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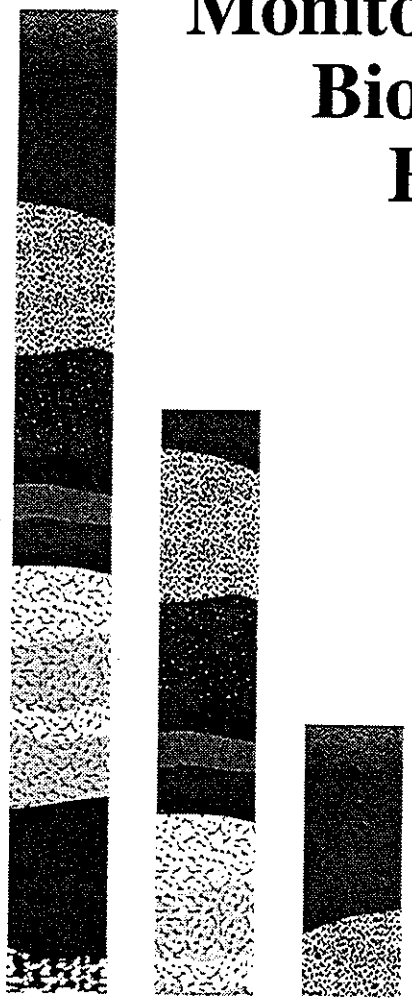

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3(5):49

BATTELLE PRESS

Library of Congress Cataloging-in-Publication Data

Hinchee, Robert E.
Monitoring and verification of bioremediation / edited by Robert E. Hinchee, Gregory S. Douglas, Say Kee Ong.
p. cm.
Includes bibliographical references and index.
ISBN 1-57477-006-3 (hc : acid-free paper)
1. Bioremediation—Congresses. I. Hinchee, Robert E. II. Douglas, Gregory S. III. Ong, Say Kee.
TD192.5.M66 1995
628.5'2—dc20 95-32266
CIP

Printed in the United States of America

Additional copies may be ordered through:
Battelle Press
505 King Avenue
Columbus, Ohio 43201, USA
1-614-424-6393 or 1-800-451-3543
Fax: 1-614-424-3819
Internet: sheldric@battelle.org

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ABSTRACT

Two sites undergoing bioremediation were studied using the signature lipid biomarker (SLB) technique. This technique isolates microbial lipid moieties specifically related to viable biomass and to both prokaryotic and eukaryotic biosynthetic pathways. The first site was a South Pacific atoll heavily contaminated with petroleum hydrocarbons. The second site was a mine waste reclamation area. The SLB technique was applied to quantitate directly the viable biomass, community structure, and nutritional/physiological status of the microbiota in the soils and subsurface sediments of these sites. All depths sampled at the Kwajalein Atoll site showed an increase in biomass that correlated with the co-addition of air, water, and nutrients. Monoenoic fatty acids increased in abundance with the nutrient amendment, which suggested an increase in gram-negative bacterial population. Ratios of specific phospholipid fatty acids indicative of nutritional stress decreased with the nutrient amendment. Samples taken from the mine reclamation site showed increases in total microbial biomass and in *Thiobacillus* biomass in the plots treated with lime and bactericide, especially when a cover soil was added. The plot treated with bactericide and buffered lime without the cover soil showed some decrease in *Thiobacillus* numbers, but was still slightly higher than that observed in the control plots.

INTRODUCTION

Bioremediation of contaminated sites offers the most cost-effective means of protecting groundwater resources. A major limitation to the application of bioremediation has been an inability to predict and monitor the effects of specific treatments on microbial communities when using classical microbiology

techniques such as plate count or most probable number (MPN) enumerations. An alternative to the classical techniques is the signature lipid biomarker (SLB) technique. This paper addresses two sites where the SLB technique was used to monitor both bioremediation and bioreclamation efforts.

Kwajalein Atoll is an island in the South Pacific that has been contaminated with petroleum hydrocarbons following years of use by the military. This site was chosen for bioremediation following feasibility studies showing that the soil conditions, temperature, and indigenous microbiota favored this type of approach. For the in situ treatments, injection wells were used to deliver combinations of air, water, and nutrients. Two plots were used as controls and received no amendments, and ten plots received amendments. Sediments were sampled from both control plots and amended plots at depths of 4 and 5 ft (1.2 and 1.5 m). Six ex situ plots also were sampled. These plots were polypropylene-lined cells that contained excavated soils from the contaminated areas. The ex situ plots were treated with the same amendments as the in situ plots.

