 0.93; $p < 0.0001$; n = 48)

Grazing rates

Total grazing on bacteria by protozoa ranged from 0.37×10^4 to 15.54×10^4 cells ml^{-1} h^{-1}, with mean value of 4.47×10^4 cells ml^{-1} h^{-1} (CV = 114%). Expressed as carbon biomass, an average grazing on bacteria was 12.32 µg C l^{-1} d^{-1} (ranged from 1.02 to 42.86 µg C l^{-1} d^{-1}). A marked increase of grazing rate was observed during the July to September period, with a maximum in September (Fig. 4A). Very low grazing rates were measured from January to April.

HNF (1 to 8 µm size fraction) accounted for 72 to 96% (mean 80%) and ciliates (8 to 100 µm size fraction) for 4 to 28% (mean 20%) of total grazing on bacteria (Fig. 4B). In the warmer part of the year (June to October) bacterial standing stock was almost completely controlled by HNF which accounted for >90% of total grazing. In the colder period (November to May), the contribution of HNF to total grazing on bacteria fell below 80%, while the contribution of ciliates increased reaching a maximum of 28% in November and December. In contrast, Sanders et al. (1989) and Iriberri et al. (1993) reported higher contribution of ciliates to total bacterivory during the warmer part of the year.

Grazing on HNF by ciliates ranged between 3.00 and 161.63 cells ml^{-1} h^{-1} with a mean of 42.09 cells ml^{-1} h^{-1}. Expressed as carbon biomass the average grazing rate on HNF was 4.40 µg C l^{-1} d^{-1} (range 0.31 to 16.92 µg C l^{-1} d^{-1}). The maximum value was measured in September (Fig. 4C). Hardly any grazing on HNF by ciliates was established in March and April

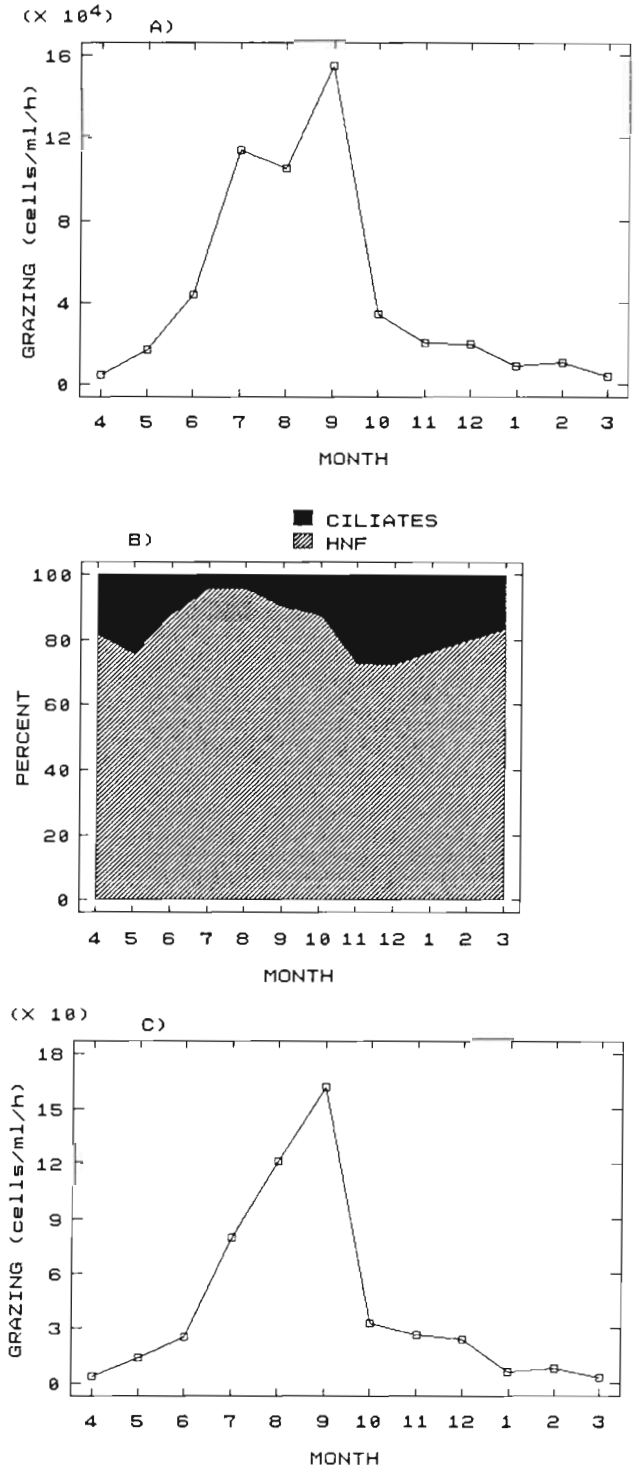

Fig. 4. (A) Seasonal fluctuations of grazing on bacteria by HNF and ciliates. (B) Contribution of HNF and ciliates to total grazing on bacteria. (C) Grazing on HNF by ciliates

suggesting that the threshold density of HNF (density of HNF below which ciliate grazing ceased) could be near 1×10^2 HNF ml^{-1}.

HNF predation parameters are listed in Table 1. Ingestion rate varied from 6.7 to 50.0 bacteria HNF^{-1} h^{-1} with a mean of 21.8 bacteria HNF^{-1} h^{-1} (CV = 69%), and clearance rate varied from 5.4 to 40.1 nl HNF^{-1} h^{-1} with a mean of 19.5 nl HNF^{-1} h^{-1} (CV = 69%). Volume-specific clearance varied from 0.27×10^6 to 2.02×10^6 body volume of HNF h^{-1} (mean 0.98×10^6 body volume of HNF h^{-1}), which corresponded to between 6.1 and 266.6% (mean 80.4%) of the water column per day. Bacterial carbon consumption per HNF biomass varied from 1.78 to 13.18% h^{-1}.

