A COMPARISON OF ACUTE TOXICITY OF BIODIESEL, BIODIESEL BLENDS AND DIESEL ON AQUATIC ORGANISMS

By

Nalissa Farrah Khan
Bachelor of Science (Honours), McMaster University, 2002

A thesis presented to
Ryerson University
in partial fulfillment of the
requirements for the degree of
Master of Applied Science
in the Program of
Environmental Applied Science and Management

Toronto, Ontario, Canada.

I hereby declare that I am the sole author of this thesis.

I authorize Ryerson University to lend this thesis to other institutions or individuals for the purpose of scholarly research

I further authorize Ryerson University to reproduce this thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research

ACKNOWLEDGEMENTS

I am truly grateful to Dr. Mostafa Warith for his assistance with every aspect of my research. His constant support and guidance throughout the course of my research is duly noted and much appreciated.

I would also like to extend my thanks to a number of individuals who assisted a great deal with experiments, including Robin Luong, Nidal Jaadlouk, Daniel Pennef and Reeyadh Khan.

Finally, I owe a tremendous amount of thanks to my parents, who have always supported and encouraged me in all that I do and to Ryan Mohammed for his ongoing understanding and encouragement.

ABSTRACT

A COMPARISON OF ACUTE TOXICITY OF BIODIESEL, BIODIESEL BLENDS AND DIESEL ON AQUATIC ORGANISMS

By Nalissa Farrah Khan

Environmental Applied Science and Management, 2005
Master of Applied Science, Ryerson University

The increasing demand of alternative energy sources has created interest in biodiesel and biodiesel blends; biodiesel is promoted as a diesel substitute. Like diesel spills, biodiesel spills can have deleterious effects on aquatic environments. The effect of neat biodiesel, biodiesel blends and diesel on *O. mykiss* and *D. magna* was evaluated using acute toxicity testing. Static non-renewal bioassays of freshwater organisms containing B100, B50, B20, B5 and conventional diesel fuel were used to compare the acute effects of biodiesel to diesel. Mortality was the significant endpoint measured in this study; percent mortality and lethal concentration (LC50) at different exposure times were determined from the acute toxicity tests performed. Trials were considered valid if the controls exhibited more than 90% survival. Based on percent mortality and LC50 values, a toxicity ranking of fuels was developed. The results of the definitive tests indicated that diesel is more toxic than neat biodiesel and biodiesel blends. This approach can provide insights into the lethality of biodiesel spills in the aquatic environment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ASTM	American Society for Testing and Materials
BOD	Biological Oxygen Demand
B50	50% Diesel, 50% Biodiesel
B20	80% Diesel, 20% Biodiesel
B5	95% Diesel, 5% Biodiesel
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
FFA	Free Fatty Acid
LC50	Lethal Concentration of 50% of a test population

GLOSSARY

Acclimation
Physiological or behavioural adaptation of organisms to one or more environmental conditions associated with the test method.

Acute test
A comparative study in which organisms are subjected to different experimental conditions that are observed for a short period of time; it usually does not constitute a substantial portion of their life span. Acute tests often use mortality as the only measured effect or endpoint.

Acute toxicity
Any poisonous effect produced by a single short-term exposure, which results in severe biological harm or death.

Additive
Material added in small amounts to finished fuel products to improve certain properties or characteristics.

Alevin
A newly hatched trout is called an alevin. At this stage, the trout has a large yolk sac which provides all nutrition for the fish in the first weeks of its life. When the yolk sac is absorbed; the alevin work their way and become free-swimming, feeding fry.

ANOVA
Analysis of variance: A parametric method used for hypothesis testing that is, to determine if statistically significant differences in a response occur among two or more groups.

Biodegradability
The rate at which compounds may be chemically broken down by bacteria and or natural environmental factors.

Biodegradation
Decomposition of a material by the action of micro-organisms.

Biochemical Oxygen Demand (BOD)
Amount of oxygen in milligrams per litre used by micro organisms to consume biodegradable organics under aerobic conditions.

Bioassay
Using living organisms to measure the effect of a substance, factor or condition.

Biodiesel
A fuel comprised of alkyl esters of long chain fatty acids derived from vegetable oils or animal fats and meeting the requirements of ASTM D 6751.

Biodiesel Blends
A blend of biodiesel fuel meeting ASTM D6751 requirements, with petroleum-based diesel fuel designated BXX, where XX is volume percent of biodiesel.

Biofuel
Any gas or liquid fuel derived either from organisms or metabolic by-products of living organisms.

Cloud Point
The temperature at which a sample of a fuel shows a cloud or haze of biodiesel or methyl ester crystals when it is cooled under standard test conditions, as defined in ASTM D2500.

Contamination
The polluting of members of the comparison or control group with any foreign material or chemical. Contamination threatens the validity of the study because the group is no longer untreated for the purposes of comparison.

Cross-contamination
Direct or indirect transfer of a pathogen from one medium to another.

Death
Is the 'effect' for determining toxicity in acute toxicity test.

Deionised
Freed from ions by a process of deionization.

Definitive Test
A test performed after the test conditions have been met and the test substance delivery system has been observed functioning properly.

Emulsification
The process that forms *emulsions*; mixtures of small droplets of oil and water. Two types of emulsions exist: water-in-oil and oil-in-water.

Endpoint
The variables that indicate the termination of a test; it also means the measurement(s) or value(s) derived that characterize the results of the test.

Energy Carrier
Any system or substance used to transfer energy from one place to another.

Evaporation
Occurs when the lighter or more volatile substances become vapours and leave the surface of the water.

Experimental Control
A group of experimental subjects that is not exposed to a chemical or treatment being investigated.

Fatty Acid
Any of the saturated or unsaturated mono-carboxylic acids that occur naturally in the form of triglycerides or as free fatty acids in fats and fatty oils.

Free fatty acids
Any saturated or unsaturated mono-carboxylic acids that occur naturally in fats, oils or greases but are not attached to glycerol backbones.

Fossil fuels
Combustibles derived from the remains of ancient organisms.

Fork length
The length of the fish, measured from the tip of the nose to the fork of the tail.

Glycerin
A neutral, sweet-tasting, colourless, thick liquid. It freezes to a gummy paste, has a high boiling point of 290°C and can be dissolved into water or alcohol, but not oils.

Immobilization
Those organisms which are not able to swim within 15 seconds after gentle agitation of the test container are considered to be immobile.

LOEC
Lowest-Observed-Effect-Concentration, the lowest concentration of toxicant to which organisms are exposed that causes adverse effects.

Lethal concentration (LC50)
Median lethal concentration; the toxicant or effluent concentration that would cause death in 50% of the test organisms.

Lethality
The quality of being deadly.

Monitoring
The routine checking of quality and or collection and reporting of information, the periodic checking and measurement of certain biological or water quality variables.

Mortality
An organism is recorded as dead when it is completely immobile.

Mortality Rate
Number of deaths in a population during a defined time period.

NOEC
No-Observed-Effect-Concentration, the highest concentration of toxicant to which organisms are exposed that causes no observable adverse effects.

Opercular
The principal opercular bone or operculum of fishes.

Oxidation
Occurs when oil comes into contact with water and oxygen combines with the oil hydrocarbons to produce water-soluble compounds.

pH
A measure related to the hydrogen ion content of a solution which represents the acidity or alkalinity of the solution.

ppm
Parts per million; a measure of concentration.

Photoperiod
The duration of illumination and darkness within a 24hr day.

Range-finding Test
Preliminary tests performed to determine the range of concentrations to be administered. Also involves tests to determine suitable experimental conditions.

Replicate
Experimental units that are tested simultaneously using the same experimental conditions.

Swim-up Fry
A young, post alevin fish which has commenced active feeding.

Test chamber
The individual containers in which test organisms are maintained during exposure to test solution.

Toxicity
The property of a chemical or combination of chemicals, to adversely affect organisms, tissues or cells.

Toxicity Test
A procedure to determine the toxicity of a certain chemical using living organisms.

Trans-Esterification
A chemical process; alcohol reacts with triglycerides contained in vegetable oils and animal fats to produce biodiesel and glycerin. The glycerin (by-product) is separated from the fat or vegetable oil.

Weathering
A series of chemical and physical changes that cause spilled oil to break down and become heavier than water.

Yellow Grease
Used in the rendering industry; often refers to frying oils from restaurants.

1. INTRODUCTION

1.1 Overview

Biodiesel is viewed as a viable alternative fuel to petroleum-based diesel. Dwindling oil reserves, expanding capitalization, increasing fuel prices, socio-economic and environmental problems have heightened the interest in renewable, affordable energy sources. Biodiesel is referred to as an energy carrier; it is also classified as a type of modified or enhanced fuel. Biodiesel is characterized as methyl or ethyl esters derived from plant oil, animal fat or yellow grease; these esters must conform to certain standards and specifications for use in diesel engines (Van Gerpen et al., 2004). Biodiesel refers to the pure fuel before blending with diesel fuel occurs; it is also referred to as neat biodiesel.

Biodiesel is also categorized as a liquid biofuel; it is a clean burning alternative fuel, produced from renewable resources, such as biomass, for example, herbaceous and woody plants, agricultural crops, forestry by-products, municipal solid and industrial wastes. Raw materials for biodiesel are mostly vegetable oils and waste animal fats; recycled cooking oils and grease are also used in biodiesel production.

Biodiesel can be blended with diesel fuel or used straight in place of fossil diesel in conventional diesel engines. Neat biodiesel contains no diesel fuel however it can be blended with diesel to produce biodiesel blends. Biodiesel can be used as additive volumes to diesel fuel or as premium fuel on its own; biodiesel blends of B5 are additive volumes and blends higher than B5 are considered premium diesel. As green fuels, particularly bioethanol and biodiesel, are becoming more commercialized, their fate in the environment is an area of concern since fuel spills

constitute a major source of aquatic ecosystem contamination (Zhang et al., 1995). Contamination of aquatic ecosystems by biodiesel may have serious consequences to organisms in these environments.

1.2 Sources of Biodiesel

Biodiesel is most commonly produced from edible oils; edible oils are oils fit for human consumption, such as vegetable oils. Biodiesel can be produced from many different types of plant oil; rapeseed oil is most common in Europe, soybean oil in North America and palm oil in Southeast Asia (Boyd et al, 2004). Biodiesel can be produced from a wide variety of feedstocks; some biodiesel feedstocks include soybeans, rapeseeds, maize, sunflower, coconut, canola oil, fish oils, waste animal fats, tallow and lard. Another feasible and realistic alternative source of biodiesel is waste cooking oils and fats from the restaurant and food processing industries (Rideout, 2004). These waste materials are becoming increasingly difficult to dispose of and create carbon dioxide emissions if they are dumped in landfills. Yellow grease can be chemically cleaned and processed; acceptable quality biodiesel can then be produced. Using waste oils and fats as a feedstock for biodiesel production may reduce further environmental pollution.

1.3 Thesis Organization

Section 2 is the literature review, which examines some pertinent issues involving biodiesel in the aquatic environment. This is followed by Section 3, the materials and method, which provides details on the methodology used for conducting all experiments. Section 4 presents the results obtained from the experiments performed on *D. magna* and *O. mykiss*. Section 5 consists

of an analysis and discussion of the results obtained in the study and Section 6 is the conclusions and recommendations for further research in the area of biodiesel toxicity.

2. LITERATURE REVIEW

2.1 Introduction

The world relies heavily on fossil fuels, such as oil and natural gas, as its main source of energy. Reserves of non-renewable resources are considered depleted when supplies are too expensive to extract, process or mine (Puppán, 2000). It is estimated that crude oil reserves may be 80% depleted within the next 35 to 90 years, depending on its demand. At the current rate of consumption, global crude oil reserves will last at least 45 more years. With increasing industrialization, global oil consumption is projected to increase by about 25% by 2010 (Puppán, 2000). Projections of future economic and energy needs will increase the focus on more renewable sources of energy, such as biomass and waste products (Tashtoush et al., 2004).

Biomass is a likely alternative source of energy, particularly liquid biofuels. Two important liquid biofuels are bioethanol and biodiesel. Biodiesel has considerable potential as a new and feasible source of renewable energy, particularly as a transportation fuel in both more developed and less developed countries.

2.2 Definition of Biodiesel

Biodiesel are alkyl esters of vegetable oils, waste animal fats and other organic materials, however, biodiesel is not oils or fats. According to the ASTM standards:

> *The term "biodiesel" means the mono-alkyl esters of long chain fatty acids derived from plant or animal matter which meet (A) the registration requirements for fuels and fuel additives established by the Environmental Protection Agency under section 211 of the Clean Air Act (42 U.S.C. 7545), and (B) the requirements of the American Society of Testing and Materials D6751*[1]

[1]Energy Policy Act of 2003, Conference Version, Section 1314

Biodiesel can be either neat ethyl or methyl esters prepared from vegetable oils, animal fats and yellow grease. Biodiesel is an ester that can be made from several different types of oils; however, it is primarily recovered from vegetable and animal fats. The process of making biodiesel is called *Trans-Esterification*, organic oils and fats are combined with alcohol to form fatty esters, such as ethyl and methyl esters. These esters can be mixed with conventional diesel fuel; these fuel mixtures are referred to as biodiesel blends. Biodiesel can be used as a neat fuel, which is commercially referred to as 100% biodiesel (B100).

Numerous studies (Boyd et., 2004), (Sheehan et al.,1998) have suggested that biodiesel is a clean burning substitute for diesel fuel; biodiesel is processed from natural, renewable materials and in terms of engine performance, biodiesel has similar properties as conventional diesel. Biodiesel operates well in compression-ignition engines and low level blends, i.e. blends of up to 20% biodiesel, can be used in almost all diesel equipment, engines and vehicles. Due to the applicability of biodiesel as a substitute for diesel fuel, there has been recent interest in the production of biodiesel as an alternative energy source for commercial and industrial processes.

2.3 Biodiesel Production

Biodiesel is commonly produced from new and even used vegetable oils and animal fats. Biodiesel is recovered from vegetable fats from such crops as soybean, rapeseed, canola, maize, sunflower, palm and coconut oil; they can also be recovered from animal fats, such as tallow and lard, as well as fish oils. In addition, waste cooking oils and fats have become a very popular feedstock in biodiesel production.

Biodiesel is made by reacting any natural oils or fats with alcohol, in most cases, methanol is used to produce fatty acid alkyl esters. These raw materials are transformed into biodiesel using a variety of *Trans-Esterification* technologies. There are three primary methods for producing biodiesel from fats and oils:

1) Base catalyzed *Trans-Esterification* of the oil with alcohol (usually methanol).
2) Direct acid catalyzed *Trans-Esterification* of the oil with methanol.
3) Conversion of the oil to its fatty acids by acid catalysis and then to biodiesel.

Oils and fats are mainly composed of triglycerides, which are chemical compounds of fatty acids, glycerin and free fatty acids (FFAs). The FFAs are bonded with methanol to produce biodiesel under acidic conditions. The triglycerides are also transformed into biodiesel and glycerin under base conditions, as indicated in Appendix A (Puppán, 2002). Base catalyzed reaction is the most common form of producing biodiesel because this type of reaction requires low temperatures and pressures, it also yields a high conversion with very few side reactions, minimal reaction time and there is direct conversion with no intermediate compounds (Van Gerpen et al., 2004).

Although there are different ways to produce alkyl esters, some important factors in the fuel production process include (i) a complete *Trans-Esterification* process, (ii) mixing intensity, (iii) reaction variables (reaction temperature, catalyst used, ratio of alcohol to oil, purity of reactants), (iv) glycerin, alcohol and any catalyst used must be removed from the end products and (v) no FFAs should be present in the alkyl esters produced (Barnwal and Sharma, 2005).

Biodiesel refers to the pure fuel; biodiesel blends (BXX) refers to a fuel that is composed of XX% biodiesel and 1- XX% diesel fuel (Tyson, 2001), for example, B100 is pure biodiesel and

B20 is a blend of 20% biodiesel and 80% diesel fuel. The diesel fuel can be no. 1, no. 2, or JP8 (Dmytryshyn, et al., 2004). At present, Europe is the largest producer and consumer of biodiesel and biodiesel blends, however, there are many potential markets in North America.

2.4 Present Uses of Biodiesel

Fuel markets that can benefit from biodiesel include bus and truck fleets, heavy construction equipment, diesel cars and boats, railways and electric generators. In 1991, biodiesel was commercially introduced in Germany, since then European biodiesel production has increased tremendously; biodiesel is the fastest developing alternative fuel source in Europe (Boyd et al., 2004). In 2000, Germany, France, Austria and Italy produced approximately one billion litres of biodiesel. Different legislations, tax incentives and oil production subsidies and grants have resulted in biodiesel being priced competitively with diesel fuel in these European countries. Also, in some European countries, marketing cooperatives have produced biodiesel on a small-scale for their own consumption and many European car manufacturers, including Mercedes Benz and Volkswagen have approved biodiesel use for their engines (Boyd et al., 2004).

2.5 Biodiesel in Canada

In Canada, biodiesel is still in the early stages of commercial and research development; in 1994, Agriculture and Agri-food Canada investigated the feasibility of biodiesel and analyzed a number of Canadian industries that may be potential biodiesel consumers (Prakash, 1998). The markets which were researched included (i) mining and petroleum exploration activities, (ii) government fleets and urban transit systems, (iii) national parks and ski resorts, (iv) forestry and (v) marine areas (Prakash, 1998). Before successful biodiesel consumption can succeed, some

concerns must be addressed. These concerns include: (i) high price of biodiesel compared to conventional diesel, (ii) lack of engine and vehicle manufacturers' approval of biodiesel usage, (iii) the performance and quality of biodiesel produced and (iv) the availability of reliable biodiesel feedstock (Prakash, 1998). Some Canadian initiatives include the Alternative Fuels Act; this act introduces legislation asserting the use of alternative fuel vehicles by all federal government departments, agencies and Crown Corporations. Unfortunately, at present, there is no major commercial use of biodiesel in Canada; however, some urban bus fleets are using biodiesel blends on an experimental basis. The practicality and environmental benefits of biodiesel have garnered attention from the Canadian government; the federal government has set a target production rate of 500 million litres per annum by 2010, under Canada's Climate Change Action Plan (Boyd et al., 2004).

2.6 Biodiesel Demand

Diesel is essential in the transportation sector of many industrial and commercial regions; diesel is used quite extensively in transporting goods and services; this demand is increasing both in more developed and less developed countries. With fluctuating oil prices, demand uncertainty and growing environmental concerns, an alternative fuel which is technically feasible, economically competitive, environmentally acceptable (Dmytryshyn et al., 2004) and readily available is needed to alleviate concerns about current and future use of non-renewable resources.

Biodiesel is not a new fuel technologically; Rudolf Diesel invented the diesel engine over 100 years ago, he suggested that the compression-ignition engine could have been operated with

peanut oil. However, as industrialization increased; petroleum became the prevailing energy source and diesel replaced vegetable oil as the fuel source for compression-ignition engines. However, interest in vegetable oils as a potential fuel source has waxed and waned over the years, with interest escalating during emergency conditions, such as World Wars I and II and the Persian Gulf War, as well as during the energy crisis of the 1970s, when petroleum supplies were interrupted (Raneses et al., 1999). In the past decade, environmental and energy security issues have led to renewed concern in alternative fuels, such as biofuels, particularly bioethanol and biodiesel.

Biodiesel can easily be substituted for petroleum diesel fuel in most diesel engines with no or only minor modifications and a slight decrease in power and fuel efficiency (Raneses et al., 1999). Biodiesel can be used as a diesel additive or a premium diesel to reduce vehicle exhaust emissions. The most common blend is 20% biodiesel and 80% petroleum diesel, popularly referred to as B20. B20 is referred to as premium blend and it also significantly reduces particulate matter emissions from diesel engines (Sheehan et al., 1998). Biodiesel demand along with expectations of increasing commercial demand has prompted great interest in biodiesel production.

In North America, more specifically, the biodiesel industry in the United States has identified three specific markets, which include: (i) public transportation (especially bus fleets), (ii) mining and (iii) marine and environmentally sensitive areas. These niche markets are considered possible candidates for potential biodiesel commercialization.

Transportation Fleets

Biodiesel production has increased rapidly over the past decade. In Europe, biodiesel blends are readily available at filling stations, however, the commercial transportation sector is the largest consumer of biodiesel fuel. In 2003, Germany, France, Austria and Italy produced over two billion litres of biodiesel and European car manufacturers, such as Mercedes Benz and Volkswagen, have approved biodiesel use for their European designed vehicles. In Europe, there is a healthy demand for biodiesel, predominantly due to the support of the European Union (EU) and its farm production and environmental health programs and also energy security concerns (Raneses et al., 1999). The EU has polices that allow farmers and farming cooperatives to grow crop feedstock for industrial and commercial uses, such as feedstock cultivation on 'set aside' land. In France, there is a 5% biodiesel blend in all commercial diesel fuel (Boyd et al., 2004); Austria and Germany have many tax benefits that promote the use of neat biodiesel in ecologically sensitive regions, such as mountainous areas and lakes (Raneses et al., 1999). In the United States, the urban transit market has been regulated as a result of the Clean Air Act Amendments of 1990; many urban fleets throughout the United States run on B20 (Prakash, 1998). One advantage of biodiesel in public transportation is practicality; the fuel demand could be met with a relatively few number of filling stations as bus fleets are fuelled at centrally located areas. However, privately owned vehicles must rely on filling stations; biodiesel pumps may not be conveniently located.

Mining

In the United States, most underground coal, metal and non-metal mines use diesel engines; diesel powered equipment is used to load, drill and transport material and personnel (Raneses et al, 1996). Diesel-powered machines are potentially less expensive to operate than other transport

systems. Biodiesel could be a very feasible form of fuel for the diesel powered equipment used in the mining industry. With greater use of diesel equipment, the potential advantages of using biodiesel as a fuel source, also has related possible health and environmental benefits. In underground and surface mining, biodiesel could be used as a fuel and a dust suppressant (Raneses et al., 1996). Biodiesel releases less particulate matter into the air (Sheehan et al., 1998) for potential inhalation by miners. In addition, biodiesel has a higher flash point, which provides a safer working environment in the mining industry than diesel (Prakash, 1998). However, before biodiesel or biodiesel blends could be used in mining, their impacts on human, environmental and ecosystem health, relative to petroleum diesel, would need to be thoroughly examined.

Marine Environments

Land transportation is not the only potential market for biodiesel use; biodiesel may have a considerable impact on marine transportation (Gustafson, 2003); many sea-going vessels rely on diesel as their primary source of fuel. Biodiesel could be used to reduce soot, odour, particulate matter and exhaust emissions (Raneses et al., 1996). Also, biodiesel and biodiesel blends maybe better able to mitigate the dangers of diesel fuel leaks and spills on lakes, rivers and estuaries. Biodiesel and biodiesel blends can also find a sustainable market as a fuel for large recreational boats and vessels.

2.7 Biodiesel Properties

Biodiesel is a reasonable alternative to diesel fuel; it is a fuel produced from renewable resources and has lower exhaust emissions than conventional petroleum diesel. One gallon of biodiesel displaces 0.95 gallons of diesel over its life cycle (Sheehan et al., 1998). It is also very energy

efficient, for every unit of fossil energy used to produce biodiesel, 3.37 units of biodiesel energy is created; it has a high energy yield when compared to fossil fuels; biodiesel also significantly reduces the amount of carbon dioxide being released into the atmosphere by diesel-engine vehicles.

Biodiesel is also an oxygenated fuel; fuel burns more efficiently and this greatly improves the emissions profile of a diesel engine. The more biodiesel used in a blend, the higher the emission reductions, in addition, biodiesel also releases less carbon dioxide than conventional diesel. Additionally, the crop feedstock used to produce biodiesel absorb large amounts of carbon dioxide as they grow (Sheehan et al, 1998). One of the unique benefits of biodiesel is that it drastically reduces air toxins, such as sulphur oxides and carbon dioxide, which are associated with petroleum diesel exhaust and are suspected of causing cancer and other human health problems.

Nitrogen oxide emissions are an exception to the rule, since biodiesel tends to increase nitrogen oxide emissions. Recent research has shown a number of ways to mitigate this problem. Through the *Trans-Esterification* process, the conversion of triglycerides into methyl or ethyl esters reduces the molecular weight of biodiesel to one-third, which reduces the viscosity by about one-eighth and slightly increases the volatility (Barnwal and Sharma, 2005). However, studies (Tyson, 2001) have suggested that some cold-flow starting problems persist in temperate conditions because biodiesel has low volumetric heating values, a high cetane number and a high flash point (Barnwal and Sharma, 2005). Nevertheless, biodiesel is rated as a strong candidate as an alternative to diesel.

Biodiesel is also safer to use than petroleum diesel; biodiesel has a higher flash point, neat biodiesel has a minimum flashpoint of 127°C, compared to 53°C for regular no. 2 diesel (Dmytryshyn, et al, 2004). It also handles like diesel and is safe to transport and store, it does not require special storage, either in its pure form or in blends. Biodiesel and biodiesel blends can be stored in the same containment areas as diesel, with the exception of concrete-lined tanks. Like petroleum diesel, biodiesel operates in diesel engines; essentially no engine modifications are required and biodiesel offers comparable lubricity, horsepower and torque to petroleum diesel (Dmytryshyn et al., 2004).

2.8 Biodiesel Spills

Biodiesel is becoming more prominent in North America; with the expected increase in use; the fate of this green fuel is an area of concern since fuel spills constitute a major source of contamination and pollution. According to the Environmental Protection Act; a spill is defined as "a discharge into a natural environment, from or out of a structure, vehicle or other container, and that is abnormal in quantity or quality in light of all the circumstances of the discharge" (Li, 2005). There are many causes of spills, including (i) vehicular and watercraft collisions, (ii) underground tank leaks (iii) discharges directly into watercourse, (iv) human error and negligence and (v) natural processes (flooding, land subsidence, earthquakes, slides). Among these concerns, water quality is one of the most important issues for living systems. Biodiesel consists of mainly fatty acids; however, it is not desirable if ethyl or methyl esters accumulate in the waterways (Tyson et al., 1998). Consequently, biodegradability and toxicity of biodiesel fuels is of interest, particularly if they accidentally enter into the aquatic environment in the course of their transportation, use, storage or disposal.

Biodiesel is an alternative fuel derived from renewable organic material such as vegetable oils or animal fats. Vegetable oil and animal fats spills do have deleterious effects on waterways; similarly, spills of biodiesel could have some negative impacts on aquatic environments. Large discharges of non-petroleum oils, as well as biodiesel, in an aquatic environment may cause significant oxygen depletion in waters due to rapid biodegradation rates. Like other petroleum-based products, discharges of these substances could pollute water and endanger organisms living in freshwater ecosystems.

A twenty-year (1982-2002) trend analysis of oil spills in EPA jurisdiction of spills of 50 gallons and more has indicated that 80% of volume spills originated from pipelines (43%) and other facilities (37%), the other 20% of spills came from other sources, for example, tank leakage and transportation accidents. Although, crude oils spills accounted for the greatest volume of oil spilled (43%), light fuels, such as no. 2 diesel, accounted for the greatest number of recorded oil spills (36%) in areas under EPA jurisdictions (Etkin, 2004). Diesel has a high frequency of recorded accidents and spills, however, a trend analysis has not been conducted on spills of biodiesel or biodiesel blends. If biodiesel becomes a commercially viable substitute for diesel, there may also be an increase of biodiesel spills into the environment.

There is not much data on recorded biodiesel spills; however, biodiesel spills can still be compared to petroleum oil spills. Biodiesel spills may generally be very small in volume due to the differences in the processing operations of crude oil and biodiesel. Petroleum tankers can exceed 250000 tonne capacity however; vegetable oils are usually carried in smaller tankers with a capacity of approximately 3500 tonnes. Consequently, a vegetable oil or waste animal fat spill

may not be comparable in magnitude to a petroleum spill; the probability is very small. Transportation processes of vegetable oils, waste animal fats and even yellow grease are also conducted at substantially different volumes and magnitudes than crude oils (Sagrans, 1998). Nevertheless, the frequency of an feedstock or biodiesel accident occurring is still high because of (i) the increasing number of biodiesel processing plants, (ii) the location of plants near freshwater environments and (iii) biodiesel and feedstock transportation routes are mainly situated inland. Consequently, the impact of a biodiesel spill in waterways may have similar consequences to a petroleum- based oil spill in marine environments.

With the expected increase in biodiesel demand as an alternative fuel source in North America, biodiesel production and biodiesel blending is also expected to increase. Increased use in the transportation sector may also lead to a rise in the number of accidental spills. Oil spills endanger public health, jeopardize drinking water supply, spoil natural resources, disturb ecosystems and disrupt the economy of a country. Nations have become more dependent upon oil-based products to help maintain a certain standard of living. Petroleum based products and non-petroleum oils, such as vegetable oils and animal fats, are increasingly being consumed. With this increased consumption, there is more biodiesel being transported from process plants to filling stations. Consequently, there is a greater chance of these fuels being spilled into the aquatic environment. Spilled biodiesel can contain toxic components that can produce chemical and physiological effects that are similar to petroleum oils; biodiesel spills may pose many threats to public health and safety. Like any other transportation fuel, biodiesel and biodiesel blends are usually stored and transported in large volumes; during storage or transport or even production, oils and other

oil-based products are sometimes spilled onto land or into waterways. When this occurs, human health and environmental quality are put at risk.

2.9 Petroleum-Based Oil Spills in the Aquatic Environment

There is limited research on biodiesel or biodiesel blend spills; however, there are studies on diesel spills and vegetable oil spills. Although spills occur on land, particularly along transportation routes, crude and petroleum-based oil spills are very prevalent in marine and freshwater ecosystems (Cripps and Shears., 1996). The short and long term risk posed from accidental spillage during transportation and storage to the environment can be particularly high, especially in aquatic ecosystems (Cripps and Shears., 1996). The product loss from leaking tanks is a significant environmental problem and may be a point source of groundwater and coastal zone contamination. These fuels may have deleterious effects on ecosystems, resulting in physical impairment of organisms and eventual mortality. According to the Alaska Administrative Code 2002, the toxicity of petroleum, petroleum products and petroleum by-products are classified as: (i) highly toxic (nos. 1 and 2 and artic diesel fuel, jet fuels A and B, motor gasoline, kerosene and stationary turbine fuels); (ii) moderately toxic (waste oil and lubricating oil); (iii) less toxic (bunker oil and hydraulic oil) and relatively non-toxic (asphalt and tars) (Mohammed, 2005). All of these oil types are transported by either trucks or tankers; both terrestrial and aquatic environments are at risk.

Most research on the fate and effects of oil entering the aquatic environment has focused on marine ecosystems, as most of the large oil spills that have received much attention have occurred in marine environments. Similar concern for freshwater aquatic ecosystems has been lacking. Nevertheless, oil spills do occur in freshwater areas as a consequence of the many oil-

related activities in this type of environment. It is important that fuel spills in freshwater habitats be addressed; one of the major sources of diesel oil spills is due to storage and transportation accidents.

Crude oil in freshwater environments is of particular concern because of its large retention time in aquatic environments. Oil retention times in marshes, swamps and other low energy environments are longer than oil spilled on high-energy coastlines (Bhattacharyya et al., 2003). Spilled crude oil may be present in sediments at low energy sites long after the occurrence of the spills; in addition, oil could be released into the water column many years after the initial spill. Water-column species and sediment-inhabiting species may both be affected by crude oil spilled in aquatic environments. Fuel may have both acute and chronic effects on freshwater ecosystems; chronic effects on water-column species include neurosensory disruption, behavioural and developmental abnormalities and reduced fertility (Bhattacharyya et al., 2003). Oil-based products spilled in waterways may limit oxygen exchange; acute effects include coating of the gills and skin, creating respiratory problems and death for many organisms.

Many studies have addressed the toxicity of crude oils and fuel oils on a variety of marine organisms. In comparison to the number of marine studies conducted on the effects of petroleum fuels, relatively few investigations have been done on freshwater habitats and organisms. Under marine conditions, wave action and surface turbulence are usually high, the volume and depth of water affected is usually large and the land-water interface comparatively small. Bhattacharyya et al (2003) have suggested that there is little surface turbulence, smaller volume and depth of water and the land-water interface is larger in freshwater environments when compared to

marine areas. Consequently, processes such as water mixing and dilution are usually limited in freshwater systems and infiltration of spills into surrounding areas may be increased. Conditions such as reduced turbulence and mixing increase the retention time of spilled hydrocarbons in the environment; the longer spilled oil is present, the greater the environmental damage. Another factor that has an effect on toxicity is the type of organisms exposed to toxins; marine and freshwater organisms are physiologically different; marine and freshwater organisms may be resistant to different types of pollutants. This can lead to a possible shift in species diversity; particularly towards a pollutant-tolerant species dominating the affected area. Unfortunately, very little research has been done on the effectiveness and toxicity implications for freshwater biodiesel oil spills on freshwater organisms.

When petroleum-based hydrocarbons are spilled into an aquatic environment, toxins can affect organisms that live in or around the water surface as well as those that live underwater. Oil spills can also harm parts of the food chain, including human food resources. Aquatic environments are seriously threatened by a variety of human activities. Many studies have shown that the extent of damage to stream flora and fauna following accidental oil spills is diverse and complex. The effect of a pollutant in aquatic environments depend on: (i) the chemical characteristics of the petrochemical involved (ii) the volume and severity of the spill (iii) the nature of the receiving water and its biota and the (iv) physical and natural conditions of the ecosystem affected, such as temperature and weather (Lytle and Peckarsky, 2001).

Oil consists of heavy insoluble compounds and lighter soluble compounds; crude oil containing heavy insoluble compounds is generally more persistent than lighter water soluble oils that

evaporate faster, however, lighter compounds are also highly toxic. The insoluble fraction can combine with the particulate organic matter or inorganic matter on the stream bed resulting in persistently toxic effects or an increased consumption of oxygen by bacteria during decomposition (Mudge, 1995). However, some studies suggest that exposure to water soluble fractions of oil causes more mortality than exposure to the products of oil degradation in sediment residues (Lytle and Peckarsky, 2001). As a result, it is very important to study the acute effects of oil and oil-based byproducts on aquatic ecosystems. Stream discharge also plays an important role in determining the extent of damage caused by oil spills; smaller streams are more susceptible than larger streams. Oil spills in streams and rivers with low discharge can create very deleterious effects to aquatic habitats; particularly areas in close vicinity to the source of the spill. Also, high volume spills cause more damage than smaller ones. However, turbulent flow at the time of a spill can enhance dispersion of chemicals, thereby affecting the spatial extent and immediate impact of the toxins on organisms. In contrast, scouring flows have been shown to accelerate recovery from oil spills not only by removing oil from the sediment but also by enhancing the recolonization of stream biota from upstream (Lytle and Peckarsky, 2001). Unfortunately, many small streams and other low energy areas have minimal scouring and erosional processes present.

Natural processes and physical conditions are present in all aquatic environments; these can reduce the severity of an oil spill and accelerate the recovery of an affected area. Some natural processes include weathering, evaporation, oxidation, photo-oxidation, biodegradation and emulsification. The intensity and extent of some of these natural processes may differ in freshwater ecosystems when compared with marine environments. Freshwater environmental

impacts can be more severe because water movement is decreased in these habitats. In standing water bodies, oil tends to pool and can remain in the environment for long periods of time. In flowing streams and rivers, oil tends to collect on sediments, as well as, plants and grasses growing on the banks and on the stream and river beds. These hydrocarbons can also interact with the sediment at the bottom of the freshwater bodies, affecting organisms that live in or feed off sediments.

Plant, animal and human exposure to toxic substances is generally reduced with time and is usually limited to the initial spill area since oil spill toxins may evaporate or biodegrade quickly. Both petroleum and non-petroleum oil can affect the environment surrounding an oil spill; all petroleum hydrocarbons possess similar chemical and physical properties that produce comparable effects on the environment. Some studies have shown that non-petroleum oil spills, such as plant and vegetable oils, can produce comparable effects as petroleum oil spills.

2.10 Vegetable Oil Spills in the Aquatic Environment

Vegetable oils are classified as non-petroleum oils; vegetable oils are commonly used as feedstock in biodiesel production (Van Gerpen, 2004). Research concerning the effects of vegetable oils in the marine and freshwater environment is limited when compared with the number of studies conducted on crude oil spills. Vegetable oils are transported and stored in similar ways to petroleum oils and the likelihood of spills of vegetable oils is no different from that of petroleum oils (Pereira et al., 2002). The lack of research on the effects of vegetable oils spills is possibly due to the assumption that vegetable oils are easily metabolized by organisms

and therefore are considered less harmful than crude oil spills or even that some renewable oils are more easily biodegraded than the more persistent petroleum-based oils (Pereira et al., 2002).

Large quantities of vegetable oils are transported from production sites to consumption areas, creating the possibility of spills to aquatic environments. Contamination can be caused by small spills during loading and unloading; acute pollution can be caused by shipping accidents. Large spills of vegetable oils have been reported all over the world. Pereira et al. (2003) have done extensive research on vegetable oil spills, some examples of spills include 2.5 million gallons of soybean oil spilled into the Minnesota River and Upper Mississippi River in 1963, a spill of 10 000 tonnes of palm and coconut oil and edible raw material occurred in 1975 on Fanning Island in the Pacific Ocean. Other examples include a palm oil spill in The Netherlands and a 400 gallon spill of rapeseed oil during the winter of 1989 in Vancouver Harbour, Canada. Although these spills caused many adverse effects to birds, fish and other aquatic organisms, vegetable oils have not been as extensively studied as those of crude oils.

Although not much research has been conducted in terms of biodiesel spills, there have been many studies involving petroleum-based oil and some on vegetable oil spills. Mudge (1995) has researched vegetable oil spills in marine environments, which has led to three main areas of concern about any such spillage. Sublethal effects on the environment may be caused by (i) Smothering (ii) Direct Toxicity and (iii) Polymerization of the oils with the sediments. Most vegetable oils float on water because of their lower density. When vegetable oils spill into waterways they reduce the oxygen exchange across the air-water interface, reducing oxygen supply by the process of smothering. Besides oxygen depletion, oils may adhere to organisms,

resulting in injury and even death. Direct toxicity may cause sublethal effects in organisms; these sublethal effects include reduced growth, suffocation, genetic variations and changes in reproductive cycles may also occur (Mudge, 1995). Polymerised oil decreases bacterial degradation; over time some polymerised oils form an impermeable cap over sediments, anoxic conditions may develop below the surface; this change in available oxygen may lead to a change in species diversity (Mudge, 1995). It can be postulated that similar effects may occur to freshwater organisms. Biodiesel is also produced from animal fats and waste cooking oils. However, research is limited on the influence of these feedstocks in aquatic environments.

