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Stable Carbon Isotope Analysis of Nucleic Acids To Trace Sources of Dissolved Substrates Used by Estuarine Bacteria

RICHARD B. COFFIN,[1]* DAVID J. VELINSKY,[2] RICHARD DEVEREUX,[3] WILLIAM ALLEN PRICE,[1] AND LUIS A. CIFUENTES[2]†

Technical Resources, Inc., c/o U.S. Environmental Protection Agency, Sabine Island, Gulf Breeze, Florida 32561[1]; Carnegie Institution of Washington Geophysical Laboratory, Washington, D.C. 20008[2]; and Department of Veterinary Pathobiology, University of Illinois, Urbana, Illinois 61801[3]

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The natural abundance of stable carbon isotopes measured in bacterial nucleic acids extracted from estuarine bacterial concentrates was used to trace sources of organic matter for bacteria in aquatic environments. The stable carbon isotope ratios of *Pseudomonas aeruginosa* and nucleic acids extracted from cultures resembled those of the carbon source on which bacteria were grown. The carbon isotope discrimination between the substrate and total cell carbon from bacterial cultures averaged 2.3‰ ± 0.6‰ (n = 13). Furthermore, the isotope discrimination between the substrate and nucleic acids extracted from bacterial cultures was 2.4‰ ± 0.4‰ (n = 10), not significantly different from the discrimination between bacteria and the substrate. Estuarine water samples were prefiltered through 1-μm-pore-size cartridge filters. Bacterium-sized particles in the filtrates were concentrated with tangential-flow filtration and centrifugation, and nucleic acids were then extracted from these concentrates. Hybridization with 16S rRNA probes showed that approximately 90% of the nucleic acids extracted on two sample dates were of eubacterial origin. Bacteria and nucleic acids from incubation experiments using estuarine water samples enriched with dissolved organic matter from *Spartina alterniflora* and *Cyclotella caspia* had stable carbon isotope values similar to those of the substrate sources. In a survey that compared diverse estuarine environments, stable carbon isotopes of bacteria grown in incubation experiments ranged from −31.9 to −20.5‰. The range in isotope values of nucleic acids extracted from indigenous bacteria from the same waters was similar, −27.9 to −20.2‰. Generally, the lack of isotope discrimination between bacteria and nucleic acids that was noted in the laboratory was observed in the field. Exceptions to this generalization were due to changes in bacterial substrate sources as a result of the incubation experiments that were used to obtain bacteria for isotope analysis. Our results from work in the field and laboratory indicate that this approach is useful for tracing sources of dissolved organic matter in aquatic environments and describing the bacterial role in its cycling.

Estuarine bacteria obtain organic matter for growth from a variety of sources, including upland vegetation, phytoplankton, salt marsh macrophytes, seagrasses, subtidal sediments, and a host of anthropogenic sources. The relative contributions of these sources to estuarine bacterial production vary temporally and spatially within an environment (8, 29, 30). Sources of organic matter for bacteria are difficult to identify because of complex biological, chemical, and physical interactions. Furthermore, since all dissolved organic matter (DOM) may not be readily metabolized by bacteria and the amount that is labile varies among sources, it is not sufficient simply to identify the sources of DOM to determine substrate sources for bacteria.

Stable isotopes are recognized as a powerful tracer of energy and elemental cycling in complex aquatic food webs (23). Carbon, nitrogen, and sulfur isotopes have been used extensively to study sources of organic matter in estuarine and coastal waters (6, 9, 24, 27). Recently, preliminary work was completed that used stable carbon isotope ratios ($\delta^{13}C$) to study sources of organic matter for estuarine bacteria (7). Bacteria grown in incubation experiments enriched with glucose and DOM extracted from *Spartina alterniflora* and oak trees had $\delta^{13}C$ values similar to that of the source of organic matter. To measure the $\delta^{13}C$ of indigenous bacteria, filtered (0.2-μm pore size) estuarine water was inoculated with filtered (1.0-μm pore size) water and incubated for 24 to 48 h. Their approach used bacteria as a bioassay for the $\delta^{13}C$ of DOM present in the water rather than direct measurement of the $\delta^{13}C$ of bacteria or DOM. This distinction is important because filtration may remove particulate sources of organic matter, such as phytoplankton, zooplankton, and detritus, that are available to indigenous bacterial populations. Furthermore, during batch culture experiments, bacteria may use substrates that are different from the natural substrates.

A method is needed that allows measurement of bacterial stable isotope ratios without an incubation step. In a study that examined the stable carbon isotope ratios of molecular components of *Escherichia coli*, nucleic acids were reported to be similar to whole cells (4). Therefore, analysis of stable isotopes of nucleic acids could be a useful indicator of estuarine bacterial isotope compositions. The advantage of this approach is that the in situ stable isotope composition of bacteria can be measured directly without incubation. Also, nucleic acids provide a specific bacterial biomarker in complex mixtures of estuarine suspended particulate matter. With nucleic acids as a biomarker, it is possible to determine the organism from which the nucleic acids were extracted by using genetic probes.

In this study, laboratory experiments were designed to illustrate isotopic continuity among bacteria, nucleic acids, and the substrate source. In the field, nucleic acids extracted from concentrated estuarine particulate matter were exam-

* Corresponding author.
† Present address: Department of Oceanography, Texas A&M University, College Station, TX 77843.

ined with kingdom level rRNA probes to confirm that the extracts contained predominantly bacterial nucleic acids. Estuarine bacteria were incubated with sources of organic matter with distinct $\delta^{13}C$ values to support comparisons of substrates, bacteria, and nucleic acids in the laboratory. The natural abundances of stable carbon isotopes in bacteria from bioassay experiments and extracted nucleic acids of indigenous bacteria sampled from several ecologically distinct estuarine environments were compared. These studies were used to demonstrate that $\delta^{13}C$ in nucleic acids is a useful tracer of bacterial substrate sources in aquatic environments.

MATERIALS AND METHODS

Bacterial cultures. *Pseudomonas aeruginosa* PA01 was grown with 20 mM glucose or 20 mM glutamate as the sole carbon source in 250 to 500 ml of minimal salts medium (13). Cultures were incubated at 37°C with vigorous mixing. Bacterial growth was monitored with a Klett-Summerson spectrophotometer. Incubations were stopped after 4 to 6 h while cells were growing exponentially. Bacteria were harvested by centrifugation at 10,000 × *g* for 10 min, resuspended, washed in phosphate buffer (1.5 mM; pH 7), and centrifuged again. Cell pellets were stored at −70°C before nucleic acid extraction.

