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Assessing Microbial Indicators for Heavy Metal Contamination using Automated Image Analysis

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1. Summary

The aim of this project was to develop and evaluate an image analysis protocol for the analysis of specific populations of bacteria in contaminated environments. The protocol was based on molecular detection procedures, i.e. *in situ* hybridization and subsequent epifluorescence microscopy. Processing of captured microscopy images allowed us to specifically enumerate hybridized bacteria, measure their cell sizes, subsequently calculate their biovolumes and estimate their biomass. This procedure was used to analyze bacterial populations in sediment samples from Kearny Marsh, some of which were artificially contaminated with nickel (Ni) and incubated under sulfate-reducing conditions. Compared to non-amended samples, Ni-amended samples generally displayed lower cell numbers, but cell size distributions in a larger range. These result indicated the development of different bacterial populations, and thus demonstrated the usefulness of the developed image analysis protocol.

A second evaluation of the image analysis protocol was attempted in a concomitant greenhouse study using Ni-amended sediment cores with *Spartina patens* obtained from Harrier Marsh. However, within the project time frame only baseline data on heavy metals in sediments and plants could be gathered. Ni concentrations in the original sediments from Harrier marsh averaged at about 20 ppm with decreasing concentrations with depth. Ni-amendment ($NiSO_4$ at 50 mg kg^{-1} core material) resulted in an increased uptake of Ni by *S. patens* with up to 250 ppm compared to 32 ppm for non-amended sediments. Uptake into roots of *S. patens* was accompanied by only little translocation into above-ground material. Similar results were obtained when plants were analyzed for heavy metals present in sediments (i.e. Cu, Cd, Cr, Pb, and Zn). These data provide perfect baseline information for studies on the interactions between different organisms and heavy metals that could be exploited for studies on key organisms with potential bioindicator function. The technologies developed in this project will be useful in achieving this goal in future research.

2. Introduction

New Jersey is one of the most densely populated areas of the U.S. with many industrial sites that border waterfronts of rivers, lakes, estuarine environments, canals, or the ocean. Due to the concentration of industries in these regions, many urban waterfront environments have been extensively contaminated. Industrial activities have generated a variety of waste streams that consist of highly heterogeneous mixtures of organic and/or inorganic material. Estuarine soils and sediments have intercepted these waste streams and are the repositories of decades of accumulated contamination with metals, petroleum hydrocarbons, polyaromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs). In New Jersey, which currently tops the list of states with EPA "Superfund Sites", numerous sites are located in densely populated urban settings with soils containing elevated concentrations of heavy metals ("Brownfields"). Contamination is not restricted to urban settings, landfills, industrial sites, or abandoned industrial areas, but extends to adjacent natural habitats. This is the case in many areas in the Northeast, such as the urban salt marshes of the Hackensack Meadowlands, which is part of the larger

New York/New Jersey Harbor Estuary. Numerous sites in the Hackensack Meadowlands have been identified to be contaminated with heavy metals such as Hg, Cr, Cu, Pb, Ni and Zn, and/or with organic pollutants such as petroleum hydrocarbons, PAHs, and PCBs. Contamination can pose not only a threat to ecosystems, but also to human health through groundwater infiltration and exposure via the food web (see (Giller et al. 1998) for review). The risks associated with accumulated contamination are especially pronounced if pollutants are recalcitrant to degradation or persistent and bioaccumulative; as such, these types of sites cannot be developed, redeveloped or restored and provide no economic benefit to industries and the government. Therefore, the management of contaminated urban settings, contaminated urban salt marshes, and contaminated shore-front properties is a major problem.

The New Jersey Meadowlands Commission (NJMC) is chartered with the restoration of several sites in the Meadowlands including wetlands and landfills. Ecological restoration, however, can change environmental conditions dramatically which may result in alterations of contaminant mobility and bio-availability. Contaminant mobility is a function of complexation and sorption interactions between the aqueous, biological, and various mineral phases in contact with that aqueous phase. Mobility and bio-availability of heavy metals depend on their speciation that is determined by various physicochemical and biological parameters such as the nature of soil and sediment particles, the amount of organic matter, the vegetation, and the presence and activity of microorganisms. The bioavailability of metals is therefore the consequence of the interactions between metals and soil/sediments, plants, and the microbial community. Thus, although ecological restoration can generally be viewed as positive, its impact on inorganic and organic contaminants in the environment must be understood for risk assessment, long-term management, and technology development.

