APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1990, p. 2572–2575
0099-2240/90/082572-04$02.00/0

Vol. 56, No. 8

Tangential Flow Filtration and Preliminary Phylogenetic Analysis of Marine Picoplankton

STEPHEN J. GIOVANNONI,† EDWARD F. DeLONG,‡ THOMAS M. SCHMIDT, AND NORMAN R. PACE*

Department of Biology and Institute for Molecular and Cellular Biology, Indiana University, Bloomington, Indiana 47405

Received 16 February 1990/Accepted 16 May 1990

A procedure was developed for harvesting gram quantities of microbial biomass from oligotrophic waters, when mixed populations are present in low abundance. Picoplankton from Atlantic Ocean (Hydrostation S, Sargasso Sea) and Pacific Ocean (Aloha Station) sites were collected in a three-stage process: (i) collection of seawater through an intake covered with 10-μm-pore Nytex; (ii) concentration by a tangential flow filtration device equipped with 10 ft² (0.929 m²) of 0.1-μm-pore fluorocarbon membrane; (iii) collection of cells from concentrate by centrifugation. The overall efficiency of picoplankton recovery was at least 37%. The cellular morphotypes recovered matched those of the original population. DNA was prepared from frozen cell pellets by enzymatic digestion, solvent extraction, and isopycnic centrifugation. As indicated by the binding of kingdom-specific hybridization probes to the purified DNA, the Sargasso Sea picoplankton in this collection were largely eubacteria.

Much of the biomass and biogeochemical activity in earth's oceans is attributed to picoplankton (1, 3), organisms between 0.2 and 2.0 μm in diameter (13). Exploring the diversity and community structure of picoplankton has been hampered by the inability to cultivate most of the population constituents. Many studies have shown that only 1 in 10^5 to 1 in 10^3 of microscopically observed picoplankton develop colonies on marine agar plates (2, 7, 8, 16).

In the absence of reliable cultivation techniques, the harvest of naturally occurring picoplankton offers a source of these organisms for studies of bulk populations or separated cell types. Methods used previously to collect picoplankton for bulk analyses include direct filtration through cylindrical membrane filters (15) and vacuum filtration onto fluorocarbon-based filters (4). These methods for collection are limited by the volume of water that can be filtered, typically tens of liters. Due to the low concentrations of cells in oligotrophic marine waters, commonly 10^5 to 10^6 cells per ml (7, 8, 16), thousands of liters of seawater must be processed to obtain sufficient biomass for many studies, such as properties of pigments, marker proteins and lipids, etc. We are particularly interested in cloning rRNA genes for phylogenetic analyses of population constituents (10, 11). In this note we describe the use of tangential flow filtration for the collection of picoplankton from oligotrophic waters of the Atlantic and Pacific oceans. We also describe a purification process for the isolation of picoplankton DNA suitable for preparation of recombinant libraries and a preliminary phylogenetic characterization of bulk DNA from the Sargasso Sea.

Collection of picoplankton by tangential flow filtration. Picoplankton were collected in the Atlantic Ocean (Sargasso Sea) from the *RV Weatherbird* at Hydrostation S (32°4'N, 64°23'W) on 3 to 4 May 1987. During the collection, the vessel was allowed to drift, ending at 32°22'N, 64°21'W. A Pacific Ocean collection was made from the RV *Moana Wave* on 1 to 3 December 1988 at the Aloha sampling site (22°45'N, 158°00'W). Picoplankton were concentrated by a tangential flow filtration apparatus, using the configuration shown in Fig. 1. In brief, ocean water was pumped into a

