

YEAR 4 ANNUAL REPORT

Period Covered by the Report: March 1, 2004 to February 28, 2005

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EPA Agreement Number: R-82867601

Title: Pacific Estuarine Ecosystem Indicator Research (PEEIR) Consortium:
Biogeochemistry and Bioavailability Component

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Research Category: EaGLE Program

Project Period: March 1, 2001 to February 28, 2006

RESEARCH OBJECTIVES

The overall aim of the proposed research of this section is to develop field indicators and the knowledge base to help assess the consequences of changes in chemical form of pollutants in tidal marshes. This section's particular emphasis is assessment of metals and organic pollutant bioavailability in relation to sedimentary lower trophic level biomarkers. This emphasis on rooted plants and sediment microbes is because they are often the entry point of pollutants into the food chain, and are major drivers of the biogeochemistry of the tidal marsh. The specific objective of this section is to uncover the chemical, biochemical, and biotic markers that herald the bioavailability, transport, and/or biotransformation of selected metal and organic pollutants. These markers, in appropriate concert with others generated by PEEIR, can comprise indicators of pollutant stress on marsh ecosystems.

PROGRESS SUMMARY

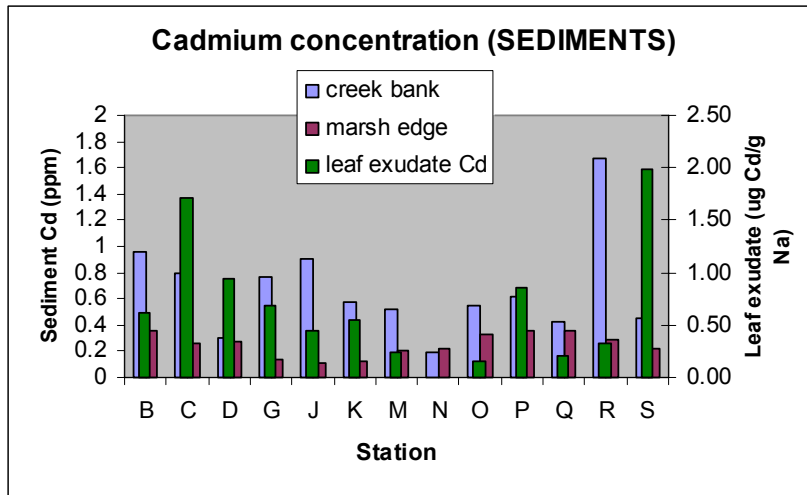
Plant biochemistry (Fan)

In this past year, major experiments were completed on growth-chamber studies with *Spartina* on Cd exposure at different N-levels, using seawater-salinity sand culture, coordinating with the Williams (EIC) group for IRGA and leaf reflectance spectra, Higashi and Green for shoot salt exudates collection and analysis, Cherr group (BRC) for toxicity testing of the exudates. Confirming results of studies from previous years, the accumulation of cadmium in roots of *Spartina* result in the induction of thiol-rich peptides such as phytochelatins (PC_n, where n=2-6). The most recent laboratory experiments showed the induction of PC₃ and PC₄ with exposure to 0.2ppm cadmium, while PC₂ is present in the control treatment. It has not been previously reported that PC₂ is constitutive in these wetland plants. As before, PC did not accumulate in the shoots.

In attempting to relate the laboratory results to field analyses of *Spartina*, roots from plants collected during the 2003 field season were analyzed for Cd and PC, and compared with Cd concentrations in the shoot exudates as well as in sediment. Figure 1 illustrates the lack of relationship between sediment Cd and shoot exudate Cd concentrations, which is not surprising given the complexity of the transport/exudation process. Likewise, sediment Cd concentrations

also did not relate to PC concentrations (data not shown); these types of results indicate a lack of bioavailability response to Cd concentration in the sediment, underscoring the well-known lack of connection between sediment metal concentrations and biological effects.

Figure 1. Cadmium concentrations in sediments and *Spartina* leaf exudate salts at Stege Marsh. Letters denote



various sampling stations.

Figure 2 summarizes the relationship between PC and Cd concentration in *Spartina* roots at various Stege Marsh stations. Phytochelatin production in roots is highest at stations P and K; station P is located near the former agrochemical plant. Station K doesn't fit the general trend of increasing PC to compensate for increasing Cd in roots; PC is known to also respond to other contaminant metals, so perhaps this is the case with station K.

