APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1983, p. 1388–1393
0099-2240/83/121388-06$02.00/0

Vol. 46, No. 6

Double-Staining Epifluorescence Technique to Assess Frequency of Dividing Cells and Bacteriovory in Natural Populations of Heterotrophic Microprotozoa†

EVELYN B. SHERR* AND BARRY F. SHERR

University of Georgia Marine Institute, Sapelo Island, Georgia 31327

Received 29 April 1983/Accepted 20 September 1983

We have developed a double-staining procedure for use with epifluorescence microscopy which allows the detection both of dividing cells and of ingested bacteria in food vacuoles of heterotrophic microprotozoa. Microprotozoan cells are stained sequentially with the DNA-specific fluorochrome DAPI (4',6-diamidino-2-phenylindole) and the nonspecific protein stain fluorescein isothiocyanate. During microscopic examination, heterotrophic microprotozoan cells are first located with fluorescein isothiocyanate fluorescence and then epifluorescence filter sets are switched to permit inspection under DAPI fluorescence of the cell nuclei and of the contents of food vacuoles. Among in situ populations of estuarine microprotozoa sampled over a tidal cycle, we found from 2.2 to 5.2% of the heterotrophic cells in a recognizable stage of division (nuclei elongated or double). Batch culture growth experiments were also carried out both with natural populations and with two isolated species of estuarine microprotozoa. In these experiments, the frequency of dividing cells ranged from 1.2 to 3.8% and appeared to be negatively correlated with growth rate. Microprotozoan populations sampled in continental shelf waters off Savannah, Ga., had mean frequencies of dividing cells ranging from 2.0 to 5.0%. A large fraction of cells in heterotrophic microprotozoan populations (an average of 27.4 ± 1.0% in estuarine water and of 30.1 ± 4.8% in shelf water) had DAPI-stained inclusions, presumably recently ingested bacteria, in their food vacuoles.

There is growing interest in the distribution and ecological roles of heterotrophic microprotozoa (2- to 20-μm diameter) in natural waters (3, 9, 20, 24–26; J. M. Sieburth, *in* J. E. Hobbie and P. J. L. Williams, ed., *Heterotrophic Activity in the Sea*, in press). These tiniest members of the zooplankton are thought to be the dominant consumers of aquatic bacteria (9, 12, 16, 24; Sieburth, in press) and could be an important trophic link between bacteria and other components of aquatic food webs (15, 20). However, because of their small size and fragility, heterotrophic microprotozoa have as yet been little studied in situ.

Recent advances in epifluorescence microscopic techniques (2, 6, 11, 23) now permit accurate enumeration of natural microprotozoan populations. Here we describe an epifluorescence method which allows the determination not only of numbers and biovolumes as described previously (23), but also of the frequency of dividing cells (FDC) in the heterotrophic microprotozoan assemblage, as well as the presence of bacteria within food vacuoles of individual cells.

† Contribution no. 494 of the University of Georgia Marine Institute.

MATERIALS AND METHODS

Epifluorescence microscopic procedures. Water samples were preserved with a final concentration of 2% formaldehyde saturated with sodium tetraborate and were stored at 5°C until being processed within 1 week of collection. Previous control tests have shown that the fragile microprotozoan cells are preserved intact for at least 2 weeks with this method of preservation (23). A 1- to 30-ml subsample was first treated with the DNA-specific strain DAPI (4',6-diamidino-2-phenylindole) by a procedure modified from that of Porter and Feig (21). Briefly, a solution of DAPI (0.1 μg ml^{-1}) was freshly made up in distilled water from a stock solution at 1 mg ml^{-1} stored at −5°C. We added 0.2 ml of the diluted DAPI solution ml^{-1} of sample and let the mixture incubate in the dark for 7 min. The sample was then filtered onto a 0.8 μm-pore-size Nuclepore membrane filter previously stained with Irgalan black. A 0.8-μm Nuclepore Membra-fil filter was used as a backing under the polycarbonate filter. Finally, the filtered sample was stained with the protein-binding dye fluorescein isothiocyanate (FITC) by a procedure (23) adapted from Fliermans and Schmidt (10).

