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Variation in toxicity during the biodegradation of various heterocyclic and homocyclic aromatic hydrocarbons in single and multi-substrate systems

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ABSTRACT

In the present study, an attempt was made to understand the variation in the toxicity during the biodegradation of aromatic hydrocarbons in single and multi-substrate system. The bacterial bioassay based on the inhibition of dehydrogenase enzyme activity of two different bacterial sp. *E.coli and Pseudomonas fluorescens* was used for toxicity assessment. Amongst the chosen pollutants, the highest acute toxicity was observed for benzothiophene followed by benzofuran having EC_{50} value of 16.60 mg/L and 19.30 mg/L respectively. Maximum residual toxicity of 30.8% was observed at the end during the degradation of benzothiophene. Due to the accumulation of transitory metabolites in both single and multisubstrate systems, reduction in toxicity was not proportional to the decrease in pollutant concentration. In multi-substrate system involving mixture of heterocyclic hydrocarbons, maximum residual toxicity of 39.5% was observed at the end of biodegradation. Enhanced degradation of benzofuran, benzothiophene and their metabolic intermediates were observed in the presence of naphthalene resulting in significant reduction in residual toxicity. 2 (1H) - quinolinone, an intermediate metabolite of quinoline was observed having significant eco-toxicity amongst all other intermediates investigated.

1. Introduction

Mixture of aromatic hydrocarbons encompassing monocyclic, polycyclic and heterocyclic hydrocarbons is the major cause for the contamination of environmental matrices such as water, soil and groundwater. Anthropogenic and industrial activities lead to the release of these contaminants including discharges from the coke oven plants (Li et al., 2003), coal gasification plants (Shi et al., 2014), oil sludge wastes (Reddy et al., 2011) and waste from wood preservation activities such as coal tar creosote. Appropriate choice of treatment technique is essential to remediate such sites without leaving any residual impact on the existing environment.

Bioremediation is an effective and widely adopted approach for degradation of various xenobiotic pollutants. The efficacy of biodegradation is often assessed in terms of decrease in contaminant concentration which is not always a direct indication of decrease in the biological activity of that particular organic pollutant. In many cases biological transformation of organic pollutants resulted in the formation of transitionary metabolites which can be more toxic than the parent compounds even at very low concentrations. To evaluate the biodegradation efficiency, assessing the reduction in toxicity is a more reliable and an effective approach than measuring the residual concentration of the parent compound. Therefore, it is important to combine chemical analysis and toxicity tests for assessing the complete mineralization of various organic pollutants.

In recent years, several biological assays have been developed and evaluated as a supplementary tool to monitor the ecotoxicity of various contaminants using aquatic organisms, bacterial and plant species. Bacterial bioassays are better indicators of ecotoxicological effects and have gained significant popularity because of their ease, reproducibility, lesser sample volume and exposure time (Parvez et al., 2006). Use of different biological assays to monitor the efficacy of bioremediation has been reported in several studies (Boyd et al., 1997; Phillips et al., 2000; Abbondanzi et al., 2003; Nunes-Halldorson et al., 2004; Nweke and Okpokwasili, 2010; Steliga et al., 2015). Phillips et al. (2000) evaluated the application of six different biological assays namely seed germination, toxi-chromotest, SOS chromotest, earthworm survival, solid-phase microtox and red blood cell (RBC) haemolysis to assess the efficacy of bioremediation of soil contaminated with polycyclic aromatic hydrocarbons, petroleum hydrocarbons and chlorophenols. They observed that the toxicity increased after bioremediation. Acute toxicity of benzene and interaction with its prominent metabolic intermediates using *Pseudomonas fluorescens* based bacterial bioassay was reported by Boyd et al. (1997). Nunes-Halldorson et al. (2004) studied the interaction between benzene, toluene and chloroform and they reported that significant residual toxicity was left in the

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system even after biodegradation. Abbondanzi et al. (2003) studied the comparison between microtox assay and dehydrogenase enzyme inhibition assay using *P. Fluorescens* to evaluate the toxicity of several heavy metals and phenol. Acute toxicity of various phenolic compounds based on inhibition of dehydrogenase enzyme activity of bacterial species such as *Pseudomonas*, *Bacillus and Escherichia* were reported by Nweke and Okpokwasili (2010). Steliga et al. (2015) reported that change in the toxicity was not directly related to the decrease in the pollutant concentration involving total petroleum hydrocarbons (TPH), polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, ethylbenzene, xylene (BTEX) compounds and phenols. A few studies (Eastmond et al., 1984; Seymour et al., 1997) evaluated not only the toxicity of parent pollutant such as benzothiophene but also the toxicity of their oxidized metabolic products. Eastmond et al. (1984) in their work observed that metabolized products of benzothiophene have much lower EC_{50} values than the parent pollutant.

Even though heterocyclic aromatic pollutants are frequently detected in the environment along with their homocylic analogues, not much information is available about their acute toxicity. Present knowledge about the toxicity variation during the biodegradation of these aromatic heterocyclic hydrocarbons is very scanty. Moreover, the evaluation of toxicity during the simultaneous degradation of pollutants belonging to different families is often less reported. Various synergistic and antagonistic effects due to substrate interactions may significantly result in higher toxicity which cannot be assessed only on the basis of chemical analysis. It is also important to evaluate the toxicity of transformation intermediates along with parent pollutants to determine the overall efficacy of bioremediation system. Degradability of intermediate metabolites in multisubstrate system may be completely different as compared to single substrate system and this may result in enhanced toxicity.

