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Frequency of Dividing Cells, a New Approach to the Determination of Bacterial Growth Rates in Aquatic Environments

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Frequency of dividing cells is suggested to be an indirect measure of the mean growth rate of an aquatic bacterial community. Seasonal changes in frequency of dividing cells were found which covariated with the bacterial uptake of ^{14}C-labeled phytoplankton exudates. Batch and continuous culture growth experiments, using brackish water bacteria in pure and mixed enrichment cultures, were performed to establish a relationship between frequency of dividing cells and growth rate. An improved technique for bacterial direct counts, using fluorescent staining and epifluorescence microscopy, is presented. Based on a 6-month survey in a coastal area of the Baltic Sea, the bacterial production in the photic zone is estimated. Compared to the total primary production in the area, the bacterial population during this period utilized approximately 25% of the amount of carbon originally fixed by the primary producers.

Heterotrophic bacteria in the pelagic system have until recently been regarded as an integrated part of the particulate organic matter in the water (10, 21, 30). This view has now gradually changed, and the importance of the free-living bacteria in the pelagic ecosystem has been recognized (1, 3, 9, 23, 28).

To understand the function of the pelagic ecosystem, an estimate of bacterial production is of fundamental importance. This requires information on both bacterial biomass and growth rate. Values of mean bacterial biomasses in different microbial communities are available in current literature (6, 19, 25). Rate of bacterial growth has hitherto been estimated by numerous methods of varying accuracy. Indirect methods based on substrate uptake, using radioactive tracers and direct methods such as the submerged-slide technique, have been used (2, 26). However, all such methods have limited general applicability because they involve an incubation procedure, which introduces interpretation difficulties.

In the gram-negative organism *Escherichia coli*, relationships relating growth rate to such directly measurable parameters as mean cell length, cell width, and frequency of dividing cells (FDC) have been established (8; B. Westling-Häggström, Ph.D. thesis, University of Umeå, Umeå, Sweden, 1978). Each bacterial cell cycle is terminated by septum formation, which eventually causes the separation of the two daughter cells. The FDC in a growing bacterial population depends on the growth rate and the time required for septum formation and cell separation. In *E. coli* FDC is positively related to growth rate. If a similar relation exists for bacteria in the aquatic environment predominantly occupied by gram-negative organisms (17, 31), one would expect seasonal variations in the FDC of the bacterial population.

The object of the present paper is to illustrate the possible use of FDC as a measure of bacterial growth rate in the phototrophic layer of the pelagic ecosystem.

MATERIALS AND METHODS

Investigation area. The work reported in this paper has been carried out at a routine station close to the Askö Laboratory (lat. 17°38′ E, long. 58°48′ N), 70 km south of Stockholm, Sweden. The investigated area can be characterized as a relatively shallow coastal zone penetrated by a canyon, approximately 10 to 15 m deeper than the surrounding bottoms. The station is situated in the middle of the canyon. The hydrography of the region has been described by Shaffer (22).

Epifluorescence microscopy. Direct counts of bacteria were performed on 0.2-μm-sieve filters (Shandon-Nuclepore Corp., Pleasanton, Calif.), using fluorescent staining according to a method modified after Zimmermann and Meyer-Reil (29). Water samples (5

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ml) were taken with a Ruttner sampler and directly preserved and stained by the addition of 0.4 ml of filtered (0.22 μm), buffered (hexamethylenediamine, 20 g/100 ml, pH 7.2) formaldehyde (20%, wt/wt) containing acridine orange (0.125 g/100 ml). The final concentrations of formaldehyde and acridine orange were 1.5 and 0.01%, respectively. To ensure an even filtration, subsamples (0.1 to 0.6 ml) were mixed with 5 ml of particle-free water in a 13-mm stainless-steel funnel (XX30 01240, Millipore Corp., Bedford, Mass.) fitted with a 0.2-μm Nuclepore filter placed on a prefilter (AP20 013 00, Millipore). The filters were rinsed with 3× 5 ml of particle-free water. To avoid the less effectively rinsed peripheral area of the dried filter, a small square was cut out and mounted on a glass slide in cinnamaldehyde and eugenol (2:1). The cover slips were sealed with clear nail varnish. The filters were counted by epifluorescence, using Zeiss WG optics (objective planapochromat, 63/1.40; oculars, kpl 12.5 W).