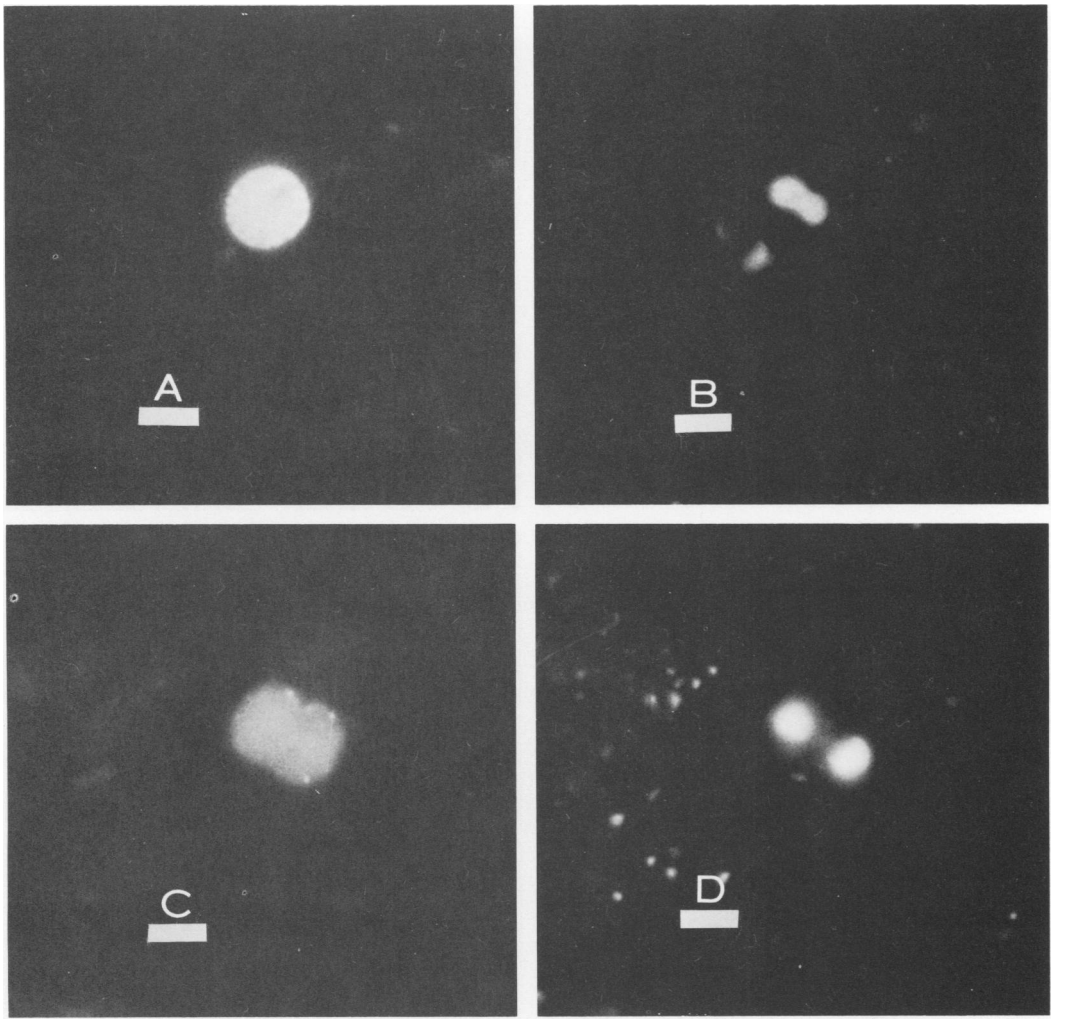

FIG. 1. Heterotrophic microprotozoa cells visualized under FITC (A, C) and under DAPI (B, D) fluorescence. (A and B) Heterotrophic cell with elongated, dividing nucleus; (C and D) dividing heterotrophic cell with double nucleus. Bar, 5 μm.

