

Relationships between Sediment Microbial Communities and Pollutants in Two California Salt Marshes

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Abstract

Salt marshes are important ecosystems whose plant and microbial communities can alter terrestrially derived pollutants prior to coastal water discharge. However, knowledge regarding relationships between anthropogenic pollutant levels and salt marsh microbial communities is limited, and salt marshes on the West Coast of the United States are rarely examined. In this study, we investigated the relationships between microbial community composition and 24 pollutants (20 metals and 4 organics) in two California salt marshes. Multivariate ordination techniques were used to assess how bacterial community composition, as determined by terminal restriction fragment length polymorphism and phospholipid fatty acid analyses, was related to pollution. Sea urchin embryo toxicity measurements and plant tissue metabolite profiles were considered two other biometrics of pollution. Spatial effects were strongly manifested across marshes and across channel elevations within marshes. Utilizing partial canonical correspondence analysis, an ordination technique new to microbial ecology, we found that several metals were strongly associated with microbial community composition after accounting for spatial effects. The major patterns in plant metabolite profiles were consistent with patterns across microbial community profiles, but sea urchin embryo assays, which are commonly used to evaluate

ecological toxicity, had no identifiable relationships with pollution. Whereas salt marshes are generally dynamic and complex habitats, microbial communities in these marshes appear to be relatively sensitive indicators of toxic pollutants.

Introduction

Salt marshes are among the most productive ecosystems, and they harbor a large variety of fish, birds, and wildlife species [78]. Salt marshes are frequently the last barriers between the coastal ocean and uplands, which are often heavily populated and developed [50]. Because they function as “buffer zones” by intercepting, stabilizing, and removing pollutants [73, 74, 78] and excessive nutrients [28], salt marshes are critical to maintaining healthy coastal ecosystems, which are, in turn, critical to human health [49]. Microbial communities in marsh sediments are particularly important because they are the major players in biogeochemical cycling, which is fundamental to the productivity, “buffering” capacity, and functional stability of salt marshes [78]. Therefore, an increased understanding of salt marsh microbial communities and their relationships with pollutants is of general interest; such understanding could also inform pollution management strategies in coastal regions.

Some relationships between soil microbial communities and either metal [48, 58, 59] or organic [39, 43] pollutants have been established. For example, in chemically polluted soils, microbial diversity is reduced, and community structure is shifted in comparison to

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unpolluted soils (for reviews, see [26, 33]). Increasing evidence shows that microorganisms are far more sensitive to heavy-metal stress than are animals or plants, with exposure resulting in loss of microbial functions [26]. Pollutant effects on salt marsh microbial communities in sediment mesocosms have received some attention [1, 67], but comparatively much less than for soils. This probably follows from the fact that, although salt marsh microbial communities have been studied generally [4, 6, 7, 22, 31, 42], they have received overall less attention than communities in soil (for reviews, see [66, 70, 94]). Apparently, a field-scale investigation of multiple pollutant effects on salt marsh microbial communities has been lacking.

The purpose of this study was to determine if salt marsh sediment microbial communities were related to the pollutants in their habitats. We examined relationships between sediment microbial communities and 24 pollutants and assessed comparative relationships to plant tissue metabolite profiles and sea urchin toxicity. Two geographically distinct, human-impacted California salt marshes were investigated at different seasons for microbial community composition and pollutant abundance. The abundances of 20 metals and 4 organics were quantified, and correlations were made to bacterial community composition after accounting for spatial variation via partial canonical correspondence analysis (pCCA). The community composition was determined by terminal restriction fragment length polymorphism (TRFLP) [13, 40, 53] and phospholipid fatty acid (PLFA) [93, 95] analyses. We hypothesized that the correlations between pollutant concentrations and microbial community composition were significant and discernable despite the dynamic and complex hydrologic and geochemical conditions in salt marshes

that also act as selective pressures on sediment microbial communities.

Materials and Methods

Site Description. The two California marshes studied were the Carpinteria Salt Marsh, a 93-ha estuarine wetland located 12 km east of Santa Barbara (34°24'N, 119°31'W), and Stege Marsh, a 100-ha wetland in Richmond (37°56'N, 122°21'W). Although these marshes are in southern and northern California, respectively, the sites are climatically similar. Carpinteria Salt Marsh is a geomorphologically mature system that consists of creek channels and tidal flats (8–13% of area). The regularly flooded tidal marsh is vegetated mainly by *Salicornia virginica* (50–60%) and by mixed species associations [55]. The climate is semiarid Mediterranean, which is typical of southern California with annual average temperatures ranging from 10.2 to 21.7°C and an annual average rainfall of 44.7 cm (Desert Research Institute, Western Regional Climate Center, <http://www.wrcc.dri.edu/index.html>). Four stations were sampled along a selected creek channel (Fig. 1) located in the west side of the marsh. This channel receives outflow from a creek that drains a small watershed, 72% of which is zoned for agriculture [55], and is known to have elevated levels of nitrate [54]. Across stations A–D (Fig. 1), dissolved nitrate concentrations in the water column ranged from 1800 to 72 μM at the time of sampling.

Stege Marsh was converted from a former mudflat to a tidal salt marsh by dumping cinder pyrite combined with sedimentation and creation of jetties. Stege Marsh is vegetated dominantly by *S. virginica* and *Spartina foliosa*. The annual average temperature of Richmond, CA,

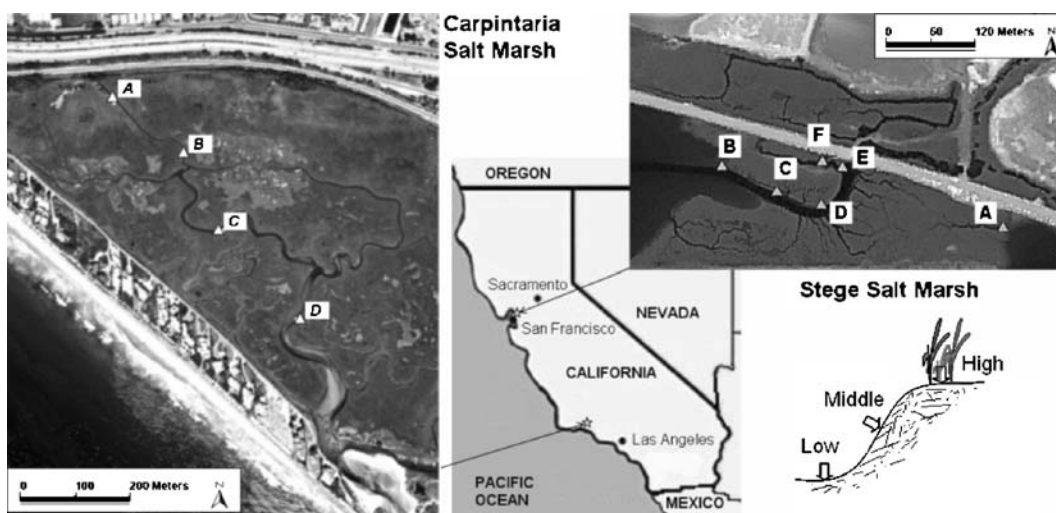


Figure 1. Map of the sampling stations and diagram of sampling elevations in Carpinteria Salt Marsh and Stege Marsh.

ranges between 10.1 and 19.3°C, and the annual average rainfall is 58.7 cm (Desert Research Institute, Western Regional Climate Center, <http://www.wrcc.dri.edu/index.html>). Stege Marsh is bordered by an upland prairie and a residential area on the west boundary and has three freshwater inputs [10]. The marsh has a long and complex history of pollutant loading [86], including by-products from a mercury fulminate facility, explosives, and other industrial chemical wastes [10]. The marsh was declared a “toxic hot spot” by the San Francisco Regional Water Quality Control Board in 1998 [45]. A paved road separates the marsh into uplands and near-ocean regions. Six stations were sampled to the southwest of the road (Fig. 1).