Nucleic acid extraction. Nucleic acids were extracted from cell pellets of bacterial cultures and concentrated bacteria (see below) by using a chloroform extraction method (4; Fig. 1). Bacterial pellets were suspended in 5 to 10 ml of 1.5 M $NaClO_4$ and shaken for 15 min. To ensure complete cell lysis, 2 g of glass beads free of organic matter were added to the bacterial suspension and cells were further broken in a bead beater (BioSpec Products, Bartlesville, Okla.) for 1 min. Lysates were extracted with an equal volume of chloroform. After separation of the aqueous layer that contained nucleic acids from the chloroform-cell debris layers by centrifugation, 5 to 10 ml of 15 mM NaCl–1.5 mM Na_2HPO_4 (pH 7.0) was added to the chloroform-cell debris layer and the extraction was repeated. After extraction, nucleic acids were precipitated at −70°C for 10 min with 0.2 M NaCl and 95% cold ethanol. Precipitated nucleic acids were concentrated by centrifugation at 17,000 × *g* for 30 min, and pellets were washed with distilled 70% cold ethanol (−20°C). Excess ethanol was removed in a vacuum oven for 30 min at room temperature. Nucleic acids were suspended in 5 ml of NaCl-phosphate buffer, extracted with chloroform, and precipitated once more.

To improve recovery of nucleic acids, phenol-chloroform extraction was also tested (17, 18; Fig. 1). Bacterial pellets were suspended in 5 to 10 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Cells were lysed by incubation with 2% (vol/vol) sodium dodecyl sulfate at 65°C for 30 min. Lysed cells were extracted twice with equal volumes of phenol, twice with a mixture of phenol-chloroform-isoamyl alcohol (50:48:2), and twice with chloroform-isoamyl alcohol (24:1). Phenol for these extractions had been distilled and frozen previously, while chloroform and isoamyl alcohol were high-pressure liquid chromatography grade reagents. Nucleic acids were precipitated as described above. The purity of the nucleic acids was determined with UV spectrophotometry at 260 and 280 nm and, on one occasion, protein analysis. Finally, nucleic acids were freeze-dried for isotope analysis.

Concentration of particles of <1 μm from environmental samples. Bacterium-sized particles were concentrated by using a method described by Barkay et al. (2). Particles greater than 1 μm in diameter were removed from water samples by filtration through Millipore cartridge filters (Fig. 1). Bacterium-sized particles in 50- and 100-liter water samples were concentrated to approximately 1-liter volumes with a Pellicon tangential-flow filtration system (Millipore Corp., Bedford, Mass.). This system used low-shear, low-pressure peristaltic pumping with filter velocities of 7 to 8 liters min^{-1}. Filtration times were minimized by using up to three ultrafiltration cassettes (nominal molecular weight, 100,000). The concentrated suspension was centrifuged at 11,000 × *g* for 30 min, and the pellet was frozen at −70°C. The efficiency of each concentration step was estimated with acridine orange direct counts (14).

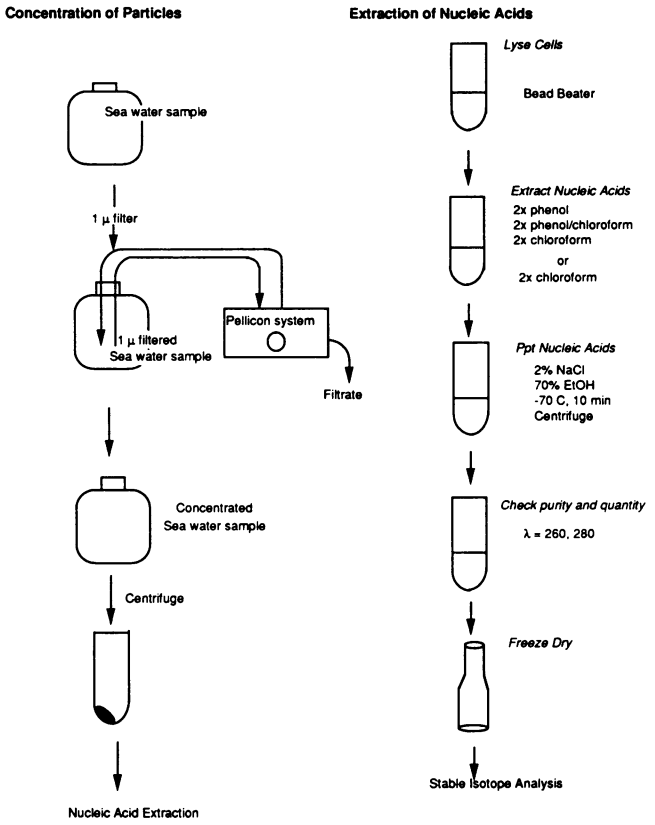

FIG. 1. Flow chart depicting concentration of <1-μm particles from water samples and extraction of nucleic acids from the concentrates. Ppt, Precipitate.

Bacterial bioassay experiments. Estuarine bacteria were incubated in filtered water samples. Particle-free water (4 to 10 liters) collected from the outflow of the Pellicon system was incubated with a 1% (vol/vol) inoculum of filtered (1.0-μm pore size) seawater for 24 to 48 h, until the bacterial biomass was sufficient for isotopic analysis (7).

Substrate addition experiments. Water-soluble DOM was collected from *S. alterniflora* and *Quercus virginiana* (live oak) by blending leaves in deionized water. DOM was separated from particulate material by centrifugation and filtration (0.2-μm pore size). DOM was collected from *Cyclotella caspia* by sonification and centrifugation and filtering out of particulate material. Substrate concentrations were 0.5, 1.7, and 1.2 g of C $liter^{-1}$ for *S. alterniflora*, *Q. virginiana*, and *C. caspia*, respectively. Samples of DOM concentrates were prepared for stable carbon isotope analysis by freeze-drying.

TABLE 1. Comparison of nucleic acid extraction techniques for stable carbon isotope ratios[a]

Source	$\delta^{13}C$ (‰) of:		
	Whole cells	Chloroform-extracted nucleic acids	Phenol-extracted nucleic acids
P. aeruginosa			
Expt. 1	−10.6	−10.7	−14.5
Expt. 2	−10.3	−10.5	−13.9
Calf thymus	−12.4	−12.1	−14.8

[a] Bacteria were grown with glucose as the sole carbon source. Nucleic acids were extracted as described by Marmur (18) (phenol) and Blair et al. (4) (chloroform). As a control, the nucleic extraction methods were tested on commercially prepared calf thymus DNA.

Bioassay experiments were prepared with water from Santa Rosa Sound as described above. Fifty-liter bioassay experiments were amended with 10 mg of C from *Spartina*, *Quercus*, or *Cyclotella* DOM liter^{-1} (final concentration). One sample received no substrate addition. Samples were incubated in the dark with aeration for 48 h. Bacterial abundance was monitored in samples with acridine orange direct counts (14). A time-zero $\delta^{13}C$ value for bacteria was obtained by using nucleic acids extracted from the cell pellet obtained with one 50-liter filtered (1-μm pore size) sample.

