APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2006, p. 3357–3366 Vol. 72, No. 5
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.5.3357–3366.2006

Diversity, Composition, and Geographical Distribution of Microbial Communities in California Salt Marsh Sediments

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Received 24 October 2005/Accepted 2 March 2006

The Pacific Estuarine Ecosystem Indicators Research Consortium seeks to develop bioindicators of toxicant-induced stress and bioavailability for wetland biota. Within this framework, the effects of environmental and pollutant variables on microbial communities were studied at different spatial scales over a 2-year period. Six salt marshes along the California coastline were characterized using phospholipid fatty acid (PLFA) analysis and terminal restriction fragment length polymorphism (TRFLP) analysis. Additionally, 27 metals, six currently used pesticides, total polychlorinated biphenyls and polycyclic aromatic hydrocarbons, chlordanes, nonachlors, dichlorodiphenyldichloroethane, and dichlorodiphenyldichloroethylene were analyzed. Sampling was performed over large (between salt marshes), medium (stations within a marsh), and small (different channel depths) spatial scales. Regression and ordination analysis suggested that the spatial variation in microbial communities exceeded the variation attributable to pollutants. PLFA analysis and TRFLP canonical correspondence analysis (CCA) explained 74 and 43% of the variation, respectively, and both methods attributed 34% of the variation to tidal cycles, marsh, year, and latitude. After accounting for spatial variation using partial CCA, we found that metals had a greater effect on microbial community composition than organic pollutants had. Organic carbon and nitrogen contents were positively correlated with PLFA biomass, whereas total metal concentrations were positively correlated with biomass and diversity. Higher concentrations of heavy metals were negatively correlated with branched PLFAs and positively correlated with methyl- and cyclo-substituted PLFAs. The strong relationships observed between pollutant concentrations and some of the microbial indicators indicated the potential for using microbial community analyses in assessments of the ecosystem health of salt marshes.

Salt marshes are among the most productive ecosystems on the planet and harbor diverse biological communities (45). Coastal estuaries provide a buffer zone between terrestrial and aquatic ecosystems in urban and industrial areas (40), and they act as protective filters and final repositories for runoff pollutants (45), pathogens (19), and nutrients (22). Many pollutants are stabilized and immobilized in sediment. Microbial communities in salt marsh sediments decompose organic matter derived from plant litter and transform pollutants (4, 43), and they can affect the availability of heavy metals (44) and perform other ecosystem services (20). Despite the importance of these ecosystems, there have been few landscape-scale studies that have described the distribution and composition of microbial communities (4a, 16, 26) or the effects of pollutant gradients on microbial communities (3, 15, 18, 39) in marsh sediments. California's 840-mile coastline encompasses numerous salt marsh ecosystems that have economic, recreational, and a esthetic importance to the state. These ecosystems are subject to unprecedented human-induced pressures resulting from urbanization, intensive agriculture, fisheries, and tourism. More knowledge about the microbial communities associated with estuaries is essential for development of effective mitigation and restoration strategies for these vulnerable ecosystems.

The Pacific Estuarine Ecosystem Indicator Research Consortium is a multidisciplinary project funded by the Estuarine and Great Lakes Environmental Protection Agency program (http://www.bml.ucdavis.edu/peeir/index.html). The main objective of this consortium is to develop indicators of the ecosystem health and integrity of estuaries along the Pacific coast. In this study, we compared sediment microbial communities in salt marshes in several estuaries over 2 years in order to assess the relative importance of environmental variables and sediment contamination in influencing the composition of these communities. We hypothesized that for salt marsh microbial community composition (i) variation is greater between different marshes than within a marsh, (ii) variation is influenced by tidal inundation cycles, and (iii) variation is related to contaminant concentrations in the sediments. The results of our study illustrate the importance of considering nonanthropogenic variables in the design and interpretation of ecosystem biomarkers, indicators, and other monitoring tools.

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MATERIALS AND METHODS

Site description and sampling. Sediments were collected in 2002 and 2003 from six marshes along the California coastline (Table 1): Stege Marsh (SM), Walker Creek (WC), Tom's Point (TP), Carpinteria Marsh (CM), Mugu Lagoon

TABLE 1. Sampling sites, sampling year and month, and coordinates of the sampling sites

Marsh	Latitude	Longitude	Yr (mo)	No. of stations/ no. of samples
CM	34°24′	119°32′	2002 (7)	4/12
			2003 (8)	12/36
CC	37°52′	122°29′	2003 (8)	3/9
ML	34°06′	119°05′	2002 (6)[a]	8/24
SM	37°46′	122°19′	2002 (2)	5/15
			2003 (8)	12/38
TP	38°04′	122°56′	2003 (9)	3/6
WC	38°04′	122°55′	2002 (8)[a]	6/18
			2003 (9)	6/7

[a] No TRFLP fingerprints were available for the samples.

(ML), and China Camp (CC). The sampling sites are part of the Pacific Estuarine Ecosystem Indicator Research Consortium sampling network. Both CC and SM are located in San Francisco Bay, WC and TP are part of the main tributaries leading into Tomales Bay (Fig. 1A), and ML and CM are the only sites located in Southern California (Fig. 1B). SM is a former mudflat converted to a tidal marsh and is bordered by an upland prairie and a residential area to the west; it has three freshwater inlets (9). SM is contaminated with heavy metals (Pb, As, Zn, and Cd) and organic compounds (polychlorinated biphenyls [PCBs] and polycyclic aromatic hydrocarbons) (47). Tomales Bay has been listed under Clean Water Act section 303(d) as being impaired due to pathogens (total and fecal coliform indicators), high nutrient levels, mercury contamination, and sedimentation (42). WC is one of the main tributaries leading into Tomales Bay. An open-pit mercury mine which ceased operation in 1972 is thought to contribute to the increased mercury levels in WC (50). TP is located near the northeast end of Tomales Bay and is relatively undisturbed by human use because of its remoteness, and CC is a relatively unpolluted site that has been a state park since 1972. Mugu Lagoon and its associated wetlands are surrounded and heavily influenced by the U.S. Navy's Point Mugu Naval Air Station and intensive, large-scale, open-field farming operations to the north. CM is influenced by greenhouse operations and open-field farms to the northwest, a light industrial park to the north, and the city of Carpinteria to the north and east, as well as the adjacent U.S. Highway 101 and Union Pacific Railroad tracks.