2.11 Biodiesel and Biodegradability

Million of tonnes of oil are spilled each year into the environment during transport, storage and processing. For example, the *Exxon Valdez* incident in 1989 released 11.2 million gallons of crude oil into the Alaskan region (Biswas et al., 2005). As interest in biodiesel increases, their fate in aquatic environments is an area of relevance since fuel spills constitute a major source of contamination of many ecosystems (Zhang et al., 1995). It is important to examine biodegradability concepts in terms of biodiesel fuels and their biodegradation rates. Research on biodiesel biodegradability is limited, however, one study was found, which dealt with comparing two types of biodiesel with diesel.

It was determined that biodiesel has desirable degradation attributes; studies at the University of Idaho have been conducted to determine the biodegradation of biodiesel in an aqueous solution. Biodiesel, in particular, rapeseed methyl and rapeseed ethyl esters, was compared to diesel fuel and dextrose (Zhang et al., 1995). The biodegradability project examined chemical oxygen

demand (COD) and biological oxygen demand (BOD), biodegradation in aqueous solutions and biodegradation in soils. It is generally better if COD and BOD data have very low values because it indicates how much of this material is in water, sewage or in an effluent (Biswas et al, 2005). However, when biodegradation is of interest and it is desirable to have the toxin break down very quickly, high BOD and COD would be desired.

According to the studies performed, rapeseed methyl and ethyl esters have high COD and BOD values; biodegradation was determined in an aqueous solution using a 28 day shake flask test. Both esters degraded more rapidly than the dextrose control, the esters degraded at almost identical rates to about 95% at the end of 28 days. The diesel fuel in this test was approximately 40% biodegraded after 28 days. Although neat biodiesel has a high biodegradability rate in comparison to conventional diesel fuel, there may be acute short term risk associated with large volumes of biodiesel and biodiesel blend spillage (Zhang et al., 1995). The two types of biodiesel used in the study were produced from a vegetable oil feedstock. Research on biodegradability of biodiesel produced from waste animal fats and spent cooking oil is very limited.

2.12 Economic Analysis

For the past decade, many countries have been confronted with fossil fuel depletion and environmental degradation; these two predicaments have sparked interest in alternative fuels. Alternative fuels, energy conservation, energy efficiency and environmental protection have become pertinent issues regarding natural resource management (Barnwal and Sharma, 2005). There is an interest in biodiesel production because of the (i) increasing demand for liquid (biofuel) energy, (ii) continuing surpluses of agricultural commodities and (iii) rural

communities seeking diversification opportunities from mainstream farming (Radich, 2004). Studies have suggested that world oil consumption will increase primarily due to the growth in consumption of transportation fuels. Increased production of biodiesel could partially alleviate this increasing crude oil deficit.

2.12.1 Economic Factors

Information on the economic feasibility of biodiesel is limited. It is difficult to determine feasibility because of the complexity of all the factors involved in biodiesel production and marketing. Several feasibility studies have evaluated the market potential and economic costs of producing biodiesel, most using vegetable oil as the primary feedstock. Others excluded cost elements such as land and property taxes, administration, transportation and market development. Nevertheless, some fundamental analysis is possible based solely on economic factors.

To determine the feasibility of biodiesel as an alternative source of energy, economic input and economic output factors should be considered. Biodiesel costs are segmented according to fixed and variable costs. Fixed costs are estimated for extracting the vegetable oil from seed and for processing the oilseed into biodiesel (Enguídanos, 2002). Variable costs depend on the (i) type of raw materials used, (ii) type of oil extraction process and (iii) size of overall biodiesel operation.

The major economic input factor in biodiesel production is the feedstock costs (Enguídanos, 2002). Feasibility of biodiesel production is predominantly determined by which type of organic feedstock is used in the *Trans-Esterification* process. Technologies and processes involved in biodiesel manufacture may vary. Nevertheless, the greatest share of the production expense is the

feedstock cost; feedstock cost is the major barrier to the feasibility of biodiesel in urban and rural markets (Bender, 1999). Raw material costs account for a major portion of the total manufacturing cost, which is approximately 80 percent of the total operating cost (Enguídanos, 2002). Reduction of the raw material cost would optimize the total manufacturing cost. The type of feedstock used also foreshadows biodiesel prices. Some studies have shown that biodiesel made from non-edible oils such as waste animal fats are less expensive than edible oils such as canola, sunflower and rapeseed oils (Bender, 1999). Virgin oil costs approximately 2–3 times more than waste oils, such as restaurant cooking oils and by-products from livestock processing plants; the use of virgin oil leads to a significant increase in total manufacturing cost (Zhang et al., 2003).

The important economic output factors for biodiesel production are the end products, break-even price and regulations and initiatives. Besides biodiesel, glycerin is the other major end product; glycerin is widely used as an ingredient or processing aid in the cosmetic, drug and food industries. The profits from reusing glycerin can be used to offset cost incurred by the feedstock as well as other raw materials used in biodiesel production (Prakash, 1998). Break-even prices may vary from one biodiesel processing facility to another depending on the scale of the operation. Plants are classified into three groups (i) community, (ii) industrial and (iii) large industrial; the size of the operation has an influence on cost (Bender, 1999), differences in fixed and variable costs create elastic break-even prices. Other important economic factors include regulations and initiatives, these procedures will vary from one country to another (Prakash, 1998).

2.12.2 Economic Feasibility of Biodiesel

Biodiesel produced from vegetable oils is currently not economically feasible; the economics of biodiesel are unstable due to the large feedstock cost. Also, factors such as capital costs, electricity and water costs and glycerin credit can significantly affect biodiesel production costs (Bender, 1999). Economic feasibility studies show that the biodiesel obtained from non-edible oils is cheaper than that from edible oils (Barnwal and Sharma, 2005). Biodiesel manufactured from non-edible oils, such as waste animal fats, is closer to being cost competitive with petroleum diesel than biodiesel produced from vegetable oils, such as soybean oil. However, the currently available supply of such yellow greases will probably limit its use for successful long-term biodiesel production (Radich, 2004). Prices of edible oils, such as vegetable oils, need to decrease considerably in order for large quantities of biodiesel to become competitive with petroleum diesel prices.

The economic feasibility of biodiesel also depends on the price of crude oil and the cost of transporting diesel over long distances to remote areas. The cost of diesel will increase in the future due to the increase in its demand and limited supply (Barnwal and Sharma, 2005). Low production costs of crude oil derivatives are another significant limitation for biodiesel success. Increasing crude oil prices may open the biodiesel market; biodiesel demand may increase biodiesel production. Production costs may be lowered to levels where biodiesel prices at the gas pumps are comparable to those of fossil diesel. Fluctuating crude oil prices may become a potential opportunity for enhancing biodiesel demand in selective niche markets (Enguídanos, 2002).

Important factors for feasible biodiesel production are in place for the industry to succeed: such as environmental concerns with diesel fuel, diminishing petroleum reserves and awareness of the increased environmental consequences of emissions from diesel and gas-fuelled engines. A viable biodiesel industry in North America has potential benefits including: (i) increased feedstock value for oilseeds, animal fats and yellow grease, (ii) increased employment rates, (particularly in rural areas) and (iii) increased tax base from plant processing operations as well as income tax benefits and investments from feedstock processing ventures.

2.13 Present Biodiesel Status

Biodiesel has been established in Europe as the leading alternative fuel source, in North America, enthusiasm for biodiesel has been slow but growing. At present, biodiesel price is the main hindrance to its success in North America. The cost of biodiesel without tax in North America is about 2 to 3 times more than the selling price of diesel (Prakash, 1998). This gap can be reduced by offering subsidies to biodiesel or by imposing additional taxes on conventional diesel fuel. Additionally, the cost of biodiesel could be reduced by employing lower cost feedstocks, such as animal fats and waste grease or through innovative technologies, such as biodiesel treated with supercritical methanol. The engine and vehicle manufacturers in North America have not yet approved the use of biodiesel blends greater than B5 in their products (Holbein et al., 2004). However, the biodiesel industry in the United States is pursuing a number of initiatives to promote and expand the use of biodiesel, such as the Biodiesel Tax Incentive and the Energy Bill. The tax incentive, established originally as part of the American JOBS Creation Act of 2004, has been extended until 2008. The excise tax credit provides an incentive to fuel

distributors to blend biodiesel into diesel fuel, such as that made from soybean oil and recycled cooking oil (ENN, 2005).

The U.S. Energy Bill provides incentives and credits for alternative fuel installations; the bill also supports demonstration and testing projects for biodiesel use. The Energy Bill also promotes the U.S. departments of the Interior, Commerce and Agriculture to use energy efficient vehicle technologies, including biodiesel. The Energy Bill states that biodiesel is eligible for the Clean Bus EPA program; for example, those implemented for school buses (ENN, 2005). These activities should have some positive impact on biodiesel use in Canada. There is great prospects for biodiesel use in Canada, particularly in the mining industry, marine and transportation sectors (Prakash, 1998).

3. MATERIALS AND METHOD

3.1 Thesis Objectives

Various types of habitats have differing sensitivities to spills, either from petroleum-based oils or oils produced from vegetable oils, animal fats or yellow grease. There has been an increased interest in the use of biodiesel as a potential fuel and a feasible alternative to diesel fuel. However, like diesel fuel, any fuel could accidentally be spilled into rivers, lakes and oceans. Aquatic environments are made up of complex interactions between plant and animal species and their physical environment. Harm to the physical environment will often lead to harm to one or more species in a food chain, which may lead to damage for other species further up the chain, particularly humans.

This thesis addresses the effect of different biodiesel blends on aquatic organisms; diesel fuel is refined, stored and transported throughout estuarine, coastal ecosystems as well as freshwater environments which include watersheds and wetlands. These ecosystems provide essential habitat for aquatic species; which are vulnerable to accidental fuel and oil spills. Even though natural oozes of crude oil occur in the ocean floor and stable marine biotic communities are associated with them, the sudden introduction of high concentrations of oil or oil-based products can potentially kill or cause sublethal effects in some aquatic organisms.

Biodiesel has been proposed as a possible alternative source of fuel; an alternative to conventional diesel fuel. Diesel spills account for many spills, particularly along transportation routes, if biodiesel is being considered as a diesel substitute; biodiesel may also prone to such spills. Consequently, it is important to study the impacts of such energy alternatives on the

environment, toxicological studies are necessary to evaluate the potential impact of biodiesel and biodiesel blends on aquatic organisms and the freshwater environment. Unfortunately, there is a discerning lack of information concerning the acute toxicity of biodiesel and biodiesel blends on freshwater organisms.

The aim of this study was to determine the effects of biodiesel and biodiesel blends on the freshwater aquatic environment. Toxic effects of neat biodiesel and biodiesel blends were compared to conventional diesel fuel. Aquatic toxicity testing used in this study involved static non-renewal bioassays. In static non-renewal tests, the toxin is introduced into the test water at the beginning of the test and then the water is not changed during the duration of the test. The duration of the test may vary with the organism being tested. The organisms used in this experiment were the water flea, *Daphnia magna* (*D. magna*) and *Oncorhynchus mykiss (O. mykiss)*, commonly referred to as rainbow trout. Acute toxicity was generally measured using a multi-concentration test, consisting of a control and a minimum of six and five toxin concentrations for *D. magna* juveniles and *O. mykiss* frys respectively.

The thesis undertaken examines the influence of different concentrations of neat biodiesel and biodiesel blends (B20, B50 and B5) on two freshwater organisms; *D. magna* juveniles and *O. mykiss* (rainbow trout) fry. Acute toxicity testing was done with different concentrations of biodiesel, biodiesel blends and diesel fuel. The purpose of this study was to determine (i) the mortality rate of biodiesel and biodiesel blends (B50, B20, and B5), (ii) the LC50 values of neat biodiesel and biodiesel blends after different exposure times and (iii) the relationship and

significance of biodiesel results obtained to conventional diesel fuel. The two endpoints of the testing were (i) mortality rate and (ii) lethal concentration (LC50) values at 24, 48, 72 and 96 hrs.

To determine the relative toxicity of certain chemicals on an aquatic organism, an acute toxicity test was conducted to estimate the lethal concentration of the toxin. The tests were designed to provide concentration-response information, expressed as the toxin concentration that is lethal to 50% of the test organisms (LC50) within the prescribed period of time (24-96 hrs). The measured endpoints of the static non-renewal test were mortality rate and LC50. A 24hr-LC50 toxicity test was conducted on *D. magna* juveniles and a 96hr-LC50 was conducted on *O. mykiss* frys, according to the EPA guidelines outlined in the *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. Most oil toxicity studies have focused on early life stages of test species because these stages are generally more sensitive to toxic effects than adults.

3.2 Acute Toxicity Test

Acute toxicity is the apparent adverse effect induced in an organism within a short time of exposure to a substance or chemical. In the tests performed, acute toxicity was expressed as the median lethal concentration (LC50); the concentration in a water medium which kills 50% of the test organisms within a continuous period of exposure. For this study, acute toxicity was measured using a multi-concentration definitive test; consisting of a control and a minimum of five sample concentrations. The tests were designed to provide concentration-response information, expressed as concentration that is lethal to 50% of the test organisms, also referred to as the LC50 within the prescribed period of time, usually between 24 and 96 hrs. Static non-

renewal tests were used in this study, this type of test involved the test organisms being exposed to the same test solution for the entire duration of the test. Static non-renewal tests were utilized because this type of test is simple and inexpensive, little space and equipment is needed and small volumes of fuel concentrations are required (EPA, 2002). This type of testing was also chosen because only toxic effects in a freshwater (small stream) situation over a short time period was considered in the study.

3.3 Bioassay

The test organisms were exposed to static non-renewal testing which involved a multi-concentration test used to determine the effects of acute toxin exposure. Bioassays were performed to determine the LC50 of both *D. magna* and *O. mykiss* under the influence of different biodiesel blends and diesel fuel. This bioassay used static non-renewal tests; this type of test involved the test organisms being exposed to the same test solution for the duration of the test. The test solutions involved are conventional diesel, neat biodiesel and biodiesel blends of B20, B50 and B5 in a water medium.

3.4 Bioassay Organisms

The two bioassay organisms used were the water flea *D. magna* and *O. mykiss*, commonly referred to as rainbow trout; young organisms are often more sensitive to toxicants than are adults. For this reason, the use of early life stages, such as daphnid juveniles and fish swim-up fry, was required for all tests. In a given test, all organisms had similar physiological characteristics and were approximately the same age and were taken from the same source. Since

age may affect the results of the tests, it enhanced the value and comparability of the data when the same species in the same life stages were used throughout the experiment (EPA, 2002).

3.4.1 Daphnia magna

Daphnia magna (*D. magna*) is a small crustacean, often referred to as a water flea, which is barely visible with the naked eye. It lives in water and has large antennae in comparison with the rest of its body. *D. magna* is used as a bio-indicator and it is often used to measure the toxicity of a chemical compound in water. This species is often used in LC50 measurements. *D. magna* has a worldwide distribution in the northern hemisphere; *D. magna* found throughout many parts of Canada and the United States (EPA, 2002).

3.4.2 Oncorhynchus mykiss

Fish may be exposed to pollutants in different ways; they may come into (i) direct or (ii) indirect contact. Toxins may contaminate their gills and other external appendages, in addition, the water column may contain toxic and volatile components of fuel that may be absorbed by their eggs and juvenile stages; fish may also be affected by eating contaminated food, which may include algae, plankton or even other fish. Fish that are exposed to petroleum-based hydrocarbons may suffer from heart and respiratory problems, fin erosion, reduced growth and reproductive and behavioural responses (EPA, 2002). Some studies have shown that chronic exposure to some chemicals present in oil may cause genetic abnormalities or cancer in some sensitive fish species. For this project, *O. mykiss* young were used; acute exposure was measured by mortality. *O. mykiss* are a variable species that differ considerably over their range. The native range of the

rainbow trout group in North America is west of the Rocky Mountains and along the eastern Pacific Ocean but *O. mykiss* has been introduced into many parts of North America. Rainbow trout has also been widely introduced and established in other cold water habitats all over the world (EPA, 2002).

The fish should be in good health and free from any apparent physical deformity or disease. The species used were selected on reason, such as availability, maintenance, testing convenience and sensitivity to chemicals (EPA, 2002). The testing used *O. mykiss* as the preferred fish species because of its worldwide availability, moderate fat content and adaptability to fresh or sea water (Environment Canada, 2000).

The procedures of the experimental work were divided into two sections; (i) toxicity effects of fuel concentrations on *D. magna* juveniles and (ii) toxicity effects of fuel concentrations on *O. mykiss* frys. Lethality and other toxic effects of a chemical are dependent on both concentration and exposure time (Lee et al., 1995). The toxicity of biodiesel and biodiesel blends was investigated using acute toxicity tests; a comparison was made to diesel fuel toxicity. For both daphnids and fish, this was done by observing lethality resulting from exposure to a series of chemical concentrations, at 24, 48, 72 or 96 hours.

3.5 Experimental Organisms

Daphnids

Two different types of daphnids were used, *D. magna* and *D. pulex.* Daphnids were obtained from Carolina Biological Supply Ltd and Ward's Natural Science. Daphnids were maintained and cultured at 20±1°C. The photoperiod consisted of 16 hours of light and 8 hours of darkness.

O. mykiss (Rainbow Trout)

Rainbow trout (*O. mykiss*) (weighing between 0.5-1.0g) were obtained from two local suppliers (Linwood Acres Trout Farm Ltd, Campbellcroft, ON, and Blue Springs Trout Farms Ltd, Hanover, ON). Although, there was two different fish stock, there was no mixing of the stocks throughout the experiments. All fish were maintained at 12±2°C. Fish acclimation, in terms of temperature, was tested for exposures between 8 and 23 °C. Fish were acclimated at 12±2°C for fourteen days prior to use. Water temperature was regulated to within 2 °C of the desired test temperature by a refrigerator. Photoperiod was 16 hours of light and 8 hours of darkness. The fish stocks were fed daily with commercially prepared trout pellets obtained from Linwood Acres Trout Farm Ltd.

3.6 Test Chemicals

The fuels used in the bioassays were conventional diesel, B100 and biodiesel blends, the biodiesel blends included B50, B20 and B5. Biodiesel blends were made from the same B100 feedstock.

3.7 Equipment & Material

Sample containers, culture units, analytical balance, test chambers, volumetric flasks & graduated cylinders (10-1000ml), volumetric pipettes (1-100ml), graduated serological pipettes (1-10ml), graduate micropipettes (1-5µl), pipette bulb & fillers, droppers, wash bottles, thermometers (for measuring water temperature and environmental temperature), DO meter, pH meter, pH buffers (4,7, 10), Sources of food for cultures and stock organisms, dip nets, test organisms: *D. magna* juveniles and *O. mykiss* frys, holding tanks, deionised distilled water (DDI), Refrigerator.

3.8 Physiochemical Parameters

The pH, temperature and dissolved oxygen content of the various concentrations and the control water were the physicochemical parameters measured in this study.

3.9 Source of Toxins

B100 was obtained from Rothsay; a rendering company, B100 was made from recycled cooking oils and fats. B20 (Topia) was a B20 blend commercially blended by Topia Energy Ltd. and obtained at a local filling station. Diesel was obtained from the same local gas station.

3.10 Biodiesel Blending

The chemical nature of biodiesel allows it to be blended with distillate and diesel fuel; this includes light fuels such as jet fuel, kerosene, no.1 diesel, or military fuels (JP8, JP5), as well as normal diesel fuel like no. 2 diesel for diesel engines. Biodiesel is blended thoroughly with diesel

fuel; if maintained at temperatures above its cloud point, biodiesel blends stay mixed (Tyson, 2001). Biodiesel mixes well with diesel fuel in any proportion and stays blended even in cold temperatures. Biodiesel tends to store as well as diesel fuel. Consequently, mixed fuels were stored at room temperature in fuel storage containers. Biodiesel blends of B20, B50 and B5 were made with B100 feedstock. Blends were made by a procedure called splash blending; specific amounts of B100 were 'splashed' (poured on top) on specific amounts of diesel fuel and then agitated.

B20 Blending
A fuel storage container was filled with 8 litres of diesel, and then 2 litres of B100 was poured into the fuel container.

B50 Blending
A fuel storage container was filled with 5 litres of diesel, and then 5 litres of B100 B was poured into the fuel container.

B5 Blending
A fuel storage container was filled with 9.5 litres of diesel, and then 0.5 litres of B100 was poured into the fuel container.

3.11 Common Method Testing

Procedures and analytical tools used in the acute toxicity tests performed with both *D. magna* and *O. mykiss* followed guidelines from the Environmental Protection Agency's (EPA) *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. Testing used in this study, solely involved B100, B50, B20, B5 and diesel; the effects of components of diesel was not tested but the effects of diesel was compared to biodiesel and biodiesel blends. Although oil spills are dynamic in nature and may change chemically and physically over a period of time; this study examined the acute toxic effects of biodiesel and diesel concentrations over a short period of time.

3.12 Daphnia magna Acute Toxicity Test

Each 24 hour *D. magna* test involved placing groups of *D. magna* juveniles into a range of test substance concentrations and environmental control to which the daphnids were acclimated. Toxicity tests with *D. magna* were conducted in 200 ml samplers. For each concentration including controls, 4 replicate test chambers were used. All tests were conducted at 20 ± 1°C. Tests were conducted under static conditions with no renewal of the test solution. For all tests, temperature and photoperiod were similar to those of culture and holding conditions and kept constant between all tests. Observations for immobility and mortality were recorded after 24 hours. A daphnid was considered to be dead if there was no movement. The fuel was stirred into the water before the *D. magna* were introduced into the test chamber. There was a slight sheen of fuel on the top of some test chambers.

3.12.1 Daphnids Test Procedure

A range-finding test was followed by a definitive test; this type of test was performed in order to obtain information about the range of concentrations to be used in the main test. A static non-renewal test was done; trials of control without any test substances were conducted as well as trials of different concentrations of diesel, B100 and B20. Daphnids were exposed to the substance for a 24 hour duration. Temperature ranged within 20±1°C among test trials, the daphnids were not fed during the 24hr test period. The daphnids were inspected after the first 2 to 4 hours and at the 24-hour interval. Daphnids were considered dead if touching did not produce any reaction and no breathing movements were visible. Dead daphnids were removed when observed and mortalities were recorded.

3.12.2 D. magna Test Concentrations

A 24hr bioassay using the water flea, *D. magna* was conducted for this study. The fuels used were diesel, biodiesel (B100) and biodiesel blends of B20, B50 and B5. Range-finding tests on different concentrations were first performed; from the results obtained; definitive concentrations were then tested. Four trials were performed for each concentration; the concentrations tested for trials 1, 2, 3 and 4 were 1.57, 3.13, 6.25, 12.5 and 50ppm with DDI water as the control. All concentrations of the fuels tested were measured in parts per million (ppm).

3.12.3 Supplier of D. magna

The *D. magna* were obtained from Carolina Biological Supply and Ward's Science Ltd. All organisms tested were fed and maintained during culturing and acclimation. The test organisms appeared vigorous and in good condition prior to testing. The *D. magna* were placed below the test surface at test initiation due to the slight sheen of some test samples observed in the range-finding tests.

3.12.4 Equipment and Test Chambers

Separate holding and toxicity testing were conducted to prevent any loss of cultures due to cross-contamination (EPA, 2002). Water used for holding and test samples were DDI water left to stand for 5 days. During culturing and testing, daphnids were shielded from external disturbances such as pedestrian traffic. All material was thoroughly rinsed before use in the test trials.

3.12.5 Test Conditions

The temperature of the test solutions was measured by placing a thermometer into the samples. Temperature was recorded at test initiation and test termination. Test solution temperatures were maintained within the limits specified for trials.

3.12.6 Number of Test Organisms

Five daphnids were exposed to each concentration; in the toxicity test performed six concentrations and one environmental control (seven concentrations x 20 organisms per concentration) were included in a test trial; four trials were performed. Daphnids were captured from a common pool and distributed sequentially to the test chambers. Excessive handling and carryover of culture water was avoided by placing the tip of the transfer pipettes below the surface of the water in the test chambers and allowing the organisms to swim out of the pipettes without discharging the contents.

3.12.7 Replicate Test Chambers

Four test chambers were provided for each substance concentration as well as the control to increase data quality. The data from these replicate chambers were combined to determine the (i) mortality rate and (ii) lethal concentration (LC50).

3.12.8 Quality of Test Organisms

The health of daphnids was assessed by the performance (survival, growth and reproduction) of *D. magna* in culture tanks and control tests; notes on such performances were maintained.

3.12.9 Food Quality

The quality of the food used in culturing and testing invertebrates is an important factor in the study, daphnid food pellets were obtained from Carolina Biological Supply Ltd.

3.12.10 Culturing Procedure

Immediately upon receiving the daphnid culture, the daphnia culture was examined to determine if the culture arrived in satisfactory condition; health was determined by actively swimming daphnids. If the *D. magna* culture arrived in satisfactory condition, the lid was opened to permit air exchange. The daphnia culture was placed in an undisturbed location that was shielded from direct sunlight. A 1-gallon plastic aquarium was filled with DDI water, the aquarium water was allowed to stabilize for 24hrs; temperature was maintained at 20± 1°C. The lids from the jars of *D. magna* were removed and the jars were slowly submerged in the aquarium.

Daphnid culturing guidelines were taken *from Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA, 2002). Once the jar was completely filled and underwater, the culture of daphnia was gently turned into the aquarium. This prevented air bubbles from becoming trapped under the carapaces of the daphnids (EPA, 2002). Dried daphnia food pellets were sprinkled into the aquarium; the daphnids were fed every other day. Aerated DDI water was used as the culture medium, DO content in the culture were maintained above 6 mg/l; aeration of the culture medium was not necessary. A minimum of 16hr of illumination was provided each day. Two culture chambers were maintained to ensure back-up cultures were present so that in the event of a population 'crash' in one of the culture chamber (EPA, 2002), the entire *D. magna* population will not be

lost. All culture containers were washed thoroughly, after the culture was established, each chamber was cleaned weekly of accumulated food, debris and dead daphnids. The medium in each stock culture vessel was replaced two times each week with fresh medium (EPA, 2002).

3.12.11 Mortality of Culture Tank

Following a 24hr acclimation period, mortalities of the stock daphnid population were recorded. Mortalities are recorded at a 24-hour interval and the concentrations killing 50% of the daphnids (LC50) at 24hrs was calculated. For acceptable results, the mortality in the culture tanks and controls did not exceed 10% by the end of the test period.

3.13 Acute Toxicity of Fish

Test procedures were similar to those for the LC50 *D. magna* toxicity test; The LC50 toxicity test involved placing groups of fish (10 per concentration) in a range of concentrations of different fuels. The tests were conducted at controlled temperatures of 12 ± 2 °C. Solutions were gently aerated throughout the 96 hour exposure period. Tests were conducted under static conditions with no renewal of the test solution. For all tests, temperature and photoperiod were similar to those of holding conditions and kept constant throughout all tests. Observations for immobility and mortality were recorded after 24, 48, 72 and 96 hours. A fish was considered dead if there was no evidence of opercular activity and it showed no response to gentle prodding.

3.13.1 O. mykiss Test Procedure

A range-finding test was followed by a definitive test, in order to obtain information about the range of concentrations to be used in the main test. A static non-renewal bioassay was performed; trials with control (DDI water), i.e., without the test substance, diesel, B100 and B20 were performed. Fish were exposed to the toxins and the control for a period of 96 hours. There was a temperature range of 12± 2°C for any particular test trial; the *O. mykiss* were not fed during the 96hr test period. The fish were inspected after the first 2 to 3 hours and at consecutive 24-hour intervals thereafter. Dead fish were removed when observed and mortalities were recorded. Visible abnormalities, such as changes in swimming behaviour and pigmentation change were noted. Measurements of pH and temperature were carried out daily.

3.13.2 O. mykiss Test Concentrations

Range-finding tests on different concentrations were first performed; from the results obtained; definitive concentrations were then tested. Two trials were done for each concentration; the concentrations tested for trials 1 and 2 were 100, 300, 600, 900 and 1200 ppm with DDI water as the control. All concentrations of the test substance were given in parts per million (ppm). The rainbow trout bioassays were conducted in 5 litre containers, with 4L test solution. The samples were performed in duplicate with 10 organisms per trial. The photoperiod was 16 hours of light and 8 hours of darkness. The temperature range was 12± 2°C. The fuel was stirred into the water before the rainbow trout were introduced into the chamber, chambers were then aerated. Mortality was recorded after 24, 48, 72 and 96 hrs.

3.13.3 Supplier of O. mykiss

The *O. mykiss* used in the first round of tests were obtained from Linwood Acres Trout Farms Ltd. Campbellcroft, Ontario and were 6 weeks old and 151±2 mm in fork length. The rainbow trout were acclimated to test conditions (control water and temperature) for 14 days prior to test initiation. All trout fry were vigorous and in good condition prior to testing. A second batch of 30 day old rainbow trout hatchlings, at the alevin stage, was obtained from Blue Springs Trout Farm Ltd, Hanover, ON. All fish appeared healthy and in good physical condition.

3.13.4 Equipment and Test Chambers

Separate holding and toxicity testing chambers were provided to prevent possible loss of fish due to cross-contamination as well as to reduce the risk of compromised test results. All material was thoroughly cleaned before use in the trials. Water temperatures for the holding tanks and test chambers were maintained by a refrigerator. During holding and testing, rainbow trout frys were shielded from external disturbances such as rapidly changing light conditions and pedestrian traffic (EPA, 2002).

3.13.5 Test Conditions

The temperature of test solutions was measured by placing a thermometer directly into the test solutions. Temperature was recorded continuously in at least one holding tank during the duration of each test; temperature of the water supply was also measured daily. Test solution temperatures were maintained within the limits specified for each test. Experimental temperature

was also measured by placing a thermometer outside the environmental chambers; environmental temperatures were also measured daily.

3.13.6 Number of Test Organisms

A minimum of 20 fish were exposed to each concentration. Toxicity test involved five concentrations and a control; fish were used from common pool and distributed to the test chambers. A minimum of two trials were performed for all concentrations; trials done in triplicate were noted.

3.13.7 Replicate Test Chambers

Two test chambers were provided for each biodiesel concentration and the control. Although the data from duplicate chambers are usually combined to determine the LC50 and confidence interval values; replicate trials were performed for each fuel concentration. This was done because it: (i) permitted easier viewing and counting trout frys, (ii) avoided possible excess loading of fish, which might occur if all of the test organisms are placed in a single test chamber and (iii) ensured against the invalidation or compromise of the test which might result from accidental loss of a test vessel, when all of the test organisms for a given treatment are in a single chamber (EPA, 2002).

3.13.8 Quality of Test Organisms

The health of the frys was assessed by the performance of rainbow trout (*O. mykiss*) in holding tanks and control tests. Rainbow trout performance was measured by survival and growth data. Mortality data was also recorded for fish in the holding tanks.

3.13.9 Food Quality

Food used in holding and testing fish was an important factor in toxicity testing; rainbow trout fish food was obtained from Linwood Acres Trout Farm Ltd.

3.13.10 Holding and Handling Test Organisms

All fish were previously exposed to water of the quality and the temperature used in the trials for at least fourteen days before they were used. Test organisms were not subjected to changes of more than 3°C in water and environmental temperatures in any 12hr period, particularly when organisms were being transferred from holding chambers to test chambers. Fish were handled as little as possible; when handling was necessary, it was done as gently, carefully and quickly as possible to minimize stress to the trout (EPA, 2002); dipnets were used to handle the swim-up frys. Holding tanks for fish were supplied with DDI water; with the aid of a recirculation system, the control water flowed through an activated carbon filter to remove dissolved metabolites; crowding was also avoided. The dissolved oxygen (DO) content was maintained at a minimum of 6.0 mg/l; the standard required for cold-water freshwater species (EPA, 2002). Fish were fed once a day with commercially prepared fish food (EPA, 2002). Excess food and fecal material was removed from the bottom of the tanks at least once every two days by siphoning. Holding

tank water was changed every two days. Each day rainbow trout frys were observed for signs of disease, stress, physical damage and mortality (EPA, 2002). Dead and abnormal specimens were removed as soon as observed. A daily record of feeding, behavioural observation and mortality was maintained.

3.13.11 Control Water & Holding Water

DDI water was used both as the control and holding water. A given batch of holding water was not used for more than five days following preparation because of the possible build-up of bacterial, fungal or algal slime growth and the problems associated with it. The control water was aerated at least 24hrs prior to use in the toxicity tests and holding water was continuously aerated and filtered. DDI water was kept at temperature of 12± 2°C.

3.13.12 Mortality of Holding Tank

Following a 48-hour acclimation period, mortalities were recorded and the following criteria applied:

(i) If after seven days, mortality is greater than 10 % of the total population, the entire batch was rejected.

(ii) If after seven days, mortality is between 5 and 10% of the total population, the holding period is to be continued for seven additional days. Then, if no further mortalities occurred; the batch is acceptable; otherwise it must be rejected.

(iii) If after seven days, mortality is less than 5 % of the total population, the batch was accepted (EPA, 2002).

The fish were exposed to water with the added test substances at a range of concentrations for a period of 96 hours. Mortalities were recorded at 24-hour intervals and the concentrations killing 50% of the fish (LC50) at each observation time are calculated where possible. The mortality in the controls did not exceed 10% of the entire population by the end of the test (EPA, 2002).

3.14 Monitoring of Bioassays

The static non-renewal tests were monitored at test initiation and termination for pH and hardness and every 24 hours thereafter for mortality. Temperature was monitored continuously throughout the test periods. A static non-renewal test was used; there was no renewal of test solution.

3.15 Data Analysis

The objective of acute toxicity testing is to identify discharges of toxins and chemicals in acutely toxic amounts. Results are derived from tests designed to determine the adverse effects of toxins in aquatic waterways on the survival of the test organisms. The toxicity test consists of a control and five or more concentrations of fuel; the endpoint of these tests was mortality. The results of the endpoint of the multi-concentration test gives an estimate of the fuel concentration which is lethal to 50% of the test organisms in the time period prescribed; the value is expressed as the LC50 (EPA, 2002). Mortality rate can also be estimated by the mortality data obtained for each fuel tested. Control survival must be 90% or greater for an acceptable test; the test is acceptable if survival in the control equals or exceeds 90% (EPA, 2002).

3.16 Acceptability of Acute Toxicity Test Results

For the test results to be acceptable for both the daphnid (*D. magna*) and rainbow trout (*O. mykiss*) experiments, the control survival must equal or exceed 90%. Tests are acceptable if temperature, DO content and other specified conditions fall within certain specifications. Any deviation from test specifications outlined in the procedure guidelines was noted when reporting data from test trials.

3.17 Statistical Analysis

(*1*) Survival raw data in replicate exposure chambers was analyzed using a one-way analysis of variance (ANOVA) statistical tool. This analysis determined whether replicates were significantly different from each other and the control. If a significant difference between replicates exists, the cause for the difference was determined (EPA, 2002), however if there was no significant difference, the trials were continued. If no significant difference was observed, replicates were pooled for further analyses. Mortality results obtained for each set of organisms were then tabulated and analysed.

(2) Concentration-mortality-time responses for all test substances were analysed for any significant trends in mortality rate as a function of time and concentration.

(3) The LC50 values for both *D. magna* and *O. mykiss* were determined by the Trimmed Spearman-Karber Method developed by Hamilton et al., 1977.

(4) Mortality rates for diesel, B100, B50, B20 and B5 were compared to each other in order to develop a rank order of toxicity among the five substances tested.

(5) Results of B20 blended in the lab were tested with B20 commercially blended and obtained from a filling station.

(6) Lethal Concentrations (LC50) for Diesel, B100, B50, B20 and B5 were compared to each other in order to develop a rank order of toxicity among the five substances tested.

3.18 Concentration-Mortality-Time Relationship

A concentration-mortality-time analysis was conducted to determine if there was any relationship between concentration and mortality and mortality and time among the five different fuels used in the toxicity tests.

3.19 LC50 Determination

The number of dead organisms per group was recorded against the time of their death. The data obtained was used to calculate the median lethal concentration (LC50) of the fuel on *D. magna* juveniles and *O. mykiss* (rainbow trout) frys (Dede and Kaglo, 2001). Mortality rates for diesel, B100, B50, B20 and B5 were compared to each other in order to develop a rank order of toxicity among the five substances tested.

4. RESULTS

DAPHNIA MAGNA

4.1 D. magna Observations

Initially, it was the intention that *Daphnia pulex* be used in the range-finding and definitive tests, however, after the initial culturing phase, it was determined that these daphnids were too small to be observed for any signs of mobility or mortality. It was very difficult to detect mobility or any other signs of death from physical observations. *Daphnia magna* were then used in the acute toxicity experiments; they responded better than the *D. pulex*. However, it was just as difficult to get viable results; 100% mortality of *D. magna* was observed at 60, 80 and 100ppm for diesel fuel as well as for B100 and B20, as indicated in Appendix B. Mortality was noted by the lack of daphnid movement, i.e., immobilization; similar observations were recorded for daphnids affected by diesel as well as those affected by neat biodiesel as well as the biodiesel blends.

Diesel

There was a slight sheen of fuel on the surface of the water and many daphnids were trapped as soon as they were placed in the test chamber (on average 6hrs), because of this a pipette was used to insert daphnids beneath the water surface.

B100, B50, B20 and B5

Neat biodiesel and biodiesel blends also had similar observations to those conducted with diesel. A sheen was present on the water surface of some samples and the daphnids experienced similar effects to those exposed to diesel. Mortality was noted by the lack of swimming and the lack of

physical movement by the daphnids. Some of the mortality seen in the tests may have been caused by the physical nature of the test substances.

4.2 D. magna Results

Results of Range-Finding Tests

A range-finding test was done to determine a range of concentrations to be administered for the definitive tests. A range of 35ppm to 100ppm was performed in the initial testing stage. There was less than 100% mortality for organisms at 35ppm and more than 100% mortality at 100ppm, as noted in Appendix B.

Control Survival

The controls met the overall survival acceptability standards; more than 90% of the control population were alive after the 24hr test period.

Significant Difference

There was a difference in the survival means of the experimental control groups when compared to the survival means from the daphnids treated with diesel, B100, B50, B20 and B5. There was no significant difference between trials.

Mortality

Mortality results are listed in Appendix B. Table 1 illustrates the average percent mortality of four trials of each concentration of the five test substances (Diesel, B100, B50, B20 and B5). There is a general increase in mortality as concentration increases. The highest mortality was

recorded at the highest concentration tested, i.e. 50ppm, for all test substance. Similarly, the lowest mortality was recorded at the lowest concentration of 1.57ppm.

Table 1: The average mortality rate for *D. magna* after 24hrs.

Concentration [ppm]	*Mean Mortality Rate (%)*				
	Diesel	**B100**	**B50**	**B20**	**B5**
1.57	40.00	34.17	42.50	40.00	45.00
3.13	75.00	45.00	55.00	40.56	60.00
6.25	85.00	55.00	75.00	60.00	80.00
12.5	90.00	60.00	80.00	65.00	90.00
25	90.00	70.00	80.00	71.67	90.00
50	90.00	71.67	85.00	69.43	100.00

Figure 1 illustrates the average mortality rate over of all fuels tested within 24hrs. There is a recorded increase in mortality rate with increasing concentrations. Only B20 showed a slight decrease in percent mortality from 71.67% to 69.43% for *D. magna* treated with 25 and 50ppm respectively.

