This document was prepared in conjunction with work accomplished under Contract No. DE-AC09-96SR18500 with the U. S. Department of Energy.

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Field Deployment for In-situ Metal and Radionuclide Stabilization by Microbial Metabolites

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September 30, 2005

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1.0 EXECUTIVE SUMMARY

A novel biotechnology is reported here that was demonstrated at SRS that facilitates metal and actinide immobilization by incorporating the physiology and ecology of indigenous bacteria. This technology is based on our previous work with pyomelanin-producing bacteria isolated from SRS soils. Through tyrosine supplementation, overproduction of pyomelanin was achieved, which lead ultimately to metal and actinide immobilization, both *in-vitro* and *in-situ*.

Pyomelanin is a recalcitrant microbial pigment and a humic type compound in the class of melanin pigments. Pyomelanin has electron shuttling and metal chelation capabilities and thus accelerates the bacterial reduction and/or immobilization of metals. Pyomelanin is produced outside the cell and either diffuses away or attaches to the cell surface. In either case, the reduced pyomelanin is capable of transferring electrons to metals as well as chelating metals. Because of its recalcitrance and redox cycling properties, pyomelanin molecules can be used over and over again for metal transformation. When produced in excess, pyomelanin produced by one bacterial species can be used by other species for metal reduction, thereby extending the utility of pyomelanin and further accelerating metal immobilization rates.

Soils contaminated with Ni and U were the focus of this study in order to develop *in-situ,* metal bio-immobilization technologies. We have demonstrated pyomelanin production in soil from the Tims Branch area of SRS as a result of tyrosine amendments. These results were documented in laboratory soil column studies and field deployment studies. The amended soils demonstrated increased redox behavior and sequestration capacity of U and transition metals following pyomelanin production. Treatments incorporating tyrosine and lactate demonstrated the highest levels of pyomelanin production.

In order to determine the potential use of this technology at other areas of SRS, pyomelanin producing bacteria were also quantified from metal contaminated soils at TNX and D areas of SRS. A bacterial culture collection from subsurface studies near P Area of SRS were also evaluated for pyomelanin production. Bacterial densities of pyomelanin producers were determined to be $>10^6$ cells/g soil at TNX and D areas. In addition, approximately 25% of isolates from P area demonstrated pyomelanin production in the presence of tyrosine.

Biogeochemical activity is an ongoing and dynamic process due, in part, to bacterial activity in the subsurface. Bacteria contribute significantly to biotransformation of metals and radionuclides. An understanding and application of the mechanisms of metal and radionuclide reduction offers tremendous potential for development into bioremedial processes and technologies. This report demonstrates the application of recent advances in bacterial physiology and soil ecology for future bioremediation activities involving metal and actinide immobilization.

2.0 INTRODUCTION

Heavy metals and radionuclides that contaminate the subsurface of Department of Energy (DOE) facilities across the country cannot be destroyed but the toxicity of these inorganic contaminants will decrease through chemical alteration. Chemical alteration (i.e. reduction) in many cases results in conversion from toxic and mobile to nontoxic and immobile metals. The use of bioremediation for biotransformation of metals and radionuclides in low concentrations in the subsurface offers considerable potential for long-term environmental cleanup.

Dissimilatory metal reducing bacteria (DMRB) have been the focus of bioremediation studies because of their unique capacity to respire using soluble and insoluble metals (Ehrlich, 1996). Some mechanisms of DMRB electron transfer to the metals, especially the solid-phase were demonstrated, particularly regarding humic-type compounds. The redox cycling properties of humics allows them to act as terminal electron acceptors for numerous subsurface bacteria (Coates et al., 1998) and as electron shuttles for soluble and insoluble metals (Lovley et al., 1996). The capacity of metal-reducing bacteria to produce the humic-type compound pyomelanin, either in solution or associated with the bacterial cell (Fuqua et al., 1991, Ruzafa et al., 1995, Turick et al 2002 and 2003), accelerates insoluble Fe(III) oxide reduction by serving as an electron shuttle (Turick et al., 2002, and 2003). Pyomelanin, a type of melanin, is structurally similar to humic acids and, thus, has similar redox cycling properties (Menter and Willis, 1997; Scott and Martin, 1990). A number of bacterial species, especially in the gamma proteobacteria produce pyomelanin (Yabuuchi and Omyama. 1972; Coon et al., 1994; Kotob et al., 1995; and Ruzafa et al., 1995 and Turick et al. 2002).

Microbial melanin has a role to play in bioremediation (McLean et al., 1998) and melanin production offers tremendous untapped potential for immobilization of metals and radionuclides because of its electron shuttling and metal sequestration capacities (McLean et al., 1998). For instance microbial melanin is responsible for enhanced adsorption of numerous metals and tributyltin chloride with the fungus *Aureobasidium pullulans* (Gadd et al., 1990; Gadd and Mowll, 1985; Gadd et al., 1987), Fe(III) reduction and subsequent Fe(II) assimilation by the yeast *Cryptococcus neoformans* (Nyhus et al., 1997) and U accumulation by a melanin-containing fungus associated with the lichen *Trapelia involuta* (McLean et al., 1998).

As a humic compound, pyomelanin has potential to increase solid phase metal reduction rates and chelation of resultant soluble metal ions. Humics bind to both metal oxides and soluble metals (Gu et al., 1995; Gu et al., 1996). The presence of hydroxyl groups, carboxyl density, and linear molecular structure of humics contribute to their strong surface complexation to iron oxides (Gu et al., 1995; Gu et al., 1996). Humic compounds form innersphere complexes with metals such as Co and Ni (Xia et al., 1997). Although humic interactions with metals such as Am(III) increase its mobility as a function of contact time between Am(III) and humic colloids, the mobility of Am(III)/humic-colloid complexes decrease as a result of humic interactions with the soil particles (Artinger et al., 2002).

Melanin is a generic term describing a ubiquitous humic-type metabolite (Ellis and Griffiths, 1974). Microbial melanins account for a measurable portion of naturally occurring soil humic compounds (Scott and Martin 1990). Melanin production is often a function of the enzymes tyrosinase (EC 1.14.18.1) or poly-phenol oxidase. These types are commonly associated with eukaryotes.

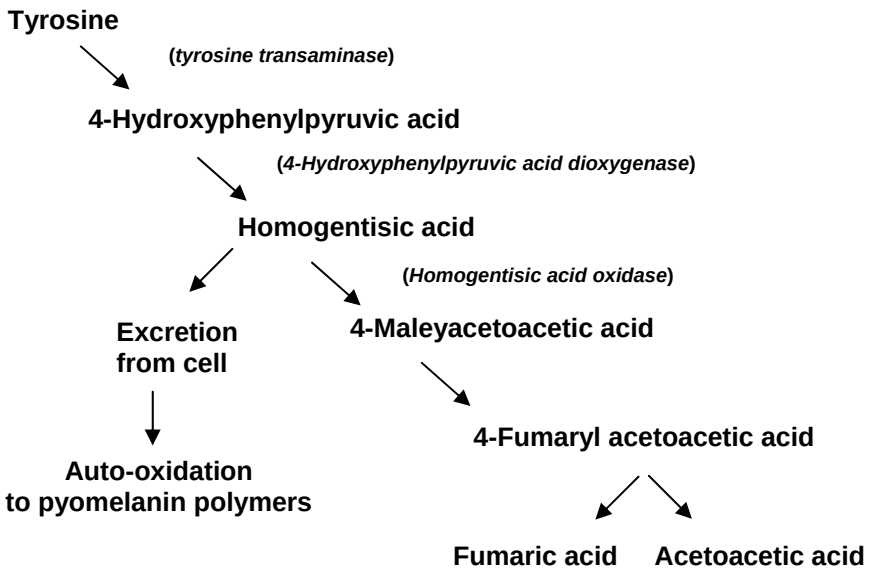


Figure 2.1. Fumarate pathway demonstrating tyrosine catabolism. Pyomelanin production can result when 4-hydroxyphenylpyruvic acid dioxygenase activity exceeds that of homogentisic acid oxidase, then a portion of tyrosine catabolism is shunted to pyomelanin production. In addition, deletion of the gene that encodes for homogentisic acid oxidase (hmgA) results in overproduction of pyomelanin while deletion of the gene that encodes for 4-hydroxyphenylpyruvic acid dioxygenase (melA) results in pyomelanin deficiency.

Another, less-studied type of melanin is pyomelanin. Pyomelanin production (Yabuuchi and Omyama. 1972) is documented in a number of bacterial species (Yabuuchi and Omyama. 1972; Coon et al., 1994; Kotob et al., 1995; and Ruzafa et al., 1995 and Turick et al. 2002). This type of melanin originates from bacterial conversion of tyrosine as part of the fumarate pathway (Fig. 2.1). Complete breakdown of tyrosine to acetylacetate and fumarate requires the enzymes 4-hydroxyphenylpyruvic acid dioxygenase (4-HPPD) and homogentisic acid oxidase (HGA-oxidase). In the absence of this enzyme [or if homogentisic acid (HGA) production exceeds that of HGA-oxidase], HGA is over-produced and excreted from the cell (Coon et al., 1994). Auto-oxidation and self-polymerization of HGA then results in pyomelanin, an aromatic polymer consisting of numerous quinone moieties (Ruzafa et al., 1995).

Pyomelanin also serves as a soluble or surface associated electron shuttle and its sorbtion onto bacterial surfaces increases the rate of electron transfer by as much as 10 fold (Turick et al. 2003). Humic sorption to bacterial surfaces has been demonstrated previously (Fein et al. 1999) and consistent with the previous work, our recent studies demonstrated that pyomelanin in solution also sorbed onto the surfaces of a broad taxonomic range of bacteria. As a result, in the presence of an electron donor, pyomelanin-sorbed bacteria demonstrated increased Fe(III)-oxide reduction. Fe(III) reduction as a means of Fe assimilation or as an electron sink but not coupled to energy conservation is well documented and is accelerated with complexing agents (Ehrlich, 2002). Pyomelanin producing bacteria offer the potential to enlist the local microbial community into facilitated metal reduction by providing pyomelanin as a complexing agent. Metal sequestration will also be accelerated as a function of pyomelanin production and subsequent bacterial sorption.

While DMRB are proving valuable in bioremediation, based on current studies, they account for a small portion of the general microbial community prior to remedial activities. Additionally, naturally occurring soil humics are ubiquitous, but their quantities and specific characteristics vary considerably. Consequently the heterogeneity of humics *in-situ* and aging effects decrease their effectiveness and dependability for remedial action. Production of pyomelanin by a portion of the microbial community

offers a potentially dependable electron shuttle and complexing agent that when "shared" enlists the local microbial community into metal and radionuclide reduction and immobilization. Pyomelanin producers are then, indeed, facilitator organisms for accelerated metal and radionuclide immobilization in metal contaminated environments.

The frequency of genetic variation in humans that results in excess HGA excretion (alkaptonuria) ranges from 1:19,000 to 1:250,000 (Janocha et al. 1994 and Milch, 1960). If this rate of genetic variability in bacteria is the same, pyomelanin-producing bacteria will be quite common in the environment. Because the amount of pyomelanin produced in-situ is probably very low in concentration, its detection could easily be overlooked.

Previous Results

Our preliminary research (funded through Soil and Groundwater Closure Projects Team) has addressed some important questions related to pyomelanin-facilitated metal reduction by the bulk bacterial population. Our results from FY03 and FY04 (Turick et al. 2003b and 2004) demonstrate that:

1) Pyomelanin-like pigments are produced by a number of species of the bacterial genus *Shewanella;*

2) The gene encoding for 4-HPPD (*melA*) is responsible for pyomelanin production and is present in numerous species of *Shewanella;*

3) The competitive inhibitor sulcotrione is specific for 4-HPPD and interferes with pyomelanin production;

4) Sulcotrione can be used to determine if pyomelanin is produced from tyrosine catabolism in various bacterial isolates;

5) Pyomelanin producing bacteria are present in numerous soils at the Savannah River Site with high numbers detected in the Tims Branch area;

6) Pyomelanin, a metal chelator and electron shuttle can be produced as a soluble metabolite by one species and "shared" by other non-pyomelanin-producers thereby promoting metal reduction and chelation by a broad range of bacteria;

7) Lactate and tyrosine amendments provide greater pyomelanin production than the same quantities of tyrosine alone;

8) Pyomelanin production is dependent on oxygen, but low oxygen concentrations are sufficient.