The prepared filter was mounted immediately on a glass slide, with a drop of FA mounting fluid (Difco Laboratories, Detroit, Mich.), pH 9, between the surface of the filter and a no. 1 cover slip. Each mounted filter was examined immediately at ×1,000 with a Zeiss Standard Microscope equipped with an XBO 75-W DC-2 lamp and a Neofluar ×100 objective with regular immersion oil (Cargille type A) between the lens and the slide. (Planachromat and Planapochromat lenses cannot be used for DAPI fluorescence). Microscope fields were examined first for FITC fluorescence with Zeiss filter set 47 77 09 (BP 450-490 excitation filter, FT 510 beam splitter, LP 520 barrier filter). When a heterotrophic microprotozoan cell, i.e., an identifiable eucaryotic cell with no autofluorescing pigments, was located, we switched to Zeiss filter set 47 77 02 (G 365 exciter filter, FT 395 beam splitter, LP 420 barrier filter) and inspected the cell for DAPI fluorescence. There was a slight FITC background fluorescence observed with the DAPI filter set, which helped to delineate the eucaryotic cells under DAPI fluorescence. Cells having elongated or double nuclei (Fig. 1B and D) were counted as actively dividing cells (5, 22, 27). We also examined the heterotrophic microprotozoa for DAPI-stained inclusions in food vacuoles. By focusing up and down, we could ascertain that DAPI-stained inclusions, presumably recently ingested bacteria, were within the cytoplasm of the cell and not simply lying over or under it. Such inclusions were generally the size and shape of aquatic bacteria, i.e., 0.1- to 1-μm coccoid or rod-shaped cells (3, 10, 19, 21), and for the most part, they were within obvious food vacuoles in the cell (Fig. 2B and D). Photomicrographs were made with