Sampling stations along the main channels were marked in each marsh, and exact coordinates were recorded using a global positioning system (Fig. 1C). At each sampling station, samples were collected at different channel depths to account for tidal inundation cycles (Fig. 1D). The following assumptions were made: the channel's deepest point was saturated at most times during the day based on low-tide levels, the intertidal bank was flooded intermittently, and the vegetated area (mostly dominated by *Spartina alterniflora* or *Salicornia* sp.) was rarely flooded and relatively unsaturated compared to the other elevations. To simplify sample identification, each sample location was designated high (unsaturated), mid (intermittently flooded), or low (saturated); this nomenclature is referred to as elevation below. At each sampling location and channel depth, 7 to 10 5-cm cores were collected with a Teflon corer, transferred to a sterile glass jar, and homogenized. The composite samples were divided into two portions, a portion that was used for phospholipid fatty acid (PLFA) analysis and a portion that was used for terminal restriction fragment length polymorphism (TRFLP) analysis. Samples were collected in the same locations for metal and organic pollutant analysis.

Sediment characterization. The organic carbon contents of a subset of 80 samples were determined using a Perkin-Elmer CHNS/O analyzer (Perkin-Elmer Corporation, Norwalk, Conn.). Sediments were combusted at 925°C in silver boats after they were exposed to concentrated hydrochloric acid fumes in a desiccator for 24 h to remove carbonate minerals. Sediments were dried to a constant weight before CHN analysis. Acetanilimide was used to calibrate the instrument for elemental carbon and nitrogen. Purified products of high-temperature combustion (CO_2 for C and N_2 for N) were detected with a thermal conductivity detector. Metal analyses were performed with 50-mg sediment samples as described by U.S. Environmental Protection Agency method 6020 (14). For the organic pollutant analyses, sediment samples were extracted with dichloromethane using Soxhlet extraction; the extracts were cleaned using alumina column chromatography to remove interfering compounds. Polycyclic aromatic hydrocarbons, PCBs, dichlorodiphenyldichloroethane (DDD), dichlorodiphenyldichloroethylene, chlordanes, and nonachlors were analyzed by gas chromatography-mass spectrometry.

Salt marshes have naturally occurring salinity gradients that can be effectively tracked by using sodium concentrations. To account for metal fluctuations associated with salinity gradients, metal concentrations were normalized by express-

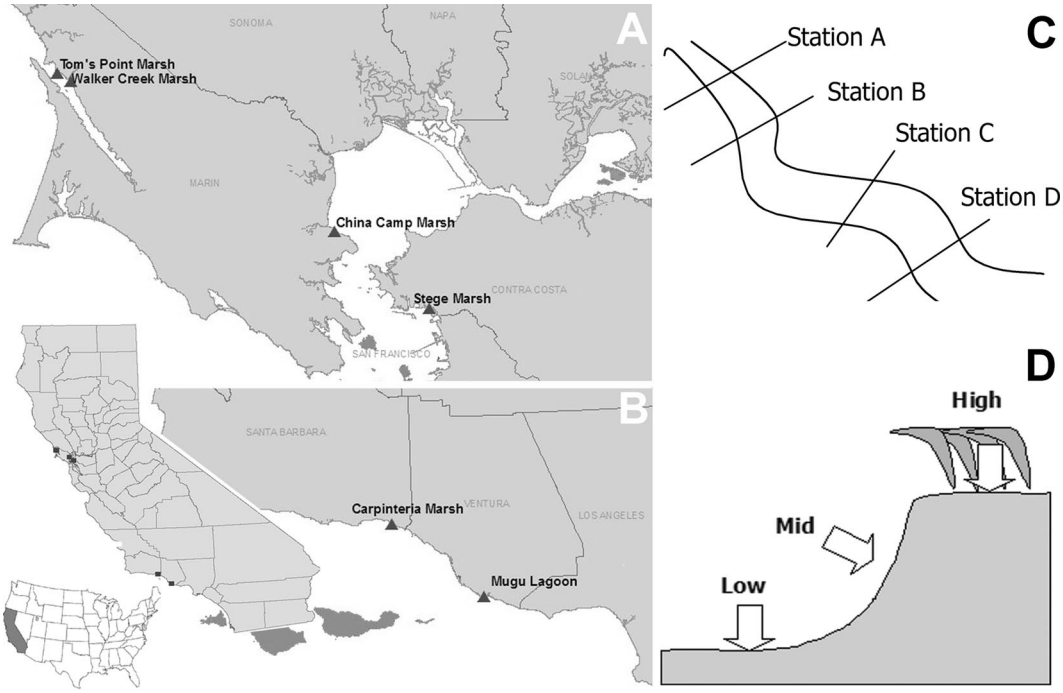

FIG. 1. Samples were collected from six salt marshes along the California coast, four in the north (A) and two in the south (B). Samples were collected along the channel (C) and at different depths (D). Table 1 shows the numbers of samples collected.

ing them relative to sodium concentrations. Overall trends in metal concentrations were not affected by this treatment.

PLFA analysis of sediments. The sediment microbial community composition was determined using PLFA analysis as described previously (5). Briefly, frozen (−80°C) sediment samples were freeze-dried, and 8 g of dry sediment was used for lipid extraction. Lipids were extracted with a one-phase chloroform-methanol-phosphate buffer solvent. Phospholipids were separated from nonpolar lipids and converted to fatty acid methyl esters before analysis. Quantification was performed using a Hewlett-Packard 6890 gas chromatograph equipped with a 25-m Ultra 2 (5% phenyl)-methylopolysiloxane column (J&W Scientific, Folsom, CA). PLFAs were identified using bacterial fatty acid standards and software from the Microbial Identification System (Microbial ID, Inc., Newark, DE). Fatty acids were designated by using the accepted convention (e.g., A:BωC).

PLFA biomass was estimated by adding the masses of all fatty acids detected (6, 17, 48) and was expressed in nanomoles of PLFA per gram (dry weight) of sediment. Biomass was averaged by location in the channel depth (i.e., high, mid, and low) for each marsh. The number of fatty acids detected was used as a proxy for diversity.

Certain PLFAs are unique biomarkers for particular functional groups of microorganisms, and other specific PLFAs or ratios of PLFAs are altered due to membrane changes in response to environmental or chemical stresses. The following biomarkers and ratios were used: 18:2ω6c,9c/bacterium ratio (18:2ω6,9c/i15:0, a15:0, 15:0, i16:0, 16:1ω5c, i17:0, a17:0, 17:0cy, 18:1ω7c, 19:0cy); actinomycetes (10Me PLFAs); sulfate reducers (sulfate-reducing bacteria) (br17:1 for *Desulfovibrio*, 10Me16:0 for *Desulfobacter*, and 17:1 for *Desulfobulbus*); eukaryotes (polyunsaturated PLFAs); gram-positive bacteria (branched PLFAs); 17cy/precursor (17:0cy/16:1ω7c); and monounsaturated PLFAs/saturated PLFAs.

