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Biodegradation of Creosote and Pentachlorophenol in Contaminated Groundwater: Chemical and Biological Assessment†

JAMES G. MUELLER,[1]* DOUGLAS P. MIDDAUGH,[2] SUZANNE E. LANTZ,[1] AND PETER J. CHAPMAN[2]

Southern Bio Products, Inc.,[1] and U.S. Environmental Protection Agency Environmental Research Laboratory,[2] Gulf Breeze, Florida 32561-3999

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Shake flask studies examined the rate and extent of biodegradation of pentachlorophenol (PCP) and 42 components of coal-tar creosote present in contaminated groundwater recovered from the American Creosote Works Superfund site, Pensacola, Fla. The ability of indigenous soil microorganisms to remove these contaminants from aqueous solutions was determined by gas chromatographic analysis of organic extracts of biotreated groundwater. Changes in potential environmental and human health hazards associated with the biodegradation of this material were determined at intervals by Microtox assays and fish toxicity and teratogenicity tests. After 14 days of incubation at 30°C, indigenous microorganisms effectively removed 100, 99, 94, 88, and 87% of measured phenolic and lower-molecular-weight polycyclic aromatic hydrocarbons (PAHs) and *S*-heterocyclic, *N*-heterocyclic, and *O*-heterocyclic constituents of creosote, respectively. However, only 53% of the higher-molecular-weight PAHs were degraded; PCP was not removed. Despite the removal of a majority of the organic contaminants through biotreatment, only a slight decrease in the toxicity and teratogenicity of biotreated groundwater was observed. Data suggest that toxicity and teratogenicity are associated with compounds difficult to treat biologically and that one may not necessarily rely on indigenous microorganisms to effectively remove these compounds in a reasonable time span; to this end, alternative or supplemental approaches may be necessary. Similar measures of the toxicity and teratogenicity of treated material may offer a simple, yet important, guide to bioremediation effectiveness.

Because of improper usage, accidental spillage, and misguided disposal of creosote and other commonly used wood preservatives such as pentachlorophenol (PCP), a large number of sites across the United States are extensively contaminated with these compounds (4, 16). As a group, polycyclic aromatic hydrocarbons (PAHs) account for 85% of coal-tar creosote; hence they are most prevalent at contaminated sites. Other compounds present include substituted phenols and *N*-, *S*-, and *O*-heterocycles (16, 21). When PCP is used, it is often present as an environmental contaminant along with chemicals associated with its use (i.e., chlorinated dioxins).

Given the requisite environmental conditions and the presence of viable microorganisms with relevant catabolic abilities to act as biocatalysts, many of these chemicals are readily biodegradable (1, 2, 12, 15–19). However, certain chemicals, such as the higher-molecular-weight (HMW) PAHs and PCP, resist biological attack and hence persist in contaminated environments (1, 2, 12, 15–18). Because of the known toxicological effects of these chemicals (8, 26) and evidence to correlate environmental contamination with bioaccumulation (6, 7) and toxic (22, 24, 25), teratogenic (13, 14), and carcinogenic (10, 28) responses in various organisms, effective site restoration is often associated with the destruction of these compounds.

For the same reasons, the potential for existing and newly emerging bioremediation technologies to treat creosote- and similarly contaminated sites is based primarily on their ability to remove these chemicals from contaminated environments. Such removal is usually determined by gas chromatographic analysis of extracts of biotreated materials. While this approach has been used for a number of biotreatability studies, there has been little attempt to correlate the disappearance of parent material with a discernible decrease in the health hazards associated with biotreated materials. Such an evaluation may not adequately assess the influence of partial biodegradation products (chemical metabolites), the cumulative effect of small amounts of residual hazardous pollutants, or the impact of contaminants not detected by the analytical procedures employed.

To address these issues, laboratory studies were performed with the following objectives: to determine, under conditions optimum for microbial growth, the ability of microorganisms indigenous to the American Creosote Works Superfund site to degrade PCP and 42 targeted constituents of creosote present in groundwater recovered from this site and to compare biodegradation data with changes in the toxicity and teratogenicity of biotreated materials. Means of enhancing the efficiency of bioremediation technologies are addressed.

MATERIALS AND METHODS

Groundwater sampling. The history of usage of various wood preservatives and the extent of environmental contamination by these chemicals at the American Creosote Works site, Pensacola, Fla., have been described previously (11, 17, 18, 23, 27). In general, groundwater contamination is limited to creosote constituents and PCP with no inorganic (i.e., metals) contamination detectable. For the studies described here, approximately 400 liters of groundwater contaminated with creosote and PCP was recovered from an on-site sampling well. Groundwater was removed from a depth of 7 m through Teflon-coated Bev-a-line tubing (15 mm inner diameter) by means of an electric pump. Groundwater

* Corresponding author.

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was delivered directly into two freshly rinsed, 208-liter steel drums (DOT-17E) and stored on site for ancillary testing. Five subsamples (1.0 liter) were collected in clean, sterile, 1.0-liter Wheaton bottles fitted with Teflon-lined screw caps and stored on ice for transport to the laboratory. Upon arrival at the laboratory, subsamples were stored in darkness at 2°C for subsequent biodegradation studies, toxicity and teratogenicity testing, and chemical analyses.

