Bioremediation
Education,
Science and
Technology
Program

Providing

training for future careers
in bioremediation.

Finding

new solutions to
old problems through
multi-disciplinary
research.

Restoring

the environment for
the next generation.

BEST: A pioneering program to restore our damaged environment, mitigate the health problems they pose, reduce clean-up costs, foster advanced research and prepare under-represented minorities and women for rewarding careers.

Contents

Executive Summary

An overview by Terry C. Hazen

The Bioremediation, Education, Science and Technology (BEST) partnership provides a sustainable and contemporary approach to developing new bioremedial technologies for U. S. Department of Defense (DoD) priority contaminants while increasing the representation of underrepresented minorities and women in an exciting new biotechnical field. This comprehensive and innovative bioremediation education program provides underrepresented groups with a cross-disciplinary bioremediation curriculum and financial support, coupled with relevant training experiences at advanced research laboratories and field sites. These programs are designed to provide a stream of highly trained minority and women professionals to meet national environmental needs.

The BEST partnership of institutions and participants benefit from a unique central strategy — shared resources across institutional boundaries. By integrating diffuse resources, BEST forms a specialized "learning institution without walls," where participants can receive advanced training at any BEST site, and where research capabilities flow freely among the participating institutions. Ongoing faculty and student exchange programs, video taped lectures, the Rotating Scholars program, and the BEST web-site ensure that all participants are empowered with opportunities to excel.

The BEST partnership consists of Lawrence Berkeley National Laboratory (LBNL) in Berkeley, Calif., Jackson State University (JSU) in Jackson, Miss., Ana G. Méndez University System (AGMUS) in Puerto Rico, University of Texas at El Paso (UTEP), University of Southern Mississippi (USM) Gulf Coast Research Lab, and University of California at Berkeley (UCB). The BEST program contract to the partnership is managed by LBNL for the Army Corps of Engineers, Waterways Experiment Station (WES) in Vicksburg, Miss. WES manages the contract for the Army Corps of Engineers and is the contracting entity for DoD.

The partnership formed by these participating institutions leverages existing institutional resources by strengthening intramural bioremediation education and research capabilities, and through outreach programs, to disseminate training and scientific enhancement to other Historically Black Colleges and Universities (HBCUs) and Minority Institutions (MIs).

The BEST institutions are focal points for the development and dissemination of cutting-edge research and technology for the bioremediation of nitro-aromatic compounds, polycyclic aromatic hydrocarbons (PAHs) and toxic metals. The multidisciplinary BEST partnership strategy creates a flask-to-field solution that develops laboratory research into technology, and technology into field-scale environmental applications required for the cost-effective restoration of damaged environments.

This year saw the addition of the University of Southern Mississippi's Gulf Coast Research Lab and the University of Texas at El Paso as partners in the BEST program. Both institutions provide significant new personnel and training opportunities for the BEST program. The USM Gulf Coast Research Lab investigators' focus on PAH and heavy metal phytoremediation along shorelines provides an exciting new focus with increased field opportunities for students. The UTEP investigators are focusing on exciting new metal phytoremediation techniques using desert plants and exciting new techniques to determine risk assessment with PAHs.

This year also saw the passage of the program directorship at LBNL from Dr. Jenny Hunter-Cevera to Dr. Terry C. Hazen in October 1999. Dr. Hunter-Cevera, who has managed the BEST program at LBNL since its inception, will be sorely missed, but her new position as president of the Maryland Biotechnology Institutes may provide increased opportunities for collaboration for the entire BEST program. Dr. Hazen, who specializes in bioremediation field applications, has demonstrated or deployed bioremediation technologies at more than 50 sites around

the United States and in Europe. He has five patents in bioremediation technologies that are licensed by more than 40 companies in the U.S. and Europe.

During the past year, the BEST program has provided minority research training for five high school students, 74 undergraduates, 32 graduate students, three post-doctoral fellows and 10 faculty. Students and faculty investigators have given 43 presentations on BEST research at scientific meetings and have published 17 scientific papers. The program produced a full color brochure and flyers in 1999 for use in recruiting more students, and also sponsored 32 lecture/seminars on bioremediation. Fourteen videotapes of BEST seminars at LBNL/UCB were distributed to the partner institutions. The BEST program also sponsored a phytoremediation workshop for BEST investigators and students that was attended by more than 60 participants. Additional workshops are planned for the coming year.

In this report, the research is organized by subject area, and two-page briefs are presented for each of 28 BEST projects. The projects presented provide a good representation of the state-of-the-science research being done with students in the BEST program – ***the best of BEST.***

Introduction

Over the next 75 years, the U.S. government will undertake what has been called the largest civil works project in world history to restore the environment damaged by previous activities at federal sites, e.g., Department of Defense (DoD) military bases and Department of Energy (DOE) nuclear facilities. Legislative action, resulting from concern over the accumulating hazards, has mandated pollution control measures and environmental restoration of hazardous waste at all sites. Estimates of total cleanup costs range from $230 billion to more than half a trillion dollars. Given the trend of diminishing budgets throughout the federal government, future generations could inherit both an environmental and budgetary disaster.

The imprecision of the cost estimates results from the lack of knowledge of how "clean" the contaminated sites will need to be. Some of the environmental damage is permanent—cleanup technologies either do not exist or are incapable of remediating the contamination. For DoD bases being closed by the Base Realignment and Closure Program, all toxic sites must be remediated before the site is returned to public use. The projected costs of site restoration using existing technologies are staggering: the estimated cleanup cost is at least $24.5 billion for the 7,313 identified U.S. sites (EPA, 1993). The pollutants at these sites include chlorinated hydrocarbons, metals, petroleum products, explosives, mixed waste and other organics. DOE also has substantial remediation costs—estimated to be from $90 billion to $200 billion (GAO, 1988; DOE, 1988). The domestic private sector presents yet another huge set of remediation problems, dwarfed only by the international problems in Eastern Europe and Russia (Glass et al., 1997).

There is clearly a need for new cost-effective treatment technologies. Bioremediation, the use of microorganisms to detoxify hazardous waste, promises to provide economical and ecologically sound clean-up strategies. An Office of Technology Assessment analysis (OTA, 1991) concluded that the U.S. does not possess a sufficient pool of qualified environmental professionals, i.e., the trained scientific personnel required to support this rapidly developing multidisciplinary field.

In response to these national environmental needs, the Bioremediation Education, Science and Technology (BEST) Program, funded by DoD, was established in 1996. In a few short years, BEST has pioneered a new and successful model for environmental science and education. This partnership has a highly integrated programmatic focus on the scientific and workforce needs of DoD.

Since the inception of the BEST program, a significant number of major milestones and deliverables have been achieved. They are described below. The BEST program has made these dramatic accomplishments by using an approach that combines a training-education element with an integrated research project, described later in this introduction.

Education and Training

The following education and training programs have been established:

- Environmental sciences Ph.D. program at JSU (first in Mississippi)
- Environmental science and environmental management M.S. program at AGMUS (first in Puerto Rico)
- Environmental science B.S. program at AGMUS and UTEP
- Microbial biology Ph.D. program at UCB

DOE, Environment, safety, and health needs of the U.S. Department of Energy, Vol. I: Assessment of needs, DOE/EH-0079. U.S.DOE, Washington, D.C., 1988.
GAO, Nuclear waste problems associated with DOE's inactive waste sites, GAO/RCED-88-169, U.S.GAO, Washington, D.C., 1988.
Glass, D.J., T. Raphael and J. Benoit, International bioremediation: recent developments in established and emerging markets, p. 307, In: In-situ and On-Site Bioremediation: Vol. 4 (B.C. Alleman and A. Leeson, eds.) Battelle Press, Columbus, Ohio, 1997.
OTA, Complex cleanup: the environmental legacy of nuclear weapons production, complex cleanup, OTA-0-485, 1991.

- Center for Environmental Biotechnology (CEB)–BEST seminar program (82 seminar presentations; 34 key seminar speakers video taped for distribution and use in special topics courses)
- Rotating Scholars program (10 outstanding CEB–BEST seminar speakers visited program member universities and taught students on-site)
- Spanish translations of CEB–BEST video seminars
- K-12 education: summer bioremediation science workshops for high school and middle school teachers
- BEST graduate education program (58 participants)
- BEST postdoctoral education program (7 participants)
- BEST "Principles of Bioremediation" video course that includes laboratory instructions (distributed to program member universities, federal agencies and DoD laboratories)
- BEST collaborative research program (42 faculty participants)
- BEST exchange research program (10 faculty participants, 40 student participants)
- BEST faculty development workshops.

Workforce Diversity

- Participants who went into academia (including graduate school): 40
- Participants who went into government (especially DOD): 2
- Participants who went into industry (especially remediation companies and new businesses): 8
- Participants who went on to professional schools: 4
- BEST program undergraduates who are currently continuing their education or are in scientific careers: 172.

New Technologies Through Scientific Accomplishments

- Developed, demonstrated and validated an in situ stable isotope monitoring system for petroleum hydrocarbon degradation at a DoD site.
- Developed, demonstrated and validated a microbial community monitoring system for industrial activated sludge hydrocarbon and toxic metal treatment systems (technology transferred to EXXON refineries at Baton Rouge, La., and Martinez, Calif.).
- Developed, demonstrated and validated an in situ x-ray absorption spectroscopy method for toxic metals speciation in plant and microbial biomass.
- Developed, demonstrated and validated an in situ low background gamma-ray spectroscopy method for quantitating toxic metals in biomass.
- Demonstrated the ability of oleophilic fertilizers to stimulate biodegradation of weathered PAHs in sediment.
- Developed metabolic engineering strategies for overcoming limitations to the microbial detoxification of toxic metals and nitrogen species (individual environmental isolate studies and microcosm models) in large-scale systems.
- Designed, constructed, demonstrated and validated an Advanced Integrated Pond System for toxic metal and nitrogen removal from waste streams at 10,000 GPD.
- Developed the first set of regulations and standards for certification of bioremedial processes.
- Developed gene chip technology for genome-wide expression analysis of hazardous metal detoxification responses.
- Developed genotyping and metabolic engineering technology to characterize and optimize the remediation of GTN explosives.
- Conducted survey of Hispanic diets and smoking habits.
- Developed method to determine Pka's in biomaterial using innovative software.
- Developed code to estimate rates of heating of ground based on input of heated humidified air to estimate rates of biodegradation in that soil.

New Knowledge Through Scientific Accomplishments

- Gave 166 BEST student and faculty presentations at national and international scientific meetings.
- Produced BEST student and faculty publications and meeting abstracts (34 papers in peer reviewed journals and 41 abstracts).
- Determined the molecular mechanisms involved in toxic metal valence biotransformation by microbial systems.
- Determined the microbial community diversity and seasonal variation in toxic metal impacted environments.
- Developed ecophysiological community models for high-pH PAH- and PCP-impacted environments.
- Developed ecophysiological models for the impacts of mercury, atrazine, chlopyrifos and UV-B on wetland microbial communities.
- Discovered the chemotactic nature of TNT compounds to large groups of bacteria.

Enhancement of Infrastructure and Capabilities

- Established BEST programs at LBNL, UCB, JSU, AGMUS, USM and UTEP.
- Established Center for Environmental Biotechnology (CEB) at LBNL.
- Established BEST–Cal/EPA Bioremediation Validation and Certification Center at UCB (first reference center in the U.S.).
- Established a new website on bioremediation for BEST (http://www-esd.lbl.gov/CEB/BEST/).

Training-Educational Element

Successful restoration of DoD hazardous waste sites and the growth of the bioremediation industry is dependent on a cadre of trained scientists, engineers and technicians. By the year 2005, thousands of trained professionals will be required to meet DoD and national environmental needs. The BEST partnership continues to build upon accomplishments and successful programs developed with DoD support.

The training-educational element continues to provide career development opportunities for underrepresented groups with bioremediation curricula, courses and fellowships. The training-educational experience is personalized to provide students with a meaningful bioremediation curriculum, financial support, an extensive mentorship network, and research and field training. The shared resources of the BEST partnership institutions aid faculty in the development of curricula, courses and environmental research projects. The training-education programs span the continuum from community college outreach to faculty development, but focuses on undergraduate and graduate students. Innovative features of the BEST program are the use of distributed learning technologies and a Rotating Scholar Program, integrated with a coordinated academic and video seminar curriculum.

BEST Integrated Research Project

The BEST two-year research program focuses on the following DoD priorities:

- Bioremediation of nitro-compounds (explosives) and polycyclic aromatic hydrocarbons;
- Interactions of plants and microorganisms (including the rhizosphere) with explosive nitro-compounds, PAHs and metals;

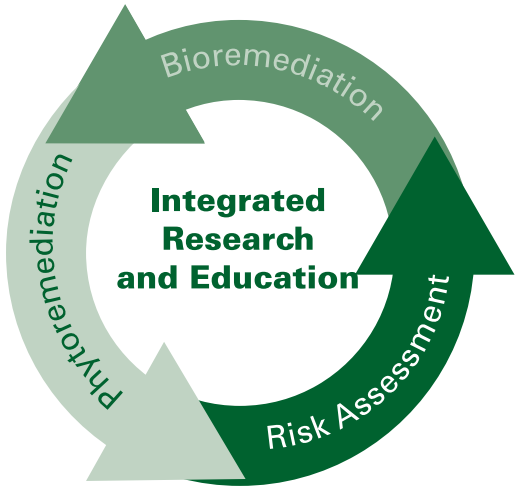

Figure 1. Concept of the integrated BEST research program

- Health effects and ecotoxicology of nitro-compounds, PAHs and metals.

This integration strategy facilitates collaboration and the use of shared resources while enabling the program to achieve its training-educational and workforce diversification goals.

Project Goals and Objectives

The primary objective of the BEST program is to provide hands-on training in bioremediation, phytoremediation and ecotox/risk assessment for underrepresented minorities. Students will obtain the necessary skills and knowledge to enable them to either enter the environmental science workforce with a bachelor's degree or enter graduate school at the master's or doctoral level. Another objective is to ensure a continued supply of skilled workers to address the diverse environmental restoration needs at DoD sites.

The BEST program goals are:

- To provide an integrated research and technology development program that clearly focuses on DoD environmental contamination concerns.
- To provide a well-rounded and stimulating research program for students that will expose them to the diversity of multidisciplined sciences required to understand and implement bioremediation, phytoremediation and ecotox/risk assessment in and at the field level.
- To provide new, cost efficient and reproducible technologies that address and solve current DoD environmental concerns.

Relevance to the DoD Mission

DoD sites throughout the United States contain highly contaminated soils, groundwater and sediments. These properties pose direct and indirect (off-site migration) exposure hazards to humans and wildlife. Conventional remedial solutions for contaminated soils and sediments (excavation, dredging and incineration) or groundwater (pump-and-treat) are slow and expensive, increase inputs to hazardous waste disposal sites, and can increase human exposure to contaminants. Bioremediation — the use of microo ganisms to destroy hazardous contaminants or to convert them to harmless forms — is an emerging treatment technology that can in many instances restore contaminated environments more quickly, at lower cost and at lower human risk than alternative remediation technologies.

Bioremediation can operate in either an in situ mode where contaminants are treated in place, or in an ex situ mode where contaminants are removed from a contaminated zone for treatment (preferably on-site). In situ bioremediation can be used when excavation is impractical — under buildings, highways, runways, etc. In situ bioremediation can simultaneously treat soil and groundwater in one step, without the generation of hazardous waste products. In situ contaminant degradation can be achieved by either intrinsic or enhanced bioremediation. Intrinsic bioremediation exploits the innate capabilities of indigenous microbial communities to degrade pollutants. Enhanced bioremediation seeks to accelerate in situ microbial activity by isolating and controlling the contaminated site so that the microbial environment can be purposely manipulated to correct nutritional or gas phase limitations. Ex situ treatment seeks to further control the remedial environment by placing the contaminants in an engineered treatment system. Phytoremediation, a process in which plants and associated microbial communities are used for contaminant biodegradation or bioimmobilization, is an important and rapidly developing mode of bioremediation.

To realize the full potential benefits of plant and microbial treatment systems at DoD sites, these biotechnologies must be developed and optimized for remediation of DoD priority contaminants by an expanded pool of qualified professionals. It was in response to these DoD environmental needs that the BEST partnership of institutions was established.

BEST Projects

Bioremediation of Explosive Nitro-Compounds, Nitrocelulose and PAHs

Plant-Assisted Phytoremediation of Soil Contaminated with Nitroaromatic and Polycyclic Aromatic Hydrocarbons

Bob Buchanan
University of California at Berkeley

Problem Being Addressed

To determine the rate of disappearance of PAHs in soil by plants.

Research Methods/Tools Employed

Plants were grown in 2-liter pots in greenhouse conditions. PAHs in soil were extracted by ultrasonication (EPA method 3550) and analyzed by GC-MSD. Bacterial counts were determined with the live/dead staining method (SYTO 9 and propium iodide) using an epifluorescence microscope.

Experimental Data

In order to determine whether plants can stimulate the degradation of PAHs in soil, plant species found in literature on phytoremediation of metal-contaminated sites were selected to measure the removal of PAHs in artificially contaminated soil over a period of 62 days. The plant species used for this experiment were alfalfa (Medicago sativa), barley (Hordeum vulgare), tall fescue (Festuca avundi - nacea) and orchard grass (Datylis glomerata). The PAHs were phenanthrene and anthracene, in a mixture of 600 ppm each.

As shown in Figures 1 and 2, phenanthrene and anthracene were removed from the soils with plants after 62 days. More than 98% of the phenanthrene was removed during that period while the anthracene removal was found to be between 70 and 90%. The results suggest that the rate of disappearance of phenanthrene in soil was greater than anthracene under the same conditions. From the results, it is also indicated that the disappearance of PAHs in soil depends on the bioavailability of the compounds. Because phenanthrene is approximately 10 times more soluble in water than anthracene, it was expected to be more readily available to microbial degradation than anthracene. Plant-assisted degradation of PAHs is thought to be more effective on PAHs with a higher number of rings and higher molecular weights, such as benzo(a)pyrene.

Anthracene removal in the soil planted with alfalfa was greater (by 40%) than in the soil without plants, while all the other plants have minimal to no effect on anthracene removal compared to the control soil. Phenanthrene was removed to a greater extent in the soil with alfalfa and tall fescue compared to the control without plants (55% and 20%, respectively). However, both barley and orchard grass showed no effects of the removal of phenanthrene during that period when compared to the soil without plants. Overall, plants had minimum effect on phenanthrene degradation while anthracene degradation was more dependent on plant species.

In order to determine the effect of PAH degradation by plants on bacterial numbers in soil, bacteria were counted in soil during the course of the experiment.

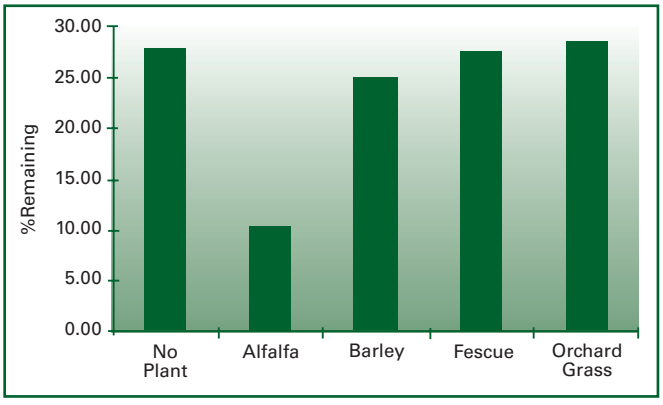

Figure 1. Percentage of anthracene remaining in the soil after two months.

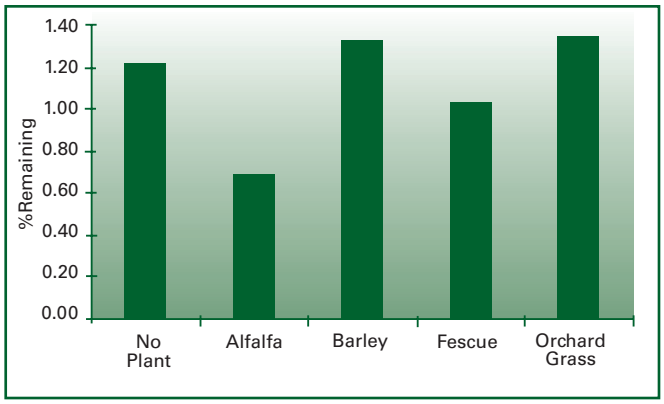

Figure 2. Percentage of phenanthrene remaining in the soil after two months.

No differences in bacterial numbers between soils with and without plants were observed. The result suggests that the degradation of PAHs by plants is not affected by differences in bacterial biomass in the soil.

Sterile controls will be used in the future in order to assess the role of bacteria in the degradation process.

Future studies

Future studies will include the following:

- Effect of bacteria: to find an appropriate sterilization method (e.g., antibiotics) and determine the sterility of soil over time.
- Effect of plants: to repeat the greenhouse experiment with alfalfa, barley and fescue.
- Mass balances: to determine recovery of PAHs in volatile, leachate, plants tissue and soil fractions.

Students involved

Michelle Coppoletta

Isolation and Characterization of Microorganisms from Contaminated Sites in the Tropics and the Study of their Biodegradation Capabilities

Doris A. Caro
Ana G. Méndez University System, San Juan, Puerto Rico

Problem Being Addressed

Highly contaminated sites in the tropical island of Puerto Rico are being examined for the isolation, purification and characterization of microorganisms that could be used as possible biodegradation agents to treat sites contaminated with toxic metals and other organic pollutants. For this research problem two sites are being studied. The first is the Juncos Landfill Superfund site, which has been closed since 1981, when it was discovered that it was receiving discharges from nearby industries containing mercury (Hg) thermometers and other pollutants. Another site under study by our laboratory is Caño Martin Peña, a site from the San Juan Bay Estuary System that has been extensively studied by the U.S. Geological Survey and found to be highly contaminated from industrial, domestic and commercial activities in the nearby San Juan metropolitan area.

Research Methods/Tools Employed

Juncos Landfill—Samples of soil, leachate and water from a near stream (Quebrada Ceiba) were obtained in order to isolate microorganisms (bacteria). Two different media were prepared for this purpose: Selective Rich Medium and minimal. The characterization of the bacterial strain was performed using the FAME technique (Table 1). Bacterial strains *Bacillus cereus* and *Paenibacillus macerans*, identified by this technique, were exposed to several concentrations of Hg(II) solutions to observe their kinetic behavior as compared with *Bacillus subtilis*, previously tested in our laboratory (Figure 1). These studies were performed using UV-Vis spectroscopy at 600 nm.

Caño Martin Peña—Soil and water samples were examined for microorganisms. Five different media were used to isolate actinomycetes, bacteria and fungi: thin pablum, marine agar, potato carrot agar, potato dextrose, multiple carbon and nitrogen agar. From these experiments 71 microorganisms have been isolated; we are now in the process of purification and Gram stain testing.

Table 1. Bacteria from Juncos Landfill identified by the FAME technique.

Bacteria	Water	Soil	Leachate
Paenibacills macerans			*
Bacillus sphaericus	*	*	
Bacillus cereus	*	*	
Paenibacills alvei		*	

Experimental Data

Figure 1 presents growth curve comparisons between the bacterial strain Paenibacillus macerans, isolated from the Juncos Landfill, and *Bacillus subtilis*, isolated from San Francisco Bay, Calif., in the presence of Hg (II) solutions. As can be observed, the bacterial strain isolated from Juncos demonstrates a higher growth in the toxic metal. Studies with the other bacteria identified are in progress.

Future Studies

Planned future studies will include the following activities:

- Characterize microorganisms isolated in the present studies using BIOLOG.
- Kinetic studies to determine the biodegradation capabilities of the microorganisms characterized in the presence of trace metals (Hg, Cr, Cd, Cu, Pb).
- Quantify the concentration of the trace metal tested in the supernatant and biomass of the microorganism used for the kinetic studies.
- Extend studies to the interaction of organic pollutants and isolated microorganisms.
- Extend this study to the Island of Vieques off the east coast of Puerto Rico, which has been highly contaminated by U.S. Navy military activities.

Students Involved

Yomarie Bernier – San Juan Bay Estuary
Arleene Rivera – San Juan Bay Estuary
Noel Diaz – Juncos Landfill Superfund
Tathianna Muñoz – (summer student)

Relevant Publications/Presentations

Rivera, A., T. Muñoz, Y. Bernier, D. Caro, D. Sauri and T. Leighton, Kinetic studies of the interaction of metallic cations and selected bacterial strains: bioremediation, presented at NCUR, Rochester, N.Y., April 8-10, 1999.

Diaz, N., Y. Bernier, J. Garmon, J.C. Hunter-Cevera and D. Caro, Isolation and characterization of bacterial strains from a superfund site and the study of their biodegradation capabilities, Pittsburg Conference, March 12-17, 2000.

Bernier, Y., J.C. Hunter-Cevera, G. Castro and T. Torok, Potential biodegradation of weathered explosives by microorganism, submitted to NCUR 2000, Montana, April 27-29, 2000.

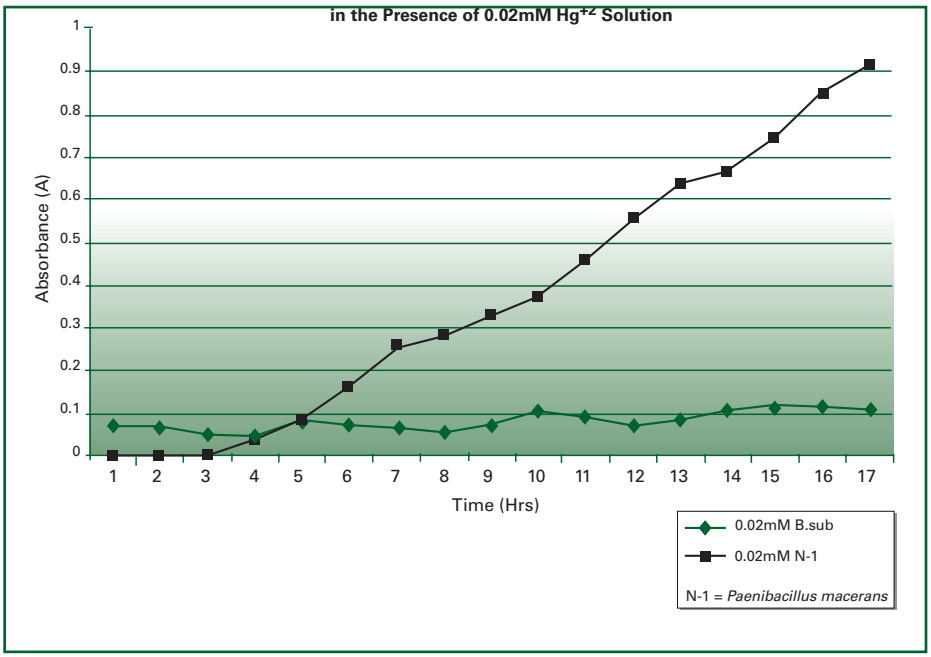

Figure 1. Growth curves between *Bacillus subtilis* and *Paenibacillus macerans* in the presence of 0/02mMhg^{+2} solution.

Development of a Coculture for the Biodegradation of Parathion

Samir Davila, Eric Gilbert and Jay Keasling
University of California at Berkeley

Introduction

Biodegradation of readily degradable contaminants has proven to be an effective treatment strategy for environmental restoration. Although bacteria have the capacity to transform a number of chemicals, many synthetic compounds have novel structures rarely found in nature and are recalcitrant to biodegradation. To expand the range of compounds that can be degraded by biological systems, the essential enzymatic reactions must be recruited, either by introducing the requisite genes into a single microorganism, or by assembling a consortium of bacteria containing the necessary enzymes. In the following work, a coculture comprised of an *Escherichia coli* and a *Pseudomonas putida* was developed to hydrolyze parathion and degrade its metabolite *p*-nitrophenol.

Problem Being Addressed

Parathion is a widely used organophosphate insecticide which can cause adverse neurological effects if ingested or after dermal exposure. No single microorganism has been isolated that is capable of completely mineralizing parathion and its metabolites. Hydrolysis of parathion significantly lowers the toxicity of the parent compound, but results in the formation of a toxic intermediate, the nitroaromatic compound *p*-nitrophenol. Parathion also contains a thioester linkage that is analogous to the chemical structure of several chemical warfare agents, including sarin. Consequently, parathion is an excellent model for studying enhanced biodegradation of environmental contaminants.

Mineralization of parathion requires three unique catabolic properties: hydrolysis of parathion, mineralization of *p*-nitrophenol, and mineralization of diethyl thiophosphate (DETP). The objective of this research was to (a) develop a coculture capable of hydrolyzing parathion and degrading its metabolite p-nitrophenol; (b) evaluate the kinetics of the reaction; and (c) test the suitability of the coculture for use in a flow-through biofilm reactor for parathion biodegradation.

Research Methods and Results

Escherichia coli strain SD2 was constructed by introducing plasmid pWM513, harboring the genes for parathion hydrolysis and ampicillin resistance, and plasmid pMAG1, carrying the green fluorescent protein gene and tetracycline resistance, into *Escherichia coli* DH10B. The plasmids were inserted by electroporation and strain SD2 was selected using media containing both ampicillin and tetracycline. Strain SD2 was used together with *Pseudomonas putida* KT2440 carrying plasmid pPNP, harboring the genes for p-nitrophenol degradation and also tetracycline resistance. Strain KT2440 is naturally resistant to ampicillin, so the two strains could be cultured in media containing both antibiotics, and consequently maintained the genes required to degrade parathion.

