BENZOTRIAZOLES: TOXICITY AND DEGRADATION

X. Wu, N. Chou, D. Lupher, and L.C. Davis

Department of Biochemistry, Kansas State University, Manhattan, KS 66502

ABSTRACT

Benzotriazole and methyl benzotriazole were studied in soil, water, and wood. As reported by various authors, benzotriazole is highly stable in soil or water. It was also found to be a potent inhibitor of plant growth, killing several species at levels of about 0.1 mg/ml. Both benzotriazoles are readily degraded by the Fenton reaction; they are also degraded slowly in the presence of horseradish plants or in cultures of the fungus *Phanerochaete chrysosporium*.

Key words: *benzotriazole, white-rot fungus, toxicity, horseradish, antifreeze*

INTRODUCTION

Benzotriazoles contain a five-membered ring with three nitrogen atoms directly bonded to one another as substituents on a benzene ring. The compounds called methyl (or tolyl) derivatives have a methyl substituent on the benzene ring. Many other derivatives are possible and a number have been used in various applications. There are three primary uses for benzotriazoles: corrosion inhibitor, ultraviolet light stabilizer for plastics, and antifoggant in photography (U.S. EPA, 1977). Because benzotriazoles are used in large quantities as a corrosion inhibitor, it is mainly through this type of use that benzotriazoles become an environmental contaminant. As a corrosion inhibitor and fire retardant, they are used in antifreeze in concentrations of 0.01-2.0% and in airplane deicing/anti-icing fluids in unknown concentrations, up to 10% (Cancilla et al.,1997). Used antifreeze may leak or be poured down drains and thence enters the environment. Also, an estimated 80% of aircraft deicing/anti-icing fluids are deposited on the ground due to spray drift, jet blast, and wind shear during taxiing and takeoff, according to a recent study (Hartwell et al., 1995).

According to a 1977 EPA report, benzotriazole is considered to be of very low toxicity and a low health hazard to humans. In the same EPA report, two benzotriazole derivatives were reported to be mutagenic in bacterial systems. A year later, NIH published a report that there was no convincing evidence that the compound is carcinogenic (NIH, 1978). It is, however, more recently well established that 1-amino benzotriazole, with an amino group attached to one of the ring nitrogens, is a potent mechanism-based inhibitor of cytochrome P-450s via a benzyne intermediate (Ortiz de Montellano and Mathews, 1981), indicating that benzotriazoles as a class may interact with the P-450s. The P450s are important both for detoxifying a broad range of xenobiotics and for activating many compounds to carcinogens in mammalian systems.

Benzotriazole has a toxic effect on plants. Several reports cited in the EPA (1977) document indicated that benzotriazole can produce distinct morphological changes in a variety of plants. Tomato plants were shown to be sensitive to both benzotriazole and benzothiadiazole (Davis,

1954). In cucumber seedlings, as low as 1 mM (0.119 mg/ml) benzotriazole causes 50% repression of root elongation. When applied to the soil of existing bushbean plants, benzotriazole produced death at 0.05 M, and at 0.01 M there was nearly complete inhibition of internodal elongation with simultaneous thickening of the stem.

Benzotriazole also has a toxic effect on the aquatic environment (Hartwell et al.,1995). The tolerance limit for bluegills and minnows is 27.5 ppm after 48 hours and 25 ppm after 96 hours (~ 0.2 mM). For trout, the tolerance limit is 15 ppm after 48 hours and 12 ppm after 96 hours. The mortality rate is higher after 96 hours than after 48 hours, suggesting that there is cumulative toxic effect on fishes. There are no reports on effects on aquatic plants.

There is limited information on the fate of benzotriazoles that are released into the environment. Due to resistance of benzotriazoles to oxidation under environmental conditions, and the uV stability of the compounds, benzotriazoles may persist in the environment for a very long time. In a 1986 study, Rollinson and Callely were not able to find any organisms that were could degrade benzotriazole as a C or N source. It was concluded that benzotriazole was nonbiodegradable. They also stated that there were no reports showing evidence for the biodegradation of benzotriazoles.