Our previous research has provided important information related to a bacterial mechanism of metal oxide reduction. Because pyomelanin production is related to microbial metal reduction, metal immobilization/sequestration and growth this is an important and exciting phenomenon that will improve our understanding and control of metal biotransformations in the environment. This work will provide us with the potential to manipulate the physiologies of autochthonous bacteria for metal and radionuclide reduction and immobilization through the production of a naturally occurring complexing agent.

3.0 MATERIALS AND METHODS

3.1 QUANTIFICATION OF MELANIN PRODUCING MICRORGANISMS

3.1.1 Site Selection, Soil Collection and Culture Selection

The Tims Branch watershed makes up a portion of SRS and parts of this watershed have been impacted in the past with U(VI) and Ni. Soils representative of this watershed were selected to study the affects of microbial metabolites on the behavior of metals in soil. In addition, sampling and research activities were coordinated with other researchers at the Savannah River Ecology Lab (SREL).

Metal contaminated soils at D area and TNX were also chosen for their potential to harbor pyomelanin-producing bacteria. These soils were quantified for pyomelanin producing bacteria due to high numbers obtained during previous studies with Tims Branch soils in the area of Pond 25. Our interest was to determine if the unexpectedly high numbers of pyomelanin producers at Tims Branch were normal for the soils at SRS or an anomaly. In addition, we sought to establish the utility of this technology if deployed at other parts of SRS in the future.

Pyomelanin production was also examined by using pure cultures of subsurface isolates obtained from the Subsurface Microbial Culture Collection (SMCC) (Balkwill et al. 1997, Benyehuda, et al. 2003 and Brigmon et al 2000) and housed at SRS. These cultures were isolated during studies conducted at SRS at borehole P 24. During the present study pyomelanin production was determined on a portion of the cultures in this collection that were well characterized previously.

3.1.2 Chemicals

Sulcotrione was supplied gratis from Zeneca Agrochemicals. All other chemicals were reagent grade and supplied by Sigma-Aldrich, unless otherwise stated below. Lactate (CH_3CHOOH) served as a carbon and energy source for bacteria. Tyrosine ($C_9H_{11}NO_3$), an aromatic amino acid was the precursor to pyomelanin when partly catabolized to homogentisic acid.

3.1.3 Most probable Number (MPN) Assay for Melanin Production.

Soil samples were collected aseptically and immediately stored on ice until delivery to the lab. Soil samples were then refrigerated until processing. Soil dilutions from $10^{-2} – 10^{-8}$ were prepared in lactate basal salt medium supplemented with 1g/l tyrosine. Controls received the same treatment except tyrosine was omitted. Each tube of the 3 tube MPN contained 10 mls of growth medium and soil. Tubes were incubated for 8 weeks at 25°C and shaken at 100 rpm. Increased pigmentation as a result of tyrosine amendments was determined spectrophotometrically by scanning the supernatant fluid of each test tube from 600-300 nm. Tubes with increased OD in this range, relative to controls were marked positive for pigment production from tyrosine. The number of positive tubes per dilution were used to calculate the most probable number of cells.

3.2 CHARACTERIZATION OF MELANIN-LIKE PIGMENTS FROM SRS SOILS

3.2.1 Isolation of Melanin-Type Pigment-Producing Microorganisms

To isolate pigment-producing microorganisms, samples were removed aseptically from the soil MPNs above and inoculated onto tryptic soy agar supplemented with tyrosine (2g/l) (TSAT), using the spread

plate method. Following incubation at 25°C for 1 week, colonies demonstrating pigmentation (relative to plates without tyrosine) were transferred from TSAT to a lactate basal salts agar with and without 2g/l tyrosine and monitored for pigmentation. Pigment production was determined by comparing coloration from tyrosine amended plates relative to those without tyrosine.

3.2.2 Characterization of Melanin-Type Microbial Pigments

The chemical sulcotrione is a specific competitive inhibitor of the enzyme 4-hydroxyphenyl pyruvate dioxygenase (4HPPD), which is the enzyme required for pyomelanin production (Secor, 1994). Methods incorporated sulcotrione to determine if pigment production was a result of 4HPPD. Pure cultures of soil isolates were grown in a defined minimal medium that contained 6 g/l lactate as the carbon and energy source. Tyrosine (1g/l) was supplemented to the defined medium to promote pigment production. Sulcotrione (18μM) was added prior to pigmentation in order to differentiate pyomelanin production from other pigments that may be produced. Pigment production was monitored and characterized spectrophotometrically in cell free culture fluid and visually. Controls for comparison were without tyrosine or without sulcotrione.

3.3 SOIL COLUMNS – LABORATORY STUDIES

3.3.1 Treatment conditions

Soil was obtained from the Tims Branch (TB) watershed in uncontaminated areas of Pond-25 at SRS. The top 5 cm of humic material was removed prior to coring. Five cores to a depth of 65 cm were removed, yielding approximately 5 Kg of soil. Soil was sorted for plant debris, homogenized and stored at 4°C for 1 week prior to distribution into soil columns. All columns contained 100 g ($\pm$ 0.5g) homogenized soil in a 0.45mM pore size sterile, capped filtration unit. Filter material was cellulose acetate. Triplicate treatments (Table 3.1) included tyrosine (0.2, 0.1 and 0.05 g/100 g soil) (10, 5.0 and 2.5 mM), and/or 70 mM lactate. Stock lactate solution contained 50 ml of 60% Na lactate in 450 ml DI water for a stock concentration of 63.0 g/l (700 mM) lactate. For soil application, 10 ml of the stock lactate solution was added to 100 g soil, resulting in 70 mM/100g soil. Tyrosine (0.2, 0.1 and 0.05 g) (10, 5.0 and 2.5 mM) was added to 10 ml of lactate prior to application to soil. Lactate-tyrosine mixtures were autoclaved prior to soil application. When lactate or DI-water alone were used, they were sterilized via filtration (0.22 μM pore size).

Controls included only lactate, only DI water, and killed soil with 70 mM lactate and 0.2 g tyrosine. For killed soil controls 350g, homogenized soil was autoclaved for 30 minutes and allowed to sit for 4 days, followed by additional autoclaving for 60 minutes.

Table 3.1. Triplicate treatments to TB soil – study I

Controls	Lactate + Tyrosine/100 g soil	Tyrosine/100g soil
Lactate (70 mM)	Lactate (70 mM) + 0.2 g Tyrosine	0.2 g Tyrosine
DI-Water	Lactate (70 mM) + 0.1 g Tyrosine	0.1 g Tyrosine
Lactate (70 mM)+0.2 g Tyrosine/100g autoclaved soil	Lactate (70 mM) + 0.05 g Tyrosine	0.05 g Tyrosine

All amendments except for the killed controls were added on 3/31/05. Amendments to the killed controls were added 4/4/05. Incubation conditions include 25°C in the dark with periodic watering with sterile DI-water. Once a week 5 mL of sterile DI-water was added to each unit. The experiment was terminated on May 31, 2005 (after two months). One day (24 hours) before the end of the experiment, 25 mL of sterile DI water was added. On May 31, 2005 from each unit the leachates were collected and send for metal analysis by Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS). Additionally, pH, H_2S and organic matter content were determined

A second column study was undertaken to examine the effects of lower lactate concentrations with and without tyrosine. Conditions and experimental protocol were identical to the previous study (above) with the exception of treatment conditions as described in Table 3.2 below.

Table 3.2. Triplicate treatments to TB soil – study II

Controls	Lactate + Tyrosine/100 g soil
Lactate (10 mM)	Lactate (10 mM) + 0.2 g Tyrosine
Lactate (1 mM)	Lactate (1 mM) + 0.2 g Tyrosine

3.3.2 Soil characteristics

After the termination of the experiment the solid phase percent organic matter (OM) was estimated by loss-on-ignition at a temperature of 375°C. The pH was determined from a 1:1 mineral/water equilibration solution. Total concentration of metals on a homogenized sample was determined before the experiment. Total concentration of metals was evaluated by a total microwave digestion of 0.6 g of sediment material with concentrated acids (10 mL of HNO_3, 4 mL of H_2SO_4, and 2 mL of HCl). The resulting extract solution was analyzed by ICP-MS.

Changes in the organic content of soils due to pyomelanin production were determined through percent organic matter determination (above), spectrophotometric determination (absorbace, 400nm) of particle free supernatants and Fourier Transform Infra-Red (FTIR) spectroscopy. FTIR was performed on soil effluents receiving 10 mM lactate +/- 10 mM tyrosine in order to discriminate the types of soil pigments produced and verify that pyomelanin or another pigment dominated this process. FTIR data provide information identifying key chemical structures such as carboxyl groups alcohols, phenolics and phenolic alcohols, quinones, etc. based on peaks at specific wavenumber designations (X-axis). Results were compared to FTIR-produced standards of Aldrich humic acids, typical fungal melanin produced from dihydroxy phenylalanine (DOPA melanin), and bacterially produced pyomelanin.

3.4 SOIL COLUMN STUDIES – FIELD DEPLOYMENT

3.4.1 Soil Sampling

On May 3, 2005 a soil sample was taken from each soil column. The collected soil samples were analyzed for moisture, organic matter content, and metal concentrations. Percent organic matter (OM) was estimated by loss-on-ignition at a temperature of 375°C. The pH was determined from a 1:1 mineral/water equilibration solution. Total concentration of metals was determined by a total microwave digestion of 0.6 g of sediment material with concentrated acids (10 mL of HNO_3, 4 mL of H_2SO_4, and 2 mL of HCl). The resulting extract solution was analyzed by ICP-MS.

3.4.2 Soil Column Construction

Soil columns were constructed to facilitate field testing of the methodology. These columns were constructed to allow rainfall to leach through the columns and be collected for analysis at different depths and at the discharge. The design consisted of an inner and outer housing with the inner housing constructed of 4" diameter Schedule 40 PVC and the outer housing constructed of 8" diameter Schedule 40 PVC. Construction details for the soil columns are shown in Figure 3.1 and a picture of the inner and outer housing is shown in Figure 3.2.

At the field site, a pit was dug by hand to facilitate installation of the soil columns. Figure 3.3a shows a picture of the soil horizon at the field site. Once the pit was dug, soil from the pit was homogenized and cleaned of any roots or other debris (Figure 3.3b). This homogenized, native soil was then placed in the inner housing of each soil column.

After the inner housings were filled with the native soil, lysimeters (Soil Moisture Equipment Corp. Rhizon lysimeters) were installed at three different depths (4, 12 and 20 inches) by inserting them through predrilled holes perpendicular to the long dimension of the housing. Figure 3.1 shows the general orientation for the lysimeters. Each lysimeter was connected to the top of the soil column using color coded nylon tubing. Each depth was assigned a color to avoid confusion during sample collection. Figure 3.4 shows a picture of the lysimeters and the color coding scheme. Black tubing was connected to the deepest lysimeter (20 inches). Blue tubing was used for the middle lysimeter (12 inches) and red tubing was used for the shallowest lysimeter (4 inches). For each soil column, the inner housing was then placed inside the outer housing.

After the lysimeters were installed, the annulus between the inner and outer housing was filled with clean sand. Nylon tubing was connected to a predrilled hole in the bottom of the outer housing and extended to the top of the soil column. This allowed for the collection of the leachate exiting the inner housing. A protective cap with holes to allow for rainfall infiltration was fitted to the top of the inner housing of each soil column (Fig. 3.5a). A cover was placed over the annulus between the inner and outer housing to prevent rainfall infiltration (Fig. 3.5b).

After assembling the soil columns, they were placed in the pit as shown in Figure 3.6. The void space between the soil columns was then filled with the remaining soil from the pit excavation. Metal sheeting was used to protect the soil columns from animal damage.