The ability of the coculture to degrade 0.5 mM parathion was evaluated during growth of the strains in a minimal medium containing glucose as the carbon source. P-nitrophenol accumulated in the medium during the growth of strain SD2 alone, resulting from parathion hydrolysis (Figure 1). In contrast, the accumulation of p-nitrophenol was only transient in the coculture, as a result of the biodegradation activity of strain KT2440. Kinetic analysis indicated that 2 mM p-

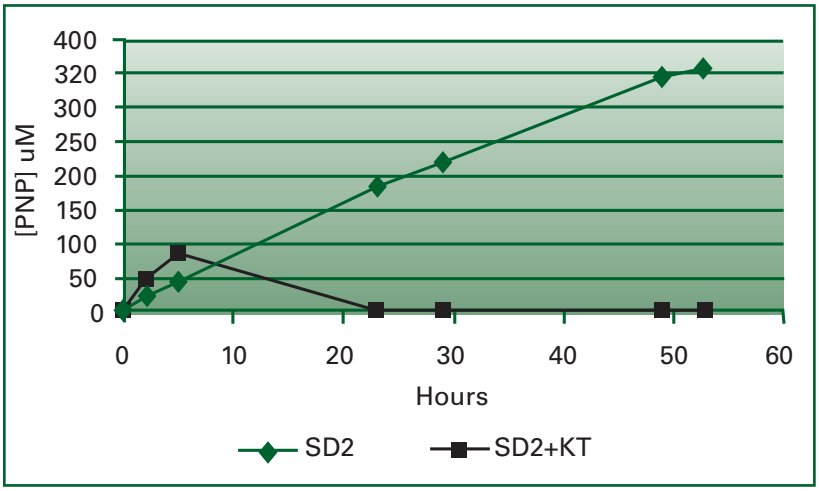

Figure 1. Effect of P-Nitrophenol (PNP) on growth.

nitrophenol was fully inhibitory to the growth of the coculture; consequently, this concentration represented the upper limit for parathion biodegradation (Figure 2).

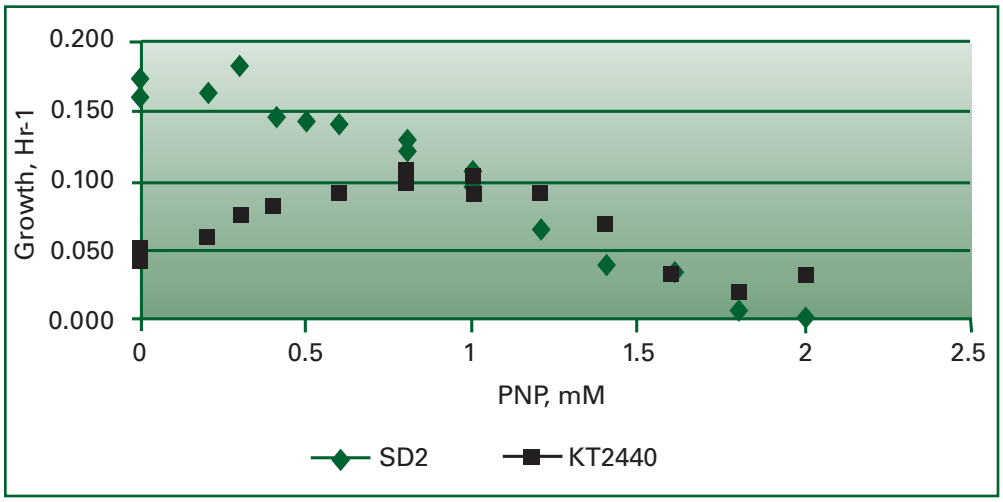

Figure 2. Effect of PNP on growth.

The coculture was used to cultivate a biofilm in a parallel plate flow cell for imaging by confocal microscopy. After 72 hours of growth in continuous mode, the biofilm was stained with a red fluorescent nucleic acid dye and imaged using a confocal microscope. The dye caused the *Pseudomonas* strain to appear red, while the *Escherichia coli* strain appeared green and yellow as a result of the colocalization of green color fluorescing from its green fluorescent protein. The images indicated that the biofilm was dominated by *Pseudomonas*, although *E. coli* was stably maintained. The results suggest that the two strains could be used as part of a flow-through biofilm reactor for detoxification of parathion.

Future Studies

The success of the current project warrants a continuation to advance the strategy developed here. Future work will focus on developing a bioreactor for degradation of parathion and p-nitrophenol, and also on the biofilm structure that forms in the reactor.

Students

Samir Davila – undergraduate student,
UC Berkeley (BEST)

Biodegradation of Nitrocellulose Filters

Luis A. Feliu and James Garmon
Ana G. Méndez University System, San Juan, Puerto Rico

Problem Being Addressed

Several industries in the United States and Puerto Rico are dedicated to the manufacture of filters of different materials, including nitrocellulose (NC). These filters are very important in ensuring the purity of ingredients in food and pharmaceutical industries. However, the nitrocellulose used for manufacturing these filters is considered a hazardous waste for its ignitability (<140°F), high flammability and oxidizing properties. The Department of Defense also has sites contaminated with NC since it is a major component in explosives. Nitrocellulose exhibits good chemical stability, which for years made incineration the preferred method for disposing of the NC wastes. Alkaline hydrolysis appears to be a rapid process for nitrocellulose degradation, but research is limited. This investigation involves the elucidation of the mechanism of alkaline hydrolysis and its combination with biodegradation.

Research Methods/Tools Employed

Table 1 shows the research methods and tools employed in this study. Every step in Table 1 seems to play an important role for either hydrolysis or biodegradation itself. Interpretation of the species left after degradation time facilitates analysis. Comparison between liquid and agar media (in a systematic experiment) is important to really determine if physical attachment is another factor for better fungus growth.

Figure 1 shows a possible precipitation of products after acidification on the 9 mL NaOH treatment. Figure 2 shows fungus growth from 10 mcL of spores suspension placed on a petri dish containing NC as sole carbon source. HPLC assays have been made on a growth curve (in liquid medium) in a culture tube (Table 2). A total of 0.1 g of NC hydrolyzed in 6 mL of SSC buffer was used. The strongest peaks are shown. The sample represents one-third of the total area. A total of 20 mcL were injected in a 50:50 acetonitrile:water mixture. Assays are being done using wavelengths of 210 nm and 214 nm, due to the abundance of probably dicarboxilic acids in the hydrolyzed NC.

Table 1.

Caustic hydrolysis conditions (for 0.1 g of NC)	Temperature (60°C vs. room temp.)
	15 mL of 1 M NaOH or 0.5 M NaOH vs. 15 mL of 1 M KOH or 0.5 M KOH
	Time (10 min)
	RPM (175 vs. 0)
	GC-MS analysis (Varian 3400; detector type: ADCB (10 volts); sample rate 10.0 Hz
Medium composition	pH (6.5 lowered with 6 M HCl)
	Inorganic salts (KH_2PO_4, K_2HPO_4, $MgSO_4$, $NaNO_3$, NH_4NO_3, $CaCl_2$)
	0.2 g of NC as C source
	Total volume of medium (100 mL)
Growth curve conditions	Liquid medium (100 mL/flask) vs. solid medium (20 mL/plate)
	Temperature (25°C)
	RPM of shaker water bath (175 for liquid medium)
Biomass measurements	Optical density at 660 nm (for liquid medium)
	Colony diameter (for solid medium)

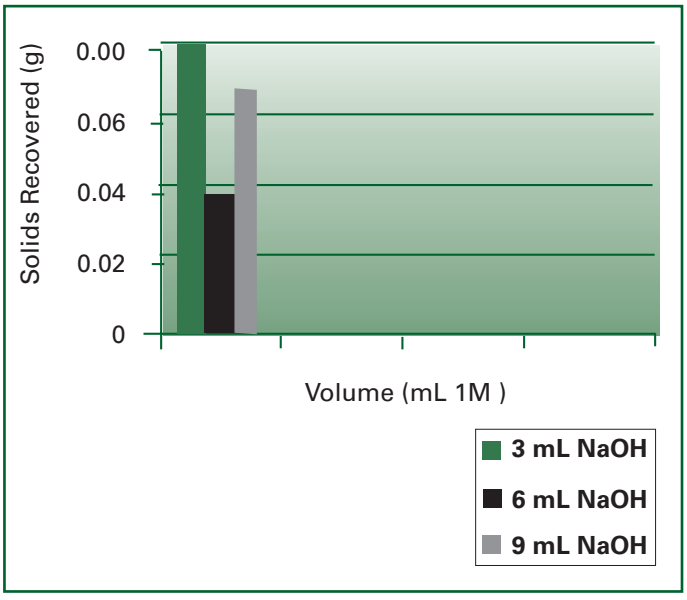

Figure 1. Dry weight vs. NaOH.

Table 2.

	Ret. Time (min)	Area	Wavelength, nm
Sample	3.18 3.92	1503461 1078821	210
Control	2.77 3.49	2328933 4040725	210

Future Studies

- GC-MS, IR and HPLC analysis correlation of the compounds formed from NC alkaline hydrolysis. HPLC detectors will be UV (210-214 nm) and fluorescence. An experiment using polar and less polar solvents in gradient will be set to try to separate the compounds in the liquor as much as possible.
- Characterization of solids formed after hydrolysis liquor acidification. Large amounts of carbon may be bound in these solids.
- Growth curves on liquid and solid media that include:
- Addition of a co-substrate as a C source.
- Individual and symbiosis growth interaction studies among various isolated fungi and bacteria from NC wastes (in characterization process at this time).
- HPLC and IR analysis on growth curve liquid medium residues to measure rate of biodegradation. HPLC detectors will be UV (210-214 nm) and fluorescence.
- Assembly and application of a chemostat.

Students Involved

James Garmon – graduate student in environmental risk assessment (UMET)

Juan Rodriguez – chemistry student (UT)

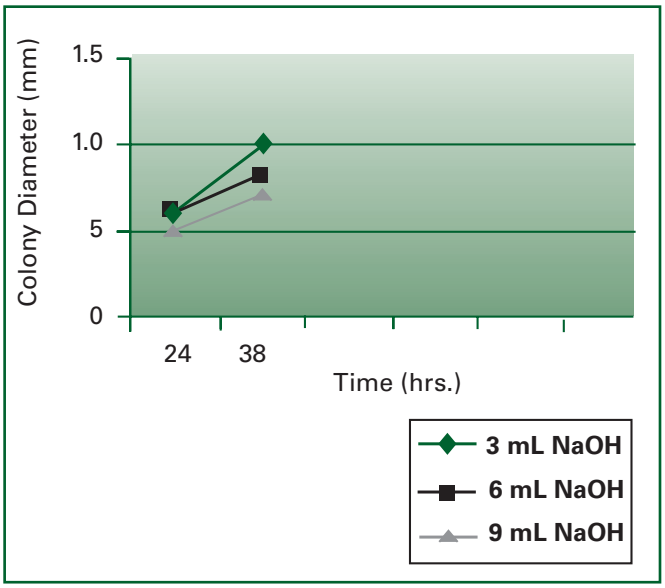

Figure 2. Preliminary fungus growth curve.

Relevant Presentations

Feliu, L.A., Biodegradation of nitrocellulose filters by endogenous microbial population, 58th Chemical Conference and Exhibition and 7th Caribbean Environmental Chemical Conference, Fajardo, PR, organized by the Puerto Rico Chemists Association, Aug. 3-6, 1999.

Feliu, L.A., Theoretical study of the conformers of nitroglycerine using semiempirical methods and density functional theory, 218th American Chemical Society National Meeting, Anaheim, Calif., August 1999.

Biostimulation of Highly Weathered PAH-Contaminated Soils Using Oleophilic Fertilizer

Terry C. Hazen, K.C. Oh and William Stringfellow
Center for Environmental Biotechnology, Lawrence Berkeley National Laboratory, Berkeley, Calif.

Problem Being Addressed

Polycyclic aromatic hydrocarbons (PAHs) are a concern in the environment because they are toxic and carcinogenic. Polycyclic aromatics are more recalcitrant in the soil than other hydrocarbons because they are hydrophobic and tend to migrate into the soil (Pothuluri and Cerniglia, 1994). Most PAHs occur as a result of fossil fuel combustion; thus, high concentrations of PAHs are found at the sites of active and inactive oil refineries (Cerniglia 1992).

This study focuses on oil and petroleum contaminated soil samples taken from Alameda Naval Air Station at Alameda Point, Calif. Pacific Coast Oil Works refinery used the site between 1879 and 1903. After the refinery closed in the 1930s, the U.S. Army and then the U.S. Navy owned the property. In 1991, jet fuel spilled from the jet engine test facility on the site. Heavy rains resulted in jet fuel in the overflow of storm drains. Damage to the storm drains during the Loma Prieta earthquake in 1989 may have caused ground water contamination. Recent studies (1996-97) showed total petroleum hydrocarbon amounts ranging from 100 to 10,000 mg/kg soil. Due to the long term and high concentration of oil in the soil it is likely that there are microorganisms capable of degrading the oil products. This study will examine the use of Inipol EAP 22, the same fertilizer used in the Exxon Valdez oil spill clean-up, to obtain optimal growth conditions of the naturally occurring bacteria using CO_2 output to monitor the degradation of PAHs and hydrocarbons. Inipol EAP 22 is particularly attractive for this site since it is oleophilic and should make the strongly sorbed PAH components more bioavailable, thereby stimulating biodegradation.

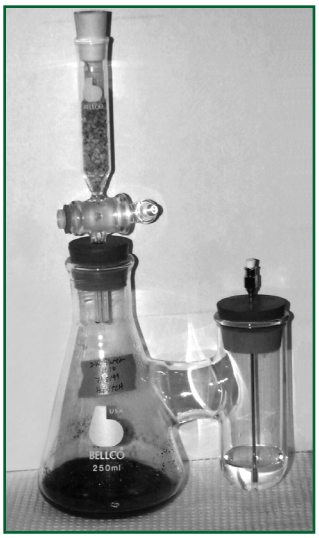

Figure 1. Biometer flask for trapping carbon dioxide generated by PAH-contaminated soil exposed to different amendments of inorganic nutrients and Inipol EAP-22.

Research Methods

Biometer flasks, 250 ml Erlenmeyer sidearm flasks with ascarite towers were used as reaction vessels (Figure 1; Bellco Company, Vineland, N.J.). The flasks contained 20 grams of oil-contaminated soil from the Alameda site as the only source of carbon and energy for the indigenous bacteria in the soil. The sidearm contained 10 ml of 0.1 N KOH to absorb the carbon dioxide produced. . The KOH was fixed with saturated BaCl and titrated with 0.05 M N HCl. The micromoles of carbon dioxide produced were then normalized based on soil weight, moisture content and time in hours. Negative controls were used in all experiments and were the same as experimental conditions, except that the bacteria were killed with three autoclave sessions to create sterile soil (Hazen and Sharp, 1989). Since carbon dioxide and water are the products of the degradation of petroleum products, the amount of carbon dioxide produced is proportional to the amount of oil degraded, if oil is the only major carbon source that is bioavailable.

Concentrations of Inipol EAP 22 were varied from 0.05% - 5% to determine the optimum conditions for the growth of the naturally occurring bacteria in the soil samples. For the treatment of the soil, 5 ml of diluted Inipol EAP 22 and 5 ml of water were added to each flask on top of the soil. The flasks were then stirred or shaken to mix the soil and water. Each experiment was conducted in triplicate. The net output of carbon dioxide was measured for each of the trials using the biometer flasks.

To confirm that the carbon dioxide production seen in the biometer flasks represented degradation of TPHs and PAHs, the samples were run through the GC/Mass Spec before and after the addition of Inipol EAP 22. One-gram samples were extracted using 1 acetone:1 hexane solvent and sonication followed by nitrogen blow down. Standards were made from

Castrol GTX 102-30 motor oil. TPH method 4 was run with 1 µl injections.

Experimental Data/Results

For the unamended soil carbon dioxide, production remained stable below 0.2 umoles/hr/g of dry soil throughout the six-day experiment. There was no significant difference in CO_2 production between the sterile and unamended test soils. Inipol EAP 22 treatments are shown in Figure 2. Both the 5% and 0.5% Inipol EAP 22 solutions were significantly different from their corresponding killed controls and also significantly different from the soil without amendments (p value less than 0.001). The lowest concentration of Inipol EAP 22 (0.05%) was not significantly different from the killed control nor from the soil without amendments. Killed controls did not remain killed for the duration of the treatments, making it difficult to compare the degradation of oil between killed and treated soils. Inipol EAP 22 was shown to give the greatest biostimulation for this highly recalcitrant soil of any nutrient mix tested, several times higher than unamended or killed controls. This suggests that it could be used as a strategy to bioremediate this site of the PAH contaminants.

Future Studies

The following future studies are planned:

- Additional tests with Inipol EAP 22 to determine the daughter products produced via this type of biostimulation.
- Repeating studies with another fumigant to maintain sterility of killed controls.
- Complete mass balance inventory of all petroleum contaminants in this soil.
- Scale-up system to demonstrate feasibility of a field demonstration.

Students Involved

Hellen Eastwood – LBNL High School Teacher Research Associate Program (summer 1999)

Marla Miller – graduate student in environmental engineering, UC Berkeley (summer and fall, 1999)

Relevant Publications

Hazen, T.C., Controlled phosphate-enhanced bioremediation tested, EPA Tech Trends 25:3-5, 1997.

Hazen, T.C., Bioremediation, in *Microbiology of the Terrestrial Subsurface* (P. Amy and D. Haldeman, eds), pp. 247-266, CRC Press, Boca Raton, 1997.

Tien, A.J., D.J. Altman, A. Worsztynowicz, K. Zacharz, K. Ulfig, T. Manko and T.C. Hazen, Bioremediation of a process waste lagoon at a southern Polish oil refinery - DOE's first demonstration project in Poland, M#9, Proceedings, Fourth International Symposium and Exhibition on Environmental Contamination in Central and Eastern Europe (Warsaw'98), Institute for International Cooperative Environmental Research at Florida State University, 1999.

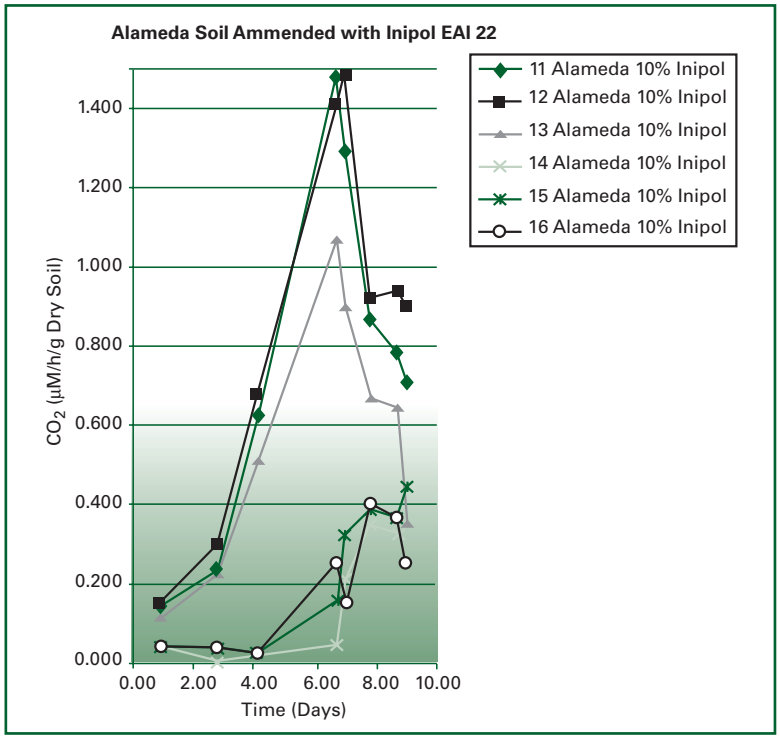

Figure 2. The effect of different concentrations of Inipol EAP 22 on the soil respiration rate as measured by carbon dioxide production for PAH-contaminated soil from Alameda Naval Air Station.

Soil Bacteria Chemotaxis to 2,4 and 2,6 Dinitrotoluene

Terry C. Hazen
Center for Environmental Biotechnology, Lawrence Berkeley National Laboratory, Berkeley, Calif.

Problem Being Addressed

More than 8,000 Department of Defense sites need clean-up efforts (DOD, 1996). TNT was the most commonly occurring compound within the contaminated samples from these sites. Fortunately, TNT is biodegradable, making in situ bioremediation a cost-effective and rapid alternative for site cleanup. The toxicity of nitroarenes and their metabolites have been studied in a variety of biological systems but we have been unable to find any studies related to chemotaxis of nitroaromatic compounds in the scientific literature. Chemotaxis allows bacteria to respond to chemical gradients, seeking higher levels of potential nutrients and lower levels of inhibitors. Organisms that have developed mechanisms with which to beneficially orient themselves with respect to these gradients of different types may have a competitive advantage over other organisms. Also, the value of motility as a survival factor for bacteria in environments where nutrients or harmful agents are discontinuously distributed, e.g., contaminated sites, seems obvious. The present study will target how bacteria can use nitroaromatic compounds as a source of nutrients and as a dispersal mechanism in soil, especially as it may apply to in situ bioremediation.

Research Methods

Bacterial strains and culture conditions: *Pseudomonas fluorecens* was incubated at room temperature in Peptone broth 10g/L. The exponentially growing cultures were harvested by centrifugation at 6000 xg for 12 minutes in a temperature of 4°C. The pellet was resuspended in an equal volume of potassium phosphate buffer or chemotactic buffer (KPB; pH 7) without EDTA and centrifuged again.

Chemotaxis assay: A modification of the capillary method (Adler, 1966-1973) and chemotaxis chamber developed by Palleroni (1976) was used for the assay (Figure 1). If the substrate is an attractant, bacteria will be attracted to the mouth and more cells will enter the capillary tube containing the chemical than the tube in the chemotactic control (KPB). If the chemical is a repellent, fewer cells will be attracted to the experimental tubes than to the KPB control. To each of the four wells and channels in the chambers, 0.4 mL of the motile bacterial suspension was added. After incubation of the chambers for 1 hr at 28°C, the capillary tubes were removed with tweezers and rinsed with distilled water. After washing, the capillary tube contents were released onto a microscope slide and counted using AODC.

Substrate solution: Substrates (2,4 and 2,6 dinitrotoluene) were diluted tenfold in the chemotaxis buffer to yield concentrations of 1M to 10^{-7} M. A motility control was also run by adding the test substrate to the cell suspension prior to incubation with a capillary tube containing KPB. A motility control was run to determine if changes in capillary tube densities were due to changes in bacteria activity caused by the substrate and not due to true chemotaxis. Each compound is tested at different concentrations and all dilutions made in KPB.

Acridine Orange Direct Counts (AODC): After fixing the contents of the capillary tubes, the cells were stained with 0.1% Acridine Orange (Difco Laboratories, Detroit, Mich.). To facilitate comparison

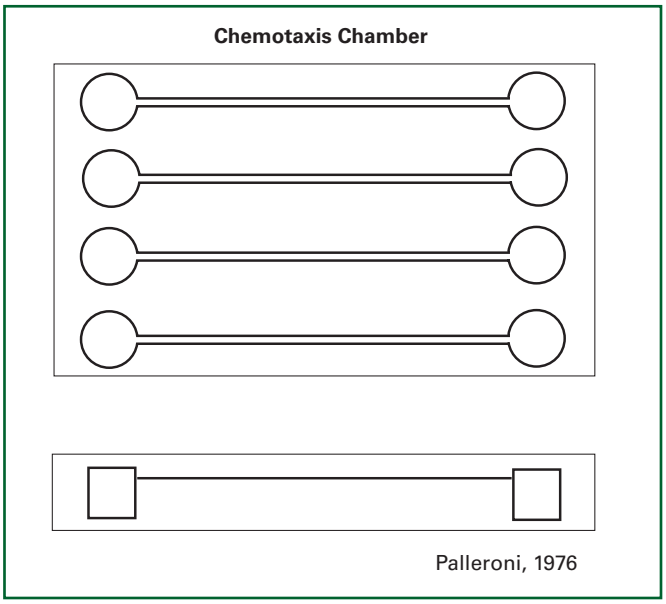

Figure 1. Chemotaxis Chamber, actual size 5.5 x 5.5 x 1.0 cm.

between experiments having differences in control cell density values, the ratio of bacterial density in the experimental assay microcapillaries to the density of the buffer controls (chemotaxis index [CI]) was also calculated (Lopez-de-Victoria, 1993). A CI value of 1 indicates no response, a value greater than one indicates a positive chemotactic response, and a value less than 1 indicates a negative response.

Experimental Data/Results

Chemotaxis provides a means for bacteria to respond to environmental gradients of potential nutrients and toxins, resulting in direct motility towards or away from these substances (Adler, 1973). The *P. fluorecens* bacterial strain used responded impressively according to our predictions (Figure 2). This is the first time that bacteria have been demonstrated to be attracted to explosive compounds. The strong attraction of soil bacteria to DNT was also verified using soil perfusion column leachate from field samples taken at Ft. Ord, Calif. In these leachate tests, the chemotactic indices observed for DNT are some of the highest rates ever observed for any type of chemotaxis, including the ones observed for *P. fluorecens* for concentrations of 2,4 and 2,6-dinitrotoluene that ranged from 0.12 M to 1.20×10^{-3} M. The results of this work have important implications for the ecology of TNT-degrading bacteria; it also may suggest ways that microenvironments containing explosives might be controlled to increase biodegradation rates in situ.

Future Studies

More work on chemotactic ability of nitrogen fixing bacteria, such as *Pseudomonas*, towards explosives like RDX, HMX and TNT can be very promising. These findings will be very helpful in future field applications that involve bioremediation and natural attenuation of nitroaromatic compounds.

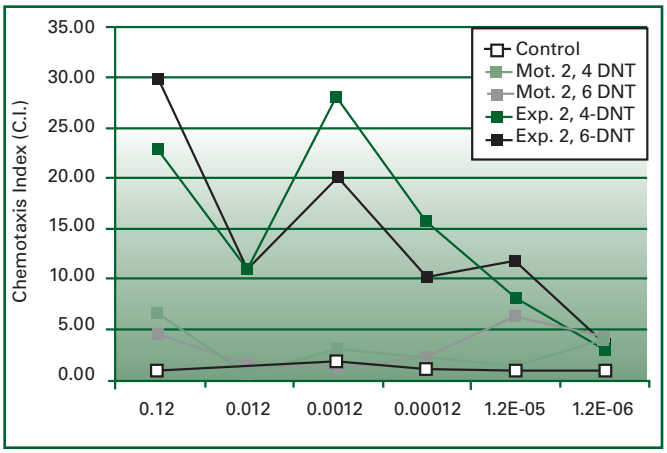

Figure 2. Chemotatic Index (CI) of Pseudomonas fluorescens to 2,4 and 2,6 dinitrotoluene (DNT), motility (Mot.), and Experimental (Exp.) CI = experimental/control.

Students Involved

Carlos M. Rivera Velázquez – undergraduate, Pontifical Catholic University, Ponce, Puerto Rico (summer 1999)

Relevant Publications

Hazen, T.C., Controlled phosphate-enhanced bioremediation tested, EPA Tech Trends 25:3-5, 1997.

Hazen, T.C., Bioremediation, In: *Microbiology of the Terrestrial Subsurface*, P. Amy and D. Haldeman (eds), pp. 247-266. CRC Press, Boca Raton, 1997.

Tien, A.J., D.J. Altman, A. Worsztynowicz, K. Zacharz, K. Ulfig, T. Manko and T.C. Hazen, Bioremediation of a process waste lagoon at a southern Polish oil refinery - DOE's first demonstration project in Poland, M#9, Proceedings Fourth International Symposium and Exhibition on Environmental Contamination in Central and Eastern Europe (Warsaw'98), Institute for International Cooperative Environmental Research at Florida State University, 1999.

The Effects of Biostimulation Agents on Respiration of Weathered and Unweathered Explosives-Contaminated Soil

Terry C. Hazen and K.C. Oh
Center for Environmental Biotechnology, Lawrence Berkeley National Laboratory, Berkeley, Calif.

Problem Being Addressed

More than 8,000 Department of Defense sites need clean-up efforts (DoD, 1996). In a compilation of soil samples collected from 44 Army ammunition plants, arsenals and depots, 28% contained detectable levels of explosives. TNT was the most commonly occurring compound within the contaminated samples, and was seen in 66% of those samples. Facilities that may be contaminated with explosives include manufacturing plants, ordnance works, Army ammunition plants, ammunition depots, Army and Naval proving grounds, burning grounds, artillery impact ranges, explosives disposal sites, bombing ranges, firing ranges, and ordnance test and evaluation facilities. Due to its toxicity and recalcitrance, the U.S. Environmental Protection Agency has listed TNT as a priority pollutant (Boopathy and Kulpa, 1994).

Bioremediation of organic contaminated soils has proven to be one of the fastest and cheapest remediation technologies available. TNT and its daughter products are highly recalcitrant, especially in highly weathered soils, i.e., soils that have been exposed to the environment for a number of years under suboptimal microbial activity conditions. The present study examines weathered and unweathered soils and looks at the ability of a number of biostimulants to increase total microbial respiration.

Figure 1. The Biometer Bucket for measuring respiration by carbon dioxide trapping of large volumes of soil.

During the course of the laboratory experiment, the following hypotheses were tested:

1. The rate of degradation in the newly contaminated soil is faster than that of the older soil sample.
2. Inorganic nutrient amendments in a minimal salts media will boost the rate of biodegradation.
3. Addition of an organic nutrient, molasses, as a cometabolic substrate will provide the greatest stimulation, especially in the highly weathered soils, where nutrients have been depleted.

Research Methods

The respiration treatablity tests done were a variation on the Bartha and Pramer (1965) Biometer Flask setup. The Biometer Bucket method that we developed represents a new use of this basic technique with several distinct advantages.

Biometer Bucket Method: Two-gallon buckets were converted to soil bioreactors (Figure 1). These reactors use the same CO_2 trapping principle as the biometer flasks, but allow for a much greater soil sample. The KOH trap was a funnel plugged at the bottom with a rubber stopper, which was held in place with epoxy. These traps rested on top of the soil, and could hold up to 80 ml of solution. Larger ascarite-top filters were made using 50 ml Corning vials. A hole was drilled into the bottom of each vial, through which a piece of plastic tubing would fit. This tube was connected to a stopcock, which let the filtered air into the bucket during the times when the KOH was changed. The attached diagram shows the biometer bucket design. The soil in each bucket was mixed well prior to the respiration measurements. After the bucket was sealed, the KOH was added to its trap, and the buckets sat for four days. In this four-day period, the KOH was changed and titrated as necessary, with intervals from an hour to a day, depending on the rate of respiration in the soil samples. These intervals change because a faster respiration rate can create enough CO_2 to saturate the KOH solution. Therefore, the KOH monitoring a more active soil sample must be titrated much

more often than in a slowly respiring sample.

