Microb Ecol (1985) 11:281–288

MICROBIAL ECOLOGY
© 1985 Springer-Verlag

The Detection and Characterization of Bacteria-sized Protists in "Protist-free" Filtrates and their Potential Impact on Experimental Marine Ecology

F. J. Cynar, K. W. Estep, and J. McN. Sieburth

Graduate School of Oceanography, University of Rhode Island, Narragansett, Rhode Island 02882

Abstract. Nuclepore filters of 0.6–1.0 μm pore size have been used to prepare "protist-free" water for a number of studies in microbial ecology. This procedure has been called into question by a recent study claiming that a significant portion of bacterial loss in filtrates could be due to uncharacterized predators passing through 0.6 μm filters. We were unable to directly observe protists in 0.6 μm filtrates using phase contrast, epifluorescence, or transmission electron microscopy. Using the culture techniques of rice grain enrichment and most probable number, however, we were able to observe and quantify several species of bacterivorous nanoflagellates that developed not only in 0.6 μm, but also in 0.4 μm seawater filtrates. The ability of predacious nanoflagellates to squeeze through bacteria-sized pores questions studies of bacterial production and chemical cycling that have assumed protist-free filtrates.

Introduction

Aplastidic (=apochlorotic) flagellates in the nanoplankton size range (2–20 μm) [28] are abundant in marine waters [3, 29] and are significant consumers of the bacteria in the picoplankton size fraction (0.2–2 μm) [12, 16]. These nanoplankters are intimately associated with changes in the total picoplankton [5, 6, 12], and their small size, feeding efficiency, and high growth yield make them a potentially important trophic link between the picoplankton and microzooplankton. Measurements of bacterial production and nutrient recycling are influenced by nanoflagellates. For example, bacterial production in the Black Sea was 45–50% lower in samples containing flagellates as compared with samples from which they were removed [22].

Filtration of seawater through 0.6–1.0 μm filters has been used to prepare "protist-free" water for studies of bacterial predation and productivity [1, 9, 17, 19, 25]. These techniques have been called into question by results of a recent study by Fuhrman and McManus [18]. This study indicated that agents responsible for 50% (= 9×10^5 bacteria/ml decrease over 22 hours) of bacterial grazing in coastal waters were able to pass through 0.6 μm Nuclepore filters.

They postulated that bacteria-sized or very flexible protists were responsible, though they were not directly observed, nor was their presence demonstrated.

Materials and Methods

Seawater was collected on 6 dates from July to September 1984 from Narragansett Bay, Rhode Island, at the Graduate School of Oceanography. Each sample was divided into 3 subsamples and filtered through 0.6, 0.4, or 0.2 μm Nuclepore polycarbonate filters, respectively, with a vacuum of <10 cm Hg. Each filtrate was immediately examined for the presence of protists using phase contrast light microscopy and epifluorescence microscopy. Live wet mounts were examined using phase-contrast with a Zeiss photomicroscope. Samples for epifluorescence microscopy were preserved with formalin (2% v/v), stained with primulin [7] or acridine orange [11, 20], concentrated onto Irgalan black-stained 0.2 μm Nuclepore filters, and viewed with an Olympus Vanox epifluorescence microscope at 400 and 1,000× magnification. Preserved subsamples of each filtrate were also dried onto formvar-coated electron microscope grids for examination using whole mount transmission electron microscopy (TEM) [13].

Protist enrichment cultures were prepared by pipetting 20 ml of each filtrate (0.2, 0.4, and 0.6 μm) into sterile, 50 ml plastic tissue-culture flasks containing sterile rice grains for enrichment of bacterial prey. Twelve enrichment cultures were made for the July 17th sample; all others consisted of 6 enrichments. Enrichments made from 0.2, 0.4, and 0.6 μm filtrates were incubated in the dark at 19°C and examined for flagellates every 1–4 days. A positive enrichment was determined by the presence of at least one motile nanoflagellate in 25 fields, using a Zeiss IM35 Photoinvertoscope at 400× magnification. Grids for whole mount TEM of enriched protist cultures were prepared as described above. Thin sections of flagellates from positive enrichment cultures were prepared as described in Johnson and Sieburth [21].