Shake flask studies. Fifteen 125-ml Erlenmeyer flasks fitted with Teflon-lined screw caps received 25 ml of groundwater medium (GWM) consisting of 12.5 ml of filtered groundwater (passed through a plug of silanized glass wool to remove undissolved solids) plus 12.5 ml of modified Bushnell-Haas medium (17). Additionally, two clean, sterile, 1.0-liter Wheaton bottles fitted with Teflon-lined screw caps received 200 ml of the same medium. No difference in organic pollutants present in filtered and unfiltered groundwater could be detected by gas chromatographic analysis or toxicity and teratogenicity tests (data not shown). The filtered medium was used to monitor the fate of organic pollutants upon exposure, under optimum conditions, to catabolic activities of indigenous microorganisms.

Microbial inoculum was prepared by mixing 25 g of creosote-contaminated surface soil (17) freshly obtained from the American Creosote Works site with 100 ml of 2.5 mM phosphate buffer (pH 7). After being mixed well, the suspension was centrifuged (2,500 rpm, 10 min) to remove larger soil particles. The resultant supernatant was decanted and used as a source of indigenous, "creosote-adapted" microorganisms for the GWM.

Each flask containing 25 ml of GWM was inoculated with 1.0 ml (27 μg of bacterial protein) of the soil microbial suspension. Extraction and analysis of this aqueous suspension showed that no contaminants were carried over to the flasks with this inoculum (data not shown). Two 1.0-liter Wheaton bottles, each containing 200 ml of GWM, received 8.0 ml of the same cell suspension. Duplicate 25-ml samples were immediately extracted (see below) for time zero analysis. Flasks were incubated at 30°C with shaking (200 rpm) in the dark for 14 days. Killed-cell controls were prepared for each sampling time point by adding 2.5 ml of a 37% formaldehyde solution to five of the shake flasks containing 25 ml of GWM.

After 1, 3, 5, 8, and 14 days of incubation, the entire contents of two active flasks and one killed-cell control flask were separately extracted and analyzed for the presence of creosote constituents (see below). After 14 days of incubation, the contents of flasks containing 200 ml of GWM were filtered (0.2-μm Teflon filter) and assessed for changes in toxicity (Microtox assay) and teratogenicity as described below (see Analytical Methods). These data were compared with those obtained from untreated (noninoculated) GWM that had been stored at 2°C during the 14-day incubation period.

Microtox assays. The toxicity of various samples was determined with a Microtox model 500 toxicity autoanalyzer (Microbics Corp., Carlsbad, Calif.) according to the manufacturer's specifications. Microtox evaluates the toxicity of a given substance by measuring the change in light level of viable luminescent bacteria upon their exposure to test substrates. These data were used in conjunction with teratogenicity data to evaluate the extent of removal of hazardous components from various media.

Teratogenicity assays. Teratological responses in inland silverside (*Menidia beryllina*) embryos exposed to GWM before and after biotreatment were evaluated. Previous studies have shown that this test organism offers a sensitive indicator for the presence of pesticides (13) and creosote contaminants in aqueous systems (13, 14). Naturally spawned embryos from an adult population of silversides, maintained in the laboratory at 25°C and 5‰ salinity in the absence of teratogenic substances, were used for all tests.

To initiate experiments, blastula-stage embryos were washed five times with sterile fresh water of moderate hardness (80 to 100 mg of $CaCO_3$ per liter), and single embryos were placed in each of 120 randomized Leighton culture tubes. Treatments were as follows: 30 control tubes each containing clean, sterile embryo incubation medium (13), 30 tubes containing undiluted GWM, 30 tubes with a 1:10 dilution of GWM, and 30 tubes with a 1:100 dilution of GWM. A six-ml amount of treatment solution was added to each tube. Tubes were sealed with Teflon-lined screw caps, placed in stainless steel racks, and incubated in a horizontal position at 25°C with a photoperiod of 14 h of light and 10 h of darkness.

On a daily basis, all tubes were removed from the incubator, and individual embryos were viewed microscopically and evaluated to determine the presence or absence of terata. A ranking system was used to assign numerical values for the severity of responses in three important organ systems within the developing embryos: the craniofacial-central nervous system, the cardiovascular-circulatory system, and the skeletal system (13, 14). Teratological responses were documented with photomicrography.

Seven to eight days after exposure, control embryos hatched. The minimum acceptable percentage hatch of control embryos was set at 80% (if less than 80%, experiments were repeated). All hatched larvae were immediately examined microscopically to determine the impact on the organ systems described above. Total test duration did not exceed 10 days, and the dissolved oxygen concentration and pH of the medium of representative tubes were determined at the end of each test. Preliminary studies showed that inland silversides are very susceptible to groundwater containing creosote residues and that this test system offered a very sensitive indicator of the presence of teratogenic or toxic chemicals.