Sample Collection. Twenty-eight samples were collected from 10 stations in the two salt marshes during 2002. Six stations in Stege Marsh and four stations in Carpinteria Salt Marsh were sampled on February 23rd and July 2nd, 2002, respectively (Fig. 1). At each station, sediment samples were taken from three elevations (Fig. 1): high (H, marsh edge, usually vegetated); middle (M, creek bank), and low (L, creek channel); plants were sampled from the high elevations where they occurred. Each elevation at each station was defined as one sampling site. Three-letter sample codes were used to identify samples where the first letter indicates the marsh (S: Stege, C: Carpinteria), the second letter indicates the station (A through D for Carpinteria, A through F for Stege), and the third letter indicates the elevation (H, M, or L). For station A in Stege, only one elevation was sampled, and its distinction as either high, middle, or low elevation was not made; the sample was thus labeled as SAN. The use of “high, middle, and low” to describe marsh elevation, although not consistent with traditional marsh ecology nomenclature, was used here to provide clear and convenient coding and presentation.

Sediment Sampling. Sediment samples were collected for both microbial community analysis and eluate chemical analysis. For each purpose, 10–12 individual sediment cores were collected within a 1-m-diameter area in each sampling site, and then the surface sediments (0–2.5 cm) were dispensed into clean sterile Mason® or Qorpak jars (Fisher Scientific, Tustin, CA) and homogenized by stirring. The sediment samples for microbial community analysis were collected by gently pushing a piston corer, modified from a 60-mL disposable polypropylene syringe (B-D, Franklin Lakes, NJ), into the sediment while maintaining minimal pressure to avoid overcompaction. The homogenized sediment was split into two portions for TRFLP and PLFA. The surface sediments used in eluate chemical analysis and for sea urchin embryo toxicity measurements were sampled using an all-Teflon piston corer (inner diameter 25 mm) (“mucksucker,” Cole-

Parmer, Vernon Hills, IL). All sediment samples were kept on wet ice during transport to the laboratory. Samples for microbial community studies were frozen until analysis; samples for eluate analysis were refrigerated for ≤ 12 h prior to eluate recovery.

Plant Sampling. Shoots and roots were sampled on-site from one dominant plant species of the marshes *S. virginica*. Samples were cleared of mud, salt, and other debris by rinsing extensively in filtered (0.45 μm) half-strength seawater followed by deionized water. The samples were then blotted dry and flash-frozen in liquid N_2 . After transport on dry ice to the laboratory, the root tissues were lyophilized directly before pulverization to $\leq 5\text{-}\mu\text{m}$ particles using a Micro Dismembrator II (Braun Scientific, Melsungen, Germany) [15], whereas the shoot tissues were ground in liquid N_2 before lyophilization.

Sediment Eluate Analysis. Eluates were recovered from as many sediment samples as possible, although the amount of recoverable pore water was highly variable across samples, and, in some cases, there was no recoverable pore water. Eluate analyses were designed for rapid, semiquantitative screening of concentrations of selected pollutants. To recover eluates, 100 g of homogenized sediment was weighed into Teflon (Nalgene) centrifuge bottles, and 100 mL of 0.45- μm filtered seawater (FSW; Nalgene) was added to the bottles. Samples were shaken (2 h, 4°C) and then centrifuged (2500 \times g, 30 min, 4°C). Eluted waters were collected and fractionated as follows: (1) 25 mL water frozen (-20°C) in clean Qorpak jars for chemical analysis; (2) 25 mL in acid-washed beakers for ammonia and sulfide analysis (to correct sea urchin embryo assay results); and (3) 50 mL for the sea urchin embryo bioassay (see below). The remaining unprocessed sediments as well as centrifuged sediments (after elution) were frozen (-20°C).

Sea Urchin Embryo Bioassay. Sea urchin (*Lytechinus anemesis*) embryo bioassays of eluate water were conducted as in Pillai *et al.* [60]. Eluate pH was measured and adjusted to approximately 7.5. Eluate ammonia and sulfides were assayed using an Accumet ammonia-ion-selective electrode (Fisher Scientific, LLC, Pittsburgh, PA) and a silver-sulfide electrode (model 9616; Orion, Inc., Beverly, MA) respectively. Adult urchins were spawned in 0.5 M KCl, and eggs from three females exhibiting at least a 90% fertilization rate were pooled, washed three times in FSW (0.45 μm), then fertilized with ca. 10^5 normal, motile sperm. Assays were performed in triplicates, using different females in each replication. Once 90–95% fertilization was achieved, the eggs were washed (3 \times , FSW), and 50 embryos/mL were incubated (17°C) with 10-mL fresh eluate or FSW (control) until the control embryos reached the pluteus

stage. The embryos were fixed in 5% glutaraldehyde and examined for developmental abnormality using an inverted compound microscope [72]. Differences in the percentage of normal plutei between samples and controls were calculated as the toxicity measures. Results from triplicates were averaged. None of the samples had ammonia and sulfides in ranges that could affect embryo development. Sea urchin embryo toxicity measurements were regarded as aggregate chemical measures and as bioassays to infer chemical effects on a selected invertebrate.

Chemical Analysis. All eluates were analyzed for selected chemical pollutants. To extract organic contaminants, a 75- μm CarboxenTM/polydimethylsiloxane solid-phase microextraction fiber device (SPME, Supelco, Inc., Bellefonte, PA) was exposed to eluate in a vapor-tight glass vial with a Teflon-coated septum for 30 min at room temperature while shaking; this procedure generally follows the manufacturer's recommendation for analysis of volatile organic compounds in water and paralleled our earlier methods [68]. For recovery studies, we used standards containing 0.01, 0.1, 1, and 10 ppm trichloromethane (CHCl_3), trichloroethylene (TCE), and dibutyl phthalate (DBP; all from ChemService, West Chester, PA) diluted into the seawater used for eluate generation. Immediately following extraction, the SPME device was inserted into the injector of a gas chromatograph-mass spectrometer system (GC-MS) consisting of an Agilent 5890 series II GC interfaced to a 5971A mass-selective detector, using electron ionization (EI-MS) at 70 eV, scanned from 46 to 400 mass units, with two full scan spectra per second averaged into one spectrum per second. The column was a 0.15 mm \times 50 m "DB-5 equivalent" (5% phenylmethyl silphenylene siloxane copolymer) coated to 0.4 μm thick (BPX-5 column, SGE, Inc., Austin, TX). The temperature regime began at 40°C with a 4-min hold and then a 10°C min^{-1} rise until reaching 300°C. Helium at 40 cm s^{-1} velocity was used as a carrier gas; the injector temperature was 280°C, and the transfer line was at 300°C. Peaks from GC-MS runs were assigned tentative identities (except for the standards, which were unequivocally identified) based on mass spectral similarity scores with spectra from a 108,000-entry NIH/EPA/NIST mass spectral library, using the search algorithm of the instrument software. Total phthalates were estimated based on peak areas of the m/z 149 ion, which is a mass spectral fragment ion common to most phthalates; the molar response factor for the m/z 149 ion from DBP was used for this estimation. Recovery of all standards at all concentrations was >95%. Concentrations of total phthalates and CHCl_3 were calculated as relative percentages, assuming concentrations in sample CDL were 100%. Concentrations of TCE and DBP were expressed in ppb.

