

# **Report for 2003NJ38B: Investigation of Design Parameters for Engineered Rhizoremediation Systems to Treat Contaminated Sediments In Situ**

- Conference Proceedings:
  - Plant-Mediated Effects on Polycyclic Aromatic Hydrocarbon (PAH) Degradation by Bacteria in the Rhizosphere of the Salt Marsh Grasses *Spartina alterniflora* and *Phragmites australis*. American Society of Microbiology, 103rd General Meeting. Washington, D.C. May 18-22, 2003.

Report Follows

### **Problem and Research Objectives**

Contamination of estuarine environments by toxic organic contaminants, such as polyaromatic hydrocarbons (PAHs) and related petroleum hydrocarbons, has become a significant environmental concern. The industrial activities in New Jersey have resulted in widespread contamination of estuarine sediments. Accordingly, there is an increased awareness in the need for restoration of these damaged habitats and the importance of wetland preservation. Despite these pressing needs, remediation of sediments in estuaries and associated wetlands is a non-trivial undertaking. *Ex situ* treatment methods, such as composting and soil washing, are by their very nature disruptive and costly. In situ remediation is an attractive alternative, but methods based on injection of surfactants or chemical oxidants (which have been employed in several large-scale sediment remediation projects) can be as expensive and ecologically disruptive as *ex situ* approaches requiring excavation. In contrast, in situ approaches that rely on the biodegradative activities of microorganisms are generally viewed as being less costly and less disruptive of the ecosystem. Nevertheless the overall rates of intrinsic biodegradation of target contaminant compounds (e.g., PAHs) in estuarine sediments is often limited both by the reduced availability of oxygen in the sediments and by the reduced bioavailability of the contaminant molecules to the biodegradative microbes. However, vascular plants that are adapted to thrive in the tidal marsh zones of such estuarine habitats establish microniches that can favor enhanced microbial activity in their rhizosphere.

Previous work in our laboratory has demonstrated that the rhizosphere of *Spartina*

sp. contains a wide variety of PAH-degrading bacteria that are distinct from those found in bulk sediment (Daane et al. 1998a,b). Identification of the isolates using a combination of phenotypic, morphologic and molecular techniques assigned them into three main bacterial groups: gram-negative pseudomonads, gram-positive non-spore forming nocardioforms, and the newly described gram-positive spore forming bacilli group, *Paenibacillus* (Daane et al., 1998a). Comparison to the classical genes (*nah* and *pah*) for naphthalene degradation from *Pseudomonas*, *Comamonas*, and *Sphingomonas* strains suggested that the nocardioform and *Paenibacillus* strains may have novel genes for PAH degradation. It is of interest to determine whether the PAH-degrading *Paenibacillus* group is specific to the *Spartina* rhizosphere, since this group has not been found in other studies examining PAH-degrading bacteria in marine and intertidal sediments (Geiselbrech et al. 1998; Dyksterhouse et al. 1995, Hedlund et al. 1999; Berardesco et al. 1998). Additional PAH-degrading bacteria have been isolated from the *Spartina* rhizosphere in work associated with this grant.

*Spartina* (Howes and Teal, 1994) is known to pump oxygen into the rhizosphere however the potential for this process enhancing biodegradation in the rhizosphere by creating more oxidized conditions has not been addressed. Experiments with *Spartina* salt marsh mesocosms indicated that biodegradation of oil was influenced by flooding and fertilization conditions (Wright et al. 1997). Of specific interest is to evaluate the role of oxygen cycling by roots of salt marsh plants relative to enhancement of biodegradative activities of rhizosphere-associated microorganisms.

The objective of this study is the investigation of the key design features of a model engineered rhizoremediation system for remediation of New Jersey sediments

contaminated with complex mixture of anthropogenic organic pollutants. Our approach will be to investigate the development of engineered rhizoremediation systems in which plants adapted to estuarine habitats, namely *Spartina alterniflora*, are combined with bioaugmented microbial consortia designed for particular suites of target contaminants. The basic hypothesis being tested is that microbial degradative activity towards target pollutants will be enhanced in the rhizosphere via an overall enhancement of microbial metabolic activity as a consequence of oxygen cycling and nutrient provision from the plant's root system.

