THE ECOLOGY AND FEEDING BEHAVIOUR OF HETEROTROPHIC MICROFLAGELLATES

by

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ABSTRACT

This thesis investigates the ecology and feeding behaviour of heterotrophic microflagellates. These organisms are considered to be a major link in the 'microbial loop', which cycles the nutrients and energy of dissolved organic matter to the conventional phytoplankton-herbivore-fish food chain in aquatic ecosystems.

Microflagellates in the water column of Howe Sound, British Columbia were studied over eighteen months to investigate factors important in determining their distribution and abundance. These vary with season. In spring, abundance is linked to the distribution and availability of their prey. While prey are available in summer, microflagellate populations are depressed, possibly due to rapid grazing by predators. In winter microflagellates seem limited by temperature.

In spring, microflagellates graze mainly free bacteria in the photic zone and aggregated bacteria in the aphotic zone. Some autofluorescent microflagellates in the photic zone in summer appear to function heterotrophically and graze bacteria. In the aphotic zone heterotrophic microflagellates appear to graze small autofluorescent organisms and bacteria. This has consequences for the passage of carbon and energy through microflagellates. The type and spatial distribution of prey may influence which microflagellate species are successful.

The ability of two microflagellates to graze free and aggregated bacteria was investigated in a laboratory study. Bodo sp. selectively grazes aggregated bacteria and Paraphysomonas sp. selectively grazes free bacteria. Spatial distribution of bacteria clearly plays a role in determining which microflagellate species predominate and also in determining the effectiveness with which the bacterial prey are captured.

The advantage conferred by a chemosensory mechanism has been estimated using a model. These calculations demonstrate that a chemosensory mechanism is important and sometimes essential for microflagellate survival in environments where prey are scarce. While not essential for survival in environments where prey are relatively abundant, such a mechanism confers a large advantage. It allows more energy to be diverted to growth and reproduction. Due to the advantage conferred, chemosensory mechanisms are probably widespread among microflagellates.

Evidence is presented that a heterotrophic microflagellate, Pseudobodo tremulans, has a chemosensory prey-finding mechanism.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

Heterotrophic microflagellates are a diverse group of protozoa generally defined by their possession of flagella and their small size (3–10 um in largest dimension, Fenchel 1982a, Sherr and Sherr 1983; 2–20 um, Sieburth et al. 1978). These small organisms have been studied independently by both botanists and zoologists and consequently some confusion exists with respect to nomenclature. The revised classification of Levine et al. (1980) will be used here. Groups of photosynthetic microflagellates, incorporating those colourless microflagellates which have close relationships with these groups, are placed in the Phytomastigophorea class e.g. Chrysomonadida and Cryptomonadida (Levine et al. 1980). Other colourless microflagellates which do not have features indicating a close relationship with any algal group are placed in the class Zoomastigophorea e.g. Choanoflagellida, Bicoecida and Kinetoplastida (Levine et al. 1980). Further confusion with respect to nomenclature arises from the many different common names used in the literature i.e. 'microflagellate' (e.g. Fenchel 1982a–d), 'nanoflagellate' (e.g. Estep et al. 1986) 'nanoplankton' (e.g. Davis and Sieburth 1982). In view of the small size of these protozoa there does seem to be justification for a separate term for this size fraction of flagellates. Nanoplankton is a general term and includes many organisms that are not microflagellates e.g. small ciliates (Sherr et al. 1986). The term microflagellate is widespread in the literature and therefore will be used here. However it should be noted that the prefix nano- may well be more accurate in describing the size of these organisms (Committee on terms and equivalents 1957, Dussart 1965).

The abundance of microflagellates

The abundance of heterotrophic microflagellates in a variety of aquatic environments (e.g. Lighthart 1969, Sieburth and Davis 1982, Davis and Sieburth 1984) together with their capacity for rapid growth (Fenchel 1982b) , potential for bacterial consumption (Haas and Webb 1979, Fenchel 1982c) and mineralization of nutrients (Fenchel and Harrison 1976, Goldman et al. 1985) indicates a central role for microflagellates in the functioning of the marine microbial ecosystem. It is clearly important to learn more about these small organisms to fully elucidate their contribution to the microbial food web.

First observed in the early 1900s (e.g. Lohrman 1911), microflagellates have since been generally overlooked, largely due to sampling methods (plankton nets with a mesh size 200 um or greater were commonly used). When investigators did consider them it was commonly assumed all microflagellates in the nanoplankton were autotrophic (e.g. Ballantine 1953, Pratt 1959). As new techniques for sampling, enumeration, and determination of grazing rates have been developed (Sherr and Sherr 1983), an important role for microflagellates in the marine food web has been revealed.

The role of microflagellates

Numerous reports have been made cataloging the abundance of microflagellates in a variety of marine environments (see review by Sherr and Sherr 1984). With the exception of Davis and Sieburth (1982) and Davis et al. (1985), little effort has been made to determine the factors responsible for the distribution of microflagellates. To evaluate the role of microflagellates it is

necessary not only to quantify them in their environment but also to gain information about the flow of carbon, inorganic nutrients and energy through these organisms. Investigation of the energy flow can be approached in two ways; (1) where is the energy coming from and going to? (i.e. what does each organism feed upon and what grazes it?) and (2) how fast is this energy utilized by microflagellates? (i.e. how fast do microflagellates grow?).

The maximum predation rate of microflagellates on non-growing bacteria has been reported to range between 25-254 bacteria microflagellate^{-1} hour^{-1} (Fenchel 1982b, Daggett and Nerad 1982). The predation rate also varies with the bacterial prey species (Sherr et al. 1983). None of these workers considered the possibility of bacterial cryptic growth i.e. growth by bacteria on by-products or degradation products of themselves or microflagellates. This suggests that the above estimates of predation rate are probably conservative. Some attempts have been made to calculate predation rates using growing bacteria (Davis and Sieburth 1984, Kopylov and Moiseev 1980). The results obtained were in the same range as those obtained previously, however these latter workers assumed that the bacteria would grow equally well in both the presence and absence of microflagellates. Evidence indicates this is not true (Sherr et al. 1983).

Many of the problems associated with understanding the passage of materials and energy through heterotrophic microflagellate populations arise from the lack of good methods that can be directly applied to assessing microflagellate productivity and grazing rates in the natural environment. The methods used by Fenchel (1982b) cannot be directly applied to natural water samples. Various modifications have been developed. One approach is to filter a water sample to remove predators larger than microflagellates and observe the growth of microflagellates and bacteria (Landry and Hassett 1982). The extent and

rate of growth of bacteria can be compared to growth of bacteria in a control with the microflagellates filtered out. The difference in bacterial numbers is assumed to be due to microflagellate predation. Other approaches using bacterial inhibitors (Newell et al. 1984), radioactively labelled bacteria (Rieman 1985) and fluorescent beads (McMannus and Fuhrman 1986) have also been proposed. Unfortunately all these methods incur problems due to 'bottle effects' (Zobell 1943). The filtration methods and the inhibitor methods may affect the activity of the populations e.g. filtration of bacteria has been noted to cause increased respiration (Ferguson and Sunda 1984). Both the labelled bacteria and the fluorescent bead methods assume there is no selection of prey by microflagellates. Using the data available Fenchel (1982b) calculated that 10 to 70% of the water column is filtered of bacteria per day and that microflagellates can potentially consume 40-60% of bacterial production.

As a result of these investigations a central role has been proposed for microflagellates in the microbial loop (Azam et al. 1983). Microflagellates may be a 'link' (Pomeroy 1974, Azam et al. 1983) that returns the carbon and energy trapped in bacterial biomass to higher trophic levels. The carbon and energy trapped in the microflagellates is passed on to the conventional food chain by the consumption of these protozoa by larger planktivores i.e. ciliates, copepods and tintinnids (Kopylov et al. 1981, Caron 1984, Nisbet 1984).

The role proposed for microflagellates is as the main grazers of free bacterioplankton (Fenchel 1984). In spite of frequent reports of significant numbers of microflagellates associated (Fenchel and Harrison 1976, Linley et al. 1981, Caron et al. 1982, Davis and Sieburth 1984), the relative importance of aggregated bacteria as a food source for microflagellates remains to be assessed. Goldman (1984) proposed a major role for microflagellates in

association with microaggregates in his 'spinning wheel' hypothesis. Although difficult to test this hypothesis has stimulated much thought and research in the field of marine microbial ecology.

A second noteworthy role for microflagellates, as mineralizers of the nutrients locked in the biomass of small organisms, is now emerging (Sherr et al. 1983, Caron et al. 1985, Goldman and Caron 1985, Goldman et al. 1985). Previously it was not clear whether protozoa stimulated increased mineralization by bacteria or were directly involved in mineralization (Johannes 1965, Barsdate et al. 1974, Fenchel and Harrison 1976). The importance of heterotrophic microflagellates in the recycling of inorganic nitrogen from grazed bacteria is now well established (Goldman et al. 1985) and evidence indicates these organisms may be important in phosphorus cycling as well (Andersen et al. 1986).

Both of these major roles of microflagellates i.e. passage of carbon and energy locked in the biomass of bacteria to higher trophic levels and mineralizing of compounds, centre around their feeding. While some workers consider heterotrophic microflagellates to be osmotrophic (Beers et al. 1980, Kopylov and Moiseev 1980), living on dissolved organic compounds, surface area to volume ratios indicate this is unlikely because bacteria can easily outcompete larger organisms for organic molecules at the low concentrations found in the water column. (Sieburth et al. 1978, Azam et al., 1983). Other workers consider microflagellates to be phagotrophic, feeding primarily on bacteria (Fenchel 1982d, Haas and Webb 1979). Most of the support for this latter view comes from laboratory studies and thus indicates that bacteria are acceptable prey. These observations do not necessarily reflect the natural food choice. Further support comes from microscope studies of microflagellates, both isolated from the

natural environment and also in culture, that have revealed the presence of bacteria in their food vacuoles (Leadbeater and Morton 1974, Sieburth and Davis 1982, Sherr and Sherr 1983). Theoretical considerations led Fenchel (1984) to suggest only the smallest suspension feeders could effectively use bacterioplankton in situ. Sheldon et al. (1972) suggested that the optimum length difference between predator and prey in the marine food web is one order of magnitude. Heterotrophic microflagellates are therefore ideally suited as grazers of bacteria. It is noteworthy that heterotrophic microflagellates have been observed ingesting objects several times as large as themselves (Suttle et al. 1986) thus this theory of the predator prey ratio may not hold for all situations. More recent evidence (e.g. Goldman and Caron 1985, Goldman et al. 1985) indicates some microflagellates can graze small autotrophs successfully. Again this work was done in laboratory culture and does not necessarily reflect the natural food choice of these protozoa.

To summarize, current knowledge of flagellate nutrition suggests microflagellates are predominantly phagotrophic. Some microflagellates graze bacteria, some graze small autotrophic organisms, and some, perhaps most, are omnivorous. The preferred food of the microflagellate may vary with the habitat, the environmental conditions and with the species. Specific questions about how these organisms locate their prey and how this might influence their distribution remain to be addressed. Since food quality and the ability of microflagellates to recognize and use food play a part in the transformation of energy and carbon flow in food webs, it is clearly important to learn more about microflagellate behaviour and feeding strategies.

The questions of how microflagellate feeding strategies and behaviour affect their distribution and abundance are addressed in this thesis. This investigation

begins with a field study of a British Columbian fjord-sound, Howe Sound. Physical, chemical and biological variables were monitored to gain insight into what regulates microflagellate populations in nature. This is followed with a consideration of microflagellate feeding strategy and behaviour, which may influence their distribution. In particular, microflagellate grazing of aggregated bacteria, the advantages conferred by possession of a chemosensory mechanism (with respect to prey location), and possible microflagellate possession of such sensory abilities are all investigated. Both theoretical and experimental approaches are employed.

CHAPTER I

MARINE MICROFLAGELLATE ABUNDANCE AND DISTRIBUTION IN A TEMPERATE COASTAL FJORD

INTRODUCTION

Autotrophic[1] and heterotrophic microflagellates are found in oceans, estuaries and fresh water bodies throughout the world (e.g. Hilliard 1971, Davis et al. 1978, Haas and Webb 1979, Sherr and Sherr 1983). Their abundance has become apparent as sampling and enumeration techniques have improved (Hobbie et al. 1977, Porter and Feig 1980, Davis and Sieburth 1982, Caron 1983, Sherr and Sherr 1983) Interest in these organisms has arisen due to the realization that microflagellates are important bacterial grazers (Fenchel 1982d, Sieburth and Davis 1982, Azam et al. 1983). In spite of many reports showing the quantitative importance of heterotrophic microflagellates in aquatic ecosystems the factors determining their distribution and abundance are not understood. Environmental studies have demonstrated correlations between the concentration of heterotrophic nanoplankton, a size class that includes microflagellates (and other organisms in the less than 20 um size class, Sieburth et al. 1978), and dissolved organic compounds, and the densities of picoplankton and autotrophic nanoplankton (Burney et al. 1981, Davis 1982, Linley et al. 1983). Few investigations of the distribution and abundance of heterotrophic microflagellates include considerations

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[1] The use of the term 'autotrophic' is widespread in the literature. It implies knowledge about the trophic mode of the organisms. The increased realization that many of these 'autotrophic' organisms also have the ability to function heterotrophically makes this term inaccurate (Estep et al. 1986). Identification of an organism as 'autotrophic' merely by the presence of chlorophyll is not strictly correct. Therefore in this thesis microflagellates with chlorophyll as identified by epifluorescent microscopy alone will be termed 'autofluorescent'. This term refers to the fact that the microflagellates contain chlorophyll but makes no assumptions concerning the trophic mode of the organism.

of temperature, depth, time and other physical, chemical and biological parameters. In addition, most studies have inevitably been restricted to relatively short time periods, usually constrained by the length of the cruise or the availability of personnel, etc. Consequently there is a need for a continuous longer term study of microflagellates, their prey and other biological, chemical and physical factors that may affect their growth and reproduction.

Autotrophic microflagellates in marine waters have previously been considered in the context of the nanoplankton size range which includes many organisms in addition to microflagellates (Ballantine 1953, Beers et al. 1975). Until the advent of epifluorescent microscopy and fluorescent staining techniques it was difficult to distinguish between microflagellates with and without chlorophyll. This is reflected in some unusually high observations of heterotrophic or 'autotrophic' microflagellate densities e.g. Sorokin 1977 and 1979. The ability of some chlorophyll containing microflagellates to also function in a heterotrophic manner and graze bacteria has been generally overlooked in investigations of the marine environment. The fact that many phytoflagellates are phagotrophic and that some can grow in the dark with bacteria as their sole carbon source has recently been emphasized (Porter et al. 1985, Estep et al. 1986). Information concerning the standing stocks of microflagellates, both with and without chlorophyll, in natural environments with respect to the abundance and growth rate of their bacterial prey is necessary to assess the potential passage of carbon and energy through this trophic step.

The spatial distribution of bacteria has not previously been investigated in any field study of the interactions of microflagellate numbers with their bacteria prey. It is likely that the bacterial prey are distributed in a complex manner in nature and this may affect their availability to grazing microflagellates. Thus, in

addition to the abundance and growth rate of bacteria, it is important to investigate the distribution of the bacterial food resource to assess its availability to microflagellates.

Phytoplankton represent an alternative potential prey source for microflagellates. This has been demonstrated in laboratory studies where, the microflagellate, Paraphysomonas, has been shown to grow well with Phaeodactylum tricornutum as a prey organism (Goldman and Caron 1985). Therefore any study of the passage of carbon and energy through microflagellates should assess the potential phytoplankton prey present. Although many studies have investigated the standing stock of phytoplankton in natural environments with respect to its effect on bacterial growth, (e.g. Straskrabova and Komavkova 1979, Bird and Kalff 1984, Iriberri et al. 1985) no environmental studies have included any consideration of phytoplankton as a potential source of prey for microflagellates.

It is not clear from past studies whether there are any significant seasonal differences in the factors effecting microflagellate distribution and abundance. If there are, they should be most obvious in temperate waters which experience significant seasonal variation. It is noteworthy that the bacterial prey of microflagellates have been demonstrated to display seasonal patterns (Straskrabova and Komavkova 1979, Iriberri et al. 1985, Albright and McCrae 1987). The most extensive study of microflagellate numbers and their bacterial prey over a period of several months was carried out by Davis et al. (1985) in Narragansett Bay. No significant correlation was found between numbers of bacteria and heterotrophic nanoplankton. They suggested this was due to interseasonal variation of the factors affecting microflagellate abundance. Seasonal analysis of data was not carried out to confirm this perhaps due to insufficient

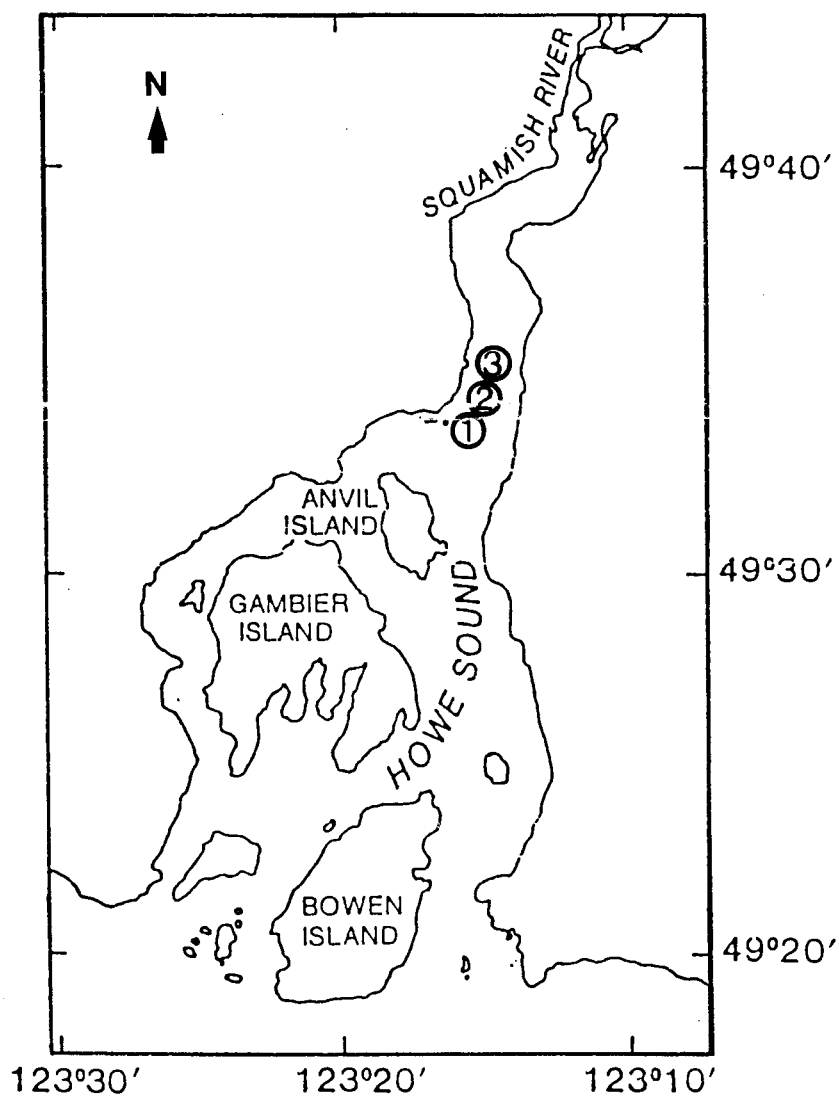
data.

Thus more continuous information about the long term spatial distribution and abundance of both pigmented and non-pigmented microflagellates and their potential prey with respect to physical factors is required to properly assess the role these organisms play in microbial food webs. Such data are also necessary as a base line for investigations of more complex interactions and to indicate the direction that future investigations should take.

The purpose of this study was to survey the abundance and vertical distribution of microflagellates during an 18 month period and to investigate factors which could be important in determining this distribution. The site chosen for this study was a temperate marine fjord-sound, Howe Sound, off the southern coast of British Columbia, Canada (Fig. 1.1). Two major reasons for selecting this site were that there is year round access to it, and its physical oceanography has been well studied (Carter 1932, 1934, Giovando 1972, Thomson 1981, Pickard 1961).

FIGURE 1.1.

A map showing the study site, Howe Sound, British Columbia. ①② and ③ are the sampling stations.



MATERIALS AND METHODS

The study site

Howe Sound, an inlet contiguous with the Strait of Georgia, is considered a combined fjord and coastal embayment/sound (Carter, 1934 Thomson, 1981). The total length of Howe Sound is ca. 45 km (Stockner et al. 1977) The fjord portion extends from Anvil Island north to the Squamish River (Fig.1.1) and is approximately 18 km long (Thomson 1981). At the southernmost end of the fjord a sill of between 30 and 70 m depth occurs. This results in an inner basin to the north with a maximum depth of approximately 290 m (Hoos and Vold 1975).

Currents in Howe Sound are a result of river runoff, tides, and winds. There is a relatively large runoff due to the Squamish river discharge. This runoff varies seasonally reaching a maximum discharge rate in early summer (due to snow melt) and is minimal in late winter (Hoos and Vold 1975). In the surface layers there is a net seaward movement of water and as a consequence the saltier oceanic water is entrained from below. Due to this there is a slow inward drift in deeper waters (Hoos and Packman 1974). The sill at the southern end of the fjord blocks the entrance and exit to the inner basin and hence the deeper waters in the fjord portion of Howe Sound tend to be poorly mixed (Mathews et al. 1966). North of the sill there is no deep anoxic region, typical of Norwegian type fjords, since although relatively poorly mixed in the deeper regions there is a biannual refreshing of the deeper waters by an overflow of oxygen rich water from the outer basin (Thomson 1981). These overflow events are apparently triggered by a combination of strong Squamish winds and abnormally high river discharge (Thomson 1981). Tides in Howe Sound are semi-diurnal and successive highs are unequal in magnitude and timing. Tidal

currents respond to the rise and fall of tides in the Strait of Georgia and are of relatively short time periods associated with tidal periodicity (Buchanan and Stockner 1976). Wind can also contribute to currents, however the thickness of the wind driven layer in Howe Sound is relatively small and hence volumetric transport is of little consequence (Buchanan and Stockner 1976). Thus the major driving force for the circulation pattern in Howe Sound and particularly in the fjord region is the Squamish river discharge.

There is some horizontal stratification of both temperature and salinity particularly in the surface waters due to the inflow of the fresh water from the Squamish river. The temperature in Howe Sound is low throughout the year due mainly to discharge of the snow fed Squamish river (Hoos and Vold 1975). Generally the temperature at all depths hovers around 10 °C but in the winter it is common for the surface waters to drop to around 5 °C (Thomson 1981).

Sampling

The sampling schedule was constrained by available boat and ship time. The three stations were chosen as they were all in the deepest area of the fjord and were suitably removed from the mouth of the Squamish river. Futhermore, another study involving these stations was already taking place and therefore the boat and ship time was available. Water samples were obtained from stations 1, 2 and 3 which were located at sites approximately 0.5, 1, and 1.5 km respectively north of the sill (Fig. 1.1). These samples were taken at 2-4 week intervals between March 1984 and October 1985. Samples from eight different depths (1, 5, 10, 20, 40, 75, 150, and 250 m) were obtained at each of these three sites using a clean Van Dorn sampler. For all assays data from each of the three stations were averaged in an attempt to obtain a better representative

value. Immediately upon retrieval the temperature and salinity of each sample were determined using a Y.S.I. salinometer model S-C-T 33.

The depth of 1% of the surface light was usually at 30m (Albright et al. 1986); hence the 0 to 30m water column was considered to be the photic zone. Thus the depths chosen to sample were equally distributed between the photic and the aphotic regions of the water column. Later observations of the level of chlorophyll a at the different depths indicated that the 40 m sample should be considered to be in the photic zone for some of the samples.

Assays

1. Bacterial numbers

Water samples to be assayed for a total bacteria count were preserved with 3.7% formaldehyde (final concentration) at the time of sampling. Immediately prior to assay each sample was treated with 0.001 M $Na_4P_2O_7$ (final concentration) for 30 min and then sonicated at 100 W for 20 s. This procedure facilitates detachment of bacteria from particulates and results in a random distribution of bacteria in the sample (Velji and Albright, 1986). After staining with acridine orange the samples were filtered onto 0.2 um pore sized, 25 mm diameter, Nuclepore membrane filters using a vacuum of less than 100 mm Hg, and the bacteria counted using epifluorescent microscopy (Daley and Hobbie 1975, Hobbie et al. 1977).

Numbers of free bacterial were determined by filtering a portion of the preserved but otherwise untreated water sample through a 2 um pore sized, 25 mm diameter Nuclepore filter. The bacteria in the filtrate were considered to be free and unattached and were stained and examined as described above.

Mean bacterial sizes were obtained using acridine orange stained samples and epifluorescent microscopy (Daley and Hobbie 1975, Hobbie et al. 1977). Calculated biovolumes (based on mean length and width of cells) were converted to bacterial carbon values as described by Valdes and Albright (1981).

2. Microflagellate numbers

Microflagellates were enumerated, using the preserved water samples, within 2-3 days of obtaining the sample. Ten ml portions were removed in triplicate from samples from each depth and each was processed by filtering the water onto 2 um pore sized, 25 mm diameter Nuclepore filters prestained with Irgalan black, at a vacuum of not more than 15 mm Hg, and staining the microflagellates with 4',6-diamidino-2-phenylindole (DAPI, Sigma). The filters were examined using an epifluorescent microscope. Microflagellates with chlorophyll emit orange fluorescence with longer wave length emissions. This is due to the presence of chlorophyll (Sherr and Sherr 1983). Therefore these cells with chlorophyll were identified as eucaryotic cells less than 10 um in diameter with flagella that emitted blue fluorescence with the filter set 48 77 01(BP 365/10, FT310 and LP395) and also emitted orange fluorescence when the Zeiss filter set 48 77 09 (BP 450-490, FT510 and LP520) was used. Heterotrophic microflagellates were identified as those eucaryotic cells less than 10 um in diameter which fluoresced blue when the Zeiss filter set 48 77 01(BP 365/10, FT310 and LP395) was used but had no autofluorescence due to their lack of chlorophyll. Total microflagellate numbers were taken to be the sum of these.

3. Bacterial production

Each bacterial production assay, as determined by Fuhrman and Azam (1980, 1982), was initiated within 30 min of sample retrieval. Two killed (3.7% formaldehyde - final concentration) and two active 10 ml portions of each water sample were each placed in a 30 ml syringe and treated with 15 nM

^{3}H-thymidine (methyl-^{3}H-thymidine, sp.act. 65 Ci mM^{-1}, New England Nuclear). The water samples were then incubated in the dark for 60 min at the same temperature as the sampled water. Incubations were stopped by addition of 10 ml of ice cold 10% TCA. Each sample was then filtered through a 0.22 um nominal pore size cellulose nitrate (Millipore) filter. Each filter was washed twice with 5 ml of ice cold TCA and then dissolved in 2 ml of ethyl acetate . When each filter was dissolved, 10 ml of Scintiverse II (Fisher Sci.) fluor was added and the contents were radioassayed using a Beckman LS8000 scintillation spectrometer. Quench corrections were by the channels ratio method. The factor used to convert rate of thymidine uptake to bacterial production rate was 1.2 x 10^{18} (see Fuhrman and Azam 1982). Albright and McCrae (1987), using water obtained from the study site in Howe Sound, noted that substantially less of the radioactive label in the TCA precipitated macromolecules enters the DNA in the samples removed from deeper water as compared to the samples removed from 1, 5, 10 and 20 m. The values of Albright and McCrae (1987) were used in this study since they had been calculated using water from Howe Sound. Thus all productivity values were calculated based upon 80% of the label entering the DNA in samples removed from 1, 5, 10, and 20 m samples and 35% in the samples removed from 40 m and deeper. This differs from the experiments of Fuhrman and Azam where a value of 80% was used for all samples.

4. Chlorophyll a assay for phytoplankton standing stock.

The chlorophyll a content of each sample was determined by filtering 1 litre water portions through Whatman GF/C glass fibre filters (nominal pore size of 1.2 um) with a small amount of $MgCO_3$ added to prevent acidification. All filters were then frozen at -15 °C until chlorophyll a was analysed (within 1 week) spectrophotometrically following acetone extraction. Extinction values were used to calculate chlorophyll a concentrations by the equation of Strickland and

Parsons (1972).

5. Statistical analysis

An analysis of the data set was carried out using the statistical package MIDAS (1976). The data were arranged in an n x m matrix where n represents the columns (parameters measured) and m represents the rows (sampling dates). Twelve different parameters were measured on 26 different days (12 x 26 matrix). The complete data set is given in Appendix 1. Summary description statistics of all the parameters measured are given in Table 1.1. The following approach was used to analyse the data:

1. The complete data set was analysed to make maximal use of all the information collected.
2. Photic and aphotic zone data were analysed separately to investigate any effects that might occur exclusively in one or the other zone.
3. An investigation of possible time-phase displacements in the data was performed.
4. The existence of a seasonal pattern in the data was investigated.
5. The total data and that for the photic and aphotic zones was re-examined on a seasonal basis.

