5-2013

Biodiesel Transesterification Byproducts as Soil Amendments

Solomon Parker
University of Arkansas, Fayetteville

BIODIESEL TRANSESTERIFICATION BYPRODUCTS AS SOIL AMENDMENTS

BIODIESEL TRANSESTERIFICATION BYPRODUCTS AS SOIL AMENDMENTS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Civil Engineering

By

Solomon William Parker
University of Arkansas
Bachelor of Science in Civil Engineering, 2011

May 2013
University of Arkansas

ABSTRACT

For every ten kilograms of biodiesel that is produced from the transesterification of vegetable oil, approximately 1 kg of glycerol is produced as a byproduct. Also known as glycerin, it is a chemical used in many products including cosmetics, foods, and desiccants. However, the crude glycerol created during biodiesel production is tainted with potassium hydroxide and methanol making it unsuitable for commercial use without costly refinement. With increase in production of biodiesel driven by rising fuel prices, the market has become glutted with glycerol and it is on the threshold of becoming a waste product. Common methods for disposing glycerol include incineration which releases carbon dioxide into the atmosphere. A more carbon neutral option is land application where glycerol can increase soil organic matter and may sequester carbon. Possible problems involved with land application include its effects on plants, microbes, and broader biological systems.

The objectives of this research project were to evaluate the effects of methanol-stripped crude glycerol on microbial, plant, soil, and animal systems in relation to soils and the potential for runoff contamination as well as incidental contact with insects during land application. Four methods were used: microbial activity in soil measured with respirometry, plant germination and growth, runoff testing by test plot application, and analysis of medium to large insect mortality.

Respirometry showed that microbial action is not inhibitory at tested concentrations between 0.03% and 10%. In plant tests, a concentration of 0.3% showed greater growth over control samples and concentrations above 1% showed inhibitory effects. In land application runoff studies, glycerol showed similar amounts of total organic carbon in runoff to controls and less than plots fertilized with $NaNO_3$. Crickets treated with glycerol in a similar manner to field application showed higher mortality than control. In conclusion, the land application of glycerol

does have an effect on the soil fauna in and on the soil upon which it is applied. In moderation, this effect is minimal and this research has demonstrated upper limits to its usage (<1% by weight). Finally, care should be taken to assure that no endangered insects are harmed if land applied.

This thesis is approved for recommendation
to the Graduate Council

Thesis Director:

____
Dr. Thomas S. Soerens

Thesis Committee:

____
Dr. Andrew N. Sharpley

____
Dr. Brian E. Haggard

THESIS DUPLICATION RELEASE

I hereby authorize the University of Arkansas Libraries to duplicate this thesis when needed for research and/or scholarship.

AGREED ________________________________

Solomon William Parker

REFUSED ________________________________

Solomon William Parker

ACKNOWLEDGEMENTS

I would like to thank the Mack Blackwell Rural Transportation Center for funding this project. Thanks to the University of Arkansas Department of Civil Engineering for the use of their laboratory space, office space, computer labs, and other facilities.

Many thanks to Dr. Thomas Soerens for his work in support, devotion, and direction of not only this project but his mentorship of my college career. Thanks to Kelley Soerens for suggesting I enter the Civil Engineering program when I enrolled at the University of Arkansas. I would like to thank Mark Kuss of Challenge Technologies for the use of the respirometer and for assistance in setting up and troubleshooting the system. Thank you to Drs. Haggard and Sharpley for donating their time to this humbled graduate student. Thanks to my team leaders at Garver for graciously allowing me to work on this thesis as much as I needed.

Finally, I want to extend my eternal gratitude to my best friend and partner in life and marriage Lisa Parker. Without her support, I would most certainly not be here today succeeding in the world of higher education. She has worked hard and supported me throughout my college career, helping with homework, raising children, and working from home while I attended classes. Words cannot describe how much I owe her.

TABLE OF CONTENTS

I. INTRODUCTION

BACKGROUND

Biodiesel is a carbon (C) neutral biofuel often made from waste vegetable oil sourced from the restaurant industry or increasingly from other sources of waste or process byproducts rich in lipids. Biodiesel is created through the transesterification of lipids such as vegetable oil or animal fat with an alcohol such as methanol and in the presence of a base such as potassium hydroxide (KOH). Transesterification involves initiating a reaction between an alcohol and an ester that results in a new alcohol and a new ester. The organic group of the alcohol is exchanged with the organic group of the ester. Biodiesel is the new ester (linoleic acid methyl ester) and glycerol is the new alcohol (glycyl alcohol).

Glycerol is also known as glycerin, glycerine (UK), and trihydroxypropane. Pure, it is a clear odorless colorless viscous liquid, but as a product of biodiesel production, it is a brown viscous liquid with an odor quite similar to the vegetable oil from which it is derived. It has the molecular formula $C_3H_8O_3$ and is very soluble in water as well as being hygroscopic meaning it attracts and absorbs water from the air. Figure 1-1 shows two models of a glycerol molecule. Its basic form is that of a propane molecule with three hydrogen atoms replaced with hydroxyl groups. As is characteristic of alcohols, hydroxyl groups allow the molecule to be miscible with water. It is poorly soluble with acetone and ether and almost insoluble with fats and volatile oils (MSDS 2012). During the transesterification process, glycerol sinks to the bottom of the container and is thus easily separated.

Crude glycerol is primarily glycerol but it contains measurable amounts of the alcohol used in transesterification and the base used as a catalyst. It also typically contains remnants of biodiesel, vegetable oil, and water. The production of one metric ton of biodiesel creates approximately 100 kg of waste glycerol (Alotaibi and Schoenau, 2011).

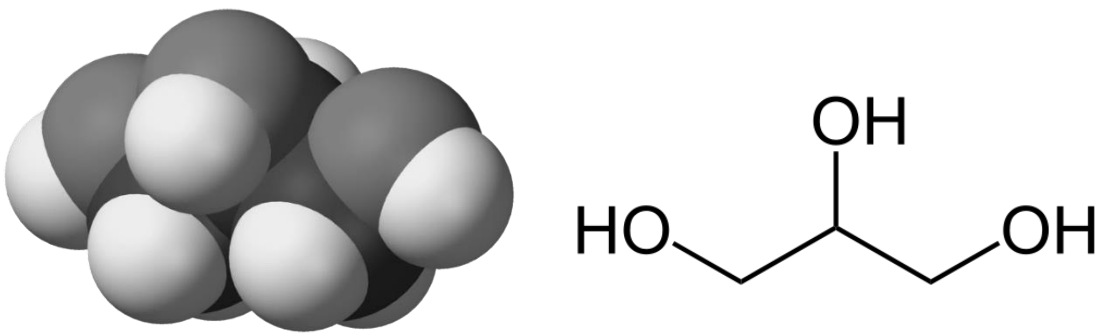


Figure 1-1. Chemical structure of glycerol ($C_3H_8O_3$).

The toxicity of glycerol is based on the concentration of methanol in the mixture. Methanol will evaporate into the atmosphere in approximately one week if the container is left open. Heating the product will increase the evaporation rate as well. After the methanol has been stripped, the resulting crude glycerol is generally considered non-toxic and biodegradable (Tickell, 2003). It is a skin and eye irritant with no known carcinogenic effects on animals or humans and is non-hazardous if ingested (EMD Chemicals Inc., 2004). It is commonly used as a sweetener or thickener in food or hygiene products respectively. Research is on the horizon exploring the use of glycerol as an alternative to anti-freeze (Shay, 2007).

Glycerol can be incinerated. However, if temperatures are not maintained beyond 1000° C, toxic acrolein fumes (commonly recognized as the smell of burnt fat) are emitted (Shay, 2007). Incineration also releases carbon dioxide (CO_2) into the atmosphere and moderates some of the CO_2 reduction biodiesel offers as a biofuel however glycerol does replace other fuels used for incineration.

Glycerol has many other uses such as an animal food supplement which has been recently researched (Groesbeck et al., 2008). There is also its aforementioned utilization in makeup, foods, and hygiene products. The demand for these uses is small compared to the increasing volume being produced in the burgeoning biofuels industry (Qian et al. 2011). Biodiesel production has modified the marketplace to the extent that Dow Chemical Company has closed the one remaining plant that produced synthetic glycerol in the United States (Crooks 2007). In 2002, it was valued at $0.103/L (Coltrain, 2002). In 2007, it sold for $0.005/L (Shay 2007). With increasing demand for biodiesel, the disposal of waste glycerol may become a substantial portion of the cost for biodiesel production which would be passed on to the purchase price of biodiesel itself (Klepàčovà et al., 2007). The cost of disposal of glycerol was estimated by Sadano et al. (2012) to be $0.067/L of biodiesel fuel produced.

It is in this market environment that the concept of disposing glycerol as a soil amendment finds its genesis. To date, the idea has not received much study. Cayuela et al. (2010) showed that glycerol as a soil amendment has implications for C sequestration and greenhouse gas emissions. They also stated that glycerol may actually show benefits for soils because it can raise the organic content of soil. However, little is yet known about the toxicity. It is generally assumed to be nontoxic due to its source in natural oils and its use in foods, but there are a limited number of studies on microbial, biological, plant and soil systems. Glycerol acts as a cryoprotectant protecting proteins from freezing damage so it finds some use as a preservative in laboratory studies (Ayala et al., 2011). Glycerol is also used as a low molecular weight plasticizer (Roh et al., 2012).

Schoenau (2009) found that glycerol was effective in increasing soil organic content but it required supplemental fertilizer due to nitrogen and phosphorus (P) tied up by microorganisms during decomposition in the soil. The immobilization of P may be of benefit in watersheds suffering from excessive P runoff due to land application of poultry litter. However, microbial uptake of P would not be a permanent sink.

PROBLEM STATEMENT

With increasing production of biodiesel and other biofuels, there is an increasing need for safe and ecological disposal of the waste products. If production of those waste products outpaces their ability to be utilized by other branches of industry, it will be necessary to dispose of them in a safe and ecologically sound way. The land application of waste glycerol may be a favorable disposal method compared to incineration which releases CO_2 and other pollutants into the atmosphere. It is therefore necessary to determine if land application of this waste product is a viable solution to its overabundance.

STUDY SITE DESCRIPTION

Thirty five small runoff test plots exist at the University of Arkansas Physiology and Parasitology Farm located east of Fayetteville Arkansas. These plots were created in 2007 and initially used for studies relating to the land application of poultry litter within the Agriculture Experiment Station. The plots have lain fallow for approximately five years. The area including the plots is characterized primarily by grazed farmland typical of the northwest Arkansas region. Grasses include fescue and Bermuda. Other plants include but are not limited to purple vetch, plumeless thistle, milkthistle, Carolina horsenettle, silverleaf nightshade, johnsongrass, and cocklebur. The ground surface slopes to the north at approximately 5%. The soil is characterized as Captina Silt Loam by the Natural Resources Conservation Service. The plots were created by driving aluminum edging into the ground without disturbing the vegetation to isolate runoff and simulate the pastures of the region.

OBJECTIVES

The overall objective of this research was to determine if land application of glycerol is a viable and acceptable alternative to incineration. Glycerol has not been studied much as a C containing soil amendment (Alotaibi and Shoenau, 2011). As an acceptable alternative, glycerol would not create stressful conditions on the soil system and its animals and cause detrimental runoff qualities. If results of testing demonstrate that glycerol is a detrimental soil additive it will be concluded that it should not be used as a soil amendment.

This research examined the effects of the application of glycerol on microbial oxygen utilization, plant germination and growth, and the Total Organic Carbon (TOC) found in runoff water, and the immediate toxicity to medium and large bodied insects using crickets as a test subject.

The specific objectives were the following:

Objective 1: A microbial respiration test was used to evaluate toxicity to microorganisms at 0.03% to 10% glycerol by weight.

Objective 2: A plant germination and growth test was conducted to evaluate the effects of germination and growth at concentrations between 0.01% and 10% glycerol by weight.

Objective 3: Runoff tests were performed in the field to compare the differences in TOC in collected runoff water samples between blank controls, fertilized controls, glycerol and fertilized glycerol applications.

Objective 4: Crickets were used as an analog for various medium/large insects and arachnids in the field to test contact lethality for the same applications as the runoff testing.

If waste glycerol from the production of biodiesel is found not to be toxic when used as a soil amendment, it should also be able to be used in dust control, replanting, and landscaping. As well as disposing of glycerol in a more ecologically sound and climate change responsible way, it could also demonstrate beneficial effects in soils and limit the need for other soil amendments. If glycerol is shown to be benign or even advantageous, guidelines for application rates can be created based on the outcomes of this research.

RESEARCH HYPOTHESES

Null Hypothesis One: There is no difference in oxygen uptake in respirometry tests between samples mixed with glycerol and control samples.

Alternative Hypothesis One: In samples mixed with glycerol, oxygen uptake in respirometry tests will increase or decrease in comparison to control samples.