In order to determine the number of cells passing through 0.6 μm filters, most probable number (MPN) estimates were calculated using enrichment cultures on four dates during August and September. MPN estimates were determined using freshly collected seawater filtered through sterile 0.6 μm Nuclepore filters. Tenfold dilutions of the filtrate were made by adding 0.2, 2, and 20 ml of the 0.6 μm filtrate to 19.8, 18, and 0 ml of autoclaved seawater, respectively. Five sterile, 50 ml tissue-culture flasks with sterile rice grains were used for each dilution. Enrichments were incubated in the dark at 19°C and examined for flagellates every 1–4 days for 2–3 weeks. MPN estimates were calculated using a computer program developed by Clarke and Owens [10].

For both enrichment cultures and MPN assays, a minimum of six sterile controls were prepared identically to cultures described previously, with the exception of the filtrate inocula. Each of these controls were examined at the same time intervals as other cultures. To insure that positive cultures did not result from contamination, all filtration apparatus was autoclaved prior to use, and all filtrations and inoculations were carried out using sterile, disposable pipettes in a laminar flow hood.

Results

Protists were never observed in freshly prepared filtrates, stained with either of the fluorescent stains. They also could not be observed using wet mount light microscopy or whole mount TEM. Abundant nanoflagellate populations were detected, however, in 71% (0–100%) of the 0.6 μm and 24% (0–67%) of the 0.4 μm, but in none of the 0.2 μm enrichment cultures (Table 1). All of the 0.6 and 0.4 and approximately 50% of the 0.2 μm enrichments contained bacteria. The lag time between inoculation and detection of nanoflagellates was generally shorter for the 0.6 μm enrichments than for the 0.4 μm enrichments

Table 1. The enrichment of bacterivorous nanoflagellates from seawater passing 0.4 and 0.6 μm Nuclepore filters

Inoculation date	No. days incubated	0.4 μm pass No. positive	percent	0.6 μm pass No. positive	percent
July 17, 1984	7	Not done		9	75
	13			3	25
July 31	2	0	0	1	17
	4	0	0	4	67
	5	0	0	5	83
	7	0	0	6	100
August 6	3	0	0	0	0
	6	4	67	4	67
	8	2	34	4	67
	11	0	0	1	17
August 15	2	0	0	0	0
	4	0	0	3	50
	6	0	0	6	100
	18	0	0	3	50
August 24	2	0	0	0	0
	5	0	0	0	0
	8	0	0	0	0
	11	1	17	1	17
September 12	2	0	0	0	0
	4	0	0	1	17
	9	1	17	3	50
	12	2	34	4	67
Mean:			23.6		71.0

suggesting that more flagellates passed through the 0.6 μm filters than the 0.4 μm filters. On two dates, positive 0.6 μm enrichments were refiltered through 0.6 μm filters and again enriched with a rice grain. Half of these enrichments contained detectable flagellate populations after only 1–2 days. Decreased time for detection of positive flagellate enrichments was probably due to a larger population, preselected for smaller forms by the original filtration.

At least eight morphologically distinct forms, including several biflagellates and a single monoflagellate, were present in the enrichment cultures examined with the light microscope. Cell sizes, measured when nanoflagellates were first detected, ranged from 1–4 μm wide and 2–7 μm long. Three of the biflagellates are tentatively described as *Bodo* species, *B. designis* and two undescribed species (Table 2). Whole mount TEM of selected cultures revealed a spherical monoflagellate with a single flagellum, without mastigonemes, ending in a terminal polar filament (Fig. 1A). This extremely small flagellate (1–2 μm) was only slightly larger than the accompanying bacteria (Fig. 1B) and closely resembles an undescribed pelagic species.

