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The fate of toxic metals in marine sediments depends on a combination of the physical, chemical, and biologic conditions encountered in any given environment. These conditions may vary dramatically, both spatially and temporally, in response to factors ranging from seasonal changes and storm events to human activities such as dredging or remediation efforts. This paper describes a program designed to evaluate the interrelationships between the microbial community and pollutants in the New Bedford Harbor, Massachusetts, area, a U.S. Environmental Protection Agency designated Superfund site. Research has focused on establishing distributional relationships between contaminant metals, fluxes of metals between sediments and the overlying water, changes in microbial diversity in response to metals, and potential use of the microbial community as a biomarker of contaminant availability. This research has shown that a significant flux of metals to the water column is mediated by benthic biologic activity, and that microbial communities may be a responsive marker of contaminant stress. A combination of biogeochemical studies and the use of molecular tools can be used to improve our understanding of the fate and effect of heavy metals released to aquatic systems. — *Environ Health Perspect* 106(Suppl 4):1033–1039 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-4/1033-1039ford/abstract.html*

Key words: marine sediments, toxic metals, microbial ecosystem, bioavailability, molecular biomarkers

Introduction

New Bedford Harbor (NBH), Massachusetts, has a long history of anthropogenic contamination with bioaccumulation of both inorganic and organic pollutants in crustaceans, fin fish, and shellfish (*1–3*). Metals discharged into aquatic ecosystems are likely to be scavenged by particles, leading to their accumulation in sediments (*4*). A large reservoir of metals in the sediments can also act as a source to the overlying water column after their input to the ecosystem has ceased (*5*), potentially leading to adverse ecologic effects (*6*). However, the extent of the risks is difficult to accurately assess because of the complexity of biologic and chemical interactions that alter the bioavailability of metals. Release from sediments may not only result from resuspension of particulates, but also through the activity of microorganisms within the sediments and at the sediment–water interface, resulting in biotransformation to more volatile/soluble forms (*7*).

Microbial communities respond rapidly to environmental change and this should be reflected in specific parameters (biomarkers) of microbial structure and function (*8*). Lack of understanding of these interactions is in part due to the difficulty of characterizing microbial communities in environmental samples and in extrapolating laboratory-based data back to natural ecosystems (*9*). Genetic exchange and selection are readily shown in laboratory cultures. However, the complexity of the natural microbial community, interactions between species, and the difficulties of *in situ* measurement with minimal disturbance to the system, have made evaluation of microbial response to chemical pollutants extremely difficult. Advances in molecular techniques are beginning to change this perspective (*10,11*).

Bioavailability of Metals

Relationships between components of the aquatic environment and anthropogenic contaminants are not easily defined by purely analytical methods or toxicity testing methods such as selective extractions and elutriate toxicity testing. Recent research has shown that contaminants in interstitial water could be environmentally more significant than the adsorbed contaminants (*12,13*). In addition, the movement of contaminants, including pesticides, heavy metals, etc., is influenced by factors such as sorption, redox gradients, and pH, which in turn are greatly influenced by microbial communities and their activities. The bacterial community metabolism can affect valence states of metals via oxidation/reduction reactions, thereby altering the chemical speciation, fate, and the ultimate toxicity of the contaminant.

Many techniques to examine toxicity of contaminated sediments rely on bulk sediment testing and treat the processes controlling bioavailability as a black box (*14*). Although these techniques can indicate site-specific links between levels of contaminants in bulk sediment and toxicologic effects, they provide no information on the underlying factors controlling toxicity. Simple methods are needed to examine the bioavailability of contaminants in sediments, which can provide information on temporal and spatial variability of the effects of contaminants on ecosystem health. Use of the microbial ecosystem structure and function is explored in this review as an alternative methodology for examining bioavailability of contaminants and biologic effect.