Null Hypothesis Two: The addition of glycerol has no effect on germination or growth of tall fescue grass seeds compared to controls.

Alternative Hypothesis Two: The addition of glycerol will increase or decrease the germination and growth of tall fescue grass seeds compared to grass seeds germinated and grown without glycerol.

Null Hypothesis Three: There is no resulting difference in the measurable TOC in runoff from a plot having received an application of glycerol to an identical plot having not received the same application

Alternative Hypothesis Three: The application of glycerol increases or decreases the measureable TOC in the runoff of a plot applied with glycerol compared to a control.

Null Hypothesis Four: There will be no difference in mortality between house crickets sprayed with a glycerol mixture compared with those sprayed only with water.

Alternative Hypothesis Four: Mortality will be increased among crickets sprayed with a glycerol mixture compared to a control.

II. LITERATURE REVIEW

BIOFUEL WASTE PRODUCTS AS SOIL AMENDMENTS

Glycerol and Similarly Sourced Products: There are several studies which evaluated a broad selection of biofuel byproducts and other byproducts like those from brewing. Schoenau et al. (2009) studied several biofuel, crop and animal processing byproducts including dry and wet distiller's grains, dehydrated alfalfa, thin stillage, and glycerol as soil amendments. They established that glycerol effectively increased soil organic content but required supplemental fertilizer to compensate for the tie up of nutrients by microorganisms in the process of respiration and decomposition in the soil. Neither glycerol nor any of the other byproducts tested had significant biological effects. At the application rates in the study, glycerol did not affect chemical soil parameters measured including pH, salinity, or soluble metals. The authors of this study noted that "glycerin addition may be of greatest benefit in increasing soil organic carbon content and carbon sequestration, compared to the alternative of incinerating the glycerin" (Shoenau et. al, 2009). They found beneficial effects of applications as high as 10,000 kg/ha (1% by weight, assuming a soil depth of 10 cm), however that rate would require excessive amounts of fertilizer to compensate for nutrient tie-up. Shoenau et al. (2009) suggested an application rate of approximately 1000 kg/ha (0.1% by weight) would be appropriate. Because of the tendency of glycerol to tie up nutrients in the soil, it may be beneficial in certain watersheds which are sensitive to loss of phosphorus (P) from the landscape.

Cayuela et al. (2010) added ten different amendments to soils (manure digestates, rapeseed meal, distilled dried grains with solubles, non-fermentables from hydrolysis of different lignocellulosic materials, and biochars) and investigated soil carbon (C) and nitrogen (N) cycling. It was found that biofuel byproducts as soil amendments contain large amounts of readily degradable C leading to short term N immobilization limiting their prospective use as fertilizers. Cayuela et al. (2010) also suggested that these products should be utilized in a way that allows them to degrade somewhat to maintain biological activity and nutrient cycling but still maintain persistence in the soil.

Qian et al. (2011) in a study closely related to the Shoenau et al. study set up some controlled environment experiments to test the effects of soil amendment with thin stillage (a byproduct of ethanol production) and glycerol in Saskatchewan, Canada. As a soil amendment, glycerol only contains C, hydrogen (H), and oxygen (O) and was effective in increasing the organic C content of the soil. Neither amendment tested showed effects due to pH, salinity, or soluble metals considered biologically significant. Thin stillage was found to be an effective fertilizer however not as effective as urea due to the fact that the N in the stillage was not biologically available within the five week testing period; however stillage is able to provide additional nutrients such as P. Glycerol was found to need supplemental fertilizer to compensate for the tie up of nutrients in the soil by microorganisms during the decomposition process.

Gell et al. (2010) evaluated crop phytotoxicity by testing 15 bioenergy rest-products from common bioenergy chains including biogas, biodiesel, bioethanol, and pyrolysis. They were mixed in sandy soil and used to evaluate the seedling root and shoot elongation of lettuce, radish, and wheat. Phytotoxic effects were noted immediately with biodiesel and bioethanol rest-products, however the effect was no longer significant after a week. The addition of biochar (pyrolysis end product) alleviated phytotoxic effects of bioethanol byproducts for radishes and wheat.

Other Biodegradable Waste Products: Other industrial waste products can be used as soil amendments. Hall (2010) studied soy based foam insulation as a soil amendment. He examined toxicity in activated sludge systems, earthworm populations, and plant environments. Hall (2010) found that it was difficult to work with the foam due to its very low density causing it not to maintain homogenous mixtures in stirred soil or activated sludge for respirometry so he proposed an alternate method. Hall's control results were highly precise, but the other studies suffered from poor repeatability due to the physical effects of foam chunks.

Dror et al. (2000) examined the effects of soil amendments including sewage sludge on the dynamics of kerosene attenuation on field plots. The plots were then leached using sprinkler irrigation. The tests lasted 100 days. The researchers discovered that soil amendments may enhance the rate of kerosene degradation and reduce the residual amount as compared to untreated soil.

A variety of bioenergy byproducts were tested by Galvez et al. (2011). They tested anaerobic digestate, rapeseed meal (also known as canola, rapeseed is a major source of vegetable oil around the word), bioethanol residue, and biochar, as well as sewage sludge and two composts. They found that "soil amendment led to a general increase in soil respiration, available N and P and microbial content and activity." The group found that rapeseed meal as well as the bioethanol byproduct led to the greatest increases in soil respiration as well as N_2O emissions. They found that enzymatic activity and respiration were higher in a slightly acidic soil while greater P availability was to be found in alkaline soil. In comparison to the control, biochar did not lead to any significant variations excluding promotion of C uptake.

Alotaibi and Schoenau (2011) examined effects of application of biofuel industry byproducts, measuring soil enzymatic activity, microbial biomass C and N content as well as microbial quotient. They used wet distiller's grains, thin stillage, and glycerol. Each of these items were added at three rates. Glycerol was added at rates of 40, 400, and 4000 kg C/ha alone and with the addition of kg N/ha as urea. They found that glycerol with N included enhanced alkaline phosphatase activity. All of the substances they used increased dehydrogenase activity but variably. Overall, Alotaibi and Shoenau (2011) noted that all of the biofuel industry byproducts increased enzyme activity and stimulated microbial growth "supporting their potential use as soil amendments to recycle plant nutrients and enhance soil biological activity."

VALUE ADDED REPURPOSING OF GLYCEROL

Pesticide: In a study by Chung et al. (2005) glycerol was used as an ingredient for a granulated biofungicide to control *Rhizoctonia solani* colonization in soil to prevent damping-off disease of Chinese cabbage. Germination of the cabbage was not negatively affected by the presence of glycerol in the growing medium. The mixture also stimulated the proliferation of actinomycetes. The study showed that the biofungicide developed was an effective method for control of damping-off of Chinese cabbage if applied at 1% with *Streptomyces padanus* prior to sowing (Chung et al. 2005).

Siddiqui and Shaukat (2002) examined the effects of zinc (Zn) and glycerol individually or in concert to improve biocontrolling activity of indigenous and non-native bacteria, namely *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. They came to the conclusion that both Zn and glycerol together and separately increased efficacy against root knot nematodes as well as improved tomato plant growth and bacterial rhizosphere colonization. Glucose alone was found to inhibit nematicidal activity of the bacteria.

Rod Rodriguez-Kabana of Auburn University has developed and patented a glycerol based product that is injected into soil to control weeds and crop destroying nematodes (AAES, 2008). The product is meant to replace methyl bromide which is now banned. Its primary ingredient is crude glycerol and may be applied with fertilizer. He suggests it could be utilized in organic farming and expects it to be widely available in a few years.

Anaerobic Digestion: In 2006, Winters (2007), reported that Gavin Reynolds of Celtic Power & Machining in Manitoba Canada received a grant from the Agri-Food Research and Development Initiative to explore the possibilities of using glycerol to make the anaerobic digestion of animal manure quicker and more efficient. He used a 1500 L stainless steel tank filled with hog manure. He found that using glycerol in the procedure improved the quality of the biogas produced by the reactor and sped up the digestion process. "We know that glycerin acts as a fuel for the microbes, offering faster, more complete digestion. It appears to be improving the quality of the gas coming out of the digester." He used the resulting methane rich biogas to replace about 96% of the diesel fuel needed to power a 150 kW generator (Winters, 2007). A small quantity of diesel is still required to provide a spark in compression ignition engines.

In a study done at the University of Cordoba in Spain, López et al. (2009) experimented with biodegradation in anaerobic laboratory-scale reactors with granular and non-granular sludges. The researchers found that the glycerol had a carbonaceous oxygen demand (COD) of 1010 g/kg. They used phosphoric acid (H_3PO_4) to recover the potassium hydroxide (KOH) catalyst as potassium phosphate ($NaPO_4$) fertilizer. They did not use crude glycerol as a control but instead distilled it to be used as a reference substrate. The reactors produced methane at a rate of 0.306 m^3/kg of acidified glycerol. They also noted an inhibition phenomenon at the highest loading of 0.38 g COD/g VSS (volatile suspended solids).

Amon et al. (2006) explored biogas production from anaerobically digested manures from several sources including dairy cows and pigs. The writers claimed that "specific parameters on the anaerobic digestibility of animal manures are unavailable which restricts the exploitation of the promising potentials" of the generation of renewable energy. They found that by supplementing pig manure and maize silage with 6% glycerol, they could increase production of methane from 569 to 679 L CH_4/(kgVS).

Composting: In a study searching for an economical method of disposal of glycerol, Sadano et al. (2012) explored the use of compost using repeated batch fermentation. The researchers used a mixture of 625 g dry sawdust, 25 g of microbial seed, 50 g of glycerol, and 0.5 g of urea. As batches were repeated, pH, N, and water contents were maintained at adequate levels for growth of the compost microbes, however oily compounds accumulated in the compost and the consumption of glycerol gradually decreased. The important finding was that even when accounting for the very high cost of purchasing the sawdust, the price for disposal of the glycerol decreased about 11% per volume of biodiesel fuel produced. They concluded that "there might be an economical advantage to compost fermentation of glycerol by-product (sic) from [biodiesel fuel] production."

Other Uses: Roh et al. (2012) explored the use of glycerol along with their main area of study, using chicken feather fibers as an ingredient in bio-plastic pellets. To measure suitability, they used low-temperature scanning electron microscopy and X-ray diffraction analysis as well as measuring the growth and flowering of *Begonia boliviensis* 'Bonfire' when grown in soil containing pellets. They found that pellets containing sodium sulfite ($NaSO_3$) and >30%

glycerol were unsuitable and not recommended for future studies. Similarly, they also found that pellets containing glycerol at 10% were unsuitable as an amendment in commercial growing media. They theorized that the release of glycerol from some of the pellets was the cause of poor growth in the begonias; however, the growth of wheat grass grown in media spiked with glycerol was better than without.

With increasing production of glycerol new ways of using it profitably will be needed. Johnson and Taconi (2007) explored a wide variety options of value-added products to be created from glycerol or using it as a feedstock for other processes. In this paper, the authors explore costs and revenues that can be reaped from using glycerol for fuel, for feed, for catalytic conversion, and for biological conversion to more valuable commodities and products. They explored the possibilities for glycerol to be converted to dozens of valuable chemicals and concluded that "the production of value-added chemicals from glycerol will play an important role in the advancement of the integrated biorefinery concept for fuel and chemical production."

ANIMALS, PLANTS AND MICROBES AS BIOASSAYS

Plant Growth: Bulluck et al. (2002) conducted field experiments to test the differences on soil microbial communities and soil physical and chemical properties of organic and synthetic soil fertility amendments on three organic and three conventional vegetable farms in Virginia and Maryland. The organic soil amendments were composted cotton-gin trash, yard waste, and cattle manure and the synthetic soil amendment was fertilizer. Thermophilic organisms and enteric bacteria were found at higher levels in soils amended with the alternative amendments and Phytophthora and Pythium species were lower in soils with the same treatments. First year yields of corn and melons were not dissimilar between the amendments at most of the farms. In the second year, all growers planted tomatoes and yields were higher on organic farms versus conventional farms regardless of the type of soil amendment. The researchers found that "alternative fertility amendments enhanced beneficial soil microorganisms, reduced pathogen populations, increased soil organic matter, total C, and cation exchange capacity, and lowered bulk density thus improving soil quality."

Wee and Obbard (2010) studied the effect of spiking compost with glycerol at rates of 0%, 10%, 20%, 30%, and 40%. These mixtures were then mixed with topsoil at a ratio of 1:2. Topsoil without compost was also included in the test. Wheat grass was grown in the samples and for eight days, growth was measured in terms of shoot height. On day eight, biomass was harvested and measured. The results showed that the best growth was to be found in samples that contained compost with no glycerol and also samples that contained compost at 10% and that all composted mixtures performed better than topsoil alone, however mixtures containing compost spiked with 40% glycerol showed an inhibitory effect on growth.

