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Epigenetic Toxicity of a Mixture of Polycyclic Aromatic Hydrocarbons on Gap Junctional Intercellular Communication Before and After Biodegradation

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Polycyclic aromatic hydrocarbons (PAHs) are known carcinogens, but most research on their toxicity in the development of human-risk assessment models has focused on genotoxicity. Many nongenotoxic PAHs, however, have been shown to be epigenetically toxic by disrupting gap junctional intercellular communication (GJIC), an effect which has been affiliated with tumor promotion. We therefore used GJIC as an epigenetic biomarker to assess the toxic effect of a nonaqueous phase liquid (NAPL) mixture of PAHs commonly found in coal tar and creosote products. The NAPL mixture consisted of toluene, naphthalene, 1-methylnaphthalene, 2-ethylnaphthalene, acenaphthene, fluorene, phenanthrene, fluoranthene, and pyrene. This mixture reversibly inhibited GJIC at a maximal and noncytotoxic dose of 60 μM. Inhibition occurred within 5 min, indicating a post-translational modification of gap junction proteins. Biodegradation of globules of this mixture suspended in mineral media by a microorganism isolated from creosote-contaminated soils resulted in the removal of all but three heavy PAHs: acenaphthene, pyrene, and fluoranthene. A reconstituted mixture of these three compounds showed results on GJIC activity identical to the original mixture relative to dose-, rate-, and time-responses, indicating that the toxicity of the PAHs was additive. The results suggest that bioremediation techniques that leave residual components of such NAPL mixtures in contaminated media can quantitatively but not qualitatively reduce their epigenetic toxic risk. Nonetheless, such bioresistant residuals may be environmentally less mobile than the biodegraded components of the precursor NAPLs.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in the environment and are known to be toxic to humans (*1*). The most significant accumulations of these compounds are commonly found at existing and former manufactured gas plant facilities, wood treatment sites, coal coking operations, and petroleum refineries. PAH compounds at these sites are often present as components of nonaqueous phase liquids (NAPLs) such as coal tars, creosotes, and petroleum distillates. Uncontrolled discharges, spills, and leaks of these NAPLs from storage facilities are often the cause for widespread contamination at many such sites (*1*). PAH compounds entrained in soil and sediment particles and contained in NAPLs are slowly but systematically released to aquatic and atmospheric systems causing a continued erosion of environmental quality. A major risk that PAHs pose to human health is cancer (*2*). Thus there is a critical need for assessing the carcinogenic potential of PAH contaminants present at such sites and the efficacy of remedial technologies in reducing carcinogenic risks.

Considerable research has been devoted to predicting the carcinogenicity of PAHs based on the genotoxic activity of these compounds (*3*, *4*). However, cancer is a multistage process that involves both genotoxic and epigenetic cellular events (*5*−*7*). The first stage of cancer, known as tumor initiation, requires irreversible genotoxic/mutagenic events (*8*, *9*), but the second stage of cancer, known as tumor promotion, is a consequence of a series of reversible, epigenetic events (*9*−*13*). Therefore, epigenetic data should also play a vital role in determining the carcinogenicity of PAHs (*14*). Ignoring the epigenetic properties of carcinogens can underestimate the risk of chemicals on human health (*15*). For example, the well-known carcinogen, benzo[*a*]-pyrene, after complete oxidation by ozone resulted in a mixture that was epigenetically more toxic than the parent compound (*16*). Simply measuring the genotoxic properties of the resultant mixture would have ignored the carcinogenic potential of this mixture by targeting only the initiating step of cancer and ignoring the tumor promoting steps of cancer. This is not a trivial oversight considering that spontaneous mutations can initiate cells, yet cancer is not expressed unless the cell is removed from growth supression via intercellular communication and mitogenic pathways are activated (*15*, *17*, *18*).

The epigenetic properties of carcinogens are often associated with the cell proliferative steps of cancer (*17*). Since many PAHs are complete carcinogens, it is assumed that these PAHs have cell proliferative properties. Unfortunately, the nongenotoxic proliferative properties and key molecular pathways of cell proliferation of most PAHs have not been well documented and nongenotoxic PAH carcinogens would therefore be overlooked. However, two studies have shown that benzo[*a*]pyrene and 7,12-dimethylbenzo[*a*]anthracene induced cell proliferation in primary human epithelial cells and vascular smooth muscle cells without involving the mutational activation of the cell proliferative gene Ha-, c-Ki-, or N-ras genes (*19*, *20*).