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Abbreviations used: BB, Buzzards Bay; cDNA, copy DNA; mRNA, messenger RNA; NBH, New Bedford Harbor; PCA, principal components analysis; PCB, polychlorinated biphenyl; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; rRNA, ribosomal RNA; SD, standard deviation; U.S. EPA, U.S. Environmental Protection Agency.

Microbial Community Responses

The response of the microbial community to environmental stress is the result of a combination of factors (*8,15*). First, short generation times result in rapid selection for strains resistant and/or adaptive to the changing physical or chemical conditions. Second, relatively high spontaneous mutation rates in microbial populations (deletions, duplications, inversions, translocations, and insertions) increase the likelihood of emergence of resistant strains. Third, rapid genetic responses to external mutagens further increase the probability of emergent strains. Fourth, genetic exchange among bacteria (transformation, transduction, and conjugation) increases the likelihood of both intra- and interspecies transfer of resistance factors. Transduction may be a particularly important process because of the recent findings of high viral abundance in marine waters and high phage infection rates (*16*).

Amann et al. (*11*) recently reviewed the applications of ribosomal RNA (rRNA) analysis for examining microbial diversity. Essentially, DNA is extracted from an environmental sample and the polymerase chain reaction (PCR) is used to selectively amplify 16S rRNA genes or gene fragments. A gene library is then established from the amplification products and sequences determined from clones. A comparison of the sequences gives some indication of genetic diversity. This technique has been applied successfully to characterization of microorganisms from a number of environments, including thermal communities (*17*), deep subsurface communities (*18*), a wide range of marine bacterioplankton communities (*19–22*), aggregate-attached and free-living marine bacterial assemblages (*23*), and more recently, marine sediment communities (*24*). Gray and Herwig's analysis (*24*) identified a phylogenetically diverse population in creosote-contaminated surficial marine sediments with six major lineages of the bacteria domain represented. None of the clones contained in their study were identical to any representatives in the Ribosomal Database Project (*25*). Other recent approaches to characterization of uncultured microorganisms have included quantitative PCR methods to estimate abundance of an uncultured soil bacterium (*26*), examination of DNA polymorphisms within the phycocyanin locus to determine genetic diversity and phylogeny of toxic cyanobacteria (*27*), and comparisons of microbial community compositions using a back-propagating neural network and cluster analysis of 5S rRNA (*28*).

As an alternative to complete sequence information, restriction fragment length polymorphism (RFLP) analysis of 16S rRNA gene sequences provides an indication of microbial community differences. Essentially, the PCR is used to selectively amplify 16S ribosomal DNA from genomic DNA extracts from environmental samples, using primers specific to conserved regions of the 16S rRNA genes. Clones derived from a library of 16S rRNA genes are then treated with site-specific restriction endonucleases and the restriction fragment length patterns are compared. For example, this technique has been used to estimate diversity and community structure of a microbial mat from a hydrothermal vent system (*29*) and uncultured microorganisms associated with seagrass (*30*).

Comparison of rRNA sequences, unfortunately, does not provide a complete picture of sediment microbial diversity and community structure. A number of problems are associated with the technique: unknown species-to-species variation in rRNA genes; PCR may preferentially amplify certain rRNA genes, underestimating overall diversity; formation of chimeric sequences may occur, resulting from sequences of different species annealing to the same primary sequence (this would tend to overestimate diversity); and lack of quantitative methods to estimate species, i.e., 16S rRNA gene sequences, dominance (evenness), etc.

To begin to address the third problem associated with this technique, sequence-specific hybridization probes are developed for *in situ* hybridization of whole fixed cells in the original sample. For example, genes have been isolated that encode for degradation of a number of organic pollutants, including naphthalene and toluene, various polychlorinated biphenyl (PCB) isomers, 3-chlorobenzoate, and 2,4-dichlorophenoxyacetic acid (*31,32*). Erb and Wagner-Dobler (*33*) used extraction of total sediment DNA, PCR amplification of *bphC* sequences, and hybridization with specific gene probes to detect PCB degradation genes in polluted sediments. The *bphC* gene encodes the *meta*-cleavage enzyme of the aerobic catabolic pathway for PCB degradation. Although these authors could detect the presence of the gene, it could not be quantified. It was recognized that using messenger RNA (mRNA) instead of DNA would be necessary to establish degradative activity.

