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Toxicity of Tolyltriazole to Bacillus Microorganisms

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TOXICITY OF TOLYLTRIAZOLE TO BACILLUS MICROORGANISMS

THESIS

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ABSTRACT (*Maximum 200 Words*)

The safety of air travel in cold weather depends upon the use of aircraft deicing fluids (ADAF). The environmental and toxic effects of the ADAF component tolyltriazole are not well understood. Tolyltriazole is a potential human carcinogen and has exhibited microbial toxicity in Microtox test.

This research used two methods to investigate the toxicity of tolyltriazole on bacillus shaped microorganisms. The first method compared the dissolved oxygen uptake of microcosms exposed to varying concentrations of tolyltriazole. When exposed to 1000 ppm tolyltriazole, all isolated species showed inhibition in respiration. There was no evidence that tolyltriazole at $\leq$ 500 ppm had a toxic effect.

The second experiment used colony counts to determine if colony formation was inhibited by previous exposure to tolyltriazole. There was no evidence to suggest that previous exposure to tolyltriazole inhibited colony formation.

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Abstract

The safety of both civilian and military aircraft in cold weather depends upon the use of aircraft deicing fluids (ADAF). The environmental and toxic effects of glycols, the primary component in ADAF, have been extensively studied. The environmental and toxic effects of minor ADAF constituents, such as the corrosion inhibitor tolyltriazole, are not well understood. Tolyltriazole is currently considered a potential human carcinogen and has exhibited microbial toxicity in Microtox tests.

This research investigated the toxicity of tolyltriazole on bacillus-shaped microorganisms. Single microbial species and a consortium containing several species were isolated from a soil sample. Toxicity of tolyltriazole to these organisms was measured by two methods. The first method grew the organisms in a liquid medium using propylene glycol and yeast extract as carbon sources. The experiment compared the dissolved oxygen uptake of microcosms exposed to varying concentrations of tolyltriazole to control microcosms. The three species of isolated organisms had similar responses to the tolyltriazole. When exposed to 1000 ppm tolyltriazole, all isolated species showed inhibition in respiration. There was no evidence that tolyltriazole at $\leq$ 500 ppm had a toxic effect. 500 ppm tolyltriazole appeared to enhance respiration for all studied organisms.

The second experiment used samples from the microcosms in the respiration experiment as an inoculum for Nutrient Agar pour plates. Colony counts were conducted after 24 hours to determine if colony formation was inhibited by previous exposure to tolyltriazole. There was no evidence to suggest that previous exposure to tolyltriazole inhibited colony formation.

TOXICITY OF TOLYLTRIAZOLE TO

BACILLUS MICROORGANISMS

I. Introduction

1.1 Overview

Safe air travel in cold climates depends upon successful deicing of aircraft. Glycol based aircraft deicing fluids (ADAF) are used in significant quantities at major airports and military installations throughout the world. A large commercial aircraft requires approximately 3,800 L (1,000 gal) of de-icing fluid per flight in sub freezing temperatures [Mericas and Wagoner 40]. Commercial airlines use up to 5.7 million liters (1.5 million gal) of ADAF per year [Strong-Gunderson *et al.* 265]. For passenger safety, the Federal Aviation Administration has developed strict requirements for deicing procedures. In addition to commercial use, ADAFs are also heavily used by the military. A typical small military base will use in excess of 95,000 L (25,000 gal) of ADAF per year [Strong-Gunderson *et al.* 265].

The formulations of most ADAFs remains proprietary information, thus the exact composition is not always available [Johnson 1-1]. Most ADAFs contain between 50-90% glycols and 10-20% other chemical additives. Extensive studies have been conducted on the effects and biodegradation of glycols while little research has been done on the chemical additives [Jank *et al.*, 1974; Kaplan *et al.*, 1982; Raja *et al.*, 1991; Strong-Gunderson *et al.*, 1995; Bausmith and Neufeld, 1996].

1.2 Problem

Approximately 80% of ADAF is deposited on the ground due to spray drift, jet blast, and wind shear during taxing and takeoff. These ADAFs either drain to storm-sewer systems, nearby creeks, or open areas where they are lost to infiltration [Hartwell *et al.* 1376]. The majority of ADAF eventually ends up in surface waters where its high Biological Oxygen Demand (BOD) results in a rapid reduction in dissolved oxygen. The carbonaceous BOD_5 of ethylene glycol ranges from 400,000 to 800,000 mg/l while propylene glycol often exceeds $1*10^6$ mg/L. Untreated BOD_5 for domestic wastewater ranges from 200 to 300 mg/L [Mericas and Wagoner 40].

The primary problem addressed in this work is the potential toxicity of minor ADAF components, in particular tolyltriazole. Formulated ADAF has a significantly higher toxicity than either pure propylene or ethylene glycol [Pillard 311]. The higher toxicity is likely the result of additives. After completing a separation analysis of ADAF Cancilla *et al.* (1997) found benzotriazoles in the most toxic fraction.

This thesis builds on experiments completed by Burke (1999). Results of soil respirometry, and other toxicity measures completed by Burke were inconsistent. Microbial plate counts and agar well diffusion tests indicated no microbial toxicity associated with tolyltriazole [Burke 4-23]. Soil respirometry data showed that high concentrations of tolyltriazole (750-1000 mg/kg) with a fixed mass of propylene glycol (1000 mg/kg) resulted in a significantly lower rate of O_2 consumption [Burke 5-1]. This result seems to indicate some toxicity from tolyltriazole. This research further analyzes these inconsistent and inconclusive results.

1.3 Research Objective

This research evaluated the toxicity, based on dissolved oxygen consumption and colony counts, of tolyltriazole to bacillus microorganisms that are capable of using propylene glycol as a substrate. This research was conducted in conjunction with complementary research completed by 1Lt. Heather Mitchell. Together, the research projects intended to compare the toxicity of tolyltriazole to bacillus and coccus shaped microorganisms.

1.4 Scope

This study focused on three isolated species of microorganisms. The microorganisms were isolated from a soil sample collected from behind Building 470 on Wright-Patterson Air Force Base. Species were selected based upon their shape and Gram stain characteristics. These species were isolated from a consortia of soil organisms grown on propylene glycol with a low concentration of tolyltriazole. Metabolic toxicity was measured by comparing changes in dissolved oxygen consumption following exposure to varying concentrations of tolyltriazole in an aqueous solution. Toxicity was also measured by a comparison of microbial plate counts. Control pour plates were compared to plates seeded with organisms previously exposed to various concentrations of tolyltriazole.

1.5 Terms Used in this Study

Biochemical Oxygen Demand (BOD) – The measurement of dissolved oxygen used in the biochemical oxidation of organic matter [Tchobanoglous and Burton 71].

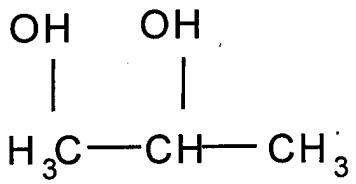
Chemical Oxygen Demand (COD) – The oxygen equivalent of organic matter that can be oxidized by using a strong chemical oxidizing agent in an acidic environment [Tchobanoglous and Burton 82].

Microcosm – The standard test unit consisting of dilution water, inoculum, and toxicant solution as required. The microcosm is contained with in a 300 ml BOD bottle.

Theoretical Oxygen Demand – A calculation of oxygen required for the complete mineralization of an organic substance [Tchobanoglous and Burton 82].

Octanol/Water Partitioning Coefficient (K_{ow}) - A measure of the hydrophobicity of chemicals [Alexander 137].

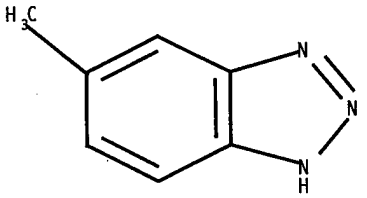
Propylene Glycol ($C_3H_8O_2$) - A compound used in ADAFs for freezing point depression.



Propylene Glycol

Statistical hypothesis – A claim about the value of a single population characteristic, or about the values of several characteristics [Devore 304].

Tolyltriazole ($C_7H_7N_3$) – Chemical frequently used as a corrosion inhibitor. Tolyltriazole is commonly found in the four, five, or six isomer. Commercial tolyltriazole generally consist of 60% six isomer and 40% five isomer [Cornell 8].



5-Tolyltriazole

II. Literature Review

2.1 Background

The composition of aircraft deicers (ADAF) changes with intended use. Type 1 formulations are deicers used to remove snow and ice from surfaces. Type 1 aircraft deicing fluid (ADF) must contain a minimum of 80% glycol. The remainder of the fluid consists of water, buffers, wetting agents, and oxidation inhibitors. Type 2 formulations are anti-icers that remove ice and remain on the treated surface to prevent future ice formation [Hartwell *et al.* 1375]. Aircraft anti-icing fluids must contain a minimum of 50% glycol. In addition to the constituents found in type 1 deicers, type 2 deicers also contain polymers that allow the fluid to adhere to the treated surface and provide residual protection against refreezing [Pillard 311]. The exact formulation of most ADAFs in use is not available because the information is proprietary.

Hartwell *et al.* (1995) found that the toxic effects of both the deicing and anti-icing solutions were significantly greater than reported values for pure glycol components. The increased toxicity is probably the result of the toxicity of the additives, or a synergistic toxicity between the additives. The results also indicate a significantly higher toxicity for the anti-icer solution than the deicer solution. This is likely the result of thickening agents and/or the particular combination of additives in the anti-icer. Recent work by Cancilla *et al.* (1997) isolated specific additives that may be responsible for some of the toxicity associated with ADAF. A comparison of the aquatic toxicity of deicers and anti-icers are seen in Table 1 [Hartwell *et al.* 1379].

Table 1. LC50s (EC 50s for *C. dubia*) (ml glycol/L) from toxicity tests of ethylene glycol de-icer and propylene glycol anti-icer solutions to fish and zooplankton

	Deicer Solution			Anti-icer Solution		
Species	48-h	96-h	7-d	48-h	96-h	7-d
Fathead minnow	9.82	9.82	9.82	0.07	0.03	0.03
Daphnia magna	13.48	3.83	-	.24	.05	-
Daphnia pulex	8.44	4.25	-	.27	.06	-
Ceriodaphnia dubia	12.85	8.95	3.02	0.44	.12	.07

Table adapted from Hartwell *et al.*

2.2 Glycol

ADAFs typically use either propylene glycol or ethylene glycol. Glycols are straight-chained alcohols with two attached hydroxyl groups [Sawyer *et al.* 203]. The glycol component provides freezing point depression. Depending upon the combination of glycols, the freezing point of water can be depressed between -13°C and -59°C [O'Connor and Douglas 22]. Propylene glycol based ADAFs are preferred because they are considered more environmentally friendly than ethylene glycol based ADAF. Ethylene glycol is a known mammalian teratogen while propylene glycol is not a known carcinogen or teratogen [Hartwell *et al.*1375]. In response to this information the USAF banned the use of ethylene-based ADAF on March 31, 1992 by a directive from Brigadier General James E. McCarthy. Table 2 summarizes some properties of propylene glycol.

Table 2. Characteristics of Propylene Glycol

Molecular Weight	76.1
Boiling Point (°C) at 760 mm Hg	188.2[a]
Freezing Point (°C) at 760 mm Hg	-59[b]
Vapor Pressure (mm Hg) at 20°C	0.2[a]
Solubility in Water	>10 g/100ml[c] at 21 C
Octanol / Water Partition Coefficient (K_{ow})	0.0389[a]

[a] Verschueren 1988
[b] Sigma Products Information Sheet
[c] Chemfinder 1999

Despite the evidence of some toxicity from the glycols, the primary environmental concern is the high oxygen demand rather than the toxicity. Glycol runoff results in a high oxygen demand that can lead to asphyxiation of aquatic and microbial life. This has resulted in numerous reports of fish kills downstream from airports [Pillard 312]. The rapid degradation rates and thus a high BOD enhance this impact. The chemical oxygen demand (COD) of ADAF is over 3,000 times the COD of typical untreated domestic wastewater [Bausmith and Neufeld 460].

Chain length and molecular weights of glycols are generally inversely proportional to the rate and extent of biodegradation. When glycols biodegrade, intermediate products such as aldehydes and organic acids can be formed; however, these products are quickly degraded to the end products of carbon dioxide and water [Raja 833-834]. Raja *et al.* (1991) used isolated strains of *Pseudomonas* and *Aerobacter* to determine pathways of degradation. *Aerobacter* strains completed further decarboxylation to CO_2. Figure 1 summarizes the proposed biodegradation of propylene glycol [Raja 833-834].

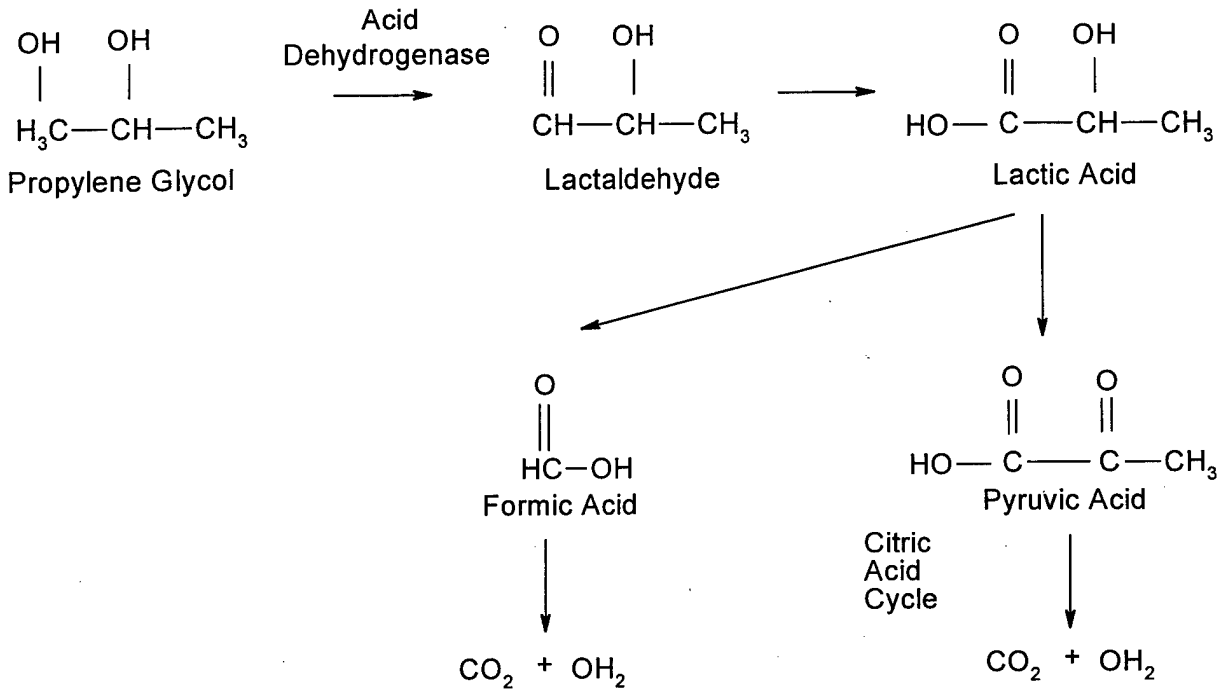


Figure 1. Proposed Biodegradation Pathway of Propylene Glycol

Assuming complete degradation allows for computation of the theoretical oxygen demand (ThOD) through stoichiometry. Table 3 summarizes the calculation of ThOD for propylene glycol; however, the BOD_5 for propylene glycol is generally only 2.2% of the ThOD [Verschueren 646].

Table 3. Calculations for Theoretical Oxygen Demand of Propylene Glycol

Basic Equation for ThOD	$C_nH_aO_bN_c + (n+a/4-b/2-3/4c)O_2 \Rightarrow$ $nCO_2 + (a/2-3/2c)H_2O + cNH_3$
Stoichiometric equation	$C_3H_8O_2 + 4O_2 \Rightarrow 3CO_2 + 4H_2O$
Molar ratio	$O_2 : C_3H_8O_2 = 4.0$
Molar ratio	$O_2 : CO_2 = 1.333$
Molecular weight	$C_3H_8O_2 = 76.094$ mg PG/mole
ThOD	128 mg O_2 / 76.094 mg PG = 1.68 mg O_2 /mg PG

2.3 Tolyltriazole

Benzotriazoles and tolyltriazoles are added to deicing fluids as corrosion inhibitors. Benzotriazoles are also commonly used in automobile antifreeze, hydraulic fluids, lacquers, and waxes. Research by Cancilla *et al.* (1997) has confirmed the presence of 1-H-Benzotriazole, 5-Methyl-1H-benzotriazole, and 5,6 Dimethyl-1H benzotriazole in deicer collected from Pearson International Airport. Tolyltriazole is a common name for benzotriazoles, which contains methyl groups on the aromatic rings [Cancilla *et al.* 431-433, 1997]. Table 4 summarizes some characteristics of commercial tolyltriazole. Commercial tolyltriazole is composed of approximately 40% of the 5 isomer and 60% of the 6 isomer [Cornell 9].

Table 4. Characteristics of Commercial Tolyltriazole

Tolyltriazole characteristics	**Result**	**Reference**
Boiling Point (°C) at 760 mm Hg	>300	PMC Specialties (1996)
Freezing Point (°C) at 760 mm Hg	76-87	PMC Specialties (1996)
Vapor Pressure (mm Hg) at 20°C	0.03	PMC Specialties (1996)
Solubility in Water at 18°C	<.01 g/100ml	Chemfinder (1999)
Octanol/Water Partition Coefficient (K_{ow})	.335	Cornell (1998)

The theoretical oxygen demand (ThOD) for tolyltriazole is calculated by stoichiometry. Table 5 shows calculations for the moles of oxygen required to convert tolyltriazole to carbon dioxide and ammonia [Burke 2-12].

Table 5. Calculations for Theoretical Oxygen Demand of Tolyltriazole

Tolyltriazole ($C_7H_7N_3$) Stoichiometric Equation:

$$C_7H_7N_3 + 6.5O_2 \Rightarrow 7CO_2 + (-1)H_2O + 3NH_3$$

Molar Ratio: O_2: $C_7H_7N_3 = 6.5$

Molar Ratio: O_2:$CO_2 = 0.9285$

Molecular weight $C_7H_7N_3 = 133$ mg TTA/mole

$$\therefore \frac{208 \text{ mg } O_2}{133 \text{mg TTA}}$$

$$= 1.564 \text{ mg } O_2/\text{mg TTA}$$

The material safety data sheet (MSDS) for tolyltriazole indicates that it presents a moderate risk to health by inhalation, ingestion, and skin absorption. For this reason, prudent safety measures are recommended to prevent inhalation or contact with the skin or eyes [PMC Specialties 1996]. Despite the information presented on the MSDS, there has been minimal research on the toxicity of tolyltriazole. Studies have shown that the toxicity of benzotriazoles depends greatly upon the molecular structure. These studies showed an increasing toxicity with increasing methylation (Table 6). Some attempts have been made to use K_{ow} as an indication of toxicity. Generally organics with log K_{ow} below 2.0 ($K_{ow} \cong 0.3$) are toxic to a majority of bacteria. Organics with log K_{ow} below 4.0 ($K_{ow} \cong 0.6$) exhibit some toxicity [Alexander 137-138]. This general statement does not apply to methylated benzotriazoles. Table 6 shows that toxicity increases with an increase in log K_{ow}. Regression analysis shows a significant linear relationship with $r^2 = 0.999$. This implies that partitioning into the microbe's bi-layer is an important aspect of the toxicity mechanism (Cornell 8).