Chemical extraction procedures. The entire volume of GWM from each flask was transferred to clean, 60-ml separatory funnels, the flasks were rinsed with 10 ml of methylene chloride, and this was added to the aqueous sample. Initially, GWM was adjusted to pH 12.0 with 1 N NaOH (Fig. 1). Media were extracted three times with 10-ml volumes of methylene chloride, resulting in the transfer of nonpolar compounds (PAHs and *O*- and *S*-heterocycles) and weakly basic creosote constituents (*N*-heterocycles) to the organic phase. The combined organic phases were washed once with 10 ml of distilled water (returned to the aqueous phase), dried by passage over a layer of anhydrous sodium sulfate (25 g), and collected in clean, 25-ml Kuderna-Danish concentrating tubes. The volume of methylene chloride was reduced to 1.0 ml by evaporating under a stream of dry nitrogen at 30°C. The organic phase was divided into two 0.5-ml aliquots, placed in glass vials, spiked with an internal standard (C_{32}-*n*-alkane, dotriacontane), and crimp-sealed for subsequent analysis for PAHs and *N*-, *S*-, and *O*-heterocycles by gas chromatographic analysis (see Analytical Methods).

The pH of the extracted aqueous phase was readjusted to 7.0 through the addition of 8.5% phosphoric acid. Aqueous solutions were then extracted three times with 10-ml volumes of methylene chloride to recover weakly acidic phe-

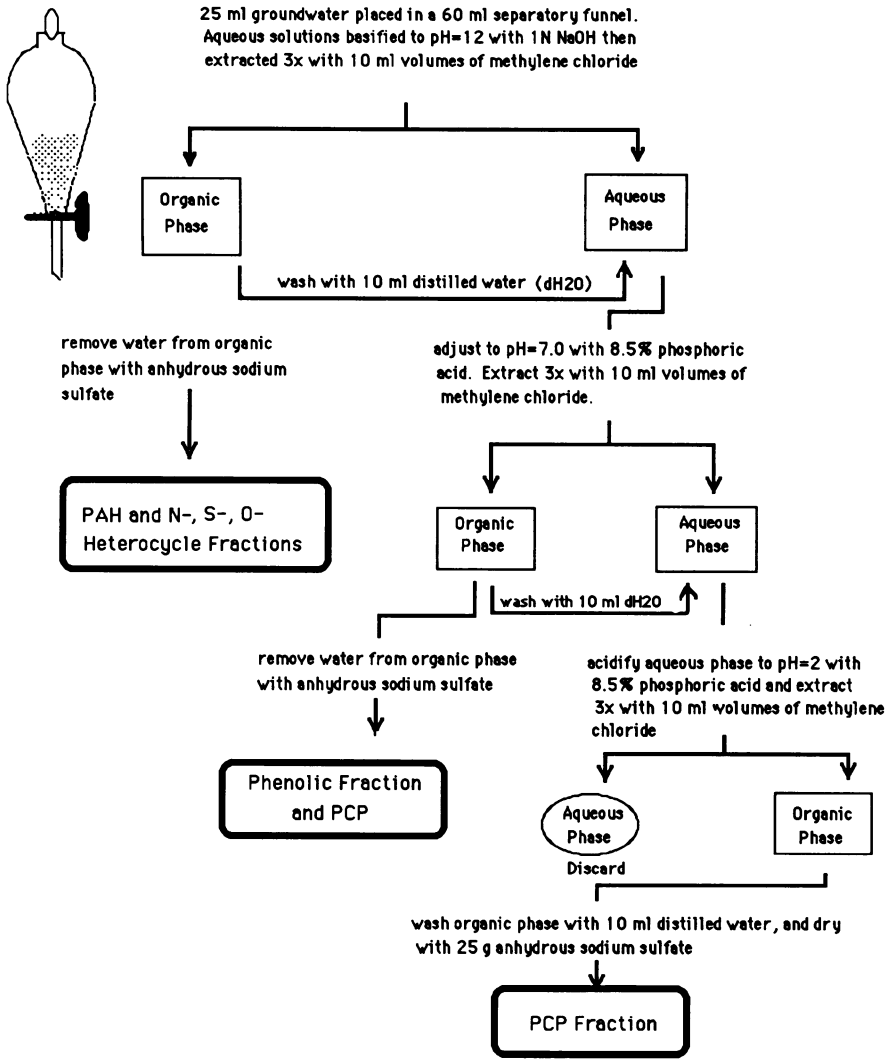

FIG. 1. Flow chart for extraction of PCP and creosote from aqueous samples.

nols and transfer them to the organic phase. The combined methylene chloride organic phases were dried by passage through a layer of anhydrous sodium sulfate (25 g) and collected into clean 25-ml Kuderna-Danish concentrating tubes. The volume of the methylene chloride organic phase was reduced to 1.0 ml under a stream of dry nitrogen at 30°C and placed in a glass vial. For analysis of phenol constituents by gas chromatographic analysis (see Analytical Methods), *o*-xylene was added as the internal standard.

The pH of the extracted aqueous phase was brought to 2.0 by the addition of 8.5% phosphoric acid. PCP (pKa, 4.7) was then extracted into methylene chloride three times (10-ml volumes). The methylene chloride organic phase was washed once with 10 ml of distilled water and then dried by passage through a layer of anhydrous sodium sulfate (25 g). The organic phase was reduced in volume to 1.0 ml under a stream of dry nitrogen at 30°C and transferred to a glass vial. PCP was derivatized (trimethylsilyl derivative) and determined by gas chromatographic analysis (see Analytical Methods). Quantitation of PCP derivatives was based on an external standard curve.