Crickets and Other Insects: Schleier and Peterson (2010) used house crickets to conduct a field bioassay for aerosol applications of insecticides used to kill adult mosquitoes implicated in the spread of West Nile Virus in North America. Ultra-low-volume aerosol sprays are used to control mosquitoes and thereby limit the transmission rate of the disease. They tested several chemicals including technical grade permethrin and naled (organophosphate insecticide). They used crickets in these tests to demonstrate that the use of these chemicals would not likely result in population impacts in medium to large bodied insects.

Walton (1983) explains how using crickets in bioassays should be a good method for testing teratogens (substances which cause birth defects or other malformations of embryos) because cricket embryos develop gross morphological abnormalities when exposed to a number of intricate organic mixtures. They also display a critical period of sensitivity to teratogens and the capability to metabolize xenobiotics during the course of development. The assay is simple, economical, impartial, and requires less than two weeks. Walton wrote “further investigation of cricket embryo responses to known teratogens is needed to establish the predictive value of this assay” (1983).

Crickets are also tested not just for survival, but growth and effects on egg production. Zhang et al. (2006) tested effects of two major hexahydro-1,3,5-trinitro-1,3,5-triazine metabolites on survival, growth, and reproduction on *Acheta domesticus*. They noted effects in egg hatching and viability as well as effects based on the developmental stage of the crickets and exposure time. They did not notice gross abnormalities in cricket nymphs and concluded that neither of the chemicals they were testing was teratogenic in the assay.

Wild cricket populations are also studied to ascertain the effects of chemicals. Hoffman et al. (2002) studied crickets affected by the deposition of sulfur dioxide (SO_2) caused by mining operations in northern Australia. They found that SO_2 affected the cricket populations but mostly in the high sulfur zone. They found that a primary correlative factor in the fitting of environmental variables was the soil SO_4 content. They concluded “crickets are sensitive to SO_2 emissions and they appear to be a good indicator group in this context.”

Lauritz Sømme explored the contents of glycerol in the bodies of ten species of insects in 1964. Evidence was gathered which demonstrated varying amounts of glycerol to be found in the bodies of insects at varying temperatures. It was found that in several species, cold hardiness was increased by the increased presence of glycerol. The amount of glycerol increased with decreasing temperatures. However, it was found that glycerol by itself cannot protect against freezing injuries in the bodies of the insects studied (Sømme 1964).

Respirometry: Respirometry is commonly used to measure a variety of parameters in a variety of settings. Chief among these is O_2 uptake which is can be a measure of microbial action in the sample. Iannotti et al. (1993) measured the stability and maturity of compost created from municipal solid waste at a full scale composting facility. They used both O_2 and CO_2 respirometry to discover process control problems and to demonstrate increasing stability with time. In concert with respirometry, they used plant growth bioassays to show inhibition based on immaturity of the compost. They found inhibition of germination in compost of all maturity levels which they hypothesized was a result of salt-related phytotoxicity. They concluded that O_2 respirometry best predicted the potential for their growth indicator species and was able to determine an acceptable level of compost maturity.

Muusze et al. (1998) used respirometry to study the effects of changing O_2 content in water on the Amazon fish *Astronotus ocellatus*. Because of large fluctuations in rainfall and flood pulses, the water experiences wide variations in O_2 content. They discovered that the cichlid could withstand severe hypoxia for 16 hours and total anoxia for four hours. They suggested that the fish's ability to withstand low O_2 levels was based mainly on suppressed metabolic rate but during deep hypoxia or anoxia, some compensation results from anaerobic glycolysis.

Møller et al. (1996) studied comparisons between rates of degradation in diesel oil contamination in unsaturated soil using respirometry and by measuring actual oil concentration. Their samples were each 6 m^3 and testing was monitored for 112 days. They used bioventing and inoculation with nutrients to enhance rates of oil degradation. Neither method alone improved the process. The results from the respirometry were used to correlate values with the actual concentration of oil in the soil.

In collaboration between respirometric and calorimetric methods of monitoring soil microbial activity, Critter et al. (2001) investigated soil microbial activity in tropical soil. Using an addition of glucose, they assayed microbial activity and measured enthalpy. They isolated bacteria and fungi, measuring enthalpic values between the cultures and measured the mass of CO_2 released over the course of the experiment. They also measured the efficiency of the bacteria and fungi in the consumption of glucose, showing the largest values found with the bacteria.

Gejlsbjerg et al. (2003) used respirometry to monitor the biodegradation of dodecyl benzene sulphonate, nonylphenol-di-ethoxylate, and tridecyl-tetra-ethoxylate added to soil at a rate of 400 mg/kg though ^{14}C experiments were amended at rates as low as 10 mg/kg. The writers stated that respirometric tests required a higher concentration in order to detect biodegradation. They wrote that respirometry could be used to estimate the biodegradation potential of the chemicals tested in soil and sludge amended soil and that respirometric experiments showed faster degradation than ^{14}C experiments.

CARBON RELATED RUNOFF TESTING AND QUALITY

Water Quality: TOC is a nonspecific measure of water quality. It represents matter that results from the degradation of plant biomass and microbial activity. Gergel et al. (1999) explored the effects of land use and land cover on aquatic systems by using the proportions of wetlands in measured watersheds to predict dissolved organic carbon (DOC) concentrations in the lakes and rivers of Wisconsin. Their study demonstrated that wetlands create considerably more variability in DOC, and especially in the fall. A higher proportion of wetlands in a watershed led to higher variability in DOC and variability in rivers more than lakes could be traced to those wetlands.

Volk et al. (2002) studied (DOC) concentrations in river water before and after conventional drinking water treatment using a Total Organic Carbon (TOC) analyzer. They discovered that after a rainfall event, DOC in the water could increase by three and a half times over that found in base-flow. Similarly, color increased by eight times, UV absorbance increased 12 times, and turbidity increased 300 times. Levels of organic matter also showed seasonal trends, being decreased in winter and increased during the warmer months between spring and fall. They also noted that "the transport of organic matter from the sediments to bulk water was minimal under low flow conditions."

Fleming and Cox (2001) measured runoff from grazed dairy pastures in Flaxely, South Australia between the years of 1996 to 1998 for P and C. They found that 98% of total P and 86% of total dissolved carbon (TDC) were lost to overland flow. They found that as much as 2.3 kg/ha of P and 10.7 kg/ha of TDC were lost in the wettest year. They noted the variability of P and C loads and found that there was no consistent trend between the proportion of TDC and TOC. They also developed predictive factors which can be used to explain a high proportion of variability in the C and P loads.

De Wit et al. (2007) sought to help explain the increase of DOC in fresh waters in the Nordic countries through comparison with acid rain deposition. They found that the increase in TOC in the long run was explained by the deposition of sulfate (SO_4) and NO_3. They discussed the solubility of organic matter in terms of the pH-dependent deprotonation of carboxylic groups and the ionic strength-dependent repulsion of organic molecules.

Jordan et al. (2003) tested runoff for TOC as well as other constituents in a restored wetland that received flows from a 14 ha agricultural watershed in Maryland. They tested for removal rates by the wetland which was less than one tenth the size of the watershed. They found that most of the removal took place during the first year of the two year study. That year, the wetland removed 41% of the TOC that flowed into it. During the two year period of the study the wetland removed 34% of the TOC it received. They concluded that the wetland reduced nonpoint-source pollution.

The land application of litter and other organic byproducts was explored by Harmel et al. (2009). The researchers used eight years of data collected from ten field scale watersheds and consisting of 574 runoff events to compare water quality differences based on litter application rate. Data showed that the nitrate (NO_3) concentration in runoff waters decreases rapidly after the application date. Immediately after application, concentrations reached between 75 to 100 mg/L-N, but after 100 days, concentrations were typically below 10 mg/L-N. They came to several interesting conclusions. First, combining organic and inorganic nutrient sources can be economically sound and environmentally responsible if the application rates are well managed. The study found that high N and P runoff events can originate from well managed fields making the regulation of edge of field water quality difficult to enforce. It also suggested that changes in the mentality of the animal production industry in the direction of viewing agriculture byproducts as marketable resources rather than waste products "could mitigate environmental problems, provide alternative fertilizer sources, and enhance animal industry revenue opportunities."

SOIL CARBON AND FERTILITY

Bationo et al. (2007) explored the lack of soil organic carbon in West Africa as it pertains to food security. Because of the low soil clay content, crops depend heavily on organic C as it is simultaneously a sink and source for nutrients. They noted that "to maintain food production for a rapidly growing population, application of mineral fertilizers and the effective recycling of organic amendments such as crop residues and manures are essential especially in the smallholder farming systems that rely predominantly on organic residues to maintain soil fertility." More than a quarter of the West African population is chronically hungry and more than half are living on less than one dollar per day as a result of soil fertility depletion.

Lambert et al. (2000) measured the effects of fertilizer treatments on soil characteristics over the course of 15 years in Hawke's Bay, New Zealand. They used a low-fertilizer treatment and a high-fertilizer treatment and treated both with superphosphate. Twice later they treated the high-treatment plots with limestone. They found that soil C decreased in both the low-fertilized and the high-fertilized at the same rate which suggested to them that soil organic matter was possibly decreasing in some New Zealand rural environments.

Wright et al. (2005) studied how organic soil amendments can increase DOC concentrations which can impact the movements of nutrients and heavy metals in soils. They investigated the influence of compost sources and application rates finding that there were few differences evident between compost sources for soil TOC. They found that DOC continued to increase over the course of the 29 month study suggesting that "compost mineralization and growth of bermudagrass contributed to DOC dynamics in soil." They found that soil NO_3-N was generally unaffected by application rate, finding that NO_3-N decreased similarly between compost applied and unamended tests. The researchers also found that the C concentrations were in part controlled by the growth patterns of the bermudagrass. TOC and extractable P did not increase over time after initial application.

Long term soil C and N was studied by Mulvaney et al. (2009) in relation to the use of synthetic fertilizers. Their results agreed with many years of studies showing decreased soil C all over the world with numerous types of soils and climates. Their concern was the loss of efficiency in raising crops for food. They decided that a major rethinking of world agricultural practices is necessary and that one viable solution is the use of legumes in rotation with other crops to restore soil N (Mulvaney et al. 2009). Using the same plots (the Morrow Plots, the oldest experimental site continuously under corn planting) and the same data but a different paper, Khan et al. (2009) pointed out the mythical nature of the commonly held assumption that soil C is increased with usage of synthetic N fertilizer. Not only is the opposite true they say, but even with the increased incorporation of crop residues into soils, soil N and C continue to decrease, resulting in stagnating or falling yields around the world.

III. MATERIALS AND METHODS

Glycerol: Glycerol used in all tests was obtained from the University of Arkansas Facilities Management department. To strip methanol, it was stored for an extended period of time in an open container in a fume hood to allow the methanol to evaporate.

Titration: A titration was performed with the glycerol to ascertain the amount of acid it would take to neutralize the high pH to a level typically found in soils. The acid used was 1 N H_2SO_4.

Microbial Toxicity Test: The microbial toxicity test was carried out using a soil mixture consisting of five parts commercially available Scotts brand topsoil, five parts commercially available composted cow manure, and one part uncomposted chicken litter. Each test batch consisting of 8 respirometer cells was made from one common quantity of soil. Soil portions were separated from the common quantity and weighed and then mixed with the respective quantity of glycerol. Soil mixtures were tested containing 0%, 0.03%, 0.1%, 0.3%, 1%, 3%, and 10% glycerol by weight.

The apparatus used was an AER-200 Respirometer system which measures oxygen (O_2) uptake (Figure 3-1). The apparatus consists of eight identical cells each containing a perforated plastic tube lined with screen that allows gas transfer from the environment of the cell to the soil sample held within. Magnetic stirrers homogenize the atmosphere in the cell, operated by the apparatus in the platform upon which all the cells sit. At the top of each tube is a plastic tube into which is placed 3 mL of potassium hydroxide (KOH) which absorbs carbon dioxide (CO_2) produced by the sample in a reaction that produces potassium bicarbonate ($KOH + CO_2 \rightarrow KHCO_3$). The cells are sealed with a rubber O-ring and a threaded cap. Attached to each cell is a thin tube which has a 0.1016 mm orifice inline and leads to a bubble counter. The bubble counter consists of eight clear plexi-glass containers which hold a known quantity of mineral oil. Each container houses a backlighting light emitting diode (LED) and a light receiving sensor. When a bubble is drawn through the oil by the negative pressure in the cell, it causes the light produced by the LED to bend around the bubble in the oil briefly ceasing interaction with the sensor. The respirometer registers this as a count and sends a signal to an attached computer which notes a volume of O_2 consumed based on an assumed bubble size. Bubbles are drawn from a common manifold that is supplied with industrial grade O_2 by an attached compressed O_2 cylinder with a regulator and a 0.1016 mm orifice. The pressure is set by allowing the O_2 to bubble through a pipe in a jar with approximately 1 mm of water head above the orifice.