For the metals, eluates were stored in the dark at 4°C until dilution and analysis. An aliquot of each eluate (250 μL) was diluted 20-fold in 1% nitric acid (Trace Metal grade, Fisher Scientific, Pittsburgh, PA) in a new polypropylene centrifuge tube. A blank tube was measured as a control, confirming negligible contamination. Metals quantification was by direct aspiration in an inductively coupled plasma-mass spectrometer (model 7500i, Agilent, Palo Alto, CA) and compared to external standards covering a range from 0.01 to 200 $\mu\text{g L}^{-1}$ for minor elements and up to 100 mg L^{-1} for major elements. Analysis was performed as prescribed by EPA method 6020 [84]. The instrument was operated with very low molecular ions—a robust plasma characterized by 0.4% CeO/Ce.

Microbial Community Analysis

Terminal Restriction Fragment Length Polymorphism. Terminal restriction fragment length polymorphism analysis was conducted similarly to LaMontagne and Holden [36, 37]. DNA was extracted from 2-g sediment samples using the UltraClean Soil DNA kit (MoBio Laboratory, Inc., Solana Beach, CA) following the manufacturer's protocol. Extracted DNA was size-exclusion-column purified, checked for quality by assessing the A260/A230 ratio, and quantified by SYBR Green fluorometry. Genes encoding 16S rRNA were amplified by polymerase chain reaction (PCR) from purified community DNA using universal eubacterial primers 8F hex (fluorescently labeled forward primer; 5'-AGAGTTTGTCTGGCTCAG [40]) and 1389R (5'-ACGGGCGGTGTGTACAAG [51]). PCR products were purified and digested with *Hha*I. The restriction enzyme was inactivated by heating (65°C, 15 min), and the lengths of fluorescently labeled terminal restriction fragments (TRFs, corresponding to peaks in the TRFLP electropherogram) were determined with an Applied Biosystems Instruments (Foster City, CA) Model 373A automated sequencer. TRFs were aligned and normalized similarly to Dunbar *et al.* [12] and were identified by their fragment length in base pairs. The abundance of each TRF was normalized to the sample with the lowest total TRF abundance, and TRFs below a relative abundance of 1% (based on the lowest total TRF abundance) were discarded. Both the presence/absence and relative abundance of TRFs were considered in data analysis.

Phospholipid Fatty Acid Analysis. Phospholipid fatty acid analysis was performed as per Bossio and Scow [5]. Samples were lyophilized, and 8-g dry sediment was extracted using a chloroform/methanol/phosphate buffer solution. Extracted fatty acids (FAs) were analyzed using a Hewlett-Packard 6890 GC. Peaks were identified using bacterial FA standards and MIDI peak identification

software (MIDI, Inc., Newark, DE). A standard FA nomenclature [93], e.g., A:B ω C, was adopted to report abundances, whereby A is the total number of carbon molecules, B is the number of double bonds, and ω C indicates the position of the double bond from the methyl end and conformation; *cis* and *trans* geometry are indicated by the suffixes c and t. The prefixes a and i refer to anteiso- and iso-branching, respectively; 10Me indicates a methyl group on the 10th carbon atom from the carboxyl end of the molecule; positions of hydroxyl (OH) groups are noted; and “cy” indicates cyclopropane FAs. Unidentified FAs were noted by the prefix “uk.” The suffix “tsba” indicates a derivative of that particular FA. A peak that includes several FAs not resolved by GC was considered a sum feature, denoted by SumD, where D is a numeric number indicating the Dth observed such feature. Analysis of the PLFA data included adding the relative abundances of specific groups of FAs together (e.g., the sum of all unsaturated FAs) and calculating ratios between specific FAs (e.g., cy17:0/16:1 ω 7c). The summed mole mass of all FAs (nanomoles of PLFA per gram of dry sediment) was regarded as a metric for microbial biomass [94]. PLFA community composition was expressed in percentages (mole percentages of individual PLFAs out of the total of all PLFAs).

Plant Tissue Metabolite Analysis. Fifty to sixty milligrams of the dry, pulverized tissue powder was extracted twice with ice-cold 10% trichloroacetic acid (TCA; w/v 40/1), as described previously [16]. TCA was removed by lyophilization, and the residue was redissolved in purified (18 M Ω) water. An aliquot of the extract was lyophilized and derivatized in the silylating agent MTBSTFA (*N*-methyl-*N*-[*tert*-butyldimethylsilyl]-trifluoroacetamide) before GC-MS analysis [16]. Metabolites were identified based on the GC retention time and mass fragmentation pattern. Quantification of metabolites was performed by comparing the peak intensity of selected mass ions for samples to those of the standards.

Statistical Analyses. All data were analyzed using either the CANOCO software (Microcomputer Power, Inc., Ithaca, NY) or S-plus 6.1 (Insightful Corp., Seattle, WA). Regression analysis was conducted in S-plus to determine associations between sea urchin embryo toxicity measurements and pollutant concentrations via the method of stepwise model selection [87].

Several multivariate statistical techniques were utilized to investigate variations in microbial community profiles and their correlation to the environmental variables. In this article, environmental variables refer to all variables except those of the microbial community and the plant metabolite profile. An indirect gradient analysis technique, detrended correspondence analysis (DCA), was used to characterize overall variation in TRFLP,

PLFA, and plant metabolite profiles [81, 82]. For both the TRFLP and the PLFA data, the distributions of TRFs and FAs were checked for skewness using scatter plots and histograms in S-plus; no need for data transformation was indicated. Indirect gradient analysis was also conducted to characterize overall differences in pollutant profiles, the major patterns of which, represented by ordination scores based on metal and organic concentrations, were regressed against sea urchin embryo toxicity measures to identify potential relationships. A direct gradient analysis technique, canonical correspondence analysis (CCA) [57, 80], was used to directly assess the relationships between microbial community profiles and environmental variables (i.e., marsh, elevation, and pollutants). Partial CCA (pCCA) [82] was used to isolate the effects of pollutants while acknowledging spatial effects (as covariables). Essentially, pCCA works by removing the variations in microbial communities contributed by designated covariables prior to performing CCA [82]. The *p* values for the canonical axes were produced by block Monte Carlo permutation tests to account for possible spatial autocorrelations [82]. The significant correlation of an FA (or a TRF) to a pollutant variable was indicated by a *t* value > 2 in CANOCO [82].

A total of 28 environmental variables were adopted in this study: 3 dummy variables were assigned to characterize spatial locations (marsh and elevation); 20 sediment eluate metals were each considered as a variable as were 4 organic pollutants and 1 toxicity measurement based on the sea urchin embryo toxicity assay. The large number of environmental variables relative to the number of samples makes constrained ordination converge to unconstrained ordination [81]. Variable selection, i.e., the lumping of variables, was thus performed manually for the metal variables by examining the correlation structure among pollutants and by continuous inspection of ordination results [56]. The correlation structure was determined by calculating pairwise Pearson's correlation coefficients in S-plus. For organic pollutants, a log transformation was performed before analysis to dampen the outlier effect [82].