Any possible time dependence of the data was ignored and the sampling dates were treated as different stations. This was justified by the facts that the investigation was of microbial populations which have relatively high turnover rates and that the samples were generally taken sufficiently far apart with respect to time for the population at time 1 to have no direct effect on the population at time 2.

TABLE 1.1

SUMMARY STATISTICS FOR THE VARIABLES MONITORED IN HOWE SOUND BETWEEN MARCH 1984 AND OCTOBER 1985

VARIABLE	N	MINIMUM	MAXIMUM	MEAN
DAY	208	1.0000	530.00	228.88
DEPTH	208	1.0000	250.00	68.97
SALINITY	176	4.5000	31.30	27.19
Chl a	200	0.2000	10.41	1.50
TEMP.	192	4.7000	18.50	9.29
BACT. PROD.	192	1.9000	18.50	7.38
BIOMASS	184	0.4900	2.27	1.02
TOT. MF	208	0.0000	19.70	1.49
AMF	133	0.0000	8.20	0.49
HMF	133	0.0000	9.66	1.12
BACT. NO.	192	2.800	20.70	7.07
ATT.BACT. NO.	168	0.0000	1097.10	66.31

TOT MF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

All the variates under consideration are dependent and therefore calculations of linear regressions are inappropriate. To investigate relationships between parameters measured, pairwise correlation coefficients were calculated. Since there was little evidence that any of the parameters were normally distributed these correlations were carried out using the non-parametric Spearman rank correlation technique. The correlations were accepted as significant when $P<0.05$ and as highly significant when $P<0.01$. The many significant relationships evident on Table 1.2 (total data) indicate many of the parameters are interacting in a complex fashion. Partial correlation coefficients were calculated in some cases to help elucidate the relationships among descriptors (Table 1.15). Partial correlations measure the degree of relationship between two variables by eliminating other variables which may simultaneously influence the relationship between the ones under study. In spite of its obvious applications for studies of this type, partial correlation analysis is uncommon in the literature.

The possible existence of phase displacements, quite common among biological data (Bolter et al. 1980), was investigated by examining the data visually (Figs. 1.2-1.10) and also by performing correlations using lagged parameters (Table 1.5).

Cluster analysis was carried out to investigate whether seasonal patterns existed in the data set. The Spearman rank correlation coefficient was employed as a measure of similarity. A complete linkage clustering method was used because this method refers to strong grouping i.e. all members of a cluster show at least the similarity (correlation coefficient) of a given threshold ($p<0.05$). Further stability of the groups was tested using the average linkage and single linkage procedures.

RESULTS

(1) Analysis of the complete data set.

Total microflagellate numbers are significantly negatively correlated with depth and salinity and positively correlated with chlorophyll a, and bacterial numbers (Table 1.2). Three way partial correlations indicate the only direct correlation is with depth (Table 1.15). Autofluorescent microflagellate numbers are similarly negatively correlated with depth and salinity and positively correlated with bacterial production, chlorophyll a, and bacterial concentration (Table 1.2). Partial correlations show depth to be the common factor in the correlations of autofluorescent microflagellates with bacterial parameters. (Table 1.15). Depth, salinity, chlorophyll a, bacterial biomass, and bacterial production are all significantly correlated with heterotrophic microflagellate numbers (Table 1.2). However 3-way partial correlations again show that depth is the only direct correlation (Table 1.15).

Bacterial concentration is significantly correlated with several parameters but it can be shown using partial correlations that depth, bacterial productivity and chlorophyll a are the direct correlations (Table 1.2).

Thus in spite of the many significant correlations apparent in Table 1.2 partial correlations demonstrate that depth is the most significant of all the parameters measured.

(2) Analysis of photic and aphotic zone data

When the data are partitioned into photic and aphotic zones and re-analysed there are fewer significant relationships between parameters. To some extent this is due to the reduction in the number of cases considered. The significant

TABLE 1.2, SPEARMAN RANK PAIRWISE CORRELATIONS FOR ALL THE DATA.

	Depth	Salinity	Chl a	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.8569** n=176	1.0000									
Chla	-0.4778** n=200	-0.2725* n=168	1.0000								
Temp.	-0.2067** n=192	-0.1018 n=170	0.5309** n=184	1.0000							
Bact.Prod.	-0.4759** n=192	-0.3603** n=160	0.6126** n=192	0.3199** n=176	1.0000						
Bact.Carbon.	-0.1772* n=192	-0.0804 n=176	0.3874** n=176	0.4440** n=176	0.4022** n=168	1.0000					
TMF	-0.3834** n=208	-0.3046** n=176	0.2727** n=200	0.0062 n=192	0.1776 n=192	0.1004 n=192	1.0000				
AMF	-0.6067** n=133	-0.5063** n=101	0.4088** n=133	0.1139 n=117	0.3651** n=133	0.1712 n=109	0.5822** n=133	1.0000			
HMF	-0.3664** n=133	-0.2929** n=101	0.1723* n=133	0.0009 n=117	0.1776* n=50	0.2546** n=109	0.5700** n=133	0.3514 n=132	1.0000		
Bact.Nos.	-0.5009** n=192	-0.3926** n=160	0.5427** n=192	**0.1803* n=176	0.7869** n=184	0.0048 n=168	0.2559** n=192	0.3101** n=133	**0.0846 n=133	1.0000	
Agg.Bact.Nos.	-0.1946 n=168	-0.1810 n=159	0.2310 n=168	0.0078 n=159	0.1270 n=160	0.1653 n=167	-0.0236 n=167	0.0212 n=109	0.0214 n=110	0.0861 n=168	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

TABLE 1.3. SPEARMAN RANK PAIRWISE CORRELATIONS FOR ALL THE PHOTIC ZONE DATA.

	Depth	Salinity	Chl a	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.8494** n=110	1.0000									
Chla	-0.4696** n=110	-0.3418** n=105	1.0000								
Temp.	-0.2041 n=120	-0.1441 n=110	0.5431** n=115	1.0000							
Bact.Prod.	-0.3944** n=120	0.2927** n=100	0.6359** n=120	0.3417** n=110	1.0000						
Bact.Carbon.	0.1529 n=115	-0.0949 n=110	0.3259** n=110	0.3750** n=110	0.2788** n=105	1.0000					
TMF	-0.1478 n=130	-0.0771 n=110	0.1298 n=125	-0.2183* n=120	0.0140 n=120	0.0662 n=115	1.0000				
AMF	-0.3030** n=82	-0.2679* n=62	0.2198* n=82	0.0203 n=72	0.0549 n=82	0.1687 n=67	0.5786** n=82	1.0000			
HMF	-0.1153 n=83	-0.1546 n=63	0.0288 n=83	-0.1311 n=73	0.0603 n=83	0.2954* n=68	0.4056** n=83	0.2562* n=82	1.0000		
Bact.Nos.	-0.3978** n=120	0.3272** n=100	0.5618** n=120	0.2985** n=110	0.8249** n=115	-0.0676 n=105	0.0717 n=120	-0.0001 n=82	-0.1238 n=83	1.0000	
Agg.Bact.Nos.	-0.0391 n=105	0.0066 n=100	-0.0550 n=105	0.1298 n=100	0.0972 n=100	-0.0489 n=105	-0.0993 n=105	-0.1433 n=67	-0.1572 n=68	0.2132* n=105	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

correlation of total microflagellate numbers with depth and salinity that is apparent in the complete data set is not present when the data of the photic and aphotic zones are analysed separately (Tables 1.3 and 1.4). The only significant correlation for total microflagellate numbers in the photic zone is a negative correlation with temperature (Table 1.3). Correlations with depth and salinity are still apparent for autofluorescent microflagellate numbers but not for heterotrophic microflagellate numbers (Table 1.3). Partial correlations indicate depth is the probable cause of the relationship of autofluorescent microflagellate numbers with salinity (Table 1.15). The only parameter with which heterotrophic microflagellate numbers are significantly correlated in the photic zone, is bacterial biomass (Table 1.3).

Bacterial numbers in the photic zone are correlated with depth, salinity, bacterial production, chlorophyll a, temperature and numbers of attached or aggregated bacteria (Table 1.3). Partial correlations indicate the common influence is bacterial production and that temperature is the only direct correlation with bacterial production (Table 1.15). Chlorophyll a in the photic zone is significantly correlated with bacterial production, bacterial concentration and temperature all of which appear to be direct correlations (Table 1.3, Table 1.15).

There are far fewer significant correlations occurring between parameters in the aphotic zone. None exist for either total microflagellate numbers or heterotrophic microflagellate numbers (Table 1.4). Autofluorescent microflagellate numbers are correlated with depth and chlorophyll a (Table 1.4). A 3 way partial correlation shows the correlation between autofluorescent microflagellate numbers and chlorophyll a to be due to a common correlation with depth (Table 1.15).

TABLE 1.4, SPEARMAN RANK PAIRWISE CORRELATIONS FOR ALL THE APHOTIC ZONE DATA.

	Depth	Salinity	Chl a	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.2296 n=66	1.0000									
Chl a	-0.1437 n=75	0.6123** n=63	1.0000								
Temp.	-0.1011 n=72	0.2290 n=66	0.3907** n=69	1.0000							
Bact.Prod.	-0.0930 n=72	0.0241 n=60	0.3004* n=72	0.1571 n=62	1.0000						
Bact.Carbon.	0.0117 n=69	0.1928 n=66	0.4357** n=66	0.6103** n=66	0.5510** n=63	1.0000					
TMF	-0.1793 n=78	-0.0597 n=66	0.1777 n=75	-0.1214 n=72	0.0821 n=72	0.0491 n=69	1.0000				
AMF	-0.4005** n=51	-0.1175 n=39	0.3031* n=51	0.0465 n=45	0.2013 n=51	0.0662 n=42	0.3621* n=51	1.0000			
HMF	-0.2571 n=50	-0.0276 n=48	0.1573 n=50	-0.0240 n=44	-0.0114 n=50	0.1714 n=41	0.5821** n=50	0.2206 n=50	1.0000		
Bact.Nos.	-0.1813 n=72	0.1357 n=60	0.2341* n=72	-0.3180* n=66	0.5638** n=69	-0.0212 n=63	0.0997 n=72	0.1404 n=51	-0.0884 n=50	1.0000	
Agg.Bact.Nos.	-0.1704 n=63	-0.1354 n=59	-0.1251 n=63	-0.0309 n=59	0.1550 n=60	-0.0354 n=62	-0.0036 n=63	-0.0851 n=42	-0.1496 n=41	0.2949* n=63	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

Pairwise correlations indicate bacterial concentration is correlated with chlorophyll a, temperature and bacterial production in the aphotic zone. Partial correlations show the correlation with chlorophyll a is indirect, a consequence of the correlation of bacterial concentration with bacterial production and the temperature of the water column (Table 1.15). Bacterial production is significantly correlated with chlorophyll a, bacterial biomass and numbers (Table 1.4). Partial correlations show bacterial concentration and biomass to be the major correlations (Table 1.4). Bacterial biomass is significantly correlated with chlorophyll a, temperature and bacterial production (Table 1.4). Temperature and bacterial production are direct correlates (Table 1.15). Salinity, temperature, bacterial production, bacterial biomass, autofluorescent microflagellate numbers and bacterial numbers are all significantly correlated with chlorophyll a both with pairwise and partial correlations (Table 1.4, Table 1.15).

(3) Time-phase displacement investigation

Visual inspection indicated that there were time-phase displacements among some of the parameters particularly bacterial parameters and microflagellate populations (Figs. 1.2-1.10). However these displacements were not constant throughout the year. It should be noted that these figures are visual summaries of the data for the photic and aphotic regions of the water column. Each point for each parameter in the photic or aphotic zone is an average of the values obtained from each different station for every depth in the photic or aphotic zone. Consequently, particularly for the photic zone, the error associated with the calculation of each point is large. These errors are not shown on the figures since a more detailed lagged correlation analysis was carried out subsequently. Correlations between the microflagellate populations and bacterial parameters were maximal using a lag of five days (Table 1.5).

FIGURE 1.2. Average total, heterotrophic and autofluorescent microflagellates with respect to time in the photic zone

■ – Total microflagellate numbers

o – Heterotrophic microflagellate numbers

□ – Autofluorescent microflagellate numbers

FIGURE 1.3. Average total, heterotrophic and autofluorescent microflagellates with respect to time in the aphotic zone

■ – Total microflagellate numbers

o – Heterotrophic microflagellate numbers

□ – Autofluorescent microflagellate numbers

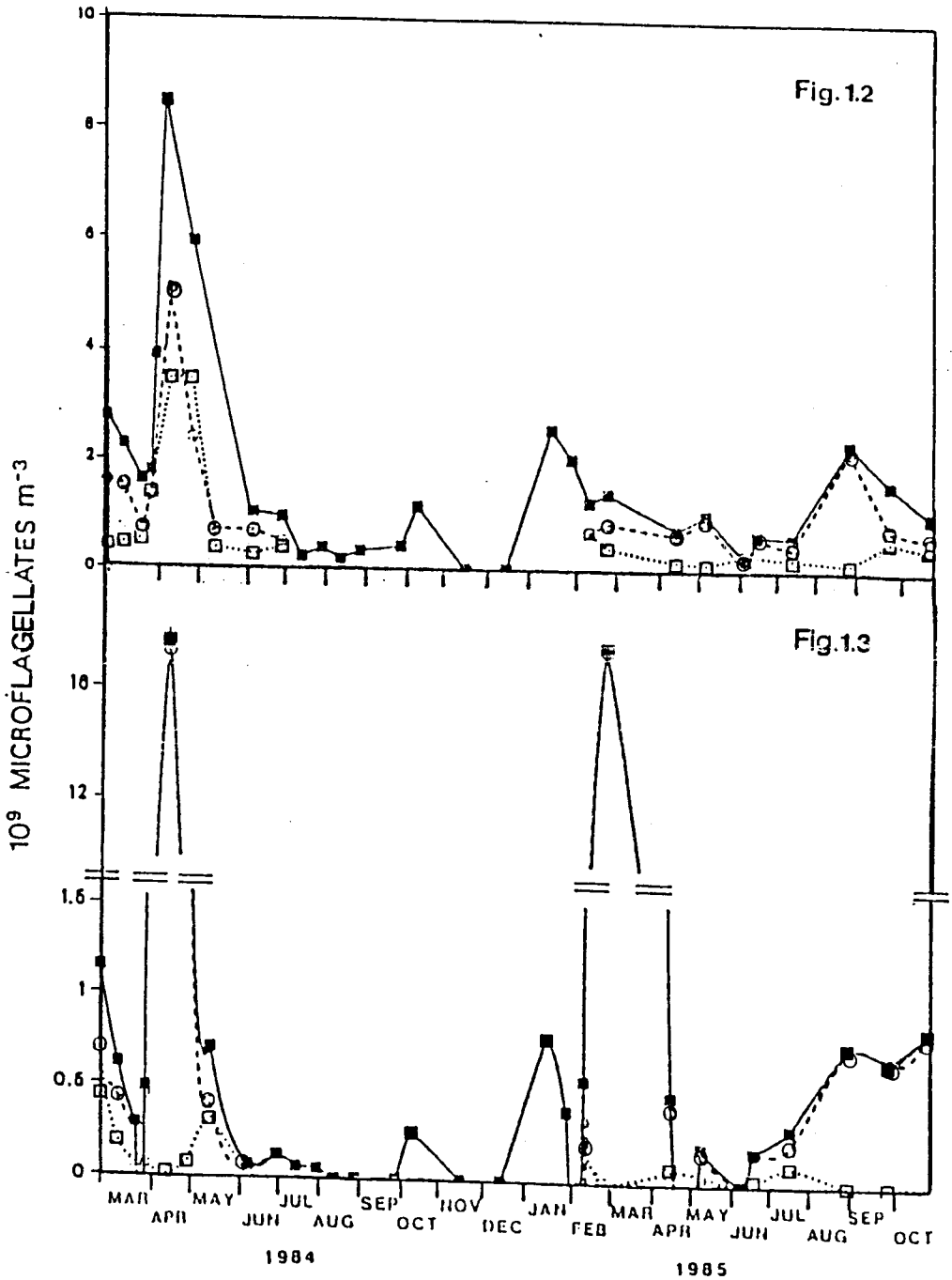


Fig.1.2
Fig.1.3
10^9 MICROFLAGELLATES m^{-3}
10
8
6
4
2
0
16
12
1.6
1
0.6
0
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
JAN
FEB
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
1984
1985

FIGURE 1.4. Average bacterial numbers in the photic and aphotic zones.

■–Bacterial numbers in photic zone

□–Bacterial numbers in aphotic zone

FIGURE 1.5. Average bacterial production in the photic and aphotic zones.

■–Bacterial production in photic zone

□–Bacterial production in the aphotic zone

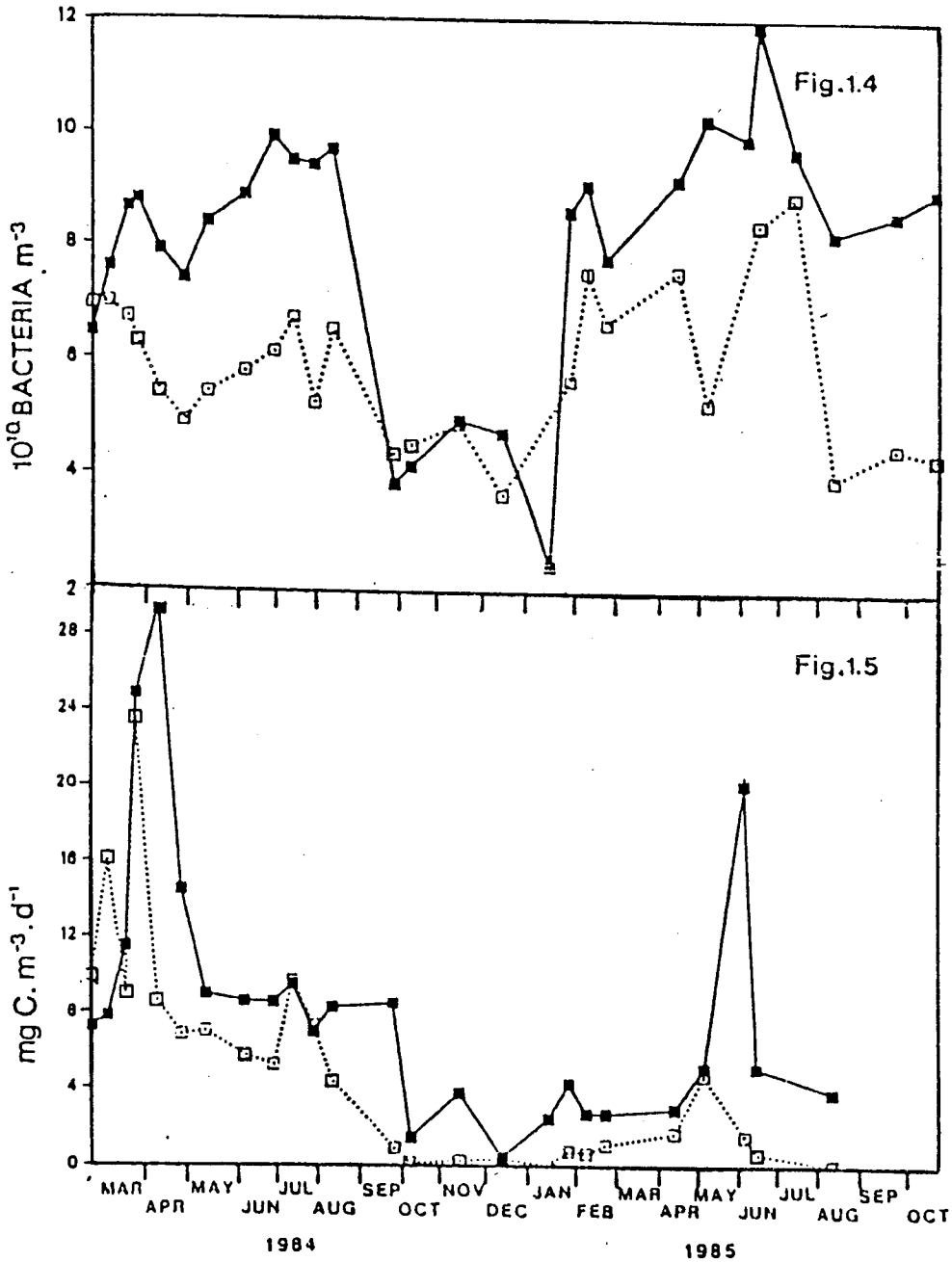


Fig.1.4
10^{10} BACTERIA m^{-3}
12
10
8
6
4
2
Fig.1.5
mg C. m^{-3}. d^{-1}
28
24
20
16
12
8
4
0
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
JAN
FEB
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
1984
1985

FIGURE 1.6. Average numbers of aggregated bacteria in the photic and aphotic zones.

■–Numbers of aggregated bacteria in photic zone

□–Numbers of aggregated bacteria in aphotic zone

FIGURE 1.7. Average bacterial biomass in the photic and aphotic zones.

■–Bacterial biomass in photic zone

□–Bacterial biomass in aphotic zone

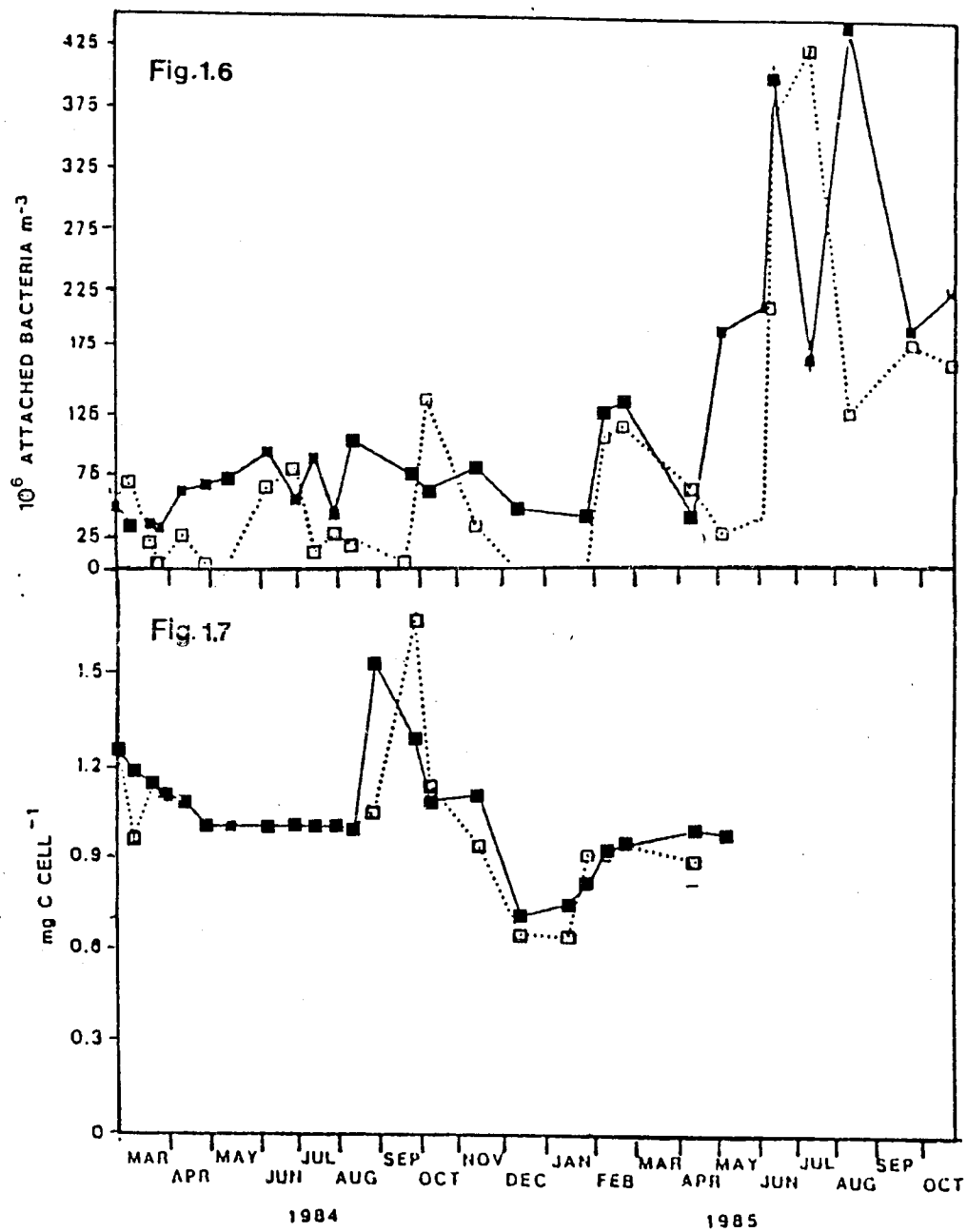


Fig.1.6
425
375
325
275
225
175
126
75
25
0
10^6 ATTACHED BACTERIA m^{-3}
Fig.1.7
1.5
1.2
0.9
0.6
0.3
0
mg C CELL $^{-1}$
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
JAN
FEB
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
1984
1985

FIGURE 1.8. Average chlorophyll a in the photic and aphotic zones.

■ - Chlorophyll a the in photic zone

□ - Chlorophyll a in the aphotic zone

FIGURE 1.9. Average temperature in the photic and aphotic zones.

■ - Temperature in the photic zone

□ - Temperature in the aphotic zone

FIGURE 1.10. Average salinity in the photic and aphotic zones.

■ - Salinity in the photic zone

□ - Salinity in the aphotic zone

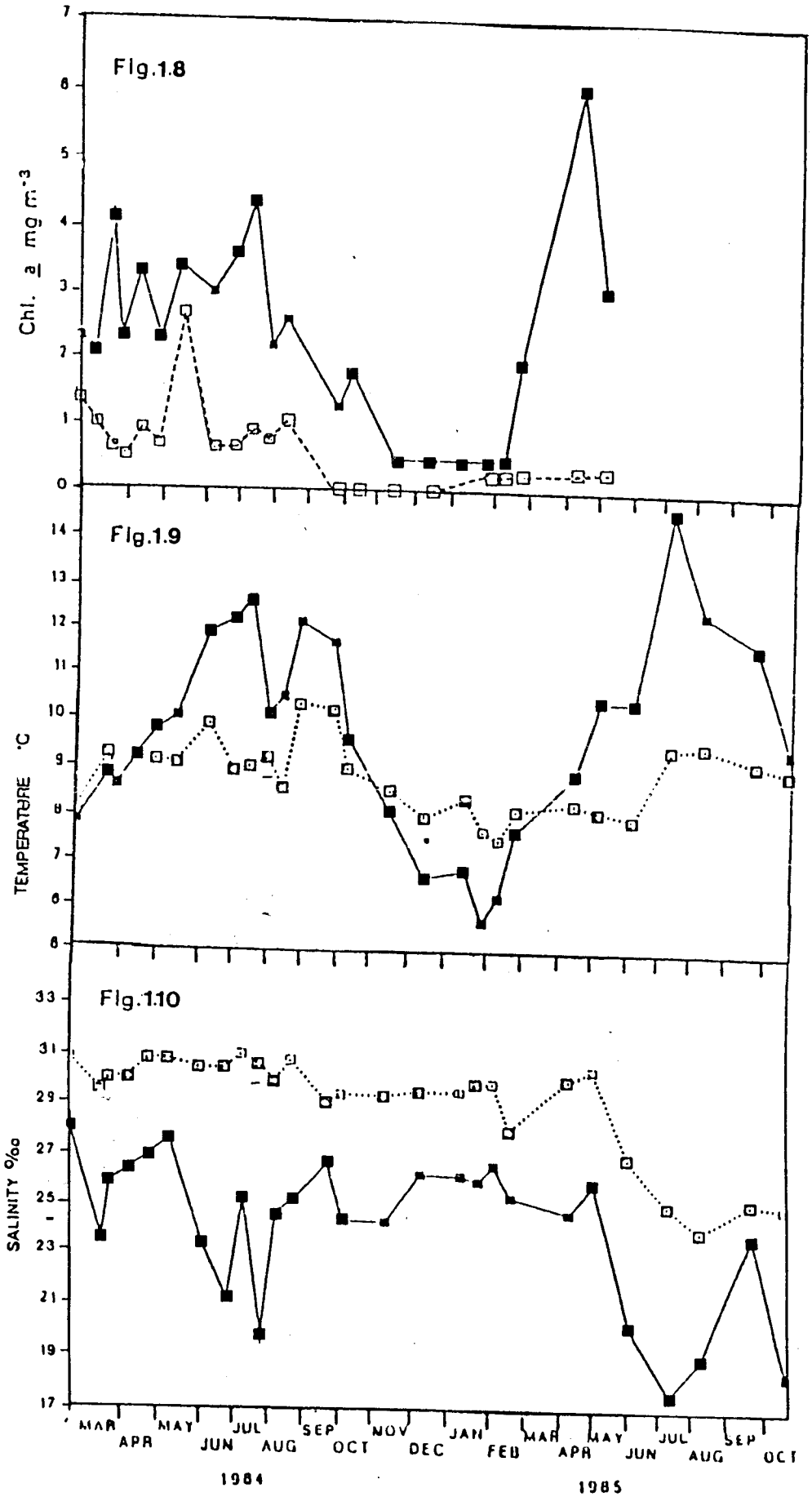


Fig.1.8
Chl. a mg m-3
Fig.1.9
TEMPERATURE °C
Fig.1.10
SALINITY ‰
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
NOV
DEC
JAN
FEB
MAR
APR
MAY
JUN
JUL
AUG
SEP
OCT
1984
1985

FIGURE 1.11. Cluster diagram of the sampling dates of parameters from the pelagic environment in Howe Sound.