Two peaks for ingestion and clearance rates were observed, one in June/July, and the other in September/October (Fig. 5A, B). A marked decrease of both rates was measured in August when a maximum abundance of HNF was not matched by an equivalent increase in grazing. Water column clearance by HNF also showed strong seasonal oscillations. In the warmer part of the year (from July to October) HNF cleared >100% of the water column per day, while in December to May less than 20% of the water column was cleared per day by HNF (Fig. 5C). Ingestion and clearance rates were in accordance with the results of several studies done with natural samples (e.g. Andersen & Sørensen 1986, Kuuppo-Leinikki 1990). Carlough & Meyer (1991) listed literature values of HNF ingestion and clearance rates from different marine and freshwater environments; both rates ranged from <1 to 100 bacteria or nl HNF^{-1} h^{-1} (values > 100 were measured very rarely). The minimum ingestion rate of only 6 bacteria HNF^{-1} h^{-1} established in November corresponded with the bacterial abundance minimum. It suggests that the threshold density of bacteria for HNF grazing was near 4×10^5 bacteria ml^{-1}. This value is in accordance with value of 3×10^5 bacteria ml^{-1} of Berninger et al. (1991) and close to the lower one (0.5 to 2×10^6 bacteria ml^{-1}) of Fenchel (1982a).

During the year the yield varied from 0.80×10^{-3} to 1.14×10^{-3} HNF cells $bacterium^{-1}$ with mean value of 1.04×10^{-3} HNF cells $bacterium^{-1}$, which means that an average HNF cell needed to consume 995 bacteria in order to divide (reciprocal of yield) (Table 1). The carbon biomass based yield or gross growth efficiency (GGE) ranged between 30.4 and 43.4% with a mean of 39.6% and was very stable during the year (CV = 8.7%). This value, which seems to be realistic (e.g. Fenchel 1982b, Ducklow 1983), suggests that bacteria were the dominant prey for HNF throughout the year. The high values of GGE (even >100%) reported in some field studies (Kuuppo-Leinikki 1990, Galvao 1990) implied that HNF used other carbon sources in addition to bacteria.

Population grazing rate, expressed as the number of bacteria grazed per population of HNF, was primarily determined by the HNF abundance ($R^2 = 0.45$; $p <$

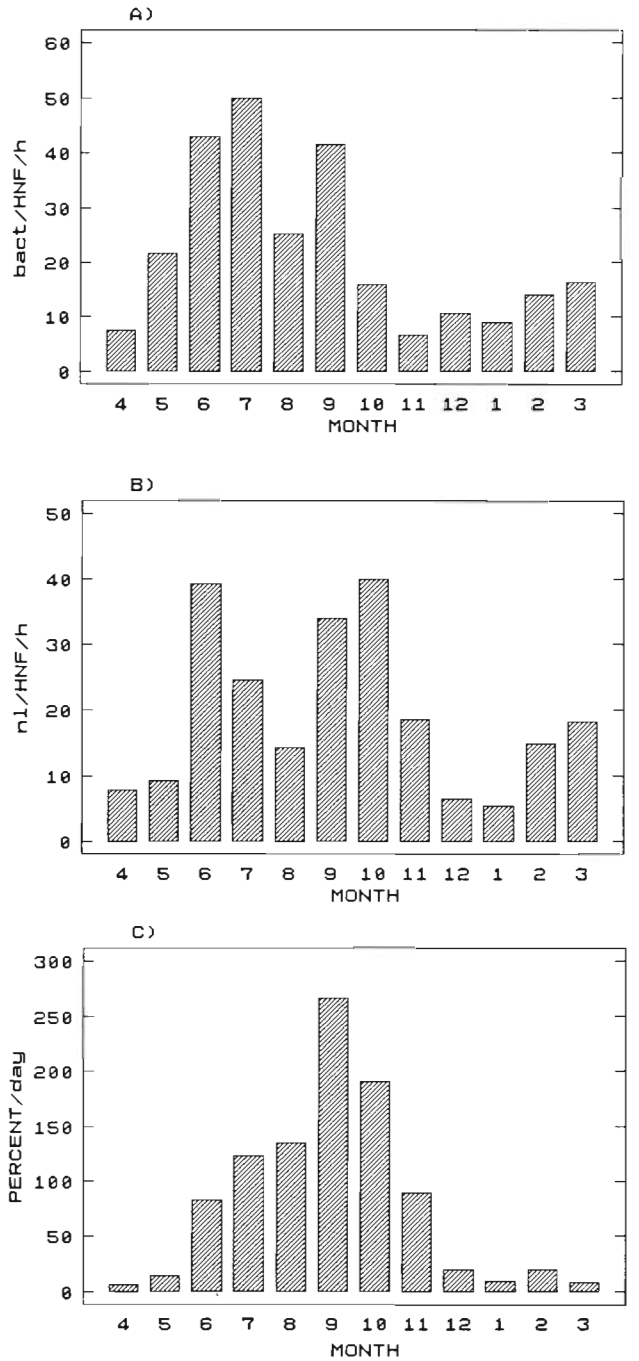

Fig. 5. Seasonal fluctuations of HNF (A) ingestion rate, (B) clearance rate and (C) clearance of water column

0.0001; n = 48). On the other hand, the oscillations of ingestion rate (I, bact. HNF^{-1} h^{-1}) can be explained by temperature and food supply expressed as bacterial production (BP, cells ml^{-1} h^{-1}):

$$I = -80.45 + 0.52\,T + 10.83 \log BP$$
$$(R^2 = 0.37;\ p < 0.0001;\ n = 48)$$

The much better relationship of both HNF grazing and growth rates with bacterial production than with bacterial abundance suggests that HNF control bacterial standing stock by direct cropping of bacterial production. This can be supported by the results of Krambeck (1988), Gonzalez et al. (1990), and Sherr et al. (1992) which suggest that bacterivores crop the production rather than simply the standing stock of bacteria because of a higher grazing pressure on more actively growing and dividing cells. Our study has also shown that during the colder part of the year the growth of HNF was very slow, corresponding not with the bacterial abundance minimum but with the bacterial production minimum. Therefore, the threshold production of bacteria (minimum production of bacteria at which HNF can grow) could be taken into account. Our results suggest that the threshold production of bacteria could be near 1 µg C l^{-1} d^{-1}.

