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BIOTRANSFORMATION POTENTIAL AND UNCOUPLING BEHAVIOR OF COMMON BENZOTRIAZOLE-BASED CORROSION INHIBITORS

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Mark Hernandez (University of Colorado, Boulder, CO)

Benzotriazole and its derivatives form strong complexes with transition metals and are the most widely used type of industrial corrosion inhibitor. Benzotriazole derivatives are present in high concentrations in deicing fluids, industrial cooling water systems, and antifreeze. Benzotriazoles are produced and applied in large quantities; total domestic production was 10^7 kg in 1977, and their use has markedly increased since. A recent USGS reconnaissance of 139 freshwater streams nationwide, found that 1/3 of them contained a single benzotriazole derivative (5-Methylbenzotriazole (MeBT)) in significant concentrations. Benzotriazole derivatives have been implicated as possible carcinogens, endocrine disrupters, and plant hormone regulators, but the literature on their biological activity is dated and tenuous. More recently, acute toxic responses to benzotriazoles have been observed in MICROTOX™ assays at concentrations less than 10 mg/L.

The environmental behavior of benzotriazoles derivatives remains largely unknown To investigate the biotransformation potential of the most commercially significant benzotriazole derivatives — 4- and 5- Methylbenzotriazole (MeBT) — biodegradation experiments were conducted with activated sludge and bacteria enriched from airport soils contaminated with aircraft deicing fluids. Below a toxic threshold (c.a. 100 mg/L), all enrichments were capable of degrading 5-MeBT under aerobic conditions; however, over a 24 month period, 5-MeBT was recalcitrant under anoxic or fermentative conditions, and 4-MeBT was recalcitrant under all conditions tested. Respirometry experiments provided evidence that MeBT may behave as an uncoupler at concentrations above 300 mg/L: oxygen uptake and biomass monitoring showed bacterial growth on propylene glycol and glucose was limited by the presence of MeBT, yet oxygen uptake rates of cell fragments remained the same or increased when MeBT levels were at or above 300 mg/L. Using fluorometric membrane potential dyes [DiBac], direct epifluorescent microscopy of bacterial cells exposed to MeBT confirmed that these benzotriazoles can attenuate the transmembrane potential of active bacterial membranes.

5-MeBT was completely mineralized by activated sludge from two different wastewater treatment plants, and enrichments of soil bacteria, all of which could use this benzotriazole derivative as a sole carbon and nitrogen source. Two pure bacteria cultures were isolated by using 5-MeBT as a sole carbon source. They were tentatively identified as *Pseudonocardia alni* and *Variovorax sp.* (16sRNA). Mass-spectral analysis of metabolic by-products separated from MeBT-degrading enrichments showed molecular weight increases consistent with the incorporation of two oxygen molecules, suggesting that oxygenase enzymes are involved in the initial stages of MeBT biotransformation.

Biotransformation Potential and Uncoupling Behavior of Common Benzotriazole-Based Corrosion Inhibitors

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A thesis submitted to the
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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

DEDICATION

To my family [redacted]. You are my inspiration, you gave me the strength to carry on. I love you.

Mami, gracias por tus sacrificios y esfuerzos. Gracias por cuidar a mis hijas cuando yo no estaba disponible. Sin ti este logro no hubiera sido posible. Te Amo.

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O'Brien, Ivette Z. (Ph.D., Environmental Engineering)

Biotransformation Potential and Uncoupling Behavior of Common Benzotriazole-Based Corrosion Inhibitors

Thesis directed by Associated Professor Mark T. Hernandez

Benzotriazoles are the most widely used type of industrial corrosion inhibitor. Benzotriazole derivatives are present in high concentrations in deicing fluids, industrial cooling water systems, and antifreeze. A recent USGS reconnaissance of 139 freshwater streams nationwide, found that 1/3 of them contained 5-Methylbenzotriazole (MeBT) in significant concentrations. Literature on the biological activity of benzotriazoles is dated and tenuous.

The environmental behavior of benzotriazoles derivatives remains largely unknown. To investigate the biotransformation potential of the most commercially significant benzotriazole derivatives — 4- and 5- MeBT — biodegradation experiments were conducted with activated sludge and bacteria enriched from airport soils contaminated with aircraft deicing fluids. Below a toxic threshold (c.a. 100 mg/L), all enrichments were capable of degrading 5-MeBT under aerobic conditions; however, over a 24-month period, 5-MeBT was recalcitrant under anoxic, and 4-MeBT was recalcitrant under all conditions tested. Respirometry experiments provided evidence that MeBT may behave as an uncoupler to pure cultures of *Psedonmonas aeruginosa*. Oxygen uptake and biomass monitoring showed bacterial growth on propylene glycol and glucose was limited by the presence of MeBT, yet oxygen uptake rates of cell fragments remained the same or increased when MeBT levels were at or above 300 mg/L. Uncoupling behavior was not evident on mixed

cultures grown from enrichments of soil bacteria taken from airport soils contaminated with aircraft deicing fluids and acclimated to propylene glycol. However, inhibitory effects were apparent in these cultures at MeBT concentrations greater than 200 mg/l suggesting that the main toxicity mechanism appears to be associated with respiratory chain activity. Using fluorometric membrane potential dyes [DiBAC], direct epifluorescent microscopy of bacterial cells exposed to MeBT confirmed that these benzotriazoles could attenuate the transmembrane potential of active bacterial membranes.

5-MeBT was completely mineralized by activated sludge and enrichments of soil bacteria. Only the acclimated soil enrichment cultures were able to use this benzotriazole derivative as a sole carbon and nitrogen source. Two pure bacteria cultures were isolated by using 5-MeBT as a sole carbon source. They were tentatively identified as *Pseudonocardia alni* and *Variovorax sp.* (16sRNA).

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CHAPTER 1

INTRODUCTION

1.1 Problem Statement

Aircraft deicing operations are regulated by the Federal Aviation Administration (FAA) and are mandatory for safe air travel. The main purpose of aircraft deicing is to remove ice and snow from control surfaces (wings, rudders, and fuselages), which can disturb airflow. Airflow disturbance results in increased drag and a decrease in stall speed (Switzenbaum et al., 1999).

A typical deicing procedure involves the application of aircraft deicing fluids (ADF) in a heated mixture with water and applied under pressure (Switzenbaum et al., 1999). On average, de-icing of a large commercial aircraft requires between 500-1000 gallons of ADF (Betts, 1999). Much of the ADF applied ends up in the stormwater systems (from over spray or drippage) or in the airfield grounds (sloughing during taxing and take-off) (Mericas and Wagoner, 1994).

In 1999, the Environmental Protection Agency (EPA) estimated that over 200 U.S. airports perform significant deicing operations. These facilities discharge approximately 21 million gallons (50% concentration) of ADF per year to surface waters and 2 million gallons to publicly owned treatment works (POTW). In total, an estimated 7 billion gallons of stormwater contaminated with deicing chemicals is generated annually (USEPA, 2000). EPA and the European Commission are currently studying the discharge of ADF to determine if regulations are warranted.

Among several other proprietary constituents, methylbenzotriazole (MeBT) is a corrosion control inhibitor commonly used in ADF formulations. In general, benzotriazoles are produced and applied in large quantities; total domestic production of benzotriazole was 10^7 kg in 1977, and their used has markedly increased since. Benzotriazoles have a variety of industrial uses: anti-corrosion agents, ultraviolet light stabilizer for plastics, and antifoggants in photography (USEPA, 1977). Manufacturers claim uses for benzotriazoles in the following applications among others: water treatment, metal working fluids, engine coolants, fuels, inks, lubricants, coatings, flooring, and cleaners (PMC Specialties Group, 2000). However, ADF operations are probably the main pathway by which MeBT enters the environment due to the large discharges associated with deicing commercial and military aircraft fleets.

Recent studies have found MeBT to be toxic at relatively low concentrations to selected ecosystems receptors. These receptors - minnows, waterflea, and Microtox® - are commonly used as models to demonstrate the response of different trophic levels to xenobiotic compounds (Pillard, 1995; Cornell et al., 2000; Pillard et al., 2001). However, the mechanisms of such toxicity are unknown. In addition, the environmental fate of MeBT under anoxic conditions, either in wastewater treatment facilities or subsurface environments, has not been investigated.

The EPA implemented broad-reaching stormwater regulations in 1990, which limited discharges and in some cases mandated permits to control discharges to stormwater outfalls. In response to such regulations, more airports are expected to improve collection systems to minimize stormwater discharges.

Currently, most airports collect ADF-containing wastewater and these collection systems often involve on-site wastewater treatment. This wastewater is sometimes processed to recycle the glycol, treated on site, or discharged to a POTW (or a combination of these methods) (USEPA, 2000). Current treatment alternatives for ADF-containing waste streams include aerobic and anaerobic biological treatment (USEPA, 2000, Betts, 1999 #65). Some activated sludge facilities have reported process upsets due to capricious ADF loadings and the effectiveness of these treatment systems could be impacted by the concentration of MeBT or other ADF additives. Therefore, it is important to gain a better understanding on the impact of MeBT to wastewater treatment systems as well as subsurface environments.

Government regulations and pollution prevention practices established by airport management have decreased the amount of ADF released to the environment from airport operations (Switzenbaum et al., 1999). But, in spite of better management practices, ADF is still expected to reach the environment in large quantities. For example, some airports will discharge diluted ADF to stormwater outfalls if the glycol concentration is below a regulated threshold (100 mg/l in Canada) (Cancilla et al., 1998). Significant amounts of ADF also drip from aircraft after leaving the gate or deicing station, and wastewater collection systems seldom collect anywhere near the amounts of ADF applied. Denver International Airport (DIA) and Albany International Airport (ALB), which operate the most state-of-the-art runoff collection systems in the U.S., reported that they at best recover 70% of all ADF applied at their deicing pads (USEPA, 2000).

Because of the current regulatory structure, the design goal for ADF treatment systems is solely to reduce wastewater chemical oxygen demand (COD) without regard for its specific constituents. In the case of ADF treatment, the consequence of this approach will at least be two fold: first, MeBT, which has been found to be more toxic than the glycol component, will still be released to the environment (Pillard, 1995; Cornell et al., 2000; Pillard et al., 2001); second, the rate at which COD is removed from a wastewater may be reduced due to MeBT, other ADF additives, and the synergistic effects of their combination (Cornell et al., 2000; Gruden et al., 2001; Gruden and Hernandez, 2002). These factors must be taken into consideration when designing industrial wastewater treatment processes that will accept ADF-containing wastes.

Finally, most major airports in the U.S. are located within three miles of an ocean, bay lake, wetland, reservoir, river or stream (NRDC, 1996), therefore, it is important to understand the fate and environmental impact of MeBT to a broad range of aquatic systems.

1.2 Research Objectives and Approach

In view of the issues discussed above, I established three goals for my research. The first goal was to obtain a better understanding of the biodegradation potential of ADF in both natural and engineered systems. Knowledge of the toxicity mechanisms of MeBT will aid in understanding the biodegradation potential of MeBT in natural systems and lead to better design of engineered systems. To address this issue my intent was to answer the following question:

1. Can MeBT act as an uncoupler of oxidative phosphorylation? This question is addressed in Chapter 3 of this dissertation.

The second goal was also to increase our knowledge regarding the biodegradation pathways MeBT may follow and determine if it can be transformed to benign by-products. This knowledge will aid in the design of effective biological treatment techniques for MeBT-containing wastewaters as well as provide evidence for the natural attenuation of this benzotriazole derivative in terrestrial environments. As mentioned before, current treatment systems are solely designed to reduce the COD of ADF-containing wastewater. In addition to uncoupling behavior, the following questions will provide useful information to help achieve this goal:

2. Does complete mineralization of 5-MeBT occur under aerobic conditions? Chapter 4 looks at this issue.

3. Can 5-MeBT act as the sole carbon and nitrogen source of a mixed aerobic bacterial consortia? This issue is investigated in Chapter 5 of this dissertation.

4. Can pure bacterial cultures be isolated that are capable of growing on 5-MeBT as their sole carbon source? Chapter 6 describes efforts to obtain these isolates and investigate their biodegradation potential.

My final goal was to obtain information that could help future researchers model the fate of ADF under common subsurface conditions. So far, all biodegradation studies of MeBT have been performed under aerobic (oxic)

conditions. It is well known that in many subsurface environments, aerobic conditions are normally only present at or near the surface. Indeed, the relatively high BOD associated with the glycol component of ADF will most likely induce anoxic conditions in shallow subsurface soils. Anoxic and anaerobic conditions often predominate in the subsurface. Therefore, in order to accurately model the fate of ADF, its fate and biodegradation potential at different redox conditions should be determined. My last question attempts to partially address this issue:

5. Are benzotriazoles degradable under anoxic conditions? Details of the experiments conducted to investigate the anoxic degradation of benzotriazoles are in Chapter 7.

1.3 Dissertation Guideline

This thesis has been designed to facilitate its reading such that each chapter can be read independently with a minimum of cross-referencing to other chapters. This chapter provides the background information for the research presented in this dissertation including problem statement, identification of research needs and a generalized literature review. In addition, each chapter contains literature reviews germane to the material presented in that particular chapter. Chapters 3 through 7 address each of the questions and topics presented above. Chapter 8 presents general conclusions, applications to engineering practice, and recommendations for future research.

CHAPTER 2

LITERATURE REVIEW

This chapter critically summarizes the available literature on the environmentally relevant behavior of MeBT including its chemistry, toxicity, and previous environmental monitoring.

2.1 Aircraft Deicing Fluid (ADF)

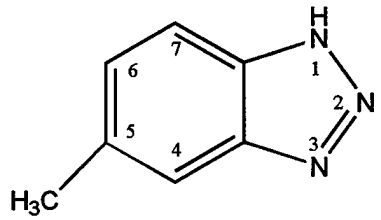
Aircraft deicers are classified by industry specifications in the US and Canada. There are four types available on the U.S. commercial market, but not all are currently used. Type I ADF is the most common formulation used by both the military and civilian sector (USEPA, 2000; Gruden et al., 2001; Cornell, 2002). However, most large U.S. carriers now use Type IV fluids as an anti-icing agent (USEPA, 2000). Type IV anti-icing fluid has a greater holdover time than Type I and requires much less mass for the same icing protection. However, Type IV deicing fluid is significantly more expensive than Type I ADF.

The main ingredient and freeze point depressant in ADF is either ethylene glycol or propylene glycol. For example, Type I propylene glycol ADF must contain at least 80% propylene (PG) or ethylene glycol by weight with the balance composed of water, buffers, thickeners and surfactants (Klecka et al., 1993; Switzenbaum et al., 1999; USEPA, 2000; Gruden et al., 2001; Cornell, 2002). This solution is typically diluted to between 30% and 70% glycol at the time of application, based on temperature and weather conditions (USEPA, 2000).

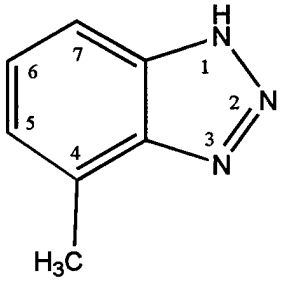
2.2 Methylbenzotriazole (MeBT) in ADF Formulations

The Federal Aviation Administration mandates aircraft deicing operations to ensure safe air travel. In the U.S. the requirements for ADF formulations are contained in the Society of Automotive Engineers (SAE) specifications. These specifications include benzotriazole compounds for corrosion control and flame suppression, the most common of which is methylbenzotriazole (MeBT, common name: tolyltriazole). In modern U.S. ADF formulations, MeBT exists as a mixture of two isomers: 4-MeBT (≈45%) and 5-MeBT (≈55%), even though 5-MeBT appears to form stronger complexes with yellow metal surfaces (Rao et al., 1996; Abu-Dalo, 2002). The main reason for this is that it is more cost effective to produce a mixture of both isomers than pure 5-MeBT (Pearson, 2000).

All ADF components other than the freeze-point depressant are commonly referred to as "additives". In this context, the term "additive" is a misnomer, because MeBT is present at a relative large concentration – 0.5-0.6% in Type I formulations. Figure 2-1 illustrates the structure of MeBT.



5-Methyl-1H-Benzotriazole



4-Methyl-1H-Benzotriazole

Figure 2-1. Chemical Structure of MeBT. MeBT is commercially available as COBRATEC® TT-100.

2.2.1 Chemistry of MeBT

Little is known regarding the environmental chemistry of MeBT. Cornell (2002) provided an excellent summary of available information on the chemical properties of benzotriazoles. Table 2-1 summarizes some of the most important physical properties of MeBT. It is noteworthy that volatilization of MeBT is not significant due to its low vapor pressure. MeBT can exhibit significant sorptive behavior in environmental systems. Castro et al (Castro et al., 2000) found that 29% of MeBT sorbed to vermiculite, whereas, in a mixture of vermiculite and soil sorption was 56%. For silty topsoil rich in plant roots up to 74% of MeBT can be sorbed to the soil or root system. In wastewater treatment systems Gruden (2001) found that up to 30% of MeBT sorbed to digesting sludge with an average total solids concentration of 1.5%. In general, sorption to soils and/or biomass will depend on organic content, surface area and charge density characteristics.

Table 2-1. Physical Properties of MeBT

Property	
Molecular Weight	133.15
Specific Gravity (25°C)	1.16[a]
Flash Point °C	182.2[c]
Bulk Density (g/cm^3)	0.56-0.80, loose[a]
	0.64-0.72, packed[a]
Melting Point, °C	83°C
Vapor Pressure, mm Hg	.03 (50°C)[a]
Boiling Point, °C	160 (2 mm Hg)[a]
pKa	8.8[b]
Log K_{ow}[b]	2.16, 1.89
Solubility (%wt)[a]	
Water	0.55 (25°C), 1.8 (60°C)
Methanol	71.6 (25°C)
Benzene	1.3 (25°C)
White Mineral Oil	0.008 (25°C)

[a] (USEPA, 1977)
[b] (Gruden, 2000)
[c] (NTP)

2.2.2 Complexation

MeBT is an excellent corrosion inhibitor because of its ability to form stable metal complexes, thereby inhibiting oxidation of metal elements, particularly copper (USEPA, 1977; Rao et al., 1996). In fact, the stability of metal complexes of triazoles follows the Irving-Williams order (Ramana et al., 1991) which states that for a given ligand, the stability of the complexes increases from Ba (Group IIA) to Cu (Group IB) and then drops for Zn (Group IIB) (Ward, 1999).

MeBT inhibits metal corrosion by forming a polymeric film on some metal surfaces, which shields the underlying metal molecules from further chemical attack (NTP). It is this surface protective property that has enabled widespread use of benzotriazoles for corrosion control. In 1977 the Environmental Protection Agency

reported that benzotriazoles are universally used in automobile antifreeze solutions, in recirculating water systems such as power plant and commercial air conditioning cooling systems, and in coatings for the protection of copper alloys in architectural and decorative applications (USEPA, 1977).

2.2.3 MeBT in the Environment

Recent monitoring investigations (1998, 1999, 2000) reported significant concentrations of benzotriazole and its derivatives in raw wastewater, stormwater runoff, and airport groundwater (Range: 0.02 to 90 mg/l) (USEPA, 2000). Cornell (Cornell, 2002) found up to 249 mg/kg of MeBT in soil samples. Cancilla et. al. estimated a MeBT concentration of 215 mg/l in a perched monitoring well (Cancilla et al., 1998). All these samples were collected at or near airports.

In 1999 and 2000 the U.S. Geological Survey, monitored 139 major streams around the U.S. to measure the concentration of 95 selected organic wastewater contaminants. This survey found detectable (> 0.10 µg/l) concentrations of 5-MeBT in 31.5% of the streams sampled (Kolpin et al., 2002). MeBT was one of the most commonly occurring anthropogenic compounds found in this reconnaissance.

2.3 Biodegradability of MeBT

The biologically mediated transformation of 5-MeBT under aerobic conditions has recently been observed, and it appears that 4-MeBT is recalcitrant (Rao et al., 1996; Cornell, 2002). Under anaerobic conditions, however, both isomers appear to persist (Gruden, 2000). In a patent application, Rao and co-workers suggested that aerobic biodegradation of 5-MeBT in cooling tower process waters containing up to 1000 mg/l resulted in significant cell growth. No cell growth was

seen in the samples containing 4-MeBT and cell counts decreased when 4-MeBT concentrations were above 400 mg/l (Hoots et al., 1997). It is not clear from this patent application what other carbon sources were present or if the cell growth reported was due solely to MeBT. Cornell (2002) observed 5-MeBT degradation with concentrations as high as 1000 mg/l however, significant growth inhibition was noted.

Cornell and co-workers (2000) found that beyond an environmentally relevant threshold (ca. 50 mg/l) the presence of MeBT had a significant inhibitory effect on propylene glycol biodegradation rates under aerobic conditions. However, the effects could not be classified into simple growth or enzymatic inhibition models because the magnitude and form of the effect on PG degradation changed as MeBT concentrations changed. In microbiological enrichments from ADF-contaminated soils, Cornell observed the following:

- Cell yield nominally decreased with increasing MeBT concentration (> 6 mg/l).
- PG removal rates decreased with increasing MeBT concentration except at concentrations near 100 mg/l MeBT where PG removal rates markedly increased.
- Soluble chemical oxygen demand (SCOD) removal efficiency decreased above 49 mg/l MeBT.

Cornell explained that the stimulatory effect observed near 100 mg/l MeBT might be similar to that observed in previous studies with benzothiazoles and benzotriazoles, were these compounds appeared to stimulate bacterial activity through a small concentration range near 100 mg/l. The mechanism(s) behind this stimulus remains unknown.

The unsubstituted form, benzotriazole, appears to be recalcitrant (Rollinson and Callely, 1986; Hem et al., 2000). Rollinson and Callely (Rollinson and Callely, 1986) tried to enrich cultures capable of using benzotriazole as sole carbon source with no success. They estimated the minimum inhibitory concentration to be near 20 mM (c.a. 2400 mg/l). Hem et al (2000) found benzotriazole to be recalcitrant through detention times common in activated sludge systems (HRT = 13.4 hrs). Wu and co-workers (1998) were able to observe benzotriazole degradation with the fungus *Phanerochaete chrysosporium* by inducing production of the lignin peroxidase from that organism.

2.4 Land Treatment of ADF Wastes

Engineered land treatment systems provide a promising alternative for the treatment of ADF waste since discharges to stormwater or surface waters will likely be stringently regulated in the near future. Constructed wetlands are an example of one of such treatments. This approach appears to be a favorable alternative since airports encompass a significant flat land area.

Constructed wetlands treatment potential is based in a complicated interaction of physical, chemical and biological processes in soil-plant systems. Researchers at Kansas State University have been studying the phytoremediation potential of benzotriazoles in model grassland and wetland systems. They investigated the effect of benzotriazoles on several plants species by applying them to the soil or established plants, or to hydroponically grown seedlings. The plants species investigated included common grass (*Festuca arundinaceous*), sunflowers (*Helianthus annuus*), alfalfa, cottonwood cuttings, pumpkins, corkscrew willow cuttings, and horseradish

root crowns. Their research has led them to the conclusion that plants systems are capable of at least partially removing various benzotriazole derivatives. They concluded that lignin peroxides and laccase might be able to incorporate and immobilize benzotriazoles into the lignin fraction. The toxicity threshold was identified near 100 mg/l for all plants species studied except horseradish, which does not express lignin peroxidases (Wu et al., 1998; Castro et al., 2000). The microbial association with mycorrhizae in these plant systems has not been isolated or studied and the symbiotic relationship of soil bacteria and plants in these systems is not yet understood.

In 1999, researchers at the University of Pittsburgh published the results of a study in which they evaluated the potential for land application alternatives to treat ADF wastes (Bausmith and Neufeld, 1999). This study only evaluated the potential of land treatment in terms of the biodegradation of propylene glycol. They found that land treatment of propylene glycol based ADF is an effective way to treat the glycol component of deicer solutions up to approximately 20% by weight. Further, they noted a significant increase in the kinetics of glycol biodegradation after multiple glycol treatments, suggesting the ability of soil microbial biomass to acclimate to ADF and accelerate its biodegradation. However, the effects of the additives were not evaluated.

2.5 Toxicity

2.5.1 Toxicity to Aquatic Vertebrates and Invertebrates

Using widely-accepted toxicological metrics, ADF formulations have been found to be one to three orders of magnitude more toxic to aquatic organisms than the

glycol component alone (Lubbers, 1993). Several studies have identified MeBT as an ADF component acutely toxic to marine microorganisms as judged by Microtox assays, water flea *(Ceriodaphnia dubia)*, and fathead minnow *(Pimephales promelas)* responses (Pillard, 1995; Cornell et al., 2000; Pillard et al., 2001). However, other ADF additives, particularly the surfactant fractions, appear to be more toxic than MeBT to higher organisms. These studies showed that microorganisms were the environmental receptors most sensitive to MeBT as judged by acute toxicity assays (Table 2-2).

Table 2-2. Summary of Toxicity Tests

	LC_{50} or EC_{50} as MeBT (mg/l)
***Ceriodaphnia dubia* (48-h LC_{50})**	18-109[a]
	108[c]
***Pimephales promelas* (96-h LC_{50})**	8-65[a]
	38[c]
Microtox (15-min EC_{50})	6-9[a]
	5.7[b]
	7.3[c]

[a] (Cornell et al., 2000). The LC50 was always lower when AdPack was present.
[b] (Cancilla et al., 1997)
[c] (Pillard et al., 2001)

2.5.2 Toxicity to bacteria

Wu et al (1998) studied the toxicity of benzotriazole to *Xanthobacter* species and *Pseudomonas putida* F1 and reported a toxicity threshold near 1 mg/l. However, the publication did not provide enough information on the materials and methods used and could not be replicated (Wu et al., 1998).

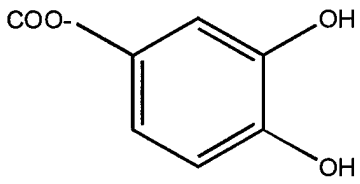
Aminobenzotriazoles have been found to inactivate cytochrome P450 (Town et al., 1993; Woodcroft et al., 1997; Sinal et al., 1998). Cytochrome P450 is a

ubiquitous heme-containing enzyme involved in the metabolism of many xenobiotic compounds (O'Sullivan et al., 2001).

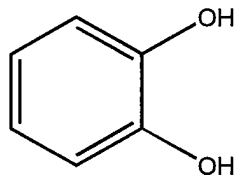
2.6 Biodegradation Pathways and By-Products

The biodegradation pathways of benzotriazoles have not been studied nor have its biodegradation byproducts been identified. Cornell (2002) tried unsuccessfully to identify some of the intermediates of MeBT biodegradation by soil bacteria. Therefore, in order to gain a better understanding of the biodegradation mechanism of MeBT and its possible byproducts, we must refer to studies in which compounds with similar structure have been studied.

The biodegradation pathways of heterocyclic compounds have been widely studied and are well understood for many. The metabolism of these compounds often involves ring hydroxylation followed by ring cleavage (Kaiser et al., 1996; De Wever et al., 1998). The hydroxylation step frequently includes the formation of either of two molecules, protocatechuate or a cathecol, shown in Figure 2-2 (Gibson, 1984; Madigan et al., 1997).



Protocatechuate



Cathecol

Figure 2-2. Structure of Protocatechuate and Cathecol

Studies of compounds similar in structure to benzotriazoles such as benzothiazoles suggest that this mechanism still holds. Benzothiazoles are heterocyclic compounds, which contain a benzene ring attached to a thiazole ring. Biodegradation studies with benzothiazoles have shown that hydroxylated compounds are formed as one of the by-products of degradation. In the case of benzothiazoles, its degradation seems to converge into the formation of 2-hydroxybenzothiazole, which is then transformed into the dihydroxy derivative (Figure 2-3) (De Wever et al., 1998). Other authors have suggested that when six-membered rings are attached to five membered rings that the latter is usually cleaved first with or without initial hydroxylation (Hill and Wright, 1978; Kaiser et al., 1996). Some of these compounds include nicotine and indole.