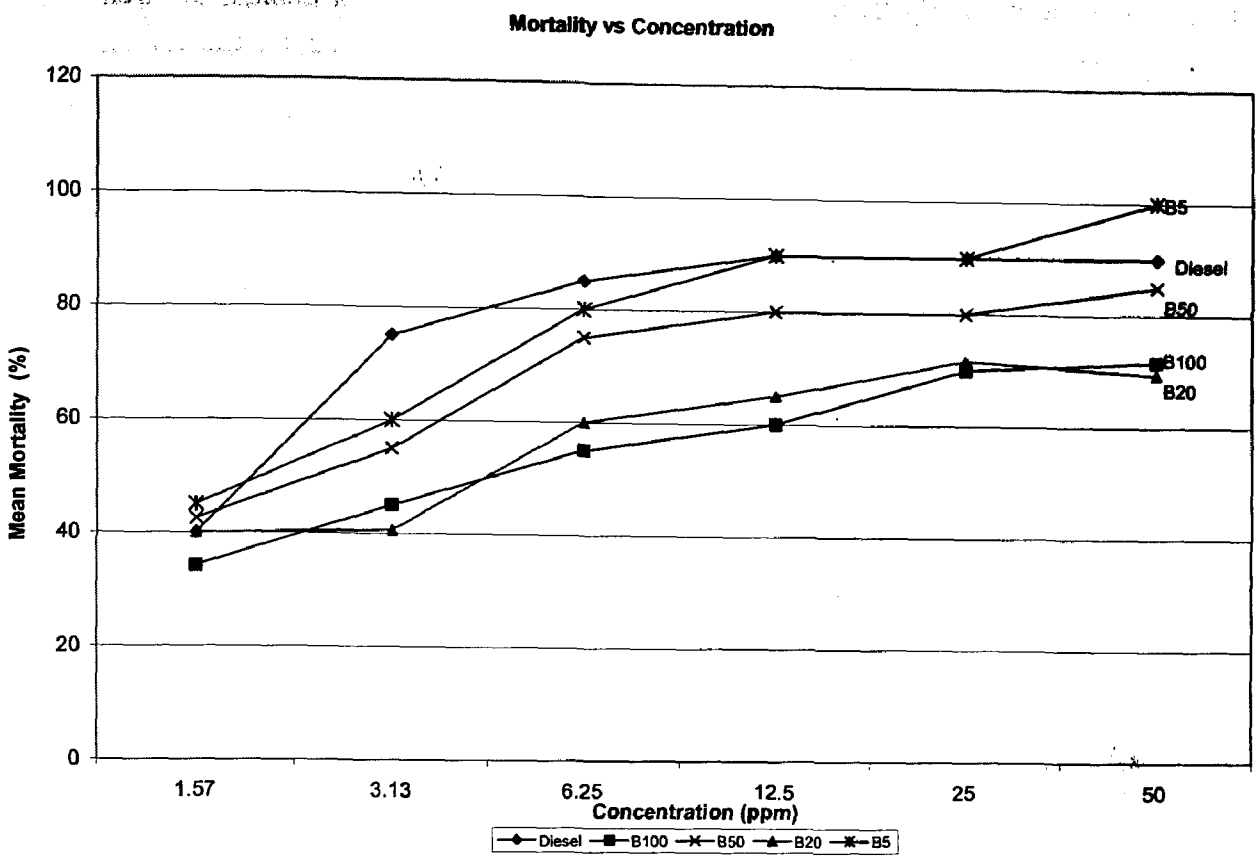

Figure 1: The average mortality rate of *D. magna* at different concentrations of different fuels over a period of 24hrs

Table 2 shows the overall mortality rate of all concentration over 24hrs; the mortality rate represents the percentage of dead daphnids over all six concentrations over the 24hr time period.

Table 2: The overall mortality rate for *D. magna*

Mean Mortality Rate (%)				
Diesel	**B100**	**B50**	**B20**	**B5**
78.34	55.98	69.58	57.78	77.50

Diesel fuel has the highest mortality rate of 78.34%; 78.34% of all daphnids treated with diesel fuel died. B100 has the lowest average mortality rate of 55.98%, which was slightly lower than B20 (57.78%).

Figure 2 shows the mortality trends for diesel, biodiesel and biodiesel blends; the trend shows that diesel has the highest mortality, closely followed by B5 (77.50%), B50, B20, while B100 has the lowest mortality.

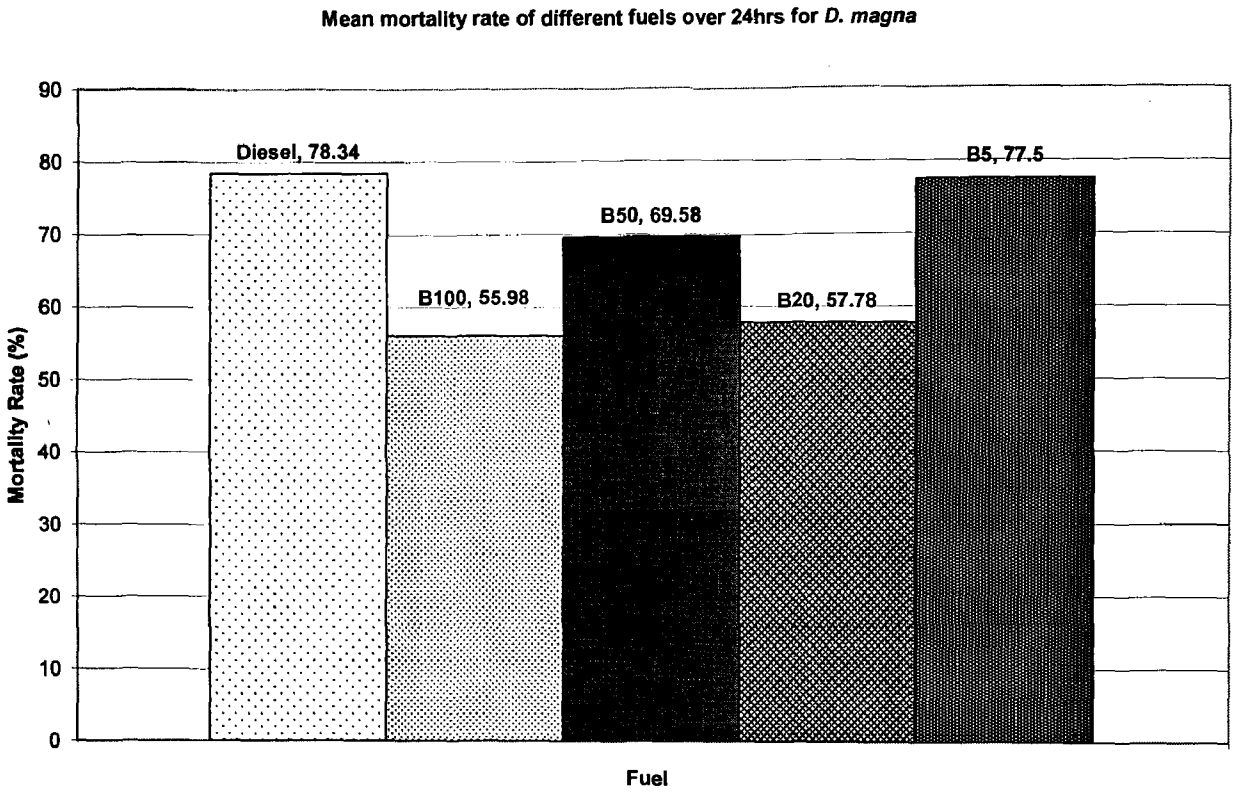

Figure 2: The mean mortality rate of *D. magna* for different concentrations over a period of 24 hrs

Lethal Concentration (LC50)

The lethal concentration that kills 50% of the test population was also calculated using the Trimmed Spearman-Karber Method. Table 3 shows the LC50 of diesel, B100, B50, B20 and B5. B100 had the highest LC50 of 4.65ppm, while diesel had the lowest of 1.78ppm.

Table 3: The LC 50 of Acute Toxicity Test Using *D. magna*

Fuel	*LC 50*	*Lower 95%*	*Upper 95%*	*% Trim*
Diesel	1.78	1.15	2.76	44.44
B100	4.65	2.22	9.72	31.82
B50	3.29	1.36	7.95	41.18
B20	4.54	2.55	8.09	38.64
B5	1.98	0.92	4.23	45.00

Figure 3 shows the LC50 trend, there are small differences in the lethal concentrations calculated for the different fuels tested. Diesel has the lowest 24hr-LC50, followed by B5, B50, B20 and B100 has the highest 24hr-LC50. The lowest static *D. magna* 24hr-LC50 is indicative of the highest toxicity level, which was 1.78ppm for Diesel, the highest static 24hr-LC50 value was determined for B100 at 4.65ppm, which was the least toxic of all the toxins tested over the 24hr period.

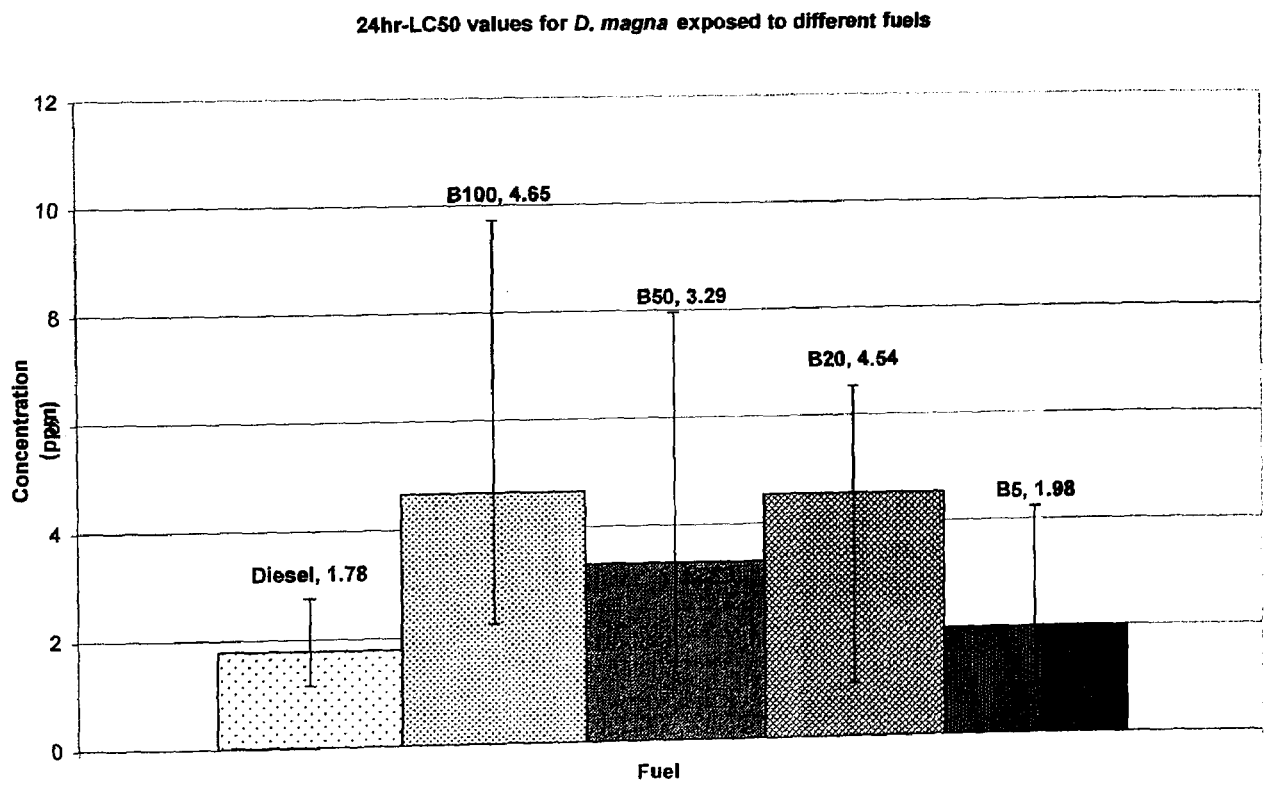

Figure 3: The 24hr- LC50 values for Diesel, B100, B50, B20 and B5

4.3 D. magna B20 Comparison

Mortality Rate

A comparison of B20 and B20 (Topia) was performed; daphnids treated with B20 (Topia) were subjected to the same concentrations as daphnids exposed to B20. There was no significant difference in mortality among the four sets of trials. Table 4 shows the average mortality rate of these 4 trials over a 24hr period. For both B20 and B20 (Topia), mortality rate increases as concentration increases.

Table 4: The average mortality rate for *D. magna* affected with B20 and B20 (Topia) for 24hrs

Concentration (ppm)	***B20***	***B20(Topia)***
1.57	40.00	35.00
3.13	40.56	39.55
6.25	60.00	55.00
12.5	65.00	55.00
25	71.67	75.00
50	69.43	69.29

Figure 4 illustrates the trend obtained from B20 (Topia) compared to those obtained from B20. Mortality rate increases with increasing concentration, however, B20 has slightly higher mortality rates for each concentration when compared to B20 (Topia).

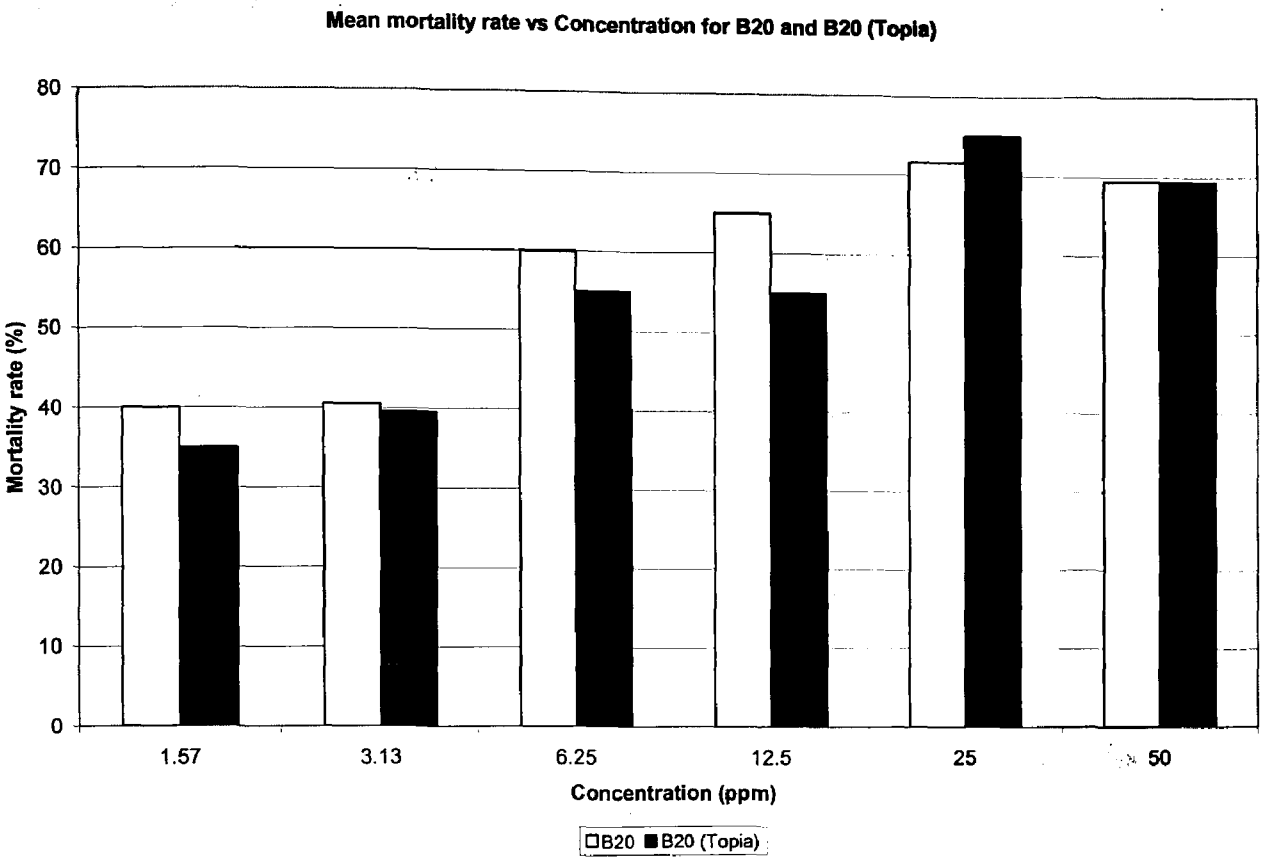

Figure 4: Comparison of B20 and B20 (Topia) mortality rates

Figure 5 shows the overall mortality rate of all concentrations, over the 24hr period, B20 has the higher mortality rate of 57.78%, while B20 (Topia) has a mortality rate of 54.81%, a difference of 2.97%

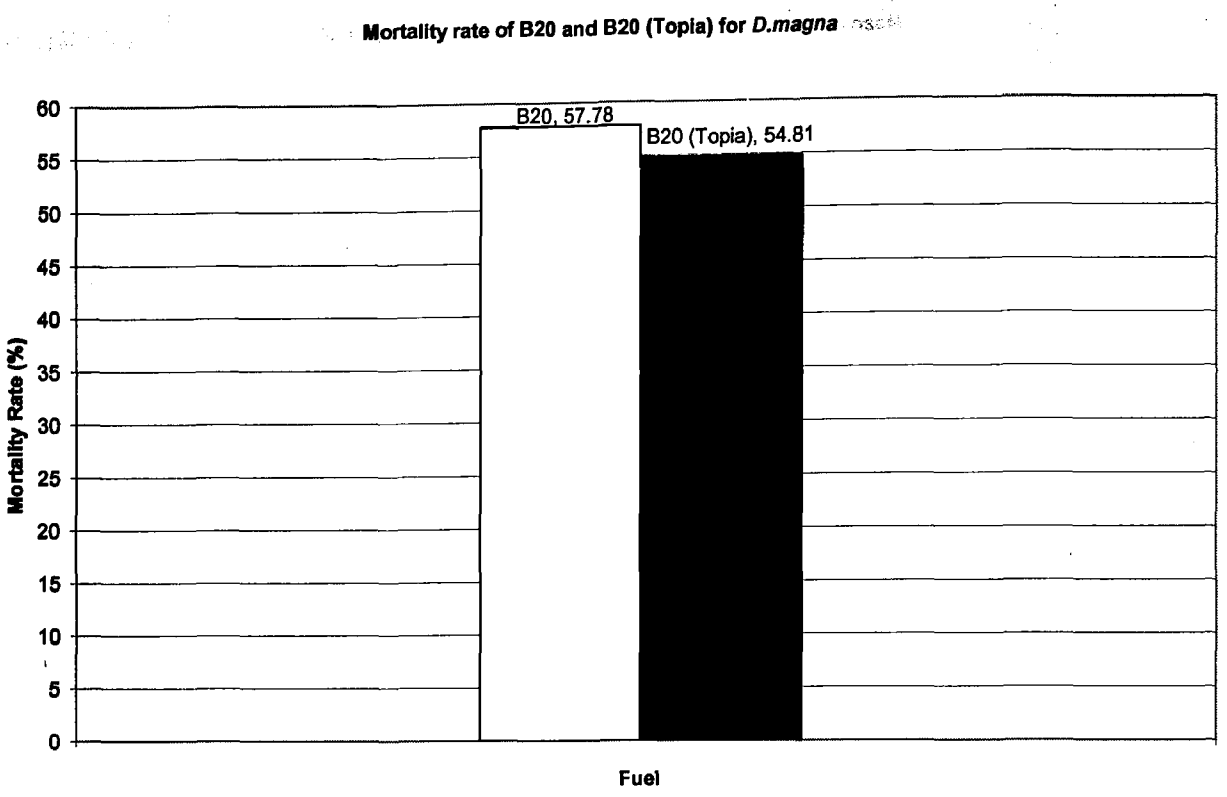

Figure 5: The average 24hr mortality rate of B20 and B20 (Topia)

Lethal Concentration

Lethal concentration (LC50) which killed 50% of organisms was calculated using the Trimmed Spearman-Karber Method. Table 5 illustrated the LC50 and confidence intervals obtained for both B20 and B20 (Topia).

Table 5: The 24hr-LC50 value for *D. magna* treated with B20 and B20 (Topia)

Fuel	*LC50*	*Lower 95%*	*Upper 95%*	*Trim*
B20	4.54	2.55	8.09	38.94
B20 (Topia)	6.74	3.25	13.96	34.78

Figure 6 illustrates the 24hr-LC50 values obtained for both B20 and B20 (Topia); the 24hr-LC50 value for B20 is 4.54ppm, while the 24hr-LC50 value for B20 (Topia) is 6.74ppm, there is a difference of 2.2ppm.

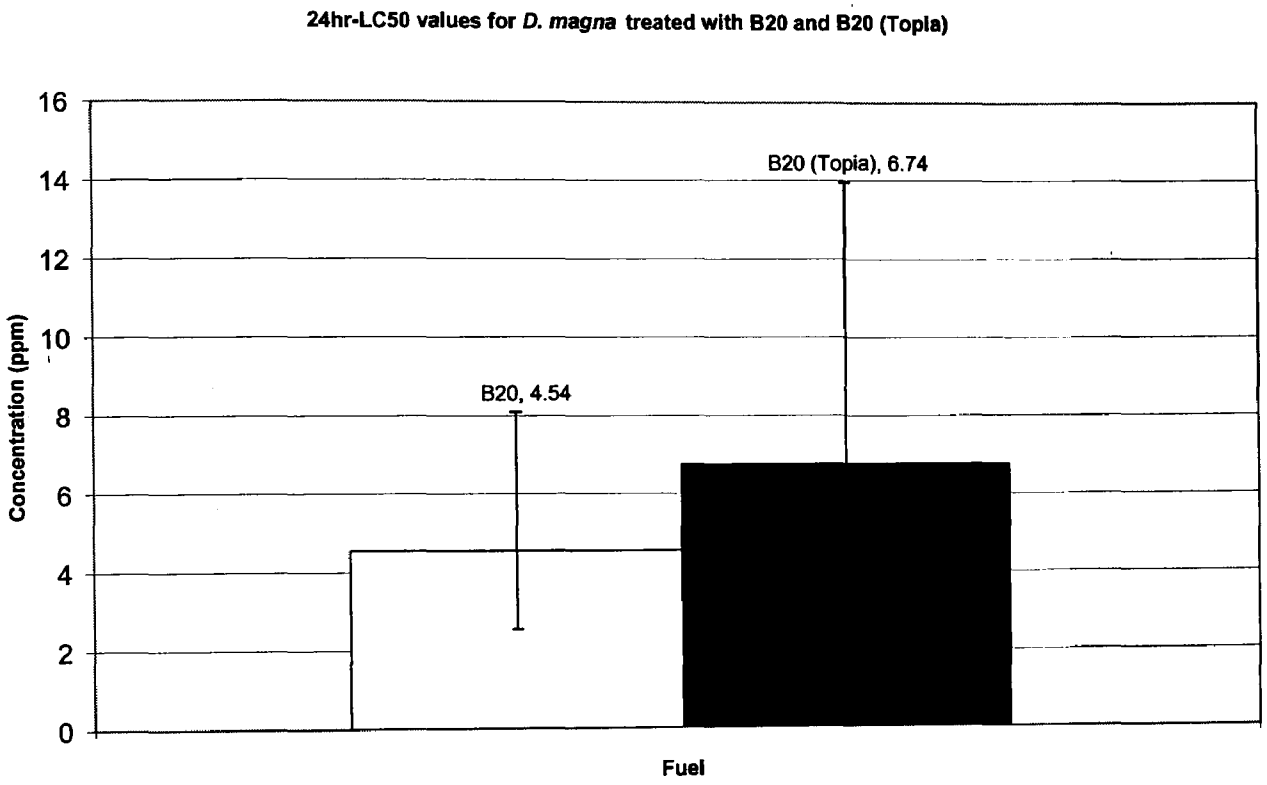

Figure 6: A comparison of B20 and B20 (Topia) 24hr-LC50 values

4.4 O. mykiss Observations

There are different stages in the life of the *O. mykiss* species; it was suggested in the *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms* (EPA, 2002) that fish between 28 and 30 days be used in toxicity experiments. However, it was determined at this stage the trout are still feeding off their embryonic (yolk) sac; they are unable to eat and do not swim but remain at the bottom of the holding tank. These fish are at alevin life stage; they are very sensitive to light, water, movement and temperature. Consequently, it was difficult to determine if fish mortality was due to ambient experimental conditions or to actual acute effects of the fuels tested. In toxicity experiments, it was too difficult to use the 15-30 day old trout results since there is uncertainty in the cause of mortality. Instead, 6-week old trout frys were used, at this stage the trout fry are able to feed off commercial fish food and are able to swim from the bottom of the holding tank to the surface. In the toxicity tests conducted with the different fuels, death or mortality was noted by lack of movement and breathing.

Diesel

Before eventual death, fish swam erratically up and down the test chamber, there appeared to be irregular breathing; there was also irregularity in the movement of their gills. The trout fry also developed slimy mucus on their body with an almost a 'half-eaten' appearance; their fins and tails were torn and tattered. Another sign of death was the bulging eyes and discolouration of the fish from a brown to greyish black. Many rainbow trout were swimming on their sides, dead fish

were found at the bottom of environmental chambers. Some fish experienced curling after being exposed to diesel concentration of 600ppm and above.

B100, B50, B20 and B5

The trout fry showed very similar signs to the affects of diesel and biodiesel blends. There was the irregularity in gill movement and haphazard swimming from the bottom of the environmental test chamber to the surface. There was a consistent bulging of the eyes which was followed by detachment from the eye socket, at death, eyes appeared white. There was also fin erosion on frys affected with the neat biodiesel and the biodiesel blends. However, fin erosion was more pronounced in fish exposed to B50 and B5, when compared to B100 and B20. Some curling was also present for B100, B50, B20 and B5 for concentrations of 600ppm and above, dead fish were also found at the bottom of environmental test chambers.

General Observations

Surviving fish were in poor condition; barely breathing with slow and erratic swimming. There was an increase in fry movements; some fry exhibited spiral swimming. Forced efforts to swallow air from the surface were also observed; the mouth and the gills of dead fish were gaping, particularly after 72 and 96hrs. Many fry had increased amounts of mucus secretion around the gills and on the body surface, as well as darkening of the fish body. For a short period of time, many fish were in a coma-like state prior to death; where there were no body motions except weak movements of the gills. At death, the mouth was usually gaping and the gills were widely extended.

4.5 O. mykiss Results

Results of Range-Finding Tests

Range-finding tests were conducted on 4 and 6-week old *O. mykiss* frys, range-finding tests are documented in Table 6 were conducted on 6-week old *O. mykiss*. A range-finding test was performed to determine a range of concentrations to use for the definitive tests. Three fuels were used; diesel, B100 and B20. A test range of 2ppm to 90 ppm was first performed on these substances. There was less than 50% mortality recorded from 2.5ppm to 50ppm for diesel, B100 and B20. A second range finding test was done, the concentrations tested were 100, 1000 and 2000ppm, as listed in Table 6. NOEC for diesel, B100 and B20 were observed between 2.5-50ppm and between 2.5-50ppm for diesel. LOEC for B100 and B20 was recorded at 100ppm and 90ppm was recorded for diesel.

Table 6: The results of the *O. mykiss* range-finding tests

Concentration (ppm)	*Average Survival of 2 Trials* after 96hrs*		
	Diesel	**B100**	**B20**
2.5	10	10	10
10	10	10	10
50	10	10	10
90	9.5	10	10
100	8.5	9	10
1000	1.5	3	2
2000	0	0	0

** 10 fish per trial*

Control Survival

All control groups met the overall survival acceptability standards; there was less than 10% mortality in all experimental control groups.

Significant difference

There was a difference in the survival means of the experimental control groups when compared to the survival means from the daphnids treated with diesel, B100, B50, B20 and B5. However, there was no significant difference between trials.

Mortality Data

Mortality results are listed in Appendix C. A concentration-mortality-time comparison was made for each test substance. Two trials were conducted for each concentration and the average of these trials was used in the comparisons.

Figure 7 shows the average mortality (in percent) for *O. mykiss* exposed to different concentrations of diesel across time. The data exhibit the typical concentration-response, with mortality increasing with increasing concentration of toxin over the exposure period. The average mortality of concentrations increased from 50.33% after 24 hr to 64.50% after 48 hr, to 80% at 72 hr and 85.33% at the end of the 96-hour exposure.

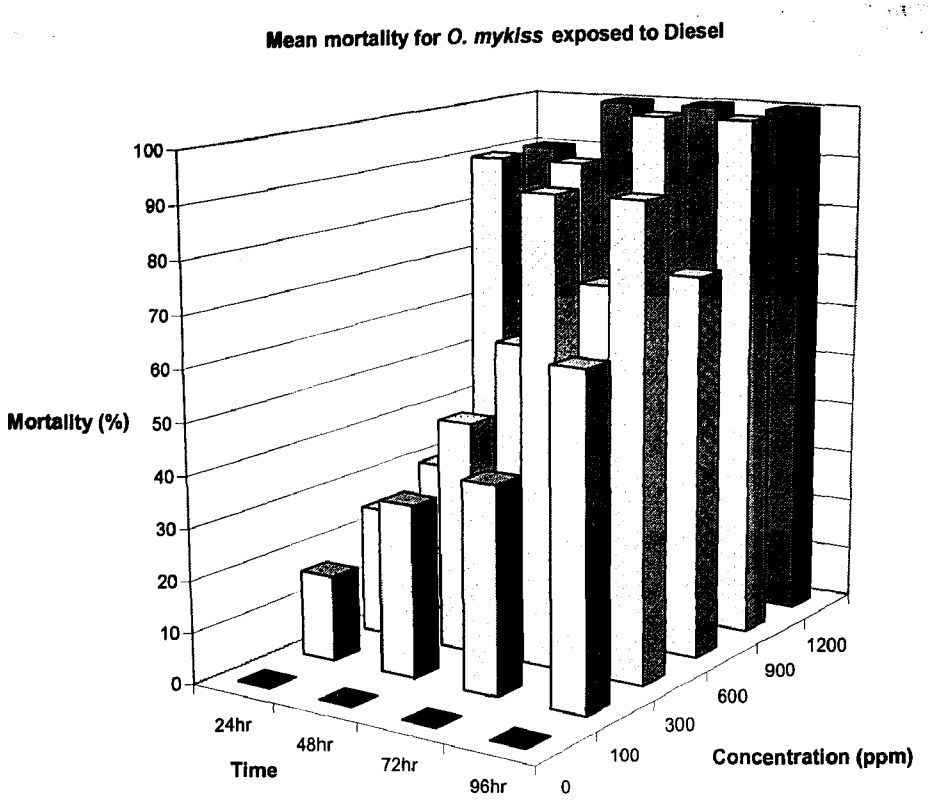

Figure 7: The results of the definitive tests with Diesel using *O. mykiss*

Figure 8 shows the average percent mortality for rainbow trout fry exposed to different concentrations of B100 across time. The data exhibit the typical concentration-response, with mortality increasing with increasing concentration of toxin over the exposure period. The average mortality increased from 25.33% after 24 hrs to 37.33% after 48 hrs, to 49.33% at 72 hrs and 57.98% at the end of the 96-hour exposure.

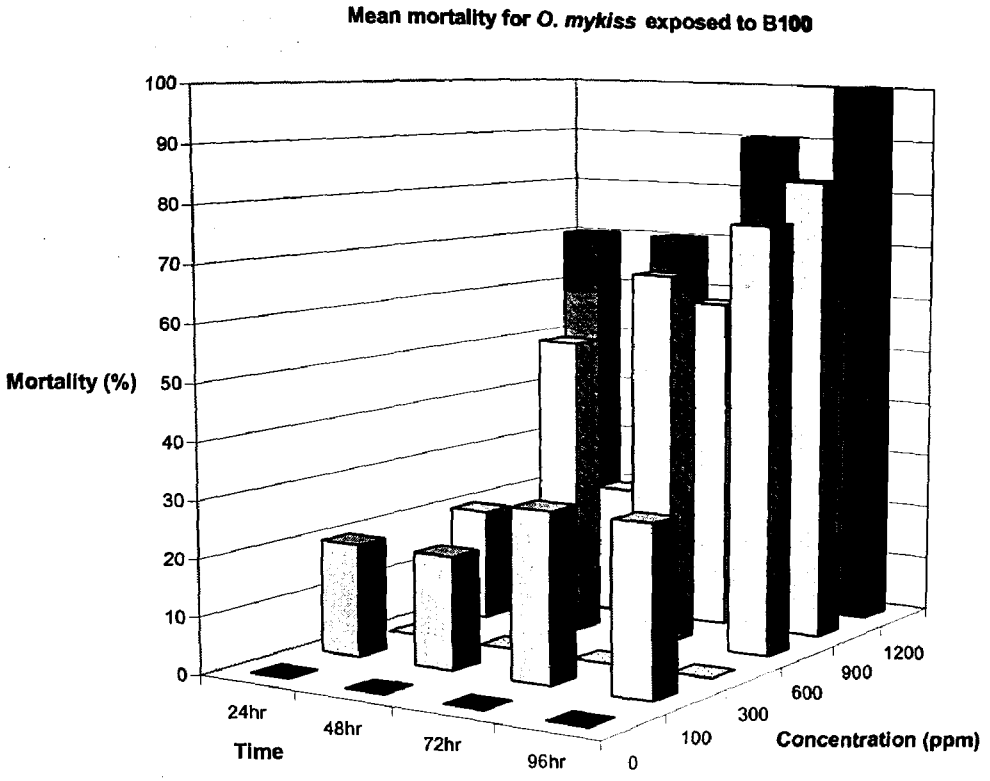

Figure 8: The results of the definitive tests with B100 using *O. mykiss*

Figure 9 shows the average percent mortality for *O. mykiss* exposed to different concentrations of B50 across time. The data exhibit the typical dose-response, with mortality increasing with increasing concentration of toxin over prolonged exposure period. The average mortality increased from 22% after 24 hrs to 50% after 48 hrs, to 64% at 72 hrs and 71% at the end of the 96-hour exposure.

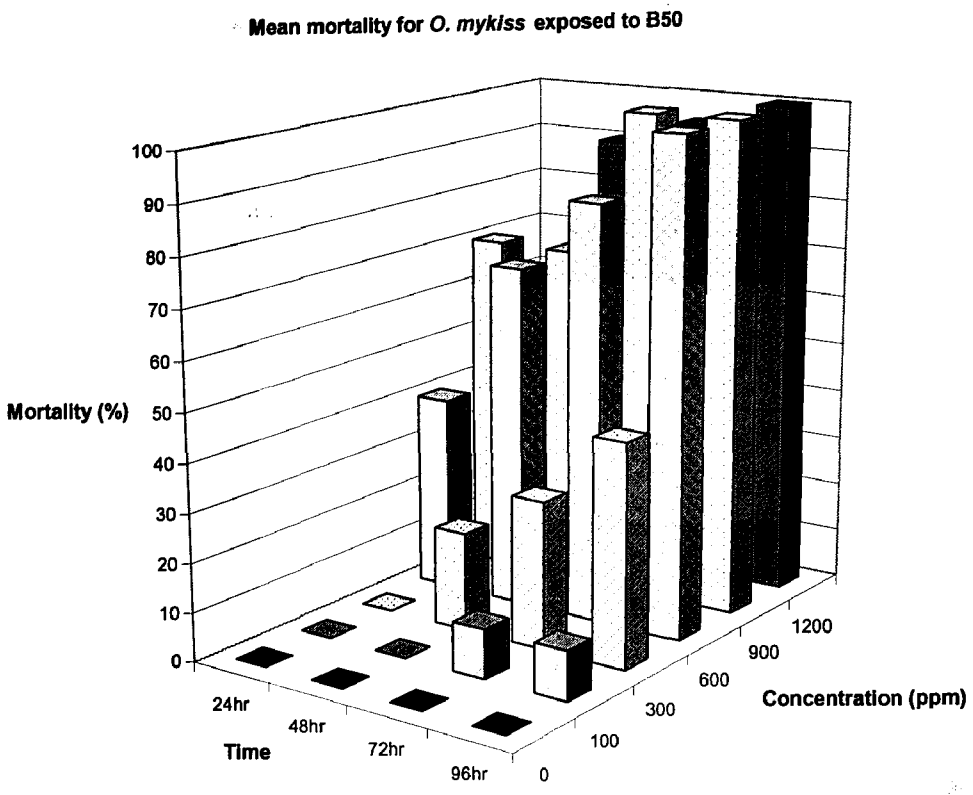

Figure 9: The results of the definitive tests with B50 using *O. mykiss*

Figure 10 shows the average percent mortality for rainbow trout fry exposed to different concentrations of B20 across time. The data exhibit the typical concentration-response, with mortality increasing with increasing concentration of toxin over prolonged exposure period. The average mortality increased from 24.67% after 24 hrs to 41.67% after 48 hrs, to 53.67% at 72 hrs and 59% at the end of the 96-hour exposure.

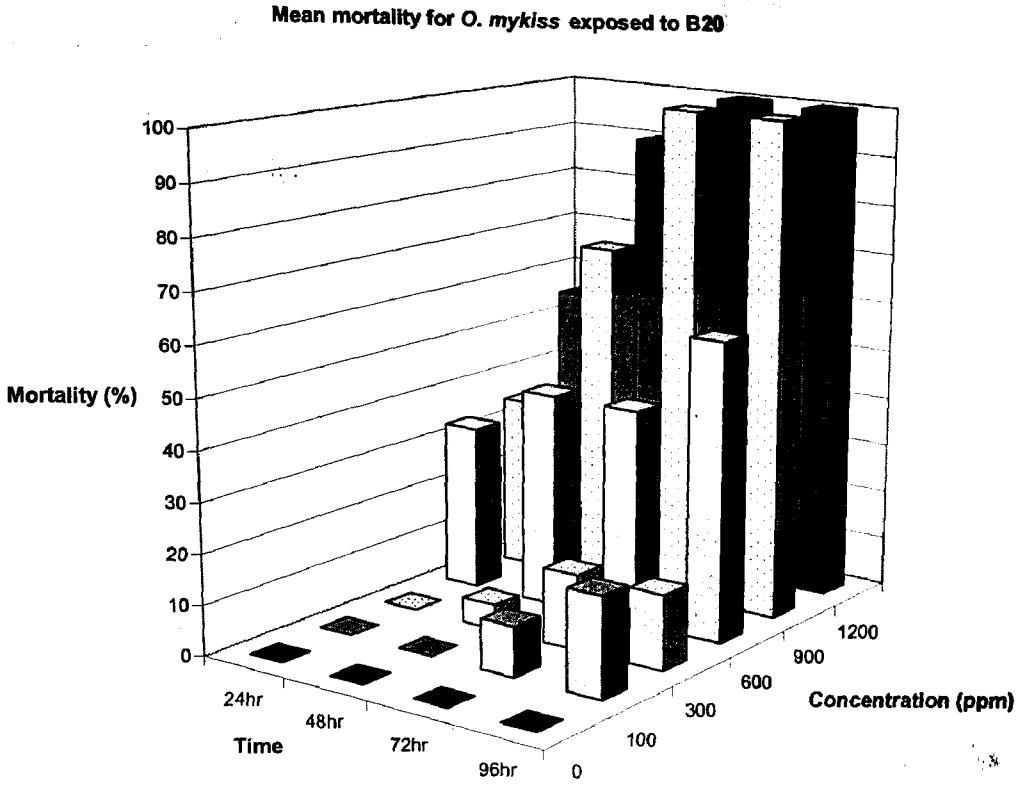

Figure 10: The results of the definitive tests with B20 using *O. mykiss*

Figure 11 shows the average mortality (in percent) for *O. mykiss* exposed to different concentrations of B5 across time. The data exhibit the typical concentration-response, with mortality increasing with increasing concentration of toxin over prolonged exposure period. The average mortality increased from 35% after 24 hrs to 53% after 48 hrs, to 70% at 72 hrs and 83% at the end of the 96-hour exposure.