Stable carbon isotope analysis. Samples were analyzed isotopically by a modified Dumas combustion that converts organic carbon and nitrogen to CO_2 and N_2 gases for mass spectral analyses (S. A. Macko, Ph.D. thesis, University of Texas at Austin, 1981). The samples were placed in quartz tubes containing Cu and CuO, and the tubes were evacuated and sealed. The tubes were heated to 900°C for 1 h and then cooled to 700°C at a rate of 4°C min^{-1} and from 700 to 500°C at 0.6°C min^{-1} to ensure that any oxides of nitrogen were converted to N_2. CO_2 gas was separated from N_2 gas by cryogenic distillation. Carbon dioxide was analyzed in a triple-collector Nuclide 6-60 isotope ratio mass spectrometer. $\delta^{13}C$ was determined by the formula $\delta^{13}C(‰) = [(R_{sample}/R_{standard})-1] \cdot 10^3$, where $R = {}^{13}C/{}^{12}C$. The standard for carbon was Pee Dee belemnite. The analytical precision of the nucleic acid extraction for $\delta^{13}C$ was ±0.2‰ (n = 5), slightly more variable than the $\delta^{13}C$ measurements of replicate bacterial cultures, ±0.1‰ (n = 5; Table 1).

Determination of relative abundances of eubacteria and eucaryotes. The proportional representation of eubacteria and eucaryotes in the microbial population was determined by hybridizations with universal and kingdom-specific rRNA-targeted oligonucleotide probes (10, 28). The sequences of the probes (mixed positions are in brackets) complementary to the following small-subunit rRNA regions were as follows: universal, *E. coli* numbered positions 1406 to 1392 (ACGGGCGGTGTGT[GA]C); eubacterial, *E. coli* numbered positions 123 to 109 (GTGTTACTCACCCGT); eucaryote, *Dictyostelium discoideum* numbered positions 557 to 542 (ACCAGACTTGCCCTCC). The universal probe hybridizes to small-subunit rRNA from every known organism, the eubacterial probe hybridizes only to eubacterial 16S rRNA, and the eucaryote probe hybridizes only to eucaryote small-subunit rRNA. Probes were labeled at their 5′ ends by using $^{32}P\gamma$-labeled ATP and polynucleotide kinase (26). Samples of 7.8 to 1,000 ng of nucleic acids extracted from the microbial populations, together with 5- to 20-ng samples of nucleic acids from reference organisms, were applied to nylon membranes by using a slot blot filtration apparatus (Schleicher & Schuell, Inc., Keene, N.H.). The membranes were dried, and the samples were hybridized with the probes as detailed previously (28). The membranes were washed once for 30 min at room temperature in 1× SSC (0.015 M NaCl, 0.015 M sodium citrate)–1% sodium dodecyl sulfate and again in 1× SSC–1% sodium dodecyl sulfate at predetermined temperatures (universal, 46°C; eubacterial, 43°C; eucaryote, 58°C). Hybridization signals obtained by autoradiography were quantified by densitometry with a Bio-Rad 60 densitometer. Probe bound to the nucleic acids from the microbial populations was compared to the reference samples. Relative abundances of eubacteria and eucaryotes were expressed as the fractional amounts of their rRNAs as determined by hybridization with the universal probe.

RESULTS

Comparison of nucleic acid extraction methods. The method used previously to extract nucleic acids from bacteria for stable carbon isotope analysis uses chloroform and inorganic buffers (4). With this protocol, *P. aeruginosa* cells grown on glucose in our laboratory had $\delta^{13}C$ values of −10.3 and −10.6‰ (Table 1). Nucleic acids isolated by chloroform extraction had values similar to those of cells, −10.5 and −10.7‰, respectively. The purity of the extracted nucleic acids was estimated by UV A_{260}/A_{280} ratios. Pure nucleic acids are expected to have a ratio of 2.0 (17). The UV absorbance ratio for replicate nucleic acid samples extracted with this protocol was 1.63 (±0.03; n = 4). This low ratio indicates that protein was present in the nucleic acid extracts. The protein content of one sample was 6.2% (weight of protein/weight of nucleic acids). To improve the purity of the extracted nucleic acids, a method using phenol, chloroform, and organic buffers (18) was tested. In contrast to the first method, the A_{260}/A_{280} ratio was 1.92 (±0.05; n = 5) and the protein content was 0.3%, suggesting that the nucleic acids were more pure. However, the $\delta^{13}C$ values of nucleic acids extracted with phenol were more negative than those of whole cells by 3.4 and 3.9‰ (Table 1). For a control, both extraction protocols were repeated with commercially purified calf thymus DNA. The carbon isotopic composition of unamended calf thymus DNA and that of DNA that was subjected to chloroform extraction were similar, −12.4 and −12.1‰, respectively (Table 1). After phenol extraction, the $\delta^{13}C$ of the calf thymus DNA was −14.8‰. It seems unlikely that removal of protein from samples by the phenol method was responsible for the shift in $\delta^{13}C$. This is because the protein content was small and the isotope discrimination between protein and bacterial cells has been found to be only 1.1‰ (4). We concluded that the phenol method caused unexplained isotopic contamination, and therefore we used chloroform extraction for all of our samples.

$\delta^{13}C$ of nucleic acids from *P. aeruginosa*. *P. aeruginosa* was cultured with glucose ($\delta^{13}C$, −13.3‰) or glutamate ($\delta^{13}C$, −27.4‰) as the carbon source. The $\delta^{13}C$ of the cells after incubation with glucose averaged −10.3 ± 0.2‰ (n = 6; Table 2). When glutamate was the carbon source, the average bacterial $\delta^{13}C$ was −25.4 ± 0.4‰ (n = 9; Table 2). When incubation experiment results obtained with both substrates were averaged, bacteria were 2.4 ± 0.6‰ (n = 15) heavier than the substrate source. Nucleic acids extracted from bacteria cultured with glucose had $\delta^{13}C$ values of −10.5 and −10.7‰ (Table 2). With glutamate as the carbon source, the $\delta^{13}C$ of the nucleic acids averaged 25.4 ± 0.4‰ (n = 9). Relative to these substrates, the average enrichment of nucleic acids was 2.4 ± 0.5‰ (n = 11). Stable carbon isotope values for bacteria and nucleic acids were not significantly different (t test; α, 0.025).