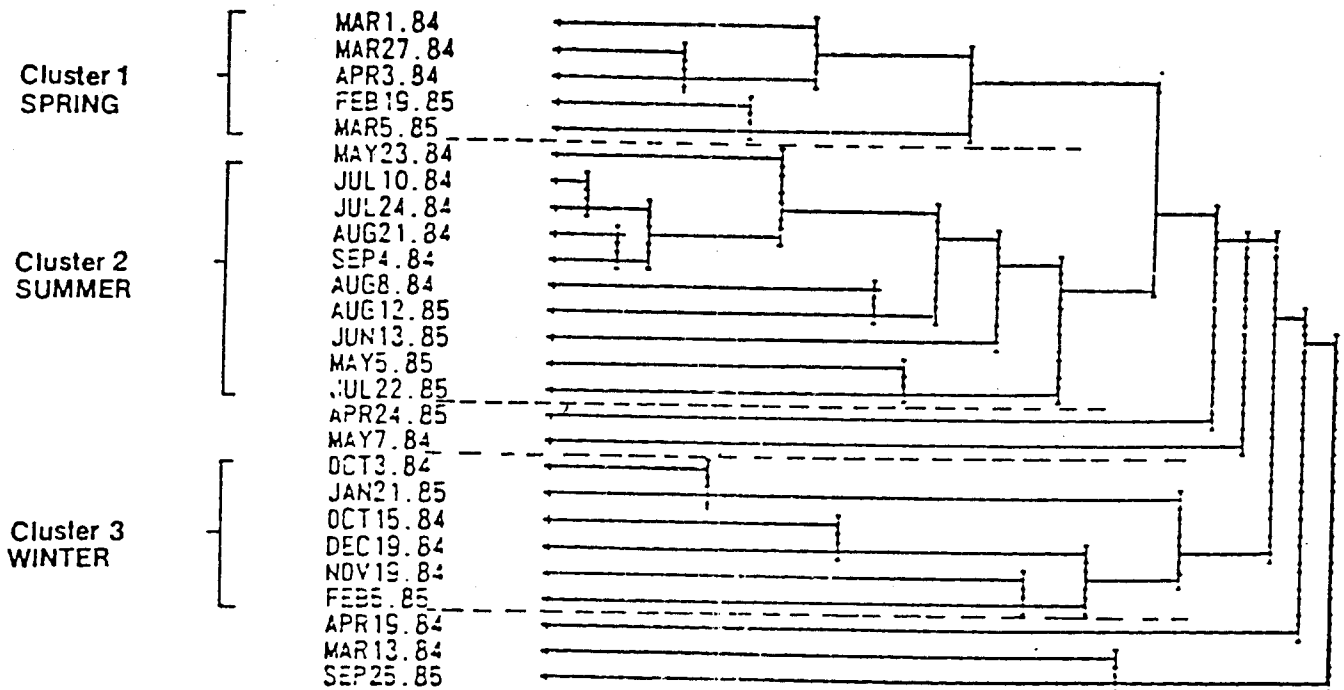


TABLE 1.5. LAGGED CORRELATIONS.

Non-parametric Spearman rank correlations of total heterotrophic and autofluorescent microflagellates, heterotrophic and autofluorescent microflagellates in the photic zone and heterotrophic and autofluorescent microflagellates in the aphotic zone with parameters of five days before.

	HMF	PHOTIC HMF	APHOTIC HMF	AMF	PHOTIC AMF	APHOTIC AMF
LAG (DAYS)	-5	-5	-5	-5	-5	-5
N	72	42	30	72	42	30
0.05	0.2326	0.3061	0.3640	0.2326	0.3061	0.3640
0.01	0.3057	0.4023	0.4783	0.3057	0.4023	0.4783
SALINITY	0.3032	NS	NS	0.3712	NS	NS
CHL a	-0.3329	NS	NS	NS	NS	NS
TEMP.	-0.2645	NS	-0.5455	NS	NS	NS
BACT. PROD.	NS	NS	NS	-0.2445	NS	NS
BIOMASS	NS	0.3579	NS	NS	0.2922	0.4480
BACT. NO.	-0.3547	NS	NS	-0.4380	NS	-0.4181
ATT. BACT. NO.	NS	NS	NS	NS	NS	NS

TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

(4) Cluster analysis

Cluster analysis revealed 3 groups corresponding to spring, summer and winter (Fig. 1.11). There are five anomalous dates that are not included in any cluster.

(5) Re-examination of the data on a seasonal basis.

The data from each of the three clusters were re-examined.

SPRING

In the spring (Table 1.6) total microflagellate, autofluorescent microflagellate and heterotrophic microflagellate numbers are all negatively correlated with depth and salinity and positively correlated with bacterial concentration. Autofluorescent microflagellate numbers are also positively correlated with chlorophyll a and bacterial production (Table 1.6). Heterotrophic microflagellate numbers are also correlated with attached bacteria (Table 1.6). Multiple partial correlations indicate that bacterial numbers and not depth are the primary correlation with total microflagellate numbers in this season (Table 1.15). The fact that this is not apparent for either autofluorescent microflagellate numbers or heterotrophic microflagellate numbers when analysed separately may be due to the reduced data sets. Partial correlation for heterotrophic microflagellate numbers with the above mentioned parameters (depth, salinity, and attached bacteria) reveal they are all direct correlations (Table 1.15). For autofluorescent microflagellate numbers however, partial correlations indicate the directly correlating parameters are depth, bacterial production and chlorophyll a (Table 1.15).

Chlorophyll a is correlated with depth, salinity, temperature, bacterial production, bacterial biomass, and autofluorescent microflagellate numbers (Table 1.6). Partial correlations show all of these except salinity and temperature to be

TABLE 1.6, SPEARMAN RANK PAIRWISE CORRELATIONS FOR ALL THE SPRING DATA

	Depth	Salinity	Chl a	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.7904** n=40	1.0000									
Chl a	-0.5387** n=40	-0.4353** n=40	1.0000								
Temp.	0.2274 n=40	0.0225 n=40	0.3864* n=40	1.0000							
Bact.Prod.	-0.3699* n=40	-0.2607 n=40	0.5138** n=40	0.4292** n=40	1.0000						
Bact.Carbon.	-0.0704 n=40	0.1100 n=40	0.5966** n=40	0.4503** n=40	0.5106** n=40	1.0000					
TMF	-0.6377** n=40	-0.6095** n=40	0.2492 n=40	-0.2175 n=40	0.2637 n=40	-0.1821 n=40	1.0000				
AMF	-0.7030** n=39	-0.4792** n=39	0.5011** n=39	-0.0313 n=39	0.5318** n=39	0.2375 n=39	0.4986** n=39	1.0000			
HMF	-0.5689** n=39	-0.4088* n=39	0.2445 n=39	-0.1321 n=39	0.2832 n=39	-0.1106 n=39	0.4416** n=39	0.3869* n=39	1.0000		
Bact.Nos.	-0.4674** n=40	-0.4645** n=40	0.1244 n=40	-0.1437 n=40	0.4948** n=40	-0.2630 n=40	0.5427 n=40	0.3454* n=40	0.4305** n=39	1.0000	
Agg.Bact.Nos.	0.0096 n=40	-0.0945 n=40	-0.2365 n=40	-0.1462 n=40	-0.0262 n=40	-0.2547 n=40	0.1926 n=40	-0.1619 n=39	0.1945 n=39	0.4025* n=40	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

direct (Table 1.15). Bacterial numbers are correlated with depth, salinity, bacterial production, total microflagellate numbers, autofluorescent microflagellate numbers, heterotrophic microflagellate numbers and numbers of attached bacteria (Table 1.6). Partial correlations indicate these are all direct correlations (Table 1.15).

When the photic and aphotic zones for the spring are considered separately the number of significant relationships are reduced, largely due to the reduced number of cases for analysis.

The photic zone- In the photic zone, depth and salinity are strongly correlated with each other and with heterotrophic microflagellate numbers (Table 1.7). Partial correlation indicates that depth is the common factor (Table 1.15). Autofluorescent microflagellate numbers decrease with increasing depth but not with salinity (Table 1.7). These small autofluorescent organisms are also correlated with chlorophyll a and bacterial production (Table 1.7). Partial correlations show bacterial production to be the common factor (Table 1.15). Total microflagellate numbers are correlated with heterotrophic microflagellate numbers but not autofluorescent microflagellate numbers (Table 1.7). Salinity and bacterial production are correlated with bacterial concentration (Table 1.7), and partial correlations demonstrate bacterial production to be direct (Table 1.15).

Chlorophyll a is correlated with several factors but multiple partial correlations indicate the underlying influences are depth and temperature (Table 1.15). Bacterial biomass is correlated with chlorophyll a, and temperature (Table 1.7), partial correlations indicate both are direct correlations. Bacterial production is correlated with chlorophyll a, temperature, autofluorescent microflagellate numbers and bacterial numbers (Table 1.7). Multiple partial correlations demonstrate all of these to be direct correlations (Table 1.15).

The aphotic zone- In the aphotic zone in spring total microflagellate numbers are

TABLE 1.7, SPEARMAN RANK PAIRWISE CORRELATIONS FOR THE SPRING PHOTIC ZONE DATA

	Depth	Salinity	Chl *a*	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.7221** n=25	1.0000									
Chl*a*	-0.5079* n=25	-0.5447** n=25	1.0000								
Temp.	0.1746 n=25	-0.1361 n=25	0.5778** n=25	1.0000							
Bact.Prod.	-0.2159 n=25	-0.2710 n=25	0.4321* n=25	0.5924** n=25	1.0000						
Bact.Carbon.	-0.0477 n=25	0.0755 n=25	0.4881* n=25	0.4830* n=25	0.3911 n=25	1.0000					
TMF	-0.3729 n=25	-0.3403 n=25	0.1947 n=25	0.0665 n=25	0.3003 n=25	0.0899 n=25	1.0000				
AMF	-0.4152* n=24	-2282 n=24	0.4280* n=24	0.1753 n=24	0.4514 n=24	0.1523 n=24	0.2958 n=24	1.0000			
HMF	-0.5302* n=24	-0.4830* n=24	0.3551 n=24	0.2152 n=24	0.4071 n=24	0.0351 n=24	0.4683* n=24	0.2989 n=24	1.0000		
Bact.Nos.	-0.3472 n=25	-0.4435* n=25	0.0911 n=25	0.1312 n=25	0.7008** n=20	-0.1993 n=25	0.3222 n=25	0.2125 n=24	0.2840 n=24	1.0000	
Agg.Bact.Nos.	-0.3116 n=20	-0.3340 n=25	-0.2999 n=25	-0.0481 n=25	0.0853 n=25	-0.1924 n=25	0.0323 n=25	-0.2886 n=24	0.0810 n=24	0.3203 n=25	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF–Total microflagellates, AMF–Autofluorescent microflagellates, HMF–Heterotrophic microflagellates.

TABLE 1.8, SPEARMAN RANK PAIRWISE CORRELATIONS FOR THE SPRING APHOTIC ZONE DATA

	Depth	Salinity	Chl *a*	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.1430 n=15	1.0000									
Chl*a*	-0.0770 n=15	-0.5523* n=15	1.0000								
Temp.	-0.0190 n=15	-0.2281 n=15	0.4658* n=15	1.0000							
Bact.Prod.	0.1993 n=15	0.5520* n=15	0.7982** n=15	0.4613* n=15	1.0000						
Bact.Carbon.	-0.0574 n=15	0.5064* n=15	0.8950** n=15	0.5141* n=15	0.8147** n=15	1.0000					
TMF	-0.6147* n=15	-0.2245 n=15	-0.1438 n=15	-0.4102 n=15	-0.3438 n=15	-0.3186 n=15	1.0000				
AMF	-0.2348> n=15	0.1750 n=15	0.3204 n=15	0.1132 n=15	0.3667 n=15	0.4596* n=15	0.0929 n=15	1.0000			
HMF	-0.5206* n=15	0.2338 n=15	-0.2532 n=15	-0.5175* n=15	-0.2723 n=15	-0.2011 n=15	0.4082 n=15	-0.0838 n=15	1.0000		
Bact.Nos.	-0.1512 n=15	0.0541 n=15	-0.0837 n=15	-0.5579* n=15	0.1453 n=15	-0.3092 n=15	0.4307 n=15	-0.0222 n=15	0.3238 n=15	1.0000	
Agg.Bact.Nos.	-0.1907 n=15	-0.1673 n=15	-0.2349 n=15	-0.4434* n=15	-0.2643 n=15	-0.3495 n=15	0.4933* n=15	-0.0743 n=15	0.4513* n=15	0.8090** n=15	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

significantly correlated with depth and the numbers of attached or aggregated bacteria (Table 1.8). Partial correlations indicate both are direct correlations (Table 1.15). Heterotrophic microflagellate numbers are likewise significantly correlated with depth and attached bacteria and also with temperature (Table 8). Partial correlations demonstrate temperature and the number of attached bacteria are direct correlations (Table 1.15). The only parameter significantly correlated with autofluorescent microflagellate numbers is bacterial carbon (Table 1.8).

The numbers of attached bacteria are significantly correlated with temperature, bacterial density, total and heterotrophic microflagellate numbers (Table 1.8). Partial correlations show all these correlations to be direct (Table 1.15). Bacterial biomass is correlated with several parameters but partial correlations clearly demonstrate chlorophyll a to be the common parameter and the only direct correlation (Table 1.15).

SUMMER

Cluster analysis indicates summer is not as discrete a cluster as spring or winter (Fig. 1.11). Overall in this season, depth in the water column appears to be the major correlation with total microflagellate numbers (Table 1.9). Heterotrophic microflagellate numbers are correlated with depth and also with temperature (Table 1.9). Partial correlations demonstrate these to both be direct correlations (Table 1.15). Autofluorescent microflagellate numbers are correlated with depth, salinity, bacterial production, temperature and chlorophyll a (Table 1.9). Again no major influence can be demonstrated with partial correlations (Table 1.15).

The photic zone - In the photic zone total microflagellate numbers are significantly correlated with autofluorescent microflagellate numbers (Table 1.10). No parameters are significantly correlated with heterotrophic microflagellates

TABLE 1.9, SPEARMAN RANK PAIRWISE CORRELATIONS FOR ALL THE SUMMER DATA

	Depth	Salinity	Chl a	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.9145** n=56	1.0000									
Chl a	-0.6840** n=72	-0.6218** n=48	1.0000								
Temp.	0.7429** n=72	-0.6596** n=56	0.6568** n=64	1.0000							
Bact.Prod.	-0.7553** n=72	0.7363** n=48	0.6247** n=72	0.6725** n=56	1.0000						
Bact.Carbon.	-0.1849 n=56	0.1196 n=56	0.2388 n=48	0.3375* n=48	0.1517 n=56	1.0000					
TMF	-0.4909** n=80	-0.4802** n=56	0.3515** n=72	0.2768* n=64	0.2609* n=64	0.0255 n=56	1.0000				
AMF	-0.5794** n=48	-0.7857** n=24	0.5369** n=48	0.5426** n=32	0.4813 n=40	0.2036 n=24	0.6832** n=40	1.0000			
HMF	-0.3438* n=48	-0.2368 n=24	0.1749 n=48	0.4536* n=32	0.1359 n=40	0.3223 n=24	0.2980 n=40	0.2400 n=40	1.0000		
Bact.Nos.	-0.7416** n=72	-0.7845** n=48	0.5785** n=72	0.6301** n=56	0.8627** n=64	0.0914 n=48	0.2245 n=64	0.5248** n=40	0.0446 n=25	1.0000	
Agg.Bact.Nos.	-0.3825** n=48	-0.3029* n=47	0.1855 n=48	0.2614 n=47	0.3820 n=48	-0.2045 n=47	0.1071 n=48	-0.1661 n=24	-0.0394 n=24	0.4594** n=48	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

TABLE 1.10, SPEARMAN RANK PAIRWISE CORRELATIONS FOR THE PHOTIC ZONE IN THE SUMMER

	Depth	Salinity	Chl a	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.9136** n=35	1.0000									
Chl a	-0.6564** n=45	-0.5911** n=30	1.0000								
Temp.	-0.7806** n=45	-0.8116** n=35	0.4949** n=35	1.0000							
Bact.Prod.	-0.6682** n=45	-0.5877** n=30	0.4514** n=40	0.6001** n=35	1.0000						
Bact.Carbon.	-0.01466 n=35	-0.0159 n=35	0.0280 n=30	0.2209 n=35	0.0596 n=30	1.0000					
TMF	-0.1723 n=50	-0.1459 n=35	0.1860 n=40	0.0307 n=40	0.0022 n=40	0.0584 n=35	1.0000				
AMF	-0.3149** n=30	0.7585** n=15	0.6755** n=25	0.3678 n=20	0.2526 n=25	0.0415 n=15	0.4272* n=25	1.0000			
HMF	0.0109 n=30	0.0424 n=15	-0.2734 n=25	0.0975 n=20	-0.0095 n=25	0.0795 n=15	-0.2191 n=25	-0.2111 n=25	1.0000		
Bact.Nos.	-0.6601** n=45	-0.6588** n=40	0.5950** n=40	0.5479** n=35	0.7972** n=40	-0.0266 n=30	-0.1238 n=40	-0.3271 n=25	-0.0446 n=25	1.0000	
Agg.Bact.Nos.	0.1310 n=30	0.0154 n=30	0.1461 n=30	0.0238 n=30	0.2949 n=30	-0.2106 n=30	-0.0567 n=30	-0.2469 n=15	0.0975 n=15	0.4141* n=30	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

(Table 1.10). In addition to their significant correlation with total microflagellates, autofluorescent microflagellates are correlated with chlorophyll a (Table 1.10). Chlorophyll a correlates significantly with depth, salinity, temperature, bacterial production, autofluorescent microflagellates and bacterial numbers (Table 1.10). Partial correlations indicate that the only significant direct correlations are with bacterial numbers, temperature and depth (Table 1.15). Bacterial production is correlated with several parameters but partial correlations indicate depth, chlorophyll a and bacteria numbers are the only direct correlations (Table 1.15). Bacterial numbers are also correlated with several different parameters and partial correlations indicate depth, chlorophyll a, bacterial production and the number of aggregated bacteria are direct correlations (Table 1.15).

The aphotic zone- In the aphotic zone total microflagellate numbers show no significant correlations (Table 1.11). Heterotrophic, and autofluorescent microflagellate numbers decrease with increasing depth (Table 1.11). This is the only significant correlation for the autofluorescent microflagellates. Heterotrophic microflagellate numbers are also significantly correlated with chlorophyll a, temperature and bacterial biomass (Table 1.11). Partial correlations demonstrate all of these factors to be direct correlations (Table 1.15). Bacterial biomass is significantly correlated with temperature, chlorophyll a, heterotrophic microflagellates and aggregated bacterial numbers (Table 1.11). Partial correlations demonstrate the correlation with aggregated bacteria is the only indirect correlation (Table 1.15). Chlorophyll a is significantly correlated with attached bacteria, bacterial biomass, temperature, and heterotrophic microflagellate numbers (Table 1.11). Partial correlations indicate the correlations with bacterial biomass, heterotrophic microflagellate numbers and aggregated bacterial numbers are direct correlations (Table 1.15).

TABLE 1.11, SPEARMAN RANK PAIRWISE CORRELATIONS FOR THE SUMMER APHOTIC ZONE DATA

	Depth	Salinity	Chl *a*	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.3582 n=21	1.0000									
Chl*a*	-0.1749 n=27	0.1270 n=18	1.0000								
Temp.	-0.1142 n=27	0.4753* n=21	0.4329* n=21	1.0000							
Bact.Prod.	-0.3766* n=27	0.0674 n=18	0.1724 n=24	0.3150 n=21	1.0000						
Bact.Carbon.	-0.24166 n=21	0.1083 n=21	0.6457** n=18	0.5728* n=21	0.1804 n=18	1.0000					
TMF	-0.0901 n=30	-0.0682 n=21	-0.0325 n=24	-0.1817 n=24	-0.2323 n=24	-0.3027 n=24	1.0000				
AMF	-0.4323* n=18	-0.4919 n=9	0.2787 n=15	0.1380 n=12	0.2598 n=15	0.2440 n=9	0.3928 n=15	1.0000			
HMF	-0.4208* n=18	0.2813 n=9	0.5110* n=15	0.6903* n=12	-0.1511 n=15	0.6981* n=9	0.4414** n=15	0.5103** n=15	1.0000		
Bact.Nos.	-0.3350* n=27	-0.1352 n=18	0.1446 n=24	0.0148 n=21	0.8139** n=24	0.0524 n=18	-0.3286 n=24	0.3504 n=15	-0.1673 n=15	1.0000	
Agg.Bact.Nos.	-0.2538 n=18	-0.2424 n=17	-0.4830 n=18	0.1778 n=17	-0.0042 n=18	-0.4249 n=17	-0.0545 n=18	-0.4813 n=9	-0.5192 n=9	-0.0242 n=18	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

WINTER

Total microflagellate numbers correlate significantly with depth, salinity, temperature and chlorophyll a (Table 1.12). Partial correlations indicate the correlation with chlorophyll a is an indirect correlation (Table 1.15). Heterotrophic microflagellates are almost significantly correlated with with bacterial production but not with any other parameter (Table 1.12). Autofluorescent microflagellate numbers are not significantly correlated with anything (Table 1.12). This absence of significant correlations for the latter two parameters may be partially due to there being too few cases available for analysis. Chlorophyll a is correlated with depth, salinity, temperature, bacterial biomass and total microflagellate numbers (Table 1.12). Partial correlations demonstrate the direct correlations are with depth and salinity (Table 1.15). Bacterial numbers are correlated with temperature and bacterial production, both of which are shown to be direct correlations with partial correlations (Table 1.15).

The photic zone- In the photic zone there are no correlations between total, heterotrophic and autofluorescent microflagellates and any other parameter (Table 1.13). This may be due once again to the reduced number of cases for analysis. Temperature correlates significantly with both bacterial numbers and bacterial biomass (Table 1.13). Bacterial numbers are also correlated with bacterial production (Table 1.13).

The aphotic zone- In the aphotic zone the number of interactions are much reduced. Total, heterotrophic and autofluorescent microflagellate numbers show no significant correlations (Table 1.14). Chlorophyll a also shows no significant correlations (Table 1.14). Bacterial production is correlated with temperature and bacterial biomass (Table 1.14). Partial correlations indicate these are both direct correlations (Table 1.15). Bacterial biomass is correlated with temperature, bacterial production,and the number of aggregated bacteria (Table 1.14). Partial

TABLE 1.12. SPEARMAN RANK PAIRWISE CORRELATIONS FOR ALL THE WINTER DATA

	Depth	Salinity	Chl a	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.9043‡ n=48	1.0000									
Chl a	-0.3608* n=48	-0.3445* n=48	1.0000								
Temp.	0.2946* n=48	0.2255 n=48	0.2844* n=48	1.0000							
Bact.Prod.	-0.2052 n=40	-0.2456 n=40	0.0642 n=40	0.1152 n=40	1.0000						
Bact.Carbon.	-0.2167 n=48	-0.3398* n=48	0.2522* n=48	0.6085‡ n=48	0.5169‡ n=40	1.0000					
TMF	-0.4337‡ n=48	-0.3016* n=48	0.2595* n=48	-0.2823* n=48	-0.0778 n=40	-0.1065 n=48	1.0000				
AMF	-0.5225 n=7	-0.3425 n=7	0.0000 n=7	0.5637 n=7	0.1261 n=7	0.4855 n=7	0.4182 n=7	1.0000			
HMF	-0.3713 n=8	-0.0478 n=8	0.0000 n=7	-0.1928 n=8	0.5150 n=8	-0.0599 n=8	0.6867‡ n=8	-0.2523 n=8	1.0000		
Bact.Nos.	-0.2052 n=40	-0.1710 n=40	-0.1269 n=40	0.4035* n=40	0.6483‡ n=32	-0.1929 n=40	0.1876 n=40	0.3424 n=7	0.1084 n=8	1.0000	
Agg.Bact.Nos.	-0.2530 n=40	-0.2848 n=40	0.0507 n=40	0.2033 n=40	0.2970 n=32	0.3533* n=40	0.0236 n=40	0.5236 n=7	-0.0574 n=8	0.3329* n=40	1.0000

‡ = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

TABLE 1.13, SPEARMAN RANK PAIRWISE CORRELATIONS FOR THE WINTER PHOTIC ZONE DATA

	Depth	Salinity	Chl a	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.8759** n=30	1.0000									
Chla	-0.3433 n=30	-0.3039 n=30	1.0000								
Temp.	0.2793 n=30	0.2798 n=30	0.3828* n=30	1.0000							
Bact.Prod.	-0.2766 n=25	-0.2679 n=25	0.0350 n=25	-0.0645 n=25	1.0000						
Bact.Carbon.	-0.3039 n=30	-0.3262 n=30	0.3221 n=30	0.6171** n=30	0.3236 n=25	1.0000					
TMF	-0.2026 n=30	-0.1270 n=30	0.1708 n=30	-0.3431 n=30	0.0494 n=25	-0.1633 n=30	1.0000				
AMF	0.0000 n=4	0.4000 n=4	0.0000 n=4	0.2000 n=4	-0.4000 n=4	0.6000 n=4	-0.4000 n=4	1.0000			
HMF	0.3591 n=5	0.2051 n=5	0.0000 n=5	0.3581 n=5	-0.0513 n=5	-0.6669 n=5	0.4617 n=5	-0.8000 n=4	1.0000		
Bact.Nos.	-0.1824 n=25	-0.2240 n=25	-0.2241 n=25	0.5423** n=25	0.8041** n=20	-0.3605 n=25	0.2207 n=27	-0.2000 n=4	-0.3684 n=5	1.0000	
Agg.Bact.Nos.	-0.1402 n=25	-0.0599 n=25	0.0127 n=25	0.1375 n=25	0.3015 n=20	0.2376 n=25	-0.1616 n=25	-0.2000 n=4	-0.2368 n=5	0.3062 n=25	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

TABLE 1.14, SPEARMAN RANK PAIRWISE CORRELATIONS FOR THE WINTER APHOTIC ZONE DATA

	Depth	Salinity	Chl a	Temp.	Bact. Prod	Bact. Carbon	TMF	AMF	HMF	Bact. Nos.	Agg. Bact.Nos.
Depth	1.0000										
Salinity	0.5454* n=18	1.0000									
Chla	0.0000 n=18	0.0000 n=18	1.0000								
Temp.	-0.2370 n=18	-0.6737* n=18	0.0000 n=18	1.0000							
Bact.Prod.	-0.0473 n=15	-0.2491 n=15	0.0000 n=18	0.6068* n=15	1.0000						
Bact.Carbon.	-0.1444 n=18	-0.5522** n=18	0.0000 n=18	0.8314** n=18	0.7619** n=15	1.0000					
TMF	-0.3781 n=18	-0.0144 n=18	0.0000 n=18	-0.1928 n=18	-0.2638 n=15	-0.2262 n=18	1.0000				
AMF	-0.8660 n=3	-0.8660 n=3	0.0000 n=3	-0.8660 n=3	-0.8660 n=3	0.0000 n=3	0.0000 n=3	1.0000			
HMF	-0.5000 n=3	-0.5000 n=3	0.0000 n=3	-0.5000 n=3	-0.5000 n=3	0.0000 n=3	0.0000 n=3	0.0000 n=3	1.0000		
Bact.Nos.	-0.0947 n=24	-0.1233 n=18	0.0000 n=15	0.0735 n=15	0.3012 n=12	0.0734 n=15	0.2268 n=15	0.0000 n=3	0.0000 n=3	1.0000	
Agg.Bact.Nos.	-0.0878 n=15	-0.3833 n=15	0.0000 n=15	0.5224 n=15	0.1972 n=12	0.5353 n=15	0.2128 n=15	0.0000 n=3	0.0000 n=3	0.3529 n=15	1.0000

** = significant at .01 level; * = signficant at .05 level
TMF-Total microflagellates, AMF-Autofluorescent microflagellates, HMF-Heterotrophic microflagellates.

correlations show that the correlation with the numbers of aggregated bacteria is indirect (Table 1.15). Aggregated bacteria are correlated with bacterial biomass and temperature and partial correlations demonstrate these are both direct correlations (Table 1.15).

DISCUSSION

(1) The complete data set

Overall the total microflagellate numbers showed the expected decrease in abundance with increasing depth. This observation is similar to that made for total nanoplankton populations in Narragansett Bay (Davis et al. 1985). Such a vertical distribution pattern may reflect the available food for microflagellates i.e. there is a better supply of potential prey (bacteria and small autotrophic organisms) in the photic zone and there is more light for photosynthesis. This is substantiated by an overall negative correlation of both bacterial and chlorophyll a concentration with depth and also by light extinction measurements (Stockner et al. 1977).