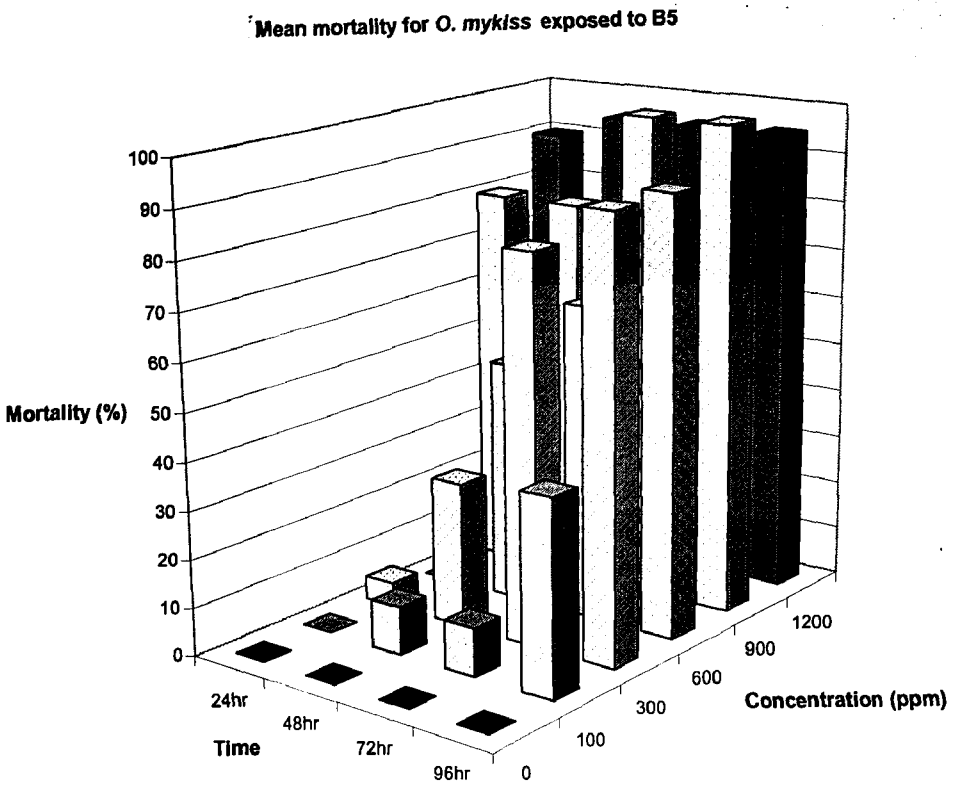

Figure 11: The results of the definitive tests with B5 using *O. mykiss*

The mean mortality of each concentration was calculated and the average over the four time periods (24hr, 48hr, 72hr, and 96hr) was tabulated, as illustrated in Table 7.

Table 7: The average mortality rate for *O. mykiss* at each fuel over 96hrs

Concentration [ppm]	*Average Mortality Rate (%) of 2 replicates over 96hrs*				
	Diesel	**B100**	**B50**	**B20**	**B5**
100	38.33	25.00	5.00	7.50	15.00
300	62.50	0.00	23.75	8.75	51.25
600	57.50	54.16	73.75	45.00	51.25
900	95.00	45.83	85.00	76.25	90.00
1200	97.50	87.50	71.25	86.25	93.75

There was a general increase in average mortality rate for each fuel had over the 96hrs; B100 had a slight decrease from 25% to 0% from 100ppm to 300ppm and a decrease from 54.16% at 600ppm to 45.83% at 900ppm as illustrated in Figure 12. B50 at 1200ppm had a slight decrease from 85% (at 900ppm) to 71.25 %. B100 had a higher mortality rate for 100, 600 and 1200ppm when compared to B20.

Figure 12 shows the regression plots for the five test substances based on mean percent mortality values over 96hrs in function of concentration. Trend analysis indicates that there is a small difference among the slopes of the five fuels; there is a relatively proportional change in percent mortality for every measured change in concentration for the different toxins.

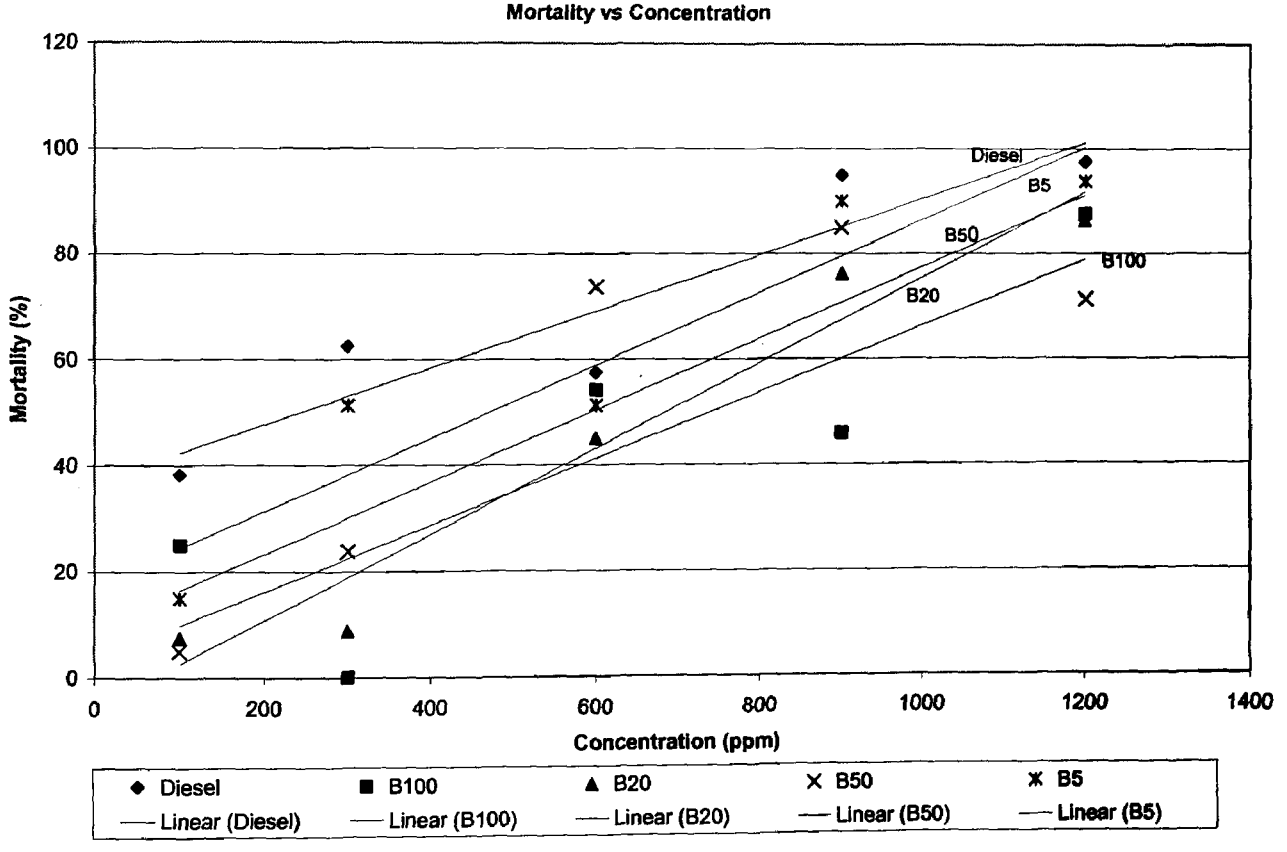

Figure 12: Regression plots for different fuel over 96hrs

Diesel has the largest mortality rate for all concentrations measured; B100 has the lowest recorded mortality for all measured concentrations. B50 has a slightly higher mortality rate for 600 and 900ppm, when compared to B20.

Mortality Rates

The mean mortality rates were calculated for the two replicates conducted for each concentration of the different fuels over a period of 96hrs; these results are summarised in the Table 8. Diesel has the highest mortality rate of 70.17%, while neat biodiesel has the lowest mortality rate of 42.5% over 96hrs.

Table 8: The overall mortality rate for *O. mykiss* over 96hrs

Mean Mortality Rate (%) of all five concentrations of the 2 replicates over 96hrs				
Diesel	**B100**	**B50**	**B20**	**B5**
70.17	42.50	51.75	44.75	60.25

Figure 13 illustrates the cumulative mortality rate of all five substances tested; neat biodiesel, B100, B20, B50 and B5. From the results obtained, diesel (70.17%) appears to be the most toxic, while B100 seems to be less toxic as B100 has the lowest recorded mortality rate (42.50%). B20 (44.75%) has a slightly lower mortality rate than B50 (51.75%); B5 has a mortality rate of 60.25%.

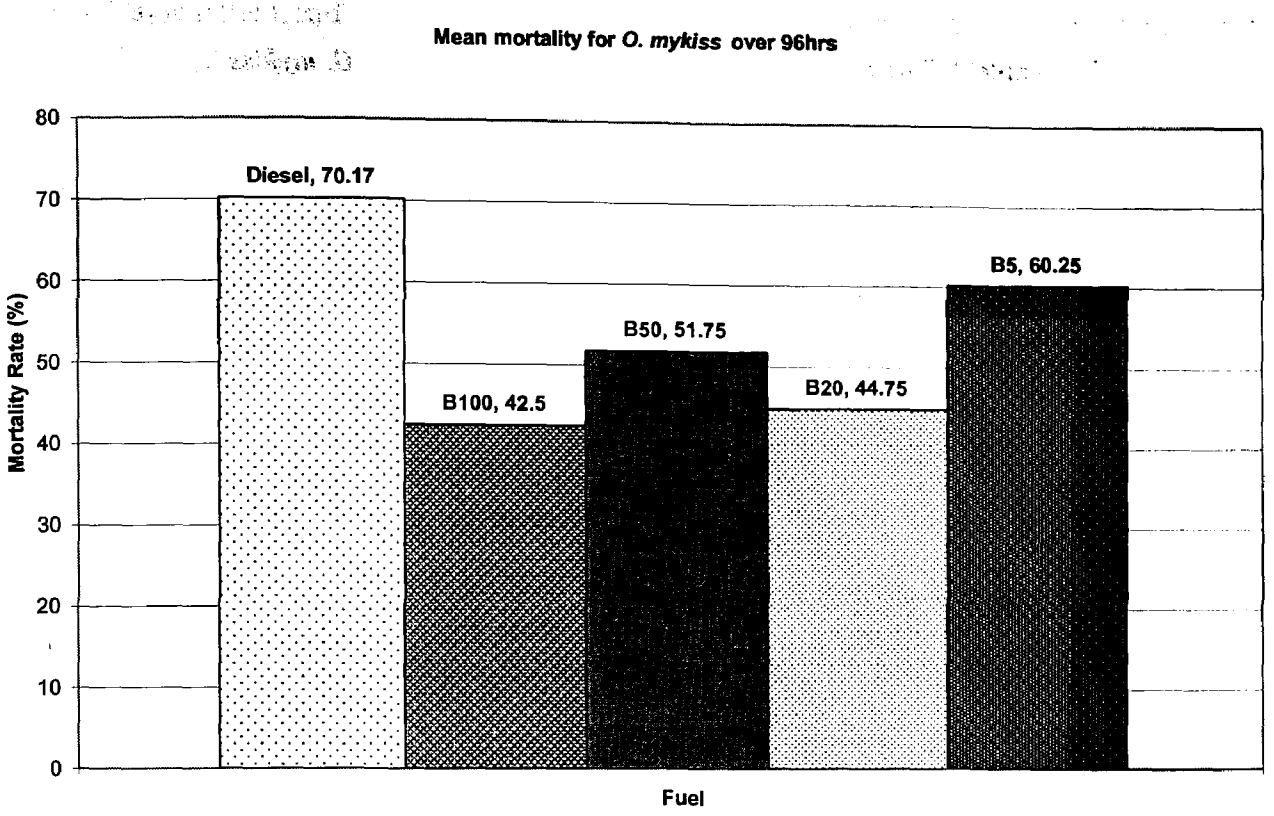

Figure 13: The mean mortality rate of *O. mykiss* for different concentrations over a period of 96hrs

Lethal Concentration

Lethal concentration that kills 50% (LC50) of the population was also calculated using the Trimmed Spearman-Karber Method. Upper and lower confidence intervals for all test substances were also calculated using the same procedure.

1) Diesel

Table 9 illustrates the LC50 calculated for *O. mykiss* treated with different concentrations of diesel fuel at 24, 48, 72 and 96hr.

Table 9: The LC 50 of Diesel acute toxicity test using *O. mykiss*

Time	*LC 50*	*Lower 95%*	*Upper 95%*	*% Trim*
24hrs	578.13	421.28	793.37	16.67
48hrs	350.38	173.04	709.47	33.33
72hrs	133.52	88.94	200.45	40.00
96hrs	NC	NC	NC	NC

*NC= Not Calculable

LC50 decreases over the 96hr period. The LC50 at the endpoint of 96hr was not calculable since no trials reported results with 50% or more survival rate. LC 50 decreases over time; with the highest resistance to the diesel fuel at 24 hrs with a LC50 of 578.13ppm. However, there is a steady decrease in LC50 over time, as illustrated in Figure 14.

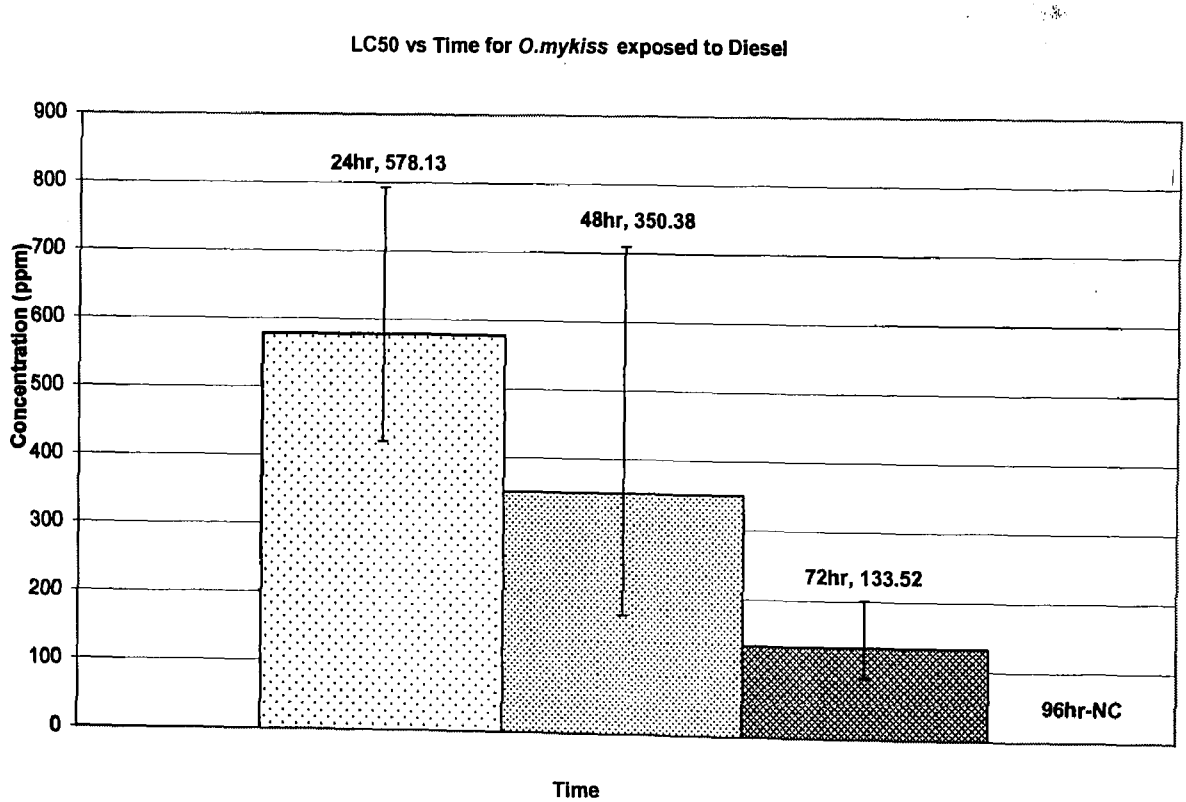

Figure 14: The LC50 values for Diesel

Figure 15 shows the trend of the mean LC50 values for diesel through time. The equation of the line offers an idea of how diesel behaves through time. A slope of –150.71 indicates that the LC50 of diesel decreases every 24 hr at a rate of 150.71ppm.

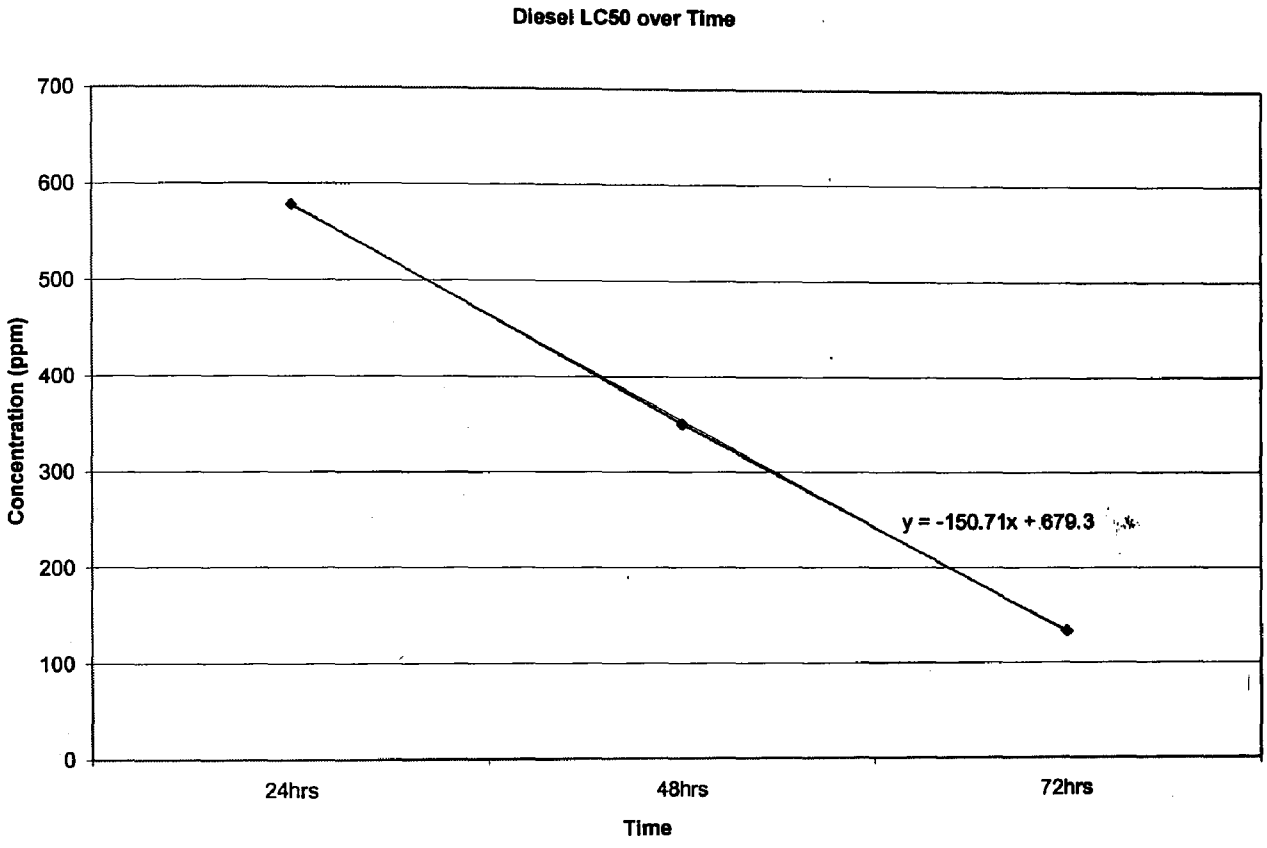

Figure 15: The mean LC50 trend through time for *O. mykiss* exposed to Diesel

2) Biodiesel

(i) B100

Table 10 shows the 50% lethal concentration calculated for *O. mykiss* treated with different concentrations of B100 fuel at 24hr, 48hr, 72 hr and 96hr.

Table 10: The LC 50 of B100 acute toxicity test using *O. mykiss*

Time	*LC 50*	*Lower 95%*	*Upper 95%*	*% Trim*
24hrs	1073.54	994.69	1158.63	30
48hrs	756.68	646.56	885.56	10
72hrs	555.19	462.94	665.82	15
96hrs	455.28	391.30	529.73	15

LC50 decreases over the 96hr period, as illustrated in Figure 16. The LC 50 at the endpoint of 96hr is approximately 455ppm. There is a consistent decrease in LC50 over the 96hours; the highest LC50 of 1073.54ppm occurs after 24hrs.

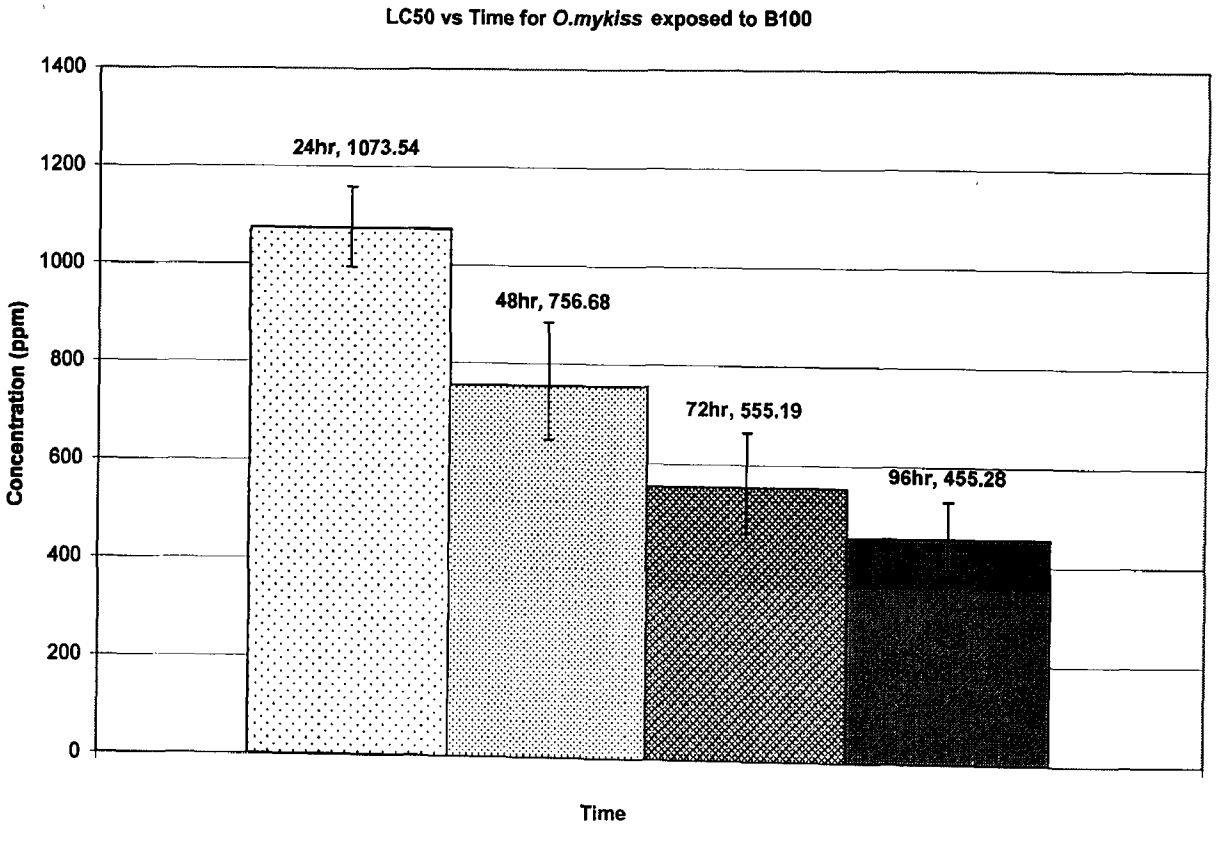

Figure 16: The LC50 values for B100

Figure 17 shows the trend of the mean LC50 values for B100 through time. The equation of the line offers an idea of how B100 behaves through time. A slope of –205.63 indicates that the LC50 of B100 decreases every 24 hr at a rate of 205.63ppm.

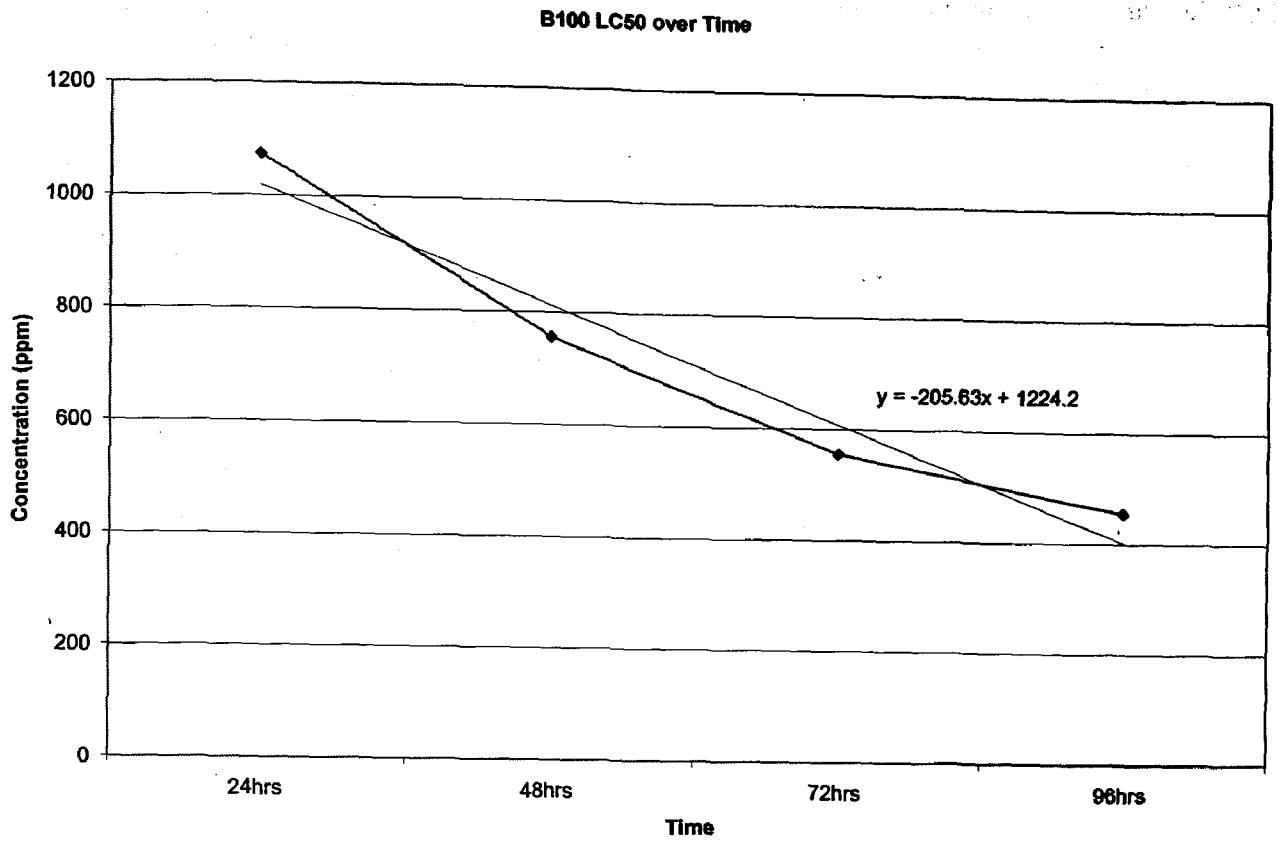

Figure 17: The mean LC50 trend through time for *O. mykiss* exposed to B100

(ii) B50

Table 11 illustrates the LC50 calculated for *O. mykiss* treated with different concentrations of B50 fuel at 24, 48, 72 and 96hrs.

Table 11: The LC 50 of B50 acute toxicity test using *O. mykiss*

Time	*LC 50*	*Lower 95%*	*Upper 95%*	*% Trim*
24hrs	NC	NC	NC	NC
48hrs	491.11	386.78	623.59	10
72hrs	348.32	267.24	453.99	10
96hrs	276.71	212.79	359.82	10

* NC = Not Calculable

LC50 decreases over the 96hr test period as shown in Figure 18. The LC50 at the endpoint of 96hr is approximately 277ppm. The LC50 for 24hrs could not be accounted for by the Trimmed Spearman-Karber Method since there were no mortalities within the 0.5 (50% mortality) bracket.

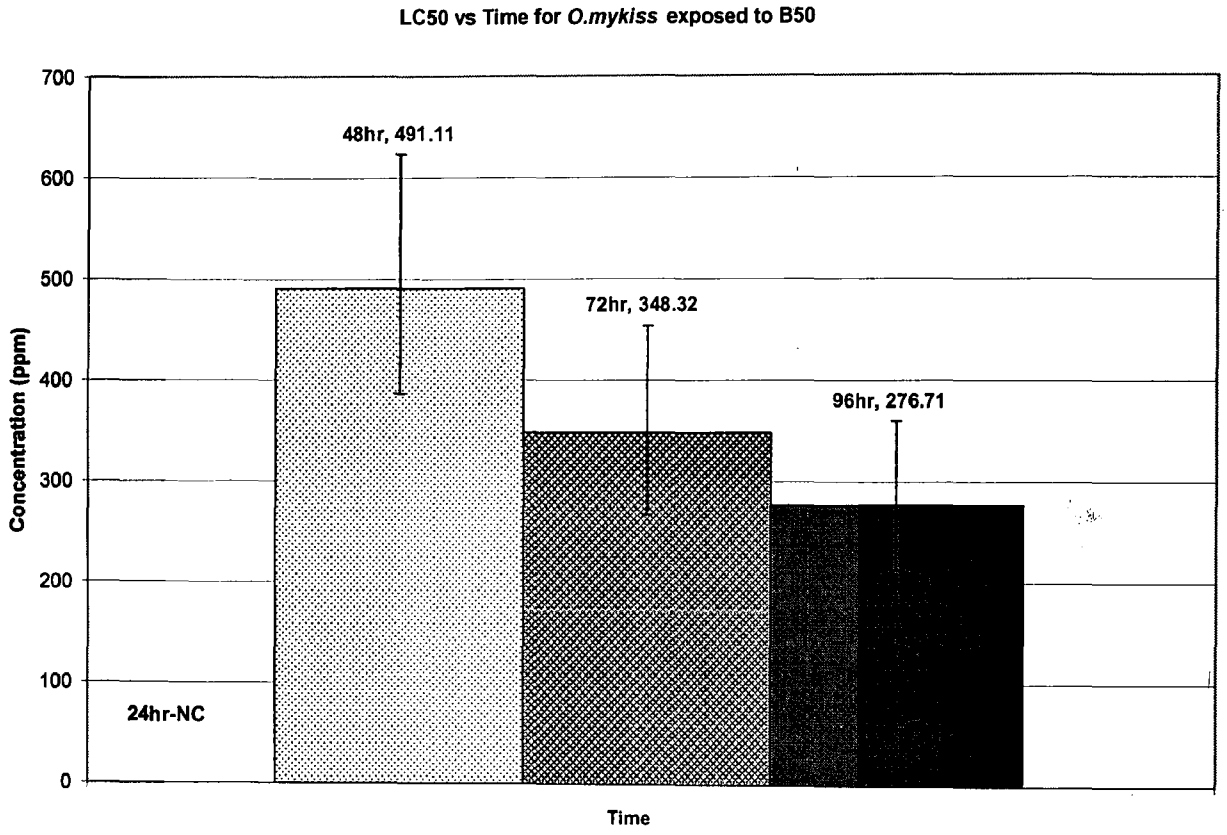

Figure 18: The LC50 values for B50

Figure 19 shows the trend of the mean LC50 values for B50 through time. The equation of the line offers an idea of how B50 behaves through time. A slope of –107.2 indicates that the LC50 of B50 decreases every 24 hr at a rate of 107.2ppm.

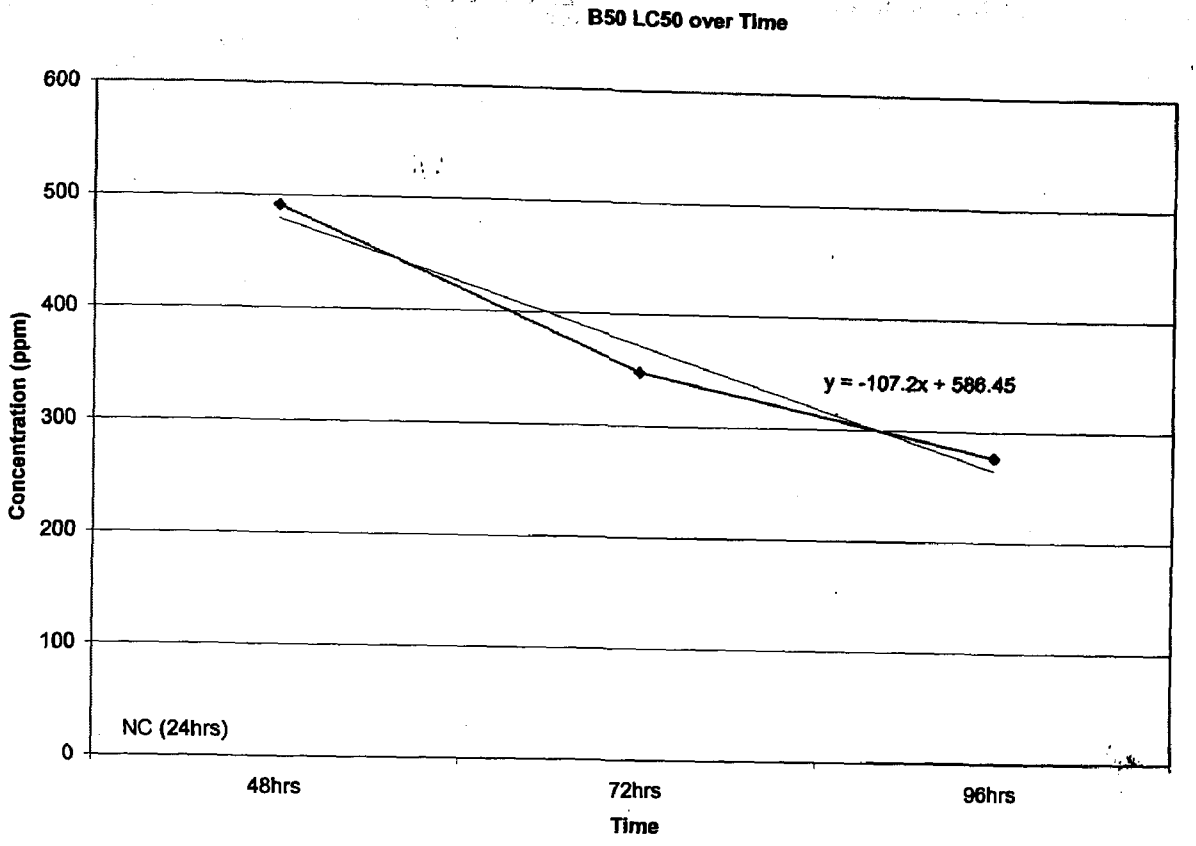

Figure 19: The mean LC50 trend through time for *O. mykiss* exposed to B50

(iii) B20

Table 12 illustrates the LC50 calculated for *O. mykiss* treated with different concentrations of B20 fuel at 24hr, 48hr, 72 hr and 96hr.

Table 12: The LC 50 of B20 acute toxicity test using *O. mykiss*

Time	*LC 50*	*Lower 95%*	*Upper 95%*	*% Trim*
24hrs	1074.31	752.15	1534.46	45
48hrs	659.02	566.51	766.64	10
72hrs	541.27	427.62	685.13	10
96hrs	497.60	421.00	588.15	17.50

LC50 decreases over the 96hr period. The LC 50 at the endpoint of 96hr is approximately 498ppm; as shown in Figure 20. There is a consistent decrease in the LC50 over the 96 hours.

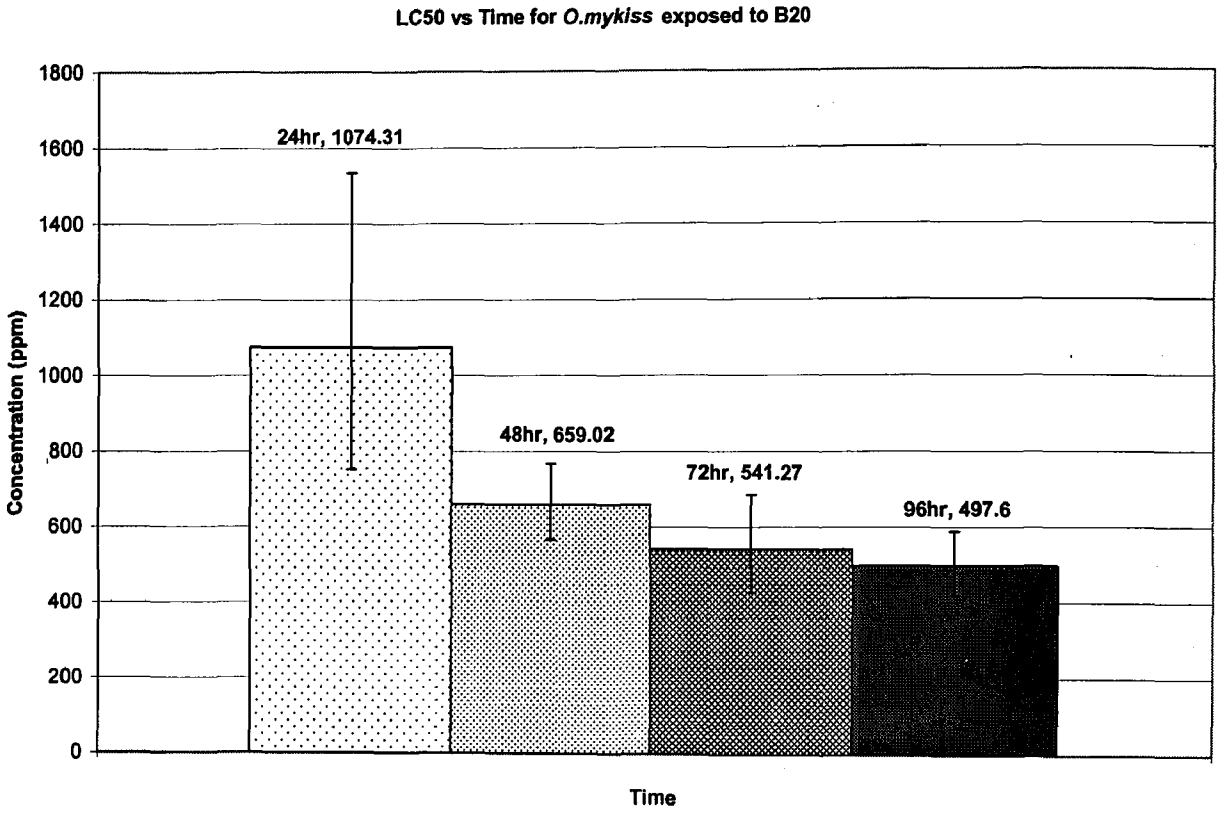

Figure 20: The LC50 values for B20

Figure 21 shows the trend of the mean LC50 values for B20 through time. The equation of the line offers an idea of how B20 behaves through time. A slope of –184.79 indicates that the LC50 of B20 decreases every 24 hr at a rate of 184.79 ppm.