TABLE 2. Stable carbon isotope fraction of whole cells and nucleic acids of *P. aeruginosa* grown on glucose[a] or glutamate[b] as the sole carbon source

Sample collection date	$\delta^{13}C$ (‰) of:				Substrate[c]
	Whole cells	Nucleic acids	$\Delta_{cells\text{-}substrate}$	$\Delta_{nucleic\ acid\text{-}substrate}$	
6/3/88	−10.2	ND[d]	3.1	ND	Glucose
	−10.4	ND	2.9	ND	Glucose
6/8/88	−10.2	ND	3.1	ND	Glucose
	−10.2	ND	3.1	ND	Glucose
10/24/88	−10.3	−10.5	3.0	2.8	Glucose
11/28/88	−25.5	−25.1	1.9	2.3	Glutamate
	−25.9	−26.3	1.5	1.1	Glutamate (100 mM)
	−26.2	−25.5	1.2	1.9	Glutamate (200 mM)
12/8/89	−10.6	−10.7	2.7	2.8	Glucose
1/23/89	−25.2	−24.5	2.2	2.9	Glutamate
	−25.0	−24.7	2.4	2.7	Glutamate
	−25.1	−24.9	2.3	2.5	Glutamate
	−25.2	−25.0	2.2	2.4	Glutamate
	−25.2	−24.9	2.2	2.5	Glutamate
5/12/89	−25.4	−24.7	2.0	2.7	Glutamate

[a] $\delta^{13}C$, −13.3‰.
[b] $\delta^{13}C$, −27.4‰.
[c] The substrate concentration was 20 mM, except when noted otherwise.
[d] ND, Not done.

Nucleic acids extracted from environmental samples. Nucleic acids were extracted from water samples taken from Santa Rosa Sound, off the dock at Gulf Breeze Environmental Research Laboratory (GBERL), and Range Point, a local enclosed salt marsh (Table 3; Fig. 2). Particles were concentrated from 50- or 100-liter water samples, depending on the season and the environment. Bacterial abundances in these samples ranged from 1.8×10^9 to 5.2×10^9 liter^{-1} (Table 3). The efficiency of each concentration step was monitored with acridine orange direct counts. Prefiltration through 1-μm-pore-size filters did not reduce bacterial abundances significantly. Upon concentration of <1-μm particles with the Pellicon system, between 30 and 88% of the bacteria remained. After centrifugation, approximately 30 to 90% of the concentrated cells were recovered. Total recoveries ranged from 10 to 45% (Table 3). For maximum bacterial recovery, centrifugation for 60 min at 27,000 × *g* was needed. Extraction of concentrated samples provided between 0.345 and 1.465 mg of nucleic acids. Nucleic acid extracts ranged from 6 to 35 fg per cell (Table 3).

We used small-subunit rRNA hybridization as an index of the relative amounts of extracted eucaryotic and eubacterial nucleic acids. Three rRNA probes were used, a universal probe with specificity for both eubacterial and eucaryotic small-subunit rRNAs (as well as archaebacterial rRNA) and eubacterium- and eucaryote-specific probes. Purified rRNAs from control organisms confirmed the specificity of each probe (Fig. 3). The eubacterial control organisms *Mycobacterium* sp., *Desulfovibrio* sp., and *Thiothrix* sp. hybridized with the universal and eubacterial probes. However, nucleic acids from *Vitreoscilla* sp. did not hybridize with the eubacterial probe. rRNAs from eucaryotic control organisms, *Dictyostelium* sp., and rat liver hybridized with the universal and eucaryotic probes but not with the eubacterial probe. Nucleic acids that had been extracted from concentrates of prefiltered (1- and 3-μm pore sizes) water samples were

TABLE 3. Recovery of bacteria and nucleic acids from estuarine waters with tangential-flow filtration and centrifugation[a]

Date	Site	Vol (liters)	Total bacteria, 10^{11}				% Remaining	Total nucleic acids (mg)	Nucleic acids/ bacterium (fg cell^{-1})
			Initial	After prefiltration	After Pellicon	After centrifugation			
6/15/88	GBERL	50	1.75	1.90	0.32	0.27[b]	15	0.46	17
6/15/88	GBERL	50	1.95	1.90	0.34	0.29[b]	15	1.01	35
6/20/88	GBERL	50	2.10		0.8	0.77[b]	37	0.47	6
6/20/88	GBERL	50	2.10		0.93	0.89[b]	42	0.57	6
11/17/88	GBERL	100	1.70	1.80		0.17	10	0.31	19
12/8/88	RP	100	3.10	3.70	2.50	1.40	45	1.47	11
1/4/89	RP	100	5.20	5.00	4.40	2.20	42	1.29	6
1/31/89	GBERL	100	1.80	1.50	0.87	0.44	24	0.35	8

[a] Water from two marine sites was collected for concentration. RP, Range Point.
[b] This calculation was based on the number of bacteria that remained in the supernatant after centrifugation. All other values were calculated from a resuspended pellet.

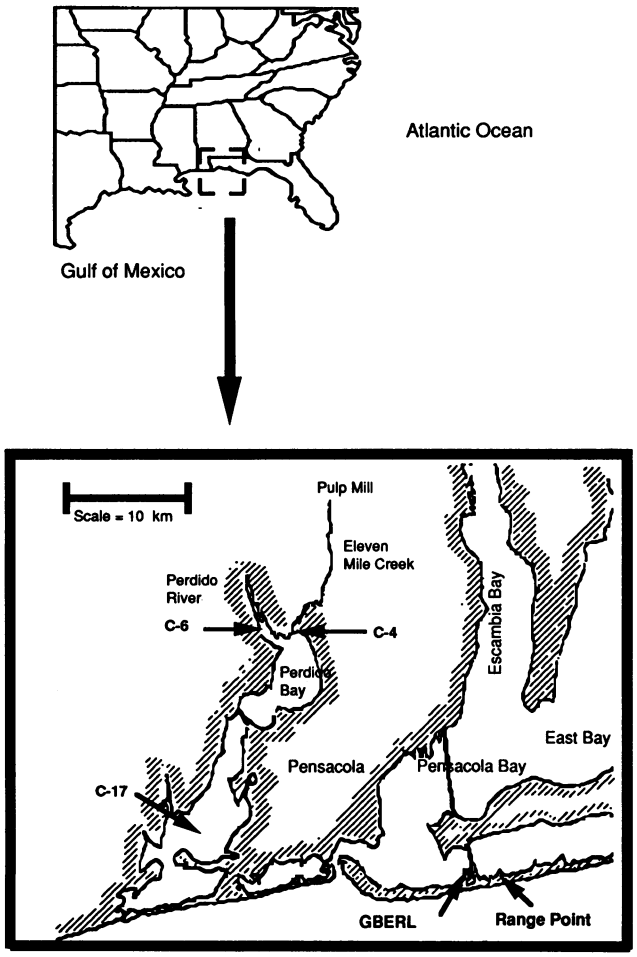

FIG. 2. Map of estuarine sampling stations near Pensacola, Fla.

hybridized with this complement of probes. The universal and eubacterial probes resulted in the strongest hybridization signals with the target RNAs. Weaker signals were observed with the eucaryotic probe, indicating that only small amounts of eucaryotic nucleic acid were present in the extracted samples (Fig. 3). Use of a densitometer to quantify the degree of hybridization showed that 90% of the nucleic acids from these samples were of eubacterial origin.