Epigenetic changes of gene expression involve extracellular, intracellular, and intercellular signaling events (*10*, *21*−*24*). In particular, intercellular communication through gap junctions is crucial in coordinating extracellular and intracellular events of an individual cell with its neighboring cells, thus enabling cells to exist communally as a multicellular organism (*17*, *21*−*25*). When cells become cancerous, they do not behave in harmony with their neighboring cells and

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reject the normal growth control checkpoints of a multicellular organism maintained via gap junctional intercellular communication (GJIC) (*25*, *26*). Therefore, it is not surprising that the down-regulation of GJIC results in uncontrolled cellular growth leading to the development of tumors (*21*, *25*). There are several lines of evidence that support the hypothesis that inhibited GJIC activity is related to carcinogenesis (*5*, *17*, *25*−*28*): (A) most if not all cancer cells have dysfunctional GJIC; (B) endogenous and exogenous tumor-promoting agents reversibly inhibit GJIC; (C) oncogenes down-regulate GJIC; (D) tumor suppressor genes and antitumor promoters up-regulate GJIC; (E) transfection of gap junction genes into GJIC-deficient and tumorigenic cells restores GJIC and normal growth regulation; and (F) gene knock-out mice that lack connexin 32, which is the gap junction protein of liver, had a 25- and 8-fold increase in spontaneous tumors in one year old male and female mice, respectively.

Altered GJIC has also been implicated in teratogenesis (*29*), reproductive dysfunction (*30*−*32*), alteration of muscle contractions in heart and uterus (*33*, *34*), and neuropathy (*35*). Obviously, the interruption of GJIC disrupts the homeostasis of the organism leading to various chronic diseases, depending on the cell- and tissue-type where communication is altered. Recently, mutated connexins have been identified in the Charcot-Marie Tooth syndrome (*35*), in hereditary nonsyndromic sensorineural deafness (*36*), in the autosomal dominant "zonular pulverulent" cataract (*37*), and visceroatrial heterotaxia syndrome (*38*). Considering the central role GJIC plays in the normal growth and maintenance of an organism, the measurement of intercellular communication serves as an important biomarker of nongenotoxic effects of environmental toxicants.

Among the technologies available for clean up of polluted sites bioremediation technologies are especially of interest because of low treatment costs and of the potential for PAHs to be mineralized to harmless constituents (*39*). Substantial laboratory research has been conducted in recent years to assess the potential for bioremediation of sites contaminated with PAH compounds. A number of bacterial species capable of degrading of PAH compounds have been isolated (*40*). However, only limited reports exist on the bacterial degradation of higher molecular weight PAHs (*41*). It appears from the existing literature that the two- and three-ring PAHs are rapidly degraded where as the higher molecular weight PAHs are recalcitrant (*42*). Reports of field scale treatment of soils contaminated with a mixture of PAHs are few in number, but in most cases the higher molecular weight PAHs constitute the residual fraction (*43*). The presence of significant residual fractions of higher molecular weight PAHs after biotreatment may result from (i) the lack of appropriate microorganisms and (ii) the poor bioavailability of such hydrophobic compounds due to their low aqueous solubility and their tendency to be sequestered in the soil organic matter matrices.

Given the supporting evidence in the literature that higher molecular weight PAHs may not be degraded extensively by bioremediation or by other water-based remediation technologies, it is important to evaluate how such remediation technologies may alter the carcinogenic risk caused by PAH mixtures at contaminated sites.

In this study, the biodegradability of a mixture of PAHs in a synthesized NAPL was evaluated under favorable conditions for biodegradation. The residual PAH fraction was analyzed, and, based on this analysis, a PAH mixture corresponding to the residual fraction was synthesized. The GJIC inhibition in cells exposed to the synthesized NAPL and the PAH mixture representing the residual fraction after biodegradation were evaluated using an epigenetic toxicity assay. This allowed an assessment of the efficacy of biodegradation in altering the carcinogenic potential of the PAHs in the NAPL form and the determination of antagonistic, synergistic, and additive effects of PAHs in the multicomponent nonaqueous liquid mixtures.