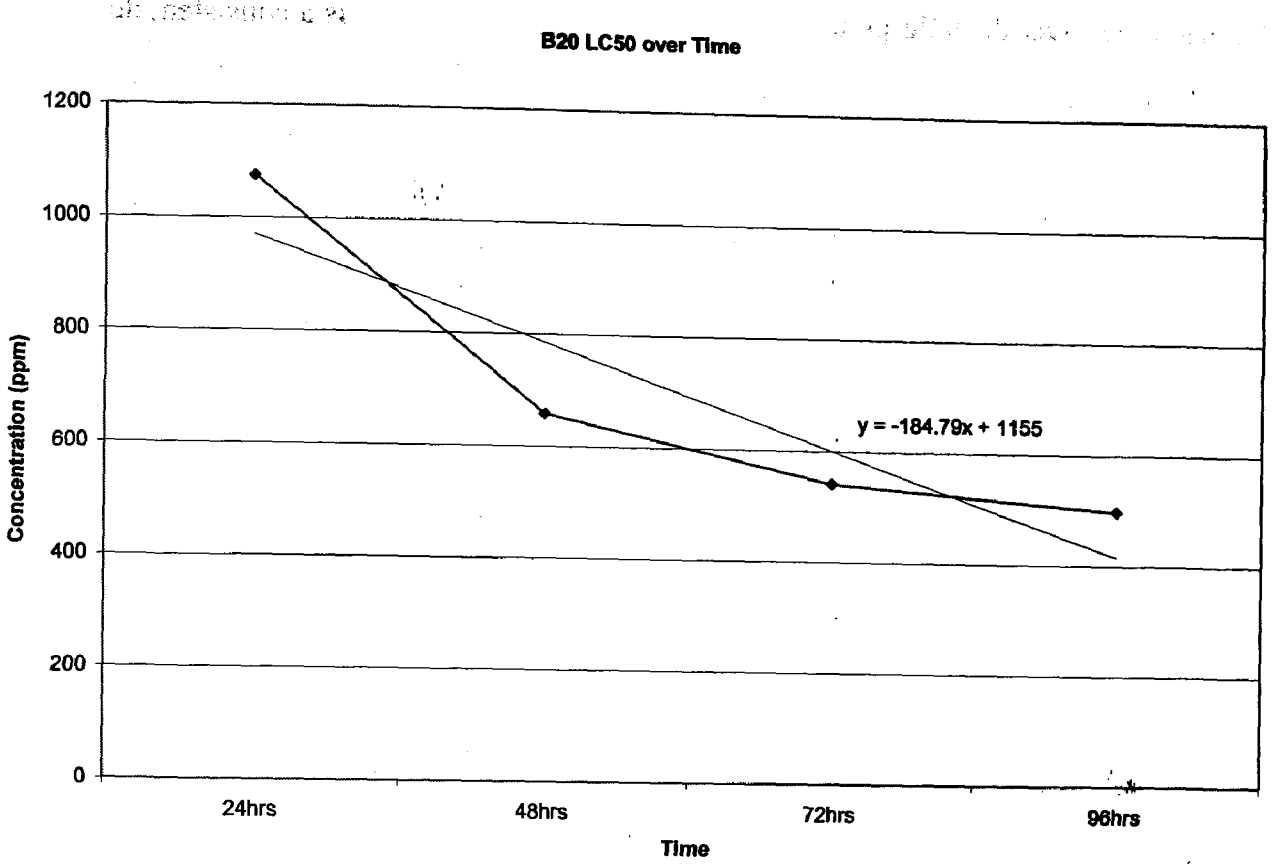

Figure 21: The mean LC50 trend through time for *O. mykiss* exposed to B20

(vi) B5

Table 13 illustrates the LC50 calculated for *O. mykiss* treated with different concentrations of B5 fuel at 24, 48, 72 and 96hrs.

Table 13: The LC 50 of B5 acute toxicity test using *O. mykiss*

Time	*LC 50*	*Lower 95%*	*Upper 95%*	*% Trim*
24hrs	780.67	718.61	848.09	10
48hrs	463.30	343.07	625.65	10
72hrs	234.47	177.93	308.97	10
96hrs	129.57	84.38	183.92	40

LC50 decreases over the 96hr period; as indicated in Figure 22. There is a consistent decrease in the LC50 over the 96hours. The LC 50 at the endpoint of 96hr is approximately 125ppm.

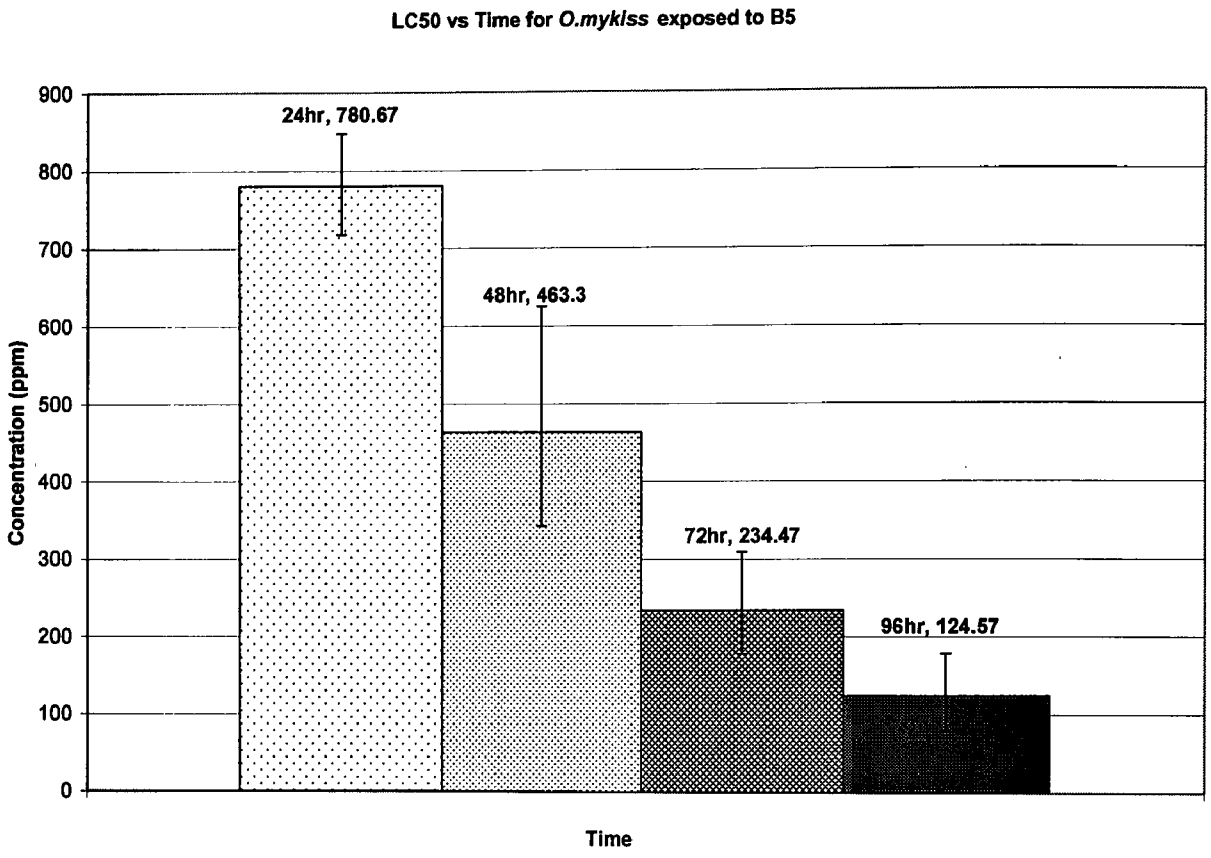

Figure 22: The LC50 values for B5

Figure 23 shows the trend of the mean LC50 values for B5 through time. The equation of the line offers an idea of how B5 behaves through time. A slope of –218.21 indicates that the LC50 of B5 decreases every 24 hr at a rate of 218.21ppm.

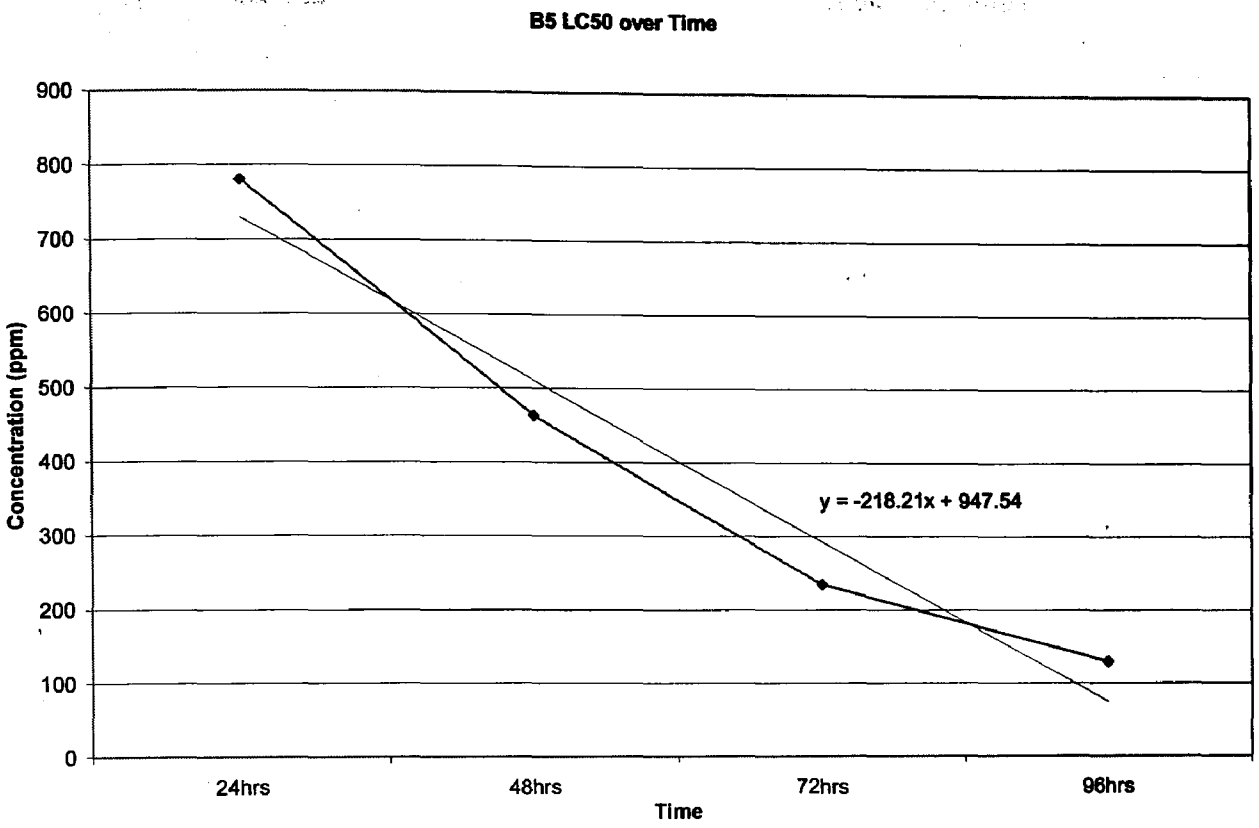

Figure 23: The mean LC50 trend through time for *O. mykiss* exposed to B5

The results of the definitive toxicity tests for the five fuels are tabulated in Table 14. The data are expressed as average LC50s of two trial runs in function of time. The mean LC50 values for diesel ranged from a minimum of 133.52ppm to a maximum of 578.13ppm, a difference of a factor of 4.33. B100 LC50 values ranged from 455.28ppm to a maximum of 1073.54ppm, a factor difference of 2.36; B50 ranged from 276.71ppm to 491.11ppm, a factor difference of 1.77. B20 ranged from 497.60ppm to 1074.31ppm, a factor difference of 2.16, B5 ranged from 129.57ppm to 780.67ppm, a factor difference of 6.03.

Table 14: The LC50 values for Diesel, B100, B50, B20 and B5

Time	Diesel	B100	B20	B50	B5
24hrs	578.13	1073.54	1074.31	NC	780.67
48hrs	350.38	756.68	659.02	491.11	463.30
72hrs	133.52	555.19	541.27	348.32	234.47
96hrs	NC	455.28	497.60	276.71	129.57

There is a consistent decrease in the LC50 for all the fuel types over the 96hr period. Table 15 shows the average LC50 over the four exposure periods. The average LC50 value for diesel and B50 are not true indications for a toxicity ranking as LC50 at 24hrs for B50 was not available since not deaths were greater than 50% of the population. Also, deaths at 96hrs for diesel exceeded 50%; the program used was not able to calculate a value for lethal concentration.

Table 15: The average LC50 over 24, 48, 72 and 96hrs

Diesel *(ppm)*	**B100** *(ppm)*	**B50** *(ppm)*	**B20** *(ppm)*	**B5** *(ppm)*
354.01± 128.36	710.17 ± 136.38	372.71 ± 63.02	693.05 ± 131.58	402.00 ±144.17

Fig 24 shows all the LC50 values obtained for diesel, biodiesel and biodiesel blends. 24hr-LC50 values are the highest recorded values for all test substances while the lowest recorded lethal concentration values were observed at the 96hr period.

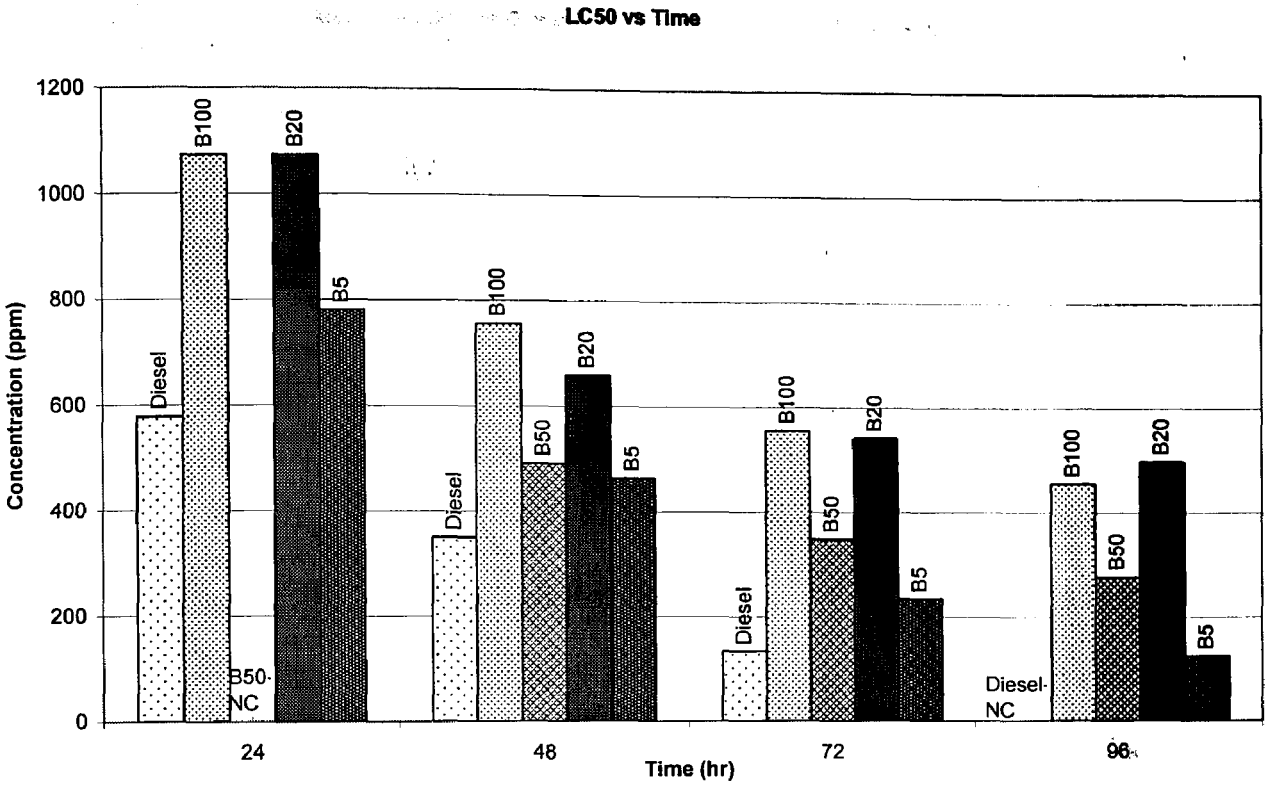

Figure 24: The LC50 values for all fuels used in the acute toxicity tests

4.6 O. mykiss B20 Comparison

A comparison of B20 and B20 (Topia) was done, *O. mykiss* treated with B20 (Topia) were subjected to the same concentrations as fish exposed to B20. There was no significant difference in mortality results among the two trials. Figure16 shows the average mortality rate of 4 trials over a 24hr period. For both B20 and B20 (Topia), mortality rate increases as concentration increases; as illustrated in Table 16.

Table 16: The average mortality rate for B20 (Topia) over 96hrs, using *O. mykiss*

Concentration (ppm)	B20 (%)	B20 (Topia) (%)
100	7.50	5.00
300	8.75	5.00
600	45.00	38.75
900	76.25	57.50
1200	86.25	82.50

Table 17 shows the overall mortality rate of all concentrations, over the 96hr period B20 has the higher mortality rate of 44.75%, while B20 (Topia) has a mortality rate of 37.75%, a difference of 7%.

Table 17: The overall mortality rate for *O. mykiss* treated with B20 and B20 (Topia)

FUELS	
B20	**B20(Topia)**
44.75 %	37.75 %

Figure 25 shows the mortality trends of laboratory blended B20 and commercially blended B20 (B20 (Topia)).

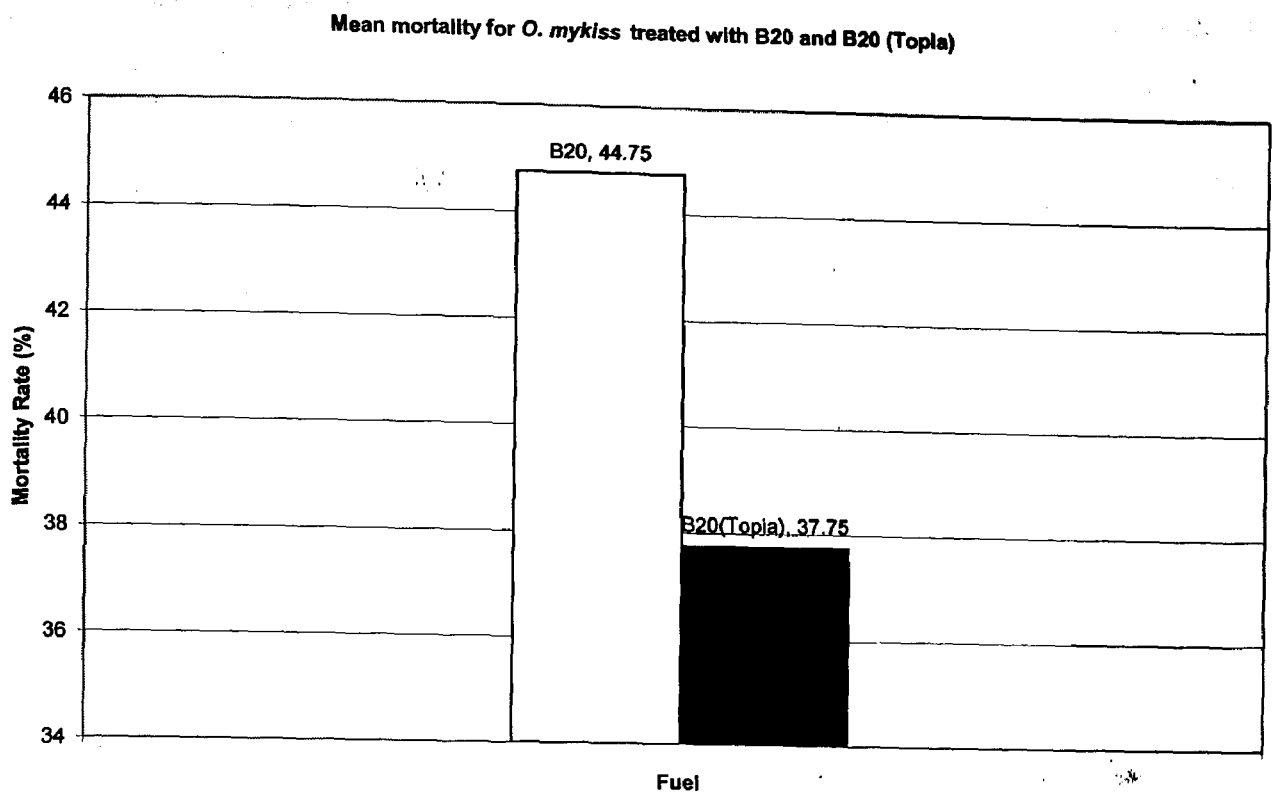

Figure 25: Comparison of mean mortality rate of B20 and B20 (Topia)

Lethal Concentration

Table 18 shows the LC50 values calculated by the Trimmed Spearman-Karber Method for B20 (Topia). Lower and upper confidence intervals were calculated by the program developed by Hamilton et al (1977).

Table 18: The LC50 values for *O. mykiss* treated with B20 (Topia)

Time	*LC 50*	*Lower 95%*	*Upper 95%*	*% Trim*
24hrs	1132.91	918.15	1397.89	45
48hrs	790.87	619.02	1010.43	25
72hrs	606.21	510.08	720.46	7.5
96hrs	527.96	406.28	686.07	10

Figure 26 illustrates the LC50 values obtained for B20 (Topia).The LC50 values for B20 (Topia) decreases over time; similar trends were obtained for the B20 blend. The highest LC50 value was obtained after 24hrs, while the lowest LC50 value of 527.96ppm was obtained for the 96hr time period.

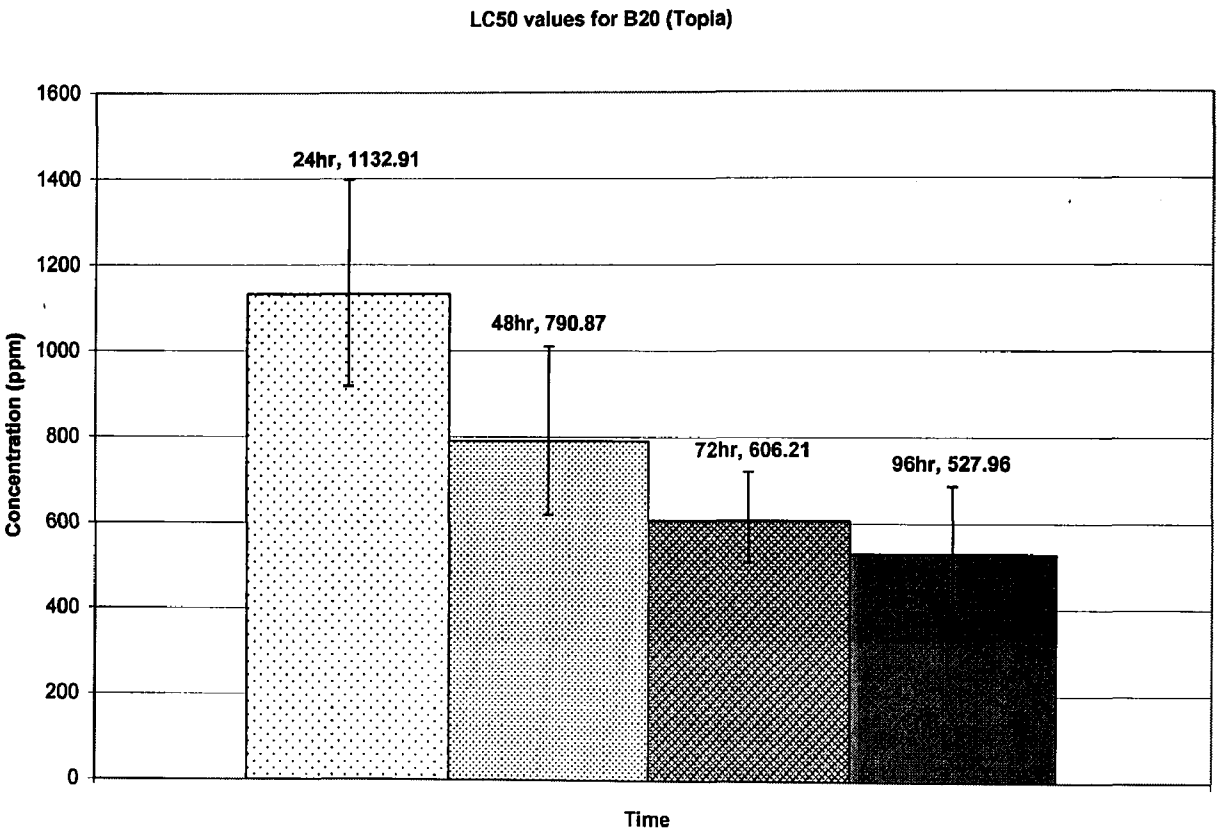

Figure 26: The LC50 values for B20 (Topia)

Figure 27 shows the trend of the mean LC50 values for B20 (Topia) through time. The equation of the line offers an idea of how B20 (Topia) behaves through time. A slope of –1995.95 indicates that the LC50 of diesel decreases every 24 hr at a rate of 199.95ppm.

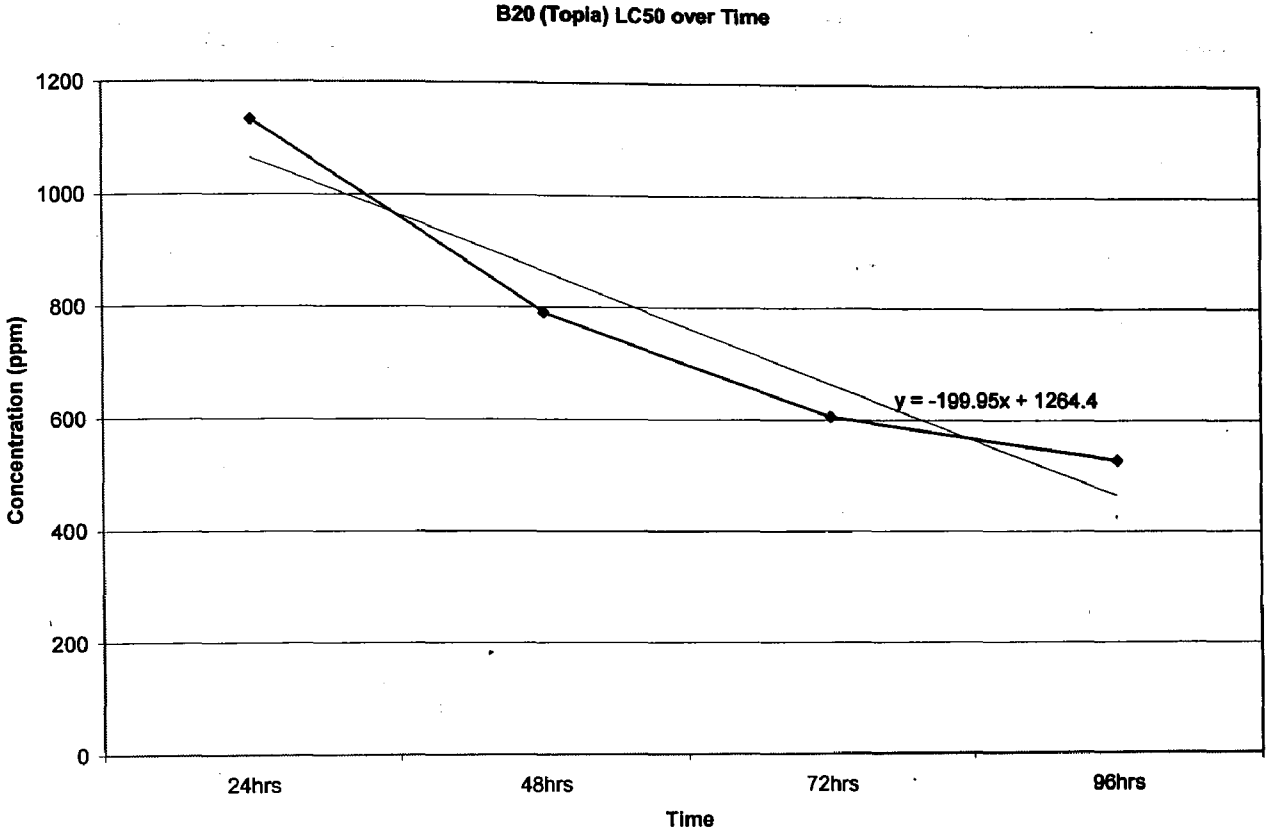

Figure 27: The mean LC50 trend through time for O. mykiss exposed to B20 (Topia)

Figure 28 shows a comparison of B20 and B20 Topia LC50 values. The average LC50 for B20 was 693.05 ± 131.58ppm, while B20 (Topia) has an average LC50 of 764.49 ± 134.61ppm. B20 has a slightly lower LC50 than B20 (Topia); a difference of 71.44ppm.

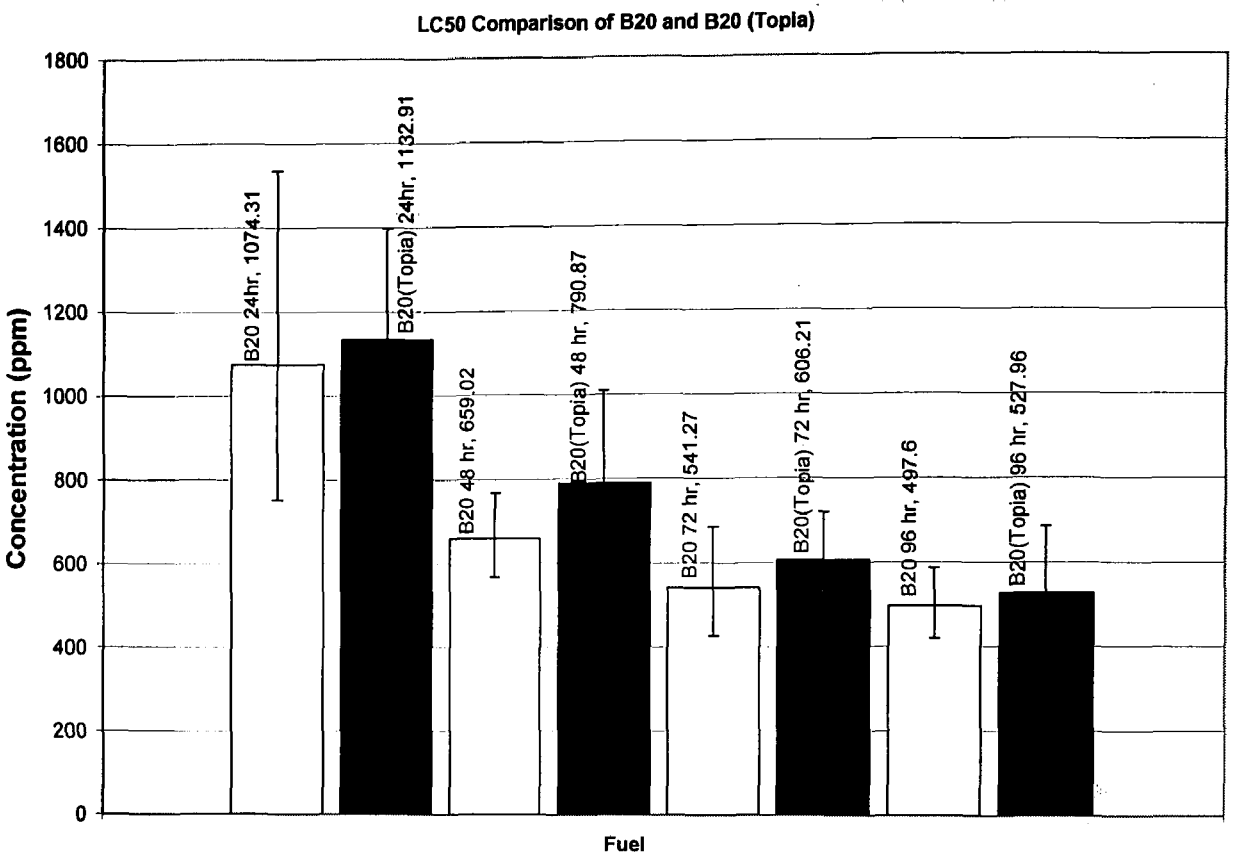

Figure 28: Comparison of the LC50 values of both B20 and B20 (Topia)

5. DISCUSSION

5.1 Experimental Parameters

Temperature

Temperature was a major controlling variable in the experiments conducted; *O. mykiss* (rainbow trout) were very sensitive to both water and environmental temperature changes. The optimal water temperature range was between 8 and 12°C and the optimal environmental temperature range was between 8 and 15°C. Fish exposed to temperatures above or below this range died within a very short time period; as noted in the range-finding results. Like water temperature, environmental temperature was also an important controlling variable; the *O. mykiss* were very sensitive to the ambient environmental temperature; the water and ambient room temperature was maintained at the same temperature to prevent warming up of control and holding water and test chambers to temperatures above 15°C.

Water

Besides temperature, another controlling variable was water type, in the control experiments, rainbow trout (*O. mykiss*) were placed in different types of water which included (i) distilled water, (ii) distilled deionised water (DDI) and (iii) tap water; one set of samples was aerated while the other set was not aerated. The tap water was allowed to stand for approximately five days before use in parameter testing. Water samples were aerated for 24 hours before test initiation. The optimal water used for the definitive tests was aerated distilled deionised (DDI) water.

Bioassay Organisms

The initial organisms to be studied were *Daphnia pulex* (*D. pulex*) and 15-30 day *O. mykiss*, however, *Daphnia pulex* was too difficult to culture, catch and use as suitable test organisms because of their small size and it was also difficult to determine mortality with 15-30 day old trout frys; as they were still attached and feeding off their embryonic sacs. Consequently, *D. magna* was used instead of *D. pulex* and 6-week old *O. mykiss* was used instead of trout at a younger life stage.

5.2 D. magna

Esters, like methyl and ethyl esters are not soluble in water; these esters formed a sheen or film on the water surface, some *D. magna* were trapped in this film. Henceforth, *D. magna* were released below the water surface; significantly less *D. magna* were trapped in the sheen. The sheen was also present in some biodiesel blend trials and similar procedures were used to remediate the problem. The mortality rate was calculated over the 24hr exposure period; diesel has the highest mortality rate (78.34%), while B100 has the lowest mortality rate of 55.98%. Besides diesel, B50, B20 and B5 all contained some amount of diesel fuel in their mixtures prepared. However, of all the prepared mixtures B5 contains the largest amount of diesel (by volume and percent), while B50 has the least amount by volume and percent; 50% biodiesel and 50% diesel. The 24hr-LC50 data was calculated using the Trimmed Spearman-Karber Method (Hamilton et al., 1977); diesel has the lowest LC50 of 1.78ppm, while B100 has the highest LC50 value of 4.65ppm. When the 24hr-LC50 values were compared, the acute aquatic toxicity of B100 was 2.6 times less toxic than diesel.

5.3 O. mykiss

For all fuels tested, mortality increases with increasing concentration and increasing time. Diesel has the highest mortality over time among all the five toxins tested. While B100 has the lowest percent mortality over time; in general the more diesel in the toxin, the higher the percent mortality; B5 has a higher percent mortality than either B50 or B20 over time.

LC50 data was calculated using the Trimmed Spearman-Karber Method; there is a general decrease in lethal concentration values for diesel, B100, B50, B20 and B5 over time. For all test substances, the lowest LC50 values were recorded at 96hrs, while the highest LC50 values were recorded at the 24hr period. The 24hr-LC50 value (578.13ppm) for diesel is the lowest LC50 value among all fuels tested, LC50 values for diesel were calculated for 24hr, 48hr and 72hr, however, no 96hr-LC50 was recorded; as more than 50% of the population were dead even at the lowest concentration. It can be inferred that this non calculable value indicates that diesel is the most toxic of all five toxins. LC50 values for B100 also decreased with increasing time, from 1073.54ppm to 455.28ppm. LC50 values for B20 also decreased with increasing time; B20 has the highest recorded LC50 value for all test substance (1074.31ppm at 24hrs).

It is also noted that B20 has the highest 96hr-LC50 of 497.60ppm, of all of the toxins. Similar trends of decreasing LC50 value with increasing time are observed for B50; however no 24hr-LC50 value was calculated as less than 50% of the test population showed no signs of lethality at the end of the 24hr period. There is also a decreasing LC50 trend for B5, the 96hr-LC50 value (129.57ppm) is also the lowest recorded lethal concentration value. B100 has the highest average LC50 value (710.17 ± 136.38ppm) while diesel has the lowest average LC50 value (354.01±

128.36ppm); however, overall average LC50 values are not a true indication of toxicity ranking. The 24hr and 96hr-LC50 values for B50 and diesel respectively were not calculable. Since no deaths were greater than 50% of the population for B50 at the 24hr time period. All deaths were greater than 50% of the population for *O. mykiss* treated with diesel after 96hrs. Consequently, a fair comparison could not be made solely on calculated lethal concentration values.