The second site is a semi-arid mine waste area undergoing bioreclamation activities. This site contained acidified soil resulting from microbial activities attributed to *Thiobacillus*. The reclamation approach was to inhibit further microbial activities through the use of lime and a bactericide. The amount of lime applied to treated plots was determined using the SMP buffer test (Shoemaker et al. 1961), and the amount of ProMac (bactericidal surfactant) was applied according to the manufacturer's recommendations. The layout of the control and treated plots is shown in Figure 1.

Lipid analysis provides a quantitative means to measure total viable microbial biomass, community structure, and nutritional status of microbial communities over the course of bioremediation activities. Phospholipid fatty acid (PLFA) analysis is based on the following: (1) phospholipids are indicative of viable biomass; (2) phospholipid fatty acids are synthesized via pathways specific to different microbial groups allowing for the establishment of SLBs; and (3) ratios of specific phospholipid fatty acids can be used to assess physiological status (Vestal and White 1989, Guckert et al. 1986). Because lipids can be directly extracted from groundwater and soil matrices (without culturing or isolating the microbes), this method has proved very useful for monitoring the effectiveness of bioremediation efforts at sites contaminated with trichloroethylene (TCE) and tetrachloroethylene (PCE) (Phelps et al. 1988, 1990, 1991; Nichols et al. 1987), polychlorinated biphenyls (PCBs) (Hill et al. 1989), and linear alkanosulfonates (LAS) (Federle et al. 1991). The SLB analysis has recently been strengthened with the discovery that the lipid extraction yields DNA suitable for gene probing (Kehrmeyer et al. 1995), which allows for the detection of specific genes involved in biodegradation.

METHODS

The Kwajalein site was treated in situ with the addition of air water and nutrients. The ex situ samples had been excavated from the site, tranferred to a plastic-lined trough, and subjected to the same treatments as the in situ plots.

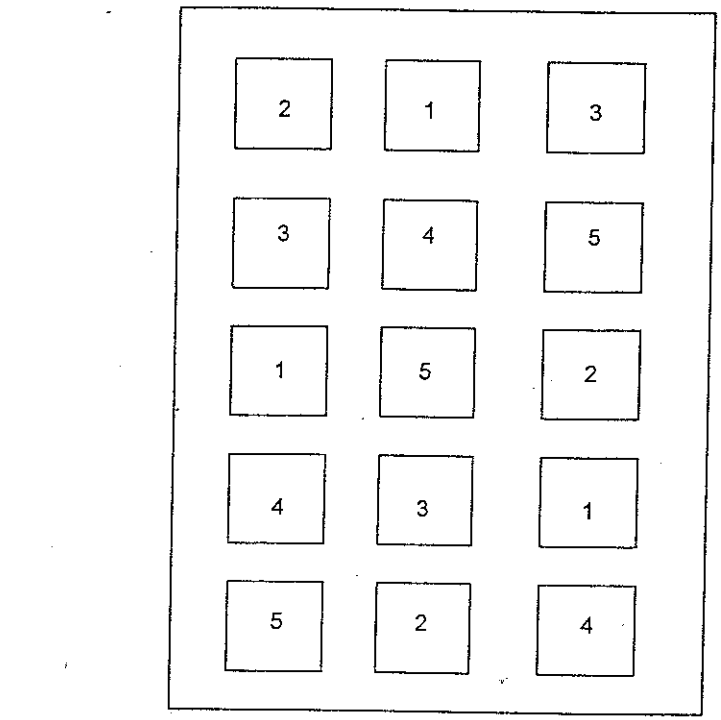

FIGURE 1. Experimental design for the mine waste reclamation site. Upper figure is a schematic representation of the layout of amended and control plots. Plot number corresponds to the treatment scheme shown in the lower plot.

The mine waste reclamation site was set up as shown in Figure 1. The number on each plot refers to the treatment applied to that area. The experimental design is shown at the bottom of the figure. Samples were collected from the cover soil region, from the amended region, and from the underlying soils. The bactericide applied to plots was ProMac, an anionic surfactant applied in the form of long-term release pellets. Lime requirements to raise the treated soils to pH 7.0 were determined by the SMP agricultural buffer method. The excess lime applied in treatment 5 was determined by acid-base account analysis, simulated weathering tests, and buffer recommendations.