TRFLP analysis. The method used for TRFLP analysis was adapted from the method of LaMontagne and Holden (29). Briefly, DNA was extracted from ~2-g sediment samples using an UltraClean soil DNA kit (MoBio, Inc., Solana Beach, CA) by following the manufacturer's protocol. The extracted DNA was then column purified, the molecular weight was determined by agarose gel electrophoresis, the quality was determined using A_{260}/A_{230}, and the quantity was determined by fluorometry. Genes encoding 16S rRNA were PCR amplified from purified samples using universal eubacterial primers 8F hex (fluorescently labeled forward primer; 5′AGAGTTTGATCCTGGCTCAG [32]) and 1389R (5′ACGGGCGGTGTGTACAAG [36]) as described previously (30). PCR products were purified with a commercially available kit (QIAGEN, Valencia, CA), and ca. 400 ng of purified DNA was digested with HhaI. After inactivation of the restriction enzyme by heating (65°C, 20 min), the lengths of fluorescently labeled fragments were determined with an Applied Biosystems Instruments (Foster City, CA) model 373A automated sequencer. Terminal restriction fragments (TRFs) were aligned and normalized to the sample with the lowest total fluorescence based on peak height, similar to the method of Dunbar et al. (13). TRFs with adjusted peak heights of <15 were discarded, as were peaks that were not present in three or more samples; the remaining peaks were summarized in matrix form. The matrix consisted of rows of samples and columns of TRFs (in base pairs), and the relative fluorescence abundance was the value in each cell. The relative abundance values were used to calculate a Shannon's diversity index.

Statistical analysis. Several multivariate statistics methods were used to explore microbial community fingerprints and correlations with environmental and pollutant variables. Correspondence analysis (CA) is an unconstrained ordination method that allows exploration of the variability in a data set related to community composition (e.g., PLFAs and TRFLPs) (10). Canonical correspondence analysis (CCA) is a direct constrained ordination method to explore the variability in a data set related to the environmental variables (e.g., latitude, elevation, etc.) and pollutant variables (e.g., metals and organic compounds) measured. Partial CCA (pCCA) was used to remove variability effects due to "background" variables (e.g., naturally occurring variables), and the remaining variability was assumed to be due to pollutants (31). The ability of PLFA and TRFLP analyses to discriminate the different microbial communities was evaluated by visually identifying clusters and comparing mean sample scores and percent explanation on the first two ordination axes.

Gradient analyses (CA, CCA, and pCCA) were carried out with the CANOCO software (Microcomputer Power, Ithaca, N.Y.). Fatty acid concentrations were expressed in nanomoles per gram (dry weight) of sediment for the analysis; TRF data were expressed as relative abundance. Alternatively, PLFA analysis and plotting were performed using a subset of 43 fatty acids to eliminate variation due to detection limits and errors associated with extraction and analysis of the samples. To determine the statistical significance of differences in microbial community composition between sites, ordination scores of samples were averaged by site on each axis, and the averages were compared using analysis of variance (ANOVA) and post hoc multiple comparisons. The same approach was used to compare average sample scores by elevation, latitude, longitude, etc., using unpaired *t* tests and ANOVAs in SAS, version 8 (SAS Institute, Cary, NC). Weighted ANOVAs were performed when means had unequal variances, as demonstrated by a significant Levene's test ($P < 0.05$). Correlations between PLFA biomass, diversity, and biomarkers for different contaminants and environmental variables were determined using linear regressions and approximate *t* values in constrained ordination plots (46). Pearson pairwise correlation coefficients were calculated for the metals in SAS (8). This allowed us to lump metal variables and reduce the complexity of the environmental variable matrix, and it facilitated interpretation of the ordination diagrams. Also, fatty acids were lumped into biomarker categories (e.g., branched, saturated, unsaturated, etc.) and added as supplementary variables to the ordination analysis. Supplementary variables did not change the ordination but were projected into the ordination space to facilitate interpretation of the results.

RESULTS

Sediment characterization. Both organic carbon and total nitrogen contents were higher in high areas than in mid and low areas (Fig. 2). To aid in interpretation of the results, covarying metals were lumped together when the paired Pearson's coefficient was greater than 0.75 and statistically significant ($P < 0.05$). The following pairs were identified: Al and Fe (Pearson's coefficient, 0.89), Pb and Zn (0.84), Zn and Cu (0.87), Ca and Sr (0.83), Co and Ni (0.97), Cr and Ni (0.92), Pb and Cu (0.90), Se and As (0.86), Co and Cr (0.89), Co and Li (0.92), and Cr and Li (0.87). Thus, the main covarying groups were Zn-Pb-Cu and Co-Cr-Li-Ni. The levels of the different metals differed from marsh to marsh. SM had the highest concentrations of As, Ca, Cu, Pb, Se, and Zn; CC (considered a relatively pristine site) had the highest concentrations of Cr, Ag, Al, Fe, Li, Co, Ni, and V. CM had the lowest overall concentrations of metals. The concentrations of Sr, Tl, Mg, and K did not differ significantly among marshes. SM also had the highest concentrations of all organic pollutants. PCBs were detected only in SM samples. Nonachlors, chlordanes, and pesticides were detected only in SM and CM samples. A more detailed description and a discussion of pollutant concentrations and sources in these marshes can be found in the study of Hwang et al. (23).

Effects of environmental variables on microbial community composition. (i) Year-to-year variation of microbial communities. The microbial community structure varied annually in SM and CM but not in WC (Fig. 3). The CA of PLFA explained up to 64% of the variation, while the TRFLP data explained only about 27% of the variation in the first two axes (Fig. 3). The microbial communities were significantly different in the 2002 and 2003 samples from SM ($P_x = 0.004$, $P_z = 0.046$) and CM ($P_y < 0.0001$) but not in the samples from WC. The TRFLP CA plots explained a smaller amount of the variation in the first and second axes (26.6% for SM and 27.5% for CM). Significant differences between years were observed for SM ($P_y = 0.012$) and CM ($P_x < 0.0001$) (Fig. 3).