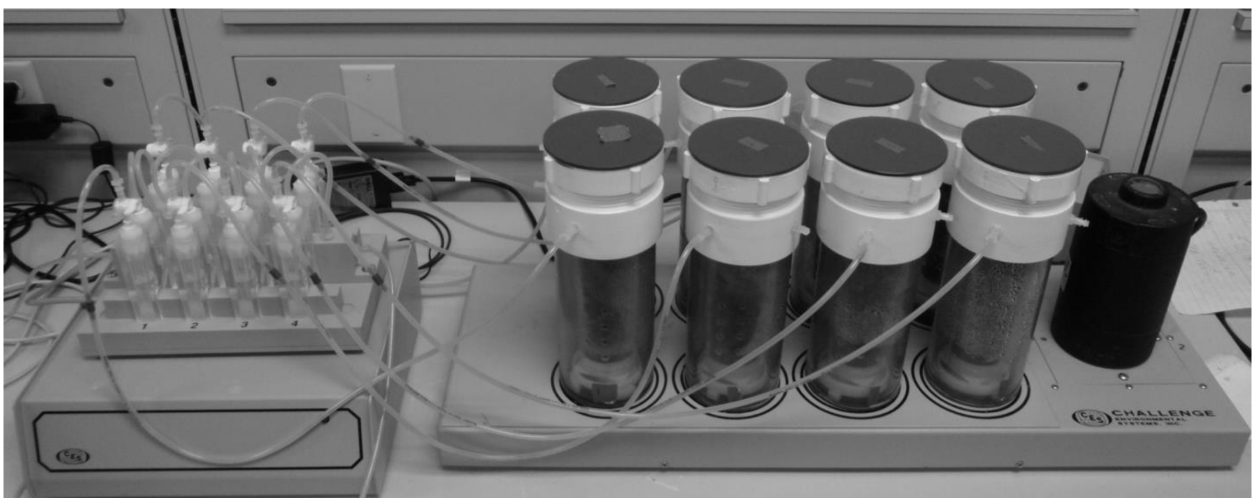


Figure 3-1. Challenge Technologies AER-200 Respirometer.

Respirometer data were recorded every minute. The length of each cycle was approximately one week but occasionally less as results warranted. The slope of the linear regression of the uptake during the main period of activity is the Oxygen Uptake Rate (OUR) which is expressed in units of mg/hr. The main period of activity is defined as the time following the lag in oxygen uptake to the point where O_2 uptake begins to taper off as the ability of the vial of KOH to absorb CO_2 is exhausted at about 800 mg. At that point, bacteria in the sample, no longer able to draw O_2 from the system, switch to anaerobic respiration and begin net production of gases which the respirometer cannot measure. For these tests, a consistent main period of activity was chosen to be six to sixteen hours from the beginning of oxygen uptake. Jumps in the data were subtracted and start times of individual cells normalized for the sake of efficient data comparison.

Grass Germination and Growth: The plant growth test was begun on September 6, 2011 using a soil mixture consisting of five parts commercially available topsoil, five parts commercially available composted cow manure, and one part uncomposted chicken litter. The containers used were commercially available plant starter kits. Each kit contained eight flats of nine pods. The total of eight pairs of flats represented the eight concentrations of glycerol tested, 0%, 0.01%, 0.03%, 0.1%, 0.3%, 1%, 3%, and 10% glycerol by mass. The soil was prepared from a common quantity mixed in a food grade plastic 5-gallon bucket. Batches were separated from the common quantity, weighed and mixed with the corresponding quantity of glycerol. In each pod were placed approximately 12 tall fescue (*Festuca arundinacea*) seeds and all were covered with a thin layer of soil unmixed with glycerol. Due to supply shortages, one flat of nine pods representing 10% glycerol by weight was replaced by a 3 inch square pot which was seeded with approximately 108 tall fescue seeds. Due to the inconsistency with the rest of the tests, this square pots was omitted from analysis. Figure 3-2 shows the method for planting seeds in the pods.

Both kits were watered with deionized water, an equal amount from below to prevent cross-contamination twice a week. The plants were kept under fluorescent lighting containing two 30 W daylight (6500K color temperature) bulbs and two 30 W soft white (3000K color temperature) bulbs. The bulbs were located approximately 46 cm above the plants.

Figure 3-2. Tall fescue seeds sown in soil pods treated with glycerol.

Four times during the course of the experiment (October 11, 2011, October 18, 2011, October 25, 2011, and November 8, 2011), the grass was trimmed using the height of a pestle as a measurement to assure an even trimming height. The same pestle was used to weigh the clippings. Figure 3-3 demonstrates the method. The clippings were weighed using an analytical balance and the values recorded to three decimal places. Throughout the experiment, pictures were taken to qualitatively discern treatment effects.

Figure 3-3. Trimming procedure for collecting and measuring biomass growth produced by tall fescue grass grown in soil treated with glycerol.

Runoff Total Organic Carbon (TOC) Analysis: The runoff test was carried out using test plots created in 2007 at the University of Arkansas Parasitology Farm (36°04’43.88” N, 94°17’04.87” W, Elev. 1240 feet). The grass was trimmed and the catchment gutters were cleaned in preparation for the tests. On May 3, 2012, the plots were dosed in four groups of four with the following applications using a handheld sprayer: Four plots with 1000 kg/ha glycerol (approximately 0.08% by weight), four plots with 300 kg/ha sodium nitrate as nitrogen ($NaNO_3$-N), four plots with the same amounts of both glycerol and $NaNO_3$, and four plots as controls with no application.

One week later the first runoff test was completed. To simulate rainfall, a movable apparatus was constructed to span the plots and provide a spray of tap water. The rainfall simulator was intended to simulate rainfall at 50 mm/hr however due to fluctuating water pressure in the available supply, rainfall application rates varied somewhat. The actual amount of precipitation applied to the plots was controlled and monitored using a water meter and hose-end ball valves. Start time, end time, and water meter readings were recorded for each plot. A second test was completed at 28 days and a third was completed at 90 days from the date of application.

The runoff was initially collected in four liter containers. When approximately three liters were collected in the container, two 200 mL samples were taken from it. This was done to achieve a consistent composite sample. One of the 200 mL sampling bottles of each plot was delivered to the fee-based water quality lab of the Arkansas Water Resources Center for TOC analysis using the APHA 5310 B (EPA 415.1) methods and the other was frozen to be utilized for possible future analysis.

120 days after application, samples of vegetation were extracted from each plot. The samples were chosen randomly by tossing a sampling ring onto each plot and manually removing all the vegetation from within the ring. The ring encircled approximately 0.063 m^2 in area. The vegetation was weighed and recorded.

Insect Mortality: On October 17, 2012, 100 "large crickets" were purchased from PetSmart and habitats created in four 19 L buckets according to commonly available online instructions for cricket care. Each container was provided a cardboard egg carton and a six cardboard toilet paper rolls to provide surface area for the crickets to occupy and hide and to hold moisture and act similarly to vegetation and thatch that might be found in the outdoor test environment. A dry wash cloth was included to serve the purpose of absorbing excess water during the application as soil would. Crickets were fed flake goldfish food and provided water using thinly sliced oranges. Each habitat was provided with fresh food and sliced oranges as necessary and equally between habitats. Crickets were divided 25 to a container but it was found that more than 100 crickets were included in the purchase so each container was apportioned approximately equally.

Applications were prepared similarly to those of the runoff test, based upon application rates of 1000 kg/ha glycerol and 300 kg/ha $NaNO_3$-N. The four containers were applied with the following: glycerol, $NaNO_3$, both glycerol and $NaNO_3$, and a control which was treated with water only. Each application was dissolved in 240 g of water and sprayed in respective containers using a spray bottle with a mist setting. Excess liquid soaked into the washcloth and was wrung out yielding an average of 87 g of water per container. The washcloth was replaced in the container after wringing to provide moisture and humidity and the opportunity for exposure of the crickets to the chemicals. Virtually all areas and surfaces in the containers were found to be soaked after application except inside the egg cartons.

Beginning one hour after application, all materials except the orange slice were removed from each container individually and dead crickets removed and counted. Shed exoskeletons were discarded. Counts were recorded at one hr, 12 hr, 24 hr and every 24 hr thereafter for a period totaling 14 days. Analyses were conducted based on the total mortality of each population.

Statistical Methods: Results of microbial toxicity tests were analyzed with a regression between the slopes of the OUR and glycerol concentration using $\alpha=0.05$ determining significance. Plant growth and toxicity results and TOC runoff results were analyzed with Analysis of Variance (ANOVA) tests with means separation by Fisher's Least Significant Difference (LSD) with $\alpha=0.05$. Results of insect mortality testing were separated by ANOVA with Fisher's LSD ($\alpha=0.05$) and with t-tests between the control and other treatments ($\alpha=0.05$).

IV. RESULTS

Titration: A titration of a sample of glycerol was performed using 1N H_2SO_4. The resulting graph is found in Figure 4-1. The pKa was found to be approximately 7.4. Incidentally, during the titration, the mixture became much more viscous than normal at times for unknown reasons.

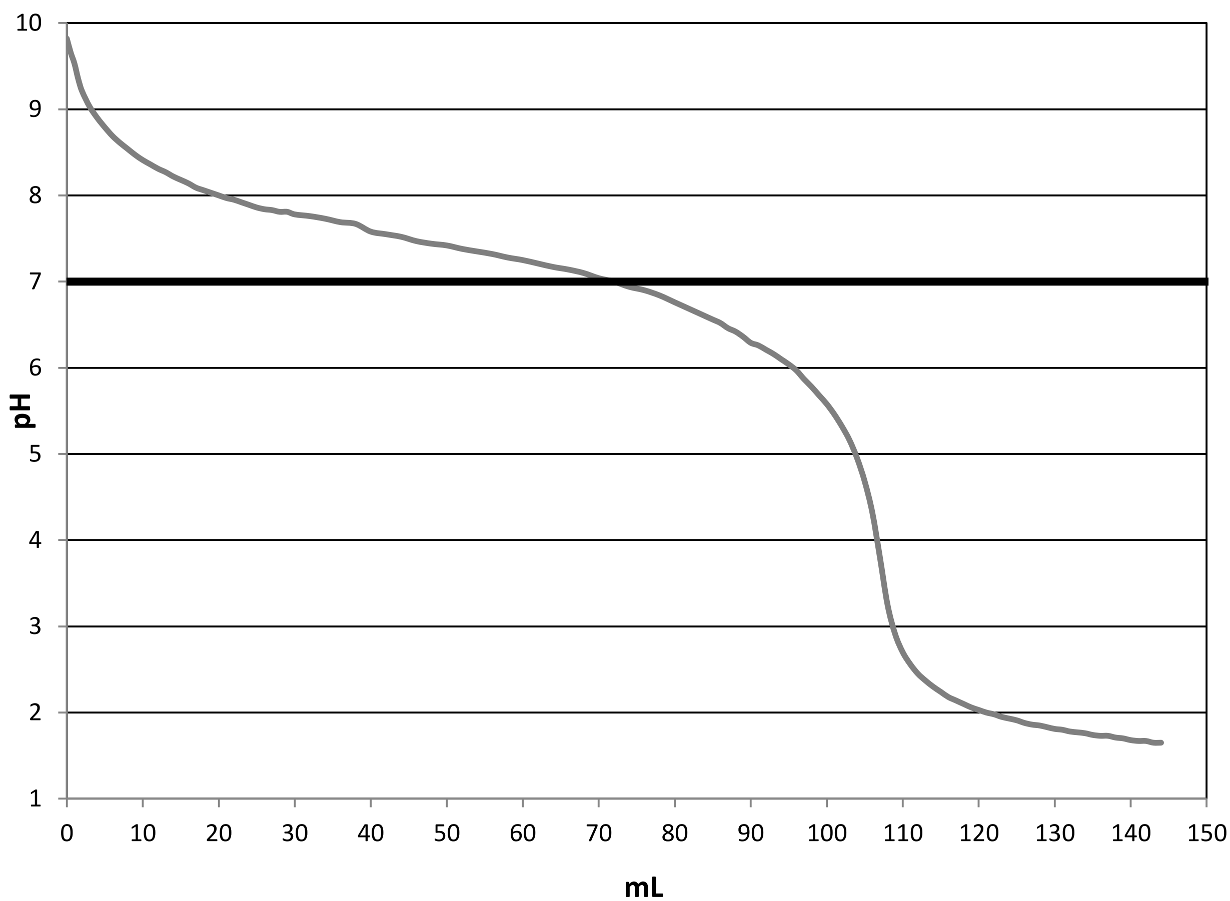


Figure 4-1. Titration curve of crude glycerol titrated with 1N H_2SO_4.

Microbial Toxicity Test Results: Figure 4-2 shows a representative respirometer cycle with seven successful control specimens. This graph shows the relative precision of the apparatus. The graph has been corrected for individual cell start time and for jumps in the data.