5.4 B20 and B20 (Topia) Comparison

The effects of B20 (Topia) were compared to those of B20; B20 (Topia) was commercially blended, while B20 was blended in the lab and the biodiesel feedstock used was composed of non-edible oils. There was a difference in the mortality rates and calculated LC50 values; B20 has a slightly higher mortality rate than B20 (Topia). B20 has a slightly lower LC50 values for all test periods than B20 (Topia), in comparison B20 is more toxic than B20 (Topia), However, there was not a substantial difference between B20 and B20 (Topia). In addition, difference in values may be a result of the difference in feedstock; the B100 feedstocks may have some influence on the results obtained.

5.5 Toxicity Ranking

5.5.1 Daphnia magna

Diesel is more toxic than biodiesel and biodiesel blends, neat biodiesel (B100) is less toxic than biodiesel blends of B100 and conventional diesel. A toxicity ranking was done based on the

mortality rate, the larger the percent mortality the more toxic the fuel. Diesel was the most toxic (78.34%), followed closely by B5, B50, B20, while B100 was the least toxic (55.98%).

A toxicity ranking was also done with the lethal concentration (LC50) values recorded, the lower the LC50 value the more toxic the substance. Diesel was the most toxic, followed closely by B5, B50 and B20; while B100 was the least toxic. Diesel is the most toxic at 1.78ppm, while B100 is the least toxic at 4.65ppm. Biodiesel blends with the larger amounts of diesel, by volume and percent, showed lower LC50 values when compared to those with less diesel blended into the test mixtures. Among the blends, B5 had the lowest calculated LC50 value when compared with B50 and B20 respectively. As with the mortality rate, fuels with diesel were more lethal than those with no diesel additive, i.e., neat biodiesel (B100). The LC50 obtained for the *D. magna* static non-renewal tests were very close, the difference for the lowest value obtained (Diesel: 1.78ppm) and the highest value (B100: 4.65ppm) was 2.87ppm. The small difference maybe due to the small concentration values tested.

Toxicity ranking based on LC50 also determined diesel is the most toxic, followed closely by B5, B50, B20 and B100 (the least toxic). In general, the more diesel in the mixture the more toxic the fuel, however B50 is ranked slightly higher than B20, in terms of toxicity. This difference can be explained by the mortality rate between B50 and B20; for all test concentrations B50 has 10±3% more deaths than B20. This difference affects both percent mortality and lethal concentration. Nevertheless, it has been proven that diesel is more toxic than neat biodiesel, as well as biodiesel blends, in terms of mortality rate and lethal concentration that kills 50% of the population.

5.5.2 O. mykiss

Similar results were obtained for experiments using *O. mykiss*: diesel is more toxic than biodiesel and biodiesel blends. A toxicity ranking was done based on percent mortality; the larger the percent mortality the more toxic the fuel. Diesel (70.17%) was the most toxic, followed closely by B5, B50, B20, while B100 (42.50%) was the least toxic for *O. mykiss*. The more diesel in the fuel mixture the more toxic the fuel; although B50 (51.75%) ranked slightly higher than B20 (44.75%). Diesel is more toxic than neat biodiesel or any other biodiesel blend, in terms of percent mortality.

A toxicity ranking was also done with the lethal concentration (LC50) values recorded, the smaller the LC50 value the more toxic the fuel. A high LC50 implies a lower toxicity because more of the chemical is required to result in death. It was difficult to obtain a definite toxicity ranking using LC50 obtained from *O. mykiss* results. For 24hr-LC50, diesel was the most toxic; however, no value was obtained for B50 because more than 50% of the population survived. The 48hr-LC50 and 72hr-LC50 values produced the same toxicity ranking; Diesel> B5>B50>B20>B100. B50 appears to be more toxic than B20, although it contains 30% less diesel. There is approximately 10± 5% more deaths of *O. mykiss* treated with B50; particularly at concentrations of 600 and 900ppm. This difference affects the LC50 values and accounts for the higher than expected toxicity ranking. It was difficult to estimate an absolute toxicity ranking based on 96hr-LC50 values; the diesel 96hr-LC50 value was not calculable because all the concentrations exhibited more than 50% mortality; even at the lowest concentration of 100ppm. In addition, B50 had a non-calculable 24hr-LC50 value, where less than 50% of the test population died. Nevertheless, an analysis of 96hr-LC50 values was done; diesel is the most

toxic (based on a non-calculable result), followed by B5, B50, B100 and B20. B20 has a higher ranking (by a difference of 42.32ppm) than B100, because slightly more fish treated with 100ppm, 600ppm and 1200ppm of B100 died when compared to similar B20 concentration trials. However, it was proven that diesel is still more toxic than neat biodiesel or biodiesel blends.

5.5.3 B20 Toxicity Ranking

A comparison of *D. magna* results has determined that B20 (Topia) is less toxic than B20 and B100. According to percent mortality results, B20 (Topia) (54.81%) is slightly less lethal than B100 (55.98%) as well as B20 (57.78%). LC50 results has determined than B20 (Topia) has a slightly higher 24hr-LC50 value (6.74ppm) than B100 (4.65ppm) and B20 (4.54ppm). An analysis of *O. mykiss* results has also revealed that B20 (Topia) is less toxic than B20 and B100. According to percent mortality results, B20 (Topia) (37.75%) is slightly less lethal than B100 (42.50%) as well as B20 (44.75%). B20 (Topia) is slightly less lethal than B100 and B20, based on LC50 values. These differences can be explained by the number of deaths recorded for each fuel type. These results may also be attributed to different B100 feedstocks; B20 (Topia) was produced from soy and canola oil, while B100 and B20 were produced from waste cooking oils and fats.

6. CONCLUSIONS

Static non-renewal tests were conducted to determine the acute toxicity of biodiesel, biodiesel blends and diesel on freshwater ecosystems; the two main endpoints were mortality rate and lethal concentration which killed 50% of the test population. The two organisms used were *D. magna* and *O. mykiss*; these organisms were used because of their sensitivity and availability. Neat biodiesel is referred to as B100, while biodiesel blends tested were B50, B20 and B5; these endpoints were compared to results obtained from conventional diesel fuel.

The toxicity tests showed that biodiesel is considerably less toxic than diesel fuel. However, like diesel, biodiesel and biodiesel blends still should be avoided in aquatic environments. Although biodiesel is less toxic than conventional diesel fuel, it can have a serious impact on aquatic organisms in the event of a large spill. Biodiesel and biodiesel blends should be handled like any other fuel to avoid contamination of watersheds. Biodiesel may have a less severe impact on freshwater organisms and the aquatic environment than petroleum diesel; however if accidentally spilled or inadvertently discharged during transportation storage or use, their impact may have similar toxic effects as those of diesel spills.

Based on the results of this study in its entirety, it can be concluded that biodiesel and biodiesel blends produced from non edible oils are less toxic than conventional diesel fuel. Biodiesel maybe a viable and environmentally-friendly alternative to diesel, in terms of acute toxicity levels to freshwater organisms. However, it should be noted that this study is limited in scope; in that the statements concluded are based solely on acute toxicity of diesel, biodiesel and biodiesel blends tested in this study. More specifically, biodiesel and biodiesel blends produced from a

non-edible oil feedstock. Other aspects of biodiesel toxicity were not addressed by this study. These include acute toxicity of biodiesel and biodiesel blends produced from edible oils, such as soybean and canola oils. With the exception of B20 (Topia), only specific non-edible biodiesel blends were tested; these were limited to B50, B20 and B5 and these blends were only mixed with conventional diesel fuel. This study only examined the effects of biodiesel and biodiesel blends over a short-term period; a study on chronic effects was not performed. Another limitation is concentration, since only a few concentrations were tested. It may be very difficult to predict or compare the extent of biodiesel spills in 'real world' situations, with limited blends and concentrations. In addition, natural processes may influence the extent and nature of any particular biodiesel spill.

Future study should examine acute toxicity of biodiesel produced from edible oils, such as vegetable oils. Long-term effects should also be considered, a comparison of chronic effects of biodiesel and biodiesel blends produced from edible and non-edible oils would also be a worthwhile study to perform.

It is difficult to definitely conclude that all neat biodiesel and all biodiesel blends are less toxic than all the different types of diesel fuel. It is also difficult to compare the results obtained on this study to previous studies, since there is a lack of existing literature pertaining to acute toxicity of biodiesel produced from non-edible oil sources on aquatic organisms.

It is also important to realise that the potential environmental impact of biodiesel spills will depend on other factors, such as (i) the location of the spill, (ii) the volume of the spill (iii) the

extent of the spill, (iv) chemical components of biodiesel and (v) other prevailing environmental factors. It should also be noted that acute effects may be species-specific, in the bioassay performed, only *D. magna* and *O. mykiss* was used. Although these two species are significant bio-indicators for acute toxicity of organisms dwelling in the water column, no sediment dwelling organisms were used in the study; these organisms may be affected differently.

There are a number of improvements that could be made to this study in order to obtain greater acceptability of the results. Increasing the number of trials in both bioassays is recommended to improve the statistical validity of the results. Different concentrations could also be tested, as well as different biodiesel blends and chronic exposure may also increase confidence in the results, such as effects on *D. magna* offspring and future generations. Different bioassay organisms can also be used to increase the validity of the results. Acute toxic effects of specific diesel and biodiesel components, as well as by-products of these toxins created by processes such as oxidation or biodegradation would offer valuable information on biodiesel toxicity.

Nevertheless, this study provides a comparison of acute toxicity of neat biodiesel to different biodiesel blends; it also provides a comparison of acute toxicity of biodiesel to diesel. As the interest in biodiesel increases, this study can serve as a baseline for further biodiesel environmental impact studies.

7. REFERENCES

Barnwal B.K. and M.P. Sharma. 2005. "Prospects of biodiesel production from vegetable oils in India;" *Renewable and Sustainable Energy Reviews* **9**: 363–378.

Bender M. 1999. "Economic feasibility review for community-scale farmer cooperatives for biodiesel." *Bioresource Technology* **70**: 81- 87.

Bhattacharyya S., Klerks P.L., and J.A. Nyman. 2003. "Toxicity to freshwater organisms from oils and oil spill chemical treatments in laboratory microcosms." *Environmental Pollution* **122**: 205–215.

Biswas S., Chaudhari S. K. and S. Mukherji. 2005. "Microbial uptake of diesel oil sorbed on soil and oil spill clean-up sorbents." *J. Chem. Technol. Biotechnol.* **80**:587–593.

Boyd M., Murray-Hill A. and K. Schaddelee. 2004. "Biodiesel in British Columbia Feasibility Study Report". *WISE Energy Co-op / Eco-Literacy Canada.*
Available from:< http://www.citygreen.ca/news_biodiesel-report.html> [Retrieved: May 2004].

Cripps G. C. and J. Shears. 1997. "The Fate in the Marine Environment of a Minor Diesel Fuel Spill from an Antarctic Research Station". *Environmental Monitoring and Assessment* **46**: 221–232.

Dede, E.B. and H.D. Kaglo. 2001. "Aqua-toxicological Effects of Water Soluble Fractions (WSF) Of Diesel Fuel On *O. Niloticus* Fingerlings." *J. Appl. Sci Environ. Mgt.* **5** (1): 93-96.

Dmytryshyn S.L., Dalai A.K., S. T. Chaudhari et al. 2004. "Synthesis and characterization of vegetable oil derived esters: evaluation for their diesel additive properties." *Bioresource Technology* **92**: 55–64.

ENN: Environmental News Network. 2005. "President Signs Energy Bill with Biodiesel Tax Incentive Extension; Numerous Other Pro-Biodiesel Measures Also Included." Available from: <www.ENN.com> [Retrieved August 9th 2005].

Enguídanos M., Soria A., Kavalov B. and P. Jensen. May 2002. "Techno-economic analysis of Bio-diesel production in the EU: A short summary for decision-makers." Report EUR 20279EN. European Union: Joint Research Centre, Institute for Prospective Studies. Available from:< http://www.europarl.eu.int/stoa/ta/renewable_energies/biomass/biodiesel(ipts).pdf> [Retrieved on June 2004].

Environment Canada. 2000. Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout. Environment Protection Series, Report EPS1/RM13, December 2000. Environment Canada, Conservation and Protection, Ottawa.

EPA. 2002. Fifth Edition. "Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms". October 2002.

Etkin, D.S. 2004. "Twenty-year Trend Analysis of Oil Spills in EPA Jurisdiction." Freshwater Spills Symposium 2004. Available from:< http://www.epa.gov/oilspill/pdfs/etkin_04.pdf>

Gustafson, C. R. "Biodiesel: An Industry Poised for Growth?" 2003. *Choices* .American Agricultural Economics Association. Third Quarter 2003. Available from: <http://www.choicesmagazine.org> [Retrieved August 2005].

Holbein B.E., Stephen J. D. and D. B. Layzell. 2005. "Canadian Biodiesel Initiative: Aligning Research Needs and Priorities with the Emerging Industry-Final Report." Available from: <http://www.biocap.ca > [Retrieved August 2005].

Kalam M.A. and H.H. Masjuki. 2002. "Biodiesel from palmoil-an analysis of its properties and potential." *Biomass and Bioenergy* **23**: 471 – 479.

Lee, G., Ellersieck N.R., Mayer F.L., and G. F. Krause. 1995. "Prediction Chronic Lethality of Chemicals to Fishes from Acute Toxicity Test Date: Multi-Probit Analysis." *Environmental Toxicology and Chemistry* **14** (2):345-349.

Li, J and C. Filteau. 2005. "2005 Oil Spill Analysis Report for Ontario. Department of Civil Engineering. Ryerson University(September, 2005).

Lytle, D. A. and B. L. Peckarsky. 2001. "Spatial and temporal impacts of a diesel fuel spill On stream invertebrates." *Freshwater biology* **46**: 693 -704.

Mohammed, A. 2005. "Toxicity of water-soluble fractions of four fuels for *Metamysidopsis insularis*, an indigenous tropical mysid species." *Environmental Monitoring and Assessment* **104**: 37–44.

Mudge. S.M. 1995. "Deleterious Effects from Accidental Spillages of Vegetable Oils." 1995. *Spill Science and Technology Bulletin* **2** (2/3): 187-191.

Pereira M. G., Mudge S. M. and J. Latchford. 2002. "Consequences of linseed oil spills in salt marsh sediments." *Marine Pollution Bulletin* **44**: 520–533.

Pereira M.G., Mudge, S. M. and J. Latchford. 2003. "Vegetable oil spills on salt marsh sediments; comparison between sunflower and linseed oils." *Marine Environmental Research* **56**: 367–385.

Prakash . C. B. (Ed). "A Critical Review Of Biodiesel As A Transportation Fuel in Canada." 1998. Environment Canada. Transportation Systems Branch Air Pollution Prevention Directorate.
Available from: < http://www.ec.gc.ca/transport/publications/biodiesel/biodeisel.pdf> [Retrieved May 2005].

Puppán D. 2002. "Environmental Evaluation of Biofuels". *Periodica Polytechnica Ser. Man. Sci.* **10** (1): 95-116.

Radich, A. "Biodiesel Performance, Costs, and Use." Environment Canada. 1990. **Biological** Test Method: Acute Lethality Test Using Rainbow Trout. Environment Protection Series,<http://www.eia.doe.gov/oiaf/analysispaper/biodiesel/pdf/biodiesel.pdf > [**Retrieved July** 2005].

Raneses A.R., Glaser L.K., Price J.M. and J.A. Duffield. 1999. "Potential Biodiesel Markets and Their Economic Effects on the Agricultural Sector of the United States." *Journal of Industrial Crops and Products* **9** (2): 151-162.

Rideout, G. (2004). "Biodiesel: the what, how, where, and why, of the Canadian use of this 'new' fuel". Air and Wastewater Management Association-Ottawa Valley Chapter May 26th 2004.

Sagrans M. 1998. "The Regulation of Biodiesel and Biodiesel Feedstocks in Aquatic Environments." Available from: <www.biodiesel.org/resources/reportsdatabase/ reports/mar/19980601_mar-004.pdf >[Retrieved June 2005]

Schumacher, L.G, S.C. Borgelt, and W.G. Hires. 1995. "Fuelling a Diesel Engine with Methyl-ester Soybean Oil". *Applied Engineering In Agriculture.* **11**(1):37-40.

Sheehan, J., V. Camobreco, J. Duffield, J, M. Graboski and H. Shapouri. 1998. "An Overview of Biodiesel and Petroleum Diesel Life Cycles." NREL/SR-580-24089 UC Category 1503. *U.S Department of Agriculture and U.S. Department of Energy*: National Renewable Energy Laboratory, Golden, CO. Available from:< http://www.nrel.gov/docs/legosti/fy98/24772.pdf> [Retrieved: June 2004].

Tashtoush G.M., Al-Widyan M.I., and M.M. Al Jarrah. 2000. "Experimental study on evaluation and optimization of conversion of waste animal fat into biodiesel." *Energy Conversion and Management* **45**: 2697-2711.

Tyson S. K. 2001. "Biodiesel Handling and Use Guidelines." NREL/TP-580-30004. U.S. Department of Energy National Renewable Energy Laboratory .Golden, Colorado Available from: <www.eere.energy.gov/biomass/pdfs/biodiesel> [Retrieved: June 1st 2004].

Tyson S. K., (Ed) Hamilton, J., J. Jones., J. Stepan. 1998. "Biodiesel Research Progress 1992–1997." Dyncorp Information & Engineering Technology." April 1998 · NREL/TP-580-24433. U.S. Department of Energy National Renewable Energy Laboratory .Golden, Colorado Available from: <www.eere.energy.gov/biomass/pdfs/biodiesel> [Retrieved: June 1st 2004].

United States House of Representatives. 2005. Energy Policy Act. Available from: < www.house.gov/rules/text_6cr.pdf > [Retrieved on July 2005].

Van Gerpen, J. 2004. "2004 Biodiesel Handling and Use Guidelines". NREL/SR-510-36240. *US Department of Energy Efficiency and Renewable Energy. National Renewable Energy Laboratory* Available from:< http://www.nrel.gov/vehiclesandfuels/npbf/feature_guidelines.html> **[Retrieved February 2005].**

Zhang X. , Peterson C. L., Reece D., Möller G. and R. Haws. 1995. "Biodegradability of Biodiesel in the Aquatic Environment." Available from: <http://www.biodiesel.org/resources/reportsdatabase/reports/mar/19950601_mar-009.pdf> [Retrieved October 2004].

Zhang Y., Dub M.A., McLean D.D. and M. Kates. 2003. "Biodiesel production from waste cooking oil: Economic assessment and sensitivity analysis." *Bioresource Technology* 90: 229–240.

8. BIBLIOGRAPHY

Agriculture and Agri-Food Canada. January 2002. "An Economic Analysis of a Major Bio-Fuel Program Undertaken By OECD Countries." Available from:< http://www.agr.gc.ca/spb/rad-dra/publications/econan/econan_e.php> [Retrieved on July 2005].

Ahouissoussi, N.B.C and M.E. Wetzstein. 1997. "A comparative cost analysis of biodiesel, compressed natural gas, methanol, and diesel for transit bus systems." *Resource and Energy Economics* **20**: 1-15.

Altin, R., Çetinkaya S., and H.S. Yücesu. 2001."The potential of using vegetable oil fuels as fuel for diesel engines." *Energy Conversion and Management* **42**: 529-538.

Anderson, R.L. and P. Shubat. 1984. "Toxicity of Flucytrinate to *Gammarus lacstris* (Amphipoda), *Pteronarcys dorsata* (Plecoptera) and *Brachycentrus americanus* (Trichoptera): Importance of Exposure Duration." *Environmental Pollution (Series A)* **35**: 353-365.

Andréasson M. and G. Dave. 1995. "Toxicity of Bile from Fish exposed to Pentachlorophenol-Spiked Sediment." *Marine Environmental Research* **39**: 335-339.

Barnwal B.K. and M.P. Sharma. 2005. "Prospects of biodiesel production from vegetable oils in India;" *Renewable and Sustainable Energy Reviews* **9**: 363–378.

Bender M. 1999. "Economic feasibility review for community-scale farmer cooperatives for biodiesel." *Bioresource Technology* **70**: 81- 87.

Beitinger T. L. and D. W. Huey. 1981. "Acute Toxicity of Nitrite to Crayfish *Procambar Us Simulans* in Varied Environmental Conditions." *Environmental Pollution (Series A) 7.6:* 305-311.

Bernd F. and G. Reinhardt. 1998. "Environmental Impacts of Biodiesel Use" *Bioenergy* 1998: Expanding Bioenergy Partnerships. Available from: <http://www.biodiesel.org/resources/reportsdatabase/reports/gen/19981001_gen-110.pdf> [Retrieved: May 2004].

Bhattacharyya S., Klerks P.L., and J.A. Nyman. 2003. "Toxicity to freshwater organisms from oils and oil spill chemical treatments in laboratory microcosms." *Environmental Pollution* **122**: 205–215.

Biswas S., Chaudhari S. K. and S. Mukherji. 2005. "Microbial uptake of diesel oil sorbed on soil and oil spill clean-up sorbents." *J Chem. Technol. Biotechnol.* **80**:587–593.

Borgelt, S.C., Kolb T. S. and L. G. Schumacher. (1994). "Biodiesel: World Status". *Proceedings of an Alternative Energy Conference, American Society of Agricultural Engineers Summer Meeting*. Kansas City, MO.

Çağlan A. Benlü K. and G. Köksal. 2005."The Acute Toxicity of Ammonia on Tilapia (*Oreochromis niloticus*) Larvae and Fingerlings." *Turk J Vet Anim Sci* **29**: 339-344.

Chandler, K., Malcosky N., Kelly K., P. Norton et al. 1996. "Alternative Fuel Transit Bus Evaluation Program Results". *International Spring Fuels and Lubricant Meeting*. Dearborn, MI. SAE Paper No. 961082. National Transportation Library. Available from:< http://ntl.bts.gov> [Retrieved June 2004].

Chung-Min L., and L. Ming-Chao. 2001. "Acute Toxicity Modeling of Rainbow Trout and Silver Sea Bream Exposed to Waterborne Metals". *Environ Toxicol.* **16** (4):349-60.

Crane M. and M. C. Newman. 2000 "What Level of Effect Is a No Observed Effect?" *Environmental Toxicology and Chemistry* **19** (2) 516–519.

Cripps G. C. and J. Shears. 1997. "The Fate in the Marine Environment of a Minor Diesel Fuel Spill from an Antarctic Research Station". *Environmental Monitoring and Assessment* **46**: 221–232.

Davis H.K., Moffat C.F. and N.J. Shepherd. 2002."Experimental Tainting of Marine Fish by Three Chemically Dispersed Petroleum Products, with Comparisons to the Braer Oil Spill." *Spill Science & Technology Bulletin*, **7** (5–6): 257–278.

Dede, E.B. and H.D. Kaglo. 2001. "Aqua-toxicological Effects of Water Soluble Fractions (WSF) Of Diesel Fuel On *O. Niloticus* Fingerlings." *J. Appl. Sci Environ. Mgt.* **5** (1): 93-96.

De Selincourt, K. (1993) "Europe's home grown fuel". 1995. *New Scientist.* **140**: 22-25.

Dmytryshyn S.L., Dalai A.K., S. T. Chaudhari et al. 2004. "Synthesis and characterization of vegetable oil derived esters: evaluation for their diesel additive properties." *Bioresource Technology* **92**: 55–64.

Enguídanos M., Soria A., Kavalov B. and P. Jensen. May 2002. *"Techno-economic analysis of Bio-diesel production in the EU: A short summary for decision-makers."* Report EUR 20279 EN. European Union: Joint Research Centre, Institute for Prospective Studies. Available from:< http://www.europarl.eu.int/stoa/ta/renewable_energies/biomass/biodiesel(ipts).pdf> [Retrieved on June 2004].

ENN: Environmental News Network. 2005. "*President Signs Energy Bill with Biodiesel Tax Incentive Extension; Numerous Other Pro-Biodiesel Measures Also Included.*" Available from: <www.ENN.com> [Retrieved August 9th 2005].

Environment Canada. 1990. Biological Test Method: Acute Lethality Test Using Rainbow Trout. Environment Protection Series, Report EPS1/RM9, May 1996 Amendments. Environment Canada, Conservation and Protection, Ottawa.

Environment Canada. 1990. Biological Test Method: Acute Lethality Test Using *Daphnia* spp. Environment Protection Series, Report EPS1/RM11, May 1996 Amendments. Environment Canada, Conservation and Protection, Ottawa.

Environment Canada. 1990. Guidance Document on Control of Toxicity Test Precision Using Reference Toxicants. Environment Protection Series, Report EPS1/RM12, August 1990. Environment Canada, Conservation and Protection, Ottawa.

Environment Canada. 2000. Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout. Environment Protection Series, Report EPS1/RM13, December 2000. Environment Canada, Conservation and Protection, Ottawa.

Environment Canada. 2000. Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*. Environment Protection Series, Report EPS1/RM14, December 2000. Environment Canada, Conservation and Protection, Ottawa.

EPA. 2002. "A Comprehensive Analysis of Biodiesel Impacts on Exhaust Emissions – Draft Technical Report". Assessment and Standards Division, Office of Transportation and Air Quality, U.S. Environmental Protection Agency. EPA420-P-02-001 (2002). Available from: <http://www.epa.gov/otaq/models/analysis/biodsl/p02001.pdf >[Retrieved: May 2004].

EPA. 2002. "Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms". Fifth Edition. October 2002.

Etkin, D.S. 2004. "Twenty-year Trend Analysis of Oil Spills in EPA Jurisdiction." Freshwater Spills Symposium 2004. Available from:< http://www.epa.gov/oilspill/pdfs/etkin_04.pdf>

Farrell A. P., Gamperl A. K., Hicks J.M.T., H. A. Shiels et al. 1996. "Maximum Cardiac Performance of Rainbow Trout (*Oncorhynchus mykiss*) At Temperatures Approaching Their Upper Lethal Limit." *The Journal of Experimental Biology* **199**: 663–672.

Feng M., Liu C., Xu J. and Q. Xu. 1998. "Modeling and Biological Implication of Time-Dose-Mortality Data for the Entomophthoralean Fungus, *Zoophthora anhuiensis*, on the Green Peach Aphid *Myzus persicae*" *Journal of Invertebrate Pathology* **72**: 246–251.

Galleger, P. W., Shapouri H., Price J. and G. Schamel. 2003. "Some long-run effects of growing markets and renewable fuel standards on additives markets and the US ethanol industry". *Journal of Policy Modelling* **25** (617): 585-609.

Gulec I. and D. A. Holdway. 1999. "The Toxicity Of Laboratory Burned Oil To The Amphipod *Allorchestes Compressa* And The Snail *Polinices Conicus*." *Spill Science & Technology Bulletin* **5** (2):135-139.

Haas, M. J. 2005"Improving the economics of biodiesel production through the use of low value lipids as feedstocks: vegetable oil soapstock." *Fuel Processing Technology* **86: 1087– 1096.**

Hamilton, M.A., Russo R. C. and R.V. Thurston. 1977. "Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays." *Environ. Sci. Technol.* **11**:714-718.

Hamoutene D., Payne J.F., Rahimtula A. and K. Lee. 2004. "Effect of Water Soluble Fractions of Diesel and an Oil Spill Dispersant (Corexit 9527) on Immune Responses in Mussels." *Bull. Environ. Contam. Toxicol.* **72**:1260–1267.

Holbein B.E., Stephen J. D. and D. B. Layzell. 2005. "Canadian Biodiesel Initiative: Aligning Research Needs and Priorities with the Emerging Industry-Final Report." Available from: <http://www.biocap.ca > [Retrieved August 2005].

Gustafson, C. R. "Biodiesel: An Industry Poised for Growth?" 2003. *Choices* .American Agricultural Economics Association. Third Quarter 2003. Available from: <http://www.choicesmagazine.org> [Retrieved August 2005].

Kalam M.A. and H.H. Masjuki. 2002. "Biodiesel from palmoil-an analysis of its properties and potential." *Biomass and Bioenergy* **23**: 471 – 479.

Keller, A.E., Ruessler D.S., Chafee C.M. 1998. "Testing the toxicity of sediments contaminated with diesel fuel using glochidia and juvenile mussels (Bivalvia, Unionidae)." *Aquatic Ecosystem Health and Management.* **1**(1): 37-47.

Kinast J.A. 2003. "Production of Biodiesel from Multiple Feedstocks and Properties of Biodiesel and Biodiesel/Diesel Blends Final Report: Report 1 in a series of 6." NREL/SR-510-31460. National Renewable Energy Laboratory. Golden, Colorado. Available from: <http://www.nrel.gov/docs/fy03osti/31460.pdf [Retrieved February 2005].

Knothe G., Dunn R.O. and M. O. Bagby. "Biodiesel: The Use of Vegetable Oils and Their Derivatives as Alternative Diesel Fuels." Oil Chemical Research. U.S. Department of Agriculture. Peoria, IL. Available from: <http://journeytoforever.org/biofuel_library/VegetableOilsKnothe.pdf> [Retrieved February 2005].

Koyama J. and A. Kakuno. 2004. "Toxicity of heavy fuel oil, dispersant, and oil-dispersant mixtures to a marine fish, *Pagrus major.*" *Fisheries Science* **70**: 587-594. ticle587594BEES Körbitz W. 1999. "Biodiesel Production in Europe and North America, An Encouraging Prospect." *Renewable Energy* **16**:1078-1083.

Kooijman, S.A.L.M and J.J.M. Bedaux. 1996. "Analysis of Toxicity Test on Daphnia Survival and Reproduction." 1996 *Wat. Res.* **30** (7): 1711-1723.

Lee, G., Ellersieck N.R., Mayer F.L., and G. F. Krause. 1995. "Prediction Chronic Lethality of Chemicals to Fishes from Acute Toxicity Test Date: Multi-Probit Analysis." *Environmental Toxicology and Chemistry* **14** (2):345-349.

Lenwood W. H. Jr., Anderson R. D. and J. V. Kilian. 1997. "Acute And Chronic Toxicity Of Copper To The Estuarine Copepod *Eurytemora Affinis:* Influence Of Organic Complexation And Speciation." *Chemosphere,* **35** (7):1567-1597.

Li, J and C. Filteau. 2005. "2005 Oil Spill Analysis Report for Ontario. Department of Civil Engineering. Ryerson University(September, 2005).

Liao, C. M. and M.C. Lin. 2001. "Acute Toxicity Modelling of Rainbow Trout and Silver Sea Bream Exposed to Waterborne Metals." *Environmental Toxicology* **16** (4): 349-60.

Lytle, D. A. and B. L. Peckarsky. 2001. "Spatial and temporal impacts of a diesel fuel spill On stream invertebrates." *Freshwater Biology* **46**:693-704.

Long S. M., and D. A. Holdway. 2002. "Acute toxicity of crude and dispersed oil to Octopus pallidus hatchlings." *Water Research* **36**: 2769–2776.

Marr J.C.A., Lipton J. and D. Cacela et al. 1999. "Bioavailability and acute toxicity of copper to rainbow trout (*Oncorhynchus mykiss*) in the presence of organic acids simulating natural dissolved organic carbon." *Can. J. Fish. Aquat. Sci.* **56**: 1471–1483.

Marr J.C.A., Hansen J.A., Meyer J.S. and D. Cacela et al. 1998. "Toxicity of cobalt and copper to rainbow trout: application of a mechanistic model for predicting survival." *Aquatic Toxicology* **43**: 225–238.

Magaud, H. B. Migeon, P. Morfin, J. Garric and E. Vindimian. 1997. "Modelling Fish Mortality due to urban storm run-off: interaction effects of Hypoxia and un-ionized Ammonia." *Wat. Res.* **31** (2): 211-218.

McCaya D.F, Rowea J. J., Whittier N., Sankaranarayanan S. and D.S. Etkin. 2004.; "Estimation of potential impacts and natural resource damages of oil." *Journal of Hazardous Materials* **107**:11–25.

McCormick R.L, Graboski M.S., T.L. Alleman, and A.M Herring. 2001. "Impact of Biodiesel Source Material and Chemical Structure on Emissions of Criteria Pollutants from a heavy-duty engine." *Environmental Science Technology* **35** (9): 1742-1747.

Meyer, J.S. Gulley D.D. and M.S Goodrich et al. 1995. "Modeling Toxicity due to intermittent exposure of rainbow trout and common shiners to monochloramine" *Environmental Toxicology* **14** (1): 165-175.

Miller, N.J and S.T Mudge. 1997. "The effect of biodiesel on the rate of removal and weathering characteristic of crude oil within artificial sand columns." *Spill Science and Technology Bulletin* **4** (1): 17-33.

Mohammed, A. 2005. "Toxicity of water-soluble fractions of four fuels for *Metamysidopsis insularis*, an indigenous tropical mysid species." *Environmental Monitoring and Assessment* **104**: 37–44.

Mudge. S.M. "Deleterious Effects from Accidental Spillages of Vegetable Oils." 1995. *Spill Science and Technology Bulletin* **2** (2/3): 187-191.

Mudge S. M. and G. Pereira. (1999). "Stimulating the Biodegradation of Crude Oil with Biodiesel Preliminary Results." *Spill Science & Technology Bulletin* **5** (5/6): 353- 355.

Mushrush, G.W., Wynne, J.H., Willauer H.D. and C.T. Lloyd et al. 2004. "Recycled Soybean Cooking Oils as Blending Stocks for Diesel fuels." *Ind. Eng. Chem.Res.* **43** (16): 4944-4946.

Nowierski R. M., Zeng Z., Jaronski S., Delgado F. and W. Swearingen. 1996. "Analysis and Modeling of Time-Dose-Mortality of *Melanoplus sanguinipes, Locusta migratoria migratorioides,* and *Schistocerca gregaria* (Orthoptera: Acrididae) from *Beauveria, Metarhizium,* and *Paecilomyces* Isolates from Madagascar." *Journal Of Invertebrate Pathology* 67, no.39: 236–252.

Ohwada K., Nishimura M., Wada M. and H. Nomura et al. 2003. "Study of the effect of water-soluble fractions of heavy-oil on coastal marine organisms using enclosed ecosystems, mesocosms." *Marine Pollution Bulletin* **47**: 78–84.

Omoregie E. "Acute Toxicity of Water Soluble Fractions of Crude Oil to the Nile Tilapia, *Oreochromis niloticus* (L.). 2002." *Bull. Environ. Contam. Toxicol.* **68**:623–629.

Paixão S. M., Mendonça E., Picado A. and A. M, Anselmo. 1999. "Acute toxicity evaluation of olive oil mill. Wastewaters: a comparative study of three Aquatic organisms." *Environmental Toxicology* **14** (2): 263-269.

Pereira M. G. and S. M. Mudge. 2004. "Cleaning oiled shores: laboratory experiments testing the potential use of vegetable oil biodiesels." *Chemosphere* **54**: 297–30.

Pereira M. G., Mudge S. M. and J. Latchford. 2002. "Consequences of linseed oil spills in salt marsh sediments." *Marine Pollution Bulletin* **44**: 520–533.

Pereira M.G., Mudge S. M. and J. Latchford. 2003. "Vegetable oil spills on salt marsh sediments; comparison between sunflower and linseed oils." *Marine Environmental Research* **56**: 367–385.

Pereira M. G., and S. M. Mudge. 2004. "Cleaning oiled shores: laboratory experiments testing the potential use of vegetable oil biodiesels." *Chemosphere* **54**: 297–304.

Pimentel, D., M. Herz, M. Glickstein and M. Zimmerman. 2002. "Renewable Energy: Current and Potential Issues". *Bioscience* **52** (12): 1111-1121.

Polat H, Ünlü Erkoc F., Rukiye V. and O. Koçak. 2002. "Investigation of acute toxicity of beta-cypermethrin on guppies *Poecilia reticulate*." *Chemosphere* **49**: 39–44.

Prakash . C. B. "A Critical Review Of Biodiesel As A Transportation Fuel in Canada." 1998. Environment Canada. Transportation Systems Branch Air Pollution Prevention Directorate. Available from: < http://www.ec.gc.ca/transport/publications/biodiesel/biodeisel.pdf> [Retrieved May 2005].

Prankl H., M. Wörgetter, and B.L.T Wieselburg. March 2000. "The Introduction of Biodiesel as a Blending Component to Diesel Fuel in Austria Final Report of NTB-net Phase IV" *Federal Institute of Agricultural Engineering*, Austria. Available from: <http://www.blt.bmlf.gv.at> [Retrieved: May 2004].

Preisler, H. K. and J. L. Robertson. 1989. "Analysis of time-dose- mortality data." *J. Econ. Entomol.* **82**: 1534-1542.

Puppán D. Environmental Evaluation of Biofuels. 2002. *Periodica Polytechnica Ser. Man. Sci.* **10** (1): 95-116.

Radich, A. "Biodiesel Performance, Costs, and Use." *US Energy Information Administration* Available from: <http://www.eia.doe.gov/oiaf/analysispaper/biodiesel/pdf/biodiesel.pdf > [Retrieved July 2005]

Raneses A.R., Glaser L.K., Price J.M. and J.A. Duffield. 1999. "Potential Biodiesel Markets and Their Economic Effects on the Agricultural Sector of the United States." *Journal of Industrial Crops and Products* **9** (2): 151-162.

Rhodes J.E., Adams W.J., Biddinger G.R. and K.A. Robillard et al. 1995. "Chronic Toxicity of 14 phthalate esters to Daphnia magna and rainbow trout (*Oncorhynchus mykiss*)." *Environmental Toxicology and Chemistry* **14** (11): 1967-1976.

Rideout, G. (2004). "Biodiesel: the what, how, where, and why, of the Canadian use of this 'new' fuel". Air and Wastewater Management Association-Ottawa Valley Chapter May 26th 2004.

Rose, J. 1994. "Biofuel benefits questioned". *Environmental Science and Technology* **28**:63.

Rosen, M.A. (2002). "Case Study on Industrial Ecology". *Chapter of Encyclopedia of Life Support Systems*, EOLSS Publishers, Oxford, U.K. (in press).

Rukiye V., Ünlü Erkoc F. Polat H., and O Koçak. 2003. "Investigation of acute toxicity of deltamethrin on guppies (*Poecilia reticulata*)." *Ecotoxicology and Environmental Safety* **55**: 82–85.

Sagrans M., 1998. "The Regulation of Biodiesel and Biodiesel Feedstocks in Aquatic Environments." Available from: <www.biodiesel.org/resources/reportsdatabase/reports/mar/19980601_mar-004.pdf> [Retrieved June 2005].

Schumacher, L. G., Weber, J. A. and J. G. Krahl. 1995. "An Alternative Fuel for Urban Buses-Biodiesel Blends". *Proceedings of the Second Biomass Conference of the Americas.* Portland, OR.

Schumacher, L G. 1995. "Biodiesel Emissions Data from Series 60 DDC Engines". *American Public Transit Association Bus Operations and Technology Conference.* Reno, NV.

Schumacher, L. and J. Van Gerpen. 1996. "Research Needs Resulting from Experiences of Fuelling Engines with Biodiesel". *Proceedings of the Third Liquid Fuel Conference.* Nashville, TN.