In the case of metal resistance, genes have been isolated that encode for resistance to a number of toxic metals including Cd, Co, Zn, Ni, Cr, Pb, Cu, and Hg (*34,35*). Barkay et al. (*36*) used a series of DNA probes to examine the abundance of the *mer* gene in the environment. The *merA* gene encodes for the nicotinamide adenine dinucleotide phosphate + H-dependent mercuric reductase that catalyzes the Hg(II) conversion to elemental Hg°, which is volatile and less toxic to the bacterium. Similar mechanisms have been proposed for other toxic metals (*37*). In some instances, the presence of a specific gene could reflect the presence of a metal-resistant community developed in response to specific pollutants. However, Nazaret et al. (*38,39*) pointed out that the presence of a specific gene does not mean that it is expressed in the environment. These authors demonstrate that sequences homologous to *mer* genes have been found in mercury-sensitive bacteria and are routinely found in uncontaminated environments. They argue that detection of *mer* gene products (mRNA transcripts and polypeptides), not the gene alone, are a better reflection of exposure to mercury contamination.

This review describes a research program designed to evaluate the interrelationships between the microbial community and pollutants in the NBH area, a U.S. Environmental Protection Agency (U.S. EPA) designated Superfund site.

Research in New Bedford Harbor

Study Site

New Bedford is a major fishing port on the eastern seaboard of the United States. The harbor was designated a U.S. EPA Superfund site in the early 1980s. Decades of industrial and municipal loadings into the Acushnet River estuary and NBH have resulted in high sediment concentrations of organic and inorganic pollutants, including PCBs, polyaromatic hydrocarbons, and heavy metals.

Distributional Relationships

A comprehensive evaluation of the distribution of toxic metals in sediments throughout the NBH area has shown marked gradients in contaminant loading from the inner harbor out into Buzzards Bay (BB) (*40*). Figure 1 provides a summary of representative values of Cr, Cu, Zn, Cd, and Pb concentrations in surficial sediments throughout the harbor. The multivariate

statistical technique principal components analysis (PCA) was used to characterize spatial and temporal patterns in the distribution of metals (Figure 2). The PCA plot calculated from the data for eight metals in 14 cores taken from NBH and BB revealed a number of characteristics. The highest concentration of metals occurs in the inner harbor and has a characteristic signature based on its Cd, Cr, and Cu content (major inputs are thought to have come from a copper/brass plant). However, sediments with this characteristic signature are confined at depth. More recent sediments, as well as all the sediments from the outer harbor area, are characterized by their Pb and Zn content. This is consistent with a model of a nonpoint source of Pb to the entire harbor, on top of which is superimposed a point source of metals to the inner harbor characterized by Cu, Cr, and Cd content. Mixing gradients are evident between these two types of sediments, as well as a gradient between the outer harbor and the clean sediments of BB (*40*).

To further understand source–receptor relationships of metal contamination in the harbor sediments, stable lead isotopes were determined in the sediment cores. The underlying assumption was that lead from different sources may have had different isotopic ratios and thus serve as chemical markers of the relative abundance of a source in a given sample. Isotope and total lead concentrations were determined by inductively coupled plasma–mass spectrometry using a procedure that yielded isotope ratios with relative standard deviations below 0.5%. This level of precision was necessary to discern small ratio differences between different sources of lead. Rather than rely on individual isotope ratios, multivariate techniques such as PCA were applied to the data to incorporate all the isotopic data. Multivariate source–receptor modeling of the data revealed three sources of lead that varied spatially between cores and temporally within cores. Results for two of the cores are shown in Figure 3, which shows the contribution of the three sources to the total lead burden. Figure 3 shows a peak in total lead at a depth of approximately 10 cm in both cores. However, the peak in the inner harbor core is due to source B, whereas the peak in the outer harbor core is due to source A. Without the isotope data, one would conclude a similar source of lead to both locations given the similar depth profiles of total lead. It should be noted that the inner harbor core was collected near the