Biflagellates present in whole mount TEM were long, narrow species (1.5×4 μm) whose smallest dimension was equal to the diameter of the spherical

Table 2. Morphology and tentative identification of nanoflagellates from enrichment cultures prepared from 0.6 and 0.4 μm seawater filtrates

Morphology and motility	Tentative identification	Size (μm)	Filtration porosity (μm)
Pleomorphic, elongate cell with 2 forward directed flagella, rotational swimming	–	2.5 × 7.0	0.6
Biflagellated, spherical cell, slow steady swimming	–	3.5 × 4.0	0.6
Bean-shaped cell with 1 forward and 2 trailing flagellum, slow steady swimming	*Bodo* sp.	2.0 × 3.0	0.6
Elongate cell with 2 forward directed flagella	*Bodo designis*	3.0 × 5.0	0.6
Spherical monoflagellate, slow tumbling swimming	–	1.0–2.0	0.4 & 0.6
Elongate, flexible cell with 2 forward directed flagella, fast swimming	–	1.5 × 5.0	0.4 × 0.6
Aspherical biflagellate, jerky, side to side swimming	*Bodo* sp.	3.5 × 5.0	0.4 & 0.6
Irregularly spherical biflagellate, jerky, side to side swimming	–	4.0–5.0	0.4

monoflagellate (Fig. 1C). The bacterivorous nature of these flagellates was confirmed using TEM of thin sections that clearly showed the presence of food vacuoles containing bacteria. Bacterial prey were identified by their typical procaryotic ultrastructure and were enclosed in typical protistan food vacuoles. Most nanoflagellate cells contained several food vacuoles, and their bacterial contents were in various states of digestion (Fig. 1D, E).

The MPN assay revealed that a small number of flagellates passed through the 0.6 μm filters. Lower dilutions of the MPN cultures were positive in as few as 6 days. MPN's averaged 0.07 cells/ml (95% confidence interval range = 0.002–0.44 cells/ml) (Table 3).

Discussion

Protists of several species appear to pass through filters that are intended to exclude them. The mechanism enabling these flagellates to pass through pores 0.4 μm in diameter is not known, but plasticity of sufficiently small cells seems