Schumacher, L.G, S.C. Borgelt, and W.G. Hires. 1995. "Fuelling a Diesel Engine with Methyl-ester Soybean Oil". *Applied Engineering In Agriculture.* Vol. 11(1):37-40.

Schumacher, L G., Weber, J. A, and J. G. Krahl, 1995. "Maintenance, Repair, and Engine Exhaust Emissions Associated with Biodiesel Fuelling of Urban Buses". *Proceedings of the American Society of Agricultural Engineers Summer Meeting.* Chicago, IL. ASAE Paper No. 956736.

Schumacher, L. G., J. A. Weber, and M. D. Russell. 1994. "Fuelling Urban Buses with Soydiesel-Diesel Fuel Blending". *Bioenergy '94- Using Biofuels for a Better Environment-Proceedings of the Sixth National Bioenergy Conference.* Reno/Sparks, NV.

Schumacher, L. G., S. Howell. 1994."Lubricating Qualities of Biodiesel and Biodiesel Blends". *Bioenergy 1994- Using Biofuels for a Better Environment- Proceedings of the Sixth National Bioenergy Conference.* Reno/Sparks, NV.

Schumacher , L. G., S.C. Borgelt, and W.G. Hires. 1993. "Fuelling Diesel Engines with Blends of Methyl Ester Soybean Oil and Diesel Fuel". Proceedings of a Conference on *Biodiesel.* Sioux Falls, SD.

Schumacher, L.G, S.C. Borgelt, and W.G. Hires. 1995. "Fuelling a Diesel Engine with Methyl-ester Soybean Oil". *Applied Engineering In Agriculture.* **11**(1):37-40.

Schumacher, L. G., Hires, W. G. and S.C. Borgelt. 1992. "Fuelling a Diesel Engine with Methyl-Ester Soybean Oil". Proceedings of an Alternative Energy Conference American *Society of Agricultural Engineers Winter Meeting.* Nashville, TN.

Schumacher, L.G. and J.V. Gerpen. 2000. "Engine Oil Analysis of Diesel Engines Fuelled with 0, 1, 2, and 100 Percent Biodiesel". *Proceedings of the American Society of Agricultural Engineers International Meeting.* Milwaukee, WI. ASAE Paper No. 006010.

Schumacher, L.G. and T. Madzura. 1997. "Lessons Learned While Fuelling With Biodiesel". *Proceedings of Commercialization of Biodiesel: Producing a Quality Fuel.* Boise, ID.

Sergio, U. 2003." A Comprehensive Energy and Economic Assessment of Biofuels: When 'green' is not enough". *Critical Reviews in Plant Sciences.* 20 (1): 71-106.

Sheehan, J., V. Camobreco, J. Duffield, J, M. Graboski and H. Shapouri. .1998. "An Overview of Biodiesel and Petroleum Diesel Life Cycles." NREL/SR-580-24089 UC Category 1503. *U.S Department of Agriculture and U.S. Department of Energy*: National Renewable Energy Laboratory, Golden, CO. Available from: <http://www.nrel.gov/docs/legosti/fy98/24772.pdf> [Retrieved: June 2004].

Smith S. D. A. and R. D. Simpson. 1998. "Recovery of benthic communities at Macquarie Island (sub-Antarctic) following a small oil spill." *Marine Biology* **131**: 567-581.

Srivastava A., and R. Prasad.2000. "Triglycerides-based diesel fuels." *Renewable and Sustainable Energy Reviews* **4**: 111-133.

Stevens D.J, Hogan E., Östman A., Segerborg-Fick A. and M Wörgetter. (November 2001). "Non-technical policy, regulatory, and market influences on the use of liquid biofuel-Final Summary Report". Available from:< www.ieabioenergy.com/library/139_ieaarweb.pdf> [Retrieved: May 2004].

Sun K., Krause G. F., Mayer Jr. F.L., Ellersieck M.R. and A. P. Basu. 1995."Predicting Chronic Lethality of Chemicals to Fishes from Acute Toxicity Test Data: Theory of Accelerated Life Testing." *Environmental Toxicology and Chemistry* **14** (10): 1745-1752.

Svecevičius G. 2001."Avoidance Response of Rainbow Trout *Oncorhynchus mykiss* to Heavy Metal Model Mixtures: A Comparison with Acute Toxicity Tests." *Bull. Environ. Contam. Toxicol.* **67**:680–687.

Tashtoush G.M., Al-Widyan M.I., and M.M. Al Jarrah. 2000. "Experimental study on evaluation and optimization of conversion of waste animal fat into biodiesel." *Energy Conversion and Management* **45**: 2697-2711.

Thomsen L. and J. Eilenberg. 2000. "Time-Concentration Mortality of *Pieris brassicae* (Lepidoptera: Pieridae) and *Agrotis segetum* (Lepidoptera: Noctuidae) Larvae from Different Destruxins." *Environ. Entomol.* **29** (5): 1041-1047.

Tyson S. K. 2001. "Biodiesel Handling and Use Guidelines." NREL/TP-580-30004. U.S. Department of Energy National Renewable Energy Laboratory .Golden, Colorado Available from: <www.eere.energy.gov/biomass/pdfs/biodiesel> [Retrieved: June 1st 2004].

Tyson S. K., (Ed) Hamilton, J., J. Jones., J. Stepan. April 1998. "Biodiesel Research Progress 1992–1997." Dyncorp Information & Engineering Technology." April 1998 · NREL/TP-580-24433. U.S. Department of Energy National Renewable Energy Laboratory .Golden, Colorado

Available from: <www.eere.energy.gov/biomass/pdfs/biodiesel> [Retrieved: June 1st 2004].

USEPA. 2005. Statistical Analysis for Biological Methods. Available from: < http://www.epa.gov/nerleerd/stat2.htm> [Retrieved on: February 2005].

United States House of Representatives. 2005. Energy Policy Act. Available from: < www.house.gov/rules/text_6cr.pdf > [Retrieved on July 2005].

Van Dyne, D. L., Weber J.A. and C.H. Braschler. 1996. " Macroeconomic Effects of a community-based biodiesel production system." *Bioresource Technology* **56**: 1-6.

Van Gerpen, J. 2004. "2004 Biodiesel Handling and Use Guidelines." NREL/SR-510-36240. *US Department of Energy Efficiency and Renewable Energy. National Renewable Energy Laboratory* Available from:< http://www.nrel.gov/vehiclesandfuels/npbf/feature_guidelines.html> [Retrieved February 2005].

Waller D. L., Rach J. J., Cope W. G., and L. L. Marking. 1993."Toxicity of Candidate Molluscicides to Zebra Mussels *(Dreissena polymorpha)* and Selected Nontarget Organisms." *J. Great Lakes Res.* **19** (4):695-702

Wörgetter, M and B.L.T Wieselburg. (2000). "Biodiesel and Environment" Liquid Biofuels-Task 27 IEA Energy. Federal Institute of Agricultural Engineering. Prepared for IEA Bioenergy Agreement IEA Bioenergy Task 27, Liquid Biofuels. Available from:< http://www.liquid-biofuels.com/FinalReport2.html#Tables> [Retrieved: February 2004].

Xu, J. and M. Feng. 2000. "The Time-Dose-Mortality Modeling and Virulence Indices for Two Entomophthoralean Species, *Pandora delphacis* and *P. neoaphidis*, against the Green Peach Aphid, *Myzus persicae*."*Biological Control* **17**: 29-34.

Yen J., Lin K. H., and Y. Wang. 2002. "Acute Lethal Toxicity of Environmental Pollutants to Aquatic Organisms." *Ecotoxicology and Environmental Safety* **52**: 113-116

Zhang J.F., Wang X.R., H .Y. Guo, et al. 2004. "Effects of water-soluble fractions of diesel oil on the antioxidant defenses of the goldfish, *Carassius auratus*." *Ecotoxicology and Environmental Safety* **58**: 110–116.

Zhang X., Peterson C. L., Reece D., Möller G. and R. Haws. 1995. "Biodegradability of Biodiesel in the Aquatic Environment." Available from: <http://www.biodiesel.org/resources/reportsdatabase/reports/mar/19950601_mar-009.pdf> [Retrieved October 2004]

Zhang Y., Dub M.A., McLean D.D. and M. Kates. 2003. "Biodiesel production from waste cooking oil: Economic assessment and sensitivity analysis." *Bioresource Technology* **90**: 229–240.

9. APPENDIX A: Experimental Parameters

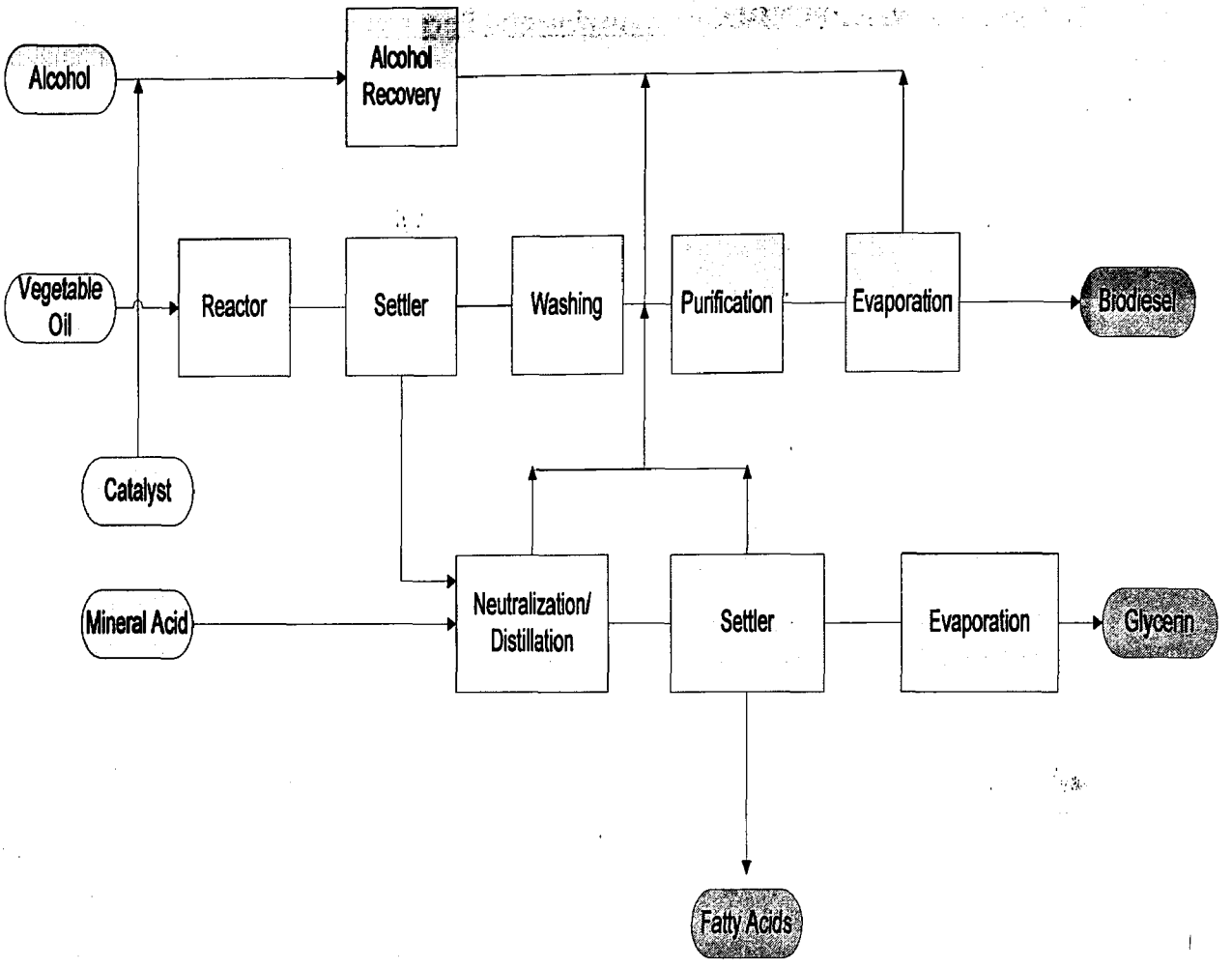

Figure 1: A Simplified Biodiesel Process Technology
Source: Puppán D., 2002. Environmental Evaluation of Biofuels

TABLE 1

TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR *DAPHNIA MAGNA* ACUTE TOXICITY WITH EFFLUENTS

Test type	Static
Test duration	48hr
Temperature	20 ± 1 C
Light Quality	Ambient laboratory illumination
Light Intensity	Ambient laboratory levels
Photoperiod	16h light, 8 hr darkness
Test Chamber Size	30 ml
Test Chamber Volume	25ml
Renewal of Test Solutions	Not Required
Age of Test Organisms	less than 24hr old
No. of Organisms per Test Chamber	5
No. of Replicate Chambers per Concentration	4
No. of organisms per Concentration	20
Feeding Regime	Feeding not required
Test Chamber Cleaning	Cleaning not required
Test Chamber Aeration	None
Dilution Water	DDI water.
Test Concentrations	6 and control
Endpoint	mortality
Test Acceptability Criterion	90% or greater survival in controls

TABLE 2

TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR RAINBOW TROUT, *ONCORHYNCHUS MYKISS* ACUTE TOXICITY WITH EFFLUENTS

Test Type	Static
Test Duration	96hr
Temperature	12 ± 1 C
Light Quality	Ambient laboratory illumination
Light Intensity	Ambient laboratory levels
Photoperiod	16h light, 8 hr darkness..
Test Chamber Size	5L
Test Chamber Volume	4L
Renewal of Test Solution	Not Required
Age of Test Organisms	45-60 days
No. of Organisms per Test Chamber	10
No. of Replicate Chambers per Concentration	2
No. of Organisms per Concentration	20
Feeding Regime	Feeding not required
Test Chamber Cleaning	Cleaning not required
Test Chamber Aeration	None
Dilution Water	DDI water
Test Concentrations	5 and control
Endpoint	Mortality
Test Acceptability Criterion	90% or greater survival in controls

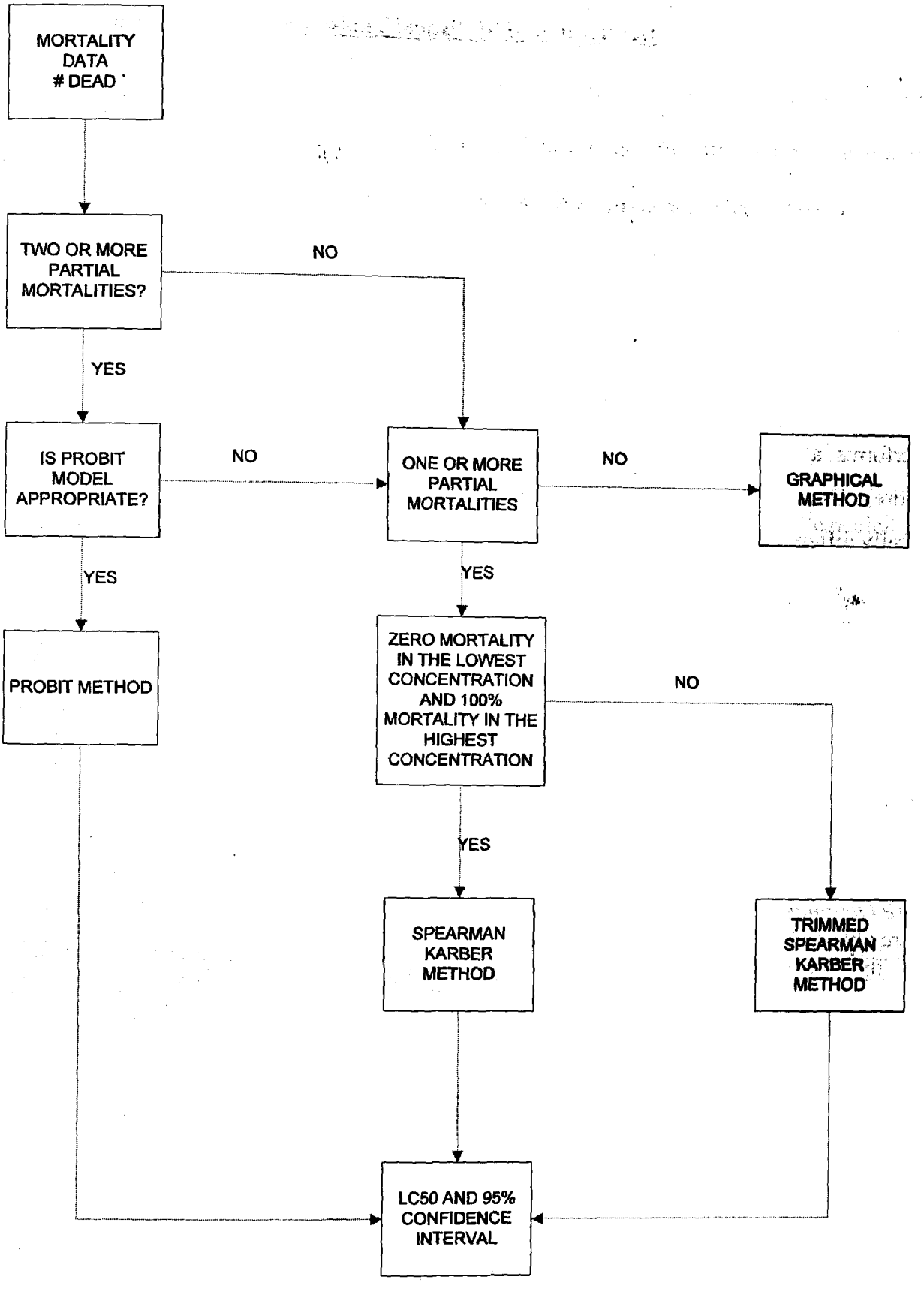

Figure 2: Flowchart for determination of LC50 for multi-toxin concentration acute toxicity tests.

Description of Statistical Tools

Dunnett Procedure

The computer program obtained from the U.S. EPA. The program was designed for the analysis of data from acute and short-term chronic toxicity tests with fish and other aquatic life, performed with effluents, receiving waters and reference toxicants. The software:

A. Performs an analysis of variance (ANOVA), which is used to obtain the error value for Dunnett's Procedure.

B. Performs a multiple comparison of treatment means with the control mean (Dunnett's Procedure). Dunnett's Procedure indicates which toxicant concentration means (if any) are statistically different from the control mean at the 5% level of significance.

C. Calculates the minimum difference between the control and treatment means that could be detected as statistically significant, and test the validity of the homogeneity of variance assumption using Bartlett's Test.

Source:
EPA. 2002. "Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms". Fifth Edition. October 2002.

Software Obtained from
Software obtained from: USEPA. 2005. Statistical Analysis for Biological Methods. Available from:< http://www.epa.gov/nerleerd/stat2.htm> [Accessed on: February, 2005].

Trimmed Spearman Karber Method

The program was designed for the analysis of mortality data from acute and chronic toxicity tests with fish and other aquatic life, performed with effluents, receiving waters, and reference toxicants. Data are input to the screen in an interactive mode. The program performs:

A. Checks the observed response proportions to determine if they are monotonically non-decreasing (i.e., the response proportion for each higher concentration of test substance is greater than or equal to the response at the previous, lower, concentration.

B. Calculates the smoothed response proportions, if necessary.

C. Automatically determines the minimum percent "trim" necessary for calculating the LC/EC50.

D. Calculates the LC/EC50 and confidence limits.

E. Provides output to the screen and printer.

Source:
Hamilton, M.A., Russo, R. C., and R.V. Thurston. 1977. "Trimmed Spearman-Karber Method for Estimating Median Lethal Concentration in Toxicity Bioassays." *Environmental Science and Technology* (11):714-719; Correction (1978), 12, pp. 417.

Software obtained from: USEPA. 2005. Statistical Analysis for Biological Methods. Available from:< http://www.epa.gov/nerleerd/stat2.htm> [Assessed December 2004].

For Example:

Trimmed Spearman Karber Method

k	The number of concentrations
n(i)	The number of individuals exposed at concentration i, i=1, ..., k
r(i)	The number of individuals that responded at concentration i, i=1, ..., k
p(i)=r(i)/n(i)	The proportion of individuals that responded at concentration i, i=1, ..., k
x(i)	Natural log of concentration i
m	Mean of the log tolerance distribution, i.e.. ln of the LC50

$$\text{Then } m = \sum_{i=1}^{K-1} \frac{(p(i) + p(i+1))(x(i) - x(i+1))}{2}$$

if p(1)=0.0 and p(k)=1.0.

For example, consider the following test:

concentration (mg/1)	0.5	1.0	2.0	4.0	8.0
number exposed	10	10	10	10	10
mortality	0	2	4	9	10
ln concentration	-0.693	0.0	0.693	1.386	2.079
mortality proportion	0.0	0.2	0.4	0.9	1.0

$$m = (0.2 - 0.0)\frac{(-0.693 + 0.0)}{2} + (0.4 - 0.2)\frac{(0.0 + 0.693)}{2} + (0.9 - 0.4)\frac{(0.693 + 1.386)}{2} + (1.0 - 0.9)\frac{(1.386 + 2.079)}{2}$$

$$= 0.693$$

LC50 = exp(m) = 2.0

TABLE 3

Dissolved Oxygen meter readings for different water samples

Water Type	Trial 1	Trial 2	Trial 3	Trial 4	Average
DDI	8.76*	8.75	8.85	8.93	8.82
DDI with O_2**	9.14	9.10	8.93	9.11	9.07
DI	8.85	8.88	8.89	8.90	8.88
DI with O_2	8.65	8.77	8.97	8.81	8.79
Tapwater	4.29	4.57	4.75	4.97	
Tapwater with O_2	8.29	8.44	8.63	8.67	4.65
Springwater	8.72	8.58	8.46	8.76	8.63
Springwater with O_2	8.87	8.88	8.90	8.85	8.88

* Readings in mg/L
** Aeration with Air Pump

DO Readings performed with DO Thermo Electron Corporation Lab DO Electrode Polargraphic 26.3°C at 102.5% saturation

REFERENCE TOXICANT

D. magna
Sodium Chloride (NaCl) used.

TABLE 4
Survival rate after 24hrs

Concentration	Trial 1	Trial 2
1.0 mg/l	5	5
3.0 mg/l	5	4
5.0 mg/l	4	3

** 5 organisms per test*

O.mykiss
Potassium Chloride (KCl) used.

TABLE 5
Survival rate after 24hrs

Concentration	Trial 1	Trial 2
3.0 mg/l	5	5
5.0 mg/l	5	5

**5 organisms per test*

10. APPENDIX B: D. Magna Bioassay Data

TABLE 1

24hr RANGE FINDING TEST FOR DAPHNIA MAGNA

Concentration (ppm)	Diesel (# Alive)					B100 (# Alive)					B20 (# Alive)				
	Trial 1	Trial 2	Trial 3	Trial 4	Ave.	Trial 1	Trial 2	Trial 3	Trial 4	Ave.	Trial 1	Trial 2	Trial 3	Trial 4	Ave.
Control	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
35	0	0	0	0	0	3	1	3	2	2.25	2	1	3	2	2
40	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

**5 daphnids per trial*

TABLE 2

DAPHNIA MAGNA PARAMETER DATA

TRIAL	TEMPERATURE (°C)	PH	GENERAL HARDNESS (mg/l)	REMARKS
Diesel 1.57 ppm T1 (24hr)	20	7.81	20	Sheen observed
Diesel 3.13 ppm T1 (24hr)	20	7.24	20	Sheen observed
Diesel 6.25 ppm T1 (24hr)	20	7.73	20	Sheen observed
Diesel 12.5 ppm T1 (24hr)	20	7.53	20	Sheen observed
Diesel 25 ppm T1 (24hr)	20	7.33	20	Sheen observed
Diesel 50 ppm T1 (24hr)	20	8.01	20	Sheen observed
Diesel 1.57 ppm T2 (24hr)	20	7.21	20	Sheen observed
Diesel 3.13 ppm T2 (24hr)	20	7.66	20	Sheen observed
Diesel 6.25 ppm T2 (24hr)	20	8.24	20	Sheen observed
Diesel 12.5 ppm T2 (24hr)	20	8.30	20	Sheen observed
Diesel 25 ppm T2 (24hr)	20	8.15	20	Sheen observed
Diesel 50 ppm T2 (24hr)	20	8.45	20	Sheen observed
Diesel 1.57 ppm T3 (24hr)	20	7.98	20	Sheen observed
Diesel 3.13 ppm T3 (24hr)	20	7.55	20	Sheen observed
Diesel 6.25 ppm T3 (24hr)	20	7.21	20	Sheen observed
Diesel 12.5 ppm T3 (24hr)	20	8.66	20	Sheen observed
Diesel 25 ppm T3 (24hr)	20	8.73	20	Sheen observed
Diesel 50 ppm T3 (24hr)	20	7.44	20	Sheen observed

Diesel 1.57 ppm T4 (0hr)	21	7.55	20	Sheen observed
Diesel 3.13 ppm T4 (24hr)	21	8.61	20	Sheen observed
Diesel 6.25 ppm T4 (24hr)	21	8.34	20	Sheen observed
Diesel 12.5 ppm T4 (24hr)	21	7.91	20	Sheen observed
Diesel 25 ppm T4 (24hr)	21	8.22	20	Sheen observed
Diesel 50 ppm T4 (24hr)	21	7.43	20	Sheen observed
AB100 1.57 ppm T1 (24hr)	20	8.64	20	
B100 3.13 ppm T1 (24hr)	20	8.32	20	
B100 6.25 ppm T1 (24hr)	20	7.12	20	
B100 12.5 ppm T1 (24hr)	20	7.23	20	
B100 25 ppm T1 (24hr)	20	7.01	20	
B100 50 ppm T1 (24hr)	21	7.44	20	
B100 1.57 ppm T2 (24hr)	20	7.21	20	
B100 3.13 ppm T2 (24hr)	20	8.11	20	
B100 6.25 ppm T2 (24hr)	20	7.49	20	
B100 12.5 ppm T2 (24hr)	20	7.11	20	
B100 25 ppm T2 (24hr)	20	7.15	20	
B100 50 ppm T2 (24hr)	21	7.32	20	
B100 1.57 ppm T3 (24hr)	20	7.23	20	
B100 3.13 ppm T3 (24hr)	20	7.44	20	
B100 6.25 ppm	20	8.61	20	

T3 (24hr)				
B100 12.5 ppm T3 (24hr)	20	7.22	20	
B100 25 ppm T3 (24hr)	21	7.33	20	
B100 50 ppm T3 (24hr)	21	7.22	20	
B100 1.57 ppm T4 (24hr)	20	7.51	20	
B100 3.13 ppm T4 (24hr)	20	7.55	20	
B100 6.25 ppm T4 (24hr)	20	7.33	20	
B100 12.5 ppm T4 (24hr)	20	7.85	20	
B100 25 ppm T4 (24hr)	21	7.88	20	
B100 50 ppm T4 (24hr)	21	7.76	20	
B20 1.57 ppm T1 (24hr)	20	7.98	20	
B20 3.13 ppm T1 (24hr)	20	7.88	20	
B20 6.25 ppm T1 (24hr)	20	7.51	20	
B20 12.5 ppm T1 (24hr)	21	7.34	20	
B20 25 ppm T1 (24hr)	21	8.35	20	
B20 50 ppm T1 (24hr)	21	8.14	20	
B20 1.57 ppm T2 (24hr)	20	8.37	20	
B20 3.13 ppm T2 (24hr)	20	8.24	20	
B20 6.25 ppm T2 (24hr)	20	8.55	20	
B20 12.5 ppm T2 (24hr)	20	8.02	20	
B20 25 ppm T2 (24hr)	20	7.44	20	
B20 50 ppm T2	20	7.61	20	

(24hr)				
B20 1.57 ppm T3 (24hr)	20	7.94	20	
B20 3.13 ppm T3 (24hr)	20	7.21	20	
B20 6.25 ppm T3 (24hr)	20	7.36	20	
B20 12.5 ppm T3 (24hr)	20	7.55	20	
B20 25 ppm T3 (24hr)	20	7.98	20	
B20 50 ppm T3 (24hr)	20	8.03	20	
B20 1.57 ppm T4 (24hr)	20	8.44	20	
B20 3.13 ppm T4 (24hr)	20	8.17	20	
B20 6.25 ppm T4 (24hr)	20	7.51	20	
B20 12.5 ppm T4 (24hr)	21	8.42	20	
B20 25 ppm T4 (24hr)	21	7.46	20	
B20 50 ppm T4 (24hr)	21	7.21	20	
B50 1.57 ppm T1 (24hr)	21	7.08	20	
B50 3.13 ppm T1 (24hr)	21	7.93	20	
B50 6.25 ppm T1 (24hr)	21	7.41	20	
B50 12.5 ppm T1 (24hr)	21	7.33	20	
B50 25 ppm T1 (24hr)	20	8.75	20	
B50 50 ppm T1 (24hr)	20	8.22	20	
B50 1.57 ppm T2 (24hr)	20	7.53	20	
B50 3.13 ppm T2 (24hr)	20	8.24	20	

B50 6.25 ppm T2 (24hr)	20	8.30	20	
B50 12.5 ppm T2 (24hr)	21	8.15	20	
B50 25 ppm T2 (24hr)	21	8.45	20	
B50 50 ppm T2 (24hr)	20	7.18	20	
B50 1.57 ppm T3 (24hr)	20	7.44	20	Slight sheen observed
B50 3.13 ppm T3 (24hr)	21	7.53	20	Slight sheen observed
B50 6.25 ppm T3 (24hr)	21	7.94	20	Slight sheen observed
B50 12.5 ppm T3 (24hr)	21	7.41	20	Slight sheen observed
B50 25 ppm T3 (24hr)	20	7.68	20	Slight sheen observed
B50 50 ppm T3 (24hr)	20	7.32	20	Slight sheen observed
B50 1.57 ppm T4 (24hr)	20	7.42	20	Slight sheen observed
B50 3.13 ppm T4 (24hr)	20	7.62	20	Slight sheen observed
B50 6.25 ppm T4 (24hr)	20	7.76	20	Slight sheen observed
B50 12.5 ppm T4 (24hr)	20	7.85	20	Slight sheen observed
B50 25 ppm T4 (24hr)	20	7.81	20	Slight sheen observed
B50 50 ppm T4 (24hr)	20	7.36	20	Slight sheen observed
B5 1.57 ppm T1 (24hr)	20	7.58	20	Slight sheen observed
B5 3.13 ppm T1 (24hr)	20	7.21	20	Slight sheen observed
B5 6.25 ppm T1 (24hr)	20	7.15	20	Slight sheen observed
B5 12.5 ppm T1 (24hr)	20	7.34	20	Slight sheen observed
B5 25 ppm T1 (24hr)	20	8.98	20	Slight sheen observed

B5 50 ppm T1 (24hr)	20	8.57	20	Slight sheen observed
B5 1.57 ppm T2 (24hr)	20	8.14	20	Slight sheen observed
B5 3.13 ppm T2 (24hr)	20	7.55	20	Slight sheen observed
B5 6.25 ppm T2 (24hr)	20	8.25	20	Slight sheen observed
B5 12.5 ppm T2 (24hr)	20	7.41	20	Slight sheen observed
B5 25 ppm T2 (24hr)	20	7.65	20	Slight sheen observed
B5 50 ppm T2 (24hr)	20	7.22	20	Slight sheen observed
B5 1.57 ppm T3 (24hr)	20	7.54	20	Slight sheen observed
B5 3.13 ppm T3 (24hr)	20	7.21	20	Slight sheen observed
B5 6.25 ppm T3 (24hr)	20	7.11	20	Slight sheen observed
B5 12.5 ppm T3 (24hr)	20	7.87	20	Slight sheen observed
B5 25 ppm T3 (24hr)	21	7.45	20	Slight sheen observed
B5 50 ppm T3 (24hr)	21	7.85	20	Slight sheen observed
B5 1.57 ppm T4 (24hr)	20	8.44	20	Slight sheen observed
B5 3.13 ppm T4 (24hr)	20	8.75	20	Slight sheen observed
B5 6.25 ppm T4 (24hr)	20	7.30	20	Slight sheen observed
B5 12.5 ppm T4 (24hr)	21	8.38	20	Slight sheen observed
B5 25 ppm T4 (24hr)	21	7.42	20	Slight sheen observed
B5 50 ppm T4 (24hr)	21	7.15	20	Slight sheen observed

TABLE 3

TOXICITY OF DIESEL ON DAPHNIA MAGNA AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	2	40	60
3.13	2	40	60
6.25	2	40	60
12.5	0	0	100
25	1	20	80
50	2	40	60

TABLE 4

TOXICITY OF DIESEL ON DAPHNIA MAGNA AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	1	20	80
3.13	1	20	80
6.25	0	0	100
12.5	1	20	80
25	1	20	80
50	0	0	100

TABLE 5

TOXICITY OF DIESEL ON DAPHNIA MAGNA AT 24HRS (TRIAL 3)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	4	80	20
3.13	1	20	80
6.25	0	0	100
12.5	0	0	100
25	0	0	100
50	0	0	100

TABLE 6

TOXICITY OF DIESEL ON DAPHNIA MAGNA AT 24HRS (TRIAL 4)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3/3	100	0
3.13	1	20	80
6.25	1	20	80
12.5	1	20	80
25	0	0	100
50	0	0	100

TABLE 7

TOXICITY OF B100 ON DAPHNIA MAGNA AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	1	20	80
3.13	2	40	60
6.25	2	40	60
12.5	3	60	40
25	2	40	60
50	2	40	60

TABLE 8

TOXICITY OF B100 ON DAPHNIA MAGNA AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	5/6	83.34	16.66
3.13	2	40	60
6.25	2	40	60
12.5	1	20	80
25	1	20	80
50	2/6	33.34	66.66

TABLE 9

TOXICITY OF B100 ON DAPHNIA MAGNA AT 24HRS (TRIAL 3)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	9/9	100	0
3.13	3	60	40
6.25	3	60	40
12.5	2	40	60
25	3	60	40
50	2	40	60

TABLE 10

TOXICITY OF B100 ON DAPHNIA MAGNA AT 24HRS (TRIAL 4)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	4	80	20
6.25	2	40	60
12.5	2	40	60
25	0	0	100
50	0	0	100

TABLE 11

TOXICITY OF B50 ON DAPHNIA MAGNA AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	3	60	40
6.25	3	60	40
12.5	1	20	80
25	1	20	80
50	1	20	80

TABLE 12

TOXICITY OF B50 ON DAPHNIA MAGNA AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	2	40	60
6.25	0	0	100
12.5	1	20	80
25	1	20	80
50	1	20	80

TABLE 13

TOXICITY OF B50 ON DAPHNIA MAGNA AT 24HRS (TRIAL 3)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	1/2	50	50
3.13	1	20	80
6.25	0	0	100
12.5	1	20	80
25	1	20	80
50	0	0	100

TABLE 14

TOXICITY OF B50 ON DAPHNIA MAGNA AT 24HRS (TRIAL 4)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	3	60	40
6.25	2	40	60
12.5	1	20	80
25	1	20	80
50	1	20	80

TABLE 15

TOXICITY OF B20 ON DAPHNIA MAGNA AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	7/9	77.78	22.22
6.25	3	60	40
12.5	3	60	40
25	2/6	33.34	66.66
50	1	20	80

TABLE 16

TOXICITY OF B20 ON DAPHNIA MAGNA AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	2	40	60
6.25	1	20	80
12.5	1	20	80
25	2	40	60
50	2	40	60

TABLE 17

TOXICITY OF B20 ON DAPHNIA MAGNA AT 24HRS (TRIAL 3)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	2	40	60
3.13	4	80	20
6.25	2	40	60
12.5	1	20	80
25	1	20	80
50	1	20	80

TABLE 18

TOXICITY OF B20 ON DAPHNIA MAGNA AT 24HRS (TRIAL 4)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	4	80	20
3.13	2	40	60
6.25	2	40	60
12.5	2	40	60
25	1	20	80
50	3/7	42.29	57.71

TABLE 19

TOXICITY OF B5 ON DAPHNIA MAGNA AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	2	40	60
6.25	2	40	60
12.5	2	40	60
25	1	20	80
50	0	0	100

TABLE 20

TOXICITY OF B5 ON DAPHNIA MAGNA AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	2	40	60
6.25	1	20	80
12.5	0	0	100
25	1	20	80
50	0	0	100

TABLE 21

TOXICITY OF B5 ON DAPHNIA MAGNA AT 24HRS (TRIAL 3)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	2	40	60
6.25	0	0	100
12.5	0	0	100
25	0	0	100
50	0	0	100

TABLE 22

TOXICITY OF B5 ON DAPHNIA MAGNA AT 24HRS (TRIAL 4)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	2	40	60
3.13	2	40	60
6.25	1	20	80
12.5	0	0	100
25	0	0	100
50	0	0	100

TABLE 23

TOXICITY OF B20 (Topia) ON DAPHNIA MAGNA AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	9/11	81.82	18.18
6.25	4	80	20
12.5	3	60	40
25	1	20	40
50	1	20	80

TABLE 24

TOXICITY OF B20 (Topia) ON DAPHNIA MAGNA AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	2	40	60
6.25	1	20	80
12.5	2	40	60
25	2	40	60
50	2	40	60

TABLE 25

TOXICITY OF B20 (Topia) ON DAPHNIA MAGNA AT 24HRS (TRIAL 3)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	3	60	40
3.13	4	80	20
6.25	2	40	60
12.5	2	40	60
25	1	20	80
50	1	20	80

TABLE 26

TOXICITY OF B20 (Topia) ON DAPHNIA MAGNA AT 24HRS (TRIAL 4)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	5	100	0
1.57	4	80	20
3.13	2	40	60
6.25	2	40	60
12.5	2	40	60
25	1	20	80
50	3/7	42.86	57.14

TABLE 27

MEAN MORTALITY OF DAPHNIA MAGNA AFFECTED WITH DIESEL

DIESEL CONCENTRATION [PPM]	MORTALITY (%) IN 4 REPLICATES IN 24HRS				MEAN MORTALITY (%)
	Trial 1	Trial 2	Trial 3	Trial 4	
1.57	60	80	20	0	40.00
3.13	60	80	80	80	75.00
6.25	60	100	100	80	85.00
12.5	100	80	100	80	90.00
25	80	80	100	100	90.00
50	60	100	100	100	90.00

TABLE 28

MEAN MORTALITY OF DAPHNIA MAGNA AFFECTED WITH B100

B100 CONCENTRATION [PPM]	MORTALITY (%) IN 4 REPLICATES IN 24HRS				MEAN MORTALITY (%)
	Trial 1	Trial 2	Trial 3	Trial 4	
1.57	80	16.66	0	40	34.17
3.13	60	60	40	20	45.00
6.25	60	60	40	60	55.00
12.5	40	80	60	60	60.00
25	60	80	40	100	70.00
50	60	66.66	60	100	71.67

TABLE 29

MEAN MORTALITY OF DAPHNIA MAGNA AFFECTED WITH B50

B50 CONCENTRATION [PPM]	MORTALITY (%) IN 4 REPLICATES IN 24HRS				MEAN MORTALITY (%)
	Trial 1	Trial 2	Trial 3	Trial 4	
1.57	40	40	50	40	42.50
3.13	40	60	80	40	55.00
6.25	40	100	100	60	75.00
12.5	80	80	80	80	80.00
25	80	80	80	80	80.00
50	80	80	100	80	85.00

TABLE 30

MEAN MORTALITY OF DAPHNIA MAGNA AFFECTED WITH B20

B20 CONCENTRATION [PPM]	MORTALITY (%) IN 4 REPLICATES IN 24HRS				MEAN MORTALITY (%)
	Trial 1	Trial 2	Trial 3	Trial 4	
1.57	40	40	60	20	40
3.13	22.22	60	20	60	40.56
6.25	40	80	60	60	60
12.5	40	80	80	60	65
25	66.66	60	80	80	71.67
50	80	60	80	57.71	69.43

TABLE 31

MEAN MORTALITY OF DAPHNIA MAGNA AFFECTED WITH B5

B5 CONCENTRATION [PPM]	MORTALITY (%) IN 4 REPLICATES IN 24HRS				MEAN MORTALITY (%)
	Trial 1	Trial 2	Trial 3	Trial 4	
1.57	40	40	40	60	45.00
3.13	60	60	60	60	60.00
6.25	60	80	100	80	80.00
12.5	60	100	100	100	90.00
25	80	80	100	100	90.00
50	100	100	100	100	100.00

TABLE 32

MEAN MORTALITY OF DAPHNIA MAGNA AFFECTED WITH B20 (Topia)

B20 CONCENTRATION [PPM]	MORTALITY (%) IN 4 REPLICATES IN 24HRS				MEAN MORTALITY (%)
	Trial 1	Trial 2	Trial 3	Trial 4	
1.57	40	40	40	20	35
3.13	18.18	60	20	60	39.55
6.25	20	80	60	60	55
12.5	40	60	60	60	55
25	80	60	80	80	75
50	80	60	80	57.14	69.29

D. magna

DIESEL

Anova: Single Factor (Diesel)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	21	3.5	0.7
Column 2	6	26	4.333333	0.266667
Column 3	6	25	4.166667	2.566667
Column 4	6	22	3.666667	3.466667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.833333	3	0.944444	0.539683	0.660586	3.098391
Within Groups	35	20	1.75			
Total	37.83333	23				

B100

Anova: Single Factor (B100)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	18	3	0.4
Column 2	6	19	3.166667	1.366667
Column 3	6	12	2	1.2
Column 4	6	19	3.166667	2.566667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.666667	3	1.888889	1.365462	0.281982	3.098391
Within Groups	27.66667	20	1.383333			
Total	33.33333	23				

B50

Anova: Single Factor (B50)

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	18	3	1.2
Column 2	6	22	3.666667	1.066667
Column 3	6	21	3.5	2.7
Column 4	6	19	3.166667	0.966667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.666667	3	0.555556	0.374532	0.772292	3.098391
Within Groups	29.66667	20	1.483333			
Total	31.33333	23				

B20

Anova:
Single
Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
T1	6	16	2.666666667	1.066666667
T2	6	19	3.166666667	0.566666667
T3	6	19	3.166666667	1.366666667
T4	6	19	3.166666667	1.766666667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.125	3	0.375	0.314685315	0.814567	3.098391
Within Groups	23.83333	20	1.191666667			
Total	24.95833	23				

B5

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	20	3.333333	1.066667
Column 2	6	23	3.833333	1.366667
Column 3	6	25	4.166667	1.766667
Column 4	6	25	4.166667	0.966667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.791667	3	0.930556	0.72043	0.551483	3.098391
Within Groups	25.83333	20	1.291667			
Total	28.625	23				

B20 (Topia)

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
T1	6	15	2.5	1.5
T2	6	18	3	0.4
T3	6	17	2.833333	1.366667
T4	6	18	3	1.2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1	3	0.333333	0.298507	0.826048	3.098391
Within Groups	22.33333	20	1.116667			
Total	23.33333	23				

Diesel 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc. (ppm)	n	Mean	s.d.	cv%
1 = control	4	1.0000	.0000	.0
1.57*	4	.6000	.3651	60.9
3.13*	4	.2500	.1000	40.0
6.25*	4	.1500	.1915	127.7
12.5*	4	.1000	.1155	115.5
25*	4	.1000	.1155	115.5
50*	4	.1000	.2000	200.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.325205
This difference corresponds to -32.52 percent of control

Between concentrations
sum of squares = 2.877143 with 6 degrees of freedom.