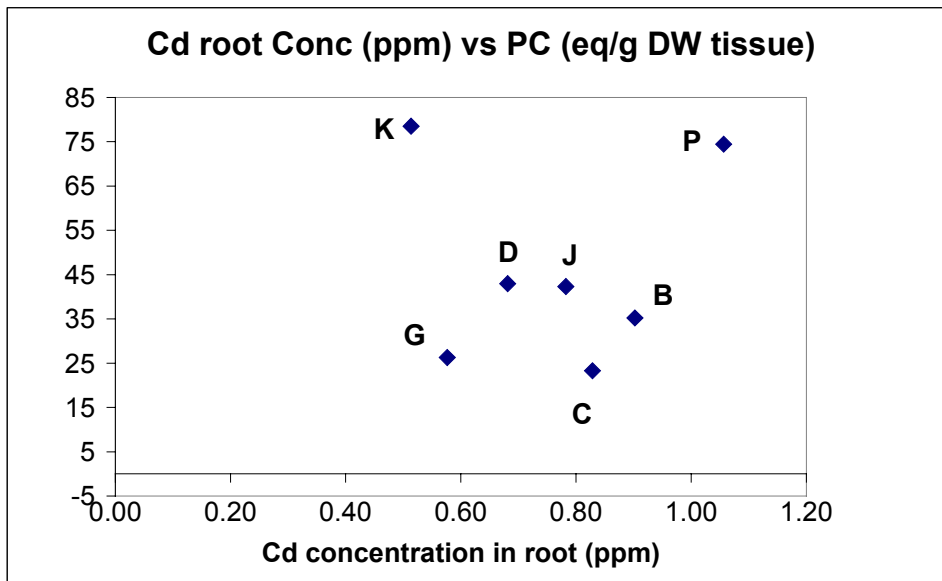


Figure 2. Relationship of PC vs Cd concentration in *Spartina* roots from Stege Marsh. Letters denote various sampling stations.

As stated in previous reports, the PC-Cd relationship is not only important as a time-integrated bioavailability indicator, but also may represent toxic metals (e.g. Cd) in an organic form,

because they are chelated as peptides. Such consideration is currently not reflected in transport, bioavailability, and toxicity models.

Organics & metals analyses & role of DOM (Higashi and Green)

In the past year, metals analysis by ICP-MS continued for a variety of samples: internal and external portions of crab embryo sacs (for Stephen Morgan's group), tissues from out-planted fish (skin, muscle and liver), clams, as well as lab-exposed *Spartina* for Cd and other elements in the nitrate-exposure study by Sarah Norris.

A small sampling team was organized in July 2004 for a sampling of Stege Marsh for *Spartina* leaf exudates, in an attempt to obtain a spatial grid of data. With 4 teams of 2, 176 samples were collected in just 2 hours to map nearly the entire 30 acre site at a level of spatial detail comparable to remote sensing imagery. The combined LIDAR data (detailed elevations) and spectral reflectance end-members for *Spartina* (AVIRIS, from NASA's JPL) was used to estimate *Spartina* density, which, when combined with the element data, apparently mapped the relative output of many important metals.

Two sediment-depth core samples were collected from the outer portion of Stege Marsh and revealed significant and interpretable trends with respect to the history of the region. The utility of this information by PEEIR researchers is pending.

Bacterial community Composition (Holden)

Salt marshes are important ecosystems whose plant and microbial communities can alter terrestrially-derived pollutants prior to coastal water discharge. However, little is known about salt marsh microbial communities including the degree to which pollutants influence their composition and diversity.

This past year we completed the investigation into relationships between microbial community composition and 24 pollutants (20 metals and 4 organics) for two California salt marshes in the PEEIR project: Carpinteria Salt Marsh and Stege Marsh. The sampling and experimental work began in 2002; the major work this past year was in data analysis and writing, including the development of new statistical approaches to tease out relationships between bacterial communities and chemical stressors when the natural marsh environment is contributing significant variation to community signals. Regression and ordination analyses were used to assess how bacterial community composition and diversity, as determined by terminal restriction fragment length polymorphism (TRFLP) and phospholipid fatty acid (PLFA) analyses, were related to pollutant abundances (please see report by Scow's group for examples). 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.

After accounting for these effects, we found that several metals were strongly associated with microbial community composition. Microbial community relationships to environmental conditions were generally consistent with those of plant metabolite profiles and sea urchin embryo toxicity. Thus, while salt marshes are likely to be stressful microbial habitats due to their dynamic and complex nature, toxic metal pollutants also appear to structure microbial communities. These findings suggest a future role for defining and using microbial communities as pollution or restoration indicators in coastal salt marshes.