(ii) Geographical distribution and small-scale spatial variation. Differences in the microbial community composition within a year for each marsh were evaluated using the 2003 data set. CA sample scores were averaged by marsh (SM, CC, CM, etc.) on each axis and used to test for differences between marshes. PLFA clustered communities into three groups (samples in each group were not significantly different from each other as determined by ANOVA; $P > 0.05$) on the first axis,

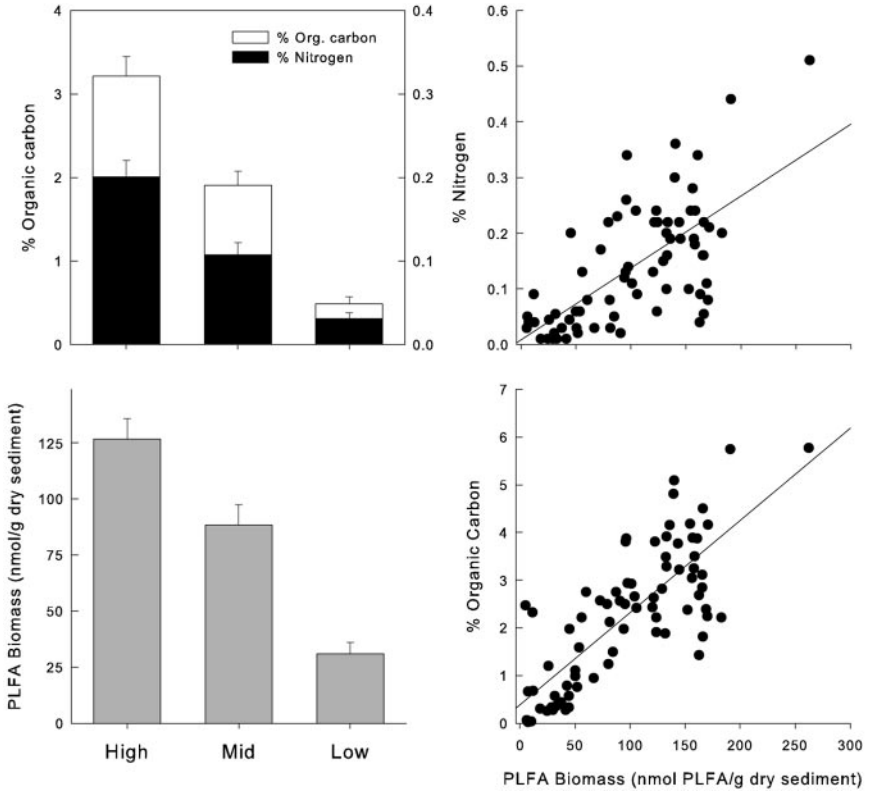

FIG. 2. Correlation of total biomass (PLFA) with organic carbon and total nitrogen contents in sediments.

one group formed by TP and WC ($P = 0.960$), a second group consisting of CM and CC ($P = 0.789$) (Fig. 4A), and a third group consisting of only SM samples. TRFLP analysis separated communities into two main groups (a group consisting of SM and CC and a group consisting of TP and WC), and SM and CC were significantly different along the second axis ($P < 0.0001$) (Fig. 4B). The average PLFA and TRFLP first-axis sample scores for the northern sites (SM, CC, TM, and WC) in 2003 were positively correlated with the latitudes of the marshes ($r^2 = 0.891$) (Fig. 4C). The within-marsh variation, which was potentially due to differences in tidal cycles or the proximity to pollutant sources, was evaluated by comparing CA scores for samples collected at different elevations of the channel. Samples from the high areas were significantly different from all other groups of samples in all marshes ($P < 0.0001$), as determined by post hoc multiple comparisons of means of the sample scores. Samples collected at the low and mid areas were not significantly different from each other ($P = 0.719$). The strongest determinants of community composition were elevation for PLFAs and year and marsh for TRFLPs. The first two axes of the PLFA CCA described 65.5% and 8.5% of the variation (Fig. 5A). The effects of the sampling year were insignificant compared to the effects of the other variables. Elevation had the greatest influence on sample distribution, as indicated by the greater lengths of the environmental variable arrows in the CCA plot (Fig. 5A). The effects of sampling location (marsh and latitude) were always smaller than the effects of elevation (high, mid, and low). Samples collected from the high areas had higher concentrations of 18:2ω6,9c,

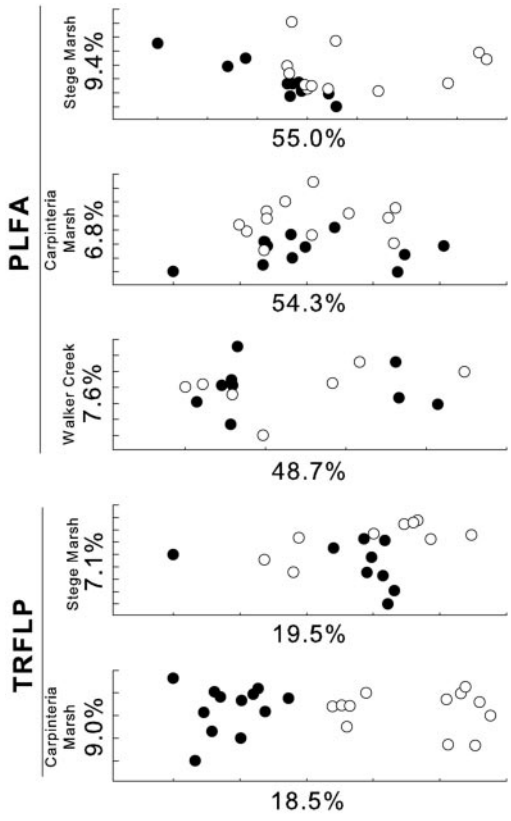

FIG. 3. Ordination plots of CA results, showing variation between 2002 samples (●) and 2003 samples (○) in PLFA and TRFLP fingerprints.