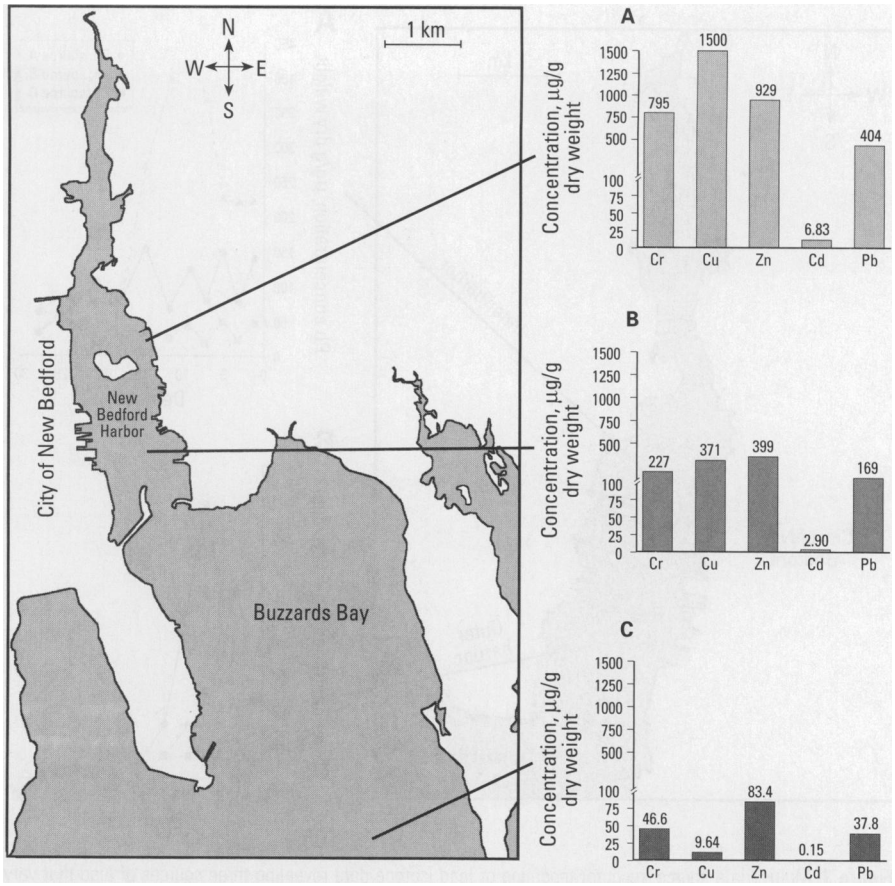

Figure 1. Map of New Bedford Harbor showing decreasing sediment metal concentration gradients out into Buzzards Bay.

