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Formation of Humus-bound Residues in the Course of BTX Biodegradation in Soil

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To examine whether the xylene component of BTX (benzene, toluene, xylene) mixture is co-metabolized and residues are produced in soil, ^{14}C-labeled-*o*-xylene was added to sandy loam in combination with unlabeled benzene and toluene. After 4 weeks of incubation in a sealed system connected to an oxygen reservoir, 55.1% of the radiocarbon was converted to $^{14}CO_2$, 3.0% was solvent-extracted, and 33~35% was recovered by wet combustion of the extracted soil, adding up to 95.8% radiocarbon recovery. Biomass incorporation of *o*-xylene radiocarbon which was detected by fumigation/extraction was usually low (5.6%), but 32.1% radiocarbon became associated with soil humus. Most of the humus-bound radiocarbon was found in humin fraction. In addition to *o*-xylene, *p*-xylene and toluene also showed similar results. The evidence shows that some of their reactive methylcatechol biodegradation intermediates attach to the humic matrix in soil in preference to mineralization and biomass incorporation.

Key words: BTX, biodegradation, mineralization, humus, humification

BTX are the mixture of benzene, toluene and xylene isomers and contained in crude oil and refined petroleum products. BTX are extensively used as solvents and feedstocks for chemical synthesis in various industry. However a lot of amounts are continuously released into natural environments accidentally or intentionally, then affect many living organisms in contaminated environments. BTX are toxic and classified as priority environmental pollutants by U. S. EPA. There have been many researches on the biodegradation and bioremediation of the contaminated sites by BTX (4, 11, 14). Each component of BTX can be completely biodegraded and the pathways of individual BTX are already well-known, but any single organism can not degrade them simultaneously (11, 20). Recently metabolic engineering for the simultaneous biodegradation of BTX mixture was reported (15). Most of these studies were carried with single or mixed cultures in laboratory media containing single component (3, 12, 16, 19). However if BTX were present together, different results could come out by competitive inhibition, sparing or cometabolism (2, 7, 9, 18). The incomplete biodegradation of xylene due to the interactions among BTX was occurred by the strains which could degrade each BTX components (9, 19). As a result, xylene was cometabolized and the metabolic intermediates were accumulated as a nonvolatile products. On the other hand, the beneficial substrate interactions can be occurred through the enzyme induction or enhancement of cometabolism (2). In terrestrial environments some other phenomena such as polymerization or binding of metabolites can take place in the presence of solid materials. The pesticide residues could be covalently bound to soil humus (13, 21) and covalent linkage of phenolic compounds to humic acids may be catalyzed by microbial enzymes in soil (8). Since reactive groups on metabolites can be formed during the biodegradation, there is a possibility of residues formation from hydrocarbon compounds. In this research the biodegradation of BTX mixture in soil was carried on. The distribution of metabolites of xylene isomers and toluene was investigated and the formation of non-degradable residues was examined.

Materials and Methods

Incubation system

The biodegradation experiment of BTX was carried in a sealed incubation system. Two sites on the plastic cap of 1 liter screw-cap Erlenmeyer flask (Bellco Glass Co.) which had Teflon cap liner was pierced and two stainless steel syringe cannulas (gauge 16, length 15 cm and 5 cm) were cemented with epoxy glue. To the end of longer cannula, a glass KOH reservoir (volume : 20 ml) was attached with silicone glue for $^{14}CO_2$ absortion. The

shorter one served for subsequent attachment to an oxygen reservoir (gasometer) which was composed of two 5-liter glass containers filled with water (Fig. 1). Several incubation systems could be connected to the oxygen reservoir using multi-place gas manifold (Kontes Glass Co.). The soil used in this experiment was Nixon sandy loam collected from Cook College Farm (New Brunswick, NJ, U.S. A.) which had no history of hydrocarbon disposal. This soil had been used in many biodegradation studies and its texture (50% sand, 21% silt, 29% clay), organic matter content (6.0%), pH (6.0) and other characteristics have been described elsewhere (6). Soil samples were always freshly collected and sieved (2 mm dia.). Lime (10 mg of $CaCO_3$ g of soil^{-1}) was added to sieved soil to raise soil pH to 7.5, and soil moisture content and water holding capacity (WHC) were measured. All concentrations in and quantities of soil are expressed in terms of oven (105°C) dry weight.