Counting procedure. Each sample was counted according to the following rules. Ten fields of magnification were counted if each field contained 30 bacteria or more. The number for each field was recorded. If less than 30 bacteria were found per field, additional fields were counted until a total of 300 cells were obtained. Dividing bacteria were counted on a minimum of 10 fields. If less than 30 dividing cells were found, a maximum of 20 additional fields were examined. Bacteria showing an invagination, but not a clear intervening zone between cells, were considered as one dividing cell.

Phase-contrast microscopy. Cells were spread on glass slides covered with 0.5 ml of seawater containing 1.5% agar. The slides were examined in a Zeiss WG photomicroscope. Microphotographs were taken using Kodak Panatomic-X film. The film was processed in Kodak D-76 developer for 7 min at 26°C.

Scanning electron microscopy (SEM). Samples were fixed in 1.5% glutaraldehyde in seawater and filtered through Nuclepore sieve filters at a constant flow rate. Two filter holders (Swinlock 25 mm, Millipore Corp.) were mounted in a tandem system retaining larger particles on a 3-μm filter while the bacteria were collected on a 0.2-μm filter. A three-way stopcock between the holders allowed separate washing of the 0.2-μm filter with 2× 10 ml of 0.1 M phosphate buffer (pH 7.4). The filters were cut and pieces approximately 8 by 8 mm were placed in containers for dehydration in ethanol and ethanol-isoamylacetate (5) and critical-point drying (Polaron E-300 critical point drying apparatus). The dry filters were mounted on aluminum stubs. Polystyrene-latex spheres (Latex, Polaron Equipment Ltd., Watford, England) with a specified diameter were used as reference for measurements of bacterial size. The filters were coated with 20 nm of gold in an Edwards vacuum coating unit during continuous rotation and tilting under a pressure of 10^{-5} torr. The filters were viewed in a Cambridge Stereoscan S-4 scanning electron microscope, operated at 20 kV with a final aperature size of 140 μm and at a 0° detector angle. Randomly selected fields were focused and photographed.

Activity in the phototrophic layer. Water samples were collected and incubated for 4 h at depths of 0, 1, 2, 4, 6, 8, 10, 15, and 20 m in 125-ml glass bottles. Four microcuries of carrier-free $^{14}CO_2$ was added to each bottle. The incubated water was filtrated through two filter holders (Swinlock 25 mm, Millipore), with a 3-μm filter in the top and a 0.2-μm filter (Nuclepore) in the bottom holder. According to Salonen (18), this yields a sufficient separation of algae, bacteria, and algal exudates. Further data to support the view that the radioactive uptake on the 0.2-μm filter is due to bacterial assimilation of phytoplankton exudates are given in a separate paper by Larsson and Hagström (12). The filters were acidified, dried, and digested using Soluene 350 (Packard Instruments). Ten-milliliter samples of the filtrates were transferred to scintillation vials. After acidification and aeration, gel-forming scintillator (Instagel, Packard Instruments) was added. All samples were counted in an Intertechnique SL 40 liquid scintillation counter. The uptake of carbon was calculated according to the Baltic Marine Biologists manual (7).

Batch culture growth experiments. The medium used was filtered seawater supplemented with 140 μg of NH_4PO_4 per liter, 40 μg of NaH_2PO_4 per liter, 80 μg of $NaNO_3$ per liter, and one of the following carbon sources: 1.5% casein hydrolysate plus 0.2% sodium acetate, 1.5% casein hydrolysate, 0.2% sodium acetate, or 0.5% glycerol. The bacteria were cultivated in a rotary shaker. Samples for determination were preserved in 1.0% formaldehyde. Growth was recorded by optical density readings at 450 nm, using a Beckman Acta II spectrophotometer. FDC was determined from phase-contrast micrographs.

Continuous culture growth experiments. Mixed enrichment cultures from seawater were used to inoculate a 400-ml chemostat (New Brunswick Inc). A medium consisting of heat-sterilized, filtered seawater supplemented with yeast extract (0.5 mg/liter) was fed at different dilution rates for a period of at least seven times the residence time. Samples for bacterial counts and FDC determination were collected and treated as natural water samples. The sterility of the inflowing media was checked after each run.

RESULTS

Field studies. The frequency of dividing bacteria in a growing population depends on the growth rate and the average time period during which bacteria are in a stage of division. We were intrigued by the possibility that this parameter, in the heterotrophic community, could reflect growth. We therefore included FDC measurements in the routine program of a coastal station in the Baltic Sea. Water samples were taken over the period August 1976 to September 1978. The following parameters were monitored biweekly: (i) direct bacterial counts, (ii) number of dividing cells, and (iii) primary production.