Analytical methods. After addition of the internal standard to the organic extracts, vials were sealed with Teflon-lined crimp caps, and samples were stored at −20°C for subsequent analysis. PCP was quantitatively analyzed as its trimethylsilyl derivative prepared by means of BSTFA (*N,O-bis*[trimethyl-silyl]trifluoroacetamide; Pierce Chemical Co., Rockford, Ill.). Analytical methods for quantitative determination of PCP and 42 creosote constituents by gas chromatography have been described previously (17).

The limit of detection for PCP, PAHs, and phenolic and heterocyclic components of creosote was set at 100 ppb. Recovery of PCP and selected creosote constituents was calculated from standard curves for identified chemicals. The ability of this extraction procedure to fractionate creosote constituents into the indicated chemical groups was verified by spiking aqueous samples with representative chemicals of each group (see below).

Quality assurance and quality control. For analysis of PAHs and *O*-, *S*-, and *N*-heterocyclic and phenolic components of creosote, various dilutions of standard mixtures of targeted chemicals in each group were used for daily calibration of gas chromatograph detectors. For PCP analysis, derivatized PCP standards were used for instrument calibra-

tion. A three-point calibration curve was established for each group of chemicals. The lowest amount of each standard was used to verify the limit of detection for individual chemicals. If the detection limit was exceeded, corrective measures were taken.

Instrument performance was verified by using standard reference materials, quality control samples, and performance evaluation samples obtained from the U.S. Environmental Protection Agency (EPA) Quality Assurance Branch, Environmental Monitoring Services Laboratory (Cincinnati, Ohio). Standards were run as unknowns every sixth sample to monitor instrument performance, and methylene chloride blanks were injected daily as contamination checks.

Quantitative analysis of targeted compounds was based on the presence of the internal standards. For PAH and *N*-, *S*-, and *O*-heterocycle analyses, exactly 10 μl of a dotriacontane stock solution (1.0 mg of C_{32} in 1.0 ml of hexane) was added to each 1.0-ml organic extract sample (or exactly 5 μl per 0.5-ml sample) immediately following extraction and concentration. Likewise, *o*-xylene was used as the internal standard for the analysis of phenolic compounds in organic extracts.

The ability to extract PCP and 42 creosote constituents from aqueous samples was verified by processing samples to which known amounts of authentic chemical standards had been added. Percent recovery for each component was subsequently determined. Likewise, the ability of the various fractionation schemes to differentially extract related groups of contaminants was verified.

RESULTS AND DISCUSSION

Compound identification numbers. The biotreatability of contaminated groundwater from the American Creosote Works site was evaluated by monitoring the fate of PCP and 42 components of creosote. For simplicity, all data tables make use of compound identification numbers rather than continually listing each of these compounds by name. Table 1 identifies the chemical which corresponds to each compound number. Compounds within each chemical class are listed in order of elution during gas chromatography (17). When two compounds coeluted, an individual number refers to the mixture (e.g., compounds 20, 30, and 33).

Extraction efficiency. Recovery of PCP and 42 creosote constituents from spiked aqueous samples ranged from 57% for lutidine (compound 34) to 165% for quinaldine (compound 38) (Table 1). Average recovery of PAH and phenolic and heterocyclic components of creosote was 92, 71, and 109%, respectively. Percent recovery for PCP was 102%. However, both neutral and acid extractions contained PCP, and hence both fractions were routinely analyzed for this compound. In general, percent recovery was within acceptable limits established for these studies (3). The differential extraction procedure was found to be much more efficient with aqueous samples than with soil or sediment samples (17, 18).

Chemical assessment of biodegradation. When requirements for inorganic nutrients and oxygen were satisfied, soil microorganisms indigenous to the creosote site demonstrated the ability to degrade a majority of the organic contaminants present in PCP- and creosote-contaminated GWM (Table 2). In all cases, recovery of monitored chemicals from killed-cell controls was consistently >90% of the recoverable material. Thus, losses due to abiotic processes (sorption, volatilization) were minimal.

While the phenolic components (compounds 22 to 30) were readily biodegraded, a short acclimation period was apparently required before the soil microorganisms degraded resident PAHs (compounds 1 to 21). With the exception of anthracene (compound 11) and 2-methylanthracene (compound 12), most PAHs with molecular weights below that of fluoranthene (compound 14) were extensively degraded after 5 days of incubation. With continued incubation, there was little change in the concentration of HMW PAHs (compounds 14 to 21), and no degradation of PCP (compound 31) was evident. Whereas certain *N*-heterocycles (compounds

