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Ecology of Heterotrophic Microflagellates. IV. Quantitative Occurrence and Importance as Bacterial Consumers

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ABSTRACT: Heterotrophic nanoplankton flagellates and bacteria were quantified at regular intervals during a month in Limfjorden, Denmark. The number of flagellates averaged about 10^3 ml^{-1} and ranged from $< 2 \times 10^2$ to about 3×10^3 ml^{-1}. The composition of this fauna was dominated by choanoflagellates, non-pigmented chrysomonads and bicoecids; other forms are rarer. A substantial part of this fauna is associated with suspended particulate matter. Bacteria occurred in concentrations ranging from 1.5 to 3×10^6 ml^{-1}. Flagellate numbers followed bacterial numbers and the bacteria-flagellate system showed a cyclical behaviour with a frequency of about 16 d during the study period. Calculations based on laboratory data on the clearance of flagellates show that such organisms on the average filter 20 % of the Limfjord water d^{-1} (range 12 to 67 % d^{-1}). This is consistent with previously published estimates of bacterial division rates in seawater provided that microflagellates are the main consumers of pelagic bacteria.

INTRODUCTION

In previous articles (Fenchel, 1982a–c) various aspects of the bioenergetics and filtration ability of some heterotrophic (bacterivorous) nanoplankton flagellates were treated. The present paper explores the qualitative composition and the quantitative occurrence of this fauna in a marine environment, the Limfjord, Denmark. The main purpose of the study is to clarify the role of these organisms as consumers of bacteria in a pelagic environment.

MATERIALS AND METHODS

Study Area and Sampling

The Limfjord is an about 150 km long sound, cutting across northern Jutland and connecting the North Sea with the Kattegat. The water body is shallow (average depth: 8 m) and the salinity decreases from west to east from about 33 ‰ S to about 25 ‰ S. Sampling was carried out in the central part, Løgstør Bredning, off the Aarhus University Marine Laboratory at Rønbjerg Harbour. Here the Limfjord is about 15 km wide and the salinity fluctuates somewhat around 27 ‰ S. Samples were taken about 1.5 km off the shore with a 0.5 l water sampler, at the surface and for every metre down to the bottom (about 7.5 m at the sampling station). Samples were taken at intervals of 2 d from August 8 to September 7, 1981. In this period water temperatures ranged from 16.0 to 18.5 °C. There was practically no thermal stratification during this period (maximum temperature difference between surface and bottom samples recorded was about 1 C°). More scattered sampling took place in the period May–July. In September samples were collected 0.5 m below the surface along a 6 km long transect parallel to the shore at two occasions. Finally, additional observations were carried out on water samples from Aarhus Bay during the period January–May, 1981.

Quantification and Qualitative Observations

Direct counts of bacteria and nanoplankton were carried out with epifluorescence microscopy following Hobbie et al. (1977). Within 15 min of sampling, 10 ml water were withdrawn and fixed in pre-filtered formaldehyde solution to give a final concentration of about

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5 %. These preserved samples can be kept for at least 1 mo. For counting, 1 ml filtered acridine orange solution was added to give a final concentration of 0.01 % The samples were then filtered through 0.2 µm Nuclepore filters prestained with irgalan black. The filters were finally mounted in immersion oil between slides and cover slips. Counting was carried out with a 100 × immersion objective on a Leitz epifluorescence microscope fitted with a mercury lamp and interference filters chosen to give blue excitation light. Counts were made with the aid of an ocular grid. With respect to bacteria, at least 200 cells were counted per sample. With respect to flagellates, compromises had to be made; in most cases 20 to 50 cells per sample were counted. The technique allows most major protistan groups and often even genera to be identified after some practice. Size distributions of bacteria and of zooflagellates were also based on the above mentioned preparations.