The number of bacteria in the water column during 1976 is shown in Fig. 1. Isolines connect dates and depths with the same range of bacterial numbers. The hydrological regime, as seen in Fig. 2, is reflected also in the bacterial counts,

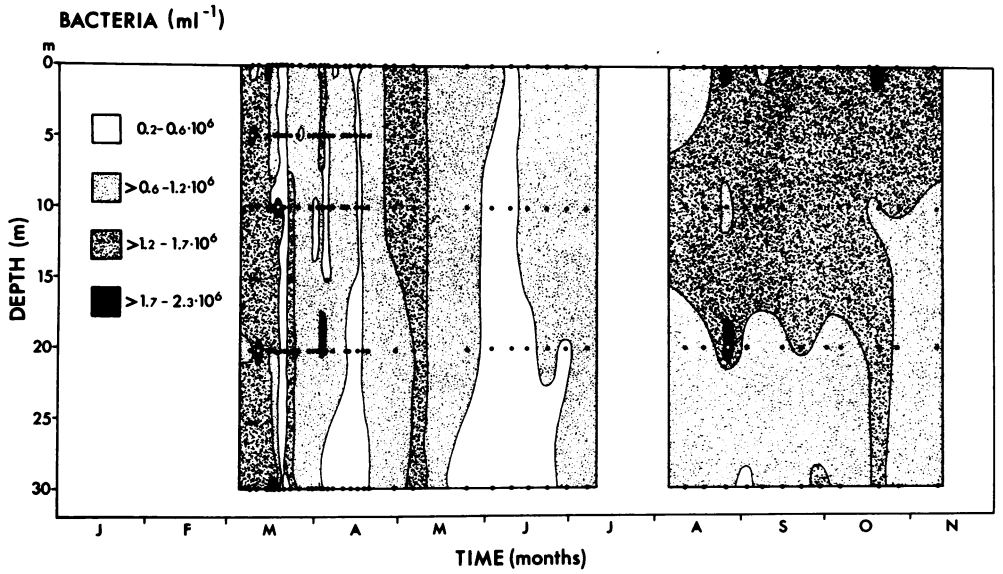

FIG. 1. *Distribution of bacteria in the water column during 1976. Numbers of bacteria are shown as differently shaded areas, as explained in the figure. Dots indicate sampling depths.*

illustrating the overall dependency of the bacterial community on common abiotic factors. However, starting in September 1976, discrete water samples (depth, 0, 5, 10, 15, and 20 m) were mixed in an Erlenmeyer flask to give an integrated value for the photic zone (0 to 20 m). The integrated bacterial numbers are shown in Fig. 3A. Numbers are given per square meter of surface area. The FDC values are shown in Fig. 3D. Fluctuations in FDC were found over the year. A marked FDC peak was found before the spring bloom.

Initially, we used the original staining technique of Zimmermann and Meyer-Reil (29), which included rinsing in propanol and xylene. As a control of this technique, samples for SEM examination were taken on five occasions during the decline of the 1977 spring bloom. Both techniques gave a decline in the FDC during this period. However, FDC determined from SEM were about twice as high as the corresponding FDC values determined by direct microscopy. The latter could have been due to inefficient washing of the filters, causing high background counts. Therefore, the staining technique was modified in October 1977 (Fig. 3, right). Instead of staining on filters, acridine orange was mixed with buffered formaldehyde and the samples were stained during preservation. Furthermore, only water was used for rinsing. This resulted in bright cells and a low background stain.

To demonstrate the precision of the counting procedure, four subsamples from each of 15 separate water samples were counted, and mean values and standard deviations in percentage of the mean values were calculated. For the 15 water samples a range of 9 ± 4% standard deviation was found. Starting in October 1977, four subsamples were routinely counted. During the period October 1977 to September 1978 the bacterial counts were, in general, lower than those of the previous corresponding seasons. This is likely due to the low background stain obtained with the modified staining technique. As a consequence, higher FDC values ranging between 0.6 and 6% were found (Fig. 3D).