Occurrence, bioavailability & effects of current-use pesticides (Kuivila)

The overall focus of the study is to examine the occurrence, bioavailability and toxic effects of current-use pesticides in wetlands. Both field sampling and laboratory studies are being used to determine the factors controlling pesticide occurrence and desorption from sediments and how these processes influence bioavailability and biological effects.

Sediment samples from all five marshes were analyzed for 34 current-use pesticides and DDT and its degradates to determine the role of toxic contaminants as stressors to wetland ecosystem health. A variety of current-use pesticides were detected in sediments at both Carpinteria and Stege Marshes but the patterns were very different. Three insecticides were detected at Carpinteria Marsh: two pyrethroids (bifenthrin and permethrin) and one organophosphate (chlorpyrifos). These results are being incorporated into the interpretation of biomarkers and biological effects in collaboration with the BRC component (Cherr and Anderson).

At Stege Marsh, the concentrations of molinate, a thiocarbamate herbicide, were correlated with developmental abnormalities observed in crabs (Morgan project, EIC). Laboratory studies were conducted to expose gravid crabs to elevated molinate concentrations under controlled conditions. Abnormalities were observed in the laboratory experiments (see EIC report) and analyses are underway to measure the molinate concentrations in the embryos.

Laboratory studies on wetland plants were focused on the effects of diuron, a urea herbicide. Diuron is a widely-used herbicide which inhibits photosynthesis, thus it impacts major functioning of plants that may be visible by remote-sensing. Although frequently detected in water, diuron also sorbs to sediments which may provide a continuous source of the pesticide to plants and aquatic organisms in wetlands. In conjunction with the Williams (EIC) project, a series of laboratory studies were conducted to measure the uptake and metabolism of diuron by native *Spartina*. Sand cultures were spiked with different concentrations of diuron and the effects estimated by measuring resulting changes in plant pigments and photosynthesis stress. Concentrations of diuron and its metabolites were measured to estimate the uptake and metabolism of diuron by the plants. These results are being compared to the response of the plants in terms of Pulse-Amplitude Modulated (PAM) fluorescence (see discussion in EIC report).

Mercury exposure & bioaccumulation (Nelson)

Estuarine production of methyl mercury from inorganic mercury occurs mainly in anoxic sediments via the action of anaerobic bacteria. Methyl mercury is typically “biomagnified” at higher trophic levels in food chains (because as much as 80% of all methyl mercury ingested is permanently retained in tissues of higher organisms) and can reach levels that trigger health warnings. It has become virtual dogma in the microbiological literature that one of these groups of microbes, i.e. the “sulfate-reducing bacteria”, is largely responsible for transforming inorganic mercury into methyl mercury in marine and freshwater sediments. In our studies of mine-impacted marine and freshwater sediments (Walker Creek Estuary and Clear Lake) we have shown that another group, iron-reducing bacteria, can reach high densities (10⁶ to 10⁸ cells gm⁻¹) in anoxic surface sediments. Sediment pore-water samples from these same two locations showed high concentrations of soluble iron (assumed to be Fe⁺⁺) that were consistent with active sediment metabolism by iron reducers. The first strain of iron-reducing bacteria that was tested produced methyl mercury from divalent mercury at a rate (per cell per day) that exceeded the corresponding rate for various sulfate-reducing bacteria. Beyond the time frame sponsored

by PEEIR funding, we will test dominant iron-reducing bacteria from Walker Marsh for their rates of mercury methylation. If we are able to establish that these bacteria are, in general, important in mercury methylation in mine-impacted sediments, the implications for remediation and management are significant.

Microbial communities (Scow)

PLFA fingerprinting of estuarine sediment microbial communities.

Sediment microbial communities from different estuaries along the California Coastline were characterized using phospholipid fatty acid analysis (PLFA) by the Scow lab. Estuaries sampled included Stege Marsh, China Camp, Carpinteria Marsh, Mugu Lagoon, Tom's Point and Walker Creek. The PLFA fingerprinting of microbial communities for both sampling years (2002 and 2003) has been completed and the data is available upon request and from the PEEIR web site. The work during the fourth year focused on the statistical analysis and exploration of the data set in conjunction with TRFLP data sets provided by the Holden Group (UCSB). PLFA fingerprint patterns were statistically analyzed using multivariate statistics including CA (correspondence analysis), CCA (canonical correspondence analysis) and pCCA (partial Canonical correspondence analysis). Comparisons were made at three spatial scales: (1) between geographical locations (e.g. marsh to marsh), (2) within marsh (e.g. between stations) and (3) within the channel profile (e.g. elevations); and between two consecutive summers (2002 and 2003). Further comparisons were made between community profiles and known pollutant gradients and other environmental factors within sites.