TABLE 1. Composition of the Synthesized NAPL

components	mole fraction
toluene	0.03
naphthalene	0.05
1-methylnaphthalene	0.22
2-ethylnaphthalene	0.11
acenaphthene	0.11
fluorene	0.05
phenanthrene	0.10
fluoranthene	0.09
pyrene	0.04

Materials and Methods

NAPL PAH Mixture. A NAPL consisting of toluene and eight other PAH components was synthesized in the laboratory with relative amounts as shown in Table 1. The relative mass distribution of the PAHs in the NAPL in Table 1 are in the range of those reported for various coal tars (*44*) and creosotes (*45*). All but toluene, 1-methylnaphthalene, and 2-ethylnaphthalene exist as solids at room temperature, and thus the synthesis of a NAPL containing all of the nine components required consideration of the liquid-phase stability criteria described by Peters et al. (*46*). The NAPL PAH mixture was prepared according to the procedure described in Mukherji et al. (*47*). Briefly, a stock NAPL mixture devoid of naphthalene was first prepared by dissolving predetermined amounts of all the components in Table 1 except naphthalene. A significant fraction of the toluene was then removed by evaporation in a rotary evaporator and then by purging the remaining mixture with nitrogen. Naphthalene crystals were then added to the NAPL stock solution in an amount sufficient to yield a mole fraction of 0.05. The NAPL phase mole fractions presented in Table 1 were determined by gas chromatographic analysis of a NAPL sample dissolved in hexane.

Microcosms. The extent of biodegradation of the PAH compounds in the NAPL was evaluated by comparing the masses of components remaining at several points in time in inoculated completely mixed batch reactors (CMBRs; comprised by continuously agitated, sealed 160-mL serum bottles) to those in sterile but otherwise identical reactors over a period of 21 days. The CMBRs contained 20 mL of mineral media and a 10-μL globule of the NAPL suspended in the mineral media. All reactors were setup in duplicate. Aseptic techniques were employed in the setup of the tests systems. The mineral media, glassware, and other accessories used were presterilized by autoclaving, and the NAPL was filtered through a 0.2-μm filter. CMBRs that were inoculated received 500 μL of 10^6 cfu/mL cell suspension. The bacterial culture used as inoculum was *Psuedomonas cepacia* CRE7, a strain isolated from creosote-contaminated soils. This microorganism is capable of rapid utilization of phenanthrene and was grown under aerobic conditions in batch cultures on phenanthrene as the sole carbon source. The growth medium was a supersaturated solution of phenanthrene in mineral media (170 mg of KH_2PO_4, 435 mg of K_2HPO_4, 850 mg of NH_4Cl, 668 mg of $Na_2HPO_4{\cdot}7H_2O$, 22.5 mg of $MgSO{\cdot}7H_2O$, 27.5 mg of $CaCl_2$, and 0.25 mg of $FeCl_3{\cdot}6H_2O$ in 1 L of deionized water). The microorganisms could also use naphthalene as a growth substrate but could not grow solely on any other substrate.

Sterile controls were not inoculated, and 500 mg/L of sodium azide was added to prevent biological growth from any contamination. All reactors were transferred to a

temperature-controlled (22 °C) rotary shaker after being closed with Teflon lined rubber stoppers and aluminum crimp-seals. The CMBRs were covered with aluminum foil to prevent exposure to light. At the time of setup, a pair of reactors was sacrificed to provide time zero data points for each compound.

At time points for which measurements were desired, a pair of inoculated and sterile CMBRs was sacrificed for analyzing the residual mass of each NAPL component. The reactors were removed from the shaker, unsealed for the addition of 15 mL of hexane, and then resealed. To facilitate extraction, the reactors were agitated in a wrist action shaker for 30 min. This resulted in the creation of an emulsion in the hexane phase. The emulsion was destabilized, and phase separation was achieved by placing the cultures in an ultrasonic bath for 10 min and subsequently storing the samples under quiescent conditions for another 20 min. Subsequently, the seals were broken, and samples from the upper hexane phase were pipetted into autosampler vials, which were then crimp-sealed and stored at about 5 °C prior to analysis. All samples from the test were analyzed simultaneously in a gas chromatograph (GC).