Table 6. Microtox EC50 Values and Log K_{ow} for Benzotriazoles [Cancilla *et al.* 431-434, 1997].

Compound	Estimated Log K_{ow}	5 min EC50 (mg/l)	15 min EC50 (mg/l)
1H-Benzotriazole	1.27	41.13 +/- 4.63	41.65 +/- 11.01
5-Methyl-1H benzotriazole	2.16	5.69 +/-1.19	5.91 +/- 1.11
5-6-Dimethyl-1H-benzotriazole	3.05	0.72 +/-.28	0.80 +/-.33

Although Microtox studies have shown tolyltriazole toxicity, tests completed at the Air Force Institute of Technology have not conclusively shown toxicity. Burke (1999) was unable to show microbial toxicity using microbial population counts (MCPC) and agar well diffusion tests. For the MCPC uncontaminated soil was compared to a plate containing 500 mg/l tolyltriazole. Observation at 48 hours showed no statistical difference in the number of colonies present. Likewise, a plate containing 1000mg/l propylene glycol and 500 mg/l tolyltriazole was compared and no statistical difference was observed. Agar well diffusion tests were conducted with 10,000 mg/l propylene glycol and 5000-10,000 mg/l tolyltriazole. No toxic effects to microbial growth were observed around the agar well [Burke 2-23,24].

The biodegradability of tolyltriazole has been studied in numerous thesis at the Air Force Institute of Technology [Burke 1999, Johnson 1997, Halterman-O'Malley 1997]. Johnson (1997) used a respiromenter to conclude that the addition of tolyltriazole to soil samples did not significantly alter the O_2 consumption. This is an indication that the microorganisms present in the soil sample are unable to degrade the tolyltriazole; however, high performance liquid chromatography analysis completed on the same samples indicated that biodegradation was occurring. When propylene glycol and tolyltriazole were combined in a soil sample, the measured degradation was more than

additive compared to separate samples of glycol and tolyltriazole. Johnson (1997) speculates that tolyltriazole may enhance the degradation of propylene glycol by making it more available to microorganisms [Johnson 5-1 to 5-3]. Burke's (1999) results differ from those of Johnson. Burke showed that the rate of O_2 consumption slowed with an increasing concentration of tolyltriazole with a fixed mass of propylene glycol. When low concentrations of tolyltriazole were added to a fixed mass of propylene glycol, little change occurred in the rate of O_2 consumption. At higher concentrations of tolyltriazole, there was a significantly slower rate of O_2 consumption. Despite the rate change as tolyltriazole increased with a fixed mass of propylene glycol, the total O_2 consumption increased.

The pathway for tolyltriazole degradation is not well understood. Cornell (1998) proposed the degradation pathway in Figure 2, following an extensive literature review.

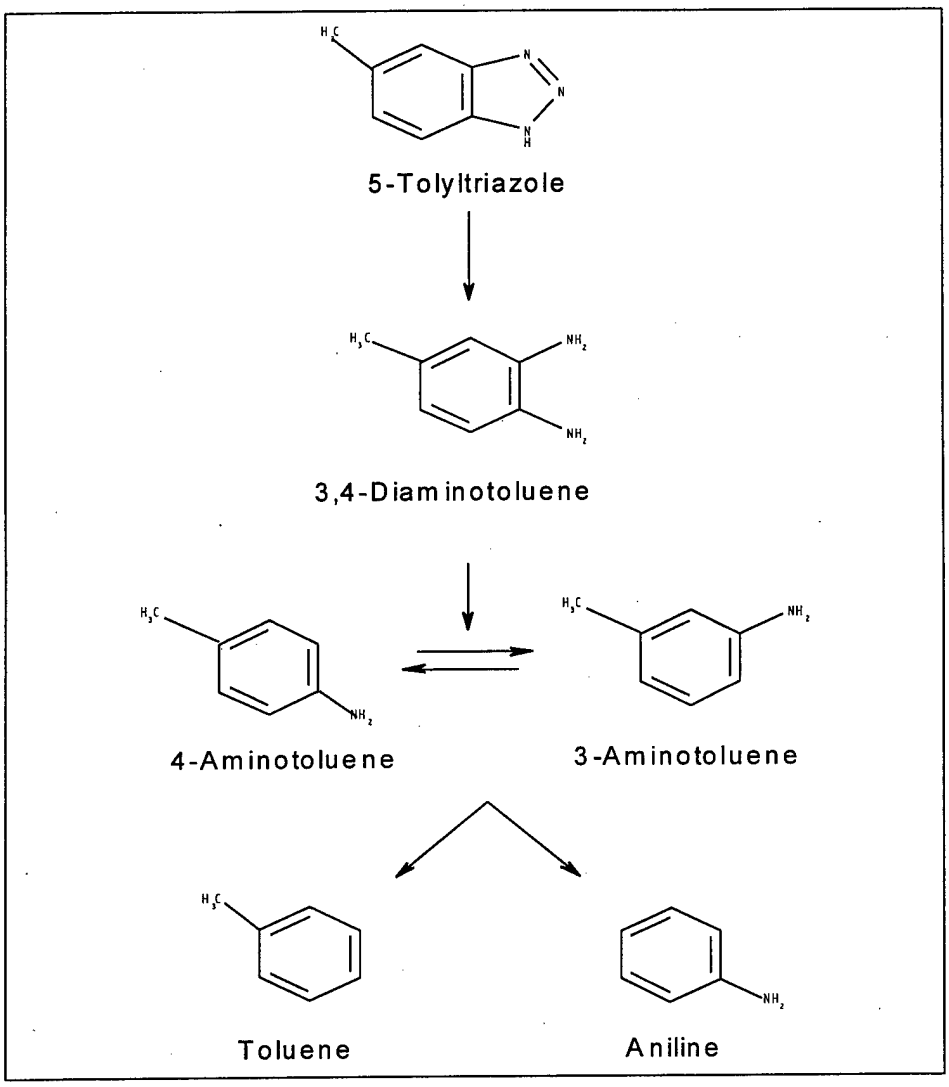


Figure 2. Biodegradation Pathway of Tolyltriazole to Toluene and Aniline

The transport of tolyltriazole through soil is not well understood. Cancilla *et al.* (1998) showed that tolyltriazole was mobile by detecting its presence in a perched aquifer near an international airport. Concentration of tolyltriazole found in the aquifer appears to be two to three times greater than Microtox EC 50 values [Cancilla *et al.* 1998: 3834-3835]. Table 7 summarizes Cancilla's estimated concentrations in the aquifer.

Table 7. Estimated Concentrations (ppm) of Benzotriazole, 5- Methylbenzotriazole, and Isomeric Methylbenzotriazole

Compound	Estimated Concentration (ppm)
Benzotriazole	126
5-Methylbenzotriazole	17
Methyl substituted benzotriazole	198
5,6 Dimethylbenzotriazole	Not detected

Kellner (1999) conducted some of the initial research on the transport and sorption of tolyltriazole. The study describes the behavior of tolyltriazole in a water saturated soil environment. Results of this research indicate that 5-Methyl-benzotriazole sorbs more than 4-Methyl-benzotriazole. The effective range of sorption coefficients ranged from 0.03 to 1.45 ml/g for the 4-MeBT to .04 to 3.24 ml/g for the 5-MeBT. This research also indicated that the application of propylene glycol made no difference on the sorption of tolyltriazole [Kellner 5-1].

2.4 Toxicity Testing

The Bacterial Bioluminescence Test (BBT) is the only applicable toxicity test mentioned in Standard Methods for the Examination of Water and WasteWater. The remaining toxicity tests are designed for macroscopic organisms. The BBT uses certain strains of luminescent bacteria, *Vibrio fischeri,* that divert up to 10% of their respiratory energy into a specific metabolic pathway that converts chemical energy into light. The light output of test organisms is measured under standard conditions. Organisms are then exposed to a test sample for a specified time and the light output is again measured. A reduction in light output is proportional to the toxicity of the sample. Consistent sensitivity and stability of the test cells is obtained by using cells in a lyophilized form [Standard Methods 8-35]. Numerous reproducibility studies have been conducted on

Microtox Rapid Toxicity Testing Systems, a commercial BBT test. Coefficient of variations average around 20% [AZUR Environmental 3]. The Microtox test has achieved standard status in ASTM Standard D5-660 and ISO Draft 11348-3.

A BBT test was not used in this thesis because it is limited to a specific organism. Although tolyltriazole may be toxic to *Vibrio fischeri*, a marine organism, this does not insure that it is toxic to common soil organisms.

2.5 Gram Staining

Differences between microbial cells can be seen by differential staining techniques. One of the most commonly used staining techniques is Gram staining. This technique floods a bacterial smear in succession with crystal violet, iodine solution, alcohol, and safranin. Based upon the results of this staining procedure, organisms are either classified as Gram positive or Gram negative. Gram positive retain the crystal violet dye and appear deep violet in color while Gram-negative organisms appear red [Pelczar *et al.* 96].

The difference in cells staining characteristics appears to be related to the differences in cell wall structure and thickness. Gram positive bacteria have a thick cell wall composed primarily of peptidoglycan, an insoluble, porous polymer with great rigidity and strength. Peptidoglycan makes up about 50% of the dry cell wall weight for Gram positive bacteria. Polymers of glycerol and ribitol phosphates called teichoic acids are often attached to the peptidoglycan. These negatively charged polymers aid in the transport of positive ions into and out of the cell [Pelczar *et al.* 121-123].

Gram negative cell walls are more complex than the walls of Gram positive cells. Gram negative cell walls have an outer membrane covering a periplasmic space that contains a thin layer of peptidoglycan. The outer membrane is a bilayered structure containing phospholipids. The membrane serves as a selective barrier controlling the passage of some substances between the cell and the environment. The membrane is selectively permeable to molecules based on charge and molecular size [Pelczar *et al.* 122-124].

III. Methodology

3.1 Overview

This chapter describes the two processes used to investigate the toxicity of tolyltriazole on selected bacillus-shaped microorganisms capable of using propylene glycol as a substrate. The first method used a dissolved oxygen (DO) probe to measure oxygen concentrations in various microcosms inoculated with isolated microorganisms. The procedures used for measuring DO were similar to those used by Marbas (1996). The oxygen uptakes of three microbial cultures, isolated from a soil sample, were measured. Oxygen consumption was compared between organisms exposed to varying concentrations of tolyltriazole. The change in DO consumption rates between control microcosms and those exposed to tolyltriazole is a measure of toxicity.

The second method of toxicity testing was a comparison of colony counts on pour plates. Control plates were inoculated with microorganisms with no exposure to tolyltriazole, additional plates were inoculated with microorganisms with previous exposure to varying concentrations of tolyltriazole. A reduction in colony forming units (CFU) would be an indication of toxicity.

3.2 Microorganism Isolation

3.2.1 Soil Collection and Microbial Selection

The microorganisms were separated from a soil sample collected in a wooded area directly behind Building 470 on Wright-Patterson Air Force Base. This was the same general area used by Johnson (1997) and Halterman-O'Malley (1997). Soil collection was completed using a steel shovel and an 8-liter plastic bucket. The soil

surface was cleared of grass and surface litter with the useable soil being collected in the next 10 to 20 centimeters. Ten grams of soil were placed in each of six 300 ml flasks along with 100 ml of water. A control bottle contained no tolyltriazole while the remaining five bottles contained up to 0.5 g of tolyltriazole in 0.1 g increments. This resulted in tolyltriazole concentrations ranging from 1000 ppm to 5000ppm. The addition of tolyltriazole allowed for selection of organisms with resistance to the substance. Following the addition of tolyltriazole, the bottles were placed on magnetic stirrers overnight.

3.2.2 Microorganism Growth and Separation

Step 1

Microorganisms were grown from each of the soil samples using agar pour plates. Pour plates were made using DIFCO Noble Agar, HACH BOD nutrient buffer pillows (Table 8), and two ppm propylene glycol. Propylene glycol was the only available carbon source added to the pour plates. The Agar mixture was sterilized in a Tuttnaur Brinkmann 3870 autoclave for 15 minutes at 121°C and 15 psi. One milliliter of the soil/tolyltriazole solution (described in 3.2.1) was added to each pour plate. Three replications were completed at each concentration of tolyltriazole. The Agar mixture was added to the pour plates after cooling to 40°C. The pour plates were then incubated in a Cole-Parmer Ecotherm Chilling Incubator at 28°C.

Table 8. Components of BOD Buffer Pillows

Component	Percent of Composition
Potassium Phosphate	<5
Magnesium Sulfate	<5
Calcium Chloride	<5
Ferric Chloride	<1
Other Components, each	<1
Demineralized Water	Up to 100

[Hach Company Material Safety Data Sheet]

Step 2

After six days, growth was observed on the control pour plates and the plates inoculated with the 1000 ppm tolyltriazole sample; noticeably more growth was observed on the control plates. No growth was observed on the remaining plates. Individual colonies were separated from the pour plates inoculated with 1000 ppm tolyltriazole, onto spread plates. This step was designed to isolate an individual species of microorganism from the consortia growing on the original pour plates. The spread plates were created using DIFCO Nutrient Agar, HACH BOD Nutrient Buffer Pillows, and 40 ppt propylene glycol. The agar solution was autoclaved and cooled to 40°C before pouring into 20 petri dishes. The petri dishes and nutrient agar solidified overnight. A platinum loop was used to separate individual colonies from the 0.1 g tolyltriazole pour plate onto the 20 spread plates. The spread plates were then incubated at 28°C. Procedures used in preparing the pour plates followed "9215 B. Pour Plate Method" found in Standard Methods for the Examination of Water and Wastewater.

Step 3

After three days, a third isolation was completed. Noticeable growth was observed on all 20 spread plates completed in step two. The same procedure as explained in step two was followed to isolate individual colonies on spread plates composed of nutrient agar, BOD buffer, and 200 ppm propylene glycol. This step was required because several different species appeared on the plates created in Step 2.

Step 4

Visual inspection of the pour plates from step 3, resulted in the identification of nine apparently distinct organisms. Each plate appeared to contain only a single species of microorganism, thus further separations were not required. The nine distinct cultures were named after the nine planets. Samples from each of the nine separated species of organisms were transferred with a platinum loop to slant tubes. The slant tubes contained solidified nutrient agar, BOD buffer, and 200 ppm propylene glycol. The agar solution had previously been autoclaved at 121°C and 15 psi. The slant tubes allowed for long term storage of the organisms in the incubator at 28°C. Two replicate slant tubes were made for each identified organism. The cultures were transferred when evidence of agar drying appeared. Organisms from the slant tubes were used as necessary to inoculate pour plates used as the working stock. This was done to help maintain the integrity of stock cultures.

3.3 Organism Identification

3.3.1 Gram staining

Gram staining was completed on both replicates from all nine organisms isolated in the slant tubes. Gram staining was completed with a Fisher Diagnostics Gram Stain

Set (Cat. No. SG 100D). Procedures supplied by Fisher Scientific were followed. Gram stained slides were examined microscopically with a Zeiss Axioskop at magnifications up to 1000x. In addition to Gram characteristics, organisms were also analyzed for shape. Table 9 summarizes the results of the Gram staining and microscopic examination. None of the organisms separated from the initial soil, water, and tolyltriazole solution appeared Gram positive. A bacterium of the genus *Bacillus* was isolated from the initial soil solution with no tolyltriazole. The *Bacillus* was isolated because it is a known Gram positive organism and appeared to be sensitive to the tolyltriazole. The remaining nine organisms had shown some resistance to tolyltriazole as they were cultivated from inoculum containing tolyltriazole.

Table 9. Results of Microscopic Gram Stain Analysis

Organism	**Gram characteristic**	**Shape**
Mercury	Negative	Rod
Venus	Negative	Rod
Earth	Negative	Coccus
Mars	Negative	Coccus
Jupiter	Negative	Rod
Saturn	Negative	Coccus
Uranus	Negative	Indeterminate
Neptune	Negative	Coccus
Pluto	Negative	Coccus

3.3.2 Lab Identification

Cultures of Mars, Saturn, Jupiter, Venus, and *Bacillus* were sent to three commercial laboratories for identification. Lab identification was completed using three different methods; 16S rRNA gene sequence profiles, 16S rDNA gene sequence profiles and fatty acid profiles. The test results, presented in Appendix A, provide a discussion of the three techniques and the laboratory findings. This thesis focused on Jupiter, Venus,

and *Bacillus*. Lt Heather Mitchell completed complementary research on Mars, Saturn, and *Bacillus*.

3.4 Lab Preparation

Prior to the execution of each DO uptake experiment it was necessary to prepare dilution water, inoculum, and a tolyltriazole solution. This section describes these processes.

3.4.1 Dilution water

Four liters of dilution water was prepared using Deionized water and HACH BOD nutrient buffer pillows. The dilution water was thoroughly stirred with a magnetic stirrer prior to use.

3.4.2 Inoculum

A new inoculum solution was prepared prior to each experimental run. Numerous attempts were made to grow organisms in a solution with propylene glycol as the sole carbon source. The organisms did not reach high enough populations to result in measurable oxygen change. The addition of yeast extract to the inoculum resulted in significantly higher organism populations.

Two liters of inoculum were prepared for each experiment. Two liters of deionized water solution containing 500 ppm propylene glycol and 500 ppm yeast extract were autoclaved. The afternoon of the day prior to an experiment, the autoclaved solution was inoculated with one species of organisms from the working spread plates. Organisms were transferred from spread plates using a sterilized platinum loop. The solution was placed on a magnetic stirrer and aerated with a standard fish tank aerator

and air stone. Air pumped into the solution was filtered with a 0.2 μm glass filter. Stirring and aeration continued until the inoculum was used in the following day's experiment. Any unused inoculum was discarded.

3.4.3 Tolyltriazole Solution

A 5000 ppm tolyltriazole solution was made by first heating deionized water to near boiling. The water was moved to a magnetic stirrer where the tolyltriazole was added. Using warm water allowed for the tolyltriazole to dissolve much quicker. A 5000 ppm solution is near the maximum solubility of tolyltriazole at 25°C [Burke 3-9].

3.5 Microcosm Setup

Each microcosm was prepared by an identical procedure in a 300 ml BOD bottle. A predetermined amount of the tolyltriazole solution was pipetted into a bottle to create the desired test concentration. 100 ml of dilution water was measured with a graduated cylinder and added to the BOD bottle. These two steps were completed for all microcosms used in the experiment before proceeding to the remaining steps. 100 ml of inoculum was then measured with the graduated cylinder and added to the bottle. The remaining headspace was filled with dilution water prior to inserting the DO probe and placing the bottle on a magnetic stirrer.

3.6 Experimental Setup

3.6.1 Dry Mass

Triplicate samples from each inoculum produced were filtered to determine the dry mass. Prior to microcosm set up, 20 ml samples of the inoculum were filtered using a 0.1μm pour-size glass filter. The filters were prepared by rinsing with deionized water,

placing them in aluminum pans and drying in the drying oven at 175°C for 4.5 hours. The filters and pans were then cooled for a minimum of 30 minutes in a desiccator before being weighted. The filters were returned to the desiccator until used to filter an inoculum sample. The used filters were again placed in the drying oven at 175°C for 4.5 hours. The filters were cooled and reweighed. The difference between the final mass and the initial mass is the mass of organics. The filtering was done in triplicate to check the assumption that the inoculum was homogenous. The dry mass was also used to normalize a comparison between the respiration rates of the different microorganisms.