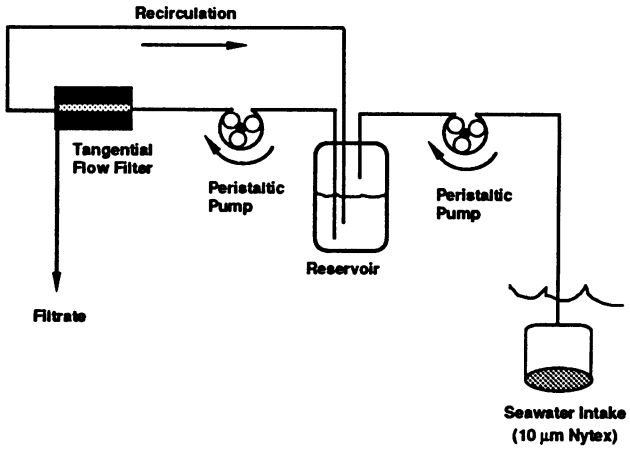

FIG. 1. Schematic representation of the tangential flow filtration system used to concentrate picoplankton. Plankton were concentrated from seawater in a three-stage process. In the first stage, a peristaltic pump was used to pump water aboard through a 28-cm-diameter intake covered with a 10-μm-mesh Nytex screen. The intake was deployed from a buoy at a depth of 1 to 2 m. Incoming water was pumped into a 30-liter carboy, the reservoir for tangential flow filtration. In the second stage, particulate matter was concentrated from the prefiltered seawater with a Pellicon tangential flow device (Millipore Corp.) equipped with 10 ft² of 0.1-μm Durapore fluorocarbon membrane. Periodically (approximately once every 4 h), the uptake pump was stopped, the suspension was concentrated in the carboy by continued tangential flow filtration, and the concentrate was removed to storage at 4°C. In the final stage, particulate material was sedimented from the concentrates by centrifugation at 16,300 × *g* (Sargasso sample) or 25,900 × *g* (Aloha sample). The cell pellets were frozen at −20°C in approximately 200-mg (wet weight) aliquots.

* Corresponding author.
† Present address: Department of Microbiology, Oregon State University, Corvallis, OR 97331.
‡ Present address: Woods Hole Oceanographic Institution, Woods Hole, MA 02543.