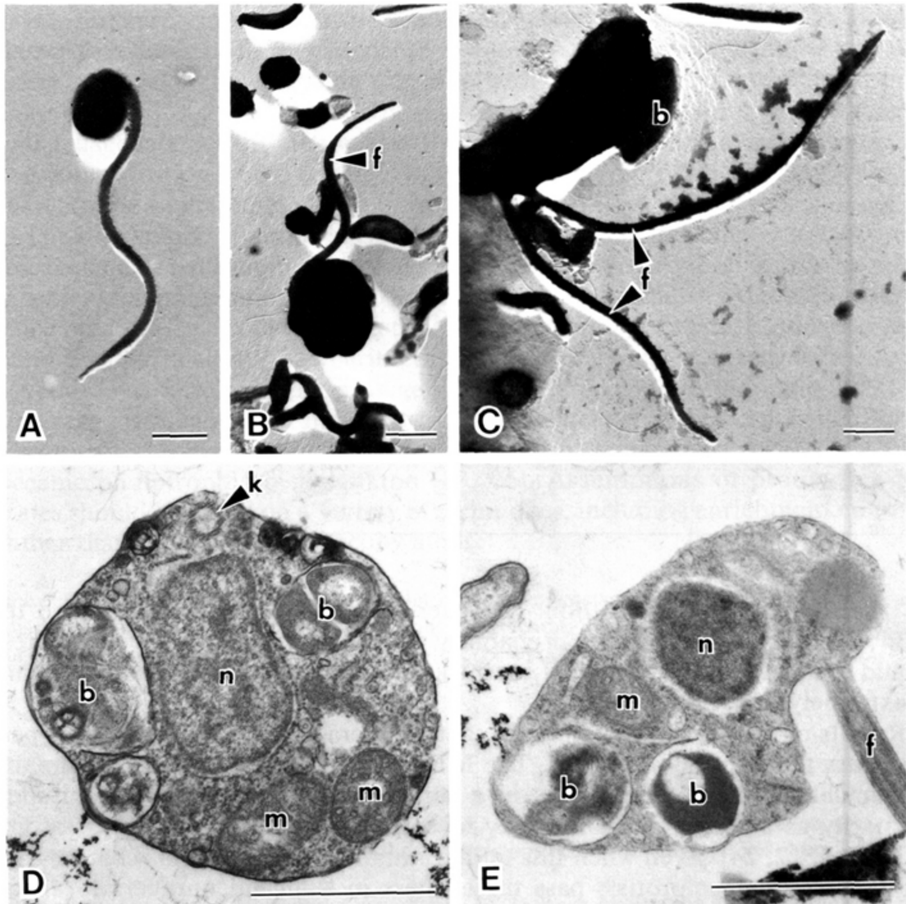

Fig. 1. Bacterivorous nanoflagellates from rice-grain enrichment cultures of 0.4 and 0.6 μm Nuclepore filtrates as shown in transmission electron micrographs of whole mounts (**A-C**) and thin sections (**D, E**). Monoflagellates from 0.4 μm filtrate (**A**) and 0.6 μm filtrate (**B**) enrichments. *Bodo*-like biflagellate from 0.4 μm filtrate enrichment (**C**) with adjacent bacterium. The ultrastructure of these nanoflagellates with their bacteria-containing food vacuoles demonstrates their phagotrophic nutrition; flagellate from 0.4 μm (**D**) and 0.6 μm (**E**) filtrate enrichments. All marker bars equal 1 μm; *b* = bacterium, *f* = flagellum, *k* = kinetosome, *m* = mitochondrion, *n* = nucleus.

very plausible. At the onset of starvation, species of *Ochromonas and Pseudobodo tremulans* undergo 1 or 2 divisions resulting in 2 or 4 smaller cells, respectively [15]. A flagellate of 2 μm diameter undergoing two divisions (without growth) would yield four smaller cells of 1.26 μm diameter. Protists of this size squeezing through 0.4 or 0.6 μm pores were probably the progenitors of the 1–5 μm cells on our TEM grids. Protistan cysts, which have been observed in a number of species [4], could have been responsible for these results, but sufficiently small cysts have never been observed. In addition, the rigidity of cyst walls makes their passage through small pores unlikely. Since overlapping pores are commonly observed on the surface of Nuclepore filters, this appears

Table 3. Most probable number estimate of nanoflagellates passing through 0.6 μm filters

Inoculation date	No. days incu-bated	MPN per 100 mls	95% confidence limits (min./max.)	
August 6, 1984	3	<1.0		
	6	6.4	2.3	17.8
	8	6.4	2.3	17.8
August 15	2	<1.0	—	—
	4	3.9	1.2	12.4
	6	15.7	5.5	44.3
August 24	8	<1.0	—	—
	11	1.0	0.2	7.3
September 12	2	<1.0	—	—
	4	1.0	0.2	7.3
	9	3.9	1.2	12.4
	12	6.4	2.3	17.8

to be an alternative explanation. However, since the pores are collimated to ±34° during manufacture, the probability that a cluster of 0.4 or 0.6 μm pores would be adjacent through the 10 μm thickness of the polycarbonate membrane is extremely low [2].

Results of the MPN assay indicate that an average of only 70 flagellates per liter pass through 0.6 μm filters. The MPN assay is known to underestimate the actual number of organisms in a sample by 1–3 orders of magnitude, primarily as a result of the inability of some species to grow in laboratory culture [8, 12, 24]. Even when this underestimation is taken into account, can we predict whether protists pass these filters in sufficient numbers to affect experimental work?

