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SUMMARY

"BACTERIAL BIODEGRADATION OF HIGH MOLECULAR WEIGHT POLYCYCLIC AROMATIC HYDROCARBONS" OR WHY OIL SPILL EATER II WORKS SO EFFECTIVELY

The University of North Carolina (Department of Environmental Sciences and Engineering), through a Grant from the U. S. Geological Survey (Grant 14-08-0001-G2103) and the UNC Water Resources Research Institute, scientifically proved that PAH's (Polycyclic Aromatic Hydrocarbons) can be Bioremediated.

These tests not only showed the Bioremediation of PAH's, but they verified The pathways and constituents needed to Bioremediate PAH's.

These pathways and constituents have been discussed in Oil Spill Eater II's Literature since 1989. This study verifies the fact that bacteria cannot be put In direct contact with a toxic contaminant without suffering a great amount Of mortality.

The study discusses that when bacteria become proximal to a contaminant, The bacteria release enzymes and surfactants to start the breakdown of the contaminant and to open pathways so the contaminant might be used as a Food source for the bacteria.

These are exactly the steps described in OSEI, Corp.'s literature.

The study also addresses the needs to have certain constituents present to act as a catalyst to induce bacteria growth and bacterial metabolism of PAH's. OSEI Corp.'s literature has addressed this since 1989.

This study proved the fact that bacteria will die if brought in direct contact with a contaminant. Bacteria need to release enzymes and surfactants to utilize contaminants as a food; and that various constituents are needed to promote the rapid colonization of bacteria that can utilize a particular contaminant as a food source.

OSEI, Corp.'s chemical process and general description has covered the methods and processes that must occur for the rapid growth of bacteria and the steps that much occur prior to a bacteria being able to utilize contaminants as a food Source.

This study backs up OSEI Corp.'s literature and proves that the very toxic PAH's can be Bioremediated as long as the correct constituents are available and the correct process occurs.

OIL SPILL EATER II contains these necessary ingredients and performs the correct process required to Bioremediate PAH's or any other organic based contaminants.

Steven R. Pedigo
Chairman

SRP/AJL

Bacterial Biodegradation Of High Molecular Weight Polycyclic Aromatic Hydrocarbons

Michael D. Aitken, Shu-Hwa Chen, Chikoma Kazunga and Randall B. Marx

Introduction

Polycyclic aromatic hydrocarbons (PAH) are among the most common pollutants found at contaminated industrial sites in the U.S. and, in fact, around the world. Although PAH are naturally occurring compounds and essentially ubiquitous at low concentrations in the terrestrial environment, high concentrations of PAH are found in contaminated soils at wood treating facilities, at sites formerly used to produce manufactured gas, and at petroleum processing operations. There are a wide range of PAH, from the simple two-ring compound naphthalene to large multi-ring compounds. We arbitrarily define those PAH with two or three rings as low molecular weight (LMW) species, and those with four or more rings as high molecular weight (HMW) species. One of the characteristic features of PAH contamination is that there is always a complex mixture of PAH, often in conjunction with a variety of other hazardous chemicals.

The U.S. Environmental Protection Agency currently regulates 16 PAH compounds as priority pollutants in water, and generally considers these same compounds as "total PAH" (tPAH) in contaminated soils. The 16 regulated PAH comprise both low and high molecular weight species, and seven of them are designated as known human carcinogens. All of the carcinogenic PAH (cPAH) are high molecular weight compounds.

In addition to their toxicity, PAH as a class are extremely hydrophobic chemicals. Naphthalene is among the most water-soluble of the PAH, and its solubility in water is only about 30 mg/L. The situation becomes much worse with increasing molecular weight: chrysene (a four-ring PAH) and benzo[a]pyrene (a five-ring compound) are soluble in water in the low part per billion (mg/L) range. By comparison benzene, normally considered to be a water-immiscible chemical, has an aqueous solubility of about 2,000 mg/L. Despite the very low solubility of PAH in water, PAH-contaminated materials have been shown to be toxic in a variety of bioassays.