Hence, the present study aimed to: (i) investigate the toxicity changes during the biodegradation of various target pollutants in single and multisubstrate system using bacterial bioassay involving two different microbial strains and (ii) determine the acute toxicity of the prominent metabolic intermediates and their significance to the overall changes in toxicity. In order to represent a heterogeneous mixture of aromatic hydrocarbons from different families, benzothiophene, benzofuran, quinoline and pyridine were chosen as representatives of heterocyclic aromatics, benzene and toluene from monocyclic and naphthalene from polycyclic group.

2. Materials and methods

2.1. Chemicals

Benzene, toluene, naphthalene, pyridine, quinoline, benzothiophene and benzofuran used in the present study had a purity of 98% or greater. Iodonitrotetrazolium chloride (INT) (97.0%) and INT formazan (INTF) used for toxicity studies were procured from Sigma Aldrich Ltd; Switzerland and TCI, Japan, respectively. N, N-dimethyl formamide used for extraction was purchased from Merck, India. Ultra purified water (Merck, Millipore) was used for all the analysis wherever required.

2.2. Microbial isolate and growth conditions

The culture used in the present study was *Chryseobacterium sp* isolated from naphthalene enriched microbial consortium (Oberoi et al., 2015a). Occurrence and isolation of *Chryseobacterium sp* . from hydrocarbon and crude oil contaminated sites were also reported in several other studies (Owsianiak et. al., 2009). For culture maintenance and all the biodegradation studies, the following composition of minimal salt medium (MSM) was used (g/L): $Na_2HPO_4{\cdot}2H_2O$ (3.0), KH_2PO_4 (1.5), $(NH_4)_2SO_4$ (0.5), $MgCl_2{\cdot}6H_2O$ (0.1), $CaCl_2$ (0.02), trace element solution 1 mL/L and pH 7.2 (Oberoi et al., 2015b). Bacterial growth was determined by measuring optical density (O.D) using UV-spectrophotometer (Shimadzu, Japan) at 600 nm. Standard calibration graph between cell dry weight and the O.D values were used to determine biomass concentration.

2.3. Bacterial strains and growth conditions for toxicity studies

Acute toxicity assessment during the biodegradation of various aromatic hydrocarbons individually or in mixtures was evaluated in terms of % inhibition of dehydrogenase enzyme activity of two different bacterial species: *E.coli* and *Pseudomonas fluorescens*. *E.coli* was used as a reference since it is the most widely used prokaryotic model organism. *P. fluorescens* is a good indicator organism to evaluate acute toxicity because of its ubiquitous occurrence in diverse environmental matrices (Abbondanzi et al., 2003) and hydrocarbon contaminated sites (Kaczorek and Olszanowski, 2011). *P. fluorescens* obtained from Department of Biotechnology, IIT Madras was previously isolated from biofilms formed on polymer and metal surfaces immersed in ocean water (Prabhawathi et al., 2012). For growth and cultivation of *E. coli,* Luria broth medium was used while for *P. fluorescens* Pseudomonas phage 3 medium was used (Nutrient Broth, 13 g; K_2HPO_4 1.11 g; KH_2PO_4 .49 g, glucose 3 g; distilled water: 1 L). *E.coli* was grown at 37 °C while *P. fluorescens* was grown at 30 °C (Abbondanzi et al., 2003). Standard growth curve was plotted between O.D values at different time intervals and the corresponding colony forming unit (CFU)/mL to determine the exponential growth phase for both the bacterial species. The standard growth curve was then used to determine initial microbial cell number and the cells were harvested at exponential growth phase to ensure maximum bacterial activity for all the toxicity studies.

2.4. Analytical procedures

2.4.1. Analysis of target pollutants and total organic carbon (TOC)

Naphthalene, pyridine, quinoline, benzothiophene and benzofuran were analyzed and quantified using UV detector, C18 column coupled HPLC (Dionex, USA) system at 254 nm. Flow rate of mobile phase consisting of acetonitrile: water (80:20) was fixed at 1 mL/minute throughout the analysis. The quantification of other two target compounds, benzene and toluene were carried out in GC (Perkin Elmer Clarus 500) coupled with flame ionization detector (FID) and PE 624 column using nitrogen (2 mL/minute) as carrier gas. Column, injector and detector temperatures were set at 120 °C, 150 °C and 300 °C, respectively. To ensure the suspension free supernatant for the analysis, centrifugation of samples were done for 8 min at 7670*g*. The organic carbon was measured using total organic carbon (TOC) analyzer (Shimadzu, Japan) coupled with NDIR detector.

2.4.2. Gas chromatography-mass spectrometry (GC–MS) analysis

The transitory intermediates were analyzed with GC–MS (Agilent, USA). The column temperature was ramped from 60 °C for 2 min to 250 °C for 1 min and held with an increment of 10 °C/minute. Other setting parameters and the detailed analytical and extraction procedure for analysis of metabolic intermediates is given elsewhere (Oberoi et al., 2015b).

2.4.3. Measurement of toxicity: dehydrogenase enzyme (DHase) inhibition assay

Aqueous samples collected at different time intervals during the biodegradation studies were centrifuged at 7670*g* for 8 min. Clear supernatant liquid obtained was further filtered through .45 µm syringe filter before conducting the assay. Dehydrogenase enzyme activity was measured as per procedure described by Awong et al. (1985) with modifications. Initial bacterial cell concentration corresponding to 4–4.5*10^6 CFU/mL was added in each of the tubes. About 1.5 mL of INT solution (.2%) was added in each tube and was