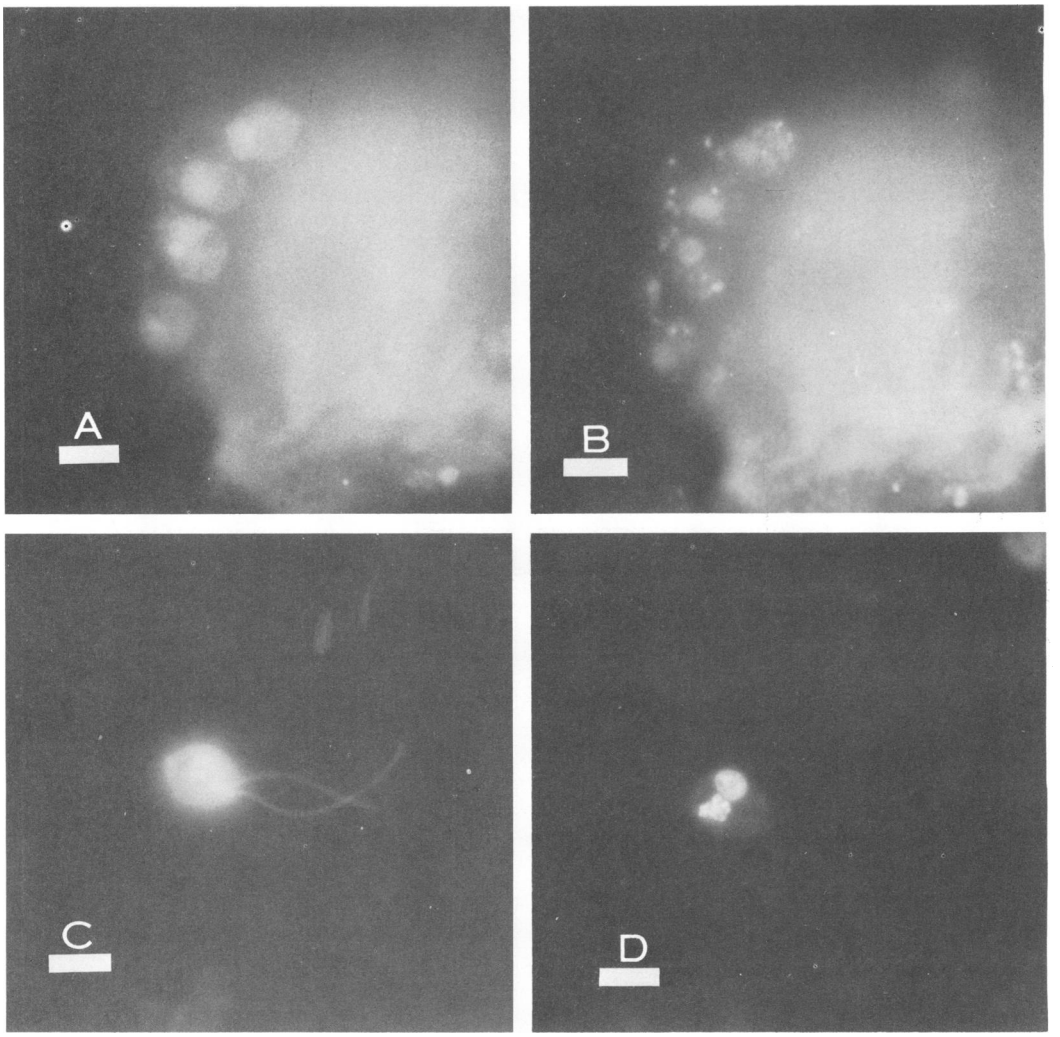

FIG. 2. Heterotrophic microprotozoa cells visualized under FITC (A, C) and under DAPI (B, D) fluorescence. (A and B) Four heterotrophic cells associated with a detrital particle and full of bacteria; (C and D) biflagellate heterotrophic cell with a food vacuole densely packed with bacteria. Bar, 5 μm.

Tri-X (ASA 400) film with exposure times of 20 to 30 s for FITC fluorescence and 60 to 80 s for DAPI fluorescence.

Field sampling. Subsurface estuarine water was collected in the mouth of the Duplin River, a tidal embayment adjacent to Sapelo Island, Ga., every 2 h over a 12-h tidal cycle in January 1983. Triplicate 5-ml subsamples of each Formalin-preserved sample were processed via the double staining procedure and were analyzed for abundance of heterotrophic microprotozoa, FDC, and percentage of the assemblage containing bacteria.

Water was also collected at depths of 3 to 60 m at five stations on the continental shelf along a transect perpendicular to Savannah, Ga., in March 1983. Subsamples (10 to 30 ml) of each 100-ml preserved shelf water sample were processed and examined as for the estuarine water samples. At least 200 microscope fields and 100 heterotrophic microprotozoan cells were inspected for each sample of estuarine or shelf water. The average coefficient of variation of the means of duplicate 100-field counts was 9.9% for abundance, 38% for FDC, and 17.4% for cells containing bacteria.

Laboratory growth experiments. A sample of subsurface estuarine water was collected in the lower Duplin River in December 1982 and was screened through 20-μm-mesh Nitex netting to remove larger zooplankton. The screened water was divided into four 500-ml samples in 1-liter glass bottles. To two bottles we added 1 mg of nutrient broth liter^{-1} to enhance bacterial growth; the other two bottles served as no-addition controls. One of each pair of bottles was incubated at either 14 or 24°C; all bottles were kept in the dark.

TABLE 1. Data on populations of heterotrophic microprotozoa in continental shelf waters along a transect off Savannah, Ga.[a]

Distance from shore (km)	Abundance ($\times 10^2$ cells ml^{-1})	FDC (%)	Cells with bacteria (%)
24.6	4.9 ± 0.3	5.0 ± 0.1	42.3 ± 0.7
36.9	3.8 ± 1.3	2.4 ± 0.0	39.7 ± 5.5
49.2	2.7 ± 0.2	2.0 ± 0.4	23.1 ± 3.0
73.8	2.3 ± 0.3	3.2 ± 0.6	17.8 ± 0.9
123	1.4 ± 0.1	4.9 ± 0.4	27.4 ± 1.0

[a] Data show mean ± standard error.

Duplicate 5-ml samples were withdrawn from each of the four bottles at 0, 12, and 22 h and were assayed for abundance of heterotrophic microprotozoa and for FDC.