Results

Sediment Eluate Analysis. Concentrations of metals and organic compounds differed greatly among samples (Table 1). There were clearly “hot spots” in the two marshes for DBP, total phthalates, CHCl₃, Cu, overall toxicity, and particularly for TCE. TCE was highest in SFH (88 ppb) and moderate in three other samples, but negligible at the other sites. Notably, CHCl₃ concentrations were much higher, up to one order of magnitude, in Stege Marsh versus Carpinteria. Pairwise Pearson's correlations were calculated for metals, and the following pairs had correlation coefficients higher

Table 1. Chemical concentrations in sediment eluates

| Pollutant | Unit | Concentration range | Mean | Standard deviation |
|-----------------------|-------------------------------|---------------------------|-------|--------------------|
| DBP | ppb | 2–786 | 122 | 165 |
| TCE | ppb | 0 ^a –88 | 6.2 | 17.0 |
| Total phthalate | % | 2–1759 | 341 | 502 |
| CHCl ₃ | % | 9–966 | 213 | 280 |
| Toxicity ^b | % | 1.7–92.7 | 36.8 | 33.5 |
| B | ppm versus Na ^c | 74.9–2281 | 629 | 425 |
| Ba | ppm versus Na | 2.7–34.5 | 10.1 | 6.8 |
| Ca | ppm versus Na | 3.41–7.42 | 6.15 | 0.98 |
| Cd | ppm versus Na | 0.003–0.170 | 0.024 | 0.038 |
| Co | ppm versus Na | 0.13–1.55 | 0.33 | 0.31 |
| Cu | ppm versus Na | 1.0–54.8 | 6.0 | 12.6 |
| Fe | ppm versus Na | 41.0–109 | 65.5 | 14.2 |
| K | 10 ³ ppm versus Na | 29.3–69.2 | 55.1 | 92.3 |
| Li | ppm versus Na | 13.2–59.1 | 20.9 | 10.4 |
| Mg | 10 ³ ppm versus Na | 74.1–213 | 137 | 28.0 |
| Mn | ppm versus Na | 0.3–228.4 | 62.8 | 64.2 |
| Mo | ppm versus Na | 1.22–6.30 | 2.44 | 1.07 |
| Ni | ppm versus Na | 0.25–3.31 | 0.82 | 0.67 |
| Pb | ppm versus Na | 0.000 ^a –0.072 | 0.019 | 0.021 |
| Sb | ppm versus Na | 0.015–0.495 | 0.151 | 0.102 |
| Se | ppm versus Na | 3.45–7.98 | 5.68 | 1.04 |
| Sr | ppm versus Na | 573–1398 | 1084 | 221 |
| Tl | ppm versus Na | 0.000 ^a –0.024 | 0.009 | 0.007 |
| V | ppm versus Na | 0.33–1.99 | 1.14 | 0.42 |
| Zn | ppm versus Na | 1.9–12.3 | 4.3 | 1.9 |

^aConcentrations reported as zero were below detection limits: 1 ppb for TCE, 0.1 µg L⁻¹ for both Pb and Tl.

^bToxicity refers to sea urchin embryo toxicity as described in [Materials and Methods](#).

^cConcentrations (ppm versus Na) were determined as relative to Na and are not absolute concentrations.

than 0.75, which was a cutoff defined for this study: K–Ca (0.91), Sr–Ca (0.81), Sr–K (0.77), B–Li (0.87), and Zn–Ni (0.86). There was no pair that exhibited a high negative correlation. The correlations were used to justify lumping variables in the manual variable selection process, as described in [Materials and Methods](#), which reduced the number of environmental variables relative to the number of samples and thus improved the robustness of the direct gradient ordination analyses. For example, Ni was not included in the analysis when Zn was, yet related results would apply to both metals.

TRFLP Profiles. Across all samples, there were 78 TRFs, ranging from 18 (SDH) to 41 (CCH). There were no significant linear relationships between the number of TRFs and either the spatial or any pollutant variable included in this study. However, DCA indicated strong differences in TRFLP patterns between Stege Marsh and the Carpinteria Salt Marsh, and between high-elevation samples and middle- or low-elevation samples, although few differences existed between middle- and low-elevation samples. These spatial effects were directly analyzed and specifically confirmed by CCA (Table 2), which showed that a large portion of community variation was explained by spatial effects. The first CCA ordination axis explained 16% of the total variation ($p = 0.002$; Table 2) and was interpreted as a combination of marsh effect and

high-elevation effect. The second axis explained 9% of total variation and was mainly a contrast between high elevation and marsh. The third axis, a contrast between middle and low elevations, was not considered important, as it only explained 2% of the total variation. Because CCA established the importance of marsh and elevation as controlling variables, these spatial variables were treated as covariables in pCCA to extract pollutant effects on microbial community TRFLP profiles.

Pollutant Effects in TRFLP Profiles. Using pCCA, a strong association ($p = 0.024$) was observed between the TRFLP profiles and five metals: zinc (Zn), cadmium (Cd), selenium (Se), thallium (Tl), and magnesium (Mg). Note that Zn and Ni concentrations were highly positively correlated; thus, Ni, although not included in the pCCA, was also strongly associated with TRFLP patterns. Indeed, in a separate analysis, Ni was found to be significantly correlated with TRFLP patterns (not shown). In the pCCA diagram (Fig. 2), more important variables are represented by longer arrows, and the associations of sites and “species” (TRFs) along each gradient can be observed by projecting perpendicular lines from the sites and TRFs to the nearest arrowed line. For example, as shown in Fig. 2, Zn, Se, and Cd are represented by relatively longer arrows, indicating that these three metals correlated more strongly with microbial co-

Table 2. Summary statistics from all ordination analyses

| Microbial community analysis | Ordination technique | Environmental variables (no. of variables) | Covariables (no. of covariables) | % inertia explained by first two canonical axes ^a | % inertia explained by all canonical axes | <i>p</i> value for the first canonical axis ^b | <i>p</i> value for all canonical axes |
|------------------------------|----------------------|---|----------------------------------|--|---|--|---------------------------------------|
| TRFLP | DCA | – | – | 32 ^c | 40 | – | – |
| | CCA | Marsh, elevation (3) | – | 25 | 26 | 0.002 | 0.002 |
| | pCCA | Zn ^d , Cd, Se; Tl, Mg (5) | Marsh, elevation (3) | 19 | 29 | 0.10 | 0.024 |
| | pCCA | Organics and toxicity (5) | Marsh, elevation (3) | 15 | 21 | 0.50 | 0.36 |
| | pCCA | Zn, Cd, Se, Tl, Mg, organics, toxicity (10) | Marsh, elevation (3) | 22 | 36 | 0.36 | 0.01 |
| PLFA | DCA | – | – | 44 | 51 | – | – |
| | CCA | Marsh, elevation (3) | – | 28 | 30 | 0.002 | 0.002 |
| | pCCA | Pb, Zn (2) | Marsh, elevation (3) | 22 | – | 0.026 | 0.006 |
| | pCCA | Organics and toxicity (5) | Marsh, elevation (3) | 11 | 16 | 0.81 | 0.87 |
| | pCCA | Pb, Zn, organics, toxicity (7) | Marsh, elevation (3) | 28 | 37 | 0.18 | 0.024 |

^aRefers to % of total inertia, which is the variance in ordination scores, or as in pCCA, refers to % of residual inertia after accounting for the spatial effects using spatial variables as covariables.