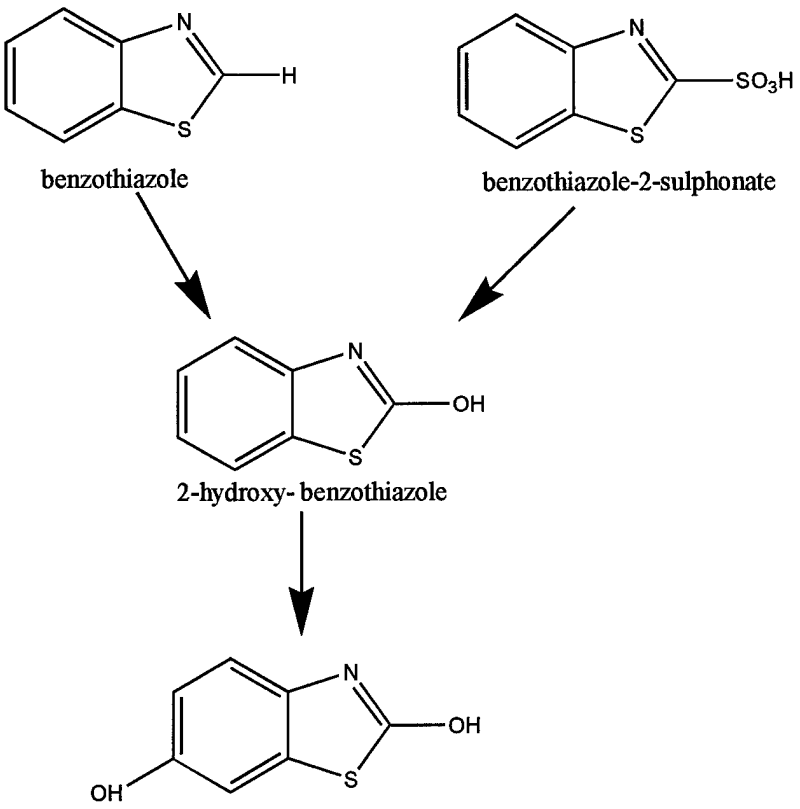


Figure 2-3. Initial transformations reported for benzothiazoles biodegradation by *Rhodococcus* isolate (De Wever et al., 1998).

In one study (Gaja and Knapp, 1997), mixed cultures capable of biodegrading benzothiazoles were also capable of degrading certain benzotriazole derivatives without a lag period, which implies that a similar mechanism could be involved. Preliminary studies by Gaja and Knapp suggest that the mechanism of ring cleavage by a *Rhodococcus* capable of growing on benzothiazoles as the sole carbon source is via the meta-cleavage pathway. In the meta-cleavage pathway the ring is broken between the hydroxylated carbon atom and an unsubstituted carbon atom (Wrenn, 1998).

These and other research were also able to demonstrate that the nitrogen in benzothiazoles is released as ammonia (Gaja and Knapp, 1997; De Wever et al., 1998). Interestingly, De Wever (1998) did not detect any nitrate or nitrite liberated into solution from a *Rhodococcus* strain degrading benzothiazole-2-sulfonate, even though only 50% of the nitrogen was released as ammonia.

Studies investigating the metabolism of aminobenzotriazoles by cytochrome P450 have identified the following metabolites: benzotriazole, benzaldehyde, glucuronide derivatives, and hydroxy derivatives (Town et al., 1993; Woodcroft et al., 1997).

Studies of the degradation mechanisms of azo dyes, provides some insight about other possible pathways for the degradation of triazoles. The bacterial metabolism of azo dyes is in most cases initiated by a reductive cleavage of the azo bond (Stolz, 2001) with the formation of amines as by-products. Interestingly, in the case of azo dyes, isolates were only capable of decolorizing the dyes when another carbon source was present. A possible explanation of the resistance of 4-MeBT to biodegradation could be the ability of the 4 isomer to form intramolecular hydrogen bonding similar to the internal bonding exhibited by selected azo-dyes (Figure 2-4) (Boyer, 1961; Pasti-Grigsby et al., 1996).

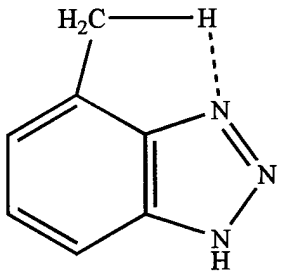


Figure 2-4. Possible configuration of intramolecular hydrogen bonding of 4-MeBT

Zimmermann et al (Zimmermann et al., 1984) isolated two azoreductases believed to catalyzed the first step in the aerobic degradation of carboxy-Orange dyes. Both enzymes were found to have a monomeric structure and specificity for NAD(P)H as co-substrates. These oxygen-insensitive enzymes were found to catalyze the reductive cleavage of the azo group with NAD(P)H as an electron donor. The aromatic amines formed by the azoreductases were subsequently mineralized by an aerobic bacterium. These enzymes were found to have high substrate specificity (Kulla, 1981; Zimmermann et al., 1982; Zimmermann et al., 1984).

CHAPTER 3

TOXICITY OF METHYLBENZOTRIAZOLE TOWARDS BACTERIAL RESPIRATION

Abstract

In this chapter, the effect of 4(5)-methylbenzotriazole (MeBT) on respiratory activity of mixed and pure bacterial cultures was studied. Respiration activity and uncoupling potential was evaluated by measuring the oxygen uptake rate (OUR) of whole cells concurrently with isolated active cell wall fragments. The ability of 4(5)-MeBT to disrupt the membrane potential of whole cells was also observed using the potentiometric dye $DIBAC_4(3)$. As judged by respiration activity and membrane depolarization, evidence of uncoupling behavior was observed in both pure and mixed cultures; while growing on propylene glycol, acute increases in the metabolic activity of soil enrichment cultures were observed immediately following exposure to 4(5)-MeBT. In pure *Pseudomonas aeruginosa* cultures, a significant decrease in oxygen uptake rates was observed in whole cells compared to their isolated cell wall fragments. Responses to the membrane potential dye $DiBAC_4(3)$ showed whole *P. aeruginosa* cells responded with acute transmembrane depolarization when challenged with 4(5)-MeBT concentrations above 100 mg/l.

3.1 Introduction

3.1.1 The potential of MeBT to act as an uncoupler of oxidative phosphorylation

Few studies have been done on the toxicity of MeBT towards bacterial cultures. Cornell (2002) obtained some of the first data on the effects of MeBT on bacterial intracellular ATP production and electron transport activity. He found that

MEBT levels near 10 mg/l could reduce ATP production in batch cultures of soil microorganisms grown on aircraft deicing fluids (ADF); however, this effect was not observed at any other concentrations studied (100 mg/l and 1000 mg/l 4(5)-MeBT). He also observed that electron transport activity did not change as judged by the reduction of tetrazolium salts by intracellular dehydrogenase activity; however, the statistical power of his experimental designs limited conclusions. His results did suggest that some benzotriazoles may confer toxicity to microorganisms by an uncoupling mechanism.

The most widely accepted definition of an uncoupler is an agent that promotes any or all of the following processes (Rottenberg, 1990): (i) stimulates basal (non-phosphorylating) electron transport; (ii) inhibits ATP synthesis without inhibition of electron transport; (iii) stimulates ATP hydrolysis; (iv) inhibits various exchange reactions catalyzed by the ATPase.

Most chemical uncouplers have been found to be weak acids with hydrophobic properties (Lehninger et al., 1993), which are both physical/chemical characteristics of MeBT. Jones (Jones and Watson, 1967) and Parker (Parker, 1965) further ascribed the most efficient uncouplers as those that are the most acidic and the most hydrophobic. Some of these compounds include: carbonyl cyanide, phenylhydrazones, some benzotriazole derivates, 3,5-dihalogeno-4-hydroxybenzonitriles, pentachlorophenol, pentafluorophenol, and, 2-trifluoromethylbenzimidazoles. Table 3-1 compares the acidity and lipophilicity of known uncouplers with the most commercially significant benzotriazoles used today. Of the benzotriazoles listed in Table 3-1, 5-chloro-4-nitro-benzotriazole, 5-

nitrobenzotriazole and, 4,5,6,7-tetrachlorobenzotriazole have been shown as uncouplers of oxidative phosphorylation (Parker, 1965).

Some researches have attempted to correlate the physiochemical properties of uncouplers with their uncoupling effects. Sturdik et al (Sturdik et al., 1987; Sturdik et al., 1988) employed a quantitative structure-activity relationship (QSAR) to correlate the partition coefficients, dissociation constants, and nucleophilic reactivity to p-substituted phenylhydrazonopropanedinitriles to their ability to disturb the membrane potential of rat liver mitochondria or *Paracoccus denitrificans.* They suggested that the most significant physical/chemical effect was the lipophilic property of the phenylhydrazonopropanedinitriles. They reported an optimal value of lipohilicity with *Paracoccus denitrificans* of 3.81 (log of octanol-buffer partition coefficient at pH=7.2). Proton dissociation (pKa) was the second most significant parameter.

Table 3-1. Acidity and Lipophilicity Data of Known and Implicated Uncouplers

Compound	Pk_a	K_{ow}
2-trifluoromethylbenzimidazole[a]	6.96	3.49
(2-methyl)hydrazono-propanedinitrile[b]	6.90	1.89[c]
pentachlorophenol[d]	4.71	5.01
pentalfluorophenol[e]	5.53	3.23
5-chloro-4-nitro-benzotriazole[f]	6.02	0.05
5-nitrobenzotriazole[f]	6.32	0.0025
4,5,6,7-tetrachlorobenzotriazole[f]	5.0	1.5
benzotriazole[d]	8.37	1.44
carboxybenzotriazole		1.18[g]
4(5)-methylbenzotriazole	8.8	2.10[g]

[a] (HSDB)
[b] (Sturdik et al., 1987)
[c] Partition coefficient in the system octanol-buffer of pH=7.2
[d] (NTP)
[e] (SRC)
[f] (Parker, 1965), the Kow listed is the hexane-water partition coefficient
[g] Leo's Fragment Method, (Hansch and Leo, 1979)

3.1.2 Other physiological effects of benzotriazoles

In the pharmacological literature, benzotriazole derivatives have been identified as potential potassium channel activators. Potassium channel activators have been identified as emerging therapy drugs for cardiovascular, respiratory or CNS diseases (Baragatti et al., 2000; Biagi et al., 2000; Biagi et al., 2001b; Biagi et al., 2001a). Benzotriazoles have also been identified as inhibitors of protein kinases CKI and CKII. CKII inhibitors are studied as potential antiviral agents (Szyszka et al., 1995; Szyszka et al., 1996; Sarno et al., 2001).

In environmental bacterial isolates, the toxic effects of MeBT have never been studied. However, the microbial toxicity of benzothiazoles, a compound structurally similar to MeBT, have been attributed to its metal chelating properties and its interference with membrane-bound (co)enzymes (De Wever and Verachtert, 1997).

3.1.3 Experimental Objectives

The objective of these experiments was to determine if 4(5)-MeBT can act as a electron transport system (ETS) inhibitor and/or an uncoupler of oxidative phosphorylation. The first part of these experiments consisted of determining the effect of MeBT on oxygen uptake rates (OUR). Isolated cell fragments have been confirmed to catalyze electron transfer from succinate or NADH to O_2, where no ATP synthesis can be coupled to this respiration (Lehninger et al., 1993). Therefore, OUR was measured in whole cells and their metabolically active cell fragments, in the presence and absence of 4(5)-MeBT.

A second experimental set consisted of determining the effects of MeBT on transmembrane potential, which is required for the generation of ATP (Novo et al.,

1999). Uncouplers can also cause membrane depolarization that can be linked to the dissipation of transmembrane electrochemical gradients. The hydrophobicity of some uncouplers allows them to diffuse across a biological membrane in a protonated form, and subsequently dissociate once inside a cell; thus, dissipating a proton gradient without the corresponding generation ATP (Lehninger et al., 1993). Therefore, in evaluating the respiratory uncoupling potential of 4(5)-MeBT, its ability to disrupt membrane potential was also evaluated.

3.2 Materials and Methods

3.2.1 Test Solutions

Methylbenzotriazole (MeBT) was obtained in kind from PMC Specialty Group Inc. (Cincinnati, OH, USA) or from Sigma-Aldrich Corporation (St Louis, MO). The MeBT used in these experiments consisted of a mixture of 4-MeBT and 5-MeBT in a 45:55 ratio (w/w), respectively. This is the same ratio found in most commercial ADF formulations. The HPLC-grade propylene glycol, succinate, and D-glucose was obtained from Fisher Scientific (Pittsburgh, PA).

3.2.2 Culture Media

Growth solution consisted of the following (per liter of MQ): NH_4SO_4, 600mg; K_2HPO_4, 440 mg; KH_2PO_4, 340 mg; $MgSO_4 \cdot 7H_2O$, 53 mg; $CaCl_2 \cdot 2H_2O$, 28 mg; $FeCl_3 \cdot 6H_2O$, 8.1 mg; $ZnSO_4 \cdot 7H_2O$, 0.68 mg; $CuSO_4 \cdot 5H_2O$, 0.17 mg; $CoCl_2 \cdot 6H_2O$, 0.17 mg; $NaMoO_4 \cdot 2H_2O$, 0.17 mg; $MnSO_4 \cdot H_2O$, 0.15 mg; H_3BO_3, 0.04 mg. The pH of this solution was 7.0. All solutions were heat sterilized prior to their use.

3.2.3 Chemical and Physical Parameter Analysis

3.2.3.1 MeBT

MeBT samples were collected using a polycarbonate pipette and centrifuged at 10,000 x g for 5 minutes. The supernatant was transferred to HPLC vials and stored at 4°C for analysis. The maximum storage time for MEBT samples was 1 month.

MeBT concentrations were measured using a SP8800 high performance liquid chromatograph (HPLC) fitted with an UV detector at 254 nm (Spectra Physics, San Jose, CA). Separation of isomers was performed isocratically using two Zorbax Rx-C8 4.6 x 250 mm columns in series (MacMod Analytical, Inc., Chadds Ford, PA, USA). The eluent consisted of a phosphate buffer mixed in a 70:30 ratio with HPLC grade acetylnitrile at a flowrate of 1.5 ml/minute incorporating a sample injection volume of 200 µl. Appendix 1 contains a sample calibration curve for 4(5)-MeBT.

3.2.3.2 Propylene Glycol

The concentration of propylene glycol was measured by a standard periodate oxidation method as previously described (Alben, 1991; Cornell, 2002). The absorbance was measured with a Hach Model 2000 (Hach Company, Loveland, CO) spectrophotometer at 412 nm. All samples were analyzed immediately upon collection. Appendix 1 contains a sample calibration curve used for propylene glycol analysis.

3.2.3.3 Quality Control Procedures

Quality control procedures are described in Appendix 2.

3.2.4 *Microorganisms*

The inoculants for the experiments consisted of two mixed cultures and one pure culture. The first mixed culture was grown from enrichments of soil bacteria taken from airport soils contaminated with aircraft deicing fluids and acclimated to propylene glycol (PG). A second mixed culture was grown in the laboratory from activated sludge and maintained with glucose as the carbon source. The pure culture was *Pseudomonas aeruginosa* (ATCC 39324).

3.2.5 *Cultures*

Mixed cultures were inoculated in 1L Erlenmeyer flasks with an initial TSS concentration between 100 to 300 mg/L and either propylene glycol (19.7 mM) or glucose (5.6 mM) as the electron donor. Once inoculated, reactors were mixed on a magnetic stirrer, allowed to equilibrate to room temperature (24°C ± 2°C) overnight achieving exponential growth conditions. *P. aeruginosa* was grown in a chemostat with glucose (2.8 mM) as the carbon source.

3.2.6 *Oxygen Uptake Measurements of Whole Cells and Cells Fragments*

Dissolved oxygen consumption was measured polarographically using an O_2 electrode (YSI, Model 5905) connected to an oxygen meter (YSI, Model 52). Before measuring oxygen consumption, all solutions were supersaturated with high purity oxygen gas by sparging for approximately 10 minutes. For the whole cell experiments, solutions were transferred to 60 ml BOD bottles prior to oxygen uptake measurements. Oxygen uptake of cell fragments was measured in 3 ml glass vials. Whole cells and cell fragments were tested for oxygen consumption at 24°C (± 2°C) in culture media at the following 4(5)-MEBT concentrations: 0, 50, 100, 300, and

500 mg/l of 4(5)-MeBT. To measure acute responses, filtered sterilized 4(5)-MeBT was added from a stock solution 10 minutes prior to measuring the OUR. Control systems consisted of reactors with no cells maintained under otherwise identical conditions. Oxygen consumption was measured for 10 minutes.

3.2.7 Preparation of Cell Fragments

Cell fragments were prepared according to methods previously reported by De Wever and coworkers (De Wever et al., 1994) and modified as follows. Cells from the mixed culture were harvested by centrifugation (30 minutes at 3,000 g) and washed with phosphate buffer (3 times). Cells were then re-suspended in phosphate buffer and broken with a French Press at 14.5 psi. Remaining cells and large debris were removed by centrifugation (as above) at 4°C. Succinate (6.5mM) was used as the electron donor for the cell fragment experiments.

3.2.8 Biomass Measurement

For whole cell experiments using propylene glycol as the carbon source, biomass concentration was determined by measuring absorbance at 490 nm (HACH Company, Model DR 2010). The absorbance was correlated to TSS concentration using a calibration curve built using these cultures raised under identical conditions (TSS = (ABS – 0.1959)/.0018, r^2 = .9776, n = 13). Prior to absorbance determination, reactors were amended with 0.1% NaN_3 to inhibit microbial activity. In addition, 10 µl of Tween 80 (Sigma-Aldrich Corporation, St Louis, MO) was added to each reactor to help homogenize the sample. Each reactor was stirred for at least 30 minutes before measuring the absorbance. For the whole cell experiments using glucose, TSS was measured directly prior to the start of the experiment.

Total suspended solids (TSS) was measured according to Standard Methods for the Analysis of Water and Wastewater Method 2540D (American Public Health Association et al., 1995) with the exception that a 45-μm Durapore filter (Fisher Scientific, Pittsburgh, PA) was used instead of glass fiber filters.

For the cell fragments, protein content was measured using a modification of the Lowry's (1951) method with a commercial kit (Bio-Rad Laboratories, Inc., Hercules, CA) using bovine serum albumin as a standard. An example standard curve for the protein method is included in Appendix 1.

3.2.9 Epifluorescent Microscopy

Two fluorescent stains were used for bacterial quantification: (i) 4'6-diamidino-2-phynylindole (DAPI) (Sigma-Aldrich Corporation, St Louis, MO), a DNA binding fluorescent stain used for total bacterial enumeration, and (ii) bis-(1,3-dibutylbarbiturid acid) trimethine oxonol ($DiBAC_4(3)$) (Molecular Probes, Inc., Eugene, OR), a fluorescent potentiometric probe which enters depolarized cells and binds to intracellular proteins (Molecular Probes, 1996). Controls consisted of heat-inactivated cells (60°C for 1 hr).

Samples for total cell counts were stained to a final concentration of 20 μg DAPI/ml. Samples for cell depolarization observations were stained to 10 ug DiBAC/ml as described by Jepras et al (1997). All cell preparations for microscopy were incubated for 2 minutes at room temperature prior to filtration through 25 mm diameter, 0.2 μm (average pore size), black polycarbonate filters (Poretics, Inc., Livermore, CA) backed with a silver membrane (25 mm diameter, 5.0 um pore size) (Osmonics, Minnetonka, MN). To ensure uniform distribution of cells on the filter

surfaces, stained cells were added to approximately 40 ml of sterile filtered (0.2 um) phosphate saline buffer (PBS) for DAPI assays and Milli-Q for the DiBAC assays. After filtration, the filters were washed with 50 ml of either PBS (DAPI) or Milli-Q (DiBAC) to remove any excess stain from the filter surfaces.

Filters were mounted using low fluorescence immersion oil and examined at 1100 x magnification using a Nikon Eclipse E400 microscope (Nikon Corp., Tokyo, Japan) fitted with a mercury lamp and polarizing filters (HBO-100 W mercury lamp; D360/40 excitation filter; 420 emission filter; 400DCLP beam splitter for DAPI, and HQ470/40 excitation filter; HQ525/50 emission filter; Q495LP beam splitter for $DiBAC_4$ (3) (ChromaTechnology Corp., Brattleboro, VT). Between ten and twelve random fields were counted per slide. All direct counts were reported as the average of all fields counted. Direct counts having a coefficient of variation (CV) greater than 30% were discarded and new sample aliquots were stained and counted until a uniform distribution was observed (Peccia, 2000).

3.2.10 Statistical Validation

All experiments were executed in random order and repeated at least three times. Results were reported as the arithmetic mean +/- standard deviation (SD). Statistical significance was determined by one-way or two-way analysis of variance (based on the design of the particular experiment) followed by the Dunnet's (1964) test. Differences were considered significant if P (two-tailed) < 0.05.

3.3 Results and Discussion

3.3.1 Respiration Inhibition with Whole Cells

3.3.1.1 Mixed Culture

The effect of 4(5)-MeBT on the respiration rate of whole cells in a mixed culture was measured four times. In these first series of experiments, the respiration rate was measured when propylene glycol was the electron donor. The solids retention time (SRT) of the batch culture used as the innocula for this experiment was 30 days. From preliminary studies suggesting that a toxicity response becomes apparent between 10 mg/l and 200 mg/l of 4(5)-MeBT, the concentrations tested in this experiment included 0, 10, 50, 100, 200, and 300 mg/l 4(5)-MeBT. Figure-1a summarizes the results for this experiment. At low MeBT concentrations (10 mg/l and 50 mg/l) there was an increase in averaged OUR compared to those cells with no MeBT with a subsequent decrease at higher concentrations. This culture had a significantly different response between the treatment with no 4(5)-MEBT and those responses with concentrations higher than 200 mg/l 4(5)-MEBT ($p = 0.0002$).

The same experiment was again repeated but with the culture at a markedly higher growth rate (SRT = 10 days) to see how it affected the respiration rates and toxicity responses. Figure 3-1b summarizes the results for this experiment. This time the respiration rates were two to three times higher than the first experiment. This experiment showed an increase in averaged OUR up to 100 mg/l 4(5)-MeBT and a decrease in OUR at 200 mg/l and 300 mg/l 4(5)-MeBT. Only the treatment with 300 mg/l 4(5)-MeBT was significantly different than the treatment with no 4(5)-MeBT ($p = 0.03$).

The third experiment consisted of a mixed culture maintained at a constant growth condition (SRT = 10 days) with glucose as the carbon source. Results are presented in Figure 3-1c. Based on the results from the previous experiments, oxygen uptake measurements were extended to include the presence of 500 mg/l 4(5)-MeBT. This experiment only showed a significant difference in oxygen uptake rates only at 500 mg/l 4(5)-MeBT ($p = 0.02$). Therefore, a fourth experiment was performed with propylene glycol as the electron donor but also including a measurement at 500 mg/l 4(5)-MeBT. Results are presented in Figure 3-1d. These results further confirmed that a significant decrease in oxygen uptake rate started at exposures of 300 mg/l 4(5)-MeBT ($p = 0.001$).

3.3.1.2 Pure Culture Response of P. Aeruginosa

For *P. aeruginosa* cultures, inhibitory responses of 4(5)-MeBT on oxygen uptake abilities were observed at 0, 10, 50, 100, 300, and 500 mg/l. Respiration was measured when glucose was the electron donor. Figure 3.2 summarizes the results of these experiments. As with the mixed culture enrichments, whole *P. aeruginosa* cells responded with an initial increase in OUR at concentrations less than 100 mg/l 4(5)-MeBT but, a statistically significant decrease in OUR was observed at 500 mg/l 4(5)-MeBT ($p = 0.03$).

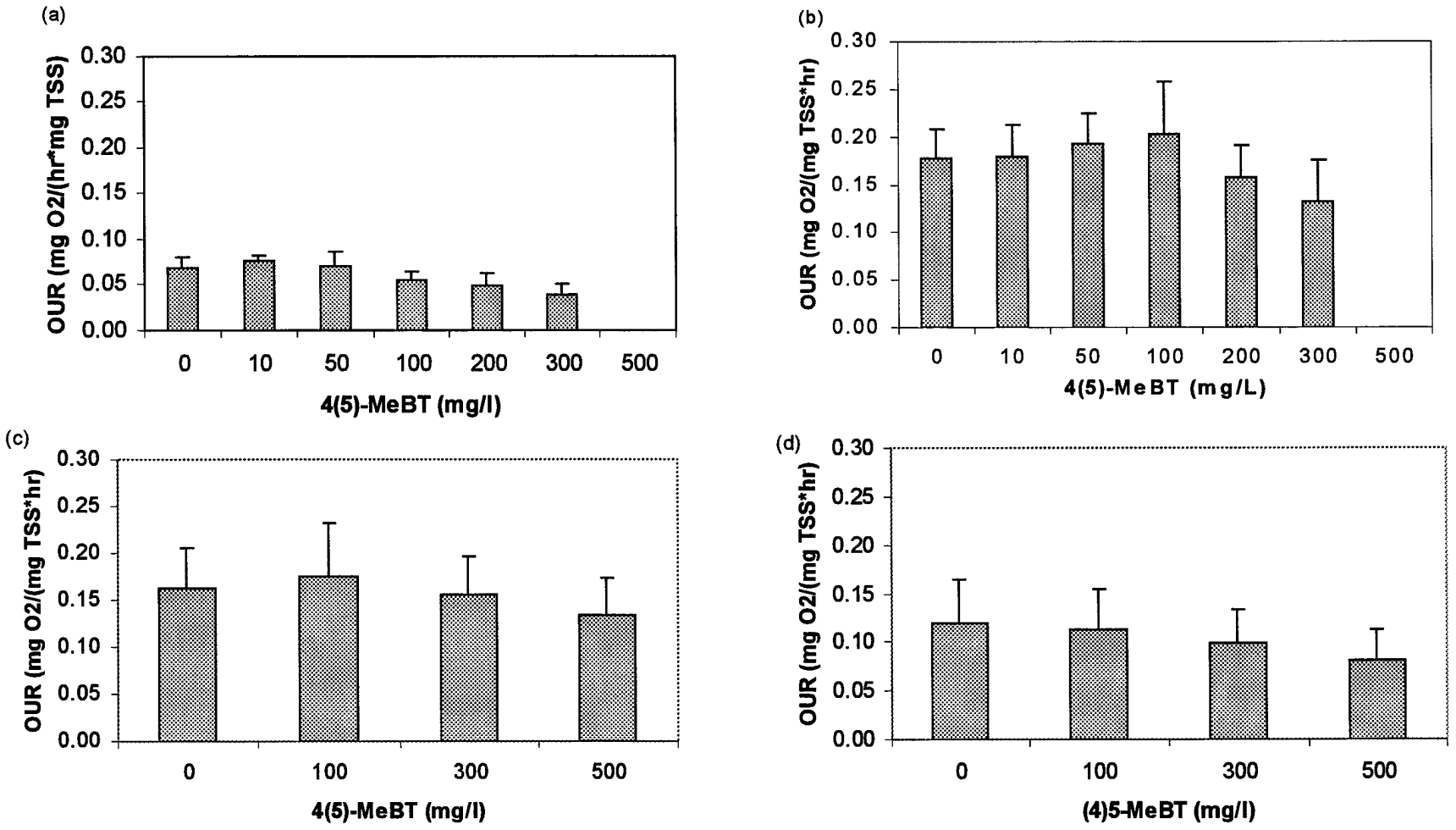


Figure 3-1. Mass normalized oxygen uptake rate of log-growth mixed cultures challenged with increasing 4(5)-MeBT concentrations. (a) Propylene glycol as substrate, SRT=30 days; (b) and (d) Propylene glycol as substrate, SRT=10 days; (c) Glucose as substrate, SRT=10 days. Bar height represents the average of five replicates. Error bars are ± 1SD.