TABLE 1. Recovery of creosote constituents and PCP from spiked samples

Compound	Compound no.[a]	Avg concn in soil[b] (μg/g of soil)	Amount added (μg/ml of water)	% Recovery[c]	
				Soil	Water
Naphthalene	1	6.4	52.5	92	90
2-Methylnaphthalene	2	5.6	50.0	93	87
1-Methylnaphthalene	3	1.8	47.0	107	87
Biphenyl	4	U[d]	49.5	95	78
2,6-Dimethylnaphthalene	5	U	49.5	100	80
2,3-Dimethylnaphthalene	6	8.5	58.0	85	80
Acenaphthylene	7	U	48.0	140	80
Acenaphthene	8	10.3	54.5	100	78
Fluorene	9	4.0	47.5	116	87
Phenanthrene	10	16.0	55.0	128	94
Anthracene	11	15.7	56.0	109	80
2-Methylanthracene	12	6.5	53.5	108	73
Anthraquinone	13	9.8	51.0	125	138
Fluoranthene	14	56.1	52.5	202	100
Pyrene	15	56.9	45.5	169	102
Benzo[*b*]fluorene	16	10.4	49.0	99	100
Chrysene	17	41.3	55.5	207	100
Benzo[*a*]pyrene	18	50.3	54.5	116	100
Benz[*a*]anthracene	19	13.2	5.0	183	107
Benzo[*b*]fluoranthene/ benzo[*k*]fluoranthene	20	61.7	14.0	586	96
Indeno[1,2,3-*c*,*d*]pyrene	21	21.5	2.8	665	97
2,6-Xylenol	22	U	20.0	36	71
o-Cresol	23	U	16.0	43	71
2,5-Xylenol	24	U	18.0	50	73
2,4-Xylenol	25	U	39.0	37	70
p-Cresol	26	U	40.0	28	71
m-Cresol	27	U	60.0	39	70
2,3-Xylenol	28	U	22.0	44	75
3,5-Xylenol	29	U	38.0	48	76
3,4-Xylenol/2,3,5-trimethylphenol	30	U	94.0	47	72
PCP	31	1.1	52.0	114	102
2-Picoline	32	—[e]	—	—	—
3-Picoline/4-picoline	33	—	—	—	—
Lutidine	34	0.3	30.0	78	57
Thianaphthene	35	0.9	31.0	29	74
Quinoline	36	0.1	28.0	90	88
Isoquinoline	37	0.3	48.0	76	78
Quinaldine	38	7.2	30.0	207	165
Lepidine	39	1.7	46.0	130	152
Dibenzofuran	40	5.6	32.0	73	90
Dibenzothiophene	41	3.2	28.0	68	101
Acridine	42	3.3	16.0	57	163
Carbazole	43	9.1	19.0	128	118

[a] Compounds listed in order of elution during gas chromatography (see text).
[b] Average of duplicate analyses on 10-g samples of soil from site, no background in distilled water.
[c] Average of triplicate independent analyses.
[d] U, Undetected (below limit of detection).
[e] —, Not analyzed.

TABLE 2. Concentrations of PCP and select creosote constituents in groundwater subjected to the action of indigenous microorganisms

Compound no.	Concn (μg/ml)[a]						
	Initial	After incubation for:					Sterile control (day 14)
		1 day	3 days	5 days	8 days	14 days	
1	28.7	17.2	0.1	U[b]	0.1	U	25.6
2	4.7	3.0	U	U	0.1	U	4.5
3	9.5	5.7	2.1	1.5	U	U	8.2
4	3.0	1.7	1.2	U	U	U	2.6
5	2.4	1.4	1.2	1.2	1.0	0.3	2.1
6	1.3	0.8	0.5	0.8	0.7	0.2	1.9
7	0.6	0.3	0.4	0.6	0.6	0.2	0.4
8	13.6	9.0	8.3	9.6	9.7	1.8	11.9
9	11.6	7.8	8.0	5.2	1.8	0.1	9.9
10	32.8	23.5	23.1	15.4	0.3	U	27.7
11	4.7	3.2	3.0	2.7	2.2	0.5	3.9
12	5.2	3.7	3.7	4.0	4.2	1.5	4.4
13	3.3	2.1	1.9	U	U	U	2.9
14	16.2	11.5	11.5	13.3	13.5	7.6	14.4
15	10.4	7.8	7.3	8.2	8.3	4.7	9.8
16	2.5	1.7	1.7	1.8	2.0	1.2	2.0
17	2.7	1.8	1.8	2.0	2.1	1.2	2.4
18	2.1	0.5	U	U	U	0.9	2.0
19	2.9	2.0	2.0	2.0	2.2	1.3	2.7
20	2.9	2.8	2.0	2.1	2.1	1.7	2.8
21	1.9	1.3	1.4	1.4	1.2	0.9	1.8
22	1.1	0.6	0.2	0.1	0.1	U	0.8
23	4.2	2.7	0.3	0.2	0.2	U	4.9
24	0.1	U	U	U	U	U	0.1
25	0.2	U	U	U	U	U	0.2
26	2.0	0.1	U	U	U	U	2.3
27	2.5	1.9	U	U	U	U	2.0
28	0.2	0.1	U	U	U	U	0.1
29	1.3	0.5	0.2	0.1	U	U	1.1
30	0.4	0.1	0.1	0.1	U	U	0.3
31	0.1	0.3	0.1	0.1	0.1	0.1	0.1
32	0.3	0.2	0.2	0.2	0.1	U	0.3
33	U	U	U	U	U	U	U
34	0.9	0.7	0.6	0.5	0.5	0.6	0.8
35	20.3	12.5	2.6	1.2	0.5	0.3	23.4
36	4.3	1.7	0.5	0.2	0.2	0.2	3.6
37	1.5	0.9	0.3	0.3	0.1	U	1.4
38	3.4	3.2	2.8	2.4	0.6	0.3	4.9
39	0.7	0.6	0.4	0.3	0.3	0.2	0.7
40	5.5	5.9	5.8	3.4	1.0	0.7	6.1
41	3.8	2.8	3.1	2.0	1.3	1.2	3.1
42	22.5	18.2	14.1	1.9	2.0	2.0	26.2
43	2.9	2.1	1.2	0.9	0.8	1.0	3.0