Comparison of methods for grazing estimation

Grazing rates obtained by the use of the radioactive-labelled bacteria method (RLB) were compared with the results obtained by the size fractionation method where grazing was estimated from differences in predation between ungrazed and grazed samples. The size fractionation method was used in 2 ways in estimation of grazing, since bacterial production was measured 2 different methods: (1) increase in cell number (dN/dt), and (2) thymidine incorporation (TI). The correlations between all methods were statistically significant ($p < 0.0001$; n = 48) and followed this order:

$$dN/dt{:}TI > dN/dt{:}RLB > TI{:}RLB$$
$$(R = 0.66) \quad (R = 0.45) \quad (R = 0.32)$$

However, some grazing parameters showed significant differences depending on the method used (Table 2). The values obtained by the RLB method were regularly higher than the values obtained by the 2 other methods. The mean population grazing rate obtained by RLB was nearly twice the mean value obtained by the dN/dt method. Further, the contribution of ciliates to the total grazing on bacteria was highest when its estimation was based on the RLB method. This is because when using the size fractionation methods (dN/dt and TI) the contribution of ciliates to total grazing was estimated as the difference between grazing rates in the 100 µm and 8 µm fractions, assuming that HNF grazing rate in the 100 µm fraction was as high as in the 8 µm fraction. However, results obtained by RLB suggest that HNF grazed a lower number of bacteria in the 100 µm fraction (in the presence of HNF predators) than in the 8 µm fraction

Table 1 Heterotrophic nanoflagellate predation parameters. CV: coefficient of variation; ingestion %: carbon consumption per flagellate biomass; GGE: carbon biomass based gross growth efficiency; TD_{50}: time required for 50% reduction of bacterial standing stock by flagellate grazing

Parameter	Minimum	Month	Maximum	Month	Mean	CV (%)
Grazing						
Bact. ml^{-1} h^{-1}	0.3×10^4	Mar	14.0×10^4	Sep	4.0×10^4	120
µg C l^{-1} d^{-1}	0.85		38.62		11.04	
Grazing/production						
%	25.9	Apr	157.0	Sep	78.5	54
Ingestion						
Bact. flag.$^{-1}$ h^{-1}	6.7		50.0		21.8	
pg C flag.$^{-1}$ h^{-1}	0.08	Nov	0.58	Jul	0.25	69
Ingestion % (h^{-1})	1.8		13.2		5.8	
Clearance						
nl flag.$^{-1}$ h^{-1}	5.4	Jan	40.0	Oct	19.5	64
Body vol. h^{-1}	2.7×10^5		20.2×10^5		9.8×10^5	
Clearance						
Water column % d^{-1}	6.1	Apr	266.6	Sep	80.4	106
Yield						
Flag. bact.$^{-1}$	0.8×10^{-3}		1.1×10^{-3}		1.0×10^{-3}	9
1/yield	1437.5	Jan	890.8	Sep	995.4	15
GGE (C/C, %)	30.4		43.3		39.6	9
Spec. grazing rate						
d^{-1}	0.06	Apr	2.24	Sep	0.69	103
T_{50} (d)	11.6		0.3		1.0	

(predator-free fraction) because in the 100 µm fraction HNF were themselves eaten by ciliates. It can also be seen from Table 2 that grazing parameters when obtained by the RLB method showed the strongest relationship with temperature. A strong exponential relationship was established between temperature and consumed radioactivity expressed as community clearance rate (ml l^{-1} h^{-1}) (Fig. 6).

The difficulties in comparing the grazing rates obtained by different methods have been well documented in the literature (e.g. Sherr et al. 1989, Nygaard & Hessen 1990). Kuuppo-Leinikki (1990) reported that fractionation method tend to give somewhat lower grazing values than the RLB method, which is in accordance with the results of this study. However, in this study grazing rates showed similar seasonal oscillations regardless of which method was used.

Growth versus predation

Bacterial production (BP, cells ml^{-1} h^{-1}) as well as HNF production (FP, cells ml^{-1} h^{-1}) showed a strong positive relationship with grazing pressure (GRZ, cells ml^{-1} h^{-1}) across the year:

$$\log GRZ = -0.93 + 1.11 \log BP$$
$$(R = 0.81;\ p < 0.0001;\ n = 48)$$

$$\log GRZ = 0.18 + 0.94 \log FP$$
$$(R = 0.95;\ p < 0.0001;\ n = 48)$$

During the year the trophic coupling of bacteria and HNF with their grazers was established. Prey-predator oscillations had a strong effect on the changes of bacterial and HNF standing stocks on a seasonal scale.

Bacterial production increased during spring, probably as a result of temperature and/or substrate concentration increase. Increase of bacterial production was not immediately matched by an equivalent increase in grazing (April to May) (Fig. 7A). The result of this was the increase of bacterial standing stock (Fig. 1A). It increased until an equilibrium between production and grazing was reached. It occured in summer (June to August) when bacterial standing stock had stabilized at higher values. Bacterial production decreased more rapidly than grazing in autumn (Fig. 7A), probably as a result of temperature and/or substrate supply decrease. However the high concentrations of HNF present during summer kept on maintaining high grazing pressure during September/October. The result was a marked decrease of bacterial standing stock during October and November (Fig. 1A). In December bacterial production increased and after this peak a new equilibrium between production and grazing was reached at lower values (January to March) (Fig. 7A). Therefore, 2 periods of stable bacterial biomass were achieved, first during

Table 2. Comparison between mean values of grazing parameters obtained by different methods (d*N*/d*t*: increase in cell number; TI: thymidine incorporation; RBL: radioactive-labelled bacteria). Coefficients of variation are reported in parentheses

Parameter	Method		
	d*N*/d*t*	TI	RLB
1. Population grazing rate (bact. ml^{-1} h^{-1})	4.47×10^4 (125.7%)	5.29×10^4 (143.3%)	8.27×10^4 (144.1%)
2. Contribution of HNF to total grazing (%)	83.0 (10.1%)	81.9 (9.1%)	75.1 (14.6%)
3. Individual grazing rate (bact. $flag.^{-1}$ h^{-1})	21.8 (82.8%)	25.7 (84.5%)	33.0 (84.0%)
4. Clearance rate (nl $flag.^{-1}$ h^{-1})	19.5 (81.2%)	21.4 (87.5%)	25.3 (64.7%)
5. Coefficient of correlation between temperature and:			
Individual grazing rate	0.38*	0.47*	0.64***
Clearance rate	0.41*	0.39*	0.84***

*$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$; (n = 48)

summer with high values of bacterial biomass, and second during winter with low values.

HNF and ciliates showed a similar trophic relationship during the year but with 2 mo time shift in comparison to the bacteria-HNF relationship (Fig. 7B); the period of positive bacterial net growth (April/May) was followed by positive HNF net growth in June/July. Likewise, negative bacterial net growth in September/October was followed by negative HNF net growth in November/December (Fig. 7A, B; Table 3). Grazing pressure on HNF by ciliates could play an important role in controlling bacterial standing stock, since the removal of HNF by predators can result in reduced HNF grazing on bacteria. This 'trophic cascade effect' (Güde 1988, Sanders et al. 1992) could partially explain the winter maximum of bacterial abundance (Fig. 1A); high grazing pressure on HNF by ciliates in November/December (Fig. 7B) was followed by a marked increase of bacterial abundance in December/January (Fig. 1A).