## **Methodology**

### *Soil Sampling*

Soil samples were collected in early July of 2000 from a petroleum-contaminated salt marsh in northeastern New Jersey. Three sample areas were selected at the sight: a stand of *Spartina alterniflora*, a stand of *Spartina patens*, and a heavily polluted pitch field. Samples were taken by carving a 10 in. X 10 in. square in the soil with a spade. These soil blocks were then pried up from the ground and removed intact. Any existing plant matter was included in the samples. Once removed, the soil blocks were transferred to plastic bags and placed on ice for transport. Three soil samples were extracted from each of the plots.

### *Enrichment*

Soil blocks were cut open aseptically in a laminar flow hood using sterile instruments for further sampling. Approximately 6 to 7 grams of soil were removed from each block in this fashion to be used in the enrichment of indigenous PAH degrading organisms.

Enrichment of bacteria was carried out in 25 ml serum vials. Clean, sterile vials were spiked with PAHs by the addition of stock solutions in acetone. The solvent was allowed to evaporate from the vials prior to further amendment. 0.5 grams of soil was then added to each spiked vial. Following soil addition, 10 ml of liquid medium was added to the vials, bringing the final concentration of PAH to 100 ppm.

Enrichment vials were then transferred to an incubator/shaker where they were shaken at 28° C at a speed of 200 rpm for a total of 4 weeks. Following the initial incubation period, a 100 ul inoculum was taken from each vial and transferred to a fresh vial containing 10 ml of liquid medium and 100 ppm of PAH. These subculture vials were then returned to the incubator shaker for an additional 4 weeks.

A total of three PAHs (phenanthrene, fluoranthene, and pyrene) were enriched for separately in this study. Chemicals were obtained from the Sigma Chemical Company. The physical and chemical properties of these compounds are summarized in Table 2. PAH stock solutions were created by dissolving solid PAHs in 5 ml of acetone to a final concentration of 5000 ppm. The stock solutions were kept at 4° C in amber glass, Teflon-capped vials. Glass Hamilton syringes were used to inoculate serum vials with the stock solutions.

A second enrichment, from soils from the same site that were aged at 4°C for 2 years, was performed using the methods described above.

#### *Isolation and Characterization*

Following incubation of the secondary enrichments, a loopful of culture medium (approximately 1 ul) was taken from each vial and streaked onto a 1:5 carbon strength LB

plate and incubated at 30° C. After a 24-hour growth period, these plates were then streaked for isolation based on colony morphology.

Individual isolates were streaked onto PAH overlay plates (50 ppm PAH in minimal medium) and incubated at 30° C for 4 to 6 weeks. Isolates showing distinct colony growth and clearing zones in the medium were scored positive for PAH degradation. Positive isolates were confirmed by streaking on minimal plates without any added PAH. Positive isolates were then frozen in 20% glycerol at -80° C.

Positive isolates were grown on TSA and tentatively identified using fatty acid esterification and the MIDI identification system (MIDI Labs, Inc.).

#### *Amplification of Dioxygenase Gene Fragments*

Genomic DNA was extracted from positive isolates using the Nucleospin Tissue Kit (Clontech Laboratories, Inc.). The PCR primers DIOX1 (5'- AGGGATCCCCANC RTGRTANSWRCA - 3') and DIOX2 (5' - GGAATTCTGYMGNCAYMGNGG - 3') were used to amplify a conserved sequence within the Reiske iron-sulfur center of the PAH initial dioxygenase gene (Cigolini & Zylstra, 2000). 50 ul reactions were set up with Promega PCR buffer (1X), 2 mM MgCl<sub>2</sub>, 0.4 mM dNTP mix, 0.2 uM each primer, 2.5 u of Promega Taq, and 1 ul of template DNA. The following program was used to amplify the conserved dioxygenase gene fragment: 95° C for 2 minutes, denaturation at 95° C for 1 minute, primer annealing at 45° C for 1 minute, elongation at 72° C for 1 minute (40 cycles), and final elongation at 72° C for 5 minutes. PCR products were electrophoretically run on a 1% agarose gel at 80 V for approximately 75-80 minutes. Agarose gels were stained with a 1 ug/ml solution of ethidium bromide and visualized under UV light using Kodak 1D (v. 3.5) image-capturing software.