Microbiologists have known for a long time that microorganisms can act on most of the PAH of concern. For this reason, PAH-contaminated soils and sediments are generally considered to be candidates for bioremediation. In many of the studies in which PAH degradation in contaminated soil or sediment has been studied, however, the higher molecular weight compounds have not been removed completely. We generally do not

exposed to a mixture of PAH, all of which are, to varying extents, structural analogues. Over evolutionary time scales such conditions would be expected to select for microorganisms with versatile metabolic capabilities. We also knew that organisms able to degrade but not grow on the higher molecular weight compounds needed to grow on something, so the low molecular weight PAH seemed like the best choice with which to begin.

We have studied each of the 11 bacterial isolates for their ability to grow on or degrade 13 PAH, ranging from two- to five-ring compounds. Most of the bacteria have a broad range of PAH substrates they can metabolize (Aitken et al., 1996). Of most interest in terms of maximizing the biodegradation of these compounds, however, is mineralization. So far we have studied the ability of nine of the strains to mineralize pyrene, chrysene, benz[a]anthracene and benzo[a]pyrene (the latter three compounds are all cPAH). The results of this work are shown in Table 1, which also summarizes the source and identity (if known) of each isolate.

Table 1. Mineralization of HMW PAH by Bacterial Cultures[a]

Strain[b]	Identification[c]	Mineralization			
		PYR[d]	CRY	BaA	BaP
G2	*Agrobacterium tumefaciens*	-	+	+	+
G3	*Pseudomonas saccharophila*	-	+	+	+
C5	*Pseudomonas cepacia*	-	+	+	+
R1	unknown	-	+	+	+
M6	unknown	-	+	+	+
P15	*Pseudomonas saccharophila*	-	+	+	+
P16	*Pseudomonas stutzeri*	-	-	-	-
P21	*Bacillus cereus*	-	-	-	-
VT1	*Pseudomonas paucimobilis*	-	+	+	+

[a] Cultures were first grown on phenanthrene, centrifuged and washed, then incubated with the test compound for 24 hours. All strains mineralized phenanthrene, the positive control.

[b] Strains G2 and G3 were isolated from soil collected at a refinery; strain C5 from a site used to treat railroad ties; strain R1 from a manufactured-gas plant site; strain M6 from a former wood treating site; strains P15 and P16 from another former wood treating site; and strain P21 from motor oil-contaminated surface soil. Strain VT1 was obtained from J. Fuglt at the University of Alberta, Canada.

[c] Identification was by fatty acid methyl ester (FAME) analysis by MIDI, Inc., Newark, DE. Strains designated as unknown did not match any strains in the MIDI database at the genus level.

[d] Abbreviations: PYR, pyrene; CRY, chrysene; BaA, benz[a]anthracene; BaP, benzo[a]pyrene.

There are two trends worth noting in Table 1. First, none of the organisms was able to mineralize pyrene under the conditions used in this experiment. Second, every organism either mineralized all three of the remaining high molecular weight substrates or did not

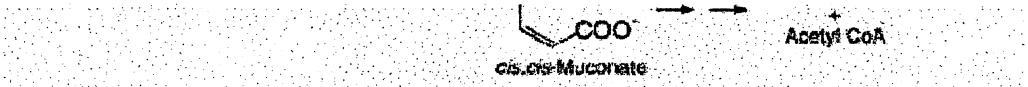


Figure 1. The principal pathway for aerobic metabolism of naphthalene by pseudomonads. Ring cleavage of catechol by either the *meta* or *ortho* route depends on the individual strain. Salicylate induces both the upper pathway (leading to salicylate) and the lower pathway (leading to catechol and subsequent ring cleavage reactions).