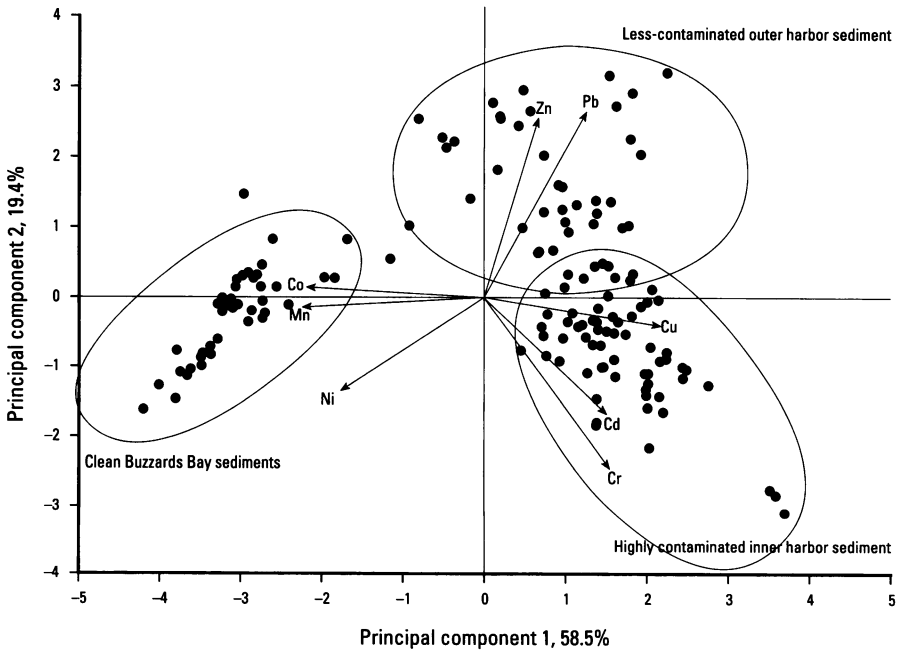

Figure 2. Biplot derived from principal components analysis illustrating spatial patterns in the distribution of metals. Adapted from Shine et al. (*40*).

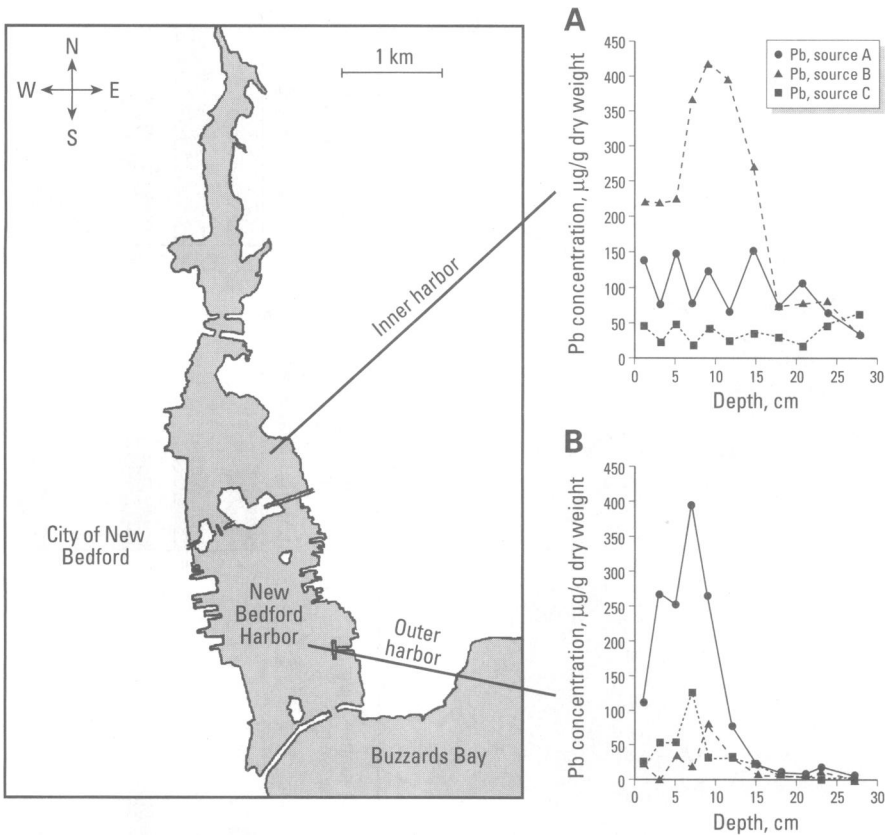

Figure 3. Multivariate source–receptor modeling of lead isotope data revealing three sources of lead that vary spatially between cores and temporally within cores. Results are shown for two of the cores.

former Revere Copper Products, Inc., factory, whereas the outer harbor core was collected near a sewage outfall. Source C, which was relatively constant (with depth in all the cores with a concentration of approximately 40 µg/g), has been tentatively identified as background crustal lead. The probable identities of sources A and B are currently being identified. In addition, the source–receptor results are being extended to the larger heavy metal data set described above.