At the same time, two isolated species of estuarine bacteriovorous microflagellates were obtained from Duplin River water through a process of serial dilutions in microtiter plates. The microflagellates were maintained at room temperature on a bacterial isolate previously obtained from estuarine water. One of the isolated species was a 3-μm-diameter monad, and the other was a 4- to 5-μm-long bodonid. Batch culture experiments were run with each of the two microflagellate species at four concentrations of the bacterial isolate, 0.02×10^7, 0.1×10^7, 0.5×10^7, and 1.0×10^7 bacteria ml^{-1}, in 100-ml volumes of 0.2-μm-filtered estuarine water incubated in 250-ml Erlenmeyer flasks shaken gently at room temperature (about 23°C). Five-milliliter subsamples were taken from each flask at 0, 26, 34, 44.5, 48.5, and 52.75 h, diluted appropriately, and processed by the double staining method to determine cell abundance and FDC. Growth rates ($hour^{-1}$) were determined during the log phase of the growth curve in each flask and were compared with average FDCs from the beginning to the middle of log-phase growth.

RESULTS AND DISCUSSION

FDC. An FDC method based on acridine orange direct counting with epifluorescence microscopy has previously been used to estimate the in situ growth of aquatic bacteria (13, 14, 18, 19). In situ growth rates of phytoplankton have also been calculated by using light microscopy to determine the fraction of cells undergoing division (17, 27, 30). More recently, Coats and Heinbokel (5) combined light and acridine orange epifluorescence microscopy to study life cycle processes in marine ciliates and dinoflagellates.

In the present study, we developed a procedure by which the FDC can be determined for in situ populations of heterotrophic microprotozoa. Our field and laboratory experiments were designed to evaluate to what extent FDC varied in these populations and whether there was a consistent relationship between FDC and microprotozoan growth rate.

Although there was approximately an order of magnitude difference in abundance between inshore and offshore heterotrophic microprotozoan populations, the range of FDCs was the same, between 2 and 5% (Table 1, Fig. 3). The average (± 1 standard error) FDC was 3.1 ± 0.4% for estuarine populations over a tidal cycle and 3.5 ± 0.6% for shelf microprotozoa from 24.6 to 123 km offshore. In the estuary, we found that the FDC decreased from low to high tide, although the abundance of heterotrophic microprotozoa did not change (Fig. 3). On the shelf, heterotrophic microprotozoa decreased in abundance with distance from the coast, but higher FDCs were obtained for populations at both ends of the transect (Table 1). These results suggest that the various microprotozoan assemblages, e.g., low-tide versus high-tide populations or inshore versus midshelf populations, were in different growth states depending on local environmental conditions.

The theoretical considerations involved in relating FDC to growth rate have been discussed by a number of authors (e.g., 1, 4, 17). Briefly, for a population of microorganisms experiencing asynchronous steady-state growth, $\mu = (1/t_d)[\log_e (1 + f)]$ (17) where μ is the growth rate, t_d is the average duration of mitosis, and f is the fraction of dividing cells (FDC/100) in the population. If t_d is constant, there should be a positive relationship between μ and f. Such relationships have been reported for natural populations of bacterioplankton (13, 14, 18, 19) and phytoplankton (27, 30).

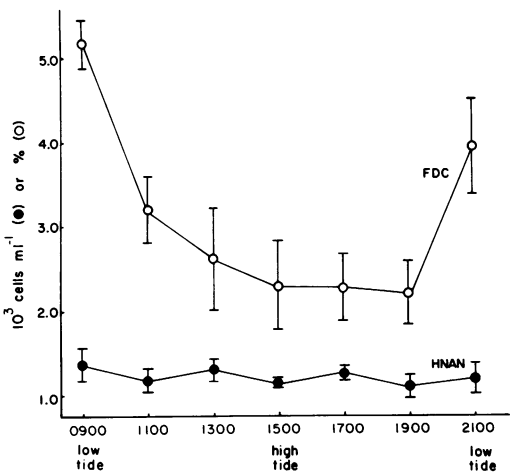

FIG. 3. Variations in the population abundance of heterotrophic microprotozoa (HNAN) cells (●) and in the FDC (○) over a tidal cycle in the lower Duplin River. Data points show mean of three counts ± 1 standard error.