3.6.2 Dissolved Oxygen

Each experiment consisted of 18 microcosms. Tolyltriazole solutions of 0 ppm, 50 ppm, 100 ppm, 500 ppm, 1000 ppm and a deionized water blank were completed in triplicate. Two DO probes were run simultaneously, with each probe continually cycling through nine of the microcosms. After a microcosm was prepared (section 3.5), the probe was allowed to stabilize for one minute before a reading was recorded. During this stabilization period a second microcosm was prepared. After one minute, the probe was removed from the initial microcosm, rinsed with deionized water, and placed in a second microcosm. The second microcosm was then placed on the magnetic stirrer while the initial microcosm was sealed and removed from the stirrer. This process was repeated until dissolved oxygen measurements were completed for nine microcosms. The probe was then returned to the initial microcosm and sequenced through the microcosms in one-minute intervals. The second probe was used simultaneously following an identical procedure on an additional nine microcosms. This procedure allowed for dissolved

oxygen measurements to be recorded for each microcosm in nine-minute intervals starting from the preparation of the microcosm. Each experiment was conducted for 8 time intervals (72 minutes).

3.6.3 Colony Counts

Colony counts were used to determine if exposure to tolyltriazole had any effect on the reproductive potential of the microorganisms. Samples taken from the microcosms were used to inoculate pour plates. Dilution of the samples was accomplished to reduce convergent growth. 20 µl were pipetted from each microcosm and diluted in separate test tubes each containing 10 ml of sterile dilution water. After addition of the sample, the test tube was shaken vigorously and 500 µl was pipetted into a petri dish. This process resulted in a dilution factor of 1000. Nutrient agar at approximately 40°C was then poured into the petri dish. The petri dishes were then placed in the incubator at 25° C. After 24 hours the colonies were counted using a Leica Quebec Darkfield Colony Counter.

3.7 Dissolved Oxygen Meter and Probe

3.7.1 Description

This experiment made use of a YSI 5010 BOD probe connected to a YSI 5100 dissolved oxygen meter and a YSI 5905 BOD probe connected to a YSI 58 dissolved oxygen meter. The two BOD probes are essentially identical except that the YSI 5010 model is specifically designed for the YSI 5100 meter. The 5905/5010 BOD probe is a voltametric sensor of dissolved oxygen. An oxygen permeable membrane covers an electrolytic cell. Oxygen entering the cell through the membrane is reduced at an applied

potential of –0.8 V referenced to a silver anode. The reduction current at a gold cathode is directly proportional to the partial pressure of oxygen in liquid [YSI 5905/5010 BOD Probe Instruction Manual 2]. To insure that the membrane was not affected by tolyltriazole, a test was performed to compare probe performance in three replicates of deionzied water and in three replicates of 1000 ppm tolyltriazole solution. Results are presented in Appendix B.

3.7.2 Calibration

Calibration of the YSI 5100 dissolved oxygen probe was completed using the auto calibration capability of the instrument. Auto calibration was completed after placing the probe in a BOD bottle containing about 1 inch of water to provide a 100% humidity environment. Following the advice of the technical support division at YSI, the percent saturation from the YSI 5100 was put into the YSI 58 which also had its probe in a 100% humidity environment. This was completed before each experiment to ensure the meters read within 0.2 mg/l for both probes.

A comparison between the two probes was completed by creating solutions of varying concentrations of Na_2SO_3. Both probes were allowed to stabilize for one minute in each of the solutions.

3.8 Statistical Analysis of Data

A two tailed, two independent sample t-test was used to determine if tolyltriazole had any significant impact on the BOD probe membrane. H_o stated that the tolyltriazole had no effect. The test was conducted with $\alpha = 0.05$. Discussion and application of the t- test are shown in Appendix B.

Statistical analysis of the oxygen consumption data and the pour count data was completed by an Analysis of Variance (ANOVA) followed by a Dunnett multiple comparisons of the means. An ANOVA can be used for comparisons of the means between two or more treatments of experimental units. Statistical differences exist between the means of the treatment if the chosen alpha value exceeds the p-value. The p-value is the tail probability associated with the F ratio in the F distribution. The ANOVA test was used to determine if further statistical analysis was required. When the ANOVA test indicated no statistical difference between the means, no further analysis was necessary. If the ANOVA indicated a statistical difference, the Dunnett multiple comparisons of the means was used. In several cases where the ANOVA failed to show a statistical difference, Dunnett multiple comparisons were still completed. This was done because it resulted in a clearer and more logical presentation of the results.

The Dunnett multiple comparison of the means is a modified t-test designed to compare a control group with other groups in a set of data. Dunnett comparisons are used because it allows for control of the familywise Type I error rate by specifying an α value. H_o states that the mean of the control group is equal to the mean of the experimental group, while H_a states that the means are not equal [Sheskin 362]. A more detailed description of the Dunnett test is presented in Appendix G.

Dunnett comparisons were completed to determine if a statistical difference existed between the mean oxygen consumption of the control and the mean oxygen consumption with various concentrations of tolyltriazole. The tests were completed using $\alpha = 0.05$. If the results of the Dunnett's test indicate that the mean oxygen consumption was higher than the control it was concluded that tolyltriazole at that concentration

enhances respiration. If oxygen consumption decreased, it was concluded that tolyltriazole inhibited respiration. The Dunnett comparisons for the three cultures are presented in Appendices H-J.

Dunnett multiple comparisons of the means were also completed on plate count data. This was completed to determine if a significant difference existed between colony counts on control pour plates with experimental pour plates. The experimental plates were inoculated with organisms that were previously exposed to tolyltriazole. H_o stated that the colony counts were equal between the control and pour plates, thus tolyltriazole had no effect on colony formation. H_a stated that a difference existed between the means, thus tolyltriazole had an effect on colony formation.

3.9 Preliminary Experiments

The development of the procedure and methodology used in this thesis were continually revised through many preliminary experiments. This section summarizes the refinements made to the experimental process.

3.9.1 Dilution Water Preparation

The original dilution water consisted of pure deionized water. This procedure was modified because the addition of inoculum to the dilution water resulted in a rapid change in osmotic gradient. The rapid change in osmotic gradient could affect the cells and mistakenly be considered an impact of the tolyltriazole. The modified procedure required that BOD buffer be added to the dilution water in equal concentration to the BOD buffer in the inoculum solution.

3.9.2 Inoculum Preparation

One of the more difficult aspects of this thesis was obtaining sufficient microbial growth in the solution used as the inoculum. Numerous attempts were made to grow the organisms in water containing only propylene glycol and BOD buffer. After several tries it was determined that significant organism growth would not occur with propylene glycol as the sole carbon source. Early experiments used beef extract as an additional carbon source; however, this addition did little to increase organism growth. Through continued experimentation it was found that the addition of yeast extract was necessary to promote organism growth in the liquid media. Inoculum containing 500 ppm yeast extract and 500 ppm propylene glycol was stirred overnight and then 300 ml was used in a BOD bottle. The DO in the BOD bottle would reach 0 mg/l in less then 2 minutes.

It was originally assumed that aeration of the inoculum during the overnight growth phase was not necessary. This resulted in initial DO reading around 4.0 mg/l. This resulted in oxygen levels approaching zero near the experiment completion. The procedure was corrected by aerating the inoculum throughout the night. Following aeration the initial DO values were generally near 7.0 mg/l.

3.9.3 Microcosm Setup

Multiple experiments were completed to determine the quantity of inoculum to use in each microcosm. Numerous experiments were completed that used between 1 and 10 ml of inoculum per BOD bottle with the remainder of the bottle filled with dilution water. The organism respiration rate was not fast enough to make these experiments

practical. After several hours DO concentrations changed by less than 0.1 mg/l. It was found that 100 ml of inoculum resulted in a reasonable DO uptake rate.

The order in which the tolyltriazole solution, the inoculum, and the dilution water were added to a microcosm was also modified. The components were originally added in the order as listed because it was the simplest set up. The cells in the inoculum were very briefly exposed to a very high concentration of tolyltriazole before the dilution water was added. This procedure was modified to add 100 ml of dilution water before the inoculum, thus preventing a higher than desired initial exposure. After the addition of the inoculum any remaining headspace was filled with the dilution water.

3.9.4 Experimental Set Up

The stirring device attached to the DO probes was not sufficient to provide adequate mixing. More consistent results were produced when the microcosms were stirred with both the attached stirring device and placed on a magnetic stirrer.

Initial experimentation was required to determine the appropriate concentrations of tolyltriazole to test. Initial tests were conducted using concentrations of 10 ppm, 50 ppm, 100 ppm, and 500 ppm. It did not appear that the 10 ppm tolyltriazole had any effect on the microorganisms, thus it was replaced by 1000 ppm microcosms in all following experiments.

One variable that was impossible to control was the number of organisms used to seed the inoculum. The platinum loop was not an accurate method for seeding the inoculum. This resulted in widely different DO consumption rates in the microcosms when identical procedures were followed between days. This made it impossible to

compare rates between days. In order to obtain true replicates at different tolyltriazole concentrations it was necessary to use both DO probes.

3.9.5 DO Probe Maintenance

Cleaning of the DO probes was essential to obtaining consistent results. Numerous experiments in this study were not reliable because the probes gave inconsistent readings. After cleaning the probes this problem was greatly reduced. The probe membranes were also suspect. Several membranes used in this experiment appeared to have defects or came off during the installation process. Reliability of the DO probes remained a concern throughout the study.

IV. Data Analysis

4.1 Overview

Results of dissolved oxygen consumption data were compared by both visual and statistical analysis. Statistical methods were used to compare microorganism growth on control pour plates with growth on pour plates inoculated with organisms from a tolyltriazole solution.

4.2 Microbial Identification

Cultures of Jupiter, Venus, and *Bacillus* were sent to three commercial labs for species identification. Lab results indicated several errors in the initial assumptions. The laboratory results indicated that all three cultures were Gram positive. Initial tests had erroneously identified Venus and Jupiter as Gram negative. The results of the initial Gram staining might have been inaccurate because the colonies stained were several days old. The reproducibility of Gram staining increases with younger rapidly growing organisms. It was also assumed that the cultures were pure, isolated species. Despite the appearance as isolated species, the lab results indicated that Venus and *Bacillus* are consortias of several organisms. Table 10 summarizes the results from the three laboratories. Complete copies of the laboratory results are presented in Appendix A. Surprisingly, the lab results indicate that one of the species in the *Bacillus* culture is the same as the species in the Jupiter culture. Visual and microscopic inspection of the colonies gave no indication that these colonies were related.

Table 10. Microbial Analysis Summary

	Laboratory (Method)		
Culture	**Microbial Insights (16S rDNA)**	**MIDI Labs (16s rDNA)**	**Microcheck (Fatty Acids)**
Venus	*Clostridium sp.* *Microbacterium* *Actinomycete*	Analysis Failed	*Bacillus coagulans* *Microbacterium lacticum*
Jupiter	*Bacillus thuringiensis*	*Bacillus thuringiensis*	*Bacillus cereus*
Bacillus	*Bacillus thuringiensis*	*Bacillus cereus*	*Bacillus cereus* *Bacillus thuringensis*

4.3 Equipment Standardization

The experimental procedure required the use of two DO probes in each experiment, thus a comparison of the probes' precision was required. This comparison was completed by creating solutions of varying concentrations of Na_2SO_3; results of this experiment are shown in Table 11. This experiment was primarily concerned with DO uptake rates rather than absolute DO values, thus the probes were considered precise as long as the difference between the DO readings remained constant at different actual DO values. This precision is measured by the standard deviation of the difference between the two probe readings. The precision between the two probes is 0.030 mg/l.

Table 11. Results of DO Probe Comparison

Na2SO3 (grams)	**DO Value YSI DO Meter 58 (mg/l)**	**DO Value YSI DO Meter 5100 (mg/l)**	**Difference**
0.0	7.19	7.14	.050
0.005	5.11	5.03	.080
0.010	4.07	4.0	.070
0.015	1.23	1.18	.050
0.020	3.37	3.29	.080
0.025	.010	.010	.000
		Average	.055
		Standard Deviation	.030

4.4 Influence of Tolyltriazole on Probe Membranes

An experiment was completed to insure that tolyltriazole, at concentrations up to 1000 ppm, did not influence the probe membrane integrity. This experiment was conducted for approximately the same time period as the toxicity tests. Results of a t-test indicated, with 95% confidence, that the membrane permeability to O_2 was not affected by the presence of tolyltriazole. Discussion and application of the t-test are shown in Appendix B.

4.5 Dry Mass of Organics

Triplicate samples from each inoculum produced were filtered to determine the dry mass. For each sample 20 ml of inoculum was filtered. The small standard deviation between the replicates is an indication that the inoculum solution was well mixed. The average dry sample mass and standard deviation are presented in Table 12.

Table 12. Dry Mass of Organics

Microorganism	Average Mass per Sample(mg)	Standard Deviation	Average Concentration (milligrams organics per liter of inoculum)
Venus	2.93	.25	146.5
Jupiter	3.23	.15	161.5
Bacillus	3.00	.5	150.0

4.6 Dissolved Oxygen Consumption

Following the experimental setup as described in section 3.6.2, dissolved oxygen consumption was measured in nine minute intervals for 72 minutes. The rate of oxygen consumption for each interval and treatment was calculated using the following equation:

$$Rate = \frac{DO_{initial} - DO_{final}}{time}$$

Graphs of DO consumption vs time intervals for Venus, Jupiter, and *Bacillus* are shown respectively in Figures 3 through 5. The raw data, averages, and standard errors are presented in Appendices C –E.

4.6.1 Analysis of Variance

An analysis of variance (ANOVA) was completed using the statistical package JMP®. The analysis of variance was completed for each microbial culture to determine if a statistical difference existed between the means of any of the treatments. Statistical difference is determined by comparing the p-value to the α level. If the p-value exceeds the alpha level (.05) the means are statistically different at the specified alpha value. Results of the ANOVA analysis are summarized in Table 13. If a statistical difference existed between the means of any treatments at a particular time interval, it is identified as a "YES" in the table. A complete listing of the P values are found in Appendix F.

Table 13. Statistical Difference Between Tolyltriazole Treatments

	Microorganism		
Time Interval	**Venus**	**Jupiter**	**Bacillus**
1	Yes	No	Yes
2	Yes	Yes	Yes
3	Yes	Yes	Yes
4	Yes	Yes	Yes
5	Yes	Yes	Yes
6	Yes	Yes	Yes
7	No	Yes	Yes

4.6.2 Venus

Visual observation of the Venus data suggests inhibition of microbial respiration at 1000 ppm tolyltriazole for the first four data points (Figure 3). After the fourth point, there appears to be microbial recovery at 1000 ppm tolyltriazole. Surprisingly, from the

fourth through the sixth point, 500 ppm tolyltriazole appears to enhance respiration. The data for the deionized water shows the inherent variability of the measurements.

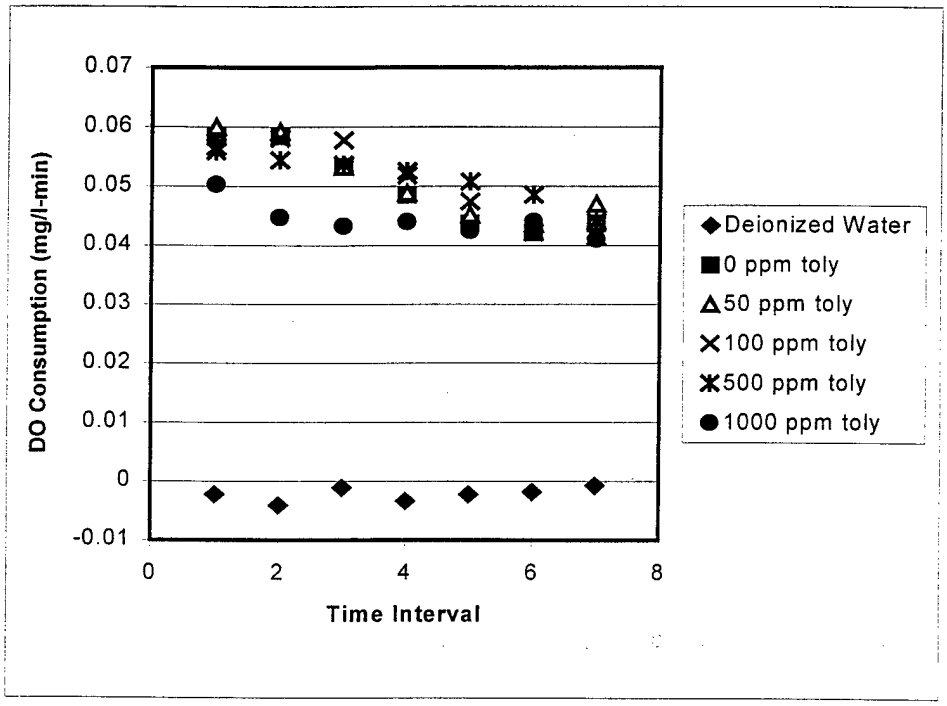


Figure 3. Averaged Rate of O_2 Consumption for Venus

Since the ANOVA analysis indicated that significant differences existed between the means of the treatments a Dunnett comparison of the means (Table 14) was completed. The Dunnett comparison of the means determined if the experimental treatments were different from the controls. This analysis confirmed the visual observation. The table shows, with 95% confidence, the impact of various concentrations of tolyltriazole on the dissolved oxygen consumption of the culture Venus.

Table 14. Dunnett Analysis of Venus

Reading	Tolyltriazole concentration (ppm)			
	50	100	500	1000
1	None	None	None	Inhibits
2	None	None	Inhibits	Inhibits
3	None	None	None	Inhibits
4	None	None	Enhances	Inhibits
5	None	Enhances	Enhances	None
6	None	None	Enhances	None
7	None	None	None	None

4.6.3 Jupiter

The data from the January 7 experiment, summarized in Figure 4, visually shows a definite inhibition of respiration at 1000 ppm tolyltriazole. Similar to the Venus data, there is some indication that tolyltriazole at 500 ppm may actually enhance respiration.

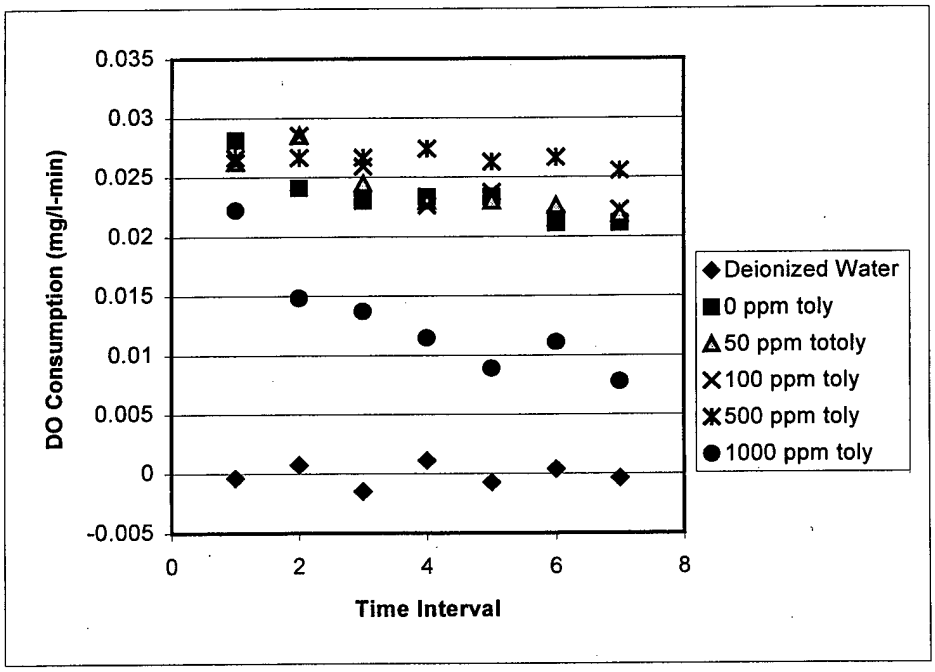


Figure 4. Averaged Rate of O_2 Consumption for Jupiter

Statistical analysis of the results using a Dunnett comparison of the means, confirmed the visual observation. This analysis is summarized in Table 15. The table, shows with 95% confidence, the impact of various concentrations of tolyltriazole on the dissolved oxygen consumption of the culture Jupiter.