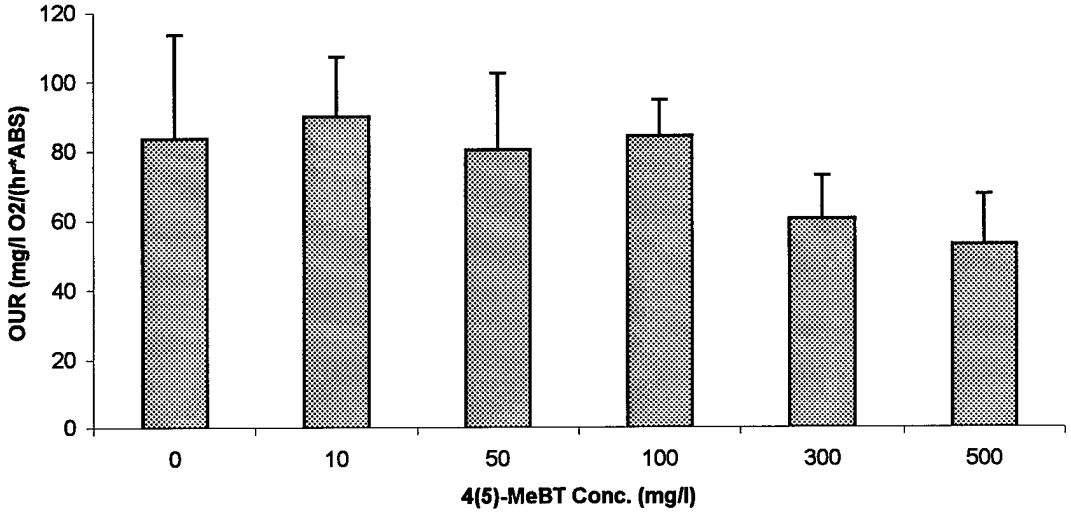


Figure 3-2. Absorbance normalized oxygen uptake rate of log-growth *P. aeruginosa* cells chanllenged with increasing 4(5)-MeBT concentrations. Glucose was used as the substrate. Bar height represents the average of five observations; error bars represent one standard deviation.

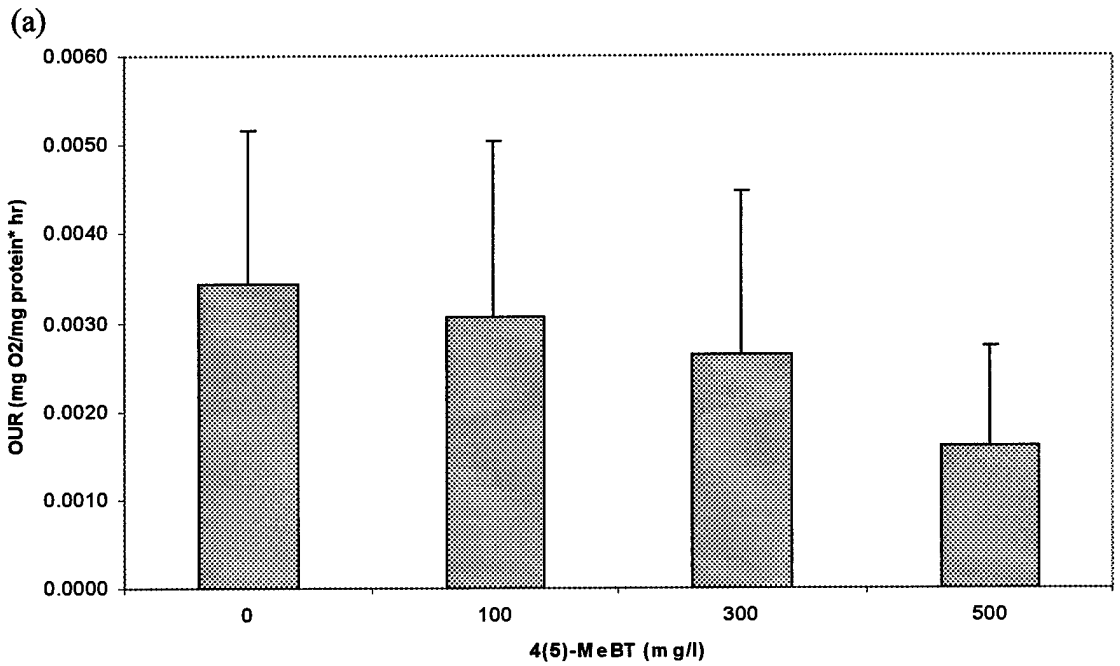
3.3.2 Respiration Inhibition with Cell Wall Fragments

The oxygen uptake rate of cell wall fragments separated and purified from the whole cells are presented in Figure 3.3a and for *P. aeruginosa* in Figure 3.3b. Succinate was used as the electron donor in these experiments.

For the mixed culture, the analysis of variance test and Dunnet's test showed a significant decrease in oxygen uptake of cell wall fragments at 500 mg/l 4(5)-MeBT ($p = 0.04$). For *P. aeruginosa* there was not a statistically significant difference between the oxygen uptake of cell wall fragments when 4(5)-MeBT was present and otherwise identical measurements with no MeBT.

3.3.3 Inhibition of Oxygen Uptake Rate of Whole Cells and Cell Wall Fragments

Figure 3.4 summarizes the response of whole cells and their cell fragments calculated as the ratio of their average oxygen consumption rate in the presence of increasing 4(5)-MeBT concentrations relative to where MeBT was not present. When compared with the relative oxygen consumption of whole cells, cell fragments of mixed cultures were essentially the same. These results indicate that the toxicity of MeBT in these mixed culture enrichments are likely governed by interactions involving the cell membrane and its constituents. However, for *P. aeruginosa* cells, the relative oxygen consumption for cell fragments was larger than whole cells when normalized by mass, indicating an inhibitory ETS response and possible uncoupling in this concentration range.



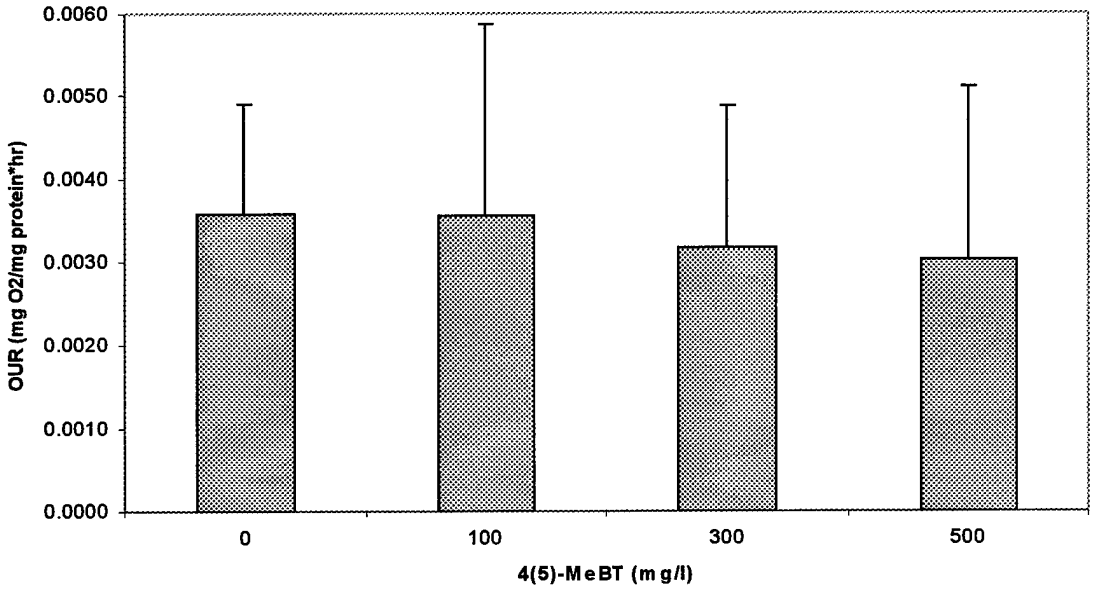


Figure 3-3 Mass normalized oxygen uptake rate of log-growth (a) mixed cultures and (b) *P. aeruginosa* cell wall fragments challenged with increasing 4(5)-MeBT concentrations. Succinate as the electron donor, SRT=10 days. Bar height represents the average of five replicates. Error bars are ± 1SD.

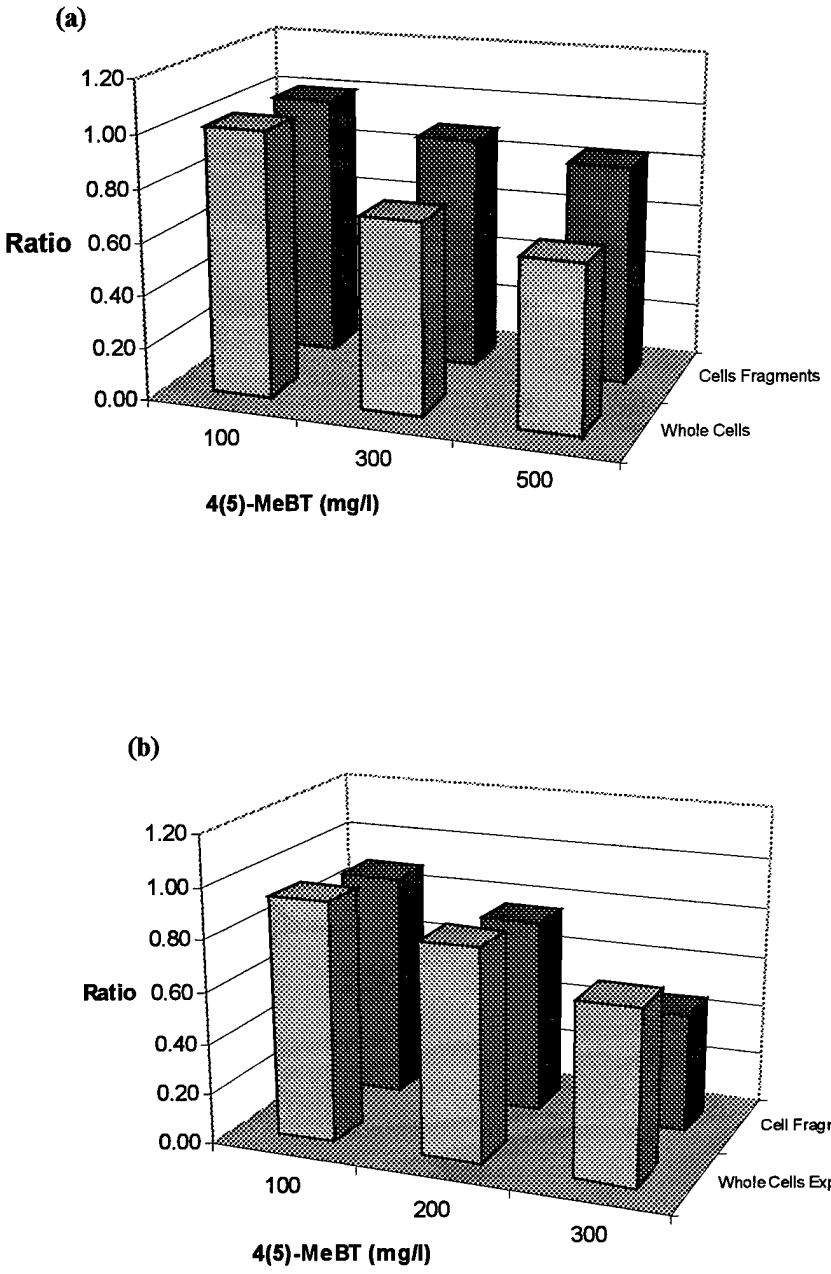


Figure 3-4. Relative oxygen consumption rate in the presence of different MeBT concentrations for (a) *P. aeruginosa* and (b) mixed culture compared to reactions without MeBT. Bar height represents the average of replicates.

3.3.4 Disruption of Membrane Potential

Oxygen uptake responses from whole cells and cell fragments suggest that both inhibition of ETS activity and uncoupling of oxidative phosphorylation are significant toxicity mechanisms in enrichments of mixed bacterial cultures and *P. aeruginosa* cells. To test the hypothesis that MeBT has the potential to disrupt ATP synthesis via an acute depolarization mechanism, a fluorescent dye, $DiBAC_4(3)$, was used to study acute membrane potential responses to increasing MeBT concentrations. Table 3-2 shows that increasing concentrations of 4(5)-MeBT (up to 500 mg/l) did not have a significant depolarization effect on soil bacteria raised in enrichment cultures using propylene glycol. However, *P. aeruginosa* cells showed significant depolarization responses to otherwise identical preparations with no MeBT. The data presented in Table 3-2 was calculated as the ratio of DiBAC positive to total cell counts (DAPI) for treatments with MeBT subtracting the ratio of DiBAC over DAPI for treatments without MeBT.

Table 3-2. Percent change in number of depolarized cells (%) compared to samples with no MeBT. Each value is the average of five replicates. Statistically significant responses are shown in bold.

MeBT (mg/l)	Mixed Culture	*P. aeruginosa*
100	0.35	-2.20
300	-0.075	**11.40**
500	-	**13.80**

3.4 Conclusions

MeBT was toxic to enrichments of soil bacteria with inhibitory oxygen uptake effects becoming apparent at MeBT concentrations greater than 200 mg/l. A main toxicity mechanism of whole cells appears to be associated with respiratory chain activity. A possible mechanism for this inhibition is MeBT complexation with membrane bound enzymes involved in the respiratory process - some of which contain redox active metals to which MeBT may coordinate. This toxicity mechanism has been previously proposed for the structurally similar benzothiazole derivatives (DeWever and Verachtert, 1997).

Pure cultures of *P. aeruginosa* responded to MeBT consistent with that of both ETS inhibition and concomitant membrane depolarization. This was demonstrated by the difference in oxygen consumption rate of whole cells and their metabolically active cell fragments. In addition, *P. aeruginosa* showed statistically significant increases in the infiltraton of potentiometric dyes compared to otherwise identical cells not challenged with MeBT. This finding furthers Cornell's previous observations where significant declines in cell yield become apparent at MeBT concentrations above 200 mg/l (Cornell, 2002).

The chemical structure and transition metal binding abilities of benzotriazoles derivatives suggest that both complexation interactions with the respiratory chain as well as uncoupling are plausible toxicity mechanisms. Indeed, they may be inseparable and occur concurrently given the appropriate concentrations. Support for an uncoupling behavior hypothesis was gained from the observation that an acute stimulatory effect on oxygen uptake rates was consistently observed when soil enrichments growing on PG were exposed to MeBT levels less than 100 mg/l;

however, uncoupling activity could not be isolated. At MeBT concentrations higher than 200 mg/l the decreases in oxygen uptake are likely due to increasing inhibition of activity of enzymes in the membrane bound respiratory chain. While uncoupling responses may indeed be occurring in the presence of relatively high MeBT concentrations, their observation may be masked by the inhibition of the ETS activity.

This study provides information useful in evaluating the potential impact on natural and engineered biological systems when MeBT is present. MeBT concentrations near the acute toxicity levels observed in this and previous studies (100 - 300 mg/l) have been found in groundwater near airports (Cancilla et al., 1998) and therefore it is possible that wastestreams at or near to toxicity levels found in these studies will be encountered by terrestrial bacterial populations and in bacteria in treatment systems receiving ADF-containing wastewater. This study further confirms that in evaluating the biodegradation potential of ADF wastes one must considered not only the toxicity that might be imparted by its main component, propylene or ethylene glycol, but the toxic effects of other components. Additives, such as MeBT, even if present at relatively small concentrations may hinder optimal performance of biodegradation processes.

CHAPTER 4

MINERALIZATION POTENTIAL OF 5-METHYLBENZOTRIAZOLE UNDER AEROBIC CONDITIONS

Abstract

Radioisotope labels can provide highly sensitive tools to study different aspects of bacterial metabolism. The objective of this study was to confirm the ability of soil bacteria to completely mineralize the common corrosion inhibitor 5-methylbenzotriazole (5-MeBT). U-^{14}C labeled 5-MeBT was added to suspensions of soil enrichment cultures that had been previously exposed to ADF and 5-MeBT. 500 mg/l TSS of biomass was exposed to 50 mg/l of U-^{14}C labeled 5-MeBT for 18 days. A significant mineralization response was observed in acclimated soil enrichments challenged with 50 mg/l 5-MeBT without notable lag period; approximately 68% of the [U-^{14}C]-5-MeBT was completely transformed to $^{14}CO_2$ under aerobic conditions. The amount of carbon assimilated as biomass (18%) compared favorably with yield measurements (0.2 ± 0.08) based on suspended solids and phospholipid increases.

4.1 Introduction

4.1.1 Radioisotope labeling

Radioisotope labels can provide highly sensitive tools to study the different aspects of bacterial metabolism. When a sample containing microorganisms or purified enzymes is incubated with a radioactively labeled compound(s), depending on the extent and type of radiolabel, different metabolic processes can be observed. In the case of carbon, ^{14} C is often used to examine the metabolic pathways of a wide

variety of substances in a microbial community or pure cultures (Mills, 1998). Both substrates as well as electron acceptors can be radiolabeled. For example, ^{35}S can be employed to confirm the potential for bacterial sulfate reduction in selected environments, as has been the case with sediment microcosms taken from San Diego Bay, which demonstrated the potential for those populations to oxidize polynuclear aromatic hydrocarbons under reducing conditions (Lovely et al, 1996).

During microbial degradation, the overall account of a carbon source supplied to microorganisms can be obtained by measuring the gaseous, soluble, and biomass fractions that result when a pure culture or community is challenged with a radiolabeled substrate (Raghavan et al., 1993):

$$C_T = C_B + C_{CO2} + C_S$$

where,
C_T = total amount of ^{14}carbon added to the culture solution
C_B = biomass ^{14}carbon
C_{CO2} = carbon recovered as $^{14}CO_2$
C_S = soluble ^{14}carbon (soluble biomass + CO_2 in solution + transformation by-products)

The initial mass is normalized by its radioactive emissions which can then be traced through metabolism (if it occurs), sorptive processes, partitioning and biomass incorporation.

The environmental significance of radiotracer biotransformation assays varies widely depending on the experimental design, the radiolabels used and incubation conditions. Most radiotracers assays simply determine the metabolic potential for a specific biotransformation process to occur – rarely can they demonstrate that a metabolic process occurs in the field. Such is the case with this investigation, where we report the first observations confirming the ability of soil microorganisms to

completely mineralize 5-methylbenzotriazole (5-MeBT) using a universal carbon label.

4.2 Materials and Methods

4.2.1 Chemicals

[U-^{14}C]-5-MEBT (500 μCi/g,) was synthesized from [U-^{14}C]-p-toluidine according to a zinc-catalyzed pathway published in U.S. Patent 4061491 and purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). The radiochemical purity of the [U-^{14}C]-5-MEBT was determined by the manufacturer and independently confirmed using high performance liquid chromatography (HPLC) to be greater than 98%.

4.2.2 Culture Media

Support media for microcosms consisted of the following (per liter of MQ): NH_4SO_4, 600mg; K_2HPO_4, 440 mg; KH_2PO_4, 340 mg; $MgSO_4 \cdot 7H_2O$, 53 mg; $CaCl_2 \cdot 2H_2O$, 28 mg; $FeCl_3 \cdot 6H_2O$, 8.1 mg; $ZnSO_4 \cdot 7H_2O$, 0.68 mg; $CuSO_4 \cdot 5H_2O$, 0.17 mg; $CoCl_2 \cdot 6H_2O$, 0.17 mg; $NaMoO_4 \cdot 2H_2O$, 0.17 mg; $MnSO_4 \cdot H_2O$, 0.15 mg; H_3BO_3, 0.04 mg. The pH of this solution was 7.0. All solutions were heat sterilized prior to their use.

4.2.3 Chemical and Physical Parameter Analysis

4.2.3.1 5-Methylbenzotriazole

^{14}C labeled 5-Methylbenzotriazole (5-MeBT) samples were collected using a polypropylene pipette and centrifuged at 10,000 x g for 5 minutes. The supernatant was transferred to HPLC vials and stored at 4°C for analysis. The maximum storage time for MeBT samples was 1 month.

MeBT concentrations were measured using a SP8800 high performance liquid chromatograph (HPLC) fitted with an UV detector at 254 nm (Spectra Physics, San Jose, CA). Separation was performed isocratically using two Zorbax Rx-C8 4.6 x 250 mm columns in series (MacMod Analytical, Inc., Chadds Ford, PA, USA). The eluent consisted of a phosphate buffer mixed in a 70:30 ratio with HPLC grade acetylnitrile at a flowrate of 1.5 ml/minute incorporating a sample injection volume of 200 µl. Appendix 1 contains a sample calibration curve for 4(5)-MeBT.

4.2.3.2 Total Suspended Solids

Total suspended solids (TSS) was measured according to Standard Methods for the Analysis of Water and Wastewater Method 2540D (American Public Health Association et al., 1995) with the exception that a 45-µm Durapore filter (Fisher Scientific, Pittsburgh, PA) was used instead of glass fiber filters.

4.2.3.3 Radioactivity measurements

^{14}C decay was measured with a Packard Instruments Model Tri-Carb 2300TR liquid scintillation counter (LSC) (Meriden, CT). Counts per minute (cpm) were converted to disintegrations per minute (dpm) by using an external standard (t-SIE) quench curve. The scintillation cocktail used for all radioactivity measurements was Ultima Gold (Packard BioScience Company, Meriden, CT).

4.2.3.4 Quality Control Procedures

Quality control procedures are described in Appendix 2.

4.2.4 Microorganisms

The innocula for these experiments consisted of an acclimated culture. The acclimated culture was grown from enrichments of soil bacteria taken from airport

soils contaminated with aircraft deicing fluids and exposed to 5-MeBT for extended periods.

4.2.5 Mineralization of ^{14}C-labeled MEBT

A mass balance on radiolabeled carbon was executed in 250 ml closed side-armed flasks (Bellco Glass, Inc, Vineland, NJ) (Figure 4-1) in which enrichment cultures were challenged with known quantities of U-^{14}C labeled 5-MeBT. Each flask contained 20 or 30 ml of culture media and biomass. The experiment was started at a biomass concentration of 500 mg/l total suspended solids (TSS). Each reactor was incubated at 25°C ± 2°C on an orbital shaker. The experiment consisted of 3 replicates and a control. The control consisted of biomass inactivated with 0.2% sodium azide. The sidearm $^{14}CO_2$ trap contained 5 ml of 1M NaOH. At each sampling interval, all the NaOH solution was removed from the trap and replaced with fresh NaOH. The radioactivity of the $^{14}CO_2$ trap was determined by diluting 1 ml aliquot in 15 ml of scintillation cocktail. The emission from this dilution was measured with 1 hour of its preparation.

4.2.6 Measurement of radioactivity in the medium and biomass

When the rates of radiolabeled carbon dioxide evolution indicated that mineralization process reached a plateau, ^{14}C which was incorporated into biomass and present in solution was determined by the following procedure: A 1ml sample of suspended biomass was obtained from each biometer and centrifuged for 15 minutes at 12,000 x g. After centrifugation, the supernatant was carefully separated from the biomass pellet. The activity of the supernatant (C_S) was measured by LSC as described above. To the biomass pellet, 250 µL of a proprietary alkaline surfactant

tissue solubilizer (ScintiGest, Fisher Scientific, Pittsburgh, PA) was added to aid in sample digestion. The pellet/tissue solubilizer mixture was then sonicated on ice for 2 hours. After which the radioactivity of the pellet (C_B) was also determined by LSC as described above.

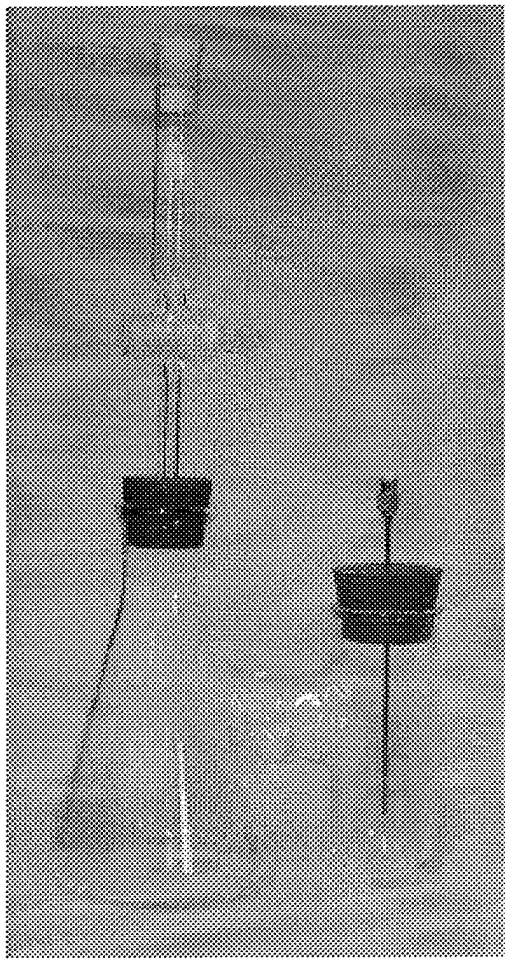


Figure 4-1. 250 mL Biometer flask used for 5 MeBT mineralization observations of soil enrichments. Photo from www.bellcoglass.com

4.3 Results and Discussion

Soil enrichment cultures, which had been previously exposed to aircraft deicing components and MeBT demonstrated a significant aerobic mineralization response degrading 50 mg/L [U-^{14}C]-5-MEBT within 5 days, most of which was $^{14}CO_2$. On average, final recoveries of 65 ± 5.6% as $^{14}CO_2$ were observed for these cultures. Degradation of [U-^{14}C]-5-MEBT to $^{14}CO_2$ was entirely attributable to biological activity because no mineralization was observed in otherwise identical systems that had been inactivated with sodium azide (Figure 4-2).

The amount of ^{14}C recovered from the biomass was 18% of the total ^{14}C added (Table 4-1). This is in agreement with yield observations estimated using standard suspended solids measurements and biomass phospholipid increases (effective yield c.a. 0.20). The ^{14}C radioactivity left in solution accounted for 11% of the total ^{14}C beta emission originally added to the biometers. As judged by HPLC, this ^{14}C could not be associated with any measurable amounts of 5-MeBT (<0.25 mg/l), and is likely some other unknown by product of bacterial metabolism, soluble biomass, and/or $^{14}CO_2$ present in solution.

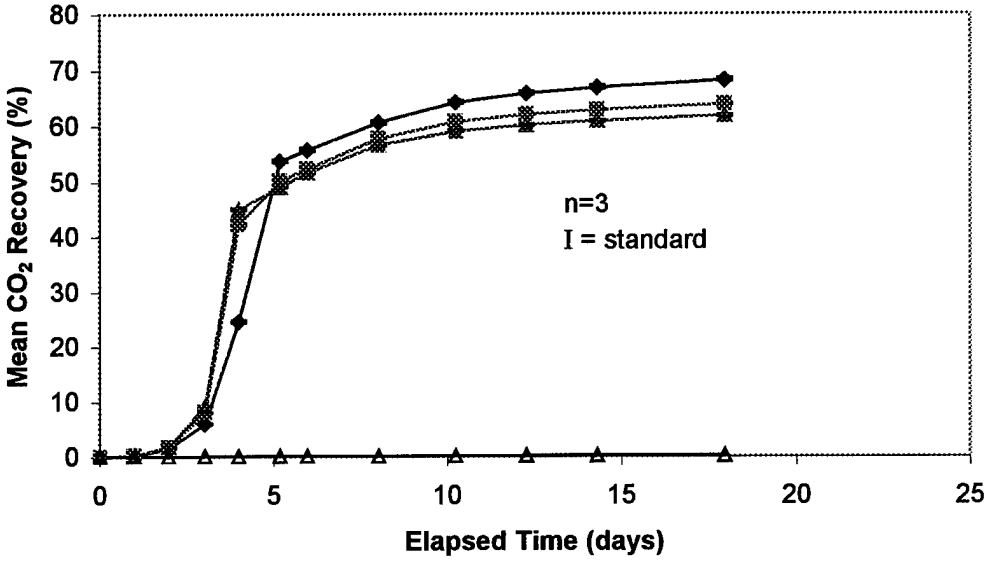


Figure 4-2. Percent $^{14}CO_2$ recovery from three biometers (■ 1, ◆ 2, ▲ 3) and a control (Δ) containing soil bacteria previously exposed to 5-MeBT and challenged with 50 mg/l 5-MeBT. Error bars are ± 1 standard deviation. Percent recovery is expressed as the percentage of total radioactivity applied at time zero.