[a] Data reported are averages of duplicate samples.
[b] U, Undetected (below detection limit).

36, 37, and 42) were readily biodegraded, quinaldine (compound 38) and carbazole (compound 43) appeared to be more persistent. PCP (compound 31) was most persistent, exhibiting no change over the course of the experiment.

Analytical chemistry data were summarized by plotting percent biodegradation versus time (Fig. 2). Biodegradation of the only *O*-heterocyclic compound monitored, dibenzofuran (compound 40), was slow to initiate, but by the end of the study over 87% of this compound was biodegraded. Likewise, biodegradation of the monitored *S*-heterocycles, thianaphthene (compound 35) and dibenzothiophene (compound 41), was extensive within the 14-day incubation period, but the initial rates of catabolism were slower than those observed with other creosote constituents.

To simplify PAH biodegradation data, these compounds were arbitrarily divided into three groups. Groups 1, 2, and 3 consist of PAHs containing 2, 3, and 4 or more fused rings, respectively. Group 1 PAHs and creosote phenolics were most readily biodegraded by indigenous microorganisms (Fig. 2). By comparison, group 2 and group 3 PAHs were biodegraded more slowly. This inverse relationship between the rate of biodegradation and the molecular weight of the substrate was also observed with the other monitored chemicals. The fact that lower-molecular-weight compounds usually have a greater aqueous solubility than the HMW chemicals suggests that this trend is related to bioavailability.

The catabolic abilities of most resident microorganisms appear to have been fully realized within 8 days of incubation, since the majority of the changes observed had occurred by this time. However, some low-level activity may have continued, since the concentrations of HMW PAHs decreased with continued incubation. A shift in the

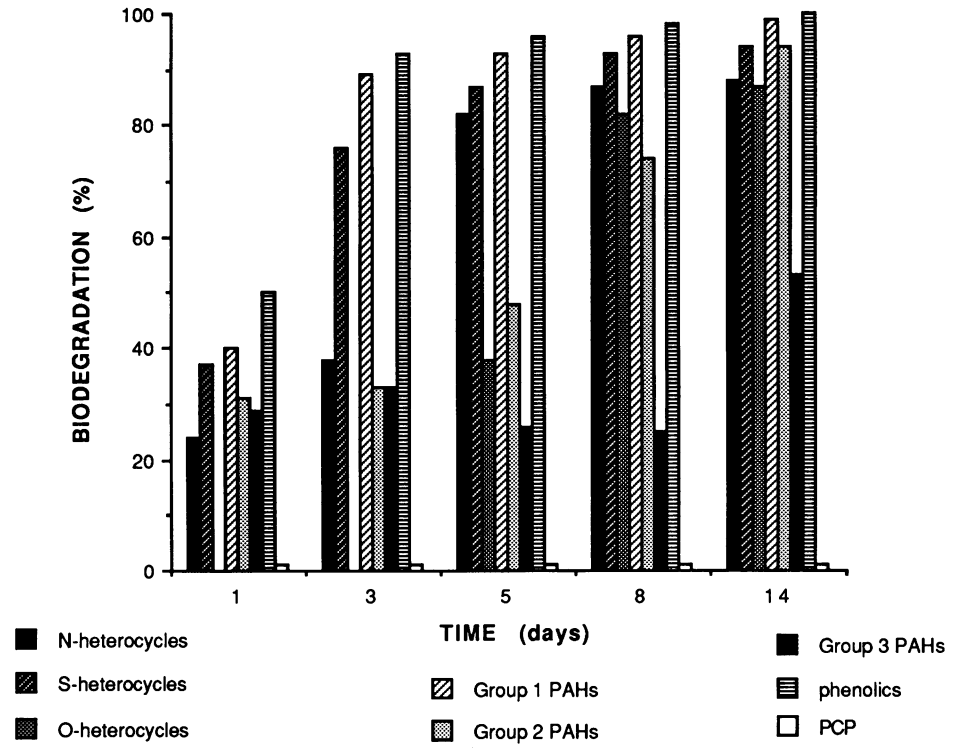

FIG. 2. Indigenous microbial activities against creosote and PCP. Percent biodegradation of *N*-heterocycles, *S*-heterocycles, *O*-heterocycles, group 1 PAHs, group 2 PAHs, group 3 PAHs, phenolics, and PCP is shown.

microbial population may also have contributed to this decrease. In general, there appeared to be an active population of indigenous microorganisms capable of degrading lower-molecular-weight creosote constituents. Conversely, the persistence of higher-molecular-weight chemicals and PCP suggests a dearth of indigenous microorganisms with relevant catabolic abilities. This is probably related to low bioavailability of these compounds, as discussed above.