In an initial data analysis, correspondence analysis (CA) was applied to explore the variability in the data set related solely to the PLFA and TRFLP composition. Both TRFLP and PLFA fingerprints showed differences in microbial community composition between different marshes (Figure 3). A consistent clustering of Tom's Point and Walker Creek samples was observed in both PLFA and TRFLP plots (Figure 3). PLFA was able to explain twice as much of the variation compared to TRFLP, a consistent trend throughout all statistical analyses performed.

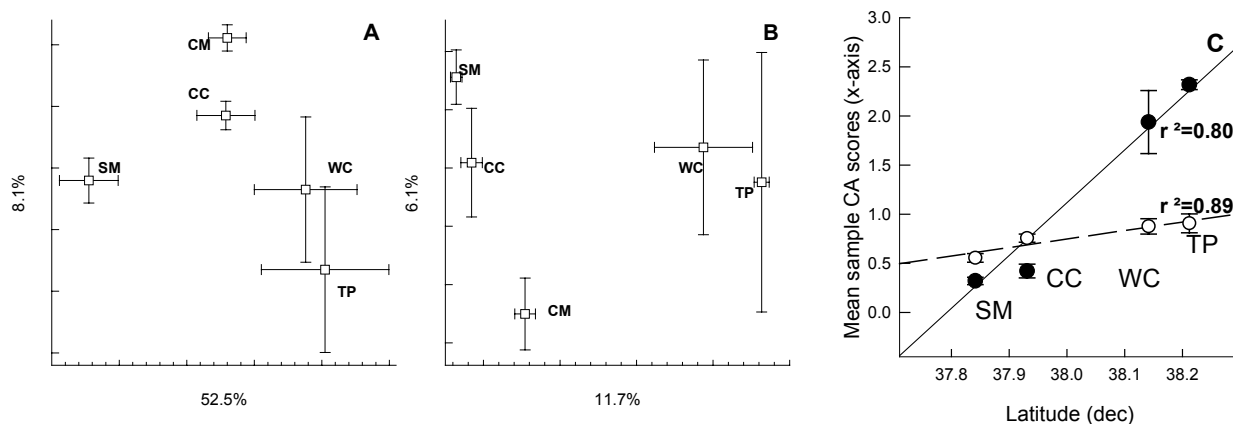


Figure 3. Ordination plot of average CA results. Geographical distribution of (A) PLFA and (B) TRFLP fingerprints of microbial communities from samples collected in 2003. Data points are means \pm SE.

The second step involved the exploration of the variation in the data set correlated to naturally occurring environmental variables (e.g. flooding regime, latitude, marsh, sampling year, etc) and pollutant variables (organic compounds and several metals) by using canonical correspondence analysis (CCA). The CCA plots showed that environmental variables had a stronger effect on community composition than did pollutant variables. Clustering in the PLFA CCA was mainly

driven by the flooding regime and in TRFLP by marsh. No pollutant effects were observed in these analyses (Figure 4).

To determine how much pollutant variables affected community composition after removing the effect of environmental variables, a partial CCA (pCCA) was performed. Several effects of pollution were observed, including an increase in cyclo- and methyl- substituted fatty acids, and a decrease in abundance of branched fatty acids, observed with higher concentrations of Pb and Cd. Organic pollutants had no significant effects.

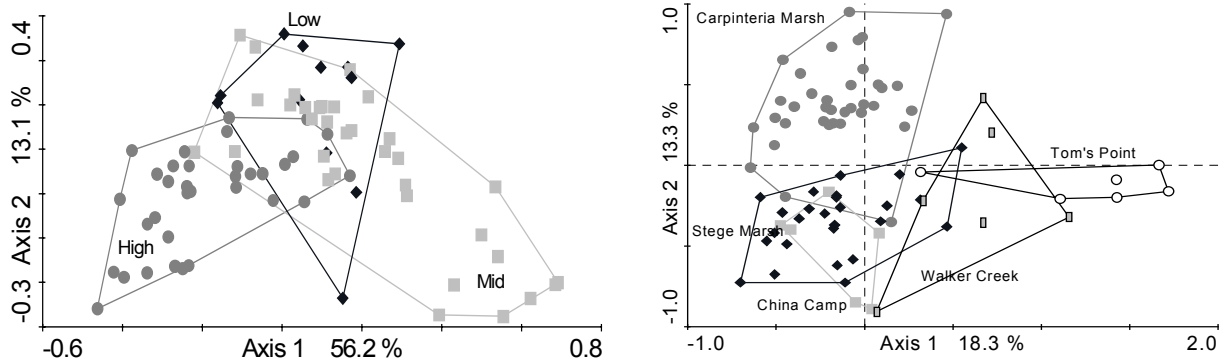


Figure 4. Ordination plot of CCA results for (left panel) PLFA and (right panel) TRFLP fingerprints. Spatial variables override potential pollutant effects.