3.4.3 Treatment conditions

Soil columns remained untreated for 2 months to allow for settling and periodic lysimeter checks. Sterile amendments were added to numbered columns chosen randomly for each treatment on August 3, 2005. Twenty four hours prior to sampling, excess water was removed from the soil columns with a peristaltic pump attached to the tubing connected to the bottom of the outer housing of the soil columns. Amendments were similar to those of the laboratory studies above, except carbon concentrations were added per 1 kg of soil instead of 100 g soil (Table 3.3). One kg of soil constituted approximately 30% of the volume of each field column.

Table 3.3. Triplicate treatments in field study.

Controls	Lactate and/or tyrosine
Lactate (10 mM)	Lactate (10 mM) + tyrosine (10 mM)
Lactate (1 mM)	Lactate (1 mM) + tyrosine (10 mM)
DI water	Tyrosine (10 mM)

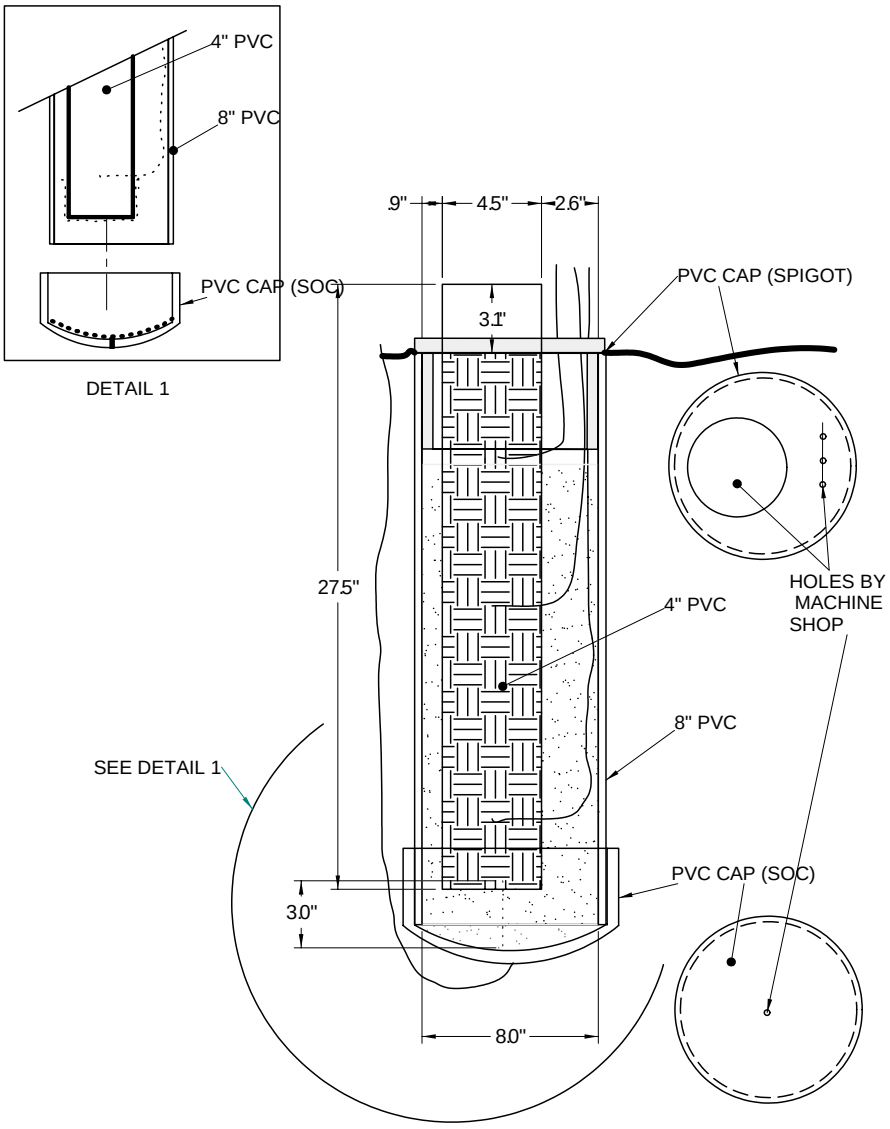


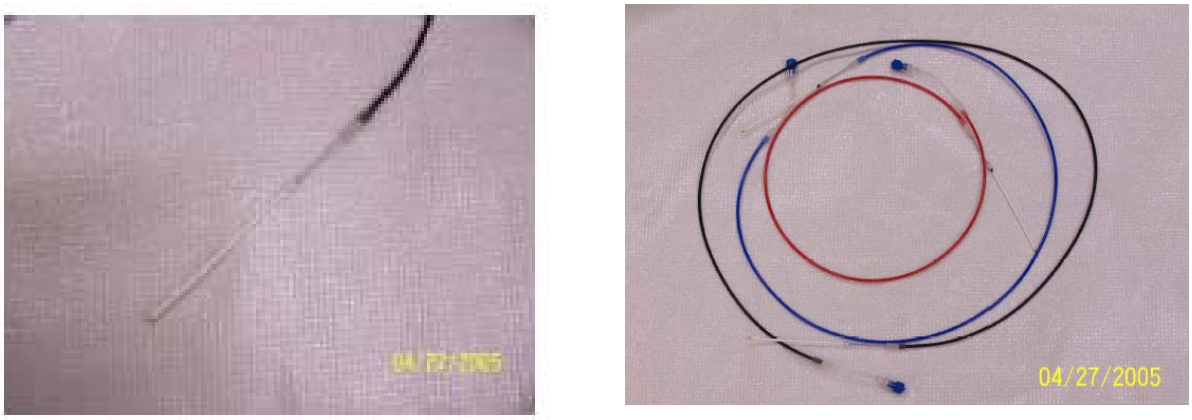
Figure 3.1. **Construction Details for the Soil Columns used at Tims Branch**.

(a) (b)

Figure 3.2. Inner (a) and Outer Housing (b) of Soil Columns used at Tims Branch..

(a) (b)

Figure 3.3. Soil Horizon (a) and soil (b) from the Tims Branch Field Site.



(a) (b)

Figure 3.4. Lysimeters for the Soil Columns used at Tim's Branch..

(a) (b)

Figure 3. 5. Caps and Screen Used on Each End of the Inner Housing for the Soil Columns used at Tims Branch.

Figure 3.6. Soil Columns used at Tims Branch Field Site.

3.4.4 Leachate Sampling

Throughout the 28 days of incubation the top 5 cm of soil of each column was mixed with sterile plastic spatulas on day 7 and 14, to assist tyrosine mixing and solubility. During field incubation rainfall was measured at 12.1 cm. After incubation soil pore water was obtained from each lysimeter (three depths per column). Samples were removed from soil by fitting each lysimeter with a 18 gauge hypodermic needle and inserting the needle into a negative-pressure, gas-tight test-tubes. The negative pressure in each 10 ml test tube pulled pore water from the soil at the lysimeters soil depth. Pore water samples were stored immediately on ice until overnight storage in the lab at 4°C. Samples were processed the next day and analyzed for metals, pH and H_2S.

4.0 RESULTS AND DISCUSSION

4.1 QUANTIFICATION OF MELANIN PRODUCING MICROORGANISMS AT SRS

4.1.1 Site Selection

Metal contaminated soils at D area and TNX were chosen for their potential to harbor pyomelanin-producing bacteria. These soils were quantified for pyomelanin producing bacteria due to high numbers obtained during previous studies with Tims Branch soils in the area of Pond 25. Our interest was to determine if the unexpectedly high numbers of pyomelanin producers at Tims Branch were normal for the soils at SRS or an anomaly. In addition, we sought to establish the utility of this technology if deployed at other parts of SRS in the future.

Bacterial isolates from the SRS subsurface culture collection were also analyzed for pyomelanin production. These pure cultures were previously isolated at various depths from the P 24 borehole at SRS during deep subsurface monitoring investigations in 1986 and characterized taxonomically during subsequent studies (Balkwill, 1997). This information (Table 4.1) provided us a framework to understand pyomelanin production across bacterial taxa as well as a function of vertical distribution in the subsurface.

4.1.2 Most probable Number (MPN) assay for melanin production

Bacterial densities of pigment producers for both soil samples from TNX and D area demonstrated MPN values of $1.1x10^6$ cells /g wet wt of soil with a 95% confidence limit of $1.1\ x10^8$ to $7.8x10^6$. Pigmentation occurred more rapidly in the higher dilutions and may have been a result of inhibitory components in the soils at the lower dilutions or a result of competition for nutrients by various bacteria.

4.2 PYOMELANIN PRODUDING BACTERIA FROM SRS CULTURE COLLECTION.

4.2.1 Characterization of Microbial Pigments

By utilizing a pre-existing bacterial culture collection, we determined the relative frequency of pyomelanin production by these isolates relative to depth of isolation and bacterial taxa. The frequency of pigment production was not a function of depth or geological strata (Table 4.1 and 4.2).

4.2.2 Characterization of Pyomelanin -Type Microbial Pigments

The enzyme inhibitor sulcotrione is specific for the enzyme responsible for pyomelanin production (Secor, 1994). As a competitive enzyme inhibitor sulcotrione affects the quantity of pyomelanin produced. When pigmentation was altered in bacterial cultures with sulcotrione, relative to pigment production without the inhibitor, it was concluded that pyomelanin was the type of melanin produced (Table 4.2). Because these isolates represent the most common pigment producers of our samples, the potential for pyomelanin production at this sight is expected to be significant.

Tables 4.1 and 4.2 provide detail about each isolate analyzed and those isolates that demonstrated pyomelanin production. Since pyomelanin production was first characterized in the genus *Pseudomonas*, it is not unexpected that members of that genus and related genera (*Comomonas* and *Aquaspirillum*) also produce pyomelanin. *In-silico* analyses of available genetic databases using computer search tools confirmed the presence of genes encoding for 4-HPPD in the organisms listed.

Table 4.1. Bacterial strains (listed by depth isolated) analyzed for pigment production from tyrosine.

Isolate #	Depth at which isolated (m)	Stratigraphic Formation isolated from	16S ID	Biolog ID	**Pigment from tyrosine**
B326	45	Dry Branch	Pseudomonas	Pseudomonas corrugata	No
B325	45	Dry Branch	Acinetobacter	Acinetobacter calcoaceticus	No
B183	58	McBean	Acinetobacter	Acinetobacter calcoaceticus	No
B211	58	McBean	Acinetobacter	Acinetobacter calcoacet/baumannii/gen	No
B173	58	McBean	Comamonas	Pseudomonas fluorescens Type A	Yes
B205	58	McBean	Pseudomonas	Pseudomonas mendocina	Yes
B259	91	Congaree	Pseudomonas	Pseudomonas mendocina	Yes
B257	91	Congaree	Acinetobacter	Acinetobacter calcoaceticus	No
B258	91	Congaree	Acinetobacter	Acinetobacter calcoaceticus	No
B253	91	Congaree	Pseudomonas	Pseudomonas stutzeri	Yes
B310	91	Congaree	Pseudomonas	Pseudomonas stutzeri	Yes
B287	91	Congaree	Pseudomonas	Pseudomonas mendocina	Yes
B307	91	Congaree	Pseudomonas	Pseudomonas mendocina	Yes
B301	91	Congaree	Pseudomonas	Pseudomonas mendocina	Yes
B141	118	Ellenton	Pseudomonas	Pseudomonas stutzeri	Yes
B113	118	Ellenton	Acinetobacter	Acinetobacter junii	Yes
B121	118	Ellenton	Acinetobacter	Acinetobacter calcoacet/baumannii/gen	No
B138	118	Ellenton	Pseudomonas	Pseudomonas stutzeri	Yes
B439	180	Pee Dee	Acinetobacter	Acinetobacter calcoaceticus	Yes
B442	180	Pee Dee	Comamonas	---	Yes
B461	180	Pee Dee	Acinetobacter	Acinetobacter calcoaceticus	No
B465	180	Pee Dee	Comamonas	Comamonas acidovorans	No
B064	200	Black Creek	Comamonas	Comamonas testosteroni	Yes
B050	200	Black Creek	Acinetobacter	Acinetobacter calcoaceticus	No
B039	200	Black Creek	Comamonas	Comamonas testosteroni	Yes
B031	200	Black Creek	Acinetobacter	Acinetobacter calcoaceticus	No
B020	200	Black Creek	Comamonas	Comamonas acidovorans	Yes
B032	200	Black Creek	Comamonas	---	No
B030	200	Black Creek	Comamonas	Acinetobacter calcoace	No
B058	200	Black Creek	Comamonas	Aquaspirillum aquaticum	Yes
B034	200	Black Creek	Comamonas	Pseudomonas corrugata	Yes
B524	204	Black Creek	Acinetobacter	Acinetobacter genospecies 15	Yes
B513	204	Black Creek	Arthrobacter	---	Yes
B623	244	Middendorf	Pseudomonas	Pseudomonas viridilivida	Yes
B575	244	Middendorf	Comamonas	Comamonas testosteroni	No
B687	259	Middendorf	Alcaligenes	Comamonas acidovorans	No
B641	259	Middendorf	Comamonas	Comamonas acidovorans	No
B716	265	Middendorf	Terrabacter	---	Yes

Table 4.2. Bacterial strains characterized as pyomelanin producers based by enzyme inhibition assays.