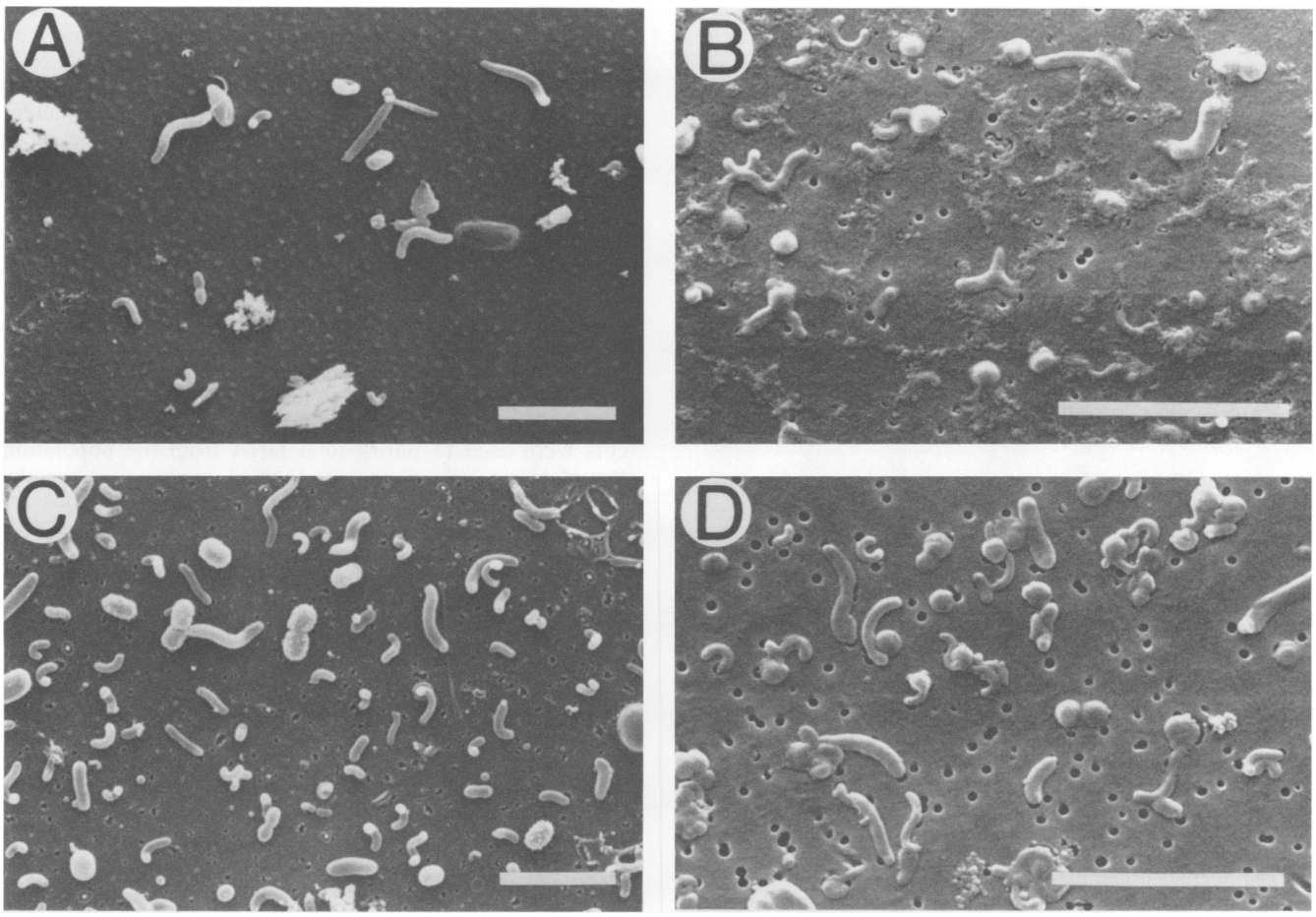

FIG. 2. Representative scanning electron micrographs of picoplankton from Hydrostation S (A and C) and from the Aloha station (B and D). Bars, 2 μm. (A and B) Prefiltration; (C and D) postfiltration. Hydrostation S samples were fixed in 1% glutaraldehyde and allowed to settle onto cover slips coated with Cel-Tak tissue adhesive (Biopolymers, Inc.). The cover slips were washed in distilled water, serially transferred through 25, 50, and 70% ethanol solutions, critical-point-dried, mounted on scanning electron micrograph stubs, and sputter coated with gold-palladium. Samples from the Aloha station were fixed in 1% glutaraldehyde and filtered onto 0.22-μm-pore polycarbonate membranes under a light vacuum. Approximately 100 ml of unconcentrated seawater or 1 ml of a 10^{-2} dilution of the 10-μm filtered, concentrated seawater was used. Samples were serially dehydrated, dried, and coated as described. Samples were viewed on a Cambridge 5250 MK2 scanning electron microscope.

reservoir and then concentrated by cycling through the tangential flow system. The resulting slurry was held in the cold (4°C) until cell pellets were collected by centrifugation. The size class of organisms collected was determined by the porosity of the intake prefilter (Nytex, nominally 10-μm pores) and the tangential flow filter (nominally 0.1 μm). These porosities bracket the picoplankton size definition (0.2 to 2 μm) (13) and permit relatively high rates of flow through the system. By using a single pump and filtration unit, 1,700 liters of seawater was filtered at Hydrostation S; with the addition of a second pump and filtration unit, 8,000 liters was filtered at Aloha Station. Each pump and filtration unit allowed the processing of about 150 liters/h. Holding tanks for ship heads were not flushed during the course of collections.

Cell concentrations in uptake and effluent waters, and in the concentrated slurries, were measured by counting acridine orange-stained preparations viewed by epifluorescence microscopy (6). Cell concentrations in raw seawater were approximately 5×10^5/ml throughout the course of both collections. Approximately 8.0×10^{11} and 4.7×10^{12} cells were collected at Hydrostation S and Aloha station, respectively. In both collections, about 37% of cells present in the raw ocean water was collected into cell pellets. This is a minimum recovery, however, because of some (10 to 20%) fluid loss due to occasional line ruptures. The apparent losses of cells during processing are puzzling. Epifluorescence microscopy of filtrates showed that <1% of picoplankton present in the prefiltered seawater passed through the entire filtration device. We think it likely that the uncollected picoplankton were excluded from the filtration unit by the Nytex intake filter. Although the pores of that filter were nominally 10 μm, loading with particulates could entrap or exclude many cells. Possibilities for cell losses related to tangential flow filtration would include incomplete recovery of concentrated cells from the interstices of the filtration cassettes or the plumbing lines and losses due to the breakage of delicate cells. All filtration techniques share the drawback that they potentially destroy fragile cells. Tangential flow filtration might seem particularly threatening because of shear forces generated by repeated passage of cells through the filter unit. However, scanning electron micros-