Table 4-1. Average percentage of radioactivity incorporated into CO_2, biomass and present in medium after 18 days. The % radioactivity represents the average number of the three replicates.

	% Radioactivity	SD
CO_2	65	3.3
Soluble	11	0.4
Biomass	18	2.8
Total	94	6.5

4.4 Conclusion

Results from this study provided evidence that bacteria in surface soils can adapt and transform the carbon in selected benzotriazole derivatives to carbon dioxide (CO_2) with a relatively small yield. However, the fate of the nitrogen-containing moiety of 5-MeBT remains uncertain. These results document the first demonstration of aerobic microbiological 5-MeBT mineralization by soil microorganisms and have important implications regarding the biodegradation of 5-MeBT in natural and engineered systems. The deicing practices and widespread use of MeBT as a corrosion inhibitor suggests that it will remain present in soils and surface waters near cold weather airports, and that engineered treatment systems designed to treat aircraft deicing fluids must consider MeBT as one of the compounds needing treatment. Recent monitoring investigations have already demonstrated the presence of benzotriazole derivates in U.S streams (Kolpin et al., 2002). These streams were not necessarily close to airports or other places were large quantities of deicing fluids are used, indicating that the widespread use of benzotriazoles as corrosion inhibitors might be affecting other environmental receptors not normally associated with deicing operations.

The results from this study indicate that microorganisms that inhabit soil, water, or wastewater where ADF is present may be acclimated to 5-MeBT and may have the metabolic potential to degrade 5-MeBT to predominantly non-toxic products.

CHAPTER 5

AEROBIC GROWTH ON METHYLBENZOTRIAZOLE AS THE SOLE CARBON AND NITROGEN SOURCE BY A MIXED BACTERIAL CULTURE

Abstract

The ability of bacteria to use a common benzotriazole derivative, 5-methylbenzotriazole (5-MeBT), as a sole source of carbon and nitrogen was assessed in microcosms containing enrichments from activated sludge and soils contaminated with aircraft deicing fluid. Under aerobic conditions, bacteria that had been previously exposed to the main components of deicing fluids (acclimated cultures), were able to grow on 5-MeBT with an effective yield of 0.24 ± 0.08 as judged by biomass increases independently confirmed by standard gravimetric analysis and a novel phospholipid assay. Relatively low-density acclimated cultures (TSS < 200mg/l) rapidly removed up to 100 mg/l 5-MeBT in the absence of supplemental carbon or nitrogen sources. Denitration of 5-MeBT resulted in significant ammonia release. After 60 days of exposure, activated sludge bacteria that had not been previously exposed to aircraft deicing fluids (non-acclimated) were not able to metabolize 50 mg/l of 5-MeBT when nitrogen was not provided from an external source. However, when non-acclimated activated sludge cultures were challenged with 100 mg/l 5-MeBT, two out of three replicates removed the 5-MeBT. These results indicate the potential for activated sludge and soil microorganisms to acclimate to relatively high concentrations of 5-MeBT. In addition, these results

demonstrate the biodegradation potential of 5-MeBT under aerobic nitrogen-limiting conditions.

5.1 Introduction

The potential for bacterial transformation of 5-methylbenzotriazole (5-MeBT) under aerobic conditions has been observed (Cornell, 2002); however, there are no studies documenting the ability of bacterial populations to use MeBT as a sole carbon and nitrogen source.

The objective of this study was to evaluate the potential for 5-MeBT to be utilized as a sole carbon and nitrogen source by naturally occurring soil and activated sludge bacteria under aerobic conditions. In addition, measurements of growth yield on 5-MeBT were made using two independent methods: biomass gravimetric total suspended solids (TSS) and phospholipid content.

The traditional gravimetric methods used in environmental engineering to measure microbiological growth can often be insensitive for low yield substrates, or when biomass concentrations are relative low. Optical methods, such as nephelometric or absorbance observations can be unsuitable for estimating the growth of many different types of cultures where their physiological states interfere with predictable light scattering response (i.e. cellular clumping, filamentous morphology or excessive polysaccharide production). Phospholipid analysis, a method routinely used for determining biomass in sediments, soils, and biofilms, can provide an accurate and sensitive way of confirming biomass growth on low-yield substrates in some bacterial cultures regardless of source or growth conditions (Findlay et al., 1989). Phospholipids, which are predominantly contained in the cell membrane, are

easily extractable and their measurement is reproducible (White et al., 1979). Phospholipids assays target only viable microbial biomass. This is because with cell death, exogenous and endogenous phospholipases rapidly transform polar lipids in the cell membranes to nonpolar neutral lipid diglycerides by removing polar phosphate-containing head groups (White et al., 1996); these non-polar lipids are not labile to extraction. A disadvantage of phospholipid analysis is that environmental stresses can change the phospholipid concentration as well as configuration in bacterial cells. White and coworkers (1996) reported that up to 50 percent changes in phospholipid concentration could occur in bacteria when environmental stresses challenge the survival of cells in nature (e.g. temperature, solvents). Thus, phospholipid assays used to assess the yields of bacterial populations are valid relative to their growth conditions and must be carefully controlled.

Findlay and coworkers (1989) modified a classical phospholipid assay to greatly increase its simplicity and sensitivity for applications to environmental systems. These modifications were able to achieve a high degree of reproducibility; coefficients of variation of less than 5% for phospholipid extraction in sediments were reported. Wang and coworkers (1995) assessed the reproducibility of Findlay's modified phospholipids assay when used to measure biomass on several types of filter media such as granulated activated carbon and anthracite. They reported coefficients of variation of less than 4% when the phospholipid concentration was greater than 5 nmoles lipid phosphate per gram of media.

5.2 Materials and Methods

5.2.1 Test Solutions

5.2.1.1 Benzotriazoles

Benzotriazoles were obtained in kind from PMC Specialty Group Inc. (Cincinnati, OH, USA) or bought from Sigma-Aldrich Corporation (St Louis, MO). HPLC-grade propylene glycol was obtained from Fisher Scientific (Pittsburgh, PA, USA).

5.2.1.2 Culture Media

The synthetic media (Accashian et al., 1998) consisted of the following (per liter of MQ): K_2HPO_4, 440 mg; KH_2PO_4, 340 mg; $MgSO_4 \cdot 7H_2O$, 53 mg; $CaCl_2 \cdot 2H_2O$, 28 mg; $FeCl_3 \cdot 6H_2O$, 8.1 mg; H_3BO_3, 0.04 mg; $CuSO_4 \cdot 5H_2O$, 0.17 mg; $MnSO_4 \cdot H_2O$, 0.15 mg; $ZnSO_4 \cdot 7H_2O$, 0.68 mg; $CoCl_2 \cdot 6H_2O$, 0.17 mg; $NaMoO_4 \cdot 2H_2O$, 0.17 mg. The pH of this solution was 7.0. All solutions were heat sterilized prior to their use.

5.2.2 Chemical and Physical Parameter Analysis

5.2.2.1 5-MeBT

5-MeBT concentrations were measured using a SP8800 high performance liquid chromatograph (HPLC) fitted with a UV detector (λ = 254 nm) (Spectra Physics, San Jose, CA). Separation of the two isomers was performed isocratically using two Zorbax Rx-C8 4.6 x 250 mm columns in series (MacMod Analytical, Inc., Chadds Ford, PA, USA). The eluent consisted of a phosphate buffer mixed in a 70:30 ratio with HPLC grade acetylnitrile at a flowrate of 1.5 ml/minute incorporating a

sample injection volume of 200 µl. Appendix 1 contains a sample calibration curve for this procedure.

5.2.2.2 Ammonia

Ammonia was measured by using HACH Method 10031 (Hach Company, Loveland, CO). An independent laboratory confirmed the nitrate, nitrate, and ammonia concentrations by ion chromatography and colorimetric analysis respectively.

5.2.2.3 Total Suspended Solids

In selected experiments, biomass was measured as total suspended solids (TSS). TSS was measured according to Standard Methods for the Analysis of Water and Wastewater Method 2540D (American Public Health Association et al., 1995) with the exception that a 45-µm Durapore filter (Fisher Scientific, Pittsburgh, PA) was used instead of a glass fiber filter.

5.2.2.4 Phospholipids

A modification of the phospholipid analysis developed by Findlay and coworkers (Findlay et al., 1989) was also used to quantify microbial growth. The phospholipid analysis included the following steps in acid washed glassware: (1) 1 ml of reactor sample, 1 ml of MilliQ water, 5 ml of methanol and, 2.5 ml of chloroform were mixed (in this order). Blanks were prepared from 1 ml of filtered (0.2 µm) reactor material. This mixture was allowed to react between 2 and 24 hours to extract the phospholipids; (2) after extraction, 2.5 ml of a sulfuric acid solution (0.0306 M) and 2.5 ml of chloroform was added to separate the lipid-containing chloroform phase. This mixture separated overnight. (3) a portion of the chloroform

layer was obtained using separatory funnels, transferred into 5 ml ampoules (Wheaton, Millville, NJ) and dried with high purity nitrogen at 37°C. (4) 0.9 ml of persulfate reagent (5 g of $K_2S_2O_8$ in 100 ml of 0.36 M H_2SO_4) was then added to each ampoule and sealed. (5) the contents of the ampoules were digested overnight at 100°C. (6) after the ampoules cooled to room temperature, 0.2 ml of 2.5% ammonium molybdate reagent (2.5% $(NH_4)_6MoO_{24}{\cdot}4H_2O$ in 5.72 N H_2SO_4) was added. (7) after 10 minutes of incubation, 0.9 ml of malachite green reagent was added (0.011% malachite green in 0.111% of polyvinyl alcohol solution) and incubated for an additional 30 minutes. (8) Absorbance was then measured in disposable cuvettes at 610 nm and compared to a standard curve developed with potassium phosphate. Findlay reported a lower detection limit for this method of 0.1 nmol of phosphate, which corresponded to 3.4×10^6 bacterial cells enriched from marine sediments and grown aerobically in liquid batch cultures (Findlay et al., 1989). Appendix 1 contains a sample standard curve for this method.

5.2.3 Microbial Enrichments

Microbial cultures consisted of both non-acclimated and acclimated cultures with respect to benzotriazole exposure. The acclimated cultures for these experiments were enriched from shallow subsurface soils within two meters of the central deicing pad at Denver International Airport; the soils in this area have been comprehensively analyzed and its sampling and enrichment previously described (Cornell, 2002). This culture has been maintained with aircraft deicing fluids components for the last four years at room temperature in batch reactors under aerobic conditions.

The non-acclimated culture was obtained from a bench-scale sequencing batch activated sludge reactor. This system was initially inoculated from a local municipal wastewater treatment plant. The reactor was operating under aerobic conditions with glucose as a substrate when biomass samples were removed for these experiments.

5.2.4 Substrate Utilization Assays

Inoculum for these experiments consisted of either acclimated or non-acclimated biomass, which were normalized by gravimetric analysis immediately prior to metabolic assays. Cells were harvested by centrifugation at 3500 rpm for 30 min washed three times in 0.85% NaCl before final suspension in 1200 ml of culture media. At this point 5-MeBT was added from a filtered-sterilized stock solution and initial parameters measured (5-MeBT, TSS, phospholipids, and ammonia). This culture dilution was subsequently divided into 300 ml aliquots deposited into autoclaved one liter Erlenmeyer flasks (three replicates and one control). All flasks were incubated either on an orbital shaker at 140 rpm or on magnetic stir plates at 22 $\pm$ 2°C. Control flasks consisted of autoclaved inocula or included 0.2% of sodium azide to quantify any potential abiotic transformations under otherwise identical conditions. No reactions between sodium azide and MeBT were observed.

Samples for 5-MeBT analysis were periodically withdrawn with a sterile polypropylene pipette and centrifuged at 10,000 x g in polypropylene microcentrifuge tubes for 5 minutes to separate biomass. After centrifugation, the supernatant was transferred to 2.0 ml amber crimp top HPLC vials (Agilent Technologies, Part # 5181-3376), stored at 4°C prior to analysis and analyzed for 5-MeBT by HPLC .

5.3 Results and Discussion

5.3.1 Removal of 5-MeBT by an acclimated culture

The utilization of 5-MeBT (100 mg/l) as the sole carbon and nitrogen source was first investigated with acclimated cultures under aerobic conditions. The first experiment (Exp 1) was conducted at an initial biomass concentration less than 200 mg/l TSS; the second (Exp 2) at 500 mg/l TSS. Rapid removal of 5-MeBT was evident in the enrichment flasks, while the corresponding removal in the control flask was not measurable (Figure 5-1).

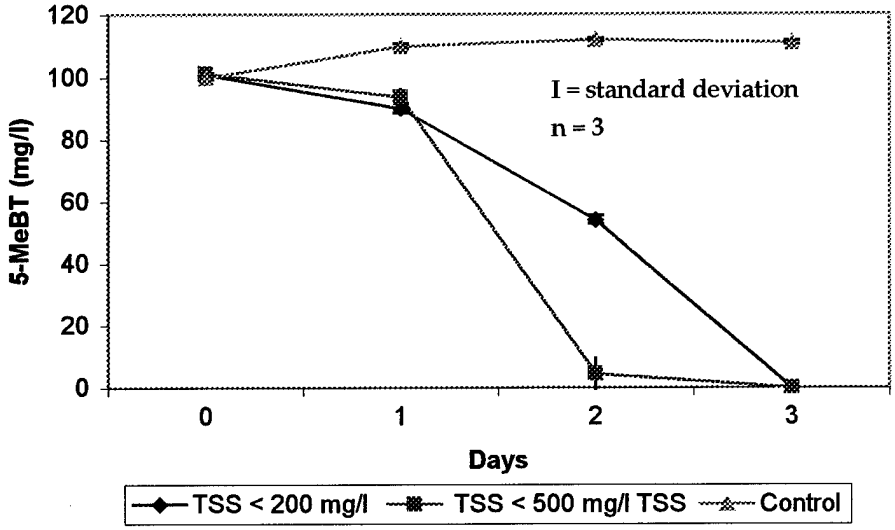


Figure 5-1. Removal of 5-MeBT by acclimated cultures at two different biomass concentrations: ■ 500 mg/l TSS, ◆ 200 mg/l TSS, ▲ Control. Each data point represents the average of three replicate reactors except for the control. 5-MeBT was the only source of carbon or nitrogen for these cultures. Error bars are ± 1SD.

5-MeBT removal was concomitant with substantial releases of ammonia to solution (Tables 5-1 and 5-2). Nitrate or nitrite was not detected. Assuming that the oxidation of 5-MeBT produces ammonia as its major nitrogen mineralization product:

$$C_7H_7N_3 + H_2O + 3H^+ + 6.5O_2 \rightarrow 7CO_2 + 3NH_4^+$$

the oxidation of 100 mg/l of 5-MeBT could liberate as much as 31.6 mg/l nitrogen as ammonia (NH_3-N); assuming a yield of 0.2 and a biomass composition containing 14% nitrogen by mass, this would correspond to a total ammonia liberation potential of 28.8 mg/l NH_3-N. Up to 41% of the nitrogen initially added to the cultures as MeBT was subsequently liberated into solution as ammonia. These results suggest the following scenarios with respect to MeBT oxidation under these conditions: (i) the near-complete mineralization of 5-MeBT did not occur, (ii) the mineralization of 5-MeBT occurs but does not results in the liberation of all its azo-bound nitrogen to ammonia, nitrate or nitrite. The potential for complete mineralization of the carbon-containing portion of 5-MeBT was reported in Chapter 4. However, those experiments were conducted with external nitrogen sources present, and were designed only to track the fate of radiolabeled carbon in MeBT, which is only present in the toluene moiety of the molecule. The fate of the heterocyclic azo-nitrogen during the metabolism of benzotriazole derivatives remains uncertain.

Table 5-1 Initial and final biomass, phospholipid and aqueous ammonia concentrations associated with a relatively low biomass concentration (< 200 mg/l) acclimated culture completely removing 100 mg/l 5-MeBT from enrichment solution (average and standard deviation). The number in parenthesis represents the number of repetitions of each test.

	Initial	**Final**
TSS (mg/l)	129 ± 8 (3)	152 ± 13 (9)
nmol lipid-phosphate/ml	4.33 ± 1.40 (2)	5.64 ± 1.07 (9)
NH_3-N mg/l	0.2 ± 0.2 (3)	8.6 ± 0.62 (9)

Table 5-2. Initial and final phospholipid and aqueous ammonia concentrations associated with a high (500 mg/l) biomass acclimated culture completely removing 100 mg/l 5-MeBT from enrichment solution (average and standard deviation). The number in parenthesis represents the number of repetitions of each test.

	Initial	**Final**
nmol lipid-phosphate/ml	16.2 ± 1.14 (2)	19.7 ± 1.98 (9)
NH_3-N mg/l	0.2 ± 0.2 (3)	12.3 ± 1.05 (9)

Growth under these conditions was confirmed by both TSS (Exp 1) and phospholipid increases (Exp 1 and 2) which were concomitant with the disappearance of 5-MeBT from solution. The effective yield for this acclimated culture with ammonium sulfate as a nitrogen source was 0.29 mg TSS/mg 5-MeBT; under

otherwise identical conditions in the absence of an exogenous nitrogen source the effective yield was 0.24 ± 0.08 mg TSS/mg 5-MeBT.

Since phospholipid analysis has been previously reported for sediments, soils, and biofilm assessments, the validity of this test to monitor suspended culture growth was investigated. With concurrent TSS and phospholipid assays (Table 5-1), it was possible to calculate the ratio of phospholipid per gram of carbon present in these MeBT degrading cultures. Assuming that the dry cell mass was 50% carbon (Dobbs and Findlay, 1993), the relative amount of phosphate per gram of biomass carbon was 67 μmol phosphate/ g carbon. Dobbs and Findlay (and references herein) reported a range of 63 to 168 μmol phosphate/ g carbon for aerobic pure cultures and 520 μmol phosphate/ g carbon for aerobic enrichment cultures (Dobbs and Findlay, 1993).

Using this relative concentration, it was possible to predict phospholipid concentration based on the final TSS concentration or yield. The predicted final phospholipid concentration for the acclimated culture growing on 5-MeBT (Experiment 1) was 5.10 nmoles/ml, which compared favorably with the average measured final phospholipid concentration of 5.64.

5.3.2 Response of non-acclimated activated sludge to 5-MeBT exposure

The response of 5-MeBT by a non-acclimated culture was investigated at two concentrations, 50 mg/l and 100 mg/l 5-MeBT, and a starting biomass concentration between 400 mg/l and 500 mg/l TSS. After 60 days, the reactors containing 50 mg/l 5-MeBT did not remove any of the 5-MeBT when present as a sole carbon and nitrogen source. However, significant liberation of ammonia was observed, which was solely due to endogenous decay. After 32 days of exposure the average NH_3-N

concentration of three replicate reactors increased from detection limit (c.a. 0.5 mg/l) to greater than 30 mg/l (Figure 5-2).

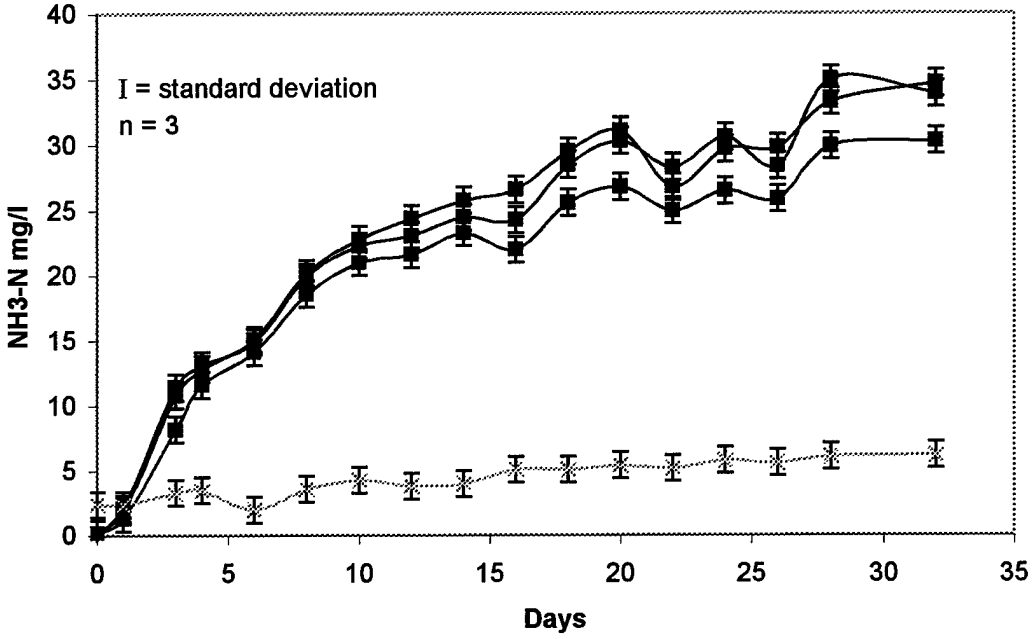


Figure 5-2 Ammonia accumulation of three non-acclimated reactors (■) and a control (✳) with an initial 5-MeBT concentration of 100 mg/l. Error bars are ± 1SD.

Phospholipid concentration was also monitored for the first 32 days of incubation. Phospholipid concentrations decreased sharply likely due to endogenous decay and the inability to biodegrade the carbon source available (5-MeBT) (Figure 5-3). The average reduction in phospholipid concentration was 78%.

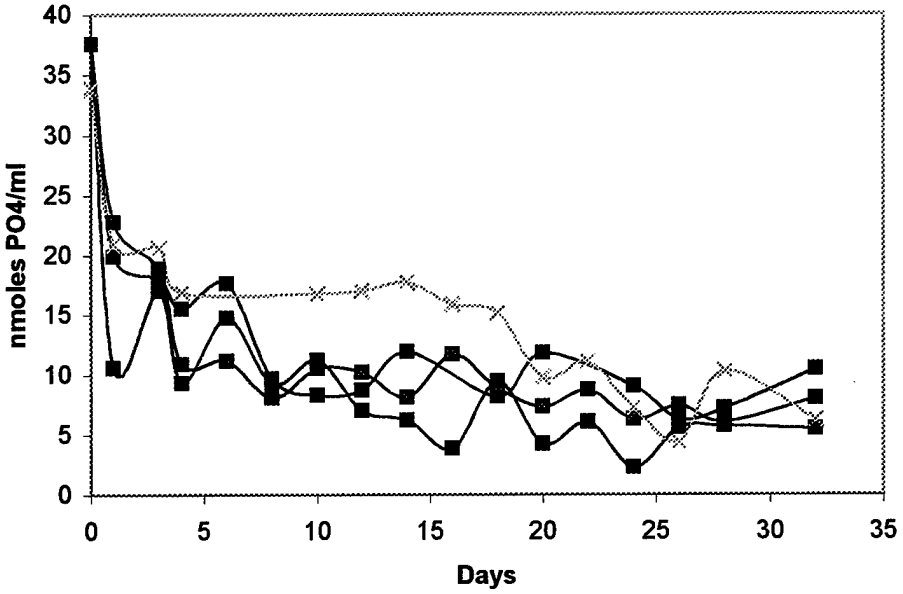


Figure 5-3. Phospholipid concentrations of three non-acclimated cultures (■) and (one control (∗) with an initial 5-MeBT concentration of 50 mg/l.

When challenged with 100 mg/l 5-MeBT, two out of three non-acclimated activated sludge enrichments were capable of using 5-MeBT as a sole carbon and nitrogen source after a 15 and 24 days, respectively (Figure 5-4). A third non-acclimated enrichment, initiated with the others that developed a 5-MeBT degradation response, was not capable of transforming the 5-MeBT after 60 days of incubation. These reactors also showed a sharp decrease in phospholipid concentration (Figure 5-5) and continuous ammonia production likely due to endogenous decay (Table 5-3).

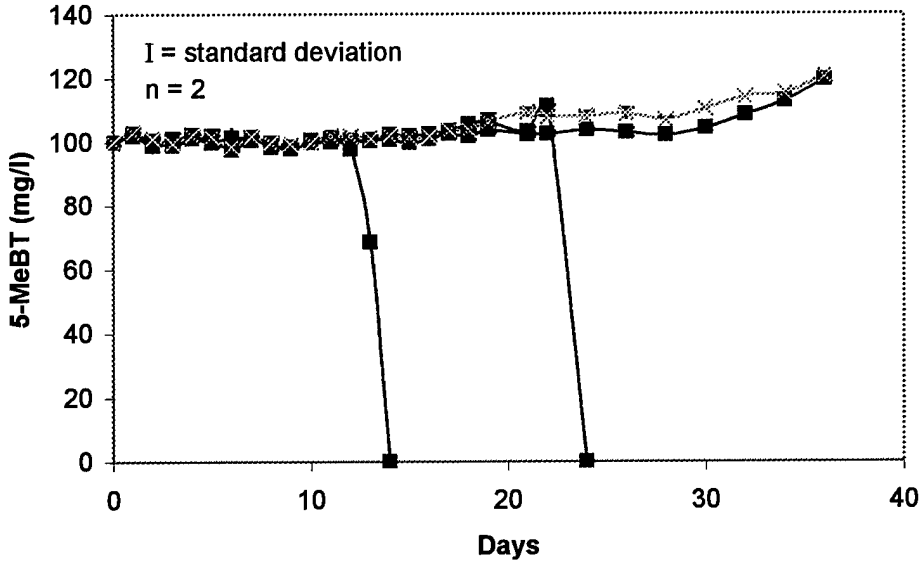


Figure 5-4. MeBT concentrations in three non-acclimated activated sludge enrichments (■) and a control (∗) with a starting concentration of 100 mg/l. The 5-MeBT concentration for the third replicate and the control were monitored for 60 days; no response to 5-MeBT was observed in that period.

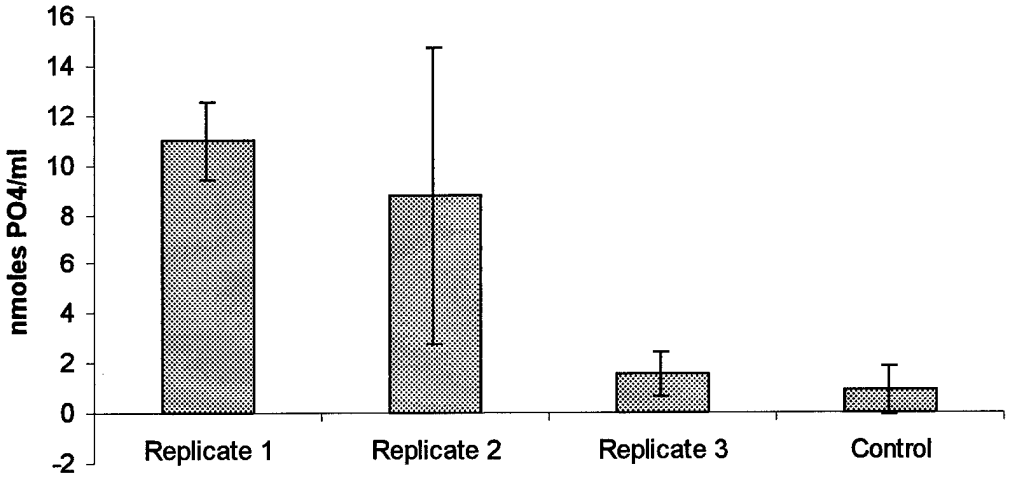


Figure 5-5 Biomass lipid phosphate concentration of three enrichments (and one control) containing a non-acclimated culture. The initial 5-MeBT concentration was 100 mg/l. For the first two replicates the phospholipid concentration was measured after all the MeBT was consumed (14 and 24 days respectively). The third reactor did not transform the MeBT, therefore, the phospholipid concentration was measured after 45 days of incubation. The initial phospholipid concentration of all reactors was 15.2 nmol PO_4/ml. All values represent the average of three measurements. Error bars are ± 1SD.