In order to confirm the qualitative composition of the fauna, the remaining (nearly 0.5 l) of the samples were on several occasions centrifuged until an about 1 ml particle concentrate remained. Part of this was observed live. By switching between phase contrast illumination and transmitted blue light from the mercury lamp, the presence or absence of chloroplasts could be determined on live cells. Another part of the centrifuged samples was fixed by the addition of an isotonic 2 % OsO_4 solution for 30 min. The sample was then rinsed 3 to 4 times with distilled H_2O by centrifugation and drops of this suspension were allowed to dry on grids with a formvar-carbon film for later observations in the transmission electron microscope.

On 3 occasions serial dilution cultures were initiated. A dilution series in steps of 1 : 10 with autoclaved seawater and in the range 10^{-1} to 10^{-5} was made. One ml of each of these dilutions was then added to each of 3 Petri dishes, each containing 25 ml seawater (filtered through 1 µm Nuclepore filters twice in order to remove all eukaryote cells but retain most bacteria) and a boiled wheat grain. Samples from these cultures were observed regularly from Day 4 to Day 10 with a 40 × phase objective. Throughout these experiments, sterile techniques were employed for transfers and sampling for microscopical preparations.

RESULTS

Observations on Centrifuged Samples

The composition of the heterotrophic microflagellate fauna varied somewhat during the study period, but the following groups remained important throughout.

Choanoflagellates, loricate as well as naked, occurred in a great variety, some examples are shown in Fig. 1 (a, c, e–g); most were associated with suspended detrital material. Among non-pigmented chrysomonads, species of the genus *Paraphysomonas* were common; based on TEM observations on silicious scales at least 2 species were present, *P. vestita* (see Fenchel, 1982a) and *P. butcheri* (Fig. 1d, h). *Pseudobodo*, a bicoecid flagellate (see Fenchel, 1982a) was constantly present, as was a somewhat smaller related type (Fig. 1b) which is likely to be identical to the *'Bodo' parvulus* of Griessmann (1914) although EM shows that it certainly is not a *Bodo* or belonging to the kinetoplastid flagellates at all. Also these flagellates tend to be attached to detrital material such as empty diatom frustules, algal threads or copepod fecal material. Species of the loricate genus *Bicoeca* were periodically common. *Actinomonas* (see Fenchel, 1982a) and kinetoplastid flagellates were rarely observed. Finally a number of forms were observed which could not be identified and which may in part represent undescribed species. In addition to the flagellates, a number of other types of heterotrophic cells were found in association with suspended particles. These cells, which are in the same size range as the flagellates (3–8 µm) and also bacterivorous as judged from vacuole contents are mainly amoebae and heliozoans. Finally, larger (10–30 µm) non-pigmented flagellates, mainly euglenoids and dinoflagellates, occurred regularly (these are not considered further as they are likely to depend on larger prey than bacteria).

Epifluorescence Counts

After some experience, it was possible to identify most major groups in the quantitative samples. Photosynthetic forms are revealed by the red autofluorescence of chlorophyll and although this may sometimes be more or less masked by the strong green fluorescence of acridine orange, one is rarely in doubt. Acridine orange binds to DNA and RNA and in particular the nucleus of eukaryotic cells is rendered luminescent. However, there is usually sufficient background luminescence to make out the position and length of flagella, the general, if somewhat distorted, cell shape, and sometimes other structural details. The main criteria for identification of major flagellate groups are shown in Table 1. In some cases flagella are difficult to see when the cells are entangled among filamentous bacteria or detrital material and occasionally cells which seem to have lysed on the filter occur. Such cases were not included in the counts.

Fig. 2 shows the quantitative occurrence for some of the most common forms during the 30 d period. It can be seen that *Paraphysomonas*, *Pseudobodo*, and