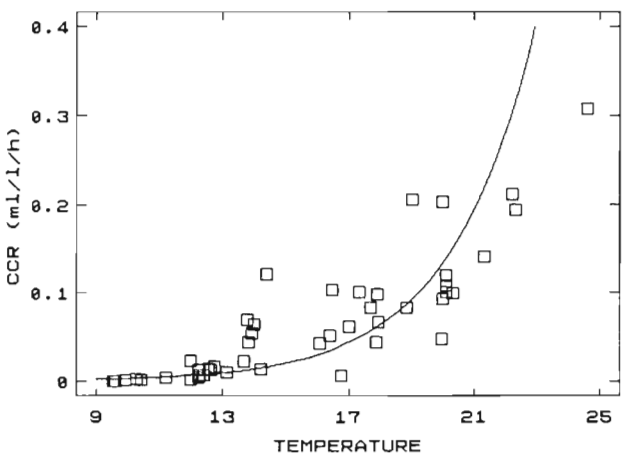

Fig. 6. Consumed radioactivity (expressed as community clearance rate, CCR) as a function of temperature [$y = \exp(-9.51 + 0.37x)$; r = 0.86; $p < 0.0001$; n = 48]

For most of the year bacterial production satisfied HNF carbon requirements (Fig. 8A). Bacterial carbon production accounted for an average for 178% of the HNF carbon requirement with maximum values of 360 to 386% recorded in spring (April/May). HNF carbon requirements exceeded bacterial production

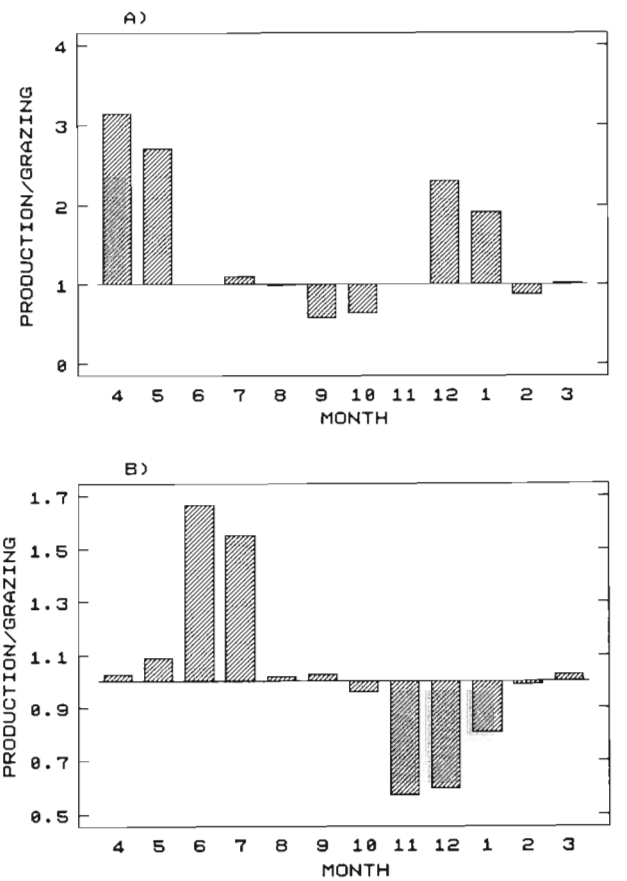

Fig. 7. (A) Ratio between bacterial production and total grazing on bacteria. (B) Ratio between HNF production and ciliate grazing on HNF

only in September/October, when bacterial production satisfied 60 to 70% of the HNF carbon requirement. In that period negative net growth rates of bacteria were recorded (Table 3). Several authors also found that in autumn HNF carbon requirement exceeded bacterial production indicating that HNF satisfied a part of their carbon requirement from sources other than bacteria in that period (Hagström et al. 1988, Kuosa & Kivi 1989). However, in Kaštela Bay bacteria were able to support HNF growth completely during most of the year. Moreover, bacteria satisfied 9 to 47% (mean 32%) of ciliate carbon requirements (Fig. 8C). Weisse et al. (1990) reported that ciliates satisfied about 10% of their food demand by bacterial ingestion which is near the lower end of our range.

HNF production accounted an average for 71% of the ciliate carbon requirement. Maximum values of about 134% were recorded in June/July (Fig. 8B). Ciliate carbon requirements were significantly higher than HNF production in November/December, when HNF production satisfied less than 40% of the ciliate carbon requirement. Thus, ciliates satisfied about two-thirds of their food demand by HNF ingestion, and one-third by bacterial ingestion (Fig. 8C).

Stimulation of bacterial growth by grazing

Thymidine incorporation rate (TI) and specific thymidine incorporation rate (STI) or thymidine incorporation per bacterial cell were higher in the presence of predators (8 µm and 100 µm fractions) than in predator-free conditions (1 µm fraction) indicating a positive effect of bacterivorous protozoa on bacterial growth (Fig. 9A, B). STI in the presence of predators was higher than in their absence as much as grazing pressure was higher (Fig. 9C). Johannes (1968) reported that the presence of bacterivores appeared to enhance the dissolved organic substrate pool. Fenchel & Harrison (1976) and Taylor et al. (1985) suggested that bacterivorous protozoa may stimulate bacterial growth by reducing growth-inhibitory bacterial competition through grazing and by supplying dissolved organic matter through excretion and through stimulation of bacterial exudation. Finally, the grazing activity of protozoa may stimulate bacterial growth indirectly, being an important source of nutrients for primary producers (Berman et al. 1987, Caron et al. 1988). Therefore, high grazing pressure on bacteria was followed by high bacterial growth resulting in the accelerated turnover of bacterial biomass (Fig. 9D). It was particularly expressed in summer when turnover time of bacterial biomass was 2 to 4 times shorter in the presence of predators.