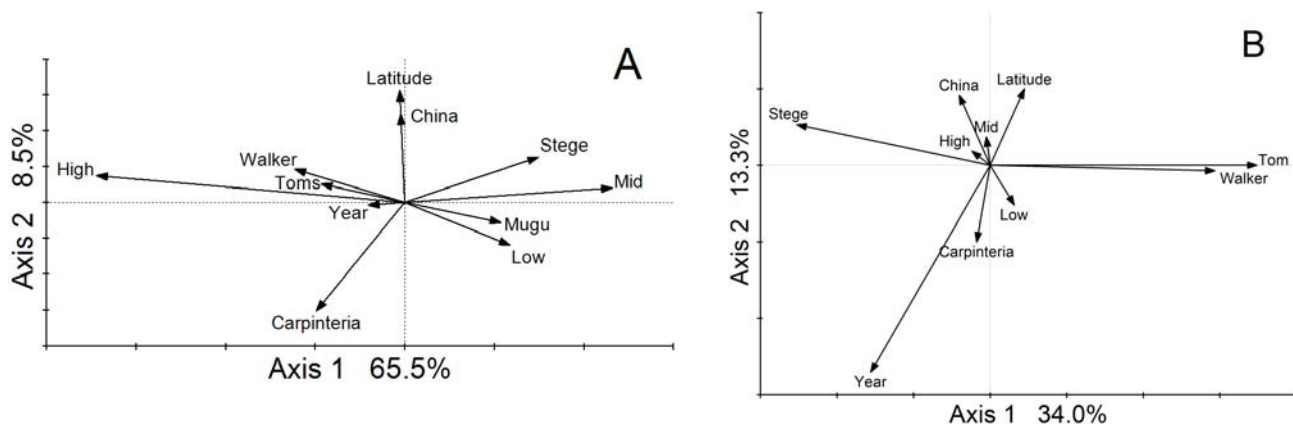


Figure 5. Ordination plot of environmental variables in CCA results for (A) PLFA and (B) TRFLP fingerprints. Only variables describing spatial variation (marsh, elevation) and sampling year were included in this analysis

We explored possible reasons why PLFA explained more variation in the data than did TRFLP. Initial CCA analysis indicated that PLFA fingerprints explained up to 74% (first two axes) of the variation in the data set associated with environmental variables (flooding regime, marsh location, latitude, year, etc). TRFLP only yielded 47.3% in the same analysis (Figure 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 and pCCA variation explained was a measure of how much variation was associated with the environmental variables. Surprisingly, approximately the same percent variation (35%) was explained by the environmental variables for both PLFA and TRFLP. This shows that both methods are equally efficient in distinguishing variation in the data set due to the flooding regime, marsh, sampling

year and latitude. The additional differences in percent variation explained must be due to other variables not previously considered.

Overall, we have shown that microbial communities from similar ecosystems have differences in composition related to biogeography. Environmental conditions associated with salt marsh ecosystems have a strong influence over the structure of the microbial communities. In the case of the estuarine sediments, the flooding regime proved to be a stronger determinant of the microbial community composition than geographical location or sampling years or even pollution. Effects of pollutants are strongly correlated with one another and are masked by environmental and spatial variables, but some of the latter can be eliminated by selection of appropriate data analyses methods. Both TRFLP and PLFA provided different types of information about microbial communities but supported the same general conclusions. The selection of one or the other will depend on the degree of resolution and the specific aims of each particular study. When possible, these techniques should be combined.

Effects of ciprofloxacin on estuarine sediment microbial communities

The purpose of these experiments was to measure the bioavailability of ciprofloxacin in sediment materials and the effects of ciprofloxacin on microbial community composition. Sediments were exposed to a ciprofloxacin concentration gradient (0 to 200 ppm ciprofloxacin) and incubated anaerobically in the dark for 30 days. PLFA community patterns were analyzed using multivariate statistics.