Table 15. Dunnett Analysis of Jupiter

Reading	Tolyltriazole concentration (ppm)			
	50	100	500	1000
1	None	None	None	None
2	None	None	None	Inhibits
3	None	None	None	Inhibits
4	None	None	Enhances	Inhibits
5	None	None	None	Inhibits
6	None	None	Enhances	Inhibits
7	None	None	None	Inhibits

4.6.4 Bacillus

An experiment comparing the oxygen consumption of *Bacillus* exposed to varying concentrations of tolyltriazole, showed that 1000 ppm tolyltriazole inhibits respiration (Figure 5). 500 ppm tolyltriazole appeared to increase respiration. An unexplained jump in oxygen consumption occurred for the 1000 ppm tolyltriazole during the sixth time interval.

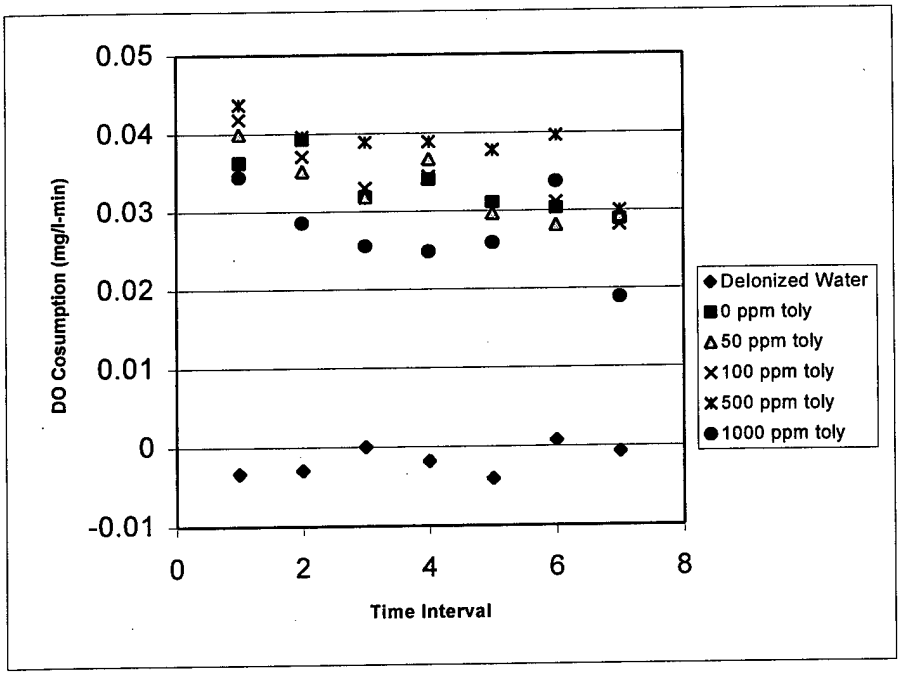


Figure 5. Averaged Rate of O_2 Consumption for *Bacillus*

Table 16 summarizes the results of the Dunnett comparison of the means for *Bacillus*. The unexplained increase in DO consumption in the 1000 ppm tolyltriazole microcosms at the sixth time interval, resulted in the conclusion that the means of the control and the treatment were not different.

Table 16. Dunnett Analysis of *Bacillus*

Reading	**Tolyltriazole concentration (ppm)**			
	50	**100**	**500**	**1000**
1	None	None	None	None
2	None	None	None	Inhibits
3	None	None	Enhances	Inhibits
4	None	None	None	Inhibits
5	None	None	Enhances	Inhibits
6	None	None	Enhances	None
7	None	None	None	Inhibits

4.7 Comparison of Normalized Respiration Rates

The respiration rates between the cultures varied by a wide range. It was expected that the differences in respiration rates could be explained by differences in dry mass of the inoculum. Figure 6 shows the respiration rates of the cultures normalized to the dry mass. The compared respiration rates are for the control microcosms that were not exposed to tolyltriazole. The coccus microbes (Saturn and Mars) exhibited a much higher respiration rate than any of the bacillus-shaped microbes. After normalizing the respiration rate Venus, was still nearly twice that of Jupiter and *Bacillus*. Jupiter and *Bacillus* were identified as being closely related species by the commercial laboratory, thus it was expected that the respiration rate should be similar. The differences between these rates could be explained by several factors. Although Jupiter and *Bacillus* were identified as similar by several laboratories, the exact strains of the microbes likely differ. The different appearance of the colonies on agar plates would support this hypothesis.

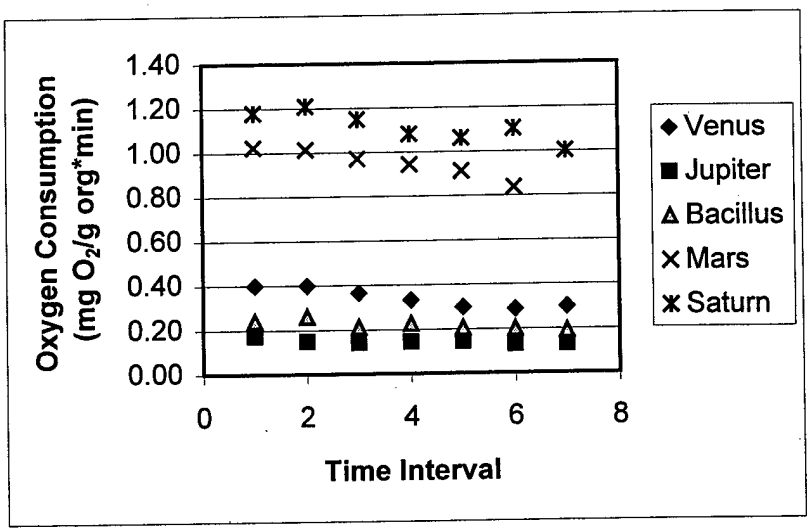


Figure 6. Averaged DO Consumption of Control Microcosms

4.8 Microbial Plate Counts

Following the completion of each dissolved oxygen experiment; an attempt was made to measure microbial toxicity through plate counts. A sample from each microcosm was used to inoculate separate pour plates. The number of colonies growing on each pour plate was counted after 24 hours. The 22 November *Bacillus* experiment resulted in convergent, wispy growth after 24 hours. No quantitative statement can be made about the impact of tolyltriazole; however, qualitatively it did not appear that the tolyltriazole had any impact on the growth. After 24 hours the pour plates for the 1 December Venus experiment and the 7 January Jupiter experiment had in excess of 1000 colonies per plate. These colonies differed from the *Bacillus* in being very small and not convergent; however, the number of colonies made accurate counting impossible. Qualitatively, it did not appear that tolyltriazole had any effect on the number of colonies.

An experiment using Jupiter was completed on 3 December that resulted in colony plate counts in an acceptable range. This data is summarized in Table 17. Dunnett analysis (Appendix K) of these results indicated no significant difference in colony growth between the controls (0 ppm tolyltriazole) and the various concentrations of tolyltriazole.

Table 17. Jupiter Colony Counts

Tolyltriazole Concentration in Inoculum (ppm)	Plate 1	Plate 2	Plate 3	Average	Standard Deviation	Standard Error of the Mean	Average Colony Forming Units/Microliter of Inoculum
Deionized water	0	0	1	.333	.567	.333	.333
0	51	53	43	49	5.30	3.06	49
50	59	34	80	57.67	23.03	13.30	57.67
100	96	71	49	72	23.51	13.58	72
500	91	232	62	128.33	90.95	52.51	128.33
1000	95	78	126	99.67	24.34	14.05	99.67

4.9 Comparison of Results With Microtox Results

The results of this experiment indicate that tolyltriazole is less toxic to common soil organisms than to *Vibrio fischer*, the microorganism species used in Microtox studies. Microtox studies show that tolyltriazole has a 15 minute EC 50 of approximately 6 ppm. This is two orders of magnitude lower than the tolyltriazole concentrations that first impacted the organisms studied in this experiment.

4.10 Potential Sources of Error

Numerous factors had potential to introduce error into this experiment. Variables that could not be controlled include: number of organisms used to seed the inoculum, laboratory temperature, and measurement error. The influences of the number of organisms used to seed the inoculum made it necessary to use multiple DO probes for data collection. This introduced error as the precision between the probes was 0.030 mg/l. Measurement error was introduced in numerous stages with the largest error being in using a 100 ml graduated cylinder for measuring the inoculum for each microcosm. The rapid set up time prevented precise use of the graduated cylinder. The actual quantity of inoculum used in the microcosms varied by several milliliters.

V. Conclusions and Recommendations

5.1 Conclusions

The research was designed to measure the toxic effects of tolyltriazole on various species of microorganisms. Previous toxicity studies involving tolyltriazole have relied heavily on Microtox. This research expanded the scope to common soil microorganisms. Dissolved oxygen and microbial plate count studies were conducted and statistical tests were performed to determine if tolyltriazole had any effect on microbial respiration and reproduction.

The results of this research indicate no measurable acute microbial toxicity associated with tolyltriazole at low concentrations. The respiration of the microorganisms did not statistically change when exposed to either 50 ppm or 100 ppm of tolyltriazole. This result matches the results found by 1st Lt. Heather Mitchell in similar research done on two different consortia of coccus organisms.

At 500 ppm tolyltriazole, the oxygen uptake of the three consortia of microorganisms increased. This would indicate that at 500 ppm tolyltriazole is a stimulant to microbial respiration. This result was not seen at all time intervals. Mitchell (2000) found that 500 ppm tolyltriazole inhibited the respiration of certain coccus organisms while having no effect on other coccus microbes.

At 1000 ppm, tolyltriazole appeared toxic to all three consortia of microorganisms. The onset of toxicity appeared rather quickly as in all cases respiration was inhibited within the first time interval (9 minutes). The Venus and Jupiter colonies appeared to recover from the initial toxicity as no statistical difference existed between

the control microcosms and the tolyltriazole microcosms after the sixth reading (54 minutes). Mitchell (2000) had mixed results finding that 1000 ppm tolyltriazole inhibited the respiration of certain coccus organisms while having no effect on other coccus microbes.

Colony counts of pour plates provided no conclusive evidence of the toxicity of tolyltriazole. The growth of Venus and *Bacillus* were too rapid to allow for accurate plate counts after 24 hours. A visual inspection was unable to determine any effects of tolyltriazole on the exposed organisms. Plate counts conducted using Jupiter appeared to indicate that organisms exposed to higher concentrations of tolyltriazole were slower in forming colonies. This qualitative observation was not supported by a Dunnett analysis, which suggested that the mean number of colonies were equal.

5.2 Improvements

5.2.1 Reproducibility

This experiment was not very reproducible because the inoculum was not consistent between days. Seeding the inoculum with organisms scrapped from agar with a platinum loop resulted in a large variably of organism population in the inoculum. Any future study must find a way to maintain a constant population inoculum. If possible, a continuous liquid culture should be maintained by removing liquid medium at the same rate that fresh medium is added.

5.2.2 Automation

The experimental procedures were very labor intensive. Two people were required to complete any DO experiment. Manual switching of the probe between

microcosms and manual data recording was required every minute during the experiment. The labor intensity greatly reduces the amount of data that can effectively be gathered.

5.2.3 Organism Isolation and Identification

Attempts to isolate individual organisms depended solely on visual appearance. This procedure was not effective in isolating individual species. The Gram staining procedures were also not accurate.

5.3 Follow on Research

Tolyltriazole at 500 ppm appears to stimulate microorganism respiration while 1000 ppm seems to inhibit respiration. Experimentation could be done to determine the concentration at which tolyltriazole switches from a stimulant to an inhibitor.

Conducting tests of longer duration would indicate whether the microorganisms are capable of adapting to tolyltriazole over time.

Similar tests could be completed using larger consortia of organism in each microcosm. This would closer match field conditions.

Identification and testing of Gram positive species by similar procedures, would provide insight into the role of the cell wall on the susceptibility to toxicity.

Appendix A: Commercial Lab Analysis of Microorganisms

Lab: Microbial Insights

AFIT/ENV

MOLECULAR ANALYSIS VIA 16S rDNA-DGGE

JANUARY 11, 2000

SUMMARY

The bacterial communities from 5 cultures from soils were characterized by denaturing gradient gel electrophoresis (DGGE). Results from the DNA profiles revealed both similarities and differences with their composition. All of the dominant bands excised from these samples fell within the Gram positive phylum. Several bands (B, C, D, and L) appeared to be present within more than one sample. Identification of individual bands are found in Table 1.

OVERVIEW:

The denaturing gradient gel electrophoresis (DGGE) approach directly determines the species composition of complex microbial populations based on the amplification of 16S rDNA fragments in polyacrylamide gels containing a linearity-increasing gradient of denaturants. DNA fragments of the same length but with different base-pair sequences are separated based on their melting behavior in an polyacrylamide gel. The banding patterns and relative intensities of the recovered bands provide a measure of changes in the community. Dominant species, which compose at least 1% of the total community in order to remain above the background level of minor bacterial amplification products, can be excised and sequenced. Fine scale sequence analysis of individual bands are used to infer the identity of the source organisms based upon database searches and phylogenetic methods (1-4).

METHOD:

Nucleic acid extraction was performed using a bead-beating system adapted from Bornemen *et al.*, with modifications (4). Sodium phosphate buffer, chaotropic reagent, glass beads, and sample were agitated in a microcentrifuge tube using a high speed bead beater. The sample mixture was centrifuged and the supernatant was collected. Chloroform was added, mixed thoroughly, and centrifuged. The aqueous supernatant was collected and combined with the first supernatant fraction. DNA was precipitated from the aqueous phase with an equal volume of isopropanol in an ice bath. DNA was pelleted by centrifugation, washed with 80% ethanol, air-dried, and re-dissolved in TE buffer. The DNA extract was purified by extraction twice with an equal volume of phenol/chloroform/isoamyl alcohol, followed by a glass-milk DNA purification protocol using a Gene Clean™ kit as described by the manufacturer.PCR amplification of 16S rDNA gene fragments was performed as described in (5) with modifications. Thermocycling consisted of 35 cycles performed on a "Robocycler™" PCR block. DGGE employed a D-Code 16/16 cm gel system maintained at a constant temperature in TAE buffer. Gradients were formed between 20-55 denaturants with the gels stained in ethidium bromide. Gel images were captured by use of an Alpha Imager™ system. The central portion of bands of were excised and soaked in purified water. The re-amplified products were purified by gel electrophoresis and isolated by use of a Gene-Clean™ kit. Purified DNA was sequenced with an ABI-Prism automatic sequencer. Sequence identifications were performed using the BLASTN 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).

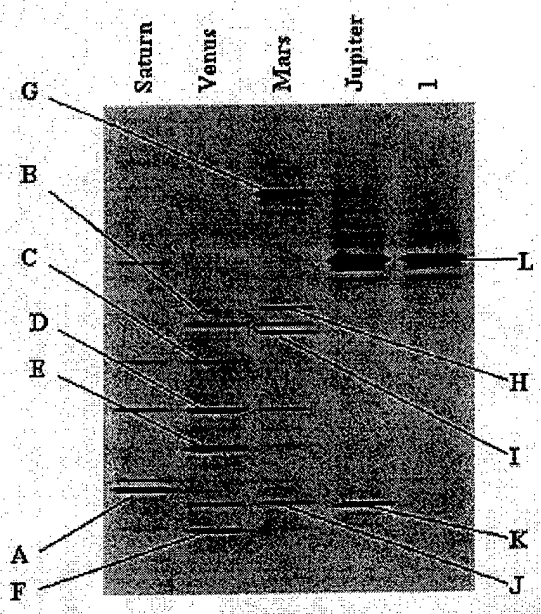


Figure 1. DGGE Gel Image.

Table 1. Sequence Results.

Band	Best Match	% Match	Phylogenetic affiliation	Ref. #
A	*Streptococcus macrosporus*	97%	Gram Positive Phylum	6
B	*Clostridium sp.*	100%	Gram Positive Phylum	-
C	Failed	-	-	-
D	*Microbacterium sp.*	95%	Gram Positive Phylum	-
E	*Uncultured actinomycete*	98%	Gram Positive Phylum	-
F	Failed	-	-	-
G	*Staphlococcus warneri*	100%	Gram Positive Phylum	-
H	*Unidentified eubacterium clone BSV45*	100%	Bacillus Like	7
I	*Unidentified eubacterium anoxic soil clone BSV77*	98%	Bacillus Like	7
J	Failed	-	-	-
K	Failed	-	-	-
L	*Bacillus thuringiensis*	100%	Gram Positive Phylum	-

Lab: MicroCheck, Inc.

LINE NO.	SAMPLE ID LABEL	✓	MICROORGANISM IDENTIFICATION	SI / SD	TYPE	MEDIA	CONFIRM TEST	LAB COMMENTS
1	Saturn		*Bacillus cereus* GC subgroup B MIXED CULTURE: see lines 1a, 1b, 1c below	0.463	B	TSBA		different colony types apparent after analysis
1a	Saturn.1 (large cream colony)	✓	*Bacillus disposauri*	0.747	B	TSBA		
1b	Saturn.2 (small yellow colony)	✓1	*Arthrobacter protophormiae/ramosus* *Paenibacillus polymyxa* *Bacillus megaterium* GC subgroup B	0.713 0.696 0.594	B	TSBA	GPR and cocco-bacilli	our spore stain result supports marked (1) FAME identification
1c	Saturn.3 (small gray colony)	✓	*Corynebacterium aquaticum*	0.682	B	CLIN		see Lab Notes at end of Results Table
2	Venus		*Xanthomonas axonopodis vitians* MIXED CULTURE: see lines 2a, 2b below	0.021	B	TSBA		different colony types apparent after analysis
2a	Venus.1 (opaque colony)	✓	*Bacillus coagulans*	0.350	B	TSBA		
2b	Venus.2 (translucent colony)	1	*Nesterenkonia halobia* *Kocuria varians* *Microbacterium lacticum*	0.633 0.497 0.382	B	TSBA	GPR	our stain result supports marked (1) FAME identification
3	Mars		*Bacillus cereus* GC subgroup B MIXED CULTURE: see lines 3a, 3b below	0.682	B	TSBA		different colony types apparent after analysis
3a	Mars.1 (large cream colony)	✓	*Bacillus disposauri*	0.719	B	TSBA		

LINE NO.	SAMPLE ID LABEL	✓	MICROORGANISM IDENTIFICATION	SI / SD	TYPE	MEDIA	CONFIRM TEST	LAB COMMENTS
3b	Mars.2 (small yellow colony)	1	*Nesterenkonia halobia* *Cellulomonas turbata* *Kocuria varians*	0.463 0.424 0.424	B	TSBA	GPR and cocco-bacilli	our stain result supports marked (1) FAME identification
4	Jupiter	✓	*Bacillus cereus* GC subgroup A	0.678	B	TSBA		
5	1		*Bacillus cereus* GC subgroup A MIXED CULTURE: see lines 5a, 5b below	0.691	B	TSBA		different colony types apparent after analysis
5a	1.1 (round colony)	✓	*Bacillus cereus* GC subgroup B	0.601	B	TSBA		
5b	1.2 (irregular colony)	✓	*Bacillus mycoides* GC subgroup B	0.406	B	TSBA		

Lab Notes:

Line No. 1c: The fatty acid profile of isolate Saturn.3 was compared to *Corynebacterium aquaticum*, a Gram positive rod which can be readily identified from growth on blood agar, and *Legionella brunensis*, a Gram negative rod which requires buffered charcoal yeast extract (BCYE) agar for growth. Since these organisms were grown on blood agar at 35°C for one day *Corynebacterium aquaticum* represents a much better choice for the identity of this isolate.