Gas Chromatography. NAPL samples were diluted in hexane prior to analysis. The diluted samples were analyzed in a Hewlett-Packard 5890A GC. The GC was fitted with a 5%-phenyl methyl DB5 (J&W Scientific) megabore column (30-m length, 0.53-mm internal diameter, and 1.5-μm film thickness). A flame ionization detector was used. The oven temperature was set at 60 °C for the first 5 min and then increased by 10 °C per min to a final temp of 250 °C. A splitless injection mode was used, and the sample volume injected was 2 μL. External PAH standards were used for calibration. External standards were prepared by dissolving individual PAH compounds in hexane. Calibration plots were generated from measurements of at least 5 concentrations. External standards were rerun after analysis of every 10 samples of unknown concentrations. All samples were analyzed in duplicate.

Gap Junction Intercellular Communication Assays. *Cell Cultures:* WB-F344 rat liver epithelial cell lines were obtained from Drs. J. W. Grisham and M. S. Tsao of the University of North Carolina (Chapel Hill, NC) (*48*) and used for the following reasons. Much of the in vivo tumor promotion assays were done in rat liver, specifically in the Fischer 344 rats, and the WB-344 cell line was designed to match in vitro work in a liver cell line from the same strain of rat. Also, the WB-344 cell line is a nontumorigenic immortalized cell line well-characterized for studies related to GJIC (*48*, *49*).

Cells were cultured in 2 mL of D-medium (Formula No. 78-5470EG, GIBCO Laboratories, Grand Island, NY) and supplemented with 5% fetal bovine serum (GIBCO Laboratories Grand Island, NY) and 50 μg/mL gentamicin (GIBCO Laboratories Grand Island, NY). The cells were grown in 35-mm diameter plastic tissue culture dishes (Corning Glass Works, Corning, NY) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 at 95% relative humidity. Bioassays were conducted on confluent cells that were obtained after 2 to 3 days of growth.

Chemical Exposures. Cells were exposed to different doses of two chemical treatments: (i) the synthesized NAPL and (ii) a mixture of acenaphthene, fluoranthene, and pyrene, the three NAPL components that were not significantly degraded during the biodegradation tests. The direct addition of the synthesized NAPL to the cell monolayer and the medium in the culture dishes posed two problems. First, the volumes of NAPL added did not readily dissolve or disperse in the culture medium (D-medium). Second, the cells were damaged or killed by the contact of the NAPL. These problems was overcome by delivering the appropriate dose of the synthesized NAPL by preparing stock solutions of the synthesized NAPL in a water soluble solvent acetonitrile (99.8% purity, Fisher Scientific Co.). Acenaphthene, fluoranthene, and pyrene (98% purity, Aldrich Chemical Co.) were dissolved in acetonitrile in appropriate concentrations for the second chemical treatment. Doses at which inhibition was measured are reported as summed concentrations (μM) of all NAPL components or of all PAH compounds added to the D-medium. The highest dose of the NAPL, 165.8 μM, was achieved by adding 5 μL of a 100x stock solution of the NAPL in acetonitrile in a culture dish containing 2 mL of D-medium and a monolayer of cells. The other doses of the NAPL correspond to further dilutions.

The volumes of the stock solutions that were added directly to the culture medium in each plate ranged from 5 to 30 μL. Vehicle controls were added to the cells at a volume of acetonitrile equivalent to the volume of PAH stock solution used. Acetonitrile is used because it is noncytotoxic and noninhibitory up to 2% (v/v) (*50*). Cells were exposed to a maximum of 1.8% (v/v) acetonitrile in culture medium. The cells were exposed to the different doses of the two chemical treatments for 30 min. The exposure time was determined based on the results of time response experiments in which cells were exposed for various lengths of time to the compound mixtures at a dose that caused inhibition of GJIC.

GJIC Measurement. GJIC was determined by using the scrape loading/dye transfer (SL/DT) assay adapted from El-Fouly et al. (*51*) and described in detail by Upham et al. (*50*). The SL/DT assay was done immediately following chemical exposure or, with respect to the time recovery experiments, following the reincubation period. After the treatment with the PAHs, cells were rinsed 3× with phosphate buffered saline (PBS), and 1 mg/mL Lucifer Yellow, lithium salt (Sigma Chemical Co, St Louis, MO) dissolved in PBS, was added. The cells were then scraped with a surgical blade scalpel to introduce the dye into the cells. Three minutes following the scrape, the cells were washed 3× with PBS and fixed with 4% formalin solution.