For each bucket, three experiments were run: (1) an unamended control, where the soil was taken directly from the buckets in which it was collected, and lasting 3.57 days, (2) an inorganic nutrient amendment, where 1000 ml of MSM was added to each bucket, and lasting 2.93 days, and (3) an organic nutrient amendment, where an aqueous molasses solution was added to each bucket, and lasting 4.80 days. Respiration was measured daily and samples for HPLC analyses of contaminants were taken initially and at the end of the treatment.

Experimental Data

The respiration analysis indicates that unweathered soil responds faster and in greater magnitudes to nutrient amendments (Figure 2). The results suggest that the weathered soils may have stressed populations and cannot react as quickly to the amendments, especially because they actually reduce their respiration rate in response to MSM addition. The molasses-amended samples all respired at a greater rate than with the other amendments. This result implies that molasses may be used as an effective nutrient source to increase bacterial activity. Three of the four molasses-amended samples are among the lowest four in concentrations of the suspected contaminant located by the HPLC. The HPLC results also suggest a correlation between bacterial activity and biodegradation.

Future Studies

We are currently analyzing the soil from the various treatments to determine what daughter products were produced and determine a mass balance on the contaminants present. We also are using the same test system to determine if combinations of chemical and biological treatment can treat weathered soils contaminated with PCBs to lower ppb concentrations.

Students Involved

Ben Runkle – undergraduate student, Princeton University (summer 1999 support from DOE EURLF)
Marla Miller – graduate student, UC Berkeley (summer and fall 1999)

Relevant Publications

Hazen, T.C., Controlled phosphate-enhanced bioremediation tested, EPA Tech Trends 25:3-5, 1997.

Hazen, T.C., Bioremediation, in Microbiology of the Terrestrial Subsurface (P. Amy and D. Haldeman, eds), pp. 247-266, CRC Press, Boca Raton, 1997.

Tien, A.J., D.J. Altman, A. Worsztynowicz, K. Zacharz, K. Ulfig, T. Manko and T.C. Hazen, Bioremediation of a process waste lagoon at a southern Polish oil refinery - DOE's first demonstration project in Poland, M#9, Proceedings, Fourth International Symposium and Exhibition on Environmental Contamination in Central and Eastern Europe (Warsaw'98), Institute for International Cooperative Environmental Research at Florida State University, 1999.

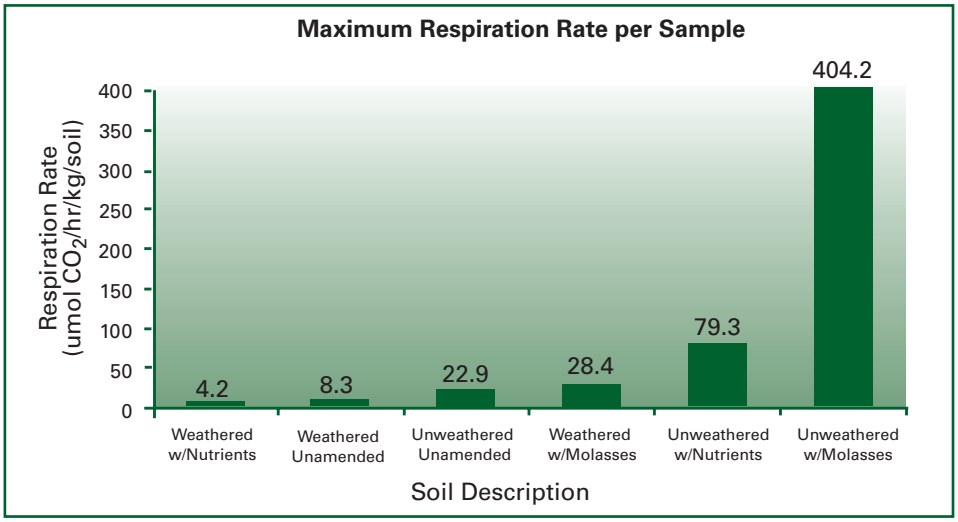

Figure 2. Comparison of the maximum respiration rate for each of the treatments for weathered and unweathered explosives-contaminated soil for Ft. Ord, Monterrey, Calif.

Degradation of Nitro-Compounds by *Bacillus spp.*

Ehab El Helow
Biochemistry and Molecular Biology Department, University of California at Berkeley

Problem Being Addressed

Glycerol trinitrate (GTN), also known as nitroglycerin, is extensively utilized for the production of explosives and pharmaceuticals. GTN is a hazardous waste product of increasing environmental concern. Current disposal techniques, such as open-air burning and incineration, are expensive and can produce hazardous waste byproducts. Bioremediation systems could remediate explosive contaminants at approximately a tenfold lower treatment cost and with increased public acceptance.

An ecological investigation of GTN-contaminated sites at the Naval Surface Warfare Station in Maryland resulted in the isolation of a *Bacillus thuringiensis/cereus* strain (ATCC 51912) able to degrade GTN. HPLC and TLC analysis by other researchers of GTN metabolism in cell-free systems suggested that there was a sequential denitration to dinitrate isomers, mononitrate isomers and ultimately to glycerol. Using classical taxonomic methods, the researchers were not able to differentially assign the isolate (ATCC 51912) to either the *B. thuringiensis* or *B. cereus* species. The main objectives of this project are to develop molecular tools that can phylogenetically position GTN-degrading bacilli and to develop economical detoxification technologies.

Research Methods/Tools Employed

In this report we describe the application of the *Bacillus sasp-B* protein-coding gene to the phylogenetic investigation of a broad range of strains from the *B. cereus/thuringiensis* group.

The requirements for a suitable molecular phylogenetic tool for these studies are:

- the ability to generate an interpretative framework for phylogenetic positioning of *B. cereus/thuringiensis* group microorganisms;
- the ability to generate emergent DNA sequence properties that enable the systematic study of genetic variation within the *B. cereus/thuringiensis* group;
- the ability to generate self-ordering systematic data properties that naturally cluster *B. cereus* group members into stratifying groups.

Clustal alignment of the ATCC 51912 *sasp-B* DNA sequence with reference sequences from *B. cereus, B. thuringiensis, B. mycoides* and *B. anthracis* species allowed, unambiguously, the assignment of this isolate to a specific *B. thuringiensis* subgroup. Once the phylogenetic position of the ATCC 51912 strain was established, a group of other *B. thuringiensis* subspecies and near-neighbor strains were screened for their ability to degrade GTN. These strains were obtained from culture collections and through collaborations. Additional *Bacillus* species outside of the *B. cereus* group were studied, including *B. subtilis, B. licheniformis, B. megaterium* and *B. pumilus*.

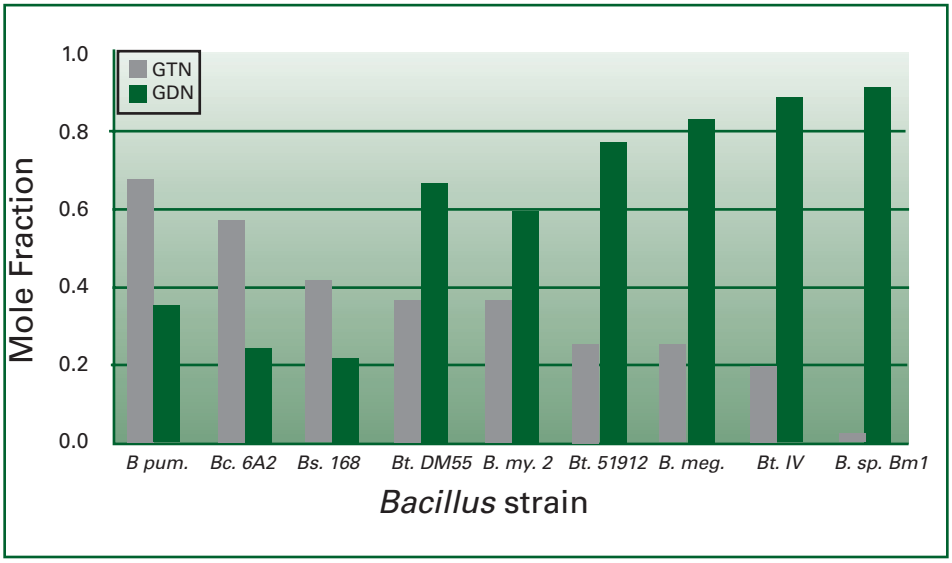

Figure 1. *Bacillus spp.* GTN degradation screening (24 h).

Experimental Data

Thirty geographically diverse *Bacillus* strains were screened for GTN degradation. Samples were collected after 24 hours of incubation. All of the *Bacillus* species examined (*B. cereus, B.*

thuringiensis, B. mycoides, B. licheniformis, B. subtilis, B. megaterium and *B. pumilus*) were able to grow in the presence of GTN. However, the rate and extent of GTN degradation by a natural isolate, Mo1, identified in this work as *B. licheniformis*, was superior to all of the other strains studied. These data, expressed as residual GDN and GMN mole fractions in the culture filtrate, are presented in Figure 1.

The effects of carbon equivalent concentrations of alternative carbon sources (sodium pyruvate, xylose, fructose, sorbitol and starch) on GTN degradation rates were examined in the minimal medium. GTN metabolism and sporulation efficiency was markedly affected by carbon source quality. Maximum GTN metabolism and minimum sporulation frequency were produced with glucose addition. On the contrary, starch reduced the rate of GTN and GDN degradation, while allowing about 80% of the Mo1 cells to develop into mature dormant spores within 48 hours. These results suggest that glucose is the most suitable carbon source for facilitating GTN degradation.

Future Studies

Several strategies for the bioremediation of GTN will be investigated:

1. Optimization of GTN degradation by *B. licheniformis* Mo1:
   - Application of a Plackett-Burman incomplete two-level factorial experimental design (Plackett and Burman, 1946) to explore the relative importance of various medium components and other environmental conditions that affect GTN metabolism by strain Mo1;
   - Investigation of the most significant independent variables, each at three levels, by applying Box-Behnken statistical design, which is a response surface methodology (Montgomery, 1991);
   - Optimization of the levels of these variables predicted using a second order polynomial model fitted to the results of the Box-Behnken experiment.
2. Examination of other soil bacteria capable of degrading explosive nitro-compounds:
   - Continued isolation, identification and characterization of environmental microorganisms that are capable of degrading GTN;
   - Use of the expanding *sasp-B* phylogenetic database to design genosensor microarrays capable of detecting nitro-compound degrading microorganisms in environmental samples;
   - Continued analysis of the physiological and genetic regulation of nitro-compound metabolism in pure bacterial cultures.

Students Involved

Yasmine Gomez – UC Berkeley BEST Program
Esperanza Nunez – UC Berkeley CSEE BEST Program
John Pool – UC Berkeley CSEE BEST Program

Degradation of TNT in Replicated Wetlands Microcosms

Alex J. Horne, Kyung-Duc Zoh and Anna Steding
Ecological Engineering Group, Civil and Environmental Engineering Department,
University of California at Berkeley

Problem Being Addressed

The Holy Grail of most microbial degradation—and wetlands phytoremediation is no exception—is complete degradation of the pollutant to its basic elements, usually water, carbon dioxide and nitrogen gas. Since nitrate is easily denitrified to nitrogen gas in properly designed treatment wetlands, TNT seems an obvious candidate for degradation. However, treatment wetlands do not need to completely degrade a pollutant to be useful. Immobilization is often adequate. For example, with heavy metals there can be no total degradation and wetlands are frequently used for metal rich mine wastes or to polish other less contaminated wastes.

Research Methods/Tools Employed

Two methods are available to determine the fate of TNT in wetlands:

- Complete assay of the degradation products;
- Use of isotope labels.

We have attempted the first route with mixed success. There are many degradation products and some may be strongly bound to the organic matrix of the wetland (dead straw, bulrush, cattail etc.; see Zoh et al., 1999). This report considers the alternative approach using radiolabelled ^{14}C TNT.

We used the same system of for other wetland experiments. A series of 1.5 L, replicated wetlands microcosms were set up using dead fragmented plant matter and a small soil inoculum. The microcosms were initially set up with nitrate to stimulate dentrifying bacteria. It is likely that bacteria that degrade nitrate will also be able to degrade TNT. The system was considered ready for TNT degradation when the nitrate concentration had fallen. The microcosms are anoxic, sparged with nitrogen gas and are slow flow-through systems. Incubations with added [U-^{14}C] TNT were used to investigate mass balance and determine metabolite production including $^{14}CO_2$. Any CO_2 from the reaction solution was trapped in a series of three bottles containing 12-mL of scintillation cocktail. At the end of the experiment, the solution was acidified to pH 1 using concentration H_2SO_4 to volatilize any CO2 that remained in the solution. ^{14}C in the final solution phase was also captured by scintillation cocktail, and then analyzed using a Beckman LS 6000 SC Scintillation Counter (Beckman Instruments, Inc., Fullerton, Calif.).

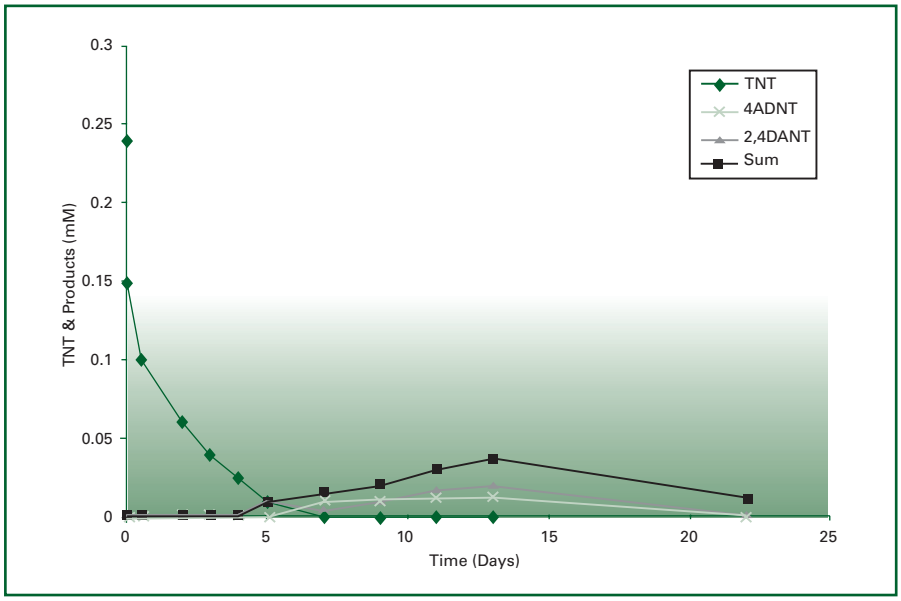

Figure 1. Degradation of TNT in wetland microcosms. Degradation products measured account for less than 30% of TNT lost.

Table 1. Summary of radioactivity recovery of the originally applied radioactivity in the straw system after anaerobic condition (1 month).

	%
Gas Phase*	0.04
Final Solution	10.3
Extraction with acetonitrile	10.2
Extraction with methanol	12.1

*The mean value of ^{14}C represents any $^{14}CO_2$ production and any volatile ^{14}C production.

Experimental data

Analytically pure 14C-labeled TNT was added to microcosms simulating typical treatment wetlands and any radiolabelled carbon dioxide produced was taken as evidence of complete degradation. The results are shown in Table 1. Mass balance calculations showed that TNT mineralization did not take place; this result agrees with at least one other published study using other kinds of plants to simulate wetland conditions (Hughes et al., 1997). Another finding is that only about 10% of ^{14}C was left in the final solution. This seems to indicate that TNT and its metabolites were leached back into the straw and became tightly bound to plant and subject to later degradation.

Conclusions

Our microcosm experiments showed that dead plant litter can reduce TNT to the level of monoaminonitrotolene and dinitrotoluene. The generally negligible TNT metabolite concentrations in plant material versus those in the incubation water suggest that most TNT degradation occurs in the plants early in the incubation period. Early degradation is followed by TNT metabolite leaching from the plants to the water. It is also possible that most TNT is degraded outside the plants by microorganisms in the water but is stimulated by plant leachates. A combination of both mechanisms are also possible. Reductive pathways appeared to be dominant.

Students Involved

Kyung-Doc Zoh – post-doctoral fellow, UCLA and University of Seoul, S. Korea
Anna Steading – graduate student, UC Berkeley
James Hauri – graduate student, UC Berkeley
Jennifer Rubrake – graduate student, UC Berkeley
Noah Hume – graduate student, UC Berkeley
Brian Lafaille – undergraduate Bio-Resource Engineering, University of Maine
Also, several undergraduates at UC Berkeley

Future Studies

Future work will include further studies on the fate and stability of TNT degradation products in wetlands, with concentration on the living and dead peat section of the wetland (as distinct from uptake into living plants).

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Hughes, J.B., Transformation of TNT by aquatic plants and plant tissue cultures, Envir. Sci. Technol. 31: 266-271, 1997.

Photolysis and Microbial Degradation of TNT and Nitro-Compounds

Huey-Min Hwang
Jackson State University, Jackson, Miss.

Problem Being Addressed

The application of in situ, either active or passive bioremediation of TNT and other nitroaromatic compounds (NACs) is an important emerging cleanup technique that holds promise of achieving both detoxification and source removal of these regulated environmental toxins. TNT is widely used as an explosive and enters subsurface environments as munition residues at nearly 17,000 Department of Defense installations that potentially need environmental cleanup. The specific objectives of this study are:

- To determine the contributions of photolysis and microbial degradation to TNT and relevant nitro-compounds removal process in a surface freshwater environment.
- To determine the effects of selected environmental factors (e.g., pH, season, photosensitizers and humic substance) on the rates and products toxicity of photolysis and microbial degradation processes.
- To assess the ecotoxicity and genotoxicity of TNT and its transformation products with microbial bioassays.
- To isolate and identify the stable transformation products, especially if photo-induced toxicity is observed.
- To develop economical detoxification technologies for remediation applications.

Research Methods/Tools Employed

A simultaneous incubation system with quartz flasks was used to study the degradation kinetics of candidate compounds under different irradiation and mediation conditions. Concurrent chemical and biological analyses were conducted to monitor the fate and effects of TNT and nitro-compounds after the simultaneous incubations. High performance liquid chromatography (HPLC) and the radiotracer technique were used to monitor the degradation process, while plate count, direct count, heterotrophic uptake and the Mutatox Test were used to measure the number/activity of bacterial populations and microbial toxicity of the test compounds.

Experimental Data

- A synergistic relationship between UV photolysis and microbial degradation of TNT is observed (Table 1). It has great potential for enhanced TNT bioremediation.
- At the concentration range examined (10 µg/L to 10 mg/L) TNT photoproducts were found to enhance bacterial viability and inhibit bacterial utilization of dissolved organic substrates such as D-glucose. The inhibition appears to be mediated through substrate competition. Genotoxicity of the test compounds is currently under study.
- At the concentration range studied (0.1-10 mg/L), riboflavin (an environmentally friendly sensitizer) was found to significantly enhance photolysis rates of TNT and chloroanilines (up to tenfold). The intermediate compounds may be different from the photolysis without the sensitizer. Identification of the intermediate compounds will be conducted with LC/MS/MS later.

Table 1. Mineralization (%) of TNT under different exposure treatments.*

Treatment	Amount mineralized into $^{14}CO_2$ (%)
Poisoned dark (1 day)	0.230 ± 0.006
Poisoned dark (3 days)	0.36 ± 0.07
Live dark (1 day)	0.10 ± 0.01
Live dark (3 days)	0.6 ± 0.4
Poisoned light (1 day)	0.100 ± 0.006
Poisoned light (2 days)	13.5 ± 9.4
Poisoned light (3 days)	40.7 ± 4.2
Live light (1 day)	0.20 ± 0.04
Live light (2 days)	63.0 ± 18.4
Live light (3 days)	73 ± 14

**TNT was added at a final concentration of 14 µg/L. Numbers are expressed as mean ± 1 standard deviation (n= 3). Poisoned (with formaldehyde) light group reflects photolysis rate and live light group reflects the combination of photolysis and microbial degradation rates.*

- Ferric compounds (photosensitizers) do not affect bacterial viability counts; however, they are not as effective as riboflavin in enhancing TNT photolysis rate.

Future Studies

- To identify the degradation pathways and mechanisms of photolysis/microbial degradation in the presence and absence of different sensitizers.
- To assess the ecotoxicity and genotoxicity of mixtures of TNT and relevant nitro-aromatic compounds.

Students Involved

Latonja Slaughter – graduated in August 1999 with a master's degree in environmental science; now pursuing doctorate in biology, Purdue University
Sean Cook – master's student in environmental science, Jackson State University
Harriet Course – undergraduate student in biology, Jackson State University

Relevant Publications

Dong, S., H.-M. Hwang, C. Harrison, L. Holloway, X. Shi and H. Yu, UVA light-induced DNA cleavage by selected polycyclic aromatic hydrocarbons, Bulletin of Environmental Contamination & Toxicology, accepted for publication.

Zappi, M., K. White, H.-M. Hwang, R. Bajpai and M. Qasim, The fate of hydrogen peroxide as an oxygen source for bioremediation activities within saturated aquifer systems, Journal of Air and Waste Management, in press.

Hwang, H.-M., L. Slaughter, S. Cook and S. Wiggins, Assessing the effects of photoproducts of 2,4,6-trinitrotoluene (TNT) on microbial assemblages in natural aquatic environment, Proc., 1999 Mississippi Water Resources Conference, Raymond, Miss., pp. 174-179, 1999.

Hwang, H.-M., 2,4,6-trinitrotoluene (TNT) in a fresh-water environment, 1999 Annual Meeting of American Society for Microbiology Meeting, Chicago, Illinois, p. 583, 1999.

Hwang, H.-M., D. McCullum and L. Slaughter, Phototransformation of 2,4-dichloroaniline in a surface freshwater environment: effects on micro-bial assemblages, Bulletin of Environmental Contamination & Toxicology, 60: 81-87, 1998.

In vitro Screening of Endemic Microorganisms for their Potential to Grow and to Degrade Munition Wastes, Organic Compounds and Metals

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Ana G. Méndez University System, San Juan, Puerto Rico

Problem Being Addressed

The projected costs of Department of Defense site restoration using existing technologies are staggering: the estimated cleanup cost is at least $24.5 billion for the 7,313 identified U.S. sites (EPA, 1993). The pollutants at these sites include chlorinated hydrocarbons, metals, petroleum products, explosives, mixed waste and other organics. There is clearly a need for new cost-effective treatment technologies. Bioremediation, the use of microorganisms to detoxify hazardous waste, promises to provide economical and ecologically sound clean-up strategies. Tropical environments may have a large number of niches where unique microorganisms might have developed that can biodegrade organic contaminants and biotransform heavy metals. This study is focused on the screening of new microorganisms in water samples from several sites in Puerto Rico and their potential use as agents for the transformation of wastes such as heavy metals, nitroaromatic compounds and other organic contaminants. The sites to be sampled include San Juan Bay in northern Puerto Rico and Yabucoa Bay to the southeast.

Experimental Design and Methods

Sampling: Samples were collected in the Yabucoa Bay, in four different sites, using a liquid-collecting device in sterile bags at 1.0 m of depth and placed in ice.

Growth of the microorganisms: Samples were taken to the laboratory and inoculated on plates containing 1/4 nutrient agar using both loop and spread techniques. Twenty (20) plates were inoculated using each technique. They were incubated at 35°C for seven days. After the incubation time, the colonies formed were transferred to new plates: 1/4 nutrient agar; 1/4 nutrient agar plus 137.14 ppm of p-Nitrophenol; 1/4 nutrient agar plus 139.11 ppm of p-Nitrotoluene; 1/4 nutrient agar plus either 0.05 mM, 0.1 mM, 0.25 mM or 0.5 mM of Cu^{2+}, Cd^{2+}, Pb^{2+} or Cr^{3+}.

Colonies that showed growth after the fourth inoculation were characterized by tcell morphology and Gram Stain reaction (Tables 1 and 2). Pure cultures of each isolate were frozen in glycerol for subsequent testing. Samples were inoculated in fresh media characterized using BIOLOG (this work is still in progress).

Contaminant degradation analysis: Calibration curves for p-Nitrophenol and p-Nitrotoluene were prepared at pH 13 using a spectrophotometer to measure the absorbance of the solution at 400 nm for p-Nitrophenol and 322 nm for p-Nitrotoluene. Then, molar absorptivity coefficients were calculated for each compound. Metal concentrations were determined by atomic absorption spectrometry (AAS).

Preliminary growth curves for several bacteria were obtained.

Table 1. Media: + p-Nitrotoluene (137.14 ppm)

Isolate	Observations (Cell morphology and Gram Stain Reaction)
2A2	N. D.
3B2	N. D.
4A1	Streptococcus, Gram Positive
4A2	Streptococcus, Gram Positive
4B2	Coccus (single), Gram Positive
4C1	Bacillus (single), Gram Positive
4D1	N. D.

Table 2. Media: + p-Nitrophenol (139.11 ppm)

Isolate	Observations (Cell morphology and Gram Stain Reaction)
2A3	Bacillus, Gram Positive
2A3(2)	N. D.
3A2	Bacillus, Gram Negative
3A3	Streptococcus, Gram Positive
3B2	Streptococcus, Gram Negative
4A1	Streptococcus, Gram Positive

Experimental Data

Twenty-five colonies were obtained from different sites. We observed different colony morphology, depending on the contaminant present in the media.

Together with Dr. Ledes' group we are investigating the effect of chelating agents on the biodegradation of metals. We have found that the presence of the ligand has different effects on the biodegradation by the bacteria (Figure 1).

Future Studies

To fulfill the milestones addressed in this proposal we need to fully characterize the microorganisms isolated and perform studies of biodegradation of the compounds. To obtain information on the mechanism of biodegradation we are going to follow the process by using HPLC and GC. Biodegradation studies of organic compounds will be followed by either the chromatographic techniques mentioned or spectrophotometric and CO_2 evolution studies. We also are going to study the effect of different carbon sources in the biodegradation of the contaminants, as well as the presence of cross-contaminants, such as heavy metals and recalcitrant halogenated organic compounds.

Students Involved

Diana Saurí – graduate student writing master's thesis about bioremediation of selenium by bacteria from San Juan Bay

Myrna Sanchez – graduate student studying biodegradation of diesel by bacteria isolated from a contaminated site in Puerto Rico

Ruth O. Vives – graduate student studying biodegradation of heavy metals by bacteria isolated from Yabucoa Bay

Diana Castro – undergraduate student studying the biodegradation of nitroaromatic compounds by bacteria isolated from Yabucoa Bay

Edwin Rodriguez – undergraduate student studying the biodegradation of heavy metals and the characterization of sub-products from biodegradation by electrochemical techniques such as cyclic voltammetry.

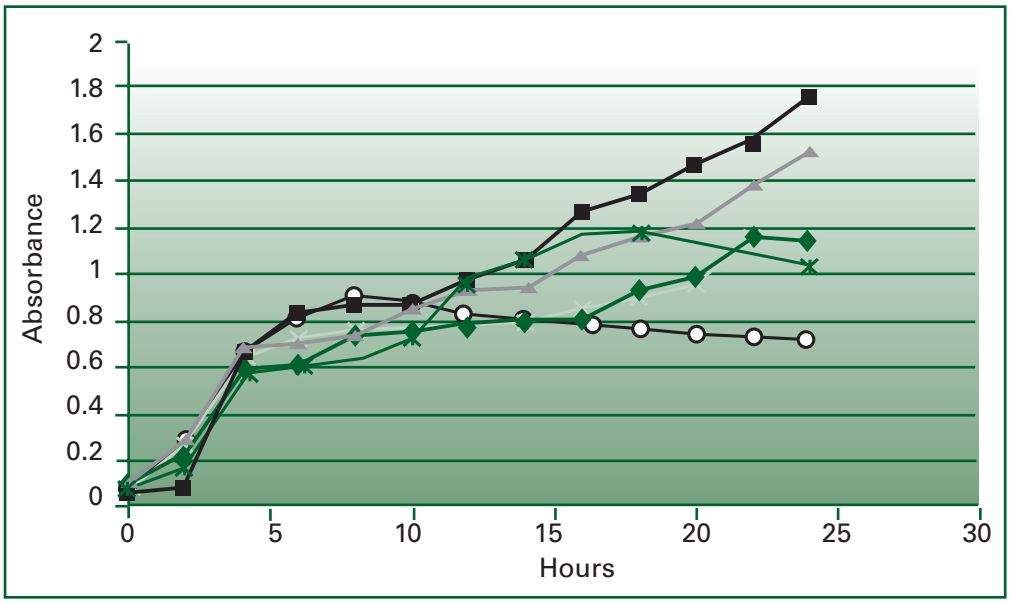

Figure 1. Growth curves for Bacillus 5(B)bl.

Warm Humidified Air Injection for Enhanced Bioremediation of Petroleum-Contaminated Soil

Dirk Schulze-Makuch and John Walton
University of Texas at El Paso

Problem Being Addressed

The hypothesis of this project is that soil contaminated with hydrocarbons can be biodegraded more rapidly and efficiently if the contaminated soil is vented with warm, humidified air.

Research Methods/Tools Employed

An experiment has been designed that will use barrels holding soil contaminated with diesel fuel. The basic design is shown in Figure 1. Warm, humidified air will be pumped into the soil barrels. The warm, humidified air is meant to accelerate rates of biodegradation within the barrels. A total of nine barrels will be used. Three barrels will have diesel contamination in the soil but will only be exposed to ambient dry air in order to provide a base line for monitored biodegradation parameters.