For your benefit, please find a brief description of our METHODOLOGY, an interpretation of the SIMILARITY INDEX, an explanation of the COMPARISON CHART, the rationale for Gram staining (GRAM STAIN), spore staining (SPORE STAIN) and how fungi (FUNGI) are identified in our laboratory in the six paragraphs below.

METHODOLOGY - The microbial identification systems at MICROCHECK consist of gas chromatographs with flame ionization detectors, autosamplers, integrators, and computers. The identification systems use five percent methyl phenyl silicone capillary columns. Retention time (RT) stability to two thousandths of a minute permits automation of the fatty acid methyl ester (FAME) peak naming. Prior to commencing analyses, there are 2 microliter injections of commercial calibration standards into the gas chromatographs. Besides calibration prior to each new method the gas chromatographs are also recalibrated every 50 samples. Once the systems have been calibrated and are operational, the autosamplers inject the cellular fatty acid extracts. The integrators process the chromatographic data and send the data to the computers. The computers name the FAME peaks and compare the fatty acid profiles of the unknown organisms to the profiles of the

7000 strains in the databases. The comparison of the fatty acid profile of an unknown to those in the databases is accomplished through use of software which uses covariance matrix, principal component analysis, and pattern recognition. Due to the large number of fatty acids produced by aerobic and anaerobic bacteria, yeast, and actinomycetes and the uniqueness of one pattern for a given taxon, the identification is very accurate. In addition to the internal standardization with the calibration fluid, an external control, *Stenotrophomonas maltophilia*, is also used. This bacterium, which has 23 fatty acid peaks, is processed daily from the streak plates with the client unknowns.

SIMILARITY INDEX - On the computer printout the similarity indices (SI) appear below the printout of the fatty acid composition and above the comparison chart in which the fatty acids of an unknown microorganism are compared to those of the reference strains. The SI value is the number following the species name (and often subspecies or pathovar name) of the library entry to which the unknown is compared. The SI value is unrelated to probability ratios and although the values range from 0.001 to 0.999 it is not a percentage. The SI is a numerical value which expresses how closely the fatty acid composition of an unknown corresponds to the fatty acids of the strains used to generate the library entry. Each library entry represents the profiles of at least 15 different microbial strains from both clinical and environmental sources. If a particular fatty acid departs slightly from the mean of the library entry either in RT or percentage the SI drops from an initial value of 1.00. A SI of 0.500 to 0.999 is an excellent match, as well as a single match comparison (no other matches listed) of 0.300 and higher. For SI between 0.100 and 0.300 the species of the unknown may not be in the current version of the database but the genus is probably correct and for SI below 0.100 the genus of the unknown is also questionable. A NO MATCH indicates there are no close comparisons to the 7000 strains in the databases. The value of the comparison is expressed in standard deviations (SD) from the mean of the strains used to generate this distantly related database entry. Infrequently an organism cannot be compared to any entry in the databases. This also results in a NO MATCH but there is not a SD measurement.

COMPARISON CHART - The comparison chart is below the results of the library search on the computer printout. The name of the library entry to which the unknown is compared is in the upper left hand corner of the chart. In the upper right hand corner of the chart is the SD that the unknown is from the mean of the strains used to generate the library entry. In the chart the x's represent the percentage of each fatty acid from the unknown, the -'s represent the range of percentages for the library entry strains, the +'s represent the mean percentage for the library entry strains, and the *'s represent peak matches of the fatty acids of the unknown with the mean of the library entry.

GRAM STAIN - A Gram stain is done on an isolate that is compared to database entries with similarity indices that are within 0.300 of each other if the cellular morphologies of the database entries are different, that has a similarity index less than 0.100, or that is a NO MATCH to any of the database entries to determine if the morphology of the isolate is consistent with that of the comparison.

SPORE STAIN - A spore stain is done, rather than a Gram stain, if the fatty acid profile of an isolate is compared to database entries with similarity indices that are within 0.300 of each other and at least two of these comparisons are G+ rods, one of which forms endospores.

FUNGI - Fungi are subcultured on plugs of potato flake agar (PFA) under coverslips on separate plates of potato dextrose agar (PDA) and on separate plates of phosphate glucose agar (PGA). Following incubation at 28°C the organisms are examined microscopically and identified using several keys to the fungi. Fungi do not have a SI / SD because they are not identified by FAME analysis.

FAME ANALYSIS Automated fatty acid methyl ester (FAME) analysis by gas liquid chromatography for identification of aerobic and anaerobic bacteria, yeast, and actinomycetes.

✓ A check mark next to a microorganism name indicates an excellent FAME match (See SI and SD)

SI The Similarity Index (SI) is a value between 0.001 and 0.999 which expresses the FAME similarity between the unknown isolate and the database match.

0.500 to 0.999	excellent match for genus and species
0.300 to 0.999	excellent for a single match to genus and species
0.100 to 0.300	good match for genus
0.001 to 0.099	weak match for genus

NO MATCH A NO MATCH analysis occurs when the unknown isolate has no close comparisons in the database.

SD The Standard Deviation (SD) value is listed for a NO MATCH analysis. The SD is a mathematical expression of the distance between the fatty acid profile of the unknown and the mean profile of the closest database entry. A NO MATCH with no SD indicates that the microorganism was not even distantly related to any of the 2,000 entries in the databases.

TYPE Microorganism TYPE

AC	actinomycete	**FAN**	facultative anaerobe
AN	anaerobic bacterium	**M**	mycobacterium
B	aerobic bacterium	**TH**	thermophilic bacterium
F	fungus	**Y**	yeast

( ) Parentheses ( ) around an entry in the TYPE column indicates that the microorganism was a different type than listed on the Test Request Form.

MEDIA The subculture MEDIA used by our laboratory to grow microorganisms.

BHIA	brain heart infusion blood agar	**PYGT**	peptone-yeast-glucose broth w/ Tween 80
CLIN	blood agar	**R2A**	defined minimal nutrient agar
MB7	Middlebrook 7H10 agar	**SDA**	Sabouraud dextrose agar
MRSA	MRS *Lactobacillus* agar	**TSB**	tryptic ase soy broth
PPP	potato dextrose agar, and phosphate glucose agar	**TSBA**	tryptic ase soy broth agar
PYG	peptone-yeast-glucose broth		

( ) Parentheses ( ) around an entry in the MEDIA column indicates that growth of the microorganism on this medium was insufficient for analysis.

CONFIRM TEST CONFIRMATION TESTING is done on an isolate whose FAME analysis result is inconclusive.

1 = stain 2 = coagulase test 3 = oxidase test 4 = API 20E test

CONFIRM TEST RESULTS

GPR	Gram positive rods	**GPC**	Gram positive cocci
GNR	Gram negative rods	**GNC**	Gram negative cocci
GVR	Gram variable rods		
coag$^+$	coagulase positive	**coag$^-$**	coagulase negative
ox$^+$	oxidase positive	**ox$^-$**	oxidase negative

API 20E Metabolic characterization which is done to confirm FAME results for members of the family Enterobacteriaceae. API 20E results often provides a more reliable identification than FAME analysis because this group of microorganisms is very similar in their FAME.

We encourage you to call our Technical Manager with any questions you may have about the analyses or the results:
Dr. Mike Sinclair - 802/485-6600 @ ext.22

Lab: Midi Labs

MIDI LABS

Enclosed are your sample results, including 16S rRNA gene alignment profiles and phylogenetic tree displays.

The bacterial identifications assigned in this report are based on 16S rRNA gene sequence similarity. Sequences analysis was preformed using PE Applied Biosystem's MicroSeq™ microbial analysis software and database. The top ten alignment matches are presented in a percent genetic distance format. In this format a low percent indicates a close match.

Also provided with the report are neighbor joining (Saitou and Nei, **Mol. Biol. Evol.** 4(4):406-425, 1987) phylogenetic trees. The trees are generated using the top ten alignment matches.

Concise alignments are also included. These illustrate positions that differ between your sample and the first match in the database. The position of the mismatch is read vertically from top to bottom and the sequences are read horizontally from left to right.

The results provided in this report are intended for research use only and will be kept confidential.

The protocol used to generate the 16S rRNA gene sequence data is as follows:

The 16S rRNA gene was PCR amplified from genomic DNA isolated from bacterial colonies. Primers used for the amplification correspond to E. coli positions 005 and 1540 (full length packages) and 005 and 531 (500 bp packages). Amplification products were purified from excess primers and dNTPs using Microcon 100 (Amicon) molecular weight cut-off membranes and checked for quality and quantity by running a portion of the products on an agarose gel.

Cycle sequencing of the 16S rRNA amplification products was carried out using AmpliTaq FS DNA polymerase and dRhodamine dye terminators. Excess dye-labeled terminators were removed from the sequencing reactions using a Sephadex G-50 spin column. The products were collected by centrifugation, dried under vacuum and frozen at -20 °C until ready to load. Samples were resuspended in a solution of formamide/ blue dextran/ EDTA and denatured prior to loading. The samples were electrophoresed on a ABI Prism 377 DNA Sequencer. Data was analyzed using PE/Applied Biosystems DNA editing and assembly software.

Thank you very much for choosing MIDI Labs for your bacterial identification needs. Do not hesitate to contact MIDI Labs should you have any questions or comments concerning the data reports.

Please keep us in mind for your future identification or sequencing needs.

MIDI LABS

Identification Report Summary

Bacterial identifications assigned by MIDI Labs are based on 16S rRNA gene sequence similarity. Sample sequences are compared against PE Applied Biosystem's MicroSeq™ database using MicroSeq sequence analysis software. The top ten alignment matches are presented in a percent genetic distance format, which is basically the percent difference between two aligned sequences. This percentage takes into account any mismatched basepairs, gaps and IUB ambiguity codes. In this format a low percent indicates a close match.

Species Level - This indicates a species level match. A 16S rRNA sequence homology of greater than 99% is indicative of a species level match (Stackebrandt and Goebel). Our experience in developing the MicroSeq database leads us to agree with this conclusion, though we feel that there is no exact cut off point that can be applied to all groups. It is our opinion that each alignment needs to be analyzed individually, taking into account the percent genetic distance between known species within that group.

Note that our results are presented in a genetic distance format, which is essentially the opposite of percent homology.

Genus Level - This indicates that the sample appears to group within a particular genus but the alignment did not produce a species level match. A genus level match indicates that the sample species is not included in the MicroSeq database

No Match - This indicates that sample did not group well within any particular genus found in the MicroSeq database. In cases such as this, we search the GenBank and Ribosomal Database Project (RDP) databases with the sample sequence to try to provide a closer match. If the sample sequence does not match well with either of these databases, it may be a new species or a species whose 16S rRNA gene sequence is not present in any of the databases.

Reference:

Stackebrandt, E. and Goebel, B. M. 1994. Taxonomic Note: A Place for DNA-DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. Int. J. Syst. Bacteriol. 44:846-849

Customer: Bleckmann

1/3/2000

MIDI LABS

500 bp Identification Summary

MicroSeq Database

C code	sample	closest match	% difference	confidence level
N267BLE	C2876 Saturn con	Arthrobacter crystallopoietes	1.98 %	Genus
N267BLE	C2878 Mars con	Microbacterium chocolatum	2.98 %	Genus
N267BLE	C2879 Jupiter con	Bacillus thuringiensis	0.00 %	Species
N267BLE	C2880 1 con	Bacillus cereus	0.65 %	Species
N267BLE	C2898 Mars white con	Staphylococcus warneri	0.19 %	Species

Key:

* - See report for additional comments concerning this field.

C code - Customer number assigned by MIDI Labs.

sample - Sample number assigned by MIDI Labs, followed by name assigned by customer.

closest match - Closest match to sample when aligned in a pairwise manner against the MicroSeq Database.

% difference - Percent difference between the sample and the closest match. Mismatched basepairs, gaps, and ambiguity codes are all accounted for in this percentage.

confidence level - This indicates the level of identification; see Identification Report Summary for additional information.

For research use only

Appendix B: Statistical Procedure for Determining Effects of Tolyltriazole on Dissolved Oxygen Probe Membrane

The data collected in Table B-1 was used to show that tolyltriazole had no effect on the DO Probe membrane, and thus probe performance. A two-sample t-test was performed using a significance level of $\alpha = 0.05$ and the following hypothesis.

H_o: There is no effect on probe performance due to tolyltriazole addition

H_a: There was an effect on probe performance due to tolyltriazole addition.

The following equation provides the pooled estimator, which is an estimator of common population variance. Where n_1 and n_2 are the sample sizes of the respective treatments, and S_1 and S_2 are the standard deviations of the respective treatments [Devore 358].

$$Sp^2 = \frac{(n_1 - 1) * S_1^{\,2} + (n_2 - 1) * S_2^{\,2}}{(n_1 + n_2) - 2}$$

The standard error was determined by the following equation [Devore 358].

$$\text{StdError} = Sp*(1/n_1+1/n_2)^{1/2}$$

The t-statistic was found by dividing the difference of the means by the standard error.

$$t = \frac{(X_{1000ppm} - X_{0ppm})}{\text{StdError}}$$

Since a two tailed test was required to determine any adverse effect, $\alpha/2$ was used to find the t critical (t_{crit}) value.

$$t_{crit} = t_{\alpha/2,n1+n2-2} = 2.776 \text{ (Sheskin 675)}$$

The decision to reject or fail to reject H_o is based on a comparison between t and t_{crit}. H_o is rejected if either $t \leq -t_{crit}$ or $t \geq t_{crit}$. As seen in table B-2, the calculated t value of 0.52 does not meet one of these conditions, thus H_o is not rejected with 95% confidence.

Table B-1. Raw Data for Determining the Effect of Tolyltriazole on DO Probe

	DO readings (mg/l)					
	0 ppm toly	0 ppm toly	0 ppm toly	1000 ppm toly	1000 ppm toly	1000 ppm toly
Minutes	Bottle1	Bottle 2	Bottle 3	Bottle 4	Bottle 5	Bottle 6
1	7.19	7.16	7.17	7.27	7.28	7.31
56	7.28	7.29	7.29	7.38	7.39	7.41
rate (mg/l-min)	0.00161	0.00232	0.00214	0.00196	0.00196	0.00179

Table B-2. Statistics for Determining the Impact of Tolyltriazole on Probe Membrane

Rate Period	Mean Ctrl (0 ppm)	Std Dev Ctrl (0 ppm)	Mean 1000 ppm	Std Dev 1000 ppm	Pooled Estimator S_p^2	Standard Error	$X_{Ctrl}-X_{1000ppm}$	Calc T Value $t_{Crit}=2.132$	Reject Ho
1	0.00202	0.00037	0.00190	0.00010	0.000000074	0.00022	0.00012	0.53452	No

Appendix C: Venus Respiration Rates (mg O_2/l-min)

Deionized Water						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	-0.0044	0.0011	-0.0033	-0.0022	0.0029	0.0017
2	-0.0011	-0.0100	-0.0011	-0.0041	0.0051	0.0030
3	-0.0011	0.0022	-0.0044	-0.0011	0.0033	0.0019
4	-0.0056	-0.0033	-0.0011	-0.0033	0.0022	0.0013
5	-0.0011	-0.0044	-0.0011	-0.0022	0.0019	0.0011
6	0.0000	-0.0033	-0.0022	-0.0019	0.0017	0.0010
7	-0.0022	0.0022	-0.0022	-0.0007	0.0026	0.0015

0 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0578	0.0567	0.0611	0.0585	0.0023	0.0013
2	0.0600	0.0578	0.0578	0.0585	0.0013	0.0007
3	0.0578	0.0511	0.0511	0.0533	0.0038	0.0022
4	0.0500	0.0467	0.0489	0.0485	0.0017	0.0010
5	0.044	0.0433	0.0433	0.0437	0.0006	0.0004
6	0.0422	0.0400	0.0444	0.0422	0.0022	0.0013
7	0.0422	0.0400	0.0489	0.0437	0.0046	0.0027

50 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0589	0.0600	0.0611	0.0600	0.0011	0.0006
2	0.0600	0.0589	0.0589	0.0593	0.0006	0.0004
3	0.0533	0.0533	0.0533	0.0533	0.0000	0.0000
4	0.0489	0.0478	0.0500	0.0489	0.0011	0.0006
5	0.0456	0.046	0.0444	0.0452	0.0006	0.0004
6	0.0422	0.0444	0.0444	0.0437	0.0013	0.0007
7	0.0411	0.0478	0.0522	0.0470	0.0056	0.0032

100 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0578	0.0556	0.0567	0.0567	0.0011	0.0006
2	0.0589	0.0578	0.0578	0.0581	0.0006	0.0004
3	0.0578	0.0589	0.0567	0.0578	0.0011	0.0006
4	0.0511	0.0533	0.0511	0.0519	0.0013	0.0007
5	0.0478	0.0467	0.0478	0.0474	0.0006	0.0004
6	0.0433	0.0433	0.0400	0.0422	0.0019	0.0011
7	0.0433	0.0411	0.0400	0.0415	0.0017	0.0010

500 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0611	0.0544	0.0522	0.0559	0.0046	0.0027
2	0.0544	0.0544	0.0544	0.0544	0.0000	0.0000
3	0.0533	0.0556	0.0522	0.0537	0.0017	0.0010
4	0.0544	0.0522	0.0511	0.0526	0.0017	0.0010
5	0.0511	0.0522	0.0489	0.0507	0.0017	0.0010
6	0.0489	0.0489	0.0478	0.0485	0.0006	0.0004
7	0.0456	0.0467	0.0400	0.0441	0.0036	0.0021

1000 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0511	0.0511	0.0489	0.0504	0.0013	0.0007
2	0.0422	0.0456	0.0467	0.0448	0.0023	0.0013
3	0.0422	0.0433	0.0444	0.0433	0.0011	0.0006
4	0.0433	0.0422	0.0467	0.0441	0.0023	0.0013
5	0.0444	0.0422	0.0411	0.0426	0.0017	0.0010
6	0.0422	0.0456	0.0444	0.0441	0.0017	0.0010
7	0.0411	0.0389	0.0433	0.0411	0.0022	0.0013

Appendix D: Jupiter Respiration Rate (mg O_2/l-min)

Deionized Water						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0000	0.0000	-0.0011	-0.0004	0.0006	0.0004
2	0.0000	0.0011	0.0011	0.0007	0.0006	0.0004
3	-0.0011	0.0011	-0.0044	-0.0015	0.0028	0.0016
4	0.0000	0.0000	0.0033	0.0011	0.0019	0.0011
5	0.0000	0.0000	-0.0022	-0.0007	0.0013	0.0007
6	-0.0011	0.0011	0.0011	0.0004	0.0013	0.0007
7	0.0011	-0.0011	-0.0011	-0.0004	0.0013	0.0007