Fluxes of Contaminant Metals

To investigate postdepositional mobility of metals, and perhaps bioavailability for correlation with microbial community parameters, sediment–water metal fluxes (Co, Ni, Cu, Zn, Cd, and Pb) were examined in laboratory microcosms. Multiple microcosms were collected seasonally from a contaminated site in the New Bedford inner harbor (depth approximately 3 m) and a control site in BB (depth approximately 17 m) to examine the temporal variability of the fluxes. Sediment samples were collected with a 225-cm^2 Soutar-style box corer and closely fitted acrylic liners used to remove sediment and overlying water with minimal disturbance. Microcosms, consisting of the acrylic liners sealed both top and bottom, contained approximately 25 cm of sediment overlaid by 20 cm (4.5 liters) of seawater. Fluxes were determined by investigating changes in the inventory of dissolved metals in the overlying water over a period of 10 to 14 days. Following determination of sediment-water exchange of metals, benthic oxygen demand was measured in each microcosm. Additional data included sediment metal content, organic carbon, oxygen, and sulfide (*41*).

The net flux of dissolved metals was generally out of the sediments into the water. However, at low rates of benthic oxygen demand (winter), the net flux of Pb and Co was into the sediments. The gross fluxes of Zn and Cu were higher than for other metals, especially in the NBH microcosms. However, this was probably due to the fact that the sediments from NBH were highly enriched in these metals. Although gross fluxes are ecologically relevant to metal cycling in NBH, normalizing the fluxes with respect to sediment metal content provided information on the efficiency with which the different metals were returned from sediments to the water column. The mean and range of the normalized flux values from the 14 microcosms are shown in Table 1. Within individual microcosms, the order of the normalized fluxes generally showed the following relationship: Cd > Zn > Co, Ni, Cu > Pb, indicating higher relative mobility for Cd and lower mobility for Pb. With respect to seasonal variation, regression of data from both sites against benthic oxygen demand was significant for each metal (r^2 = 0.6–0.8). Thus, as benthic oxygen demand changed between seasons (high in summer, low in winter), the fluxes of metals also varied directly with these changes. In addition, because slopes for the relationship were different, the relative amounts of metals released varied in different seasons. The slope of the Cd relationship was the highest, whereas the slope for Pb fluxes was relatively low. Because metals in porewaters are thought to be more bioavailable, this highlights the importance of temporal variability in benthic activity in altering the potential bioavailability of metals in sediments as well as fluxes back to the overlying water column.

Microbial Community Responses

As part of a preliminary survey, initial work on pollutant effects on the structure and function of the microbial community examined colony formation as a function of metal concentration (*42*). Certain contaminated sites within the NBH showed an apparent lower diversity of colony types than less contaminated sites; however, the data were inconclusive because of small

Table 1. Sediment–water fluxes of dissolved heavy metals normalized to sediment metal/organic carbon content measured in microcosms collected from New Bedford Harbor and Buzzards Bay.

Metal	Sediment–water flux[a]	
	Mean (n=14)[b]	Range[c]
Co	0.58	–0.42–2.0
Ni	2.0	0.097–6.4
Cu	0.91	0.26–2.5
Zn	9.3	–1.4–35
Cd	14	1.2–42
Pb	0.079	–0.14–0.46

[a] Normalized to sediment metal content. To allow for comparison between metals, metal fluxes are adjusted for sediment metal content, which in turn is expressed on an organic carbon normalized basis (*4,39*). [b] $(mmol/m^2/d)/(mol/molC)_{sediment}$. [c] $(mmol/m^2/d)/(mol/mol\ C)_{sediment}$. A positive value indicates a flux out of the sediment to the overlying water, a negative value indicates a flux into the sediments.

sample size. Most recent results for samples taken during the summer of 1995 indicate that microbial diversity is actually greater in NBH than in BB. Shannon–Wiener Indices (*43*) were 1.71 (standard deviation [SD] 0.2; $n = 5$) for NBH and 1.46 (SD 0.22; $n = 6$) for BB (*44*). These results may be attributed to more available nutrients in the harbor relative to the bay. The inconsistency between these and earlier results is not surprising considering the now well-documented limitations associated with culturing environmental isolates (*8,10,11*).