Assuming published mean values for grazing rate, G (119.3 bacteria/flagellate/hour), growth rate, μ (0.192 flagellates/hour) [14], and the grazing observed by Fuhrman and McManus [18] (9×10^5 bacteria/ml/22 hours), the total amount of grazing, G^T, by flagellates in a 0.6 μm filtrate can be modeled using the equation:

$$G^T = \sum_{t=0}^{22} C_f G / 2^{(\mu t)}$$

When this equation is summed over values of t from 0–22 hours, it is satisfied with values of C_f (concentration after 22 hours) = 975 flagellates/ml. Assuming this value for C_f, the initial concentration, C_i of nanoflagellates passing a filter can be calculated using the equation:

$$C_i = C_f/(2^{(\mu t)}) = 52 \text{ flagellates/ml}$$

Considering the inherent underestimation of the MPN assay, this value of C_i is reasonably close to our MPN value of 0.07 flagellates/ml. Even when present at these low numbers, flagellates present in 0.6 μm filtrates accounted for up to 60% of the total whole water bacterial predation at 22 hours [18].

It is not surprising that neither Fuhrman and McManus nor we were able to detect flagellates in freshly prepared filtrates. At the concentration calculated using the model above, a slide prepared with 10 ml of the 0.6 μm filtrate and examined at 1,000× with the light microscope would contain 7 × 10^4 bacteria and 1 flagellate per 20 microscope fields. Bacterial-sized flagellates present at this concentration would be masked by the more numerous bacteria and would therefore be undetectable by microscopy [27, 30].

The occurrence of eucaryotes in bacterial-dominated filtrates is not entirely unexpected, as plastidic eucaryotes have been reported previously in fixed picoplankton preparations [21]. Bacterivorous nanoflagellates in filtrates thought to be predator-free could affect estimates of bacterial numbers, production and activities, particularly in experiments whose time courses are measured over several days. Similarly, the passage of plastidic protists in the nanoplankton size range through filters intended to exclude them may be responsible for a portion of the high primary production ascribed to natural populations of oceanic phototrophic picoplankton [23, 26]. Assumptions of protist-free filtrates should be based on a variety of techniques, including enrichment culture, rather than relying on microscopy alone.

Acknowledgments. We wish to thank M. McCray for her assistance, P. W. Johnson for the transmission electron microscopy of the thin sections and preparation of the figure, and George McManus for providing raw data from his study. This work was supported by NSF Grant No. OCE-8121881.

References

1. Ammerman JW, Fuhrman JA, Hagstrom A, Azam F (1984) Bacterioplankton growth in seawater: I. Growth kinetics and cellular characteristics in seawater cultures. Mar Ecol Prog Ser 18:31–39
2. Ballew HW, Staff of Nuclepore Corporation (1978) Basics of filtration and separation. Nuclepore Corp, p 8
3. Beers JR, Stewart GL (1970) Numerical abundance and estimated biomass of microzooplankton. Bull Scripps Inst Oceanogr 17:67–87
4. Brooker BE, Ogden CG (1972) Encystment of *Bodo caudatus*. Protoplasma 74:397–409
5. Burney CM, Davis PG, Johnson KM, Sieburth JMcN (1981) Dependence of dissolved carbohydrate concentrations upon small scale nanoplankton and bacterioplankton distributions in the Western Sargasso Sea. Mar Biol 65:289–296
6. Burney CM, Davis PG, Johnson KM, Sieburth JMcN (1982) Diel relationships of microbial trophic groups and *in situ* dissolved carbohydrate dynamics in the Caribbean Sea. Mar Biol 67:311–322
7. Caron DA (1983) Technique for enumeration of heterotrophic and phototrophic nanoplankton, using epifluorescence microscopy, and comparison with other procedures. Appl Environ Microbiol 46:491–498
8. Caron DA, Davis PG, Madin LP, Sieburth JMcN (1982) Heterotrophic bacteria and bacterivorous protozoa in oceanic macroaggregates. Sci 218:795–797
9. Christian RR, Hanson RB, Newell SY (1982) Comparison of methods for measurement of bacterial growth rates in mixed batch cultures. Appl Environ Microbiol 43:1160–1165
10. Clarke KR, Owens NJP (1983) A simple and versatile micro-computer program for the determination of 'most probable number.' J Microbiol Methods 1:133–137
11. Davis PG, Sieburth JMcN (1982) Differentiation of phototrophic and heterotrophic nanoplankton populations in marine waters by epifluorescence microscopy. Ann Inst Oceanogr 58(S):249–260