A functional relationship between bacterial and algal production can be seen from Fig. 3. The total assimilation of carbon in the water column, the algal uptake, and the bacterial incorporation of algal exudates are presented in separate curves (Fig. 3B and C; data from Larsson and Hagström [12]). High FDC values were noticed before and during the spring bloom (Fig. 3D). However, it should be noted that due to the low temperature high FDC values do not result in a high specific growth rate, as will be shown below. Hence the bacterial uptake of exudates during the spring bloom is also low. At the end of the bloom the available nutrient pool is exhausted, which coincides with low FDC values. During the summer season an apparent covariation between FDC and bacterial uptake of ^{14}C-labeled exudates can be seen (Fig. 3C and D).

Laboratory studies. The relationship between FDC and growth rate was sought under controlled in vitro conditions. A number of brackish water bacteria were isolated from the

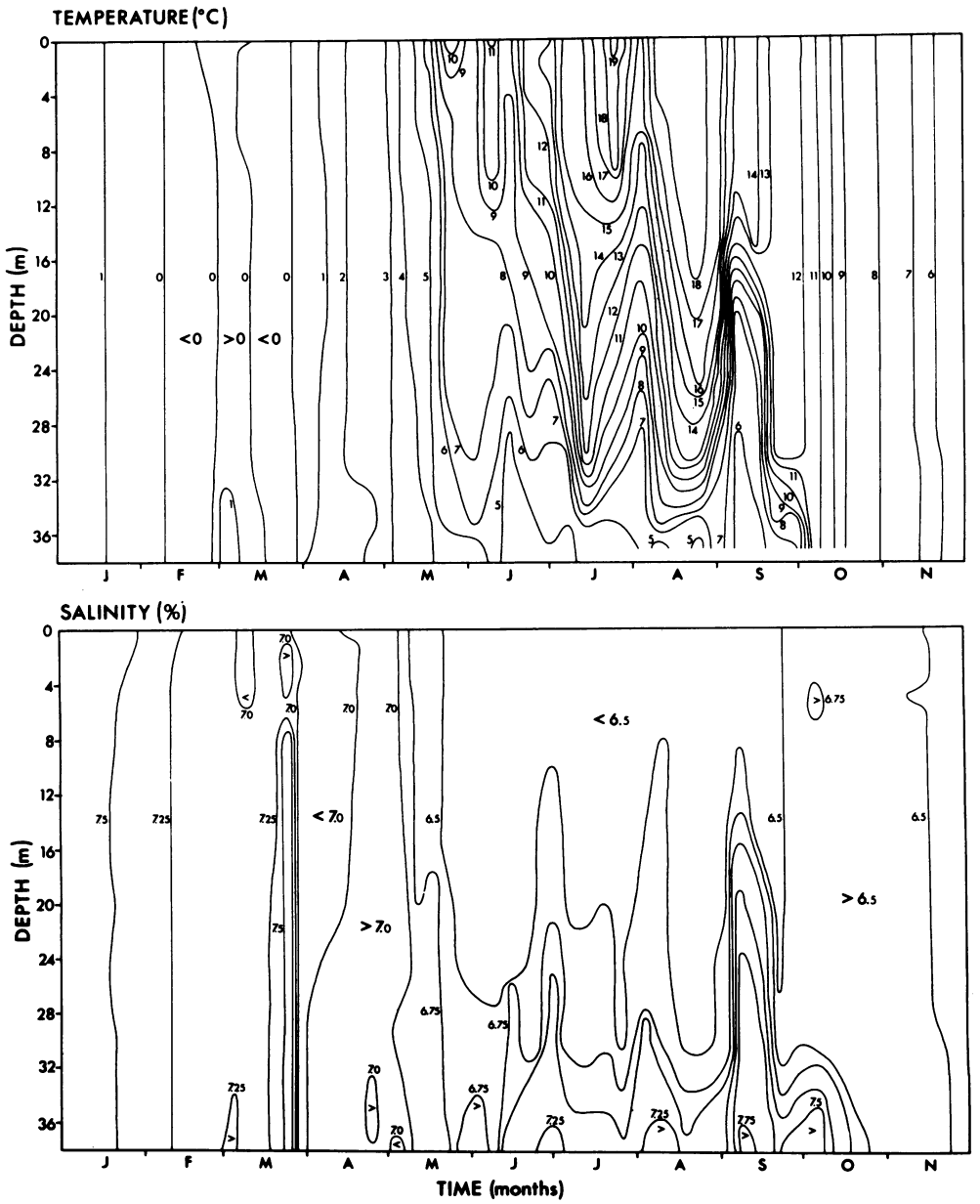

FIG. 2. *Isopleth diagrams of salinity and temperature illustrating the overall hydrological regime of the studied area in 1976.*

investigated area and grown to steady state in media giving different growth rates. Growth rate and FDC were recorded during incubation at 5, 10, and 15°C (Fig. 4). For each isolate and for a given temperature, FDC increased with growth rate. Growth in identical medium but at different temperatures did not significantly affect FDC. The FDC values for the batch cultures were obtained from photomicrographs in phase-contrast microscopy. However, no significant difference in FDC was found between the phase-contrast and the epifluorescence techniques when three strains were counted using both methods.