The diesel fuel will be mixed into the sand at a concentration of about 1,000 ppm. The sand, with a grain size of about 1 mm, will be spread out on a plastic liner. Diesel fuel will be dropped onto the sand while the sand is stirred to obtain a homogeneously contaminated sand. Then the contaminated sand will be put into the barrels.

The other three barrels with diesel-contaminated soil will be vented with warm, humidified air. The last three barrels will have a nitrogen-based fertilizer added to the diesel-contaminated soil to ensure that there is not a lack of nitrogen acting as a limiting factor to the rate of biodegradation that can occur. Biodegradation parameters will be monitored using the sampling ports as shown in Figure 1.

Material and supplies needed for the project have been ordered and delivered to the project site, the University of Texas at El Paso Solar Pond facility. The barrels are currently assembled at the project site. The experiment started Jan. 10, 2000, with a six-month monitoring phase. Analysis and interpretation of results will start shortly after the first monitoring data are obtained.

Students Involved

Alfonso Munoz – master's student, Department of Geological Sciences, UTEP
Everardo Vega – senior undergraduate student, Department of Biological Sciences, UTEP.

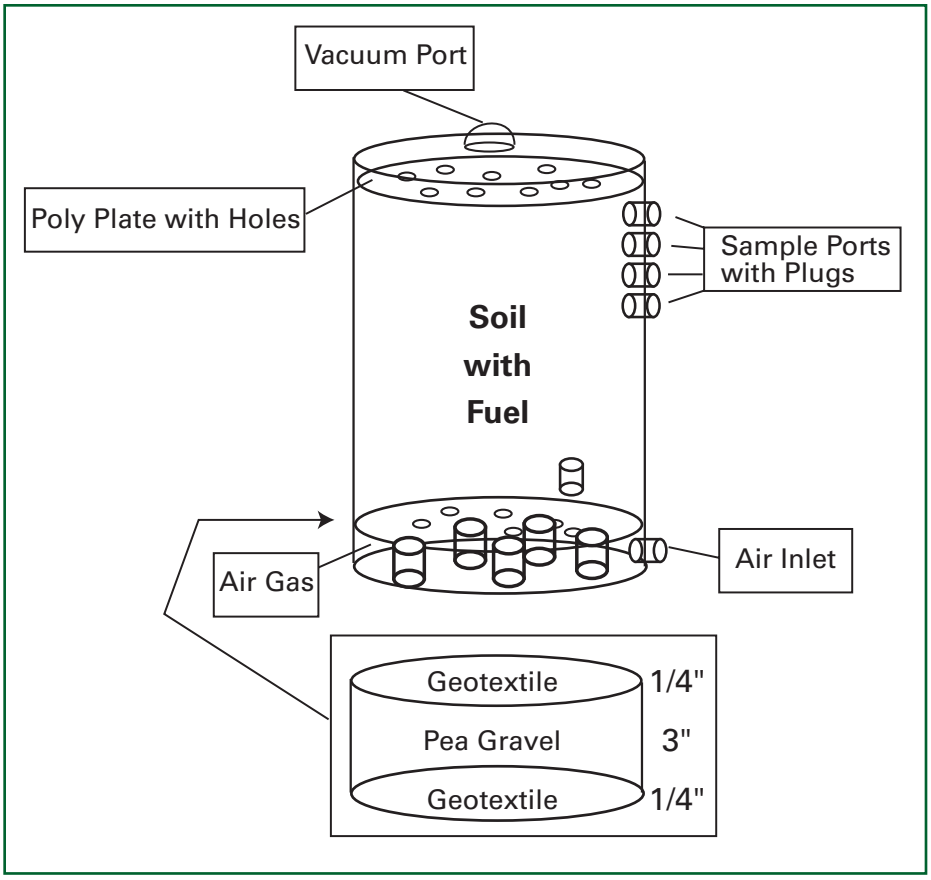

Figure 1. Schematic of barrel holding soil contaminated with diesel fuel.

Biodegradation of Polycyclic Aromatic Hydrocarbons

William T. Stringfellow
Lawrence Berkeley National Laboratory, Berkeley, Calif.

Problem Being Addressed

Most Department of Defense (DoD) sites are contaminated with polycyclic aromatic hydrocarbons (PAHs). PAHs contaminate military property as a result of open burning, incomplete fossil fuel combustion and accidental or deliberate hydrocarbon spills. PAHs rank among the most important environmental contaminants because of their persistence in the environment and their carcinogenic and mutagenic properties. Bioremediation has been demonstrated to remove PAHs from contaminated soil. However there is evidence that the PAHs are not always mineralized (to carbon dioxide and water) during bioremediation, but are transformed to stable intermediates that may remain in the soil after treatment. The identification of these intermediates is incomplete. Our task, the biodegradation of PAHs, is an investigation of how bacteria transform high molecular weight PAHs in both laboratory culture and in the field. Our hypothesis is that we can use metabolite turnover to measure intrinsic PAH degradation.

The scientific goals of the project are to develop a greater understanding of how bacteria that are able to grow on light PAHs (such as phenanthrene) can also oxidize heavier PAHs, such as pyrene and benz[a]pyrene. The technology or application goals of the project are to develop tools that allow us to study PAH degradation and PAH metabolite turnover in contaminated soils. If we can link metabolite turnover and PAH degradation, we believe we can make a strong argument for an intrinsic degradation procedure based on monitoring metabolite turnover. The application of intrinsic remediation to fuel hydrocarbon contamination has proven a very cost effective approach to site clean-up and it would be beneficial if this technology could be applied to PAHs as well.

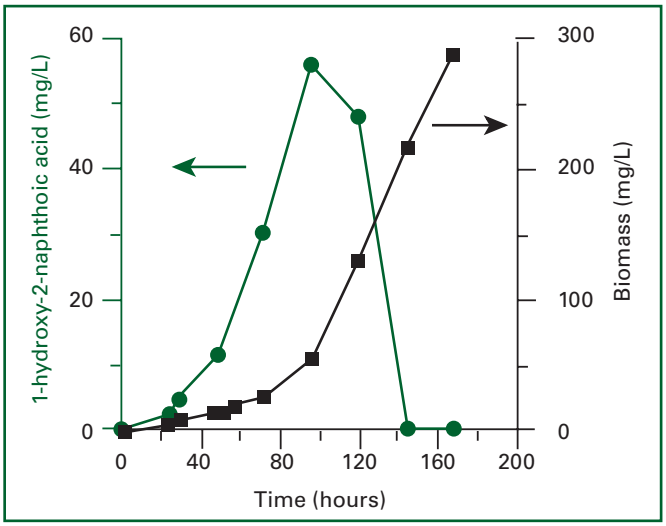

Figure 1. Production of metabolites by pure culture bacteria during growth on phenanthrene.

Research Methods/Tools Employed

The central focus of this research so far has been the development of gas chromatography (GC) and high performance liquid chromatography (HPLC) methods for monitoring PAH transformations by bacteria. The methods are then applied to pure and mixed cultures of bacteria to investigate patterns of metabolite formation as they relate to PAH degradation (Figure 1). These methods are then applied to PAH-contaminated soils undergoing remediation to see if similar patterns can be observed in the environment (Figure 2).

Experimental Data

A method for the extraction and analysis of PAH metabolites has been developed. This method uses an acetone extraction followed by a fluoracil column clean-up procedure that produces a methylene chloride fraction for GC analysis and an ethyl acetate fraction for HPLC analysis. This procedure was tested against known phenanthrene metabolites (1-hydroxy-2-naphthoic acid and salicylic acid) and found to be effective for their analysis in soil. Our current results suggest that this method is selective for carboxylic acids and excludes many alcohols. An HPLC method with fluorescence detection is used to selectively analyze polynuclear compounds.

Metabolite production was studied in a pure, well-characterized culture (Figure 1). As the bacteria grow on phenanthrene (a PAH), we see a transitory accumulation of a metabolite. This metabolite is present only during active PAH turnover.

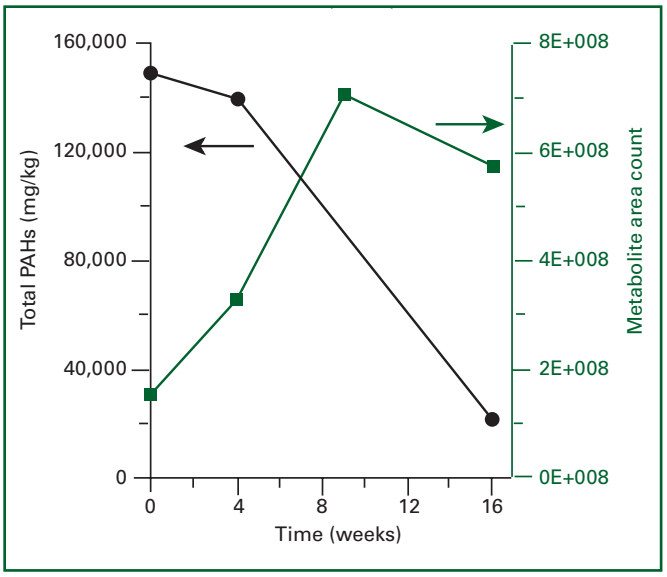

Figure 2. Relationship between metabolite production and PAH concentration in a soil undergoing active remediation.

When we examined PAH-contaminated soils undergoing active remediation (supplied by Chevron Research and Development Company) we saw a similar pattern of transitory metabolite accumulation (Figure 2). The amount of metabolite extracted from soils undergoing active biological remediation was inversely correlated with the removal of PAHs.

Future Studies

This coming year's program will include analysis of a complete set of soil samples from a remediation test site to be provided by the Gas Research Institute and DoD facilities. The metabolite extraction protocol will be further refined and standards for the metabolites of heavier PAHs will be developed. Further work will be done with pure cultures and heavier PAHs to characterize specific metabolites that may be observed in the field.

Students Involved

Berenice Villatoro – undergraduate, Laney Jr. College, Oakland, Calif.
Kyianna Merchant – undergraduate, Laney Jr. College, Oakland, Calif.
Andre Adams – graduating senior, Grambling State University
Samir Davila – undergraduate student, UC Berkeley

Relevant Publications

Stringfellow, W.T., and G.M. Castro, Using bacterial metabolite production to monitor the natural attenuation of PAHs in contaminated soils, 15th Annual Conference on Contaminated Soils and Water, Amherst, Mass., Oct. 18-21, 1999.

Immobilization of TNT in Wetlands Using Replicated Microcosms

Kyung-Duc Zoh, Alex J. Horne and Anna Steding
Ecological Engineering Group, Civil and Environmental Engineering Department,
University of California at Berkeley

Problem Being Addressed

Trinitrotoluene (TNT) is chiefly used in military operations because it is a high explosive that is unaffected by shock or abrasion. TNT is also used in certain types of film processing. The compound has been manufactured and used for over a century with concomitant accumulation of TNT waste and unexploded ordinance. Although TNT is flammable, it is readily soluble in water and has accumulated in the groundwater of many military sites. In addition, TNT can also be found in small pellets, which have become coated and are quite resistant to water. As with many hazardous wastes, TNT can be excavated from soil and incinerated, but costs are high and air pollution can be a problem. Thus, less costly alternatives are needed to remove TNT from a large number of sites covering a large area of the United States and elsewhere. It would prevent much trouble if the removal of TNT occurred on-site, since this would avoid shipping large volumes of contaminated soil or water on public highways.

In the last two decades, wetlands have shown great promise for treatment of wastes, and continue to do so. In particular, there are now several large (50-500 acre) treatment wetlands for nitrate. These have operated for several years and are part of the water treatment system for several large public agencies. A number of investigators have already made important findings showing that TNT and other explosives could be taken up and transformed by living plants. Some of the plants tested may be amenable to full-scale wetlands treatment systems.

Wetlands are characterized as reducing systems but treatment wetlands are quite different from natural wetlands. In particular, treatment wetlands use plants grown in the wetland to provide carbon and habitat for bacteria that carry out the desired process. The soil underlying the dead leaf litter layer and the live plants play little role in the degradation or immobilization processes. It is not that these two components are not active, but that the flow rate and reaction rate in the leaf litter is engineered to dwarf that elsewhere.

Research Methods/Tools Employed

Understanding the mechanisms involved in the removal of pollutants requires two tools: molecular (skin-in) or whole-organism (skin-out) methods. At UC Berkeley, the Molecular and Cell Biology Group of Professor Leighton use these tools, and, together with the published literature, defines what is possible for the Ecological Engineering Group.

In order to design an engineered wetland capable of treating TNT, multiple species tests must be run under conditions that simulate wetlands conditions. The first task is translating laboratory molecular and single-species. The methods possible are microcosms, mesocosms and macrocosms (pilot-plant scale), usually employed in that order.

We have approached the system in the following way initially using microcosm studies:

- Natural mixtures of microbes
- Anoxic conditions that simulate wetlands leaf litter layers
- Natural sources and levels of organic carbon
- Likely levels of TNT found in contaminated sites.

The 1.5 L replicated wetlands microcosms were set up using dead fragmented plant matter. This is a very important difference from most reported literature, which describes the use of live plants. Both live and dead plants may take up or degrade TNT. However, dead leaf litter is the site of choice for wetlands pollutant degradation. The litter is much smaller in volume for the same weight and is more easily shielded from sensitive biota (especially birds) that might eat contaminated matter. Although the live plants themselves may provide a slow degradation method for TNT, the concept of living plant tissue rich in TNT and degradation products is hard to sell to post-Kesterson environmental groups and regulators.

Experimental Data

TNT was added to microcosms simulating typical treatment wetlands. The difference from other systems published in the literature was the use of dead straw

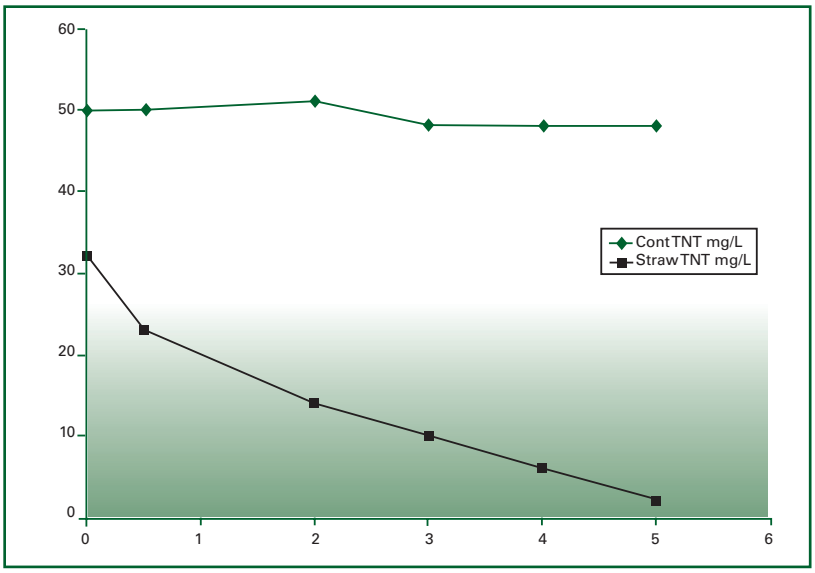

Figure 1. Removal of TNT by wetlands micrococms using straw as the substrate.

to represent the active wetland components (see above). Analytically pure TNT was added at a typical groundwater concentration of 50 mg/L to anoxic nitrogen-sparged 1.5 L microcosms. TNT was rapidly lost from solution (Figure 1) so that it was virtually absent from free solution in five days.

Extraction of the straw with various solvents did not produce any TNT. However, the TNT may have been bonded too strongly with the organic matter. Alternatively, the TNT may have been only partially degraded to its various reductive byproducts. A search for the degradation products was made and the results are shown in Figure 2. The most prominent TNT degradation products were 4-ADNT and 2,4-DANT, as have been found by other researchers. Importantly, only about 30% at best of the original TNT added could be accounted for by the byproducts measured. The remainder of the TNT must be either bound very strongly to the straw organic matrix or present in other byproducts that were not detected.

Future Studies

The fate of TNT and its degradation products will be investigated in several ways:

- Radiolabelled TNT will be added to the wetlands microcosms and any gaseous product such as carbon dioxide will be evidence for complete degradation of TNT.
- Different kinds of wetland plants, as dead material, will be investigated to determine the best sources for immobilization and degradation of TNT.
- Toxicity of TNT degradation products in relationship to wetland design will be tested. The degradation products found in the free water will be tested for toxicity to *Ceriodaphnia*, as sensitive zooplankter. The effect of humic acids will be assessed as a likely component that can be manipulated in full-scale wetlands.

Students Involved

Kyung-Doc Zoh - post doctoral fellow, UCLA and University of Seoul, S. Korea
Anna Steading - graduate student, UC Berkeley

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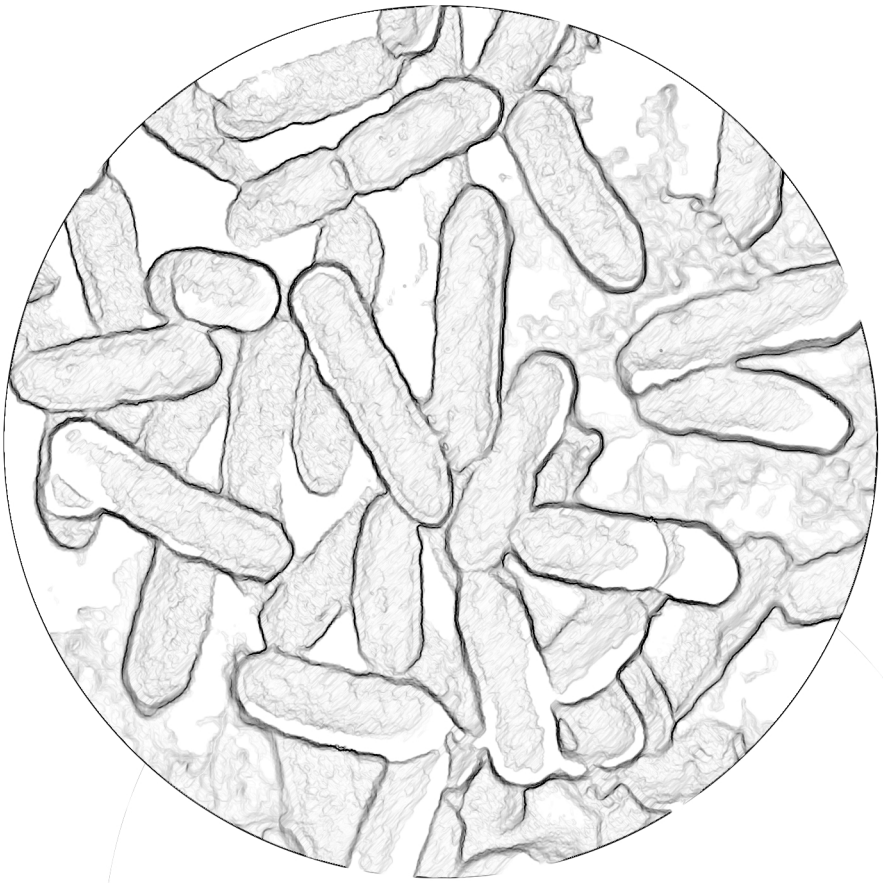
Interactions of Plants and Microorganisms with Metals



Phytoremediation of Lead in Contaminated Soils

Gregorio B. Begonia, Maria F.T. Begonia, Charles Rhyne and Y.M. Liu
Jackson State University, Jackson, Miss.

Problem Being Addressed

Lead is a common contaminant of surface soils at Department of Defense installations, especially the very large areas used as firing ranges. Given the high toxicity of lead to plants, animals and man, there is a great need for techniques that reduce the lead concentrations in large areal extents of surface soil at DoD sites. Concentrating lead from the soil in plants and harvesting those plants to remove lead from the soil is a very cost effective and esthetically pleasing remediation technique, ie., phytoremediation. Our main objectives in this project were to (1) determine whether plant species that demonstrated metal-accumulating attributes under hydroponic systems showed similar metal uptake rates when grown in metal-contaminated soils; (2) investigate how phytoextraction of metals can be enhanced using synthetic chelates and soil pH manipulations; and (3) assess the rates of disappearance of metals from contaminated soils through sequential cropping of selected species.

Research Methods

Experiments were conducted in a naturally lighted greenhouse with the photo-period extended to 12 hours using high intensity (1000 W) lamps. Plants were grown from seed in 1.8 L plastic pots containing soils amended with known concentrations of Pb (applied before planting as aqueous solutions of $Pb(NO_3)_2$). In another experiment, known amounts of Pb were mixed with the soil one week before seeding. Plants were irrigated with either nutrient solution or distilled water whenever necessary. After 6-8 weeks of growth, plants were harvested, dried and ground. Subsampled ground tissues were digested using nitric acid-hydrogen peroxide procedures (EPA method # 305A). Metal concentrations were quantified using atomic absorption spectrophotometry.

Experimental Data/Results

- Phytoextraction of Pb by morning glory (*Ipomoea lacunosa* L.) was enhanced through the addition of 5 mM EDTA; with increasing concentration of applied EDTA, there was a corresponding increase in the amount of Pb translocated to the shoots (Figure 1). Uptake of Pb was evident only in roots of *Sesbania exaltata* with a 5 mM EDTA amendment.
- For each level of applied Pb, residual soil Pb after plant harvest generally decreased with increasing concentration of soil-applied chelate (Figure 2). Effects of soil pH manipulation and chelate application time on Pb phytoextraction from a contaminated soil were also evaluated using *I. lacunosa and S. exaltata*. Plant and soil samples are currently being acid-digested.
- Bioavailability of metal for plant uptake depends on the geochemical phase of the metal situated in the soil. Sequential extraction of a Pb-contaminated soil revealed that Pb was found 34.5% in free status, 43.5% in carbonates, 11.5% in

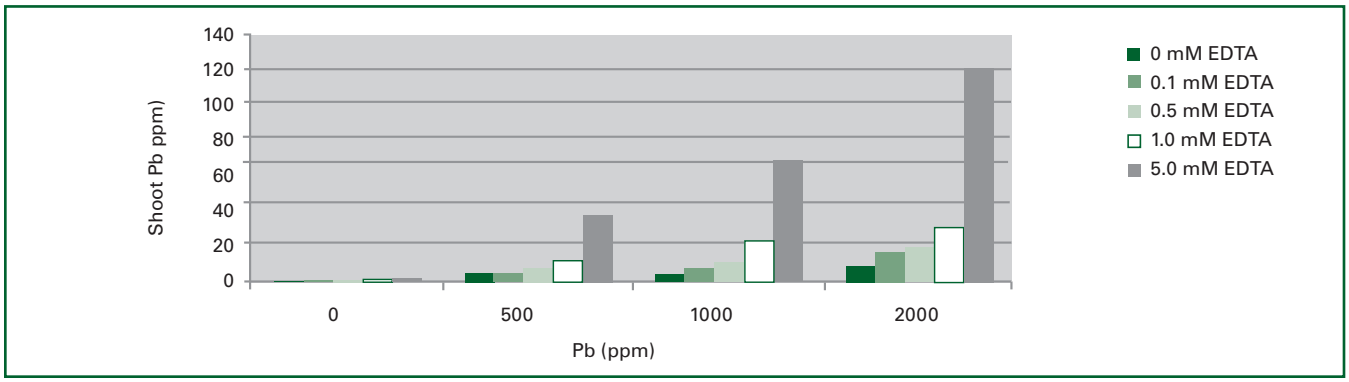


Figure 1. Effects of different concentrations of lead (Pb) and EDTA on shoot lead contents (ppm) of morning glory. Vertical bars indicate standard error of the mean (n=5).

oxides, 4.3% in organic matter and 6.2% in residue.
- In lieu of a DoD-contaminated soil, soils were amended with known amounts of Pb to determine how efficient hyperaccumulator species are in reducing metal levels in soils. It is expected that Pb levels in contaminated soils will be reduced to environmentally acceptable levels after several sequential cropping cycles.

Future Studies

Future studies include the following:
- Continued selection of more efficient plant species/varieties and soil amendments.
- Optimization of agronomic/horticultural practices for plant cultivation, especially for exotic hyperaccumulator species.
- Isolation of genes from various plant, bacterial and animal sources that can enhance metal accumulation or degradation of organics.
- Manipulation of metal tolerance in plants through chelation, compartmentalization or biotransformation.
- Manipulation of rhizospheric bacteria/other microorganisms to enhance their role in phytoremediation.

Students Involved

Murty S. Kambhampati – Ph.D. student, graduated August 1998
Gloria L. Miller – master's student, biology-environmental science, BEST
Miriam Ighoavodha – master's student, biology-environmental science, BEST
Keyuna Seals – master's student, biology-environmental science, BEST
Corey Burks –undergraduate student, biology, BEST
Valencia Payne – undergraduate student, biology, BEST
Michael Johnson – undergraduate student, biology, ONR-SEMEP
Melicia Brown – undergraduate student, biology, MARC
Joshuanda Owens – undergraduate student, biology, MARC

Relevant Publications

Kambhampati, M.S., G.B. Begonia, M.F.T. Begonia and Y. Bufford, Phytoremediation of lead-contaminated soils using morning glory (*Ipomoea lacunosa* L.), Environ. Sci. Technol., submitted.

Begonia, G.B., C.D. Davis, M.F.T. Begonia and C.N. Gray, Growth responses of Indian mustard [*Brassica juncea* (L.) Czern.] and its phytoextraction of lead from a contaminated soil, Bull. Environ. Contam. Toxicol, 61(1): 38-43, 1998.

Begonia, M.F.T., G.B. Begonia, G. Miller, J. Owens, M. Brown, M. Johnson and C. Burks, EDTA-assisted phytoextraction of lead from contaminated soils using coffeeweed (*Sesbania exaltata* Raf.), National Minority Research Symposium Abstracts, p. 74, Phoenix, Ariz., Nov. 10-13, 1999.

Begonia, G.B., M.F.T. Begonia, C. Rhyne, G. Miller and M. Johnson, Chelate-enhanced phytoextraction of lead from contaminated soils using *Sesbania exaltata* (Raf.), Agronomy Abstracts, p. 30, American Soc. Agronomy/Crop Science Soc. America, Salt Lake City, Utah, Oct. 31-Nov. 4, 1999.

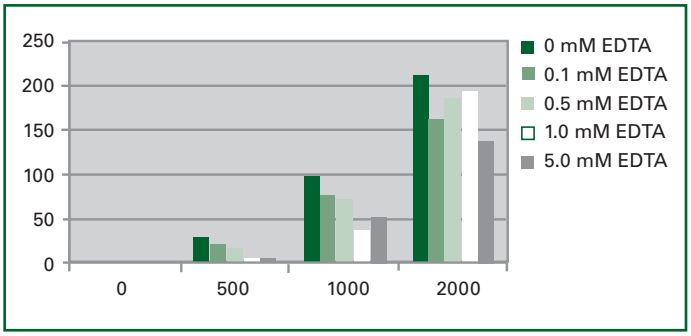


Figure 1. Remaining lead (ppm) in soil treated with different concentrations of lead and EDTA. Vertical bars indicate standard error of the mean (n=5).

A Novel Bioremediation Approach to Clean Heavy Metal Ions from Contaminated Waters with Inactivated Tissues of Desert Plants

Jorge Gardea-Torresdey
Chemistry Department, University of Texas at El Paso

Problem Being Addressed

The main objective of this project is to study the ability of desert plants to adsorb toxic heavy metal ions from contaminated waters, thus reducing the threat to the public's health. Metals such as lead (Pb), copper (Cu), nickel (Ni) and chromium (Cr) have been determined to cause acute health effects in humans as well as ecological damage. However, many desert plants have been found to survive in heavy metal contaminated soils. This resistance may be in part due to the development of chemical binding sites found on the exposed desert plant's cell walls. Therefore, nature could hold the answer to removal of heavy metal ions from aqueous solutions. By utilizing the plant tissues that have evolved a natural affinity for heavy metal ions, we may be able to develop a method to remove these contaminants in a low-cost and highly effective manner. However, before a method can be developed to remove the heavy metal ions using desert plant tissues, the binding mechanism must be better understood.

Research Methods/Tools Employed

At the present time we are performing experiments with the desert plant *Solanum ealeagnifolium* (silverleaf nightshade). The plants were collected, dried and ground in preparation for the various metal binding studies. Batch laboratory methods are being employed to characterize the metal binding abilities of the desert plant. In addition, chemical modification experiments have been utilized to further study the metal binding mechanism(s) of *Solanum ealeagnifolium* with the different metal ions being studied. Metal analysis for the batch experimentation has been performed utilizing flame atomic absorption spectroscopy (FAAS) and graphite atomic absorption spectroscopy (GFAAS) with Zeeman background correction.

In addition, samples have been analyzed utilizing synchrotron X-ray Absorption Spectroscopy (XAS) at Stanford Synchrotron Radiation Laboratories (SSRL) at Stanford University in California. The XAS data are currently being examined to determine the X-ray Absorption Near Edge Structure (XANES) of bound metals such as Cu(II) and Cr(III). By using the computer software, Fourier transformation of the spectra can be performed, which provides the Extended X-ray Absorption Fine Structure (EXAFS) of the metal bound on the biomass. These data can then be compared with model compounds to delineate the nearest neighbor atom bound to the metal ion on the biomass. The XAS data along with the chemical modification will help us pin down the actual chemical functional groups responsible for binding of the metal ions and help to find the most effective method to remove metal ions from contaminated waters.