PCR product bands corresponding to the expected product size were excised from the gel and eluted using the QIAquick Gel Extraction Kit (QIAGEN, Inc.). Eluted product was then reamplified under identical conditions to increase DNA yield for sequencing.

#### *Indole Assay*

The conversion of indole to indigo was used as an indicator of dioxygenase activity in further plant model system experiments. Several isolates were assayed for indigo production. Bacterial isolates were inoculated at high density ( $\sim 10^8$  cells/ml) into serum vials containing M9 minimal medium, 100  $\mu$ M PAH for induction, and 1 mM indole. After the vials were sealed, they were shaken at 30° C at a speed of 200 RPM and monitored for production of indigo (blue precipitate). Isolate PCpG-3 (*Pseudomonas putida*) was selected for use in rhizosphere model experiments based on its ability to produce a substantial indigo precipitate after a period of 12 hours.

#### *Rhizosphere Model System*

A rhizosphere model system was constructed in order to show the effects of plant root activities on PAH catabolism in these zones. Essentially, this system uses the indole reaction as an indicator of dioxygenase activity, providing a visual confirmation of plant-microbe interactions.

Live *Spartina alterniflora* and *Phragmites australis* grasses were collected from stands adjacent to an unnamed creek in Cheesequake State Park in Matawan, New Jersey, and transported back to the greenhouse. The plants were then separated and gently washed with clean water. The grasses were transplanted to washed sand and irrigated with tap water for 7 to 10 days, in order to acclimate them to the artificial experimental conditions.

Acclimated plants were removed from the sand and fully separated. The roots were surface sterilized with a 5% bleach solution to remove as much biomass as possible. Each individual plant root mass was then inserted into a 50 ml plastic centrifuge tube with small (2 mm) holes drilled along the length on all sides. These tubes were then filled with washed sand and saturated with a 1% Miracle-Grow® solution. These tubes prolonged the viability of the plants in the experimental system.

These plant tubes were then used to create the experimental system. A glass tank (16 in. wide X 8 in. long X 10 in. deep) was filled with approximately 1.5 inches of washed sand. This served as a support for the tubes, which were inserted into it. A layer of 0.4% agar approximately 0.5 inches thick was then poured on top of the sand and allowed to set. Indole crystals were added directly on top of this support agar.

Next, approximately 2 to 3 inches of a 0.2% agar solution were added on top of the indole crystals. This agar (made from the mineral salts medium) contained a high-density inoculum of PCpG-3 and 100 uM phenanthrene for enzyme induction. The indole crystals dispersed throughout this layer upon addition to the tank. This cell layer was then allowed to partially set before further amendment.

Finally, a 2-inch thick layer of 0.4% agar was added to the tank on top of the cell layer. This layer served to impede the diffusion of atmospheric oxygen into the lower levels of the system, leaving the plant roots as the only source of oxygen flux. Additionally, it served to confine all necessary experimental components to the middle layer within the tank.

A total of 8 plant vials were included in the system: 2 *S. alterniflora* (light), 2 *S. alterniflora* (dark), 2 *P. australis* (light), and 2 *P. australis* (dark). Dark controls were

performed by covering the aerial portions of the plants with opaque black plastic. The rhizosphere system was transported to the greenhouse and observed for an indicator reaction over a period of three weeks.