Mineralization of BTX

First, 50 g of soil was added to 1 liter screw-cap flask, spreaded evenly, then the flask was capped. To this, 50 µl of cold benzene, toluene and ^{14}C-ring labeled *o*-xylene (1.4×10^5 dpm, Sigma Chem. Co.) were added through a short canular with GC syringe and mixed well. Distilled water was sprayed to adjust soil moisture content to 60% of WHC.

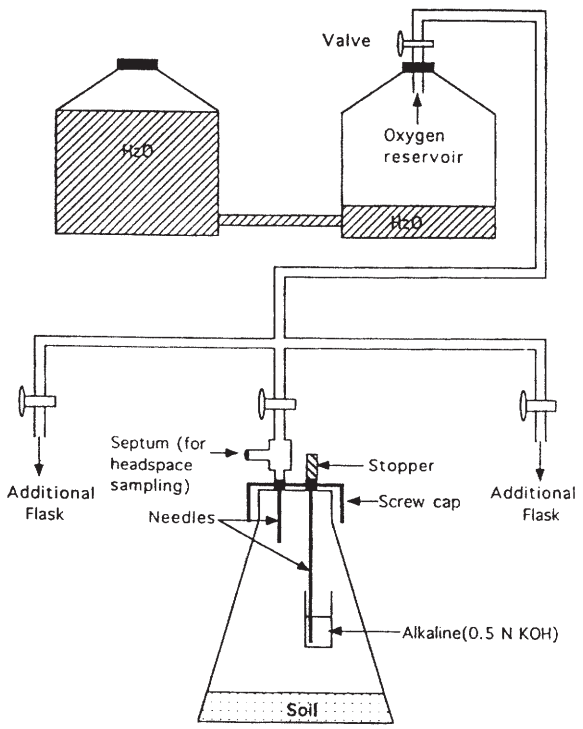

Fig. 1. Sealed aerobic incubation system for BTX compounds in soil.

The KOH reservoir was filled with 10 ml of 0.5 N CO_2-free KOH solution and the syringe port of longer cannular was sealed with syringe stopper. The stainless-steel 3-way valve was placed on the syringe port of shorter cannula and the flask was connected to the oxygen reservoir which already filled with pure oxygen. After checking the leakage, the whole incubation system was incubated at 28°C. On every 3-4 days during the incubation, 100 µl of headspace gas of the flask was sampled through the 3-way valve with gas-tight GC syringe and analyzed using a gas chromatograph equipped flame ionization detector (Hewlett-Packard Co., Model 5890) (19). After this, the KOH solution was replaced with new KOH solution and 1 ml of withdrawn KOH was put into Scintiverse BD cocktail (Fisher Scientific Co.) and the radioactivity was quantified using a Beta-Track Model 6895 liquid scintillation counter (TM Analytic). All ^{14}C-counts were corrected for background and quenching using the external standard ratio method. All treatments were in triplicate and each experiment was repeated at least once. Instead of radioactive *o*-xylene, ^{14}C-ring labeled *p*-xylene, and toluene (Sigma Chemical Co.) were also tested in same procedures. After 3~5 weeks of incubation, the flask was connected to gas trapping device (17) and the remaining radioactive volatile compounds and CO_2 in flask headspace were trapped using Scintiverse BD and Oxosol cocktail (National Diagnostics Co., Atlanta, GA), respectively and the radioactivities of each group were counted.

Fate of radiocarbons in residual soil

After termination of incubation, the whole soil sample was transferred into glass bottle, and the moisture content was measured. To investigate the fate of radioactive xylenes and toluene, the soil sample was divided into 3 fractions and treated as follows (Fig. 2);

(1) Solvent extraction: A 20 g of soil sample was mixed with 70 g of anhydrous sodium sulfate and put into cellulose thimble. The remaining hydrophobic hydrocarbon compounds were extracted by soxhlet extraction for 6 hours using ethylacetate. The extract was concentrated to 10 ml with a rotary evaporator and 1 ml was added to Scintiverse BD cocktail and the radioactivity was measured. To detect the formation of polymerized residue which could be extracted by polar solvents, 5 g each of soil was put into 20 ml of various solutions (distilled water, 0.2 M ammonium acetate, DW: methanol(1:1), methanol), and shaked for 1 hour on a rotary shaker (300 rpm). The soil suspension was centrifuged (12,000 g, 15 min.), and the radioactivi-

ty of supernatant was measured.