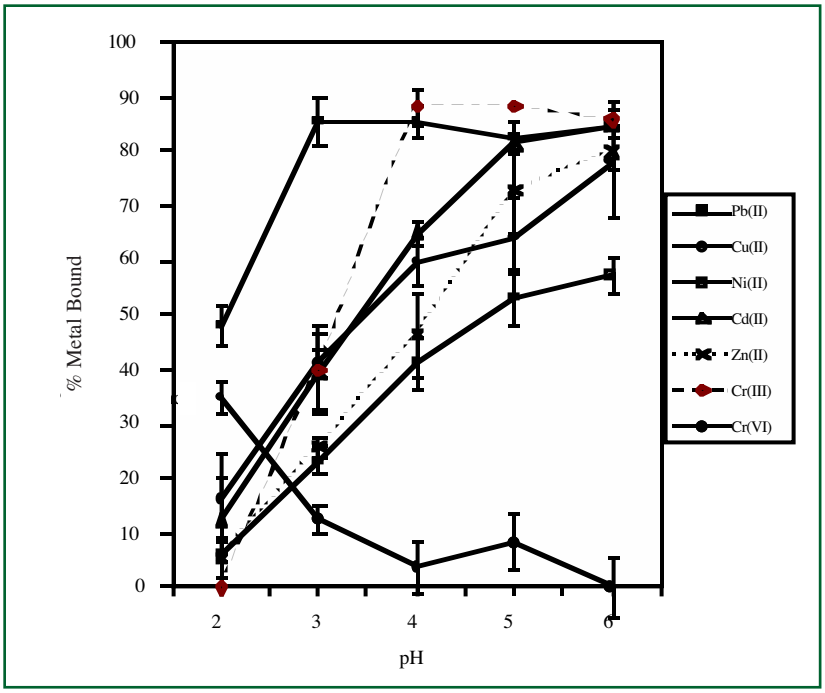


Figure 1. Percent of metal bound at various pHs.

Experimental Results

Thus far, we have determined that *Solanum ealeagnifolium* is able to bind appreciable amounts of metals tested. Figure 1 shows the percent of metal bound

Table 1. Chemical Modification Results

Metal studied	Unmodified capacity (mg metal/g biomass)	Esterified capacity (mg metal/g biomass)	% decrease in binding	Hydrolyzed capacity (mg metal/g biomass)	% increase in binding
Cu(II)	45.9	10.3	**77.5**	70.5	**153.6**
Cr(III)	43.1	3.4	**92.1**	78.3	**181.8**
Pb(II)	31.9	12.6	**60.5**	80.9	**253.3**
Ni(II)	13.3	1.8	**86.8**	35.7	**268.4**
Zn(II)	11.5	2.4	**79.6**	32.7	**284.0**

at various pHs. It is clear from Figure 1 that the binding of these metals is pH-dependent, with maximum binding occurring at or near pH 5.0. Since carboxyl ligands are known to have pKa values in the 3-4 region, we believe that carboxylic acids found on the cell walls may be involved in the binding of these metal ions. However, the binding of Cr(VI) did not follow the same trend. This indicates that Cr(VI) may be binding through a different mechanism.

In order to better understand how carboxyl ligands on the cell walls of *Solanum ealeagnifolium* are involved in the metal binding process, we chemically modified the plant tissues and performed batch experiments with modified biomass. The results of the esterification and hydrolyzation capacity experiments are shown in Table 1. From the table, it is clear that the esterification of the cell walls of Solanum ealeagnifolium blocked the majority of the binding for these metals. These data support the finding from the pH profile, which indicates that carboxyl ligands are involved in the metal binding (by hydrolysis) process. In addition, it can be seen form the table that by creating additional carboxyl ligands, there is an increase in the binding of these metals, which further supports that carboxyl groups found on the cell walls of *Solanum ealeagnifolium* are involved in the binding of these metal ions. However, further investigation will be necessary. We plan to utilize XAS to corroborate these results.

Future Studies

Since not all of the binding of these metals by *Solanum ealeagnifolium* was blocked, future work will involve further investigation of the mechanism(s) to determine if other groups may be involved. This will require further chemical modification studies along with batch experimentation. Also, examination of the XAS data obtained from SSRL may help to elucidate which ligands are involved with the metal binding. We will also conduct pH profile experiments with the esterified biomass and compare the data with the unesterified *Solanum ealeagnifolium* biomass.

Students Involved

Kenneth Dokken – graduate student in chemistry, UTEP
Estela Rascon – graduate student, UTEP

Relevant Publication

Gardea-Torresdey, J., K. Dokken and E. Rascon, Determination of Au(III) binding and bioreduction by alfalfa biomass using EXAFS, XANES and UV-Vis spectroscopies, submitted to Environmental Science and Technology; paper presented at workshop on phytoremediation, Jackson, Miss.

Biodegradation of Heavy Metals Using New Chelating Agents

Desiderio Ledes
Universidad del Turabo, Ana G. Méndez University System, Puerto Rico

Problem Being Addressed

Synthetic chelators such as EDTA and NTA can form stable, soluble complexes with heavy metals and were commonly used as cleaning agents during industrial processing at DoD sites. Metal–chelate complexes have entered the environment and may migrate freely in groundwater. When conditions necessitate immobilization of the contaminant, one approach for limiting the migration of the metal is to biodegrade the organic ligand. The resulting free metal ions are likely to adsorb to mineral surfaces or form oxide mineral precipitates that would transport poorly in groundwater. A number of EDTA- and NTA-degrading organisms have been identified. However, little is known about the enzymes that catalyze the degradation reactions and how these reactions proceed in the environment. In one study, microbial degradation of EDTA by the environmental isolate BNC1, was influenced by the complexed metal. Similar fundamental research focusing on the mechanisms of enzymatic degradation of synthetic chelators is expected to provide useful information for including these enzymes in engineered bioremediation technologies.

The current study focuses on the interaction of bacterial strains with copper chelates. We look at the effect of Cu(II) biotransformation in the presence of different copper chelates. Metal concentrations in growth media-biomass and pellets are analyzed using AA spectroscopy. The results of these studies of toxic metals and microorganism interaction will enable us to determine bioremediation capabilities for copper and similar metals.

Research Methods

Equimolar amounts of the ligands (EDTA, 1,2-CDTA, 1,3-CDTA, 1,4-CDTA or 1,4-CDTP) and of copper (II) nitrate were mixed in enough water to make solutions ranging from 0.010 M to 3.0x10-3 M of the complexes and stirred for two hours. The pH of the solution was adjusted to 5.6. The solution obtained was used to prepare the culture media. IR spectra in solution were obtained in a Nicolet Magna FTIR-750 spectrophotometer in BaF2. A total of 64 scans were taken. UV-Visible spectra were taken on a Beckman 4B spectrophotometer using 1.0 cm quartz cells. NMR spectra were taken using a Bruker 300 NMR spectrometer. Cultured cells from selected strains *Streptococcus, Staphylococcus* and *Bacillus*) were inoculated into 100 mL of the liquid culture media spiked with each of the copper solutions to a final concentration of 20 ppm copper. They were placed in a shaker at 32°C at 200 rpm for 24 hrs. Every two hours 5 ml aliquots were removed and their absorbance measured at 520 nm. A control containing copper (II) nitrate was prepared for comparison. After 24 hours of incubation, 10 mL of the liquid media were removed and centrifuged at 5000 rpm for 10 min. The cells were washed five times with 20 mL of buffer solution. The amount of copper remaining in the supernatant and in the washing was determined by ICP.

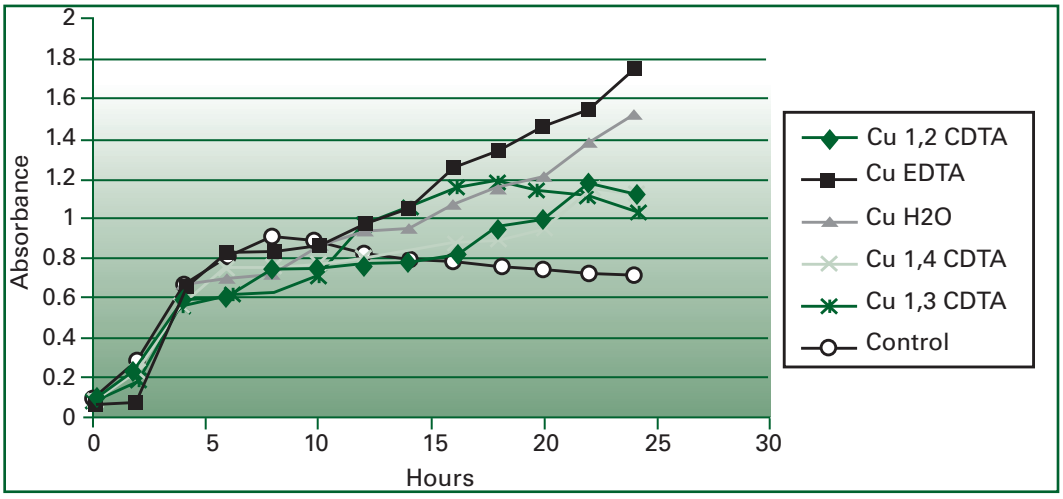


Figure 1. Growth curves for Bacillus 5(B) bl.

Experimental data: Figure 1 shows the growth curves for the *bacillus* studied in the presence of the com-

Table 1.

% Copper uptake	Aqueous	Cu(II)(EDTA)	Cu(II)(1,2-CDTA)	Cu(II)(1,3-CDTA)	Cu(II)(1,4-CDTA)
bacteria 5(b)1	10.25	4.21	26.24	16.54	5.74
bacteria 2(d)1	2.94	24.43	6.61	1.3	0
bacteria 4(d)1	6.51	11.38	1.35	0	0

plexes Cu(II)(EDTA), Cu(II)(aqueous), Cu(II)(1,2-CDTA), Cu(II)(1,3-CDTA) and Cu(II)(1,4-CDTA). The curve for Cu(II)(1,4-CDTP) is not shown because no growth was observed at 24 hours. Lower concentrations are currently being studied. Previous work in our lab has shown that the bacteria studied grow very slowly at concentrations higher than 0.35 mM of the complexes of the other ligands. From the curves, a similar pattern is observed for each complex, so no evidence for a dramatic effect was found. Nevertheless, the presence of copper in the concentration studied seems to increase the amount of cells growing at longer times (Table 1). We summarize the results obtained in percent of copper for the different bacteria in different copper complexes.

Future Studies

We plan to continue our studies and design bench-scale pilot experiments to elucidate possible mechanisms of biotransformation of Cd, Cr, Hg and Pb. Metal concentration in the growth media and cell pellet extracts will be measured by atomic absorption spectroscopy (EPA-SW-846-7130). Conditions of the analysis of target metals will be optimized. Quantification will be performed using calibration curves obtained at the same analytical conditions with standard solutions. To test our findings under field-related conditions, soil samples containing mixed contaminants (i.e., metals and organics) will be used in the bench-scale pilot experiments. Biodegradation of organic co-contaminants will be tracked by intermediate metabolites using HPLC and a combination of HPLC and GC-MS.

Students Involved

Marcel Jaenz – 1998, AGMUS
Melvin Medina – research student until August 1999
Martínez Dayna – research student from August to December 1999
Segarra Justiniano – summer 1999
William Del Valle – graduated with thesis entitled "Comparative analysis between EDTA, 1,4CYDTA and aqueous complexes in the mitigation of metallic ions and their effects in bioremediation processes by bacteria"

Relevant Publication/Presentations

Ledes, D., M. Perez and C. Lozano, Chelating agents on the bioremediation of heavy metals: synthesis and characterization of 1,4-cyclohexanodiamine N,N,N^1,N^1-tetracetate and complexes with divalent cations, EPSOR Meeting, Ponce, P.R., 1999.

Ledes, D., M. Nieves and C. Lozano, Bioremediation of heavy metals by fungi: synthesis, characterization and biodegradability studies of metals complexes with EDTA-type chelating agents, SIM Annual Meeting, Arlington, Va., 1999.

Ledes, D., M. Nieves and C. Lozano, Bioremediation of heavy metals by bacteria: synthesis, characterization and biodegradability studies of metals complexes with EDTA-type chelating agents, SIM Annual Meeting, Arlington, Va., 1999.

Estuarine Plant Responses to Contaminated Sediments

Julia S. Lytle and Thomas F. Lytle
The University of Southern Mississippi, Ocean Springs, Miss.

Research Problem

Tidal marsh plants form an expansive fringe around our coastal ecosystems, serving as a buffer against storms, a habitat for wildlife, an anchor for sediments and a phytoremediator of contaminated sediments. Their capacity to take up contaminants and possibly degrade them has had little attention in comparison to other plant systems. To better understand the role of estuarine plants in reducing contamination in coastal sediments, three dominant estuarine plants from two sites of known contamination were chosen for biochemical adaptive responses.

Juncus roemerianus and *Spartina alterniflora* are the most abundant plant species found in Mississippi tidal marshes and are major salt marsh species found in estuaries on the Gulf and southern Atlantic coasts. Both have extensive rhizome systems. *Sagittaria lancifolia*, called arrowhead or duck potato, also has stout, deeply buried rhizomes and is found abundantly in fresh to brackish marshes. These three species were chosen for study because of their abundance in the estuary and their tolerance to contaminants.

The objective of the study was to compare the ability of the three marsh plants to adapt to elevated contaminant levels by examining glutathione and peroxidase responses. Glutathione acts as an antioxidant to protect labile macromolecules against attack by free radicals and hydrogen peroxide, which form as a result of oxidative stress. Peroxidase activity increases when plants are under toxic stress as the plant transforms or degrades the toxicant causing the stress. From previous studies, it appears that those plants with strong peroxidase systems can better cope when exposed to toxic chemicals.

Three locations were chosen for study: the mouth of an estuarine small boat harbor located in Ocean Springs, Miss., a small inlet on East Beach in Ocean Springs which served as a control, and an inlet adjacent to Keesler Air Force Base on Back Bay in Biloxi, Miss. Analysis of sediments in the Ocean Springs Harbor indicated one area relatively high in silver and another high in cadmium (other metals were present but in lesser concentrations) and an extensive area of petroleum impacted sediments. The inlet adjacent to Keesler AFB had high levels of sediment petroleum hydrocarbons. This inland site was the only site colonized by all three plant species.

Research Methods

Plants and sediment were collected for metal analysis and measurement of plant responses from the three contaminated areas in the boat harbor site, from the inlet adjacent to Keesler AFB and the control site. Root and leaf tissue were analyzed for peroxidase activity and glutathione levels. Biochemical assays utilized both uv/visible and fluorescence spectrophotometry.

Experimental Data

S. alterniflora and *J. roemerianus* had similar glutathione response patterns, with high GSH concentrations in leaf tissue and low responses in roots at contaminated areas except at the Cd site, where *S. alterniflora* had no measurable GSH in leaves or roots. Loss may be due to synthesis of phytochelatins (see related report). Though the same response patterns are seen for peroxidase (POD) activity (Figure 1), POD activity is relatively low in J. roemerianus and extremely high in *S. alterniflora.* High POD responses in *S. alterniflora* may be one attribute of this species that allows it to tolerate high levels of certain toxins. *S. lancifolia* did not grow in the higher salinity sites in Ocean Springs but was collected from Keesler AFB in sediments having high polynuclear aromatic hydrocarbons (PAHs). This species also showed extremely high POD response. In this study as well as other recent studies in our laboratory, we find that plant species that are dominant in contaminated sediments have evolved one or more adaptation responses to protect them from cellular toxicity.

Future studies

Plant responses will be used as a tool in other studies to assess plant species' ability to adapt to specific chemical stress. Understanding how plants adapt to

toxicants and determining which species have stronger adaptive mechanisms will help in choosing plants most likely to sustain high levels of specific toxicants and still exercise their role in the bioremediation of sediments.

Students Involved

Larry Stewart – high school student, Ocean Springs High School
Jeff Lyons – undergraduate, University of Southern Mississippi
Nathaniel Smith – undergraduate, University of Southern Mississippi
Ashley Trahan – undergraduate, University of Southern Mississippi

Related Publication

Stuart, L., Emergent macrophyte responses to oxidative stress, poster presented at the National Meeting of the American Chemical Society, New Orleans, La., Aug. 23, 1999.

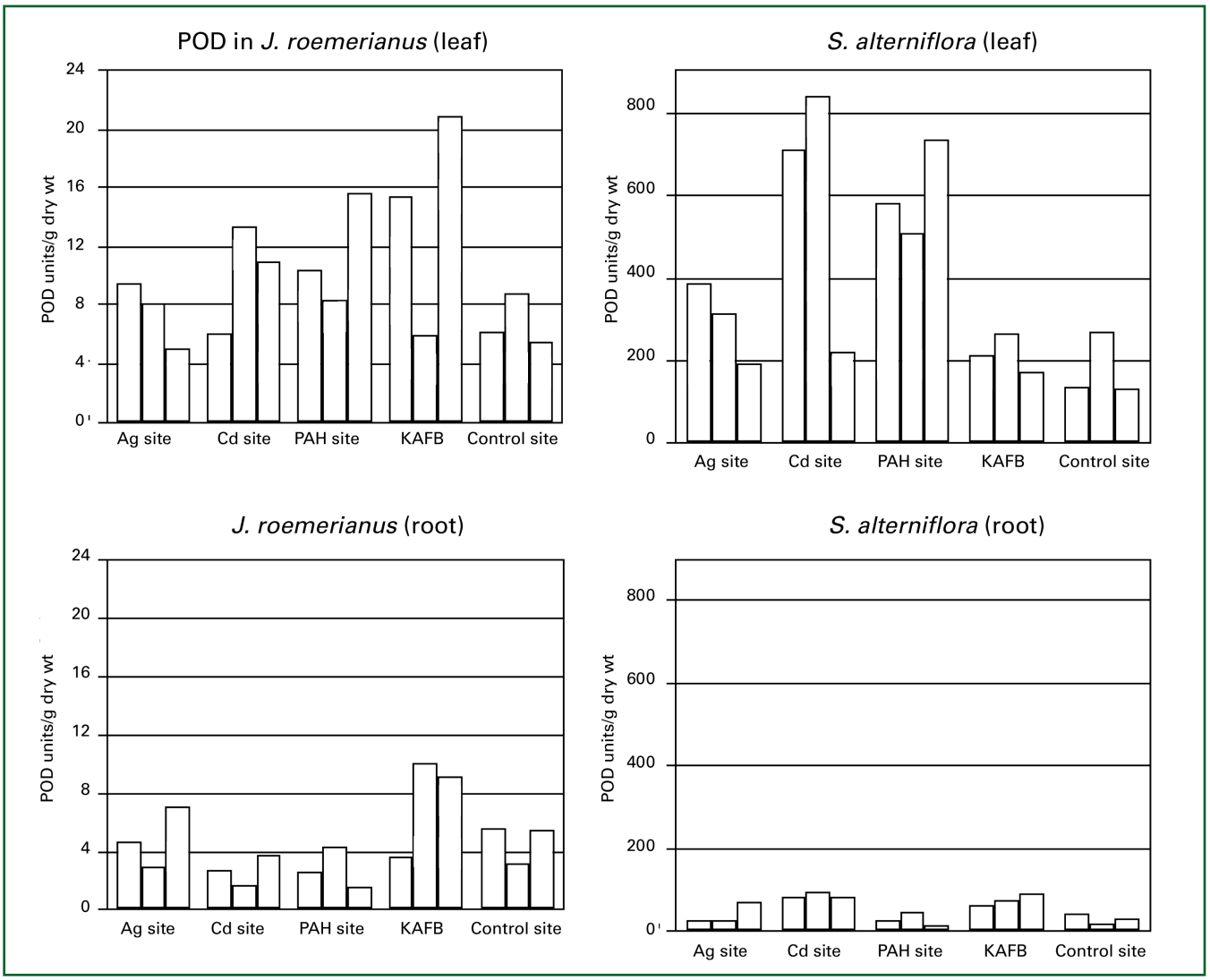


Figure 1. Peroxidase activity in *S. alterniflora* and *J. roemerianus* at five sites.

The Role of Estuarine Plant Exudates in Phytoremediation

Julia S. Lytle and Thomas F. Lytle
The University of Southern Mississippi, Ocean Springs, Miss.

Problem Being Studied

Contaminated sediments pose a significant threat to environmental and human health. They are a sink for a wide range of pollutants. Cleanup of contaminated sediments by physical means can threaten sensitive habitats. Phytoremediation offers an alternative method of sediment remediation. In naturally occurring ecosystems, a significant fraction of toxic organic compounds are sorbed either on the mineral solids or the organic matter in sediments. The resistance to contaminant desorption from the sediments affects bioavailability, toxicity and the efficiency of bioremediation technology. Plants ooze materials from their roots called exudates. Little is known regarding what affects exudate concentrations and chemical composition or what role they play in possibly desorbing contaminants from sediment particles, making them more bioavailable to microbes and plants. One objective of the exudate research is to quantify surfactant characteristics of exudates from estuarine plants with a new technique using a surfactant electrode developed by Orion. Another objective is to compare plant exudate patterns under different environmental conditions, and finally, to compare exudates when plants are exposed to polynuclear aromatic hydrocarbons (PAHs).

Spartina alterniflora, commonly known as smooth cord grass, is an important salt marsh plant which generally occurs in extensive stands in saline areas along the Gulf as well as the Atlantic and Pacific coastlines. Besides acting as a buffer against storm surges and as a habitat for young fish and wildlife, *S. alterniflora* most certainly plays a role in phytoremediation. Though there are not yet supportive data regarding this plant's ability to degrade sediment contaminants, a massive root system supplies large amounts of exudates to the sediment for microbial utilization. The authors postulate that these exudates also act as a surfactant, solubilizing soil-bound contaminants and thus allowing microbial degradation and possibly plant uptake and degradation/transformation.

Research Methods

A study was initiated in summer 1999 to collect exudates from *S. alterniflora* and to measure surfactant characteristics. *S. alterniflora* seedlings were collected from a relatively clean estuary and carefully placed into jars with a 1% nutrient solution. Sterile filtering fiber was placed around the mouth of each jar to prevent evaporation and contamination. Identical systems were prepared for controls, except without plants. All jars (microcosms) were placed outside each day for 9 hours and brought inside overnight. Initial water levels were marked, and distilled water was added to the mark as needed to maintain the water level. Plants appeared healthy throughout the six-week experiment. Water containing plant exudates was removed from one set of microcosms weekly for analysis.

Exudate solutions were placed in vials and flushed with nitrogen and refrigerated until analyzed using an Orion 960/940 Autochemistry Titration System. The instrument was calibrated using two industrial standard surfactants, sodium lauryl sulfate and hyamine or benzethonium chloride. Ionic and nonionic standards were used to develop a technique for characterizing surfactants.

Experimental Data

No evidence of cationic and anionic surfactants was found, but further purification of surfactants from the exudates will be required before nonionic surfactants can be characterized.

Future Studies

Future studies will include the use of other techniques for concentrating exudates such as lyophilization. Seedlings will be replanted and allowed to grow for a longer time period so that a higher concentration can be obtained. In addition, *Juncus roemerianus*, the most abundant salt marsh plant in Mississippi that has the capacity to take up large quantities of petroleum hydrocarbons, will be

included in these studies. Exudates will be separated into ionic and nonionic fractions to obtain a better nonionic electrode measurement. In addition, experiments will be conducted using exudates as an extraction solution of aged PAH sediments to determine if there is enhanced solubility of certain PAH compounds.

Students Involved

Ashley Trahan – undergraduate, University of Southern Mississippi
Larry Stewart – high school student, Ocean Springs High School

Related Publications

Lytle, J.S., T.F. Lytle and L. Stewart, Role of surfactants in phytoremediation, Journal of Mississippi Academy of Sciences 45, No. 1:72, 2000.

Trahan, A., L. Stewart, J.S. Lytle and T.F. Lytle, Technique development for surfactant characterization of plant exudates, Journal of Mississippi Academy of Sciences 45 No. 1:74, 2000.

BEST students at the University of Southern Mississippi, with researchers Tom and Julia Lytle, lower left.

Phytochelatins: Metal Binding Agents in Wetland Plants

Thomas F. Lytle and Julia S. Lytle
The University of Southern Mississippi, Ocean Springs, Miss.

Research Problem

The use of plants to remove/immobilize metals in soils is effective in many applications and non-destructive to natural environmental systems. We have examined the ability of coastal marsh plants to remove metals from contaminated sediments and the mechanisms of removal and storage in the plants. Many terrestrial and aquatic plants produce metal-binding phytochelatins, synthesized from glutathione in response to heavy metals. Phytochelatins in wetland marsh plants have not previously been identified as a means these plants may employ in coping with elevated levels of heavy metals that are often found in coastal sediments.

Phytochelatins are derived from glutathione, a tripeptide that serves as an antioxidant and has other important biochemical functions in plant and animal cells. Its structure is shown in Figure 1. In the presence of toxic heavy metals, many plants (in a manner analogous to metallothionein production in animals and some plants) synthesize phytochelatins, which are chains with repeated links of two of the glutathione peptides, with each link containing a chelating S group and ending with a terminal glycine. Shorter chain phytochelatins are thought usually to result from low-level metal exposure with longer chains elicited as metal levels increase. Objectives of this study are to determine whether marsh plants produce these compounds to sequester and store excessive heavy metals from their rhizosphere and determine if this particular defense mechanism continues to operate for plants that have been exposed to heavy metals long-term. In the first phase of this study we have tested three prominent marsh plants and associated sediments that were collected from sites with a documented history of heavy metal contamination.

Collection and Analytical Methods

Three sites at Ocean Springs Harbor (OSH) were identified, which from previous work in this laboratory, were known to contain elevated levels of either Ag, Cd or polynuclear aromatic hydrocarbons (PAHs). A site at Keesler Air Force Base (KAFB) with a previous history of metal contaminant disposal was also identified and included in this study. Three species of dominant marsh plants were collected at each site: *Juncus roemerianus, Spartina alterniflora* and *Sagittaria lancifolia*. Replicate samples of leaves and roots from the three plants were collected; sediment from the top 5 cm was also collected for metal analysis (with results described elsewhere in this annual report). Entire plant clumps were brought back to the laboratory and processed there.

Samples of leaves and roots were flash frozen in liquid N_2 and lyophylized to prevent the immediate degradation of phytochelatins and its precursor, glutathione, which accompanies extraction of fresh tissue. Freeze-dried material was extracted with trifluoroacetic acid; sulfur groups were derivatized with monobromobimane and analysis proceeded HPLC with a C^{18} bonded phase column and methanol water eluant with fluorescence detection.

Results

Evidence of phytochelatin production can be deduced from loss of glutathione (precursor of phytochelatin as seen in Figure 2, where *S. alterniflora* growing in sediments contaminated with Cd con-

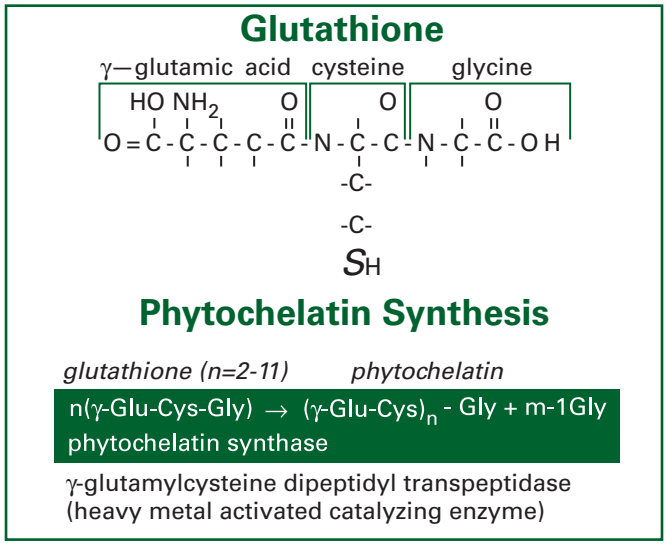


Figure 1. Structure of glutathione.

tained no detectable glutathione in roots or leaves. At all contaminated sites, roots of *J. roemerianus* showed suppressed levels compared to controls, but this suppression was not seen in leaves.

Thus far no phytochelatins have been identified in tissues collected from these marsh plants, at first suggesting to us that additional analytical refinements may be necessary or additional testing conducted to confirm the ability of these plants to form phytochelatins. However, previous studies that have demonstrated a phytochelatin response to heavy metal exposure have all relied upon acute exposure. Evidence suggests that during chronic exposure, phytochelatins may be metabolized after metals are transported to vacuoles, cell walls or other plant regions where they pose little harm to the plant. To clarify the role that phytochelatins may play in safeguarding marsh plants from heavy metals, acute exposure tests will be needed.

Future Studies

Presently a study is underway in which supplemental amounts of Cd have been added to the root zones of *J. roemerianus* and *S. alterniflora* to see if short-term exposure to enhanced levels of Cd in a readily available form elicits a phytochelatin production response. Other aquatic plants are also being tested in this treatment.

Students

Ashley Trahan – undergraduate, University of Southern Mississippi
Larry Stewart – high school student, Ocean Springs High School
Nicole Housley – undergraduate, University of Southern Mississippi

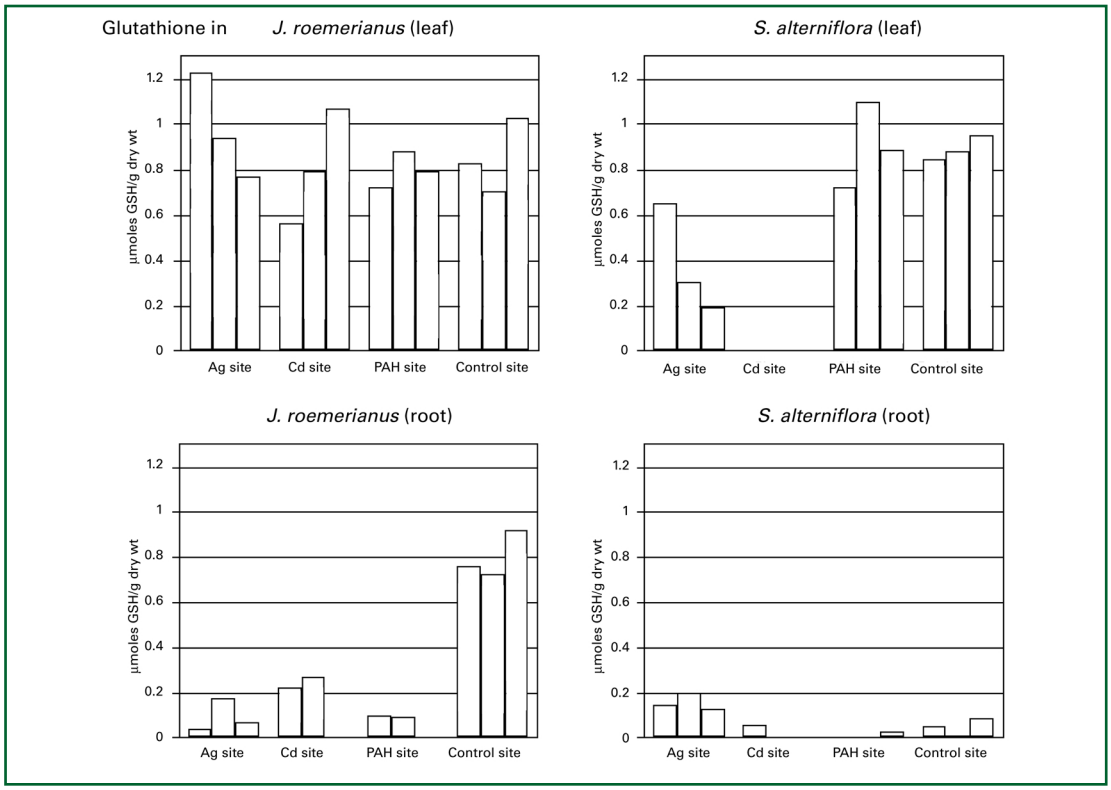


Figure 2. Glutathione in marsh plants from sites with varying metal contamination. The Ag site, Cd site and PAH site are all from Ocean Springs Harbor with control site from an East Ocean Springs marsh site far removed from known metal sources.