Table 5-3. Ammonia liberated to solution during the removal of 5-MeBT of three replicates (and one control) reactors with an initial 5-MeBT concentration of 100 mg/l. The numbers in bold represents those reactors that were able to consume the MeBT. The final concentration for the third replicate and the control were measured after 45 days.

	NH_3-N (mg/l)	
	Initial	**Final**
Replicate #1	**0.1 ± 0.3 (6)**	**26.8 ± 1.2 (3)**
Replicate #2	**0.1 ± 0.3 (6)**	-
Replicate #3	0.1 ± 0.3 (6)	33.5 ± 4.7 (3)
Control	0.1 ± 0.3 (6)	10.6 ± 0.6 (3)

The final phospholipid concentration of the activated sludge cultures used in these experiments showed that the biomass concentration could decrease significantly during the acclimation period (prior to any MeBT biodegradation response). However, by measuring the phospholipid concentration continually during the incubation period it was possible to determine if after the acclimation period these cultures were capable of using 5-MeBT for growth. Figure 5-6 summarizes the results of one of the non-acclimated enrichments containing an initial 5-MeBT concentration of 100 mg/l. This culture was capable of using 5-MeBT for growth following an extended period (12 days) of endogenous decay in the presence of 5-MeBT. Ammonia release, consistent with acclimated cultures able to rapidly degrade 5-MeBT, was observed. 5-MeBT removal rates (after the acclimation period) for

these activated sludge non-acclimated enrichments were also consistent with removal rates measured for acclimated enrichments (0.1 mg 5-MeBT/(mg TSS*day).

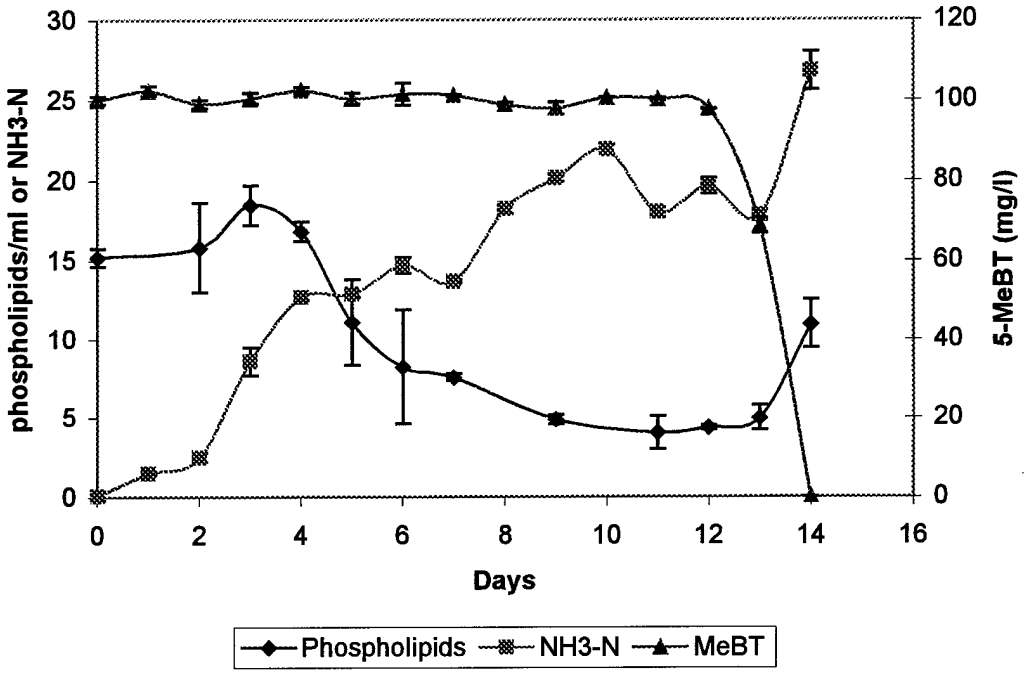


Figure 5-6. Phospholipid, ammonia and 5-MeBT concentrations patterns of an activated sludge culture, which has not been previously exposed to 5-MeBT. The initial biomass concentration for this culture was 400 mg/l TSS. Error bars are ± 1SD. Each value represents the average of 3 measurements.

5.4 Conclusions

Phospholipid analysis, a method originally developed to measured biomass concentrations in subsurface soils was successfully adapted for use with liquid cultures with very few modifications. The phospholipid method was sensitive enough to estimate biomass growth with a benzotriazole derivative that produces a relatively low biomass yield (0.2-0.3).

An acclimated soil enrichment culture was capable of using 5-MeBT as a sole carbon and nitrogen source and removing as much as 100 mg/l of 5-MeBT from solution. The removal of 5-MeBT by an acclimated culture was relatively rapid (substrate uptake rate) and resulted in ammonia nitrogen accumulation and measurable biomass growth. Non-acclimated activated sludge cultures were not always capable of using 5-MeBT as a sole carbon and nitrogen source. However, in those instances were MeBT removal was observed; growth and ammonia release was evident. The effective yields observed as well as the ammonia liberated were near that of acclimated cultures. The amount of ammonia released was less than 40% of the amount predicted by the stoichiometry of complete MeBT mineralization. Other forms of inorganic nitrogen (nitrate and nitrate) were not detected in solution. In contrast, studies with benzothiazoles, a compound structurally similar to MeBT, found that ammonia was produced close to the expected stoichiometric amounts (De Wever and Verachtert, 1997; Gaja and Knapp, 1997). These results suggest that 5-

MeBT biodegradation is possible in environmental situations where nitrogen is limited.

CHAPTER 6

CHARACTERIZATION OF BENZOTRIAZOLE TOLERANT ENVIRONMENTAL ISOLATES.

Abstract

Two pure bacterial cultures, capable of growing in the presence of 5-methylbenzotriazole (5-MeBT) were isolated from enrichments of shallow subsurface soils contaminated with aircraft deicing fluids (ADF). Both cultures were isolated on Noble agar (Difco Laboratories, Detroit, MI) with 25 mg/l 5-MeBT as the only carbon source. Colonies grew to typical size and morphology described by systematic sources; however neither isolate could grow in solution with 5-MeBT as the main carbon source. As judged by PCR amplification and sequencing of small subunit ribosomal RNA using broad-spectrum primers, these isolates were tentatively identified as members of the genera *Pseudonocardia* and *Variovorax*. Using statistically-based electronic alignment tools available from the National Center for Biotechnology Information BLAST network, the *Pseudonocardia sp.* and *Variovorax sp.* isolated were respectively assigned 88.4% and 87.6% as their highest similarity indices for homology to RNA sequences catalogued with the Ribosomal Database Project (RDP). This similarity was based on a 492 bp amplicon of 16sDNA using 27f-519r Bacterial primer set.

6.1 Introduction

Aerobic degradation of 5-MeBT by mixed bacterial cultures has been recently documented (Cornell, 2002), but reports on the biodegradation of 5-MeBT by pure cultures remain tenuous and the byproducts and mineralization potential of

benzotriazole derivatives remains unknown. One US patent application claimed to have isolated two microorganisms capable of degrading MeBT from industrial cooling water towers. These were identified as members of the genus *Xanthomonas* by fatty acid analysis. However, this investigation did not report species identification when comparing with a trypticase soy broth agar (TBSA) database and did not report any genetic basis their classification. Further, this work did not attempt to study the growth and biodegradability characteristics of the isolates and did not present evidence for the ability of these isolates to use MeBT as their sole carbon source (Rao et al., 1996).

Because of their widespread uses in tire manufacturing, benzothiazoles, which are structurally similar to MeBT, have been more extensively studied. Gaja and Knapp (1997) and De Wever and co-workers (1998) isolated *Rhodococcus* strains capable of growing with benzothiazoles as their sole carbon source. Gaja and Knapp (1997) found that this strain could sustain its growth on benzothiazoles and 2-hydroxybenzothiazoles but was not observed to grow on glucose, thiazole, 2-mercaptobenzothiazole, 2-amino benzothiazole or benzotriazole among others. De Wever and coworkers (1998) isolated a *Rhodococcus erythropolis* strain capable of degrading benzothiazole (BT), 2-hydroxybenzothiazole (OBT), and benzothiazole-2-sulfonate ($BTSO_3$) and a *Rhodococcus rhodochrous* strain (De Wever et al., 1997) capable of degrading BT and OBT but not $BTSO_3$. Neither isolate was capable of degrading 2-mercaptobenzothiazole (MBT). In the same 1998 study, De Wever and coworkers also identified 2-hydroxybenzothiazole and a dihydroxybenzothiazole as aerobic by-products of benzo-2-sulfonate degradation and benzothiazole degradation.

Benzothiazole-2-sulfonate was also transformed in near stoichiometric amounts to 2-hydroxybenzothiazole under anaerobic conditions. As described in the literature review (Chapter 2), the biodegradation mechanisms used by these isolates have been implicated to involve oxygenases in the initial steps of the process. In the case of benzothiazole degradation by a *R. erythropolis* strain, the initial steps of the biodegradation are thought to involve hydroxylation by monooxygenases (Figure 6-1).

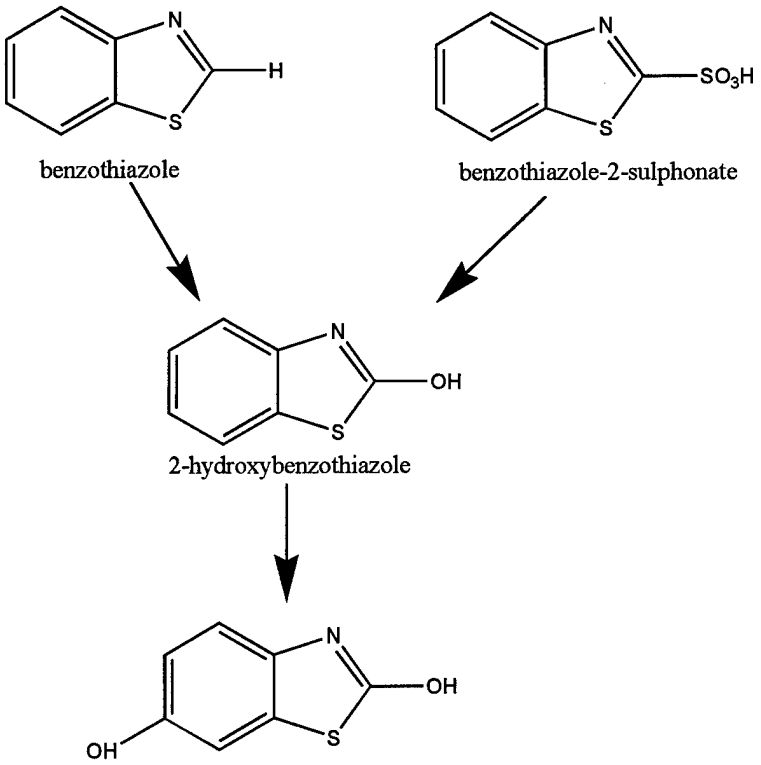


Figure 6-1. Initial transformations in benzothiazole biodegradation by an *R. erythropolis* strain. From (De Wever et al., 1998)

Like the previous reports on benzothiazole transformation, isolation and characterization of pure cultures capable of using MeBT as a carbon source is the first step in gaining a better understanding of the potential catabolic pathways for MeBT

degradation that have evolved for this xenobiotic compound. The objective of this work was to enrich MEBT-degrading microorganisms and to study MeBT biodegradation by two pure cultures.

6.2 Material and Methods

6.2.1 Test Solutions

Methylbenzotriazole was obtained in kind from PMC Specialty Group Inc. (Cincinnati, OH, USA) or bought from Sigma-Aldrich Corporation (St Louis, MO). HPLC-grade propylene glycol and D-glucose were obtained from Fisher Scientific (Pittsburgh, PA, USA).

6.2.2 Culture Media

Support media consisted of the following (per liter of MQ): NH_4SO_4, 600mg; K_2HPO_4, 440 mg; KH_2PO_4, 340 mg; $MgSO_4 \cdot 7H_2O$, 53 mg; $CaCl_2 \cdot 2H_2O$, 28 mg; $FeCl_3 \cdot 6H_2O$, 8.1 mg; $ZnSO_4 \cdot 7H_2O$, 0.68 mg; $CuSO_4 \cdot 5H_2O$, 0.17 mg; $CoCl_2 \cdot 6H_2O$, 0.17 mg; $NaMoO_4 \cdot 2H_2O$, 0.17 mg; $MnSO_4 \cdot H_2O$, 0.15 mg; H_3BO_3, 0.04 mg. The pH of this solution was 7.0. All solutions were heat sterilized prior to their use.

6.2.3 Chemical and Physical Parameter Analysis

6.2.3.1 5-MeBT

5-MeBT samples were collected using a polypropylene pipette and centrifuged at 10,000 x g for 5 minutes. The supernatant was transferred to HPLC vials and stored at 4°C for analysis. The maximum storage time for MeBT samples was 1 month.

5-MeBT concentrations were measured using a Spectra Physics Model SP8800 high performance liquid chromatograph (HPLC) fitted with a UV detector at

254 nm (Spectra Physics, San Jose, CA). Separation of the two isomers was performed isocratically using two Zorbax Rx-C8 4.6 x 250 mm columns in series (MacMod Analytical, Inc., Chadds Ford, PA, USA). The eluent consisted of a phosphate buffer mixed in a 70:30 ratio with HPLC grade acetylnitrile at a flowrate of 1.5 ml/minute incorporating a sample injection volume of 200 µl. Appendix 1 contains a sample calibration curve for 5-MeBT.

6.2.3.2 Quality Control Procedures

Quality control procedures are described in Appendix 2.

6.2.4 Isolation and bacterial characterization

The innoculum for these experiments consisted of two mixed cultures acclimated to the presence of relatively high MeBT concentrations. The first culture was enriched from shallow subsurface soils within two meters of the central deicing pad at Denver International Airport and previously described (Cornell, 2002). This culture has been maintained for the last four years in batch reactors at the University of Colorado under aerobic conditions. A second enrichment culture was obtained from ADF-contaminated grassland near the Manhattan, Kansas airport, and maintained at Kansas State University. Isolates were obtained by repeated streaking on agar plates incorporating 5-MeBT. Each agar plate consisted of autoclaved Noble Agar (Difco Laboratories, Detroit, MI) prepared with culture media and supplemented with 5-MeBT (20 mg/l) as the only carbon source. 5-MeBT was added to the noble agar after autoclaving from a filter sterilized stock solution. Plates were incubated either at room temperature or 28°C. Colonies growing on these plates were periodically streaked on new plates. This selection method was repeated until a pure

culture was obtained as judged by direct microscopy and genetic amplification analysis.

6.2.5 MEBT Transformation Studies

Pure culture colonies growing on agar plates surfaces were transferred to sterile media using aseptic techniques. 250 ml Erlenmeyer flasks, containing 10 mg/l or 25 mg/l of 5-MEBT and 10 mg/l TNP-10 in 50 ml of autoclaved culture media, were used to support aqueous growth of these isolates. The TNP-10 was used to homogenize the biomass.

All incubations were performed on a rotary shaker. Cultures were initially left at room temperature and then transferred to a 28°C incubator. Controls consisted of reactors with autoclaved biomass. Periodically, the purity of these cultures was assessed by streaking 0.1 ml in Tryptic Soy Agar (TSA) (Becton Dickinson, Sparks, MD) and observing the resulting colonies by direct microscopy.

6.2.6 Identification of pure cultures

Identification of pure cultures was performed by Microbial Insights (Rockford, TN). Nucleic acids separation was performed using a zirconium bead-beating method (Stephen et al., 1999). Sodium phosphate buffer, chaotropic reagent, glass beads, and the sample were agitated in a microcentrifuge tube using a high-speed bead beater. Chloroform was added, mixed thoroughly, and the tube was recentrifuged. The aqueous supernatant was collected and phenol/cholroform/isoamyl alcohol (24:24:1) extracted. Glycogen was added and the DNA was precipitated from the aqueous phase with an equal volume of isopropanol. DNA was pelletted by centrifugation, washed with 80% ethanol, air-

dried, and re-dissolved in Tris buffer, pH 8.0. The DNA was purified by a glass-milk DNA purification protocol using a Gene Clean™ kit as described by the manufacturer. PCR amplification of 16S rRNA gene fragments was performed as described in Muyzer et al. (Muyzer et al., 1993) with modifications as follows. Thermocycling consisted of 35 cycles of 92°C for 45 sec., 55°C for 30 sec., and 68°C for 45 sec. Using 0.44 units of Clontech Advantage™ 2 polymerase and 12.5 pmole each primer (forward primer contained a 40 bp GC-clamp) in a total volume of 25 µL, thermocycling was performed using a "Robocycler™" PCR block. Two primer sets were used in a nested PCR approach. The first primer set used primers corresponding to *E. coli* bp positions 27 and 1492 of the 16s rRNA gene. The second set of primers targeted Bacterial 16s rDNA regions corresponding to *E. coli* positions 27-519. A portion (20%) of each PCR product was analyzed by agarose gel electrophoresis (1.5% agarose, 1x TAE buffer) and ethidium bromide fluorescence. The amount of DNA used for DGGE was standardized to 150 ng by comparison to molecular weight standards using Alpha Imager™ software. DGGE was performed on a D-Code 16/16 cm gel system maintained at a constant temperature of 60°C in 6L of 0.5 x TAE buffer (20mM Tris actate, 0.5mM EDTA, pH 8.0). Denaturing gradients were formed at 30 – 65 % denaturant (with 100% denaturant defined as 7 M urea, 40% v/v formamide). A size gradient was imposed on the denaturing gradient by forming an 8 – 10 % acrylamide gradient (i.e., double gradient – DGGE) as described by Cremonesi et al. (Cremonesi et al., 1997). Gels were electrophoresis was done at 35V for 16 hr. Gels were stained with ethidium bromide (0.5 mg/L) and destained twice in 0.5 x TAE for 15 min. each. Gel images were captured using an

Alpha Imager™ system. The central 1mm portion of intensely fluorescing DGGE bands were excised using a razor blade and soaked in 50 μL of purified water overnight. A portion (15 μL) was used as the template in a PCR reaction as described above. The products were purified by electrophoresis through a 1.2% agarose/TAE gel followed by glass-milk extraction (Gene-Clean™ kit). Purified DNA was sequenced with an ABI-Prism automatic sequencer model 377 with dye terminators. Sequence identifications were performed using the BLAST facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/Blast) and the "Sequence Match" facility of the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.html).

6.2.7 Test for Toluene Oxygenase Activity against 5-MeBT

Two bacterial isolates, RR1 and RR2, known for their ability to rapidly oxidize toluene, were obtained from University of California at Berkeley. RR1 was isolated from a BTEX-degrading consortium indigenous to a gasoline-contaminated aquifer and enriched on toluene (Deeb and Alvarez-Cohen, 1999; Deeb et al., 2002). Initially, RR1 and RR2 enrichments were grown aseptically in 250 ml side-armed culture flasks with 50 ml of culture media. Each flask was sealed with Teflon-lined Mininert valves. Sequential additions of 50 mg/l toluene were added to each flasks until an optical density of 0.3 was achieved. Then, the reactors were uncapped and allowed to sit overnight to dissipate any remaining toluene. The next day 5-MeBT (25 mg/l) was added to each reactor from a filtered sterilized stock solution. These cultures were grown at room temperature (22 ± 2°C) on a shaker table.

6.3 Results and Discussion

6.3.1 Isolation and characterization of 5-MEBT-degrading bacteria

Two 5-MEBT-degrading strains, with distinct colony types, were isolated from the batch reactors. These colonies were visible on Noble agar plates after approximately 15 days of incubation at room temperature. Using phase contrast microscopy, enhanced with Nomarski-type interference lenses (1000x), the first isolate was described as true branching filamentous morphology with average trichrome diameter of 1.2-1.5 μm and averaged several hundred microns in length (Figures 6-1 and 6-2a). On agar they formed tight spherical colonies and exhibited behavior consistent with other hydrophobic bacteria. This isolate was designated G3. The second isolate, G3KS, were translucent bacillus-like, non-filamentous bacteria with a yellow halo around the colony. These strains were also culturable on TSA (Figure 6-2b).

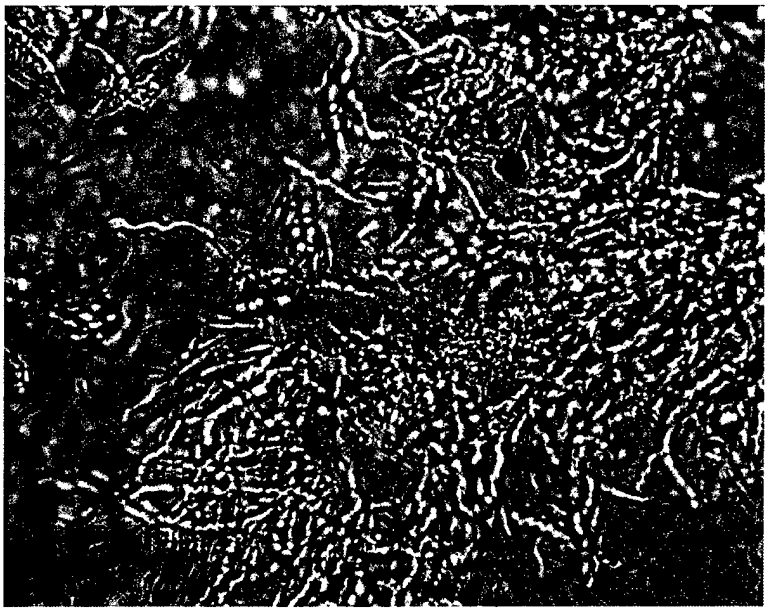


Figure 6-2. Microscope photograph of isolate G3. Wet mount at 1000x.

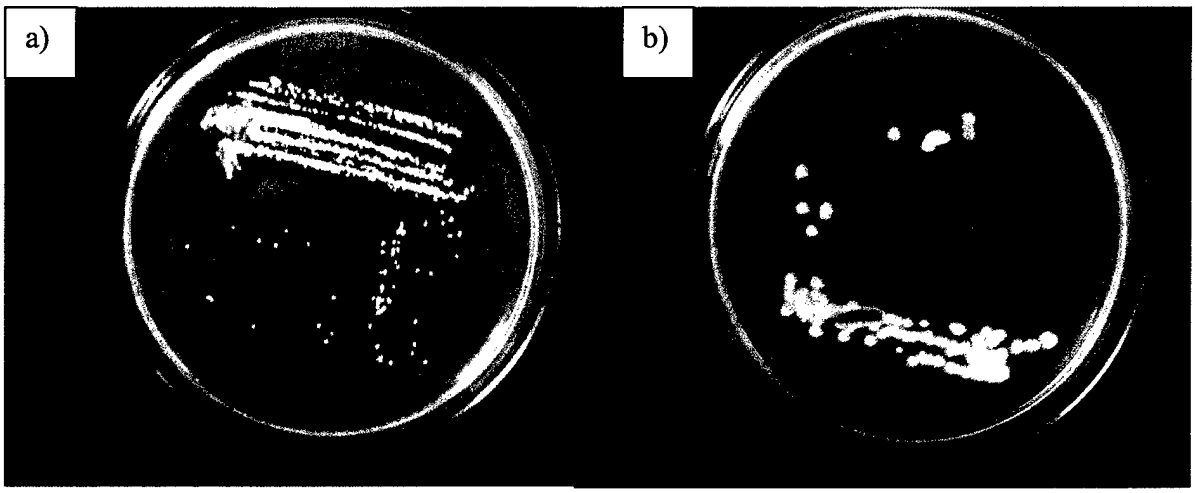


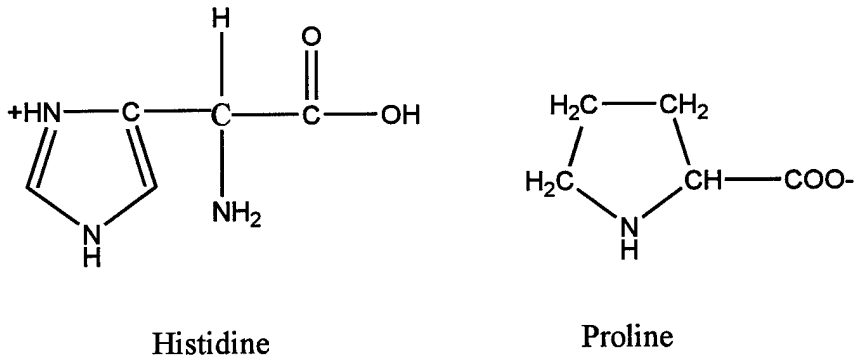
Figure 6-3. a) Isolate G3 on TSA after 10 days of incubation b) Isolate G3KS on TSA after 10 days of incubation on Difco Noble agar made with culture media and 25 mg/l 5-MeBT.

In solution G3 showed very hydrophobic properties, with all visible growth floating on the solution; very few filaments could be observed free in solution. G3KS grew as heavy conglomerates that settled at the bottom of the reactor even with rapid agitation.

Isolates G3 and G3KS were sent to an independent laboratory, Microbial Insights (Rockford, TN), for amplification and sequencing of 16s rDNA. The closest corresponding 16sRNA sequence match to those amplified from isolate G3 was *Pseudonocardia alni* with a similarity index of 0.884. For G3KS the closest sequence match was *Variovorax sp.* (0.876). Similarity indices of 0.800 or better are considered excellent (Appendix 3). Identification was based upon BLAST network and the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.html).

6.3.1.1 Pseudonocardia

Pseudonocardia strains belong to the class Actinomycetes, order Actinomycetales, and the Pseudonocardiaceae family. The bacterial systematics describing this genera are tenuous and rapidly changing. One source described all currently recognized members of this family as strictly aerobic (Embley, 2002). Another source claims that some members of this genus are facultative autotrophs (Reichert et al., 1998). The first *Pseudonocardia alni* strain catalogued was originally isolated from root nodules and rhizosphere of alder trees and was identified as capable of using L-Histidine and L-Proline as nitrogen sources (Figure 6-4) (Evtushenko et al., 1989). These compounds are N-containing cyclic compounds with some structural similarities to the azo containing moiety in benzotriazoles.



Histidine Proline

Figure 6-4. Chemical structures of the amino acid Histidine and the imino Proline. From (Madigan et al., 1997).

Recent reviews of actinomycetes-mediated biodegradation of benzoates and other xenobiotic heterocyclic benzene suggest that dioxygenases play a major role in the initial order of catabolism (Hammann and Kutzner, 1998). In fact, actinomycetes in general have been cited for the ability to catalyze hydroxylations; O, N, and S oxidations; O- and N-dealkylation reactions against various xenobiotic compounds (Paszczynski et al., 1992). Paszcynski and coworkers identified cytchrome P450 as a catalyst involved with the electron transfer on most of those reactions. Other studies, with compounds structurally similar to MeBT, identified isolates from the genus *Pseudonocardia* as capable of biodegrading 5-methylbenzothiophene (Bressler et al., 1999). As with the isolates described herein, these isolates did not grow on 5-methylbenzothiophene as a sole carbon source. *Pseudonocardia* has also been isolated from compost biofilters treating toluene vapors (Juteau et al., 1999).