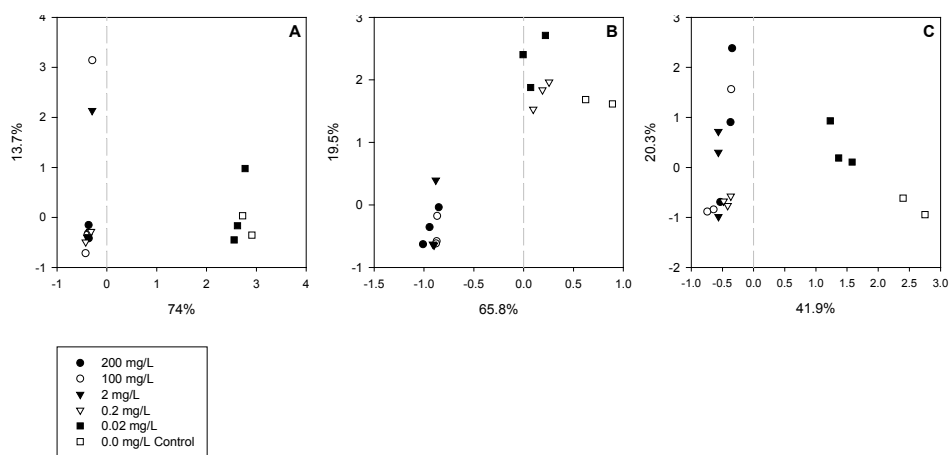


Figure 6. CA plots of (A) China Camp, (B) Walker Creek, and (C) Stege Marsh. Sorption capacity increases from left to right.

A total of 79 lipids were detected in the microcosm samples and used for individual CA analysis by sediment (Figure 6). Explanation of the variation on the first axis decreased (from 74% to 41.9%) with increasing sorption capacity of the sediments. The second axis had the opposite response, increasing from 13.7% for China Camp to 20.3% for SM.

The total PLFA content for the live controls was in the range of 8.3 ± 0.31 ($n=3$) to 12.43 ± 0.63 ($n=3$) nanomoles per gram of dry sediment. Both PLFA biomass and diversity increased in all sediments with increasing ciprofloxacin concentrations. The magnitude of the increase varied by sediment. SM had a smaller net increase of biomass and diversity compared to WC. CC had the largest net increase of biomass and diversity (Figure 3).

Diversity, measured as number of peaks detected, also increased at higher ciprofloxacin concentrations. The average total number of PLFAs detected in the live controls ranged from 25 ± 2 ($n=3$) to 31 ± 1 ($n=3$). Diversity increased by at least 50% in CC and WC sediments when ciprofloxacin concentrations were 2mg/L or higher (Figure 7).

The largest increase in diversity was observed in WC, followed by CC and the lowest increase was observed in SM. The PLFA fingerprint for the sulfate reducer community also indicates an increase in diversity. Effects on sulfate reducers are being explored more deeply using DNA based techniques, such as DGGE (denaturing gradient gel electrophoresis) and TRFLP (terminal restriction fragment length polymorphisms) with 16s rDNA universal bacterial primers and primers specific for sulfate reducing bacteria.

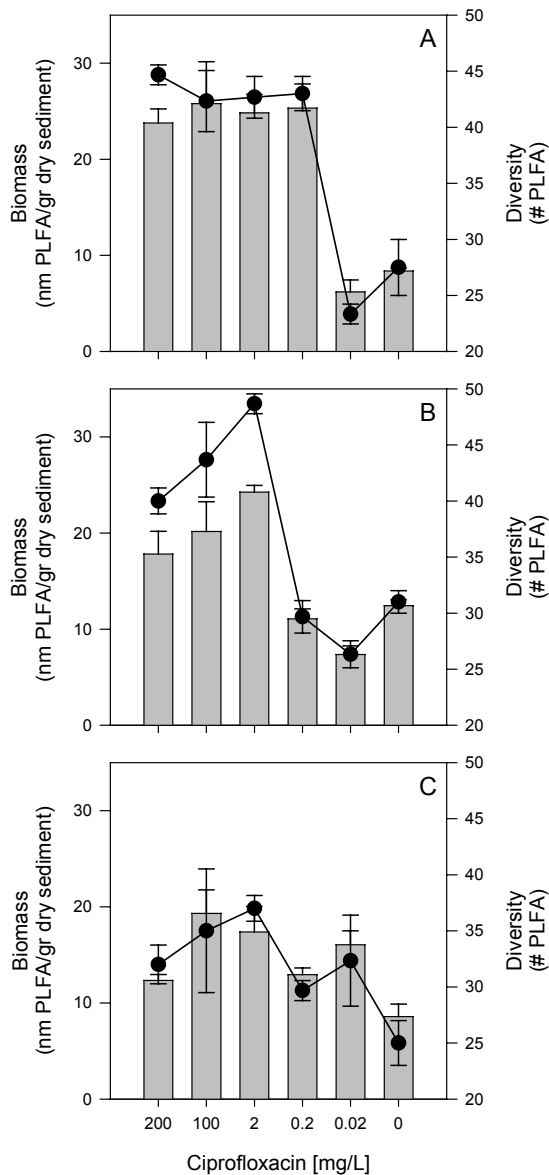


Figure 7. Effects on diversity and biomass. (A) China Camp, (B) Walker Creek, (C) Stege Marsh. Biomass and diversity are represented with bars and lines respectively. Error bars correspond to standard error.