These studies show that, under optimum conditions, many of the contaminants present in groundwater at the American Creosote Works site are susceptible to biodegradation by indigenous microorganisms. However, the following points must be considered: (i) studies were performed under controlled laboratory conditions (well mixed, aerobic, 30°C), (ii) inorganic nutrients were optimally available, (iii) relatively high concentrations (27 μg of bacterial protein per 25 ml of medium) of surface soil microorganisms were used to inoculate each flask, and (iv) the tests were performed within a closed system. Therefore, these rates and extents of degradation cannot be used directly to predict accurately those occurring in situ. However, the rate and extent of biodegradation of monitored contaminants observed in these studies are comparable to those generated during slurry-phase bioremediation of similarly contaminated material (18).

Biological assessment of biodegradation. From the analytical chemistry data described above, it was determined that, with the exception of PCP and certain HMW PAHs (compounds 12 and 14 to 21), most contaminants monitored were extensively biodegraded by the indigenous microflora within 14 days of incubation under controlled conditions. However, data generated from both the Microtox and the fish toxicity and teratogenicity assays showed that the bioremediated groundwater was still capable of eliciting a positive response. Microtox assays demonstrated a 50% effective concentration (EC_{50}) of 0.72 (a solution containing 0.72% parent material killed 50% of the test organisms) for filtered (silanized glass wool), untreated groundwater freshly recovered from the creosote-contaminated site. After 14 days of exposure to the catabolic activities of indigenous, "creosote-adapted" microorganisms, an EC_{50} of 3.8 was still observed. This result represents a relatively minor decrease (<10-fold) in the toxicity of the starting material.

Fish toxicity and teratogenicity assays showed essentially the same response. Filtered, untreated groundwater freshly obtained from the American Creosote Works site was toxic to embryos at 100% and teratogenic at 10 and 1% concentrations (Table 3). At the 1% concentration, all hatched larvae had terata, including stunted skeletal axes and de-

TABLE 3. Response of embryonic *Menidia beryllina* to untreated and biotreated filtered groundwater

Groundwater concn (%)	% of embryos dead		% of larvae hatched	
	With terata	No terata	Normal	With terata
Untreated groundwater				
0	0	3	97	0
100	0	100	0	0
10	100	0	0	0
1	67	13	0	20
Biotreated groundwater				
0	0	14	83	3
100	0	100	0	0
10	97	3	0	0
1	0	11	78	11