Pseudonocardia species have also been characterized as slow-growing K-strategists. K-strategists, in general, could be explained using the Monod's growth rate model:

$$\mu = \mu_{max} \frac{[S]}{K_S + [S]}$$

where,

m = specific growth rate (1/time)

μ_{max} = maximum specific growth rate (1/time)

K_s = half saturation constant (mg/l)

[S] = growth limiting substrate concentration

Low K_s values suggest that the organism can compete successfully at low substrate concentration, while high K_s value suggest that successful competition occurs a higher substrate concentrations. Organisms with low K_s values are termed "K-strategists"; they compete by having an efficient substrate uptake mechanism. K-strategists normally possess high collective affinity for substrates or can access selected substrates because of their physiologic niche (i.e. hydrophobicity) (Juteau et al., 1999). Organisms with high K_s values are termed μ_{max} strategists; they have both high K_s and μ_{max} values. Figure 6-5 illustrates the two different growth strategies according to the Monod model. Previous research discussed earlier in this dissertation has identified mixed cultures growing on 5-MeBT as the sole carbon source as slow growing with low yields, which is in accordance with K-strategist population characteristics.

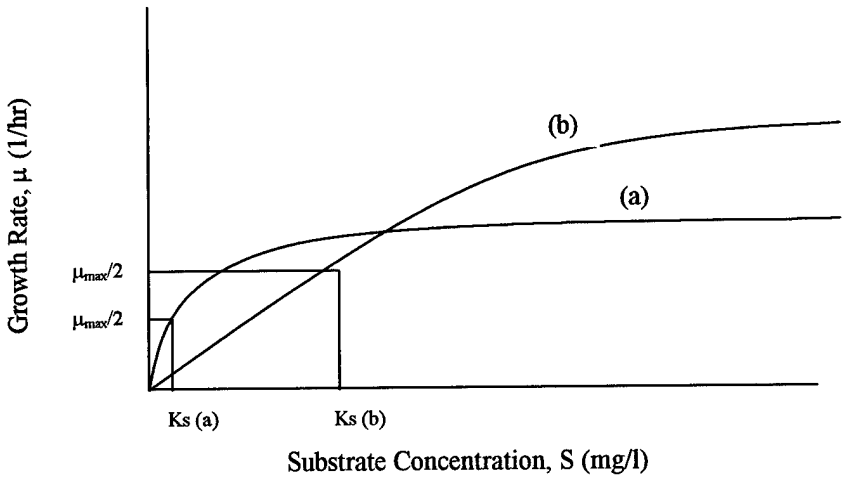


Figure 6-5. Growth rate (μ) as a function of substrate concentration (S) as given by the Monod equation for (a) K-strategist and (b) μ_{max} - strategist.

6.3.1.2 Variovorax

Variovorax is a gram-negative bacteria that belongs to the class Proteobacteria (beta subclass). It is one of the most common gram-negative bacteria reportedly cultured from subsurface environments (Balkwill et al., 1997) and it has been identified as one of the dominant bacteria in toluene, nitrobenzene, and pyrene biodegradation studies (Fries et al., 1997; Zhao et al., 2001; Eriksson et al., 2002). An isolate capable of degrading the azo compond 1-(4-carboxyphenylazo)-2-naphtol was also identified as belonging to the beta-subclass Proteobacteria (Blumel et al., 2001). The biodegradation of BTEX compounds by *Variovorax* species have also been implicated to an oxygenase-initiated pathway. *Variovorax sp.* has been isolated from cultures grown on aliphatic polycarbonates, 2-phenylpropionitrile, and sodium monofluoracetate (Wong et al., 1992; Layh et al., 1997; Suyama et al., 1998). It appears that this genera is incapable of denitrification.

Most of the compounds mentioned above have structural similarities with the MeBT molecule, or have toluene moieties that some may be labile to some enzymatic pathways used for the breakdown of MeBT.

6.3.2 5-MEBT biodegradation potential of isolates G3 and G3KS

In batch reactors (< 5 mg/l initial TSS), visible growth, as judged by a turbidity increase, was evident after 30 days of incubation. However, the concentration of 5-MeBT remained unchanged during that same period. This small but visible growth may have been due to the cultures using the surfactant TNP-10 as a growth substrate.

After 90 days of incubation in liquid culture neither isolate had used 5-MeBT as a carbon source. After this period, 5 mg/l of yeast extract was added to isolates to provide and external source of vitamin B. After adding the yeast extract, the 5-MeBT concentrations were monitored for an additional 90 days with no change.

The capability of these isolates to use other carbon sources was also tested. In batch cultures, G3 and G3KS grew on propylene glycol and glucose.

6.3.3 Transformation of 5-MeBT by other pure cultures

Assuming that the initial stages of 5-MeBT degradation follows a pathway similar to toluene degradation, in which the first step could be catalyzed by a dioxygenase or a monoxygenase, the biodegradation potential of pure cultures capable of transforming toluene was investigated. Isolates RR has been identified a member of the Rhodococcus family (Deeb et al., 2002) capable of expressing enzymes that can rapidly mineralize toluene. Two reactors were started for each isolate, RR1 and RR2 and 5-MeBT concentration was monitored by HPLC as

previously described. After, 15 days, neither isolate was capable of using 5-MeBT as a sole carbon source.

The results of this experiment did not provide any additional information regarding the biodegradation pathways of 5-MeBT except that the enzymes expressed by these organisms for toluene degradation did not recognize MeBT as a substrate. At least five different pathways are known to initiate toluene biodegradation. Those pathways involve the following enzymes: 2,3-dioxygenase, 2-, 3- or 4-monooxygenase and TOL (plasmid) pathway (Parales et al., 2000). RR1 apparently uses a monooxygenase-initiated pathway for the degradation of benzene, but information on the specific mechanism these isolates use for toluene degradation is scarce. Due to the structural characteristics of 5-MeBT, a candidate biodegradation pathway is initiated on the benzene ring of the molecule by a mechanism similar to toluene oxygenase-mediated pathways. Due to the presence of the triazole moiety, the most likely enzymes that might be involved in the initial step of 5-MeBT biodegradation are i) a 2,3-dioxygenase forming an alkyl cathecol, ii) a 2-monoxygenase which will form the corresponding cresol, or iii) a 1-monoxygenase in which MeBT is converted to a benzyl alcohol (Figure 6-6). This is in contrast to biotransformation pathways previously reported for benzothiazoles where initial oxidation occurs via hydroxylation of the anomeric carbon in the thiazole ring.

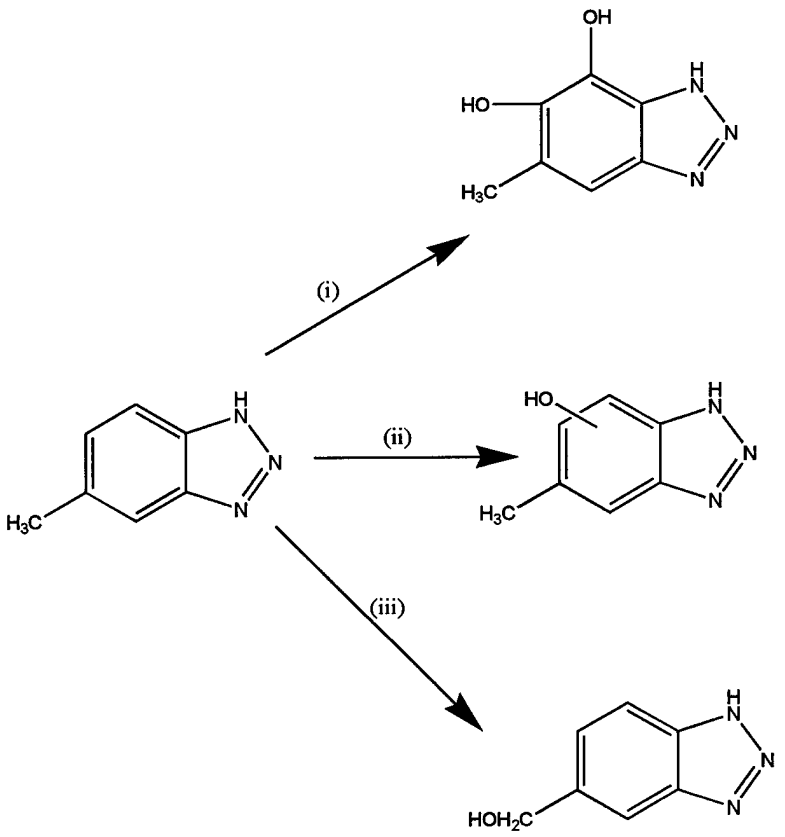


Figure 6-6 . Proposed aerobic pathways for the biodegradation of 5-MeBT as catalyzed by: (i) a 2,3-dioxygenase forming an alkyl cathecol, (ii) a monooxygenase which will form the corresponding cresol, and (iii) a 1-monooxygenase in which MeBT is converted to a benzyl alcohol. These pathways are analogous to the well-established pathway for the aerobic degradation of toluene.

6.4 Conclusions

Two bacterial strains capable of growing on Noble agar supplemented with 5-MeBT as the sole carbon source had been isolated. These isolates were tentatively identified as members of the genus *Pseudonardia* and *Variovorax*. An earlier study also isolated a member of the Proteobacteria class from 5-MeBT containing enrichments (Rao et al., 1996). In liquid batch cultures however, these isolates were not capable of biodegrading 5-MeBT, but both isolates were able to grow on glucose and propylene glycol. Strains of *Pseudonocardia* and *Variovorax* have been found to

degrade compounds similar to MeBT such as aryl benzenes and many other compounds containing heterocyclic azo bonds.

The degradation of xenobiotics, containing moieties similar to those in benzotriazole derivates, by actinomycetes has been widely studied. The degradation of compounds with critical structural similarities to 5-MeBT suggests that some members of this bacterial class can catalyze hydroxylation reactions that may be critical to the environmental biodegradation of MeBT. As described in the literature review (Chapter 2), hydroxylation is believed to be one of the first steps in the biotransformation of 5-MeBT. However, other benzotriazoles derivates has been shown to partially inactivate cytochrome P-450, the enzyme more likely responsible for the hydroxylation step (Town et al., 1993; Woodcroft et al., 1997; Sinal et al., 1998).

This work attempted to grow and characterize biodegradation kinetics in culture media following aseptic transfer from noble agar plates. The small yield on 5-MeBT, combined with the inability to optimize growth conditions yielded indeterminate results. The extremely hydrophobic properties of the isolates could have also limited access to the 5-MeBT as a substrate in solution. Future work with these isolates should focus on optimizing liquid phase growth conditions

This chapter also included preliminary attempts to elucidate the biodegradation pathway of 5-MeBT. Two pure cultures initially grown on toluene were not capable of using 5-MeBT as the sole carbon source. However, information regarding the degradation pathways of these isolates is limited. Degradation pathways among toluene degraders can vary significantly and future experimental

designs should isolate the five known toluene oxygenase-mediated pathways as the process variable against 5-MeBT biodegradation.

CHAPTER 7

BIODEGRADATION POTENTIAL OF BENZOTRIAZOLE DERIVATIVES UNDER ANOXIC CONDITIONS

Abstract

Determining the degradation potential of xenobiotic compounds under anoxic conditions is essential to understanding their fate in the environment. This research examined the denitrification potential of the most commercially significant corrosion inhibitors: 4- and 5-carboxybenzotriazole and 5-methylbenzotriazole. Microcosms, containing suspensions of soil microorganisms, were used as carefully controlled experimental systems to model environmental denitrification conditions; enrichments were derived from aircraft deicing fluids (ADF) contaminated soils, which contained mixed bacterial cultures capable of utilizing selected benzotriazole derivatives as a sole carbon source under aerobic conditions. After 1 year of incubation under denitrifying conditions, soil enrichments did not utilize any benzotriazole as a carbon source, but could readily denitrify propylene glycol (PG) when challenged with this common constituent of ADF. Relatively long term observations (>1 year) under nitrate reducing, sulfate reducing and strict anaerobic (fermentative) conditions produced similar results, suggesting that some benzotriazoles might only be biodegradable at significant rates under aerobic conditions.

7.1 Introduction

The fate and biodegradability of ADF additives under anoxic conditions, particularly the corrosion inhibitors, remain unclear. Therefore, the true environmental impact of ADF cannot be determined until the fate of these additives

under expected environmental conditions is resolved. Anoxic conditions can be induced in both natural systems and engineered treatment systems accepting industrial wastes; indeed, many wastewater treatments systems rely on denitrifying anoxic conditions for treatment (Grady et al., 1999).

In many natural environments anoxic conditions often prevail in subsurface soils due to the relatively low solubility of oxygen in water, and diffusion limitations in subsurface soils (Madigan et al., 1997). In biologically active soils, aircraft deicing fluids (ADF) induce anoxic conditions fairly rapidly due to the high biological oxygen demand of its main component, propylene glycol (PG).

The biodegradation potential of the most commercially significant benzotriazole derivatives appears be linked to oxidation/reduction conditions. This and other research has demonstrated the biodegradability of 5-methylbenzotriazole (5-MeBT) under aerobic conditions; however, recalcitrance under sustained anaerobic conditions has been previously observed (Gruden, 2000; Cornell, 2002). The literature suggests benzotriazoles derivatives will not serve as a fermentation substrate for bacterial consortia enriched in high rate anaerobic digesters, which are commonly found in activated sludge wastewater treatment plants.

Preliminary studies with ADF-acclimated enrichment cultures, under both denitrification and sulfate reducing conditions, have not demonstrated any anoxic degradation potential for MeBT at concentrations that are readily degradable under aerobic conditions.

Researchers at Kansas State University believed to have isolated some microorganisms capable of denitrifying MeBT. This chapter describes the

experiments performed from enrichment of these and other soil cultures to test for their potential to degrade MEBT under denitrifying conditions. It also provides initial observations of the biodegradability potential of carboxybenzotriazole isomers (CBT) under denitrifying conditions.

7.1.1 Benzotriazoles as Nitrification Inhibitors

Most fertilizers applied to the ground are in the form of ammonium or ammonium producing compounds such as urea. In many biologically active soils, ammonium is quickly oxidized to nitrate by nitrifying microorganisms. This nitrate has the potential of contaminating potable water supplies. Therefore, a significant body of research has focused on the study of compounds that are associated with land applications, which can effectively inhibit nitrification. Some of the most potent nitrification inhibitors have been found to be heterocyclic nitrogen (N)-containing compounds (McCarty and Bremner, 1989; McCarty, 1999; Puttanna et al., 1999, 2001a, b).

McCarty (1989) studied the effects of 12 unsubstituted heterocyclic N-containing compounds on the nitrification of ammonium in soil, and found that the compounds containing two or three adjacent N atoms in their rings (azo-bonds) significantly inhibited nitrification, whereas, those containing two or three non-adjacent ring N atoms or only included one N atoms in the ring had little or no effect on nitrification. In fact, benzotriazole was one of the most potent nitrification inhibitors observed. In more recent publications, McCarty (1999) suggests that the mechanism of nitrification inhibition involves complexation with Cu-containing enzymes, which are directly involved in the nitrification process (McCarty, 1999).

7.1.2 Biodegradation Under Anoxic Conditions

In subsurface environments that are microbiologically active, oxygen is usually available as a terminal electron acceptor for respiration processes at or near the surface. Under common terrestrial conditions, several other inorganic ions can serve as terminal electron acceptors. These include nitrate (NO_3^-), sulfate (SO_4^{2-}), and Fe^{+3} among others. Of these, nitrate is of most importance because of its prevalence in groundwater, and the fact that most denitrifiers are facultative (Averill, 1996; Madigan et al., 1997). In bioremediation processes, denitrification is seen as great potential for the mitigation, since dissolved nitrate is relatively ubiquitous, and in engineering applications, its concentration is easier to control than oxygen. (Averill, 1996).

7.1.2.1 Dissimilative Pathways of Nitrate Reduction

The dissimilative pathways of nitrate reduction are fairly well understood. Figure 7-1 summarizes the nitrate reduction pathways, including the generic name for the class of enzyme involved in each step. These enzymes are only synthesized under anoxic conditions.

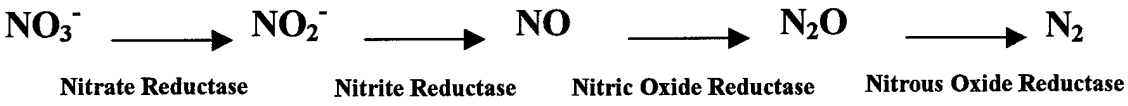


Figure 7-1. Dinitrification pathways and enzyme involved.

The first enzyme involved in denitrification, nitrate reductase, has been recognized as a molybdenum-containing enzyme bound to the cell membrane. The

second enzyme, nitrite reductase, has two distinct types: those containing heme cd_1 chromophores and those containing copper. Of all the organisms studied thus far, about 75% percent appear to contain the cd_1 heme-containing enzyme. But it appears that there is no correlation between microbial taxonomy and the type of dissimilatory nitrite reductase present in an organism. In bacteria, a third enzyme involved in denitrification, nitric oxide reductase, consists of membrane bound cytochrome *b* and cytochrome *c*. Little is know about the last enzyme, nitrous reductase, but recent studies suggest it is structurally similar to cytochrome c, which has a copper center (Averill, 1996).

7.1.3 Denitrification Potential of MeBT

Chapter 6 of this thesis identified an actinomycete, *Pseudonocardia*, as an isolate of MeBT enrichments. Many members of this class are known to be capable of dissimilatory nitrate reduction (Betlach, 1982; Shoun et al., 1998). Shoun et al tested 40 actinomycetes of various genera for their denitrifying activity. Twenty-two of the actinomycetes were able to denitrify, but with distinct levels of denitrification as judged by N_2O evolution since none of the organisms tested were not capable of producing nitrogen gas (N_2) as the final product. Each actinomycete in that study was tested with two different electron acceptors, nitrate and nitrate, and two environmental conditions: (1) with the headspace initially purged with helium and, (2) with residual oxygen left in the headspace (no purging). For some of the strains tested, N_2O evolution varied significantly among the four conditions tested, producing near stoichiometric amounts of N_2O under one or more conditions but below detection limits under other(s). There was significant variability in

denitrification among the different strains of actinomycetes tested and among the conditions of the experiments. In this same study, Shoun et al (1998) used *Streptomyces thioluteus* as a model actinomycete to investigate its denitrifying system in more detail. They identified the nitrite reductase as being the copper-containing form, suggesting that other actinomycetes capable of dentirification may carry this form of nitrite reductase (Shoun et al., 1998).

While these findings demonstrate that denitrification by bacteria found in MeBT enrichments is possible, there are no studies that specifically address the denitrification potential of benzotriazole derivatives themselves. Other studies, however, have addressed the denitrification potential of benzothiazole derivatives compounds, which are structurally similar to MeBT. For example, while studying the inhibitory effects of 2-mercaptobenzothiazoles (MBT), De Wever (1997) and colleagues found that MBT slowed down the growth of *E. coli* and *Paracoccus denitrificans* under denitrification conditions. However, they did not mention if these cultures were actually able to utilize MBT as a carbon source for dissimilatory denitrification. Other heterocyclic nitrogen-containing compounds such as indole, quinoline, 2-methylquinoline and isoquinoline are biodegraded under nitrate-reducing, sulphate-reducing and methanogenic conditions (Licht et al., 1996; Li et al., 2001).

7.2 Materials and Methods

7.2.1 Culture Media

The culture media (Accashian et al., 1998) consisted of the following (per liter of MQ): K_2HPO_4, 440 mg; KH_2PO_4, 340 mg; $MgSO_4 \cdot 7H_2O$, 53 mg; $CaCl_2 \cdot 2H_2O$, 28

mg; $FeCl_3 \cdot 6H_2O$, 8.1 mg; H_3BO_3, 0.04 mg; $CuSO_4 \cdot 5H_2O$, 0.17 mg; $MnSO_4 \cdot H_2O$, 0.15 mg; $ZnSO_4 \cdot 7H_2O$, 0.68 mg; $CoCl_2 \cdot 6H_2O$, 0.17 mg; $NaMoO_4 \cdot 2H_2O$, 0.17 mg. The pH of all media used in these experiments was 7.0. All solutions were heat sterilized prior to their use.

7.2.2 Environmental Microcosms

Environmental microcosms that imitate natural settings are extremely useful as experimental laboratory models to monitor microbial processes under pseudo-environmental conditions (Atlas, 1997). Microorganisms for these experiments consisted of soil bacteria enriched from ADF-contaminated airport sites near the Manhattan, Kansas airport, and Denver International Airport (DIA). Prior to these experiments the culture was grown aerobically using MeBT as the sole carbon source. Hoagland's (Hoagland and Arnon, 1960) solution was used as the basal medium for the enrichments from the Manhattan airport. Culture media was used as the basal medium from the DIA enrichments.

To test the biodegradation potential of benzotriazole derivatives, three sets of experiments were designed and tested (Table 7-1). The microcosms for each experimental set consisted of four 125 ml serum bottles (Wheaton No. 223748), three replicates and a control, inoculated with aerobically enriched cultures, which were previously acclimated to MeBT. First, cultures were harvested by centrifugation at 2000 x g for 30 minutes and washed with a 0.85% solution of sodium chloride. This procedure was repeated three times after which the cells were suspended in approximately 130 ml of culture media. Prior to suspension, the culture media solution was stripped with helium to oxygen levels < 1.0 mg/l O_2 in an anaerobic

chamber. Serum bottles were then capped with gray butyl rubber stoppers and 20 mm aluminum crimp seals. After capping, each serum bottle was purged with either helium or 20% carbon dioxide balanced with hydrogen using sterile syringes; propylene glycol, MeBT, and carboxybenzotriazole (CBT) were then added as the carbon sources. Using Manhattan Kansas airport soils as the enrichment source, a fourth experimental set was also started, under conditions identical to those listed above but with the inclusion of a microcosm series where the pH of the solution adjusted to 9.0 with Na_2HPO_4.

Table 7-1. Summary of conditions and soil sources used to evaluate the biodegradation potential of selected benzotriazoles derivatives under anoxic conditions.

	Denitrification	**Sulfate Reduction**
Manhattan Airport	4(5)-CBT, 5-MeBT (pH 7 and 9)	-
DIA	4(5)-MeBT	4(5)-MeBT

The electron acceptor for the denitrification experiments consisted of nitrate, from $NaNO_3$; for the sulfate reduction experiments the electron acceptor consisted of sulfate from $(NH_4)_2SO_4$. Both electron acceptors were added in at least 10% excess of the stoichiometric amounts needed to oxidize amount all carbon sources added and reduce nitrate to nitrogen or sulfate to hydrogen sulfide. Microcosms were incubated on a shaker table at 140 rpm in an inverted position at room temperature. Controls were autoclaved prior to incubation.

In these experiments, propylene glycol served as an appropriate readily degradable carbon source to ensure that anoxic conditions were achieved and maintained in these reactors, and to indicate that enriched biomass was readily capable of ADF denitrification or sulfate reduction (glycol). The initial conditions are summarized in Table 7-2. After the initial mass of PG was consumed, the microcosms were spiked a second time with a similar mass of PG and were subsequently monitored to ensure the complete removal of PG and that MeBT and CBT (when present) were left as the only carbon source.

With the exception of autoclaving, all the controls were treated exactly as the experimental microcosms including purging of headspace, initial concentrations of carbon source, and addition of electron acceptors (i.e. nitrate or sulfate).

Table 7-2. Initial conditions of anoxic reactors. Nitrate was only added to microcosms monitored for denitrification activity, and sulfate was only added to those microcosms monitored for sulfate reduction activity. CBT was only used in selected experiments (See Table 7-1).

Analyte	**(mM)**
Propylene Glycol (PG)	1.3
5-Methylbenzotriazole (5-MEBT)	0.1
4(5)-Carboxybenzotriazole (4(5)-CBT)	0.1
Nitrate (as $NaNO_3^-$)	6.0
Sulfate (as $(NH_4)_2SO_4$	3.0

7.2.3 Bench-scale packed bed reactor

The denitrification potential of MeBT was also tested in bench-scale packed bed, attached growth reactors (Figure 7-3). These packed bed reactors were constructed of acrylic tubing, 38 centimeter (cm) in height and 14 cm in diameter, with 1.30 cm acrylic plate caps for the top and bottom. Ninety percent of the total

reactor volume was packed with trickling filter media (Koch Flexiring, Whichita, Kansas). This packing provided approximately 17000 cm^2 of surface area for biological attachment in each reactor (Carlson, 1995). Initially, the feed for this reactor consisted of culture media containing PG (1.3 mM) and 5-MeBT (0.1 mM) as carbon sources and nitrate (6.0 mM) as the electron electron. This feed solution was added to the reactor with a fixed-speed peristaltic pump (Cole-Parmer, Chicago, IL). The direction of flow was from top to bottom, providing uniform mixing but with sufficiently low velocity to avoid biomass shearing. The feed solution was continuously purged with helium to remove any oxygen left in solution prior to be added to the reactor.

7.2.1 Test Solutions

4- and 5-methylbenzotriazole (MeBT) and 4- and 5-carboxybenzotriazole (CBT) were obtained in kind from PMC Specialty Group Inc. (Cincinnati, OH, USA) or bought from Sigma-Aldrich Corporation (St Louis, MO). HPLC-grade propylene glycol was obtained from Fisher Scientific (Pittsburgh, PA, USA).

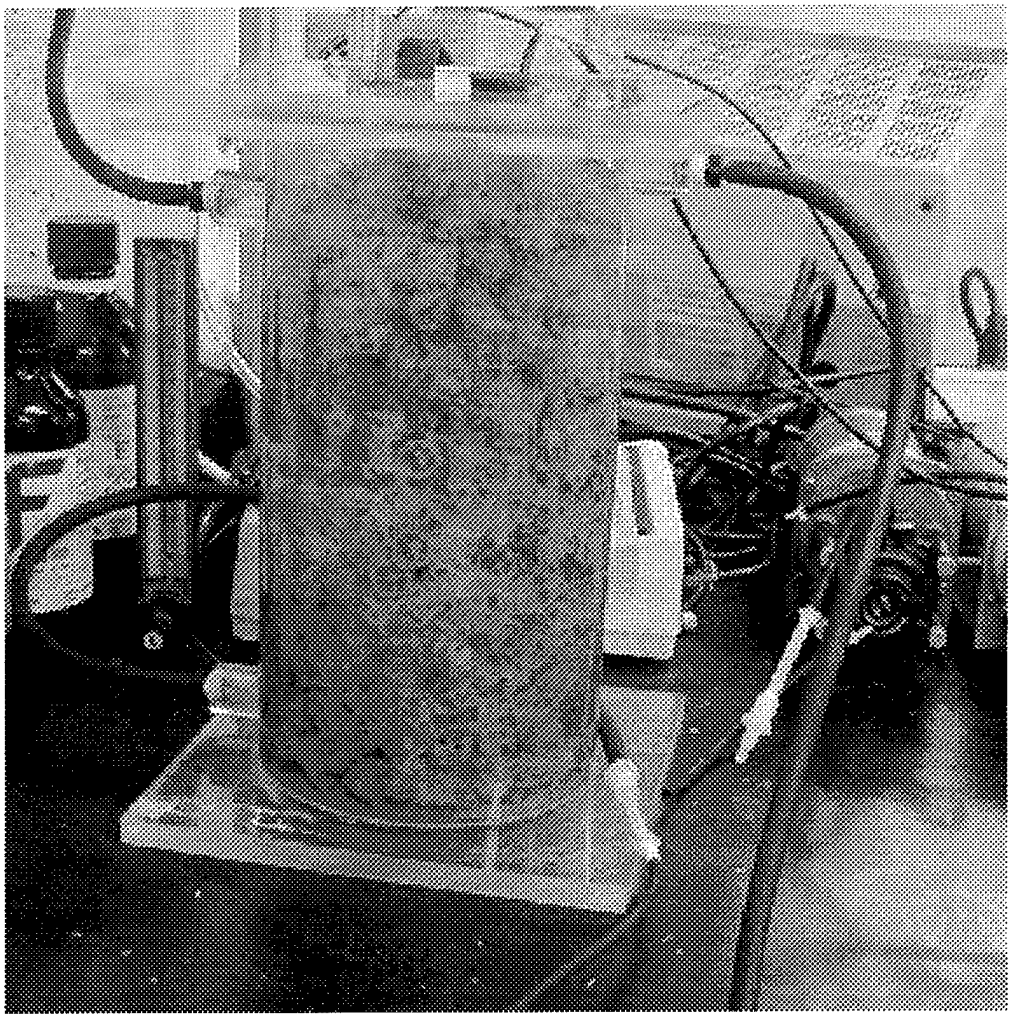


Figure 7-2. Bench-scale packed bed reactor used for denitrification experiments.