^b*p* values based on block Monte Carlo permutation test, as described in Materials and Methods.

^cEntries italicized refer to those in indirect gradient.

^dNi was highly correlated to Zn and was thus not included in the analysis, but had similar strong correlations to community profiles as described in Results.

community patterns than Tl and Mg. The direction of an arrow indicates the direction of increase in the corresponding environmental variable. By projecting imaginary perpendicular lines to the arrowed line for Zn, one would infer that Zn concentrations decreased from SCH, CAM, and CCH to CBH (Fig. 2). Similarly, TRFs 529 and 190 are more likely to appear with high Zn concentration, whereas TRFs 556 and 551 are more likely to appear when Zn levels are low. Thus, Zn-tolerant bacterial species may have contributed to TRFs 529 and 190, whereas Zn-sensitive species may have contributed to TRFs 556 and 551. Overall, nine and five TRFs were positively and negatively correlated with Zn concentration, respectively (*t* value > 2). A forward selection procedure in CANOCO also identified Zn, Cd, and Se as the three most important metals in explaining variation in the TRFLP profiles. As for Cd, sample CDH had a high Cd concentration (Table 1). Six and two TRFs were positively and negatively correlated with Cd, respectively (*t* value > 2). There was no peak or sample that was strongly correlated with Se.

The four organic pollutants and the sea urchin toxicity measurement were only weakly correlated with TRFLP patterns, as these five variables explained little of the TRFLP data (21% of the residual variation after removing spatial effect, *p* = 0.36; Table 2). However, among the five variables, the sea urchin toxicity was relatively more important. Consistently, in the pCCA ordination diagram including all pollutant variables

(not shown), the organic pollutants and toxicity measurement were represented by relatively shorter arrows. Overall, after acknowledging the spatial effects via pCCA using marsh and elevation as covariables, metals (Zn, Cd, Se, Tl, and Mg) played a more important role in explaining variation in TRFLP data than other pollutants.

PLFA Profiles. There were 75 total FAs identified for all samples, and the number of FAs per sample ranged from 35 (CDL) to 57 (CAH). The number of FAs was higher in vegetated sites (H, high elevation) and lower in channel bottoms (L, low elevation; *p* < 0.05). The total PLFA abundance, as an approximation of microbial biomass [94], differed greatly across samples and was lowest at low elevations (*p* = 0.0018). As analyzed by DCA, microbial communities differed greatly between high-elevation samples and either middle- or low-elevation samples, but distinctions between the two marshes and between middle and low elevations were slight. The first two DCA axes together explained 44% (36 and 8% from the first and second axis, respectively) of the total variation in PLFA patterns (Table 2). The first axis explained the major portion and separated high-elevation samples from the rest except for sample SBH.

The spatial effects on PLFA profiles were assessed and confirmed by CCA. With the first two axes together

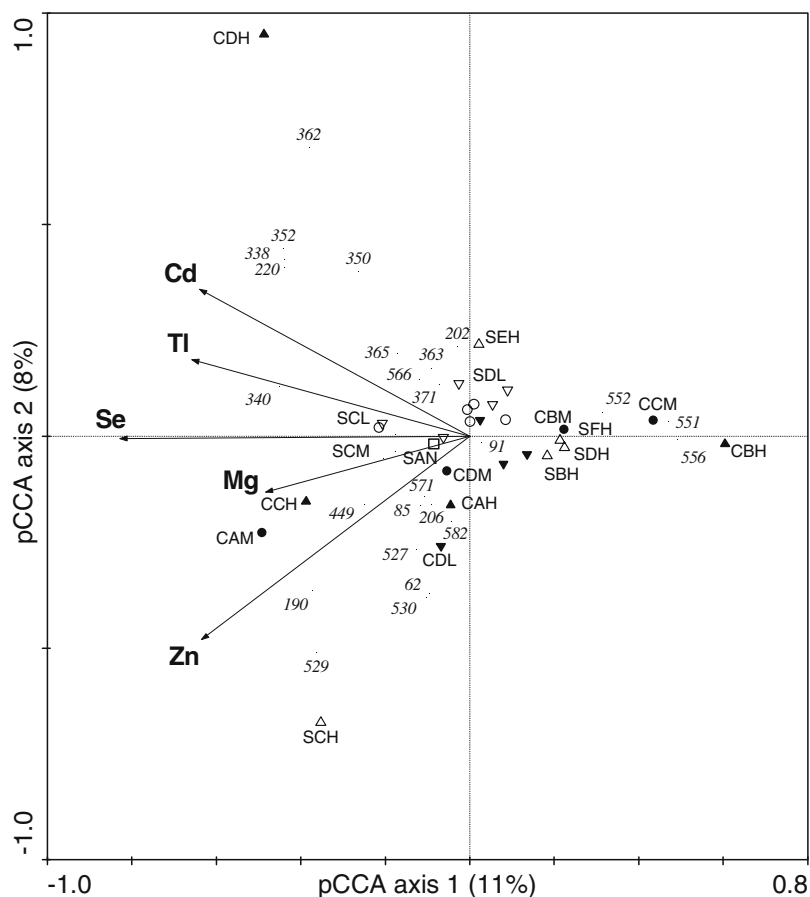


Figure 2. Ordination diagrams from pCCA of TRFLP community data where selected metals were environmental variables, and spatial locations were covariables. Samples were labeled using the three-letter sample codes as per [Materials and Methods](#) and denoted by *triangles*, *circles*, or a *square*. *Open symbols* are for Stege Marsh; *filled symbols* are for Carpinteria Salt Marsh. *Upward triangles* indicate the high-elevation samples, *downward triangles* indicate the low elevation, and *circles* indicate the middle elevation. Sample SAN is denoted by an *open square*. TRFs were labeled by *numeric numbers* representing fragment length in base pairs. Only TRFs that were significant (species fit > 0.15) are displayed. Labels for symbols nearest the origin were omitted to reduce clutter.

explaining 30% of the total variation (Table 2), the first CCA ordination axis explained 22% ($p = 0.002$) and was mainly contributed from high elevation (coefficient = 0.80). The second and third axes were trivial, explaining only 5 and 2% of the total variation in PLFA data, respectively. Because of their importance in explaining PLFA profiles and to extract pollutant effects, marsh and elevation were treated as covariables in pCCA.