Overall autofluorescent microflagellate numbers are more strongly correlated with depth than are heterotrophic microflagellate numbers. This is due to many of the autofluorescent microflagellates being totally dependent on photosynthesis and hence light to grow and reproduce (e.g. Aaronson and Baker 1959). Although bacterial numbers are negatively correlated with depth, bacteria are still available as potential prey throughout the water column. This may help explain the weaker correlation of heterotrophic microflagellate numbers with depth.

Clearly depth in the water column has a major influence on all the parameters measured. To reduce this overwhelming effect and to reveal other

TABLE 1.15 PARTIAL CORRELATION COEFFICIENTS FOR SELECTED CASES OF THE DATA SET.

Partial correlation coeffients were calculated where parameters of interest were noted to be correlated (using Spearman rank correlations, Tables 2-24) with more than one parameter. The coefficient of partial correlation measures the degree of linear association between two dependent variates, taking into account their common association with a certain number of other variates which have a simultaneous influence on these variates.

TMF-Total microflagellates, AMF-autofluorescent microflagellates HMF-Heterotrophic microflagellates.

PARAMETER 1	PARAMETER 2 & 3	PARTIAL CORRELATION	SIGNIFICANCE
ALL DATA			
TMF	Depth	-0.2299	0.0102
	Salinity	0.0966	0.3387
TMF	Depth	-0.3018	0.0002
	Chl a	-0.1373	0.1007
TMF	Depth	-0.2781	0.0010
	Bact. nos.	-0.1085	0..2070
AMF	Depth	-0.5368	0.0000
	Salinity	0.1473	0.1436
AMF	Depth	-0.4200	0.001
	Chl a	0.0860	0.3264
AMF	Depth	-0.4681	0.0001
	Bact. Prod	0.0860	0.3264
AMF	Depth	-0.4671	0.0001
	Bact. nos.	-0.1051	0.2304
HMF	Depth	-0.2280	-0.0326
	Salinity	0.0986	0.3606
HMF	Depth	-0.2566	0.0052
	Chl a	0.0363	0.6975
HMF	Depth	-0.2068	0.0431
	Bact. Biom.	0.1595	0.1205
HMF	Depth	-0.2010	0.0297
	Bact. Prod.	0.1595	0.1205
Bact. ncs.	Depth	-0.2883	0.0002
	Salinity	-0.0453	0.5705
Bact. nos.	Depth	-0.2491	0.0005
	Chl a	0.4041	0.0000
Bact. nos.	Depth	-0.4010	0.0000
	Temp	0.1211	0.1103
Bact. nos.	Depth	-0.1434	0.0529
	Bact. Prod.	0.7496	0.0000

continued on next page

PARAMETER 1	PARAMETER 2 & 3	PARTIAL CORRELATION	SIGNIFICANCE
	AMF	0.1953	0.0805
Chl a	Bact. Prod.	0.5513	0.0000
	Temp.	0.4070	0.0000
Chl a	Temp.	0.5462	0.0000
	Depth	-0.3289	0.0004
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APHOTIC ZONE			
AMF	Depth	-0.3752	0.0072
	Chl a	0.1576	0.2744
Bact. nos.	Temp.	-0.3863	0.0015
	Chl a	0.2381	0.0859
Bact. nos.	Temp.	-0.6041	0.0000
	Bact. Prod.	0.6399	0.0000
Bact. nos.	Chl a	0.0118	0.9233
	Bact. Prod.	0.5641	0.0000
Bact. Prod.	Bact. Biom.	0.8300	0.0000
	Bact. nos.	0.8448	0.0000
Bact. Prod.	Chl a	0.1325	0.2812
	Bact. nos.	0.5641	0.0000
Bact. Prod.	Chl a	0.2249	0.0788
	Bact. Biom.	0.6310	0.0000
Bact. Biom.	Temp.	0.5739	0.0000
	Bact. Prod.	0.6879	0.0000
Bact. Biom.	Chl a	0.0288	0.8240
	Bact. Prod.	0.6310	0.0000
Bact. Biom.	Temp.	0.5162	0.0000
	Chl a	0.1732	0.1782
Chl a	Bact. nos.	-0.0411	0.8173
	Temp.	0.3616	0.0356
	Salinity	0.5142	0.0019
	Bact. Prod.	-0.0472	0.7907
	Bact. Biom.	0.4168	0.0142
	AMF	0.4262	0.0119
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SPRING			
TMF	Salinity	0.0067	0.9680
	Bact. nos.	0.3154	0.0537
	Depth	-0.2774	0.0917
HMF	Depth	0.1398	0.5146
	Salinity	-0.2439	0.2507
	Bact. nos.	0.2686	0.2043
	Att. Bact. nos.	0.0034	0.9876
AMF	Depth	-0.4131	0.0099
	Salinity	-0.1701	0.3072

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PARAMETER 1	PARAMETER 2 & 3	PARTIAL CORRELATION	SIGNIFICANCE
Bact. nos.	Depth	-0.4598	0.0000
	TMF	-0.1085	0.2070
Bact. nos.	Depth	-0.5677	0.0000
	AMF	-0.1051	0.2304
---	---	---	---
PHOTIC ZONE DATA			
AMF	Depth	-0.3177	0.0126
	Salinity	0.1192	0.3599
AMF	Depth	-0.2142	0.0548
	Chl a	0.0802	0.4765
Bact. nos	Depth	-0.1269	0.2105
	Salinity	-0.0905	0.3730
Bact. nos	Bact. Prod	0.8860	0.0000
	Temp.	-0.2136	0.0295
Bact. nos	Bact. Prod.	0.8609	0.0000
	Att. Bact. nos.	0.2240	0.0501
Bact. Prod.	Depth	0.1395	0.1685
	Salinity	-0.0724	0.4758
Bact. Prod.	Depth	-0.0959	0.2995
	Chl a	0.5587	0.0000
Bact. Prod.	Depth	-0.2495	0.0089
	Temp.	0.3320	0.0004
Bact. Prod.	Depth	-0.2431	0.0129
	Bact. Biom.	0.3208	0.0009
Bact. Prod.	Chl a	0.5513	0.0000
	Temp.	0.0539	0.5775
Bact. Prod.	Depth	-0.1975	0.3541
	Bact.nos.	0.8349	0.0000
Bact. Prod.	Bact. Biom.	0.5924	0.0000
	Chl a	0.2213	0.0239
Bact. Prod.	Bact. nos.	0.2874	0.0019
	Chl a	0.7964	0.0000
Bact. Prod.	Bact. nos.	0.9373	0.0000
	Bact. Biom.	0.7190	0.0000
Chl a	Depth	0.1775	0.0713
	Salinity	0.1004	0.3105
Chl a	Depth	-0.2786	0.0021
	Bact. Prod.	0.5587	0.0000
Chl a	Bact. prod.	0.2873	0.0019
	Bact. nos.	0.0596	0.5286
Chl a	Bact. prod.	0.5924	0.0000
	Bact. Biom.	0.0930	0.3474
Chl a	Bact. Prod	0.3025	0.0000

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PARAMETER 1	PARAMETER 2 & 3	PARTIAL CORRELATION	SIGNIFICANCE
AMF	Depth	-0.5757	0.0002
	Bact. nos.	0.1446	0.3863
AMF	Depth	-0.5226	0.0008
	Chl a	0.3329	0.0411
AMF	Depth	-0.5688	0.0002
	Bact. Prod.	0.4363	0.0062
AMF	Chl a	0.3379	0.0379
	Bact. Prod.	0.3651	0.242
Chl a	Depth	-0.5800	0.0003
	Salinity	0.0815	0.6468
	Bact. Prod.	0.0107	0.9519
	Bact. Biom.	0.5394	0.0010
	AMF	0.1930	0.2741
	Temp.	0.6518	0.0000
Bact. nos.	Depth	-0.2817	0.2040
	Salinity	0.0772	0.7328
	Bact. Prod.	0.7750	0.0000
	AMF	-0.3422	0.1189
	HMF	0.3668	0.0931
	Att. Bact. nos.	0.5045	0.0166
-----	-----	-----	------
SPRING PHOTIC			
HMF	Salinity	-0.1837	0.4015
	Depth	0.1570	0.4741
AMF	Depth	-0.2932	0.1854
	Chl a	0.2040	0.3625
	Bact. Prod.	0.4020	0.0636
Bact. nos.	Salinity	-0.2607	0.2185
	Bact. Prod.	0.6832	0.0002
Chl a	salinity	0.1581	0.5175
	Depth	-0.5952	0.0072
	Temp.	0.7561	0.0002
	Bact. Prod.	-0.1705	0.4852
	Bact. Biom.	0.4106	0.0807
	AMF	0.2646	0.2735
Bact. Biom.	Chl a	0.3608	0.0833
	Temp.	0.2732	0.1964
Bact. Prod.	Chl a	-0.0224	0.9477
	Temp.	0.3646	0.2702
	AMF	0.6091	0.0667
	Bact. nos.	0.3148	0.3456
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SPRING APHOTIC			
Bact. Biom.	Salinity	0.2189	0.5024

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PARAMETER 1	PARAMETER 2 & 3	PARTIAL CORRELATION	SIGNIFICANCE
	Chl a	0.8058	0.0016
	Temp.	0.3411	0.2778
	Bact. Prod.	0.2300	0.4719
TMF	Depth	-0.3579	0.3098
	Att. Bact. nos.	0.3691	0.2938
HMF	Depth	-0.2410	0.5652
	Temp.	-0.6236	0.0985
	Att. Bact. nos.	0.1109	0.7937
Att. Bact. nos.	Temp.	-0.3688	0.4155
	Bact. Nos.	-0.0620	0.8949
	TMF	0.0766	0.8702
	HMF	0.1621	0.7274
-----	-----	-----	-----
SUMMER			
TMF	Depth	-0.3138	0.0707
	Salinity	0.0171	0.9235
TMF	Depth	-0.3878	0.0134
	Chl a	0.0153	0.9251
TMF	Depth	-0.4691	0.0034
	Temp.	-0.2732	0.1018
TMF	Depth	-0.3523	0.0257
	Bact. Prod.	-0.0358	0.8262
HMF	Depth	-0.1438	0.5338
	Temp.	0.1022	0.6592
AMF	Depth	-0.2308	0.3275
	Salinity	-0.0426	0.8584
	Chl a	0.3055	0.1856
	Temp.	0.1100	0.6443
	Bact. nos.	-0.0488	0.8379
-----	-----	------	-------
SUMMER PHOTIC			
AMF	Chl a	0.4592	0.0316
	TMF	0.4073	0.0598
Chl a	Depth	-0.2802	0.1085
	Temp.	0.2322	0.1863
Chl a	Depth	-0.3557	0.0262
	Bact. Prod.	0.3972	0.0123
Chl a	Depth	-0.4126	0.0451
	AMF	0.3071	0.1443
Chl a	Depth	-0.2809	0.0832
	Bact. nos.	0.5343	0.0005
Chl a	Bact. Prod.	0.0977	0.5538
	Bact. nos	0.4508	0.0040
Chl a	Temp.	0.4195	0.0137

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PARAMETER 1	PARAMETER 2 & 3	PARTIAL CORRELATION	SIGNIFICANCE
	Bact. nos.	0.5911	0.0002
Chl a	Temp.	0.4132	0.0151
	Bact. Prod	0.5583	0.0006
Bact. nos	Depth	-0.6450	0.0007
	Att. Bact. nos.	0.5737	0.0034
Bact. nos.	Bact. Prod.	0.8275	0.0000
	Att. Bact. nos.	0.4027	0.0510
Bact. nos	Bact. Prod.	0.6702	0.0000
	Chl a	0.4508	0.0040
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SUMMER APHOTIC			
Bact. nos.	Bact. Prod.	0.7546	0.0000
	Chl a	-0.3243	0.1311
HMF	Depth	-0.7277	0.0262
	Chl a	0.1002	0.7974
HMF	Depth	-0.7820	0.0561
	Temp.	0.5742	0.2334
HMF	Depth	0.7485	0.0213
	Bact. Prod.	0.2217	0.5663
Bact. Biom.	Temp.	0.4547	0.0812
	Chl a	0.3345	0.1893
Bact. Biom.	Chl a		
	HMF		
Bact. Biom.	Temp.		
	HMF		
Bact. Biom.	Temp.	0.6536	0.0404
	Att. Bact. nos.	-0.3241	0.3609
Chl a	Temp.	0.3247	0.2035
	Bact. Biom.	0.3345	0.1893
Chl a	Temp.	-0.65941	0.1260
	TMF	0.2083	0.0921
Chl a	Temp.	0.49771	0.1432
	Att. Bact. nos.	-0.7073	0.0221
Chl a	TMF	-0.1316	0.9160
	Att. Bact. nos.	-0.4066	0.7332
Chl a	Bact. Biom.	0.5969	0.0685
	Att. Bact. nos.	-0.6536	0.0404
Chl a	Bact. Biom.		
	TMF		
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WINTER			
TMF	Depth	-0.3835	0.0861
	Salinity	0.1181	0.6100
TMF	Depth	-0.6694	0.0009

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PARAMETER 1	PARAMETER 2 & 3	PARTIAL CORRELATION	SIGNIFICANCE
	Chl a	-0.5559	0.0089
TMF	Depth	-0.4811	0.0272
	Temp.	-0.6589	0.0012
TMF	Chl a	0.1679	0.4669
	Temp.	-0.6546	0.0013
Bact. nos.	Temp.	-0.7384	0.0000
	Bact. Prod.	0.6011	0.0003
Chl a	Depth	-0.0457	0.7603
	Salinity	-0.2345	0.1126
Chl a	Depth	-0.5389	0.0001
	Temp.	0.5015	0.0003
Chl a	Salinity	-0.6003	0.0000
	Temp.	0.5362	0.0001
Chl a	Depth	-0.3742	0.0096
	Bact. Biom.	0.1508	0.3116
Chl a	Depth	0.6726	0.0008
	TMF	0.5559	0.0089
Chl a	Temp.	0.4543	0.0385
	TMF	0.1679	0.4669
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WIN PHOTIC			
Temp.	Bact. nos.	-0.5272	0.0081
	Bact. Biom.	0.4936	0.0142
Bact. nos	Temp.	-0.7123	0.0006
	Bact. Biom.	0.7505	0.0002
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WINTER APHOTIC			
Bact. Prod.	Temp.	-0.1770	0.5447
	Bact. Biom.	0.7291	0.0031
Bact. Biom.	Temp.	0.6522	0.0115
	Bact. Prod.	0.7291	0.0031
Bact. Biom.	Temp.	0.6765	0.0664
	Att. Bact. nos.	0.1366	0.7470
Bact. Biom..	Bact. Prod.	0.8998	0.0022
	Att. Bact. nos.	-0.1260	0.7661
Att. Bact. nos.	Temp.	0.1693	0.6886
	Bact. Biom.	0.1366	0.7470

important relationships among the parameters measured the data set was divided into photic and aphotic zone and re-analysed.

(2a) The photic zone

Total microflagellate numbers increase with decreasing temperature in the photic zone. This is a relatively weak correlation (.05 level) and is contrary to what is generally expected. Increasing temperature generally increases the rates of chemical reactions and hence to a certain extent generally results in increased growth rate. This suggests that for much of the year the water temperature in the photic zone of Howe Sound is higher than the optimum growth temperature of the microflagellate populations. However this is hard to believe in view of what is known about the growth temperature of various microflagellate species (Fenchel 1982a, Sherr et al. 1983) and also about the average temperatures in Howe Sound (Thomson 1981). Alternatively this relationship could be spurious. The latter suggestion is more likely since this relationship is not apparent in any seasonal analysis of the photic zone. It should be noted that in studies of this type where many correlations are carried out, significance tests can be of doubtful validity since a number of correlations can be expected to reach a 5% significance level by chance.

The increase of autofluorescent microflagellate concentration in the photic zone with decreasing depth is probably, as already mentioned, due to the decreasing light intensity with increasing depth. The absence of any correlation with bacterial parameters, such as that noted for bacterial production when the total data were analysed, suggests that in the photic zone autofluorescent microflagellate numbers do not employ heterotrophy as a mode of nutrition. This is probably a result of there being sufficient light to allow adequate photosynthesis. Caution must be used when interpreting lack of significant

correlations. For example an autofluorescent microflagellate that displays heterotrophy may only feed on a few species of bacteria and therefore not correlate with total bacterial population parameters.

Heterotrophic microflagellate numbers display no significant correlation with depth or salinity in the photic zone. This serves to emphasize the difference between their mode of nutrition and that of autofluorescent microflagellates i.e. they can not use light. The only parameter measured with which heterotrophic microflagellate numbers show any significant correlation in the photic zone is bacterial biomass. The absence of any correlation with chlorophyll _a_ indicates that, in the photic zone at least, heterotrophic microflagellate are predominantly bactivorous and not herbivorous. The correlation with bacterial biomass suggests it is total available bacterial carbon that is limiting growth of heterotrophic microflagellate numbers in the photic zone.

A number of different parameters are correlated with bacterial concentration in the photic zone but partial correlations imply the only direct influence is bacterial production. Temperature is apparently an important influence on bacterial production in the photic zone. This is consistent with the findings of other workers investigating factors influencing the activity of bacteria (Bolter 1982, Iriberri _et al_. 1985, Pomeroy and Deibel 1986). Partial correlations demonstrate bacterial production, bacterial density and temperature to be direct influences on chlorophyll _a_ levels in the photic zone. This correlation of chlorophyll _a_ with bacterial parameters is probably a consequence of the ability of bacteria to graze phytoplankton extracellular products and also in some situations to graze decaying phytoplankton. The temperature of the water column probably influences this interaction. It also has a direct influence on the rate of phytoplankton growth. Furthermore the temperature of the water often reflects the hours of

sunlight and this may affect chlorophyll a levels.

(2b) The aphotic zone

The very limited number of significant interactions occurring in the aphotic zone serves to emphasize the generally reduced microbial activity in this region of deeper water where little light penetrates. Unexpectedly, in view of this latter point, there are two significant correlations for autofluorescent microflagellate numbers but none for either total microflagellate numbers or heterotrophic microflagellate numbers. As before, autofluorescent microflagellate numbers are significantly correlated with depth and chlorophyll a. This is consistent with the observation that some photosynthetic microflagellates have been demonstrated to be photosynthetically active at light intensities of below 1% of surface illumination e.g. cryptomonads (Rodhe 1955, Takahashi and Ichimura 1968, Morgen and Kalff 1975, Wetzel 1983, Gavrielli 1984). The absence of correlation of these autofluorescent microflagellates with any bacterial parameters further indicates that they are functioning in a autotrophic manner.

Pairwise correlations indicate bacterial standing stocks in the aphotic region are influenced by several parameters, however partial correlation indicates this is a consequence of their relationship with bacterial production and the temperature of the water column.

(3) Time-phase displacements

Figures 1.2 and 1.3 show an increase in total, autofluorescent and heterotrophic microflagellate numbers, during late March to April in each year of the study. An early spring phytoplankton bloom is a recognized event in Howe Sound (Stockner et al. 1977). Presumably the increased energy input to the system as a result of increased day length and sunlight hours (that generally

occur in early spring) acts as a switch on primary productivity which increases rapidly (Stockner et al. 1977). Subsequently increases in other parameters occur including an increase in bacterial numbers (Fig. 1.4) followed by an increase in total microflagellate numbers (Figs. 1.2 and 1.3). The increase in total microflagellate numbers is significantly correlated with a decrease in bacterial numbers five days earlier (Table 1.5). This suggests this decrease in bacterial concentration is due to grazing by microflagellates. This type of 'lag effect' has previously been observed in culture (e.g. Andersen and Fenchel 1986), and to some extent in natural water bodies (e.g. Sorokin 1977,1979). It has been noted to have a period ranging from 3-8 days (Linley et al. 1981, Fenchel 1982d, Andersen and Fenchel 1986, Sieburth and Davis 1982). This significant correlation with bacterial density five days previously holds for both heterotrophic microflagellate numbers and autofluorescent microflagellate numbers (Table 1.5). Upon analysing the data for the photic and the aphotic zone separately it is clear that the relationship observed for the autofluorescent microflagellates is due to the autofluorescent microflagellate populations in the aphotic zone (Table 1.5). In the photic zone autofluorescent microflagellate numbers are not correlated with bacterial densities five days previously (Table 1.5). The reduced numbers of total microflagellate numbers in the summer months in Howe Sound (Fig. 1.2 and 1.3) could be due to grazing of microflagellate numbers by ciliates or other predator populations. This was suggested by Sorensen and Andersen (1986) to account for a similar observation of reduced microflagellate populations in the summer in Lim fjord (Norway). It must, however, be noted that Lim fjord is relatively shallow (mean depth 8 m, Fenchel 1977), and as such may be strongly influenced by interactions with the sediment (Bolter et al. 1981) which is not likely to be the case for Howe Sound (maximum depth at sampling site is approximately 290 m). Alternatively, the reduced number of microflagellate numbers could be due to

some nutrient being limiting.

It is probably a partial result of reduced grazing pressure due to depressed microflagellate populations that bacterial numbers increase in June in both years of the study (Fig. 1.4). It is also significant however that in June the Squamish river generally reaches its maximum discharge rate (Hoos and Vold 1975). This discharge has a heavy glacial silt load and consequently there is an opaque brackish surface layer at this time of year that limits phytoplankton growth (Hoos and Vold 1975). Hence senescent phytoplankton may be available as bacterial substrate at this time. Figures 1.2 and 1.3 also demonstrate how the proportion of total microflagellates that are autofluorescent varies with time and with depth (photic vs aphotic). This observation is important since many studies do not distinguish between microflagellates that contain chlorophyll and those that do not (Ballantine 1953, Beers et al. 1975, Hannah and Bonney 1983). Obviously the ability to photosynthesize and/or function heterotrophically can affect the role played by the microflagellate population in the microbial food web. In Howe Sound heterotrophic microflagellate numbers predominate relative to autofluorescent microflagellate numbers. This is unusual as in other studies autofluorescent microflagellates generally predominate at some time during the summer months (e.g. Davis et al. 1985). The predominance of heterotrophic microflagellates in Howe Sound may be a consequence of light limitation by the opaque brackish layer produced by the Squamish river discharge at this time of year.

(4) Seasonal patterns

The observations of Figures 1.2-1.10 show seasonal differences among the data. To define these seasons more clearly cluster analysis based on rank correlation coefficients of the data for different sampling dates was carried out.

Thus the seasonal aspects and the recurring pattern of the annual cycle were established. The result revealed three clusters which corresponded to spring, summer and winter (Fig. 1.11). These seasonal subunits of the total annual cycle can be regarded as different 'ecological situations' and the major factors affecting microflagellate numbers may differ in each case. All the parameters measured were re-analysed on a seasonal basis. It is possible that interseasonal variation may obscure or even alter relationships that may be present when the data from one season is examined. A consequence of dividing the data into seasonal subsets is that the data sub-set to be analysed in each case is reduced and hence comparisons are weaker.

(5) Re-examination of data on a seasonal basis

SPRING

Cluster (1) (Fig.1.11) approximately corresponds to the season spring. This period extends from 1/3/84 to 3/4/84 and from 19/2/85 to 5/3/85. During this time total microflagellate numbers, autofluorescent microflagellate numbers, and heterotrophic microflagellate numbers are all negatively correlated with depth and salinity and positively correlated with bacterial numbers. Partial correlations indicate that for total microflagellate numbers, bacterial concentration is the major influence. Interestingly, primary productivity is high in the early part of this season (Stockner et al. 1977) and consequently it is likely extracellular organic material for use as bacterial substrate may also be high. Heterotrophic microflagellate numbers are also positively correlated with the number of attached or aggregated bacteria. Partial correlations indicate all the correlations to be direct. Alldredge et al. (1986) have noted large aggregates of 'marine snow' to commonly occur in this season. These aggregates have been demonstrated to have concentrations of bacteria and microflagellate numbers associated with them that are generally higher than the concentrations of these organisms found in the

surrounding water (Caron et al. 1982). It has been suggested that the marine snow aggregates represent localized high concentrations of nutrients for bacteria and hence heterotrophic microflagellates (Goldman 1984). Such marine snow aggregates have been observed in spring in Howe Sound (M. Barth pers. comm.). The observed correlation of aggregated bacteria and heterotrophic microflagellate numbers may reflect the presence of marine snow or smaller particulates in the water column in spring. Microelectrode studies have demonstrated that gradients of oxygen and pH exist around marine snow despite advection of water past these particles. Such gradients may significantly influence the distribution of marine micro-organisms (Alldredge and Cohen 1987). Alternatively this increase of attached or aggregated bacteria with increasing numbers of heterotrophic microflagellates could imply the latter organisms are secreting some substance that results in aggregation of bacteria. Such a phenomenon has been documented by Aaronson (1973) for Ochromonas danica growing heterotrophically in laboratory culture. There is no evidence in the literature that this is a widespread occurrence. It is suggested that this aggregation of bacteria by Ochromonas facilitates the ingestion of bacteria. This could be occurring in this case.

In addition to depth, salinity and bacterial concentration, autofluorescent microflagellate numbers are significantly correlated with chlorophyll a and bacterial production. The correlation of these small autofluorescent organisms with depth is stronger than that of the heterotrophic microflagellates for reasons already discussed. Their correlation with bacterial concentration is weaker than that of the heterotrophic microflagellate numbers, however they are significantly correlated with bacterial production. Perhaps when the bacteria are productive they release some compound that is required by the autofluorescent microflagellates for growth or vice versa. Alternatively, the autofluorescent microflagellates may

be functioning heterotrophically and grazing bacteria.

Overall during the spring, bacterial standing stocks are correlated with depth, temperature, autofluorescent and heterotrophic microflagellate numbers and bacterial production. Partial correlations do not reveal any common cause of these correlations.

The photic zone

In the photic zone autofluorescent microflagellate numbers are correlated with depth, chlorophyll a and bacterial production. Partial correlations indicate the correlation with bacterial production is the only direct correlation. The rapid increase in autofluorescent microflagellate numbers that generally occurs at this time of year, due to increased sunlight hours and increased temperature, may have resulted in some substance other than light now limiting autofluorescent microflagellate growth. Such a substance could be produced by bacteria e.g. vitamins or growth factors. Some Cryptomonads have specific requirements for vitamin B_{12}, thiamine or biotin (Hutchinson 1967, Provasoli and Carlucci 1974, Wetzel 1983). Alternatively, some autofluorescent microflagellates could be functioning heterotrophically and grazing bacteria.

Bacterial production in the photic zone is the most significant direct correlation with bacterial concentration. An increase in bacterial productivity generally leads to an increase in bacterial concentration but what is interesting here is that apparently the increase in bacterial production affects bacterial numbers more than any grazing by predators such as microflagellates. That is, the bacterial populations are growing faster than the microflagellate populations or other predators can graze them. It is significant that this occurs after the spring algal bloom when there generally is an abundance of extracellular material

produced by the phytoplankton available for bacterial utilization (Fogg 1983).

The aphotic zone

The significant correlation of autofluorescent microflagellate numbers with bacterial biomass implies that in this light-limited region, autofluorescent microflagellates are functioning in a heterotrophic manner. Bacterial densities increase with decreasing temperature and are also significantly correlated with increasing numbers of bacteria that are attached or aggregated. The correlation with temperature is hard to explain but may indicate that the bacterial populations present in the water column at this time of year are cold adapted i.e. grow better at lower temperatures. This would be consistent with the bacterial populations present having derived from those that successfully survived the cold winter period. The correlation between bacterial numbers and the number of attached/aggregated bacteria suggests that many of the bacteria present are attached or aggregated. Significantly heterotrophic microflagellate populations existing in this region in the spring are correlated with the number of attached bacteria. This implies these aggregated bacteria may be an important food source for the heterotrophic microflagellate numbers. In particular they could be utilizing bacteria that are attached to or grazing debris sinking from the more active photic zone. This may also explain the higher than usual number of microflagellate numbers found in the aphotic zone in spring. The settling rate of free bacteria has been observed to be 1-2 mm d^{-1}. However when the bacteria are in clumps the settling rates increase considerably to 0.1-1 m d^{-1} (Ducklow et al. 1982). Thus bacteria that are attached to debris or aggregated would sink to the aphotic zone faster and microflagellates that could utilize these aggregates would have a distinct advantage in this zone. Any chlorophyll a in the aphotic zone unless associated with a mixotrophic organism is probably derived from a

decaying or moribund phytoplankter that has sunk from the photic zone. This is consistent with observations in laboratory culture that show mainly free bacteria are associated with living phytoplankton but upon senescence and death of the phytoplankter the numbers of associated attached bacteria increases (Fukami et al. 1983, Albright et al. 1986)

SUMMER

The summer cluster is not as clearly defined as that of spring (Fig. 1.11) and includes the periods from 7/5/84 to 4/9/84 and 24/4/85 to 12/8/85 During this period, particularly in 1984, some microflagellate counts were not carried out sufficiently soon after sampling for the autofluorescent microflagellate numbers and heterotrophic microflagellate numbers to be differentiated by the presence or absence of autofluorescence due to chlorophyll. Consequently there are some missing data for heterotrophic and autofluorescent microflagellate counts.