12. Davis PG, Sieburth JMcN (1984) Estuarine and oceanic microflagellate predation of actively growing bacteria: estimation by frequency of dividing-divided bacteria. Mar Ecol Prog Ser 19: 237–246
13. Estep KW, Hargraves PE, Davis PE, Sieburth JMcN (1984) Chloroplast-containing microflagellates in natural populations of North Atlantic nanoplankton: their identification and distribution, including a description of five new species of *Chrysochromulina* (Prymnesiophyceae). Protistologica 20:613–634
14. Fenchel T (1982) Ecology of heterotrophic microflagellates. II. Bioenergetics and growth. Mar Ecol Prog Ser 8:225–231
15. Fenchel T (1982) Ecology of heterotrophic microflagellates. III. Adaptations to heterogeneous environments. Mar Ecol Prog Ser 9:25–33
16. Fenchel T (1982) Ecology of heterotrophic microflagellates. IV. Quantitative occurrence and importance as bacterial consumers. Mar Ecol Prog Ser 9:35–42
17. Fuhrman JA, Azam FA (1980) Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Appl Environ Microbiol 39:1085–1095
18. Fuhrman JA, McManus GB (1984) Do bacteria-sized marine eukaryotes consume significant bacterial production? Sci 224:1257–1260
19. Hagström A, Ammerman JW, Henrichs S, Azam F (1984) Bacterioplankton growth in seawater. II. Organic matter utilization during steady-state growth in seawater cultures. Mar Ecol Prog Ser 18:41–48
20. Hobbie JE, Daley RJ, Jasper S (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225–1228
21. Johnson PW, Sieburth JMcN (1982) In-situ morphology and occurrence of eucaryotic phototrophs of bacterial size in the picoplankton of estuarine and oceanic waters. J Phycol 18: 318–327
22. Kopylov AI, Moiseev ES (1980) Effect of colorless flagellates on the determination of bacterial production in seawater. Doklady Biol Sci 252:272–274
23. Li WKW, Scubba Rao DV, Harrison WG, Smith JC, Cullen JJ, Irwin B, Platt T (1983) Autotrophic picoplankton in the tropical ocean. Sci 219:292–295
24. Lighthart B (1969) Planktonic and benthic bacterivorous protozoa at eleven stations in Puget Sound and adjacent Pacific Ocean. J Fish Res Bd Can 26:299–304
25. Newell SY, Christian RR (1981) Frequency of dividing cells as an estimator of bacterial productivity. Appl Environ Microbiol 42:23–31
26. Platt T, Subba Rao DV, Irvin B (1983) Photosynthesis of picoplankton in the oligotrophic ocean. Nature 301:702–704
27. Sieburth JMcN (1979) Sea microbes. Oxford University Press, New York, pp 112–114
28. Sieburth JMcN, Smetacek V, Lenz J (1978) Pelagic ecosystem structure: heterotrophic compartments and their relationship to plankton size fractions. Limnol Oceanogr 23:1256–1263
29. Sorokin YI (1979) Zooflagellates as a component of the community of eutrophic and oligotrophic waters in the Pacific Ocean. Oceanology 19:316–319
30. Takahashi M, Hori T (1984) Abundance of picoplankton in the subsurface chlorophyll maximum layer in subtropical and tropical waters. Mar Biol 79:177–186