The migration of the dye in the cells was observed using a Nikon epifluorescence phase microscope, illuminated with an Osram HBO 200-W lamp and equipped with a 35-mm camera (Nikon Inc., Nikon, Japan). Photographs of the cells were taken at a magnification of 200×. GJIC was assessed by comparing the distance the dye traveled in the chemically treated cells to the distance the dye traveled in the vehicle controls and reported as a fraction of the control (FOC). The rate of dye migration in the control was measured by dividing the average distance the dye traveled in micrometers by 3 min, which is the time allowed for dye migration through gap junctions. Experiments were done at least in triplicate, and the results were reported as an average ± the standard deviation at the 95% confidence interval.

Cytotoxicity. Cytotoxicity was determined by a viable dye uptake assay using neutral red, developed by Borenfreund and Puerner (*52*) and adapted to measuring cytotoxicity in WB-cells according to the method of Weis et al. (*53*). Cytotoxicity was reported as a fraction of the control (FOC).

Results

Biodegradation Experiments. Mass depletion in excess of the control and the presence of abundant populations of the microorganism in the inoculated CMBRs during the test period were attributed to biodegradation. The component mass in the inoculated and control microcosms at the beginning (day 0) and the end of the test period (day 21) are presented in Figure 1. Variations for duplicate measurements of each sample were less than 2%, and variations for measurements from duplicate reactors were less than 4%. Almost complete depletions of naphthalene, 1-methylnaphthalene, 2-ethylnaphthalene, fluorene, and phenanthrene were observed in the inoculated reactors. No significant

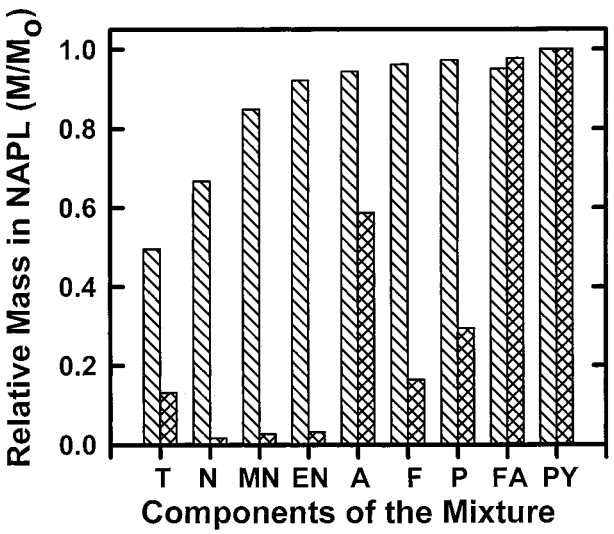

FIGURE 1. The relative mass of each PAH component in the NAPL prior to biodegradation (hatched) and after biodegradation (cross-hatched) (T = toluene, N = naphthalene, MN = 1-methylnaphthalene, EN = 2-ethylnaphthalene, A = acenaphthene, F = fluorene, P = phenanthrene, FA = fluoranthene, and PY = pyrene). Variations for measurements from duplicate reactors were less than 4%.

depletions of mass for pyrene and fluoranthene and only partial degradation of acenaphthene occurred over the test period. Some losses of components were noted from the controls over time, especially for toluene and naphthalene, most likely due to volatilization during setup and sample handling.

The residuals of the biodegradation tests were not directly used for the GJIC tests because the presence of degradation products, bacterial cell fragments, various proteins, etc. could affect GJIC in addition to the effects of the residual compounds. Instead a solution of acenaphthene, fluorene, and pyrene in acetonitrile was prepared to represent the residual NAPL components in the serum bottles. Based on the results presented in Figure 1, endpoints of biodegradation were assigned to determine the appropriate dose for biodegradation residuals to be used in the GJIC test. The biodegradation endpoints were assigned to be 50% of the original mass of acenaphthene and 100% of the mass of the fluoranthene and pyrene in the synthesized NAPL. The three compounds were dissolved at these mass proportions in a volume of acetonitrile equal to the corresponding NAPL volume.