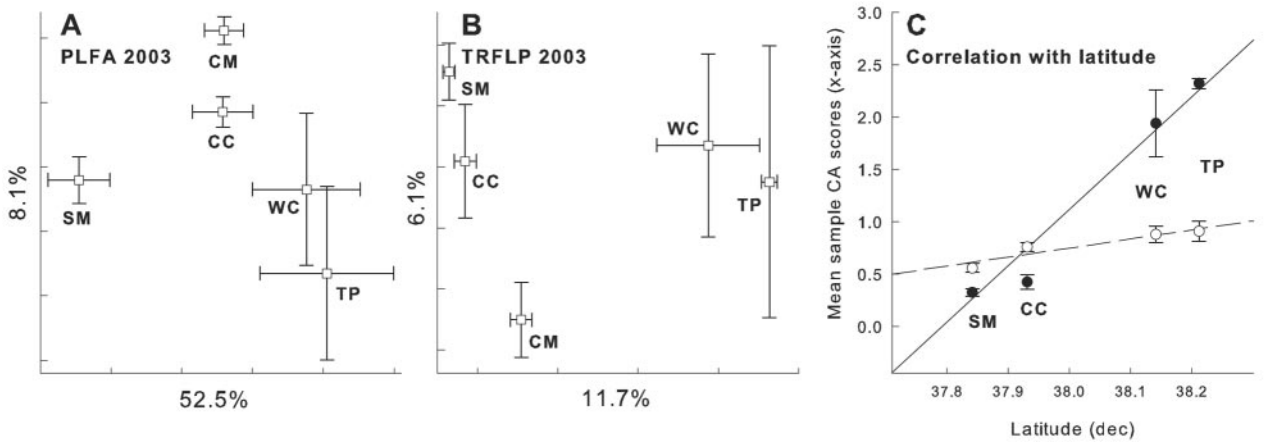

FIG. 4. Ordination plots of CA of PLFA (A) and TRFLP (B) fingerprints by marsh and correlation of CA scores to latitude (C). Only samples collected in 2003 were included. The data are means ± standard errors; the numbers of samples are shown in Table 1.

actinomycete biomarkers (10Me PLFAs), and polyunsaturated indicators of eukaryotes (20:2ω6,9c). Samples from the mid and low areas of the channel were enriched in unsaturated PLFAs, which are associated with gram-negative bacteria.

Based on TRFLP analysis, using the environmental variables described above, a CCA explained 34% and 13.3% of the variation on the first two axes, which was one-half of the variation described by the PLFA analysis (Fig. 5B). Samples were clearly separated into two groups, a group consisting of SM, CC, and CM and a group consisting of WC and TP. Latitude, marsh, and year had the strongest influence on sample distribution, whereas, unlike the PLFA data, elevation did not. When CCA plots for PLFAs and TRFLPs were compared, several similarities were found. In both cases TP and WC grouped closely together and in the direction opposite to SM.

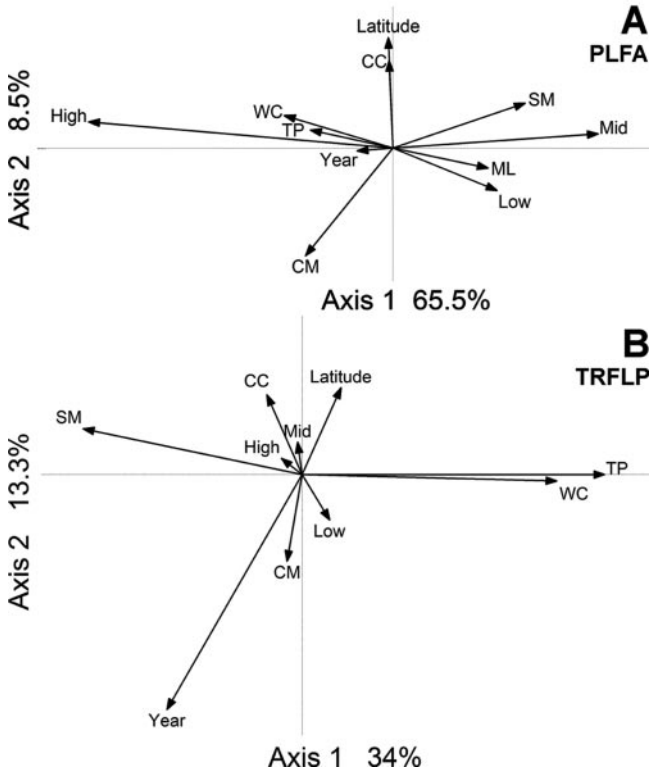

FIG. 5. Ordination plots of CCA results for PLFA (A) and TRFLP (B) fingerprints. Only variables describing spatial variation (marsh, elevation) and sampling year were included in this analysis. The direction of an arrow indicates the steepest increase in the variable, and the length indicates the strength relative to other variables.

(iii) PLFA biomass and diversity. Total biomass levels, as estimated from the total PLFA abundance, were consistently higher for the high-elevation samples, followed by the mid- and low-elevation samples (Fig. 6A and B). Total biomass levels were also positively correlated with organic carbon levels (r^2 = 0.628, $P < 0.0001$) and nitrogen content (r^2 = 0.481, $P < 0.0001$) in the sediments (Fig. 2). This trend was observed in all marshes and in both years. Although the difference between the means for high and mid elevations or for mid and low elevations was not significant in all cases, the difference between the means for high and low elevations was significant according to post hoc multiple comparisons ($P < 0.05$) in most cases.

Both seasonal variation and marsh variation were observed in diversity metrics. Both PLFA number and TRFLP Shannon diversity showed similar trends; the diversity was consistently higher in samples from the high area, whereas the lowest diversity was observed in samples from the low area (data not shown).

(iv) PLFA biomarkers. The ratios of monounsaturated PLFAs to saturated PLFAs were also consistently higher in samples from the unsaturated area in all marshes (Fig. 6C). 18:2ω6c,9c/bacterium ratios were two to three times higher in high areas than in low or mid areas for all marshes (Fig. 6D). No significant differences were detected in the other biomarkers analyzed (see Materials and Methods).

Effects of pollutant gradients on microbial community composition. The sum of all the metal concentrations in sediment samples was positively correlated with total PLFA biomass (r^2 = 0.50, $P < 0.0001$, n = 80). The number of fatty acids (PLFAs) detected was also positively correlated with the total metal concentration ($r^2 = 0.51, P < 0.0001, n = 80$). A multiple