NJMCs current wetland restoration work in the Meadowlands involves restoration and cleanup of moderately contaminated salt marshes such as Harrier Meadow (North Arlington) that was used as disposal site for shot rock from construction of U.S Route 280 in the past. At the Harrier Meadow wetlands site construction work was completed during September 1998. At the moment, portions of the area support a mixture of native high salt marsh vegetation, dominated by salt hay (*Spartina patens*), and open water. Other areas consist of dense monocultures of invasive species, common reed (*Phragmites australis*) and purple loosestrife (*Lythrum salicaria*), that are rapidly replacing native wetland species throughout the northeastern US. During the last year, several baseline studies were conducted at Harrier Meadow and there are plans to install an environmental monitoring system for long-term-monitoring of basic environmental data. In one of these baseline studies conducted in our laboratory, sediment from Harrier Meadow was found to be only moderately contaminated with levels of metal contamination close to regulatory action levels (25 ppm Ni, 45 ppm Cr, 3 ppm Cd, 72 ppm Cu, 270 ppm Pb, 670 ppm Zn). *S. patens* plants growing in this sediment, however, were found to take up these heavy metals. Similar results had been obtained with *S. alterniflora* and *P. australis* that were shown to be involved in the translocation of heavy metals with different rates and modes of uptake and release (Burke et al. 2000). *S. alterniflora* takes up more heavy metals than *P. australis* (esp. chromium and lead) and more readily releases them through plant detritus or direct excretion on leaf and stem surfaces (Burke et al. 2000). *S. patens*, *S. alterniflora* and *P. australis* are fast-growing at different salinities, produce large biomass yields and are known to change environmental conditions in bulk soil and rhizosphere. They also stimulate microbial activities by releasing chelating root exudates, oxygen through their aerenchyma system (Sundby et al. 1998), and salts and organic material through leaf litter deposition (Burke et al. 2000, Kraus et al. 1986, Kraus 1988).

The activity of soil microorganisms is generally stimulated in the rhizosphere of plants. The rhizosphere microbial community is comprised of microorganisms with distinct metabolism and adaptive responses to environmental conditions. Exudation of a variety of soluble organic compounds by plant roots plays an important part in microbial growth and activity in the rhizosphere. Since root exudation provides microorganisms with a readily available carbon source (Patriquin and Keddy 1978, Whiting et al. 1986), the metabolic activity for some groups is greatest when they were physically associated with root surfaces (Hines et al. 1999). Activity of root-associated bacteria, however, also depends on interrelationships with other organisms. In salt marshes, *S. patens* usually forms a mutualistic association with arbuscular mycorrhizal fungi (AMF). AMF are highly effective in acquiring and supplying nutrients

and trace elements (including heavy metals) from the sediment to the host plant (Hayman 1986). In a recent study, we found that the presence of AMF on plant roots affects microbial biomass and community structure in soil, probably through competition for carbon supplied by the plant (Burke et al. submitted). The presence of AMF might therefore increase heavy metal uptake by *S. patens*, but decrease bacterial activity in the rhizosphere. Therefore, the presence of *S. patens* and associated AMF symbionts may affect not only the fate of heavy metals and organic contaminants in the environment directly, but also indirectly by changing environmental conditions for microorganisms.

Since microorganisms play an intimate role in transformations of both inorganic and organic compounds in nature, contamination with heavy metals affects ecosystem health by altering community structure and function of microorganisms (Dahlin et al. 1997, Sandaa et al. 1999). Heavy metals, for example, have large effects on processes important for maintaining fertility and primary production of soils (Giller et al. 1998). Their accumulated presence leads to a reduction of total microbial biomass (Brookes and McGrath 1984, Fliessbach et al. 1994), to a decrease in numbers of specific populations such as infecting rhizobia (Chaudri et al. 1993) or mycorrhizae (Koomen et al. 1990), or to shifts in microbial community structure and a reduction of total microbial diversity (Bååth et al. 1998, Griffiths et al. 1997). Key functions in soil processes such as N mineralization and immobilization, mineralization of organic material, and nitrogen fixation are inhibited even by levels of metal contamination below regulatory action levels (Chaudri et al. 1993). Analysis of microorganisms at the community level in contaminated environments is therefore ecologically relevant and reflects responses of a large number of species that have the same function or a range of sensitivities to the contaminants present. Community analysis also reflects integrated conditions over a relatively long period of time.