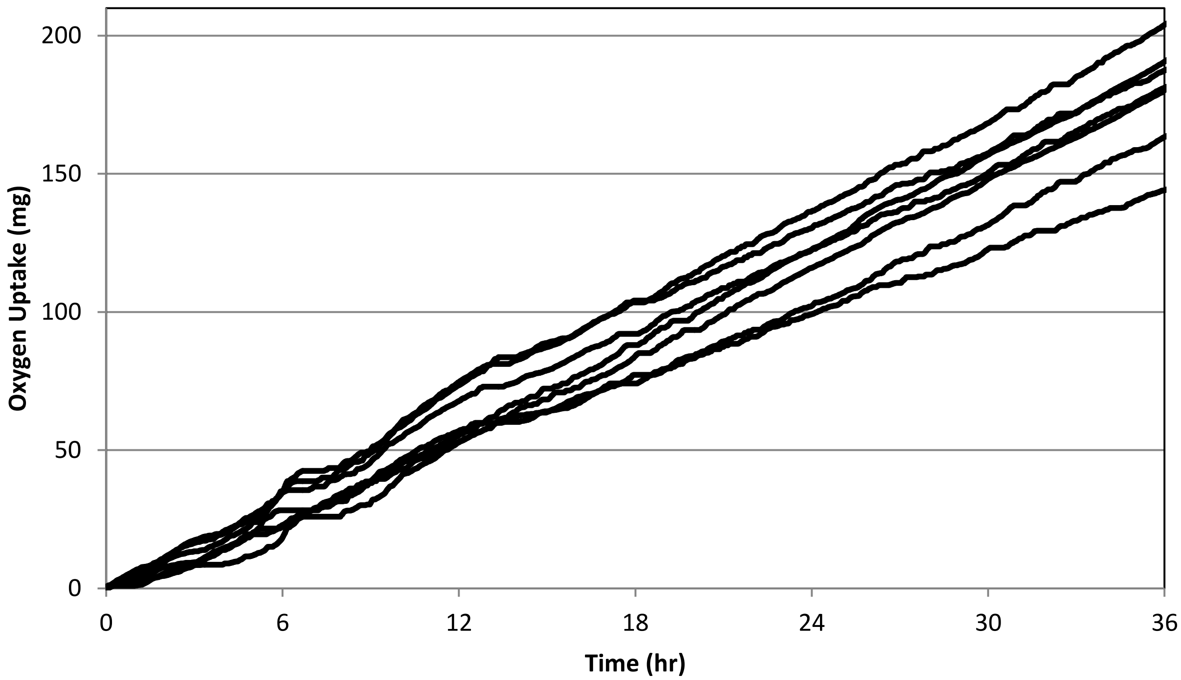


Figure 4-2. Representative respirometer run consisting of seven control tests.

Successful respirometer results were collected from a total of 24 individual cells. A linear regression of those data points falling between times from 6 to 16 hours after first sign of oxygen uptake yielded oxygen uptake rates (OUR) which are compared in Figure 4-3. There was a significant regression (α=0.05) between OUR and glycerol concentration (p=0.0078) and between OUR and log-transformed glycerol concentration (p=7.3x10^{-5}).

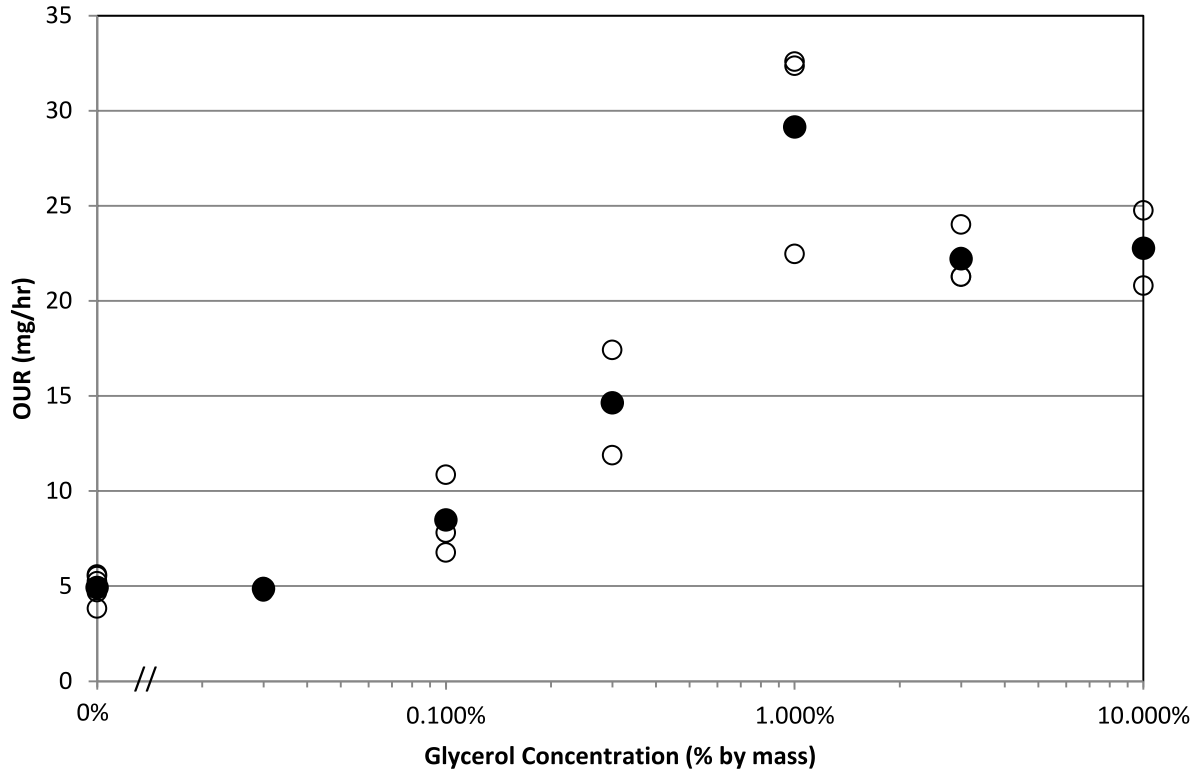
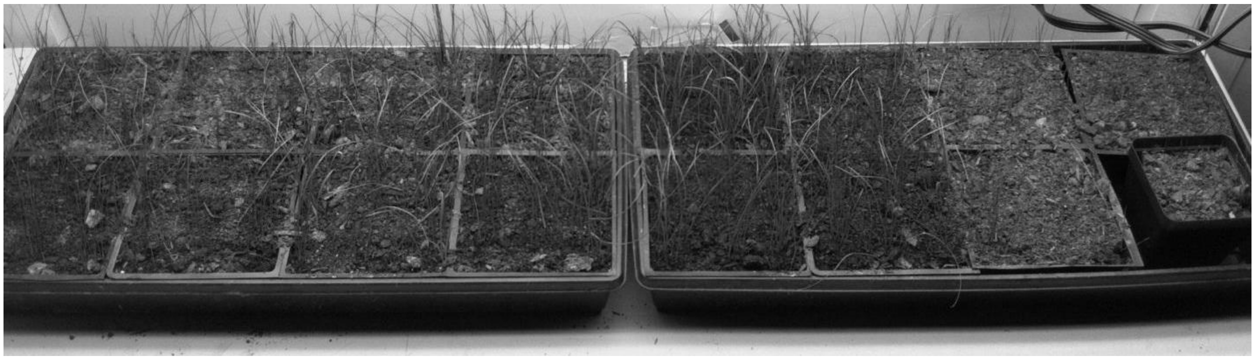


Figure 4-3. Respirometer Results: OUR versus glycerol concentration (log scale), circles represent individual cell results and black dots represent the average OUR for each glycerol concentration.

Plant Growth and Toxicity: Figure 4-4 demonstrates the differences between the different soil concentrations visually. From the control to higher concentrations, the soil becomes darker as more water is apparently absorbed from the air and made available to the plants. The soil in the pods containing 3% and 10% glycerol was desiccated and firm to the touch. In Figure 4-5 can be found the mass growth data collected from the trimmings and organized with high, low, and average values. One result for 10% is included. Growth rates were for the 57 day duration of the test.



0% 0.01% 0.03% 0.1% 0.3% 1.0% 3.0% 10

Figure 4-4. Photograph of early growth showing soil moisture effects, glycerol amended in percent by weight.

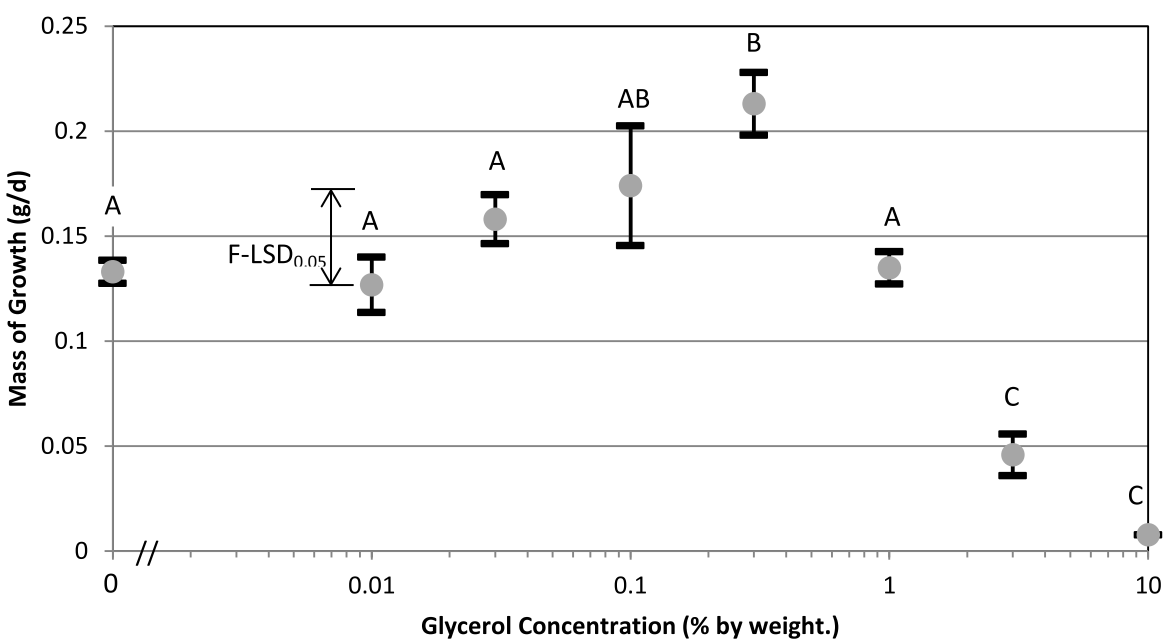


Figure 4-5. Biomass growth collected in grams per day by glycerol concentration, two cell values (bars) with average (grey dot). Treatments with the same letter are not significantly different from each other (α=0.05) but are statistically different from groups with other letters.

ANOVA tests with means separation by Fisher's LSD were performed on the trimmings data demonstrating no significant difference between the control and the soils bearing concentrations of 0.01%, 0.03%, 0.1% and 1%. The group treated with the concentration of

0.3% showed the greatest growth though not statistically different from the concentration of 0.1%. Significant inhibitory effects were found at concentrations of 3% and 10%.

At the conclusion of the plant germination and growth test, a sample of soil from each glycerol concentration group was removed from the test area, mixed with deionized water and tested for pH. The pH values of all samples including control were approximately 8.0.

Runoff Tests: Runoff tests show correlations between Total Organic Carbon (TOC) in the runoff water and the applied chemicals. Figure 4-6 shows the results of runoff water testing from plots designated as controls, for $NaNO_3$ application, for glycerol application, and for the 7 day test and Figure 4-7 shows the same for the 28 day test.

ANOVA with separation by Fisher's LSD was performed on sets of log transformed data from both runoff tests. Results one week after treatment showed significant difference between the control and $NaNO_3$ plots. Significant difference was not found between the control and the other two groups. Similarly, the glycerol and the group with both treatments were not significantly different from the $NaNO_3$ group. For the 28 day test, LSD showed significant difference between control plots and plots amended with $NaNO_3$ and with both treatments. The glycerol group was found not to be different from the control or the group with both treatments but significantly different from the group of plots amended with $NaNO_3$.

Between the two tests, TOC measurements of the runoff water increased by an average 3.57 mg/L.   t-tests comparing the differences between the 7 day test and the 28 day test found that the differences were significant between the two tests in the $NaNO_3$ group ($p=0.028$) and the glycerol group ($p=0.006$) but not in the control group or the group with both treatments.