We experimentally varied the growth rates of heterotrophic microprotozoa by altering temperature and concentration of bacterial food. Results of experiments with both the natural microprotozoan assemblage and two isolated species of microflagellate are summarized in Fig. 4. Over a range of growth rates from 0.036 to 0.225 h^{-1}, the FDC varied between 1.5 and 3.9%. However, there appeared to be a generally negative relationship between μ and FDC (Fig. 4). This result is at variance with both the theoretical expectation and with most empirically determined relationships between μ and FDC for bacteria and algae.

A nonpositive relationship between μ and FDC could result from variations in t_d, the duration of mitosis. McDuff and Chisholm (17) addressed the problem of nonconstant t_d when FDC (or, in their terminology, the mitotic index) is used as a measure of phytoplankton growth. They concluded that if "t_d is highly variable with growth conditions, or if it is solely a function of μ, the technique has limited applicability other than to put bounds on the growth rates of *in situ* populations."

We calculated t_d for our growth experiments with the equation presented above from McDuff and Chisholm (17), using the values of μ and f previously determined for each of the microprotozoan growth curves. The calculated t_d values varied considerably, from 3.3 to 52 min, the smaller t_d values being associated with the faster growth rates. The mean t_d value, 21 min, was close to the average duration of mitosis of 15 min which Warner (29) reported for the rumen protozoan *Entodinium* sp. The wide range in t_d values estimated by us for microprotozoans under various growth conditions suggests that nonconstant t_d may indeed be one cause of the results depicted in Fig. 4.

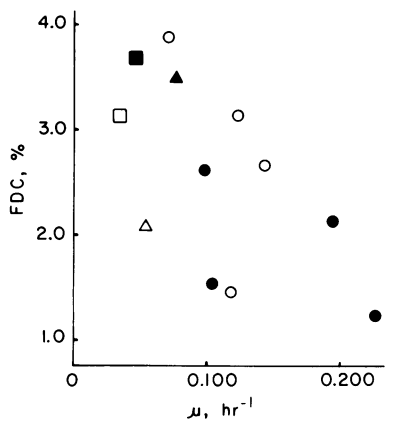

FIG. 4. Relation between FDC and growth rate (μ) for heterotrophic microprotozoa in laboratory experiments. Symbols: ○, A series (monad isolate); ●, B series (bodonid isolate). Both isolates were grown with various concentrations of food bacteria. □, 14°C control; ▲, 24°C control; ■, 14°C culture with nutrient broth; △, 24°C culture with nutrient broth. These last four cultures were grown from 20-μm-screened estuarine water incubated in the dark at the two temperatures indicated with and without nutrient broth addition to stimulate bacterial growth.

Problems in relating μ and FDC have also appeared in studies involving bacteria. Trueba et al. (28) reported shifts in t_d for *Escherichia coli* grown at different temperatures. Furthermore, the FDC method may not always yield a reasonable estimate of in situ bacterial growth; for instance, in marine sediments, it appears that the FDC grossly overestimates bacterial production (7, 19). Our experimental results now additionally question the usefulness of the FDC as an indicator of the in situ growth rate of heterotrophic microprotozoa. More research is clearly required to determine whether FDC can provide meaningful information about the productivity of natural microbial populations.

Percentage of cells containing bacteria. The double-stain epifluorescence method enabled us to visualize DNA-rich, bacterium-sized particles in the cytoplasm of heterotrophic microprotozoa (Fig. 2). These particles were generally located within obvious food vacuoles, which were identified with FITC fluorescence as clear or less densely stained areas of the microprotozoan cell. In some cases, a spherical food vacuole engorged with bacteria superficially resembled the cell nucleus; however, on closer inspection the two could be differentiated by the grainier texture of the food vacuole (see Fig. 2D).