Within the harbor, chemical analysis of sediments is clearly sufficient to establish the extreme levels of pollution. However, biomarkers may be used to provide both a rapid evaluation of bioavailability and biologic effect. The NBH provides a highly polluted environment with a complex mixture of contaminants to begin to examine sediment microbial community DNA for specific patterns rather than specific markers. These patterns can then be compared to microbial community DNA along pollution gradients out into BB. Further analysis will allow identification of genetic markers of metabolic potential that might exist in this particular system. For example, based on initial bioavailability studies, selection for a Cd efflux system might be advantageous in the microbial population.

Molecular approaches are currently being used to test the hypothesis that bacterial species diversity in NBH sediments has changed because of input of heavy metals and synthetic organic compounds. Genetic diversity of microbial communities has been systematically characterized in highly polluted marine sediments along a contamination gradient from NBH out into BB (*44*). Procedures entailed extracting DNA from surface sediments using adaptations of the techniques used by Tsai and Olson (*45*). The methods involve amplification of 16S rRNA genes from purified sediment DNA using specific eu-bacterial primers via the PCR, subcloning the genes into *Escherichia coli* with the vector pCRII (Invitrogen, San Diego, CA) arbitrary endonuclease digestions of unique 16S rRNA gene sequences, and resolution of the varying size DNA fragments by gel electrophoresis. These fragment patterns, RFLPs, were used to describe the genetic diversity. Figure 4 shows a representative RFLP pattern from a contaminated site in NBH and a cleaner site in BB. In agreement with the most recent plate-culturing data, initial data on the RFLP pattern from NBH suggest a greater diversity of genotypes relative to BB. Phylogenetic analyses using fragment data from several hundred site-specific operational taxonomic units have been performed and distance matrix methods used for tree reconstruction to reveal degrees of similarity in genetic diversity, both between different environmental sites and between different seasons within a given site. Further statistical analysis has been performed to determine whether specific pollutants are correlated with bacterial genetic diversity in this ecosystem.

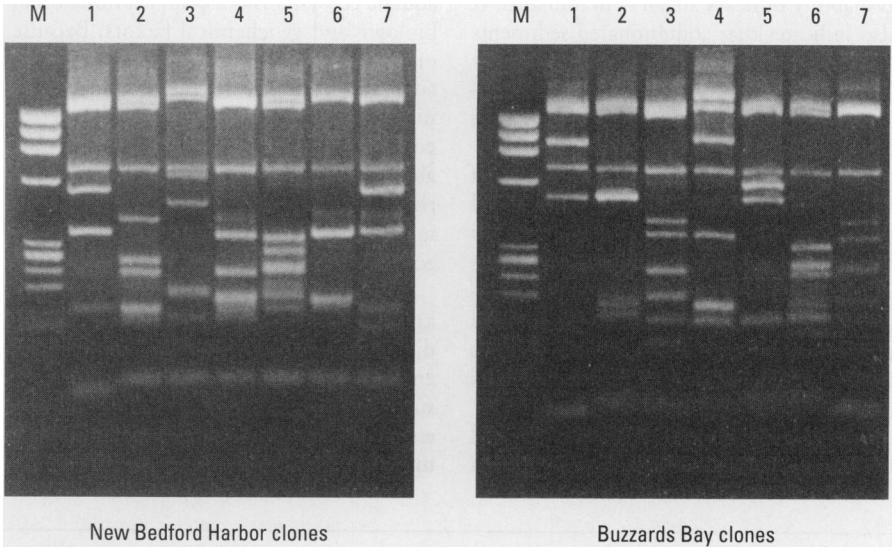

Figure 4. Endonuclease digestion of 16S rRNA gene sequences showing RFLP patterns from New Bedford Harbor and Buzzards Bay sediment extractions. Modified from Sorci (*44*).