Studies on the toxic effects of benzotriazoles on different plant species have been conducted by us in order to determine whether a plant-based bioremediation system is feasible for benzotriazole-contaminated solutions. Degradation of benzotriazoles, especially benzotriazole and methylbenzotriazole, through microbiological or chemical approaches has been investigated.

MATERIALS AND METHODS

Plants were grown under continuous cool white fluorescent light. Pumpkins were grown from seed in a 1:1 mixture of perlite:vermiculite and watered with Hoagland's solution. After the first true leaves had appeared, they were transferred to test tubes containing different concentrations of benzotriazole or methylbenzotriazole.

Alfalfa plants were grown from seed and then transferred to a sandy soil obtained from the Riley County Landfill area. Containers were plastic tennis ball cans that held 500 ml. The plants had been established for two months, and had been treated with a watering solution containing propylene glycol for one month. Then the watering solution was dosed with the indicated level of contaminants, without further propylene glycol.

Cottonwood cuttings (about 20 cm long and <1 cm diameter) were obtained in the early spring and rooted in the same type of sandy soil used for alfalfa plant growth. They were grown for several weeks and then treated as indicated.

Corkscrew willow cuttings (about 30 cm long and 1 cm diameter) were rooted in water and grown for several weeks until shoots and roots were a few inches long. Then the contaminants

were added to obtain the desired concentration.

Horseradish root crowns, about 2-3 cm in diameter and 3-4 cm long, were placed in a 1:1 perlite:vermiculite mixture watered with Hoagland's solution and allowed to initiate root and shoot growth. They were then transferred to a fresh medium which was watered with the indicated concentration of contaminant. Samples of water were taken for analysis on several occasions. Hoagland's solution was supplied after several weeks, on one occasion.

To study the horseradish peroxidase enzyme directly, 5 g of root was ground with 10 ml water and 10 ml of 1 mg/ml benzotriazole, using a mortar and pestle. It was incubated at room temperature (39°C) and assayed at intervals. Another sample was prepared with a final concentration of 0.75% hydrogen peroxide, by replacing half the water with a pharmacy grade 3% peroxide solution.

The fungus *Phanerochaete chrysosporium* was cultured on a malt agar medium for maintenance and on a defined medium with veratryl alcohol added as an inducer when lignin peroxidase activity was to be expressed (Capdevila et al.,1990). An inoculum of spores was used in each case. For the induction of peroxidase, cultures were grown at 39°C with 10 ml of culture medium in a 125 ml flask as described. Indicated levels of contaminant were added to the culture during the induction process. Disappearance of the contaminant was monitored by taking 1/2 ml samples, which were centrifuged briefly at 13,000 x g prior to measurement by HPLC.

The HPLC column used in these studies was Hamilton PRP-1 which is a polystyrene polymer. The eluting solvent was usually 70:30 methanol:water and the flow rate was typically 2 ml/min. Higher methanol concentration speeds up the elution of the methylbenzotriazole but decreases its separation from benzotriazole. Veratryl alcohol and more polar metabolites elute at approximately the same volume as nonretained ions, in front of benzotriazole. To assure that altered pH did not change the mobility of benzotriazole, which has a pK near 9, 1 mM phosphate buffer pH 6.8 was used in some investigations in place of the water phase.

RESULTS AND DISCUSSION

The effect of benzotriazole on several plant species was investigated by applying different concentrations of benzotriazole to the soil of established plants, or to hydroponically grown seedlings. Typical results are shown in Table 1. All but one of the tested plant species died sooner or later following the introduction of benzotriazole, which indicates the toxicity of benzotriazole on plants. The important exception was horseradish, discussed further below.

Benzotriazole has a structure that resembles auxin, which may account for its toxicity to plants (Davis, 1954). It may also be an analog of purines and indoles. It shows long-term stability in water in the presence of soil. We observed that low levels (0.25 mg/ml) do not prevent the growth of (presumably unicellular) green algae or cyanobacteria, although they may affect the rate of growth. We tested its effect on a *Xanthobacter* species, which possesses a monooxygenase that

metabolizes propylene glycol, and in *Pseudomonas putida* F1, which has an inducible toluene dioxygenase. Benzotriazole was toxic to both species at concentrations around 1 mg/ml and showed no evidence of degradation, even when the toluene dioxygenase was induced by growth in the presence of toluene.

