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Combined toxicities of methyl *tert*-butyl ether and its metabolite *tert*-butyl alcohol on earthworms via different exposure routes

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HIGHLIGHTS

- MTBE and TBA are among the major soil contaminants that threaten the soil health.
- The combined toxicities of MTBE and TBA were studied using two earthworm species.
- The combined toxicity showed weak antagonistic effects.
- Sensitivity toward same pollutants differed in the earthworm species and exposure routes.
- The results provide important information for future assessments of soil ecosystem risk.

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ABSTRACT

Methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA) are among the major soil contaminants that threaten the health of soil ecosystems. Many MTBE-contaminated sites accumulate TBA, because TBA is the intermediate of MTBE biodegradation. To access the risk of MTBE and TBA in soil, we investigated the combined toxicities of MTBE and TBA using two earthworm species, *Perionyx excavatus* and *Eisenia andrei*, as well as the toxic effects via different exposure routes. The combined toxicity showed weak antagonistic effects ($LC50_{mix}$ values were slightly greater than 1.0), and sensitivity toward same pollutants differed in the two earthworm species. Moreover, the toxicity of MTBE and TBA was also affected by the exposure route; both filter paper and artificial soil tests showed that dermal-only exposure to MTBE had an even greater toxic effect than combined dermal and oral exposure. Thus, we suggest that diverse environmental factors including organic materials, the physicochemical properties of the contact media, and the exposure routes of the organism, should be taken into consideration when assessing the effects of pollutants on organisms in diverse environmental systems.

1. Introduction

The fuel oxygenates methyl *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA), its major metabolite, are major environmental contaminants in soils and groundwater because of their widespread production and use as gasoline additives. MTBE has been found in subsurface systems and is released into the environment mainly through the leakage of gasoline from underground storage tanks (Schmidt et al., 2004). Moreover, MTBE is a known animal carcinogen and classified as a potential human carcinogen by the United States Environmental Protection Agency (U.S. EPA) (Mennear, 1997; Mehlman, 1999). The effects of MTBE contamination in groundwater have been studied by measuring its toxicity on aquatic organisms (Werner et al., 2001; Rausina et al., 2002) and conducting risk assessments of drinking water (Brown, 1997; Stern and Tardiff, 1997; EPA, 1999). According to previous studies on the toxic effects of MTBE on aquatic organisms, 57–1000 mg L^{-1} of MTBE is toxic to invertebrate freshwater organisms and 388–2600 mg L^{-1} toxic to vertebrates; among saltwater biota, the acute median lethal concentration (LC50) or effective half-maximal concentration (EC50) values of the compound ranges from 166 mg L^{-1} for grass shrimp to 1950 mg L^{-1} for mussels. However, studies on the toxicity of MTBE to terrestrial organisms are relatively limited, despite the current widespread soil contamination by MTBE (An et al., 2002; Venkateswara Rao et al., 2003; Dodd and Addison, 2010).

TBA, the major metabolite of MTBE, is also widely used as a solvent in many industries and as a fuel oxygenate in association with

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MTBE (Schmidt et al., 2004). TBA is a recalcitrant dead-end product of MTBE, and therefore, often detected in association with MTBE at gasoline release sites (Schmidt et al., 2004; Shih et al., 2004). Despite the likely coexistence of MTBE and TBA, toxicity studies have primarily focused on MTBE, although many studies have noted the toxicity of TBA to environmental systems or living organisms. A weak tumorigenic response has also been reported for both MTBE and TBA, and chronic exposure to TBA via drinking water causes tumors in rats and mice (Cirvello et al., 1995; Cruzan et al., 2007). Generally, chemicals in mixtures have known to interact with biological systems and alter the toxicity of each chemical greatly (Faust et al., 1994; Fernandez-Alba et al., 2002). Previously, the toxicity of MTBE in combination with several pesticides has been examined, MTBE combined with certain pesticides, such as Diuron, TBT, and Linuron, showed synergetic effects, whereas combinations with other pesticides, such as SEA-NINE and dichlofluanid, showed no synergistic effects (Hernando et al., 2003). However, the combined effects of MTBE and TBA are not well studied. Therefore, it is necessary to assess the risk of the combined effects of MTBE and TBA because of their likely coexistence in environmental systems. Although we have previously reported the combined effects of MTBE and TBA on terrestrial plants, that study was insufficient for soil risk assessment because of the complexity of the environmental system (An and Lee, 2007). Therefore, we focused on the combined effects of MTBE and TBA on soil invertebrates by using earthworms as a model system.