Depth appears to be the major influence on total microflagellate numbers throughout the water column. Heterotrophic microflagellate numbers increase with decreasing depth and with increasing chlorophyll a and temperature. Clearly the latter two parameters are affected by depth but partial correlations do not demonstrate depth to be the common factor. This could be partly due to too few cases being available for analysis. Another interpretation of these correlations is that both chlorophyll a and temperature are somehow limiting microflagellate growth. The temperature of the water column could be less than optimal for the microflagellates. This may be caused by the influx of snow melt water from the Squamish River that occurs at this time of year (Hoos and Vold 1975). The correlation with chlorophyll a suggests the heterotrophic microflagellates may be grazing small autotrophic organisms. The availability of such organisms for prey may be limited due to the reduced light penetration as

a consequence of the opaque brackish layer of snow melt water on the surface. Numbers of autofluorescent microflagellates are correlated with several parameters all of which are also correlated with depth, however as with heterotrophic microflagellate numbers this common factor is not demonstrated by partial correlations, possibly for the same reason - too few data. Particularly interesting is the increase in autofluorescent microflagellates with increasing bacterial production and bacterial concentration. It is possible some of the autofluorescent microflagellate population are functioning heterotrophically due to the reduced light levels in the water column.

The photic zone

In the photic zone, total microflagellate numbers increase with increasing numbers of autofluorescent microflagellates. Although these parameters are not strictly independent this may indicate many of the active microflagellates in this region are autofluorescent. The increase of autofluorescent microflagellates with increasing chlorophyll a probably reflects the presence of this substance in these organisms as already mentioned. The level of chlorophyll a in this upper part of the water column increases with increasing temperature and with decreasing depth. Clearly, both temperature and the intensity of light (which decreases with increasing depth) affect the rate of growth of organisms that contain chlorophyll a. Chlorophyll a also increases with increasing bacterial numbers. This latter correlation may indicate bacteria are involved in recycling of compounds such as phosphorus and nitrogen that are required for growth by the phytoplankton. Thus the increase in bacterial numbers in this season (Fig. 1.4) is probably partially triggered by the overall lower level of microflagellate numbers (Fig. 1.2) together with an increase in available substrate from phytoplankton that are secreting organic material or from decaying phytoplankton.

The aphotic zone

In the aphotic region of the water column the levels of the microflagellate populations are further depressed (Fig. 1.3). There are no parameters significantly correlated with the concentration of total microflagellates. Heterotrophic and autofluorescent microflagellate numbers decrease with increasing depth. This is the only correlation for autofluorescent microflagellates and probably once again reflects the decreasing light intensity with increasing depth. Heterotrophic microflagellates also increase with temperature, chlorophyll *a* and bacterial biomass. This suggests heterotrophic microflagellates in the aphotic zone are using bacteria as prey and are limited by the amount of bacterial biomass available and implies the temperature in the aphotic zone in the summer is less than optimal for the microflagellate populations that exist there. Increasing heterotrophic microflagellates with chlorophyll *a* again indicates these microflagellates may be using small autotrophic organisms as prey as well as bacteria. This is consistent with the hypothesis that the amount of bacterial biomass is limiting and indicates that under such conditions small phytoplankton represent an important potential prey source.

WINTER

The winter cluster consists of fewer sampling dates than that of summer or spring. It includes the October, November, and December sampling dates in 1984 and the January and February sampling dates in 1985. Unfortunately samples were not obtained during the 1985-1986 winter period. Total numbers of microflagellates in the winter increase with decreasing depth and increasing temperature. This increase in microflagellate concentration with increasing temperature suggests that the temperature in this season is lower than the optimal growth temperature of the heterotrophic microflagellate population present.

Thus in winter an important limiting factor for microflagellate numbers is apparently temperature. Neither heterotrophic nor autofluorescent microflagellates show any significant correlations. This lack of correlation is probably due to there being very few microflagellates present in this season and due to the reduced number of cases available for analysis. Heterotrophic microflagellates do increase with increasing bacterial production but this relationship is not statistically significant. It does however, imply that bacteria still represent the major food source for these small phagotrophic microflagellates. Both bacterial concentration and bacterial biomass increase with increasing temperature indicating that this microflagellate food source is limited by the temperature of the water column in the winter. Bacterial biomass is significantly correlated with the level of chlorophyll a suggesting that chlorophyll a containing organisms may represent a limiting food resource for bacteria. Chlorophyll a is significantly negatively correlated with depth and salinity, again, reflecting the decreasing light intensities in the water column with increasing depth.

In the photic zone and in the aphotic zone there are no significant correlations between total, heterotrophic and autofluorescent microflagellates and any other parameters. This is probably due to the reduced number of cases available for analysis. The number of bacteria in the upper and lower parts of the water column increase with increasing temperature as does bacterial biomass. In the aphotic zone bacterial numbers increase with numbers of aggregated bacteria suggesting that many of the bacteria in this region are aggregated. This is not the case in the photic zone. This is of interest since it has been suggested that bacterial aggregation could be a method of survival for bacteria in nutrient poor environments (Marshall 1979).

Thus in the winter months microflagellate numbers are apparently limited by the abiotic factor, temperature. This also appears to be an important factor in regulating the growth of bacteria, a major prey source of microflagellates. The increase of bacteria with chlorophyll a suggests that the available nutrients in the water column are limiting.

Some dates, 24/4/85, 7/5/84, 19/4/84, 13/3/84, and 25/9/84, do not fit any of the seasonal clusters. These anomalous dates may occur due to storms, abnormal temperature conditions or other disturbances immediately preceding the sampling. It is interesting to note however that the first four dates (24/4/85, 7/5/84, 19/4/84, 13/3/84) fall approximately during the transition period between spring and summer and the last date (25/9/84) falls during the transition between summer and winter. These samples may represent the marginal conditions of the seasons. If this is the case further investigation around these transition periods may lead to a better understanding of the 'switches' between seasons.

Clearly there are substantial differences in the factors affecting microflagellate populations throughout the year. If the data had not been analysed seasonally many of these differences would have been overlooked. This could be a problem in interpreting studies where the seasonal distinction has not been made.

In conclusion, the spring is a season of high microbial activity. In the photic zone heterotrophic microflagellates may be grazing bacteria as demonstrated by the correlation with bacterial concentration and bacterial biomass five days previously. Autotrophic microflagellate numbers are apparently influenced by depth and to some extent bacterial production indicating there may be some cycling of nutrients by the bacteria. An interesting interaction seems to

be occurring in the aphotic zone in spring between the heterotrophic microflagellate numbers and attached bacterial numbers. This is not apparent during the rest of the year. These heterotrophic microflagellates are apparently grazing attached bacteria as a carbon source. The autofluorescent microflagellates in the aphotic region are correlated with bacterial biomass indicating that in this light limited region autofluorescent microflagellates may be functioning in a heterotrophic manner. Bacterial numbers appear to be controlled by their growth rate which is affected by temperature. Significantly, grazing by microflagellate numbers does not control bacterial numbers at this time of year. Possibly the excess of available nutrients produced as a result of the spring phytoplankton bloom (Fogg 1983) allows the bacteria to grow faster than the microflagellates can graze them.

In contrast, the populations of microflagellates are significantly depressed in the 'summer' season. This triggers an increase in the bacterial standing stock. The bacteria seem to be utilizing the phytoplankton extracellular products and or decaying phytoplankton at a rate controlled to some extent by temperature (correlations with chlorophyll a and temperature). Although not demonstrated by the cluster analysis, Figs. 1.2, 1.3 and 1.4 show that in late August to September in both years of study particularly in the photic zone there is a drop in bacterial numbers closely followed by an increase in total microflagellate numbers to reach a peak in October in 1984 and late August/early September 1985. This is further demonstration of the out of phase cycling of bacterial numbers and microflagellate numbers and corresponds to a small autumn bloom previously noted to occur in other temperate sites (Davis et al. 1985, Stewart and Wetzel 1986). This oscillation may be initiated by an increase in organic nutrients e.g. decaying phytoplankton.

Populations are further reduced in winter. In November and December microflagellate populations fall below countable numbers. In winter, temperature appears to be the dominant limiting factor for microflagellate and bacterial populations. The reduced temperature reflects to a certain extent the reduction in light levels that occur at this time of year. These reduced light levels result in the phytoplankton population decreasing and hence there is no longer extracellular material secreted by these organisms. Thus the bacteria are nutrient limited and consequently the microflagellates grazing them also are limited.

The results of this study indicate that the factors affecting microflagellate abundance and distribution vary from season to season. The abundance of these organisms in spring is clearly linked to the availability of their prey. However, this does not appear to be the case in the summer when although prey are available the microflagellate populations are significantly depressed. This could be due to some nutrient other than the bacteria being limiting. A more likely possibility is that during this season the microflagellates may be grazed rapidly by various predators e.g. ciliates (Nisbet 1984), and copepods (Caron 1984). There is some evidence that these organisms graze microflagellates but, as yet, there has been no field study comparing the seasonal populations of copepods, ciliates, microflagellates and bacteria. The microflagellates show a slight increase in numbers in October 1984 and late August/early September 1985. This could be due to the phytoplankton populations that have developed over the summer months senescing and decaying and thus providing a food source for bacteria and, hence, for microflagellates. Alternatively it could be due to a decrease in the populations of organisms that graze microflagellates. The limiting factor for microflagellates during the winter months in Howe Sound appears to be temperature. Temperature does not generally act in isolation; it interacts with

other factors. This is demonstrated in this study by the fact that the bacterial prey of the microflagellates are apparently limited by temperature.

This study serves to emphasize that the availability of prey is an important factor in determining the abundance and distribution of microflagellate populations providing the physico-chemical characteristics are within tolerable limits. This is consistent with studies of other protozoan populations (Jackson and Berger 1984). Seasonal cycles of specific nutrient availability, e.g. aggregated bacteria in the spring aphotic zone, chlorophyll containing organisms in the summer, may affect which species of microflagellates dominate the total microflagellate population. If all the microflagellate species are considered as one group, interesting differences in function and feeding requirements with respect to their role in the microbial food web may be overlooked. Although species identification of these small fragile organisms is problematic this area holds potential for future research. Questions such as whether different species dominate at different times of year and why this might be the case remain to be addressed. The results presented in this chapter imply also the spatial distribution of bacterial prey may influence which types of microflagellates are successful at any one time. For example, in spring many of the bacteria are aggregated and only those microflagellate numbers that are able to utilize bacteria packaged in this manner will be successful. This is also an area that requires further research.

CHAPTER II

AGGREGATED AND FREE BACTERIA AS FOOD SOURCES FOR HETEROTROPHIC MICROFLAGELLATES

INTRODUCTION

Bacterioplankton are among the major prey of heterotrophic microflagellates in aquatic environments (Haas and Webb 1979, Linley et al. 1981, Fenchel 1982b, Davis and Sieburth 1984) and clearly plays a role in determining the abundance and distribution of microflagellates (Chap. 1). Fenchel (1982a) has proposed that heterotrophic microflagellates are uniquely able to feed on free bacterioplankton since they are among the few predators sufficiently small to efficiently graze such minute prey. In spite of frequent reports (Caron et al. 1982, Fenchel 1982a, 1986, Davis and Sieburth 1984) of observations of microflagellates associated with bacterial aggregates[1] and attached bacteria[2] the relative importance of aggregated bacteria as a food source for microflagellates has not been assessed. To date there has been no detailed investigation of the ability of microflagellates to graze aggregated bacteria and only limited investigation of their abilities to graze bacteria attached to surfaces (Gude 1979, Newell et al. 1981, Caron 1984). These studies of grazing of attached bacteria by microflagellates have concentrated on bacteria attached to relatively large particles e.g. 400-1600 um^2 sheets of chitin (Caron 1984) and dead phytoplankton (Newell et al. 1981). Recent work (Pearl 1984, Goldman 1984) emphasizes the importance of microaggregates in oligotrophic systems and indicates a need for the study of the growth of microflagellates associated with small aggregates of

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[1] Aggregated bacteria are defined here as bacteria in intimate association with other bacteria in the absence of another surface.

[2] Attached bacteria are defined here as bacteria attached to a surface.

bacteria.

In communities containing heterotrophic microflagellates several apparently competing species often occur simultaneously. For example both Fenchel (1975) in his study of an Arctic pond and Lighthart (1969) in his study of the Juan de Fuca Strait found species of Bodo, Monas, Rhynchromonas, and Actinomonas , all bacterivorous microflagellates, occurring together. An understanding of: (1) the feeding mechanisms of these small protozoa, and (2) how the spatial distribution of the bacterial food resource affects its availability to different microflagellates may help explain the high species diversity found in their communities. If different feeding mechanisms exist among microflagellates, interpretation of microflagellate abundance data in different environments will need to be re-assessed. This is because the passage of carbon and energy from the bacterial food source through the 'microbial loop' via microflagellates (Azam et al. 1983) may be dependent on both the feeding behaviour of the predator and the spacial distribution of the prey. Evidence indicates that prey species is also important (Sherr et al. 1983) and this may have implications when assessing predation rates for different species.

The study of Howe Sound reported in Chapter 1 indicates a role for aggregated bacteria as an important prey source for microflagellates, particularly in spring. This together with previous observations of microflagellates associated with aggregates and bacteria attached to surfaces (Gude 1979, Newell et al. 1981, Caron 1984) demonstrates a need for further investigation in this area. The purpose of this study is to investigate the ability of two different genera of microflagellates to graze free and aggregated bacteria. Bodo sp. and Paraphysomonas sp. were chosen as experimental organisms as they are commonly found in marine environments (Lighthart 1969, Davis 1982). Bodo spp.

have been frequently observed in association with aggregates (Davis and Sieburth 1984, Fenchel 1986) and Paraphysomonas spp. is generally considered to be a free swimmer that encounters its prey by direct interception (Fenchel 1982b, Davis and Sieburth 1984). This study set out to provide more quantitative evidence about the feeding mechanisms of these organisms with respect to their grazing of free and attached bacteria.

Bodo spp. and Paraphysomonas spp. are members of the Protozoan phylum Sarcomastigophorea (Levine et al.1980). Both microflagellates are small (approx. 10 um in largest diameter) and have two flagella. Bodo spp. differ in that they possess a kinetoplastid at the base of their flagella and are consequently placed in the order Kinetoplastida, class Zoomastigophorea (Levine et al. 1980). Members of this order are always colourless. Paraphysomonas spp. although not pigmented are members of the order Chrysomonadida (Levine et al. 1980), a group belonging to the class Phytomastigophorea.

METHODS AND MATERIALS

The two microflagellates studied, Bodo sp., and Paraphysomonas sp. were donated by David Caron (Woods Hole Oceanographic Institute, Woods Hole, MA.)

Artificial seawater (ASW)(Harrison et al. 1980) was used in all laboratory experiments. Total, aggregated, and free bacteria were prepared from a late exponential culture of a Gram-negative marine bacterium isolate grown in Marine Broth 2216 (Difco) at 22 °C. Cells (total bacteria) were harvested (5900 x g for 20 min), washed and resuspended in ASW . Free bacteria were prepared by filtering these resuspended cells through a 2 um pore size Nuclepore membrane. Aggregated bacteria were retained on the membrane. Where killed cells were

required the culture was treated with formaldehyde (1% final concentration) for 3 h and then washed in three times in ASW to remove formaldehyde. Other methods of killing bacterial cells were investigated but found to be unsatisfactory e.g. ultraviolet light killed cells (265 nm, 30 min. exposure in a shallow Petri dish) rapidly clumped and heat killed (autoclaved) cells lysed. Siliconized flasks were used in all the experiments to minimize bacterial wall growth (Maniatis et al. 1982).

Total, free, and aggregated bacteria, as prepared above, were each resuspended in ASW (200 ml, initial average bacterial concentration in all flasks = 3-6 X 10^6 ml^{-1}). Each preparation was then inoculated with 10 ml of a mid-exponential culture of Bodo sp. and incubated at 22 °C for approximately 75 h. Numbers of free and total bacteria as well as microflagellates were monitored with time. These experiments were repeated using formaldehyde killed bacterial cells. Both the experiments with the live bacteria and those with the formaldehyde killed bacterial cells were repeated with the microflagellate Paraphysomonas sp. Control experiments without microflagellates but with free, aggregated and total bacteria respectively were also performed with both live bacteria and formaldehyde killed and washed bacteria. All experiments were performed in triplicate.

At each sampling time a 20 ml portion was removed from each flask and immediately fixed (3.7% formaldehyde final concentration). For microflagellate counts 3-2.5 ml samples were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) and filtered through a 2 um pore sized 25 mm diameter Nuclepore membrane, prestained with Irgalan black, at a vacuum of not more than 15 mm Hg and examined using epifluorescent microcopy (Porter and Feig 1980)

Total bacteria counts were determined by treating 0.5 ml samples with pyrophosphate (0.001 M) and sonication (100 W for 30 s., Velji and Albright 1986). This dispersed the bacterial aggregates and randomly distributed the bacteria on the filter. The bacteria were then stained with DAPI as described by Porter and Feig (1980). Free bacteria counts were determined by first passing the samples through a 2 um pore sized Nuclepore filter; the filtrate was then treated as above. Numbers of aggregated bacteria were calculated from the difference between numbers of free and total bacteria.

RESULTS

Figs. 2.1 and 2.2 show the growth of Bodo sp. and Paraphysomonas sp. on mixed free and aggregated bacteria. The microflagellate Bodo sp. selectively grazed aggregated bacteria (Fig. 2.1) whereas the microflagellate Paraphysomonas sp. selectively grazed free bacteria (Fig. 2.2). Bodo sp. reduced the density of the aggregated bacteria to 5 X 10^5 ml^{-1} and Paraphysomonas sp. reduced the density of the free bacteria to 0.5 X 10^5 ml^{-1}. In all the figures the aggregated bacteria are expressed as a percentage of the total bacteria at that sampling time. The total bacteria for each sampling time are given in Table 2.1. The reasons for this were two-fold: 1) the investigation was of the relative importance of aggregated bacteria as a food source for microflagellates and 2) some growth of bacteria did take place during the experiment and this confused the results when expressed as numbers of aggregated bacteria.

Bodo sp. grew to a maximum density of 7.50 X 10^3 ml^{-1} when presented with mixed bacteria and 1.40 X 10^4 ml^{-1} with predominantly aggregated bacteria (Fig. 2.3a). Its maximum specific growth rate[3] (umax=0.12 h^{-1}) was higher when given predominantly aggregated bacteria than when presented with mixed aggregated and free bacteria (umax=0.07 h^{-1}). With predominantly free bacteria its maximum specific growth rate was slower (umax=0.05 h^{-1}) and a much lower maximum density was reached (2.01 X 10^3 ml^{-1}). Bodo sp. reduced the percent of aggregated bacteria in both the mixed and the aggregated treatment but in the treatment with predominantly free bacteria the percent of aggregated bacteria began to increase after 20 h (Fig.2.3b).

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[3]u = (ln conc.of microflagellates at time 1 - ln conc. of microflagellates at time zero) / (time 1 - time zero) (Stanier, Adelberg and Ingraham 1976)

FIGURE 2.1. Growth of Bodo sp. (●) on a mixture of free (■) and aggregated bacteria (○).

FIGURE 2.2. Growth of Paraphysomonas sp. (□) on a mixture of free (■) and aggregated bacteria (○).

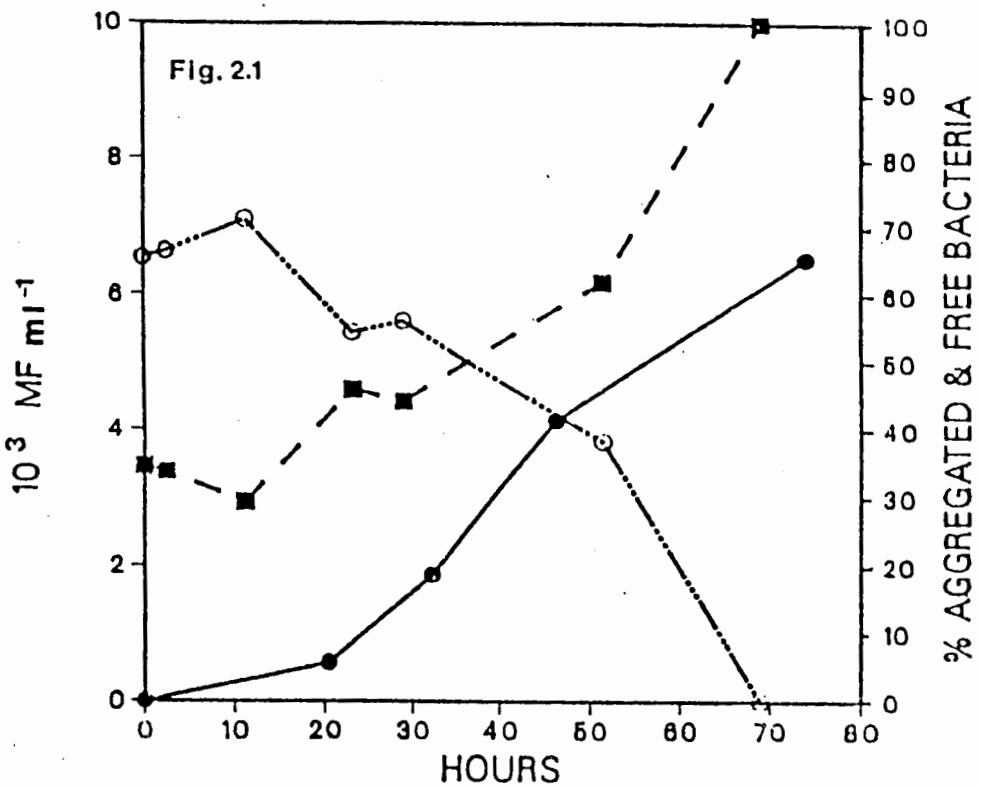


Fig. 2.1
10 ^{3} MF ml^{-1}
% AGGREGATED & FREE BACTERIA
HOURS
0
2
4
6
8
10
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
70
80

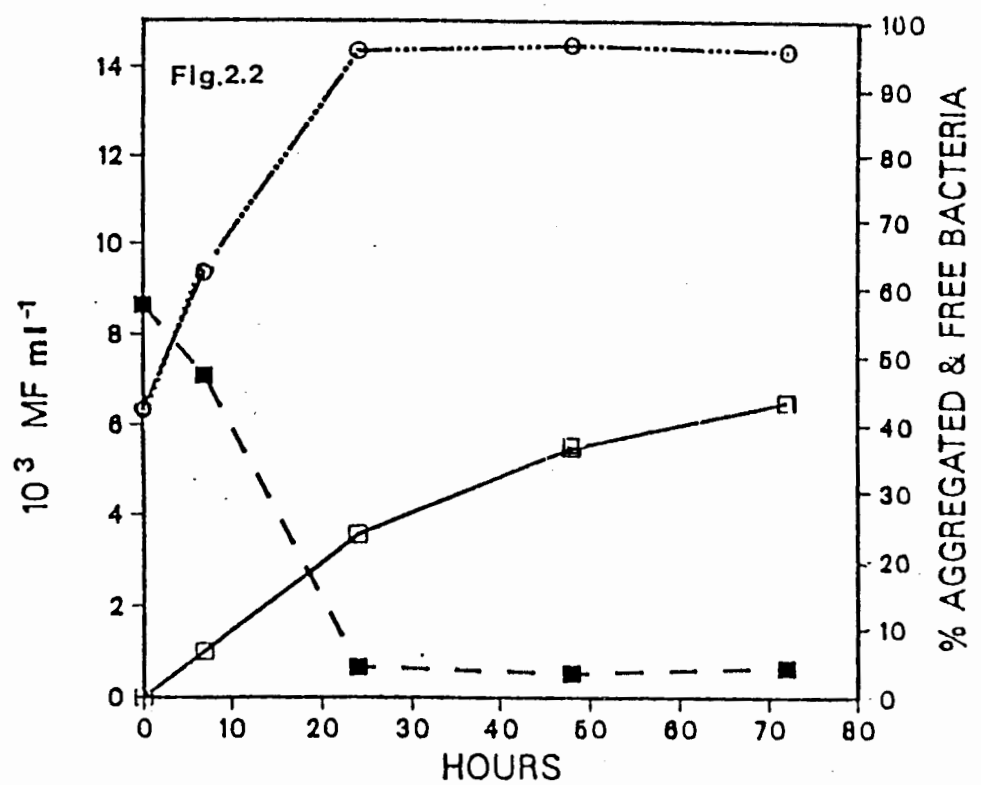


Fig.2.2
10 ^{3} MF ml^{-1}
% AGGREGATED & FREE BACTERIA
HOURS
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
70
80

FIGURE 2.3a. Comparison of the growth of Bodo sp. on predominantly free (●), predominantly aggregated (o) and mixed bacteria respectively (□).

FIGURE 2.3b. Comparison of the percent aggregated bacteria with time in each of the differently treated Bodo sp. cultures.

(●) % aggregated bacteria in the predominantly free bacteria treatment.

(o) % aggregated bacteria in the predominantly aggregated bacteria treatment.

(□) % aggregated bacteria in the mixed free and aggregated bacteria treatment.

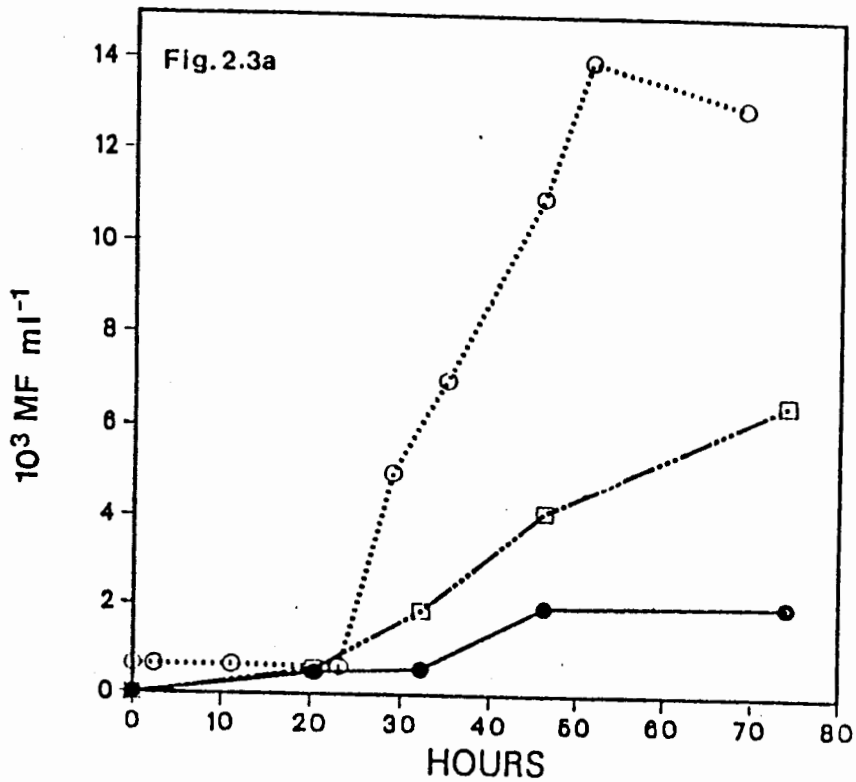


Fig. 2.3a
10^3 MF ml^{-1}
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
70
80
HOURS

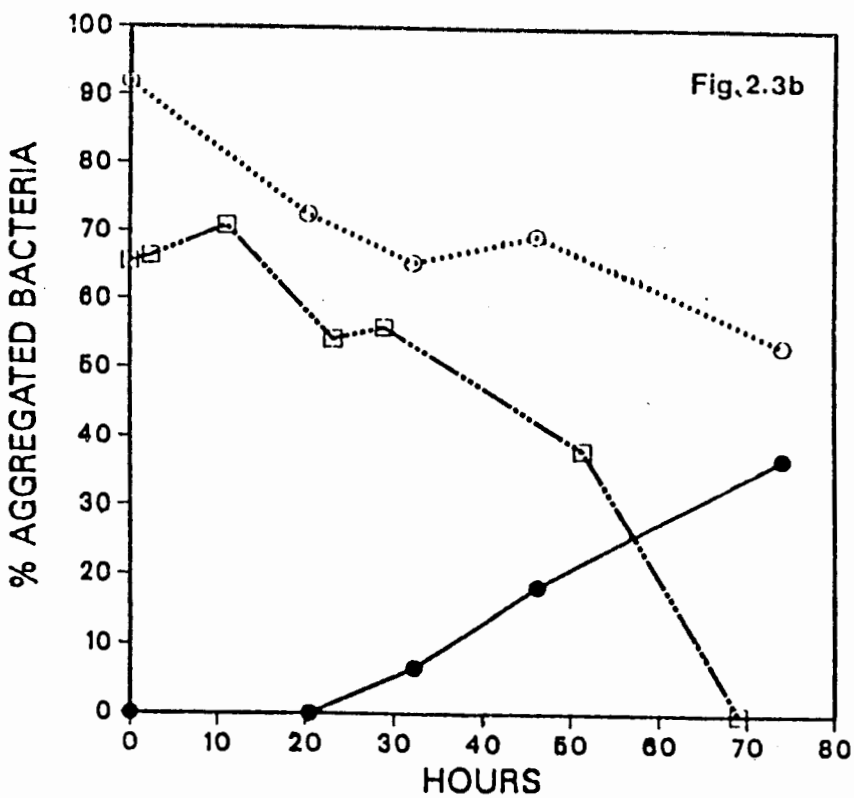


Fig. 2.3b
% AGGREGATED BACTERIA
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
HOURS

Paraphysomonas sp. reached its maximum density (9.90 X 10^3 ml^{-1}) when growing on predominantly free bacteria (Fig. 2.4a). In all treatments the percent of aggregated bacteria increased with time because Paraphysomonas sp. seemed to almost exclusively graze free bacteria. When presented with a mixture of aggregated and free bacteria it did not reach as high a maximum density as with predominantly free bacteria (6.5 X 10^3 ml^{-1}). When growing on predominantly aggregated bacteria its maximum density was further depressed (3.3 X 10^3 ml^{-1}). However in all cases its maximum specific growth rate was similar (umax=0.036, 0.040, 0.039 h^{-1} on free, mixed and aggregated bacterial treatments respectively).