Because the benzotriazoles have been observed to be readily degraded by a Fenton reaction in the presence of peroxide and iron (Chou, unpublished), we tested the fungus *Phanerochaete chrysosporium* under several conditions of growth known to induce the lignin peroxidase of that organism. Fungi were grown as stationary culture at 39°C by inoculating fungal culturing media with spore suspensions. Three days later, contaminants of benzotriazole and/or methylbenzotriazole were introduced. Fungal cultures were kept and samples were withdrawn over a period of time to monitor the remaining amounts of contaminants in the culture. In the first set of experiments, (Fig. 1) up to 0.125 mg/ml (~1 mM) benzotriazole or 0.11 mg/ml methylbenzotriazole (~0.8mM) was introduced into fungal cultures. Obvious degradation of benzotriazole was observed in each case. In the second set of experiments, up to 0.21 mg/ml benzotriazole was introduced into fungal cultures and consistent degradation was observed (Fig. 2). When as little as 0.01 to 0.05 mg/ml (up to 0.4 mM) of methylbenzotriazole was introduced, there was no degradation. In the third experiment (Fig. 3), methylbenzotriazole and benzotriazole were introduced together into the same fungal culture. Degradation of both benzotriazole and methylbenzotriazole was observed.

Horseradish has been reported to supply an effective peroxidase that can degrade a number of compounds, although it does not operate at such a high redox potential as does lignin peroxidase and is therefore not such a strong oxidant (Barr and Aust, 1994). At an initial concentration of 0.1 mg/ml benzotriazole the horseradish plant grew moderately well though not so well as a control plant. Over time it appeared to recover and grow more vigorously. At an initial concentration of 0.2 mg/ml there was very little growth of the plant and after 1.5 or 3 months a large portion of the crown had decayed and there were few roots. Assays of the water in the culture containers at 3 months indicated that more than half of the starting 0.2 mg/ml concentration of benzotriazole had disappeared, while from a starting concentration of 0.1 mg/ml more than 95% was gone. Further experiments are in progress to more fully describe the time course of degradation starting with known amounts of plant biomass.

An experiment was carried out using ground horseradish root, with and without added peroxide. Within two days half of the input level of benzotriazole or methylbenzotriazole (0.5 mg/ml) disappeared at room temperature. A sample incubated at 39°C showed no such decrease. It may be possible to take advantage of the peroxidatic activity of horseradish to degrade the benzotriazoles through use of either intact plants or the extracted enzyme. Further experiments are needed to optimize conditions for degradation.

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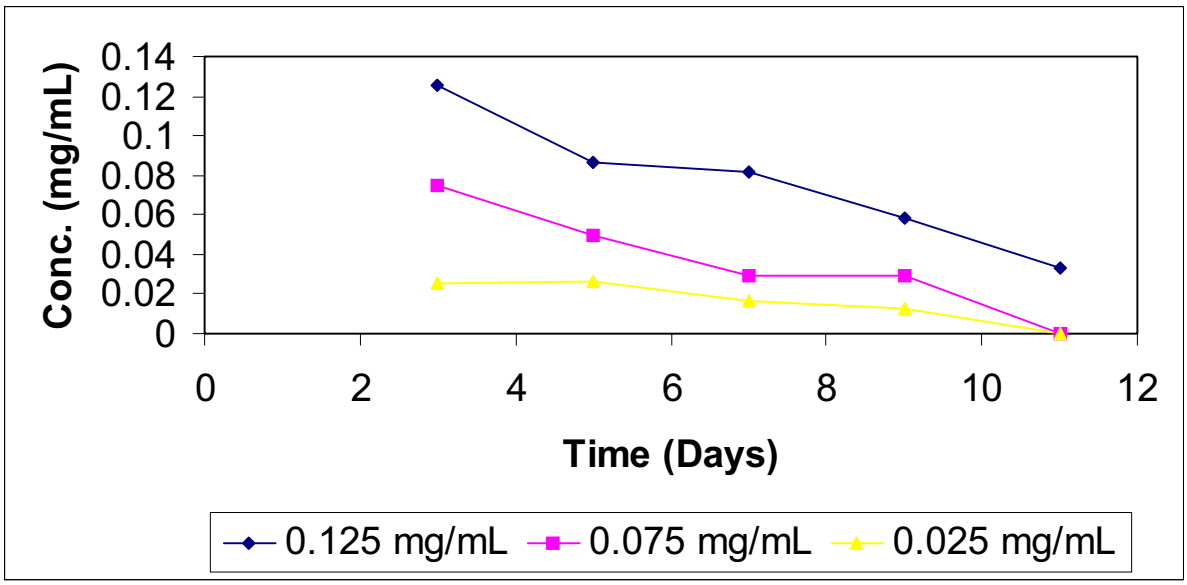
Table 1. Survival of plants under different treatments.