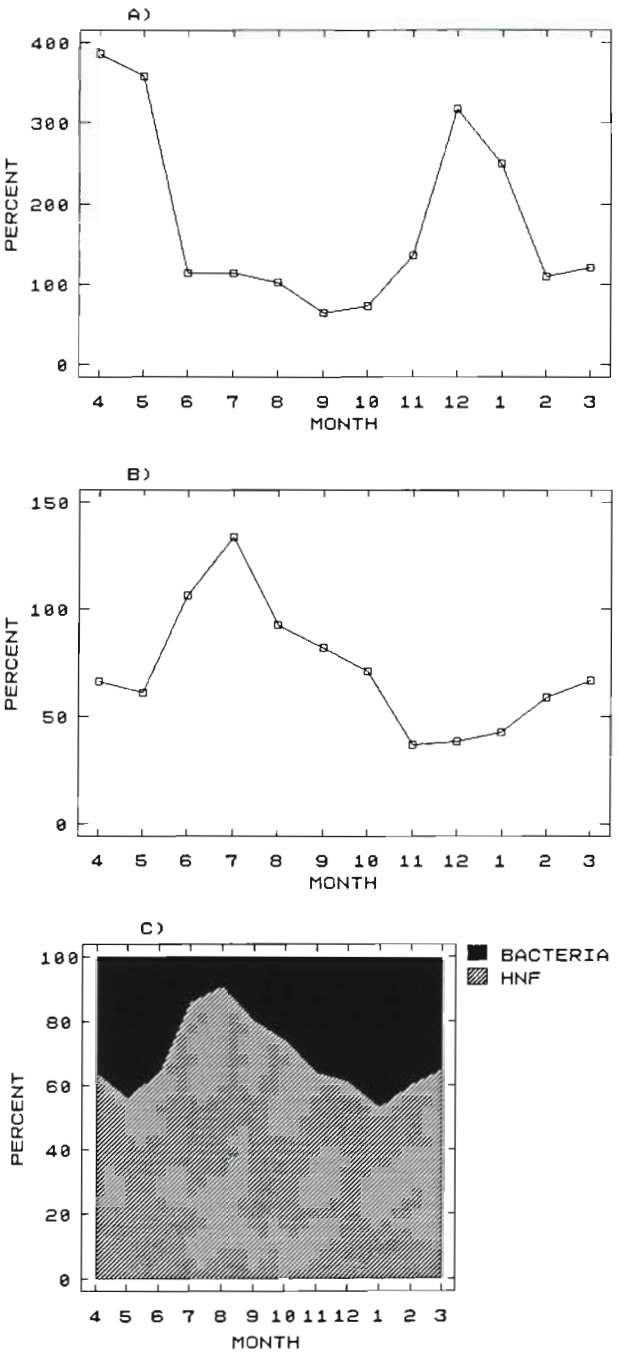

Fig. 8. (A) Bacterial production expressed as percentage of HNF carbon requirement. (B) HNF production expressed as percentage of ciliate carbon requirement. (C) Contribution of bacteria and HNF in satisfying ciliate carbon requirement

Bacterial carbon flux

HNF removed 23 to 154% of the bacterial production (mean 78%) or 6 to 236% d^{-1} of bacterial standing stock (mean 78% d^{-1}) (Fig 10A, B). Another 5 to 27%

Table 3. Specific growth and grazing loss rates of bacteria and HNF. k: gross growth rate; g_F: HNF grazing rate; g_C: ciliate grazing rate, μ: net growth rate; Tendency: changes of bacterial and HNF standing stock (+, significant increase; –, significant decrease; 0, no change or insignificant change)

Month	Group	Specific growth and grazing rate (d^{-1})				Tendency
		k	g_F	g_C	μ	
Apr	Bacteria	0.28	0.06	0.01	0.21	+
	HNF	0.20	–	0.19	0.01	0
May	Bacteria	0.50	0.13	0.04	0.33	+
	HNF	0.61	–	0.51	0.10	0
Jun	Bacteria	0.84	0.73	0.12	−0.01	0
	HNF	1.10	–	0.64	0.46	+
Jul	Bacteria	1.19	1.03	0.06	0.10	0
	HNF	1.26	–	0.80	0.46	+
Aug	Bacteria	1.15	1.12	0.05	−0.02	0
	HNF	0.71	–	0.70	0.01	0
Sep	Bacteria	1.28	2.24	0.35	−1.31	–
	HNF	1.14	–	1.10	0.04	0
Oct	Bacteria	1.13	1.65	0.30	0.82	–
	HNF	0.38	–	0.40	−0.02	0
Nov	Bacteria	1.04	0.76	0.34	−0.06	0
	HNF	0.16	–	0.29	−0.13	–
Dec	Bacteria	0.60	0.18	0.07	0.35	+
	HNF	0.25	–	0.42	−0.17	–
Jan	Bacteria	0.23	0.09	0.03	0.11	0
	HNF	0.17	–	0.21	−0.04	0
Feb	Bacteria	0.21	0.20	0.05	−0.04	0
	HNF	0.33	–	0.33	0.00	0
Mar	Bacteria	0.10	0.08	0.02	0.00	0
	HNF	0.40	–	0.39	0.01	0

(mean 14 %) of bacterial production or 1 to 35 % (mean 11 %) of bacterial standing stock was cropped by ciliate grazing. Therefore, bacterial growth was approximately balanced by HNF plus ciliate grazing. Removal rates of bacterial production by their grazers obtained in this study were comparable in magnitude to those reported in the literature (e.g. Wright & Coffin 1984, Taylor & Pace 1987). Wikner et al. (1990) listed literature values of the grazing/production ratio which showed that predators removed 40 to 430 % of bacterial production.

The maximum rates of both bacterial production and removal of standing stock were observed in September corresponding with the shortest turnover time of bacterial biomass (Fig. 10C). Turnover time of bacterial biomass varied from 0.7 to 11.2 d with a mean value of 2.9 d. This value was in accordance with literature values obtained in different marine and freshwater environments (Linley et al. 1983, Pace et al. 1990). In most of these studies turnover time of bacterial biomass ranged from 0.5 to 16 d.

Ciliates removed 63 to 175 % of the HNF production (mean 106 %) or 20 to 130 % d^{-1} of HNF standing stock (mean 57 % d^{-1}) (Fig. 10D, E). This supports the results of Weisse et al. (1990) who reported that HNF production was entirely used by ciliates.

Maximum HNF standing stock removal was recorded in July to September corresponding with the shortest turnover time of HNF biomass (Fig. 10F). On the other hand, removal of HNF production reached a maximum in November to December. However, due to low HNF production in this period, the HNF biomass actually removed was very low. Turnover time of HNF biomass varied from 0.8 to 8.0 d with a mean of 3.7 d (Fig. 10F).

