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TOXICITY, PERSISTENCE AND INFLUENCE OF PYRENE-4,5-DIONE ON PAH BIODEGRADATION

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ABSTRACT: Multi-ring PAHs are metabolized by soil microorganisms through a series of oxidation steps, initially producing quinone type compounds. Toxicity and persistence of one oxidized metabolite, pyrene-4,5-dione (P45D), an intermediate metabolite of pyrene degradation, was investigated. Methods for the synthesis, handling and analysis of P45D were developed in the laboratory and its toxicity, persistence and effect on parent PAH degradation were evaluated. Water solubility of P45D was estimated by high performance liquid chromatography (HPLC) analysis and found to be 1.66 (±0.34) mg/L. Toxicity of P45D in saturated solution was examined by Microtox, returning an EC50 of 0.46 to0.63 mg/L. Degradation of pyrene in the presence of P45D was monitored in spiked soil microcosms. Pyrene showed greatly reduced initial mineralization kinetics in the presence of P45D, but total mineralization over 180 days was not significantly different from the control microcosm. Recovery of spiked P45D from soil microcosms indicated that 15 to 18% of the initial concentration of P45D in the soil could be solvent-extracted after 180 days of biological treatment.

INTRODUCTION

The following work was designed to evaluate the toxicity and persistence in aerobic soils of pyrene –4,5 dione (P45D), an oxidized byproduct of pyrene metabolism by soil microorganisms (Kazunga and Aitken 2000). Methods for the synthesis, handling and analysis of P45D were developed and a known quantity of the quinone was added to soil for degradation studies. Additionally, the effect of P45D on biodegradation of pyrene in soil was investigated. The fate and persistence of quinone metabolites of polycyclic aromatic hydrocarbons (PAHs) in soils is significant to determining the hazard presented by PAH contaminated media, as well as possible eco-toxicity and inhibition of parent compound degradation. The possible effect of PAH metabolites must be considered in developing an integrated risk profile of contaminated soils and sediments and predicting the long-term safety and productivity of contaminated media.

PAHs exert their toxic, mutagenic and carcinogenic effects after activation by oxidizing enzymes (Fouchecourt et al. 1999). Several enzyme systems produce potentially hazardous PAH-quinones (Flowers-Geary et al. 1996). Research has indicated that the genotoxicity of PAH contaminated soils may be increased by partial biodegradation of the parent PAH compounds (Belkin et al. 1994). Likewise, the presence of these compounds may inhibit further degradation of parent PAHs and contribute to PAH persistence.

Few studies have looked at the effect of different concentrations of intermediate metabolites on PAH degradation in soils (Ogunseitan and Olson 1993; Wischmann and Steinhart 1997). PAH metabolites may exert toxic effects on sensitive soil organisms or

serve as alternate substrates for microbial carbon and energy needs. The partially oxidized metabolites of PAHs may be more prone to chemical condensation with soil organic matter or the formation of large insoluble polymers. Evaluation of metabolite persistence and toxicity is an essential first step in determining the overall impact of PAH metabolites on the hazard posed by contaminated media.

MATERIAL AND METHODS

Pyrene-4,5-dione (Figure 1) was synthesized in the laboratory by oxidation of pyrene with ruthenium dioxide and sodium periodate (Kazunga and Aitken 2000). Purity was checked by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) against an authentic standard synthesized by C. Kazunga and identified by NMR.

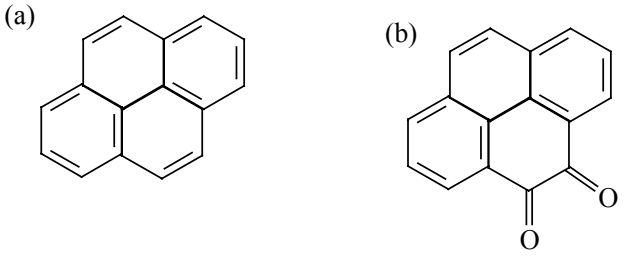

FIGURE 1. Chemical structures of pyrene (a) and pyrene-4,5- dione (b).

Uncontaminated Schenck Forest (SF) soil (mineral soil, pH 4.8, CEC 3.4, f_{oc} 3.5), was collected from North Carolina State University, Raleigh, NC in the upper 15 cm of the top horizon. Characteristics of this soil have been reported elsewhere (Guthrie and Pfaender 1998). The uncontaminated forest soil neither exhibits evidence of prior PAH contamination nor mineralizes PAH over a six-month period (Guthrie and Pfaender 1998). PAH contaminated soil was collected from Reilly Tar and Chemical Company, St. Louis Park, MN (MN soil), a former Superfund site with historic PAH contamination and a microbial community known to be capable of mineralizing PAHs (Guthrie and Pfaender 1998). The MN soil was used to seed the spiked pristine soil with a PAH metabolizing community.