7.2.2 Chemical and Physical Parameter Analysis

7.2.2.1 Propylene Glycol

The concentration of propylene glycol was measured by a periodate oxidation method as previously described (Alben, 1991; Cornell, 2002). The absorbance was measured with a Hach Model 2000 (Hach Company, Loveland, CO) spectrophotometer at 412 nm. All samples were analyzed immediately upon collection. Appendix 1 contains a sample calibration curve used for propylene glycol analysis.

7.2.2.2 Methylbenzotriazole

MeBT and CBT concentrations were measured using a SP8800 high performance liquid chromatograph (HPLC) fitted with a UV detector (λ = 254 nm) (Spectra Physics, San Jose, CA). Separation of the two isomers was performed isocratically using two Zorbax Rx-C8 4.6 x 250 mm columns in series (MacMod Analytical, Inc., Chadds Ford, PA, USA). The eluent consisted of a phosphate buffer mixed in a 70:30 ratio with HPLC grade acetylnitrile at a flowrate of 1.5 ml/minute incorporating a sample injection volume of 200 µl. Appendix 1 contains a sample calibration curve for this procedure.

7.2.2.3 Nitrogen and Hydrogen Sulfide

Nitrate consumption and nitrogen production were used as an indicator that dinitrification conditions had been achieved in microcosms targeted for this activity. Sulfate consumption and hydrogen sulfide production were the indicators used to verify sulfate reduction had been achieved in those microcosms targeted for that activity. Headspace nitrogen and hydrogen sulfide concentrations were analyzed using a gas chromatograph (GC) fitted with a thermal conductivity detector (TCD) (Gow-Mac Instrument Co., Bethlehem, PA). The GC was fitted with a 1/8" x 8' Haysep Q capillary column (Supelco, Inc., Bellefonte, PA) and column temperature of 80°C. Helium was used as the carrier gas at a flowrate of 30 ml/min. The TCD temperature was maintained at 100°C. Injections were made with a 250 µL gas-tight syringe (Hamilton, Inc., Reno, NV).

7.2.2.4 Nitrate, Nitrate and Sulfate

Ion chromatography (IC) was used to measure the concentration of nitrate, nitrite and sulfate according to Standard Methods for the Analysis of Water and Wastewater Method 4110B (American Public Health Association et al., 1995). The IC equipment consisted of a Dionex 300 Series liquid chromatograph fitted with a conductivity detector. Anion separation was performed isocratically using an IonPac AS9-SC 4mm column. (Dionex Corporation, Sunnyvale, CA, USA). The method used a 0.17/0.18 M sodium bicarbonate/sodium carbonate eluent at a flowrate of 1 ml/minute incorporating a sample injection volume of 200 µl.

7.3 Results and Discussion

7.3.1 Environmental Microcosms

Enrichments from ADF contaminated soils at Denver International Airport and Kansas State University were not able to utilize 5-MeBT or 4(5)-CBT as carbon sources under conditions defined by microbiological denitrification or sulfate reduction. Given oxygen, these enrichments s degraded 5-MeBT under otherwise identical conditions. As described earlier, a readily biodegradable substrate, PG, was added in the initial stage of the experiments to promote denitrification and sulfate reducing conditions in each microcosm.

7.3.1.1 Manhattan, Kansas airport enrichment

Under denitrifying conditions, enrichments obtained from grassland near Manhattan, Kansas Airport, and maintained at pH 7, took 13 days to completely consume the initial mass of PG added. After addition and consumption of the initial PG mass, a second PG spike was added; this second addition, which was equivalent

in mass to the original conditions, dropped below detection limit in less than 7 days (Figure 7-3). After day 21, the PG had been completely consumed, 5-MeBT and 4(5)-CBT, were left as the only significant carbon sources. After 120 days of incubation the concentration of the benzotriazoles remained unchanged (Figure 7-4). The benzotriazoles concentration was monitored periodically for another year without significant change in concentration.

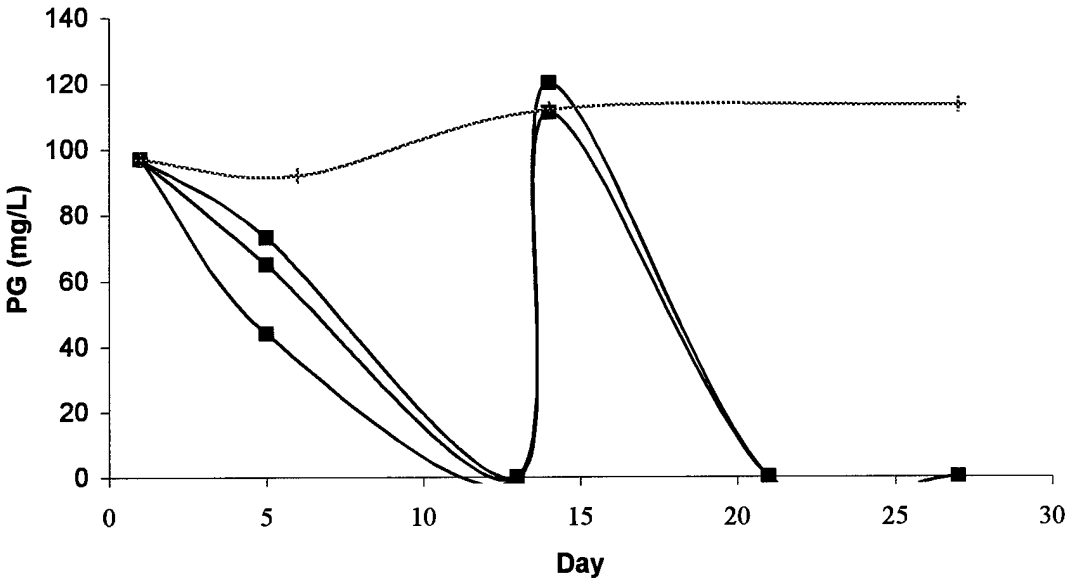


Figure 7-3. Propylene glycol concentrations in three replicate microcosms (■) and a control (+) containing enrichments cultures derived from ADF-contaminated grassland near the Manhattan Kansas Airport and maintained under denitrification conditions at pH 7.

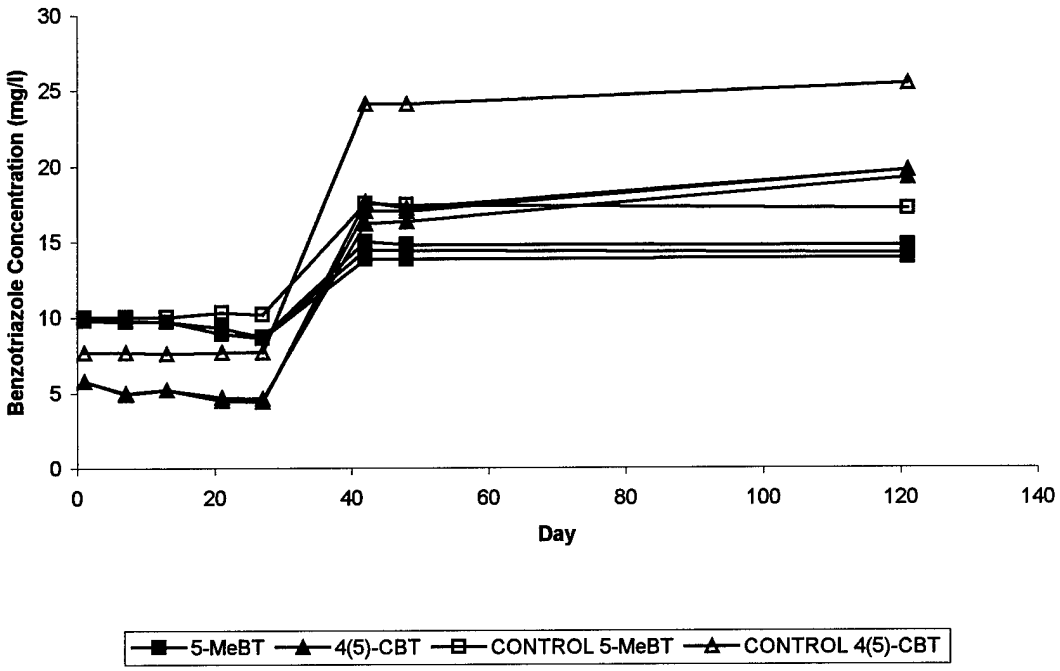


Figure 7-4. Concentration of benzotriazoles in microcosms enrichments cultures derived from ADF-contaminated grassland near the Manhattan Kansas Airport and maintained under denitrification conditions at pH 7. On Day 42 more culture media benzotriazole derivatives were added to each reactor to increase the analytical resolution.

In order to ensure that anoxic conditions were maintained and denitrification could in fact occur, nitrate consumption (Figure 7-5) and nitrogen production was monitored (data not shown) throughout the experiment. From the initial inoculation, nitrate disappeared faster than would be predicted from stochiometry. This became more obvious after the benzotriazoles were the only carbon source present. Even thought the concentration of benzotriazoles was not changing, large amounts of nitrate (up to 550 mg/l) were being consumed in a couple of days. A likely explanation for this phenomenon was that enrichments were using the hydrogen in the

headspace as electron donor. To prove this theory, the headspace of each reactor was purged with high purity helium instead of the H_2/CO_2 mixture initially provided. After purging with helium (Day 42) the nitrate concentrations remained essentially constant (Figure 7-5).

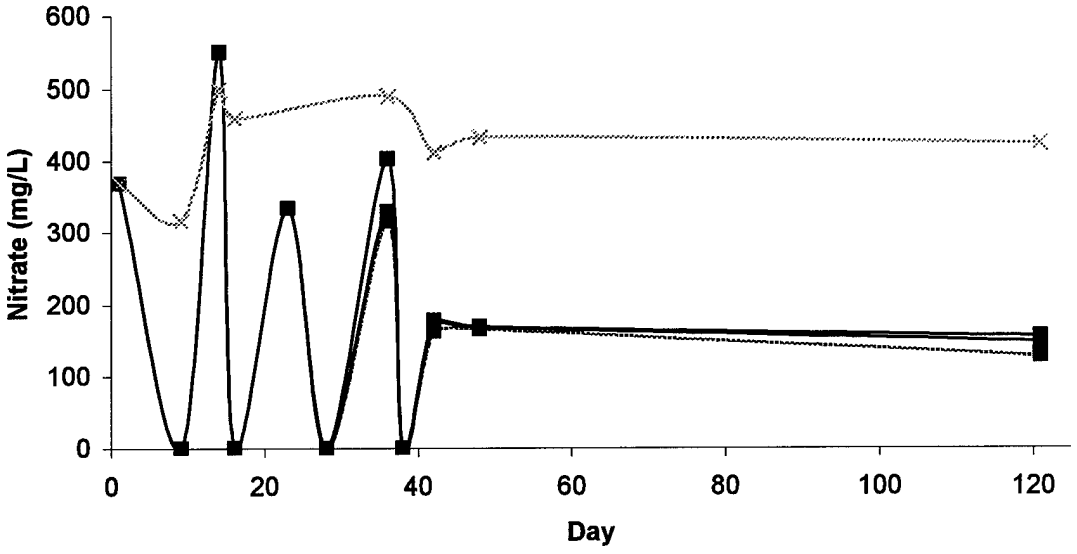


Figure 7-5. Nitrate concentrations in three replicate microcosms (■) and a control (x) containing enrichments cultures derived from ADF-contaminated grassland near the Manhattan Kansas Airport and maintained under denitrification conditions at pH 7.

The reactors maintained at pH 9 resulted in similar results (data not shown). However, after the first 42 days of incubation, when additional culture media was added, pH = 7 culture media was inadvertently added, instead of the media adjusted to pH 9. The resulting pH was 8.2. These reactors were also periodically monitored for benzotriazole concentrations for another year with no significant change.

7.3.1.2 DIA Enrichments

After denitrification and sulfate reducing conditions were achieved in reactors enriched from subsurface soil obtained from DIA (as judge by nitrogen and hydrogen sulfide production and nitrate and sulfate consumption), these enrichments were not capable of transforming 4- or 5-MeBT after 228 days of incubation (data not shown).

7.3.2 Packed-Bed Bench Scale Reactor

The strategy used for the environmental microcosms was also applied to the continuous flow packed bed reactor. At the initial stage of the experiment, PG was added to promote denitrification. Denitrification was initially monitored by measuring PG and nitrate consumption. After 15 days, 5-MeBT was left as the only carbon substrate. Experimental results from this experiment yielded similar results (data not shown); no 5-MeBT transformations occurred after 120 days of contiuous exposure to biofilms immobilized in the anoxic packed bed reactor..

7.4 Conclusions

When evaluating biodegradation potential of xenobiotic compounds in natural environments, the effects of changing redox conditions and the role of alternate electron acceptors must be evaluated. Previous research has shown that 5-MeBT and 4(5) CBT are readily biodegradable under aerobic conditions (Cornell, 2002; Abu-Dalo, 2003). The rapid, concomitant removal of nitrate (or sulfate) and propylene glycol, and production of nitrogen gas (or hydrogen sulfide) suggest that the biomass enriched in these microcosms were readily capable of denitrification and sulfate reduction. However, MeBT acclimated cultures reduced and maintained under anoxic conditions were not able to degrade 5-MEBT or CBT isomers after more than

12 months of incubation. Denitrification using 5-MeBT as the sole carbon source was also attempted in a continuous flow packed-bed reactor with no success.

Some of the enrichments tested, which were capable of the heterotrophic denitrification of propylene glycol, were also capable of oxidizing hydrogen in the presence of benzotriazole derivatives (> 25 mg/l). The difference in free energy available from the heterotrophic oxidation of aromatic carbon in benzotriazole (-2397 kJ for benzene) and the autotrophic oxidation of hydrogen (-961 kJ) is markedly different and favors the biodegradation of benzotriazole given that enzymatic pathway is available and expressed, and that this substrate does not inhibit enzymes involved in the process.

Whether or not those members, either symbiotically or individually, could metabolize these benzotriazole derivatives is unknown. The fact that this ADF acclimated consortia included a significant facultative population that could denitrify glycol in the presence of benzotriazole suggest any (or all) of the following possibilities with respect to benzotriazole metabolism under these (anoxic) conditions: (1) These microcosms may not have enriched the microorganisms capable of producing the enzymes necessary to oxidize benzotriazoles under anoxic conditions. (2) Benzotriazoles could be particularly toxic to denitrifiers and or sulfate reducers. For example, copper-containing enzymes are particularly predominant in the nitrate reduction pathway coupled to heterotrophic catabolism. Research has shown that MeBT particularly binds to copper more than any other metal studied. The calculated conditional stability constant of 4(5)-MEBT:Cu complex was determined to be $10^{15.27}$ (Abu-Dalo, 2003). Complexation between MeBT and Cu-

containing enzymes could inhibit the denitrification process. Given that complexation between MeBT and some metal containing enzymes in fact occur at environmentally relevant pH. (3) The experimental design used in these experiments were not adequate for enriching denitrifers or sulfate reducers capable of oxidizing benzotriazoles.

As Shoun and co-workers demonstrated in the study of denitrification of actinomyces, the initial conditions could have a determining effect on the capability of these cultures to denitrify (Shoun et al., 1998). Their studies found that factors such as the initial electron acceptor (nitrate or nitrite) or headspace conditions at the start of the experiment (with or without residual oxygen) could dramatically affect the denitrification potential.

In summary, these results demonstrated that some benzotriazoles are likely to prevail in environments with low oxygen concentrations. The implications of this are two fold: first, when evaluating the fate of benzotriazoles in natural environments, benzotriazole biodegradation may only occur at or near the surface. In the deeper subsurface near ADF applications, benzotriazoles will more likely prevail. Some benzotriazoles will be expected to attach to sediments, but most will more likely be transported with groundwater flow (if present) due to their high water solubility, possibly ending in natural water systems or potable water supplies; (2) second, many airports have implemented anaerobic or anoxic wastewater treatment systems, or soil aquifer treatment systems for the treatment of ADF wastewater. Based on the information provided here and elsewhere (Gruden, 2000), those systems are more likely ineffective in removing benzotriazole derivatives from solution. Therefore, one

of the most toxic components of aircraft deicing fluids, will likely be unaffected in many subsurface environmental settings.

CHAPTER 8

RESEARCH CONCLUSIONS, RECOMMENDATIONSAND APPLICATION TO PRACTICE

8.1 Conclusions

Aircraft deicing operations are necessary to ensure safe air travel and efficient military operations. In the past decade, increasing regulatory pressures in North America and Europe have driven a market movement to develop more environmentally friendly deicers, and to develop deicing techniques that will minimize the discharge of aircraft deicing fluids (ADF) to the environment. In addition, more stringent requirements for the US National Pollution Discharge Elimination Systems (NPDES) and increased enforcement of the Total Maximum Daily Load (TMDL) requirements, are forcing airports to implement theses changes rapidly (Switzenbaum et al., 1999). Knowledge of the environmental effect of ADF components is essential in understanding and developing treatment techniques. This research investigated the degradation and microbial toxicity of one of the most prevalent corrosion control inhibitors, methylbenzotriazole. Results from these investigations led to the following conclusions:

Hypothesis #1: MeBT can act as an uncoupler of oxidative phosphorylation

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Conclusion #1: The physical and chemical characteristics of the MeBT molecule, a weak acid with strong ligand characteristics, suggest that it will be toxic to respiration processes of bacterial cells. The toxicity of MeBT to model bacterial cultures is likely conferred by two major mechanisms: (i) the uncoupling of oxidative phosphorylation, and (ii) toxicity to membrane bound enzymes involved in respiration.

Hypothesis #2: Below a toxic concentration, 5-MeBT can be completely mineralized by bacteria common to soil and wastewater treatment systems under aerobic conditions.

Conclusion #2: An aerobic metabolic pathway for the mineralization of 5–MeBT exists in some soil bacteria. When challenged with 50 mg/l 5-MeBT, a concentration which does not induce any obvious toxic effects, these bacteria were capable of mineralizing nearly all the carbon in the MeBT to common non-toxic products: CO_2 and biomass.

Hypothesis #3: 5-MeBT can be used as a sole carbon and nitrogen source to support terrestrial aerobic bacterial consortia

Conclusion #3: Aerobic bacterial cultures acclimated to 5-MeBT can metabolize relatively high concentrations of this compound when it was present as a sole carbon and nitrogen source. Significant release of ammonic nitrogen is associated with the metabolism of MeBT. Growth yields appear relatively low for this substrate (0.2 – 0.3). Non-acclimated cultures were not always capable of metabolizing 5-MeBT; however, in those instances where non-acclimated bacteria were associated with its removal; growth and ammonia released was on the order of MeBT-acclimated bacteria.

Hypothesis #4: Pure bacterial cultures capable of growing on 5-MeBT as their sole carbon source can be isolated from bacterial enrichments contaminated with aircraft deicing fluids.

Conclusion #4: Two bacterial strains capable of growing in the presence of 5-MeBT as the only significant carbon source were isolated from enrichments derived from shallow subsurface soils contaminated with aircraft deicing fluids. Using genetic methods, these bacterial strains were tentatively identified as members of the genera Pseudonocardia and Variovorax. Previous observations of these genera, together with models of xenobiotic metabolism of compounds similar in structure to MeBT, are consistent with the ability of these organisms to use MeBT as a substrate.

Hypothesis #5: Benzotriazoles are biodegradable under anoxic conditions

Conclusion #5: Long-term studies in reducing environments that have enriched facultative and anaerobic bacteria acclimated to aircraft deicing fluids, suggest that benzotriazoles derivatives that can be completely mineralized under aerobic conditions, appear to persist under anoxic and anaerobic conditions.

8.2 Applications to Practice and Recommendations for Future Research

This research provides critical information for researchers and engineers to consider when dealing with the biotransformation potential for MeBT in many natural systems or engineered treatment systems. However, much work is still needed to better understand its environmental behavior, longevity and toxicity mechanisms to different ecosystem receptors as well as to accurately assign risk to its presence. The original motivation for this research was the pending regulatory pressures associated with runoff containing aircraft deicing fluids and subsequent point-source water pollution events. However, a recent nationwide reconnaissance executed by the USGS, demonstrated that the presence of benzotriazoles derivatives is not limited to environments close to airports, and that the frequency, levels and occurrence of these corrosion inhibitors in our nation's surface waters are much higher than previously anticipated (Kolpin et al., 2002). It is noteworthy to mention that the recent USGS reconnaissance focused only on surface waters, and included only a single benzotriazole derivative in the survey – 5-Methylbenzotriazole — the only methylated benzotriazole derivative that has thus far been judged as biodegradable under aerobic conditions. Several other benzotriazole derivatives that have been in wide spread

commercial use for many years, were not included in this survey because standards were not available; thus, the environmental loading of these compounds is likely higher than recent reconnaissance may suggest. This work focused primarily on 5-methylbenzotriazole; the environmental fate of benzotriazole and many of its derivatives remain unknown.

Results from this research consistently showed that obvious and predictable toxic responses were elicited in soil and activated sludge enrichment cultures under aerobic conditions when challenged with MeBT concentrations above 200 mg/l. Below 100 mg/l 5-MeBT, bacterial cultures, especially those previously exposed and already acclimated to 5-MeBT degradation, appear to effectively remove it from solution, even in nutrient-limiting environments. These findings provide useful trends for chemical manufacturers, the regulatory community, and environmental engineers to better predict the fate of this compound in selected environmental situations and engineering works.

The role chemical manufacturers play when producing and marketing benzotriazole-containing products is to ensure that the concentration present in each particular formulation is sufficient to effectively perform the intended function, which in the case of MeBT is, in most cases, corrosion inhibition. For the airline industry, this factor is critical to ensure flame suppression during deicing operations, as well as protect alloys that are labile to corrosion. Chemical manufacturers will hopefully use this information when balancing the chemical effectiveness of benzotriazole derivatives against deleterious environmental risks by considering the concentrations

that will be potentially released to the environment, and marketing the most biodegradable and environmentally benign benzotriazole derivatives.

Environmental regulators have the difficult task of minimizing environmental and health risks juxtaposed to restricting the use of this highly specialized chemical, which has extremely high commercial use, protection utility, and value. When considering regulatory action, they must consider many issues that will help them evaluate and rank the risk of this particular chemical. This research gives environmental regulators some information necessary to better evaluate such risks given the protection benzotriazoles offer.

To environmental engineers this research will help them to effectively evaluate the bioremediation potential of MeBT. It also provides essential toxicity and degradation potential information that must be considered when designing systems to treat waste streams containing methylbenzotriazoles.

Below is a list of recommendations for future research, which will take the research community a step closer in understanding this fascinating compound:

1. *Toxicity of MeBT to model ecosystem receptors*- Much work is still needed in order to fully understand the toxicity mechanisms MeBT can exert against many different aquatic organisms; this should not be limited to bacteria, although it was the focus of this research. This work demonstrated the potential for MeBT to uncouple oxidative phosphorylation from bacterial respiration. Investigations are currently underway to provide a better understanding for other toxicity mechanisms proposed; complexation to metal-containing enzymes and subsequent inhibition of their function. However, a third probable toxicity

mechanism, inactivation of cytochrome P450 has not been investigated for methylbenzotriazole. Future efforts in these are should concentrate in investigating the inactivation potential of methylbenzotriazole to cytochromes since they contain transition metals and are widely conserved among microorganisms. Also, the study of combined effects of these mechanisms on different bacterial strains will provide further information regarding synergistic effects against mixed bacterial cultures.

2. *MeBT Mineralization* –This research also elucidated the potential for microorganisms convert the carbon in MeBT molecules to non-toxic by-products, carbon dioxide and biomass. However, the fate of the nitrogen through this metabolism is not completely understood. Investigations on the potential of mixed enrichment cultures to use MeBT as the sole carbon and nitrogen source resulted in less than 40% of total nitrogen converted to ammonia; no other common forms of inorganic nitrogen were found. Future work in this area should focus on elucidating the fate of azo-bound nitrogen through a microbiological metabolism.
3. *By-products* - Another area to be investigated is by-products formation during the biotransformation of 5-MeBT. This information is essential to further elucidate the biodegradation pathways of MeBT biodegradation. A combination of thin-layer chromatography and gas chromatography-mass spectrometry could be used to further characterize the by-products of MEBT degradation (Heitkamp and Cerniglia, 1987). Enzyme assays could be used to test for the presence of key

enzymes involved in ring cleavage and the formation of the associated cathecol (Gaja and Knapp, 1997).

4. *Fate and Biodegradation Potential in Soil Systems* -Most research regarding benzotriazoles derivates under aerobic conditions had involved suspended enrichment cultures. The biodegradation potential of MeBT in soil systems should be monitored. This will provide a better understanding of the fate of this corrosion control inhibitor in the subsurface environments were varying redox conditions and transport are important. Also the effects of temperature on MeBT biodegradations should also be investigated, since most MeBT contamination will be found in cold climates.
5. *Denitrification Potential* - As discussed in Chapter 7, the denitrification potential of actinomycetes implicated to participate in MeBT mineralization, appear to be linked to the initial environmental conditions at the onset of xenobiotic exposure. Further work in this area should focus in trying different initial experimental conditions such as electron acceptor and headspace environment (Shoun et al., 1998).
6. *Biodegradation Potential by Pure Bacterial Cultures* - This research isolated two bacterial strains capable of growing in agar plates with 5-MeBT as the only carbon source. However, they were not able to transform 5-MeBT when suspended liquid. Further work in this area should focus in attempting to facilitate biotransformation of these isolates under different growth conditions. Other work could involve degradation studies of pure cultures known to biodegrade compounds that are structurally similar to MeBT. For example, the

actinomycete, *Streptomyces chromofuscus,* has been shown to mineralize azo dyes and might be able to use MeBT as the sole carbon source (Paszczynski et al., 1992). Other isolates that could be tested are those capable of using benzothiazoles as a carbon source (Gaja and Knapp, 1997; De Wever et al., 1998). Other studies could focus on gaining a better understanding of the predominant populations present on MeBT acclimated cultures and to determine if these populations act independently, or in concert, to metabolize MeBT.

7. *Complexation* – The capability of MeBT to form strong complexes with transition metals raise many questions regarding its biodegradability. In theory, if MeBT form strong complexes with metal in solutions, those metals might not be available to microorganisms. On the other hand, since 5-MeBT is biodegradable, is it the complex or the free MeBT molecule that is biodegradable? Thomas and co-workers (Thomas et al., 1998) investigated the biodegradability of metal-EDTA complexes by microbial enrichments. They found that the biodegradation rate of these complexes vary with the metal used to form the complex. Therefore, it is possible that for MeBT a similar effect occurs. In addition, the effects of MeBT on the electron transport system of cells should be further investigated.