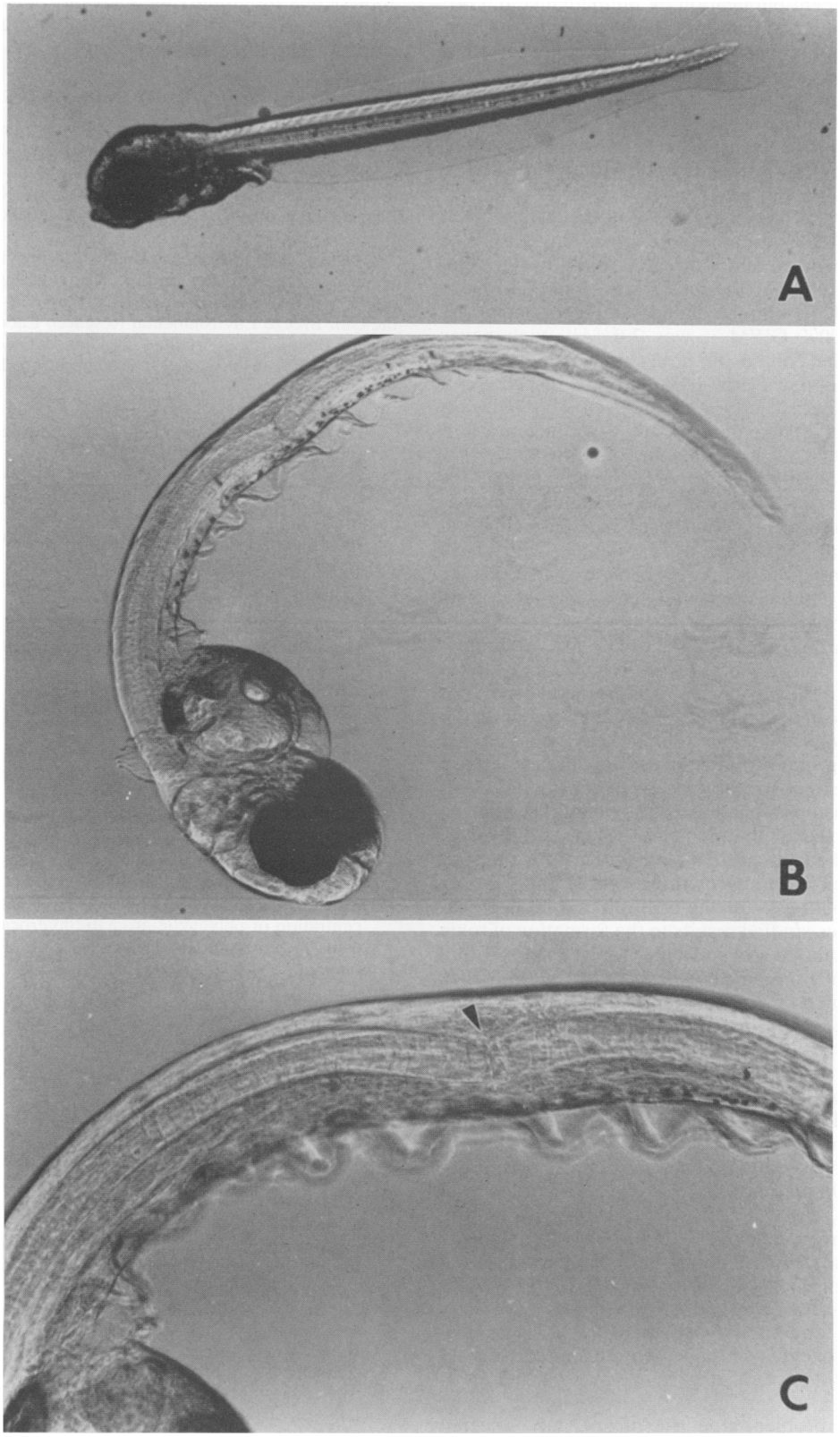

FIG. 3. Response of inland silversides exposed to creosote- and PCP-contaminated groundwater. (A) Newly hatched larvae from control embryo showing normal vertebral development. (B) Individual larvae exposed to 1% biotreated groundwater solution, exhibiting scoliosis and back aberration. (C) Detail of back aberration (arrow). Magnifications: A, ×25; B, ×50; C, ×100.

formed hearts. Typical examples of these effects are shown in Fig. 3 and in a recent publication by Middaugh et al. (14). Following biotreatment, reduction of embryo toxicity and teratogenicity of groundwater was not evident at the 100 and 10% groundwater concentrations, but at the 1% level marked improvement could be seen: 78% of the embryos that hatched produced normal larvae, while only 11% developed observable terata. This contrasts sharply with that observed with untreated groundwater at the 1% dilution level (no normal larvae, 20% terata).

Checks of dissolved oxygen at the end of the tests showed that levels were >89% saturation in controls and all GWM preparations of untreated and biotreated groundwater. The mean pH in untreated GWM ranged from 7.9 in controls to 5.5 in 100% GWM. In bioremediated samples, control pH was 8.0 and in 100% GWM it was 7.0. Although never observed during the course of these studies, if dissolved oxygen and pH dropped below 60% and 5.0, respectively, the tests were repeated. Therefore, responses observed were not due to effects of change in pH or oxygen concentration.

Based on analytical chemistry data, bioremediation appeared to offer a potentially effective treatment strategy for restoration of creosote- and PCP-contaminated groundwater. However, biotreatment did not result in a total reduction in the toxicity and teratogenicity associated with these materials. There are at least three possible explanations for these results: (i) catabolism of biodegradable chemicals resulted in the formation of toxic metabolites; (ii) the observed response is due to the cumulative effect of nonmetabolized, residual constituents; or (iii) toxicity and teratogenicity were due to the presence of chemicals that were not analyzed (i.e., chlorinated dioxins, inorganic compounds). The observed responses were not due to heavy metal toxicity, since previous groundwater analyses did not detect these compounds. A fourth possibility, although considerably less likely, is that the bacteria synthesized toxic and teratogenic compounds during the incubation period. There is, however, no precedent for the formation of teratogenic substrates by bacteria during their natural growth processes.

There is no strong basis for the position that biodegradation of the substrates studied results in the formation of toxic or teratogenic metabolites (4a). While it is recognized that fungi and eucaryotic organisms may transform PAHs to toxic metabolites such as *trans*-diol epoxides, some of which have been identified as ultimate carcinogens (8, 26), there is no reason to think that the action of bacteria will lead to the formation of such metabolites (5, 9). On the other hand, there are no detailed studies to show whether the bacterial metabolites of PAHs possess inherent toxicity beyond that of the parent chemical.

Alternatively, observed toxicological responses are probably related to the limited activity of indigenous microorganisms towards PCP, HMW PAHs, and other persistent contaminants. While an average of 53% of the HMW PAHs were degraded, a concentration of 19.5 ppm of cumulative, carcinogenic PAHs (compounds 14 to 21) remained after 14 days of incubation. Thus, the observed toxicity and teratogenicity response is likely due to the presence of these residual carcinogenic, teratogenic, and mutagenic chemicals. Dose-response experiments are in progress to resolve this question.

To address this problem, the use of specially selected biocatalysts with demonstrated ability to attack these compounds has been proposed (19, 20). Moreover, physical polishing (extraction) steps have been shown to be very effective in removing these residual contaminants (14). The effectiveness of a treatment strategy that integrates both approaches by employing such biocatalysts in conjunction with physical separation technology is currently being evaluated at the pilot-scale level.

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