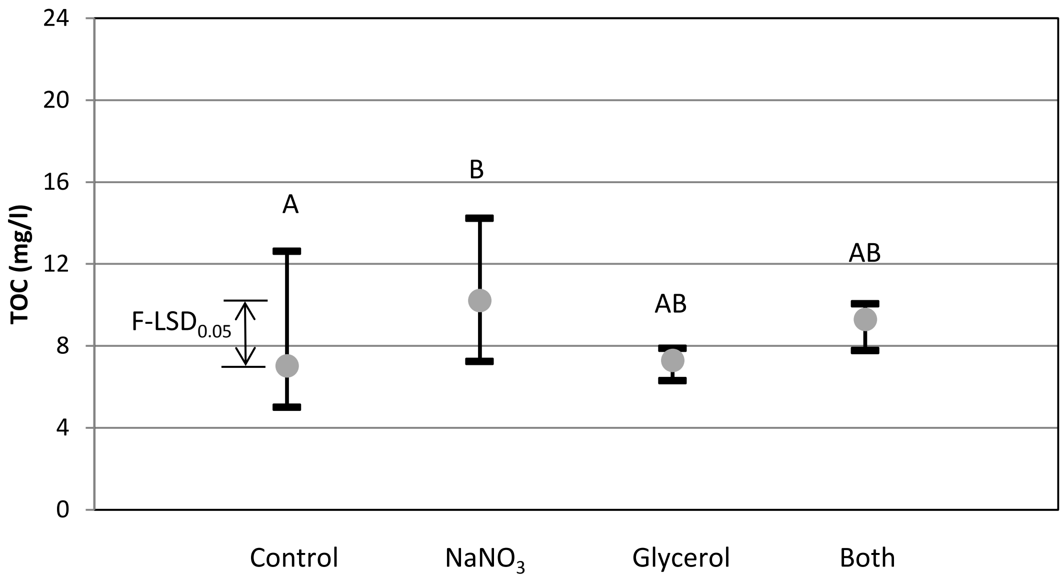


Figure 4-6. Total Organic Carbon (mg/L) results for runoff tests performed one week after application. The grey dot is the mean and bars represent the minimum and maximum values. Treatments with the same letter are not significantly different from each other (α=0.05) but are statistically different from groups with other letters.

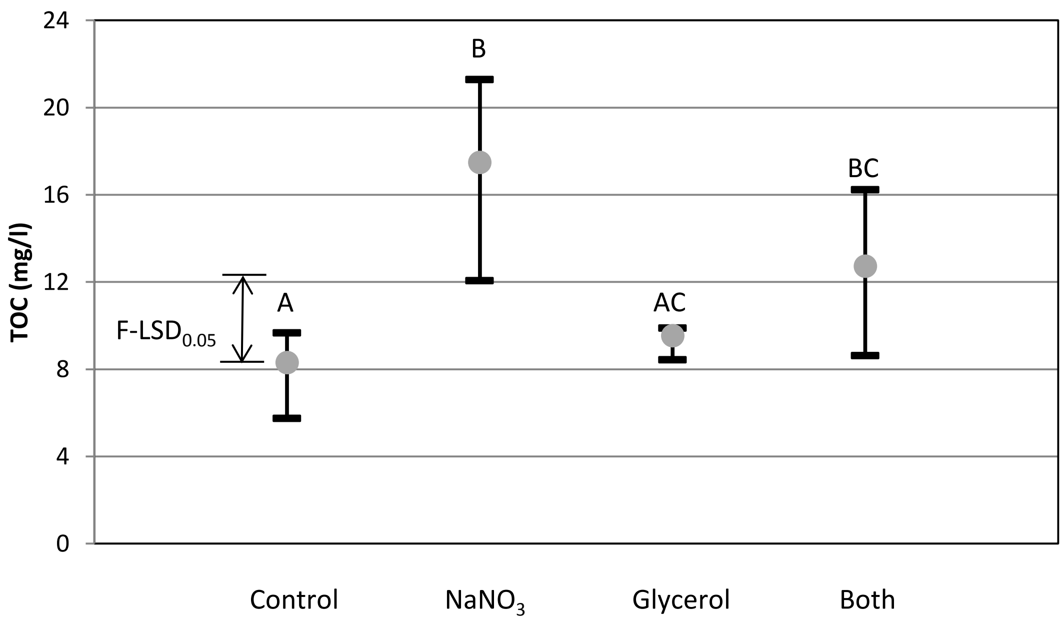


Figure 4-7. Total Organic Carbon (mg/L) results for runoff tests performed 28 days after application. The grey dot is the mean and bars represent the minimum and maximum values. Treatments with the same letter are not significantly different from each other (α=0.05) but are statistically different from groups with other letters.

120 days after application, one sample of vegetation (0.063 m^2) from each plot was collected and weighed. Using ANOVA with LSD, significant differences were found between the glycerol and other groups. Figure 4-8 shows the average mass collected as well as the high and low range for each group.

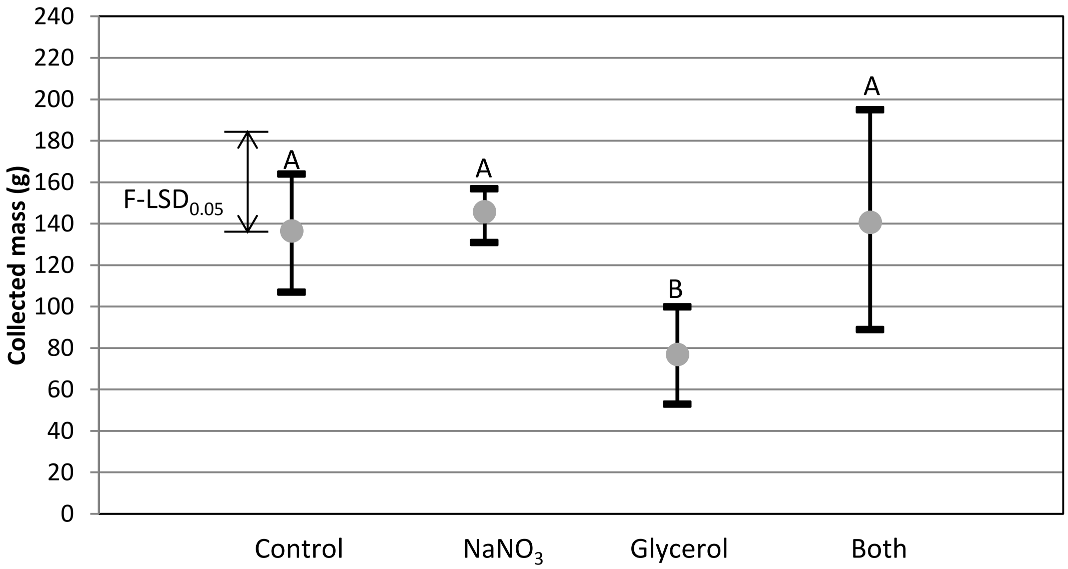


Figure 4-8. Mass of vegetation collected from runoff plots 120 days after application. The grey dot is the average and bars represent the minimum and maximum values. Treatments with the same letter are not significantly different from each other (α=0.05) but are statistically different from groups with other letters.

Insect Mortality: Initial mortality for the cricket test was striking especially for the container applied with glycerol and $NaNO_3$. After one hour, only 44% of crickets remained living in the group with both glycerol and $NaNO_3$ while the glycerol only container retained 85%, the $NaNO_3$ only container retained 90% and the control retained 100%. Figure 4-9 demonstrates the mortality of the crickets calculated as a percent of the total population at the beginning of the test.

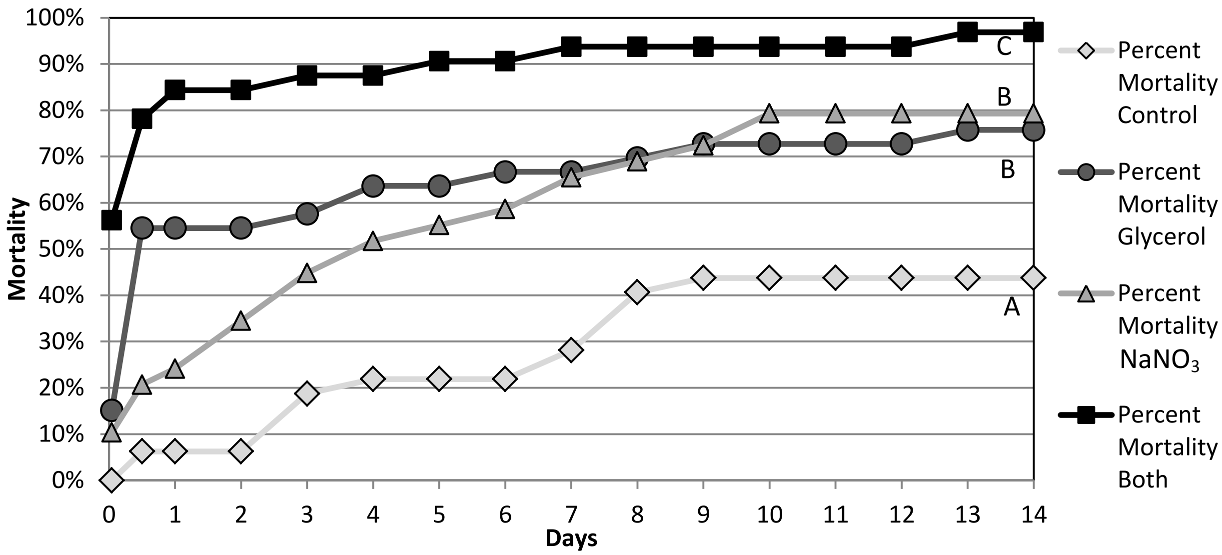


Figure 4-9. Total cricket (*Acheta domesticus*) mortality as a percent of original population by day. Treatments with the same letter are not significantly different from each other ($\alpha=0.05$) but are statistically different from groups with other letters.

ANOVA with LSD on total mortality was used to determine significant differences between treatments concluding that each group was different from each other group except for glycerol and $NaNO_3$ which were not different. T-tests also demonstrated that significant difference existed between the control group and the other groups.

V. DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

DISCUSSION

Titration: The titration of glycerol demonstrated by the steep negative slope at the beginning shows how little acid is required to reduce the high pH of the crude product. Even so, the high pH may be of benefit to acidic soils such as are found in Northwest Arkansas where this research was conducted. It has yet to be demonstrated how much this effect will benefit these soils and how persistent it is. The plant growth testing showed at the end of the experiment that the soil pH was around 8 which is within acceptable levels for natural and cultivated soil and was the same as the control group soil. Schoenau et al. (2009) similarly found pH and other chemical soil parameters were not affected by the addition of glycerol.

Microbial Toxicity: The general shape and form of the graphs are similar to results found in other respirometric experiments such as published by Critter et al. (2001). In the respirometer testing, no test group showed significant inhibition and oxygen uptake rate (OUR) was shown to increase with increased glycerol concentration, however this research shows that this same trend is only valid up to a certain concentration as Wee and Obbard (2010) discovered using concentrations of 40% by weight and Shoenau et al. (2009) demonstrated with concentrations up to 1%. Low and moderate concentrations of glycerol did not kill microbial cultures or negatively affect them. Johnson and Taconi (2007) stated that there are many bacteria able to metabolize glycerol and in fact, glycerol is an intermediate chemical in many bacterial metabolic processes. What should be avoided is the overuse of the chemical which this research showed could lead to anaerobic conditions and could cause unappealing smells as were noted in these tests.

Plant Germination and Growth: Shoenau et al. (2009) showed beneficial effects of concentrations up to 1% by weight which agrees with the trend in this research. One thing noted was how soil at different concentrations appeared to attract or repel water. This is possibly due to the fact that glycerol is hygroscopic which means it attracts and holds water molecules from the surrounding environment. This could have made the water unavailable to the plants and demonstrating a clear maximum concentration in this non-nutrient-limited soil. In nutrient limited soils such as found in the humid subtropical region of Northwest Arkansas, more pronounced inhibition effects are seen as demonstrated in the runoff testing and in studies by Shoenau et al. (2009) and Qian (2011) due to nitrogen tie-up.

Runoff Total Organic Carbon (TOC) Analysis: The seven day runoff test did not show a large amount of variability among the groups likely due to the fact that in biological systems it takes some time for both the glycerol and fertilizer to show biologically significant effects. What was important to note at the seven day testing time was that the glycerol itself did not significantly contribute to the runoff TOC and in fact was found to produce lower levels of TOC than other test groups. Later testing showed increased TOC in runoff water which agrees with the studies done by Khan et al., (2009) and Mulvaney et al., (2009) as fertilized soil loses increased amounts of nutrients. Soils treated with synthetic fertilizer lose C and P (Fleming and Cox, 2001).

During field testing, it was noted that cows became very interested in glycerol that had been spilled on the ground. Such was the case that the cows actually ate holes in the turf as they sought the sweet glycerol that had soaked in. Fortunately, the test plots had been fenced off prior to the commencement of the experiments and turf damage was limited to areas outside the confines of the testing range. It may be necessary to separate cattle from areas where glycerol is being land applied temporarily so that they may not cause damage to the turf and consequently cause increased erosion and sediment in runoff.