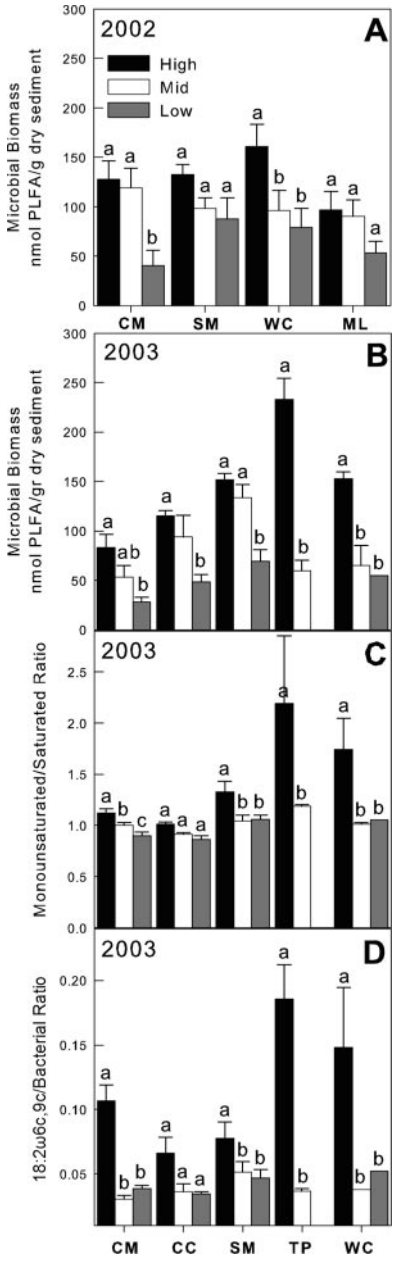

FIG. 6. (A and B) Total PLFA biomass at different elevations for sites sampled in 2002 (A) and 2003 (B). (C and D) PLFA biomarker ratios for monounsaturated and saturated fatty acids (C) and 18:2ω6c,9c and bacteria (D) in 2003. Different letters above bars for the same marsh indicate that the values are significantly different at a level of $P = 0.05$.

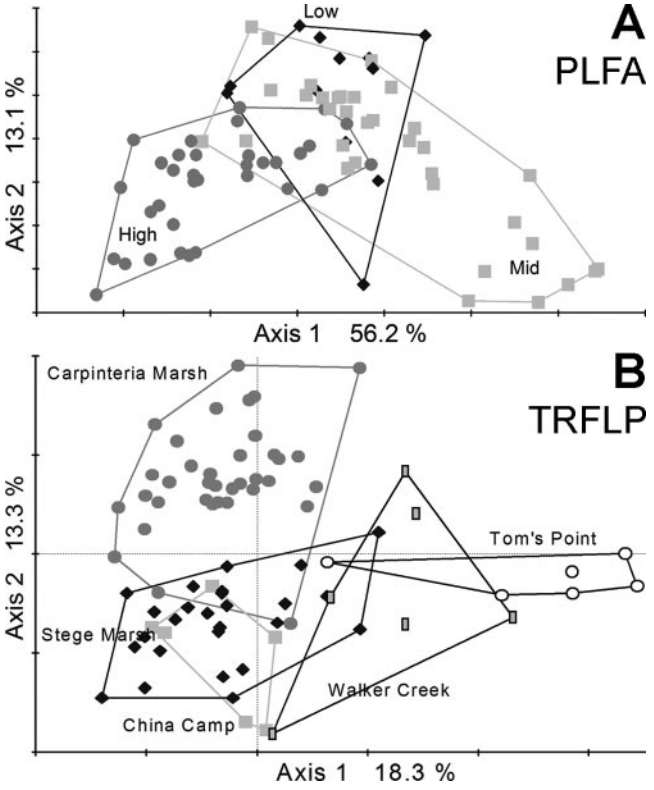

FIG. 7. Sample ordination plots of CCA results for PLFA (A) and TRFLP (B) fingerprints. The analysis included all variables, before pCCA. Polygons indicate sample groups.

regression analysis selected Al ($P < 0.0001$), Mg ($P < 0.0001$), and Cd ($P = 0.011$) as the most significant variables correlated with increased PLFA biomass ($r^2 = 0.44$).

Elevation (i.e., high, mid, and low) was the strongest determinant of microbial community composition based on a CCA analysis that included metal and organic pollutant concentrations as pollutant variables (Fig. 7). The CCA explained 63.9% of the variation on the first two axes. Dummy variables for elevation and marsh were included as covariables in a pCCA, effectively eliminating the variability associated with them. The remaining variation was assumed to be associated only with pollutants. The pCCA (Fig. 8) explained 29.1% and 18% of the variation on the first two axes. The automated forward selection procedure in CANOCO identified the 10 best variables (Al, As, Ca, Cd, Cs, Cu, Fe, Rb, U, and total PCBs) that significantly contributed to the pCCA model according to the Monte-Carlo permutation test. Total PCBs were detected only in SM, and they skewed the ordination toward SM samples. Thus, PCBs were dropped from the analysis, and the pCCA was repeated. The automated forward selection procedure in the pCCA without PCBs identified As, Ca, Cs, Cs, Cu, Pb, Rb, U, *p,p′*-DDD, and pesticides (lumped variable) as the best variables describing the microbial community PLFA fingerprint. Branched (including iso- and anteiso-substituted) fatty acids were positively correlated with U and negatively correlated with *p,p′*-DDD, Pb and Cd. Unsaturated fatty acids were positively correlated with Cd. Monounsaturated fatty acids were negatively correlated with U and Cu. Cyclo- and methyl-substituted fatty acids were positively correlated with Cu and negatively correlated with Cd. Additionally, cyclo-substituted fatty acids were also negatively correlated with Pb and pesticides (Table 2).

The same analysis procedure was used for the TRFLP microbial fingerprints. Use of the pCCA procedure with spatial dummy variables as covariables effectively eliminated differences due to marsh and elevation (Fig. 7B), so the residual variation was assumed to be due to pollutants. Each metal or