Cross-cutting Work by BBC members

1. Scow's group participated in EIC's litterbag experiments, where microbial communities were analyzed using PLFA. Data was provided to Susan Williams, for which the results are found in the EIC annual report.

2. Holden's and Scow's research groups collaborated on a study (resulting in 2 manuscripts) investigating the relative effects of environmental and pollution variables on microbial community composition and diversity in marsh sediments. Two different yet complementary methods, targeting different biochemical constituents (lipids by phospholipid fatty acid PLFA and 16S rDNA by TRFLP), were used to characterize microbial communities. PLFAs represent living organisms because these molecules are readily degraded on death; they provide information about both taxonomy and physiology. DNA can persist for some time outside of cells and 16S rDNA provides taxonomic information of greater resolution than does PLFA. For both methods, environmental factors (such as flooding regime and marsh type) had larger effects on microorganisms than did pollution concentration. PLFA explained about two times the variation in microbial community composition that DNA methods did, most likely due to the rapid response of PLFA to short-term environmental variations (e.g. flooding regime), which was captured in the sampling. On the other hand, annual variations in community composition could be better discriminated by TRFLP than PLFA, most likely because biomarkers of microbial communities over all seasons were preserved as DNA. We concluded that both methods provided valuable information about how microbial communities respond to their environment and, in addition, individual PLFAs and TRFLP bands can serve as biomarkers indicative of environmental stress.

3. The spatial grid of leaf exudate metals conducted by Green has leapt across several spatial scales to combine chemical analysis with remote-sensing on the landscape scale. This joint effort by BBC and Ustin's Remote Sensing portion, funded by NASA, plans for publication of results that will include leaf salt exudation eco-physiology modeling by Dr. Morris of CEER-GOM.

FUTURE ACTIVITIES

The BBC group will spend much of the remaining no-cost-extension time wrapping up analyses, data interpretation, and manuscript production.

Fan. Interpretation regarding metabolite data sets, and how they relate to the metals and PC data, will continue. Work on manuscripts will be a top priority; Ms. Norris is currently writing up her M.S. thesis, and at least one manuscript is planned from that work in conjunction with the chamber work conducted by William's group.

Higashi and Green. We are wrapping up analyses and writing manuscripts in the coming year. In conjunction with other funding, a new M.S. student is tackling Hg concentrations in *Salicornia* with regards to spatial distribution, seasonal variation, and chemical form. Preliminary sampling showed elevated total Hg concentrations in the range of animals such as fish.

Holden. We are in the process of investigating possible relationships between denitrification and chemical gradients. Two manuscripts are under preparation, to be first-authored by Yiping Cao (doctoral candidate); tentative titles are "Significance of Particle-Associated Bacterial

Communities in Denitrification in Carpinteria Salt Marsh” and “Phylogenetic and denitrification community analysis along chemical gradients in Carpinteria Salt Marsh”.

Kuivila. In the coming no-cost extension period, we are completing data analysis/interpretation and participating in manuscripts. In particular, a manuscript is in preparation regarding toxicity of diuron to *Spartina* in collaboration with William’s group.

Nelson. With the limited time left under current funding we are finishing up our survey of total mercury (mainly methyl form) in the shore crab, *Pachygrapsus crassipes*. Between various PEEIR estuarine sites and a pristine control site this indicator showed more than a 50-fold difference in concentrations, and there were very different tendencies for trophic magnification in larger crabs depending on the site. Stege Marsh crabs showed moderate Hg levels (200 ppb) and no tendency toward biomagnification in larger individuals. Similar concentrations occurred in small crabs from Walker Creek and Tom’s Point estuaries, but larger individuals from these sites contained up to 1100 ppb and showed equivalent strong tendencies toward trophic magnification. These very different patterns of biomagnification may highlight food-chain differences that contribute to the relative impairment or health of a specific estuary. In our earlier surveys we used muscle mass of a crab leg as a surrogate for size. We are now using additional crab samples from these same sites to calibrate muscle mass against carapace width, a more widely accepted measure of crab size. We are finalizing *Pachygrapsus* surveys that will to assess the extent of impact of the Gambonini Mine at sites around Tomales Bay.

