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Characterization of Benthic Microbial Community Structure by High-Resolution Gas Chromatography of Fatty Acid Methyl Esters

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Fatty acids are a widely studied group of lipids of sufficient taxonomic diversity to be useful in defining microbial community structure. The extraordinary resolution of glass capillary gas-liquid chromatography can be utilized to separate and tentatively identify large numbers of fatty acid methyl esters derived from the lipids of estuarine detritus and marine benthic microbiota without the bias of selective methods requiring culture or recovery of the microbes. The gas-liquid chromatographic analyses are both reproducible and highly sensitive, and the recovery of fatty acids is quantitative. The analyses can be automated, and the diagnostic technique of mass spectral fragmentation analysis can be readily applied. Splitless injection on glass capillary gas chromatographic columns detected by mass spectral selective ion monitoring provides an ultrasensitive and definitive monitoring system. Reciprocal mixtures of bacteria and fungi, when extracted and analyzed, showed progressive changes of distinctive fatty acid methyl esters derived from the lipids. By manipulating the environment of an estuarine detrital microbial community with antibiotics and culture conditions, it was possible to produce a community greatly enriched in eucaryotic fungi, as evidenced by scanning electron microscopic morphology. The fatty acid methyl esters from the lipids in the fungus-enriched detritus showed enrichment of the C_{18} dienoic and the C_{18} and C_{20} polyenoic esters. Manipulation of the detrital microbiota that increased the procaryotic population resulted in an absence of large structures typical of fungal mycelia or diatoms, as evidenced by scanning electron microscopy, and a significantly larger proportion of anteiso- and iso-branched C_{15} fatty acid esters, C_{17} cyclopropane fatty acid esters, and the *cis*-vaccenic isomer of the C_{18} monoenoic fatty acid esters. As determined by these techniques, a marine settling community showed greater differences in bacterial as contrasted to microeucaryotic populations when compared with the microbial communities of benthic cores.

The microbial community forms an essential element in the trophodynamics of detrital and benthic ecosystems (18). Changes in the structure and functional interrelationships in this vital community have proved difficult to measure without the bias of methods that require separation of the microbes from their microenvironment. Staining and epifluorescent microscopic examination required either release of the microbes from the detrital or sedimentary substrates, which is not quantitative (D. J. W. Moriarty, *in* M. R. Walter, P. A. Trudinger, and B. J. Ralph, ed., *Proceedings of the Fourth International Symposium on Environmental Biochemistry*, in press), or assumptions as to the concentration on the particulate faces not visible in the preparation (12, 13). Scanning electron microscopy shows morphology that is useful in defining the more structurally diverse microbes, but the technique is not quantitative. Unfortunately, for most of the important bacterial components of the community, morphology gives no indication of the function or state of metabolic activity.

Chemical analysis of sediments or detritus for microbe-derived constituents and activities is a potential solution to the problem that morphology does not often indicate function or metabolic activity in procaryotes (17, 30, 31, 54, 56, 57).

Lipids, especially fatty acids, have been recognized to be of great value in the understanding of phylogenic and taxonomic classifications (36, 43, 48). Fatty acid analysis has been utilized in environmental studies to compare different marine depositional environments (34), as a criterion for pollution (47), and for tracing the origin of detritus and marine organic surface deposits (5, 39, 46, 53). Specific branched monoenoic and

hydroxy fatty acids have been used to implicate *Desulfovibrio* in marine oozes (6), and the ratio of palmitoleic to palmitic acids was linked to seasonal succession in phytoplankton communities (23).

More recently, lipids and fatty acids have been integrated into more classsical ecological studies. Extractable lipid phosphate has been proposed as an indicator of biomass in marine and estuarine sediments (58), and changes in total lipid composition and biosynthesis have reflected successional changes in the microbiota associated with detritus (31, 53, 55, 56). Fatty acid patterns have been investigated as possible organic tracers in establishing estuarine feeding relationships (24) and as indicators of the rise and fall of seasonal species within plankton and fouling communities (25). Furthermore, the relative proportions of fatty acids derived from the microflora of artificial detritus have been shown to be highly correlated with the types of macroorganisms associated with the detritus (59).

The recent introduction of a highly polar cyanosiloxane stationary phase that has sufficient high-temperature stability to be used in open tubular capillary gas chromatographic columns, the increase in sensitivity with splitless sample introduction (20), the improved sensitivity of flame ionization detectors, and the improvement of autosampling and data processing techniques have greatly facilitated the analysis of fatty acids derived from the lipids of detrital and sedimentary microbial communities. This paper will show that the extraordinary resolution of branched, cyclopropane, and isomeric unsaturated fatty acid methyl esters by glass capillary gas-liquid chromatography can be very useful in determining the community structure of estuarine detrital and benthic marine microbial assemblies.

MATERIALS AND METHODS

Materials. Nanograde hexane and freshly redistilled analytical-grade chloroform (Mallinckrodt, St. Louis, Mo.) were used. Other analytical-grade solvents were used without further purification. Fatty acid standards were supplied by Supelco, Inc. (Bellefonte, Pa.) and Applied Science Laboratories (State College, Pa.).

Scanning electron microscopy. Samples were prepared for scanning electron microscopy immediately upon removal from experimental tanks with a modification of the method described earlier (41). Artificial seawater of equivalent salinity was substituted for Krebs-Ringer solution during the initial fixation with glutaraldehyde. A Cambridge Stereoscan S4-10 microscope (Cambridge Instrument Co., Ossining, N.Y.) equipped with a Polaroid series 125 camera was utilized.

Extraction of lipids. Lipids were quantitatively extracted by the modified Bligh and Dyer procedure (58). The lipids were recovered in the chloroform phase, the solvents were removed in vacuo, and the lipids were stored under nitrogen at −20°C.