In all the bacterial preparations without microflagellates there was some growth of bacteria. This is in direct contrast with observations made by Fenchel (1982c). Fenchel (1982b) used aged filtered seawater in his experiments; here ASW was used. It is possible that the filtration of the bacteria to separate them into free and aggregated bacteria may have resulted in the release of nutrients from stressed or ruptured cells. Filtration of bacteria through 3 um Nuclepore filters has been demonstrated to result in the release of amines (Ferguson and Sunda 1984). There was more bacterial growth in the experiments where microflagellates were present than in the controls with bacteria alone. A rapid increase in bacterial concentration in the treatments with microflagellates did not take place until approximately 40 h after the start of the experiments. After this length of time in a closed system (batch culture) many interactions and limitations for bacteria and microflagellates may be taking place and hence confuse the result.

Attempts were made to avoid some of the complications arising from bacterial growth in these experiments by repeating them using formaldehyde killed bacterial cells. Unfortunately it was difficult to eliminate all bacteria associated

FIGURE 2.4a. Comparison of the growth of Paraphysomonas sp. on predominantly free (●), predominantly aggregated (o) and mixed bacteria respectively (□).

FIGURE 2.4b. Comparison of the percent free bacteria with time in each of the differently treated Paraphysomonas sp. cultures.

(●) % free bacteria in the predominantly free bacteria treatment.

(o) % free bacteria in the predominantly aggregated bacteria treatment.

(□) % free bacteria in the mixed free and aggregated bacteria treatment.

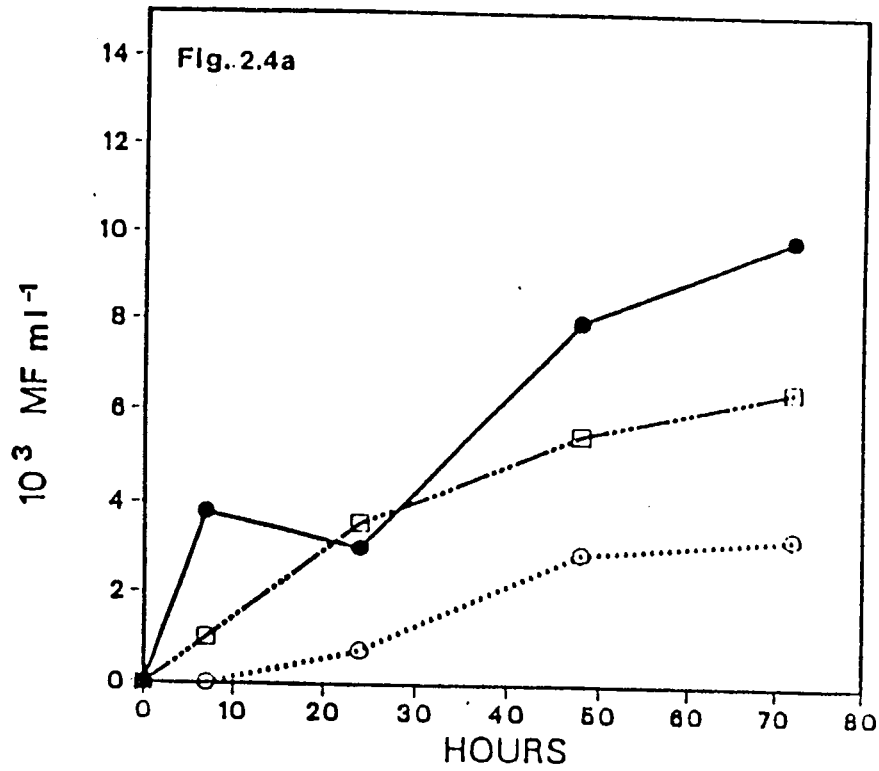


Fig..2.4a
14
12
10
8
6
4
2
0
10^3 MF ml^{-1}
0
10
20
30
40
50
60
70
80
HOURS

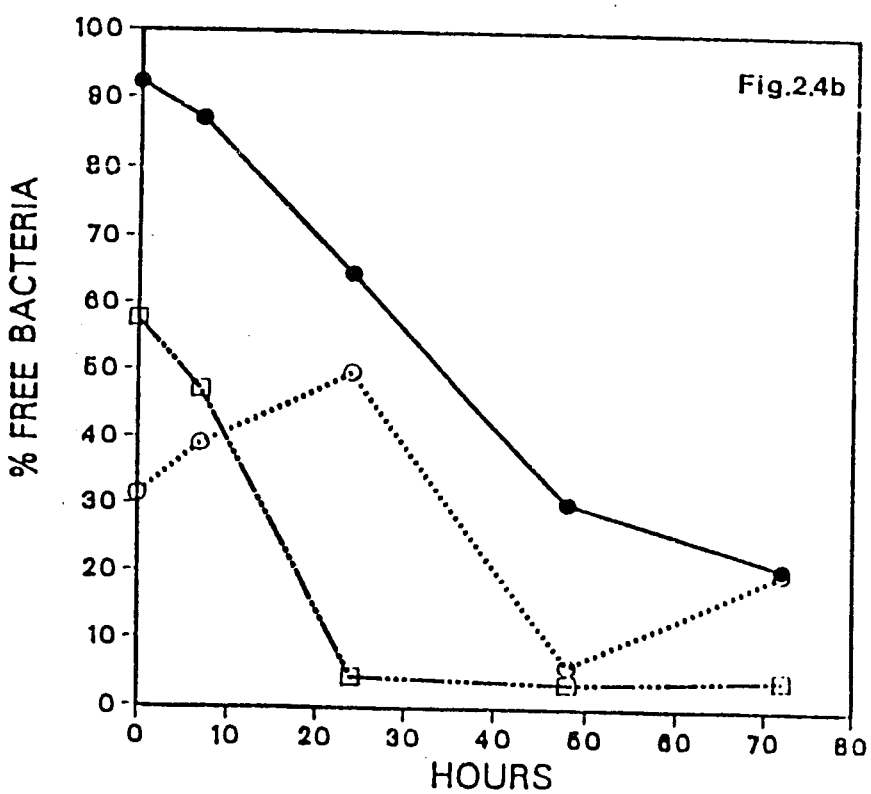


Fig.2.4b
100
90
80
70
60
50
40
30
20
10
0
% FREE BACTERIA
0
10
20
30
40
50
60
70
80
HOURS

FIGURE 2.5a. Percent of aggregated bacteria with respect to time for each treatment with no microflagellates present.

(●) % aggregated bacteria in the predominantly free bacteria treatment.

(o) % aggregated bacteria in the predominantly aggregated bacteria treatment.

(■) % aggregated bacteria in the mixed free and aggregated bacteria treatment.

FIGURE 2.5b. Total number of bacteria with time for each treatment with no microflagellates present.

(●) predominantly free bacteria treatment.

(o) predominantly aggregated bacteria treatment.

(■) free and aggregated bacteria treatment.

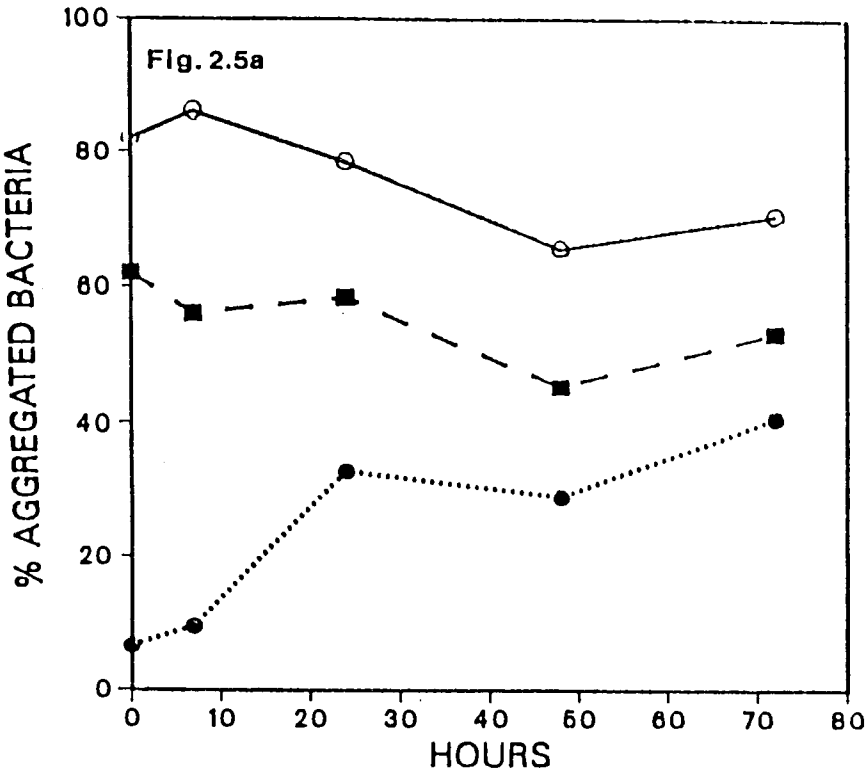


Fig. 2.5a
% AGGREGATED BACTERIA
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
HOURS

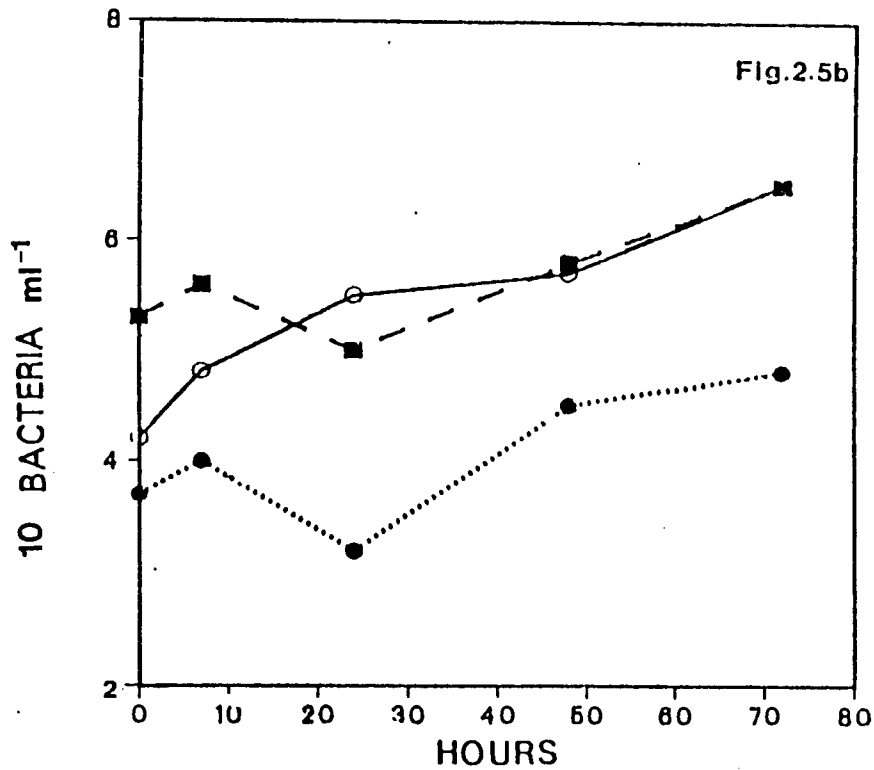


Fig.2.5b
10 BACTERIA ml-1
8
6
4
2
0
10
20
30
40
50
60
70
80
HOURS

TABLE 2.1. TOTAL NUMBER OF BACTERIA IN EACH DIFFERENT TREATMENT FOR BODO SP. AND PARAPHYSOMONAS SP. RESPECTIVELY.

Bacterial numbers are all X 10^6

			BODO SP.					
SAMPLE TIME (h)	0.00	2.50	10.00	20.45	29.00	46.25	51.50	74.00
TREATMENT								
Bodo sp. + free bacteria	3.60	-	2.50	1.78	-	1.30	-	6.20
Bodo sp. + aggregated bacteria	1.60	1.90	2.40	4.72	4.08	-	7.60	1.84
Bodo sp. + mixed free and aggregated bacteria	3.37	-	-	3.56	3.70	3.93	-	8.77
			PARAPHYSOMONAS SP.					
SAMPLE TIME	0.00	-	7.00	24.00	-	48.00	-	72.00
TREATMENT								
Paraphysomonas sp. + free bacteria	6.40	-	9.20	11.90	-	5.90	-	6.80
Paraphysomonas sp. + aggregated bacteria	7.80	-	4.10	3.71	-	10.00	-	7.60
Paraphysomonas sp. + mixed free and aggregated bacteria	6.07	-	9.70	8.08	-	5.70	-	12.40

with the microflagellate cultures and still retain viable microflagellates. Therefore complications due to bacterial growth still occurred and the results were substantially the same.

Both Bodo sp. and Paraphysomonas sp. were frequently seen in association with aggregates of bacteria when samples were observed under the epifluorescent microscope.

DISCUSSION

Aggregates of bacteria represent high concentrations of prey and as such could be an important microflagellate food source. This work demonstrates that at least one microflagellate species selectively grazes aggregated bacteria. It further suggests that aggregation of bacteria may even play a role in the feeding mechanisms of microflagellates that selectively graze free bacteria. This is the first study to show quantitatively, using defined microflagellates, that different feeding mechanisms exist among these small bactivores with respect to their grazing of aggregated bacteria. The results indicate caution should be used in selecting a microflagellate species and their food source for estimation of predator feeding rates, since these rates may be dependent on the predator species and their prey preferences. For example, if Bodo sp. was used to estimate predator feeding rates a different result would be obtained depending on what percentage of the prey was aggregated. These results would be different from those obtained when Paraphysomonas sp. was used as the test organism.

It may be significant that in the microflagellate treatments the number of bacteria, although complicated by cryptic growth, are reduced to around 10^5 ml^{-1} approximately equivalent to levels generally found in a variety of marine

environments (estuaries- 50 x 10^5 bacteria ml^{-1}, coastal waters- 10-50 x 10^5 bacteria ml^{-1}, offshore waters 0.5-10 x 10^5 bacteria ml^{-1}, and deep waters 0.1 x 10^5 bacteria ml^{-1}, as summarized by Azam et al. 1983). This further implicates microflagellates as important factors in controlling bacterial populations. The relatively low bacterial concentrations observed here and in natural waters suggests there is a threshold below which it is difficult for the microflagellates to catch bacteria. Some evidence suggests that such a population threshold level may be due to size selective grazing on the part of the microflagellates (Andersson, Larsson, and Hagstrom 1986).

The specific growth rates calculated here are comparable with those found in other studies (e.g. Rivier et al. 1986, Fenchel 1982b, Davis and Sieburth 1984, Sherr and Sherr 1984). The specific growth rate for Bodo sp. when growing on predominantly aggregated bacteria is higher than observed with predominantly free or mixed free and aggregated bacteria. This could be because its bacterial food source is in the 'preferred' aggregated state. The fact that the specific growth rate of Paraphysomonas sp. does not increase when provided with predominantly free bacteria (its 'preferred' food source) may indicate its feeding mechanism is more complicated.

There was less bacterial growth in the treatments with bacteria alone than in the treatments with both bacteria and microflagellates. This suggests there is some cycling of nutrients occurring in the presence of the microflagellates that results in enhanced bacterial growth. This is consistent with observations concerning the role of microflagellates in the cycling of nitrogen and phosphorus (Andersen et al. 1986, Goldman et al. 1985).

One possible explanation for the fact that even the free bacteria grazer, Paraphysomonas sp. is frequently associated with aggregates is that this microflagellate may use aggregates as "attachment sites" from which to graze free bacteria. The bacterial aggregates may act as 'bacteria factories' continually producing and releasing free bacteria, hence association with a bacterial aggregate would be advantageous for a microflagellate grazing free bacteria.

Lighthill (1976) has shown that if a flagellum is attached to a solid surface the associated disturbance fields in the fluid caused by flagellar movement are much larger than they would normally be, i.e. the microflagellate would be able to draw prey from a much larger area when it is associated with a bacterial aggregate. It may be significant that movement by an organism of low Reynolds number near a surface, such as an aggregate, results in both higher swimming velocity and propulsive efficiency (Lighthill 1976). This would enable a microflagellate to swim over a larger area for less cost in terms of energy.

This evidence of an association of these microflagellates with bacterial aggregates emphasizes the potential implications of the aggregate spinning wheel hypothesis (Goldman 1984) for food chain dynamics. A preference for grazing bacterial aggregates rather than free bacteria makes microflagellates such as Bodo sp. suited to graze in bacteria enriched microenvironments.

These results demonstrate aggregation of bacteria can play a major role in determining which microflagellate species predominate in an environment. An implication of these results is that some microflagellate species selectively graze aggregated bacteria whereas others selectively graze free bacteria. Qualitative observations made here and elsewhere indicate that bacterial aggregates can be important in both feeding mechanisms. There is much evidence that the bacterial

prey of microflagellates is not homogeneously distributed, as is commonly assumed, but is patchily distributed throughout the water column due to aggregation of bacteria and the attachment of bacteria to surfaces (Marshall 1979). This would have important consequences not only with respect to the feeding mechanism of microflagellates but also with respect to the location of bacterial prey by microflagellates in natural systems. When the bacterial food resource is patchily distributed a chemosensory mechanism would be one method of locating these enriched microenvironments. Chemosensory mechanisms are known to exist among protozoa (e.g. Jennings 1906, Levandowsky et al. 1984) but there is little evidence that they are present in microflagellates. Concentrations of solutes greater than in the bulk water would occur around aggregates as the solutes diffuse from these centres of concentrated microbiological biomass and/or activity (Fletcher 1984). Thus possession of a chemosensory mechanism would explain in part why microflagellates are so often found in association with bacterial aggregates. Cairns and Ruthren (1970) and Cairns (1982) have show microflagellates to be well adapted to locating substrates and colonizing them before other protozoa. Both substrate size and distance from the substrate have been demonstrated to be important factors in protozoan colonization of particulates (Henebry and Cairns 1980). These factors would affect the rate of diffusion of chemicals from the aggregate and therefore the ability of the protozoa to sense it.

The results presented here can also be interpreted as an example of food specialization that allows a form of resource partitioning. This may enable apparently competing genera/species to exist in the same environment thus explaining the microflagellate species diversity found in many environments.

Clearly these different feeding mechanisms and the relative aggregation of the prey should be taken into account when considering the significance of microflagellates in the natural environment. These factors are obviously important in determining the effectiveness with which the bacterial prey resource is captured by a given microflagellate. The existence of different feeding mechanisms among microflagellates will affect the passage of carbon and energy from bacteria to microflagellates in the 'microbial loop' (Azam et al. 1983).

CHAPTER III

THE ADVANTAGE OF A CHEMOSENSORY MECHANISM IN MICROFLAGELLATE PREY DETECTION

INTRODUCTION

The observation that aggregated and attached bacteria are important in the feeding of some heterotrophic microflagellates emphasizes the significance of the distribution of bacterial prey in space with respect to their microflagellate predators (Caron et al. 1982, Davis and Sieburth 1984, Chapter 2, this thesis). In marine waters bacteria are normally present at densities of 10^4 to 10^6 ml^{-1} (Sieburth 1984). This is an average concentration which does not account for bacterial distribution in water. Clearly, spatial heterogeneity of prey can be vitally important and may affect the ability of microflagellates to locate their bacterial prey (e.g. Chap. 2, this thesis, Alldredge and Cohen 1987). Chemosensory mechanisms are generally supposed to be a powerful way in which predatory protozoa can identify and find their prey (Seravin and Orlovskaja 1977). It has been suggested that microflagellates possess a chemosensory mechanism that enables them to locate their prey but there is little evidence to support this (Goldman 1984). It is not clear if such a food-finding mechanism is essential for their survival. It could be argued that since swimming costs little in terms of energy for microflagellates (Fenchel 1987) they may be able to locate sufficient food by swimming at random. To investigate this the advantage conferred on a microflagellate by a chemosensory mechanism has been estimated. This calculated advantage factor is applicable in particular to microflagellates that encounter their prey when swimming and more generally to any other organism existing in a low Reynolds number ($Re<1$) environment that locates their prey by direct

encounter.

MODEL

To create the model, the important interactions between factors considered to influence microflagellate energy gain were diagrammed in a flow chart (Fig. 3.1). Inspection of Fig. 3.1 shows that the microflagellate cannot influence many of the parameters in the model and has only a few ways to increase net energy obtained per unit time. These include decreasing expenditures that go into growth and reproduction, or increasing energy intake by swimming faster or by finding prey more effectively.

It is convenient to rearrange the model in Fig. 3.1 from:

Net energy/unit time gained by a microflagellate

	energy		energy		energy		energy		
=	gained	-	used	-	used	-	used	-	used (1)
	from		for		for		for		
	food		swimming		maintainance		growth		reproduction

to

Net	energy		energy		energy	
energy/unit=	gained	-	used	-	used	(2)
time	from		for		for	
	food		swimming		maintainance	

where net energy/unit time is now understood to be the energy available for growth and reproduction. An assumption implicit in this idea is that all energy in excess of swimming and maintainance is used for growth and reproduction. Prior to developing the model, units and abbreviations were chosen for each quantity.

FIGURE 3.1. Factors affecting microflagellate net energy gain per unit time.

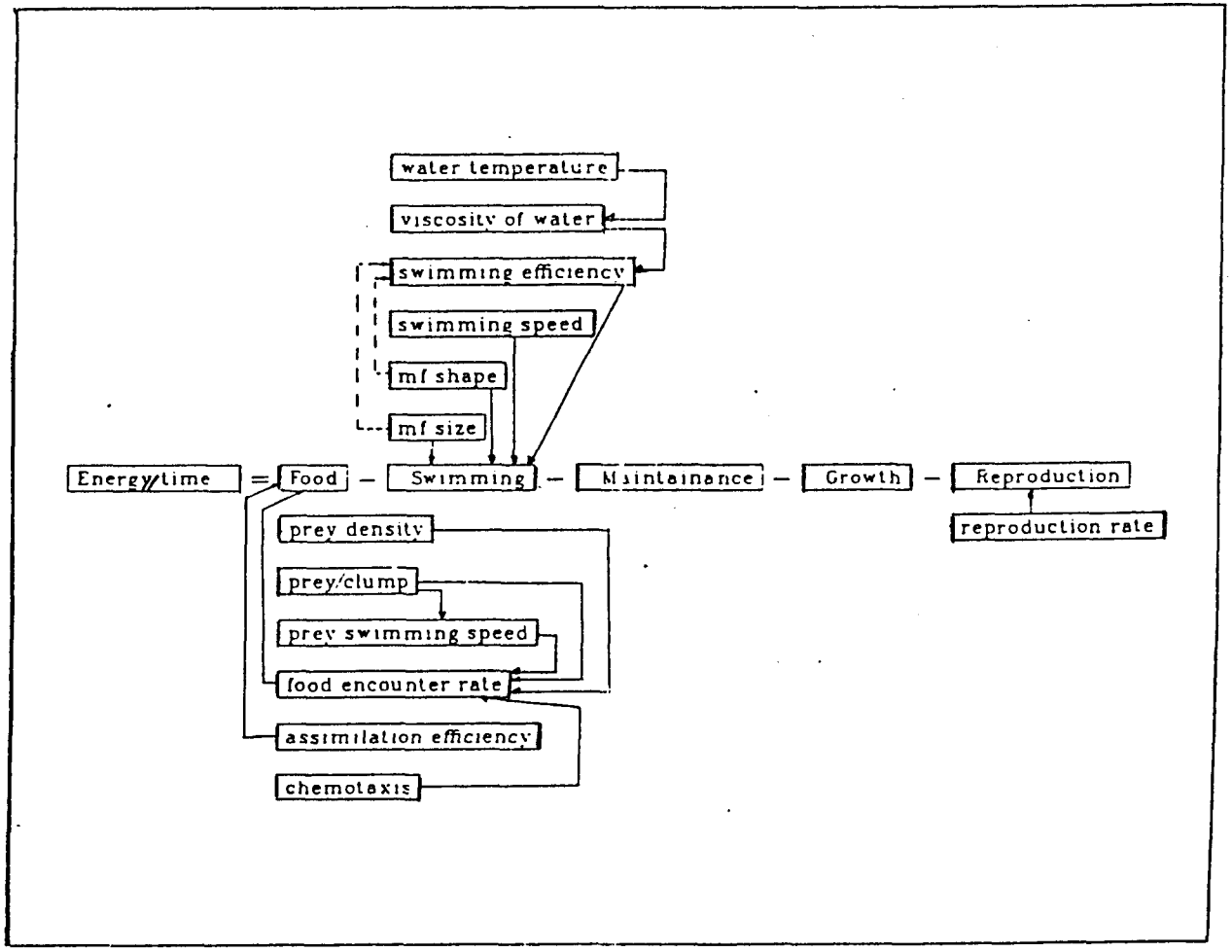


TABLE 3.1 VALUES FROM THE LITERATURE AND ABBREVIATIONS FOR THE VALUES IN THE MODEL.

Friction coefficient mf refers to the frictional coefficient for a sphere the size of a microflagellate (many microflagellates are spherical (Cynar et al. 1985, own observations)) moving through water of some viscosity. It is possible to also calculate f for ellipsoids from formulas found in physics texts. Viscosity is a function of water temperature. The value is provided for 10°C.

Quantity	Abbrev.	Units	Value	Reference
radius of microflagellate	rmf	cm	1-5 um	Sherr et al. 1983, Fenchel 1982a
			1-10 um	Sieburth et al. 1978
radius of a bacterial clump	rc	cm	varies with bcl	
number of clumps mL^{-1}	cmL	cm^{-3}	variable	
bact $clump^{-1}$	bcl	unitless	variable	
time to eat a bacterium	tb	sec	no data	
velocity of microflageliate	v	cm sec^{-1}	5-350E-4	Goldman 1984
			75-370E-4	Throndsen 1970
energy value of a bacterium	eb	J	—	
assimilation efficiency	a	unitless	14-40% (based on bacteria)	Fenchel 1982b, Kopylov and Moiseev 1980, Sherr et al. 1983, Caron et al. 1985
swimming efficiency	S	unitless	1-2% (based on bacteria)	Purcell 1977
maintainance energy	M	J sec^{-1}	—	
frict. coeff. of a microflag.	f	g sec^{-1}	$6\pi(rfm)(vis)$	
viscosity	vis	centipoise	1.50	

These, in addition to reported values from the literature for each quantity, where available, are shown in Table 3.1.

Below, the model is elaborated, first, for a microflagellate predator without a chemosensory mechanism, and then for a microflagellate with a chemosensory mechanism. In both cases net energy increase per second is calculated for a single microflagellate. The assumptions of this model will be discussed later.

Without a Chemosensory mechanism (Pac-man approach)

It is assumed the microflagellate swims in a linear fashion and eats every clump of prey it touches (Bacteria can occur clumped or not; when not clumped bcl=1.). The microflagellate touches a clump if the centre of the clump is within (rmf+rc) of the centre of the microflagellate as the microflagellate passes the clump. In swimming a distance D cm, the microflagellate sweeps out a volume $\pi(rmf+rc)^2D$. Entirely or partially in this volume there are clumps containing $\pi(rmf+rc)^2D(cmL)(bcl)$ bacteria. The time spent eating in this D is $\pi(rmf+rc)^2D(cmL)(bcl)(tb)$ seconds. The time to swim D is Dv^{-1} seconds so the total time to travel D is:

$$\pi(rmf+rc)^2D(cmL)(bcl)(tb) + Dv^{-1} \ (s) \qquad (3)$$

Food intake in D is

$$\pi(rmf+rc)^2D(cmL)(eb)(a) \ (J) \qquad (4)$$

The energy required to swim D at some velocity v is (Stokes law):

$$fvDS^{-1}(10^{-9}) \qquad (5)$$

where $f=6\ \pi(rfm)(vis)$. The 10^{-9} factor is the product of 10^{-2} needed to convert

centipoise to poise and 10^{-7} needed to convert ergs to Joules.