Scow. The final, no-cost extension period will be used to participate in manuscripts, and wrapping up data interpretation. Three of these manuscripts are listed below.

PUBLICATIONS & PRESENTATIONS

Publications

Cao, et al. Relationships between sediment microbial communities and pollutants in two California salt marshes. Manuscript by the Holden and Scow groups and other members of the Stressor Core. Manuscript is completed and undergoing final author review/comments.

Fleming, Mack, Green and Nelson (to be submitted by 8/1/05, Applied and Environmental Microbiology). Iron-reducing bacteria, novel methylators of mercury in pure culture and possible activity in mercury mine-impacted sediments.

Green, et al. Landscape-scale Contaminant Metal Emissions Estimates via *Spartina* Leaf Exudation in a Salt Marsh (tentative title). This is a multi-funding agency (EPA & NASA), cross-EaGLes manuscript with Dr. Ustin of the PEEIR Remote Sensing Component and Dr. Morris of CEER-COM. Manuscript data analysis is nearly complete, essentially awaiting tidal inundation data which is needed for the *Spartina* salt exudate production modeling.

Scow, et al. Diversity, composition and geographical distribution of estuarine sediment microbial communities along the California coastline. Manuscript in collaboration with Holden group UCSB). Manuscript is completed and going through final editing before submission for publication.

Presentations

Cao, Y and Holden, P.A. 2005 EPS Composition of Denitrifying Biofilms from Wild-Type and Quorum Sensing Mutants of *Pseudomonas aeruginosa* as a Function of Nitrate Concentration. Poster presentation and abstract. American Society for Microbiology, 2nd Conference on Cell-Cell Communication in Bacteria. July 23rd - 27th, 2004. Banff, Alberta, Canada.

Cordova, A.L and Scow, K.M. 2004. Effects of Ciprofloxacin on Microbial Communities in Marsh Sediment. Ana Lucia Cordova (presenter) and Kate M. Scow. 4th SETAC World Congress. November 14-18, 2004. Portland, Oregon.

Cordova, A.L and Scow, K.M. 2004. Fate and Effects of Ciprofloxacin on Estuarine Sediments. 4th International Conference of Pharmaceuticals and Endocrine Disruptors in Water, organized by NGWA. October 13-15, 2004. Minneapolis, Minnesota.

Cordova, A.L and Scow, K.M. 2004. Diversity of microbial Communities along pollutant gradients in California coastal marshes. 10th International Symposium on Microbial Ecology. August 22-27, 2004. Cancun, Mexico.

Cordova, A.L and Scow, K.M. 2004. Characterization of sediment microbial communities using PLFA: microbiology for the environmental chemistry lab. Agricultural and Environmental Chemistry Graduate Group Annual Colloquium. February 14-15, 2005. University of California, Davis.

Datta, S., Kuivila, K. 2004. Bioavailability of Pesticide contaminants in California Wetlands. Poster presentation at the Society of Environmental Toxicology and Chemistry NorCal Chapter Meeting, Berkeley, CA. April, 2004.

Datta, S., Smith, S., Kuivila, K. 2004. Determination of diuron and its metabolites in various matrices by solid phase extraction and HPLC-DAD. Platform presentation at the Society of Environmental Toxicology and Chemistry National Meeting. Portland, OR. November, 2004.

Green P.G., Norris A.S., Fan T.W-M., Vines C.A., Cherr G.N., Higashi, R.M.. 2004. Bio-assay of soil metals mobilized by salt marsh plants. 4th SETAC World Congress. Portland, OR. November, 2004.

Green P.G., Norris A.S., Fan T.W-M., Vines C.A., Cherr G.N., Higashi, R.M.. 2004. Rapid Analysis of Salt Exudates from Marsh Plants: Implications to Ecotoxicity and Bioavailability. Platform presentation at the CALFED Science Meeting, Sacramento, CA. October, 2004.

Higashi, R.M., Ustin, S.L., Williams, S.L., Fan, T.W-M. 2004. Interplay of tidal marsh plant biogeochemical and landscape ecology with nutrient and contaminant loads. 4th SETAC World Congress, Portland, OR. November 2004.

Kuivila, K., Datta, S. 2004. Pesticides in California Wetlands: Integrating Exposure and Effects. Platform presentation at the CALFED Science Meeting, Sacramento, CA. October, 2004.

Higashi, R.M. Tidal marsh plants as integrators of biogeochemistry, landscape ecology, contaminant mobilization. 4th Annual EaGLes Meeting. Duluth, MN. September 30 – October 1, 2004.

SUPPLEMENTAL KEYWORDS:

bioavailability, transformations, biogeochemistry, selenium, mercury, pesticides.