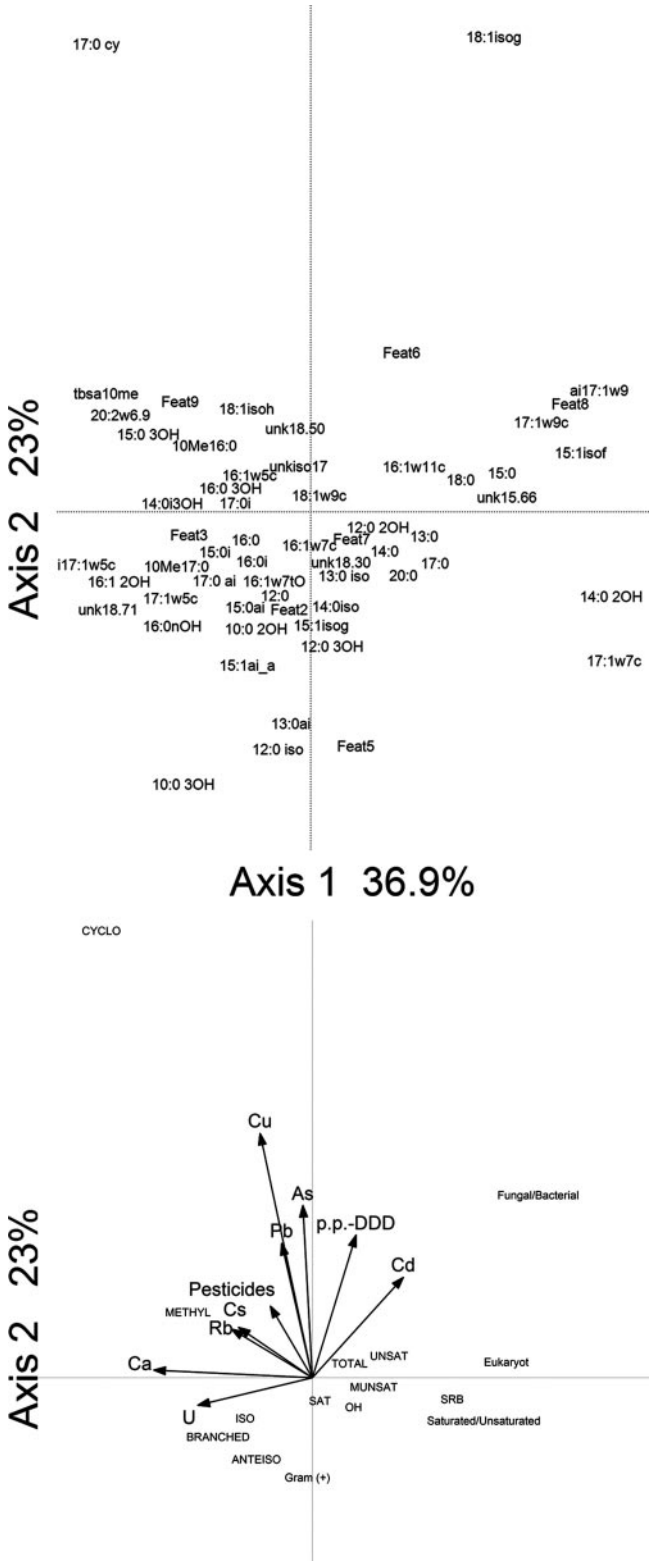

FIG. 8. Ordination plots of pCCA results for PLFA fingerprints. Elevation, latitude, and year were used as covariables. The arrows represent the best 10 variables by automatic forward selection in CANOCO. Fatty acid groups (branched, saturated, cyclo, etc.) were included as supplementary variables and are not determinants of the sample ordination. UNSAT, unsaturated fatty acids; MUNSAT, monounsaturated fatty acids; SAT, saturated fatty acids; Eukaryot, eukaryote; SRB, sulfate-reducing bacteria.

TABLE 2. Significant correlations between PLFA biomarker groups and pollutants detected in sediment samples, as observed in ordination plots (Van Dobeen circles) or determined by multiple or linear regression

Biomarker group	Pollutant variable(s)	Direction	*PI*
Total PLFA	% Organic carbon	+	<0.0001
Biomass	% Total nitrogen	+	<0.0001
	Total metals	+	<0.0001
	Al	+	<0.0001
	Mg	+	<0.0001
	Cd	+	0.011
Branched fatty acids[a]	U	+	
	p,p′-DDD, Cd, Pb	−	
Unsaturated fatty acids	Cd	+	
Monounsaturated fatty acids[b]	U, Cd	−	
Cyclo-substituted fatty acids	Pesticides, Cd, U	−	
	Cu	+	
Methyl-substituted fatty acids	Cu	+	

[a] Fatty acids indicative of gram-positive bacteria.
[b] Fatty acids indicative of gram-negative bacteria.

organic pollutant variable was tested for significant correlation with TRFLP data using the Monte-Carlo permutation test, and only Se ($P = 0.038$), Cu ($P = 0.042$), and As ($P = 0.050$) were statistically significant variables. The automated forward selection procedure in CANOCO selected 10 metals (Se, Cu, As, Zn, Cs, Ag, Mn, Li, U, and Cr) as the best variables describing differences in the model. No organic pollutants were identified by the manual single-selection approach or automated forward selection. The combination of these variables explained 11.1% and 8.3% of the variation on the first and second axes, respectively (data not shown).

PLFA analysis versus TRFLP analysis. The initial CCA analysis indicated that PLFA fingerprints were capable of explaining up to 74% (first two axes) of the variation in the data set associated with environmental variables (elevation, marsh location, latitude, year, etc.). TRFLP data explained 47.3% of the variation on the first two axes in the same analysis (Fig. 5). The residual variation for both fingerprinting methods was determined by including all the environmental variables as covariables in a pCCA. The difference between total CCA variation and pCCA variation explained was a measure of how much variation was associated with the environmental variables. Based on this analysis, both PLFAs and TRFLPs explained the same amount of variation (approximately 35%) associated with elevation, geographic location, and year.

DISCUSSION

Both environmental and pollutant variables had strong effects on the sediment microbial community composition in all marshes. The inevitable interactions of these two categories of variables, however, pose a challenge for statistical analysis and interpretation of microbial community fingerprints and stress biomarkers and make it difficult to determine how much contaminants change microbial communities. Thus, a stepwise or layer-by-layer approach was used to remove more dominant variable effects with partial ordination methods, which allowed

smaller effects, in this case the effects of the pollutants, to become evident while an acceptable level of the variability in the data set was still explained.

In comparisons of microbial communities across marshes, TP and WC were more similar to one another than to other marshes, as determined by both PLFA and TRFLP analyses. TP and WC share two characteristics: both are influenced by tributaries draining into the Tomales Bay watershed and have higher concentrations of mercury than the other marshes have. This is an example of how geographic location and pollution effects cannot be clearly separated. The CA plots of both PLFA and TRFLP data showed that SM was distinct from the other marshes. SM is unique among the marshes studied in having the highest concentrations of organic pollutants and the metals Zn, Pb, As, Cu, and Cd. Although it is tempting to conclude that the high degree of contamination contributed to differences in the communities, this hypothesis cannot be tested in the field, where several factors vary concurrently. Controlled contaminant exposure experiments in microcosms could test this hypothesis.