In the batch culture experiments high substrate concentrations were used to enable steady-state growth. The low substrate conditions occurring naturally were investigated in continuous growth experiments. The chemostat

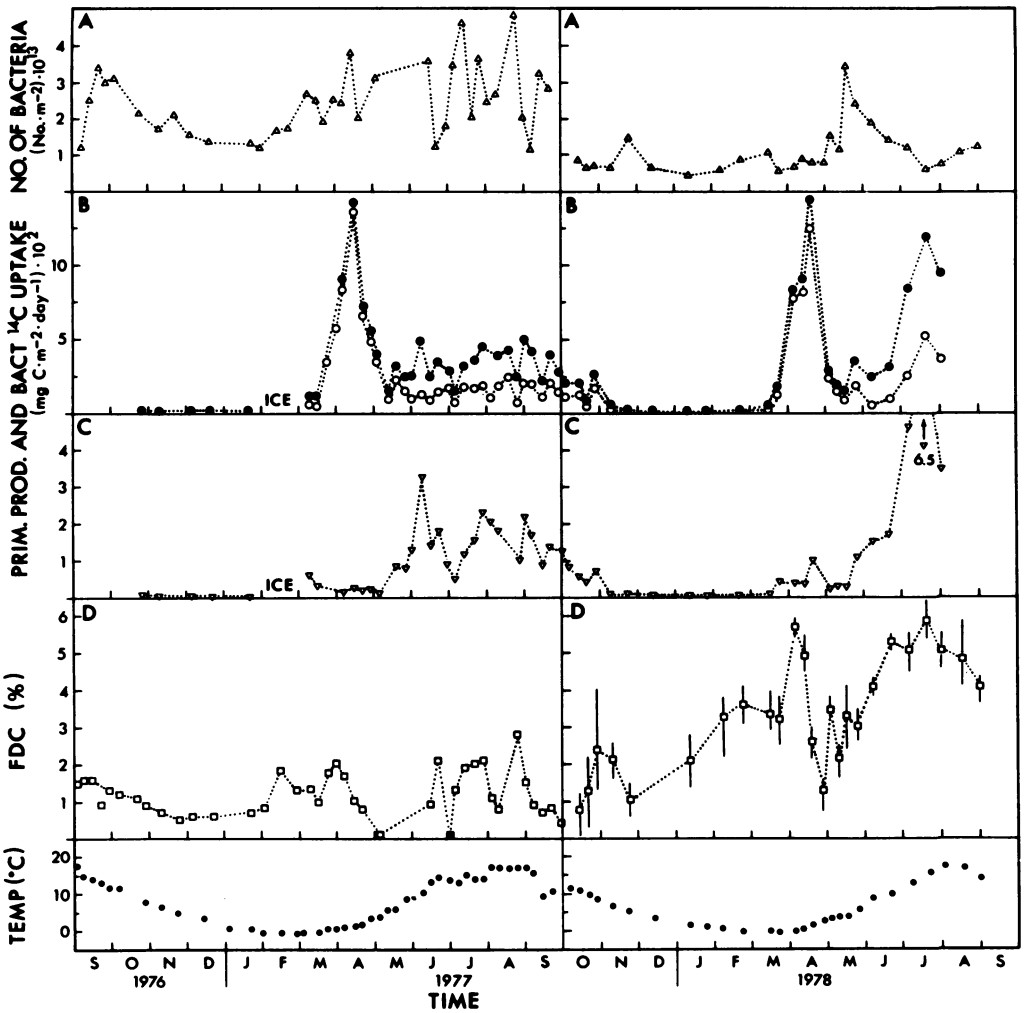

FIG. 3. *Algal and bacterial activity in the trophogenic layer (0 to 20 m) of a Baltic Sea coastal area. (A) Total bacterial number per square meter determined by epifluorescence microscopy. (B and C) Primary production (●) calculated from the sum of algal $^{14}CO_2$ uptake (○), bacterial uptake of ^{14}C-labeled phytoplankton exudates (▽), and the exudate pool. (D) FDC. From September 1976 to October 1977 two samples were counted at each occasion. The number of bacteria and FDC were determined from 10 fields of magnification. From October 1977 the counting procedure was modified as described in the text. Vertical bars indicate the range of four subsamples. Transformation of inorganic $^{14}CO_2$ to organically bound ^{14}C in algae, bacteria, and phytoplankton exudates was obtained by selective filtration (3- and 0.2-μm Nuclepore filters) of water samples incubated in vivo.*