(2) Fumigation/extraction: The biomass incorporation of radiocarbon was detected by fumigation/extraction method (10). A 10 g of soil was put into 150 ml beaker and placed in a vacuum desiccator with a 50 ml of ethanol-free chloroform containing beaker. The desiccator was evacuated until boiling of the chloroform was observed and then sealed. After 24 h at 28°C the chloroform was removed and the desiccator was evacuated at least 5 times for periods of 3 minutes to remove chloroform residues from the soil. A 50 ml of 0.5 N K_2SO_4 was added to fumigated sample and unfumigated control soil sample, respectively. These soil suspensions were shaked for 1 h with a rotary shaker (200 rpm), and centrifuged (12,000 g, 15 min.). A 1 ml of supernatant was put into Scintiverse BD and the radioactivity was counted. If the radioactivity was low, it was measured by the wet combustion (1). A 3 ml of supernatant was mixed with 1.5 g of $K_2Cr_2O_7$ and 30 ml of digestion acid (sulfuric acid : phosphoric acid=6:4). The remaining radiocarbon was completely transformed to $^{14}CO_2$ and trapped into Oxosol cocktail and the radioactivity was measured.

(3) Pyrophosphate extraction: To detect the humus-bound radioactivity without the destruction of humus structure, pyrophosphate extraction was carried. A 20 g of soil sample was placed into 60 ml of 0.15 M sodium pyrophosphate solution in 250 ml centrifuge bottle. This sample was shaken for 8 h at 200 rpm and centrifuged (12,000 g, 15 min). The radioactivity of the supernatant was measured by wet combustion.

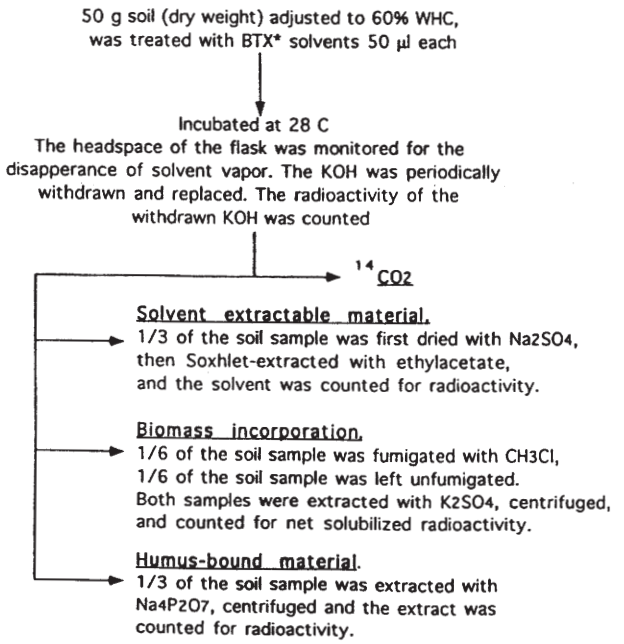

Fig. 2. Flow chart for the analysis of radioactivity distribution in soil.

Fractionation of humus-bound radioactivity

The soil humus of the leftover soil after fumigation/extraction was fractionated into 3 groups (Fig. 3). A 30 ml of 0.5 M NaOH was added to 10 g of soil in 250 ml centrifuge bottle. The headspace was flushed with nitrogen gas to prevent the oxidation of organics and closed. The soil suspension was shaken for 2 h at 200 rpm and centrifuged (12,000 g, 15 min). The radioactivity of precipitated solids which was humin fraction was detected using a wet combustion. The supernatant was acidified to pH 1 and stored in cold room (4°C) for overnight, and centrifuged (12,000 g, 30 min). The precipitated solids, humic acid fraction was oxidized by wet combustion and the radioactivity was measured. To the remaining supernatant, 0.6 g of $FeCl_3$ was added and the pH was raised to 2.5. This sample was stored in cold room for overnight and centrifuged (12,000 g, 1 h). The radioactivity of the precipitated solids, which was fulvic acid fraction was counted after a wet combustion. A 1 ml of final supernatant was put into Scintiverse and the remaining radioactivity was detected.