Elevation directly controls many sediment characteristics that strongly influence microbial community structure (16). Tidal cycles create distinct habitats within marsh creeks in saturated, intermittently flooded, and unsaturated zones. Unsaturated areas, which are vegetated, are expected to have higher levels of available oxygen (due to plant-driven gas exchange) and inputs of carbon and other nutrients from living root exudates, while saturated areas are expected to be anaerobic and to have lower redox levels, with carbon and other nutrients derived largely from detritus. Based on these characteristics, hypotheses can be formulated regarding the presence and abundance of certain fatty acid biomarkers. For example, a higher level of 18:2ω6c,9c biomarkers in a higher unsaturated area can be attributed to an environment that is more aerobic than the environments in saturated zones (low). These fatty acids are often attributed to fungi but may also originate from plants. These fatty acids, along with 18:2ω3c, accounted for more than 60% of the fatty acids in axenic *Spartina* roots (33), a plant that is abundant in the unsaturated zones in all of the marshes sampled. Franklin et al. (16) found significant small-scale differences due to tidal cycles in east coast salt marshes comparable to the marshes selected for this study. Microbial communities in this study were fingerprinted using a DNA-based method, and geostatistical analyses identified spatial patterns in microbial communities in salt marsh sediments. The variation in the composition of the communities was greater along the vertical axis (i.e., elevation) than at different horizontal locations at the same elevation in a creek.

Certain PLFA biomarkers reflect the physiological status of microorganisms because microbes respond to various stresses by modifying cell membrane fluidity through increasing the branching and degree of saturation (monounsaturated versus polyunsaturated) of membrane lipids. Changes in these biomarkers have been measured in order to investigate the responses of microbial communities to stress (35, 37, 49). Increased levels of saturated straight-chain and branched PLFAs were observed in response to flooding of previously aerobic soils in controlled microcosm studies (5). This report is consistent with our results obtained in the field, which showed that samples from the saturated areas of the tidal channels had lower ratios of monounsaturated PLFAs to saturated PLFAs.

Many studies, primarily studies of soils, have demonstrated the effects of heavy metal pollution on soil microbial communities using PLFA analysis (1, 2, 12, 18). In our study, branched, unsaturated, and monounsaturated fatty acids showed the strongest association with high concentrations of heavy metals (Table 2), and these fatty acids warrant further exploration as potential biomarkers of metal pollution in salt marsh sediment. Metals appeared to have greater effects than organic pollutants on microbial community composition in our study; however, with the cooccurrence of organic and metallic wastes, the potential for synergistic effects must be considered when biomarkers of stress are interpreted.

The decreases in levels of branched fatty acids with higher concentrations of Pb and Cd are similar to the decreases reported in other studies (18, 41). In a study of 14 different soil samples exposed to metals and hydrocarbons (41), branched fatty acids were less abundant in the samples with higher concentrations of Pb and Cr, and a higher level of 18:2ω6, interpreted in this case to be a fungal biomarker, was associated with more contaminated soils (elevated levels of petroleum hydrocarbons, Cr, and Pb). Previous studies have also reported that there were increases in cyclo- and methyl-substituted fatty acids in soils amended with metals (Cd, Cu, Ni, Pb, and Zn) (18).

Fungal biomarkers were not strongly associated with any pollutant gradients in our study, in contrast to what has been reported for many soils (18, 25, 27, 38). The absence of measurable responses may reflect the fact that fungi made up only a minor part of the PLFA biomass, making differences difficult to detect, or the fact that the origin of most of the 18:2ω6c in these samples was plants.

PLFA and TRFLP analyses revealed differences in the sensitivity of microbial communities to environmental and pollutant parameters. PLFAs represent only the viable members of a community because phospholipids are rapidly dephosphorylated and thus no longer detectable when the associated organisms die (21). TRFLP analysis, on the other hand, targets DNA sequences, and these molecules persist longer than PLFAs in the environment, even when the associated organisms are no longer viable (7, 24). Another difference is the range of taxonomic groups potentially captured by each method; whereas PLFA analysis can detect bacteria, fungi, and eukaryote fatty acids, the TRFLP method, using universal bacterial primers, can detect only bacterial DNA. Of the parameters measured, the PLFA and TRFLP profiles were most strongly related to elevation (e.g., high versus medium or low) and marsh or year, respectively.

Among the environmental conditions associated with different elevations are, perhaps most importantly, differences in redox conditions, carbon and nutrient availability, and vegetation. For PLFAs, the types of microbial communities associated with different elevations had much in common across all marshes. Some of the fatty acids that determined these differences were, at the highest elevation, fungal or plant-derived (18:2ω6c,9c) and actinomycete (10Me18:0) biomarkers. Both of these biomarkers are more common in unsaturated soils than in flooded soils (5, 11). The mid and low elevations were enriched with biomarkers for two groups of sulfate-reducing

bacteria (17:1ω7c, ai17:1ω9) (34) and gram-positive bacteria (saturated and terminally branched fatty acids). These differences were not reflected in the TRFLP patterns, in part because the contribution of eukaryotes to the community was not captured and possibly because intermittent inundation of the higher-elevation areas with water may have mixed communities in the different habitats. Persistence of DNA from organisms selected by previous and current environmental conditions would obfuscate detection of only the currently active organisms.

In conclusion, microbial communities in salt marsh sediments respond to naturally occurring environmental cycles by changing composition (as reflected by PLFAs and TRFLPs) and the physiology of specific members of the communities (shown by PLFAs but not by TRFLPs). The nature and behavior of the molecules targeted by the two methods, DNA and fatty acids, are a factor that should be considered when microbial communities are interpreted and compared. The combination of different fingerprinting methods and statistical analyses provided different perspectives for the microbial community. Both fingerprinting methods indicated that As, Cu, Cs, and U had significant effects, while branched, unsaturated, monounsaturated, and cyclo- and methyl-substituted fatty acids had significant correlations with several metals, particularly Cd and Cu. However, none of these pollutants had stronger effects than geographic and tide-associated variables.

ACKNOWLEDGMENTS

This research was supported in part by the CONACyT and UC-Mexus programs and by the UC Toxic Substances Research and Teaching Program. Additional support was provided by a grant from the U.S. Environmental Protection Agency's Science to Achieve Results Estuarine and Great Lakes Coastal Initiative through funding to the Pacific Estuarine Ecosystem Indicator Research Consortium (U.S. EPA agreement EPA/R-82867601) and by grant 5 P42 ES04699 from the National Institute of Environmental Health Sciences, NIH.

The contents of this paper are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NIH.

We thank Rebecca Drenovsky for her useful comments and advice regarding statistical analysis, Kevin Feris for insightful discussions, and the undergraduate staff of Kate Scow's lab for their skills in PLFA analysis. Jane Choe, James Yi, and Dan Brooks contributed to the TRFLP analyses at UCSB.

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