With the biomass collected and weighed from these plots, conditions must be taken into account. While obvious growth and lush green color was noted in the fertilized plots during the first two months of the runoff testing period, by the time the samples were taken, it was the end of August and there had been relatively little rainfall during the course of the summer. Consequently, positive effects of fertilizer addition would have been largely negated as the grass had gone to seed and much of it had withered and died. If the fertilizer amended soil is considered to be not nutrient limited similar to the soil from the laboratory germination and growth testing, it is consistent with the 0.1% amended soil in that it was not different from the control.

Insect Mortality: Cricket testing was undertaken after the close of the runoff testing as it was discovered during the application that some invertebrates were killed by the combined $NaNO_3$ and glycerol application. Spiders and caterpillars were seen to be killed within minutes during the application. It was theorized that there was some sort of effect resulting from the combination of glycerol and $NaNO_3$ that caused invertebrates to suffocate similar to the way water mixed with a surfactant does but it may in fact be due to the hygroscopic nature of glycerol and the salty nature of $NaNO_3$.

The house cricket (*Acheta domesticus*) is often used as an analog for medium to large bodied ground dwelling insects in lethality tests for a variety of chemicals and insecticides (Schleier and Peterson, 2010). They are ubiquitously available and have a total lifespan from egg to death of only a few months. They are similar to grasshoppers which makes them a good replacement for wild insects. Cricket testing demonstrated a contrast between respective applications agreeing with the apparent conditions observed in the field. Glycerol applied by itself can cause increased mortality in medium and large bodied insects and glycerol mixed with $NaNO_3$ shows further increased mortality.

CONCLUSIONS

The first objective of this research was to test microbial response to incorporation of glycerol into the soil by measuring oxygen uptake using a respirometer. Results show that the Alternative Hypothesis was correct. The incorporation of glycerol into the soil increased oxygen uptake in some treatments. It is clear that glycerol did not inhibit microbial respiration in the soil as demonstrated by increased microbial respiration with increasing glycerol concentration ($p=7.3x10^{-5}$).

The second objective of this research was to test the germination and growth of tall fescue grass seeds grown in soil treated with varying concentrations of glycerol. The results show that the Alternative Hypothesis was correct. For concentrations up to 0.3%, glycerol addition in the soil appeared to increase moisture available to the seedlings, allowing them to grow more than the control; however, a significant ($p<\alpha$) increase was observed in only one treatment. At the concentrations of 3 and 10% by weight, the glycerol in the soil demonstrated a significant ($p<\alpha$) inhibitory effect likely because water was made unavailable to the plants.

The third objective of this research was to test the total organic carbon in runoff water from plots treated with glycerol and or fertilizer. The Null Hypothesis was not rejected. Analysis of runoff tests show no significant ($p<\alpha$) difference between the amount of total organic carbon in the runoff water between control plots and plots amended with glycerol. Testing did show an increase in total organic carbon in plots amended with $NaNO_3$.

The fourth objective was to test mortality among field insects and arachnids using crickets as a test analog. The results have shown that the Alternative Hypothesis was correct. Mortality was increased among the population of crickets sprayed with a glycerol mixture compared to the group sprayed with only water. Mortality was very high among the test group sprayed with both glycerol and $NaNO_3$.

In conclusion, the land application of glycerol does have an effect on the soil fauna in and on the soil upon which glycerol is applied. In moderation, this effect is minimal and this research has demonstrated upper limits to its usage. In land application, glycerol should not be applied in concentrations above 1% by weight and care should be taken to assure that no endangered insects are harmed by its application.

RECOMMENDATIONS

With the continuing increases in biodiesel production, glycerol is set to remain in a state of declining price. Land application is only one option, but there are numerous others. One promising option is the conversion of glycerol into a usable fuel alongside biodiesel. While it may not be able to be burned in the same engines as biodiesel, it offers significant promise in offsetting other dirtier fossil fuels like natural gas and coal. According to Biodiesel Magazine, a company called Xcelplus Global Holdings Inc. has been working on new fuels utilizing crude glycerol as a base stock. One new fuel, GlyClene, can be burned in a standard gas turbine engine, similarly to the way natural gas is burned in power plants and was found to contain 119,000 British Thermal Units of energy per gallon. Another liquid fuel they have developed as a byproduct of GlyClene is GlyCoal, which is said to be able to displace coal in coal fired power plants. In one test, Glycoal was able to offset 10% of coal consumption in an 80 megawatt plant (Voegele 2009).

As the price of any commodity decreases, new uses are found to compensate for the relative cheapness of the source. Crude glycerol is no exception. The food industry has seen numerous examples of products evolving to fill a need. As an example, the Green Revolution has created a system in which massive amounts of corn are produced at a very low price. Corn can be chemically converted into high fructose corn syrup which has found its way into new and varying food products. As it is 75% sweeter and much cheaper than cane sugar, many food producers have altered their recipes to include high fructose corn syrup, especially manufacturers of soda (Ryan 2011). Along this same line of thought, it has been shown "that crude glycerol can be used as a safe and effective substrate for algal culture to produce high levels of omega-3 fatty acids" (Pyle 2008). The food industry is adapting and will continue to do so with changing prices in commodities. The price of glycerol nearing zero is certain to spawn new and expanded use in the food and cosmetics industry. It is already used as a non-sugar sweetener and a thickener. Those uses are likely to expand. It is even possible that glycerol could displace some use of high fructose corn syrup as a source of sweetness in foodstuffs. One benefit of glycerol over high fructose corn syrup is how as a sweetener, it does not cause tooth decay. That could create new opportunities for sweetening food products and in advertising for those products.

A viable option for disposal or even beneficial reuse of glycerol which cannot be ignored is in its addition to anaerobic digestion processes. These processes are used in many places to reduce the volume and volatility of waste sludges resulting from municipal waste water treatment or animal farming (Winters 2007). Its benefit in significantly increasing methane output cannot be ignored as methane can be used as fuel for electrical power generation to offset power use in waste water treatment plants or sold to utility companies for renewable energy credits. Additionally, waste glycerol does not need its methanol content removed as methanol is easily metabolized by microbes. Its normally high pH may also be of benefit in an anaerobic digester's acidic environment.

When exploring recommendations for disposal methods for glycerol, one must consider governmental regulations. Under the Resource Conservation and Recovery Act (RCRA), there are a number of regulations which are of interest in disposal of this product. RCRA governs the disposal of solid waste and hazardous waste. Glycerol is not considered toxic as it does not contain any of the contaminants listed in Table 1 on page 58 of Title 40 – Protection of the environment. Glycerol also does not qualify for restrictions under the class of reactivity. It is also not corrosive under the corrosivity qualification. Glycerol's flashpoint is well below the limit under the qualification for ignitability and it contains less than 24% alcohol by volume which is below that limit as well (Malhotra and Randolph 2007).

Surface water regulations are varied depending oftentimes on the existing conditions of the waters of the state in which the product is being disposed. Minimum regulations stipulate that glycerol with a concentration higher than 30% must be treated at a wastewater facility. Similarly, groundwater is expected to be free from pollutants in concentrations that are dangerous to humans (Malhotra and Randolph 2007). This leaves open the possibility for land application, however, individual watersheds will vary in their promulgation and application of regulations.

Biodiesel is already a carbon neutral fuel. The responsible use, reuse, or disposal of glycerol can expand the benefits of biofuels or hinder them. Probably the most beneficial use of glycerol in the realm of global climate change is its use as an aid to carbon sequestration in composting and soil amending applications. However, at the same time, its use as a fuel directly displacing coal in electrical generation also presents an excellent opportunity to reduce the net release of carbon dioxide.

Glycerol could be used as a soil amendment only if more economical options do not exist. The situation should be investigated carefully in all situations because of the emerging possibilities for other more beneficial and value-added uses. Applications as a soil amendment must also be carefully monitored to assure limited contamination of surface and ground waters. It must also be assured that the application of glycerol will not cause detrimental harm to animals such as insects and arachnids which with some species in some areas may be endangered or protected.

Future research in this area is necessary. This research is limited in scope and much study remains to be done as it pertains to the effects of glycerol land application. Especially necessary is the need to more closely study nutrient utilization and mobilization. This research drew its glycerol and fertilizer application rates from that of other researchers and it was not the purpose of this research to discover the best rate of fertilizer application. But it was demonstrated the application rate used in this research more than compensated for the nutrient tie-up effects of glycerol. This demonstrates the ability of glycerol to be disposed of in this manner alongside an application of fertilizer, but does not define the exact application rate that compensates for nutrient tie up. It is also not definitively shown what effects will result from chemical interactions with combined applications.

CHAPTER 6: REFERENCES

Alabama Agricultural Experiment Station (AAES) (2008) Biofuel by-product shows promise on crops. AG Illus 6:1 College of Agriculture, Auburn University, Auburn, Alabama.

Alotaibi D, Shoenau JJ (2011) Enzymatic activity and microbial biomass in soil amended with biofuel production byproducts. App Soil Ecol doi: 10.1016/j.apsoil.2011.03.002

Amon T, Amon B, Kryvoruchko V, Bodiroza V, Pötsh E, Zollitsch W (2006) Optimizing methane yield from anaerobic digestion of manure: Effects of dairy system and of glycerine supplementation. Int Congr Ser 1923:216-220

Ayala M, González-Muñoz SS, Pinos-Rodriquez JM, Vázquez C, Meneses M, Loera O, Medoza GD (2011) Fibrolytic potential of spent compost of *Agaricus bisporus* to degrade forages for ruminants. Afr J Microbiol Res 5(6):643-650

Bationo A, Kihara J, Vanlauwe B, Waswa B, Kimetu J (2007) Soil organic carbon dynamics, functions and management in West African agro-ecosystems. Agric Syst 94:13-25

Bulluck LR, Brosius M, Evanlyo GK, Ristiano JB (2002) Organic and synthetic fertility amendments influence soil microbial, physical and chemical properties on organic and conventional farms. App Soil Ecol 19:147-160

Cayuela ML, Oenema O, Kuikman PJ, Bakker RR (2010) Bioenergy by-products as soil amendments? Implications for carbon sequestration and greenhouse gas emissions. GCB Bioenerg, 2:201-213

Chung W, Huang J, Huang H (2005) Formulation of a soil biofungicide for control of damping-off of Chinese cabbage (*Brassica chinensis*) caused by *Rhizoctonia solani.* Biol Control 32(2):287-294

Coltrain D (2002) Biodiesel, Is It Worth Considering. Risk and Profit Conference. Kansas State University

Critter SAM, Freitas SS, Airoldi C (2001) Calorimetry versus respirometry for the monitoring of microbial activity in a tropical soil. App Soil Ecol 18:3, 217-227

Crooks A, (2007) Evolving technology may generate profit from biodiesel glycerin glut. Rural Cooperatives 74:4

De Wit HA, Mulder J, Hindar A, Hole L (2007) Long-term increase in dissolved organic carbon in streamwaters in Norway is response to reduced acid deposition. Environ Sci Technol 41:7706-7713

Dror I, Gerstl Z, Braester C, Rubin H, Yaron B (2000) In situ effect of soil amendments on the dynamics of kerosene attenuation. L Contam Reclam 8:4

EMD Chemicals Inc (2004) Material Safety Data Sheet for Glycerin. https://www.vwrsp.com/msds/10/VW5/VW5712-7.pdf. Accessed 13 April 2010

Fleming NK, Cox JW (2001) Carbon and phosphorus losses from dairy pasture in South Australia. Aust J Soil Res 39:969-978

Galvez A, Sinicco T, Cayuela ML, Mingorance MD, Fomasier F, Mondini C (2011) Short term effects of bioenergy by-products on soil C and N dynamics, nutrient availability and biochemical properties. Agric Ecosyst Environ. doi:10.1016/j.agee.2011.06.015

Gejlsbjerg B, Madsen T, Andersen TT (2003) Comparison of biodegradation of surfactants in soils and sludge-soil mixtures by use of 14C-labelled compounds and automated respirometry. Chemosphere 50:3, 321-331

Gell K, van Groenigen JW, Cayuela ML (2010) Residues of bioenergy production chains as soil amendments: Immediate and temporal phytotoxicity. J Hazard Mater. doi:10.1016/j.jhazmat.2010.12.105

Gergel SE, Turner MG, Kratz TK (1999) Dissolved organic carbon as a indicator of the scale of watershed influence on lakes and rivers. Ecol Appl 9(4):1377-1390

Groesbeck CN, McKinniy LJ, DeRouchey J M, Tokach MD, Goodband RD, Dritz SS, Nelssen JL, Duttlinger AW, Fahrenholz AC, Behnke KC, (2008) Effect of crude glycerol on pellet mill production and nursery pig growth performance. J Anim Sci 86:2228-2236.