These results, however, are entirely based on studies conducted on agricultural systems. But even for these systems, data available on structure and function of microbial communities are only of limited value since most of them have been obtained by growth-dependent detection methods. Studies on microbial communities, however, are generally impeded by the fact that most microorganisms resist cultivation, which is an essential prelude to characterization by many traditional methods. Detection methods relying on the isolation of microorganisms can therefore be extremely selective and usually underestimate numbers of microorganisms and diversity of microbial communities (Richaume et al. 1993, Sorheim et al. 1989). Recently, however, growth-dependent methods in studies on microbial communities in the environment were increasingly supplemented or even replaced by molecular methods (see Amann et al. (1995) for review). Because molecular methods target sequences of macromolecules (DNA, mRNA or rRNA) that characterize organisms and their potential metabolic activities, rather than the organisms themselves, these methods allow researchers to study microbial communities unaffected by the limitations of culturability and to get an indication of their *in situ* abundance and activity. Such methods, for example, revealed the abundance in soil of yet uncultured bacterial populations in numbers that by far exceeded those of all bacteria usually detected by growth-dependent protocols (Chatzinotas et al. 1998, Zarda et al. 1997). In earlier studies we have shown that molecular methods are sensitive tools for the analysis of impacts of heavy metal contamination on microbial communities (Sandaa et al. 1999). Levels of metal contamination below regulatory action levels already reduced biodiversity, induced shifts in community structure and impaired activities such as nitrogen fixation.

Different molecular techniques have been developed and employed in our laboratory for the analysis of specific microbial populations in terrestrial systems (Chatzinotas et al. 1998; Schönholzer et al. 1999). Most of these methods, however, are still used at the experimental level. Studies generally demonstrate the applicability of the methods for the analysis but information on the significance of the results in the environment is usually not provided. Since the methods are usually time- and labor-consuming researchers generally apply one method of detection focusing on methodological problems, on the analysis of single pristine or extreme environments or on short-term ecological studies conducted under laboratory conditions. In order to increase the significance of the studies and the content of information, experimental formats are needed that reduce time and labor and provide for greater sample capacity and greater sensitivity. Recent developments in technology have provided the foundation for such formats with automated image analysis as one of the most promising technologies. Image analysis technologies are based on the analysis of stained microorganisms in epifluorescence microscopy images (Fry 1990).

Recently, they have become increasingly important tools for the localization and quantification of fungi, bacteria and protozoa in aquatic and terrestrial environments since criteria for the specific detection and quantification can be defined and standardized, and size measurements be used to estimate microbial biomass (Bloem et al. 1995, Daniel et al. 1995). In combination with the *in situ* hybridization technique in which fluorescent oligonucleotide probes hybridize to rRNA sequences in fixed whole cells (see Amann et al. (1995) for review), measurements of signal intensities can be used to demonstrate activities of specific populations.

In a previous study, we developed an image analysis protocol for the analysis of DAPI-stained bacteria in soils (Schönholzer et al. 1999). High-quality images were obtained by the optimizing the sample preparation ensuring an equal dispersion of the target cells in thin layers (Daniel et al. 1995). Image quality was enhanced by the use of DAPI as high contrast fluorochrome, and image analysis optimized by the definition and standardization of specific criteria for the automated image analysis protocol (Schönholzer et al. 1998, 1999). In addition to DAPI-stained bacteria, this image analysis protocol also allowed us to quantify filamentous fungi that were visualized by *in situ* hybridization with Cy3-labeled oligonucleotide probes (Schönholzer et al. 1999). Cy3 is a very photostable carbocyanine dye that produces bright signals due to a high molar extinction coefficient (150000 $M^{-1}cm^{-1}$) and a high quantum yield (Mujumdar et al. 1989). Analyzed with optimized high quality filter systems, signals from Cy3-labeled probes were easily distinguishable from background autofluorescence of organic material. Detectability of bacterial populations in soil was also significantly enhanced when Cy3-labeled probes and the respective optimized filter system was used for the analysis rather than the commonly available dyes FITC and TRITC (Zarda et al. 1997).