was inoculated with mixed enrichment cultures isolated from seawater. The dilution rate was set to give the desired growth rate. Samples for FDC determinations were taken after a steady state was reached, and the samples were treated according to the routines for the in vivo samples. The relation between FDC and specific growth rate found in the continuous culture is presented in Fig. 4. Depending on the dilution rate, the chemostat was subjected to 5 to 20 μg of yeast extract per liter per h. Deduced from data shown in Fig. 3, this energy input is of the same order of magnitude as that provided by the release of phytoplankton (up to 3 μg of carbon per liter per h). The cell densities of the continuous culture were also found to be within the same range as that found in the trophogenic layer of the sampled area (0.3×10^6 to 3.0×10^6 bacteria per ml).

DISCUSSION

The production of bacterial cell mass for a

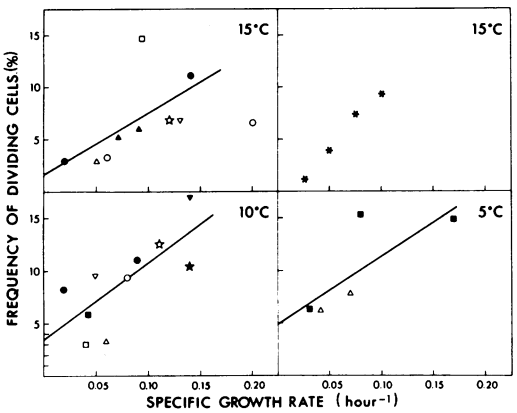

FIG. 4. *Relationship between FDC and specific growth rate obtained from pure cultures grown at steady state in 5, 10, and 15° C. Symbols indicate separate bacterial isolates grown in different media. Regression lines were drawn using the least-squares method. (Upper right) Mixed cultures isolated from the Baltic Sea grown in continuous culture at different dilution rates at 15° C.*

given time period is a function of bacterial abundance, cell volume, and growth rate. The first two parameters can be determined through direct measurements in the aquatic environment (6, 27). Determination of growth rate in a population where the amount of grazing on the bacteria is unknown is a considerably more complex problem. Methods for growth estimations reported so far usually involved incubations, which may bias the results.

In an effort to obtain a measure of bacterial production, we have tried to relate growth rate to a specific event in the bacterial cell cycle, namely, septum formation, a process which is reflected in the FDC (20). The monitoring of dividing cells was done by epifluorescence microscopy, where cells showing a visible invagination were considered to be in a stage of division. When a clear zone between individual cells was observed, the cells were assumed to be divided but not separated. These criteria emanate from morphological studies on a chain-forming mutant of *E. coli* K-12 which divides but does not separate properly (13). The accuracy by which the FDC can be determined is indicated in Fig. 3D, where the FDC values of four subsamples were usually within ±0.5% FDC. We have experienced a good correlation between the FDC counts of different operators when analyzing identical samples. The frequencies of dividing cells using both SEM and epifluorescence microscopy are very similar. The difficulties in detecting dividing cells with SEM are minor, but the technique is too tedious to be used in a routine program.

To establish a relationship between FDC and growth rate, pure cultures of a number of Baltic Sea isolates were grown to steady state in different media and temperatures. For a given temperature a positive correlation between FDC and growth rate was found, although different isolates showed individual variations. For a given medium only small differences in FDC were found during incubation at 5, 10, or 15°C. It therefore seems likely that the fraction of the cell cycle during which a bacterium is under division is independent of temperature but dependent on the nutritional value of the medium (Westling-Häggström, Ph.D. thesis). However, results obtained in batch cultures with single bacterial isolates grown to steady state at high substrate concentration cannot a priori be assumed to be relevant for mixed populations growing at much lower substrate concentrations in the natural habitat. In an attempt to mimic natural conditions, mixed cultures were grown in continuous culture at a low substrate concentration. In the continuous culture the growth rate is determined by the medium supply, that is, the dilution rate of the chemostat. The results obtained from continuous culture confirmed the positive correlation between FDC and growth rate found in the batch cultures.