Biodegradation of humus-bound radiocarbon

A 15 g of the leftover soil after fumigation/extraction was placed into the biometer flask (Bellco Glass Co.). Distilled water was added to adjust the soil moisture content to 60% of WHC. To the sidearm of biometer flask, 5 ml of 0.5 N CO_2-free KOH solution was injected. After flushing with pure oxygen, the biometer flask was incubated at

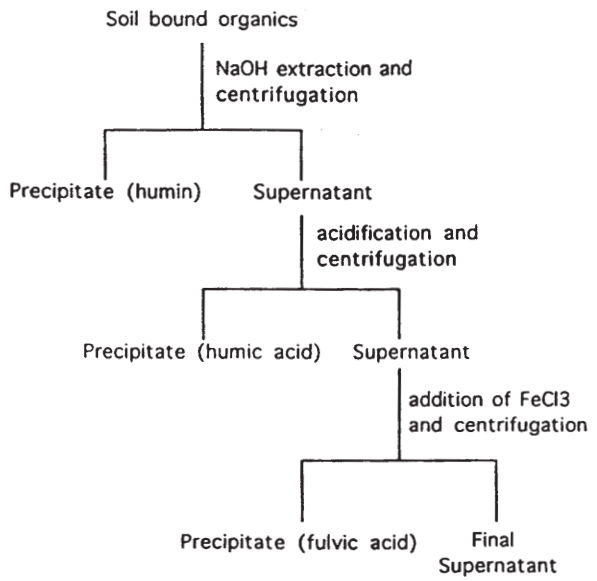

Fig. 3. Fractionation of humus-bound residues.

28°C. On every 7 days the KOH solution was replaced with new KOH solution and the radioactivity in the withdrawn KOH was measured.

Results and Discussion

Fate of xylene or toluene during the biodegradation of BTX in soil

To make a closed system for volatile hydrocarbons and maintain aerobic condition for the aerobic metabolism, a special incubation system (Fig. 1) was designed. When the radioactive o-xylene was added to soil with cold benzene and toluene, 55.1% of $^{14}CO_2$ was evolved during 1 month of incubation (Table 1), which was a higher mineralization rate than expected. It has been known that inhibitory substrate interactions such as competitive inhibition, cometabolism and others can occur when BTX were present together. Due to the inhibitory interactions, xylene was not completely mineralized, but cometabolized or metabolite of xylene was polymerized (2, 9, 19). In this experiment, *o*-xylene had been also expected to be cometabolized, however there was a extensive and rapid mineralization. It might be due to the presence of various degrading organisms, which was different conditions from above reports. This result indicates that some unexpectable phenomena can take place in natural environments in which many kinds of organisms and substrates are present together. The solvent-extractable radiocarbon which was detected by Soxhlet extraction was low (3.0%), which means that most *o*-xylene was metabolized or transformed. The extraction with several polar solvents showed a negligible amount of radioactivity (data not shown). The results of both solvent extractions indicated that the polymerization of metabolic intermediates of *o*-xylene was not occurred. Different from the high mineralization, a small percentage (5. 6%) of the added *o*-xylene was converted to the biomass-associated fraction. Unexpectedly, 32.1% of radiocarbon was bound to soil humus. During the metabolism of *o*-xylene, dihydrodiol, phenolics and dimethylcatechol were produced, which have reactive side group such as hydroxyl group (19). In pure or mixed culture in a chemical medium, those intermediates could be cometabolized (9, 12) or polymerized (19), but some of them were bound to humic material in soil in this experiment. Although the humification of metabolic intermediates was reported in pesticide biodegradation (6), this is the first report of the humification of hydrocarbon which can be easily biodegraded.