The objective of this study was to develop an automated image analysis protocol for the detection and quantification of bacteria indicative of heavy metal contamination in aquatic systems. The protocol was based on images obtained after staining with 4'-6-diamidino-2-phenylindole (DAPI) as a universal stain intercalating into DNA, and after *in situ* hybridization with Cy3-labeled, rRNA-targeted oligonucleotide probes. The applicability of this protocol was evaluated in two environmental systems with different complexity. The first system was an artifical aquatic system with limited complexity with respect to environmental factors represented by laboratory microcosms. In this system we used the image analysis protocol to study shifts in microbial populations in time after artificially increasing heavy metal availability (nickel). The second was a highly complex terrestrial system represented by soil cores with plants (*Spartina patens*) that were kept in the greenhouse. In this systems the image analysis protocol was used to investigate seasonal and vegetational effects on heavy metal bioavailability (nickel) in contaminated sediments and its concomitant influence on microbial community dynamics.

3. Image Analysis Protocol

3.1. Epifluorescence Microscopy

Aliquots (10 µl) of fixed and dispersed samples spotted onto gelatine-coated slides (Glöckner et al. 1996) were hybridized and concomitantly stained with DAPI according to Zarda et al. (1997). Slides were mounted with Citifluor solution and the preparations were examined at 400 x magnification (Nikon CFI Plan-Apo 40x/1.30 Oil) with a Nikon High Resolution Inverted Microscope (Eclipse TE200) fitted for epifluorescence with a high-pressure mercury bulb (100 W) and filter sets UV-3A (EX330-380, DM400, BA435-485 for DAPI) and HQ-Cy3 (G535/50, FT565, BP610/75 for Cy3) (Schönholzer et al. 1999). For the analysis of pure cultures of bacteria, samples on slides were scanned on a continuous area of about 0.4 mm^2 (about 30 images). Bacteria from environmental samples were analyzed in 21 images per sample with up to 400 cells per image.

DAPI- and Cy3-images were captured separately from the same scenery (exposure time 0.64 and 1.20 sec, respectively) with a cooled digital video camera (DEI-750TD, Optronics) and with three CCD-chips for the basic colors red (R), green (G) and blue (B). For the analysis of DAPI-stained cells, both green and blue channels were used with the red channel shut down, using the filter set UV-3A, while Cy3-stained cells were captured with the red channel with the green and blue channels shut down, using the filter set HQ-Cy3 to obtain separate signals. DAPI- and Cy3-images were subsequently merged to produce a virtual RGB-image.

3.2. Image Processing

For processing the images from fluorescence microscopy, the software IDL (Research Syst., Inc.) and the image analysis tools described by Russ (Russ 1990) were used. Intensity relations and gradients in the color channels were used for a differential detection of objects based on i) the detection of DAPI-signals, and ii) the detection of Cy3-signals. Three categories of objects were detected: i) objects stained by DAPI, ii) objects stained by both DAPI- and Cy3, and iii) objects stained basically only by Cy3, since the corresponding DAPI-signal had only a low intensity due to quenching effects.

DAPI-stained bacteria were detected following an improved version of our image processing protocol described previously using three separate pathways for the differentiation between bacteria and debris particles (Schönholzer et al. 1999). This process is based on i) the preliminary detection of all objects in the green channel (both bacteria and debris), ii) the differentiation between bacteria and debris, based on processing the green and the blue channel, and iii) the combination of the outputs of i and ii by eliminating the debris particles from the image. The detected and segmented DAPI-signals were used as baseline data for Cy3-signal detection, since *in situ*-hybridized bacteria make up a portion of the DAPI-stained bacteria. The detection limit for numbers of *in situ*-hybridized bacteria was set to 3% of the number of DAPI-stained cells. This corresponded to about 6 bacterial cells for a typical image with about 200 DAPI-stained bacteria. Below this limit, the high impact of every debris particle that was counted erroneously hindered an appropriate counting of *in situ*-hybridized bacteria. In the initial detection step, the red channel was processed to obtain all objects that came in consideration to be *in situ*-hybridized bacteria. This was basically done by setting a grey value threshold to separate objects (brighter than the threshold value) from the background (darker than the threshold value). However, neither the objects nor the background had a consistent brightness over the whole image area due to e.g. autofluorescence effects or inhomogeneous illumination. Therefore, a reliable differentiation between objects and background required a special grey value transformation, the dog-filter (Difference of Gaussians; Ò1 = 1.0, Ò2 = 0.625) (Marr and Hildreth 1980, Russ 1990) which was used to achieve a homogeneous background and to quench large areas of fluorescence. The calculation of the threshold for image segmentation was performed following a procedure based on the grey value with the maximum frequency and described previously (Schönholzer et al. 1999).