Phytoremediation of Heavy Metals Using Coastal Wetland Plants

Thomas F. Lytle and Julia S. Lytle
The University of Southern Mississippi, Ocean Springs, Miss.

Research Problem

Phytoremediation (vegetation-enhanced bioremediation) may offer an improved solution to cleanup of contaminated soils. Plants can reach contaminants effectively and often can withstand high concentrations of toxic substances. They also serve as hosts to biodegrading microorganisms by providing suitable environments for their habitation in the soil rhizosphere. Three species of marsh plants were chosen and metal-uptake capacity examined at a metal-contaminated site at Keesler Air Force Base in Biloxi, Miss.

Metals associated with various components or phases of sediments show varying degrees of extractability, and presumably, bioavailability. There have been several analytical approaches used to estimate how various metals are partitioned among the sediment fractions. The metals removed by mild extractions are thought to represent that which is bioavailable. In this study we chose a simultaneous extraction technique on separate sediment aliquots, preventing the cross contamination and loss problems inherent with other sequential extraction procedures. Results of plant tissue metal analysis are interpreted in terms of the distribution of metals in the bioavailable sedimentary fractions.

Research Methods

Clumps with intact roots and associated sediments of three prominent marsh plants of coastal Mississippi, *Spartina alterniflora, Juncus roemerianus*, and *Sagittaria lancifolia* were collected from several sites, including Keesler AFB. Metals associated with Mn oxides were removed with a 30-minute digestion with hydoxylamine, with metals associated with Fe oxides removed with a 6-hr treatment. A 7-day extraction with ammonia removed those metals associated with organics and the residual (or "non-bioavailable") metals were those requiring strong acid digestion. These latter metals are primarily those bound in clay lattice sites. Plant samples were digested with minimal quantities of concentrated sulfuric acid. Cu, Zn, Pb, Ni, Cr, Cd, Co and Ag analysis utilized flame and furnace atomic absorption spectrophotometry.

Experimental Data

Results for sediment and tissue of S. lancifolia and J. roemerianus from Keesler AFB are shown in Figures 1 and 2. Sediment metals are shown as % of total in the sediments, and plant tissue values are bioconcentration factors (BFs), ratios of tissue metal levels to the bioavailable metals in sediments.

Pb, Zn, Ni and Ag in sediments mostly occurred in Fe oxide fractions, which may not be readily available to plant uptake and account for low metal transfer (low BF) from sediment to leaves. Similarly, most Cd in sediments of *J. romerianus* and *S. alterniflora* was bound to Fe oxides, with little Cd in tissues of these plants. However, Cd in *S. lancifolia* sediments was found mostly in the easily leachable Mn oxide fraction and was accompanied by a very high BF in leaves of this plant. This finding suggests that metals in Mn oxides may be of much greater importance in uptake ability than those bound to Fe oxides.

Bioavailable Cu and Cr in these sediments was mostly bound to organics. *S. lancifolia* showed greater facility in accumulating Cu, though neither it nor *S. alterniflora* showed any capacity to accumulate Cr. *J. roemerianus* leaves showed a BF that not only exceeds the BF in the roots, indicating translocation to leaves, but is much higher than for the other plants. We have found that *J. roemerianus* can move highly insoluble organic compounds from sediments through roots and into the leaves. Possibly the Cr, bound in specific organic residues, is readily available for uptake by *J. romerianus*. This finding may suggest that this plant holds promise for phytoremediating certain Cr-contaminated sediments.

Future Studies

Continuing studies of metal uptake and response to metals derived from sediments will include fractionation of sediment-bound metals and further refinement of the techniques used in separately analyzing these fractions. The mechanisms of Cr uptake by *J. roemerianus* will also be explored.

Students Involved

Jeff Lyons
Nathaniel Smith

Related Publication

Stewart, A.R., and D.F. Malley, Effect of metal mixture (Cu, Zn, Pb, and Ni) on cadmium partitioning in littoral sediments and its accumulation by the freshwater macrophyte Eriocaulon septangulare, Environmental Toxicology and Chemistry, Vol. 18, No. 3, pp. 436-447, 1999.

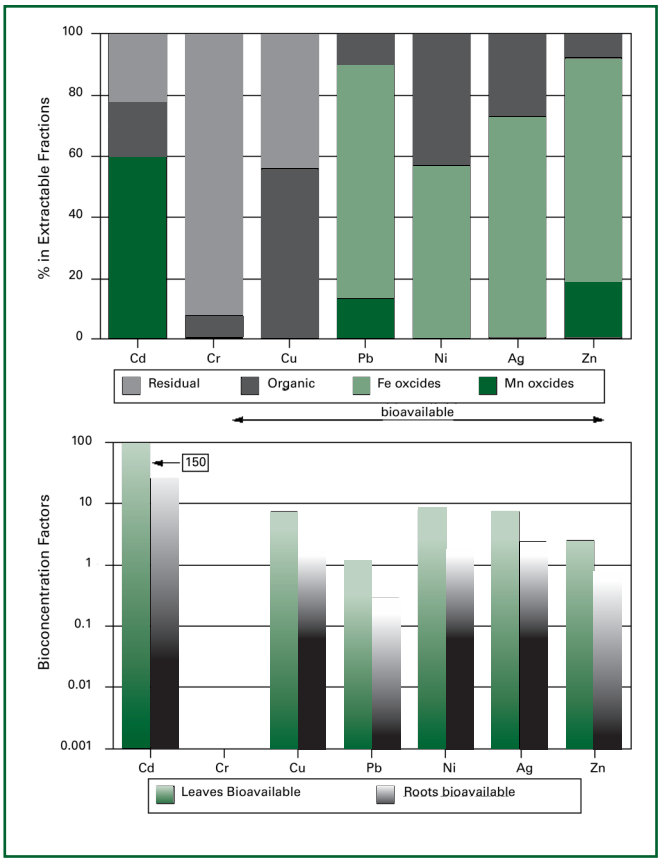


Figure 1. Heavy metals in *S. lancifolia*. Metal bound in either Mn oxide, Fe oxide or organic phases of sediments (bioavailable) is represented as % of total. Bioconcentration factors are computed as dry wt concentration in leaves or roots divided by dry wt bioavailable fractions.

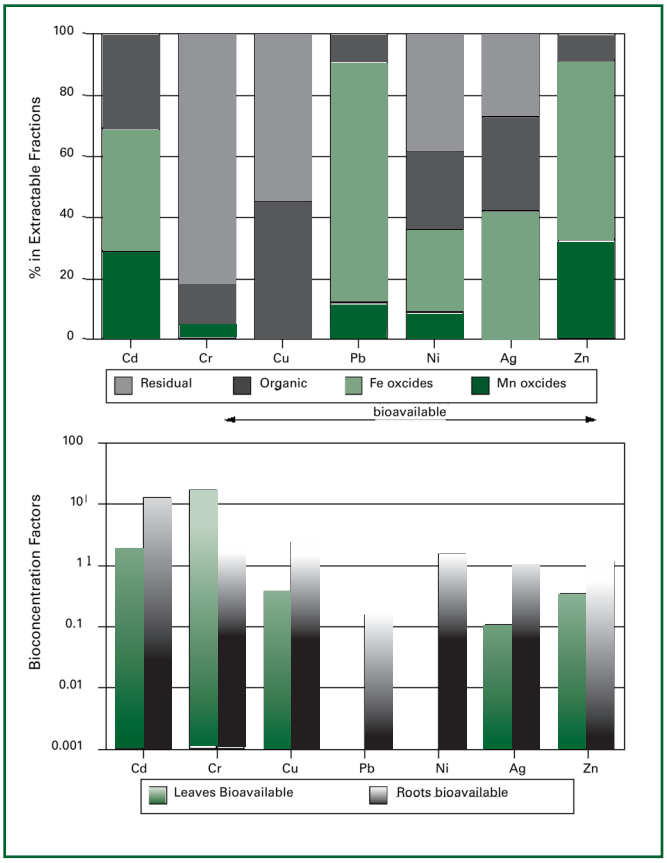


Figure 2. Heavy metals in *J. roemerianus*.

Isolation, Identification and Characterization of Endemic Fungi and their Potential to Grow and to Degrade Munition Wastes, Organic Compounds and Metals

Pedro Luis Melendez
Ana G. Méndez University System, San Juan, Puerto Rico

Problem Being Addressed

In Puerto Rico, the U.S. Armed Forces, the National Guard and the Police Department use and store large quantities of ammunitions for their field-range firing maneuvers. Many of the areas used (including Vieques and Cieba) are adjacent to agricultural fields and water resources, which eventually become contaminated with TNT, RDX, or both. Moreover, many sites in Puerto Rico are contaminated with organic compounds, including halogenated solvents, aromatic hydrocarbons and metal ions. These compounds are known to be toxic and mutagenic to plants, animals and humans. Therefore, bioremediation of these wastes in soils and water is very important.

Previous studies from abroad have demonstrated that both fungi and bacteria are capable of degrading these compounds. In addition, several plant species have been found capable of absorbing heavy metals from the soil. It is of interest to study the fungal flora predominant in Puerto Rico; few or none are capable of flourishing on media containing these compounds.

Since lignin and cellulose-degrading fungi have been reported as potential candidates for the bioremediation of areas contaminated by munition wastes, this project will place great emphasis on fungal genera within the class Basidiomycetes, which includes several aggressive wood rotters. *Aspergillus* and *Penicillium* will also be tested.

Chemical compounds and metals such as those mentioned above will be incorporated into culture media, both solid and liquid. Each fungal genus to be tested will be added to the media and incubated, and the extent of growth determined. Growth will be based on fungal dry weights as well as colony diameter in petri plates. The extent of biodegradation, if any, will be determined by conventional laboratory analysis (atomic absorption, chromatography, etc.). Fungal genera found to be capable of degrading the compounds under study will be tested further, using more sophisticated experiments and equipment.

Research Methods/Materials

Fungal were originally isolated in pure culture using culture media specific for fungal isolation, including potato dextrose agar (PDA); malt extract agar (MEA); and sabouraud agar modified (SAM).

Fungi such as wood rotters were obtained in pure cultures and preserved on MEA throughout this research. These fungi included: *Junhnia straminea, Phlebia chrysocrea, Ganoderma, australe, Tinctosporellus epimiltimus, Programme albocincta, Lactiporus persicinus, Rogodoporus microporus* and *Antrodierella sp*. Other fungi, such as species of *Aspergillus* and *Penicillium*, were obtained from contaminated bacterial cultures mixed with heavy metals and preserved on fresh media (PDA, MEA or SAM).

The chemicals tested were selenite, arsenite and p- concentrations of 98 mM for selenite and arsenite and 50 mM for p-nitrophenol. There were three different concentrations: 79.0 ppm, 158 ppm and 316 ppm for selenite; 37.5 ppm, 75.0 ppm and 150 ppm for arsenite; and 69.5 ppm, 139 ppm and 278 ppm for p-nitrophenol.

The effects of selenite, arsenite and p-nitrophenol on growth of the fungi was determined in liquid media mixed with the chemicals. A volume of each chemical corresponding to these concentrations (described above) was dispensed into each flask containing 50 ml of the media.

Table 1.

Isolate	Chemicals					
	Arsenite			Selenite		
	37.5ppm	75ppm	150ppm	79ppm	158ppm	316ppm
L009**	380	250	150	330	180	200
DS005***	580	500	580	300	120	120

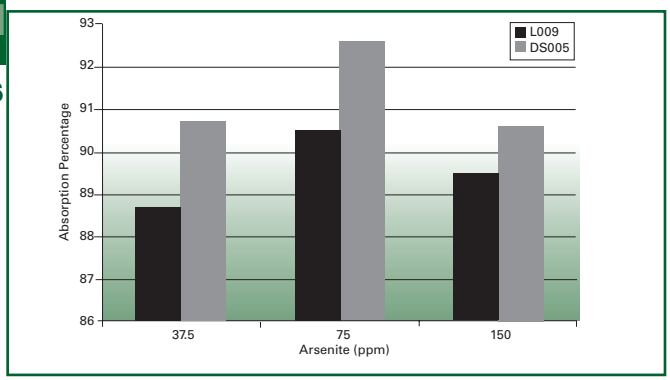


Figure 1. Absorption percentage of penicillium at three different concentrations of arsenite.

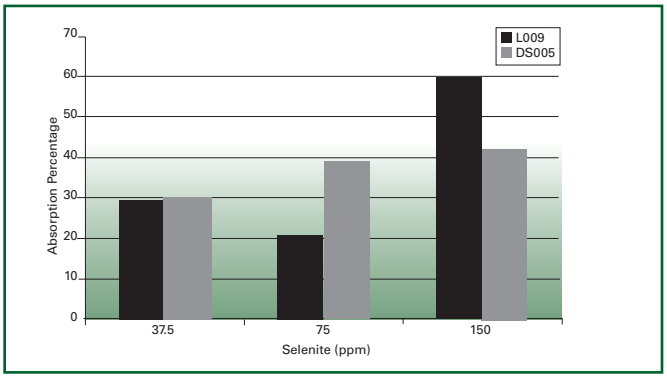


Figure 2. Absorption percentage of two isolates of penicillium at three different concentrations of selenite.

Culture filtrates of *Penicillium* growing in liquid media were preserved in a refrigerator (8°C) for further atomic absorption analysis. Prior to analysis, each filtrate was diluted in deionized distilled water.

For the dilution of the filtrates containing arsenite, 50 ml of the filtrate were diluted in 50 ml of deionized water for the 37.7 ppm concentration and 23 µl for the 75 ppm concentration and 12 µl for the 150 ppm concentration of the chemical.

Experimental Results

- Data obtained from *Penicillium* isolates DS005 and L009 grown in liquid media containing either arsenite or selenite at three different concentrations are summarized in Table 1. Both isolates were capable of growing in liquid media containing arsenite, even at 150 ppm.
- The most remarkable growth was displayed by isolate DS005, when compared to the control (400 mg).
- Growth of both isolates in selenite is considered to be moderate and decreasing with increasing concentration of the chemical.
- There was a marked change in color—from gray to red—of the isolates when exposed to selenite.
- Figure 1 shows a higher absorption percentage from DS005 vs L009. However, the absorption percentages are over 90% for every case. Just a little difference is noticed when trying to establish a relationship between the mycelial dry weight and the absorption percentage at 75 ppm.

Figure 2 shows a higher absorption percentage from L009 vs DS005 for all the analyzed concentrations. From the data it is not possible to establish a relationship between the mycelial dry weight and the absorption percentage.

Future Research

- Continue and repeat some trials run with Aspergillus and Penincillium in liquid media with chemicals compounds and metals.
- Process the soil, sand and water samples collected at Vieques and follow a soil and sand analysis for the presence of TNT and/or aromatic hydrocarbons and metals.
- Run additional atomic absorption spectrophotometry analysis.
- Run digestion trials with mycelial mats exposed to metals or aromatics compounds.
- Identify to the species level those fungal genera found to be of interest as biodegraders.

Students Involved

Francisco Rivera
Juan Rodriguez
Antonia Lopez
Yolanda Guerrios

Research Presentations

Results of the work conducted in bioremediation with fungi were displayed in a poster by student Antonia Lopez at the SIM Annual Meeting held in Arlington, Va., July 29 – Aug. 6, 1999.

Phytoremediation of Lead and Cadmium in Hydroponic Systems

Charles Rhyne and Sumita Ghosh
Jackson State University, Jackson, Miss.

Problem Being Addressed

Lead and cadmium are common contaminants of surface soils at Department of Defense installations, especially the very large areas used as firing ranges. Given the high toxicity of lead to plants, animals and man, there is a great need for techniques that reduce the lead and cadmium concentrations in large aerial extents of surface soil at DoD sites. Concentrating lead and cadmium from the soil in plants and harvesting those plants to remove these heavy metals from the soil is a very cost-effective and esthetically pleasing remediation technique, ie., phytoremediation. As part of the overall phytoremediation program in BEST, we are part of the laboratory effort that screens possible candidate species for their attributes in being hyperaccumulating for the metals Pb and Cd. Our work looks at accumulation and biomagnification of these metals using a modified hydroponic growing system. Plants that show at least a 1% accumulation of these metals will then be used at the next stage of phytoremediation research, incorporating contaminated soil in the growing process.

Research Methods/Tools Employed

Selection and growing procedures: Plants are selected through some understanding of their basic uptake and translocation properties based on the literature as well as some "guesstimation" of a plant's physiological chances of being a hyperaccumulator.

Light energy: Plants are grown under artificial lights, 400 W metal halide and 320 W fluorescent natural spectrum bulbs at approximately $0.8x10^{13}$ quanta cm^2 sec or 5% of full sunlight.

Growing medium: Sand, lava rock mix in plastic trays

Nutrient solution: Hoagland's solution

Heavy metals: $Pb(NO_3)_2$ and $Cd(NO_3)_2$

Metal analysis of plant tissue: Hot-plate digestion using EPA method #3050A; metal analysis using Thermo-Jarell Ash AA-Scan4 Furnace Atomic Absorption Spectrometer.

Experimental Data

Sesbania exaltata and *Ipomoea lacunosa* can tolerate well up to 1000 ppm Pb in solution. Pb accumulation increases with Pb concentration in both species. Shoot tissue Pb concentrations in *Ipomoea* were maximum at 15,000 mg/kg when grown at 500 mg/l Pb, while root tissue Pb was maximum at 20,000 mg/kg when grown at 1000 mg/l Pb.

Shoot tissue Pb concentrations in *Sesbania* were maximum at 10,000 mg/kg Pb when grown at both 250 and 500 mg/l Pb, while root tissue concentrations were maximum at 22,000 mg/kg Pb when grown at 500 mg/l Pb. EDTA could stimulate Pb accumulation in shoots in both species and roots in *Sesbania* (Figure 1).

Root-shoot transport of Pb occurred in both species with or without EDTA addition, but EDTA could significantly increase Pb transport in several cases (Figure 2).

Future Studies

Our team would like to look at a series of temperate and semi-tropical grasses for their possible application in removing Pb and Cd at upper soil surfaces where grass roots and rhizomes are thick. If a suitable lawn-type grass were available as a hyperaccumulator, harvesting using a standard lawn mower and grass catcher could greatly enhance large area metal removal.

We would like to revisit another series of weed species for their applicability as hyperaccumulators. The last series yielded two good candidates.

We believe a study into the exudate chemistry of the root of established hyperaccumulators with and without the stress of a Pb and Cd environment would be important. The hydroponic environment could make this investigation more accurate in detecting indicator compounds used in the initial uptake of these metals.

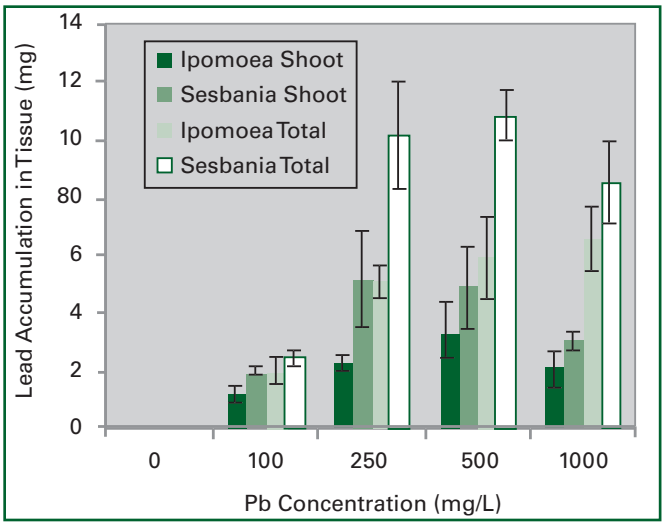


Figure 1. comparison of the values of the tissue lead accumulation in *Ipomoea lacunosa* and *Sesbania exaltata*. Vertical bars indicate SEM (n=4 for Ipomoea and n=5 for Sesbania).

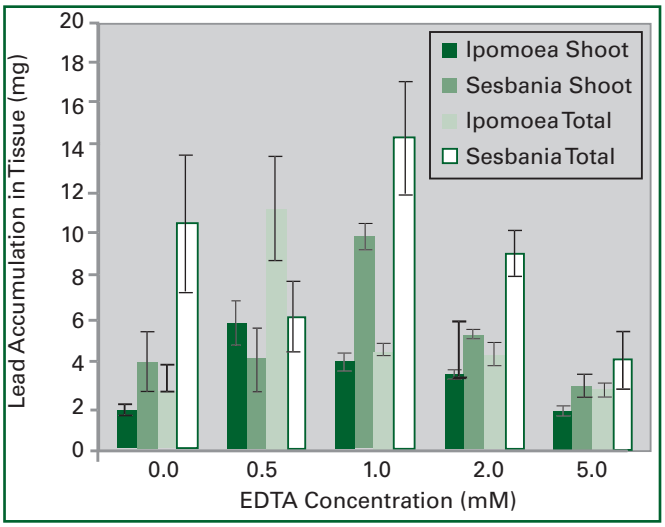


Figure 2. Comparison of the values of the tissue lead accumulation in *Ipomoea lacunoa* and *Sesbania exaltata* in the presence of EDTA. The lead concentration in the solution is 100 mg/L. Vertical bars indicate SEM (n=5).

Students Involved

Jennifer Ntoni – master's student, environmental science
Amelia Hardaway – undergraduate student, biology

Relevant Publications

Ghosh, S., and C. Rhyne, A search for hyperaccumulator plants in phytoremediation studies of lead contaminated soils, 59th Annual MS Academy of Science Meeting, Jackson, Miss., February 1995.

Ghosh, S., and C. Rhyne, Phytoremediation studies with lead hyperaccumulating plants, 61st Annual MS Academy of Science Meeting, Biloxi, Miss., February 1997.

Ghosh, S., and C. Rhyne, A search for lead hyperaccumulating plants in the laboratory, 62nd Annual MS Academy of Sciences Meeting, Biloxi, Miss., Feb. 27, 1998.

Ghosh, S., and C. Rhyne, Influence of EDTA on Pb uptake in two weed species, *Sesbania* and Ipomea, in hydroponic culture, 63rd Annual MS Academy of Sciences Meeting, Tupelo, Miss., Feb. 26, 1999.

Ghosh, S., and C. Rhyne, Lead accumulation and effects of EDTA in *Sesbania exaltata*, a weed species in hydroponic culture, 6th Internat. Symp. on Metal Ions in Biol. & Med., San Juan, P.R., May 7-10, 2000 (to be presented).

Metabolic and Genetic Regulation of Toxic Metal Biovalence Transformation in Bacillus subtilis

Sasha Shafikhani, Biochemistry and Molecular Biology
University of California at Berkeley

Problem Being Addressed

The subsurface of central California, including the San Joaquin valley, is highly contaminated with toxic metal oxyanions. The processing of San Joaquin Valley crude oil and agricultural drainage extracted from the subsurface results in the discharge of approximately 2200 kg/year of selenium oxyanions into the San Francisco Bay Area ecosystem. Selenium is toxic to fish, shellfish, waterfowl and small mammals. There is currently no cost-effective technology to treat high-volume selenium-contaminated waste streams. The absence of such technology threatens the environmental quality of San Francisco Bay and other aquatic ecosystems.

Certain soil and aquatic bacteria are able to biotransform selenium and other toxic metal oxyanions into non-toxic metal precipitates that are immobilized within the bacterial biomass. *Bacillus subtilis* is one of these selenium-detoxifying soil bacteria. One goal of this project is to identify and characterize the gene/s involved in hazardous metal detoxification in *B. subtilis*. This should allow us to investigate the mechanism/s of detoxification and provide us with the necessary knowledge to improve the detoxification capacity of *B. subtilis* through genetic/metabolic engineering and directed evolution. We expect that these findings will also have considerable value in understanding the biovalence transformation of other toxic metal oxyanions, such as chromate and arsenite.

Research Methods/Tools Employed

A defined minimal growth medium developed in our laboratory was used for these studies. A genome-wide array technique was used to profile the expression of all of the identified open reading frames (ORFs) in *B. subtilis* (~4000 genes). Total RNA was extracted from cultures of unexposed 168 cells (n - naïve or wild type), and selenium-induced 168 cells (168 - i) at three time points: (1) prior to selenite addition at 95 Klett (T_0); (2) two hours after exposure to selenite (T_2); and (3) 45 hours after exposure to selenite when 168(n) had fully recovered (T_3). ^{33}P-labeled cDNAs were prepared from these RNA samples by reverse transcription. The labeled RNA was hybridized to genome-wide arrays obtained from Eurogentec, containing a duplicate set of oligonucleotides for all of the *B. subtilis* ORFs. The expression profile of each gene was determined by the ratio of the digitized intensity values for control and experimental time points.

Experimental Results

1. The initial growth arrest of 168(n) following exposure to selenite is primarily due to the formation and incorporation of seleno-cysteine and seleno-methionine into proteins. Selenium is very similar in chemical properties to sulfur and is capable of participating in similar biochemical reactions. To investigate the biochemical pathways affecting selenite toxicity in *B.subtilis*, the following sulfur amino acid biosynthetic mutants, *cysA, metC* and *cysA metC*, were constructed. If selenite toxicity is due to the incorporation of seleno-cysteine and seleno-methionine into proteins, then selenite toxicity should be reduced in *cysA* and *metC* single mutants. These mutants are defective in the early stages of de novo cysteine and methionine biosynthesis. The *cysA metC* double mutant should completely block de novo synthesis of sulfur amino acids. Addition of cys-

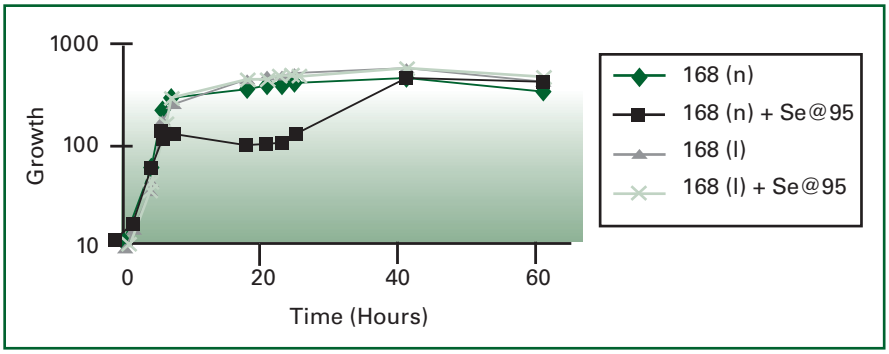


Figure 1. Effect of selenite stress on the physiology of B. subtilis.

teine and/or methionine to the mutant and wild type strains might also physiologically mitigate selenite toxicity. The results shown in Figure 1 demonstrate that de novo sulfur amino acid biosynthetic pathways are indeed the primary targets for selenite toxicity.

2. The adaptation to selenite stress observed in 168 (i) is irreversible. Induced *B. subtilis* no longer undergo growth arrest or cell lysis upon subsequent selenite exposure. We wished to examine whether this induction was permanent or transient. A single induced clonal population was grown for 12, 48 and 70 generations in the absence of selenite stress. These cells were then allowed to sporulate. All of these lineages maintained the induced phenotype. These results indicated that adaptation persisted over a large number of generations in the absence of selenite stress.

3. Genome-wide expression profiling of selenite stress has identified genes involved in general stress response, protein turnover, energy production and operons with no previously known functions. We have employed genome-wide expression profiling techniques to identify genes that are involved in toxic metal adaptation and detoxification processes. Analysis of these data suggests that upon initial exposure to selenite, there is an increase the expression of damaged protein turnover and refolding genes (*hsp70* and *hsp100* genes). There are also significant increases in the expression of genes involved in DNA recombination and repair (*recA, sms, uvrA/B/C*) suggesting that selenite-induced growth arrest may adversely affect cell cycle events.

A number of genes involved in ion efflux and heavy metal binding (*czd, ygvW*) are overproduced in 168 (i). Following recovery from selenite stress, genes involved in energy production (*cydA/B/C/D, glcB/C/D*) and components of the electron transport chain (*etfA/B*) are highly induced. This may indicate that selenite detoxification is energetically intensive.

The expression of thioredoxin and thioredoxin reductase increased several fold by T_3. These genes have been shown to catalyze the in vivo reduction of selenite to nontoxic elemental selenium. Previous proteomic analysis has also implicated these proteins in the management of selenite stress.

We have identified a number of operons with no previously known function that could be involved in hazardous metal adaptation and/or detoxification. For example, *yxlA* through *yxlJ* are found within an operon that also includes a sigma factor of unknown function, sigY (Figure 2). The expression of the genes in this operon increased up to 28-fold during the recovery from selenite stress (T_3).