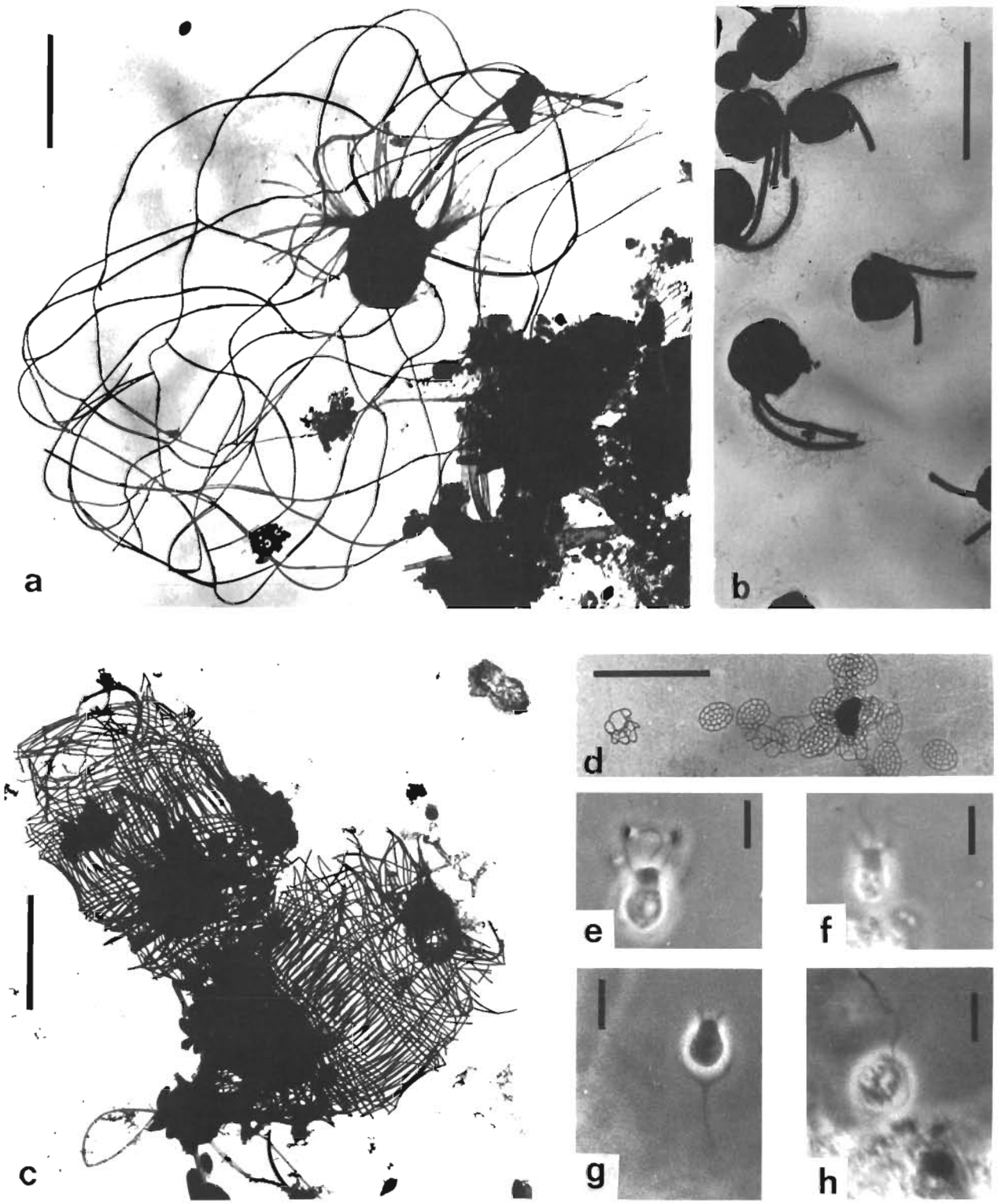

Fig. 1. Examples of diversity of microflagellates. a: choanoflagellate *Diaphanoeca grandis* Ellis, centrifuged water sample, Limfjorden, whole mount, TEM; b: *'Bodo' parvulus* Griessmann, enrichment culture, Limfjorden, whole mount, TEM; c: choanoflagellate *Stephanoeca norrisii* Thomsen, enrichment culture, Aarhus Bay, whole mount, TEM; d: dimorphic scales of *Paraphysomonas butcheri* Pennick and Clarke, enrichment culture, Limfjorden, TEM; e–h: micrographs of live cells from centrifuged water sample, Limfjorden, e–g: choanoflagellates (e: *Stephanoeca* sp., g: *Codosiga* sp.), h: *Paraphysomonas* sp. Scales: a–c and e–h: 5μm, d: 1 μm