Substrate addition experiment. Bioassay experiments with GBERL dock water were amended with DOM from *S. alterniflora*, *C. caspia*, or *Q. virginiana*. Figure 4 reviews the experimental design and results. Stable carbon isotope ratios were measured in whole plants, DOM from the plants, bacteria incubated with the sources of DOM, and nucleic acids extracted from the same bacteria. The stable carbon isotope ratio of nucleic acids from the initial water sample was −21.8‰. With *S. alterniflora* or *C. caspia* as the DOM source, carbon isotope values for bacteria reflected the source (Fig. 4). With DOM from *S. alterniflora* as the substrate source, the $\delta^{13}C$ of extracted nucleic acids also reflected the substrate source. With no addition of substrate to bioassay experiments, bacteria and extracted nucleic acids changed little. DOM from *Q. virginiana* leaves did not appear to be taken up by bacteria; the $\delta^{13}C$ was similar to that of the samples that received no substrate addition. After 48 h of incubation, the bacterial abundance in waters amended with *Quercus* DOM was only 2.4×10^9 cells liter^{-1}, compared with 11×10^9 and 6.5×10^9 cells liter^{-1} for samples incubated with organic matter from *S. alterniflora* and *C. caspia*.

Stable carbon isotopes of nucleic acids extracted from environmental samples. Nucleic acids extracted from field samples were compared with bacteria cultured in bioassay experiments (7; see Materials and Methods). The $\delta^{13}C$ of Range Point and GBERL dock bacteria grown in the bioassay experiments ranged from −20.5 to −24.2‰ (Table 4). The $\delta^{13}C$ ratios for nucleic acids varied between −20.2 and −22.6‰. Isotopic discrimination between the nucleic acids and bacteria ranged from +0.3 to +1.6‰.

In the Perdido Estuary (Fig. 2), three stations were examined; C-4 is at the mouth of a tributary that carries pulp mill waste to the Perdido estuary, C-17 is located 17 km downstream from the mouth of the tributary, and C-6 is 1 km upstream in the Perdido River. At C-4, the $\delta^{13}C$'s of bacteria from bioassays and extracted nucleic acids were −31.9 and −27.5‰ (Table 4). Farther away from the source of pulp mill waste, bacteria and nucleic acids were enriched in ^{13}C; downstream at C-17, the values were −29.9 and −27.9‰, respectively, and upstream at C-6, the ratios were −26.8 and −26.4‰, respectively.

DISCUSSION

To trace sources of bacterial carbon successfully with the $\delta^{13}C$ of nucleic acids, several criteria must be met. Nucleic acid extractions must yield enough material for isotope analysis, bacteria must be separated from other microorganisms, and the $\delta^{13}C$ of nucleic acids must be similar to that of the whole bacterium. We showed that prefiltration removed most eucaryotic microorganisms and that tangential-flow filtration concentrated sufficient bacterial biomass for extraction of nucleic acids for isotope analysis. The analytical minimum for stable carbon isotope analysis in this study was 100 mg of C. The smallest amount of nucleic acid recovered from estuarine samples was 310 μg (Table 2). Assuming an average nucleoside carbon content of 45.1%, the lowest recovery of nucleic acid was equivalent to 140 μg of C. Furthermore, the amount of nucleic acid that was extracted per cell concentrated was consistent with the results of other laboratory and field studies. RNA/DNA ratios for *Vibrio proteolyticus* range from 0.83 to 5.06 and are highest during log-phase growth (W. H. Jeffrey, M.S. thesis, University of South Florida, Tampa, 1985), and the DNA contents of aquatic bacteria vary from 5.66 to 14.4 fg cell^{-1} (21, 22). In our study (Table 3), there was 6 to 35 fg of nucleic acids cell^{-1} (including RNA and DNA).

Bacterial recovery ranged from 10 to 45%. Recovery of bacteria, and therefore nucleic acids, can be improved at two steps in the concentration protocol. (i) Recovery of bacteria with the tangential-flow system varies with the filtration rate. Higher recoveries were observed when the ratio of recycled water to filtrate was above 15. Later in the study, bacterial recoveries were improved by increasing this ratio (Table 3). (ii) Although centrifugation is necessary because the Pellicon system can only concentrate cells in a final volume of 1 liter, centrifugation is not an efficient concentration step. Centrifugation removed most estuarine bacteria (80 to 90%) from the supernatant. However, it is difficult to resuspend the bacterial pellet quantitatively. To overcome this problem, we have recently tested a smaller tangential-flow system that is designed to concentrate cells to 10 to 30 ml. This system eliminates the need for large-volume centrifugation and increases bacterial recoveries.