Acid methanolysis. An internal standard, usually methyl nonadecanoate, was added to the lipids which were suspended in 2 ml of anhydrous methanol-concentrated HCl-chloroform (9:1:1, vol/vol) in a test tube with a Teflon-lined screw cap and heated at 100°C for 60 min. Chloroform and water (2 ml of each) were added, and the suspension was mixed with a Vortex mixer for 5 min and then centrifuged for 5 min. The aqueous layer was removed and discarded. The test tube was washed by adding an additional 2 ml of water along the sides of the test tube without mixing with the organic layer. This second layer was then removed and discarded. The chloroform was evaporated in a gentle stream of nitrogen at 25°C, and the lipids were stored at −20°C. The acid methanolysis procedure was suggested by W. Mayberry (East Tennessee State University, Johnson City).

Thin-layer chromatography. The esterified lipid mixture was partitioned on a channeled thin-layer plate (Whatman K6 silica gel, 4.0 nm, 250 μm thick) with a solvent mixture of petroleum ether-ethyl ether-acetic acid (80:20:1, vol/vol). The band containing the fatty acid methyl esters (R_f, 5.5 to 6.5) was collected, eluted in 5 ml of $CHCl_3$, dried under nitrogen, and dissolved in hexane for analysis. An authentic standard of methyl nonadecanoate was developed separately on each side of the plate and visualized under ultraviolet light after spraying with 0.01% rhodamine 6G.

Gas-liquid chromatography. A Varian model 3700 gas chromatograph, equipped with two flame ionization detectors, a model 8000 autosampler, and a CDS-111 data system, was used for all analyses, except where noted. The chromatograph was equipped with a 50-m glass capillary column of 0.24-mm inside diameter coated with Silar 10C, a polysiloxane linked to phenyl and cyanoalkyl functional groups (Applied Science Laboratories). Samples were introduced without stream splitting, with a 0.5-min venting time for the hexane solvent. The oven temperature was programmed from 42 to 192°C, heating for 60 min at a rate of 2°C/min, followed by a 30-min isothermal period at 162°C, and finally heating for 30 min at a rate of 1°C/min up to the maximum temperature of 192°C, which was maintained until the components were eluted. The analysis time for environmental samples was 240 min. The column was operated with helium as the carrier gas at a flow rate of about 1 ml/min at 16 lb/in^2. The injector and detector temperatures were 225°C.

The autosampler was programmed to deliver an injection volume of 2 μl. The entire delivery system was washed between samples with two 50-μl volumes of hexane. Carry-over determined by gas-liquid chromatographic response was negligible.

Identification of fatty acid methyl esters. The fatty acid methyl esters were identified by gas-liquid chromatography by comparing retention times with authentic standards on the polar (Silar 10C) and nonpolar (OV-101) capillary columns before and after hydrogenation (57). Structures of the major components were confirmed by using gas chromatography-

mass spectral analysis by electron impact and chemical ionization fragmentation.

Fatty acid methyl ester nomenclature. The fatty acid methyl esters are designated as the number of carbon atoms in the chain:the number of double bonds. With polyenoic esters, the position of the ultimate double bond (i.e., the double bond nearest the ω end of the molecule) is designated as $\omega 3$, $\omega 6$, etc. Special structural designations are prefixes a, i, br, and Δ for anteiso, iso, branched, and cyclopropane structures, respectively.

Lipid phosphate analysis. Lipids were digested in 23% perchloric acid for 2 h at 200°C and were analyzed for phosphate by a modification of the Bartlett method (2, 58).

Growth of cultures. A strain of wild-type *Neurospora crassa* was grown by using 500 ml of Vogel's N broth (52) in 3.8-liter parrot flasks. The culture was incubated at 35°C for the first 48 h and then held at 25°C. After 6 days, the culture was harvested by Büchner filtration and then quick-frozen and lyophilized.

A strain of *Escherichia coli* was grown by using 1,000 ml of nutrient broth (Difco Laboratories, Detroit, Mich.) in 3.8-liter parrot flasks on a gyratory shaker at 100 rpm. After 48 h at 35°C, the cells were harvested by centrifugation and then quick-frozen and lyophilized.

Incubation of detrital microbes. Strings of 5-cm squares of Nitex screen were incubated in Apalachicola Bay (Franklin County, Florida) for 2 weeks in the spring of 1979 to establish a natural mixed community (4, 41). The Nitex surfaces were then transported to the laboratory in oxygenated estuarine water and maintained in 15-liter plastic tanks containing aerated estuarine water (3‰ salinity) at a temperature of 21°C.

To inhibit eucaryotic growth, one tank was maintained at pH 7.8 and supplemented daily with 0.01 mM NaH_2PO_4, 0.1 mM glutamine, and 25 mg of cyclohexylimide per liter (final concentration). To inhibit procaryotic organisms, a second tank was maintained at pH 5.5 by adding HCl and was supplemented daily with 0.10 mM sucrose, 0.001% (wt/vol) nutrient broth (Difco Laboratories), 50 mg of streptomycin per liter, and 4 mg of penicillin per liter. A third tank was maintained as a control. After 8 days, 18 Nitex squares were recovered from each tank and analyzed.

Benthic marine microbial communities. Sandy marine sediments, recovered from the Gulf of Mexico 14 miles south of Panama City, Fla., at the U.S. Navy Stage I in 35 m of water, were air dried and then exposed to unfiltered running seawater in tanks similar to those described by Cantelmo and Rao (8) for a period of 10 weeks during the summer of 1978 to study the effects of oil drilling muds on benthic recolonization (N. L. Richards, *in Effects of Chemicals Used in Oil and Gas Well Drilling Operations in Aquatic Environments*, in press). At the end of the experiments, the sediments were sieved through 1-mm mesh sieves, and the macrofauna were recovered. The sands from this recolonization experiment were then quick-frozen. Control cores (10 cm in diameter) of the top 3 to 5 cm of undisturbed sandy bottom were treated similarly. Forty-gram samples were thawed, extracted, and analyzed for benthic microbial and meiofaunal fatty acid content.