Since the segmented image of the red channel contained both bacteria and debris particles, additional procedures were necessary to identify bacterial cells, resulting in a subdivision of objects into two categories. The first category of objects consisted of objects that were found to have the same image co-ordinates as bacteria identified previously by the DAPI-detection procedure. Their locations were identified based on Feature-AND operation. The second category was the remainder of objects. As a result of quenching effects, DAPI signals of these objects were too weak to be detected by the DAPI-detection procedure. These objects made up to 25% of all *in situ*-hybridized bacteria. They were identified by a procedure using information from all of the three colour channels, based on i) RG-ratio intensity and ii) signal intensity of the blue channel. The RG-ratio was found to be a valuable indicator for *in situ*-hybridized cells in the presence of quenched, weak DAPI-signals, since high intensities in the red channel combined with low intensities in the green channel indicated the absence of the green fluorescence typical for debris particles. Spots with this property were identified by processing the red and green channel by background subtraction (dark rank filters; (Anonymous 1992)), followed by division, dog-filtering to obtain a homogenous background, automated threshold calculation and image segmentation. The signal intensity of the blue channel was used to detect spots considered to indicate weak DAPI signals. These spots were identified by processing the blue channel by background subtraction (dark rank filters), automated threshold calculation and segmentation. Afterwards, the results of both RG-ratio and blue channel processing were combined by Feature-AND operation, limiting the number of image spots to those indicating both signals from *in situ* hybridization and low DAPI intensity. They were used as information to identify objects of the second category. Objects of the segmented image of the red channel, which did not belong to either the first or second category were classified as debris particles.

4. Image Analysis Protocol Evaluation

4.1. Microcosm Study

5-g-sediment samples (fresh wt.) from Kearny Marsh were incubated in airtight serum bottles with 100 ml medium C for sulfate-reducing bacteria (lactate medium for *Desulfovibrio* and *Desulfotomaculum*) (Widdel and Bak 1992) under a N_2/CO_2 headspace (n = 28) (Fig. 1). These conditions support fermentative growth of bacteria as well as sulfate reduction.

Fig. 1. Microcosm setup

Two third of the microcosms were spiked with nickel sulfate (160 ppm Ni), one third kept unspiked. In half of the Ni-amended microcosms microbial activity was suppressed with a cocktail of different antibiotics during incubation for 21 days at 25°C (final conc.: Penicillin, 100 µg ml^{-1}; Streptomycin, 100 µg ml^{-1}; Tetracycline, 50 µg ml^{-1}; Chloramphenicol, 170 µg ml^{-1};). This treatment with antibiotics was repeated after 3 days to ensure complete suppression. Microcosms (n = 4) were destructively sampled at different times (t = 0, 1, 2, 3, 6, 14, and 21 days) and analyzed for sulfate and sulfide (Spectrophotometry), Ni (Atomic Absorption Spectroscopy), and microorganisms (*In situ* hybridization and image analysis).

Sulfate was reduced during incubation in both Ni-amended and non-amended microcosms (Fig. 2), but not in those amended with Ni and antibiotics (data not shown). This suggested microbial activity in Ni-amended and non-amended microcosms and an effective suppression of microbial growth by the antibiotics in the control microcosms. Sulfate reduction, however, could not be correlated to sulfide production. Sulfide could only be detected in non-amended, but not in Ni-amended microcosms (Fig. 2). This failure in detection might be due to the immediate removal of the biogenic H_2S by precipitation with heavy metals added. During incubation, the concentration of dissolved Ni was decreasing (Fig. 3) which was mainly attributed to sorption of dissolved Ni to particulate matter in the microcosms. Small amounts, however, might have been precipitated as NiS after reaction with biogenic H_2S.