When the same experiment was carried with ring-labeled *p*-xylene which is more reactive than *o*-xylene, the mineralization (53.6%) and humification (31.3%) took place in a similar rate. However, the solvent-extractable (1.9%) and biomass-bound fraction (1.8%) showed lower levels of radioactivity. One of the reasons for the low figures might be the reduction from the conversion of data with low radioactivities. More accurate experiment is necessary to figure out this result. Another reason seemed to be from the difference of the bioavailability and reuse as a respiration substrate. *o*-Xylene is usually reported to be the most recalcitrant of the BTX compounds, and some of the biomass-incorporated carbon might be mineralized after depletion of intact substrates (2). This was supported by the evolution curve of $^{14}CO_2$ (Fig. 4). Even after the depletion of *p*-xylene vapor from the headspace, the evolution of $^{14}CO_2$ was continued. During the first 8 days of period most *p*-xylene was removed, then the evolution of $^{14}CO_2$ was accelerated. Trace amount of *p*-xylene was remained in the headspace after 15 days, but the evolution of $^{14}CO_2$ was maintained at a constant rate, which might be from the respiration of the biomass-bound and remaining radiocarbon in soil.

It had been expected that most of radioactive toluene could be mineralized in the presence of benzene and *o*-xylene, however it showed quite similar result with *o*-xylene. The $^{14}CO_2$ evolution, 48.6% was a little bit lower, but the solvent-extractable (3.

Table 1. Distribution of radioactivity after one month of incubation in soil of BTX mixtures, with one radiolabeled component in each mixture. Radiolabel is indicated by asterisk (*)

BTX mixture	$^{14}CO_2$ evolved	Solvent-extractable	Biomass-associated	Humus-bound	Mass balance
BT*o*-X*	55.1	3.0	5.6	32.1	95.8
BT*p*-X*	53.6	1.9	1.8	31.3	88.6
BT**o*-X	48.6	3.3	9.6	33.4	94.9

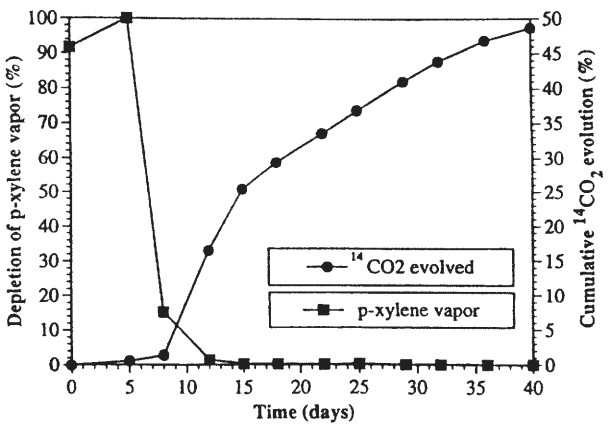

Fig. 4. Fate of ring-labeled *p*-xylene vapor and evolution of $^{14}CO_2$ with time.

3%) and humus-bound fraction (33.4%) were similar to those of *o*-xylene. Biomass incorporation (9.6%) was higher than those of xylene isomers, which indicates that toluene might be better substrate for the biosynthesis than xylene isomers. Unexpectedly, 33.4% of radiocarbon was bound to soil humus. The major metabolic intermediates of toluene is methylcatechol and it also has hydroxyl group which can be attached to the humic compounds. The amount of humus-bound radioactivity of toluene in the presence of benzene and *o*-xylene was similar to the result with toluene alone (data not shown). It indicates that the metabolism of toluene and the production of intermediates which were bound to humus subsequently were always same in soil whether toluene was present alone or benzene and xylene were existed. The competitive inhibition or cometabolism of BTX mixture reported in other studies with pure cultures (3, 9, 19) did not occurred or might occur in a less scale in this experiment.

The formation of bound residues to soil humus from xylene isomers and toluene is very important because they are not completely removed, but remained in natural environment in modified forms. Therefore the characteristics including toxicity of bound residues should be investigated.