Microbial lipids were extracted from soil and sediment samples using the method of Guckert et al. (1986). The polar lipid fraction (containing the phospholipids) was separated from the other lipid fractions by silicic acid column chromatography. Phospholipid fatty acid methyl esters were prepared by transesterification of the phospholipids. The methyl esters of the fatty acids were then separated and quantified by capillary gas-liquid chromatography and identified by electron impact mass spectrometry (GC/MS). Cluster analysis was accomplished using Ein*Sight (Infometrix, Seattle, Washington) pattern recognition software.

RESULTS

Kwajalein Site

Initial characterization of the Kwajalein site using PLFA analysis indicated a diverse microbial community existed and consisted primarily of actinomycetes (evidenced by tuberculostearic acid) and gram-negative organisms (evidenced by monoenoic PLFA). During the bioremedial activities, SLB analysis showed an overall increase in the mean biomass estimate in the nutrient-amended plots, and the ex situ plots as compared to the controls (Figure 2). Multivariate statistics showed that the control sediments differed slightly from the nutrient-amended plots, and both of these sample groups differed from the ex situ treatments with regards to PLFA composition. These results indicate that the treatments had an effect on the community structure of the extant microbiota. Analysis of individual PLFA indicated that the community which showed the greatest response to nutrient amendments was primarily gram-negative, as evidenced by the increases in the percentages of the monoenoic fatty acids 16:1ω7*cis* and 18:1ω7*cis*, terminal points in the anaerobic desaturase pathway utilized by the gram-negative bacteria. Changes in the nutritional status of the gram-negative population were noted as well. Although no change in *trans/cis* ratios (indicator of environmental toxicity) occurred between the control and nutrient-amended plots, the percentage of cyclopropyl 17:0 decreased in the nutrient-amended plots as compared to the controls, indicating some alleviation of nutritional stress during the course of the remediation effort. Although the production of cyclopropyl PLFA has been related to metabolic stress, certain bacterial types have been shown to contain more of this

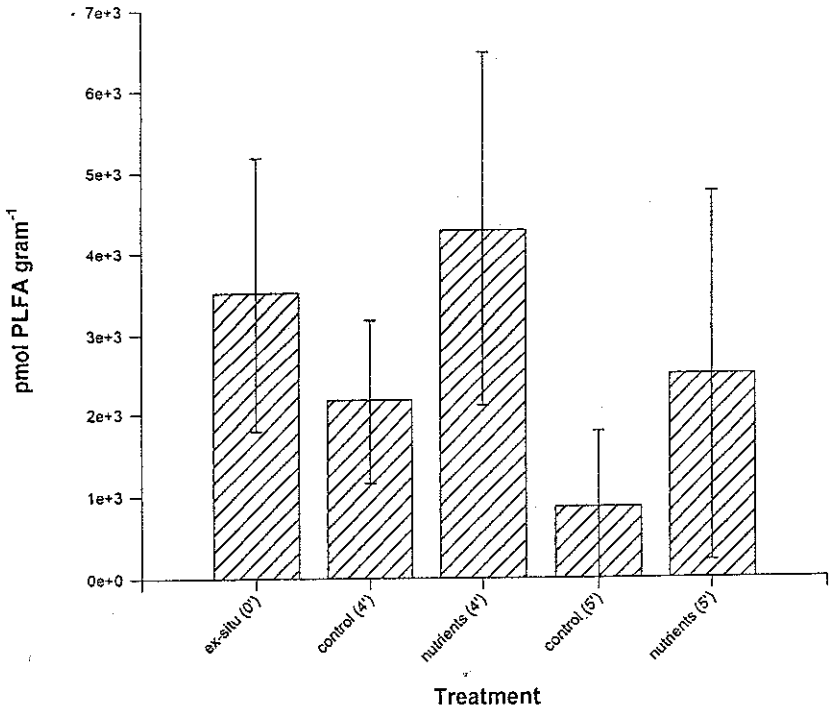

FIGURE 2. Biomass estimations for the Kwajalein Atoll control plots ex situ plots, and nutrient-amended plots.

type of fatty acid than others under ideal growth conditions. Therefore, the observed decrease in this fatty acid could also be related to a shift in community structure.