#### *Plant Root Extracts*

Roots from *Spartina alterniflora* individuals were washed in deionized water and dried overnight in a convection oven. Samples of fine roots (5.0 g) were ground to a fine powder in a mortar and pestle and boiled in 150 ml of deionized water for 30 minutes. Boiled extracts were filter sterilized and stored at 4°C.

#### *Phenolic Content of Extracts*

Total phenolic assessment was made using a variant of the Folin-Ciocalteu phenol reaction (Kramling and Singleton 1969, Singleton and Rossi 1965, Somers and Ziemelis 1980). Extract samples (1 ml) were added to glass test tubes. Five (5.0) ml of a 1:10 dilution of Folin-Ciocalteu reagent was added, and the mixture was vortexed and incubated at room temperature for 5 minutes. Four (4.0) ml of a 7.5% Na<sub>2</sub>CO<sub>3</sub> solution was added, and the mixture is vortexed. The reaction was allowed to incubate at room temperature for 2 hours. Phenolic content was assessed by spectrophotometric quantitation at 740 nm.

#### *Chromatographic Analysis of Extracts*

For HPLC analysis, extracts were diluted to 25% in deionized water and run on a Shimadzu HPLC with the following conditions: 95% aqueous to 40% aqueous over 60 minutes, aqueous = 0.3% H<sub>3</sub>PO<sub>4</sub>, organic = methanol, detection at 275 nm.

For GC/MS analysis, extracts were extracted with an equal volume of hexane in sealed serum vials for 24 hours (30°C, 200 RPM shaking). The organic fraction was

transferred to GC vials and run on an HP 5800 series GC/MS under the following conditions: HP5 column (30m X 0.25mm), initial temp = 70°C, ramp to 280°C over 45 minutes, carrier = He (5 ml/min), MS detection.

#### *Culture Growth on Extracts*

Triplicate cultures of each isolate were set up in root extract, glucose, and p-hydroxybenzoate media, each adjusted to a concentration of 100 mg/L in mineral salts (phenolic concentration was used for extracts). Cultures were incubated at 30°C with shaking for 3 days. Cell density was measured spectrophotometrically at 600 nm.

### **Principle Findings / Significance**

#### *Isolates*

First round isolation:

<b>Isolate</b>	<b>Soil Type<sup>1</sup></b>	<b>Substrate</b>	<b>MIDI match</b>	<b>SI<sup>2</sup></b>
ABy52	rhizosphere	pyrene	<i>Phenylobacterium immobile</i>	0.15
PAyG1	rhizosphere	pyrene	<i>Bacillus</i> GC grp. 22	0.22
PR-P3	rhizosphere	phenanthrene	<i>Alcaligenes oxydans</i>	0.46
ACpM3	rhizosphere	phenanthrene	<i>Alcaligenes xylooxidans</i>	0.53
PCfM3	rhizosphere	fluoranthene	<i>Pseudomonas balearica</i>	0.49
TPpG1	bulk	phenanthrene	<i>Agrobacterium radiobacter</i>	0.85
TPpG3	bulk	phenanthrene	<i>Nocardia asteroides</i>	0.64
ABpM2	rhizosphere	phenanthrene	<i>Methylobacterium radiotolerans</i>	0.82
PAy51	rhizosphere	pyrene	<i>Pseudomonas putida</i>	0.56
PBfG2	rhizosphere	fluoranthene	<i>Bacillus pumilis</i>	0.44
PCpM2	rhizosphere	phenanthrene	<i>Arthrobacter atrocyaneus</i>	0.62
PByG3	rhizosphere	pyrene	<i>Rhodococcus equi</i>	0.33
PCpG4	rhizosphere	phenanthrene	<i>Acinetobacter calcoaceticus</i>	0.34

<sup>1</sup> Rhizosphere soil was isolated from the root zones of *Spartina alterniflora*.

<sup>2</sup> SI = similarity index, as calculated by the MIDI software. This is a measure of the extent to which the putative identification matches the typical characteristics for the named strain.