We have focused our attention on the inducibility of HMW PAH degradation in one of the organisms in our collection, Pseudomonas saccharophila P15. This organism was isolated several years ago by Will Stringfellow, who also conducted in-depth studies on its physiology (Stringfellow and Aitken, 1994; Stringfellow and Aitken, 1995). Both phenanthrene and salicylate induce the mineralization of benz[a]anthracene, chrysene, and benzo[a]pyrene by P. saccharophila P15 (Figure 2). None of the three compounds are used as growth substrates by the organism, so these results suggest that the metabolism of high molecular weight PAH in P. saccharophila P15 is linked to the metabolism of a low molecular weight compound. We do not yet know, however, if the different PAH are metabolized by a common pathway or if different pathways are regulated through a common mechanism.

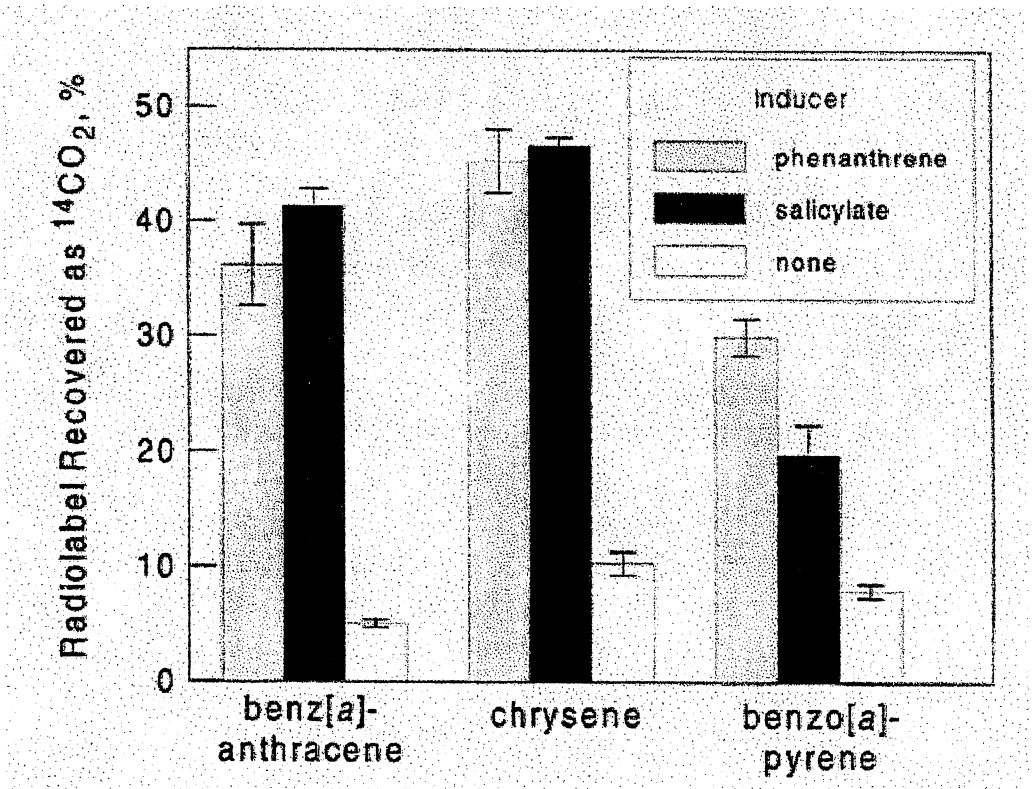
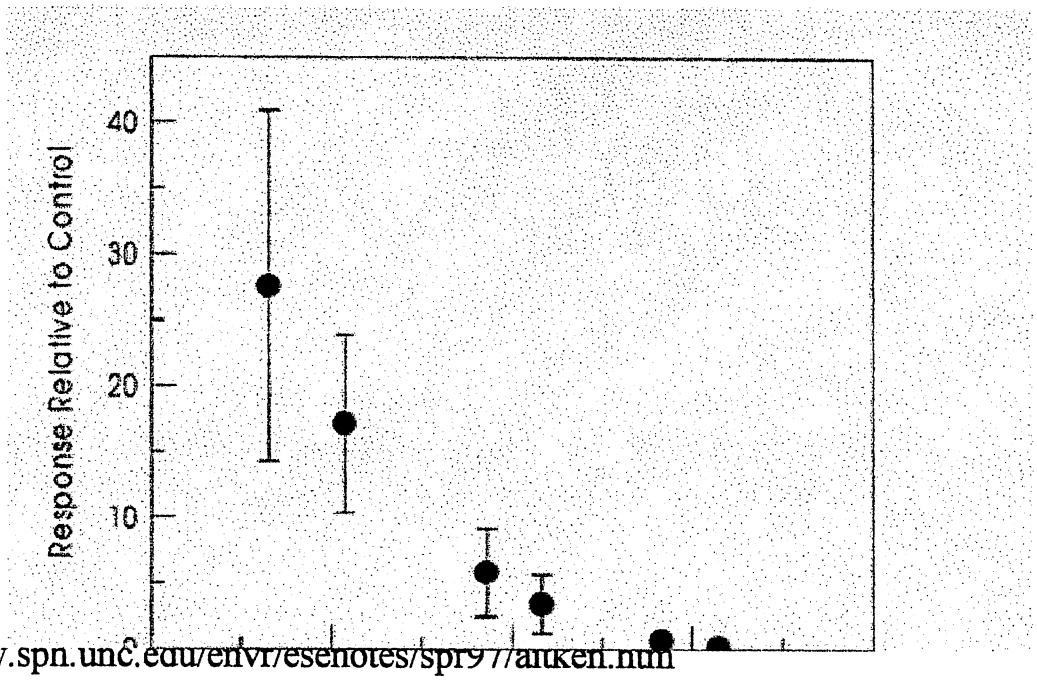