Future Studies

A number of the aforementioned genes will be inactivated in both 168 (n) and 168 (i) strains to determine their role in toxic metal adaptation and detoxification processes. We will also investigate the possibility that exposure to selenite endows *B. subtilis* with cross-protection against other toxic metal oxyanions such as arsenite, molybdate, chromate, etc.

Students Involved

Anatoly Urisman — undergraduate, UC Berkeley

Maureen — undergraduate, UC Berkeley

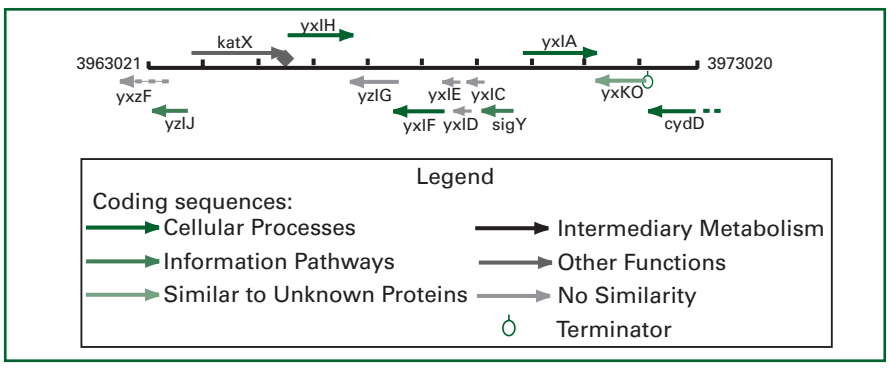


Figure 2. *sigY* operon and fluctuation in gene expression in this operon resulting from selenite exposure.

Microbial Community Structure in Explosives-Contaminated Soils

Tamas Torok, Terry Hazen and Jennie C. Hunter-Cevera
Center for Environmental Biotechnology, Lawrence Berkeley National Laboratory, Berkeley, Calif.

Problem Being Addressed

Around the world there are many sites contaminated with explosives as a consequence of wars, military practices and industrial processes. These explosives are toxic to humans and the environment. Bioremediation, and specifically biostimulation, may be one of the best strategies for cleaning up these sites.

The objective of the ongoing project is to investigate the biodegradation of 2,4,6-trinitrotoluene (TNT) under aerobic conditions. The following goals were set:

- Collect explosives contaminated soil samples at the now closed Army base at Fort Ord, Calif.;
- Characterize the microbial communities present in recently contaminated and weathered soil samples, respectively;
- Study the changes in the microbial community structure caused by different biostimulation treatments.

Fort Ord, Calif., was established in 1917 as a maneuver area and field artillery target range. Currently, 14,000 acres of the 29,440-acre installation are contaminated with ordnance and explosives and with unexploded bombs, shells, missiles and grenades (Figure 1). In 1990, Fort Ord was placed on the U.S. Environmental Protection Agency's National Priorities List. Thus, the underlying assumption was that by understanding the structure and function of the microbial communities, cost-effective and efficient bioremediation processes could be developed.

Figure 1. Unexploded ordnance at Fort Ord, Calif.

Research Methods

Recently contaminated and weathered soil samples were collected at Fort Ord. Following the microbial community characterization for base line establishing, four perfusion columns (Figure 2) for each soil were filled and treated with minimal salts solution, Tween 80, 0.3% of molasses, and water, respectively. The molasses provided carbon, phosphate and nitrogen sources to the soils. The minimal salts medium added nitrogen and phosphate, while Tween 80 was used as a surfactant.

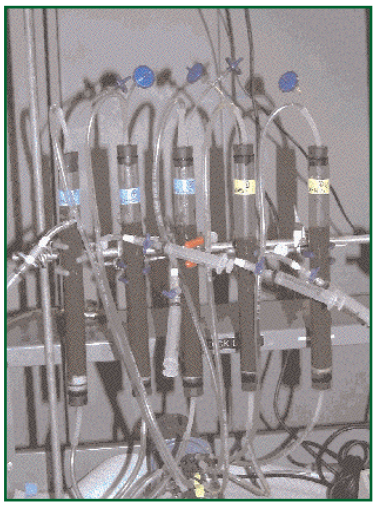


Figure 2. Perfusion columns.

Two community level techniques were employed in the characterization of the microbial community structure: (1) whole cell fatty acid methyl ester (FAME) analysis, and (2) analysis of carbon source utilization, BIOLOG.

Community structure of the soils before treatment was compared with samples obtained during the biostimulation process, including the water-treated controls.

Experimental Data/Results

Community level FAME and BIOLOG data were evaluated by principle component analysis (PCA). Typical results are described below:

Twenty-eight different fatty acids from the soils were identified, with 16:0, 17:0 cyclic and 18:2 w6 9c being more abundant in the weathered soils. The 16:0 fatty acid has been reported characteristic for microbial biomass, while the 18:2 6w 9c was characteristic for fungal biomass. The principle component analysis showed that the first two components explain 99% of the variability. The first component alone explains 83%

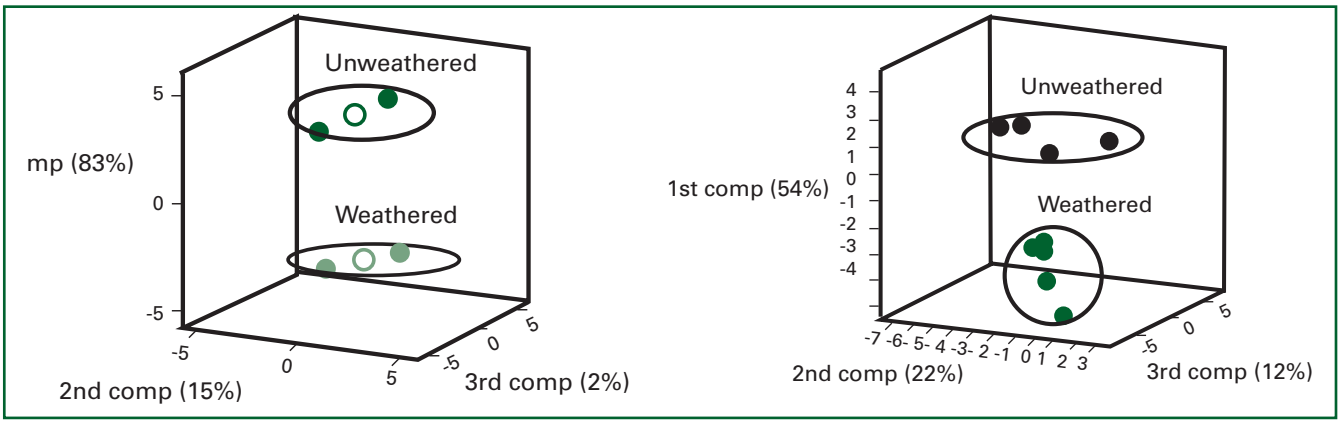


Figure 3. Results of BIOLOG analysis.

of the variability and is correlated with biomass. The second component explains 15% of the variability and is correlated with variability between the replicates.

In the BIOLOG analysis, PCA was different in both samples; the six replicates of each sample formed close groups (Figure 3). The first two components describe 78% of the variability. Our results indicate that the soils are different in carbon utilization pattern. The weathered soils had more biomass than the recently contaminated soils.

Future Studies

The microbial community characterization of explosives-contaminated soils and the investigation of TNT biodegradation are parts of an ongoing BEST project at the Center for Environmental Biotechnology at Lawrence Berkeley National Laboratory. Currently, the nitro-aromatic biodegradation capability of isolated microorganisms is being verified and validated. Biodegradation intermediates and end products are being chemically analyzed. The isolation and investigation of aerobic TNT biodegraders would be a major scientific breakthrough.

Students Involved

Roberto A. Rodriguez-Martinez – graduate student, University of Puerto Rico, studying soil microbiology and the response of microbial populations to chemical pollutants

Melissa B. Clark – undergraduate student, Humboldt State University, Calif., summer 1999.

Relevant Publications

Rodriguez-Martinez et al., Microbial community studies of differently treated explosives contaminated soils, abstract accepted for ASM General Meeting, Los Angeles, Calif., May 21-25, 2000.

Ecotoxicology and Risk Assessment of Explosive Nitro-Compounds

Advanced Detection of Exposures and Biological Responses to Organic Toxins

Hoi-Ying N. Holman, Regine Goth-Goldstein, Michael C. Martin, Marion Russell and Wayne R. McKinney
Lawrence Berkeley National Laboratory, Berkeley, Calif.

Problem Being Addressed

Organochlorines (OCs) and polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants (especially at Department of Defense sites) that are toxic and suspected human carcinogens. Traditional assessment of human exposures to these organic toxins and subsequent biological response relies primarily on high-dose and short-term animal experiments. A major uncertainty inherent to this approach is the extrapolation from the high-dose animal experiments to the low-dose and long-term human exposures. To overcome this uncertainty, we recently have directed our research towards exploring the use of synchrotron radiation-based (SR) Fourier transform infrared (FTIR) spectromicroscopy for identifying chemical changes in cellular nucleic acids and proteins as a result of OC and PAH exposures.

To date, the primary research objective has been to identify SR FTIR spectroscopic signals of human cell culture systems that are indicative of low-dose exposures to OCs and PAHs and could be used as biomarkers. SR FTIR spectromicroscopy is used because this is a sensitive and nondestructive technique capable of providing direct biochemical information at molecular levels. The fine spatial resolution of 5-10 microns and strong signal-to-noise levels of SR FTIR spectromicroscopy allow detection of the subtle changes in intracellular biochemical processes as the cells are exposed to environmental stimuli.

Research Methods/Tools Employed

The biomarker considered for OC and PAH exposures is the induction of the cytochrome P4501A1 *CYP1A1* gene expression and the increase in the associated enzyme activity. It is well established that *CYP1A1* transcript levels increase in response to exposure to OCs and PAHs through their binding to the Ah receptors.

HepG2 (human hepatoma-derived) cells were used as model human epithelial cells that can metabolize PAHs; 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) modeled OCs; BaP modeled PAHs and coal tars modeled mixtures of PAHs. HepG2 cells were maintained in Eagle's Minimum Essential Medium with non-essential amino acids and Earle's BSS supplemented with 10% fetal calf serum, 1 mM L-glutamine, 10 mM Hepes and antibiotics. Cells were sub-cultured and then treated for 2-20 hours with TCDD, BaP and coal tars at environmentally relevant concentrations.

The effect of TCDD was monitored and mapped by the SR FTIR spectromicroscopy technique in the mid-IR region (4000-400 cm^{-1}). SR FTIR signals (from individual cells) that are specific to the intracellular response after their exposure to these organic toxins were identified. To validate the SR FTIR spectromicroscopy technique, results from the TCDD experiments were compared with the *CYP1A1* transcript levels measured by the widely accepted yet more time-consuming reverse transcription-polymerase chain reaction (RT-PCR).

Experimental Data

Dimensionless SR FTIR spectra were recorded at the proximity of a cell nucleus of HepG2 cells that were exposed to TCDD of different concentrations (0, 0.01, 0.1, 0.5, and 1.0 nM) for 20 hours. There are considerable differences in the SR FTIR spectra associated with the *CYP1A1* gene expression and the increase in the associated enzyme activity, with one difference being the increased absorption of the vibration band 1180 - 1160 cm^{-1}, centered at ~1170 cm^{-1}. Here, the normalized absorbance intensity for individual cells increased from 0.007 to 0.21 when the TCDD concentration increased from 10-11 to 10-9 M (Figure 1a). The normalized absorbance intensity at ~1170 cm^{-1} for individual control cells was 0.005, a 42-fold increase in the absorbance intensity. This systematic spectral change might be related to the alteration in the DNA base structure and will be the subject of future investigation.

A comparison of the dose response described above with that obtained using the RT-PCR technique is shown in Figure 1b. The solid line was the least-squares fit to the data. The excellent agreement (with

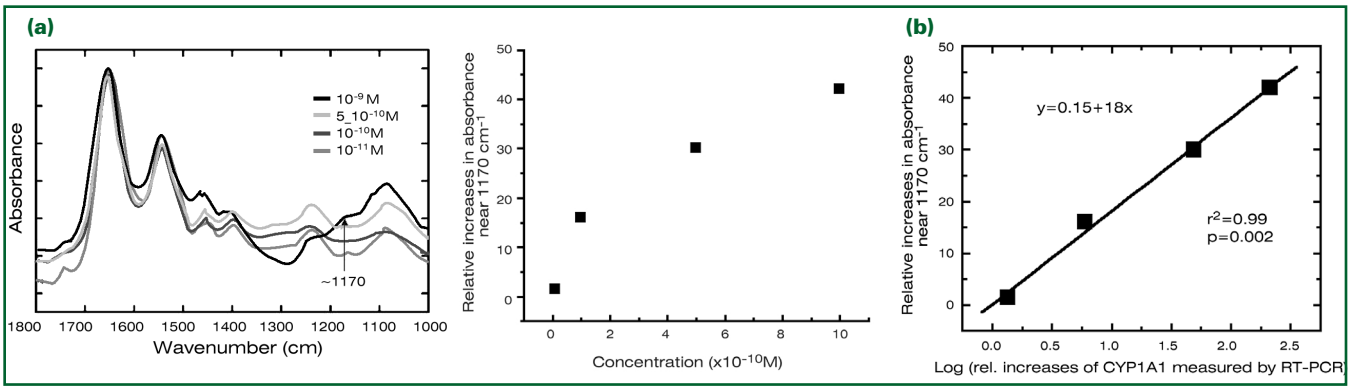


Figure 2. Development of a micro-fabricated device for detecting human exposure to environmental organic toxins. (a) Dose response of individual living HepG2 cells after 20 hour exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin, as measured by the new IR technique (after hours of work). (b) New IR technique versus RT-PCR assay.

r^2 = 0.99) for measurements from the two methods indicated that the fast and direct SR FTIR spectromicroscopy technique was indeed comparable to the more time-consuming and widely accepted RT-PCR technique that specifically measures increases in the *CY1A1* transcript levels.

The SR FTIR spectra recorded at the proximity of a cell nucleus of HepG2 cells that have been exposed to BaP and coal tars at environmentally relevant concentrations showed similar spectral characteristics at ~1170 cm^{-1}. The recorded dose-response behavior was also similar to those reported in literature.

The agreement between the SR FTIR spectromicroscopic data for dioxin exposures and the RT-PCR results, and the agreement between the PAH measurements and those reported in the literature indicate that the intracellular biological responses to low-dose exposures to these organic toxins are well represented by our specific spectral changes. These changes are associated with *CYP1A1* gene expression and the increase in the associated enzyme activity with different types of damage. The capabilities of SR FTIR spectromicroscopy for the direct detection of intracellular biochemical responses to exposures to dilute concentrations of OCs and PAHs will have significant impacts in future research methodology of environmental toxicology.

Future Studies

The capabilities of SR FTIR spectromicroscopy for the direct detection of intracellular biochemical responses to exposures to dilute concentrations of OCs and PAHs will have significant impacts in future research methodology of environmental toxicology. We will continue to expand the number and type of DoD- important contaminants that can be measured using this technique.

Student

Elsa Olvettei – University of Virginia

Related Publications

Holman, H.-Y.N.,M. Zhang, R. Goth-Goldstein, M.C. Martin, M. Russell, W.R. McKinney, M. Ferrari and J.C. Hunter-Cevera, Detecting exposure to environmental organic toxins in individual cells: Towards development of a micro-fabricated device, Proc. Intl. Symp. Biomed. Optics, in press.

H.-Y.N. Holman, D.L. Perry, M.C. Martin and W.R. McKinney, Applications of synchrotron infrared microspectroscopy to the study of inorganic-organic interactions at the bacterial-mineral interface, Application of Synchrotron Radiation Techniques to Material Science IV (S.M. Mini, S.R. Stock, D.L. Perry and L.J. Terminello, eds.), 524, pp. 17-23, 1998.

Applications of Microbial Assays in the Assessment of Metal Toxicity

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Problem Being Addressed

Bioassays used in aquatic toxicology have taken a prominent position among analytical tests for identifying and measuring environmental hazards. Such bioassays have been developed for testing a variety of organic and inorganic chemicals, as well as effluents, surface waters and sediment samples for acute and chronic toxicity. Many bioassays using higher organisms such as fish, protozoa and algae have been executed, but are labor- and equipment-intensive, costly and complex. More importantly, these aquatic bioassays do not provide quantitative information on the impact of pollutants on biological treatment systems. In the view of national and international regulations, regulatory agencies are also supporting the development of new toxicity screening procedures that are sensitive, inexpensive and easier to perform.

The use of bacterial in vitro assays such as the Microtox Assay has become an attractive alternative to traditional fish and invertebrate methods for toxicological screening. These new assays have been developed to assess the toxicity of various environmental agents, validated and recognized by several standards organizations. The purpose of this study was to apply selected microbial test protocols (bioluminescence, growth and oxygen uptake measurements) to assessing the toxicity of hazardous metals such as cadmium and lead. These metals have been reported to pose a high level of hazard to ecological and human health.

Research Methods

A Microtox assay was carried out to measure the relative acute toxicity of metal (Cd or Pb) producing data for the calculation of lead concentration effecting 50% reduction in light output (EC_{50}). For each test run, two controls without lead, eight sample/lead dilutions and two replicates were done. Tests were carried out on various percentages of the original lead concentration (4 ppm). The sensitivity of the strain of bioluminescent bacteria (*Vibrio fischeri*) was tested for quality control purposes. Growth and oxygen uptake experiments were performed following previously described protocols.

Descriptive statistics were applied to calculate the means+SD of all data sets associated with specific metal concentrations. Specific growth and oxygen depletion rates were computed as slopes of graphical representations of raw data versus times. The toxic end-points expressed as 50% growth inhibition concentration or as 50% oxygen depletion concentration (EC_{50}s) were next derived from graphical presentations of these specific rates versus metal concentrations. Activity quotients were calculated to determine the degree of toxicity associated with lead exposure. Linear regression analysis was performed to determine the relationship between lead concentrations and the times required for 50% reduction in oxygen uptake (TD_{50}s).

Experimental Data

Bioluminescence was used as an endpoint for measuring the effect of Cd and Pb *to Vibrio fischeri*. For both Cd and Pb, a strong dose-response relationship was determined. The concentrations of Cd and Pb effecting 50% reduction in bioluminescence (EC_{50}) were computed to be 0.79+0.12 mg/L and 0.34+0.03 mg/L, respectively; indicating that Pb was more toxic than Cd. A strong dose-response relationship was also found in the tests with the mixed population of microorganisms. Figure 1 shows the growth patterns

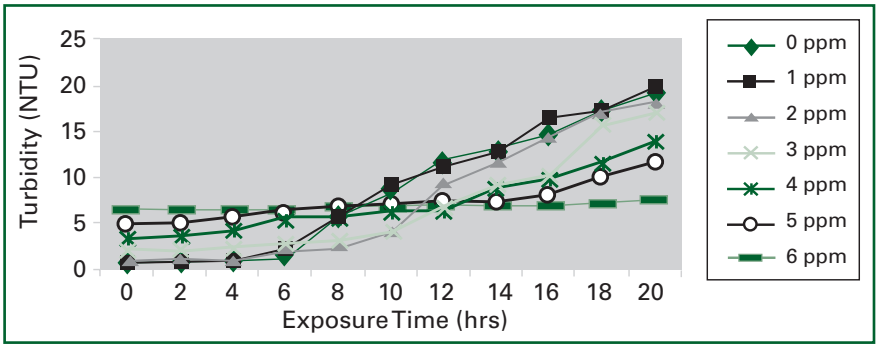

Figure 1. Effect of lead on the growth of microorganisms.

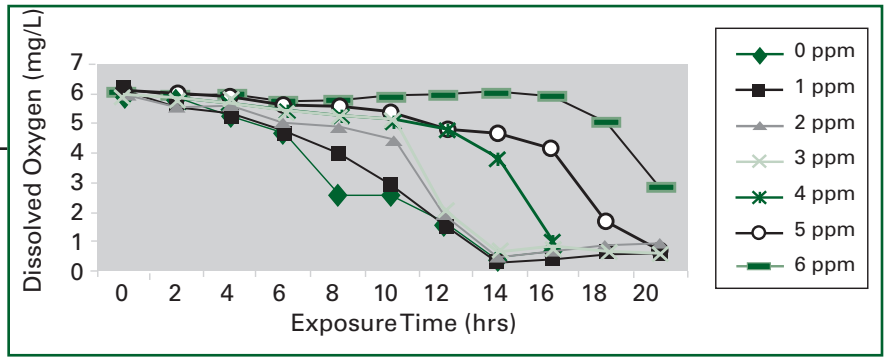

Figure 2. Effect of lead on the respiration of microorganisms.

obtained from exposure to lead of the mixed population of microorganisms. Data presented in this figure show an overall increase in bacterial growth with the increase in holding/incubation time. These data also show significant reductions in maximum growths with increasing concentrations of lead. EC_{50} values were computed to be 4.50+0.04 mg/L, and 3.50+0.02 mg/L for Cd and Pb, respectively.

Figure 2 presents the dissolved oxygen uptake rates by the mixed population of microorganisms exposed to various concentrations of lead. In general, curves presented in this figure indicated that individual rates of oxygen uptake decreased as lead concentrations increased. The mean values of EC50 were 5.00+0.42 mg/L for Cd, and 3.80+0.04 mg/L for Pb. These data indicated that the mixed population of microorganisms was about 10 times (one order of magnitude) less sensitive to lead toxicity than the marine bacterium, *Vibrio fischeri*. Data also showed a strong correlation (r^2=0.98) between TD50s (times required to produce 50% reduction in oxygen uptake) and lead concentrations, indicating a time-response relationship with regard to lead toxicity. A similar result was obtained in experiments with cadmium.

Data obtained from this research clearly point out the significance of using microbiological systems for acute toxicity testing in aquatic toxicology. Bioassays employed in the present investigation fulfilled the requirement criteria of fast toxicity screening based on their simplicity, speed, cost effectiveness and the fact that bacteria grow rapidly, represent a low trophic level, and thus provide sensitive early warning data of environmental impacts at higher trophic levels. Of the three biosystems evaluated, the Microtox was the most sensitive; yielding an EC_{50s} that was only about one tenth of values recorded in batch cultures (growth inhibition and oxygen depletion tests). Although batch systems were time-consuming (20 hrs exposure time), and relatively less sensitive than the Microtox, they provided valuable information on the toxic effects of lead on microbial growth and respiration. Also, they were easier to perform, and required less expensive equipment compared to the high cost of the Microtox analyzer.

Future Studies

- Apply microbial bioassays validated in this study to assess the acute toxicity of other chemicals (other toxic metals, munitions chemicals, etc.) of importance to the U.S. Army.
- Use these bioassays as valuable surrogates to predict the potential acute ecological impacts of Army-related chemicals.

Students Involved

Lamar Reed– master's student, Environmental Science Program, JSU

Relevant Publications

Tchounwou, P.B., Health risk assessment and management of toxic metals, paper presented at the Workshop on Environmental Pathology and Toxicology '99: Environmental and Health Effects of Trace Elements and Metal Ions, Jackson State University, Jackson, Miss., April 29-30, 1999.

Tchounwou, P.B., and L. Reed, Assessment of lead toxicity to the marine bacterium, Vibrio fischeri, and to a heterogeneous population of microorganisms derived from the Pearl River in Jackson, Mississippi, USA, Reviews on Environmental Health 14(2):51-61, 1999a.

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Toxicity and Risk Assessment of 2,4,6-Trinitrotoluene, 2,4-Dinitrotoluene and 2,6-Dinitrotoluene

Paul B. Tchounwou, Barbara Wilson and Ali Ishaque
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Problem Being Addressed

Trinitrotoluene (TNT) is a munitions chemical that was produced and used on an enormous scale during World Wars I and II in shells, bombs, grenades, demolition explosives and propellant compositions. 2,4-Dinitrotoluene (2,4-DNT), and 2,6-Dinitrotoluene (2,6-DNT), on the other hand, are used in the manufacture of dyes, in munitions as smokeless propellant powders, and as gelatinizing and plasticizing agents in both commercial and military explosive compositions. Both 2,4-DNT, and 2,6-DNT are produced through denitration of toluene with nitric acid in the presence of concentrated sulfuric acid. Small amounts of DNT isomers also occur as byproducts in the production of TNT. Significant amounts of TNT- and DNT-containing wastewaters arising from their preparation and production at Army ammunition plants have been identified in soils, surface water and ground water after leaching from disposal sites. Exposure to TNT and DNTs has been associated with numerous health effects. However, limited scientific information is available regarding the environmental fate, ecotoxicity and health effects of these nitroaromatic compounds. We have performed the Microtox, Mutatox and CAT-Tox(L) assays to determine the acute toxicity, genotoxicity and molecular mechanisms by which these munitions chemicals exert their toxicity.

Research Methods

Acute and genotoxicity tests were carried out, using a Microtox/Mutatox Model 500 Toxicity Analyzer System. The Microtox procedure measured the relative acute toxicity of lead, producing data for the calculation of lead concentration effecting 50% reduction in light output (EC_{50}). For each test run, two controls without lead, eight samples/chemical dilutions and two replicates were done. The Mutatox Assay was conducted according to the standard test protocol. Nonglowing or dark mutant strains of luminescent bacteria were exposed to the test substance (TNT, 2,4-DNT or 2,6-DNT), and the amount of light emitted was measured with the Mutatox Analyzer. The sample-induced reversion from nonglowing to luminescent phenotype was used to indicate the genotoxicity of the sample. Prepared samples were mixed and preincubated in a water bath at 35 ± 0.5°C for 45 minutes. After preincubation, samples were incubated at 27 ± 5°C for 16, 20 and 24 hours, and the potential genotoxic response of the luminescent bacteria was determined at each time period by measuring the light intensity of each cuvette using the Mutatox Model 500 Analyzer. The positive response was defined as the light output of at least two times the light intensity of the reagent control blank.

The mammalian Gene Profile Assay was performed for measuring differential gene expression in the human liver hepatoma cell line, HepG2. Thirteen recombinant cell lines and the parental HepG2 Cell line were plated over two 96-well microplates. The cell lines were dosed at five TNT concentrations (0, 19, 38, 75 and 150 ug/mL in 1% DMSO) and incubated at 37°C, 5% CO_2, for 48 hours. After the incubation period, the total protein was measured by the Bradford method, at 600 nm using a microplate reader. A standard sandwich ELISA was performed and in the final step horseradish peroxidase catalyzed a color change reaction that was measured at 405 nm. The parental HepG2 cell line was dosed in the same manner as the recombinant cell lines, and was used to perform a MTT-based cellular viability assay at 550 nm.

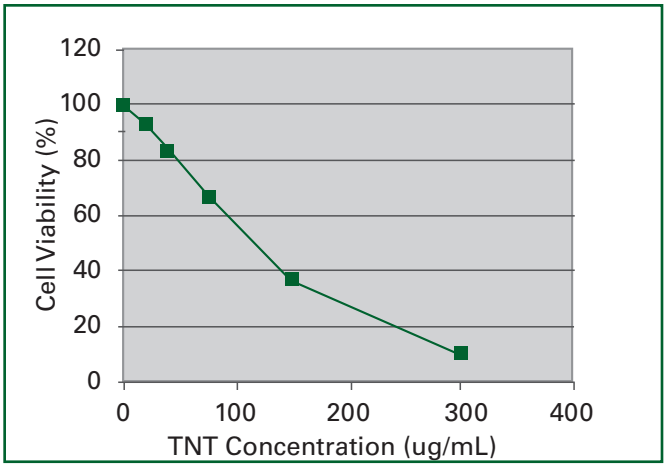

Figure 1. Cytotoxicity of TNT to HepG2 cells.

Experimental Data

The Microtox results yielded EC_{50} values of 0.96 (0.80-1.14) ppm for 2,4,6-Trinitrotoluene, 3.46 (3.18-3.77) ppm for 2,6-Dinitrotoluene, and 35.22 (32.98-37.62) ppm for 2,4-Dinitrotoluene, indicating the 2,4,6-TNT was the most toxic, while 2,4-DNT was the least toxic of tested chemicals. The order of decreasing toxicity was 2,4,6-TNT, 2,6-DNT, and 2,4-DNT. For the Mutatox assay, tests without S9 activation showed that all the three nitroaromatic compounds had a weak genotoxic response after 20 hours of exposure. However, experiments involving S9 activation produced a positive genotoxic response for 2,6-Dinitrotoluene, and a negative genotoxic response for both 2,4,6-TNT, and 2,4-DNT. On the basis of these results, it can be concluded that 2,4,6-TNT, and 2,4-DNT are weak mutagens that are biotransformed to non-mutagenic metabolites by S9 activation. 2,6-DNT on the other hand is a weak mutagen showing a genotoxicity that is potentiated by metabolic activation.

A cytoxicity test with HepG2 cells using the MTT assay for cell viability yielded an LC50 of about 105 ug/mL for 2,4,6-TNT in 1% DMSO, upon 48 hours of exposure (Figure 1). For most constructs evaluated, the induction of stress genes was concentration-dependent. For example, fold inductions of HSP70 were 1.00±0.00, 2.41± 1.04, 2.05±1.30, 5.55±3.69, and 11.24±3.94 at 0, 18.8, 37.5, 75 and 150 ug/mL TNT, respectively (Figure 2). At 150 ug/mL TNT, six out of the 13 constructs showed a twofold or higher level of induction. These included XRE, HMTIIA, *c-fos*, HSP70, GADD153, GADD45, and GRP78. These results indicate the potential of TNT to cause of protein damage and/or perturbations of protein biosynthesis (HSP70 and GRP78), alterations in DNA sequence or its helical structure (*c-fos*, GADD153, GADD45), and its potential involvement in the biotransformation process (XRE), and in the toxicokinetics of metals (HMTIIA). No significant induction of CYP IA1, GST Ya, NFKBRE, CRE, p53RE and RARE was found at the maximum exposure level of 150 ug/mL TNT.