Pollutant Effects in PLFA Profiles. Using pCCA, a strong correlation between PLFA profiles and two metals was observed (Table 2). The arrows representing Pb and Zn were almost perpendicular to each other (Fig. 3), which indicates that although lead and zinc both strongly influence PLFA microbial community composition, they do so dissimilarly. A forward selection procedure in CANOCO also identified Pb, Zn, and Ni as the most influential metals on microbial community composition as determined by PLFA. Positively associated with high Pb levels (t value > 2) were FAs 20:4 ω 6,9,12,15c, 15:1 ω 8c, uk18.5, and the ratio of all unsaturated FAs over the total FAs. Negatively correlated with Pb were FAs *i*14:0, 16:1 ω 7t OH, *a*15:0, *i*16:0, *a*12:0, Sum1 (the first sum feature, including 14:1 ω 5c and 14:1 ω 5t), *i*15:0, 16:1 2OH, as well as the percentages of sum of all iso-branched, all

anteiso-branched, all branched, and all hydroxylated FAs out of the total FAs. Positively correlated with Zn were FAs Sum6 (the sixth sum feature, including *a*18:0 and 18:2 ω 6,9c), uk12.4, *a*16:0, *i*15:0, 16:1 2OH, 10Me16:0, the ratio cy17:0/16:1 ω 7c, the ratio of all methylated over the total FAs, and the ratio of all iso-branched over the total FAs. Negatively correlated with Zn were FAs *i*14:0, 16:1 ω 7c, *i*15:1g, the ratio of all unsaturated over the total FAs, and the ratio of all monounsaturated over the total FAs.

The four organic pollutants and the sea urchin toxicity measurements explained little of the PLFA patterns (Table 2). However, of these five variables, CHCl_3 , total phthalates, and sea urchin embryo toxicity appeared more important (not shown). In the pCCA ordination diagram including all pollutant variables (Fig. 3), the organic pollutants and toxicity measurement appeared to be relatively less important pollutant variables. Overall, Zn and Pb were the most important pollutants in explaining the variation in PLFA data after accounting for the spatial effects via pCCA where marsh and elevation were covariables.

Other Biological Metrics. Plants have been known to produce organic acids, amino acids, or peptides for

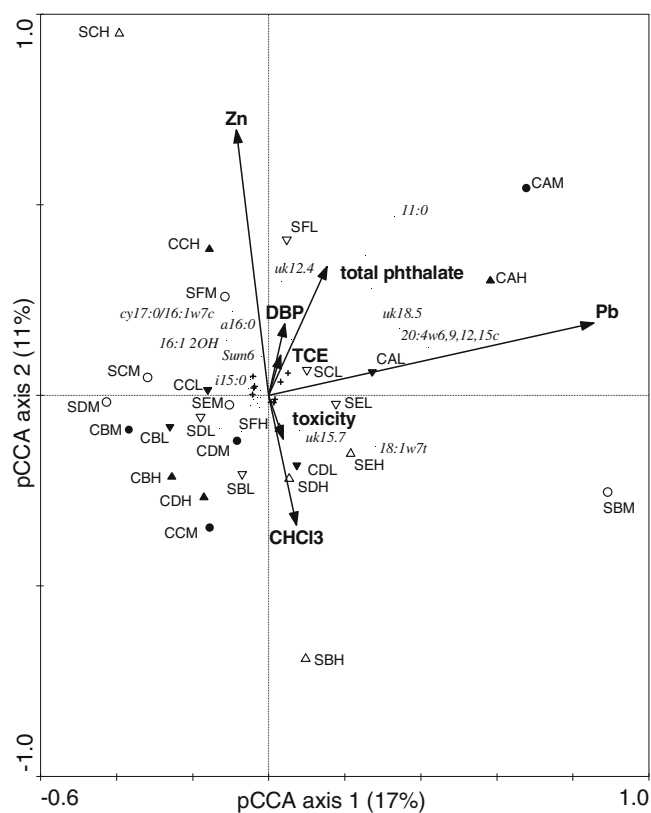


Figure 3. Ordination diagrams from pCCA of PLFA where all pollutant-related variables were environmental variables, and spatial locations were covariables. Samples were labeled using the three-letter sample codes as per the Materials and Methods and denoted by triangles or circles. Symbols are as in Fig. 2. Only the fatty acids and PLFA composite variables (crosses) that were well represented in pCCA (species fit >0.15) are displayed. Labels for symbols nearest the origin, and SAN, were omitted to reduce clutter.

heavy-metal detoxification and tolerance [27]. Thus, changes in tissue metabolite profiles may reflect environmental conditions such as stresses from heavy metals. DCA diagrams (not shown) of *S. virginica* shoot and root tissue metabolite profiles both suggested that there was a marsh effect dominating the differences. Sample scores from the first DCA axis of plant tissue metabolite profiles were significantly correlated with sample scores from the first DCA axis of the microbial TRFLP profiles (Fig. 4), indicating that both plants and microbes were responding to similar selective pressures. However, a complete multivariate analysis of the relationships between plant metabolite profiles and the multiple pollutants was beyond the scope of this study.

Sea urchin embryo toxicity was considered as an integrated measurement of chemical stressors and was thus treated as an environmental variable in the previous pCCAs of microbial community data. However, it was also a biological metric for overall toxicity caused by metals, organic pollutants, and/or any other chemicals in the sampling sites. No spatial pattern (i.e., marsh and elevation) was evident in the toxicity measures. No relationship was identifiable between the toxicity measures and any of the 24 measured metal and organic pollutants whether analyzed singly or in groups.

Discussion

Despite the inherently dynamic and complex nature of the salt marsh environment, the structures of microbial communities in the California marshes we studied were significantly related to marsh pollutant levels. To discern pollutant effects from other environmental effects on

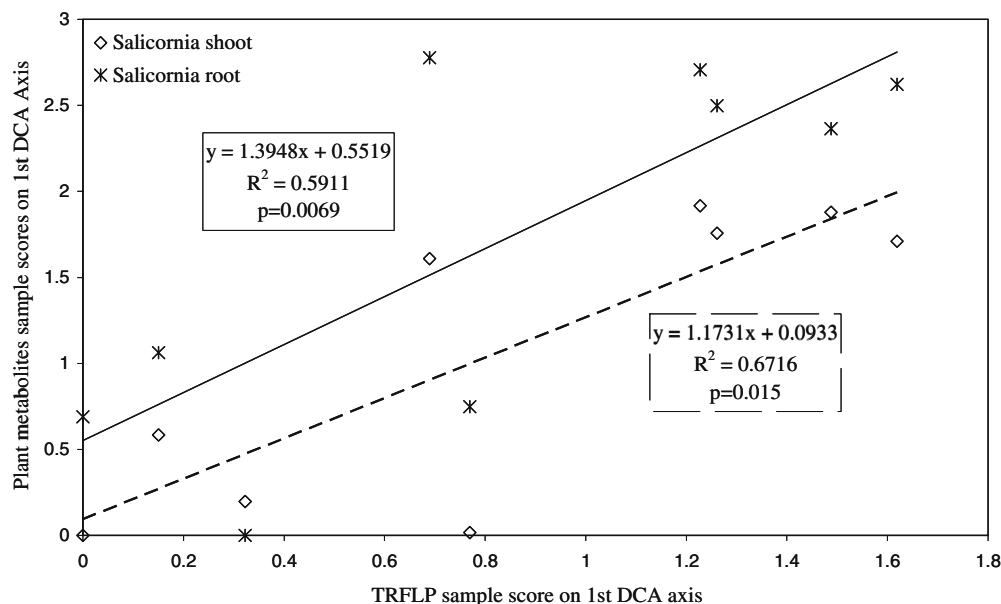


Figure 4. Regression of DCA axis 1 scores for plant metabolite profiles versus DCA axis 1 scores for the TRFLP microbial community profiles.

communities, we employed pCCA, an ordination technique rarely used in microbial ecology, for statistically separating location effects from pollutant effects. This enabled identification of several key stressor pollutants for which microbial community composition shifts appeared significantly related.