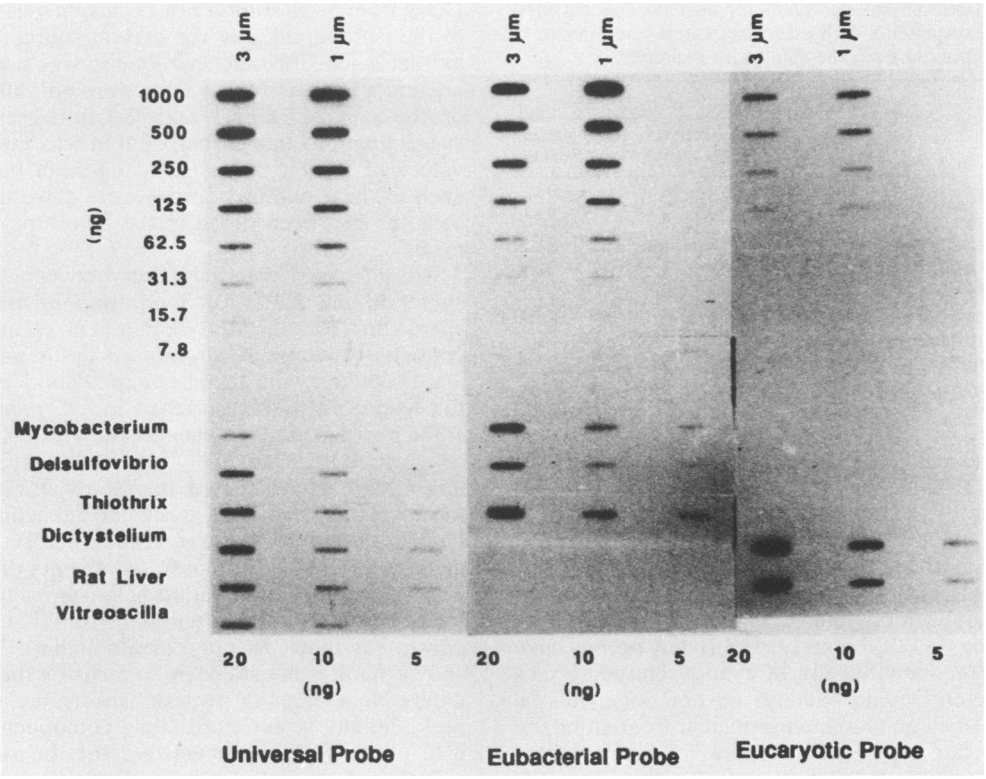

FIG. 3. Small-subunit rRNA-targeted hybridization of nucleic acids extracted from estuarine samples.

We used 16S rRNA hybridization as an index of the relative proportion of extracted procaryotic and eucaryotic nucleic acids. Prefiltration through a 1- or 3-µm-pore-size cartridge selectively removed larger eucaryotic microorganisms from water samples. The contribution of nucleic acids from eucaryotes in prefiltered water was estimated to be <10% on the two dates when the 16S rRNA probes were used. Recently, new probes specific for eubacterial rRNA have been developed (D. Stahl, personal communication); these probes will be used in the future for more quantitative analysis of the sources of nucleic acids. The results of our study are consistent with other work in which most of the organisms in the 0.2- to 1.0-µm size range were bacteria (21). However, the probes used for this survey do not address the

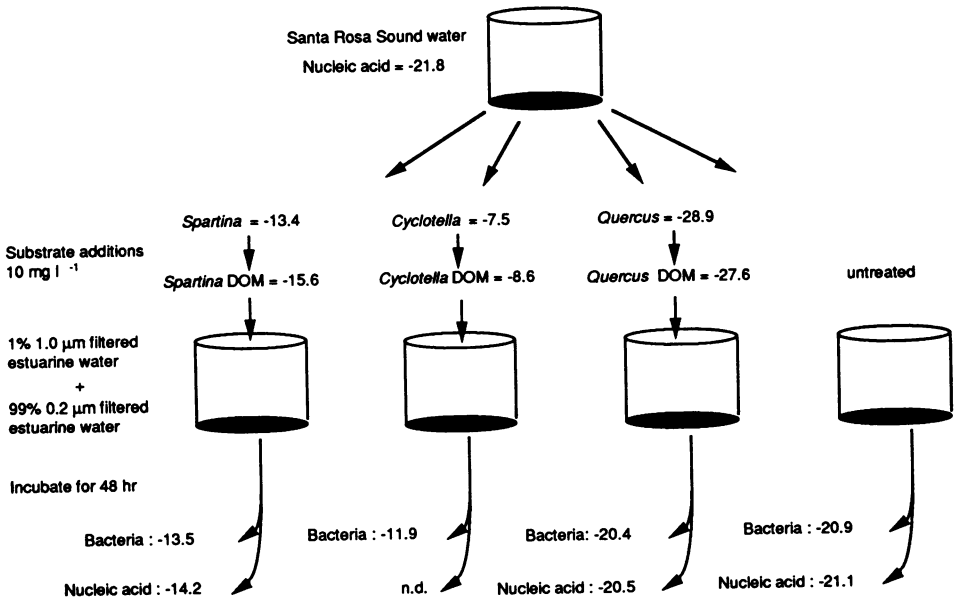

FIG. 4. Incubation experiment tracing the $\delta^{13}C$ (‰) of extracts leached from *S. alterniflora*, *C. caspia*, or *Q. virginiana* into bacteria and extracted nucleic acids. n.d., Not done.

TABLE 4. Stable carbon isotope values for bacteria concentrated from bioassay experiments with estuarine waters and nucleic acids extracted from the same water samples

Date	Sample location	$\delta^{13}C$ (‰) of:	
		Bacteria (bioassay)	Nucleic acids
11/17/88	GBERL	−24.2	−22.6
12/8/88	Range Point	−20.5	−20.2
1/4/89	Range Point	−21.4	−21.2
1/31/89	GBERL	−22.3	−21.1
6/24/89	Perdido C-4 (mouth)	−31.9	−27.5
	Perdido C-6 (1 km upstream)	−26.8	−26.4
	Perdido C-17 (5 km downstream)	−29.9	−27.9

contribution of nucleic acids from phototrophic procaryotes (e.g., references 5, 11, and 12). Upon microscopic examination of concentrates from the estuarine samples, autofluorescent cells did not appear to be a significant proportion of the microorganisms that were present. However, data presented in a recent study in the Mediterranean Sea indicate that coccoid cyanobacteria could account for up 37% of the procaryotic biomass (12). Recently, 16S rRNA probes have been developed for identification of cyanobacterial species (11). Clearly, heterotrophic bacteria do not dominate the <1-μm biomass in all aquatic environments. Combining the probes used in our study with probes for phototrophic bacteria would provide a quantitative screening tool for environments where phototrophic organisms contribute significantly to the picoplankton biomass.

To establish isotopic continuity among substrates, bacteria, and nucleic acids, *P. aeruginosa* was grown on glucose or glutamate as the sole carbon source. Bacterial cells and nucleic acids were consistently 2.4‰ enriched in ^{13}C relative to the substrate sources. This result is at the high end of the range reported for discrimination between bacteria and carbon sources, 1.9 to −1.9‰ (1, 4, 7, 20). In the laboratory, *E. coli* $\delta^{13}C$ was depleted in ^{13}C with respect to glucose by 0.6‰ (4). With glutamate as the carbon source, *V. harveyi* was 0.1 to 2.5‰ enriched in ^{13}C (16). However, in the same study, $\delta^{13}C$ fractionation by *V. harveyi* ranged from −5.5 to 11.1‰ with other amino acids. Therefore, the amount of fractionation appears to be related to the carbon source and possibly to the bacterial genus. However, natural bacterial populations incubated with extract leached from *S. alterniflora* and oak leaves were 1.7 to 1.9‰ enriched with ^{13}C relative to the substrate (7). As a result of the close fidelity between estuarine bacteria and natural substrate sources, Coffin et al. (7) suggested that species- and substrate-specific isotope fractionation is averaged in field samples. Results of our experiments with natural substrates provide further support for this conclusion (discussed below).