GJIC Assays. There was no difference in dose response on GJIC between the NAPL and the residual PAH fraction after biodegradation (Figure 2). Complete inhibition occurred within the total mixture concentration of 60 μM. The PAH mixtures were not very cytotoxic up to a total concentration of 400 μM (Figure 3). An ANOVA of the data and a Dunettes post-hoc test indicated no significant difference in the cytotoxicity of the mixtures vs the control below the total concentration of 150 μM for both the original NAPL and the residual NAPL samples. When the cells were allowed to recover in fresh media, in the absence of the PAH mixtures, GJIC was restored significantly within the first 80 min. GJIC was completely restored within 2 h (Figure 4). Recovery was similar for the NAPL samples and the residual PAH fraction samples from biodegradation of the NAPL. The kinetic response of the cell's ability to communicate when treated with these two samples was identical (Figure 5). Significant inhibition occurred within 5 min.

Acenaphthene, pyrene, and fluoranthene, the PAHs remaining after biodegradation of the mixture, were used to test for antagonistic, synergistic, and additive effects on GJIC

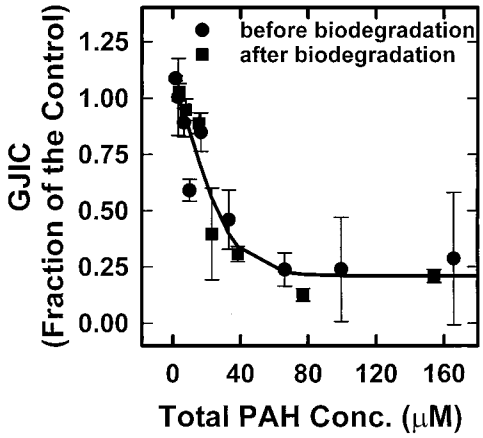

FIGURE 2. The dose response of PAHs on GJIC before and after bioremediation. The incubation time was 30 min, and the rate of dye migration in the control was 55.3 ± 4.3 μm/min. Each value represents an average of at least three replicates ± the standard deviation at the 95% confidence interval.

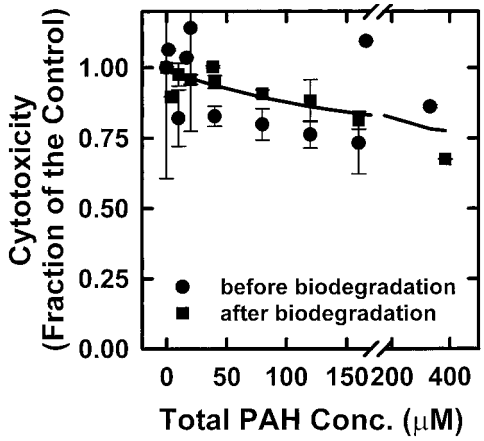

FIGURE 3. The cytotoxic response of PAHs before and after bioremediation. The incubation time was 30 min. Each value represents an average of at least three replicates ± the standard deviation at the 95% confidence interval.

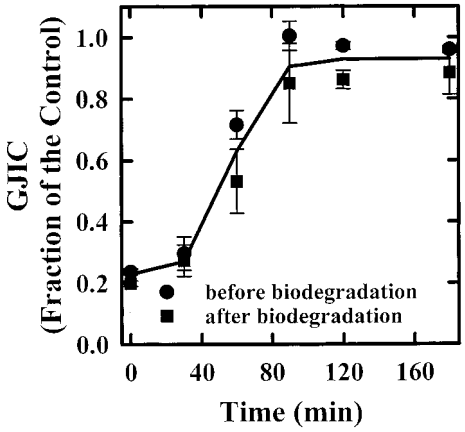

FIGURE 4. The recovery from GJIC-inhibition after the PAH-containing media was replaced with PAH-free media. Cells were initially incubated with the PAH-containing media for 30 min. The control rate was 59.0 ± 4.3 μm/min. Each value represents an average of at least three replicates ± the standard deviation at the 95% confidence interval.

activity. A total concentration of 20 μM was used to study the one-, two-, and three-way interactions because at this concentration the mixtures inhibited GJIC approximately 50%