0 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0333	0.0244	0.0267	0.0281	0.0046	0.0027
2	0.0267	0.0244	0.0211	0.0241	0.0028	0.0016
3	0.0233	0.0244	0.0211	0.0230	0.0017	0.0010
4	0.0233	0.0222	0.0244	0.0233	0.0011	0.0006
5	0.0233	0.0244	0.0222	0.0233	0.0011	0.0006
6	0.0200	0.0244	0.0189	0.0211	0.0029	0.0017
7	0.0233	0.0211	0.0189	0.0211	0.0022	0.0013

50 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0267	0.0211	0.0367	0.0281	0.0079	0.0046
2	0.0300	0.0267	0.0289	0.0285	0.0017	0.0010
3	0.0278	0.0222	0.0233	0.0244	0.0029	0.0017
4	0.0233	0.0211	0.0244	0.0230	0.0017	0.0010
5	0.0256	0.0222	0.0211	0.0230	0.0023	0.0013
6	0.0222	0.0211	0.0244	0.0226	0.0017	0.0010
7	0.0222	0.0211	0.0222	0.0219	0.0006	0.0004

100 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0267	0.0222	0.0300	0.0263	0.0039	0.0023
2	0.0322	0.0278	0.0256	0.0285	0.0034	0.0020
3	0.0267	0.0244	0.0267	0.0259	0.0013	0.0007
4	0.0233	0.0211	0.0233	0.0226	0.0013	0.0007
5	0.0233	0.0233	0.0244	0.0237	0.0006	0.0004
6	0.0211	0.0200	0.0233	0.0215	0.0017	0.0010
7	0.0233	0.0211	0.0222	0.0222	0.0011	0.0006

500 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0278	0.0244	0.0278	0.0267	0.0019	0.00111111
2	0.0267	0.0256	0.0278	0.0267	0.0011	0.0006415
3	0.0267	0.0244	0.0289	0.0267	0.0022	0.001283
4	0.0278	0.0267	0.0278	0.0274	0.0006	0.00037037
5	0.0289	0.0244	0.0256	0.0263	0.0023	0.00133539
6	0.0267	0.0256	0.0278	0.0267	0.0011	0.0006415
7	0.0267	0.0244	0.0256	0.0256	0.0011	0.0006415

1000 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0211	0.0222	0.0233	0.0222	0.0011	0.0006415
2	0.0156	0.0133	0.0156	0.0148	0.0013	0.00074074
3	0.0133	0.0144	0.0133	0.0137	0.0006	0.00037037
4	0.0111	0.0122	0.0111	0.0115	0.0006	0.00037037
5	0.0100	0.0089	0.0078	0.0089	0.0011	0.0006415
6	0.0111	0.0100	0.0122	0.0111	0.0011	0.0006415
7	0.0067	0.0044	0.0122	0.0078	0.0040	0.00231296

Appendix E: *Bacillus* Respiration Rate Experiment (mg O_2/l-min)

Deionized Water						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	-0.0033	-0.0044	-0.0022	-0.0033	0.0011	0.0006
2	-0.0033	-0.0033	-0.0022	-0.0030	0.0006	0.0004
3	-0.0022	0.0056	-0.0033	0.0000	0.0048	0.0028
4	-0.0022	-0.0022	-0.0011	-0.0019	0.0006	0.0004
5	-0.0011	-0.0089	-0.0022	-0.0041	0.0042	0.0024
6	-0.0022	0.0056	-0.0011	0.0007	0.0042	0.0024
7	-0.0022	0.0011	-0.0011	-0.0007	0.0017	0.0010

0 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0333	0.0356	0.0400	0.0363	0.0034	0.0020
2	0.0411	0.0389	0.0378	0.0393	0.0017	0.0010
3	0.0344	0.0333	0.0278	0.0319	0.0036	0.0021
4	0.0311	0.0367	0.0344	0.0341	0.0028	0.0016
5	0.0322	0.0311	0.0300	0.0311	0.0011	0.0006
6	0.0311	0.0322	0.0278	0.0304	0.0023	0.0013
7	0.0278	0.0289	0.0300	0.0289	0.0011	0.0006

50 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0411	0.0400	0.0389	0.0400	0.0011	0.0006
2	0.0344	0.0367	0.0344	0.0352	0.0013	0.0007
3	0.0344	0.0322	0.0289	0.0319	0.0028	0.0016
4	0.0311	0.0400	0.0389	0.0367	0.0048	0.0028
5	0.0322	0.0267	0.0300	0.0296	0.0028	0.0016
6	0.0289	0.0300	0.0256	0.0281	0.0023	0.0013
7	0.0256	0.0311	0.0311	0.0293	0.0032	0.0019

100 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0400	0.0422	0.0433	0.0419	0.0017	0.0010
2	0.0356	0.0400	0.0356	0.0370	0.0026	0.0015
3	0.0333	0.0322	0.0333	0.0330	0.0006	0.0004
4	0.0300	0.0378	0.0356	0.0344	0.0040	0.0023
5	0.0322	0.0289	0.0322	0.0311	0.0019	0.0011
6	0.0322	0.0322	0.0289	0.0311	0.0019	0.0011
7	0.0256	0.0267	0.0322	0.0281	0.0036	0.0021

500 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0500	0.0422	0.0389	0.0437	0.0057	0.0033
2	0.0411	0.0422	0.0356	0.0396	0.0036	0.0021
3	0.0389	0.0400	0.0378	0.0389	0.0011	0.0006
4	0.0400	0.0400	0.0367	0.0389	0.0019	0.0011
5	0.0378	0.0367	0.0389	0.0378	0.0011	0.0006
6	0.0400	0.0389	0.0400	0.0396	0.0006	0.0004
7	0.0300	0.0322	0.0278	0.0300	0.0022	0.0013

1000 PPM Tolyltriazole						
Time Interval	Replicate 1	Replicate 2	Replicate 3	Average	Std Dev	Std Error
1	0.0367	0.0333	0.0333	0.0344	0.0019	0.0011
2	0.0311	0.0278	0.0267	0.0285	0.0023	0.0013
3	0.0278	0.0267	0.0222	0.0256	0.0029	0.0017
4	0.0244	0.0267	0.0233	0.0248	0.0017	0.0010
5	0.0289	0.0244	0.0244	0.0259	0.0026	0.0015
6	0.0289	0.0389	0.0333	0.0337	0.0050	0.0029
7	0.0256	0.0133	0.0178	0.0189	0.0062	0.0036

Appendix F: ANOVA Results

An ANOVA was completed for each microorganism culture, at each time interval, to determine if a statistical difference existed between any of the experimental treatments. If the P-value is less then the alpha level of .05, there is a statistical difference. Table 18 summarizes the P-Values calculated by the statistical package JMP®.

Table 18. ANOVA P-Values

	Microorganism		
Time Interval	**Venus**	**Jupiter**	**Bacillus**
1	.0074	.5206	.0288
2	<.0001	<.0001	.0013
3	<.0001	<.0001	.0011
4	.0008	<.0001	.0037
5	<.0001	<.0001	.0005
6	.0046	<.0001	.005
7	.3815	<.0001	.0213

Appendix G: Description of Dunnett Multiple Comparison of the Means

The Dunnett multiple comparison of means is a modified t-test designed to compare a control group with other groups in a set of data. Dunnett's test is considered a superior for comparisons to a control group because it allows for control of the familywise Type I error rate by specifying an alpha value. H_o states that the mean of the control group is equal to the mean of the experimental group, while H_a states that the means are not equal [Sheskin 362].

The test statistic (t_D) for the Dunnett test is calculated with the following equation.

$$t_D = \frac{\overline{X}_a - \overline{X}_b}{\sqrt{\frac{2MS_{WG}}{n_h}}}$$

$\overline{X}_a$ = Mean of experimental group
$\overline{X}_b$ = Mean of control group
MS_{WG} = Mean Square within groups
n_h = harmonic mean

The harmonic mean is the mean of the sample size. The harmonic mean is calculated by the following where there are k samples of size n.

$$n_h = \frac{k}{\frac{1}{n_1} + \frac{1}{n_2} + \cdots + \frac{1}{n_k}}$$

The mean square within groups is calculated from the within-groups sum of squares (SS_{WG}) and the within-groups degrees of freedom.

$$MS_{WG} = \frac{SS_{WG}}{df_{WG}}$$

If the calculated value of t_D exceeds the tabled critical value, the alternative hypothesis is supported. The following equation is used to calculate the minimum required difference between the means to state that they differ significantly.

$$CD_D = t_{D_{(k,dfWG)}} \sqrt{\frac{2MS_{WG}}{n_h}}$$

$t_{D(k,dfWG)}$ is the tabled critical value for the Dunnett's modified t statistic for k groups and dfWG at the prespecified value of α_{FW}. A significance difference between the means is supported when:

$$\overline{X}_a - \overline{X}_b \geq CD_D$$

The statistical program JMP® was used to complete the Dunnett Analysis for this thesis. JMP represents CD_D as LSD, thus JMP® output states that a significant difference exist between the means if:

$$[\overline{X}_a - \overline{X}_b] - LSD \geq 0$$

A final method used to compare the means for statistical difference was comparison circles. The angle of intersection between the circles is an indication of statistical difference. If the angle exceeds 90 degrees this is an indication that the means are not significantly different, while an angle less then 90 degrees indicates statistical difference.

Appendix H: Statistical Analysis of Venus

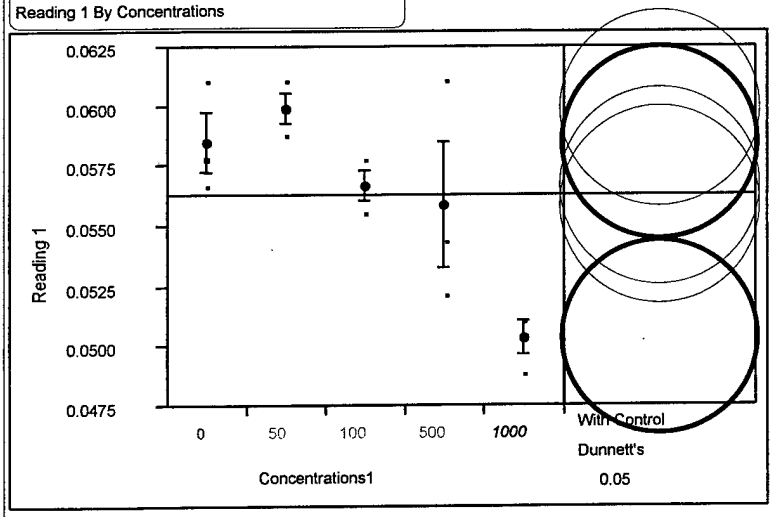
This appendix shows the statistical analysis of the Venus data. All data collected from each time period is plotted on a separate graph. The mean rate for each concentration was then calculated and plotted. The error bars shown on the graphs represent one standard error above and below the mean value.

The circles to the right of the graphs are comparison circles. The angle of intersection between the circles is an indication of statistical difference. If the angle does not exceed 90 degrees this is an indication that the means are significantly different.

Dunnett multiple comparison of the means were completed using an α of 0.05. A positive difference between the difference of the means and the LSD is an indication of statistical difference between the means. Further information on the Dunnett comparison can be found in Appendix E.

Dunnett Analysis of Venus, Reading 1

Reading 1 By Concentrations



Means Comparisons

Dif=Mean[i]-Mean[j]	50	0	100	500	1000
50	0.000000	0.001433	0.003267	0.004067	0.009600
0	-0.00143	0.000000	0.001833	0.002633	0.008167
100	-0.00327	-0.00183	0.000000	0.000800	0.006333
500	-0.00407	-0.00263	-0.0008	0.000000	0.005533
1000	-0.0096	-0.00817	-0.00633	-0.00553	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

|d|

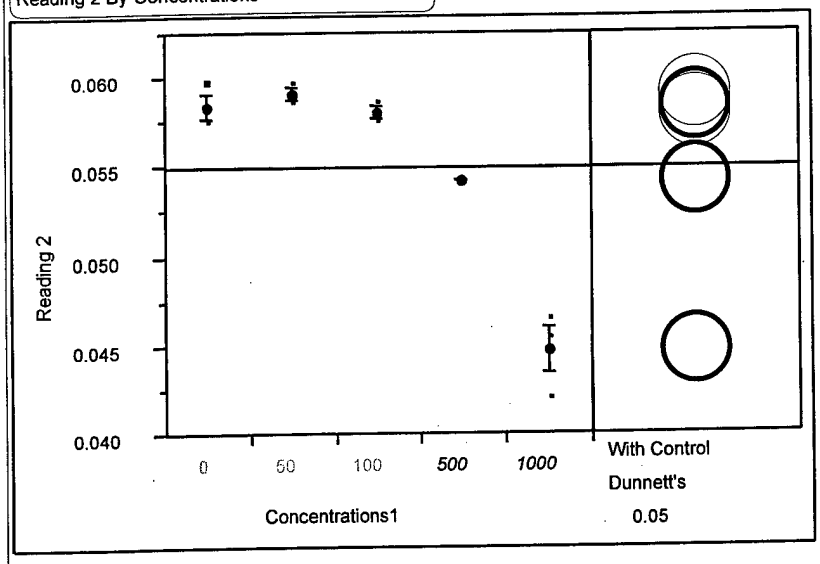
2.89051

Abs(Dif)-LSD	0
50	-0.00443
0	-0.00587
100	-0.00403
500	-0.00323
1000	0.002301

Positive values show pairs of means that are significantly different.

Dunnet Analysis of Venus, Reading 2

Reading 2 By Concentrations



Means Comparisons

Dif=Mean[i]-Mean[j]	50	0	100	500	1000
50	0.000000	0.000733	0.001100	0.004867	0.014433
0	-0.00073	0.000000	0.000367	0.004133	0.013700
100	-0.0011	-0.00037	0.000000	0.003767	0.013333
500	-0.00487	-0.00413	-0.00377	0.000000	0.009567
1000	-0.01443	-0.0137	-0.01333	-0.00957	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

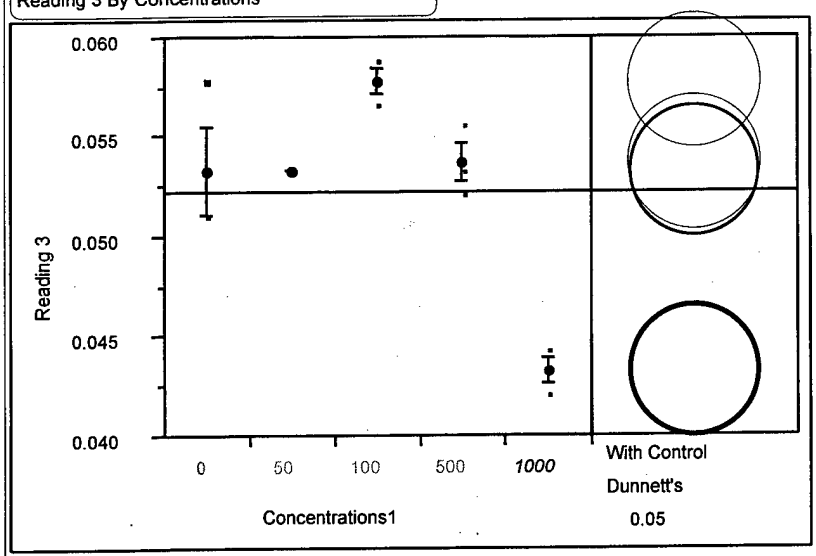
|d|
2.89051

Abs(Dif)-LSD	0
50	-0.00224
0	-0.00297
100	-0.0026
500	0.001162
1000	0.010729

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Venus, Reading 3

Reading 3 By Concentrations



Means Comparisons

Dif=Mean[i]-Mean[j]	100	500	0	50	1000
100	0.000000	0.004100	0.004467	0.004500	0.014500
500	-0.0041	0.000000	0.000367	0.000400	0.010400
0	-0.00447	-0.00037	0.000000	0.000033	0.010033
50	-0.0045	-0.0004	-0.00003	0.000000	0.010000
1000	-0.0145	-0.0104	-0.01003	-0.01	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

|d|

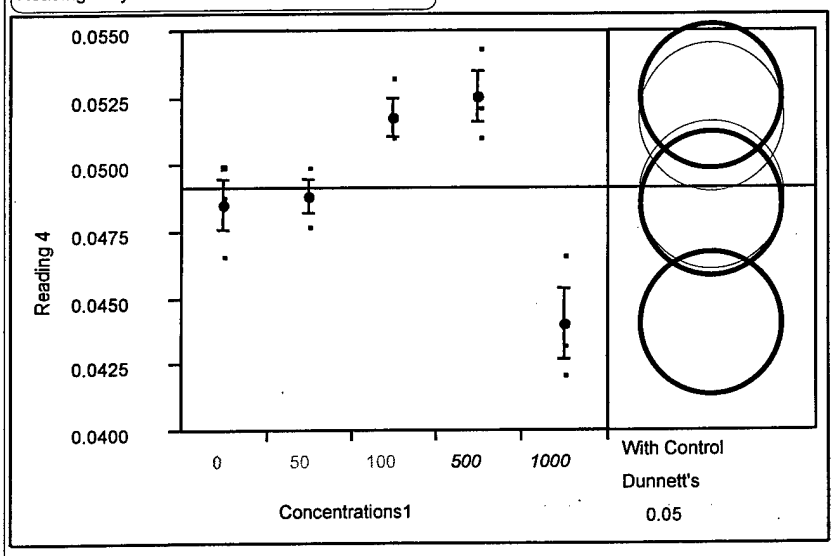
2.89051

Abs(Dif)-LSD	0
100	-0.0003
500	-0.0044
0	-0.00477
50	-0.00473
1000	0.005267

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Venus, Reading 4

Reading 4 By Concentrations



Means Comparisons

Dif=Mean[i]-Mean[j]	500	100	50	0	1000
500	0.000000	0.000733	0.003700	0.004033	0.008500
100	-0.00073	0.000000	0.002967	0.003300	0.007767
50	-0.0037	-0.00297	0.000000	0.000333	0.004800
0	-0.00403	-0.0033	-0.00033	0.000000	0.004467
1000	-0.0085	-0.00777	-0.0048	-0.00447	0.000000

Alpha= 0.05

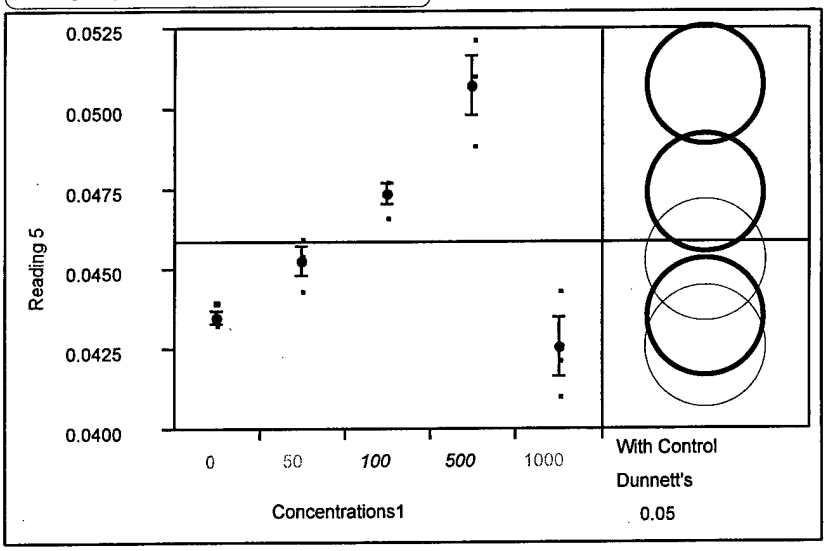
Comparisons with a control using Dunnett's Method

	|d
	2.89051
Abs(Dif)-LSD	0
500	0.000087
100	-0.00065
50	-0.00361
0	-0.00395
1000	0.000521