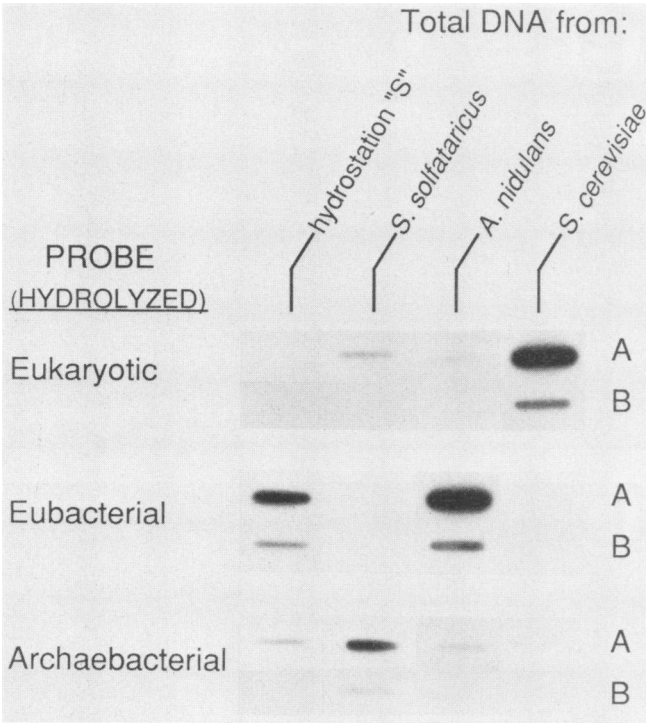

FIG. 3. Phylogenetic analysis of bulk Sargasso Sea picoplankton DNA. A 1.0-μg (A) or 0.1-μg (B) portion of total DNA extracted from, as indicated, picoplankton collected at Hydrostation S, *Sulfolobus solfataricus*, *Saccharomyces cerevisiae*, or *Anacystis nidulans*, was denatured in 0.3 N NaOH at 65°C for 1 h, diluted by addition of 1.4 volumes of 10× SET (1.5 M NaCl, 10 mM EDTA, 0.2 M Tris hydrochloride, pH 7.8), and applied to a nylon membrane (GeneScreen-Plus, DuPont) with a Schleicher & Schuell filtration manifold. The filters were dried in vacuo at 80°C for 1 h. Hybridization probes were prepared from 16S or 18S rRNA isolated from *Saccharomyces cerevisiae* (a eucaryote), *Oceanospirillum linum* (a eubacterium), and *Sulfolobus solfataricus* (an archaebacterium). The RNAs were purified by electrophoresis in a 3% polyacrylamide gel (11) and partially hydrolyzed by incubation at 90°C in 100 mM $NaHCO_3$–Na_2CO_3, pH 9.0, containing 2 mM EDTA. One-third of the reactions was removed at 10, 20, and 30 min, and the aliquots were mixed following the incubation. This procedure ensured a broad spectrum of sizes of rRNA fragments. The fragmented RNAs were precipitated with ethanol, and the fragments were labeled (12) at their 5′ ends with [γ-^{32}P]ATP and polynucleotide kinase (Pharmacia LKB Biotechnology Co.). Filters loaded with DNA were incubated in hybridization buffer (5) overnight at 45°C in the presence of approximately 10^7 cpm of each labeled probe. The filters were removed from the hybridization mix and washed successively (20 min each wash) in 5× SET at 25°C, 1× SET at 25°C, and 0.2× SET at 50°C. After drying, filters were exposed to preflashed X-ray film. The resulting autoradiogram is shown.

copy of cells in raw ocean water compared with cells in concentrates (examples in Fig. 2) revealed the presence of the same morphotypes in about the same proportions. Thus, processing by tangential flow filtration did not seem to bias the collection of any particular types of cells.

Another potential problem for recovery of cells during tangential flow processing is loss due to grazing of picoplankton by phagic organisms (1). We believe that this was not a significant influence in these collections because only a few eucaryotes were present in the concentrate and seawater, and cell concentrates were held no more than a few hours before transfer to 4°C. The majority of cells visible before and after concentration were 0.4 to 1.8 μm in size. Few morphotypes that might be attributed to eucaryotes were visible before or after concentration.

It has been suggested that collection strategies that rely on centrifugation might bias the collections of marine bacteria toward larger cells (4). Although centrifugation was incorporated as a final step in our protocol (see above), such selection at the centrifugal forces used would be expected only if picoplankton have densities well below the values typical of bacteria. In contrast, biomass and biovolume estimates for marine bacteria suggest that they are unusually dense (9). The final supernatants from which these picoplankton cell pellets were sedimented were clear and by light microscopy did not contain significant numbers of cells.