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Appendix 1

Standard Curves

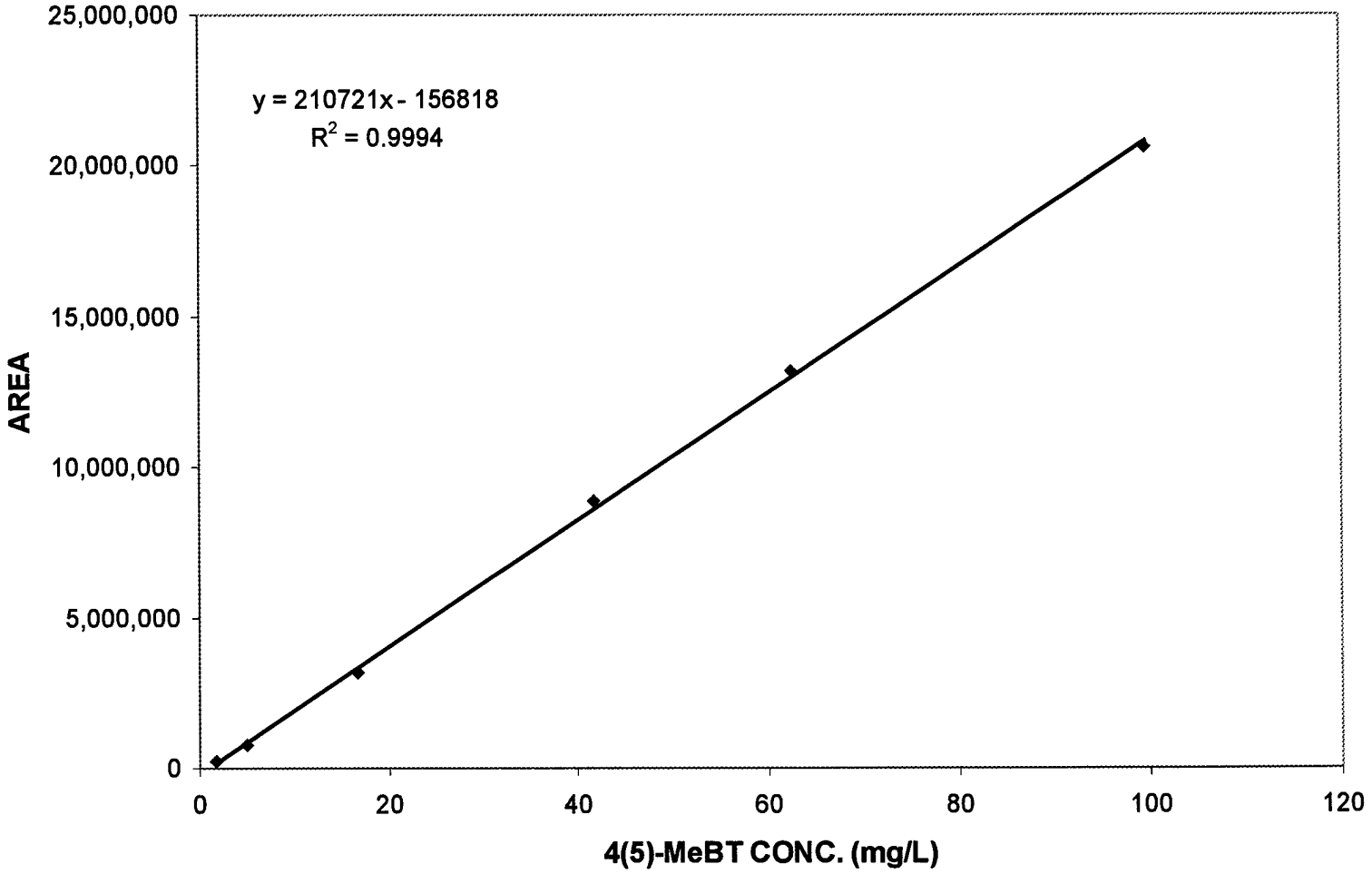

Figure A1-1. Example of calibration curve used to measure 4(5)-MeBT concentrations by high performance liquid chromatography

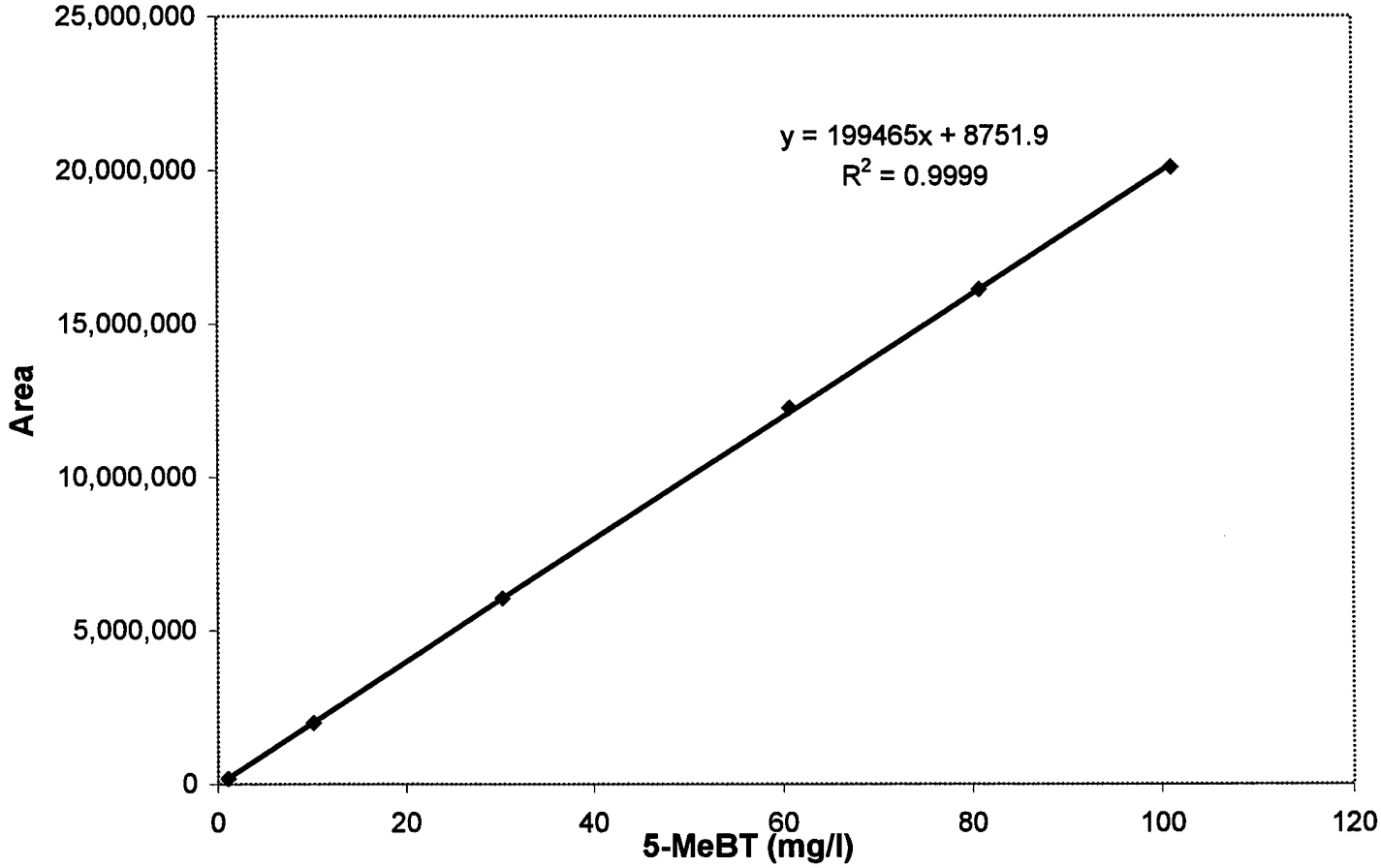

Figure A1-2. Example of calibration curve used to measure 5-MeBT concentrations by high performance liquid chromatography.

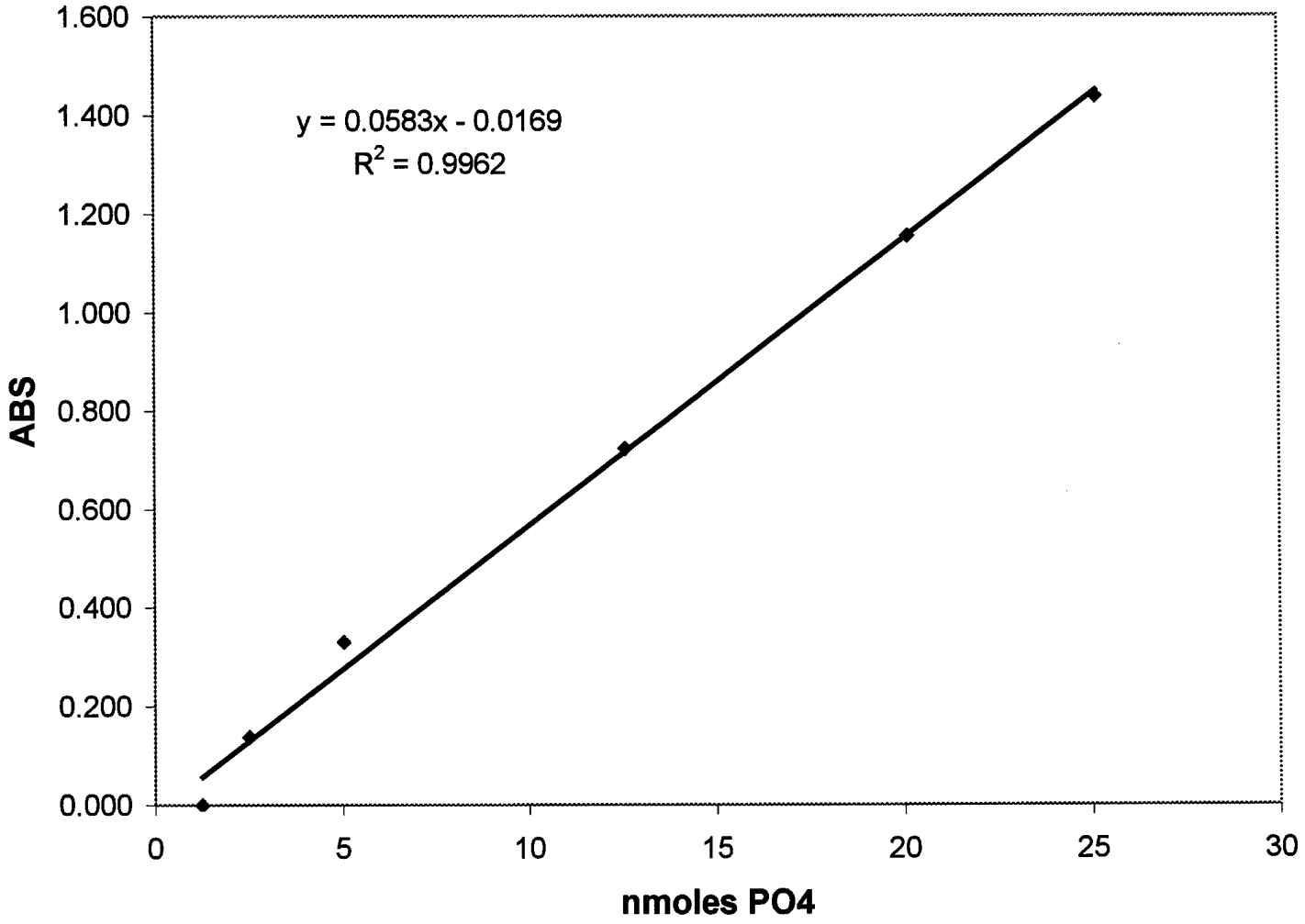

Figure A1-3. Example of calibration curve used for phospholipids analysis.

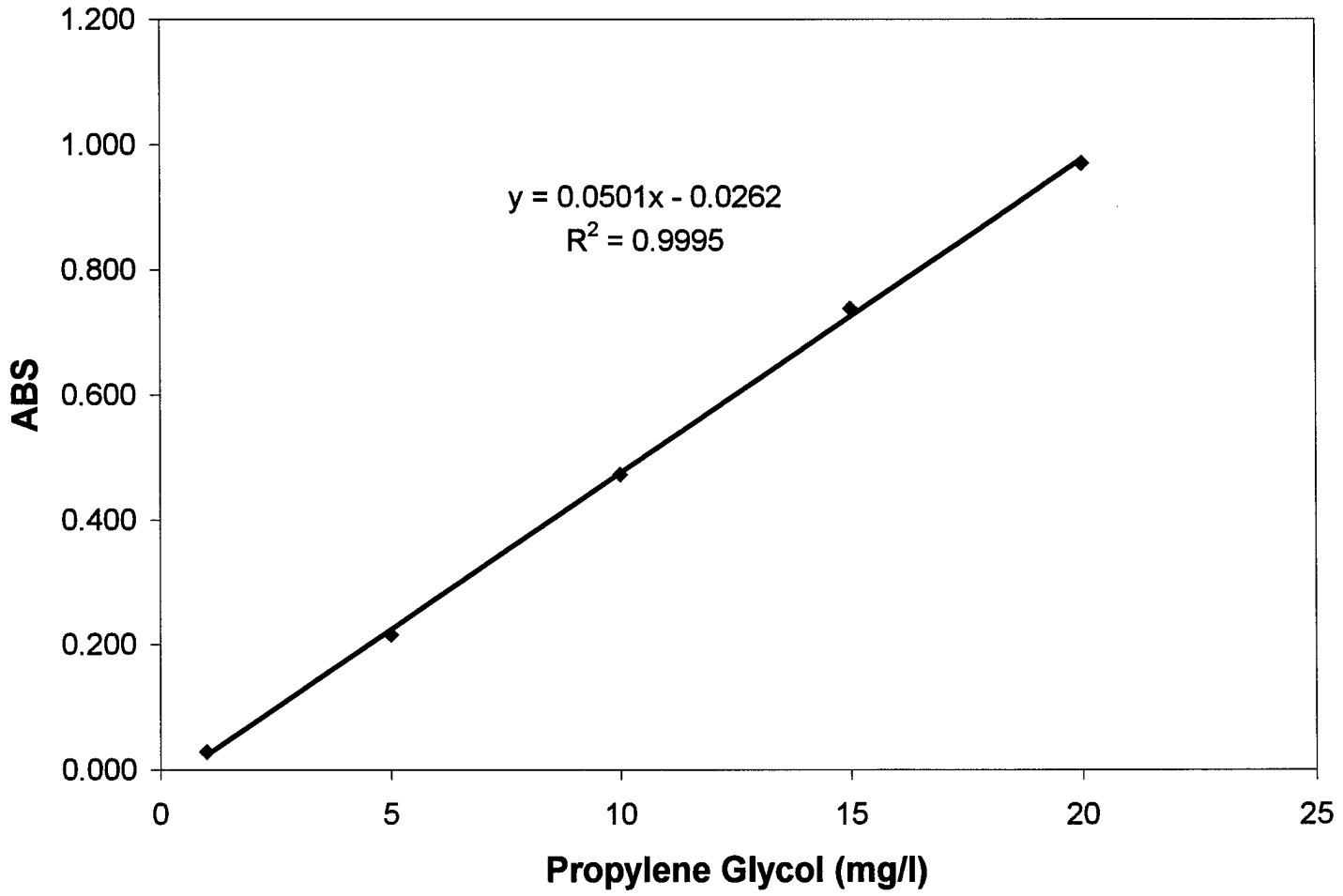

Figure A1-4. Example of calibration curve used to calculate propylene glycol concentrations.

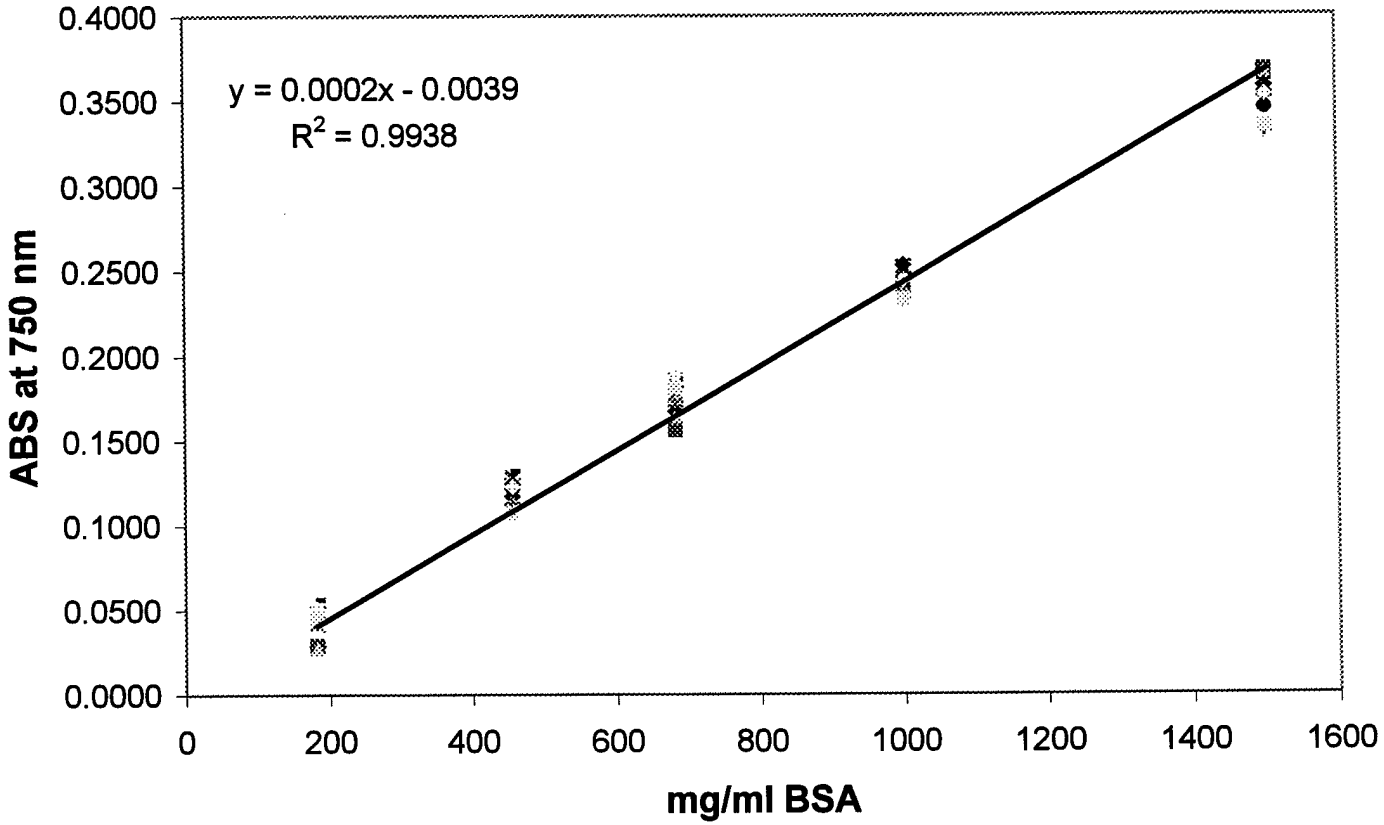

Figure A1-5. Calibration curve used to calculate protein concentration of cell fragments.

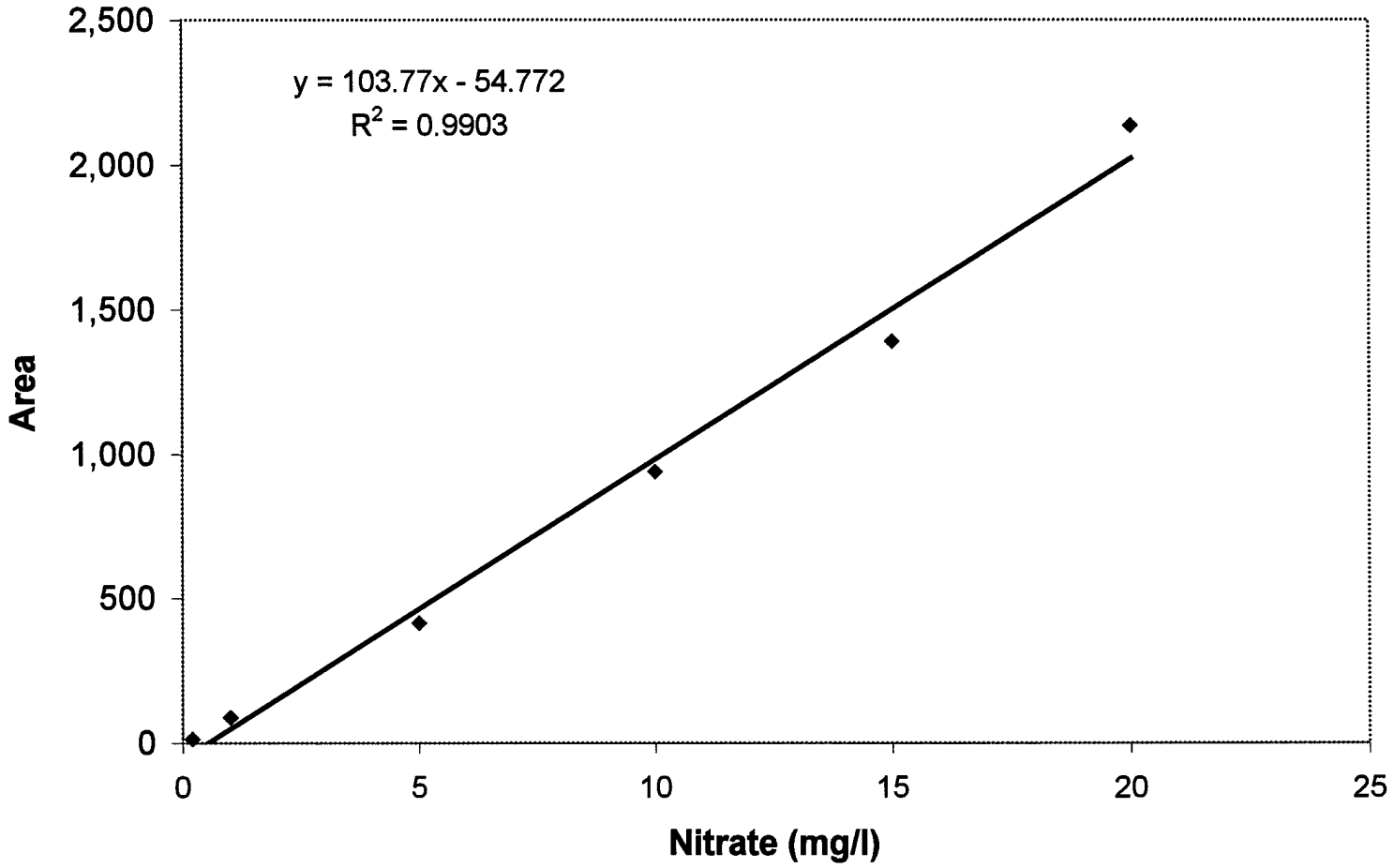

Figure A1-6. Example of calibration curve used to calculate nitrate concentration by ion chromatography.

Appendix 2

Chemical Analysis and Quality Control Procedures

Strict quality control procedures were followed during sample collection and analysis. Whenever, possible the elements of a good quality control (QC) program were followed: recovery of known additions, analysis of reagents blanks, calibration with standards, and analysis of duplicates as discussed in Standards Methods for the Examination of Water and Wastewater (American Public Health Association et al., 1995). Table A2-1 summarizes the QC protocols followed for each analytical procedure. Tables A2-2 to A2-7 summarizes the precision and accuracy data for the analytical methods presented in this thesis.

Table A2-1. Quality Control Procedures

Instrument	**Analytes(s)**	**Standards**	**Spikes**	**Blanks or Control Samples**	**# Replicates**
HPLC	MeBT	5	10%	5%	2
IC	Nitrate	5	10%	5%	3
Microscope	DAPI & DiBAC	N/A	N/A	1	5
Oxygen meter	O_2	N/A	N/A	1	5
Spectrometer	PG	5	10%	1	3
Spectrometer	Phospholipids	6	5%	1	3
Spectrometer	Protein	5	5%	1	3
Scintillation Counter	Radioactivity	1	N/A	1	3

Table A2-2. Precision and accuracy of the HPLC determination of 4(5)-MeBT

4(5)-MeBT added (mg/l)	*4(5)-MeBT found (mg/l)*	*Average (mg/l)*	*Error (%)*	*Precision (% std dev)*	*Recovery (%)*
1.7	1.9, 1.9, 1.0, .97	1.4	15	31	85
42	42, 43, 42, 42, 42	42	1.5	1.0	101
100	100, 99, 100, 99, 98	99.0	0.46	0.9	100

Table A2-3. Precision and accuracy of the HPLC determination of 5-MeBT

5-MeBT added (mg/l)	*5-MeBT found (mg/l)*	*Average (mg/l)*	*Error (%)*	*Precision (% std dev)*	*Recovery (%)*
0.99	0.65, 0.88, 0.83	0.79	20	12	79
25	26, 26, 27, 24, 24	25	0.96	4.4	101
72	71, 71, 70, 75, 74	72	0.25	2.9	100

Table A2-4. Precision and accuracy of the lipid-phosphate method as modified by Findlay and co-workers (Findlay et al., 1989).

Phosphate added (mg/l)	*Phosphate found (mg/l)*	*Average (mg/l)*	*Error (%)*	*Precision (% std dev)*	*Recovery (%)*
1.3	1.0, 0.51, 1.4, 0.92, 0.90	0.94	25	24	75
13	13, 13, 12, 13, 12	13	0.72	3.1	101
25	25, 24, 25, 25, 26	25	0.23	2.3	100

Table A2-5. Precision and accuracy of the standard periodate oxidation method for the determination of propylene glycol.

PG added (mg/l)	*PG found (mg/l)*	*Average (mg/l)*	*Error (%)*	*Precision (% std dev)*	*Recovery (%)*
1.0	1.0, 1.2, 1.0, 1.1, 1.1	1.1	8.0	8.4	108
10	10, 9.9, 10, 9.7, 10	10	1.2	3.6	101
20	20, 20, 20, 20, 20	20	0.1	1.4	100

Table A2-6. Precision and accuracy of the modification of the Lowry's (1951) method for the determination of protein content.

BSA added (mg/l)	*BSA found (mg/l)*	*Average (mg/l)*	*Error (%)*	*Precision (% std dev)*	*Recovery (%)*
182	182, 168, 232, 208, 208	200	9.6	14	110
683	858, 802, 882, 818, 892	850	24	5.8	125
1502	1622, 1608, 1708, 1708, 1718	1672	11	3.5	111

Table A2-7. Precision and accuracy for the ion chromatography determination of nitrate.

Nitrate added (mg/l)	*Nitrate found (mg/l)*	*Average (mg/l)*	*Error (%)*	*Precision (% std dev)*	*Recovery (%)*
1.0	1.4, 1.1, 1.3, 1.4, 0.78	1.2	20	26	120
10	10, 10, 9.2, 9.2, 12	10	0.30	12	100
20	21, 20, 21, 21, 20	21	3.5	3.0	104

Appendix 3

microbial insights

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Microbial Analysis Report

Client: Mark Hernandez
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Fax: 303-492-7317

MI Identifier: 3ucb **Date Rec.:** 8/29/01 **Report Date:** 9/14/01

Analysis Requested: Isolate Identification

Project:

Comments:

microbial insights

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Microbial Analysis Report

Sample Name	Date Sampled	Similarity Index (0.0 – 1.0)	Closest Match	Phylogenetic Analysis	Reference
G3	7/12/01	.884	Pseudonocardia alni	Gram Positive bacteria	A
G3 KS	8/14/01	.876	Variovorax sp.	Beta Proteobacteria	B

Identifications are based upon the Ribosomal Database Project (RDP). Similarity indecies above .800 are considered excellent, .600-.700 are good and below .500 are considered to be unique sequences.

References:

A. Reichert,K., Lipski,A., Pradella,S., Stackebrandt,E. and Altendrf,K. Pseudonocardia asaccharolytica sp. nov. and Pseudonocardia sulfidoxydans sp. nov., two new dimethyl disulfide-degrading actinomycetes and emended description of the genus Pseudonocardia. Int.J.Syst.Bacteriol. 48 Pt 2, 441-449 (1998)

B. Suyama,T., Hosoya,H. and Tokiwa,Y. Bacterial isolates degrading aliphatic polycarbonates. FEMS Microbiol.Lett. 161 (2), 255-261 (1998)