Equations (3)-(5) have been stated in terms of distance. To solve with respect to time (5) is subtracted from (4) and divided by (3). Then the maintainance energy which has units of J s^{-1} can simply be subtracted from the result. D cancels and the net energy gain per second without a chemosensory mechanism is:

$$\text{energy/time}_{\text{NOCHEMO}} = \frac{\pi(rmf+rcl)^2(cmL)(bcl)(eb)(a)-fvS^{-1}(10^{-1})}{\pi(rmf+rcl)^2(cmL)(bcl)(tb) + v^{-1}} - M \ (J\ s^{-1}) \qquad (6)$$

With a chemosensory mechanism

One way to model a chemosensory mechanism is to assume that the microflagellate swims to the nearest bacterial clump, eats it, and then repeats this process. The average distance from a randomly chosen point, the predator, to the nearest prey is determined as follows: Consider one ml which contains cmL clumps of bacteria. Treat the clumps as points. The ml can be divided up into x small volumes such that x>>cmL. Each small volume is 1/x ml. If a small volume is chosen at random the probability P that it contains exactly one clump is cmL/x. The P that it contains exactly two clumps is $(cmL/x)^2$, exactly three clumps $(cmL/x)^3$ etc. The P that this small volume contains no clumps is 1 minus the P that it contains at least one clump, i.e.

$$P(\text{no clumps in small volume}) = 1-cmL/x-(cmL/x)^2 \ldots\ldots \qquad (7)$$

However for any series $1 + p + p^2\ldots\ldots = (1 - p)^{-1}$ so equation (7) can be simplified:

$$P(\text{no clumps in small volume}) = (1 + cmL/x)^{-1} \qquad (8)$$

Now consider that the predator is in the centre of a small sphere, the size of

which is y small volumes. Thus the sphere has a volume y/x. The probability that this sphere contains no clumps of prey is

$$P(\text{no clumps in sphere}) = (1 + cmL/x)^{-y} \quad (9)$$

But if the volume of the sphere is y/x then

$$y/x = 4/3\pi R^3 \quad (10)$$

where R is the radius of the sphere. Rearrange to

$$y = (4/3)x\pi R^3 \quad (11)$$

Therefore the probability that a sphere, with a radius R, contains no prey is

$$P = (1+cmL/x)^{-4/3\pi xR^3} \quad (12)$$

The base of natural logs, e, equals $(1+1/x)^x$ as x goes to infinity. So let the number of small volumes x, go to infinity and the expression (12) becomes

$$P = e^{-4/3cmL\pi R^3} \quad (13)$$

This is the probability that the predator will have to swim further than R to find a clump of prey. If P=0.5, then $R/cmL^{-1/3} = 0.55$. So the distance that the predator has to swim half the time to get the nearest prey is $0.55cml^{-1/3}$. This result is used below for the purpose of calculating the time between clumps. The time to swim between clumps is then $(0.55)cmL^{-1/3}v^{-1}$. The time spent at each clump is bcl(tb). So the total time spent getting from one clump to the next and eating it is:

$$(0.55)cmL^{-1/3}v^{-1} + bcl(tb) \quad (s)$$

(14)

The energy obtained in this time is:

$$bcl(eb)(a) \quad (J) \tag{15}$$

Energy required for swimming is:

$$fv(0.55)cmL^{-1/3}S^{-1}(10^{-9}) \quad (J) \tag{16}$$

So the net energy gain with a chemosensory mechanism is ((15) - (16))/(14) -M. This is:

$$\text{energy/time}_{CHEMO} = \frac{(bcl)(eb)(a) - fvS^{-1}(10^{-9})(0.55)cmL^{-1/3}}{(0.55)cmL^{-1/3}v^{-1} + bcl(tb)} - M \quad (J\ s^{-1}) \tag{17}$$

RESULTS

The relative advantage conferred by a chemosensory mechanism is estimated by comparing the $\text{energy/time}_{NOCHEMO}$ to $\text{energy/time}_{CHEMO}$. This has been done by creating a parameter called the advantage factor;

$$\text{Advantage factor} = \frac{\text{energy/time}_{CHEMO}}{\text{energy/time}_{NOCHEMO}}$$

The larger this factor the greater the benefit, in terms of energy, conferred upon the microflagellate which possesses a chemosensory mechanism. It should be obvious that the advantage factor will vary depending upon the environment of the microflagellate. The advantage factor as well as $\text{energy/time}_{CHEMO}$ and $\text{energy/time}_{NOCHEMO}$ are shown in Table 3.2 for a variety of environmental situations.

TABLE 3.2. THE ENERGY GAIN PER SECOND FOR A MICROFLAGELLATE WITH AND WITHOUT A CHEMOSENSORY MECHANISM.

The table is arranged so that total bacterial densities of 10^3 appear followed by 10^4, 10^5 and 10^6. Bacterial clump radius was calculated by assuming that the bacteria in each clump were packed without spaces into a sphere. The radius of the sphere was then calculated. The other parameters used were rmf=.0005, tb=0, v=.015, eb=4.67 x 10^{-4}, S=1, a=.4, and vis=1.50 (as defined in Table 3.1).

cml[1]	bcl[1]	ENERGY CHEMO[2]	ENERGY NOCHEMO[2]	ADVANTAGE FACTOR[2]
1	1000	5.09E-7	6.20E-13	821699
10	100	1.10E-7	3.78E-13	289998
100	10	2.36E-8	2.86E-13	82567
1000	1	5.09E-9	2.48E-13	20545
1	1E4	5.09E-6	1.34E-11	379227
10	1000	1.10E-6	6.22E-12	176216
100	100	2.36E-7	3.81E-12	62009
1000	10	5.09E-8	2.89E-12	17612
1E4	1	1.10E-8	2.51E-12	4376
1	1E5	5.09E-5	3.83E-10	133181
10	1E4	1.10E-5	1.34E-10	81684
100	1000	2.36E-6	6.23E-11	37947
1000	100	5.09E-7	3.82E-11	13349
1E4	10	1.10E-7	2.90E-11	3790
1E5	1	2.36E-8	2.51E-11	941
1	1E6	5.09E-4	1.35E-8	37780
10	1E5	1.10E-4	3.83E-9	28692
100	1E4	2.36E-5	1.34E-9	17598
1000	1000	5.09E-6	6.23E-10	8175
1E4	100	1.10E-6	3.82E-10	2876
1E5	10	2.36E-7	2.90E-10	817
1E6	1	5.09E-8	2.51E-10	203

1 - as defined in Table 3.1

2 - as defined in text.

The advantage factor is greater than 1 for all situations modelled. Even when there are 10^6 individual (non-clumped) bacteria per ml, the chemosensory predator is 203 times better at obtaining food.

DISCUSSION

The trends shown by the model are striking. When the average density of bacteria is constant, the degree of clumping affects the ability of both chemosensory and non-chemosensory predators to find them. In both cases clumping increases the ability of the predator to locate bacteria but the chemosensory predator is most strongly affected. This indicates that clumping is not an effective strategy, by bacteria, under the conditions of the model to avoid predation. A second trend is that as the average bacterial density decreases, it is more of an advantage for the microflagellate to be chemosensory. This is intuitively reasonable; when prey are scarce the ability to locate them is at a premium.

The model allows the calculation, I believe for the first time, of the advantage conferred by a chemosensory mechanism. It is surprising that the advantage is so large. Even at prey densities of 10^6 it is still advantageous to possess such a mechanism. Although such a mechanism is not essential for survival in all environments it would generally enable a microflagellate to use food energy more efficiently. It is probable that natural selection would perpetuate species that are able to interact with their environment in a near optimal manner. The possession of a chemosensory mechanism increases the chance of survival by the microflagellate when food is scarce and when food is plentiful it enables the microflagellate to divert more energy to reproduction.

These are qualities that improve fitness and hence are likely to be transmitted to subsequent generations (Alexander 1982).

Several assumptions are contained in the model and affect interpretation of the results generated. These are listed below.

1. It is assumed that if the microflagellate encounters a clump it eats all the bacteria in that clump. While this is reasonable for a chemosensory microflagellate (it can 'sense' the presence of the other bacteria and hence remains to graze them) it may not be totally applicable to microflagellates that have no chemosensory mechanism. If this is the case the relative advantage of possession of a chemosensory mechanism is underestimated.

Charnov (1976) suggests it would be most profitable for an organism to find patches where the density of food is higher than the average density of food items in the entire habitat but indicates once feeding has reduced the food density in that patch to the average value it is more profitable to move on. This would violate the above assumption (1). However, it is not clear whether this hypothesis would hold for small organisms with low Reynolds numbers and relatively short generation times. Furthermore if the prey are stationary the number of prey removed has no effect on finding the remainder. This is the case for the model proposed here so it is unlikely Charnov's hypothesis is applicable.

2. The radius of a microflagellate, swimming velocity, energy per bacterium and assimilation and swimmming efficiencies of a microflagellate are assumed to be constant. These factors would actually vary with the physiological state of the microflagellate or bacterium and probably also with species. Constant 'average' values have been used to keep the model simple.

3. The predator is indifferent to its condition. It does not change its strategy if

it is starving for example. Although this assumption is violated with some microflagellates, (Fenchel 1982b) which undergo transformation into 'swarmers', small rapidly motile cells, when starving, I have used it for the sake of simplicity. Furthermore, if both chemosensory and nonchemosensory microflagellates can swarm, this ability has a negligible effect on the model.

4. The microflagellate ingests and assimilates food as rapidly as it encounters it (tb = 0). A maximum ingestion rate of 254 bacteria h^{-1} has been reported (Fenchel 1982b) under laboratory conditions. The relevance of this number to field situations is not clear though. It is possible, for example, that bacteria in laboratory cultures are larger than those normally encountered by the microflagellate. If this were true the ingestion rate of smaller bacteria could be considerably higher than 250 h^{-1}. The value of tb however does have a significant effect on the chemosensory microflagellates. If tb is 14 s (approximately 257 bacteria h^{-1}), then the chemosensory predator encounters prey more rapidly than it can ingest them. Two important consequences of this are 1) that the energy taken in by the chemosensory microflagellate is constant for most of the situations shown in Table 3.2 and because of this the advantage of chemosensory ability is considerable lower than it is when tb is 0 and 2) since prey are being encountered more rapidly than they can be ingested, the chemosensory microflagellate can exercise much more choice in the prey it ingests than can the nonchemosensory microflagellate.

5. All prey are of equal value. Certain bacterial prey, particularly pigmented ones, have been demonstrated to be toxic to protozoa (Skinner and Shewan 1976). Bacteria are not uniform in size (e.g. Pedros Alio and Brock 1982, Andersson et al. 1986) nor in energy value (Laybourn-Parry 1984) and hence are not all of equal nutritional value. This assumption (5) would result in a conservative estimate of the advantage of a chemosensory mechanism since possession of

such a mechanism may enable a microflagellate to take advantage of chemical cues about the palatability of the food. Such cues may also convey information about the nutritional adequacy of the food. Hence time and energy would not be expended on non-edible, non-nutritional food.

6. The prey have zero velocity. For a clump this is reasonable but can be violated for single prey. The assumption even for single prey however is justified because motile bacteria have an average swimming speed of around 30 um sec^{-1} (Purcell 1977, Roberts 1981) which is much less than the average velocity of microflagellates (Table I).

7. Implicit in assumption 6 is that the prey have no avoidance behaviour.

8. The bacterial clumps are spherical. While this is not true in natural waters, it is unclear that deviations from sphericity change the result. If for example the clumps were oblate ellipsoids, they would appear to the microflagellate sometimes "on edge" and other times would present a maximum area. Similar arguments can be applied to other shapes to suggest that if the shapes are oriented without respect to the position of the microflagellate, then this parameter is not important.

9. It is assumed that the cost in energy to an organism of a chemosensory response is negligible. I do not know if this is reasonable since this is an area that recieved little study in past investigations of chemosensensory mechanisms. This is clearly an area that deserves further consideration.

10. Maintenance energy of a microflagellate is also assumed to be negligible. This is consistent with observations made by Fenchel (1982a).

11. It is assumed the chemosensory microflagellate can always detect the nearest prey. This is dependent on the distance to the nearest prey, the sensitivity of the microflagellate, the rate of production and diffusion of the compound(s) to which the microflagellate is chemosensory, turbulence of the surrounding water

and the size of the bacterial clump (source). The distance from the chemosensory microflagellate to the nearest prey half of the time as calculated for the model is 0.55 x $cmL^{-1/3}$. Therefore for the cases considered in Table 2 (10^3–10^6 cmL) this lies between 55–550 um. It seems reasonable to suggest that a chemosensory microflagellate could detect chemicals at these distances from the source.

These calculations demonstrate that possession of a chemosensory mechanism gives a microflagellate an advantage both in terms of energy and prey selection as compared with a microflagellate without such an ability. This advantage is present both when prey are abundant and when the prey are scarce. However, a chemosensory mechanism confers a far greater advantage when prey are scarce. The model also suggests that clumping by bacteria is not an effective strategy to avoid predation. A chemosensory mechanism allows a microflagellate to efficiently exploit available food. When food is scarce or patchily distributed such a mechanism would enable microflagellates to locate it more efficiently and when food is plentiful microflagellates would be able to divert more energy to reproduction. Thus a chemosensory mechanism would be important to a microflagellate for locating food items and may also play a role in prey discrimination. It is probable that natural selection would perpetuate species of microflagellates that possess such a mechanism since it would increase the chance of survival of a microflagellate and also increase the energy availible for growth and reproduction.

CHAPTER IV

PSEUDOBODO TREMULANS, A HETEROTROPHIC MICROFLAGELLATE, HAS A CHEMOSENSORY RESPONSE

INTRODUCTION

The existence of chemosensory mechanisms among protozoa has been recognized since the turn of the century (Jennings 1906). Chemosensory reactions have been demonstrated in a number of protozoa, e.g. Tetrahymena thermophila is attracted to complex peptide mixtures and amino acid mixtures (Leick and Hellung-Larson 1985) and Paramecium tetraurelia is attracted to acetate (Van Houten 1977). Much of this work has been done using 'weed species', i.e. species kept for many years in laboratory culture and consequently subject to genetic drift away from the wild-type genome. There are few reports in the literature of chemosensory mechanisms in protozoa recently isolated from the natural environment. Engelmann (1882) and Pfeffer (1888) demonstrated a response by Bodo sp., a kinetoplastid, to meat extract and other substances. Bodo sp. was also observed to aggregate at an oxygen source (Fox 1921). Since these pioneer studies with the microflagellate Bodo sp. chemosensory mechanisms have been demonstrated among the larger structurally distinct dinoflagellates. For example both Gymnodinium fungiforme and Crypthecodinium cohnii, non-photosynthetic dinoflagellates, are attracted to certain amino acids (Hauser et al 1975, Spero 1985). Goldman (1984) recently hypothesized that heterotrophic microflagellates might locate enriched microenvironments by a chemosensory mechanism. However, there is little evidence that marine heterotrophic microflagellates possess such a mechanism. Theoretical calculations (Chap 3) indicate that a chemosensory mechanism would confer a tremendous advantage on a microflagellate existing

not only in a nutrient poor environment or an environment where prey are patchily distributed but also in environments where bacterial prey are relatively abundant. Possession of a chemosensory mechanism would enable a microflagellate to find prey in the former environments and to divert more energy to growth and reproduction in the latter cases. These considerations, together with the observations made concerning the role of bacterial aggregates in the feeding strategies of different microflagellates (Chap 2), led to this investigation of whether the heterotrophic microflagellate Pseudobodo tremulans has a chemosensory response .

METHODS AND MATERIALS

Cultures

Pseudobodo tremulans was recently isolated from the Atlantic Ocean by P.G. Davis and obtained for this work from the American Type Culture collection (ATCC #50061). The culture was maintained in ASW with added bacteria as described by Fenchel (1982a). The bacteria added were isolated from Howe Sound seawater by the conventional technique of streak plating a sample on Marine agar (DIFCO 2216). Pure cultures were obtained by subculturing individual colonies. Preliminary identification of bacteria was attempted and the bacteria were determined to be Gram-negative motile rods. Unfortunately since the previous laboratory study (Chap. 2) both Paraphysomonas sp. and Bodo sp. had been lost from culture. Attempts to obtain these organisms from the original source (David Caron) and other laboratories working with microflagellates were unsuccessful. Pseudobodo tremulans was used for these experiments as it is supposed to closely resemble Paraphysomonas sp.

Solutions

The base solution in all experiments was artificial seawater (Harrison et al. 1980). The pH unless otherwise noted was adjusted to pH 7.5 as this was the pH at which Pseudobodo tremulans was cultured in the laboratory. In each set of experiments, there was only one ion species different between the test and the control solution, e.g. If potassium chloride was added to the test solution then some equivalent amount of some other potassium salt was added to the control solution, thus the potassium ion can not be held responsible for any attraction or repulsion as it is in equivalent concentration in both test and control

solutions. All chemicals used were from Sigma or BDH.

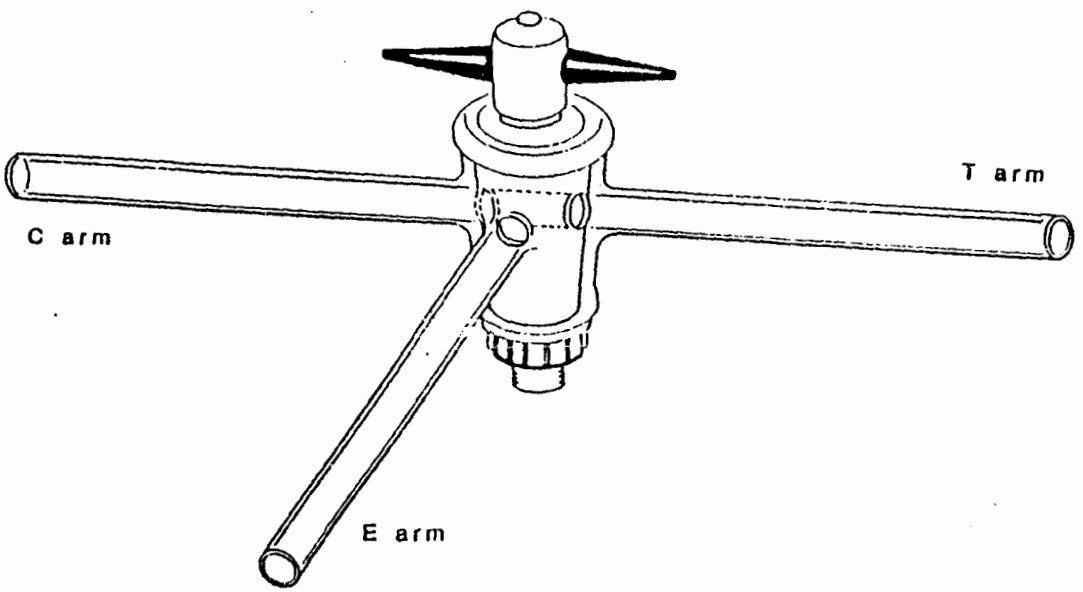
Assay procedure

To test for a chemosensory mechanism, modified T maze assays were carried out using the protocol of Van Houten et al. (1975) (see Fig. 4.1). A T maze consists of a three way stopcock with a two way plug. Test solution fills one arm (T) and control solution another (C). The suspension of organisms to be tested fills the plug. When the plug is turned 90° the organisms interface with the test and the control solutions creating a gradient of test solution. The cells are allowed to migrate for 1 h in the dark, (to avoid complications of phototaxis) in a box (to avoid convection currents) at room temperature. The stopcock is then gently shut and each arm of the T maze gently decanted into vials and fixed with formaldehyde (1.5% final concentration). Cells were stained with 4'6-diamidino-2phenylindole (DAPI, Sigma) and filtered onto a 2 um pore sized, 25mm diameter, Nuclepore filter, prestained with Irgalan black. Cells on the filters were enumerated using epifluorescent microscopy (Filter set 48 77 01 [BP 365/10, FT 310, and LP 395]). The microflagellate culture was concentrated by reverse filtration through a 1 um filter to ensure at least 300 organisms were used per test.

The equations of Van Houten et al., (1975) were used to calculate an index of chemosensory response and an index of motility.

$$\text{Index of Chemosensory response} = \frac{\text{No. of cells in the T arm}}{\text{No. of cells in the T and the C arm}}$$

FIGURE 4.1. The T-maze apparatus.



$$\text{Index of Motility} = \frac{\text{No. of cells in the T and the C arms}}{\text{Total No. of cells used in the assay}}$$

These indices have values between 0 and 1. An index of chemosensory response (Ic) equivalent to 0.5 indicates that the organism is indifferent to the substance in the test arm. An Ic of greater than 0.5 implies the organism is attracted to the test substance, and an Ic of less than 0.5 indicates the organism is repelled from the test substance. The index of motility was also calculated to check whether the cells moved at all. If this index was less than 0.5 we took this to indicate the cells had been damaged in preparation and the data were rejected.

Preliminary experiments were run to determine the time required for microflagellates to migrate to the test arm, and the density and age of microflagellate cells to use in the assays.

RESULTS

There were no net migrations of microflagellates to the test arm after one hour. Even when no attractant was used 1 h was sufficient time to reach equilibrium. Therefore all experiments were run for 1 h.

No significant difference in the index of chemosensory response or the index of motility were noted when different cell densities were used (Table 4.1). Low densities increase the chance of error due to a small sample size, therefore

TABLE 4.1 CHEMOSENSORY RESPONSE TO BACTERIA IN ASW[1] WITH THREE DIFFERENT DENSITIES OF MICROFLAGELLATES.

Data given ± s.d.

No. of experiments in each case=4.

No. of Microflagellates mL^{-1}	Ic^2	Im^3
100	0.86±.06	0.89±.08
300	0.90±.05	0.94±.03
600	0.85±.10	0.87±.08

1,2 & 3 as defined in text

TABLE 4.2 COMPARISON OF THE Ic AND Im OF MID-LOG PHASE MICROFLAGELLATES AND STATIONARY PHASE MICROFLAGELLATES

Data given ± s.d.

No. of experiments in each case=4.

Growth stage	Ic	Im
mid-log	0.88±.04	0.86±.05
stationary	0.92±.06	0.89±.10

for routine work tests were run with a cell concentration of approximately 300 ml^{-1}.

No significant difference in the index of chemosensory response was found when cells of different ages were used (Table 4.2). Therefore in all subsequent experiments cells in mid-exponential phase were used.

P. tremulans was strongly attracted to the bacteria, as indicated by an Ic of 0.8 to 0.9 (Table 4.3). It is not clear from this experiment whether the microflagellates were attracted to the bacteria themselves via mechanoreception or to some bacterial product via chemoreception. To address this question bacterial culture was filtered through a 0.2 um pore size Nuclepore filter to remove bacterial cells and the T-maze assay was repeated using the filtrate as the test solution. The results indicate that P. tremulans was attracted to a substance produced by the bacteria. In an additional experiment the bacteria were washed, spun and resuspended in ASW five times and then used as the test solution. P. tremulans were not attracted to these bacteria (Table 4.3).

The possible stimulatory effects of chemicals that are either bacterial products or microflagellate nutrient requirements, or that had been shown to play a role in chemosensory responses of other small eucaryotes were investigated (Table 4.4). P. tremulans was attracted to ammonium, histidine, glycine, and threonine and repelled from nitrate and nitrite (Table 4.4.). Aspartate, proline, phenylalanine, serine, glucose, acetate, urea, and folic acid were all tested, at concentrations ranging between 0.001 and 10 mM and found to have no stimulatory effect. The particular amino acids tested were chosen because each represented a different type. For example aspartate has an acidic side chain, histidine has a basic one, glycine is uncharged, phenylalanine is an uncharged aromatic amino acid, proline has a secondary amino group.

TABLE 4.3 CHEMOSENSORY RESPONSE OF PSEUDOBODO TREMULANS TO BACTERIA.

Assayed as described in the text. Results are means ±1 standard deviation.

Chemical tested	Conc.	Index of chemotaxis	No. of replicates
Bacteria in spent medium[1] (ASW + gluc.)	Orig. conc.	0.90 ± 0.11	8
	1/10 diln.	0.86 ± 0.09	8
	1/1000 diln.	0.84 ± 0.16	8
Bacterized medium[1] (filtered to remove bacteria)	Orig. conc.	0.92 ± 0.10	8
	1/10 diln.	0.89 ± 0.09	8
	1/1000 diln.	0.45 ± 0.05	8
Soaked and washed bacteria[2] suspended in ASW	Orig. conc.	0.46 ± 0.07	8
	1/10 diln.	0.52 ± 0.05	8
	1/1000 diln.	0.48 ± 0.09	8

1. control solution-fresh medium 2. control solution-ASW

TABLE 4.4 THE CHEMOSENSORY RESPONSE OF PSEUDOBODO TREMULANS.

Assayed as described in the text. Artificial seawater was used as the base solution and as the control solution in all cases. All amino acids are L amino acids. Results are means ± standard deviation.

T-arm		No. of replicates	Index of chemotaxis	Index of motility
10 mM	NH_4Cl	5	0.78 ± 0.13	0.89 ± 0.06
1 mM	NH_4Cl	5	0.90 ± 0.07	0.86 ± 0.08
0.1 mM	NH_4Cl	5	0.88 ± 0.06	0.89 ± 0.12
0.05mM	NH_4Cl	5	0.92 ± 0.07	0.90 ± 0.14
10 mM	Histidine	5	0.80 ± 0.19	0.61 ± 0.12
5 mM	Histidine	5	0.43 ± 0.08	0.58 ± 0.11
0.1 mM	Histidine	5	0.35 ± 0.09	0.74 ± 0 08
10 mM	Glycine	5	0.94 ± 0.06	0.62 ± 0.06
5 mM	Glycine	5	0.46 ± 0.10	0.74 ± 0.06
0.1 mM	Glycine	5	0.39 ± 0.05	0.73 ± 0.03
10 mM	Threonine	5	0.72 ± 0.11	0.74 ± 0.08
5 mM	Threonine	5	0.69 ± 0.08	0.76 ± 0.04
0.1 mM	Threonine	5	0.54 ± 0.06	0.59 ± 0.07
100 mM	$NaNO_2$	5	0.10 ± 0.04	0.60 ± 0.08
10 mM	$NaNO_2$	5	0.42 ± 0.07	0.75 ± 0.06
0.1 mM	$NaNO_2$	5	0.45 ± 0.12	0.74 ± 0.08
100 mM	$NaNO_3$	5	0.08 ± 0.04	0.76 ± 0.04
10 mM	$NaNO_3$	5	0.26 ± 0.14	0.77 ± 0.11
0.1 mM	$NaNO_3$	5	0.35 ± 0.08	0.61 ± 0.13

DISCUSSION

This is the first evidence that a chemosensory mechanism exists in Pseudobodo tremulans, a marine heterotrophic microflagellate. The fact that this microflagellate is attracted to unwashed bacteria, and bacterized culture medium and not attracted to washed bacteria implies the chemical properties of food are important. 'Food' without, an apparently water soluble, odour or flavour is not attractive. As bacteria are considered to be a major food source of microflagellates in the natural environment it would seem beneficial to these animals to evolve an attraction mechanism to substance released extracellularly by their prey. This is clearly the case for P. tremulans.

The attraction of P. tremulans to ammonium, histidine, glycine, and threonine and repulsion from nitrate and nitrite (Table 4) establishes that the heterotrophic microflagellate, P. tremulans does exhibit a chemosensory response to several compounds. These attractants are all chemical species that could signal the presence of prey in the marine environment. There was no apparent pattern among the amino acids to which P. tremulans was attracted. This may indicate that the charge of the molecule is not important in the chemosensory mechanism.

The factor(s) produced extracellularly by bacteria signals the presence of these procaryotes and could include amino acids (Azam and Ammerman 1984). Amino acids could also be produced extracellularly by other micro-organisms e.g. phytoplankton (Aaronson et al. 1971) , released by sloppy zooplankton feeders or released during autolysis of dead organisms (Azam and Ammerman 1984). As suggested by Azam and Ammerman (1984), these are all situations were bacterial growth and accumulation could occur and thus represent potential sources of bacterial prey for microflagellates.

Ammonium is an excretion product of zooplankton (Russell-Hunter 1970) including heterotrophic microflagellates (Sherr et al. 1983). In a recent paper Goldman et al. (1985) indicated that where the prey is nitrogen limited (i.e. a high C:N ratio) the heterotrophic microflagellate Paraphysomonas sp. appeared to excrete less ammonia; thus ammonium might signal healthy prey or feeding microflagellates. Marine aggregates have been reported as sites of elevated nutrient concentrations e.g. ammonium is 10^3 x more concentrated (5mM NH_4^+) in aggregates than in the surrounding water (Shanks and Trent 1979). The attraction of P. tremulans to ammonium supports Goldman's hypothesis (1984) that chemotaxis would enable microflagellates to locate these enriched microenvironments.

Some of the chemicals documented here as attractants for P. tremulans are also attractants for bacteria e.g. amino acids have been documented as bacterial attractants (Adler 1975). It is possible P. tremulans and its bacterial prey are attracted to some of the same signals. Some microflagellates are free swimming while others have been observed to attach to particulates (Fenchel 1984, Davis and Sieburth 1984). Chemosensory behaviour would help the free swimming microflagellates to detect the presence of prey and also the attaching microflagellates to locate new bacterial enriched locations for attachment.

The concentrations of chemicals used in this study were higher than those generally found in marine pelagic environments. However, the concentrations of chemicals actually eliciting responses were probably lower than the concentrations used to fill the T-maze, since the cells were responding to the gradient of diffusing chemical. Moreover, the concentrations of chemicals used represent localized high concentrations such as those found in marine aggregates (Shanks and Trent 1979).