Figure 2. Mineralization of HMW PAH by *Pseudomonas saccharophila* P15. A culture of strain P15 was grown in a succinate medium, then incubated with the indicated inducer (or no inducer) at a 5 μM concentration for three hours. After the induction period, the cells were transferred to a medium containing radiolabeled benz[a]anthracene, chrysene or

such mechanisms in in situ biodegradation reactions and whether such mechanisms can eventually be manipulated to our advantage. If, for example, motility and/or chemotaxis are found to be important aspects of PAH (or other pollutant) biodegradation by soil bacteria, then we need to begin incorporating such phenomena into models that purport to simulate biodegradation processes in the subsurface.

Most of the bacteria identified in Table 1 are motile, and we screened most of them for chemotaxis to naphthalene, phenanthrene and known metabolites of these low molecular weight PAH. Only one of them, Pseudomonas stutzeri P16, seemed to have a chemotactic response to the chemicals of interest. Interestingly, strain P16 appears to be chemotactic to 1-hydroxy-2-naphthoate and to salicylate, both metabolites that have been found extracellularly in media containing active PAH degraders. However, we are learning that some of the methods traditionally used to study chemotaxis are not well suited to the study of chemotaxis towards compounds of low aqueous solubility. To test our methods with a known chemotactic organism, we recently obtained the strain Pseudomonas putida G7 from Professor Caroline Harwood at the University of Iowa, who recently identified this strain's chemotaxis towards naphthalene using a qualitative assay (reported at the 1996 American Society for Microbiology meeting). We learned that the chemotactic response of strain G7 in a standard quantitative assay becomes increasingly detectable as the starting concentration of bacterial cells is decreased (Figure 3). The optimum response we observed was at a cell concentration nearly two orders of magnitude lower than the concentration typically used in the assay, which we attribute to the rapid loss of the naphthalene concentration gradient at high cell concentrations and the necessarily low starting concentration of naphthalene. Overall the response of strain G7 to naphthalene was variable yet strong. Consequently, we will focus on this organism in our subsequent efforts to understand the relevance of chemotaxis to PAH biodegradation.



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