A strong equilibrium between annual production and grazing was established for both bacteria and HNF. Therefore, the entire annual bacterial (4.47 g C m^{-3} yr^{-1}) and HNF (1.75 g C m^{-3} yr^{-1}) production was consumed by their predators. HNF accounted on average for 80 %, and ciliates for 20 % of the total grazing of bacteria. About 32 % (1.43 g C m^{-3} yr^{-1}) of bacterial production was incorporated in HNF biomass (average gross growth efficiency 40 %). Ciliates incorporated about 8 % (0.36 g C m^{-3} yr^{-1}) of bacterial production by direct grazing and about 13 % (0.58 g C m^{-3} yr^{-1}) indirectly through HNF, assuming a gross growth efficiency of 40 % (Ducklow 1983, Hagström et

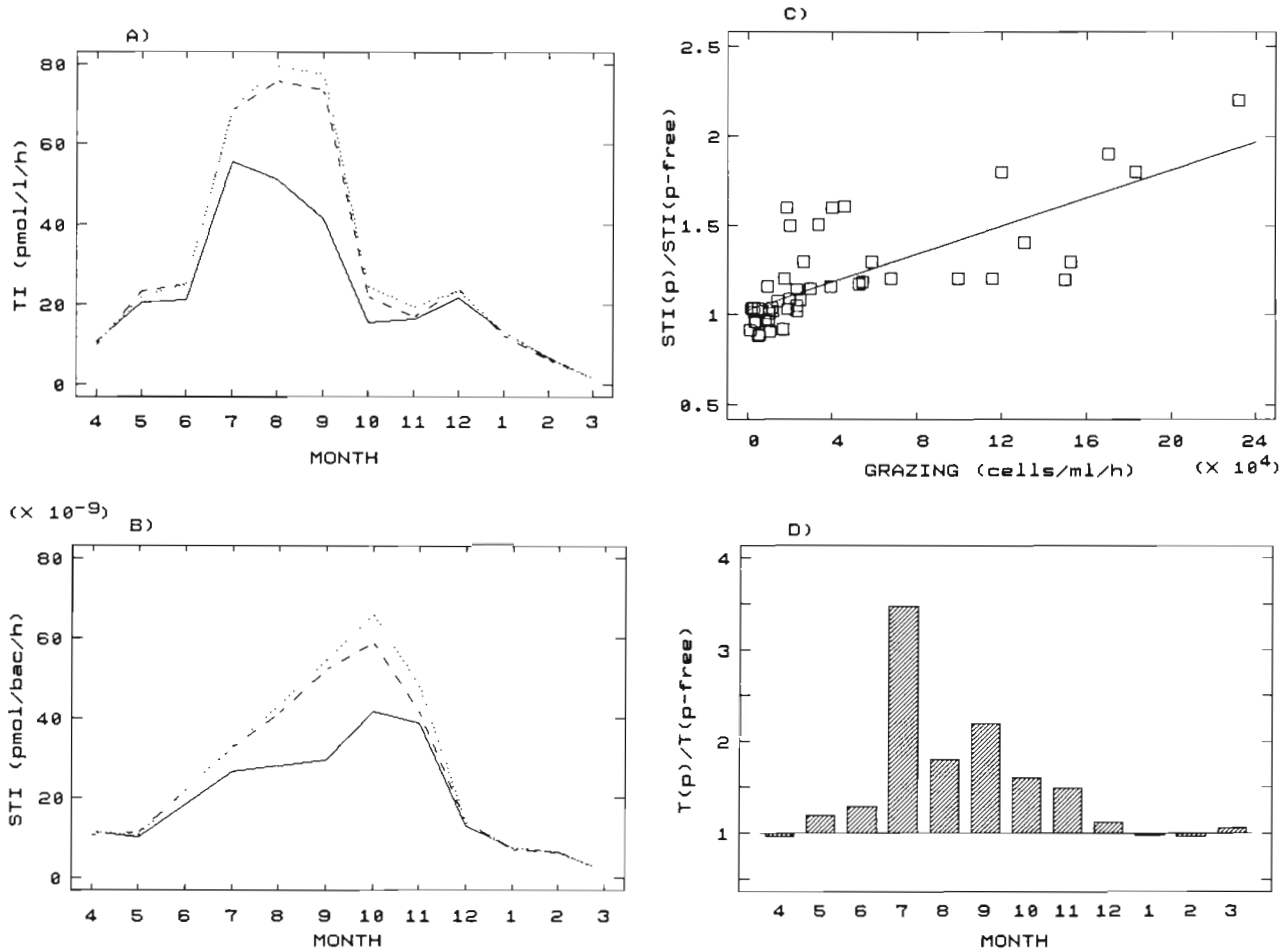

Fig. 9. (A) Thymidine incorporation (TI) and (B) specific thymidine incorporation (STI) in 1 µm (solid lines), 8 µm (dashed lines) and 100 µm (dotted lines) fractions. (C) Ratio between STI in 100 µm predators fraction and STI in 1 µm predator-free fraction as a function of grazing pressure ($y = 1.03 + 3.90 \times 10^{-6}\ x$; $r = 0.75$; $p < 0.0001$; $n = 48$). (D) Ratio between turnover time of bacterial biomass in 100 µm predators fraction and in 1 µm predator-free fraction (turnover time is calculated from estimates of carbon biomass and production)

al. 1988). This means that of the total annual bacterial production about 21% (0.94 g C m^{-3} yr^{-1}) is available to the metazoan food web. Ciliates incorporated about 40% of the annual HNF production or 0.70 g C m^{-3} yr^{-1}.

This annual carbon-flow model cannot completely explain the ecological importance of the microbial loop due to marked seasonal oscillations of bacterial carbon flux through the protozoan predator components.

In spring, relatively high bacterial production corresponded with low grazing. The result of this was the increase of bacterial biomass but its flux through the microbial loop was very low. Only 10 to 12% of bacterial production (0.36 to 1.50 µg C l^{-1} d^{-1}) was incorporated into HNF biomass (Fig. 11A, B), and 6 to 8% (0.23 to 1.05 µg C l^{-1} d^{-1}) into ciliate biomass (Fig. 11C, D). In this period turnover time of bacterial biomass ranged between 2 and 4 d (Fig. 10C), and less than 20% of bacterial standing stock was removed per day by grazers (Fig. 10B).

In summer, bacterial production and grazing on bacteria balanced at high values. Turnover time of bacterial biomass became faster (ca 1 d) (Fig. 10C), and bacterial carbon flux was at a maximum. More than 100% of bacterial standing stock was removed per day by grazers (Fig. 10B). During this period, HNF incorporated into its own biomass about 12 µg C l^{-1} d^{-1} or about 40% of bacterial production (Fig. 11A, B). Ciliates incorporated 2 to 5 µg C l^{-1} d^{-1} or 16 to 19% of bacterial production (Fig. 11C, D).