Air-dry Schenck Forest (SF) soil was spiked with approximately 833.33 Bq of [^{14}C] pyrene and 400 μg g^{-1} unlabeled pyrene. The PAH spiked soils were aged 2-3 months before addition of P45D and the microbial inoculum from the MN soil. P45D was dissolved in DCM and added to the PAH spiked SF soil, then mixed with the inoculum. The final P45D concentration used in both pyrene-spiked microcosms was 0.40 mg P45D g^{-1} wet weight of soil. Control microcosms were constructed as above, without P45D addition. All microcosms were constructed in triplicate, with triplicate untreated control microcosms set up simultaneously.

For each treatment, 12 g. soil was placed in 3-60 mL serum vials and sealed with Teflon/butyl rubber crimp seals. Vials were stored at 26°C in the dark. Soil from each treatment was combusted in three replicate 0.3 to 0.5 g samples in an RJ. Harvey Biological Oxidizer (R. J. Harvey Instrument Corporation, Hilldale, NJ) for 4 min to determine initial activity. Trapping efficiencies were calculated between 95 and 100% for each run. Activity was counted on a Packard TriCarb 1900TR liquid scintillation counter

(Packard Instruments, Meriden, Ct.) for 10 min each.

The soil treatments were analyzed for $^{14}CO_2$ production over time. Microcosms were sampled every week for one month and thereafter, every two weeks. Headspace gas was purged through the serum vials and $^{14}CO_2$ was trapped in 8 mL ethylamine scintillation cocktail (R. J. Harvey) and counted as above.

After 180 days, the microcosms were sacrificed. Headspace gas was sampled as above to recover residual $^{14}CO_2$. The soils were extracted with acid then rinsed with 10 mL of DI H_2O and were subjected to a base/ humic acid extraction protocol as per (Schnitzer and Schuppli 1989). The microcosms were then extracted in with dichloromethane (DCM) to recover residual pyrene. The extracts were scintillation counted to determine solvent extractable ^{14}C in each fraction. The DCM extracts were subsequently analyzed by HPLC. Mass balances were then calculated by totaling the activity in the acid/aqueous fraction, the base fraction, the DCM fraction, the residual soil and the fraction mineralized.

Solvent extracts of microcosms were analyzed by HPLC (Waters 600E System controller and 717 Autosampler, Supelco LC-PAH column (250mmx4.6mm id) Millennium 2010 software Millipore Corporation Milford, MA) against authentic standards (Supelco). Pyrene eluted at approximately 23 minutes. Pyrene was detected by fluorescence (Waters 470 Scanning Fluorescence; 260 and 374 nm) P45D eluted at approximately 10 min and was identified by UV absorbance (Spectroflow 757 Absorbance Detector 254 nm, Kratos Analytical) and identified by comparison with authentic standards synthesized by C. Kazunga. BaP eluted at 34.8 min and was also identified by UV absorbance.

A saturated aqueous solution of P45D was made by adding 4 mg of P45D to 5 mL of DI H_2O in a sealed amber vial with a Teflon stir-bar. The solution was stirred vigorously for 1 hr under an N_2 headspace and then passed through a 0.2 μm pore nylon filter. The resulting solution was then analyzed by six replicate HPLC injections against an external standard and immediately used in the Microtox evaluation of toxicity.

Toxicity of the P45D aqueous solution was evaluated using the Microtox Basic Test for Pure Compounds (Azure Environmental, Carlsbad, CA.) using the Microtox system and software. The test was conducted in duplicate as per manufacturer instructions using reduction in luminescence of the test organism *Vibrio fischeri* as an indicator of toxicity. Toxicity was calculated as EC50, for sample concentration causing a 50% decrease in light output. An EC50 was calculated for both 5 and 15 min exposures.

RESULTS AND DISCUSSION

HPLC analysis of an aqueous solution of P45D indicated that the concentration of P45 D in the aqueous solution was 1.66 ±0.34 mg/ L (7.15 ± 1.4 μM). Pyrene solubility is evaluated between 0.135 and 0.175 mg/L (0.68 μM to 0.865 μM) (Mackay et al. 1992). The above aqueous solution was evaluated immediately after preparation using the Microtox system. The Basic Toxicity test involves exposing a series of compound dilutions to *Vibrio fischeri*. Duplicate tests indicated an average EC50 of 0.63 mg/L for the 5 min. toxicity and 0.46 mg/L for 15 min. value. The EC50 for this compound is well below the aqueous solubility.

The effect of P45D on the degradation of pyrene in sample microcosms indicates that there is initial inhibition of mineralization and a significant decrease in overall

mineralization of pyrene after 180 days of incubation ($P \leq 0.05$) (Figure 2). Conversely, there is a small but significant increase in radioactivity in the DCM extract of the P45D treated microcosm relative to the control (data not shown). There is no significant difference in the amount ^{14}C from pyrene in the residual soil compared to the control. The difference in mineralization between the two treatments is split between the DCM extractable fraction and the combusted residual and the difference obscured by the variance in the fractions.