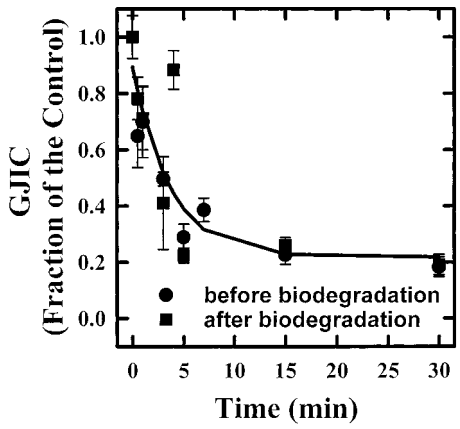

FIGURE 5. Time response of cells to inhibition of GJIC by the PAH mixtures. The total concentration of the PAH mixture was 80 μM. The rate of dye migration in the control was 76.3 $\pm$ 4.9 μm/min. Each value represents an average of at least three replicates $\pm$ the standard deviation at the 95% confidence interval.

TABLE 2. Interactive Effects of PAHs on GJIC Activity

compound	fraction of the control[a] (foc)
acenaphthene	$0.49 \pm 0.02^{a,b}$
pyrene	$0.49 \pm 0.11^{a,b}$
fluoranthene	0.43 ± 0.05^{a}
acenaphthene + fluoranthene	$0.56 \pm 0.10^{a,b}$
fluoranthene + pyrene	$0.61 \pm 0.08^{b,c}$
acenaphthene + pyrene	$0.51 \pm 0.06^{a,b}$
acenaphthene + pyrene + fluoranthene	$0.61 \pm 0.06^{b,c}$

[a] Values with the same letters were not found to be significantly different at the $p \leq 0.05$ level as determined using the Tukey-pairwise multiple comparison test following an ANOVA. Each value is an average of three replicates.

(Figure 2). No significant synergistic effects were observed between the PAHs (Table 2). A statistically significant antagonistic effect was observed between the two-way interaction of fluoranthene and acenaphthene vs fluoranthene alone and the three-way interaction of acenaphthene, pyrene, and fluoranthene vs fluoranthene alone (Table 2).

Discussion

Our primary objective was to determine the epigenetic toxicity of an environmentally relevant mixture of aromatic hydrocarbons before and after biodegradation. The biodegradation patterns of the components of our NAPL mixture were similar to those reported by Mukherji and Weber (*54*) using the same NAPL mixture with other bacterial cultures. In those experiments, the microorganisms used were a pure culture, *Psuedomonas putida*, strain ATCC 17484, in one set of experiments, and a mixed culture RET PA101 in another set of experiments. These microorganisms were grown on naphthalene prior to inoculation of the NAPL-water systems. The microorganisms transformed all compounds in the NAPL other than fluoranthene and pyrene. In the experiments discussed in this study as well as those reported by Mukherji (*54*), the mineral medium contained significant amounts of the macronutrients (available nitrogen and phosphorus), the headspace contained sufficient O_2 to allow total complete chemical oxidation of all the NAPL components, and abundant microorganisms were present in the systems. Film transfer coefficients for all of the NAPL components have been determined to be similar in magnitude (*47*), and thus mass transfer resistance *per se* is unlikely to be the cause for limited biodegradation of the three residual components. Thus the degradation of only a fraction of the acenaphthene and the absence of degradation of fluoranthene and pyrene is likely due either to the very low aqueous solubility of the compounds, which in itself would of course lower the actual rate of mass transfer to solution, and/or to the absence of microorganisms capable of degrading these compounds.

The carcinogenicity of nongenotoxic carcinogens has been attributed to epigenetic changes via perturbations of cell signaling pathways (*15*, *18*, *49*). Our results showed that mixtures of PAHs, which include those found in coal tar extracts (*42*), can reversibly inhibit GJIC at noncytotoxic doses (Figures 2−4). These epigenetic effects in addition to or instead of their genotoxic properties imply that our PAH mixtures could be potentially carcinogenic. The most notable result from the GJIC assays is that there is very little change in the pattern of GJIC inhibition relative to dose (Figure 2), time (Figure 4), and time of recovery (Figure 5) caused by the PAH fraction that was found to be recalcitrant to biodegradation in comparison to the entire suite of NAPL components.