Montana Mine Waste Site

The investigation of this site was conducted over a 2-year period to determine if the addition of bactericide to these sites could reduce the population of *Thiobacillus ferrooxidans*, the assumed causative agent of soil acidification at this site. Biomarkers specific to the *Thiobacillus* genus were used to determine whether the bactericide was having any effect on the *Thiobacillus* population. The biomarkers used were a cyclopropyl 18:0, a branched cyclopropyl 20:0, and 3-hydroxy-16:0, a hydroxy fatty acid prominent in the lipopolysaccharide (LPS) of *Thiobacillus* species. Values for PLFA recovered from extracted mine wastes are shown in Table 1. Interestingly, it was found that the plot to which no treatments were given showed the lowest overall biomass, and the lowest numbers

TABLE 1. Biomass estimations for the mine waste reclamation site. Biomass is expressed in picomoles PLFA per gram of soil extracted.

Total PLFA (pmol/gram soil)

	Topsoil 1991	Topsoil 1992	Middle soil 1991	Middle soil 1992	Bottom soil 1991	Bottom soil 1992
Plot 1	2.25×10^{15} (± 1.61)	1.26×10^{15} (± 0.06)	2.26×10^{15} (± 1.71)	1.71×10^{15} (± 0.75)	8.47×10^{13} (± 11.5)	1.86×10^{15} (± 1.32)
Plot 2	5.94×10^{15} (± 5.23)	2.17×10^{16} (± 0.67)	5.83×10^{14} (± 4.76)	6.42×10^{15} (± 1.38)	1.41×10^{14} (± 1.25)	2.37×10^{15} (± 1.47)
Plot 3	5.46×10^{16} (± 4.81)	1.91×10^{16} (± 0.42)	9.60×10^{15} (± 1.08)	6.53×10^{15} (± 2.0)	1.43×10^{14} (± 0.64)	2.74×10^{15} (± 1.73)
Plot 4	1.42×10^{16} (± 1.31)	4.71×10^{15} (± 1.16)	3.71×10^{15} (± 1.18)	1.49×10^{15} (± 0.61)	1.02×10^{14} (± 1.01)	2.36×10^{15} (± 1.53)
Plot 5	5.55×10^{16} (± 4.08)	1.98×10^{16} (± 0.40)	9.71×10^{15} (± 2.47)	5.76×10^{15} (± 0.66)	1.65×10^{14} (± 1.15)	3.45×10^{15} (± 0.99)

Thiobacillus ferrooxidans PLFA (pmol/gram soil)

	Topsoil 1991	Topsoil 1992	Middle soil 1991	Middle soil 1992	Bottom soil 1991	Bottom soil 1992
Plot 1	7.48×10^{12}(± 6.59)	2.78×10^{13} (± 2.08)	N/D	1.85×10^{13} (± 1.41)	4.32×10^{12} (± 2.49)	5.96×10^{14} (± 4.15)
Plot 2	8.63×10^{13} (± 3.54)	2.35×10^{14} (± 0.92)	1.91×10^{13} (± 1.66)	2.48×10^{14} (± 0.19)	9.14×10^{12} (± 11.8)	7.31×10^{14} (± 4.62)
Plot 3	3.93×10^{14} (± 3.48)	2.45×10^{14} (± 0.89)	4.33×10^{13} (± 1.47)	2.54×10^{14} (± 0.50)	6.59×10^{12} (± 4.98)	1.12×10^{14} (± 0.84)
Plot 4	N/D	2.29×10^{14} (±0.98)	2.88×10^{12} (± 1.66)	1.43×10^{14} (± 0.55)	8.31×10^{11} (±14.4)	9.07×10^{14} (± 6.96)
Plot 5	3.42×10^{14} (± 2.14)	2.28×10^{14} (± 0.97)	3.73×10^{13} (±1.08)	2.54×10^{14} (± 0.39)	9.14×10^{12} (± 8.01)	1.31×10^{15} (± 0.65)

Thiobacillus genus PLFA (pmol/gram soil)