In autumn, bacterial production decreased rapidly, while grazing pressure still stayed high. Grazers removed >200% of bacterial standing stock per day (Fig. 10B), and turnover time of bacterial biomass was

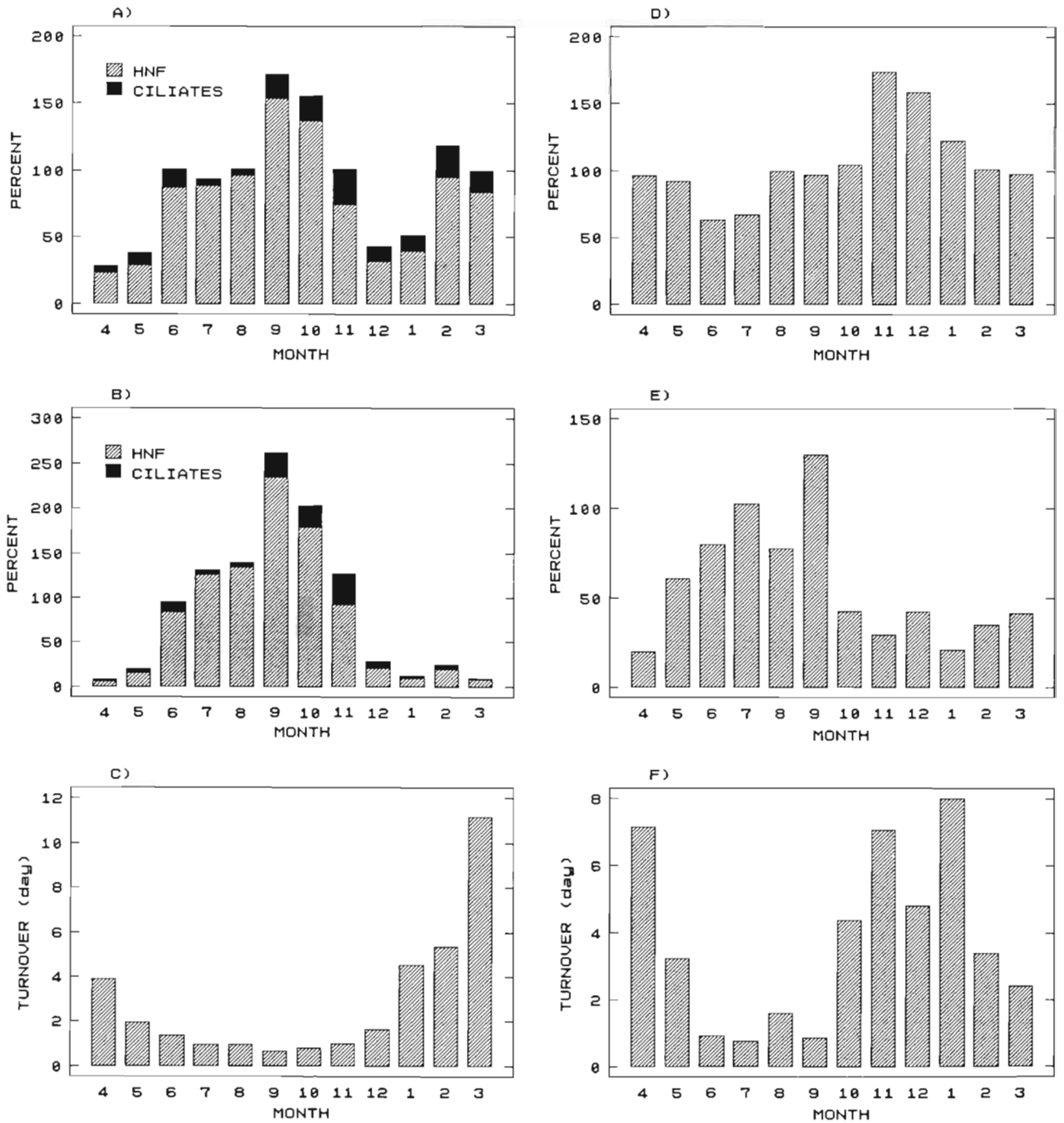

Fig. 10. Removal of (A) bacterial production, (B) bacterial standing stock, (D) HNF production and (E) HNF standing stock by grazers throughout the year. Seasonal fluctuations of (C) bacterial and (F) HNF biomass turnover time (turnover time is calculated from estimates of carbon biomass and production)

less than 1 d (Fig. 10C). Although bacterial carbon flux through the microbial loop, expressed as a percentage of bacterial production, was as high as or even higher than in summer (Fig. 11B, D), due to considerably lower bacterial production in autumn, the real flux was lower than in summer (Fig. 11A, C).

In winter, after the bacterial production peak in December, bacterial losses again equaled bacterial production at low values. Turnover time of bacterial biomass was considerably longer (4 to 11 d) (Fig. 10C). Grazers removed <30 % of bacterial standing stock per day (Fig. 10B). Bacterial carbon flux was very low