Table 1. Identification of flagellate types with epifluorescence microscopy

Type	Diagnostic features	Remarks
Kinetoplastids	Kinetoplast, flagellation	Often difficult, mostly rare in plankton
Choanoflagellates	Flagellation, radial symmetry, collar and lorica sometimes visible	
Bicoecids	Lorica, flagellation	
Paraphysomonas	Spherical shape, flagellation	Small flagellum not always visible
Pseudobodo	Cell shape, flagellation	
Actinomonas		Not distinguishable from other zooflagellates with one flagellum
Dinoflagellates +/− chloropl.	Structure of nucleus, test, shape, flagellation	
Euglenoids +/− chloropl.	Flagellation, shape, pellicula striations	
Cryptomonads	Shape, flagellation	
Photosynthetic 'monads' sensu lato	Flagellation, size, presence of chloroplasts	Many individual genera – e.g. *Ochromonas, Stenocalyx, Pyramimonas* – are relatively easy to recognize

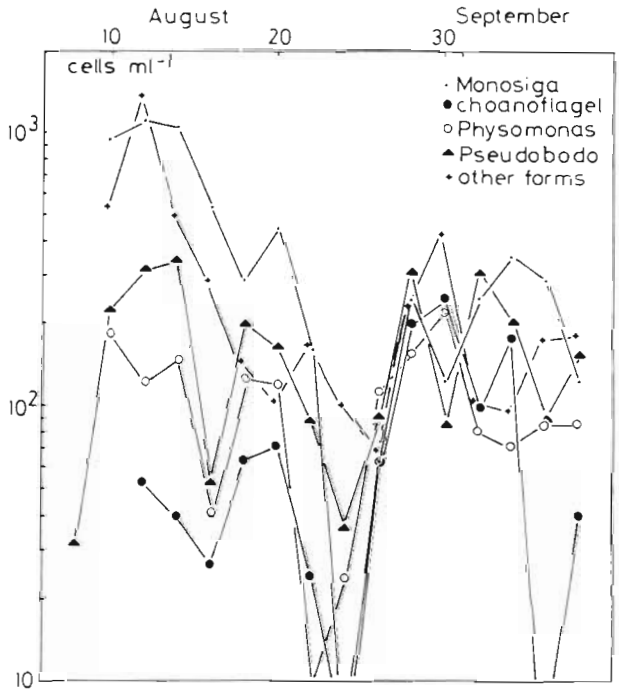

Fig. 2. Quantitative occurrence of the most important forms of microflagellates

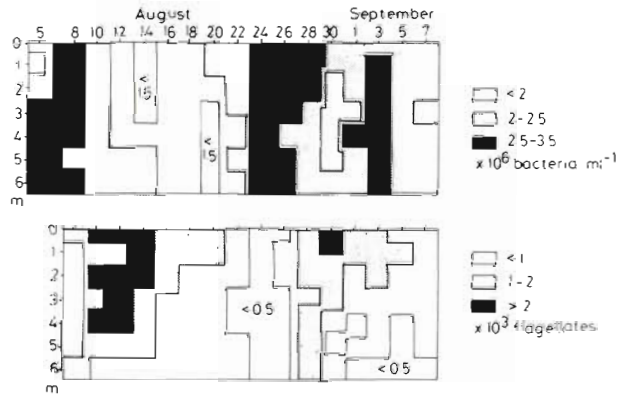

Fig. 3. Quantitative occurrence of bacteria and microflagellates from surface to bottom during 1 mo