Extraction of DNA from bulk picoplankton. We anticipated that the diverse organisms collected might be differentially susceptible to lysis, so rigorous lysis and extraction protocols were used to purify total DNA from the population. Picoplankton cell pellets were thawed on ice and suspended in 40 mM EDTA–400 mM NaCl–0.75 M sucrose–50 mM Tris hydrochloride, pH 9.0. Lysozyme (Sigma Chemical Co.) was then added to 1 mg/ml, and the suspension was incubated at 37°C for 20 min, followed by the addition of sodium dodecyl sulfate to 0.5% and proteinase K (Sigma) to 160 μg/ml. The cell suspension was then incubated for 2 h at 37°C. Examination of samples by phase-contrast microscopy showed no evidence of remaining, intact cells. Lysates were extracted once with an equal volume of phenol (saturated with 10 mM Tris hydrochloride [pH 9.0], 100 mM NaCl, 1 mM EDTA) at 56°C followed by reextraction of the aqueous phase with $CHCl_3$-isoamyl alcohol (24:1) for 20 min at 56°C. Following the extractions, cesium trifluoroacetate (Pharmacia LKB Biotechnology Co.) was added to the aqueous phase to a final density of 1.65 g/ml. The mixture was centrifuged at 35,000 rpm for 48 h in an SW41 rotor (Beckman Instruments) at 15°C. DNA-containing fractions were dialyzed against 10 mM Tris hydrochloride [pH 7.8]–1 mM EDTA for 48 h at 4°C. The resulting DNA was of high molecular weight and was readily digested with the restriction enzyme *Sau*3a (data not shown). In one preparation, 1.4 mg of DNA (based on A_{260} and ethidium bromide spot tests) was recovered from ca. 3×10^{11} cells. If it is assumed that picoplankton have a DNA content of ca. 5 fg/cell (14), then 3×10^{11} cells would contain ca. 1.5 mg of DNA. The recovery of 1.4 mg is very close to this value, suggesting that minimal bias was introduced during the preparation of DNA.

Although the purified DNA was digested readily by restriction endonucleases, the resulting fragments from the Sargasso Sea sample could not readily be ligated with bacteriophage λ DNA in cloning experiments. A limited recombinant library of only several thousand clones was generated and three rRNA gene-containing clones were identified. In contrast, restriction fragments of DNA purified from the Pacific picoplankton readily participated in ligation with vector DNA, and a satisfactory recombinant library resulted. The analysis of rRNA gene-containing clones from both libraries will be presented elsewhere. The inability of the Sargasso Sea DNA to ligate was not a property of the DNA; rather, it was due to an unknown inhibitory substance in the sample, which could be removed as follows. The isolated DNA (above) was treated with RNase A (3 μg/ml) for 1 h at 37°C; then sodium dodecyl sulfate was added to 0.1% and the sample was held at 55°C for 2 min. Proteinase K then was added to 0.5 μg/ml, and incubation was continued at 37°C for 1 h. The sample was extracted with phenol-$CHCl_3$-isoamyl alcohol (25:24:1, saturated with 10 mM Tris

hydrochloride [pH 9.0], 100 mM NaCl, 1 mM EDTA), the aqueous phase was precipitated with ethanol, and DNA was dissolved in 10 mM Tris hydrochloride (pH 7.8)–1 mM EDTA. The solution was adjusted to 2.5 M ammonium acetate and held at 65°C for 2 min, and then DNA was precipitated by addition of 1 volume of isopropanol. The DNA pellet was rinsed with 70% ethanol and dissolved in 10 mM Tris hydrochloride (pH 7.8)–1 mM EDTA.

Preliminary phylogenetic analysis of Hydrostation S picoplankton. To gain a preliminary phylogenetic characterization of the DNA purified from Sargasso Sea picoplankton, we carried out nucleic acid hybridization experiments with the mixed-population DNA and ^{32}P-labeled, 16S-like rRNAs purified from representatives of each of the three primary kingdoms: archaebacteria, eubacteria, and eucaryotes (17). Under appropriate hybridization conditions, such probes selectively hybridize to DNA from the kingdoms they represent (11). However, because even intrakingdom DNA-RNA hybridization involves formation of imperfect hybrids, such probes are most useful for qualitative analyses.