Isotopic discrimination between nucleic acids and bacteria is small when glucose and glutamate are used as carbon sources (Table 2). This result is consistent with those of another study in which the $\delta^{13}C$ of nucleic acids was not significantly different from that of whole *E. coli* cells when glucose was the carbon source (4). The low stable carbon isotope fractionation between bacteria and nucleic acids in our laboratory studies supports the use of nucleic acids as a tracer of bacterial substrate sources. Our results are further supported by those of experiments that traced $\delta^{13}C$ ratios of substrate sources into bacteria and bacterial nucleic acids in estuarine water samples. The $\delta^{13}C$ of bacteria incubated with DOM from *S. alterniflora* or *C. caspia* was distinctly related to the isotope value of the carbon source. However, when extract leached from *Q. virginiana* was used as a source of organic matter, isotope values were not different from those of the control sample and the initial estuarine sample, suggesting that this carbon source was not used. The $\delta^{13}C$ values of nucleic acids extracted from bacteria grown on each of these carbon sources were close to those of whole cells, as had been noted in the laboratory with *P. aeruginosa*.

Isotope-based discrimination between bacteria and the substrate was 2.1‰ for *S. alterniflora* and −3.3‰ for *C. caspis*. In a recent study, Benner et al. (3) compared the $\delta^{13}C$ contents of whole *S. alterniflora* tissue and the hemicellulose, cellulose, and lignin components. They reported that the whole plant was enriched in ^{13}C relative to lignin by 4.3‰ and depleted in comparison with cellulose and hemicellulose by 1.5 to 1.7‰. In our study, DOM from *S. alterniflora* was depleted in ^{13}C by 2.2‰ relative to the whole plant, suggesting a significant lignin content in the DOM. Since bacteria preferentially select the more labile cellulose and hemicellulose components of *Spartina* DOM, isotope-based discrimination between bacteria and the substrate was considerably different from that measured for *C. caspia* (diatoms do not contain lignin). This experiment shows that it is not sufficient to measure the $\delta^{13}C$ of DOM to determine sources of bacterial substrates, because bacteria preferentially select more labile components of DOM. The $\delta^{13}C$ of the bacterium must therefore be measured.

Potential sources of dissolved organic matter for estuaries and coastal waters have a broad range of $\delta^{13}C$ values. The $\delta^{13}C$ of aquatic carbon sources is a function of the isotopic composition of CO_2 that is fixed by the primary producer (aquatic or atmospheric) and the pathway and physiological conditions associated with CO_2 fixation (C_3 or C_4 metabolism). As a result, typical $\delta^{13}C$ values of upland vegetation range from −31.0 to −24.0‰ (3, 24, 27), those of phytoplankton range from −21.0 to −16.6‰ (6, 23, 24), those of C-4 salt marsh plants (e.g., *S. alterniflora*) range from −13.2 to −12.4‰ (3, 9, 24), and those of seagrasses range from −24 to −3‰ (19, 27). Stable carbon isotope values of seagrasses overlap those of marsh grasses and phytoplankton. A multiple-isotope approach using stable carbon, nitrogen, and sulfur would be useful to distinguish substrate sources in environments where carbon isotope signals overlap (24).

We surveyed $\delta^{13}C$ values of bacteria and nucleic acids in different estuarine environments (Table 4). Bacteria grown in bioassay experiments (7) were compared with nucleic acids extracted from estuarine concentrates. At Range Point, the dominant seagrass species include *Syringodium filiforme*, *Thalassia testudinim*, and *Halodule wrightii* (15, 25). Stable carbon isotope values of these seagrasses range from −3 to −13‰ in Gulf of Mexico coastal waters (19). As mentioned above, phytoplankton $\delta^{13}C$ values range from −16.6 to −21.0‰ (6, 23, 24). On two dates, the $\delta^{13}C$ values of bacteria were −20.5 and −21.4‰ and for extracted nucleic acids they were −20.2 and −21.2‰, respectively. Our preliminary analysis of bacterial $\delta^{13}C$ from Range Point suggests that phytoplankton could have been the major source of carbon. If seagrasses were an important substrate, we would expect lower isotope values in bacteria, and *S. alterniflora* is not abundant around the marsh source. In Santa Rosa Sound, we expected that organic matter from terrestrial sources would be important because this system receives large freshwater inputs. Bacterial $\delta^{13}C$ values on two different dates were −24.2 and −22.3‰, and those of the

corresponding nucleic acids were −22.6 and −21.1‰ (Table 4). These $\delta^{13}C$ values of nucleic acids indicate that DOM from phytoplankton was probably the dominant carbon source at the station in Santa Rosa Sound. However, we are continuing our field work in both the Range Point marsh and Santa Rosa Sound to confirm the relative importance of carbon sources.

In the Perdido Estuary, station C-4 is located at the mouth of Eleven Mile Creek, which carries pulp mill waste effluent into Perdido Bay; C-6 is located 1 km upstream in the Perdido River; and C-17 is 17 km downstream from station C-4 in the Perdido Bay (Fig. 2). Nucleic acid $\delta^{13}C$ data from these stations indicated a larger contribution from terrestrial carbon sources than at Range Point or in Santa Rosa Sound. Closest to the source of pulp mill effluent, the $\delta^{13}C$ for nucleic acids was −27.5‰, upriver it was −26.4‰, and downriver it was −27.9‰. Although the $\delta^{13}C$ values of nucleic acids did not vary greatly among these stations, carbon isotopes in bacteria from bioassays did vary. Above the pulp mill input, the nucleic acids were only 0.4‰ heavier isotopically than bacteria grown in a bioassay experiment; this value is similar to the discrimination measured in laboratory studies and at Range Point (Table 4). Closest to the pulp mill, nucleic acids were 4.4‰ heavier isotopically than the bacteria. Further downstream, at C-17, nucleic acids were 2.0‰ enriched in ^{13}C relative to bacteria. Therefore, at C-6 the $\delta^{13}C$ values of both bacteria and nucleic acids suggested that terrestrial sources were important. However, at C-4 and C-17 identification of bacterial carbon sources was more difficult. Simply examining the nucleic acids extracted from these stations indicated that the carbon source was the same as that of station C-6. Results of the bioassay experiments alone indicated that the source of organic matter was isotopically lighter than that at station C-6. Dissolved organic carbon concentrations in and at the mouth of Eleven Mile Creek suggested that the pulp mill was the source of the isotopically lighter carbon; concentrations measured recently were 17.9 mg of C liter^{-1} at a station 7 km up Eleven Mile Creek, 12.9 mg of C liter^{-1} at station C-4, and 6.9 mg of C liter^{-1} at station C-6 (R. B. Coffin, L. A. Cifuentes, and R. Benner, unpublished data). A possible explanation for the difference between the stable isotope values of bacteria and nucleic acids is that bacteria in nature were growing on two different substrate sources at stations C-4 and C-17. The $\delta^{13}C$ for seston measured at C-17 was −21.6‰ (Coffin et al., unpublished data), suggesting that phytoplankton was a major component of the seston. Since phytoplankton is excluded by filtration from bioassay experiments, the difference between the bioassay and nucleic acid data could result from removal of this carbon source. We are continuing our examination of carbon sources in the Perdido Estuary to confirm our hypothesis.