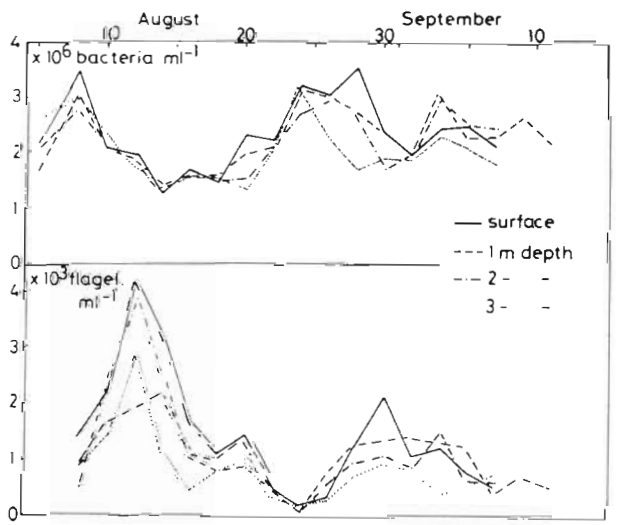

Fig. 4. Quantitative occurrence at the upper 3 m of bacteria and microflagellates during 1 mo

choanoflagellates (including the naked *Monosiga* which totally dominated the fauna during the first half of the period) together made up for more than 50 % of the fauna.

The concentration of bacteria and of the heterotrophic flagellates are shown for all depths in Fig. 3 and for the upper 3 m only, in Fig. 4. There was never any strong tendency for vertical zonation of the cell counts although there were more flagellates in the uppermost metres of the water column. Over time,

however, both bacterial and flagellate concentrations varied considerably. Bacterial numbers ranged between 1.5 and 3×10^6 ml^{-1}, those of flagellates between $< 2 \times 10^2$ and 3×10^3 ml^{-1}. The temporal variation followed a characteristic and repeated pattern. A bacterial peak in the beginning of August was followed by an increase in numbers of flagellates which peaked some 4 d after the bacteria. The decline in bacterial numbers continued until the middle of the month and the flagellates showed a minimum during the fourth week of the month. Following peaks of bacterial and of flagellate numbers were evident around the 26th and the 30th of August, respectively.

Two transects sampled at 1 km intervals over a 6 km long stretch parallel to the coast from Rønbjerg Harbour and north, showed very little spatial variation in bacterial numbers and somewhat more for flagellate numbers (Fig. 5). Flagellate counts had a much larger

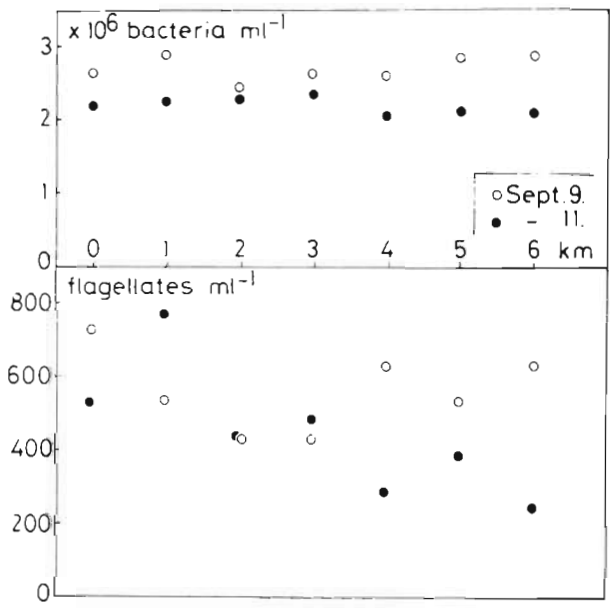

Fig. 5. Numbers of bacteria and microflagellates in the upper 0.5 m along a 6 km transect, collected within 4 h

sample variance than the corresponding counts of bacteria (SE: about 20 and 7 %, respectively) and this may account for the seemingly higher variation in flagellate numbers.

In an attempt to estimate the fraction of flagellates associated with suspended particles, 2 parallel samples, 1 of which was first filtered through a 20 µm sieve, were counted on August 5. The unsieved and the sieved samples yielded, respectively: for bacteria, 3.14 and 3.04×10^6 ml^{-1}; for photosynthetic nanoplankton cells, 2.66 and 2.46×10^3 ml^{-1}; but for heterotrophic flagellates, 8.47×10^2 and 3.62×10^2 ml^{-1}. Since many detrital particles are likely to pass a 20 µm sieve, the difference underestimates the fraction of flagellates associated with particles.