DNA from the Sargasso Sea (Hydrostation S) picoplankton predominantly binds the probe representative of eubacteria (Fig. 3). Some hybridization of the DNA with the probes representing eucaryotes and archaebacteria was observed. The low intensities of the signals may be due to cross-kingdom hybridization, so the actual presence of those types of organisms is uncertain. It is clear, however, that the DNA extracted from this collection of Sargasso Sea picoplankton is derived primarily from eubacteria. A similar analysis has not been performed with DNA isolated from picoplankton collected at the Aloha station. However, phylogenetic analysis of cloned rRNA genes confirms these hybridization results: >90% of unique rRNA genes recovered are eubacterial (unpublished data).

This investigation demonstrates the feasibility of harvesting large quantities of naturally occurring picoplankton by using tangential flow filtration. In work under way, the sequencing of rRNA genes from recombinant libraries will provide a phylogenetic identification of picoplankton species. The use of species-specific hybridization probes based on those rRNA sequences will reveal the distribution of the species. Taken together, these approaches will lead to a broader understanding of picoplankton community structure.

This research was supported by a grant from the National Science Foundation (BSR-8600170, to S.J.G.), a contract from the Office of Naval Research (N14-87-K-0813, to N.R.P.), and a Public Health Service grant from the National Institutes of Health (GM34527, to N.R.P.).

We thank the staff of the Bermuda Biological Station and the crew of the RV *Weatherbird* for assistance with the collection at Hydrostation S, and we thank David Karl (University of Hawaii) and the crew of the RV *Moana Wave* for assistance with the collection at the Aloha site.

LITERATURE CITED

1. **Fenchel, T.** 1988. Marine plankton food chains. Annu. Rev. Ecol. Syst. **19**:19–38.
2. **Ferguson, R. L., E. N. Buckley, and A. V. Palumbo.** 1984. Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. **47**:49–55.
3. **Fogg, G. E.** 1986. Picoplankton. Proc. R. Soc. London Ser. B **228**:1–30.
4. **Fuhrman, J. A., D. E. Comeau, A. Hagström, and A. M. Chan.** 1988. Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. Appl. Environ. Microbiol. **54**:1426–1429.
5. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170**: 720–726.
6. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
7. **Jannasch, H. W., and G. E. Jones.** 1959. Bacterial populations in seawater as determined by different methods of enumeration. Limnol. Oceanogr. **4**:128–139.
8. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25**:415–420.
9. **Lee, S., and J. A. Fuhrman.** 1987. Relationships between biovolume and biomass of naturally derived bacterioplankton. Appl. Environ. Microbiol. **53**:1298–1303.
10. **Olsen, G. J., D. J. Lane, S. J. Giovannoni, N. R. Pace, and D. A. Stahl.** 1986. Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. **40**:337–365.
11. **Pace, N. R., D. A. Stahl, D. J. Lane, and G. J. Olsen.** 1986. The analysis of natural microbial populations by ribosomal RNA sequences. Adv. Microb. Ecol. **9**:1–55.
12. **Sgaramella, V., and H. G. Khorana.** 1972. Total synthesis of the structural gene for an alanine transfer RNA from yeast. Enzymatic joining of the chemically synthesized polydeoxynucleotides to form the DNA duplex representing nucleotide sequence 1 to 20. J. Mol. Biol. **72**:427–444.
13. **Sieburth, J. M., V. Smetacek, and J. Lenz.** 1978. Pelagic ecosystem structure: heterotrophic compartments of the plankton and their relationship to plankton size fractions. Limnol. Oceanogr. **23**:1256–1263.
14. **Simon, M., and F. Azam.** 1989. Protein content and protein synthesis rates of planktonic marine bacteria. Mar. Ecol. Prog. Ser. **51**:201–213.
15. **Somerville, C. C., I. T. Knight, W. L. Straube, and R. R. Colwell.** 1989. Simple, rapid method for direct isolation of nucleic acids from aquatic environments. Appl. Environ. Microbiol. **55**:548–554.
16. **Watson, S. W., T. J. Novitsky, H. L. Quinby, and F. W. Valois.** 1977. Determination of bacterial number and biomass in the marine environment. Appl. Environ. Microbiol. **33**:940–946.
17. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**: 221–271.