Combined $\delta^{13}C$ measurements of extracted nucleic acids and bacteria grown in bioassay experiments revealed several important observations regarding sources of DOM for aquatic bacteria. The $\delta^{13}C$ of bacteria grown in bioassay experiments reflects the source of consumable dissolved substrates. If particulate substrate sources (i.e., phytoplankton) are not important, this approach is satisfactory. The $\delta^{13}C$ of nucleic acids is an in situ measurement of the averaged bacterial substrate. As was demonstrated in the Perdido Estuary, when multiple substrate sources are important, isotope analysis of the nucleic acids alone may not provide adequate resolution. A combination of stable carbon isotope analyses of both extracted nucleic acids and bacteria grown in bioassay experiments provided the most descriptive approach for identification of bacterial substrate sources.

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LITERATURE CITED

1. **Abelson, P. H., and T. C. Hoering.** 1961. Carbon isotope fractionation in formation of amino acids by photosynthetic organisms. Proc. Natl. Acad. Sci. USA **47**:623–632.
2. **Barkay, T., C. Liebert, and M. Gillman.** 1989. Hybridization of DNA probes with whole-community genome for detection of genes that encode microbial responses to pollutants: mer genes and Hg^{2+} resistance. Appl. Environ. Microbiol. **55**:1574–1577.
3. **Benner, R., M. L. Fogel, E. K. Sprague, and R. E. Hodson.** 1987. Depletion of ^{13}C in lignin and its implication for stable carbon isotope studies. Nature (London) **329**:708–710.
4. **Blair, N., A. Leu, E. Muñoz, J. Olsen, E. Kwong, and D. Des Marais.** 1985. Carbon isotopic fractionation in heterotrophic microbial metabolism. Appl. Environ. Microbiol. **50**:996–1001.
5. **Chisolm, S. W., R. J. Olsen, E. R. Zettler, R. Goericke, J. B. Waterbury, and N. A. Welshmeyer.** 1988. A novel free-living prochlorophyte abundant in the oceanic euphotic zone. Nature (London) **334**:340–343.
6. **Cifuentes, L. A., J. H. Sharp, and M. L. Fogel.** 1988. Stable carbon and nitrogen isotope biogeochemistry in the Delaware Estuary. Limnol. Oceanogr. **33**:1102–1115.
7. **Coffin, R. B., B. Fry, B. J. Peterson, and R. T. Wright.** 1989. Carbon isotopic compositions of estuarine bacteria. Limnol. Oceanogr. **34**:1305–1310.
8. **Coffin, R. B., and J. H. Sharp.** 1987. Microbiol trophodynamics in the Delaware Estuary. Mar. Ecol. Prog. Ser. **41**:253–266.
9. **Fogel, M. L., E. K. Sprague, A. P. Gize, and R. W. Frey.** 1989. Diagenesis of organic matter in Georgia salt marshes. Estuarine Coastal Shelf Sci. **28**:211–230.
10. **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.
11. **Giovannoni, S. J., S. Turner, G. J. Olsen, S. Barns, D. J. Lane, and N. R. Pace.** 1988. Evoluntionary relationships among cyanobacteria and green chloroplasts. J. Bacteriol. **170**:3584–3592.
12. **Hagstrom, A., F. Azam, A. Andersson, J. Wikner, and F. Rassoulzadegan.** 1988. Microbial loop in an oligotrophic pelagic ecosystem: possible roles of cyanobacteria and nanoflagellates in the organic fluxes. Mar. Ecol. Prog. Ser. **49**:171–178.
13. **Hareland, W., R. L. Crawford, P. J. Chapman, and S. Sagley.** 1975. Metabolic function and properties of 4-hydroxyphenylacetic acid 1-hydroxylase from *Pseudomonas acidovorans*. J. Bacteriol. **121**:272–281.
14. **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.
15. **Humm, H. J.** 1956. Seagrasses of the northern Gulf Coast. Bull. Mar. Sci. Gulf Caribb. **6**:305–308.
16. **Macko, S. A., and M. L. F. Estep.** 1984. Microbial alteration of stable nitrogen and carbon isotopic compositions of organic matter. Org. Geochem. **6**:787–790.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 109–112. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
19. **McMillan, C., P. L. Parker, and B. Fry.** 1980. $^{13}C/^{12}C$ ratios in

seagrasses. Aquat. Bot. **9**:237–249.
20. **Monson, K. D., and J. M. Hayes.** 1982. Carbon isotopic fractionation in the biosynthesis of bacterial fatty acids. Ozonolysis of unsaturated fatty acids as a means of determining the intramolecular distribution of carbon isotopes. Geochim. Cosmochim. Acta **46**:139–149.
21. **Paul, J. H., and D. J. Carlson.** 1984. Genetic material in the marine environment: implication for baterial DNA. Limnol. Oceanogr. **29**:1091–1097.
22. **Paul, J. H., W. H. Jeffrey, and M. DeFlaun.** 1985. Particulate DNA in subtropical oceanic and estuarine planktonic environments. Mar. Biol. **90**:95–101.
23. **Peterson, B. J., and B. Fry.** 1987. Stable isotopes in ecosystem studies. Annu. Rev. Ecol. Syst. **18**:293–320.
24. **Peterson, B. J., R. W. Howarth, and R. H. Garrit.** 1985. Multiple stable isotopes used to trace the flow of organic matter in estuarine food webs. Science **277**:1361–1363.
25. **Phillips, R. C.** 1960. Observations on the ecology and distribution of the Florida seagrasses. Florida State Board of Conservation Marine Laboratory.
26. **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.
27. **Simenstad, C. A., and R. C. Wissmar.** 1985. $\delta^{13}C$ evidence of the origins and fate of organic carbon in estuarine and nearshore food webs. Mar. Ecol. Prog. Ser. **22**:141–152.
28. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54**:1079–1084.
29. **Wright, R. T., and R. B. Coffin.** 1983. Planktonic bacteria in estuaries and coastal waters of northern Massachusetts: spatial and temporal distribution. Mar. Ecol. Prog. Ser. **11**:205–215.
30. **Wright, R. T., R. B. Coffin, and M. Lebo.** 1987. Dynamics of planktonic bacteria and heterotrophic microflagellates in the Parker Estuary, Northern Massachusetts. Continental Shelf Sci. **7**:1383–1397.