The introduction of the modified staining technique resulted in in vivo values of FDC ranging from 0.6 to 6%. The covariation between FDC and bacterial uptake of ^{14}C-labeled exudates seems to support the view that FDC reflects bacterial growth. However, the validity of the in vitro relationship between FDC and specific growth rate would be endangered in vivo if factors other than growth rate could influence the FDC of the bacterial community. From the batch culture experiment it was found that different isolates showed different FDC values for the same medium and temperature. These isolates all belonged to different morphological groups. Therefore, a virtually constant relation between different morphological groups throughout the year is essential when using FDC as a measure of growth. Sieburth (24) found a seasonal succession of bacteria with different temperature optima during the year but no changes in the relative composition of morphological groups. Changing nutrient levels may also select for different bacterial types. However, we have not observed any marked shifts in morphological groups during periods of high FDC values, such as the period preceding the spring bloom (data not shown). The existence of dormant cells could cause an underestimation of the FDC values. Available data, however, suggest that the portion of dormant cells is less than 40% (9). Furthermore, the relatively constant

bacterial concentration maintained during periods of high bacterial productivity suggests grazing (4, 16) and thus a turnover, which should reduce the influence of dormant cells on the FDC values. It has recently been shown that psychrophilic marine vibrios under starvation conditions may increase in cell number without mass increase by successive divisions (14). During the low productive period preceding the spring bloom and during winter, such conditions could prevail. At present we do not know to what extent this process will affect our production estimates. Studies on the variation of mean bacterial cell volume during the year would, however, give valuable information on the importance of starvation-induced cell division.

Using the relationship between FDC and growth rate presented, generation times ranging between 10 and 100 h were found. The bacterial biomass can be obtained from the bacterial abundance and the mean cell volume. Usually mean cell volumes of about 0.06 μm^3 are reported (19, 27, 28). In our study we found the same value, based on the measurement of 800 cells from SEM micrographs of five separate water samples (unpublished data). A value for the bacterial cell density of 1.1 g/cm^3 (11) and a carbon-to-wet weight ratio of 0.15 were used to calculate a carbon content from the bacterial abundance. Using the constants above, bacterial numbers and FDC from the in situ measurements made during the first half of 1978 gave a bacterial production of 7.5 g of C per m^2. Assuming a growth efficiency of 60% (15), the corresponding bacterial consumption would be 13 g of C. The bacterial incorporation of carbon calculated from the uptake of phytoplankton ^{14}C-labeled exudates during the same time was 14.5 g of C per m^2. The primary production during this period was 51 g of C per m^2. Thus, with both methods about 25% of the amount of carbon originally fixed by phytoplankton is channeled through bacteria.