Pigmented microflagellates occurred during the first ten days of the study period in numbers around 10^3 ml^{-1}. In the middle of the month they increased to about 2×10^3 ml^{-1} whereafter they decreased again to 5×10^2 to 10^3 ml^{-1} for the remaining period. The dominating forms were cryptomonads, a variety of pigmented 'monads' and somewhat fewer euglenoids and dinoflagellates. Ciliates were much rarer, the average for all samples is 17 ml^{-1} ranging between 5 and 40 ml^{-1}. However, due to a large sampling error associated with low numbers, no significance can be attributed to variation between samples. The ciliates were dominantly small (about 20 µm) non-loricate oligotrichs and more rarely *Mesodinium* and *Uronema*-like holotrichs. Tintinnids were only occassionally observed in the quantitative samples and probably occurred at densities around 1 ml^{-1}.

Dilution Cultures

From a qualitative view point, the dilution cultures gave results in accordance with direct observations: the dominating forms growing in the cultures were *Paraphysomonas* spp., *Pseudobodo tremulans*, *'Bodo' parvulus*, *Bicoeca* spp., and a variety of choanoflagellates dominated by the non-loricate *Monosiga*. With one exception, kinetoplastid flagellates occurred only in the least diluted samples. Species distributions in the cultures indicated non-randomness; some species occurred most frequently in cultures with more diluted inoculates. This indicates that some species were rare (and thus overlooked) or became extinct due to competitive interactions in cultures which were initially inoculated with several species. If, in spite of this, Poisson distribution is assumed, then tables of 'most probable numbers' (e.g. Jacobs and Gerstein, 1960) for many species gave results in reasonable agreement with direct counts. For serial dilution cultures initiated from water samples collected on August 16, tables of MPN gave the following estimates: *Pseudobodo*, 90 ml^{-1}; *Paraphysomonas*, 40 ml^{-1}; *'Bodo' parvulus*, 460 ml^{-1}; *Monosiga*, 40 ml^{-1}; other choanoflagellates, 4 ml^{-1}. Comparisons with Fig. 2 show reasonable agreement except for choanoflagellates where direct counts gave much higher numbers.

DISCUSSION AND CONCLUSIONS

The present study gives a general picture of the composition of the microflagellate fauna as a combination of loricate or spine bearing forms described

mainly by electron microscopists (Leadbeater, 1972a, b, 1974; Thomsen, 1973, 1975, 1976; Throndsen, 1974) and naked forms which are mainly known from enrichment cultures (Griessmann, 1914; Lighthart, 1969; Haas and Webb, 1979). Based on this literature it would seem that the composition of this fauna in the Limfjord is not untypical of that of other seas.

Serial dilution cultures did not prove very suitable for the estimation of absolute numbers. However, the agreement between the species composition of the dilution cultures with that of the quantitative samples shows that the quantitatively important species in nature can be grown in the laboratory and that the species studied experimentally (Fenchel, 1982a, b) do not represent rare or specialized forms. The fact that estimates based on MPN rarely exceeded direct counts significantly would suggest that resting cysts do not play an important role.

Estimates based on direct counts must also be considered with some reservation. The fact that some cells may be overlooked or misinterpreted tends to yield minimum estimates. When the capacity of this fauna as bacterial consumers is to be considered it must be kept in mind that some pigmented forms like *Ochromonas* (see Fenchel, 1982a, b) may dominantly be phagotrophs. However, such forms are all classified as phototrophs in the following.