Variation Related to Spatial Locations. In this study, a large amount of variation in microbial community profiles was associated with differences between the two marshes; similar spatial effects were also detected in the metabolite profiles of plant tissues. Sediment microbial communities are inherently spatially heterogeneous on the scales of centimeters, meters, and kilometers [22, 23, 65], and the spatial scale of heterogeneity could differ from location to location [18]. Factors affecting heterogeneity include differences in nutrient inputs, temperature, salinity [3], tidal influence [29], grain size [65], biotic disturbance [20], and even deposit feeding [91]. The differences between marshes in this study were mainly a result of geographical location and seasonal effects, which would include temperature, tide and thus salinity, and nutrient effects. Specifically, Stege Marsh and Carpinteria Salt Marsh were sampled in the winter and the summer of 2002, respectively, and Carpinteria Salt Marsh is known for its elevated nutrient loading [55]. However, organic pollutants could also contribute to the marsh differences because Stege Marsh had much higher CHCl_3 levels and lower total phthalate and DBP levels, as compared to Carpinteria Salt Marsh.

There were also, within marshes, elevation effects attributable to factors such as vegetation coverage and sediment saturation. It has been reported previously that, for salt marsh microbial communities, compositional variations across vertical elevation gradients were greater than along longitudinal gradients [22]. In this study, the middle- and low-elevation communities were similar owing to the fact that most of the channels were shallow and narrow. On the other hand, communities from high elevation, vegetated sites, were distinct. *Actinomyces* and fungi are common in the rhizosphere, and thus, it was not surprising to find that the biomarkers (*Actinomyces*: 10Me branched FAs [52]; fungi: 18:2 ω 6,9c and 18:3 ω 6,9,12c [5, 88]) were much more abundant at the high-elevation sites but relatively absent from low- and middle-elevation sites. The lower percentage of sulfate reducer biomarkers (17:1 ω 8c and 17:1 ω 7c [5]) at high-elevation sites and the high percentage of 10Me16:0 (*Desulfobacter* biomarker [90]) at channel bottom sites (low elevation) were also consistent with the fact that sulfate reducers are anaerobic.

However, the main goal of this research was not to define spatial heterogeneity *per se*, but to discern relationships between pollutants and microbial communities irrespective of spatial variation. Partial ordination

techniques (i.e., pCCA) were thus adopted to identify potential stressors to microbial communities after accounting for spatial variations. Beasley and Kneale [2] used pCCA to investigate the influence of heavy metals on macroinvertebrate communities while physical habitat variables also had an influence. To our knowledge, the current study is new in microbial ecology to take advantage of pCCA, which is a partial direct gradient analysis that has been successfully used in macroecology to separate effects from different sets of factors influencing biological community profiles [9, 19, 35, 61]. Other indirect “partial” techniques, which calculate single correlation coefficients between distance matrices, have also been applied in microbial ecology [30].

Potential Metal Stressors. In this study, metal concentrations were measured in sediment eluates as described in **Materials and Methods**. Assuming a mass of 36 g NaCl per liter of FSW in this study, estimated concentrations of metals on a per-kilogram-sediment basis were generally much lower as compared to the 10- to 100-mg kg^{-1} ranges previously reported for soils [26]. Yet, strong correlations between metal concentrations and microbial community shifts were still observed. Metals in sediment eluates were water-soluble and presumably bioavailable. Indeed, long-term exposure of microbes to metal concentrations in the ranges of 1.5–10 $\mu\text{g L}^{-1}$ for Zn, 0.6–2.7 $\mu\text{g L}^{-1}$ for Cu, 5–10 mg L^{-1} for Ni, and 0.3–0.4 $\mu\text{g L}^{-1}$ for Cd has been shown to be toxic when free metal ion concentrations were considered [26]. These ranges were comparable to those estimated in the current study. Thus, although metals were neither measured nor reported on a per-mass-of-sediment basis, the amounts and fractions reported here were ecotoxicologically important.

As above, several metals appeared as stressors to the microbial communities as assessed either by TRFLP or by PLFA, but only Zn, and thus also Ni, had a consistent effect on microbial communities independently of the profiling method. Zinc has been reported to alter microbial community patterns in near-shore Antarctic sediments [64] and soils [14, 25, 47, 75] and decrease microbial community diversity as assessed by clone library analysis [47]. Also, Frostegard *et al.* observed a strong increase of 18:2 ω 6,9 in response to Zn pollution [25] and other metals [24], which is consistent with the observation that fungi tend to be more resistant to heavy metals than bacteria [83]. In this study, the percentage of fungi increased with these metals, and Sum6 (the sixth sum feature), which is partly comprised of the 18:2 ω 6,9c signature FAs for fungi [5, 88], was strongly associated with high Zn and Ni contents. Additionally, the ordination analyses revealed strong correlations between several specific TRFs and FAs and Zn or Ni. Whereas this suggests that metal indicators may reside within the

PLFA or TRFLP profiles, the observation that some signature FA biomarkers are related differently to metal gradients depending on soil type, i.e., arable versus forest [24], somewhat tempers this expectation. Nonetheless, the influence of metal pollution on the microbial communities in this study appears to be unequivocal and indeed separable from other, more general, differences between marshes.

Organic Pollutants. Microbial community responses to organic pollutants have been studied before, although mostly for soils [34, 43]. Community structure shifts have been observed in soil upon the addition of 2,4-dichlorophenoxyacetic acid and in petroleum-contaminated sand [33]. In this study, however, organic pollutants were not important, as compared to metals, in determining microbial community structure. Nevertheless, organic pollutants may still have influenced microbial communities at these sites. Concentrations of CHCl_3 were much higher in Stege than in Carpinteria, which would have contributed to overall differences in the marshes. Whereas pCCA removed the variation associated with marsh and elevation and thus revealed the variation caused by pollutant variables, the differences in the concentrations of CHCl_3 were strongly associated with marshes, and thus, the residual variation was unlikely to strongly correlate with CHCl_3 . Similarly, when the “partialing” procedure removed marsh effects, possible effects of DBP and total phthalates were eliminated because the presence of these pollutants was so strongly correlated with the marshes and not along gradients within each marsh. This is evident in the ordination plot where arrows representing these three organics were either along or opposite the direction of dummy variable “Msh” (Fig. 5). It is possible that a separate, more involved study of each marsh could have revealed a within-marsh importance of organic pollutants to microbial community composition, but the sample size in this study was insufficient for that analysis.

Other environmental variables, including organic matter, nutrients, salinity, and other physical conditions known to influence microbial growth [3, 21], were not measured in this study but could be important in explaining variations in microbial community profiles. Yet, the absence of these measures does not hinder our ability to investigate the effects of the pollutant variables at hand. One merit of CCA and pCCA is that they allow an investigator to explore the correlations between the biological data (i.e., microbial communities) and a subset of environmental variables of interest (i.e., metal and organic pollutants), without exhaustively measuring all potentially relevant environmental variables [57, 79]. In addition to its effect as being a carbon source, organic matter could influence the toxicity effects of metals by

changing their bioavailability. However, in this study, metals were measured in eluates thus representing the soluble and bioavailable portions. Ultimately, although we cannot establish cause–effect relationships between microbial communities and pollutants in these marshes, the work reveals testable relationships upon which to base future, more detailed studies; our results are also consistent with previously identified relationships between the potential stressors (Zn, Ni, and other metals) and changes in microbial communities across a number of different habitats (see above).