Mass spectrometry. Gas chromatography-mass spectrometry of each sample was performed with a Hewlett Packard 5983 instrument, using the same capillary column under identical conditions. Electron impact mass spectrometry was performed at 70 eV.

The ion source was held at 200°C, with the electron multiplier voltage at 3,000 V. Scans from 50 to 450 amu were run at a rate of 180 amu/s, with a scan time of 22 s and five A-to-D conversions per datum. Chemical ionization mass spectrometry was performed with methane at a pressure of 0.4^{-1} torr in the ion source with the same electron multiplier voltage. Scans from 200 to 450 amu were run at a rate of 112.5 amu/s, with a scan time of 2.7 s giving eight A-to-D conversions per datum.

RESULTS

Chromatographic separation. Excellent resolution of the fatty acid methyl esters was given by a 50-m Silar 10C open tubular support coated-glass capillary (Fig. 1). The Trenzahl (Tz) separation number (26), measured with methyl-*n*-octadecanoate and methyl-*n*-nonadecanoate, was 10.67 (mean of three analyses), with $Tz/m = 0.21$ ($m = 50$ m). The calculated theoretical plates gave $n = 13{,}619$ or $n/m = 272$, which is typical of cyanosiloxane-coated capillaries (21).

Recovery. Acid methanolysis of the lipid formed fatty acid methyl esters quantitatively. Recovery of the methyl esters after partitioning the methanolysate in the chloroform was greater than 99% (Table 1). Quantitative recovery of saturated, monoenoic, and branched fatty acid was demonstrated. The slight loss of polyenoic fatty acid esters (recovery of 93 ± 8.2%) can be further decreased by careful flushing of the thin-layer chromatograph chamber with argon.

Sensitivity. The sensitivity was determined by measuring the response of dilutions of methyl nonadeconoate in hexane; 1 ng gave a response four times the noise level at an electrometer sensitivity of 8×10^{-12} A/mV.

Reproducibility. The accuracy of the gas chromatographic analysis of the samples was evaluated by using both standard mixtures and environmental samples containing fatty acid methyl esters after isolation by thin-layer chromatography. Peaks were normalized to methyl nonadecanoate. Trimmed means were computed in a method similar to that of Herb and Martin (22).

The standard deviation ($\pm 1\sigma$) was 4.6% for repeated analyses (10 replicates) on a mixture of 11 common bacterial fatty acids, including saturated branched and straight-chained, cyclopropane, and monoenoic fatty acid methyl esters. Typically, sand samples were much more complex, with over 240 components commonly detected at maximum sensitivity. The standard deviation ($\pm 1\sigma$) was 10.6% for repeated analysis

(10 replicates) with 26 of the esters present in the greatest amounts.

Fatty acid methyl esters and microbial community structure. To show the utility of fatty acid methyl esters in defining the microbial community structure, we analyzed mixtures of