Significant inhibition of GJIC by the PAH mixtures occurred within 5 min (Figure 4), which is a very short time period and suggests that the regulation of gap junctions was post-translational. Also, 5 min is probably not enough time for the metabolic activation of a PAH. These results contrast with the genotoxic properties of PAHs in which the parent compound must be metabolically converted to a strong electrophile (*55*, *56*). Also, rat liver epithelial cell lines such as WB-F344 have very low cytochrome p450 activity and are probably incapable of metabolizing PAHs (*57*−*59*). This time period of inhibition is very similar to results reported for single-component PAH solutions (*15*, *16*, *50*, *53*, *57*) of naphthalene, 1-methylnaphthalene, fluorene, phenanthrene, fluoranthene, and pyrene. Likewise the dose response and time of recovery of the NAPL PAH mixture was also similar to the results of single-component PAH solutions (*15*, *16*, *50*, *53*, *60*). The similar biological activity between single vs multicomponent PAH solutions suggests that the effects of PAHs on GJIC inhibition are additive for doses up to 60 μM.

The additive effect of PAH mixtures is further supported by the tests of GJIC inhibition by acenaphthene, fluoranthene, pyrene, and two- and three-component mixtures of these compounds at a total dose of 20 μM. These PAHs were residuals of the NAPL PAH mixture after biodegradation (Figure 1). A small statistically significant difference was found between the three component system (foc = 0.61) vs fluoranthene (foc = 0.43) and the two component system containing fluoranthene and pyrene (foc = 0.61) vs fluoranthene (foc = 0.43) (Table 2). However, based on the dose response of the PAH mixtures (Figure 2) and the dose responses reported by Weis et al. (*53*) the estimated difference in dose between the foc values of 0.43 and 0.61 is only 5−10 μM, which is probably not biologically significant.

The environmental significance of our results is severalfold. One is the assessment of the epigenetic toxicity of an environmentally relevant chemical mixture. Risk assessments of toxicants on human health historically have relied primarily on an individual environmental agent; yet, human exposure is rarely limited to a single chemical. Strategies to limit human exposure to environmental toxicants, i.e., the removal of toxicants by remediation methods such as bioremediation, must therefore incorporate a mechanistic understanding of the toxicity of "mixtures" rather than single-component systems.

We demonstrated that the effect of our PAH mixtures was additive, which means that the epigenetic toxicity of the mixture depends primarily on the total concentration of the compounds with no biologically significant antagonistic or synergistic effects of a component within the mixture. Therefore, there was no qualitative change in the toxicity of the mixtures on GJIC either before or after partial biodeg-

radation, i.e., the toxicity of the three-component mixture was the same as that of the nine-component mixture (Figure 2). However, there was a quantitative change in that all but three of the PAHs were removed by bioremediation, thereby decreasing the total dose of PAHs, hence decreasing the potency of the original mixture.

Another environmental factor to consider for risk assessment is the epigenetic toxicity of the PAH metabolites. Although our residual NAPL extract did not contain any metabolites of PAHs, these metabolites could potentially be more or less toxic than the parent compounds. For example, ozonated mixtures of benzo[*a*]pyrene, pyrene, and fluoranthene, in which the parent compound was completely removed, resulted in mixtures that were more toxic than the starting material (*16*, *50*). Therefore, a complete risk-analysis of a biodegraded NAPL mixture will eventually need to assess the epigenetic toxicity of the byproducts.

In summary, the study demonstrates that *Pseudomonas cepacia CRE7* grown on phenanthrene was able to degrade most of the two- and three-ring PAHs in a synthesized NAPL comprised of nine aromatic hydrocarbons. Significant amounts of acenaphthene, fluoranthene, and pyrene, the higher molecular-weight or "heavy" PAHs in the mixture, were not degraded. There was no qualitative difference in the epigenetic toxicity of the NAPL residual after biodegradation over an extended time period, but there was a quantitative reduction in the total mass of NAPL, thereby diminishing the overall risk to humans.

Based on the results, it may be argued that evaluations of remediation performance should include consideration of epigenetic bioassays such as GJIC inhibition in the determination of cleanup criteria.

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