Second round isolation:

Isolate	Soil Type	Substrate	MIDI Match	SI
A3YSY	rhizosphere	pyrene	<i>Bacillus globisporus</i>	
A3NY1	rhizosphere	pyrene	<i>Nocardia carnea</i>	
A3PRY	rhizosphere	phenanthrene	<i>Bacillus globisporus</i>	
A3PRW	rhizosphere	phenanthrene	<i>Bacillus cereus</i>	
TP2P	bulk	phenanthrene	<i>Rhodococcus rhodochrous</i>	
P2P1	rhizosphere	phenanthrene	<i>Yersinia pseudotuberculosis</i>	
A3YSW	rhizosphere	pyrene	<i>Pseudomonas fluorescens</i>	
A3PRB	rhizosphere	phenanthrene	<i>Bacillus globisporus</i>	
A3PWW	rhizosphere	phenanthrene	<i>Bacillus subtilis</i>	
TP2Y	bulk	phenanthrene	<i>Hydrogenophaga palleronii</i>	
A3NY2	rhizosphere	pyrene	No Match	
A3NY3	rhizosphere	pyrene	No Match	
P2Y1	rhizosphere	pyrene	<i>Yersinia pseudotuberculosis</i>	
A3NY4	rhizosphere	pyrene	<i>Rhodococcus equi</i>	

*Dioxygenase Amplification*

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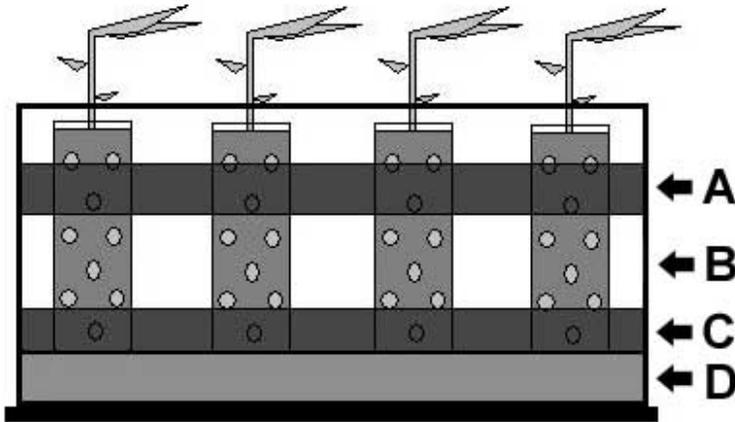


**Row 1**

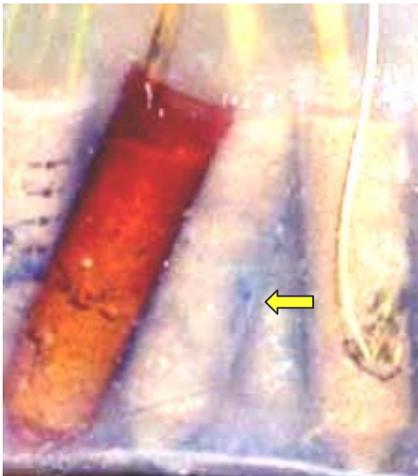
Amplification of dioxygenase gene fragments using degenerate PCR primers. Key (Row 1): Lane (1)1kb ladder, (2)*B. cepacia* LB400, (3)ABpM-2, (4)ACpM-2 large fragment, (5)ACpM-2 small fragment, (6)TPpG-1, (7)ACpG-1, (8)PBfG-2, (9)PCfM-3, (10)PCpG-4, (11)ACfM-2. Row 2: Lane (1)1kb ladder, (2)PAyG-1, (3)TPyG-1 large fragment, (4)TPyG-1 small fragment, (5)PAy5-1, (6)PCy5 large fragment, (7)PCy5 small fragment, (8)H<sub>2</sub>O blank. The red arrows indicate the approximate position of the expected product.