The second aim of the microbiologic research has attempted to elucidate gene sequences induced in NBH isolates, which are potentially responsible for certain mechanisms of metal resistance. Culturable microorganisms from NBH sediments were isolated on marine agar plates amended with various heavy metals at differing concentrations (*45*). A bacterium has been isolated from the harbor sediments capable of growth on marine agar buffered with Tris (pH 7.8) and amended with the following individual concentrations of metals: 1 mM Cd, 10 mM Cr, 5 mM Co, 10 mM Cu, 1 mM Pb, 0.1 mM Hg, 5 mM Ni, and 2.5 mM Zn, respectively. Laboratory simulations culturing this isolate under stressed and nonstressed conditions, i.e., in the presence and absence of metals, respectively, were performed. mRNA was extracted from these cultures and has been used in differential display PCR experiments to identify specific inducible genes responsible for metal resistance. This method, originally designed to isolate and clone differentially expressed copy DNAs (cDNAs) from eukaryotic cells (*46*), has been modified to use procaryotic mRNA to produce differing amplification profiles reflecting RNA levels and diversity. Total RNA extracted from microbial populations is reverse transcribed to cDNA. Short sequences are then amplified (subpopulations) using defined sets of random oligonucleotide primers and the polymerase chain reaction, as defined previously. The cDNA products resulting from each set of primers are then resolved simultaneously on denaturing polyacrylamide gels and visualized by autoradiography. A major strength of this procedure is that only genes being expressed, not merely present, contribute to the fingerprint. In addition, unique cDNAs can be subcloned and sequenced to identify genes that convey differential survival among microbial populations and can be compared to specific metal-resistance genes in extant databases. Although differential expression of mRNA has been shown in stressed and nonstressed isolates from NBH, function of differentially expressed genes has yet to be confirmed (*45*).

Discussion

The biogeochemical study of metal recycling at the sediment–water interface shows the role of biologic communities in the flux of metals from contaminated sediments to the overlying water and identifies different ranges of effects for different metals. This has broad implications for the role of wetlands and coastal marine waters as traps for specific contaminants and highlights the role of biologic activity in altering the

availability of heavy metal contaminants. It also indicates that contaminated sediments can remain a source of metals after their input to the environment has ceased, perhaps attenuating anticipated improvements in water quality.

The detailed study of partitioning of metals in sediments leading to uptake and bioaccumulation in the food chain is continuing. Metal speciation experiments are beginning to add to the understanding of bioavailable forms of metals and future research will address relationships between contaminant speciation and microbial response.

The distribution, transport, fate, and effects of heavy metal contaminants in aquatic ecosystems is a complex function of biologic and geochemical factors. Because of rapid selection pressures and/or genetic adaptation, the natural microbial community may provide a sensitive biomarker of environmental stress. However, considerable work is still necessary to identify either the key molecular tools or the specific markers that can be used to evaluate ecosystem health.

It is clear that any specific molecular approach is not necessarily going to directly reflect effects of exposure to an environmental pollutant or other form of stress. A combination of techniques is necessary to develop an approach to using the microbial community as an indicator of stress, including molecular approaches outlined above. In some cases, the more classical approach of enriching for specific organisms with resistive or degradative abilities may be useful. Subsequent analysis of these isolates can then lead to construction of function-specific probes.

Research in this area promises to provide a clearer understanding of microbial community responses to environmental stress. A combination of the fingerprint approach (rRNA), markers of microbial activity (mRNA), specific markers of catabolic or resistance genes, and better knowledge of metal speciation and availability will improve the understanding of these complex coastal ecosystems.

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