Two species of earthworms, *Perionyx excavatus* and *Eisenia andrei*, were selected as model systems to understand the effects of MTBE and TBA on soil invertebrates because earthworms have been widely used as test organisms in soil toxicity assessments and their growth and reproduction are well known (An, 2005). Because earthworms take up pollutants both through their skin (dermally) and by ingestion (orally), both exposure routes were considered to assess the risk of soil pollution (Jager et al., 2003). To test the effects of dermal and oral MTBE and TBA exposure, earthworms with sealed and unsealed mouths were used in present study. The LC50 and toxic unit (TU = c_i/LCx_i) were established to determine the individual and combined toxic levels of MTBE and TBA, respectively. These data provide the first insights into the combined effects of MTBE and TBA on soil invertebrates. Additionally, this study provides evidence for differences in the toxicity to earthworms of dermal and oral exposure. Therefore, the results are valuable to for assessing the risk of invertebrates exposed to soil contaminated with MTBE and TBA.

2. Materials and methods

2.1. Chemicals

Methyl *tert*-butyl ether (MTBE, 99% purity; ACROS Organics, NJ, USA) and *tert*-butyl alcohol (TBA, 99% purity; Sigma–Aldrich, St. Louis, MO, USA) were used without further purification. MTBE and TBA were diluted with distilled water to obtain a range of exposure concentrations in the test soil.

2.2. Test soils

Natural field soil and OECD artificial soil were used for the earthworm assay. The natural field soil was a loamy sand soil collected from the Konkuk University campus (Seoul, Korea). Collected soils were sieved (1.4 mm) and dried for 24 h at 105 °C in the oven. The pH and soil organic matter (SOM) content were 5.61 ± 0.13 and 2.20% ± 1.01%, respectively. The OECD artificial soil consisted of 69.5% sand, 20% kaolin clay, 10% sphagnum peat moss (Daesin Co., Sungnam, Korea), and 0.5% $CaCO_3$, which was added to adjust the initial pH to 6.0 ± 0.5. The percent organic matter content of the peat moss was 95% according to the manufacturer. Soil pH was measured using a soil pH meter (Model CP 411, ELMETRON sp.j., Zabrze, ul. Witosa 10, Poland). The percent organic matter content of the test soil was measured using an organic matter soil test kit (Model ST-OR 5020, LaMotte Co., Chestertown, MD, USA) according to the manufacturer's specifications. The kit followed the Walkley–Black wet oxidation method for measurement of the active or decomposable organic matter in the soil. The physicochemical properties of the test soils are listed in Table 1.

2.3. Test earthworm species

Two earthworm species, *P. excavatus* and *E. andrei*, were selected as the test organisms in this study. *E. andrei* was test species recommended by the OECD, and *P. excavatus* has been employed in previous toxicity tests. The culture of *P. excavatus* was obtained from an earthworm-breeding farm located at the Nanji wastewater treatment plant (Ilsan, Korea), and *E. andrei* was purchased from the Seobu earthworm-breeding farm (Seoul, Korea). The cultures were maintained in the laboratory at 25 °C in darkness on a culture soil consisting of a 1:1 mixture of OECD artificial soil and earthworm bedding (Carolina Co., USA). The culture soil was replaced once every two weeks, and the worms were fed once a week with ground grain mixed with soil. The earthworms were separated from the cultures and rinsed with distilled water 3 h prior to use, and then kept on damp filter paper at 25 °C in darkness to remove their gut contents (OECD, 1984).

2.4. Filter paper contact test

To characterize the toxicities of MTBE and TBA to earthworms, we employed several toxicity tests with various experimental conditions. The overall experimental design is illustrated in Fig. S1 of SI. A filter paper contact test was conducted following method No. 207 described in the OECD Guidelines for the Testing of Chemicals (OECD, 1984), with slight modifications. The test unit was closed tightly a with 1 mm silicon-lined cap, instead of perforated plastic film, to prevent the volatilization of MTBE and TBA. The test unit was a flat-bottomed glass vial (ID 26 mm, length 75 mm, volume 34 mL), and each vial was lined with a 85 × 50 mm filter paper (Whatman No. 2). Concentrations of MTBE and TBA were prepared at 0% (control), 0.5%, 1%, 1.5%, 2%, 3%, and 4%, corresponding to 0, 88, 175, 263, 350, 525, 700 μg cm^{-2}, and 0, 93, 185, 278, 371, 556, 742 μg cm^{-2}, respectively. Then, 1 mL of solution was added to each test unit using a micropipette, and 1 mL of distilled water was applied to the control vial. The filter paper contact test was performed with one earthworm in each test unit, and each concentration of MTBE and TBA was replicated 10 times. The exposure durations were set as 24, 48, and 72 h because our preliminary tests showed that the earthworms could survive for up to 4 days. The test unit was placed in a temperature-controlled incubator (25 °C) in darkness. After the incubation periods of 24, 48, and 72 h, mortality and abnormality were recorded. Earthworms were presumed dead when no responses to light and/or mechanical stimulation with a needle were observed.

2.5. Soil toxicity tests

Soil toxicity tests were performed in a closed soil microcosm (CSM) using a protocol previously described by An and Lee (2007). Natural field soil and OECD artificial soil were used for these soil toxicity tests. The moisture contents were adjusted to 35% and 30% for the natural field soil and OECD artificial soil, respectively, and each concentration of MTBE and TBA was added to 10 g of soil in each CSM. One adult earthworm was added to each