Isolate #	Depth at which isolated (m)	Stratigraphic Formation isolated from	16S ID	Biolog ID
B205	58	McBean	Pseudomonas	Pseudomonas mendocina
B259	91	Congaree	Pseudomonas	Pseudomonas mendocina
B253	91	Congaree	Pseudomonas	Pseudomonas stutzeri
B287	91	Congaree	Pseudomonas	Pseudomonas mendocina
B307	91	Congaree	Pseudomonas	Pseudomonas mendocina
B301	91	Congaree	Pseudomonas	Pseudomonas mendocina
B141	118	Ellenton	Pseudomonas	Pseudomonas stutzeri
B113	118	Ellenton	Acinetobacter	Acinetobacter junii
B439	180	Pee Dee	Acinetobacter	Acinetobacter calcoaceticus
B442	180	Pee Dee	Comamonas	---
B064	200	Black Creek	Comamonas	Comamonas testosteroni
B039	200	Black Creek	Comamonas	Comamonas testosteroni
B058	200	Black Creek	Comamonas	Aquaspirillum aquaticum
B034	200	Black Creek	Comamonas	Pseudomonas corrugata
B513	204	Black Creek	Arthrobacter	---
B623	244	Middendorf	Pseudomonas	Pseudomonas viridilivida
B716	265	Middendorf	Terrabacter	---

4.3 LABORATORY SOIL COLUMN STUDIES

4.3.1 Carbon Amendments to Soil

Tyrosine amendments to soils and pure bacterial cultures previously showed a direct correlation to pyomelanin production and tyrosine content of growth media (Turick, et al. 2002). Additionally, growth in the presence of tyrosine and lactate resulted in greater pyomelanin production than tyrosine alone. The present study incorporated lactate as a means to accelerate and enhance pyomelanin production from tyrosine. Lactate was used at three concentrations in order to determine a near-optimal concentration range.

In all cases, lactate stimulated bacterial activity resulting in an increase in the soil pH. Growth and pigment production also occurred in the autoclaved soils containing lactate and tyrosine. Given the difficulty to completely sterilize soil and previous studies (Turick and Kritzas, 2004) which identified a strain of the spore forming bacterial genus *Bacillus* as a common pyomelanin producer in these soils, it is not unlikely that some growth occurred. As a result these soils were no longer valid as controls. The highest concentration of lactate resulted in microbial activity that raised the pH over 9, which is higher than acceptable (Table 4.3). Because of the low buffering capacity of the soil, the resulting high pH caused dissolution of bound humics from the soils (Fig. 4.1). This was confirmed abiotically by adjusting the soil pH with dilute NaOH and measuring absorbance of the particle free liquid. Addition of tyrosine alone (without lactate) did not result in as high a pH as 70 mM lactate (Table 4.3). No significant change in pH occurred with the higher tyrosine concentration. This result was unexpected and suggests tyrosine

at this concentration did not support bacterial growth but was utilized for pigment production, based on the increased absorbance of the soil effluents (Table 4.3).

Table 4.3. pH ranges of triplicate samples receiving various treatments.

Treatment	pH Range
DI Control	4.45 - 4.55
70 mM Lactate	9.37 – 9.49
70 mM Lactate + 2.5 mM Tyrosine	9.55 – 9.67
70 mM Lactate + 5 mM Tyrosine	9.47 – 9.63
70 mM Lactate + 10 mM Tyrosine	9.62 – 9.86
2.5 mM Tyrosine	6.08 – 6.83
10 mM Tyrosine	4.43 – 4.47
10 mM Lactate + 10 mM Tyrosine	6.65 – 6.90
1 mM Lactate + 10 mM Tyrosine	5.76 – 6.40
10 mM Lactate + 10 mM Tyrosine	6.29 – 6.59
1 mM Lactate + 10 mM Tyrosine	5.57 – 6.41

4.3.2 Quantitative pyomelanin Determination in Soil Effluents

Absorbance at 400 nm was used to quantify relative changes in bacterial pigment production (Fig.4.1). The highest lactate concentration was responsible for the greatest increase in absorbance, due to humic desorption resulting from increased pH. Absorbance increased in other treatments as a function of tyrosine concentration. It is important to point out that tyrosine appears to have diminished the amount of humic desorption resulting from lactate (Fig.4.1). This may be a result of recently produced bacterial pigments combining with desorbed humics to form submicron colloids. While colloidal development was beyond the scope of this research, we did encounter drastic decreases in soil porosity with lactate amended soils but not with DI water controls or with only tyrosine amendments. This suggests that lactate utilization by bacteria decreases soil porosity, possibly through colloid production.

Soils receiving 10 or 1 mM lactate with or without tyrosine had an increase in pH not exceeding pH 7 and did not result in any dissolution of previously bound humics (Table 4.3, Fig. 4.4). Both of these results create favorable conditions for metal immobilization via pyomelanin production. The increase of pH to near 7 is favorable because pyomelanin production is optimal at neutral pH (Coon et al. 1994). Bound humics were not desorbed at neutral pH as compared to higher resulting pH values from the 70 mM lactate treatments. The desorption of humics at high pH will likely mobilize metals previously bound to humic-mineral complexes.

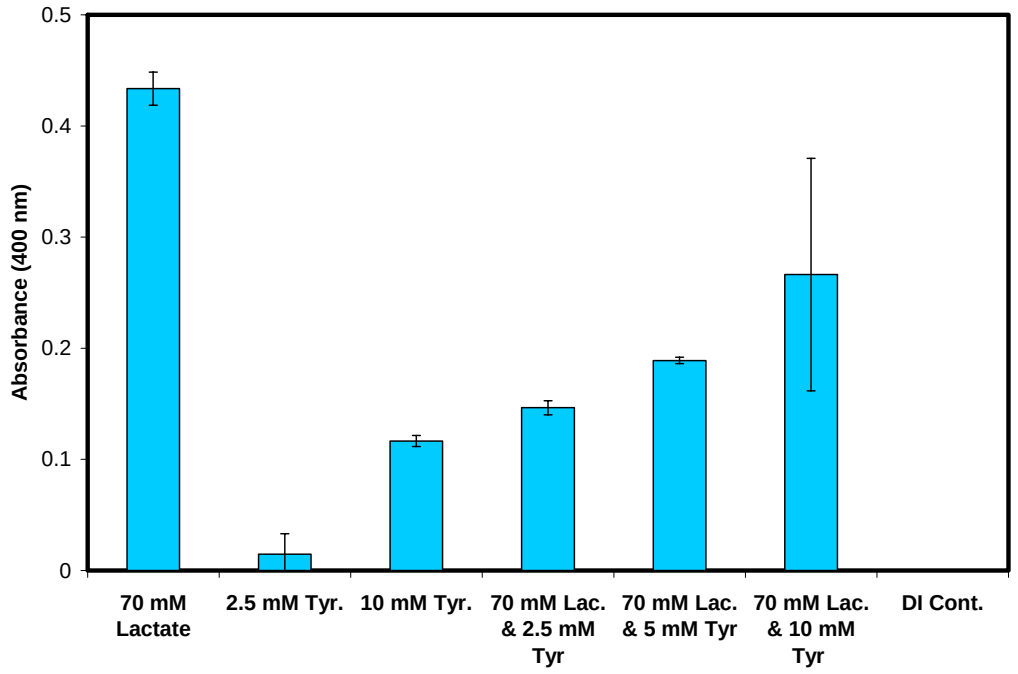


Figure 4.1. Absorbance (400nm) of particle free soil effluents (1/10 dilution) demonstrating changes in OD as a function of treatment.

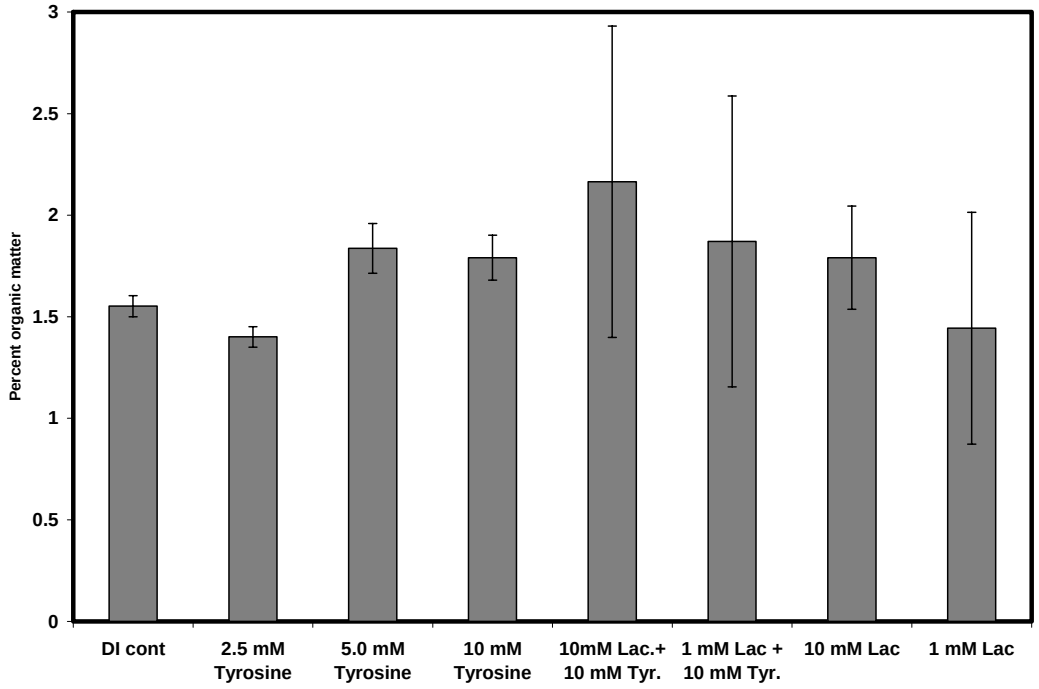


Figure 4.2. Percent organic matter of soils after removal of soil effluents, as a function of treatment.

Differences in percent organic matter (Fig.4.2) demonstrated a trend toward increased organic matter as a function of treatment but the trends were not significantly different (P>0.1). The differences between the results of Figures 4.1 and 4.2 likely are related to the degree of bound organics after effluents were

removed. Soils amended with lactate plus tyrosine demonstrated obvious darkening (Fig. 4.3) as well as increased absorbance at 400nm when effluents were measured spectrophotometrically (Fig.4.4). No obvious changes in soil coloration were obvious with only lactate amended soils, even though the absorbance (400 nm) of the aqueous effluents were higher than controls (Figs. 4.1 and 4.4).