Fenchel (8) commented that dead bacteria could be recognized in food vacuoles of protozoa stained with acridine orange. At times, we saw what appeared to be bacteria in microprotozoan cells with FITC fluorescence. Such inclusions were frequently, but not always, also stained with DAPI. It is possible that bacterial DNA is rapidly denatured, or that binding of DAPI to bacterial DNA is inhibited, during digestion within food vacuoles. We counted only bacterium-sized, DAPI-stained particles which were clearly contained within the cytoplasm of the heterotrophic cells, and we assumed these inclusions to be evidence for recent ingestion of bacteria. We suggest that this method for determining the percentage of microprotozoan cells containing bacteria in a given population can be used as an index of bacteriovory. As such, the method may provide information regarding the proportion of the heterotrophic microprotozoan population which is bacteriovorous; the specific component of the population which is consuming bacteria, e.g., a certain size range or species assemblage; and, combined with experimental

manipulations, perhaps also rates of bacterial consumption.

A significant fraction of in situ heterotrophic microprotozoa in Georgia estuarine and shelf waters contained what appeared to be recently ingested bacteria. During the tidal cycle sampled in the Duplin River, the percentage of cells with bacteria ranged from 25.1 to 32.4% and averaged 27.4 ± 1.0%. The percentage of cells with bacteria was more variable for shelf microprotozoan populations, ranging from 42.3% at 24.6 km to 17.8% at 73.8 km (Table 1). There was no apparent correlation of this index of bacteriovory with either population abundance or FDC. We did find bacteria within cells throughout the size range of the heterotrophic microprotozoan assemblage. At present, we are using the DAPI-FITC double staining procedure outlined here to study estuarine microprotozoan populations in conjunction with simultaneous analysis of bacterioplankton abundance and productivity. Results of this work should allow a more critical evaluation of the usefulness of the technique.

ACKNOWLEDGMENTS

This work was supported by a University of Georgia Faculty Research Grant, by National Science Foundation grant no. OCE-8219866, and by grants from the Sapelo Island Research Foundation.

We thank Steve Newell and David Caron for reviewing the manuscript. Lea Kneib and Lorene Gassert prepared the figures.