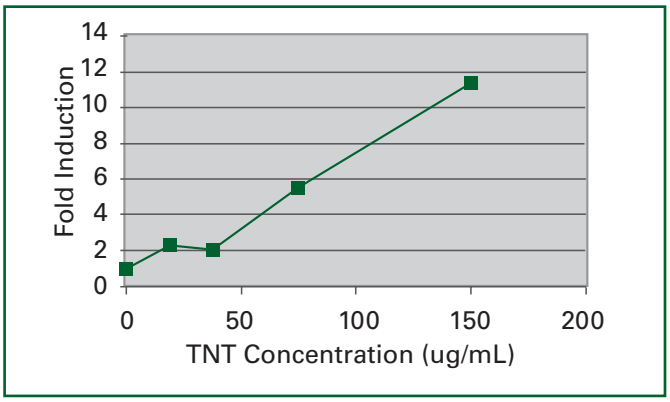

Figure 2. Fold induction of HSP70 in HepG2 cells exposed to TNT.

Future Studies

Study the molecular mechanisms of DNT-induced toxicity;

Carry out *in-vivo* experiments to study the biomarkers of exposure, sensitivity and effect associated with the toxicity of nitro-aromatic compounds.

Students Involved

George Ray – undergraduate student, Biology, JSU
Rosalyn Ransome – undergraduate student, Biology, JSU
Lamar Reed – master's student, Environmental Science Program, JSU

Relevant Publication

Tchounwou, P.B., B. Wilson and A. Ishaque, Acute toxicity, genotoxicity and cytotoxicity assessment of three munition chemicals, paper accepted for presentation at the Tenth Symposium on Environmental Toxicology and Risk Assessment, American Society for Testing and Materials, Toronto, Ontario, Canada, April 10-12, 2000.

Validation of the Use of 1-hydroxypyrene as a Biomarker for Exposure to Polycyclic Aromatic Hydrocarbons: The Role of Genetic Polymorphisms

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[1]University of Texas at El Paso; [2]Lawrence Berkeley National Laboratory, Berkeley, Calif.

Problem Being Addressed

Polycyclic aromatic hydrocarbons (PAHs) are a family of compounds that includes some potent carcinogens that are ubiquitous in the environment. The major metabolic pathway for ingested or inhaled PAHs to water-soluble derivatives is oxidative activation by cytochrome P4501A1 (CYP1A1) followed by detoxification by phase II enzymes like glutathione S-transferases, especially GSTM1. Interindividual variation in PAH metabolism exists due to genetic polymorphisms in the genes coding for these enzymes. The GSTM1 gene is frequently deleted in individuals, resulting in reduced detoxification. Several single-base changes have been identified in the CYP1A1 gene that appear to result in increased susceptibility to various cancers in these individuals.

Because PAHs present a threat to human health, human exposure to PAHs has to be monitored in occupational settings. While PAHs consist of hundreds of different aromatic compounds, pyrene is typically present in all of these mixtures. Pyrene is metabolized primarily to 1-hydroxypyrene (1OHP) and detoxified as 1OHP sulfate or glucuronide conjugate and excreted via the urine. Through simple enzymatic methods, these conjugating molecules can be cleaved. Therefore urinary 1OHP is the most commonly used biomarker of exposure to PAHs. Recently, a number of investigators have reported differences in the quantity of urinary PAH metabolites in individuals with polymorphisms or variations in key enzymes involved in the metabolism of xenobiotics. These findings suggest the need for clarification of the effects of polymorphisms on the metabolism of pyrene. To investigate the role of these polymorphisms, we have undertaken a study to measure 1OHP levels.

Research Methods/Tools Employed

Subject recruitment: A survey has been developed to identify individuals with similar smoking and dietary habits for our study. Inclusion criteria are: Hispanic, cigarette consumption of one pack a day, charbroiled food consumption less than three times per week, alcohol consumption less than 10 drinks per week. Urinary cotinine levels will be used to validate survey responses for tobacco use. Participants are asked to donate a blood and urine sample. Urine samples are separated into two 25 ml aliquots and stored at –80°C for 1OHP analysis.

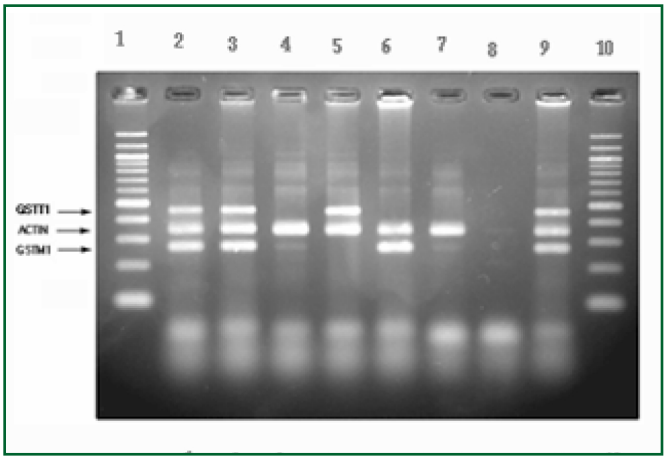

Figure 1. 3% agarose gel showing GST multiplex system. Lanes 1 and 10 are standards. Lanes 2 and 3 show wildtype GSTM and GSTT. Lanes 4 and 7 show a deletion in both genes. Lane 5 shows a deletion in the GSTM gene while lane 6 shows a deletion in the GSTT gene. Lane 8 is a negative control and lane 9 is a positive control.

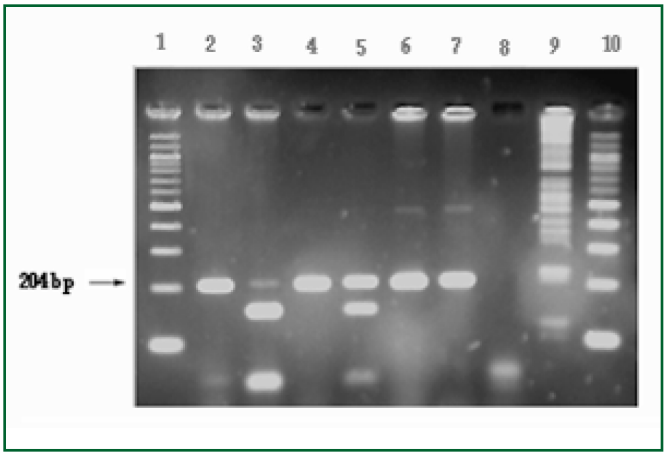

Figure 2. 4% agarose gel showing CYP1A1 M2 polymorphisms. Lanes 1 and 10 are standards. Lanes 2 (undigested product) and 3 (digested product) show a wildtype sample. Lanes 4 and 5 show a heterozygote. Lanes 6 and 7 show a homozygote m2 variant. Lane 8 is a negative control and lane 9 is a digestion of lambda DNA.

Genetic polymorphisms: Peripheral blood lymphocytes (PBLs) are isolated from the blood samples using Histoprep density separation media (Sigma). DNA is extracted from the PBLs using standard phenol chloroform extraction methods. Polymorphisms are analyzed by published procedures: For CYP1A1, the procedure described by Cascorbi et al. (1996) is followed. For identification of CYP1A1 M1, a 899 bp fragment is amplified, then digested with MspI which cuts the variant fragment into a 693 and 206 bp fragment. For the identification of the CYP1A1 M2 polymorphism, a 204 bp DNA fragment is amplified, then subjected to digestion with BsrDI, which cuts the wildtype into a 149 and 55 bp fragment. Restriction enzyme digested PCR products are separated by agarose gel electrophoresis. GST analysis is performed using a multiplex PCR that co-amplifies the GSTM1 and GSTT1 genes (Nelson et al., 1995). An actin DNA fragment is co-amplified as an internal control. The absence of a GSTM1 or GSTT1 band in the presence of the actin band indicates a GST gene deletion.

Analysis of 1OHP: Methods for analysis of 1OHP follow the protocol by Whiton et al. (1995), which involves overnight enzymatic digestion of all conjugated forms of pyrene in a 25 ml urine sample, organic extraction of 1OHP, and reverse phase HPLC analysis and quantitation of the 1OHP peak.

Experimental Data

A survey was administered to Hispanic smokers to determine smoking and dietary habits in this group. Thirty healthy men and women smokers who reported smoking one pack a day were chosen based on similar dietary and smoking habits, hence similar PAH intake. However, we have detected some discrepancies in the survey responses, and have found that the wording of some of the questions on the survey was ambiguous and that certain issues regarding meat consumption should be included in the survey. As a consequence, we have reservations about the information collected. We have revised the original survey and pre-tested it on seven people. Because the whole success of the project hinges on comparing individuals with similar PAH exposure, we plan to recruit 40 new smokers with improved survey instrument and recruitment methods.

We are in the process of analyzing the CYP1A1 and GSTM1 genetic polymorphisms (see Figures 1 and 2). The graduate student working on this project is learning how to perform the 1OHP analysis as well.

Future Studies

The initial collection of biological samples and administration of the survey of dietary and smoking habits will be repeated due to questions regarding the validity of the response. We are presently in the process of identifying a large population of Hispanics in El Paso from which we will be able to recruit study participants. Efforts are being made gain access to the facilities at the local general hospital. We view this initial work as a pilot study that provided the opportunity for all of us to learn more about survey work and some of the problems of molecular epidemiological research.

Students Involved

Gabriela Sanchez – graduate student, UTEP
Cuau Vital – undergraduate student, UTEP

Publications, Presentations and Abstract

Publications

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Gardea-Torresdey, J., Adsorption of Silver Ions from Aqueous Solutions Using Medicago sativa (Alfalfa) Biomass, Joint 55th Southwest/15th Rocky Mountain Regional Meeting (SWARM99) of the American Chemical Society, El Paso, Texas, 1999.

Gardea-Torresdey, J., The Determination of Trace Level Metal Binding to Alfalfa (Medicago sativa) Biomass by Graphite Furnace Atomic Absorption Spectroscopy (GFAAS), Joint 55th Southwest/15th Rocky Mountain Regional Meeting (SWARM99) of the American Chemical, El Paso, Texas, 1999.

Gardea-Torresdey, J., The Use of X-ray Absorption Spectroscopy to Determine the Toxic Heavy Metal Chemical Environment Upon Adsorption to Alfalfa Biomass, Joint 55th Southwest/15th Rocky Mountain Regional Meeting (SWARM99) of the American Chemical Society, El Paso, Texas, 1999.

Gardea-Torresdey, J., Effects of Oxidation on Metal Ion Binding by Medicago sativa (Alfalfa): Atomic and X-ray Absorption Spectroscopic Studies with Fe(II) and Fe(III), submitted to Environmental Science and Technology, 1999.

Gardea-Torresdey, J., Biosorption and Bioreduction of Au (III) Ions by alfalfa Biomass: Characterization through Spectroscopic Techniques, Joint 55th Southwest/15th rocky Mountain Regional meeting of the American Chemical Society, El Paso, Texas, 1999.

Gardea-Torresdey, J., Recovery of Au (III) Ions from Aqueous Solution Through Adsorption and Reduction by Alfalfa Biomass, UTEP Student Research Exposition, El Paso, Texas, 1999.

Gardea-Torresdey, J., A Study of the Removal of Silver Ions from Aqueous Solutions Through adsorption by Medicago sativa (Alfalfa) biomass, UTEP Student research Exposition, El Paso, Texas, 1999.

Gardea-Torresdey, J., Determination of Trace Level Copper Binding to Alfalfa (Medicago sativa) Biomass with Graphite Furnace Atomic Absorption Spectroscopy (GFAAS), UTEP Student Research Exposition, El Paso, Texas, 1999.

Ghosh, S., and C. Rhyne, Influence of EDTA on Pb Accumulation in Two Weed Species, Sesbania and Ipomoea in Hydroponic Culture, Mississippi Academy of Sciences Meeting, Tupelo, Miss., 1999.

Goldman, S., Mechanisms for Hydrogen Fractionation in Microbial Systems, DOE Technology Exchange Conference, Oakland, Calif., 1999.

Hazen, T.C., Recent Advances in Bioremediation, presented at the Universidad Metropolitana, San Juan, P.R., 1999.

Hazen, T.C., Methane Injection Pilot Study for Bioremediation of Old Town Solvent Plume, California Regional Water Quality Board, Berkeley, Calif., 1999.

Hazen, T.C., Critical Biogeochemical Parameters for Bioremediation of Solvents in Fractured Rock, International Symposium on Deep Subsurface Microbiology, Vail, Colo., 1999.

Hazen, T.C., Field Studies of Aerobic Bioremediation of Groundwater Contaminated by a Sanitary Landfill, International Symposium on Deep Subsurface Microbiology, Vail, Colo., 1999.

Hunter-Cevera, J.C., The Value of Biotechnology, presented at the Universidad Metropolitana, San Juan, P.R., 1999.

Hwang, H.M., Photodegradation of Carcinogenic Polycyclic Aromatic Hydrocarbons, Research Center for Minority Institutions (RCMI) Scientific Conference, Los Angeles, Calif., 1999.

Hwang, H.M., DNA Photocleavage by Carcinogenic Polycyclic Aromatic Hydrocarbons, Research Center for Minority Institutions (RCMI) Scientific Conference, Los Angeles, Calif., 1999.

Kerr, J.B., et al., Electrokinetic Acceleration of Bioremediation of Metal Ion and Organic Pollutants In Soil, ACS Spring Meeting, Anaheim. Calif., 1999.

Ledes, D., and C.M. Lozano, Synthesis and Characterization of Ligands 1,2-CDTA, 1,3-CTA and 1,4-CDTA and their Complex Formation with Heavy Metals, XXI Scientific Research Congress, San Juan, P.R., 1999.

Leighton, T.J., et al., Development of a Pilot-Scale Selenium Waste Treatment System, Emerging Technologies in Hazardous Waste Treatment, Industrial and Engineering Chemistry Division, American Chemical Society Annual Meeting, New Orleans, La., 1999.

Leszczynski, J., Molecular Basis of Biological Processes: A Computational Approach, Research Center for Minority Institutions (RCMI) Scientific Conference, Los Angeles, Calif., 1999.

Lytle et al., Emergent Macrophyte Responses to Oxidative Stress, National Meeting of the American Chemical Society in New Orleans, La., 1999.

Martin, M.C., The first synchrotron infrared beamlines at the Advanced Light Source: Spectromicroscopy and Fast Timing, CEB-BEST seminar, 1999.

Olivetti, E., and H.Y. Holman, Mining for Organisms, 1999.

Pool, Molecular Evolution of Spore-Forming Bacteria, CSEE Undergraduate Poster Session, 1999.

Rhyne, C., Influence of EDTA on Pb Uptake in Two Weed Species, Sesbania and Ipomoea in Hydroponic Culture, Mississippi Academy of Sciences 63rd Annual Meeting, 1999.

Rivera and T.C. Hazen, Chemotaxis of Pseudomonas fluorecens to 2,4 and 2,6-dinitrotoluene, 1999. (http://csee.lbl.gov/cup/Su99/RiveraC/page2.htm)

Rivera and J.C. Hunter-Cevera, Bioremediation of Explosives Found in Freshly Contaminated Soil, 1999.

Rodriguez and J.C. Hunter-Cevera, Molecular Mechanisms of Metal Sorption and Desorption by Microbial Sink: Bacterial Cell Wall and Spore Coat Proteins Biopolymers, 1999.

Runkle and T.C. Hazen, Biological Degradation Rates at Weathered and Recent Explosives Contaminated Sites, 1999. (http://csee.lbl.gov/cup/Su99/Runkle)

Sanchez and J.C. Hunter-Cevera, The Biodegradation of methyl tert-butyl ether, 1999.

Slaughter, L. and H.M. Hwang, Assessing the Microbial and Photochemical Effects of 2,4,6-trinitrotoluene (TNT) in Mississippi River Water, Mississippi Academy of Sciences Meeting, Tupelo, Miss., 1999.

Syed and W.T. Stringfellow, Isolation and Characterization of Microorganisms Present in Diesel Fuel Crude Oil Contaminated Soils, 1999.

Tchounwou, P.B., Effects of Cadmium and Lead on the Bioluminescence of Vibrio fischeri and Growth and Oxygen Uptake Rate of Heterogeneous Population Microorganisms, Eleventh International Symposium on Biodeterioriation and Biodegradation, Arlington, Va., 1999.

Torok, T.T., and Repin, Bioprospecting in Siberia, CEB-BEST seminar, 1999.

Abstracts

Begonia, G.B., Chelate-Enhanced Phytoextraction of Lead from Contaminated Soils Using Morning Glory (Ipomoea lacunosa), Mississippi Academy of Sciences 63rd Annual Meeting, 1999.

Feliu, L.A., Kinetic Studies of the Interaction of Metallic Cations and Selected Bacterial Strain Bioremediation, MIE Annual Conference, 1999.

Feliu, L.A., Theoretical Studies of the Conformers of Nitroglycerine Using Semi-Empirical Methods and Density Functional, New Orleans, La., 1999.

Gardea-Torresdey, J., Study of the Binding Mechanisms of Heavy Metals by Inactivated Tissues of Solanum elaeagnifolium, 2000 Conference on Hazardous Waste Research: Environmental Changes and Solutions to Resource Development, Production, and Use, Denver, Colo., 1999.

Gardea-Torresdey, J., Removal and Recovery of Cr(III) and Cr(VI) with Alfalfa Biomass, 2000 Conference on Hazardous Waste Research: Environmental Changes and Solutions to Resource Development, Production, and Use, Denver, Colo., 1999.

Gardea-Torresdey, J., Mechanisms of Au(III) Binding and Bioreduction by Alfalfa Biomass, 2000 Conference on Hazardous Waste Research: Environmental Changes and Solutions to resource Development, Production and Use, Denver, Colo., 1999.

Gardea-Torresdey, J., Adsorption and Recovery of Silver Ions from Aqueous Solutions by Medicago Sativa (Alfalfa) Biomass, 2000 Conference on Hazardous Waste Research: Environmental Changes and Solutions to resource Development, Production, and Use, Denver, Colo., 1999.

Gardea-Torresdey, J., Determination of Trace Level Gold (III) Binding to Alfalfa Biomass Using GFAAS with Zeeman Background, 2000 Conference on Hazardous Waste Research: Environmental Changes and Solutions to Resource Development, Production, and Use, Denver, Colo., 1999.

Gardea-Torresdey, J., Electrochemical and X-ray Absorption Spectroscopic Studies of Cu (II) and Pb (ii) binding to Alfalfa Biomass, 2000 conference on Hazardous Waste research: Environmental Changes and solutions to Resource Development, Production, and Use, Denver, Colo., 1999.

Hwang, H.M., Photodegradation of Carcinogenic Polycyclic Aromatic Hydrocarbons, Annual NIH-RCMI Program Director's Meeting and Scientific Conference, 1999.

Hwang, H.M., DNA Photocleavage by Carcinogenic Polycyclic Aromatic Hydrocarbons, Annual NIH-RCMI Program Director's Meeting and Scientific Conference, 1999.

Ledes, D., and C.M. Lozano, Bioremediation of Heavy Metals by Bacteria: Synthesis, Characterization and Biodegradability Studies of Metal Complexes with EDTA-Type Chelating Agents, SIM, Arlington, VA, 1999.

Ledes, D., and C.M. Lozano, Bioremediation of Heavy Metals and Organic Compounds by Fungi: Isolation, Identification and Characterization of Endemic Fugal Genera and their Potential to Grow on and Degrade Selenite, Arsenite and p-Nitrophenol, 1999.

Ledes, D., and C.M. Lozano, Molecular Basis of Biological Processes: A Computational Approach, Annual NIH-RCMI Program Director's Meeting and Scientific Conference, 1999.

Leszczynski, J., Molecular Basis of Biological Processes: A Computational Approach, Annual NIH-RCMI Program Director's Meeting and Scientific Conference, 1999.

Rhyne, C., Influence of EDTA on Pb Uptake in Two Weed Species, Sesbania and Ipomoea in Hydroponic Culture. Mississippi Academy of Sciences 63rd Annual Meeting, 1999.

BEST Trainees

Student	Mentor(s)	Research Topic
Ana G. Méndez University System (AGMUS)		
Undergraduates		
D. Bacelo	L. Feliu	Structure comparison among organic nitrates
D. Castro	C. Lozano	Nitroaromatic degradation by environmental isolates
I. Cordova	L. Feliu	Structure comparison among organic nitrates
T. DeLa Mora*	R. Webb	PCR amplification of YDAE, a class II MT in E. coli
M.V. Gamez*	R. Webb	Copper resistance in cyanobacteria
Y. Guerrio	P. Melendez	Collection of fungi, growth of Penicillium sp. in the presence of selenite, arsenite, and p-nitrophenol
A. Lopez	P. Melendez	Collection of fungi, growth in the presence of selenite, arsenite, and p nitrophenol
D. Martinez	P. Melendez	Fungi and bacteria tolerance to copper
M. Medina	D. Ledes	NMR, RAMAN, and FT-IR data collection, ligand selection
I. Ramos	L. Feliu	Biodegradation of nitrocellulose
F. Rivera	D. Caro	Bacteria isolation and characterization, metal tolerance
E. Rodriguez	D. Caro	Bacteria isolation and characterization, metal tolerance
Y. Vasquez	R. Webb	Copper resistance in wild-type and recombinant E. coli
Graduates		
Y. Bernier	D. Caro	Growth kinetics of Bacillus subtilis in the presence of metals
W. Del Valle*	D. Caro	Isolation of microorganisms from landfill leachates
N. Diaz	D. Caro	Isolation of microorganisms from landfill leachates
J. Garmon	L. Feliu	Biodegradation of nitrocellulose
X.C. Kretschmer	R. Webb	Interactions between copper and cyanobacterial whole cells and cell walls
T. Munoz	D. Caro	Growth kinetics of Bacillus subtilis in the presence of metals
M. Nieves	D. Caro	Growth kinetics of Bacillus subtilis in the presence of metals
A. Rivera	D. Caro	Isolation of microorganisms from landfill leachates
J. Rodriguez	P. Melendez C. Lozano	Collection of fungi, growth of Penicillium sp. in the presence of selenite, arsenite, and p-nitropheno
M. Sanchez	C. Lozano	Media preparation, sampling, isolation of microorganisms
D. Sauri	C. Lozano	BIOLOG training, sampling, isolation of microorganisms
R. Vives	C. Lozano	BIOLOG training, sampling, isolation of microorganisms

Student	Mentor(s)	Research Topic

Jackson State University (JSU)

Undergraduates

C. Burks	G. Begonia	Media preparation, plant care and maintenance, plant growth measurement, metal extraction and analysis
C. Carter	G. Begonia	Media preparation, plant care and maintenance, plant growth measurement, metal extraction and analysis
H. Course	Y. Liu	Training in analytical instrument and AA spectrometer
J. Green	J. Leszczynski	Mass balance studies on TNT
A. Hardaway	C. Rhyne	Mass balance studies on TNT
C. Howard	P. Tchounwou	CAT-TOX assay
L. Hughes	P. Tchounwou	CAT-TOX assay
K. Johnson	W-H. Yang	CAT-TOX assay
R. Ransome	P. Tchounwou	Microtox assay and TNT
G. Ray	C. Rhyne B. Wilson	Phytoremediation of Pb and analysis/Toxicological effects of TNT
E. Stamps	P. Tchounwou	CAT-TOX assay
R. Thomas	J. Leszczynski	Mass balance studies on TNT

Graduates

S. Cook	H. Hwang	Photolysis of TNT
S. Ghosh	C. Rhyne	Influence of EDTA on Pb accumulation in plants
G. Miller	H. Hwang	Microtox and nitroaromatics
J. Rawls	H. Hwang	Microtox and nitroaromatics
L. Reed	P. Tchounwou	Mutatox assay of TNT and related compounds
L. Slaughter	H. Hwang	Photolysis of TNT
K. Terry	W-H. Yang	Genotoxic effect of nitrobenzene and related benzene derivatives

Lawrence Berkeley National Laboratory (LBNL)

Undergraduates

D. Anghelescu	T. Torok	16S rRNA gene sequencing of San Juan Bay isolates
R. Bruinsma	R. Lupu	Qualitative immunohistochemical assay for Erß protein
S. Cheng	W. Stringfellow M. Conrad	Monitoring MTBE biodegradation
M. Clark	T. Hazen	Soil perfusion treatment of explosive-contaminated soils from Fort Ord, CA
S. Datta	T. Torok	Characterization of a subsurface microbial community
S. Davila Lopez	J. Kerr	Analytical techniques to monitoring electrokinetic treatment of soil

Student	Mentor(s)	Research Topic
H. Eastwood*	T. Hazen	Biostimulation of PAHs-contaminated soil from Alameda Naval Air Station
S. Lee	W. Stringfellow	MIBK degradation in samples from the Hanford site
A. Maldonana*	R. Lupu	Expression of estrogen and progesterone receptors
K. Merchant	T. Torok	Isolation, FAME analysis, strain preservation
E. Olivetti	J. Hunter-Cevera H-Y. Holman	Presence of microorganisms in coal cores
C. Rivera	T. Hazen	Chemotaxis in explosive-contaminated soils from Fort Ord, CA
F. Rivera	J. Hunter-Cevera G. Castro T. Torok	Preferential metal binding by Bacillus subtilis strain 168
R. Rodriguez	J. Hunter-Cevera	Characterization of microbial communities in explosive contaminated soils from Fort Ord, CA
B. Runkle*	T. Hazen	Effect of biostimulants on the intrinsic biodegradation of explosives contaminated soils from Fort Ord, CA
G. Sanchez	R. Goth-Goldstein	Urinary 10HP biomarker study of exposure to PAHs in Hispanic smokers and the influence of polymorphisms
F. Syed	W. Stringfellow	Solid phase microinjection of MTBE
B. Villatoro	W. Stringfellow T. Torok	PAHs biodegradation, chemical analysis/Isolation, FAME analysis, strain preservation
Graduates		
Y. Bernier	J. Hunter-Cevera G. Castro	Isolation and characterization of intrinsic microorganisms from explosive contaminated soils from Fort Ord, CA
M. Miller*	T. Hazen	Biostimulation of UXO-contaminated soil
R. O'Brien	T. Hazen	Chemical and biological treatment of PCB-contaminated soil
A. Rivera	J. Hunter-Cevera G. Castro	Isolation and characterization of intrinsic microorganisms from explosive contaminated soils from Fort Ord, CA
J. Rodriguez	J. Hunter-Cevera G. Castro	Metal biosorption in Bacillus subtilis strain 168
R. Rodriguez	T.C. Hazen T. Torok J. Hunter-Cevera	Microbial communities in weathered and unweathered soils contaminated with explosives
M. Sanchez-Olivero*	J. Hunter-Cevera G. Castro	Isolation and characterization of intrinsic microorganisms from , crude-oil-contaminated soil
Post-Doctoral Students		
F. Rabbi*	J. Kerr	Soil analysis of explosive-contaminated samples

Student	Mentor(s)	Research Topic
University of California at Berkeley (UCB)		
Undergraduates		
R. Abadeer*	T. Leighton	Spore laccase assay, biochemical characterization
L. Chu*	T. Leighton	Spore laccase assay, biochemical characterization
M. Coppoletta*	B. Buchanan	Phytoremediation of PAHs
C. Edmerson	T. Leighton	BEST web page update
N. Glebova*	T. Leighton	Genetics of hazardous metal detoxification
Y. Gomez	T. Leighton	GTN degradation by B. subtilis
C. Huang	T. Leighton	GTN degradation by B. subtilis
A. Kemp*	T. Leighton	Advanced Integrated Pond treatment plant for the biological treatment of selenium and nitrate contaminated waste streams
K. Kwan*	T. Leighton	Spore laccase assay, biochemical characterization
R. Lee*	T. Leighton	Spore laccase assay, biochemical characterization, copper biosorption by bacillus spores
J. Lopez*	T. Leighton	GTN degradation by B. subtilis
J. Pool	T. Leighton	GTN degradation by B. subtilis
M. Price*	A. Horne	Nitroaromatics in constructed wetlands
A. Silber	T. Leighton	BEST web page update
A.J. Southwell*	A. Horne	Nitroaromatics in constructed wetlands
A. Stammets*	A. Horne	Nitroaromatics in constructed wetlands
J. Sunahara*	A. Horne	Nitroaromatics in constructed wetlands
A. Urisman*	T. Leighton	Genetics of hazardous metal detoxification
R. Yu*	T. Leighton	Genetics of hazardous metal detoxification
Graduates		
M. Beutel	A. Horne	Conceptual design for TNT removal wetlands
M. Flemming*	A. Horne	Conceptual design for TNT removal wetlands
N. Hauri*	A. Horne	Mesocosms for TNT biodegradation
N. Hume*	A. Horne	Conceptual design for TNT removal wetlands
S. Shafikhani	T. Leighton	Mechanisms of hazardous metal detoxification in Bacillus subtilis
A.M. Stending*	A. Horne	Mesocosms for TNT biodegradation
Post-Doctoral Students		
Y.B. Kim*	B. Buchanan K.C. Oh	Phytoremediation of PAHs and heavy metals
D-K. Zoh	A. Horne	TNT analysis by HPLC and TNT degradation in microcosms

Student	Mentor(s)	Research Topic

University of Southern Mississippi (USM)

Undergraduates

N. Housley*	J. Lytle, T. Lytle	Cadmium uptake in estuarine plants
J. Lyons	J. Lytle, T. Lytle	Metal uptake in plants
N. Smith*	J. Lytle, T. Lytle	Metal uptake in plants
L. Stewart*	J. Lytle, T. Lytle	Phytoremediation
A. Trahan	J. Lytle, T. Lytle	Phytoremediation
S. Ward*	J. Lytle, T. Lytle	Effect of petroleum hydrocarbons on Sesbania vesicaria

University of Texas at El paso (UTEP)

Undergraduates

K. Dokken	J. Gardea-Torresday	Field collection of Solanum ealeagnifolium and chemical analysis
E. Rascon	J. Gardea-Torresday	Plant material sampling, metal binding experiments, FAA analysis

*Partially supported by BEST or working on BEST research with other students.

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Published by the Earth Sciences Division
at Lawrence Berkeley National Laboratory

Editing: Mary Pratt
Design and Production: Maria Fink

http://www-esd.lbl.gov/CEB/BEST