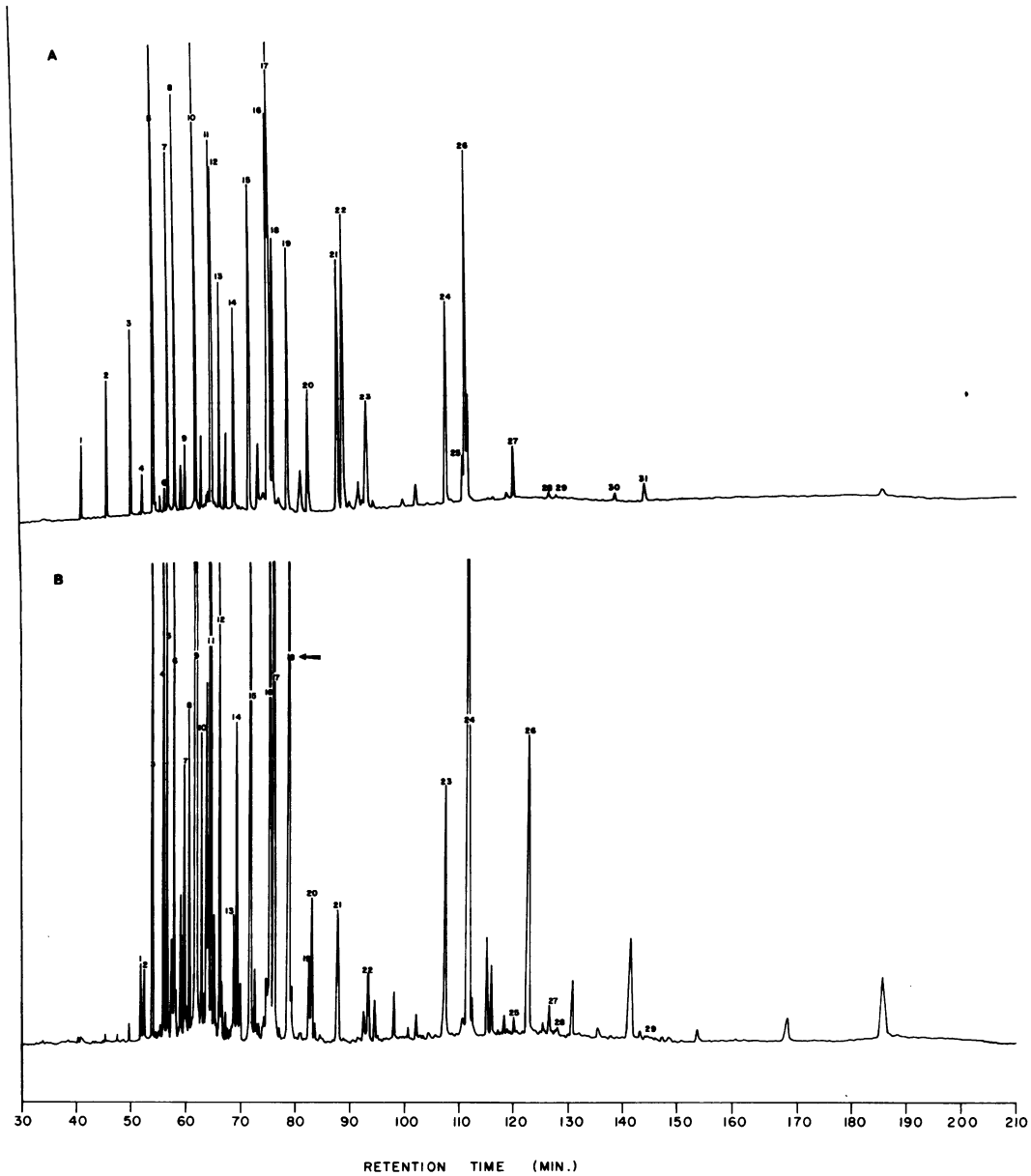

FIG. 1. *Capillary gas chromatographic separation of fatty acid methyl esters performed with a 50-m Silar 10C column. (A) Analysis of a standard mixture of fatty acid methyl esters measured with the flame ionization detector at a sensitivity of 16 × 10^{-12} A/mV. Peaks: 1, 11:0; 2, 12:0; 3, 13:0; 4, i14:0; 5, 14:0; 6, i15:0; 7, a15:0; 8, 15:0; 9, i16:0; 10, 16:0; 11, 16:1ω7; 12, a17:0; 13, 17:0; 14, Δ17:0; 15, 18:0; 16, 18:1ω9; 17, 18:1ω11; 18, 18:1ω7; 19, 19:0; 20, 18:2ω6; 21, 18:3ω6; 22, 20:0; 23, 18:3(ω3); 24, 20:3ω6; 25, 22:1ω9; 26, 20:4ω6; 27, 20:5ω3; 28, 22:4ω6; 29, 24:1ω9; 30, 22:5ω3; 31, 22:6ω3. (B) Sample of marine sand sediment analyzed as in (A). Peaks: 1, i14:0; 2, a14:0; 3, 14:0; 4, i15:0; 5, a15:0; 6, 15:0; 7, i16:0; 8, br16:1; 9, 16:0; 10, br17:0; 11, 16:1ω7; 12, 17:0; 13, Δ17:0; 14, 17:1; 15, 18:0; 16, 18:1ω9; 17, 18:1ω7; 18, 19:0; 19, 18:2ω6; 20, Δ19:0; 21, 20:0; 22, 20:1; 23, 22:0; 24, 22:1ω9; 25, 20:5ω3; 26, 24:0; 27, 22:4ω6; 28, 24:1ω9; 29, 22:6ω3.*

lyophilized *N. crassa* and *E. coli* (Fig. 2). Increasing the proportion of bacteria increased the ratio of *cis*-vaccenic acid to oleic acid (18:ω7/18:1ω9) from 0.24 to 89.8. The polyenoic fatty acids (primarily 18:2ω6 and 18:3ω3) increased from 0 to 48% of the total fatty acids as the proportions of *N. crassa* increased. The ratios of cyclopropane fatty acids to the internal standard (Δ17:0 and Δ19:0 to 19:0) increased with increasing proportions of *E. coli*, paralleling the ratio of *cis*-vaccenic acid to oleic acid. The ratio of 24:0 to 19:0 increased with increasing proportions of *N. crassa*, as did the total polyenoic fatty acids.

Manipulation of the estuarine detrital microbial community structure. To establish whether the fatty acid methyl esters could reflect changes in natural community structures, nylon sheets were incubated in a subtropical estuary and transferred either to tanks where eucaryotic growth was inhibited by cycloheximide and procaryotic growth was stimulated by addition of phosphate and glutamine or to tanks where procaryotic growth was inhibited by penicillin and streptomycin and eucaryotic growth was stimulated by acid pH, sucrose, and nutrient broth.

Scanning electron micrographs show clear morphological differences in the microbial community structure (Fig. 3). The stimulation of eucaryotic and inhibition of procaryotic growth resulted in the proliferation of fungal mycelia, as observed at both high and low magnifications (Fig. 3A and B). These mycelia were completely

TABLE 1. *Recovery of fatty acid methyl esters after acid methanolysis, fractionation by thin-layer chromatography, and analysis by gas-liquid chromatography*

Fatty acid[a]	Initial composition[b]	After methanolysis[c]	After thin-layer chromatography[d]	Recovery (%)[e]
16:0	44,000	44,000	45,300	103
18:0	61,500	61,200	62,200	101
18:1	28,300	28,600	29,200	103
18:2	68,500	68,200	62,900	92
20:0	62,300	62,500	69,500	111
20:4	56,600	56,200	61,400	109
20:5	42,700	42,400	43,900	100
22:6	40,900	40,700	37,000	91

[a] Fatty acids are designated as the number of carbon atoms:number of double bonds.

[b] Results of the integrated areas from the responses of an authentic sample by gas-liquid chromatography.

[c] Recovery after acid methanolysis.

[d] Recovery after thin-layer chromatography of the esters.

[e] Percent recovery of the initial standard mixture. Each area represents the average of six replicate analyses.

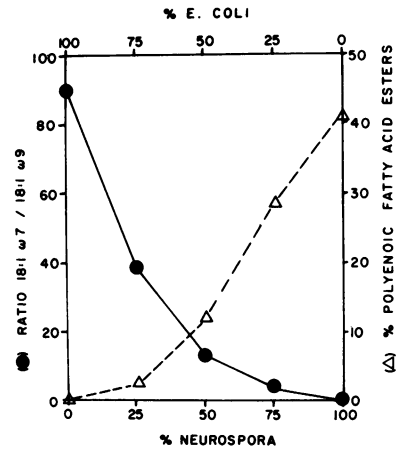

FIG. 2. *Ratios of critical fatty acid methyl esters extracted from reciprocal mixtures of N. crassa and E. coli. Symbols: ●, ratio of 18:1ω7/18:1ω9; △, ratio of the polyenoic fatty acids (18:2ω6 + 18:3ω3)/total fatty acid methyl esters.*

absent in the cycloheximide-inhibited preparation (Fig. 3E and F). Some filaments were visible on the procaryote-stimulated detritus, but they were sparsely distributed and were three to four times thinner than those in the strips where fungal growth was enhanced. The detritus incubated in the control had a mixed community of bacteria, fungi, algae, and diatoms (Fig. 3C and D).