Treatment	Length of Time Plants Survived
Benzotriazole or Methylbenzotriazole at 0.05% (0.5 mg/ml) applied daily to soil	Alfalfa: two to three weeks Poplar: two to four weeks
0.05% (0.5 mg/ml) benzotriazole	Pumpkin: 2 days
0.005% (0.05 mg/ml) in water	Pumpkin: 10 days
0.02% (0.2 mg/ml) benzotriazole	Corkscrew willow: 10 days
0.01% (0.1 mg/ml) in water	Corkscrew willow: 3 weeks
0.02% (0.2 mg/ml) benzotriazole	Horseradish root: 3 months
0.01% (0.1 mg/ml) in water	Horseradish root: > 3 months

Figure 1. Degradation of benzotriazoles added to fungal cultures after induction with veratryl alcohol.



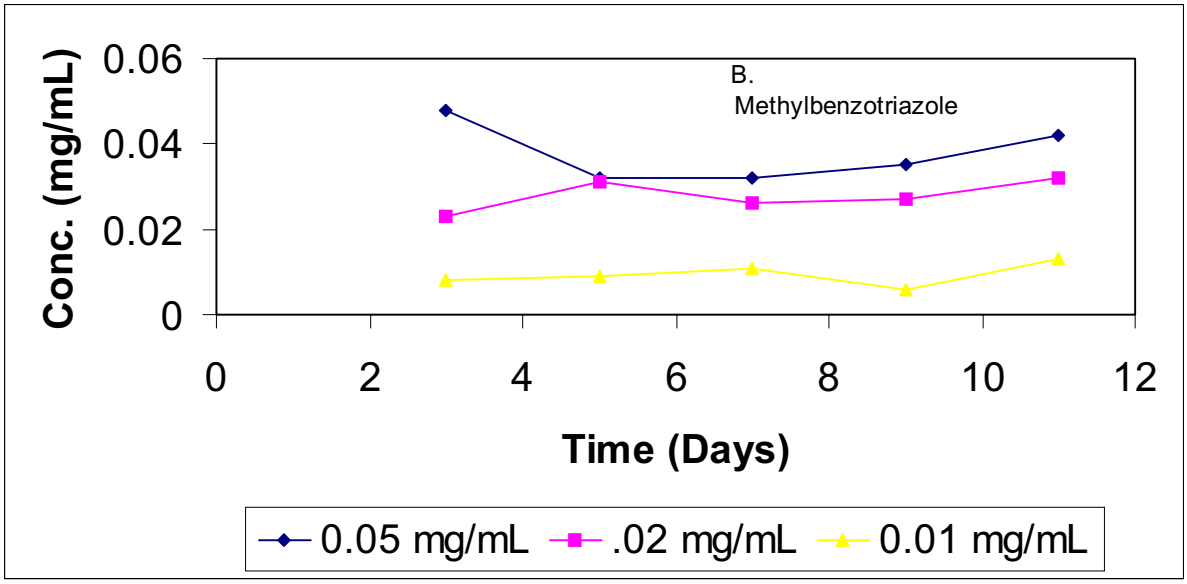


Figure 2. Degradation of benzotriazoles added with veratryl alcohol.