TRFLP versus PLFA in Assessing Microbial Community Responses. Although overall patterns in TRFLP and PLFA profiles appeared to consistently vary with pollutants, the discrepancies found may indicate the magnitude of stresses. TRFLP and PLFA characterize microbial community structure on the levels of genotype, i.e., sequences of genes encoding 16S ribosomal RNA, and phenotype, i.e., membrane FA composition, respectively. Phospholipids, essential membrane components of all living cells, differ in composition for different organisms [94]. However, changing membrane FA composition is also an important survival strategy for microorganisms

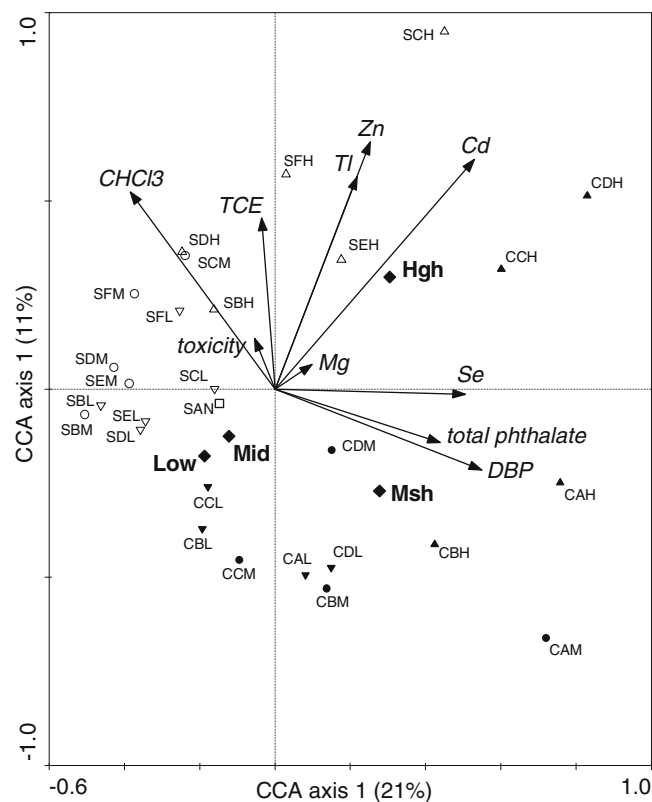


Figure 5. An example of effects of pollutants (arrows) coinciding with effects of spatial variables (diamonds) in CCA of TRFLP data. Three-letter sample codes are as per Materials and Methods. Symbols and interpretations are as in Figs. 2 and 3.

facing environmental stress [41]. Hence, different species from one sample could have similar FA composition as a result of similarly adapting to common stresses. Pb, clearly an important influencer on microbial communities based on PLFA data (major component of the first pCCA axis, Fig. 3, $p = 0.026$), was not important in correlation to TRFLP profiles. The magnitude of stress from lead might be moderate enough such that microbial communities in the study could tolerate the stress and preserve genotypic structures but would have to adjust phenotypically and change membrane lipid compositions. Zn, on the other hand, likely represented a higher magnitude of stress that influenced both the genotypic and phenotypic profiles of the microbial communities.

In the analysis of microbial communities, most methods are imperfect in some important ways. As described above, it is well understood that PLFA profiles can vary not only because of between-population differences but also because of within-population changes to FA composition in response to stresses. Similarly, we acknowledge that TRFLP analysis, although a high-throughput and reproducible community profiling technique [44, 53], is subject to PCR bias [92], which may include differential amplification [62, 69] and template annealing [76, 77]. These issues could be problematic when trying to interpret TRFLP data quantitatively. However, based on at least three lines of evidence, we conclude that bias was minimal in this study. First, our statistical assessments of community differences were the same whether we employed TRFLP data on only a presence/absence basis or on the basis of relative abundance of TRFs. This lack of apparent bias, at least when applied to comparing communities along gradients or across similar ecosystems, is consistent with other PCR-based microbial ecological studies [46] and with previous studies using the same primer sets and the same PCR conditions in our laboratory [36–38]. Although Denaro *et al.* [11] reported a discrepancy in results based on either abundance data or binary data, they were actually comparing diversity indices and not community profiles. Second, bias caused by template annealing mostly depends on the PCR product concentration and is less likely to occur for environmental DNA samples where the amplification of any one template is unlikely to produce products at a high-enough concentration to cause reannealing inhibition [76, 77]. Template annealing changes the relative peak heights in a TRFLP profile with increasing number of cycles in PCR. However, with the same PCR protocol as in this study (same primer, template concentration, etc.), LaMontagne *et al.* [38] showed that the relative peak height in TRFLP changed minimally with the number of amplification cycles. Thus, template annealing was not likely to have caused bias in the current study. Template GC% content, another potential cause for preferential amplification, has been investigated, but

studies into this factor yielded contradictory findings [17, 69]. Similarly, genomic size and DNA copy number have been reported as both influential [17] and not [62] to template-to-product ratios in multitemplate PCR. Ultimately, however, the two profiling techniques (PLFA and TRFLP) utilized in the current study gave consistent results: that marsh and elevation were important in explaining variations in microbial communities, that metals were more strongly correlated than organic pollutants to microbial communities, and that zinc and nickel were the most important metals in explaining community variations. Because of this excellent agreement between TRFLP and PLFA results, we must conclude that PCR and other TRFLP-related biases did not exert a strong influence on our community analyses.

Microbial Communities as Indicators of Stress. Salt marsh pollution management is facilitated by sound metrics of pollution including direct chemical analysis and indicators of chemical stressors [63]. Bio-indicators are of great interest when the endpoint of management is to protect biological and ecological resources and functions [89]. This is particularly important when pollutant speciation or unresolved dose response relationships confound the ability to extrapolate biological effects from field pollutant concentration data [71]. For instance, plant root exudates have been shown to dampen the toxicity effects of certain heavy metals [32]. Substrate availability has also been reported to interfere with heavy-metal toxicity effects [8]. In this study, three biological metrics were compared. The major patterns in plant metabolite profiles were consistent with microbial community profiles, which may imply similar marsh effects (i.e., effects of geological location and season). However, sea urchin embryo toxicity appeared to be an aggregate chemical measure that had no major pattern or discernable relationship with individual or groups of pollutants in this study of two human-impacted salt marshes. Being an acute method conducted using nonindigenous invertebrates [85], although commonly performed to infer toxicity to eukaryotes, sea urchin embryo toxicity measurements are potentially less informative to understanding chronic effects of pollutants. Sediment microbial communities, on the other hand, are exposed *in situ* and chronically to pollutants in the marsh. Also, increasing evidence has suggested that microorganisms are far more sensitive to heavy-metal stress than are animals or plants, with exposure resulting in a loss of microbial functions [26].

In that microbes respond rapidly and are relatively easy to sample [32], and that microbial communities are direct assessments of the biological community fundamental to the biogeochemical cycling, productivity, and “buffering” capacities in salt marshes [78], we thus emphasize the potential advantages of including whole

microbial communities within a portfolio of indicators needed for salt marsh health diagnosis and for coastal environmental management generally [49].

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