**Row 2**

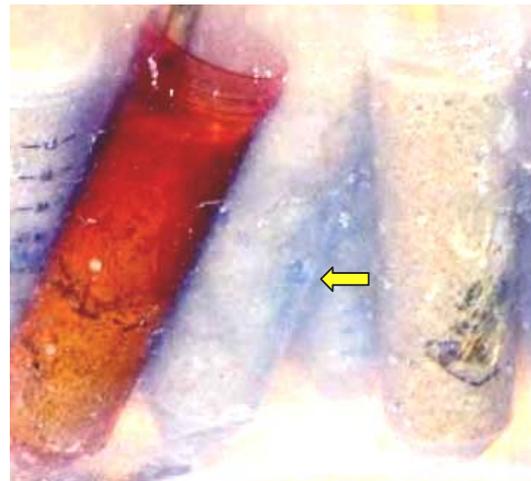
## Model Rhizosphere



Setup of the bench-scale model rhizosphere apparatus. Individual plant tubes are inserted into the base sand layer (D). The lower layer of 0.4% agar (C) is poured directly on top of the sand. The cell layer, containing the culture inoculum, indigo, and phenanthrene (B) is added on top of this layer. The system is then capped with another layer of 0.4% agar (A). Dark controls were made by covering the aerial portions of the plants with black plastic.



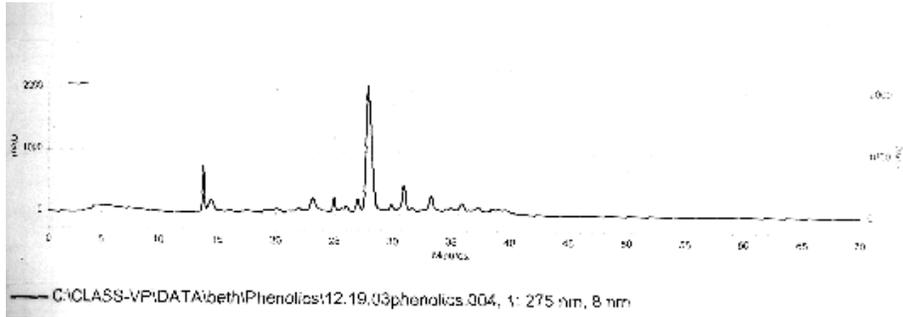
A.



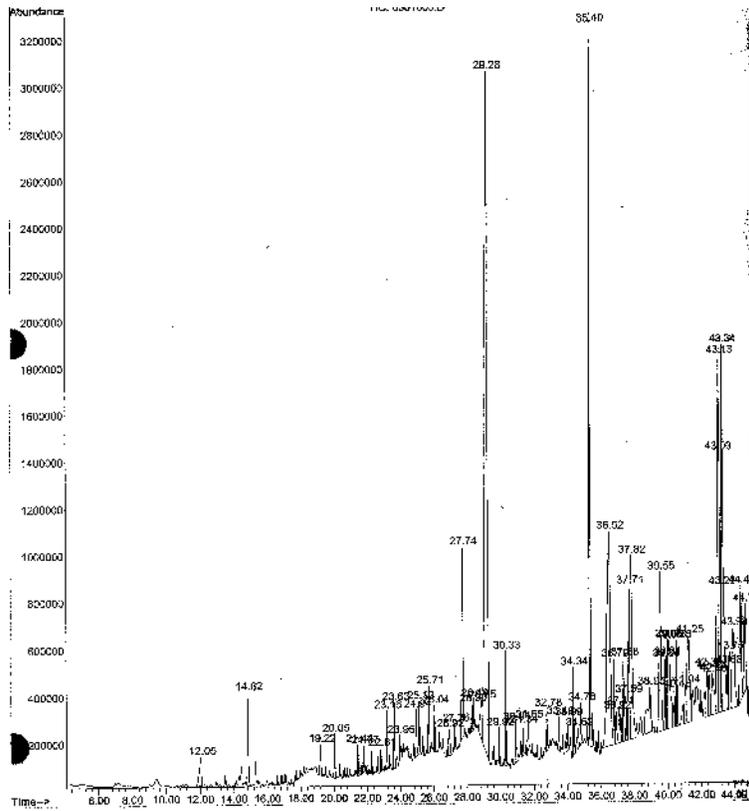
B.