In summary , the results demonstrate that P. tremulans , a heterotrophic microflagellate, exhibits a chemosensory response and some of the stimuli that attract this microflagellate have been defined. The positive stimulants are all chemicals that could signal the presence of prey or of feeding microflagellates. This chemosensory ability enable P. tremulans to locate food and gives this microflagellate a distinct feeding advantage when existing in a nutrient limited environment where prey are scarce or where prey are patchily distributed and even in environments where prey are relatively abundant (Chap. 3). Together with their relatively high swimming speed the ability to perceive chemical stimuli in the environment gives microflagellates additional opportunity for finding favourable living conditions and food.

CONCLUSIONS

The study of microflagellate abundance and distribution in Howe Sound indicates that different factors may be important in regulating microflagellate distribution and abundance at different times of year. For example prey availability and distribution appears to be important in the spring, predation on microflagellates seems important in the summer and physical factors such as temperature appear to be important in the winter.

Temperature may affect the seasonal distribution directly, particularly in the winter months or indirectly by affecting the food resources of microflagellates such as bacteria. Food availability is important to some extent throughout the year as demonstrated by the increase in microflagellate densities following increases in bacterial densities. At certain times of year, such as the spring, the spatial heterogeneity of the bacterial prey plays a role in determining whether or not the microflagellate population present can graze the bacterial prey. The significance of this observation is reinforced by laboratory studies described in Chapter 2. These laboratory studies demonstrated a difference in the ability of two microflagellates to graze free and aggregated bacteria. The presence of microflagellates in the water column throughout most of the year is probably partially a result of different species responding to changes in abundance of their preferred food source.

There is some indication that microflagellates under nutrient stress graze small autotrophic organisms. This is consistent with recent laboratory studies, e.g. Goldman and Caron (1985), and has important consequences for the passage of carbon and energy through microflagellates.

The indication that many autofluorescent microflagellates function in both a heterotrophic and also an autotrophic manner under certain conditions is consistent with other observations, both recent and not so recent. (e.g. Aaronson and Baker 1959, Estep et al. 1986). The adaptive advantage of this is clear - such microflagellates can survive by feeding heterotrophically when light levels are inadequate for photosynthesis. Thus the role played by such mixotrophic organisms in the passage of carbon and energy through the microbial food web and in mineralization may be more significant than previously considered.

The absence of countable microflagellates in the November and December months of this study corresponds to periods of low temperatures. This suggests that physical factors such as temperature set broad limits on microflagellate abundance and distribution. Other controlling factors such as food availability apparently act within the range of tolerable physical factors.

Although not measured directly factors such as predation are also likely to have modified microflagellate abundance and distribution. This appears to be the case in the early summer when microflagellate abundance does not increase in spite of an abundance of available prey. There is a notable absence of studies of grazers of microflagellates particularly in field situations and this is clearly an area that warrants further research.

An interesting ecological aspect arising from the observation of the importance of prey distribution both in the field and in the laboratory studies, is whether microflagellates can efficiently exploit such patchy habitats. In general a small organism, such as a microflagellate, will not experience an 'average resource density', instead their environment is spatially heterogeneous with respect to prey distribution (Fenchel 1987). Consequently mechanisms such as behavioural responses that enable microflagellates to exploit patches of prey

would be important adaptations. Estimations, presented here, of the relative advantage of a chemosensory mechanism to a microflagellate demonstrate that this is the case. A chemosensensory mechanism is particularly important and sometimes essential for survival of a microflagellate in prey depleted environments. This is as expected. The calculations do indicate that possession of such a mechanism confers a great advantage. In addition to this and perhaps surprisingly the calculations indicate that although not essential for survival the possession of such a mechanism in environments where prey is relatively abundant still confers a large advantage on the microflagellate. It allows a microflagellate to find prey more efficiently.

Until this study, the possession of such an ability by a microflagellate was a subject of considerable speculation. The evidence presented in Chapter 4 indicates that at least one microflagellate has a chemosensory mechanism. The calculation of the advantage conferred by such an ability demonstrates that even at high prey densities the advantage conferred by such a mechanism is great and at low prey densities it may be necessary for survival. Thus it seems likely that possession of a chemosensory mechanism is widespread among microflagellate species since it enables them to better interact with their environment.

There is no clear pattern among the chemicals to which P. tremulans is attracted. Both the attraction to bacterial secretions and to certain amino acids and ammonium have implications with respect to prey location. However whether the concentrations of chemicals necessary for a chemosensory response by a microflagellate are present for any length of time in the water column is a source of debate (Mitchell et al. 1985, Alldredge and Cohen 1987) and a challenge for further research.

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APPENDIX 1, THE DATA FROM HOWE SOUND

Sampling date	Day
1/3/84	1
13/3/84	13
27/3/84	27
3/4/84	34
19/4/84	50
7/5/67	67
23/5/84	84
10/7/84	132
24/7/84	146
8/8/84	162
21/8/84	174
4/9/84	188
3/10/84	217
15/10/84	229
19/11/84	264
19/12/84	294
21/1/85	327
5/2/85	342
19/2/85	356
5/3/85	370
24/4/85	420
15/5/85	441
13/6/85	470
22/7/85	510
12/8/85	530
25/9/85	574
23/10/85	602

1. DAY	2. DEPTH	3. SALIN	5. CHLA	6. TEMP	10. BIOB	11. BIOM	12. TOT.MF	15. AMF	16 HMF	19. BACT.NO	20 ATT.BACT
1	1	24.500	3.1200	7.3000	8.8000	1.2500	1.5300	.76000	.76000	6.8200	0.
1	5	28.100	2.9500	7.3000	7.5000	1.2500	.68000	.27000	.41000	5.8400	0.
1	10	28.700	1.5300	7.7000	8.5000	1.2500	7.9100	.20000	5.9000	6.5900	0.
1	20	29.300	1.4500	8.2000	8.6000	1.2500	1.0000	.57000	.43000	6.6500	126.35
1	40	29.500	1.2200	8.7000	9.9000	1.2500	.68000	.27000	.40000	7.6800	138.24
1	75	30.800	1.3100	8.2000	9.0000	1.2500	.73000	.29000	.44000	6.9800	125.64
1	150	30.800	1.8100	8.3000	8.8000	1.2500	.67000	.13000	.54000	6.8000	34.000
1	250	30.800	.84000	8.0000	8.2000	1.2500	.39000	0.	.39000	6.3200	25.280
13	1	-0.	3.1300	-0.	9.5000	1.2000	1.1500	.58000	.57000	7.7500	62.000
13	5	-0.	2.6300	-0.	9.6000	1.2000	2.2300	0.	2.2300	7.7700	31.080
13	10	-0.	1.6300	-0.	8.6000	1.1700	3.1500	.95000	2.2000	7.1600	21.480
13	20	-0.	1.0400	-0.	9.4000	1.1800	2.4900	.84000	1.6600	7.7500	15.500
13	40	-0.	.51000	-0.	8.4000	1.1500	.95000	0.	.95000	7.1700	35.850
13	75	-0.	1.2700	-0.	6.4000	.85000	1.8100	.20000	.16000	7.3900	214.31
13	150	-0.	.85000	-0.	4.8000	.71000	1.0900	.38000	.73000	6.6100	0.
13	250	-0.	.70000	-0.	7.8000	1.1300	.40000	0.	.40000	6.7400	0.
27	1	13.600	4.7500	8.7000	10.400	1.1400	3.1700	1.3900	1.7900	8.9100	0.
27	5	21.800	5.0400	9.3000	9.8000	1.1400	1.1100	.42000	6.9700	8.3100	24.930
27	10	26.500	4.5500	8.7000	10.700	1.1400	1.2400	.49000	.74000	9.0600	72.480
27	20	26.500	2.1300	8.7000	9.7000	1.1400	.97000	.14000	.83000	8.3700	58.590
27	40	23.200	1.0600	8.7000	10.100	1.1400	.74000	.25000	.49000	8.7000	8.7000
27	75	29.500	.77000	8.8000	7.7000	1.1400	.40000	.11000	.29000	6.6300	19.890
27	150	29.500	.73000	9.0000	8.0000	1.1400	.36000	0.	.36000	6.9500	41.700
27	250	29.500	.59000	9.8000	10.400	1.1400	.11000	.11000	0.	4.6000	0.
34	1	21.600	4.6400	8.8000	11.100	1.1100	3.7700	2.1600	1.6200	8.6800	43.400
34	5	22.500	4.0200	8.7000	12.900	1.1100	3.0100	1.9600	1.0600	10.020	0.
34	10	27.700	3.8700	8.3000	11.000	1.1100	4.2400	2.1200	2.1200	8.6200	86.200
34	20	28.000	.63000	8.5000	9.9000	1.1100	1.0000	.14000	.86000	7.6800	15.360

34	40	29.800	.97000	8.7000	8.7000	1.1100	1.6700	.68000	.99000	6.7700	6.7700
34	75	30.000	.67000	8.7000	7.9000	1.1100	.78000	0.	.78000	6.1100	6.1100
34	150	30.000	.68000	8.7000	7.8000	1.1100	.39000	0.	.39000	6.0800	0.
34	250	30.100	.41000	8.5000	7.8000	1.1100	.29000	.15000	.14000	6.0900	0.
50	1	20.000	4.6400	9.3000	7.8000	1.0800	6.7800	2.9100	4.0700	7.0300	21.090
50	5	26.500	4.0200	9.5000	9.7000	1.0800	6.7600	3.7800	2.9700	8.7300	52.380
50	10	27.600	3.8700	9.2000	8.8000	1.0800	6.7600	4.3800	3.2900	8.0000	40.000
50	20	28.000	.63000	9.0000	8.5000	1.0800	10.680	2.8400	9.6600	7.7100	92.520
50	40	30.000	.97000	9.0000	6.9000	1.0800	12.500	-0.	-0.	6.2200	80.860
50	75	30.000	1.6400	9.0000	6.2000	1.0800	14.000	.50000 -1	1.3900	5.6000	56.000
50	150	30.100	.68000	9.3000	6.3000	1.0800	18.900	0.	8.9000	4.9100	19.640
50	250	30.100	.41000	9.2000	5.6000	1.0800	19.700	0.	7.5000	5.0300	0.
67	1	20.800	3.1500	10.700	7.7000	1.0000	13.800	8.2000	4.3800	7.0600	14.120
67	5	26.000	2.9800	10.700	8.6000	1.0000	13.800	7.1000	.22000	8.4600	67.680
67	10	29.200	2.3200	9.3000	7.0000	1.0000	2.9800	.78000	1.7700	6.9000	0.
67	20	29.300	.99000	9.0000	7.2000	1.0000	3.4000	3.0600	3.2000	7.1200	92.560
67	40	29.600	.92000	9.0000	6.1000	1.0000	3.6000	.35000	2.3900	6.0700	133.54
67	75	30.500	.83000	9.0000	4.9000	1.0000	2.8200	.22000	.95000	4.7900	0.
67	150	31.000	.79000	9.3000	4.6000	1.0000	1.5900	0.	2.3400	4.5300	9.0600
67	250	31.000	.71000	9.0000	4.4000	1.0000	3.9800	0.	-0.	4.5300	0.
84	1	22.300	3.2600	11.000	8.7000	1.0000	1.1200	.42000	.20000	8.6700	60.690
84	5	26.800	2.9900	10.800	9.6000	1.0000	1.0200	.44000	.58000	9.5900	115.08
84	10	29.400	4.4200	10.300	8.3000	1.0000	1.0700	.35000	.71000	8.2600	74.340
84	20	29.600	3.1000	9.0000	7.2000	1.0000	.96000	.48000	.48000	7.2100	79.310
84	40	30.000	2.2200	9.0000	6.2000	1.0000	1.2700	.21000	.80000 -1	6.1900	24.760
84	75	30.200	2.4100	9.0000	5.7000	1.0000	2.0500	.94000	1.1000	5.6300	0.
84	150	31.000	3.0800	9.2000	5.5000	1.0000	0.	0.	.40000	5.4400	0.
84	250	31.200	3.0700	9.0000	4.6000	1.0000	.12000	0.	.11000	4.5200	18.080
110	1	4.5000	4.4700	13.300	10.300	1.0000	1.0500	.35000	.69000	10.160	81.280
110	5	24.000	4.9000	13.300	9.9000	1.0000	1.3100	.16000	1.1400	9.8400	49.200

110	10	29.300	1.7500	12.300	6.5000	1.0000	.85000	.42000	.42000	8.4600	8.4600
110	20	29.500	.73000	10.600	7.1000	1.0000	.95000	.41000	.55000	7.0100	154.22
110	40	29.600	.73000	10.000	6.8000	1.0000	.63000	0.	.69000	6.6800	120.24
110	75	30.000	.71000	10.300	5.7000	1.0000	0.	0.	0.	5.6300	50.670
110	150	30.800	.69000	10.000	5.9000	1.0000	0.	0.	.70000 -1	5.8200	58.200
110	250	30.600	.61000	9.3000	5.5000	1.0000	.20000	0.	.20000	5.5600	83.400
132	1	5.0000	2.6700	16.800	10.600	1.0000	.1200	.67000	.45000	10.430	20.860
132	5	18.500	4.9200	14.200	9.7000	1.0000	.86000	.57000	.28000	9.5400	38.160
132	10	25.300	3.9700	11.000	9.3000	1.0000	1.0200	.38000	.64000	9.1400	54.840
132	20	28.700	1.6500	9.7000	6.9000	1.0000	.82000	.16000	.66000	6.8000	102.00
132	40	28.300	.53000	9.0000	6.2000	1.0000	.62000	.20000	.48000	6.1000	6.1000
132	75	30.200	.70000	8.8000	6.0000	1.0000	0.	0.	0.	5.8800	64.680
132	150	30.600	.68000	9.0000	5.8000	1.0000	.35000	0.	.35000	5.6800	96.560
132	250	30.600	.62000	9.0000	5.6000	1.0000	0.	0.	.12000	5.2800	63.360
146	1	14.500	4.4400	15.000	10.200	1.0000	0.	-0.	-0.	10.190	101.90
146	5	23.500	5.1500	14.000	9.5000	1.0000	.19000	-0.	-0.	9.3700	9.3700
146	10	28.000	3.0000	13.300	9.8000	1.0000	.50000	-0.	-0.	9.1900	64.330
146	20	29.500	1.5600	11.000	7.2000	1.0000	.19000	-0.	-0.	7.7500	139.50
146	40	30.600	.86000	10.000	6.9000	1.0000	0.	-0.	-0.	6.7800	74.580
146	75	31.000	1.0100	9.0000	6.2000	1.0000	0.	-0.	-0.	6.1100	36.660
146	150	31.000	.89000	9.0000	6.5000	1.0000	.20000	-0.	-0.	6.3500	0.
146	250	31.000	.60000	9.0000	6.2000	1.0000	0.	-0.	-0.	6.2000	0.
162	1	16.800	2.2400	11.500	11.000	1.0000	.48000	-0.	-0.	11.000	99.000
162	5	22.800	2.2400	11.000	8.3000	1.0000	.60000	-0.	-0.	8.2000	0.
162	10	29.000	2.4400	10.000	9.8000	1.0000	.25000	-0.	-0.	9.8000	39.200
162	20	29.500	1.2000	9.0000	7.8000	1.0000	.19000	-0.	-0.	7.8000	0.
162	40	30.100	.66000	9.0000	5.5000	1.0000	0.	-0.	-0.	5.4000	54.000
162	75	31.000	.72000	10.000	7.0000	1.0000	0.	-0.	-0.	6.9000	41.400
162	150	30.500	.62000	9.0000	4.6000	1.0000	0.	-0.	-0.	4.6000	46.000
162	250	30.500	.60000	8.5000	3.7000	1.0000	.16000	-0.	-0.	3.7000	0.

174	1	11.800	4.0000	12.500	9.8000	.99000	0.	-0.	-0.	9.7300	107.03
174	5	25.000	2.5100	12.000	5.3000	1.0000	.48000	-0.	-0.	10.530	126.36
174	10	28.600	2.3800	10.000	10.000	1.0000	0.	-0.	-0.	9.9100	158.56
174	20	28.700	1.5300	9.5000	8.6000	1.0000	.20000	-0.	-0.	8.4900	127.35
174	40	29.000	1.5200	8.3000	6.6000	1.0000	0.	-0.	-0.	6.5800	0.
174	75	29.400	1.0400	8.7000	7.0000	1.0000	0.	-0.	-0.	6.9400	41.640
174	150	30.400	.97000	8.5000	7.0000	1.0000	0.	-0.	-0.	6.9000	20.700
174	250	30.500	.97000	8.5000	5.8000	1.0000	0.	-0.	-0.	5.7600	0.
188	1	15.300	-0.	14.500	-0.	1.9000	.70000	-0.	-0.	-0.	-0.
188	5	25.500	-0.	13.000	-0.	1.7600	.20000	-0.	-0.	-0.	-0.
188	10	27.100	-0.	12.300	-0.	1.3200	0.	-0.	-0.	-0.	-0.
188	20	27.300	-0.	10.100	-0.	1.1400	.41000	-0.	-0.	-0.	-0.
188	40	30.600	-0.	10.800	-0.	1.1100	.22000	-0.	-0.	-0.	-0.
188	75	30.300	-0.	10.500	-0.	1.0900	0.	-0.	-0.	-0.	-0.
188	150	31.300	-0.	10.200	-0.	1.0100	0.	-0.	-0.	-0.	-0.
188	250	30.800	-0.	10.200	-0.	.98000	0.	-0.	-0.	-0.	-0.
217	1	23.800	5.1600	13.200	4.6000	1.0200	.86000	-0.	-0.	4.5000	67.500
217	5	25.300	.20000	12.500	6.4000	1.2800	0.	-0.	-0.	5.0000	90.000
217	10	28.000	.20000	12.200	4.3000	1.5400	.76000	-0.	-0.	2.8000	159.60
217	20	27.200	.20000	10.800	4.5000	1.5500	.76000	-0.	-0.	2.9000	0.
217	40	29.300	.20000	9.8000	4.1000	1.0800	0.	-0.	-0.	3.8000	38.000
217	75	29.100	.20000	10.800	5.8000	1.2300	0.	-0.	-0.	4.7000	18.800
217	150	29.100	.20000	9.9000	5.9000	1.4800	0.	-0.	-0.	4.0000	0.
217	250	29.100	.20000	9.9000	10.900	2.2700	0.	-0.	-0.	4.8000	81.600
229	1	14.300	1.9400	6.8000	3.2000	.97000	1.1100	-0.	-0.	3.3000	0.
229	5	23.500	2.2500	11.000	5.0000	1.1900	1.4100	-0.	-0.	4.2000	71.400
229	10	27.200	2.3900	10.900	6.3000	1.3100	.91000	-0.	-0.	4.8000	129.60
229	20	27.600	.20000	10.100	4.0000	1.0000	1.1400	-0.	-0.	4.0000	60.000
229	40	29.100	.20000	9.2000	3.8000	.97000	.59000	-0.	-0.	3.9000	78.000
229	75	29.600	.20000	9.2000	6.1000	1.4500	.71000	-0.	-0.	4.2000	121.80

229	150	29.100	.20000	8.7000	3.8000	.86000	0.	-0.	-0.	4.4000	158.40
229	250	29.600	.20000	9.0000	6.0000	1.1100	0.	-0.	-0.	5.4000	151.20
264	1	20.000	.20000	7.5000	5.9000	1.1800	0.	-0.	-0.	5.1000	96.900
264	5	23.800	.20000	7.8000	5.7000	1.2000	0.	-0.	-0.	4.9000	68.600
264	10	24.500	.20000	8.2000	6.1000	1.1200	0.	-0.	-0.	5.6000	123.20
264	20	25.500	.20000	8.3000	3.9000	.95000	0.	-0.	-0.	4.6000	0.
264	40	27.700	.20000	9.0000	4.9000	.89000	0.	-0.	-0.	6.6000	191.40
264	75	29.100	.20000	9.0000	4.4000	.95000	0.	-0.	-0.	4.6000	73.600
264	150	29.500	.20000	8.5000	3.9000	.95000	0.	-0.	-0.	4.1000	20.500
264	250	29.700	.20000	8.2000	3.6000	.98000	0.	-0.	-0.	4.0000	4.0000
294	1	25.300	.20000	5.0000	-0.	.76000	0.	-0.	-0.	5.2700	89.590
294	5	26.000	.20000	6.3000	-0.	.74000	0.	-0.	-0.	4.1000	8.2000
294	10	25.800	.20000	7.7000	-0.	.85000	0.	-0.	-0.	4.4300	22.150
294	20	27.500	.20000	6.7000	-0.	.49000	0.	-0.	-0.	4.9400	143.26
294	40	27.000	.20000	7.7000	-0.	.78000	0.	-0.	-0.	3.5000	0.
294	75	29.000	.20000	8.3000	-0.	.79000	0.	-0.	-0.	3.6000	0.
294	150	29.700	.20000	8.0000	-0.	.67000	0.	-0.	-0.	4.2000	0.
294	250	30.000	.20000	7.7000	-0.	.58000	0.	-0.	-0.	3.2700	0.
327	1	22.500	.20000	6.2000	4.0000	1.0200	3.4800	-0.	-0.	-0.	-0.
327	5	24.200	.20000	5.7000	3.0000	.78000	2.2800	-0.	-0.	-0.	-0.
327	10	26.500	.20000	6.7000	3.8000	.65000	2.2800	-0.	-0.	-0.	-0.
327	20	28.900	.20000	7.5000	2.4000	.55000	1.6400	-0.	-0.	-0.	-0.
327	40	29.200	.20000	8.0000	2.7000	.65000	.64000	-0.	-0.	-0.	-0.
327	75	28.900	.20000	8.5000	2.8000	.66000	1.7000	-0.	-0.	-0.	-0.
327	150	29.800	.20000	8.7000	2.8000	.70000	0.	-0.	-0.	-0.	-0.
327	250	30.000	.20000	8.0000	1.9000	.62000	.65000	-0.	-0.	-0.	-0.
342	1	23.000	.20000	5.0000	7.0000	1.0100	2.8100	.48000	.80000	7.8000	31.200
342	5	22.200	.20000	4.7000	7.5000	.81000	2.6100	.37000	.87000	9.6000	124.80
342	10	25.600	.20000	4.8000	6.0000	.83000	1.3000	1.4700	.13000	9.1000	81.900
342	20	28.900	.20000	6.7000	6.9000	.65000	1.3900	-0.	.80000	7.8000	0.

342	40	30.600	.20000	7.3000	5.1000	.61000	1.8900	.45000	.89000	5.3000	0.
342	75	29.800	.20000	7.3000	4.2000	.70000	1.1400	.65000	.26000	6.0000	12.000
342	150	29.900	.20000	8.0000	4.9000	.75000	0.	0.	.55000	6.6000	0.
342	250	30.000	.20000	7.8000	4.3000	.54000	0.	0.	.19000	4.5000	0.
356	1	23.200	.20000	5.2000	8.7000	.96000	1.2900	.60000	1.0700	9.0500	271.50
356	5	29.800	.20000	5.3000	8.9000	.90000	1.2400	.54000	.90000	9.8900	69.230
356	10	23.600	.20000	6.2000	9.9000	.95000	1.6100	.16000	1.1300	10.480	377.28
356	20	27.600	.20000	7.0000	6.5000	.89000	.80000	.40000	.80000	6.8100	0.
356	40	29.500	.20000	7.5000	6.7000	.95000	1.3400	.36000	.36000	7.0400	0.
356	75	29.800	.20000	7.7000	6.4000	.87000	.91000	0.	8.2500	7.4000	214.60
356	150	29.900	.20000	7.3000	6.0000	.91000	.55000	0.	.40000	6.5200	0.
356	250	30.100	.20000	7.5000	7.7000	.91000	.19000	0.	3.4400	9.0100	135.15
370	1	23.300	2.4900	8.0000	7.8000	1.0000	2.2100	0.	1.1400	7.8100	179.63
370	5	23.800	3.2300	7.0000	6.6000	.91000	.91000	-0.	-0.	7.2900	0.
370	10	25.200	2.3300	7.5000	6.9000	.95000	1.2900	.42000	.42000	7.2000	43.200
370	20	26.800	.38000	7.8000	8.0000	.92000	12.210	0.	.22000	8.6700	208.08
370	40	27.800	.95000	8.2000	5.3000	.95000	.72000	.16000	.80000	5.6000	207.20
370	75	28.000	.20000	7.8000	6.5000	.95000	12.250	.19000	.77000	6.8300	122.94
370	150	27.500	.20000	8.7000	4.6000	.95000	.41000	0.	.42000	4.8800	87.840
370	250	28.800	.23000	8.0000	7.7000	.84000	3.4800	0.	0.	9.1400	164.52
420	1	17.200	5.8400	9.2000	11.400	1.0200	1.1400	0.	.17000	11.100	222.00
420	5	21.500	10.410	9.3000	9.9000	1.0600	.85000	.26000	1.2700	9.1000	0.
420	10	26.800	6.9900	9.0000	10.000	.95000	.23000	.29000	.60000	10.500	52.500
420	20	28.800	.96000	8.7000	5.5000	.95000	.97000	0.	.98000	5.9000	0.
420	40	29.300	.39000	8.3000	6.2000	.95000	2.9600	0.	.51000	6.6000	0.
420	75	29.500	.49000	8.5000	7.0000	.93000	.42000	0.	0.	7.4000	0.
420	150	30.200	.71000	8.2000	6.5000	.96000	0.	.13000	.27000	7.4000	51.800
420	250	30.500	.42000	8.2000	8.2000	.95000	0.	0.	.18000	8.7000	174.00
441	1	16.500	3.0400	15.000	8.8000	1.0400	1.1700	.55000	0.	7.8000	0.
441	5	24.000	5.8800	12.000	18.200	.88000	.53000	.51000	0.	20.700	1097.1

441	10	28.600	.88000	9.0000	7.2000	.94000	1.9000	.16000	.17000	7.6000	197.60
441	20	30.000	2.5200	8.0000	10.100	1.0700	.98000	0.	0.	9.4000	169.20
441	40	30.500	.94000	8.0000	5.4000	.99000	.51000	.19000	0.	5.5000	0.
441	75	30.500	.20000	8.5000	7.9000	.98000	0.	0.	0.	8.0000	56.000
441	150	30.500	.20000	8.0000	4.6000	.89000	.40000	0.	0.	5.2000	72.800
441	250	30.500	.20000	8.0000	3.8000	.71000	.19000	0.	0.	5.3000	-0.
470	1	-0.	4.1800	13.000	18.500	-0.	0.	0.	0.	14.500	-0.
470	5	-0.	4.0100	13.000	16.600	-0.	.52000	0.	1.0700	14.200	-0.
470	10	-0.	6.7400	10.000	12.300	-0.	.33000	.19000	1.3700	12.100	-0.
470	20	-0.	2.0200	8.0000	8.1000	-0.	0.	0.	.24000	9.7000	-0.
470	40	-0.	1.0300	8.0000	8.0000	-0.	.19000	0.	.36000	9.0000	-0.
470	75	-0.	.54000	8.0000	5.5000	-0.	0.	0.	.18000	9.3000	-0.
470	150	-0.	.41000	8.0000	4.6000	-0.	0.	0.	.32000	8.0000	-0.
470	250	-0.	.51000	8.0000	4.2000	-0.	0.	0.	0.	7.7000	-0.
503	1	-0.	2.1200	18.000	15.600	-0.	0.	.13000	.40000	12.400	-0.
503	5	-0.	1.8500	18.500	9.7000	-0.	1.0700	.11000	.46000	7.0800	-0.
503	10	-0.	2.0900	14.000	8.7000	-0.	1.5700	.48000	.48000	6.9000	-0.
503	20	-0.	1.2700	12.000	16.700	-0.	.24000	.32000	.48000	13.800	-0.
503	40	-0.	.69000	10.800	8.0000	-0.	.36000	0.	.18000	8.0400	-0.
503	75	-0.	.95000	9.0000	9.0000	-0.	.18000	.29000	.44000	8.4000	-0.
503	150	-0.	.74000	10.200	9.3000	-0.	.32000	0.	.16000	4.2000	-0.
503	250	-0.	1.1900	9.0000	9.4000	-0.	0.	0.	0.	10.300	-0.
530	1	-0.	2.1700	-0.	13.700	-0.	.54000	.16000	3.2200	11.300	-0.
530	5	-0.	1.2000	-0.	16.600	-0.	.57000	0.	1.6100	8.2000	-0.
530	10	-0.	1.0500	-0.	15.900	-0.	.97000	.44000	1.9300	8.8000	-0.
530	20	-0.	.49000	-0.	4.8000	-0.	.80000	0.	1.6100	6.8000	-0.
530	40	-0.	.21000	-0.	4.2000	-0.	.18000	0.	2.6100	5.9000	-0.
530	75	-0.	.92000	-0.	4.3000	-0.	.73000	0.	1.7700	4.9000	-0.
630	150	-0.	2.9400	-0.	2.9000	-0.	.16000	0.	.27000	3.7000	-0.
530	250	-0.	.23000	-0.	2.5000	-0.	0.	0.	.21000	3.1000	43.400