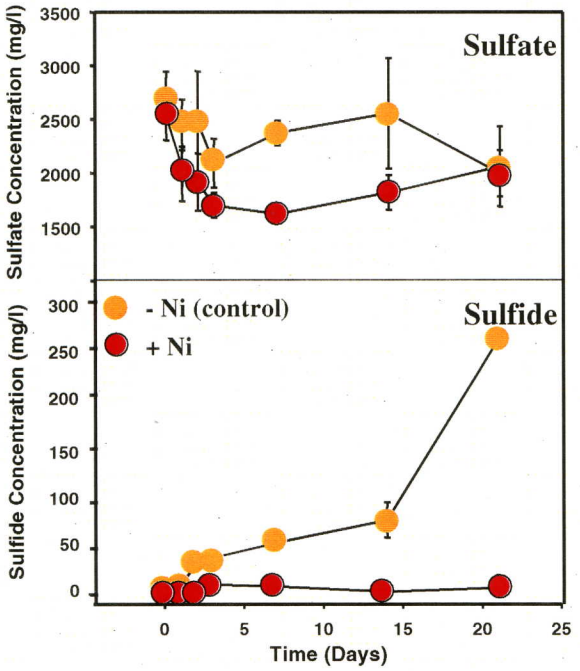

Fig. 2. Sulfate and sulfide profiles in microcosms (0 to 21 days)

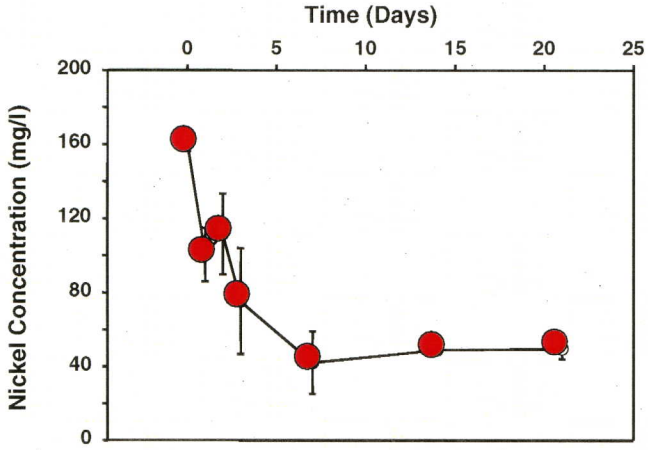

Fig. 3. Concentration of dissolved Ni in Ni-amended microcosms during incubation (0 to 21 days)

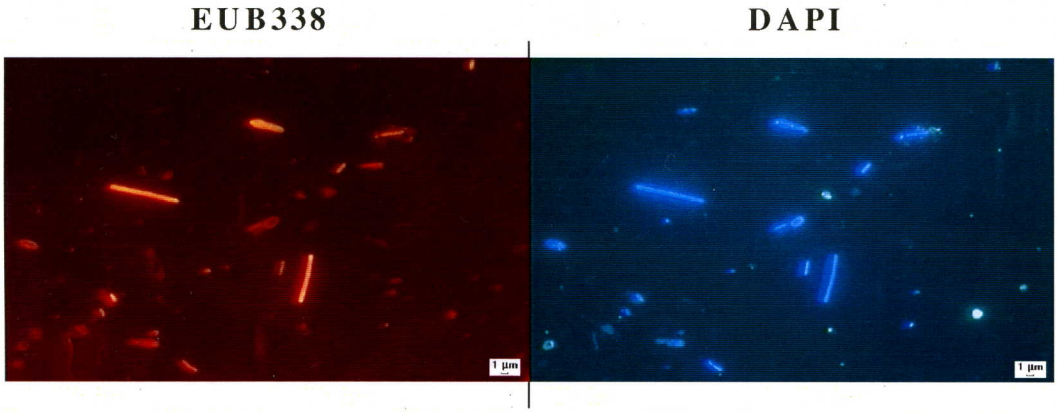

Fig. 4. Bacterial cells detected after in situ hybridization with Cy3-labeled probe EUB338 targeting all members of the Domain Bacteria (left panel) and after concomitant DAPI staining detecting all organisms (right panel)