Fractionation of humus-bound radioactivity

To examine the distribution of humus-bound radiocarbon, the humus was fractionated into 3 groups, humin, humic acid and fulvic acid based on acid-base solubility (Fig. 3). Among 32.1% of humus-bound radioactivity from *o*-xylene, 27.4% was found in humin fraction (Table 2). The humic acid and fulvic acid fractions contained 2.0 and 2.7% of radioactivity, respectively. The humin is a alkali-insoluble fraction of soil organic matter and has high molecular weight (5). This fraction is regarded as a strongly bound complex of fulvic and humic acids to mineral material and has numerous binding sites, so the most of metabolic intermediates of *o*-xylene also seemed to be attached tightly. The other two groups are alkali-soluble and have lower molecular weight than humin but still can bound the reactive side group of xylene metabolites. Since all of the residual metabolites were bound to soil organic matter, the final product of fractionation did not contain any radioactivity. This results can help to investigate the long-term fate of the aromatic contaminants although they were modified and bound to the soil organic matter.

Table 2. Distribution of bound *o*-xylene radioactivity among the fractions of soil organic matter

Fulvic acid	Humic acid	Humin	Final supernatant
2.0	2.7	27.4	0

All numbers indicate % of original radioactivity applied.

Biodegradation of humus-bound radiocarbon

To examine the biodegradability of humus-bound radiocarbon, the soil sample after K_2SO_4 extraction which removed the biomass-bound portion was incubated with fresh soil. This experiment tested the stability and persistence of humus-bound metabolites of aromatic hydrocarbons. From the soils in which radioactive toluene or *o*-xylene were added, 0.49 and 0.65% of radioactivity were detected as $^{14}CO_2$ during 4 weeks of period (Fig. 5). These amounts are very small when compared with 32~33% of radioactivity in parent materials. Moreover, most of them seemed to be produced from the residual biomass-bound radiocarbon because the extraction efficiency of K_2SO_4 extraction is just 40% in previous control experiment. Therefore, some radiocarbons which could be more easily mineralized than soil humus might be left. Actually 0.22~0.29% of $^{14}CO_2$ were produced during initial 7 days, then the evolution rates were reduced. Considering this fact, the biodegradation rates of humus-bound substances assumed to be 5~10% which is the range of biodegradation rate of normal soil organic matters. However long-term studies are necessary to investigate more accurate biodegradability of humus-bound materials.

Until now xylene isomers and toluene have been considered to be easily biodegradable. However, this study founds that they could not be completely mineralized in soil although most of original forms were disappeared, rather about 1/3 of their metabolites was bound to soil humus. The humification of metabolites of pesticide was already reported (6, 21), but this is the first report of humification of 1

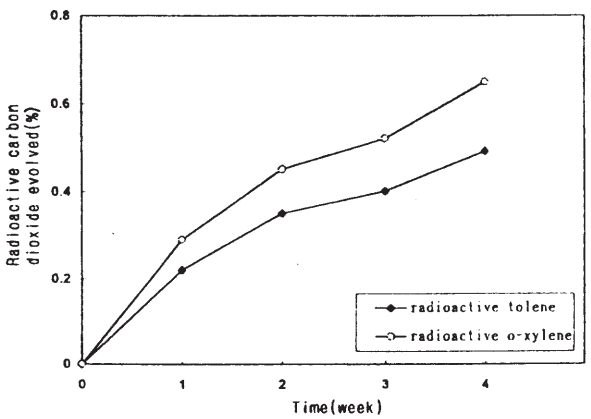

Fig. 5. Evolution of radioactive carbon dioxide from mixture of fresh soil and residual soil after fumigation/extraction.

ring hydrocarbons. Also there is a possibility of humification of other hydrocarbons, such as polycyclic aromatic hydrocarbons, phenolic compounds, substituted aromatic compounds and others. The bound residues seemed to become a part of soil organic matter and showed lower biodegradation rate and leachability by hydrophilic and hydrophobic solvents. However more researches are necessary to investigate the precise structure and characteristics such as biotoxicity, persistence and mobility in soil. The humification capacity of soil also should be examined for the application of this phenomenon. If a strong humification would occur with recalcitrant hydrocarbon contaminants in soil, the effects of those contaminants might be lower than expected, and this reaction could be used as a treatment process of various pollutants in soil.

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