The clear morphological differences in estuarine detrital community structure are reflected in the fatty acid methyl ester concentration (Table 2). Stimulation of procaryotic and depression of eucaryotic growth (Fig. 3E and F) showed marked increases in the ratios of the branched to normal C_{15} esters and the proportion of the *cis*-vaccenic to oleic C_{18} monoenoic esters and some increase in the C_{17} cyclopropane ester (Table 2, column C). Stimulation of eucaryotic growth and inhibition of procaryotic growth (Fig. 3A and B) increased the ratios of dienoic to normal saturated C_{18} and of total polyenoic esters of both the α- and γ-linolenic series (Table 2, column A). The control detrital community (Fig. 3C and D) contained a mixture of typical procaryotic and eucaryotic fatty acid methyl esters (Table 2, column B).

This experiment was repeated three times with Teflon and polyethylene surfaces. In each case, the ratios of fatty acids were similar to those given in Table 2.

Benthic marine microbial community. Analysis of the sediment microbiota has proved difficult (55). However, the lipids of the microbial and meiofaunal communities yielded a diverse collection of fatty acid methyl esters (Fig.

1B). Microbes colonizing sun-bleached sands incubated on a platform in 35 m of water were compared with the benthic microbial community from that site (Table 3). The colonizing community was exposed to seawater from that site at the same light intensity as that at the

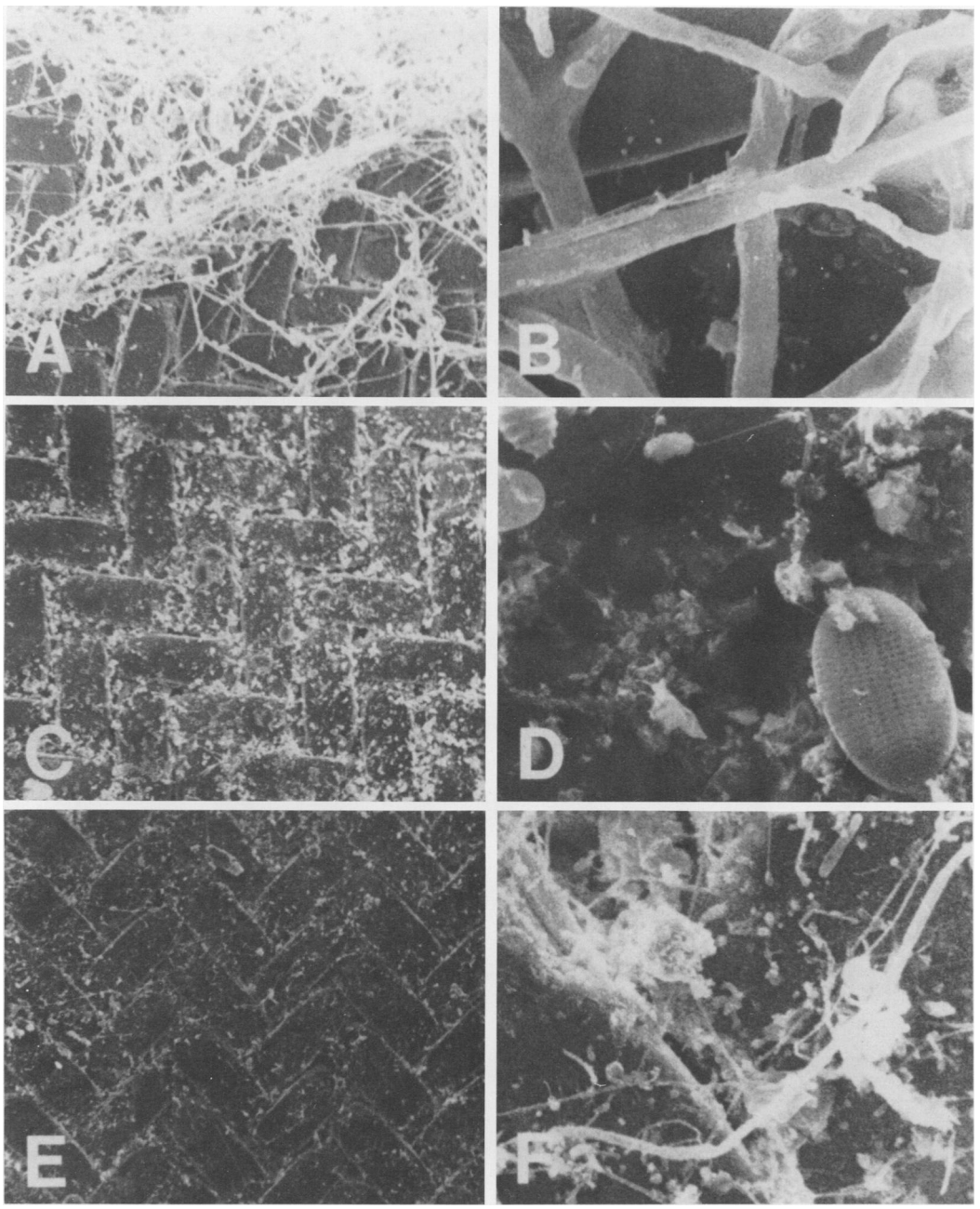

FIG. 3. *Scanning electron micrographs of the Nitex surfaces taken from the estuary and incubated for 8 days at 21°C in aerated estuarine water supplemented daily with 50 mg of streptomycin per liter, 4 mg of penicillin per liter, 10 μM sucrose, and 0.01% (wt/vol) nutrient broth, maintained at pH 5.5 (A and B); estuarine water (C and D); and estuarine water supplemented daily with 0.01 mM NaH_2PO_4, 0.01 mM glutamic acid, and 25 mg of cycloheximide per liter, maintained at pH 7.8 (E and F). Picture widths (in micrometers) are as follows: (A) 370; (B) 40; (C) 365; (D) 37; (E) 360; (F) 37.*