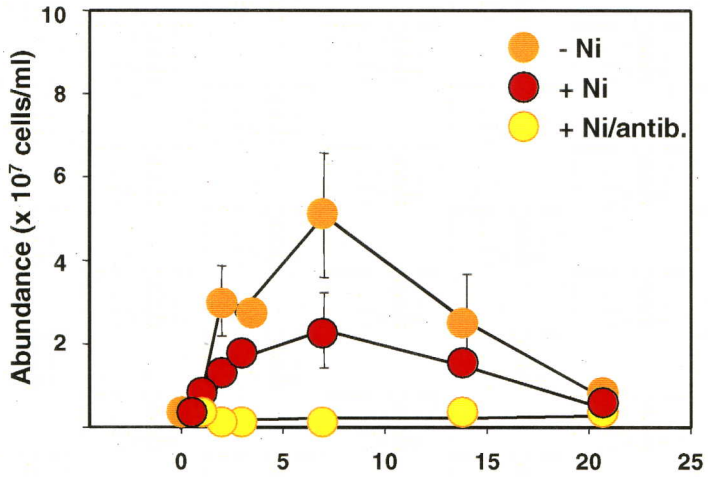

Fig. 5. Number of bacterial cells detected after in situ hybridization with Cy3-labeled probe EUB338 targeting all members of the Domain Bacteria in non-amended, Ni-amended and Ni- and antibiotics-amended microcosms

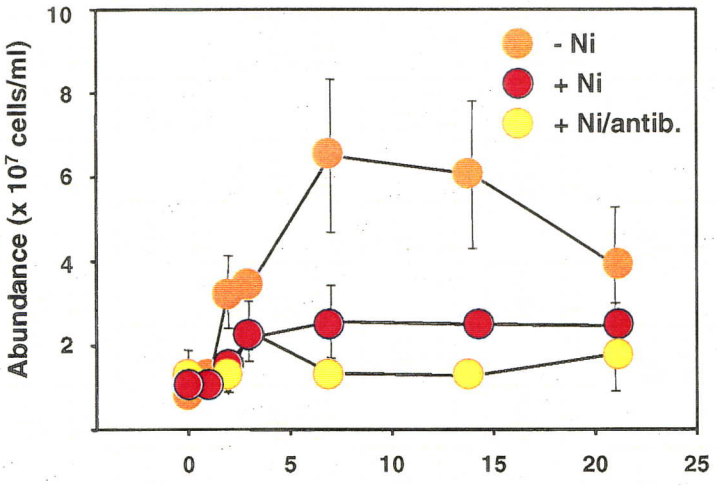

Fig. 6. Number of all organisms after DAPI staining in non-amended, Ni-amended and Ni- and antibiotics-amended microcosms

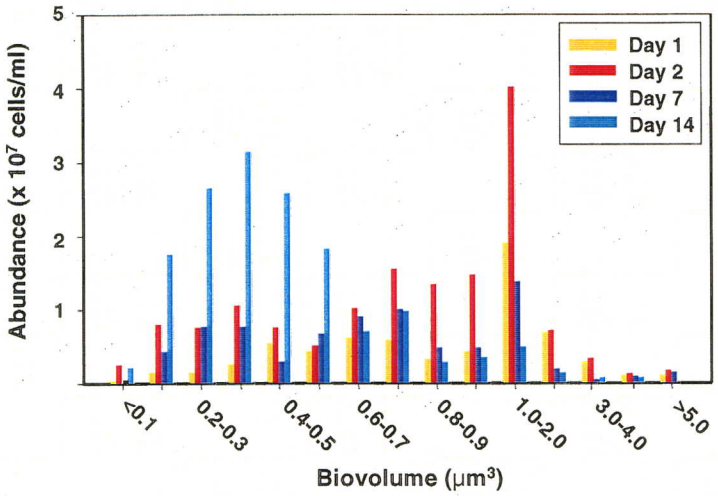

Fig. 7. Cell size distribution of bacterial cells detected after in situ hybridization with Cy3-labeled probe EUB338 targeting all members of the Domain Bacteria in Ni-free control microcosms