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Venus, Reading 5

Reading 5 By Concentrations1



Means Comparisons

Dif=Mean[i]-Mean[j]	500	100	50	0	1000
500	0.000000	0.003300	0.005433	0.007200	0.008167
100	-0.0033	0.000000	0.002133	0.003900	0.004867
50	-0.00543	-0.00213	0.000000	0.001767	0.002733
0	-0.0072	-0.0039	-0.00177	0.000000	0.000967
1000	-0.00817	-0.00487	-0.00273	-0.00097	0.000000

Alpha= 0.05

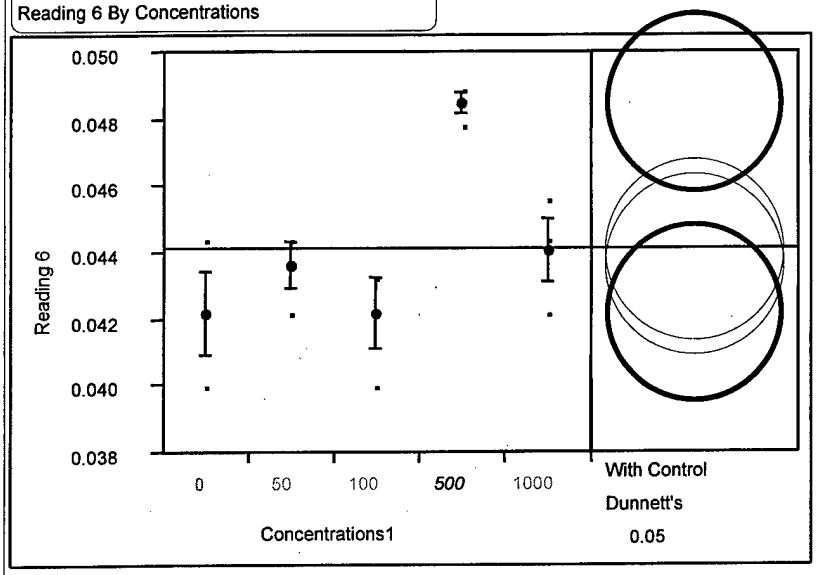
Comparisons with a control using Dunnett's Method

	|d|
	2.89051
Abs(Dif)-LSD	0
500	0.004431
100	0.001131
50	-0.001
0	-0.00277
1000	-0.0018

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Venus, Reading 6

Reading 6 By Concentrations



Means Comparisons

Dif=Mean[i]-Mean[j]	500	1000	50	0	100
500	0.000000	0.004467	0.004867	0.006333	0.006333
1000	-0.00447	0.000000	0.000400	0.001867	0.001867
50	-0.00487	-0.0004	0.000000	0.001467	0.001467
0	-0.00633	-0.00187	-0.00147	0.000000	0.000000
100	-0.00633	-0.00187	-0.00147	0.000000	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

|d|

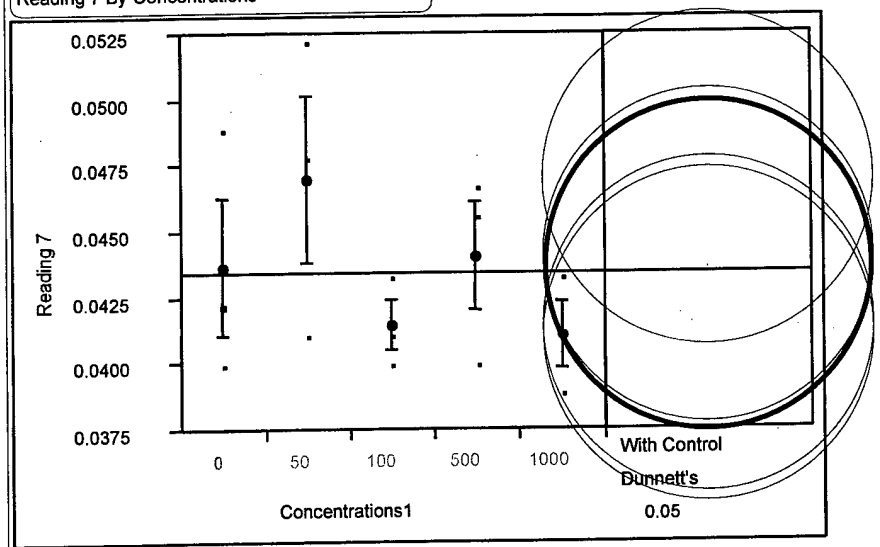
2.89051

Abs(Dif)-LSD	0
500	0.002461
1000	-0.00201
50	-0.00241
0	-0.00387
100	-0.00387

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Venus, Reading 7

Reading 7 By Concentrations



Means Comparisons

Dif=Mean[i]-Mean[j]	50	500	0	100	1000
50	0.000000	0.002937	0.003337	0.005570	0.005937
500	-0.00294	0.000000	0.000400	0.002633	0.003000
0	-0.00334	-0.0004	0.000000	0.002233	0.002600
100	-0.00557	-0.00263	-0.00223	0.000000	0.000367
1000	-0.00594	-0.003	-0.0026	-0.00037	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

|d|

2.89051

Abs(Dif)-LSD	0
50	-0.0057
500	-0.00863
0	-0.00903
100	-0.0068
1000	-0.00643

Positive values show pairs of means that are significantly different.

Appendix I: Statistical Analysis of Jupiter

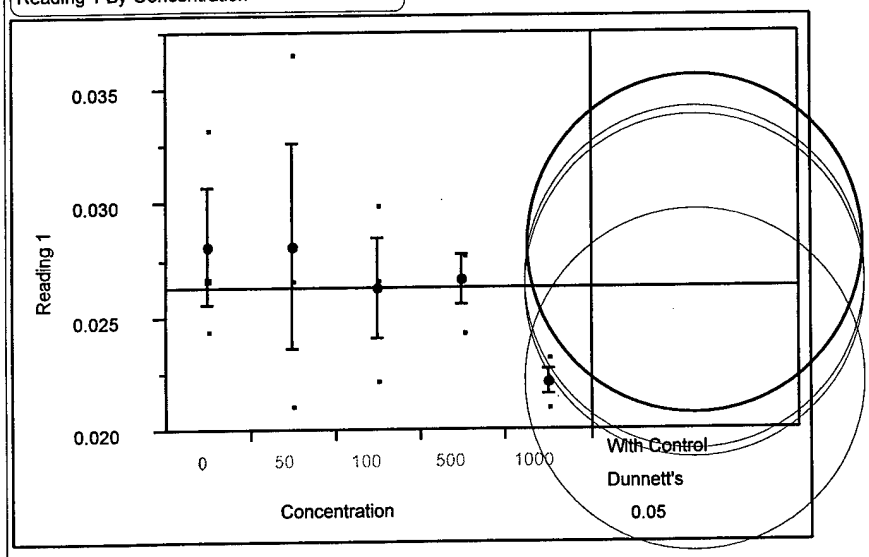
This appendix shows the statistical analysis of the Jupiter data. All data collected from each time period is plotted on a separate graph. The mean rate for each concentration was then calculated and plotted. The error bars shown on the graphs represent one standard error above and below the mean value.

The circles to the right of the graphs are comparison circles. The angle of intersection between the circles is an indication of statistical difference. If the angle is less then 90 degrees this is an indication that the means are significantly different.

Dunnett multiple comparison of the means were completed using an α of 0.05. A positive difference between the difference of the means and the LSD is an indication of statistical difference between the means. Further information on the Dunnett comparison can be found in Appendix E.

Dunnett Analysis of Jupiter, Reading 1

Reading 1 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	50	0	500	100	1000
50	0.000000	0.000011	0.001481	0.001852	0.005948
0	-0.00001	0.000000	0.001470	0.001841	0.005937
500	-0.00148	-0.00147	0.000000	0.000370	0.004467
100	-0.00185	-0.00184	-0.00037	0.000000	0.004096
1000	-0.00595	-0.00594	-0.00447	-0.0041	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

|d|

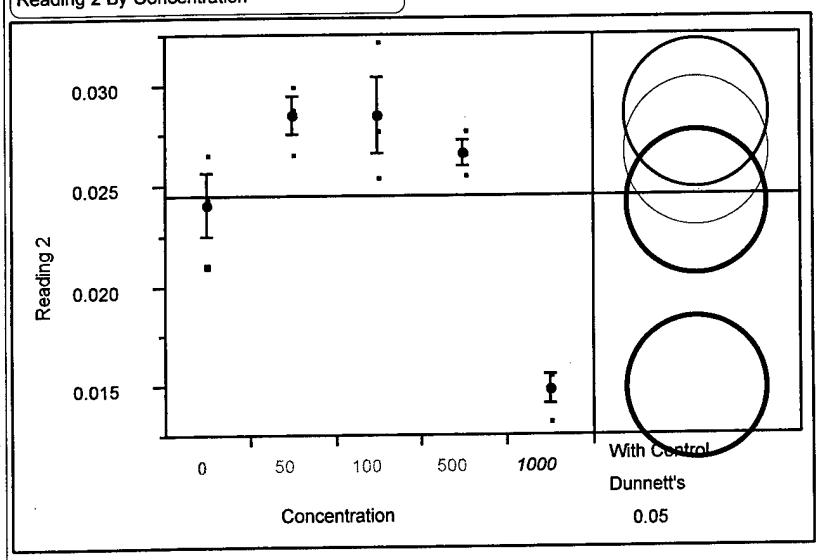
2.89051

Abs(Dif)-LSD	0
50	-0.01073
0	-0.01075
500	-0.00928
100	-0.00891
1000	-0.00481

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Jupiter, Reading 2

Reading 2 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	100	50	500	0	1000
100	0.000000	0.000000	0.001819	0.004433	0.013685
50	0.000000	0.000000	0.001819	0.004433	0.013685
500	-0.00182	-0.00182	0.000000	0.002615	0.011867
0	-0.00443	-0.00443	-0.00261	0.000000	0.009252
1000	-0.01369	-0.01369	-0.01187	-0.00925	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

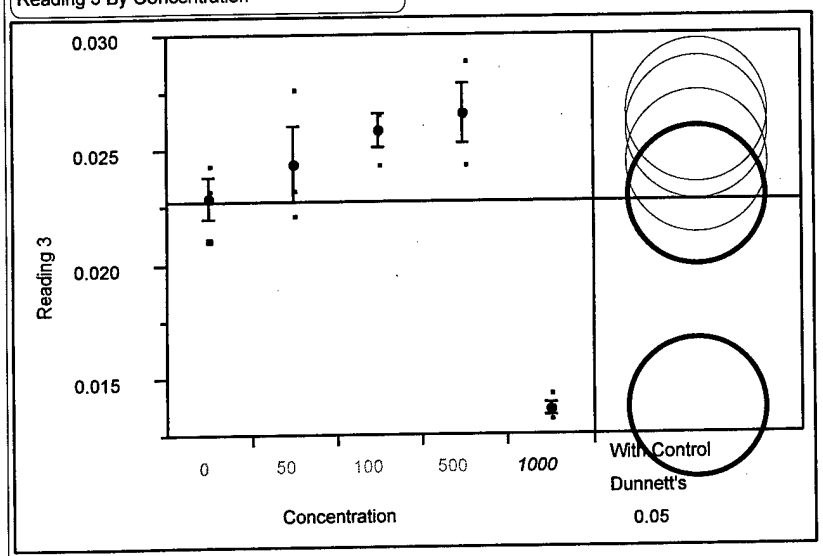
|d|
2.89051

Abs(Dif)-LSD	0
100	-0.00087
50	-0.00087
500	-0.00269
0	-0.00531
1000	0.003945

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Jupiter, Reading 3

Reading 3 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	100	50	0	1000
500	0.000000	0.000741	0.002222	0.003715	0.013000
100	-0.00074	0.000000	0.001481	0.002974	0.012259
50	-0.00222	-0.00148	0.000000	0.001493	0.010778
0	-0.00371	-0.00297	-0.00149	0.000000	0.009285
1000	-0.013	-0.01226	-0.01078	-0.00929	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

|d|

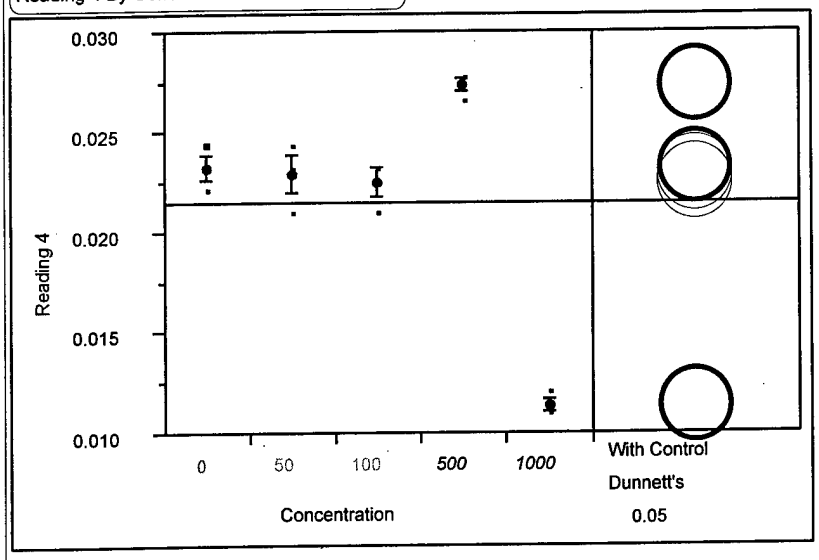
2.89051

Abs(Dif)-LSD	0
500	-0.00084
100	-0.00158
50	-0.00306
0	-0.00455
1000	0.004730

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Jupiter, Reading 4

Reading 4 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	0	50	100	1000
500	0.000000	0.004111	0.004470	0.004841	0.015967
0	-0.00411	0.000000	0.000359	0.000730	0.011856
50	-0.00447	-0.00036	0.000000	0.000370	0.011496
100	-0.00484	-0.00073	-0.00037	0.000000	0.011126
1000	-0.01597	-0.01186	-0.0115	-0.01113	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

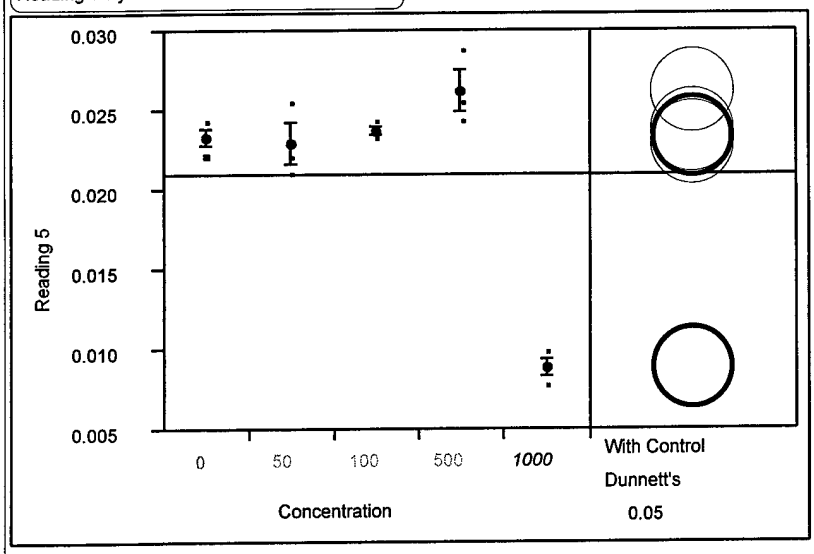
|d|
2.89051

Abs(Dif)-LSD	0
500	0.001406
0	-0.00271
50	-0.00235
100	-0.00198
1000	0.009151

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Jupiter, Reading 5

Reading 5 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	100	0	50	1000
500	0.000000	0.002596	0.002978	0.003337	0.017400
100	-0.0026	0.000000	0.000381	0.000741	0.014804
0	-0.00298	-0.00038	0.000000	0.000359	0.014422
50	-0.00334	-0.00074	-0.00036	0.000000	0.014063
1000	-0.0174	-0.0148	-0.01442	-0.01406	0.000000

Alpha= 0.05

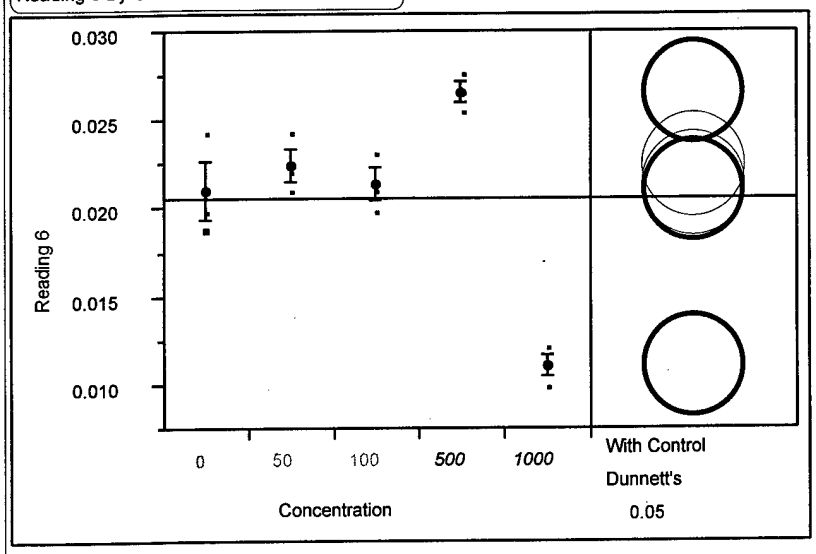
Comparisons with a control using Dunnett's Method

	|d
	2.89051
Abs(Dif)-LSD	0
500	-0.00092
100	-0.00352
0	-0.0039
50	-0.00354
1000	0.010525

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Jupiter, Reading 6

Reading 6 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	50	100	0	1000
500	0.000000	0.004107	0.005219	0.005589	0.015600
50	-0.00411	0.000000	0.001111	0.001481	0.011493
100	-0.00522	-0.00111	0.000000	0.000370	0.010381
0	-0.00559	-0.00148	-0.00037	0.000000	0.010011
1000	-0.0156	-0.01149	-0.01038	-0.01001	0.000000

Alpha= 0.05

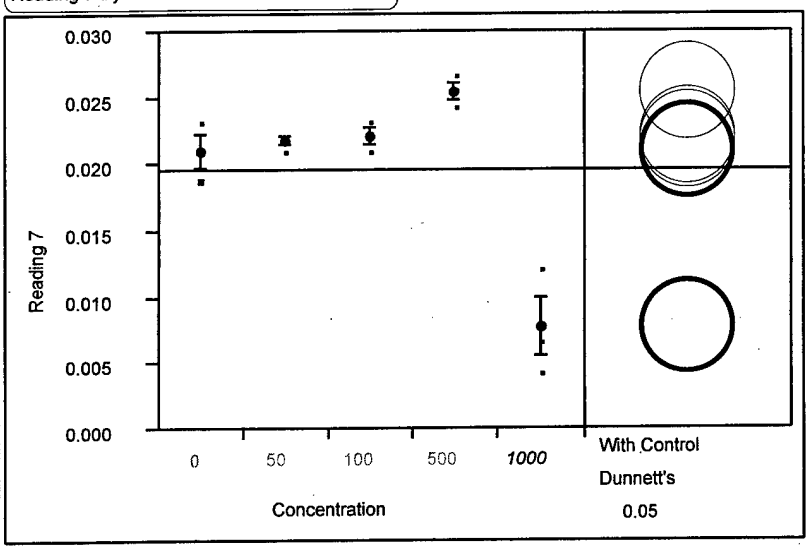
Comparisons with a control using Dunnett's Method

	|d|
	2.89051
Abs(Dif)-LSD	0
500	0.001260
50	-0.00285
100	-0.00396
0	-0.00433
1000	0.005682

Positive values show pairs of means that are significantly different.