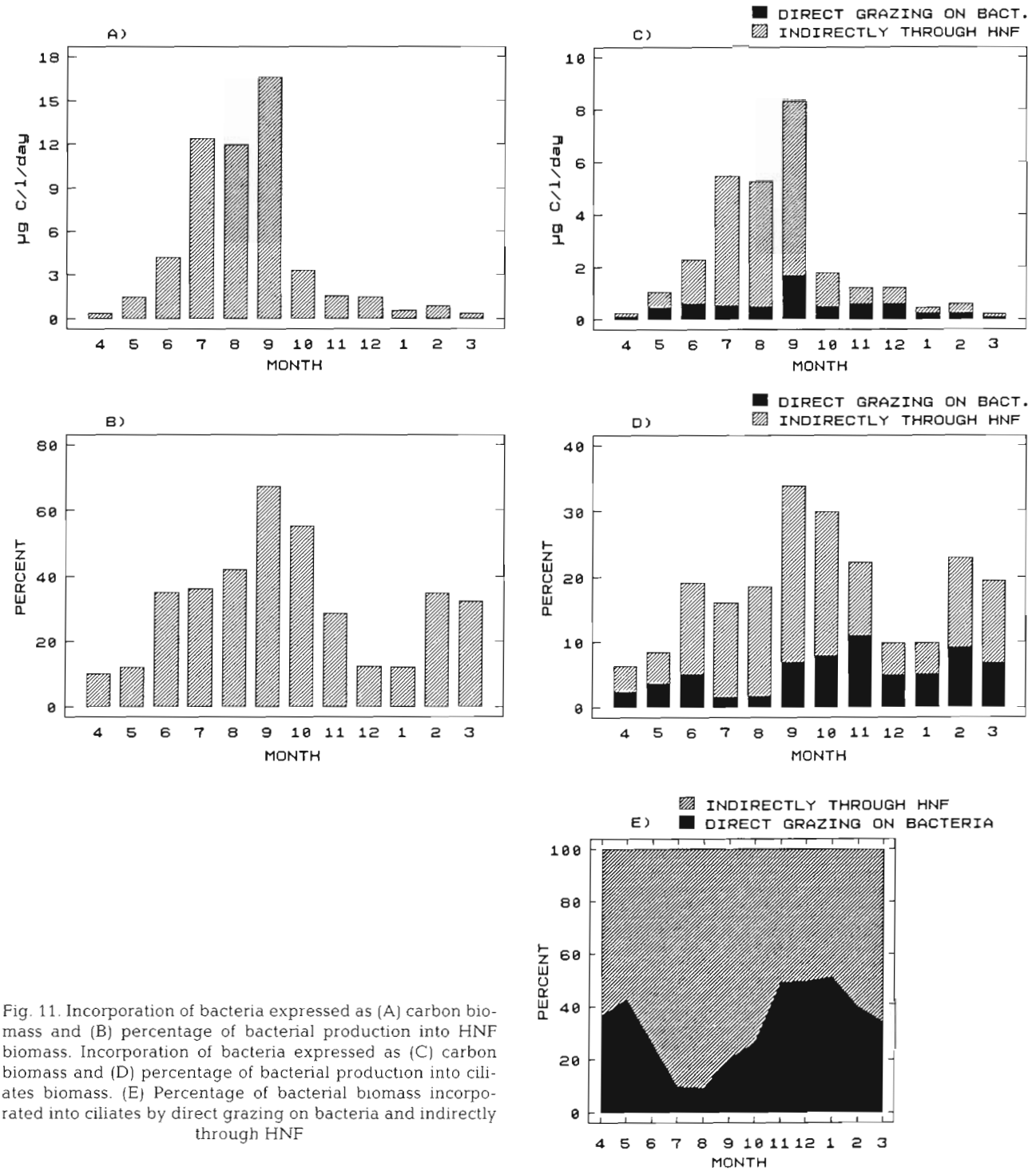

Fig. 11. Incorporation of bacteria expressed as (A) carbon biomass and (B) percentage of bacterial production into HNF biomass. Incorporation of bacteria expressed as (C) carbon biomass and (D) percentage of bacterial production into ciliates biomass. (E) Percentage of bacterial biomass incorporated into ciliates by direct grazing on bacteria and indirectly through HNF

(<1 µg C l^{-1} d^{-1} was incorporated in both HNF and ciliate biomass) (Fig. 11A, C).

Therefore, according to the ecological importance of the microbial loop, 2 different periods of the year were distinguished.

During the warmer part of the year (June to November) a significant part of bacterial carbon was channelled to higher trophic levels. Thus, besides the classical food chain, the microbial loop could be an important additional link between primary production and higher trophic levels. In this period the grazing pressure on bacteria by their predators was very strong, and grazing could be a major factor in controlling bacterial standing stock (top-down control).

During the colder part of the year (December to May) the bacterial carbon flux was very low. Therefore, the microbial loop was not important as a link to higher trophic levels. Although the grazing pressure on bacteria was reduced, bacteria were not capable of rapid increase of production. It could be concluded that, in this period, low temperature and/or substrate limitation were more important than grazing in controlling bacterial standing stock (bottom-up control).

The efficiency of bacterial carbon transfer to the ciliate component depended on the contribution of ciliates to the total grazing on bacteria. Namely, if bacteria are consumed by ciliates, the efficiency of bacterial biomass transfer will be higher than if bacteria are mostly consumed by HNF, when the efficiency of transfer is much reduced and vertical flux of bacterial biomass is slow. On average, 20% of total grazed bacterial biomass was fixed in ciliate biomass. Of this, 38% was the result of direct ciliate grazing on bacteria, whereas 62% of ciliate biomass of bacterial origin previously passed through the HNF component. The maximum efficiency of bacterial carbon transfer was established in November to January when 50% of incorporated bacterial biomass was result of direct grazing by ciliates (Fig. 11E). However, due to low grazing pressure the bacterial carbon flux in this period was very low (Fig. 11A, C). On the other hand, during the period of maximum bacterial carbon flux (July to September) (Fig. 11A, C) the efficiency of bacterial carbon transfer to the ciliate component was at a minimum: in that period >90% of bacterial biomass incorporated in the ciliate component previously passed through the HNF component (Fig. 11E). Therefore, the efficiency of carbon transfer from bacteria to ciliates was inversely proportional to the amount of carbon transfered.

CONCLUSIONS

In this study, small HNF (<8 µm) were the most important grazers of bacteria, controlling bacterial abundance and production, whereas ciliates were the most important grazers of HNF. Bacteria were the dominant prey for HNF, satisfying HNF carbon demand through most of the year. Ciliates satisfied about two-thirds of their carbon demand by HNF ingestion and one-third by bacterial ingestion.

In general the microbial loop was not broken by higher consumers. The result was that bacterivorous protozoa (HNF and ciliates) dissipated a significant part of bacterial carbon production in respiration. However, bacterial carbon flux through the microbial loop showed strong seasonal oscillations. During the warmer part of the year (June to November) a significant part of bacterial carbon was channelled through the microbial loop suggesting that it could be an important link between primary production and higher trophic levels. On the other hand, during the colder part of the year (December to May), bacterial carbon flux was very low and the microbial loop acted as a mineralization system rather than as a link. Therefore, this study emphasizes the great importance of seasonal scales for carbon-flow models.

Bacterivorous protozoa stimulated bacterial growth and contributed to an enhanced turnover of bacterial biomass.

This study affirms temperature as a primary mechanism in controlling activity of the protozoan community. Thus, the growth of bacterial and their predator populations, as well as grazing on bacteria, were strongly affected by temperature.

Grazing on bacteria depended on the method used. Thus, grazing values obtained by the RLB method were higher than values obtained by the 2 other methods (size fractionation methods). However, the correlations between all methods were high, and grazing showed same seasonal dynamics regardless of the method used.

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