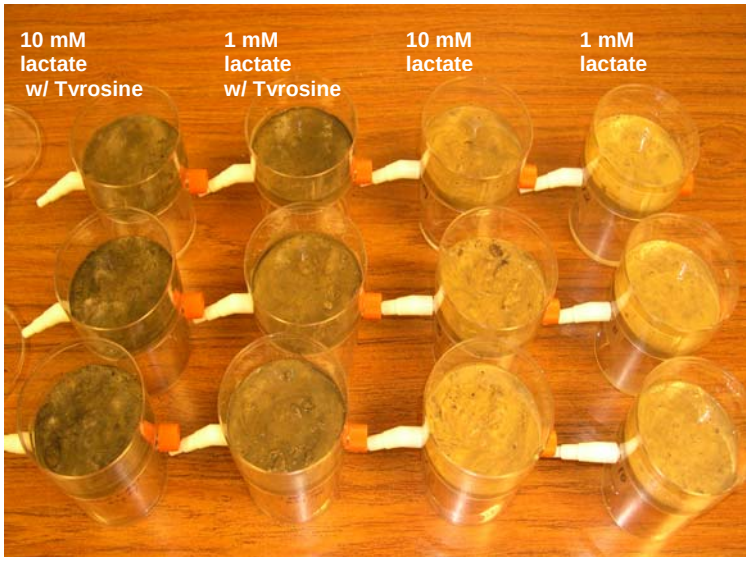


Figure 4.3. Photograph demonstrating visual darkening of soils receiving tyrosine amendments compared to those without tyrosine.

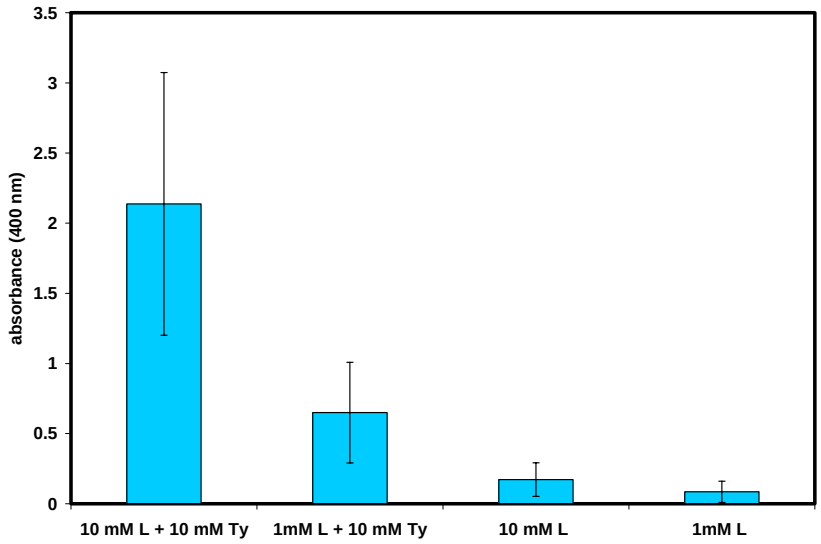


Figure 4.4. Absorbance (400nm) of particle free soil effluents demonstrating changes in OD as a function of treatment.

Pigmentation was more obvious at higher rather than lower soil dilutions in previous MPN studies with SRS soils. These results suggested potential inhibitory conditions in soils that would limit bacterial pigment production. Based on the soil column results presented here, pigmentation was not inhibited in these soils. Previous MPN results may be due to medium composition or culture conditions.

FTIR scans of pyomelanin were different from the other compounds used as standards (Fig. 4.5), demonstrating the possibility to discriminate between pyomelanin and other common soil organics. Effluents from soils incubated for 30 days with 10 mM lactate with and without tyrosine were analyzed to determine if differences existed in FTIR response relative to treatments (Fig. 4.6). Similar FTIR scans were observed with treatment replicates but differences were evident with and without tyrosine amendments. Tyrosine amended soils demonstrated a characteristic response similar to that of pyomelanin (Fig. 4.7) but dissimilar to Aldrich soil humics or DOPA melanin (Fig. 4.5).

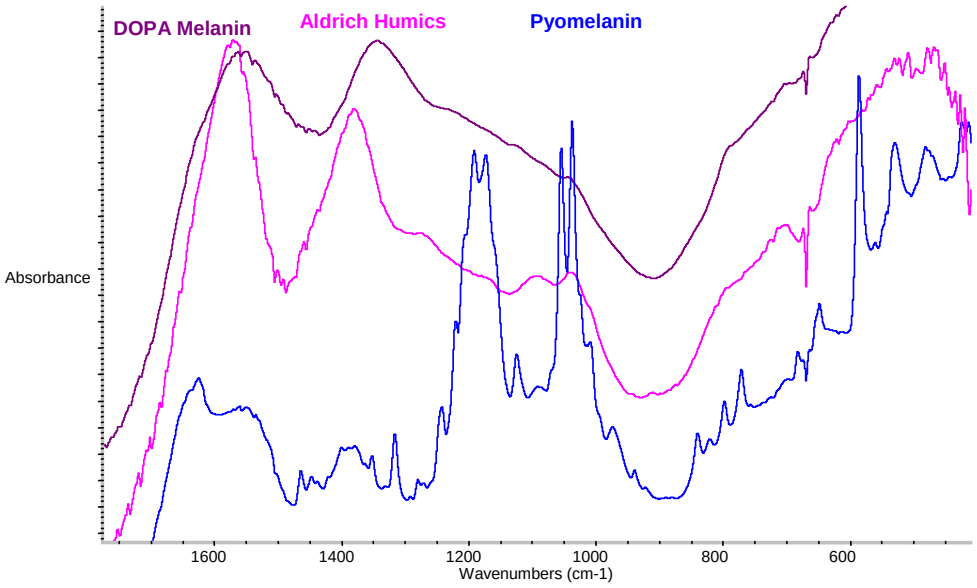


Figure 4. 5. FTIR scans demonstrating differences between pyomelanin, Aldrich humics and DOPA melanin.

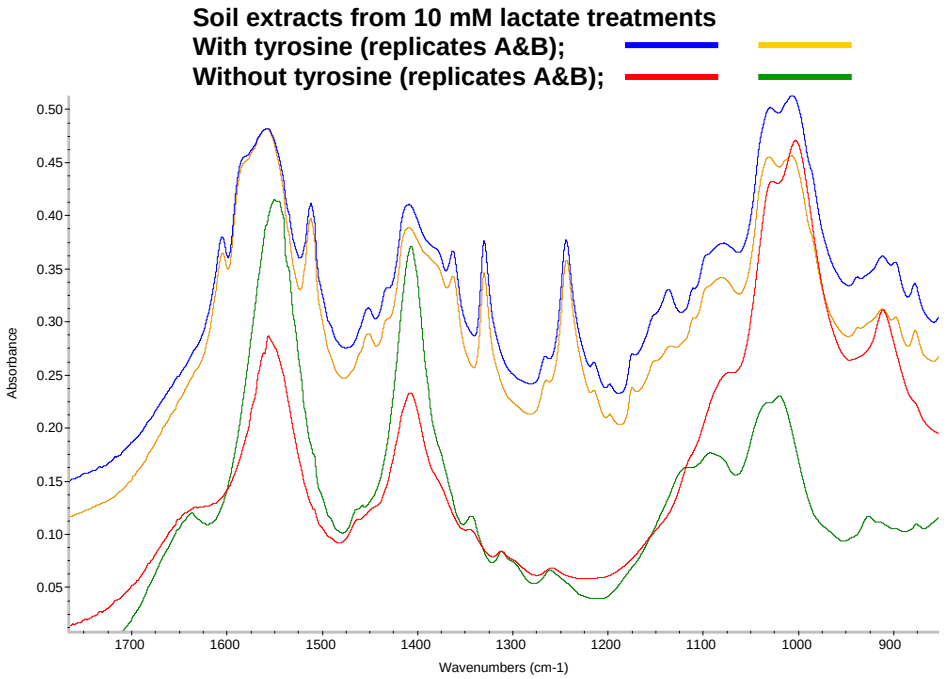


Figure 4.6. FTIR scans comparing and contrasting duplicate soil effluents from soil column studies with and without tyrosine.

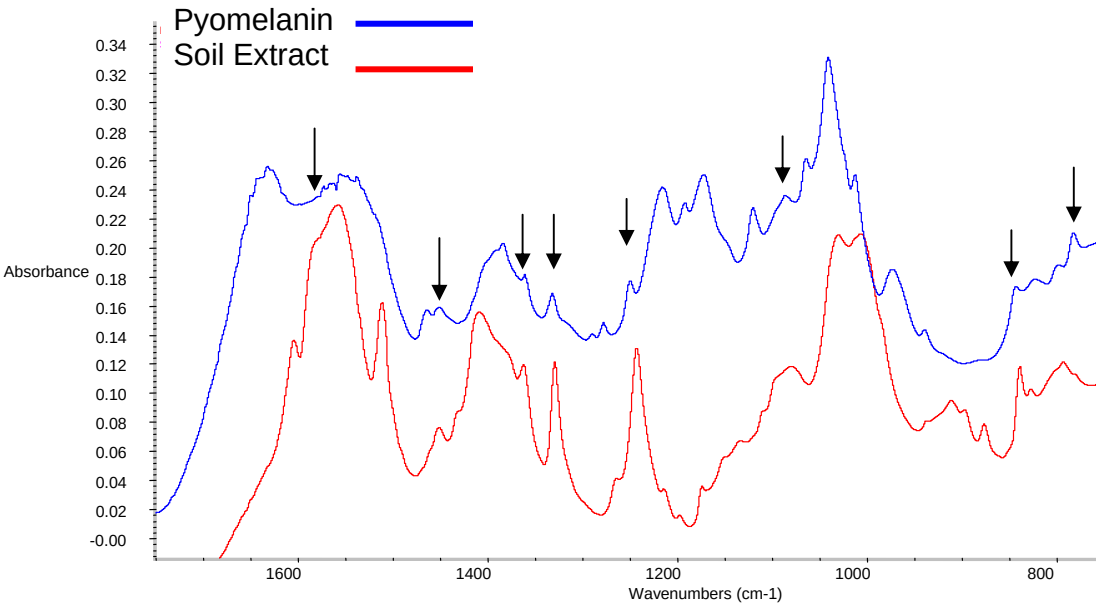


Figure 4.7. FTIR scans demonstrating similarities between pyomelanin and effluents from soils incubated with tyrosine. Arrows designate peaks from pyomelanin that coincide with those of soil effluents with tyrosine but not those without tyrosine (Fig. 4.5)

4.3.3 Effects of Pyomelanin on Reduction Capacity of Soil.

Pyomelanin contributes to metal reduction by bacteria due to its electron shuttling. Both the rates and the degree of metal reduction increase in the presence of pyomelanin (Turick et al. 2002 and 2003). Previous SGCP funded work also demonstrated pyomelanin sharing by a variety of bacterial genera commonly associated with soils, increased metal reduction capacity by the same bacteria (Turick and Kritzas, 2004). In the present study, after 3 weeks incubation, lactate and tyrosine-amended soils demonstrated increased metal reduction capacity relative to soils amended with lactate only (Fig. 4.8). These results (DI controls subtracted) reflect the degrees of difference in the redox activity in this soil as a function of treatments with 39% and 48% increases in metal reduction in tyrosine treated soils during incubation.

In kinetic studies, hydrous ferric oxide-spiked soil samples demonstrated metal reduction from lactate and tyrosine to be 15.9 mM compared to 13.4 from lactate treated soils after 50 hrs of incubation. Thus, resulting in a 15.7% increase in metal reduction by tyrosine treated soils. The increased rate (hr^{-1})in metal reduction was calculated to be 3.4% greater for tyrosine-amended soils relative to soils treated only with lactate.

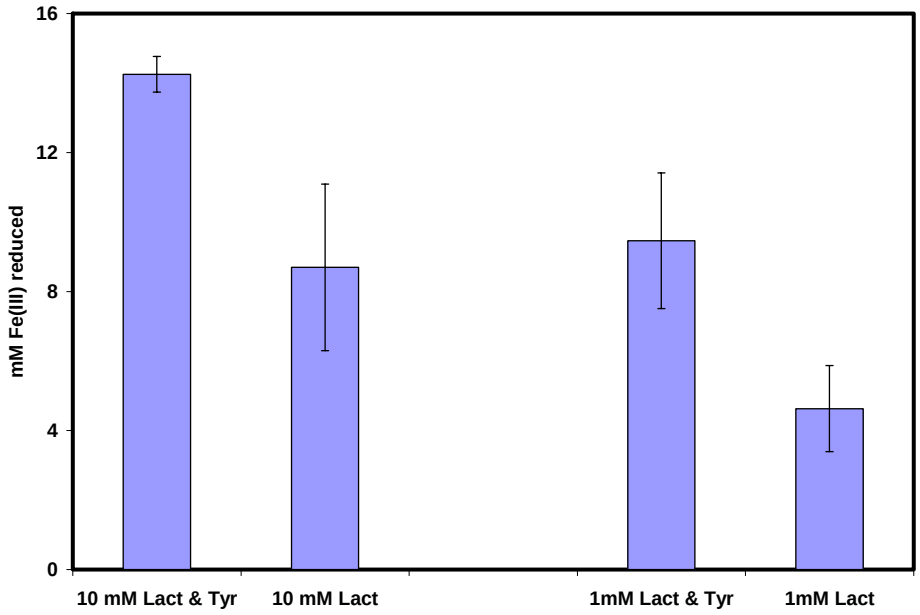


Figure 4.8. Relative amounts of Fe(III) reduced [measured as Fe(II)] after soils were incubated with various treatments. A greater reduction potential was evident in soils with tyrosine.