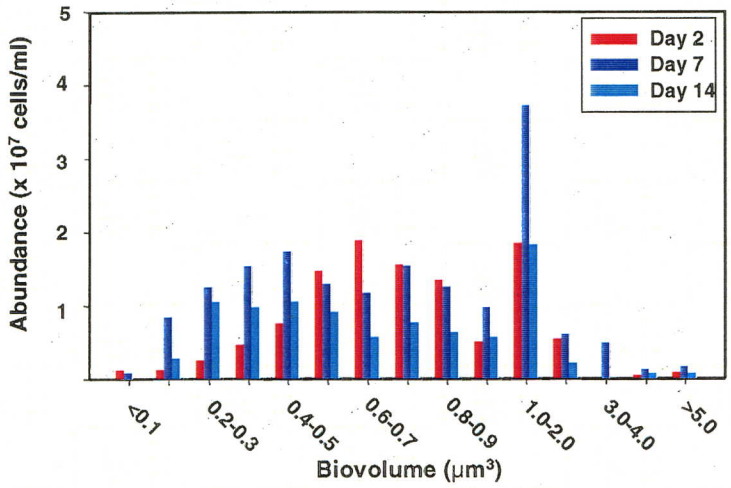

Fig. 8. Cell size distribution of bacterial cells detected after in situ hybridization with Cy3-labeled probe EUB338 targeting all members of the Domain Bacteria in Ni-amended microcosms

Microbial growth is smaller in Ni-amended microcosms than in non-amended ones (Fig. 5, 6), and there is no growth in controls amended with Ni and antibiotic. Detectability of bacteria is decreasing after 7 days of incubation in both amended and non-amended microcosms which might be due to changes in nutrient availability and concomitant shift from fermentative to sulfate-reducing metabolism. Microbial communities in Ni-amended and non-amended microcosms are different with respect to cell sizes and densities (Fig. 7, 8). Both contain endospore-forming bacteria (see Fig. 4 for example) that differ from each other. Cell size distributions in Ni-amended microcosms are in a larger range than those in non-amended microcosms.

These results show that the image analysis protocol developed allowed us to analyze specific bacterial populations that grow under sulfate-reducing conditions in the presence of high concentrations of Ni. Ni is removed from the aqueous phase during incubation. These initial data, however, require future studies to identify members of the microbial community as potential bioindicators, and to address questions on the mechanism of the Ni removal from the aqueous phase (e.g. precipitation by biogenic H_2S or by binding to media components).

4.2. Greenhouse Study

Cores of intact plants and soil were collected from Harrier Meadow (North Arlington) that was used as disposal site for shot rock from construction of U.S Route 280 in the past and restored recently. Construction work was completed during September 1998. It is a tidal marsh that experiences a tidal range of 1 m and a salinity between 5-15 ppt located in the Hackensack Meadowlands approximately 5 km north of Newark, New Jersey, and 10 km west of New York City. At the moment, portions of the area support a mixture of native high salt marsh vegetation, dominated by salt hay (*Spartina patens*), and open water. Soil cores with *S. patens* (10 x 10 x 10 cm, n = 40) weighing approximately 1kg fresh weight, were collected at the end of February 2000 similar to a previous study (Burke et al. 2002). Cores were transported back to a greenhouse at Rutgers University-Newark, fitted into plastic pots and placed in individual pails containing 5 ppt artificial seawater (Instant Ocean® Mentor, OH, USA) amended with 5 ppm NH_4^+ and PO_4^{3-}, respectively. Half of the cores were then treated with benomyl (50% WP; Bonide Products, Inc. Yorkville, NY, USA; 0.1 g per kg of soil dissolved in 100 ml water) to suppress AMF colonization (Hetrick et al. 1994). Pails containing potted cores were arranged randomly on a greenhouse bench and artifical seawater was added to each pail so that standing water was present 5 cm below the top of the core. This correlated to the level of water observed in holes remaining in the field after core removal. Previous studies in our laboratory demonstrated a nearly complete suppression of AMF by a single benomyl-treatment, and the generation of large redox gradients between surface and bottom of the cores (Burke et al. 2002). In one half of the non-treated and benomyl-treated cores the availability of Ni was artificially increased through amendments with $NiSO_4$ (50 mg kg^{-1} core material). The second half of the non-treated and benomyl-treated cores received NaCl and served as controls for heavy metal contaminated cores.

Fig. 9 Sampling location in Harrier Meadow (red-marked spot)