Photos of model rhizosphere root tubes following three weeks of greenhouse incubation. On the left, a *P. australis* light tube (note the deep red color). The middle tube is a *S. alterniflora* light tube. The yellow arrow indicates a drilled hole where formation of indigo was observed. The tube on the right is a *S. alterniflora* dark control tube, with no visible color reaction. 6B gives a closer view of the system, where the diffusion of indigo from the drill hole area is more evident.

Root Extracts

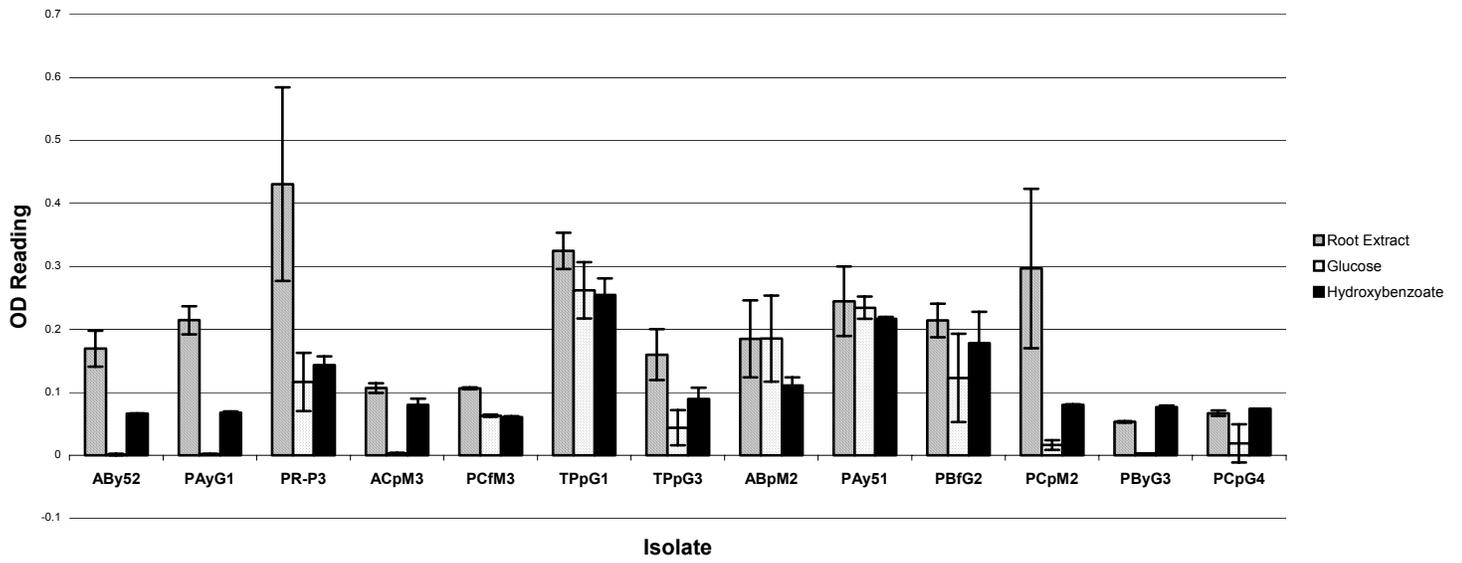


HPLC chromatogram of *Spartina alterniflora* root extracts (275 nm)



GC chromatogram of hexane extraction from aqueous *Spartina alterniflora* root extracts

### Root Exudate Growth Assay



Results of growth experiments involving primary PAH isolates growing on root extracts, p-hydroxybenzoate, and glucose. Data points represent triplicate values.

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