4.3.4 Effects of Pyomelanin Production on Metal Mobility in Soil

Filtered soil effluents were analyzed for metal content to determine the effect of tyrosine addition and subsequent pyomelanin production in soils. Samples were analyzed after 30 days incubation and compared to DI water controls. Soils that demonstrated high pH and humic desorption were not analyzed. These results represent metal behavior in the top 5 cm of soil.

A decrease in U in soil effluent was evident with 2.5 and 10 mM tyrosine amendments, indicating U immobilization (Fig. 4.9). Lactate and lactate plus tyrosine amendments however demonstrated increased U in effluents, with highest U concentrations occurring with lactate and tyrosine together relative to only

lactate. Possible production of colloids resulting from these treatments may be responsible for this trend of U mobility. Alternatively, excess pyomelanin production in the first 5 cm of soil could have complexed with metals prior to sorption to soil, thereby increasing the metal concentrations in the soil effluent. Field studies (below) were designed to provide data related to pyomelanin/metal complexes in the soil column.

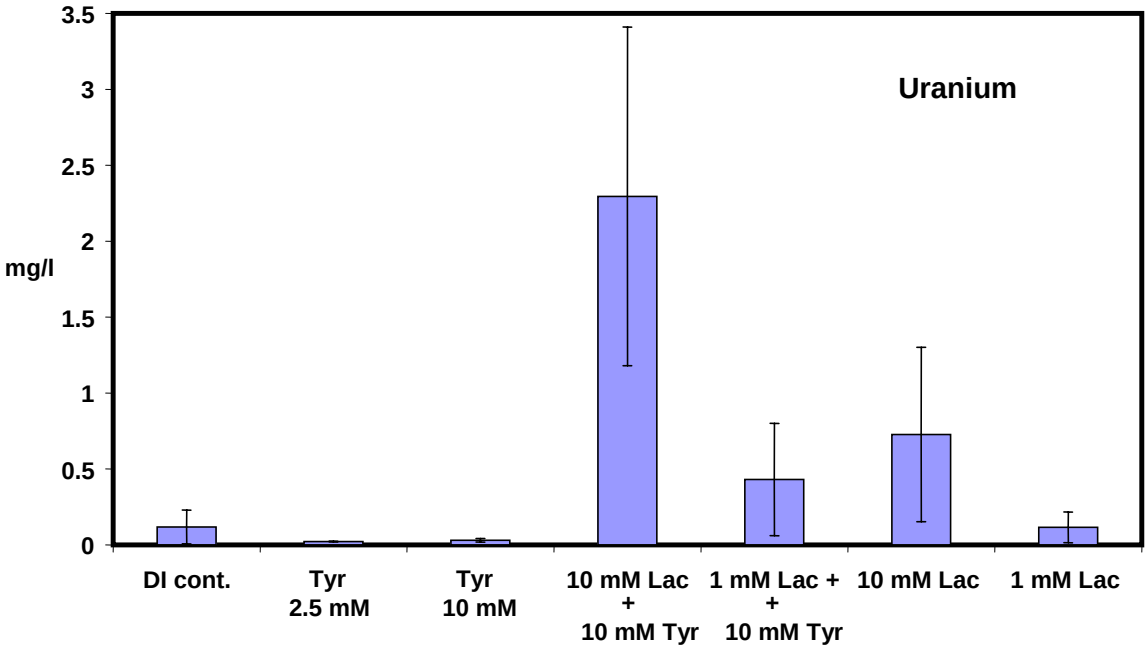


Figure 4.9. Uranium concentrations of soil effluents after incubation with various treatments.

Nickel behaved very differently than U where Ni was lower in concentration in all effluents studied. All treatments demonstrated significant immobilization except for 10 mM tyrosine, which demonstrated only about 30% immobilization (Fig. 4.10). It is unlikely that excess pyomelanin played a role here. The lower pH of the 10 mM tyrosine treatment may have played a larger factor in Ni mobility.

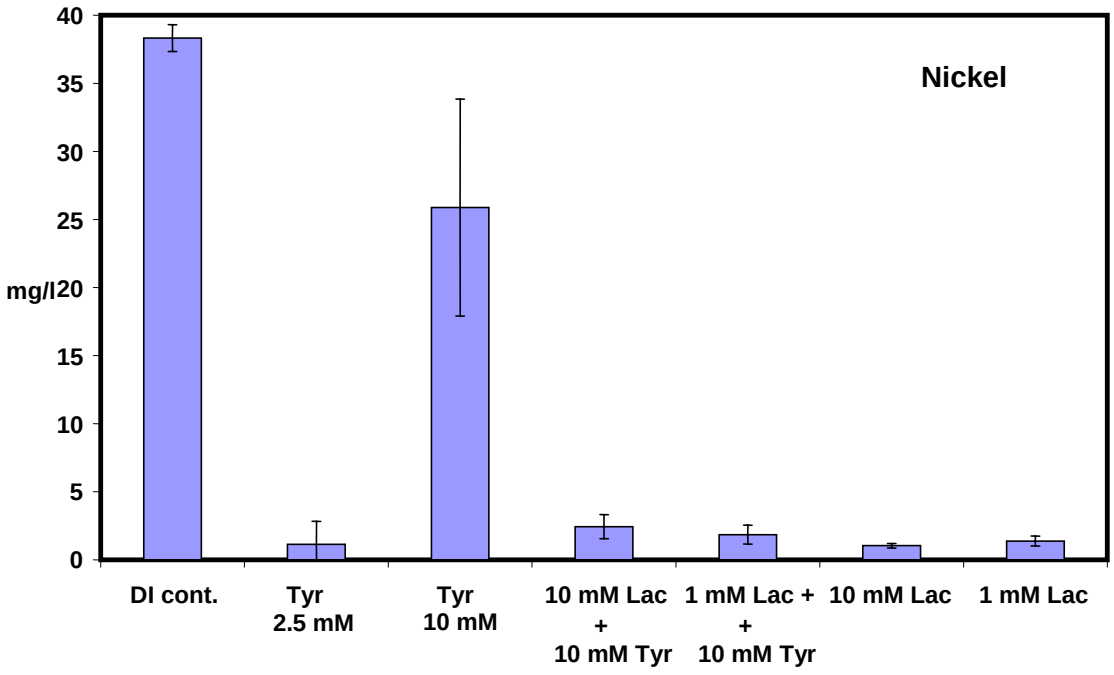


Figure 4.10. Nickel concentrations of soil effluents after incubation with various treatments.

Because biogeochemical activities are complex and affect the soil chemistry as a whole, we also evaluated several other metals to determine the role of pyomelanin production on the bulk geochemistry. Arsenic and lead were chosen in order to determine the fate of other metals of potential concern in order to understand more fully the mechanistic role of pyomelanin on metal immobilization. Although these metals are present at Tims Branch watershed soils they are not in high enough concentrations to be considered environmentally problematic.

Pb and As were both immobilized with tyrosine amendments, relative to controls (Fig. 4.11). In contrast, lactate-only amendments demonstrated increased mobilization of both metals as a function of lactate concentration. When tyrosine was added with lactate the relative degrees of metal mobilization decreased, relative to lactate-only treatments.

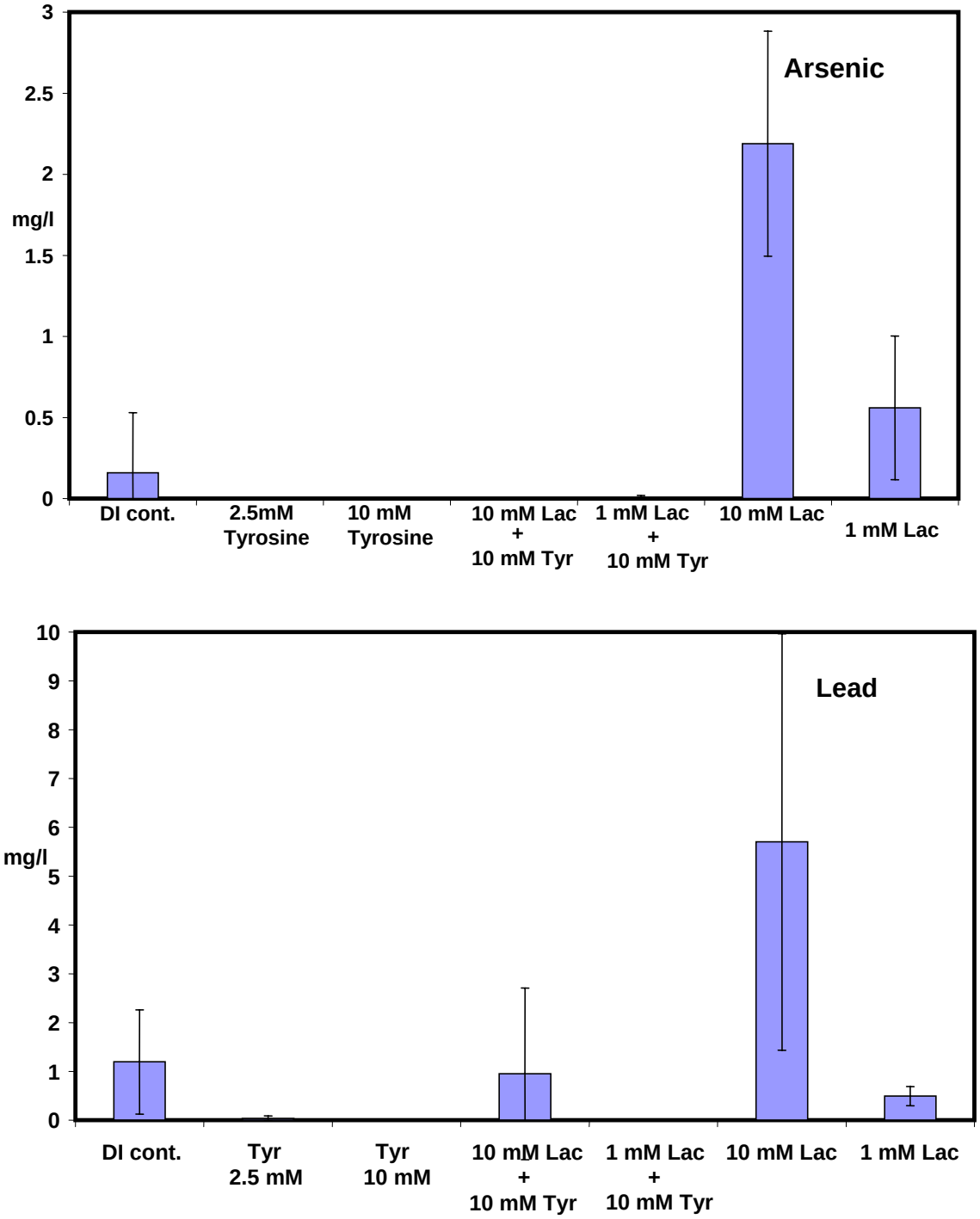


Figure 4.11. Arsenic and lead concentrations of soil effluents after incubation with various treatments.