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LITERATURE CITED

1. **Azam, F., and R. E. Hodson.** 1977. Size distribution and activity of marine microheterotrophs. Limnol. Oceanogr. **22**:492-501.
2. **Brock, T. D.** 1967. Bacterial growth rate in the sea: direct analysis by thymidine autoradiography. Science **155**: 81-83.
3. **Derenbach, J. B., and P. J. le B. Williams.** 1974. Autotrophic and bacterial production: fractionation of plankton populations by differential filtration of samples from the English Channel. Mar. Biol. **25**:263-269.
4. **Drake, J. F., and H. M. Tsuchiya.** 1976. Predation on *Escherichia coli* by *Colpoda steinii*. Appl. Environ. Microbiol. **31**:870-874.
5. **Elmros, T., P. Hörstedt, and B. Winblad.** 1975. Scanning electron microscopic study of virulent and avirulent colonies of *Neisseria gonorrhoeae*. Infect. Immun. **12**:630-637.
6. **Ferguson, R. L., and P. Ruble.** 1976. Contribution of bacteria to standing crop of coastal plankton. Limnol. Oceanogr. **21**:141-144.
7. **Gargas, E.** 1975. A manual for phytoplankton primary production studies in the Baltic. Baltic Marine Biologists Publ. no. 2. Danish Water Quality Institute, Hørsholm.
8. **Grover, N. B., C. L. Woldringh, A. Zaritsky, and R. F. Rosenberger.** 1977. Elongation of rodshaped bacteria. J. Theor. Biol. **67**:181-193.
9. **Hoppe, H. G.** 1976. Determination and properties of actively metabolizing heterotrophic bacteria in the sea, investigated by means of micro-autoradiography. Mar. Biol. **36**:291-302.
10. **Jannash, H. W.** 1967. Growth of marine bacteria at limiting concentrations of carbon in seawater. Limnol. Oceanogr. **12**:264-271.
11. **Lamanna, C., M. F. Mallette, and L. Zimmerman.** 1973. Basic bacteriology, 4th ed., p. 68. The Williams & Wilkins Co., Baltimore.
12. **Larsson, U., and Å. Hagström.** 1979. Phytoplankton extracellular release as an energy source for bacterial growth in a pelagic ecosystem. Mar. Biol., in press.
13. **Normark, S., H. G. Boman, and G. D. Bloom.** 1971. Cell division in a chain-forming *envA* mutant of *Escherichia coli* K-12. Acta Pathol. Microbiol. Scand. Sect. B **79**:651-664.
14. **Novitsky, J. A., and R. Y. Morita.** 1978. Possible strategy for the survival of marine bacteria under starvation conditions. Mar. Biol. **48**:289-295.
15. **Payne, W. J.** 1970. Energy yields and growth of heterotrophs. Annu. Rev. Microbiol. **24**:17-52.
16. **Pike, E. B., and C. R. Curds.** 1970. The microbial ecology of the activated sludge process. *In* Microbial aspects of pollution. Soc. Appl. Bacteriol. Symp. Ser. **1**:123-147.
17. **Rheinheimer, G.** 1974. Aquatic microbiology, p. 35. John Wiley & Sons Inc., New York.
18. **Salonen, K.** 1977. Effectiveness of cellulose ester and perforated polycarbonate membrane filters in separating bacteria and phytoplankton. Ann. Bot. Fenn. **11**: 133-135.
19. **Salonen, K.** 1977. The estimation of bacterioplankton numbers and biomass by phase contrast microscopy. Ann. Bot. Fenn. **14**:25-28.
20. **Sargent, M. G.** 1975. Control of cell length in *Bacillus subtilis*. J. Bacteriol. **123**:7-13.
21. **Seki, H.** 1970. Microbial biomass on particulate organic matter in seawater of the euphotic zone. Appl. Microbiol. **19**:960-962.
22. **Shaffer, G.** 1975. Baltic coastal dynamics project—the fall Downwelling regime of Askö. Contrib. Askö Lab. Univ. Stockholm **7**:1-61.
23. **Sheldon, R. W.** 1972. Size separation of marine seston by membrane and glass-fiber filters. Limnol. Oceanogr. **17**: 494-498.
24. **Sieburth, J. McN.** 1967. Seasonal selection of estuarine bacteria by water temperature. Exp. Mar. Biol. Ecol. **1**: 98-121.
25. **Straskrabova, V., and Y. I. Sorokin.** 1972. Determi-

nation of cell size of microorganisms for the calculation of biomass, p. 48–50. *In* Y. I. Sorokin and H. Kadota (ed.), Techniques for the assessment of microbial production and decomposition in fresh waters. IBP Handbook no. 23. International Biological Programme, Oxford.

26. **Strickland, J. D. H.** 1970. Microbial activity in aquatic environments. *In* Microbes and biological activity. Symp. Soc. Gen. Microbiol. **21**:231–253.
27. **Watson, S. W., T. J. Novitsky, H. L. Quinby, and F. W. Valois.** 1977. Determination of bacterial numbers and biomass in the marine environment. Appl. Environ. Microbiol. **33**:940–946.
28. **Zimmermann, R.** 1977. Estimation of bacterial number and biomass by epifluorescence microscopy and scanning electron microscopy. *In* G. Rheimheimer (ed.), Microbial ecology of a brackish water environment, p. 103. Springer-Verlag, Berlin.
29. **Zimmermann, R., and L. A. Meyer-Reil.** 1974. A new method for fluorescence staining of bacterial populations on membrane filters. Kiel. Meeresforsch. **30**:24–27.
30. **ZoBell, C. E.** 1946. Marine microbiology, p. 140. Chronica Botanica Co., Waltham, Mass.
31. **ZoBell, C. E., and H. C. Upham.** 1944. A list of marine bacteria including description of sixty new species. Bull. Scripps Inst. Oceanogr. **5**:239–292.