Hall L (2010) Biobased foam as a soil additive. Thesis, University of Arkansas

Harmel RD, Smith DR, Haney RL Dozier M (2009) Nitrogen and phosphorus runoff from cropland and pasture fields fertilized with poultry litter. J Soil Water Conserv 64(6):400-412.

Hoffman BD, Lowe LM, Griffiths AD (2002) Reduction in cricket (Orthoptera: Ensifera) populations along a gradient of sulphur dioxide from mining emissions in northern Australia. Aust J Entomol, 41:2, 182-186

Iannotti DA, Grebus ME, Toth BL, Madden LV, Hoitnik HAJ (1994) Oxygen respirometry to assess stability and maturity of composted municipal solid waste. J Environ Qual 23:6, 1177-1183

Johnson T, Taconi KA (2007) The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. doi:10.1003/ep.10225

Jordan TE, Whigham DF, Hofmockel KH, Pittek MA (2003) Nutrient and sediment removal by a restored wetland receiving agricultural runoff, J Environ Qual 32:4, 1534-1547

Kerr BJ, Dozier WA, Bregendahl K (2007) Nutritional value of glycerin for nonruminants. http://www.ars.usda.gov/research/publications/publications.htm?SEQ_NO_115=214725. Accessed 14 April 2010

Khan SA, Mulvaney RL, Ellsworth TR, Boast CW (2007) The myth of nitrogen fertilization for soil carbon sequestration. J Environ Qual Oct 24;36(6):1821-32

Klepàčovà K, Mravec D, Kaszonyi A, Bajus M (2007) Etherification of glycerol and ethylene glycol by isobutylene. App Catal 328:1, 1-13

Lambert MG, Clark DA, Mackay AD Costall DA (2000) Effects of fertilizer application on nutrient status and organic matter content of hill soils. N Z J Agric Res 43:1, 127-128

López S, Santos M, Pérez C, Martín MA (2009) Anaerobic digestion of glycerol derived from biodiesel manufacturing. Bioresour Technol 100(23):5609-15

Material Safety Data Sheet (2012) Glycerol. http://www.sciencelab.com/msds.php?msdsId=9927350. Accessed 20 September 2012

Malhotra R, Randolph M (2012) Developing markets for biodiesel co-products in Wyoming, A report for the Wyoming Department of Agriculture. http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELPRDC5064918. Accessed 3 September 2012

Møller J, Winther P, Lund B, Kirkebjerg K, Westermann P (1996) Bioventing of diesel oil-contaminated soil: Comparison of degradation rates in soil based on actual oil concentration and on respirometric data. J Ind Microbiol Biotechnol 16:2

Mulvaney RL, Khan SA, Ellsworth TR (2009) Synthetic nitrogen fertilizers deplete soil nitrogen: a global dilemma for sustainable cereal production. J Environ Qual Oct 29;38(6):2295-314

Muusze B, Marcon J, van den Thillart G, Almeida-Val V (1998) Hypoxia tolerance of Amazon fish: Respirometry and energy metabolism of the cichlid *Astronotus Ocellatus*. Comp Biochem Physiol 120:1, 151-156

Pyle DJ, (2008) Use of biodiesel-derived crude glycerol for the production of omega-3 polyunsaturated fatty acids by the microalga *Schizochytrium limacinum*. Thesis, Virginia Polytechnic Institute

Qian P, Shoenau J, Urton R (2011) Effect of soil amendment with thin stillage and glycerol on plant growth and soil properties. J Plant Nutr 34:14, 2206-2221

Roh MS, Bauchan GR, Huda MS (2012) Physical and chemical properties of biobased plastic resins containing chicken feather fibers. Hort Environ Biotechnol 53(1):72-80

Ryan DB (2012) List of foods containing fructose corn syrup. LiveStrong. http://www.livestrong.com/article/35128-list-foods-containing-fructose-corn/. Accessed 3 September 2012

Sadano Y, Toshimitsu R, Kodha J, Nakano Y, Yano T (2012) Optimization of compost fermentation of glycerol by-product discharged from biodiesel fuel production process. J Mater Cycl Waste Manag 12(4):308-313

Schleier JJ, Peterson RKD (2010) Toxicity and risk of permethrin and naled to non-target insects after adult mosquito management. Ecotoxicol. doi:10.1007/s10646-010-0497-9

Schoenau J, Qian P, Alotaibi K, Urton R (2009) Potential for using biofuel, crop and animal processing by-products as soil amendments to increase fertility: effect of soil amendment with dehydrated alfalfa, dry and wet distillers grains, thin stillage and glycerol on growth of canola, soil properties, and microbial activity final report, Department of Soil Science, College of Agriculture, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Shay R, (2007) National biodiesel surge creates bittersweet co-product with glycerol, renewable energy world. http://www.renewableenergyworld.com/rea/news/article/2007/12/national-biodiesel-surge-creates-bittersweet-co-product-with-glycerol-50702. Accessed 3 September 2012

Siddiqui S, Shaukat I (2002) Zinc and glycerol enhance the production of nematicidal compounds in vitro and improve the biocontrol of Meloidogyne javanica in tomato by fluorescent pseudomonads. Lett Appl Microbiol 35:212-217

Sømme L (1964) Effects of glycerol on cold-hardiness of insects. Can J Zool 42:87-101

Taylor P, (2008) Synthetic glycerine is back (but never really went away)! in-Pharma Technologist. http://www.in-pharmatechnologist.com/Ingredients/Synthetic-glycerine-is-back-but-never-really-went-away-!. Accessed 2 October 2012

Tickell J (2003) From the fryer to the fuel tank: The complete guide to using vegetable oil as an alternative fuel. Tickell, New Orleans

Urbanchuk JM (2008) Economic contribution of the biodiesel industry, National Biodiesel Board.

Volk C, Wood L, Johnson B, Robinson J, Zhu, HW, Kaplan L (2002), Monitoring dissolved organic carbon in surface and drinking waters. J Environ Monit 4:43-47

Walton BT (1983) Use of the cricket embryo (*Acheta domesticus*) as an invertebrate teratology model. Fundam Appl Toxicol 3:4, 233-236

Wee MH, Obbard J (2010) Effect of glycerine spiked compost-soil mixture on the growth of wheat grass. Division of Environmental Science and Engineering, National University of Singapore.

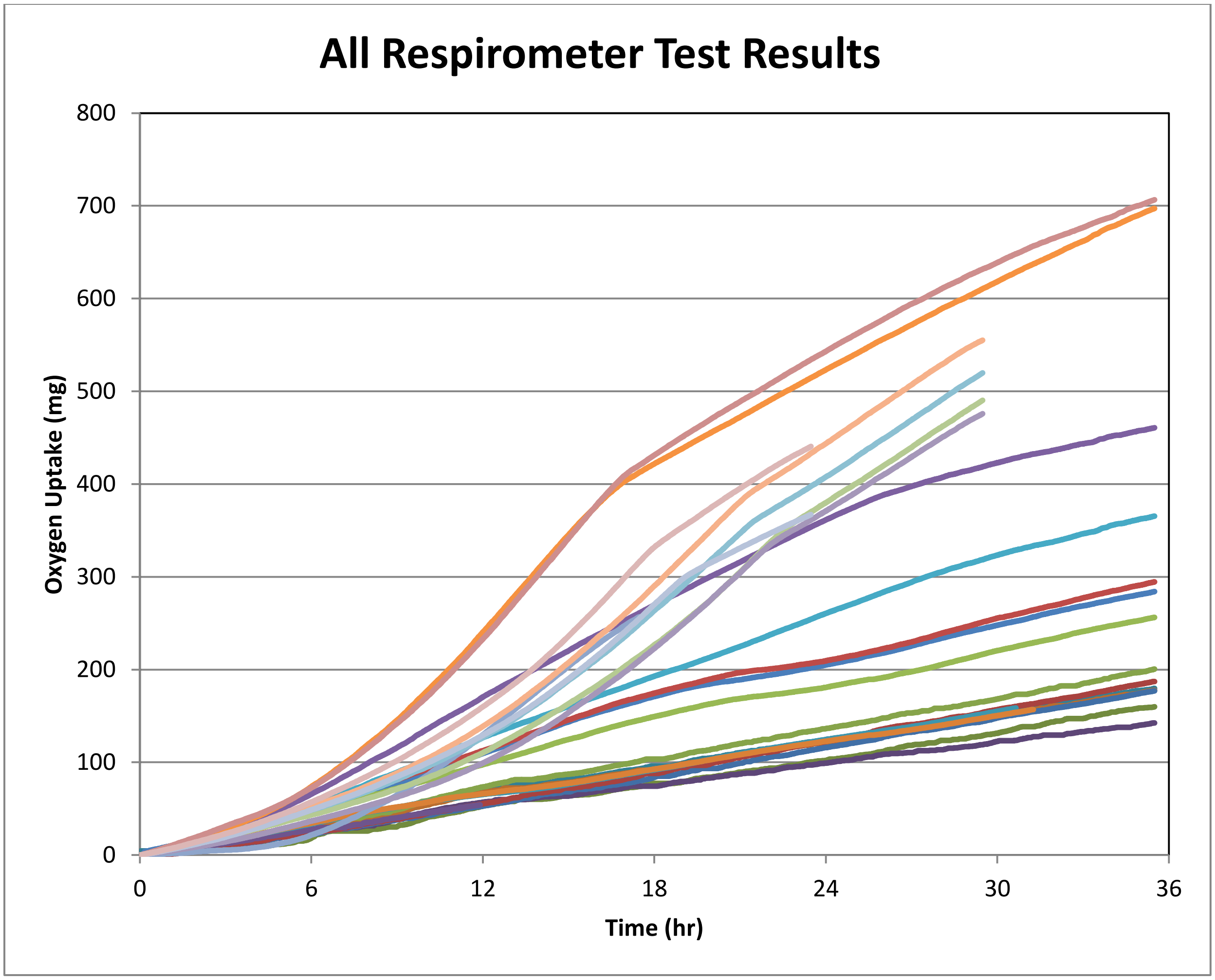
Winters D (2007) Manitoba family designs biodiesel factory. http://www.producer.com/2007/01/man-family-designs-biodiesel-factory/. Accessed 20 August 2012

Wright AL, Provin TL, Hons FM, Zuberer DA (2005) Dissolved organic carbon in soil from compost-amended bermudagrass turf. Hort Sci 40(3)

Zhang B, Freitag CM, Cañas JE, Cheng Q, Anderson TA (2006) Effects of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) metabolites on cricket (*Acheta domesticus*) survival and reproductive success. Environ Pollut 144:2, 540-544

APPENDIX A: RESPIROMETER DATA

Due to the nature and extensive volume of data from respirometer tests (approximately 50,000 points); they cannot be included in their entirety here. Digital data files are available free of charge upon request.



APPENDIX B – GROWTH DATA

Trimming Mass (g)								
Conc. (%)	1		2		3		4	
10		0.1795		0.0787		0.0438		0.1392
3	0.7708	1.1653	0.5555	1.1325	0.2938	0.4105	0.4265	0.4693
1	4.3929	5.1333	1.4298	1.4402	0.7528	0.7601	0.6741	0.7916
0.3	7.1517	8.6486	1.9549	2.0406	1.1013	1.1949	1.0798	1.1079
0.1	4.2421	6.5323	1.6894	1.8396	1.1195	1.1825	1.2384	1.988
0.03	4.9521	5.5897	1.4206	1.7779	1.0021	1.2409	0.9683	1.0631
0.01	3.422	4.3167	1.2795	1.4624	0.8136	1.1151	0.9598	1.0841
0	3.7227	4.1468	1.481	1.6184	0.9318	0.9403	1.1295	1.1841

APPENDIX C: RUNOFF DATA

Total Organic Carbon Test			
Plot	Designation	Test 1	Test 2
1	C1	12.63	9.68
2	F1	14.23	21.30
3	G1	7.15	10.41
4	B1	7.77	8.64
5	C2	5.11	5.75
6	F2	9.42	12.07
7	F3	10.00	20.07
8	B2	10.06	14.64
9	C3	5.33	9.28
10	F4	7.24	16.55
11	G2	7.81	9.42
12	B3	9.73	16.25
13	C4	5.00	8.51
14	G3	7.89	9.90
15	G4	6.30	8.44
16	B4	9.60	11.40

APPENDIX D: CRICKET MORTALITY DATA

Mortality					
Days	Control	Glycerol	Fertilizer	Both	Total
0.04	0	5	3	18	26
0.5	2	13	3	7	25
1	0	0	1	2	3
2	0	0	3	0	3
3	4	1	3	1	9
4	1	2	2	0	5
5	0	0	1	1	2
6	0	1	1	0	2
7	2	0	2	1	5
8	4	1	1	0	6
9	1	1	1	0	3
10	0	0	2	0	2
11	0	0	0	0	0
12	0	0	0	0	0
13	0	1	0	1	2
14	0	0	0	0	0
				∑=	93
Total pop.	32	33	29	32	126

5