4.4 FIELD DEPLOYMENT STUDIES

4.4.1 H_2S Production and pH changes

Soil columns packed with well mixed soil from Pond 25 were amended in triplicate with DI water, 10 and 1 mM lactate with and without 10 mM tyrosine and 10 mM tyrosine alone. Soil effluents from the field-deployed lysimeters were analyzed 3 weeks after amended at 3 levels; 4, 12 and 20 inches, in order to determine the effects of soil treatments on metal mobility. In addition to ICP-MS analysis effluents were also analyzed for pH and hydrogen sulfide production. This was done to determine if changes in the soil pH contributed to metal mobility and if sulfate reducing bacteria contributed to metal immobilization as a result of sulfate reduction to hydrogen sulfide. Table 4.4 demonstrates changes in pH as a function of treatment. The pH values were not significantly impacted by the treatments and likely did not contribute to differences in metal mobility. Figure 4.12 shows that negligible hydrogen sulfide originated, indicating that, relative to controls, treatments did not stimulate growth of sulfate reducing bacteria and thus hydrogen sulfide had little to no effect on the fate of metals in this study.

Table 4.4. Soil pH of field study as a result of treatment conditions.

Treatments	pH		
	Depth (inches)		
	4	12	20
DI control	3.9 – 4.1	3.8 – 4.0	4.8 – 5.2
1 mM Lactate + 10 mM Tyrosine	3.9 – 4.0	3.9 – 4.0	4.9 – 5.2
1 mM Lactate	4.0	3.9 – 4.1	4.8 – 5.3
10 mM Lactate + 10 mM Tyrosine	4.2 – 5.7	4.1 – 5.2	4.6 – 5.5
10 mM Lactate	4.6 – 5.0	4.0 – 4.3	5.0 – 5.2
10 mM Tyrosine	3.8	4.0 – 4.4	4.5 – 5.1

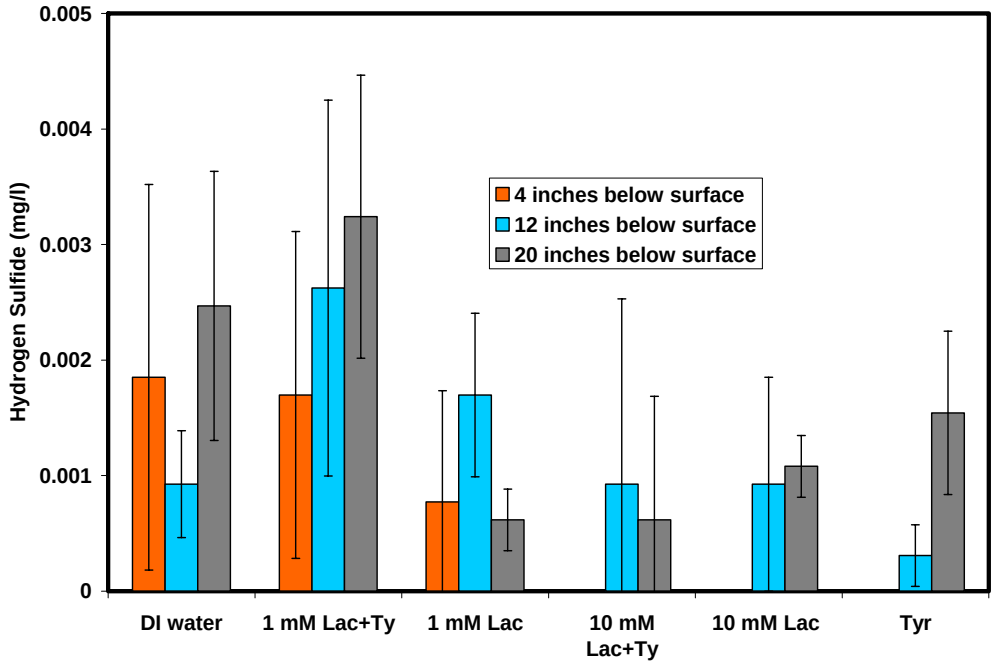


Figure 4.12. Hydrogen sulfide concentrations in pore water from lysimeters after incubation with various treatments.

4.4.2 U and Ni Immobilization

Uranium immobilization occurred in the three levels of the lysimeter study relative to controls (Fig. 4.13) with results similar to that of soil column studies (Fig. 4.9). In all cases tyrosine as the sole amendment demonstrated the greatest degree of U immobilization. The higher lactate concentrations demonstrated less U immobilization with the least attributed to 10 mM lactate plus 10 mM tyrosine. Since all tyrosine concentrations in this study were 10 mM, lactate in combination with tyrosine contributed to a decrease in U immobilization. This may be a result of increased pyomelanin production from lactate and tyrosine and hence an overall higher concentration of soluble pyomelanin as a function of depth. Sorption kinetics are required for a more informed understanding of these data. This topic is currently the focus of another project. Upon its conclusion we will be able to define more fully the fate of U in this system.

Nickel immobilization was most enhanced as a function of lactate concentration in the top portion of the soil column while Ni concentrations increased, relative to controls with tyrosine amendments (Fig. 4.14). Ni concentrations were higher with tyrosine alone and tyrosine with 1 mM lactate than controls. In contrast, Ni was substantially immobilized in soil column studies by all treatments but tyrosine (Fig. 4.14). The 20 fold increase of Ni in the lysimeters may account for the difference between these two sets of data.

Overall, U demonstrated a decrease in concentration with soil depth for all treatments but the control, (Fig. 4.15) indicating that all amendments contributed to U immobilization. The most pronounced degree of U immobilization occurred with 10 mM tyrosine alone. The overall trend for Ni was also a decrease in concentration with soil depth (Fig. 4.15), however it is unlikely that the soil amendments contributed to immobilization in this case. The mechanism of Ni immobilization in this study is unknown but worthy of further study.

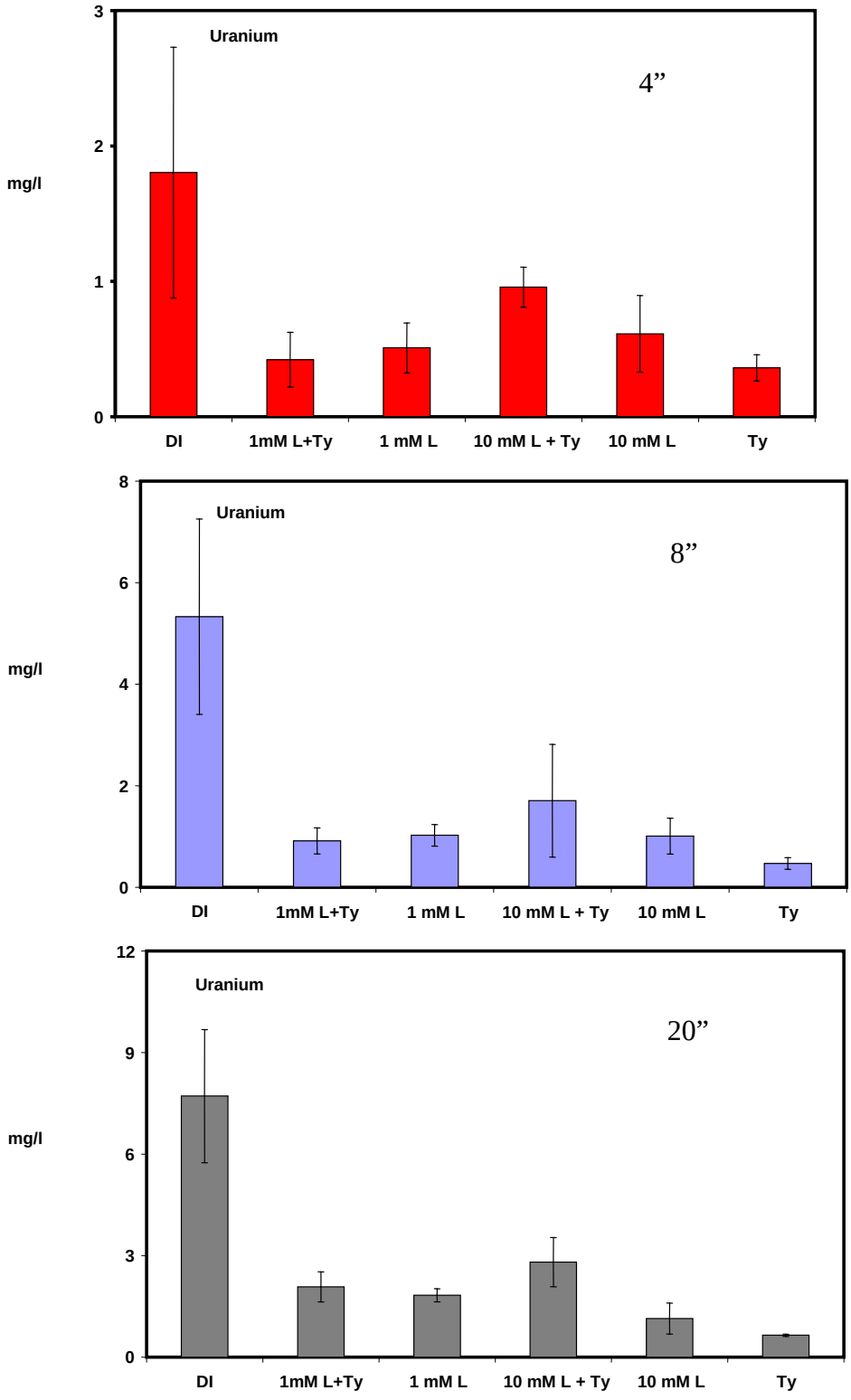


Figure 4.13. Uranium concentrations of soil effluents at 3 depths after incubation with various treatments.

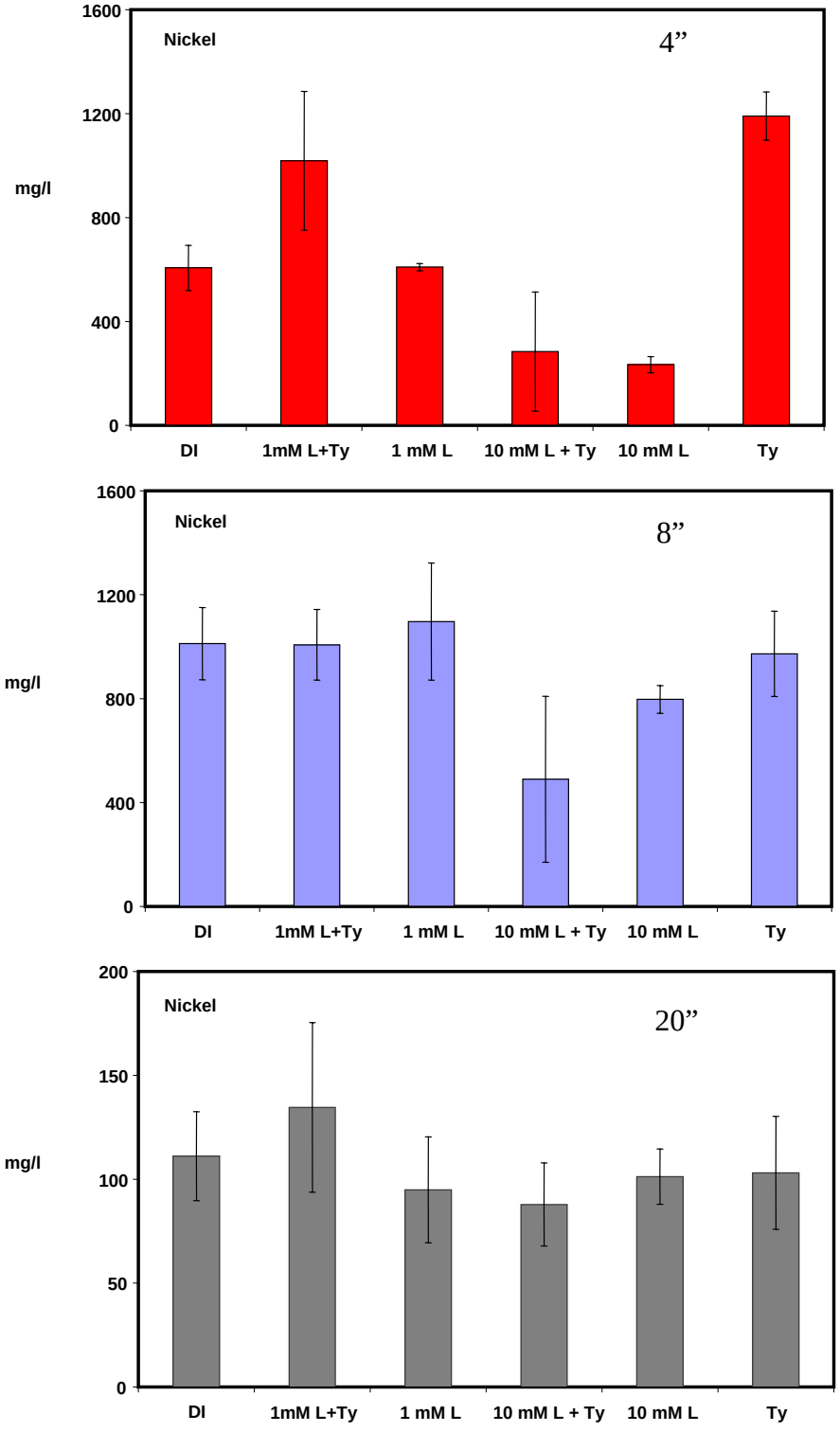


Figure 4.14. Nickel concentrations of soil effluents at 3 depths after incubation with various treatments.