bottom. There was a significantly larger community in the cores, which contained more than twice the lipid phosphate. The bacterial component, particularly the component containing the short-branched fatty acids, was depressed in the sand-colonizing community as compared with the undisturbed benthic community. The same was true for the bacterial component containing cyclopropane fatty acids. The microbes utilizing the anaerobic microbial fatty acid synthetic pathway (18:1ω) and the eucaryotic communities contributing 18:2, α- and γ-linolenic polyenoic series (ω3 and ω6), and 24:0 were not significantly different.

Identification of specific components. The fragmentation patterns of fatty acid methyl esters in the electron impact mass spectrometry often give specific structural information. For methyl stearate (Fig. 1A, component 15) the base peak at *m/e* 74 represents an odd electron ion fragment formed by β cleavage after hydrogen radical transfer to the hydrogen in the McLafferty rearrangement (7), and prominent even electron ions at *m/e* 87, 143, 199, and 255 are formed after hydrogen radical transfer from C-6 to the carbonyl oxygen with homolytic cleavage between C-6 and C-7 (*m/e* 143), radical transfer from C-6 to C-2 with homolytic cleavage between C-3 and C-4 (*m/e* 87), or radical transfer from C-6 to C-10 with homolytic cleavage between C-11 and C-12 (*m/e* 199) (7). The acylium ion formed by the cleavage of the bond adjacent to the carbonyl forms the M-31 ion at *m/e* 267.

The mass spectrum of 18:2ω6 (Fig. 1A, component 20) carbon shows a M^+ ion at *m/e* 296 of 4 mass units less than that of the unsaturated ester. It also shows prominent hydrocarbon fragments of C-5, C-6, C-7, C-8, and C-9, which are larger than the odd electron ion at *m/e* 74 from the McLafferty rearrangement or the radical transfer fragments at *m/e* 87, 143, 199, and 255, which are so characteristic of the saturated fatty acid methyl esters (15). The position of the double bonds in this sample was inferred from its retention time on the gas chromatograph compared with an authentic standard.

The i15:0, a15:0, and n15:0 (Fig. 1A, components 6, 7, and 8) show fragments at *m/e* 84, 87, 143, and 199, which identify the components as saturated fatty acid methyl esters. The increased likelihood of sigma bond cleavage at a branched carbon allows differentiation. In anteiso esters the M-57 (loss of $C_4H_9^+$) is larger than the M-43 (loss of $C_3H_7^+$) or the molecular ion M^+. The iso and normal esters can be differentiated because the M-43 in the iso-branched ester is always larger than the M-31 or M^+ (45).

The increased fragmentation at a tertiary carbon allows the structural determination of the unusual fatty acid methyl ester (Fig. 1B, component 10). The prominence of M-141 (loss of $C_{10}H_{21}^+$), M-85 (loss of $C_6H_{13}^+$) and M-43 (loss of $C_3H_7^+$) define the branching. The fact that M-141 and M-85 also correspond to the radical transfer fragments at *m/e* 143 and 199 intensifies the yields of these fragments.

The position of the unsaturation in the 18:1

TABLE 2. *Changes in fatty acid methyl esters recovered from Nitex screens removed from the estuary and incubated as described in the legend to Fig. 3*

Fatty acid ratio[a]	A[b]	B[c]	C[d]
(i15:0 + a15:0)/15:0	1.04	1.08	4.42
Δ17:0/16:0	0.004	0.065	0.009
18:1ω7/18:1ω9	0.30	0.39	1.30
16:1/16:0	0.15	0.13	0.13
18:2/18:0	5.98	0.23	0.03
Polyenoics (%)/total[e]	0.390	0.083	0.024

[a] Fatty acids are designated as a or i (anteiso or iso branched), Δ (cyclopropane), number of carbon atoms: number of double bonds, position of the unsaturation nearest the ω end of the ester.
[b] Ratios in the microeucaryote-stimulated environment (Fig. 3A and B).
[c] Ratios in the control (Fig. 3C and D).
[d] Ratios in the microprocaryote-stimulated environment (Fig. 3E and F).
[e] Ratio of the sum of 18:2, 18:3, 20:4, and 20:5 to the total fatty acids.

TABLE 3. *Differences between the population structure of the microbiota in a marine benthic settling community and undisturbed cores, shown as the ratios of the fatty acid methyl esters derived from the lipids.*

Lipid	Colonizing community[a]	Core[b]	Significance[c]
Lipid phosphate[d]	0.0074 ± 0.008	0.0186 ± 0.002	*
Fatty acids[e]			
(i15:0 + a15:0)/15:0	0.98 ± 0.08	2.64 ± 0.21	*
Δ17:0/16:0	0.06 ± 0.007	0.02 ± 0.006	*
Δ19:0/16:0	0.065 ± 0.013	0.015 ± 0.002	
16:1/16:0	0.36 ± 0.08	0.62 ± 0.15	
18:1ω7/18:1ω9	1.40 ± 0.37	1.67 ± 0.52	
18:2ω6/18:0	0.26 ± 0.07	0.13 ± 0.08	
Polyω6/19:0[f]	0.06 ± 0.008	0.06 ± 0.02	
Polyω3/19:0[g]	0.12 ± 0.03	0.16 ± 0.09	
24:0	0.12 ± 0.05	0.09 ± 0.004	