	Topsoil 1991	Topsoil 1992	Middle soil 991	Middle soil 1992	Bottom soil 1991	Bottom soil 1992
Plot 1	7.35×10^{14} (± 5.55)	3.30×10^{14} (± 0.49)	6.82×10^{14} (± 5.37)	5.88×10^{14} (±3.73)	7.37×10^{14} (± 6.14)	5.96×10^{14} (± 4.15)
Plot 2	2.53×10^{15} (± 0.77)	1.0×10^{16} (± 0.32)	2.49×10^{15} (± 1.96)	3.08×10^{15} (± 0.73)	1.16×10^{15} (± 1.25)	7.31×10^{14} (± 4.62)
Plot 3	1.62×10^{16} (± 1.40)	9.21×10^{15} (± 2.11)	3.75×10^{15} (± 0.24)	3.07×10^{15} (± 1.07)	1.56×10^{15} (± 1.22)	1.12×10^{15} (± 0.84)
Plot 4	6.97×10^{15} (±1.55)	2.34×10^{15} (± 0.95)	1.13×10^{15} (± 0.55)	6.14×10^{14} (± 2.50)	9.29×10^{14} (± 6.56)	9.07×10^{14} (± 6.96)
Plot 5	2.35×10^{15} (± 1.76)	$9.15 \times \times 10^{15}$ (±1.85)	3.99×10^{15} (± 1.22)	2.65×10^{15} (± 0.44)	2.06×10^{15} (± 0.48)	1.31×10^{15} (± 0.65)

of *Thiobacillus* as determined by SLB. Treatment 2 (bactericide, lime, and cover soil) showed the highest biomass. The only treatment to show decreases in total biomass and in *Thiobacillus* numbers was treatment 4, i.e., bactericide and lime with no cover soil. Treatments 3 and 5 both showed increases in total microbial biomass and *Thiobacillus* numbers. Cluster analysis showed distinct community structure changes between sample plots indicating that the treatments were, in fact, influencing the microbial communities (Figure 3). All

FIGURE 3. Hierarchical cluster analysis of PLFA profiles from treated and untreated soils from the Mine Waste Reclamation Site.

control soils clustered together. The bottom soil of treatment 2 samples also clustered with the control plot, indicating that the treatments applied to this plot had little or no effect at this soil depth. The middle depth soils of all plots treated with a cover soil clustered together, indicating that the cover soil may make a significant contribution to the microbial community structure. All topsoils of the treated plots clustered away from the control plots, suggesting that soil treatments resulted in a change in the microbial community structure. With the exception of treatment 2, the bottom soils of the treated plots clustered away from the control plot, indicating that the treatments could influence the microbial community structure.

DISCUSSION

At the Kwajalein site, SLB analysis indicated an initial microbial community structure containing both actinomycete and gram-negative organisms. These results were supported by other investigators through their use of gene probes and classical microbiological techniques (Adler et al. 1992). The PLFA-based biomass estimations paralleled those of investigators using classical methods, and changes in the nutritional status followed nutrient amendments over the course of the bioremediation effort. By the use of SLB, it was determined that the nutrient amendments successfully increased the extant microbial biomass on site, with an associated shift in the community composition.

At the mine reclamation site, the SLB technique proved very useful for showing the lack of effectiveness of the traditional technique of simply using buffering lime to reclaim acid soil. It also showed that the application of cover soil seemed to enhance both total microbial biomass and that of the *Thiobacillus*. The fact that the control samples contained less biomass suggests a disturbance effect resulting from the stimulation of the microbial populations. These data also suggest that the application of a cover soil not only serves to increase the microbial population, but perhaps diminishes the effect of the lime and bactericide by dilution. This is supported by the clustering of the bottom soils of all the plots away from the treated soils.

The data reported in the peer-reviewed literature clearly demonstrate the efficacy of the SLB in correlating the microbial ecology with bioremediation potential both in the laboratory and the field. This efficacy for prediction between microbial activity and ecology has been demonstrated for TCE and PCE (Phelps et al. 1988, 1990, 1991), PCBs (Hill et al. 1989), linear alkane sulfonate degradation (Federle et al. 1991), and alkanes (Ringelberg et al. 1988). The analysis of other lipids such as the diglycerides (showing nonviable biomass), sterols (for the microeukaryotes—nematodes, algae, protozoa), glycolipids (phototrophs, gram-positive bacteria) (Vestal & White 1989), or the hydroxy fatty acids in the lipid A of lipopolysaccharide (gram-negative bacteria) (Parker et al. 1982) can provide an even more detailed community structure analysis.

ACKNOWLEDGMENTS

The authors wish to acknowledge the support of DOE—Martin Marietta subcontract # 11X-SP830V for its support of the Kwajalein research, and Dr. Douglas J. Dollhopf and the Montana State University College of Agriculture Reclamation Unit for their support of the Montana Mine Waste project. The authors also gratefully acknowledge the technical assistance of Stephen J. Nold.

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