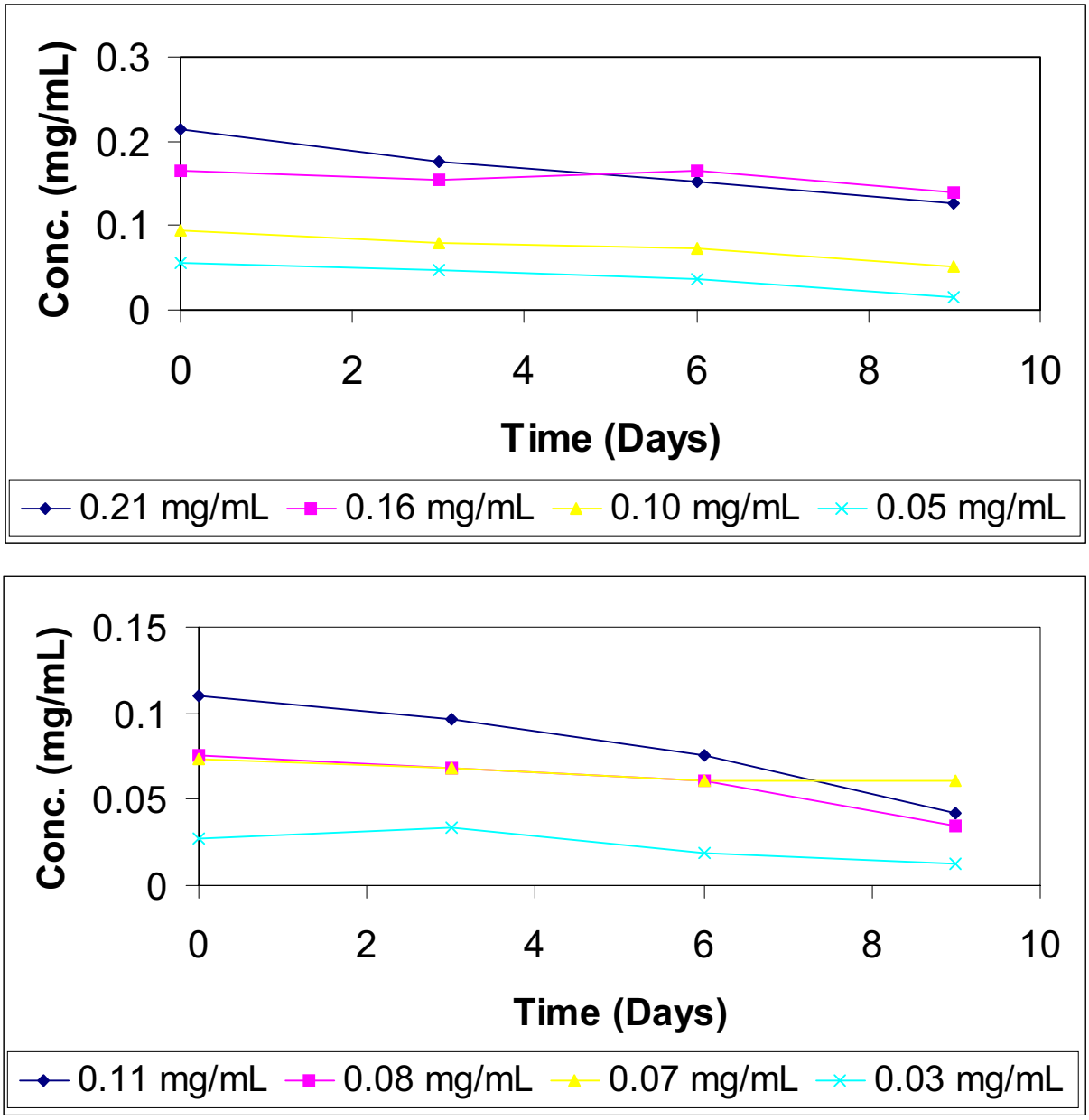


Figure 3. Codegradation of benzotriazoles added with veratryl alcohol.