[a] Population colonizing dried baked sand after 10 weeks in flowing seawater given as mean ± 50 for five replicate analyses.
[b] Core of undisturbed sediment under 35 m of water.
[c] Significance (*) indicates $t > 3.5$, $df = 7$, and $P = 0.005$.
[d] Lipid phosphate in micromoles per gram of dry weight (mean ± standard deviation).
[e] Fatty acids are designated as indicated in Table 2, footnote a.
[f] Polyω6 = 18:3ω6 + 20:4ω6 + 22:4ω6/19:0.
[g] Polyω3 = 20:5ω3 + 22:6ω3/19:0.

has important consequences in the definition of the microbial population structure, as the *cis*-vaccenic acid 18:1ω7 (Fig. 1A, component 18) is formed exclusively by the bacterial anaerobic pathway. Other eucaryotic and procaryotic organisms form oleic acid 18:1ω9 (Fig. 1A, component 16), which shows a rearrangement fragment at *m/e* 192, which is not in the *cis*-vaccenic ester.

Unsaturation is very easily defined in the chemical ionization mass spectrometry spectra. The prominent M+1 quasi-molecular ion for the 16:0, *n* (Fig. 1A, component 10), is at *m/e* 271, and that for 16:1 is at *m/e* 269. The M−1 peak is prominent in the 16:0 at *m/e* 269, but the M−1 peak for 16:0 (Fig. 1A, component 11) at *m/e* 267 is low. When methane is used in chemical ionization, there is more prominent fragmentation in the unsaturated esters. These techniques distinguish the number of double bonds by the *m/e* for the M−1 quasi-molecular ion.

The chemical ionization mass spectrometry technique is useful in detecting the cyclopropane fatty acid methyl esters as the M+1 ion is 2 *m/e* units less than the normal ester, but the M−1 ion is present.

Selective ion monitoring. Since it is known that specific fragmentation patterns yield ions at *m/e* 74, 87, 143, etc., for fatty acid methyl esters, it is possible to rapidly define the composition of an environmental sample by following the specific ion concentrations. It is also possible to establish whether the maximum intensity of specific ions corresponds to the maxima in the total ion current. In Fig. 4, the relative intensities of ions *m/e* 199, 213, and 225 [corresponding to $(CH_2)_{10}COOCH_3^+$, M-43, and M-31] have a maximum intensity at the same point as the total ion current corresponding to a component between components 3 and 4 in Fig. 1B. This information, coupled with the retention time and resistance to hydrogenation, suggests that it is a br15:0 fatty acid methyl ester which is present in too low a concentration to give a definitive mass spectrum.

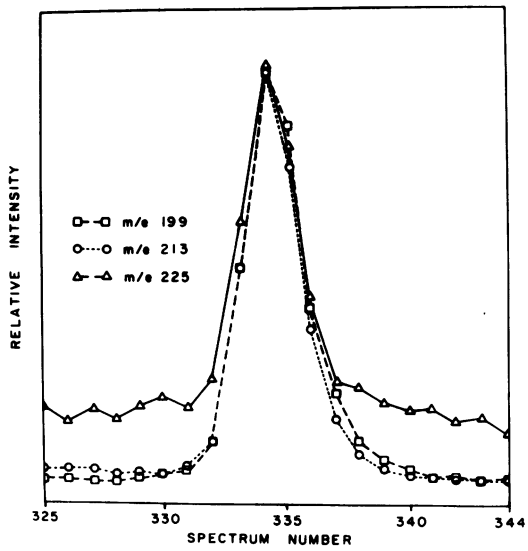

FIG. 4. *Plot of the intensity of the ions versus the number of the spectrum which corresponds to the maximum intensity of the total ion current for a fatty acid methyl ester derived from marine sediment corresponding to a br15:0 ester between components 3 and 4 (see Fig. 1A).*

DISCUSSION

Capillary gas-liquid chromatographic analysis. The analysis of the fatty acid methyl esters by the polar Silar 10C coated-glass capillary columns gives excellent resolution (Fig. 1) and great sensitivity with the splitless injection technique. The long analysis time despite the temperature programming can be compensated for by the use of an autosampler, with automated electronics and a data processing system, which allows unattended 24-h operation. The lipid extraction, acid methanolysis, and thin-layer chromatographic fractionation allow for essentially quantitative recovery (Table 1). This allows for not only fatty acid analysis but also the potential use of other lipid fragments such as alcohols, aldehydes, steroids, sphinganines, and ethers, as well as the glycerol, carbohydrates, amino acids, and amines recovered from the aqueous phase after partitioning the methanolysis reactants, and, after suitable derivatization, can help specify the microbial population structure. For example, fatty aldehydes derived from plasmalogens in the absence of polyenoic fatty acids indicate the presence of anaerobic procaryotes, which, surprisingly, are much more concentrated in the aerobic portion of estuarine sediments (55).

Significance of the fatty acid analysis. From studies of microbial monocultures, the significance of selected fatty acids can be postulated.

Short C_{13} to C_{21} iso- and anteiso-branched fatty acids are characteristic of bacteria (36, 44, 51). Protozoa and fungi form significant amounts of these branched fatty acids only when supplied with high concentrations of branched precursors in the growth milieu (16, 33, 40). The short-chain, anteiso- and iso-branched fatty acids correlate well with the bacterial colonization of dead *Spartina* marsh grass (46). Studies by Cranwell (9–11) suggest that bacterially-derived short-chain branched and cyclopropane fatty

acids of productive lakes result from microbial decomposition of autochthonous detritus. Iso- and anteiso-branched fatty acids may accumulate in the stationary growth phase of some bacterial monocultures (1) or at the surface of recent sediments (39).