There are few data in the literature with which to compare the present enumerations of heterotrophic flagellates. Throndsen (1969) estimated numbers of non-pigmented flagellates which appeared as contaminants in serial dilution cultures set up in order to quantify phototrophic nanoplankton. His figures are lower than mine, which – considering purpose and the culture conditions – may not be surprising. Lighthart (1969) tried to quantify this fauna in water and sediments from the west coast of America by a serial dilution culture technique. His results for the water column are considerably lower than mine (usually $< 10\ ml^{-1}$), but his species lists suggest that he overlooked many of the smallest forms. Two studies employing direct counts are quite comparable to my data. Chretiennot (1974) mainly studied pigmented nanoplankton, but also included choanoflagellates occurring in the water off Marseille during a year. Usually between 10^2 and $10^3\ ml^{-1}$ were found, and at one occasion even around $10^4\ ml^{-1}$. Sorokin (1977) studied the succession of the heterotrophic organisms following a plankton bloom in the Japan Sea. As a response to a high concentration of bacteria, a peak of microflagellates with concentrations in the range 10^3 to $10^4\ ml^{-1}$ was observed.

With respect to bacteria, there are more data with which to compare the present findings; these are not at variance with numbers recorded by Ferguson and Rublee (1976), Hagström et al. (1979) or Meyer-Reil et al. (1979). The present findings are also not inconsistent with previous enumerations of planktonic ciliates (Rassoulzadegan, 1977; Hargraves, 1981). My counts of pigmented nanoplankton lie within the ranges reported by Throndsen (1969), Chretiennot (1974), and Ibanez and Rassoulzadegan (1977).

The importance of the heterotrophic microflagellates as consumers of bacteria may be assessed by multiplying their numbers in nature with experimentally determined values of clearance. In a previous paper (Fenchel, 1982b) it was shown that 6 different species may clear about 10^5 (5×10^4 to 10^6) times their own body volume of water h^{-1} or from 2×10^{-6} (for the smallest) to 8×10^{-5} (for the largest species) ml h^{-1} (20 °C). The amount of water filtered in the field was estimated in 2 ways: multiplying the average clearance per cell volume with the total flagellate volume estimated from the direct counts and by estimating the clearance for individual species. The 2 methods yield the same result: on the average 20 % (ranging from about 10 to 70 %) of the water of Limfjorden is filtered by microflagellates d^{-1}. As discussed above, this estimate is likely to represent a minimum value. The figures do impose a minimum growth rate for pelagic bacteria to doubling times in the range 30 to 200 h if there are no other mortality factors than grazing by flagellates, which, however, is not likely. There are very few estimates of bacterial growth rates in marine plankton. Hagström et al. (1979), based on the frequency of dividing bacteria, found that bacterial generation times in the Baltic Sea vary between 10 and 100 h, not totally inconsistent with the present findings.

Hagström et al. (1979), and Larsson and Hagström (1979) estimated that some 25 % of the primary production of the phytoplankton occurs as exudates which are utilized by bacteria. In the Limfjord, the phytoplankton production in late summer has been estimated to be about 7×10^{-5} g C l^{-1} 24 h^{-1} (Anonymous, 1976). The bacterial biomass (based on the present enumerations and a measured average volume of 0.22 μm^3) represents on the average 8×10^{-5} (5 to 10×10^{-5}) g C l^{-1}, so if 25 % of the production is chanelled through the bacteria and yield is about 50 %, this would only account for a doubling every 8 days or so. However, in the shallow Limfjorden, the phytoplankton accounts only for a part of the primary production and therefore does not represent the only source of dissolved organics available to bacteria. The heterotrophic microflagellates represented on the average about 3.7×10^{-5} g ww l^{-1} or about 6×10^{-6} g C l^{-1}. If their average mean consumption of the standing crop of bacteria is 20 % d^{-1} and if their gross growth efficiency is 35 % (Fenchel, 1982b), this would account for 1 doubling of the flagellates d^{-1}.

A characteristic feature of the findings is the apparently coupled cycling of bacterial and flagellate numbers. While it is strictly speaking not possible to sample the same water mass over time in the sea, there is little evidence to suggest that the findings represent large-scale spatial patchiness. The turnover of the water in the Limfjord is (mainly due to a net flow of water from the North Sea to the Kattegat) only about 75 % y^{-1}. Although the hydrography of the studied section of the fjord (Løgstør Bredning) is not known in detail, measurements of net flow and tidal flow at the rather narrow entrances to this section show that for all practical purposes it was the same water mass sampled during the study period (Anonymous, 1976). The fact that bacterial and flagellate numbers remained rather constant along a 6 km stretch (a distance much longer than the tidal movements in the system) also suggests that the patterns observed represent a temporal succession of events with a frequency of some 16 to 18 d.