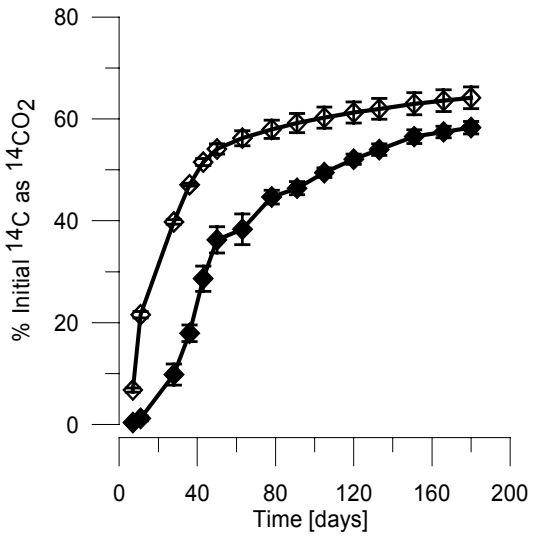

FIGURE 2. Mineralization of pyrene in soil microcosms. Pyrene incubated with P45D 0.4mg/g (♦) and without (◊). Data are the means and standard deviations of triplicate microcosms at each time point.

The mechanism by which P45D inhibits the degradation of pyrene is unknown. P45D could exert a toxic effect on sensitive species, such as with the *Vibrio fischeri* in the Microtox experiment. However, Kazunga and Aitken (2000) observed no toxic effect of P45D on pure cultures of microorganisms. Flowers-Geary et al. (1996) also observed no direct toxic effect on pure culture organisms after treatment with other PAH quinones. The lack of toxicity to these microorganisms may indicate that P45D may function as a competitive substrate, or a metabolic inhibitor that does not affect overall cell viability.

DCM extracts of the soil microcosms were analyzed by HPLC to determine recovery of P45D from the soil at various times after spiking. Sample soil from the [^{14}C] pyrene and P45D spiked microcosm was extracted immediately after set up and again after 180 days of incubation. Some of the pyrene and P45D spiked soil was placed in vials without PAH degrading microorganisms and incubated for 1 month in the absence of a PAH degrading community.

Data from the solvent extracts of the microcosms and subsequent HPLC analyses are presented in Table 1. The recovery of P45D from soil almost immediately after spiking was 78.36% of the initial addition, but after 30 days in the absence of PAH metabolizing organisms, the recovery dropped to 33.59 ± 1.70% based on extraction of triplicate microcosms. The uninoculated microcosms showed no pyrene mineralization.

By contrast, recovery of [^{14}C] pyrene from pyrene spiked SF soil aged 30 days in the same manner was 90.13%.

After 180 days of incubation with a PAH degrading community, the recovery of P45D was about 15%, whereas [^{14}C] pyrene recovery in the DCM extract was 8.44% of initial ^{14}C. It is unknown whether the remaining P45D was mineralized, humified or irreversibly sorbed to soil particles. In any case, P45D appears to be more persistent than pyrene in the solvent extractable portion of the microcosm.

TABLE 1. Percentage of initial P45D determined by HPLC in DCM extracts of spiked soils. The Day 0 soil was a single extraction; other values are an average (n=3) and standard deviation.

Soil Incubation	% of initial P45D Recovered
Microcosm Day 0	78.36 %
No degrading community (1 month)	33.59 ± 1.70%
Pyrene Microcosm 180 days	14.99 ± 1.27%

CONCLUSIONS

Results from the above experiments indicate that P45D is toxic to at least a subset of microbes. The compound displays significant toxicity in the Microtox test at concentrations well below its experimentally determined aqueous solubility. Based on solvent extraction experiments, spiked P45D is also more persistent in soil than freshly added pyrene. Solvent extractability has been linked to the 'reversibility' of sorption and may indicate that this fraction of the total mass of compound is more mobile than the unextractable mass. Initial pyrene mineralization is significantly inhibited in the presence of P45D; however, long term inhibition of mineralization was not found.

Extractable P45D disappeared from the soil system over time. Close to 22% of the compound was unextractable after less than 24 hours incubation. As yet, it is unclear if the disappearance of P45D is from degradation, sorption, covalent reaction or incorporation into microbial biomass. Judging from the disposition of pyrene and other PAH, P45D probably undergoes all of these processes to varying extents. Incubation of P45D and its subsequent disappearance in the absence of a PAH degrading community may indicate that P45D is significantly sorbed or spontaneously humified. However, based on the results of the microcosm extractions, P45D is more persistent in the solvent extractable fraction than pyrene.

Prediction of release, transport and transfer of toxic compounds is essential to risk assessment of contaminated sites. As clean-up standards transition from a strict numerical basis to a risk-based standard the identification of significant compounds and the evaluation of toxin mobility is essential. Consequently, by virtue of the fact that P45D is more water soluble and persistent in the solvent extractable fraction of the soil, is a catalyst for generation of reactive oxygen species, and displays toxicity to sensitive organisms, it may pose a significant hazard at contaminated sites.

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