Cyclopropane fatty acids in bacteria are restricted to certain lipids because they are formed from monoenoic fatty acids only when they are esterified with certain lipids (50). The cyclopropane fatty acids are found in certain classes of gram-negative bacteria, as well as in the lactobacilli and clostridia (27, 36, 37, 48). They are also found in the kinetosomes of some protozoa (40) and in terrestrial plants. In some bacterial species, the cyclopropane fatty acids accumulate in the stationary phase (27, 35, 38) or with adverse growth conditions (32), resulting in a decrease in C_{18} mono-unsaturated fatty acids and an increase in C_{19} cyclopropane fatty acids.

It is possible to infer community structure from the position of the double bond in octadecanoic mono-unsaturated fatty acids (5). The anaerobic system that is found predominantly in bacteria (3) results in *cis*-vaccenic acid (18:1ω7) (16).

Polyenoic fatty acids are not found in bacteria (16, 32, 36, 48). An exception is a conjugated dienoic fatty acid found in a bacterium under unnatural growth conditions (19). Certain complex blue-green algae contain C_{18} trienoic fatty acids (16, 28, 29, 42), but no polyenoic acids with more than 18 carbon atoms. Polyenoic acids with longer chains are typical of eucaryotes, especially photosynthetic eucaryotes (16, 48). By noting the positions of the double bonds in these fatty acids, phylogenetic associations and further differentiation of the populations are possible (5, 14, 16, 49, 53).

With the excellent separation of the capillary column (Fig. 1), the α-linolenic series (double bonds begin at the ω3 position), which are characteristic of yeasts, higher fungi, and algae, were readily distinguished from the γ-linolenic series (unsaturation begins at the ω6 position), which are characteristic of protozoa and microeucaryotic animals.

Fatty acid analysis of the detrital microbiota. In this study, the relatively simple demonstration of the usefulness of fatty acid analysis in determining the microbial eucaryote-procaryote relationship can be documented for monocultures (Fig. 2) and, by manipulation of the environment, for the detrital microbiota (Fig. 3 and Table 2). The eucaryote/procaryote ratio of the detrital microbiota has been shown to correlate with the number of species, biomass, and Margalef species richness of the macrofauna attracted to baskets containing detritus in a subtropical estuary (59).

Fatty acid analysis of the sedimentary microbiota. Analysis of benthic microbes has proved difficult because cultural and extractive methods necessitate quantitative removal of microbes from the sedimentary substrate (55). Indirect chemical methods are effective; for example, lipid phosphate is a measure of the total sedimentary microbial biomass (58). Fatty acid analysis can show subtle differences in the community structure. The bacterial, rather than the microeucaryotic, population appeared different in a colonizing community as opposed to a mature benthic marine community (Table 3). The benthic core samples showed significantly higher proportions of short-branched fatty acids and lower proportions of cyclopropane fatty acids than did those of the community colonizing the sunbleached sand. The short-branched fatty acids are more a measure of bacterial biomass, and the proportions of cyclopropane fatty acids are a measure of physiological status, as these fatty acids may accumulate in stressed organisms (32). The bacteria utilizing the anaerobic unsaturated fatty acid pathway (measured as 18:1ω7) and the cyanophytes and microeucaryotes (measured as polyenoic and long-chain fatty acids) were not significantly different.

Effects of animal predation on fatty acid composition. In a preliminary experiment performed in collaboration with Roy Robertson of the Sapelo Island Laboratory of the University of Georgia, the effect of excluding snails from or increasing the number of snails on estuarine muds was examined. Snail predation decreased the microbial biomass as measured by the lipid phosphate. The microeucaryotes disappeared, as indicated by the absence of polyenoic fatty acids. The proportion of short-branched fatty acids increased, indicating an increase in the relative proportions of bacteria with predation. A decrease in the proportions of cyclopropane fatty acids in the plots subjected to snail bioturbation suggested that these bacteria were growing too rapidly to accumulate cyclopropane fatty acids.

S. J. Morrison and D. C. White of this laboratory have recently demonstrated that grazing amphipods cause an increase in the metabolic activity of the grazed detrital population (unpublished observations). The fatty acid analysis thus appears useful in the analysis of benthic microbial communities.

Predation by the sand dollar (*Mellita quinquiesperforata*) on marine sandy sediments resulted in little change in the total cellular and membrane biomass or metabolic activity (total adenosine nucleotides, lipid phosphate, chloro-

phyll *a*, and rates of lipid synthesis) or in procaryotic biomass and activity (muramic acid and thymidine incorporation into deoxyribonucleic acid). There was, however, marked diminution of microeucaryotic activity and biomass (sulfate incorporation into sulfolipids and wall glucosamine, inositol, and lipid glycerol). The selective predation on microeucaryotes was reflected in significantly decreased ratios of both $\omega 3$ and $\omega 6$ polyenoic fatty acids and the long-chain fatty acids and increased proportions of the short-chain branched, cyclopropane, and *cis*-vaccenic fatty acids in sands processed by the sand dollars (D. C. White, R. H. Findlay, S. D. Fazio, R. J. Bobbie, J. S. Nickels, W. M. Davis, G. A. Smith, and R. F. Martz, *in* V. A. Kennedy, ed., *Estuarine Perspectives*, in press).

Mass spectrometry. The usefulness of capillary column gas chromatography in the analysis of detrital or sedimentary microbial communities can be greatly extended by coupling the chromatograph to a mass spectrometer. The combined gas chromatograph-mass spectrometer can give definitive structural identifications of the esters. Selective ion monitoring can not only give structural information, but can also greatly increase the sensitivity of the detection (Fig. 4). In addition, the use of selective ion monitoring of extracts from organisms exposed to ^{13}C, ^{15}N-, or ^{18}O-labeled precursors allows metabolic processes to be followed using precursor molecules with much higher specific activities than radioactive isotopes and with a much higher sensitivity in the analysis. The high specific activities should be particularly useful in studies of the oligotrophic environment in the ocean.

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