One may ask whether microflagellate grazing and subsequent starvation can account for this behaviour when experimentally determined parameter values and functional forms of the prey/predator system are

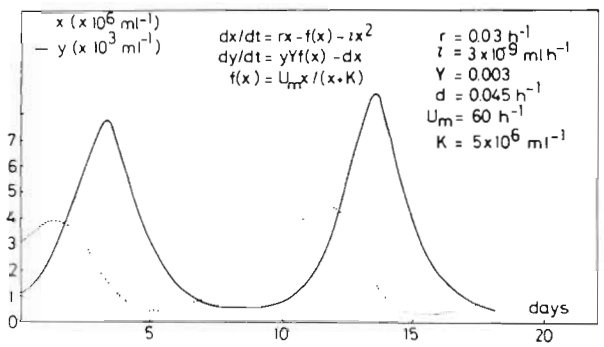

Fig. 6. Numerical simulation of a model of interaction between bacteria and microflagellates. For explanation see text

taken into account. An attempt to answer this is shown in Fig. 6. Here it is assumed that the flagellates, y, have a maximum consumption rate of 60 bacteria (x) h^{-1}, a gross growth efficiency of 3×10^{-3} flagellates per bacterium, and that the functional response can be described by a Michaelis-Menten type equation with a half-saturation constant of 5×10^6 bacteria ml^{-1}. All these values and relationships are supported experimentally (Fenchel, 1982b). The following, on the other hand, only represents educated guesses. The bacterial population is assumed to grow logistically with a growth rate, $r = 0.03\ h^{-1}$ and a carrying capacity, $K = 10^7\ ml^{-1}$ ($r/K = \iota = 3 \times 10^{-9}$). Finally a density independent death rate, $d = 0.045\ h^{-1}$ is assumed for the flagellates. A simulation run of the coupled differential equations initiated arbitrarily with 10^6 bacteria and 3×10^3 flagellates ml^{-1} is shown in Fig. 6. It would be an easy, if a rather meaningless pasttime, to adjust the parameter values to give a very close fit to the observed data. The important point, however, is that the experimentally determined growth and consumption rates of the flagellates may account for such prey/predator cycles in the field with frequencies in the range of 1 to 3 wk and with the predators lagging about ¼ cycle behind the prey. This result is quite robust to variation in the guessed parameter values (within reasonable limits) which mainly leads to changes in amplitude.

Since sufficiently frequent sampling has rarely been carried out, there are few investigations with which to compare the present findings. Ibanez and Rassoulzadegan (1977) found by spectral analysis of plankton data from the Mediterranean Sea that ciliates and (pigmented and non-pigmented) flagellates cycle with a frequency of about 30 d. They concluded that this must be explained as an example of a prey/predator relationship. Sorokin (1977) described from the Japan Sea how a peak in primary production was followed by a peak in bacterial biomass which again stimulated protozoan growth leading to a decline in bacterial numbers. The present data give no information on how the cycles are initially generated. The functional form of the equations in Fig. 6 yields stable-limit cycles (Christiansen and Fenchel, 1977) so the system might show an inherent tendency for cyclic behaviour even if constant bacterial productivity is assumed. However, it is most likely that the cycles were initiated by a summer peak in primary production and that the amplitudes tend to decrease over time.

Several recent studies (e.g. Hagström et al., 1979; Meyer-Reil et al., 1979) have shown that bacteria represent a dynamic component of marine plankton. The present investigation suggests how bacteria are removed and explain the relatively constant and low bacterial concentration usually found in seawater. The study also suggests that the heterotrophic microflagellates represent the 'missing link' between bacteria and larger suspension feeders (ciliates, microcrustaceans, etc.) in pelagic food chains.

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