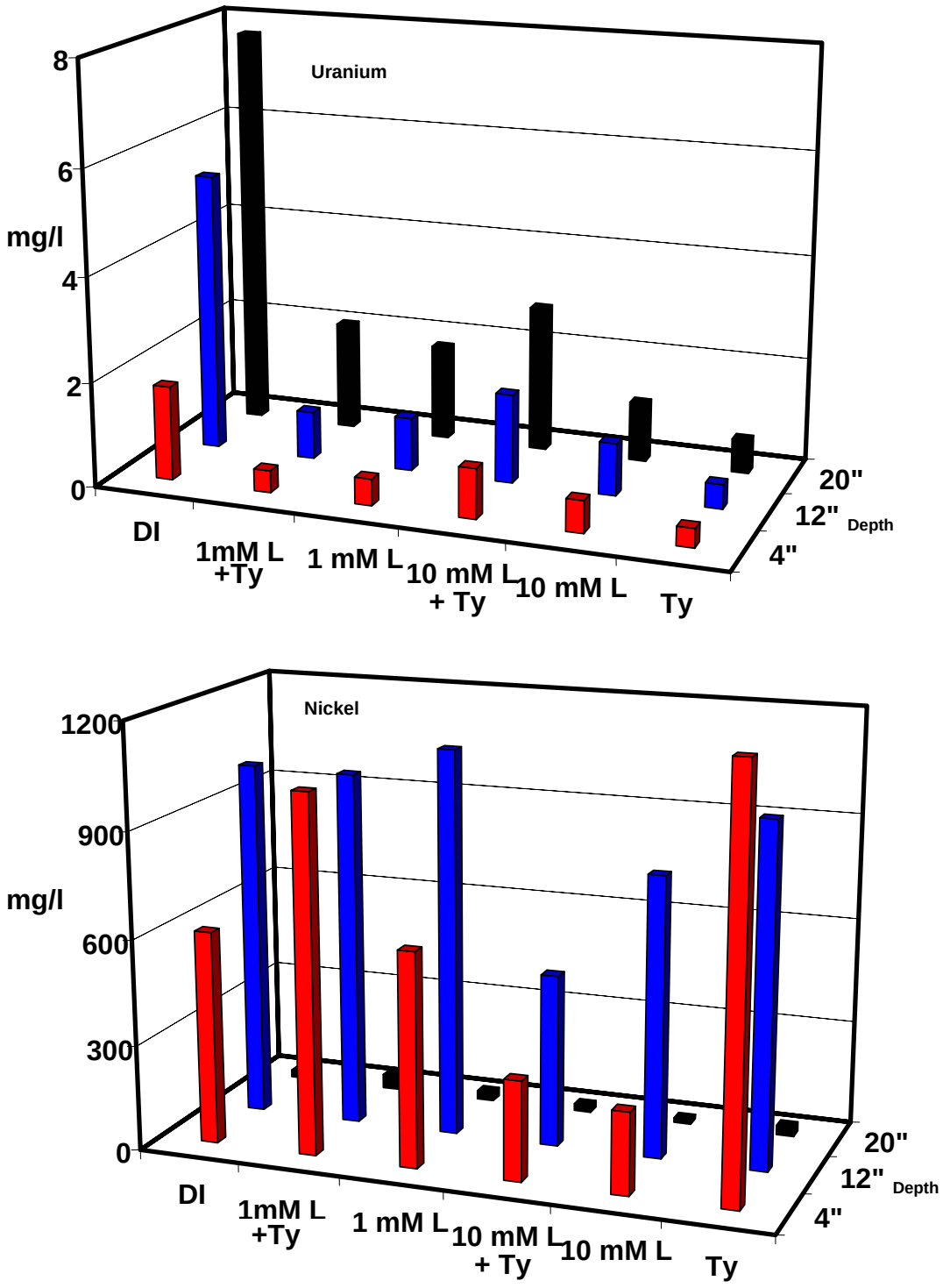


Figure 4.15. 3D graphs demonstrating U and Ni concentrations as a function of depth and soil treatment.

4.4.3 Pb and As Immobilization

Lead immobilization was not detected with depth in the controls but was evident with 1 mM lactate with and without tyrosine as well as tyrosine alone (Fig. 4.16). These results were consistent with those of the soil column study (Fig. 4.11). While 10 mM lactate with tyrosine demonstrated a decrease in Pb in the top portion of the lysimeter, the lower portion had a higher Pb concentration than controls. Since Pb concentrations were higher in the lysimeter study, this could account for the differences in Pb behavior. However, Pb concentrations in the treated soils were not significantly higher than the controls, indicating that these treatments did not contribute to substantial Pb immobilization.

Arsenic was not detected in the top portion of the soil but was in the lower two depths (Fig. 4.16). The soil amendments had no significant effect on As mobilization. The low concentration and high heterogeneity of As in this soil does not permit a conclusion about the effects of the treatments on its fate.

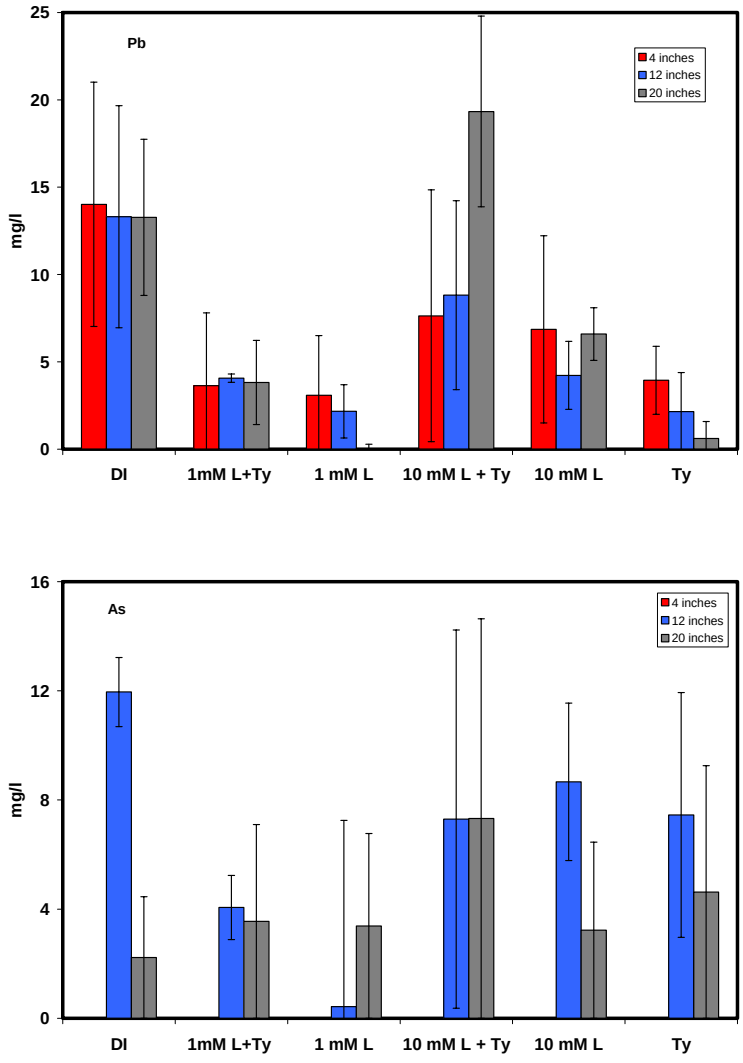


Figure 4.16. Lead and arsenic concentrations of soil effluents at 3 depths after incubation with various treatments.

5.0 SUMMARY AND CONCLUSIONS

Contamination from metals and radionuclides exists throughout the DOE system including SRS. Remediation technologies exist for treating these contaminants but when metals and radionuclides are present in low concentrations these technologies may cease to be cost effective. This project addressed research to support environmental restoration and stabilization needs. The ultimate goal of this work was to develop and deploy an environmental biotechnology-based application for accelerated metal and radionuclide reduction and *in-situ* stabilization.

Melanin-type pigments are common metabolites of many subsurface bacteria. The redox behavior, metal chelation properties and the recalcitrance of these compounds suggests that they influence metal behavior in soils for extended time periods. In particular, pyomelanin, a class of melanin pigments, was shown to be common in SRS soils, including the surface soils of Tims Branch watershed, D area wetland and the TNX wetland. A vertical distribution of pyomelanin producing bacteria at SRS was shown to extend below 200 meters, based on studies with bacteria isolated from the P 24 borehole. These results demonstrate the ubiquity of pyomelanin producing bacteria at SRS and indicate that this physiology can be exploited for bioremediation.

Pyomelanin is produced through the partial catabolism of tyrosine. Therefore, pyomelanin production was stimulated with tyrosine amendments *in-vitro* and *in-situ* with Tims Branch soils containing low actinide and metal levels . Pyomelanin production in soils was tentatively identified spectrophotometrically and confirmed through FTIR. Because pyomelanin is an electron shuttle and accelerates metal reduction, the rate and degree of metal reduction was documented in soils with pyomelanin compared to controls.

Metal and actinide immobilization were enhanced as a result of pyomelanin produced by indigenous bacteria when soils were amended with tyrosine. The addition of lactate with tyrosine enhanced the degree of pyomelanin production but did not always improve metal immobilization efficiency relative to amendments with only tyrosine. This was most evident with U immobilization where the greatest degree of immobilization, both *in-vitro* and *in-situ* resulted from only supplemental tyrosine at several concentrations. While U immobilization was accomplished with both lactate and tyrosine amendments as well as tyrosine alone, tyrosine without lactate was most successful.

Similar results of pyomelanin induced immobilization were demonstrated with Pb and As. Lactate-only amendments resulted in increased Pb and As mobility with *in-vitro* studies, relative to no carbon controls. Minimal effects by any treatment were documented for Ni immobilization *in-vitro* with tyrosine-only amendments demonstrating a slight trend towards increased Ni mobilization. Field studies demonstrated Ni immobilization occurring within the first 20 inches of the soil column will all treatments.

Lactate supplementation resulted in decreased porosity of soils. This along with increased mobility as a result of some lactate additions suggests that other mechanisms are involved in actinide and metal behavior in these soils. Such mechanisms may include colloid production, which was beyond the scope of this project. The demonstration of Ni immobilization, even with no carbon controls also indicates another possible mechanism for Ni sequestration in these soils.

This work demonstrates the potential of exploiting indigenous microbial populations for metabolite production aimed at metal and actinide immobilization. Microbial production of pyomelanin as a result of tyrosine supplementation resulted in metal immobilization. Pyomelanin-based metal immobilization does not occur equally for all metals. Based on the biogeochemical complexity of specific sites, preliminary studies should accompany any remedial activity in order to comprehend the behavior of all metals from a holistic perspective.

6.0 ACKNOWLEDGMENTS

We would like to thank SCURF for providing student assistance and Dr. Chad Leverette at USC Aiken for student-mentoring as well as assistance in FTIR analysis. We are also grateful to Dr. T.H Cromartie of Zeneca Ag. Products, Richmond, CA for providing sulcotrione. This work was funded by Soil and Groundwater Closure Projects at SRS.

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