Dunnett Analysis of Jupiter, Reading 7

Reading 7 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	100	50	0	1000
500	0.000000	0.003344	0.003715	0.004467	0.017800
100	-0.00334	0.000000	0.000370	0.001122	0.014456
50	-0.00371	-0.00037	0.000000	0.000752	0.014085
0	-0.00447	-0.00112	-0.00075	0.000000	0.013333
1000	-0.0178	-0.01446	-0.01409	-0.01333	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

	|d
	2.89051
Abs(Dif)-LSD	0
500	-0.00069
100	-0.00404
50	-0.00441
0	-0.00516
1000	0.008174

Positive values show pairs of means that are significantly different.

Appendix J: Statistical Analysis of *Bacillus*

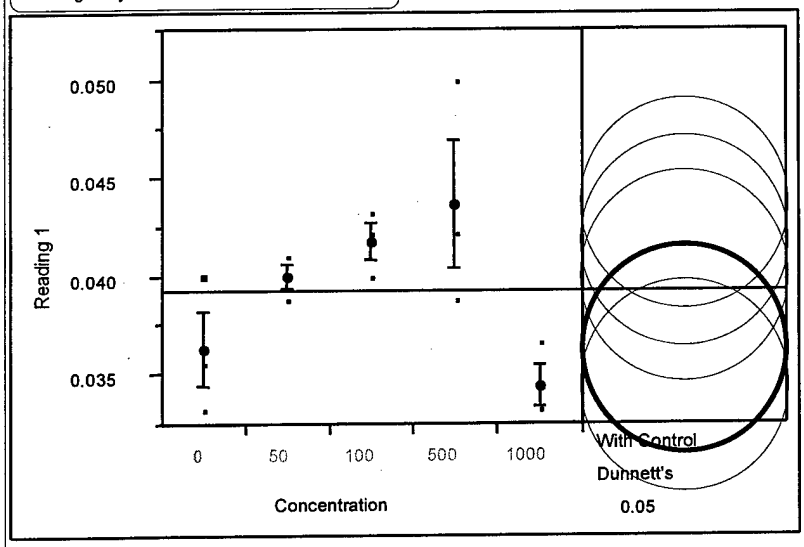
This appendix shows the statistical analysis of the *Bacillus* data. All data collected from each time period is plotted on a separate graph. The mean rate for each concentration was then calculated and plotted. The error bars shown on the graphs represent one standard error above and below the mean value.

The circles to the right of the graphs are comparison circles. The angle of intersection between the circles is an indication of statistical difference. If the angle does not exceed 90 degrees this is an indication that the means are significantly different.

Dunnett multiple comparison of the means were completed using an α of 0.05. A positive difference between the difference of the means and the LSD is an indication of statistical difference between the means. Further information on the Dunnett comparison can be found in Appendix E.

Dunnett Analysis of *Bacillus*, Reading 1

Reading 1 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	100	50	0	1000
500	0.000000	0.001867	0.003700	0.007400	0.009267
100	-0.00187	0.000000	0.001833	0.005533	0.007400
50	-0.0037	-0.00183	0.000000	0.003700	0.005567
0	-0.0074	-0.00553	-0.0037	0.000000	0.001867
1000	-0.00927	-0.0074	-0.00557	-0.00187	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

|d|

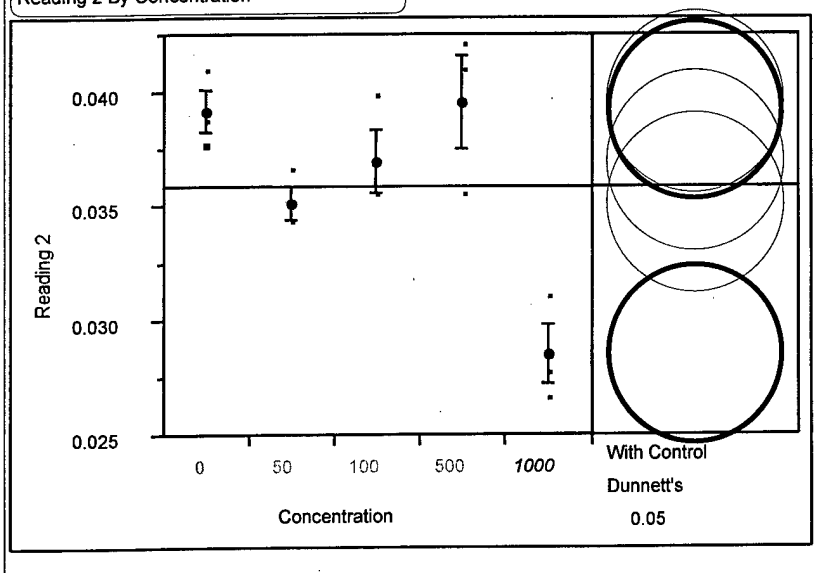
2.89051

Abs(Dif)-LSD	0
500	-0.00021
100	-0.00208
50	-0.00391
0	-0.00761
1000	-0.00574

Positive values show pairs of means that are significantly different.

Dunnett Analysis of *Bacillus*, Reading 2

Reading 2 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	0	100	50	1000
500	0.000000	0.000367	0.002567	0.004467	0.011100
0	-0.00037	0.000000	0.002200	0.004100	0.010733
100	-0.00257	-0.0022	0.000000	0.001900	0.008533
50	-0.00447	-0.0041	-0.0019	0.000000	0.006633
1000	-0.0111	-0.01073	-0.00853	-0.00663	0.000000

Alpha= 0.05

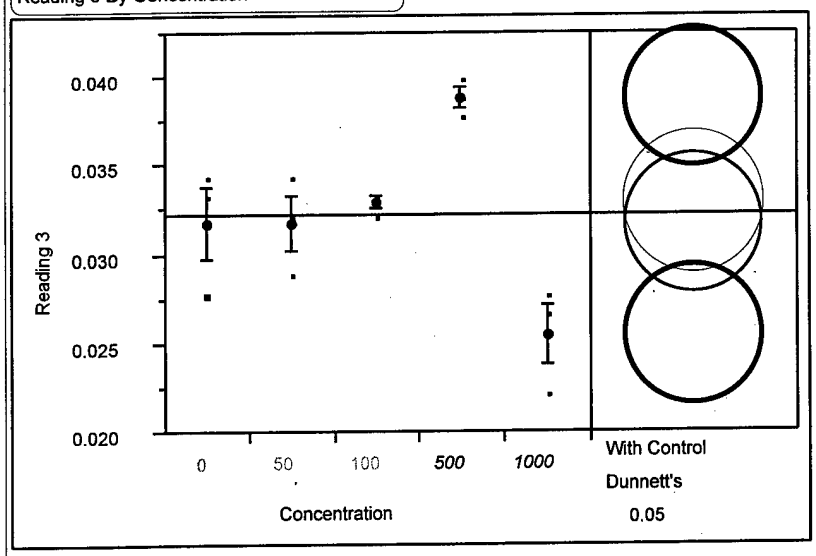
Comparisons with a control using Dunnett's Method

|d|	
2.89051	
Abs(Dif)-LSD	0
500	-0.0053
0	-0.00566
100	-0.00346
50	-0.00156
1000	0.005070

Positive values show pairs of means that are significantly different.

Dunnett Analysis of *Bacillus*, Reading 3

Reading 3 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	100	0	50	1000
500	0.000000	0.005967	0.007067	0.007067	0.013333
100	-0.00597	0.000000	0.001100	0.001100	0.007367
0	-0.00707	-0.0011	0.000000	0.000000	0.006267
50	-0.00707	-0.0011	0.000000	0.000000	0.006267
1000	-0.01333	-0.00737	-0.00627	-0.00627	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

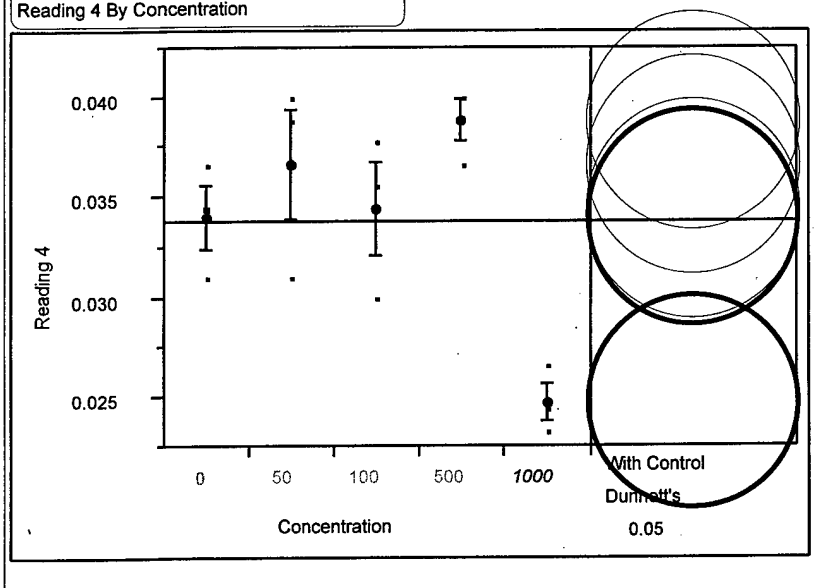
|d|
2.89051

Abs(Dif)-LSD	0
500	0.001230
100	-0.00474
0	-0.00584
50	-0.00584
1000	0.000430

Positive values show pairs of means that are significantly different.

Dunnett Analysis of *Bacillus*, Reading 4

Reading 4 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	50	100	0	1000
500	0.000000	0.002233	0.004433	0.004833	0.014100
50	-0.00223	0.000000	0.002200	0.002600	0.011867
100	-0.00443	-0.0022	0.000000	0.000400	0.009667
0	-0.00483	-0.0026	-0.0004	0.000000	0.009267
1000	-0.0141	-0.01187	-0.00967	-0.00927	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

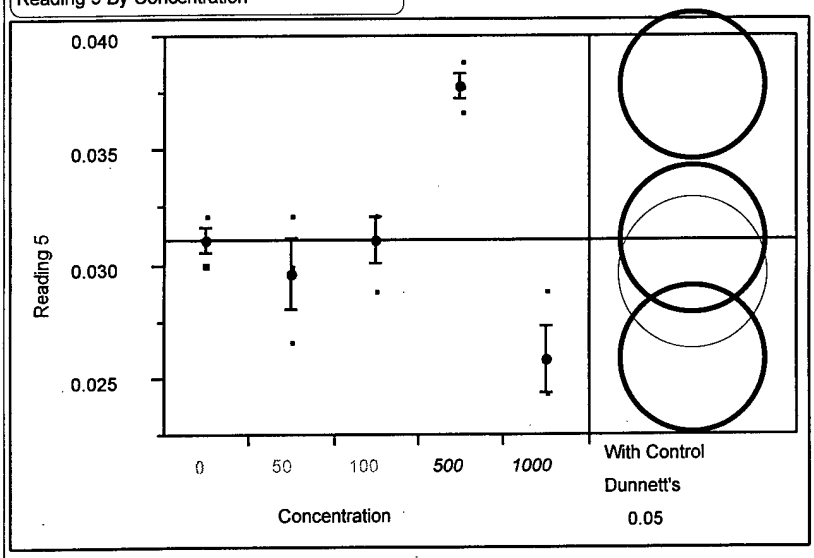
|d|
2.89051

Abs(Dif)-LSD	0
500	-0.00294
50	-0.00518
100	-0.00738
0	-0.00778
1000	0.001491

Positive values show pairs of means that are significantly different.

Dunnett Analysis of *Bacillus*, Reading 5

Reading 5 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	0	100	50	1000
500	0.000000	0.006700	0.006700	0.008167	0.011900
0	-0.0067	0.000000	0.000000	0.001467	0.005200
100	-0.0067	0.000000	0.000000	0.001467	0.005200
50	-0.00817	-0.00147	-0.00147	0.000000	0.003733
1000	-0.0119	-0.0052	-0.0052	-0.00373	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

|d|

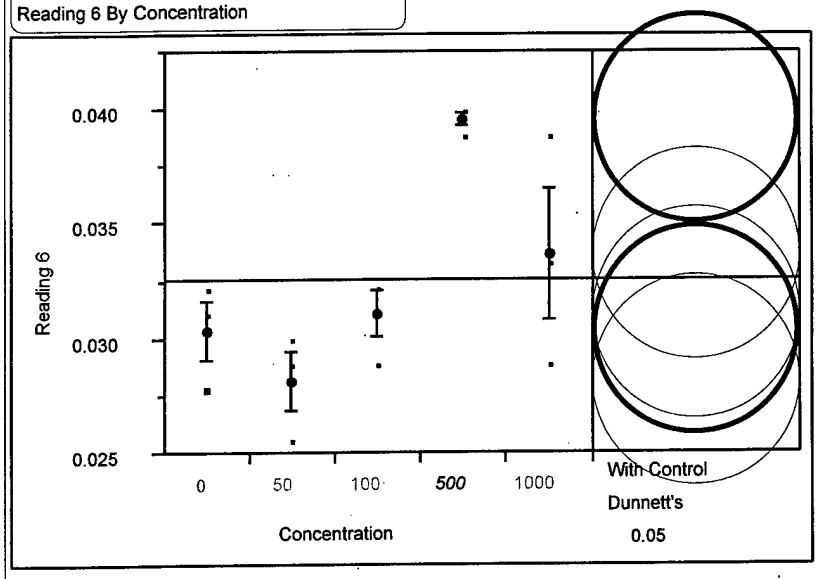
2.89051

Abs(Dif)-LSD	0
500	0.001925
0	-0.00477
100	-0.00477
50	-0.00331
1000	0.000425

Positive values show pairs of means that are significantly different.

Dunnett Analysis of *Bacillus*, Reading 6

Reading 6 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	1000	100	0	50
500	0.000000	0.005933	0.008533	0.009267	0.011467
1000	-0.00593	0.000000	0.002600	0.003333	0.005533
100	-0.00853	-0.0026	0.000000	0.000733	0.002933
0	-0.00927	-0.00333	-0.00073	0.000000	0.002200
50	-0.01147	-0.00553	-0.00293	-0.0022	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

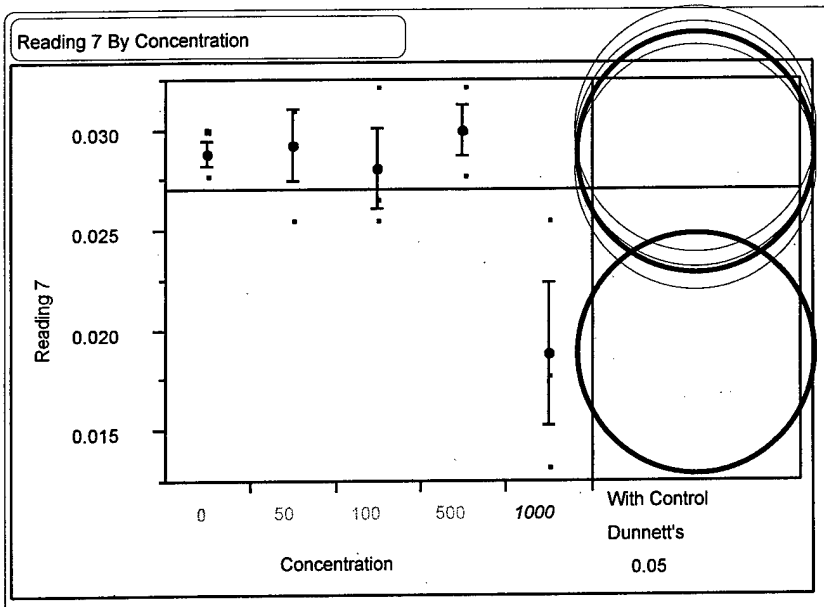
|d|
2.89051

Abs(Dif)-LSD	0
500	0.002621
1000	-0.00331
100	-0.00591
0	-0.00665
50	-0.00445

Positive values show pairs of means that are significantly different.

Dunnett Analysis of *Bacillus*, Reading 7

Reading 7 By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	50	0	100	1000
500	0.000000	0.000733	0.001100	0.001833	0.011100
50	-0.00073	0.000000	0.000367	0.001100	0.010367
0	-0.0011	-0.00037	0.000000	0.000733	0.010000
100	-0.00183	-0.0011	-0.00073	0.000000	0.009267
1000	-0.0111	-0.01037	-0.01	-0.00927	0.000000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

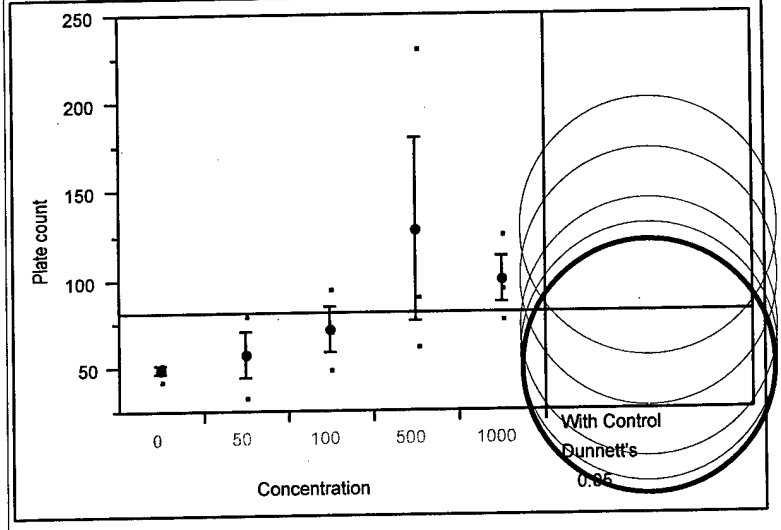
|d|	
2.89051	
Abs(Dif)-LSD	0
500	-0.00756
50	-0.0083
0	-0.00866
100	-0.00793
1000	0.001337

Positive values show pairs of means that are significantly different.

Appendix K: Microbial Plate Count Comparison of Jupiter

This appendix compares the results of pour plate colony counts of Jupiter. The innocolum was taken from the DO microcosms exposed to the varying concentrations of tolyltriazole. As seen by the Dunnett's analysis there is no statistical difference between the plate counts.

Plate count By Concentration



Means Comparisons

Dif=Mean[i]-Mean[j]	500	1000	100	50	0
500	0.0000	28.6667	56.3333	70.6667	79.3333
1000	-28.6667	0.0000	27.6667	42.0000	50.6667
100	-56.3333	-27.6667	0.0000	14.3333	23.0000
50	-70.6667	-42.0000	-14.3333	0.0000	8.6667
0	-79.3333	-50.6667	-23.0000	-8.6667	0.0000

Alpha= 0.05

Comparisons with a control using Dunnett's Method

|d|

2.89051

Abs(Dif)-LSD	0
500	-26.076
1000	-54.743
100	-82.409
50	-96.743
0	-105.409

Positive values show pairs of means that are significantly different.

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