LITERATURE CITED

1. **Brock, T. D.** 1971. Microbial growth rates in nature. Bacteriol. Revi. **35**:39–58.
2. **Caron, D. A.** 1983. Technique for the enumeration of heterotrophic and phototrophic nanoplankton, using epifluorescence microscopy, and comparison with other procedures. Appl. Environ. Microbiol. **46**:491–498.
3. **Caron, D. A., P. G. Davis, L. P. Madin, and J. M. Sieburth.** 1982. Heterotrophic bacteria and bacteriovorous protozoa in oceanic microaggregates. Science **218**:795–797.
4. **Chung, K-T., E. H. Nilson, M. J. Case, A. G. Marr, and R. E. Hungate.** 1973. Estimation of growth rate from the mitotic index. Appl. Microbiol. **25**:778–780.
5. **Coats, D. W., and J. F. Heinbokel.** 1982. A study of reproduction and other life cycle phenomena in planktonic protists using an acridine orange fluorescence technique. Mar. Biol. **67**:71–79.
6. **Davis, P. G., and J. M. Sieburth.** 1982. Differentiation of the photosynthetic and heterotrophic populations of nanoplankton by epifluorescence microscopy. Ann. Inst. Oceanogr. **58**(Suppl.):249–260.
7. **Fallon, R. D., S. Y. Newell, and C. S. Hopkinson.** 1983. Bacterial production in marine sediments: will cell-specific measure agree with whole-system metabolism? Mar. Ecol. Prog. Ser. **11**:119–127.
8. **Fenchel, T.** 1970. Studies on the decomposition of organic detritus derived from the turtlegrass *T. testudinum*. Limnol. Oceanogr. **15**:14–20.
9. **Fenchel, T.** 1982. Ecology of heterotrophic microflagellates. IV. Quantitative occurrence and importance as bacterial consumers. Mar. Ecol. Prog. Ser. **9**:35–42.
10. **Fliermans, C. B., and E. L. Schmidt.** 1975. Fluorescence microscopy: direct detection, enumeration, and spatial distribution of bacteria in aquatic systems. Arch. Hydrobiol. **76**:33–42.
11. **Haas, L. W.** 1982. Improved epifluorescent microscopic technique for observing planktonic micro-organisms. Ann. Inst. Oceanogr. **58**(Suppl.):261–266.
12. **Haas, L. W., and K. L. Webb.** 1979. Nutritional mode of several non-pigmented microflagellates from the York River Estuary, Virginia. J. Exp. Mar. Biol. Ecol. **39**:125–134.
13. **Hagström, Å., U. Larsson, P. Hörstedt, and S. Normark.** 1979. Frequency of dividing cells, a new approach to the determination of bacterial growth rates in aquatic environments. Appl. Environ. Microbiol. **37**:805–812.
14. **Hanson, R. B., D. Shafer, T. Ryan, D. H. Pope, and H. K. Lowery.** 1983. Bacterioplankton in Antarctic Ocean waters during the late austral winter: abundance, frequency of dividing cells, and estimates of production. Appl. Environ. Microbiol. **45**:1622–1632.
15. **Kopylov, A. I., A. F. Pasternak, and Y. V. Moiseyev.** 1981. Consumption of zooflagellates by planktonic organisms. Oceanology **21**:269–271.
16. **Lighthart, B.** 1969. Planktonic and benthic bacteriovorous protozoa at eleven stations in Puget Sound and adjacent Pacific Ocean. J. Fish. Res. Board Can. **26**:299–304.
17. **McDuff, R. E., and S. W. Chisholm.** 1982. The calculation of *in situ* growth rates of phytoplankton populations from fractions of cells undergoing mitosis: a clarification. Limnol. Oceanogr. **27**:783–788.
18. **Newell, S. Y., and R. R. Christian.** 1981. Frequency of dividing cells as an estimator of bacterial productivity. Appl. Environ. Microbiol. **42**:23–31.
19. **Newell, S. Y., and R. D. Fallon.** 1982. Bacterial productivity in the water column and sediments of the Georgia (USA) coastal zone: estimates via direct counting and parallel measurement of thymidine incorporation. Microb. Ecol. **8**:33–46.
20. **Pomeroy, L. R.** 1979. Secondary production mechanisms of continental shelf communities, p. 163–186. *In* R. J. Livingston (ed.), Ecological processes in coastal and marine systems. Plenum Publishing Corp., New York.
21. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25**:943–948.
22. **Prescott, D. M., and G. E. Stone.** 1967. Replication and function of the protozoan nucleus, p. 117–146. *In* T. T. Chen (ed.), Research in protozoology, vol. 2. Pergamon Press, Oxford.
23. **Sherr, B., and E. Sherr.** 1983. Enumeration of heterotrophic microprotozoa by epifluorescence microscopy. Est. Coast. Shelf Sci. **16**:1–7.
24. **Sieburth, J. M., and P. G. Davis.** 1982. The role of heterotrophic nanoplankton in the grazing and nurturing of planktonic bacteria in the Sargasso and Caribbean Sea. Ann. Inst. Oceanogr. **58**(Suppl.):285–296.
25. **Sorokin, Y. I.** 1977. The heterotrophic phase of plankton succession in the Japan Sea. Mar. Biol. **41**:107–117.
26. **Sorokin, Y. I.** 1981. Microheterotrophic organisms in marine ecosystems, p. 293–342. *In* A. R. Longhurst (ed.), Analysis of marine ecosystems. Academic Press, Inc. (London) Ltd., London.
27. **Swift, E., and E. G. Durbin.** 1972. The phased division and cytological characteristics of *Pyrocystis* spp. can be used to estimate doubling times of their populations in the sea. Deep-Sea Res. **19**:189–198.
28. **Trueba, F. J., E. A. van Spronsen, J. Traas, and C. L. Woldringh.** 1982. Effects of temperature on the size and shape of *Escherichia coli* cells. Arch. Microbiol. **131**:235–240.
29. **Warner, A. C. I.** 1962. Some factors influencing the rumen microbial population. J. Gen. Microbiol. **28**:129–146.
30. **Weiler, C. S., and R. W. Eppley.** 1979. Temporal pattern of division in the dinoflagellate genus *Ceratium* and its application to the determination of growth rate. J. Exp. Mar. Biol. Ecol. **39**:1–24.