Error mean square = .035238 with 21 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances are zero.

B100 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc. (ppm)	n	Mean	s.d.	cv%
1 = control	4	1.0000	.0000	.0
1.57	4	.6583	.3468	52.7
3.13*	4	.5500	.1915	34.8
6.25*	4	.4500	.1000	22.2
12.5*	4	.4000	.1633	40.8
25*	4	.3000	.2582	86.1
50*	4	.2833	.1915	67.6

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.356812
This difference corresponds to -35.68 percent of control

Between concentrations
sum of squares = 1.496587 with 6 degrees of freedom.

Error mean square = .042421 with 21 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances are zero.

B50 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc. (ppm)	n	Mean	s.d.	cv%
1 = control	4	1.0000	.0000	.0
1.57*	4	.5750	.0500	8.7
3.13*	4	.4500	.1915	42.6
6.25*	4	.2500	.3000	120.0
12.5*	4	.2000	.0000	.0
25*	4	.2000	.0000	.0
50*	4	.1500	.1000	66.7

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.244270
This difference corresponds to -24.43 percent of control

Between concentrations
sum of squares = 2.232143 with 6 degrees of freedom.

Error mean square = .019881 with 21 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances are zero.

B20 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc.	n	Mean	s.d.	cv%
1 = control	4	1.0000	.0000	.0
2*	4	.6000	.1633	27.2
3*	4	.6444	.1859	28.9
4*	4	.4000	.1633	40.8
5*	4	.3500	.1915	54.7
6*	4	.2833	.1000	35.3
7*	4	.2417	.1067	44.2

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.250158
This difference corresponds to -25.02 percent of control

Between concentrations
sum of squares = 1.707963 with 6 degrees of freedom.

Error mean square = .020851 with 21 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances are zero.

B5 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc. (ppm)	n	Mean	s.d.	cv%
1 = control	4	1.0000	.0000	.0
1.57*	4	.5500	.1000	18.2
3.13*	4	.4000	.0000	.0
6.25*	4	.2000	.1633	81.6
12.5*	4	.1000	.2000	200.0
25*	4	.1000	.1155	115.5
50*	4	.0000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.196437
This difference corresponds to -19.64 percent of control

Between concentrations
sum of squares = 2.934286 with 6 degrees of freedom.

Error mean square = .012857 with 21 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances are zero.

B20 (Topia) 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc. (ppm)	n	Mean	s.d.	cv%
1 = control	4	1.0000	.0000	.0
1.57*	4	.6500	.1000	15.4
3.13*	4	.6045	.2363	39.1
6.25*	4	.4500	.2517	55.9
12.5*	4	.4500	.1000	22.2
25*	4	.2833	.1000	35.3
50*	4	.3071	.1243	40.5

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.265667
This difference corresponds to -26.57 percent of control

Between concentrations
sum of squares = 1.455966 with 6 degrees of freedom.

Error mean square = .023517 with 21 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances are zero.

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 3-15-2005
TEST NUMBER: T1+T2+T3+T4
CHEMICAL: DIESEL
SPECIES: DAPHNIA MAGNA

RAW DATA:

CONCENTRATION(PPM)	1.57	3.13	6.25	12.50	25.00	50.00
NUMBER EXPOSED:	18	20	20	20	20	20

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	%TRIM
24	1.78	1.15	2.76	44.44

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 3-15-2005
TEST NUMBER: T1+T2+T3+T4
CHEMICAL: B100
SPECIES: DAPHNIA MAGNA

RAW DATA:

CONCENTRATION(PPM)	1.57	3.13	6.25	12.50	25.00	50.00
NUMBER EXPOSED:	22	20	20	20	20	20

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	%TRIM
24	4.65	2.22	9.72	31.82

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 3-15-2005
TEST NUMBER: T1+T2+T3+T4
CHEMICAL: B50
SPECIES: DAPHNIA MAGNA

RAW DATA:

CONCENTRATION (PPM)	1.57	3.13	6.25	12.50	25.00	50.00
NUMBER EXPOSED:	17	20	20	20	20	20

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	%TRIM
24	3.29	1.36	7.95	41.18

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: TEST NUMBER:
CHEMICAL: B20 SPECIES: DM

RAW DATA:

CONCENTRATION (PPM)	1.57	3.13	6.25	12.50	25.00	50.00
NUMBER EXPOSED:	20	24	20	20	21	21

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	%TRIM
24	4.54	2.55	8.09	38.64

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 3-15-2005
TEST NUMBER: T1+T2+T3+T4
CHEMICAL: B5
SPECIES: DAPHNIA MAGNA

RAW DATA:

CONCENTRATION(PPM)	1.57	3.13	6.25	12.50	25.00	50.00
NUMBER EXPOSED:	20	20	20	20	20	20

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	% TRIM
24	1.98	0.92	4.23	45.00

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 3-15-2005
TEST NUMBER: T1+T2+T3+T4
CHEMICAL: B20 (TOPIA)
SPECIES: DAPHNIA MAGNA

RAW DATA:

CONCENTRATION (PPM)	1.57	3.13	6.25	12.50	25.00	50.00
NUMBER EXPOSED:	20	26	20	20	20	22

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	%TRIM
24	6.74	3.25	13.96	34.78

11. APPENDIX C: O. mykiss Bioassay Data

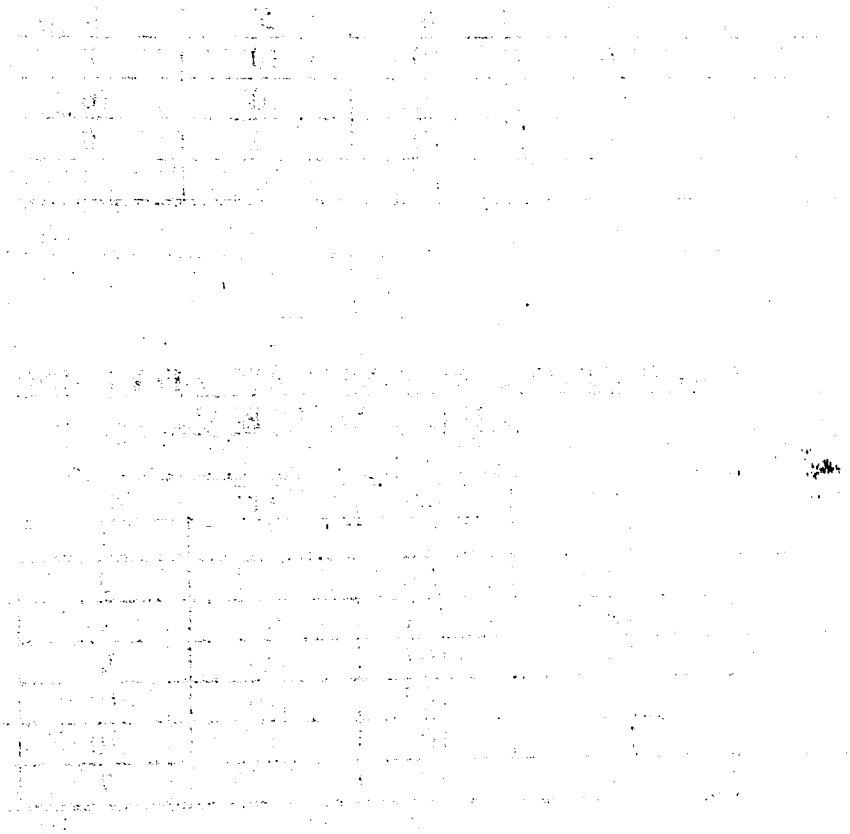

TABLE 1

TEMPERATURE AFFECT ON O. MYKISS MORTALITY USING CONTROL WATER TRIAL #1 OF 4 TRIALS

Temperature (°C)	0 hrs	6 hrs	12hrs	24hrs	48hrs	72hrs
25	9*	6	3	0	0	0
22	9	6	4	1	0	0
18	10	8	5	1	1	0
15	10	10	10	9	8	8
12	10	10	10	10	10	10
8	10	10	10	10	10	10
4	10	10	10	10	6	5

TABLE 2

TEMPERATURE AFFECT ON O. MYKISS MORTALITY USING CONTROL WATER TRIAL #2 OF 4 TRIALS

Temperature (°C)	0 hrs	6 hrs	12hrs	24hrs	48hrs	72hrs
25	10	5	3	1	0	0
22	10	7	4	3	1	0
18	10	8	6	4	1	1
15	10	10	10	9	8	8
12	10	10	10	10	10	10
8	10	10	10	10	10	10
4	10	9	9	7	6	5

TABLE 3

TEMPERATURE AFFECT ON O. MYKISS MORTALITY USING CONTROL WATER TRIAL #3 OF 4 TRIALS

Temperature (°C)	0 hrs	6 hrs	12hrs	24hrs	48hrs	72hrs
25	10	6	5	2	0	0
22	10	7	4	2	1	0
18	10	7	5	1	1	0
15	10	10	9	9	9	9
12	10	10	10	10	10	10
8	9	9	9	9	9	8
4	10	10	10	10	6	5

TABLE 4

TEMPERATURE AFFECT ON O. MYKISS MORTALITY USING CONTROL WATER TRIAL #4 OF 4 TRIALS

Temperature (°C)	0 hrs	6 hrs	12hrs	24hrs	48hrs	72hrs
25	10	7	4	2	0	0
22	9	5	4	2	0	0
18	10	8	5	1	1	0
15	9	9	9	8	8	8
12	10	10	10	10	10	10
8	10	10	10	10	10	10
4	10	10	10	10	6	5

*Number of live trout frys

TABLE 5

RANGE FINDING TEST FOR (4 week old) O. MYKISS

Concentration (ppm)	Survival of 2 Trials* after 96hrs								
	Diesel			B100			B20		
	Trial 1	Trial 2	Average	Trial 1	Trial 2	Average	Trial 1	Trial 2	Average
Control	7	9	8	9	10	9.5	7	7	7
10	4	3	3.5	3	5	4	6	4	5
50	0	1	0.5	1	1	1	0	0	0

** 10 fish fry per trial*

TABLE 6

RANGE FINDING TEST FOR (6 week old) O. MYKISS

Concentration (ppm)	Survival of 2 Trials* after 96hrs								
	Diesel			B100			B20		
	Trial 1	Trial 2	Average	Trial 1	Trial 2	Average	Trial 1	Trial 2	Average
Control	10	10	10	10	10	10	10	10	10
2.5	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10
50	10	10	10	10	10	10	10	10	10
90	10	9	9.5	10	10	10	10	10	10
100	9	8	8.5	9	9	9	10	10	10
1000	2	1	1.5	3	3	3	2	2	2
2000	0	0	0	0	0	0	0	0	0

** 10 fish fry per trial*

TABLE 7

O. MYKISS PARAMETER DATA

TRIAL	TEMPERATURE (°C)	pH	GENERAL HARDNESS (mg/l)
Diesel 100 ppm T1 (0hr)	14	8.46	40
Diesel 100 ppm T1 (96hr)	14	8.44	40
Diesel 300 ppm T1 (0hr)	14	7.79	20
Diesel 300 ppm T1 (96hr)	14	7.95	20
Diesel 600 ppm T1 (0hr)	12	8.31	20
Diesel 600 ppm T1 (96hr)	12	7.84	20
Diesel 900 ppm T1 (0hr)	12	7.81	20
Diesel 900 ppm T1 (96hr)	12	7.94	20
Diesel 1200 ppm T1 (0hr)	11	7.86	20
Diesel 1200 ppm T1 (96hr)	11	8.03	20
Diesel 100 ppm T2 (0hr)	12	8.71	20
Diesel 100 ppm T2 (96hr)	12	7.44	20
Diesel 300 ppm T2 (0hr)	12	7.72	20
Diesel 300 ppm T2 (96hr)	12	7.05	20
Diesel 600 ppm T2 (0hr)	14	8.11	20
Diesel 600 ppm T2 (96hr)	14	7.64	20
Diesel 900 ppm T2 (0hr)	14	7.39	20
Diesel 900 ppm T2 (96hr)	14	7.44	20
Diesel 1200 ppm	14	7.69	20

T2 (0hr)			
Diesel 1200 ppm T2 (96hr)	14	8.13	20
B100 100 ppm T1 (0hr)	14	8.27	20
B100 100 ppm T1 (96hr)	14	8.39	20
B100 300 ppm T1 (0hr)	14	7.55	20
B100 300 ppm T1 (96hr)	14	7.81	20
B100 600 ppm T1 (0hr)	11	7.64	20
B100 600 ppm T1 (96hr)	11	7.40	20
B100 900 ppm T1 (0hr)	11	7.08	20
B100 900 ppm T1 (96hr)	14	7.19	20
B100 1200 ppm T1 (0hr)	14	7.25	20
B100 1200 ppm T1 (96hr)	14	7.44	20
B100 100 ppm T2 (0hr)	14	7.46	20
B100 100 ppm T2 (96hr)	12	8.45	20
B100 300 ppm T2 (0hr)	12	7.31	20
B100 300 ppm T2 (96hr)	12	7.07	20
B100 600 ppm T2 (0hr)	11	8.21	20
B100 600 ppm T2 (96hr)	11	7.94	20
B100 900 ppm T2 (0hr)	12	7.61	20
B100 900 ppm T2 (96hr)	12	7.54	20
B1001200 ppm T2 (0hr)	12	7.16	20
B100 1200 ppm T2 (96hr)	12	8.23	20

B100 1200 ppm T2 (96hr)	12	8.41	20
B50 100 ppm T1 (0hr)	12	8.39	20
B50 100 ppm T1 (96hr)	12	6.93	40
B50 300 ppm T1 (0hr)	12	7.52	20
B50 300 ppm T1 (96hr)	14	7.72	20
B50 600 ppm T1 (0hr)	11	7.69	100
B50 600 ppm T1 (96hr)	11	7.29	100
B50 900 ppm T1 (0hr)	14	7.71	100
B50 900 ppm T1 (96hr)	14	7.52	100
B50 1200 ppm T1 (0hr)	12	7.14	20
B50 1200 ppm T1 (96hr)	12	6.93	20
B50 100 ppm T2 (0hr)	12	7.78	100
B50 100 ppm T2 (96hr)	12	7.31	100
B50 300 ppm T2 (0hr)	12	7.80	20
B50 300 ppm T2 (96hr)	12	7.65	20
B50 600 ppm T2 (0hr)	14	7.42	100
B50 600 ppm T2 (96hr)	14	7.37	100
B50 900 ppm T2 (0hr)	14	7.63	20
B50 900 ppm T2 (96hr)	14	7.40	20
B50 1200 ppm T2 (0hr)	12	7.98	40
B50 1200 ppm T2 (96hr)	12	8.31	40

B20 100 ppm T1 (0hr)	12	7.41	20
B20 100 ppm T1 (96hr)	12	8.46	20
B20 300 ppm T1 (0hr)	12	8.24	20
B20 300 ppm T1 (96hr)	12	7.40	20
B20 600 ppm T1 (0hr)	12	8.26	20
B20 600 ppm T1 (96hr)	12	8.15	20
B20 900 ppm T1 (0hr)	12	7.96	20
B20 900 ppm T1 (96hr)	12	8.03	20
B20 1200 ppm T1 (0hr)	12	8.21	20
B20 1200 ppm T1 (96hr)	12	7.48	20
B20 100 ppm T2 (0hr)	14	7.21	20
B20 100 ppm T2 (96hr)	14	7.54	20
B20 300 ppm T2 (0hr)	14	7.97	20
B20 300 ppm T2 (96hr)	14	7.64	20
B20 600 ppm T2 (0hr)	11	7.35	20
B20 600 ppm T2 (96hr)	11	7.31	20
B20 900 ppm T2 (0hr)	12	7.89	20
B20 900 ppm T2 (96hr)	12	8.02	20
B20 1200 ppm T2 (0hr)	12	8.35	20
B5 100 ppm T1 (0hr)	12	8.39	20
B5 100 ppm T1 (96hr)	12	7.40	20
B5 300 ppm T1	12	8.49	20

(0hr)			
B5 300 ppm T1 (96hr)	12	8.76	20
B5 600 ppm T1 (0hr)	12	8.52	20
B5 600 ppm T1 (96hr)	12	7.74	40
B5 900 ppm T1 (0hr)	12	8.35	20
B5 900 ppm T1 (96hr)	14	7.81	20
B5 1200 ppm T1 (0hr)	14	7.68	20
B5 1200 ppm T1 (96hr)	14	7.73	20
B5 100 ppm T2 (0hr)	14	8.51	40
B5 100 ppm T2 (96hr)	14	8.49	40
B5 300 ppm T2 (0hr)	14	8.76	20
B5 300 ppm T2 (96hr)	14	8.41	20
B5 600 ppm T2 (0hr)	14	7.49	20
B5 600 ppm T2 (96hr)	14	6.97	20
B5 900 ppm T2 (0hr)	11	5.13	20
B5 900 ppm T2 (96hr)	12	6.62	20
B5 1200 ppm T2 (0hr)	12	6.39	20
B5 1200 ppm T2 (96hr)	12	7.41	20

TABLE 8

TOXICITY OF DIESEL ON O. MYKISS AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	7	70	30
300	8	80	20
600	7	70	30
900	1	10	90
1200	1	10	90
100(ii)	10	100	0

TABLE 9

TOXICITY OF DIESEL ON O. MYKISS AT 48HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	5	50	50
300	6	60	40
600	5	50	50
900	1	10	90
1200	0	0	100
100(ii)	9	90	10

TABLE 10

TOXICITY OF DIESEL ON O. MYKISS AT 72HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	5	50	50
300	2	20	80
600	3	30	70
900	0	0	100
1200	0	0	100
100 (ii)	8	80	20

TABLE 11

TOXICITY OF DIESEL ON O. MYKISS AT 96HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	2	20	80
300	2	20	80
600	3	30	70
900	0	0	100
1200	0	0	100
100 (ii)	8	80	20

TABLE 12

TOXICITY OF DIESEL ON O. MYKISS AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	8	80	20
300	7	70	30
600	7	70	30
900	1	10	90
1200	1	10	90
600(ii)	7	70	30

TABLE 13

TOXICITY OF DIESEL ON O. MYKISS AT 48HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	6	60	40
300	5	50	50
600	4	40	60
900	1	10	90
1200	0	0	100
600(ii)	4	40	60

TABLE 14

TOXICITY OF DIESEL ON O. MYKISS AT 72HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	5	50	50
300	0	0	100
600	3	30	70
900	0	0	100
1200	0	0	100
600(ii)	3	30	70

TABLE 15

TOXICITY OF DIESEL ON O. MYKISS AT 96HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	1	10	90
300	0	0	100
600	3	30	70
900	0	0	100
1200	0	0	100
600(ii)	2	20	80

TABLE 16

TOXICITY OF B100 ON O. MYKISS AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	10	100	0
600	7	70	30
900	9	90	10
1200	3	30	70
600(ii)	10	100	0

TABLE 17

TOXICITY OF B100 ON O. MYKISS AT 48HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	10	100	0
600	3	30	70
900	9	90	10
1200	1	10	90
600(ii)	8	80	20

TABLE 18

TOXICITY OF B100 ON O. MYKISS AT 72HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	10	100	0
600	1	10	90
900	4	40	60
1200	1	10	90
600(ii)	8	80	20

TABLE 19

TOXICITY OFB100 ON O. MYKISS AT 96HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	10	100	0
600	1	10	90
900	2	20	80
1200	0	0	100
600(ii)	5	50	50

TABLE 20

TOXICITY OF B100 ON O. MYKISS AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	7	70	30
300	10	100	0
600	7	70	30
900	9	90	10
1200	3	30	70
900(ii)	7	70	30

TABLE 21

TOXICITY OF B100 ON O. MYKISS AT 48HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	7	70	30
300	10	100	0
600	3	30	70
900	9	90	10
1200	1	10	90
900(ii)	5	50	50

TABLE 22

TOXICITY OF B100 ON O. MYKISS AT 72HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	5	50	50
300	10	100	0
600	1	10	90
900	4	40	60
1200	1	10	90
900(ii)	4	40	60

TABLE 23

TOXICITY OF B100 ON O. MYKISS AT 96HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	5	50	50
300	10	100	0
600	1	10	90
900	1	10	90
1200	0	0	100
900(ii)	2	20	80

TABLE 24

TOXICITY OF B50 ON O. MYKISS AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	6	60	40
900	3	30	70
1200	10	100	0

TABLE 25

TOXICITY OF B50 ON O. MYKISS AT 48HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	6	60	40
600	3	30	70
900	3	30	70
1200	1	10	90

TABLE 26

TOXICITY OF B50 ON O. MYKISS AT 72HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	6	60	40
600	0	0	100
900	0	0	100
1200	0	0	100

TABLE 27

TOXICITY OF B50 ON O. MYKISS AT 96HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	3	30	70
600	0	0	100
900	0	0	100
1200	0	0	100

TABLE 28

TOXICITY OF B50 ON O. MYKISS AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	6	60	40
900	3	30	70
1200	10	100	0

TABLE 29

TOXICITY OF B50 ON O. MYKISS AT 48HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	3	30	70
900	3	30	70
1200	1	10	90

TABLE 30

TOXICITY OF B50 ON O. MYKISS AT 72HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	8	80	20
600	3	30	70
900	0	0	100
1200	1	10	90

TABLE 31

TOXICITY OF B50 ON O. MYKISS AT 96HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	8	80	20
600	0	0	100
900	0	0	100
1200	0	0	100

TABLE 32

TOXICITY OF B20 ON O. MYKISS AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	7	70	30
900	6	60	40
1200	5	50	50
600(ii)	6	60	40

TABLE 33

TOXICITY OF B20 ON O. MYKISS AT 48HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	9	90	10
600	7	70	30
900	3	30	70
1200	1	10	90
600(ii)	3	30	70

TABLE 34

TOXICITY OF B20 ON O. MYKISS AT 72HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	100	0
100	10	100	0
300	7	70	30
600	5	0	50
900	0	0	100
1200	3	30	100
600(ii)	7	70	30

TABLE 35

TOXICITY OF B20 ON O. MYKISS AT 96HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	7	70	30
600	5	50	50
900	0	0	100
1200	0	0	100
600(ii)	2	20	80

TABLE 36

TOXICITY OF B20 ON O. MYKISS AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	7	70	30
900	7	70	30
1200	4	40	60

TABLE 37

TOXICITY OF B20 ON O. MYKISS AT 48HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	7	70	30
900	3	30	30
1200	1	10	90

TABLE 38

TOXICITY OF B20 ON O. MYKISS AT 72HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	8	80	20
300	10	100	0
600	5	50	50
900	0	0	100
1200	0	0	100

TABLE 39

TOXICITY OF B20 ON O. MYKISS AT 96HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	6	60	40
300	10	100	0
600	5	50	50
900	0	0	100
1200	0	0	100

TABLE 40

TOXICITY OF B5 ON O. MYKISS AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	9	90	10
600	10	100	0
900	2	20	80
1200	1	10	90

TABLE 41

TOXICITY OF B5 ON O. MYKISS AT 48HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	7	70	30
600	5	50	50
900	2	20	80
1200	0	0	100

TABLE 42

TOXICITY OF B5 ON O. MYKISS AT 72HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	2	20	80
600	2	20	80
900	0	0	100
1200	0	0	100

TABLE 43

TOXICITY OF B5 ON O. MYKISS AT 96HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	2	20	80
600	2	20	80
900	0	0	100
1200	0	0	100

TABLE 44

TOXICITY OF B5 ON O. MYKISS AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	10	100	0
900	2	20	80
1200	1	10	90

TABLE 45

TOXICITY OF B5 ON O. MYKISS AT 48HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	7	70	30
600	5	50	50
900	2	20	80
1200	1	10	90

TABLE 46

TOXICITY OF B5 ON O. MYKISS AT 72HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	9	90	10
300	2	20	80
600	5	50	50
900	0	0	100
1200	1	10	90

TABLE 47

TOXICITY OF B5 ON O. MYKISS AT 96HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	3	30	70
300	0	0	100
600	0	0	100
900	0	0	100
1200	1	10	90

TABLE 48

TOXICITY OF B20 (Topia) ON O. MYKISS AT 24HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	9	90	10
900	8	80	20
1200	5	50	50

TABLE 49

TOXICITY OF B20 (Topia) ON O. MYKISS AT 48HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	7	70	30
900	5	50	50
1200	3	30	70

TABLE 50

TOXICITY OF B20 (Topia) ON O. MYKISS AT 72HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	100	0
100	10	100	0
300	9	90	10
600	5	50	50
900	3	30	70
1200	0	0	100

TABLE 51

TOXICITY OF B20 (Topia) ON O. MYKISS AT 96HRS (TRIAL 1)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	7	70	30
600	5	50	50
900	3	30	70
1200	0	0	100

TABLE 52

TOXICITY OF B20 (Topia) ON O. MYKISS AT 24HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	90	90	90
900	60	60	40
1200	40	40	60

TABLE 53

TOXICITY OF B20 (Topia) ON O. MYKISS AT 48HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	10	100	0
300	10	100	0
600	5	50	50
900	5	50	50
1200	2	20	80

TABLE 54

TOXICITY OF B20 (Topia) ON O. MYKISS AT 72HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	8	80	20
300	10	100	0
600	5	50	50
900	3	30	30
1200	0	0	100

TABLE 55

TOXICITY OF B20 (Topia) ON O. MYKISS AT 96HRS (TRIAL 2)

CONCENTRATION (PPM)	NO. SURVIVING	% ALIVE	% DEAD
Control	10	10	0
100	8	80	20
300	10	100	0
600	4	40	60
900	1	10	90
1200	0	0	100

TABLE 56

MEAN MORTALITY OF O. MYKISS AFFECTED WITH DIESEL

Diesel Concentration [ppm]	*Mean Mortality (%) in 2 Replicates*				**Mean Mortality (%) over 96hrs**
	24hr	48hr	72hr	96hr	
100	16.66	33.33	40.00	63.33	38.33
300	25.00	45.00	90.00	90.00	62.50
600	30.00	56.66	70.00	73.33	57.50
900	90.00	90.00	100.00	100.00	95.00
1200	90.00	100.00	100.00	100.00	97.50
Average Mortality for each 24hr period	50.33	64.50	80	85.33	

TABLE 57

MEAN MORTALITY OF O. MYKISS AFFECTED WITH B100

B100 Concentration [ppm]	*Mean Mortality(%) in 2 Replicates*				**Mean Mortality (%)**
	24hr	48hr	72hr	96hr	
100	20.00	20.00	30.00	30.00	25.00
300	0.00	0.00	0.00	0.00	0.00
600	20.00	53.33	66.66	76.66	54.16
900	16.66	23.33	60.00	83.33	45.83
1200	70.00	90.00	90.00	100.00	87.50
Average Mortality for each 24hr period	25.33	37.33	49.33	57.98	

TABLE 58

MEAN MORTALITY OF O. MYKISS AFFECTED WITH B50

B50 Concentration [ppm]	*Mean Mortality(%) in 2 Replicates*				**Mean Mortality (%)**
	24hr	48hr	72hr	96hr	
100	0.00	0.00	10.00	10.00	5.00
300	0.00	20.00	30.00	45.00	23.75
600	40.00	70.00	85.00	100.00	73.75
900	70.00	70.00	100.00	100.00	85.00
1200	0.00	90.00	95.00	100.00	71.25
Average Mortality for each 24hr period	22	50	64	71	

TABLE 59

MEAN MORTALITY OF O. MYKISS AFFECTED WITH B20

B20 Concentration [ppm]	*Mean Mortality (%) in 2 Replicates*				**Mean Mortality (%)**
	24hr	48hr	72hr	96hr	
100	0.00	0.00	10.00	20.00	7.50
300	0.00	5.00	15.00	15.00	8.75
600	33.33	43.33	43.33	60.00	45.00
900	35.00	70.00	100.00	100.00	76.25
1200	55.00	90.00	100.00	100.00	86.25
Average Mortality for each 24hr period	24.67	41.67	53.67	59	

TABLE 60

MEAN MORTALITY OF O. MYKISS AFFECTED WITH B5

B5 Concentration [ppm]	*Mean Mortality in 2 Replicates*				**Mean Mortality (%)**
	24hr	48hr	72hr	96hr	
100	0.00	10.00	10.00	40.00	15.00
300	5.00	30.00	80.00	90.00	51.25
600	0.00	50.00	65.00	90.00	51.25
900	80.00	80.00	100.00	100.00	90.00
1200	90.00	95.00	95.00	95.00	93.75
Average Mortality for each 24hr period	35	53	70	83	

TABLE 61

MEAN MORTALITY OF O. MYKISS AFFECTED WITH B20 (Topia)

B20 Concentration [ppm]	*Mean Mortality (%) in 2 Replicates*				**Mean Mortality (%)**
	24hr	48hr	72hr	96hr	
100	0	0	10	10	5.00
300	0	0	5	15	5.00
600	10	40	50	55	38.75
900	30	50	70	80	57.5
1200	55	75	100	100	82.5
Average Mortality for each 24hr period	19	33	47	52	

O. mykiss

DIESEL

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Diesel 24hrs T1	6	26	4.333333	14.26667
Diesel 24hrs T2	6	29	4.833333	10.56667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.75	1	0.75	0.060403	0.810831	4.964603
Within Groups	124.1667	10	12.41667			
Total	124.9167	11				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Diesel 48hrs T1	6	34	5.666667	11.06667
Diesel 48hrs T2	6	40	6.666667	5.466667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3	1	3	0.362903	0.56031	4.964603
Within Groups	82.66667	10	8.266667			
Total	85.66667	11				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Diesel 72hrs T1	6	42	7	9.6
Diesel 72hrs T2	6	49	8.166667	4.566667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.083333	1	4.083333	0.576471	0.46522	4.964603
Within Groups	70.83333	10	7.083333			
Total	74.91667	11				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Diesel 96hrs T1	6	45	7.5	8.7
Diesel 96hrs T2	6	54	9	1.6

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.75	1	6.75	1.31068	0.278933	4.964603
Within Groups	51.5	10	5.15			
Total	58.25	11				

B100

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B100 24hrs T1	6	12	2	7.2
B100 24hrs T2	6	12	2	7.2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0	1	0	0	1	4.964603
Within Groups	72	10	7.2			
Total	72	11				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B100 48hrs T1	6	20	3.333333	13.86667
B100 48hrs T2	6	25	4.166667	12.16667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.083333	1	2.083333	0.160051	0.697522	4.964603
Within Groups	130.1667	10	13.01667			
Total	132.25	11				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B100 72hrs T1	6	27	4.5	16.3
B100 72hrs T2	6	35	5.833333	10.96667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.333333	1	5.333333	0.391198	0.545679	**4.964603**
Within Groups	136.3333	10	13.63333			
Total	141.6667	11				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B100 96hrs T1	6	33	5.5	17.9
B100 96hrs T2	6	41	6.833333	14.16667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.333333	1	5.333333	0.33264	0.57686	**4.964603**
Within Groups	160.3333	10	16.03333			
Total	165.6667	11				

B50

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B50 24hrs T1	5	11	2.2	10.2
B50 24hrs T2	5	11	2.2	10.2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*F crit*
Between Groups	-1.4E-14	1	-1.4E-14	-1.4E-15	**5.317655**
Within Groups	81.6	8	10.2		
Total	81.6	9			

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B50 48hrs T1	5	27	5.4	12.3
B50 48hrs T2	5	23	4.6	18.3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.6	1	1.6	0.104575	0.754698	5.317655
Within Groups	122.4	8	15.3			
Total	124	9				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B50 72hrs T1	5	35	7	18
B50 72hrs T2	5	29	5.8	16.7

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.6	1	3.6	0.207493	0.660849	5.317655
Within Groups	138.8	8	17.35			
Total	142.4	9				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B50 96hrs T1	5	38	7.6	15.3
B50 96hrs T2	5	33	6.6	21.8

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.5	1	2.5	0.134771	0.723057	5.317655
Within Groups	148.4	8	18.55			
Total	150.9	9				

B20

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B20 24hrs T1	6	16	2.666667	4.666667
B20 24hrs T2	5	12	2.4	6.3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.193939	1	0.193939	0.035964	0.853798	**5.117355**
Within Groups	48.53333	9	5.392593			
Total	48.72727	10				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B20 48hrs T1	6	27	4.5	13.5
B20 48hrs T2	5	19	3.8	16.7

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.336364	1	1.336364	0.089555	0.771539	**5.117355**
Within Groups	134.3	9	14.92222			
Total	135.6364	10				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B20 72hrs T1	6	31	5.166667	16.56667
B20 72hrs T2	5	27	5.4	20.8

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.148485	1	0.148485	0.008049	0.930479	**5.117355**
Within Groups	166.0333	9	18.44815			
Total	166.1818	10				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B20 96hrs T1	6	36	6	16.4
B20 96hrs T2	5	29	5.8	18.2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.109091	1	0.109091	0.006342	0.938266	5.117355
Within Groups	154.8	9	17.2			
Total	154.9091	10				

B5

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B5 24hrs T1	5	18	3.6	20.3
B5 24hrs T2	5	17	3.4	21.8

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1	1	0.1	0.004751	0.946741	5.317655
Within Groups	168.4	8	21.05			
Total	168.5	9				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B5 48hrs T1	5	27	5.4	13.3
B5 48hrs T2	5	26	5.2	11.2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1	1	0.1	0.008163	0.93023	5.317655
Within Groups	98	8	12.25			
Total	98.1	9				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B5 72hrs T1	5	37	7.4	13.8
B5 72hrs T2	5	33	6.6	13.3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.6	1	1.6	0.118081	0.739982	5.317655
Within Groups	108.4	8	13.55			
Total	110	9				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B5 96hrs T1	5	37	7.4	13.8
B5 96hrs T2	5	46	9.2	1.7

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.1	1	8.1	1.045161	0.336541	5.317655
Within Groups	62	8	7.75			
Total	70.1	9				

B20 (Topia)

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B20(Topia)T1 24hrs	5	8	1.6	4.3
B20(Topia)T2 24hrs	5	11	2.2	7.2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.9	1	0.9	0.156522	0.70272	5.317655
Within Groups	46	8	5.75			
Total	46.9	9				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B20(Topia)T1 48hrs	5	15	3	9.5
B20(Topia)T2 48hrs	5	18	3.6	12.3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.9	1	0.9	0.082569	0.781149	5.317655
Within Groups	87.2	8	10.9			
Total	88.1	9				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B20(Topia)T1 72hrs	5	23	4.6	17.3
B20(Topia)T2 72hrs	5	24	4.8	15.7

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1	1	0.1	0.006061	0.939859	5.317655
Within Groups	132	8	16.5			
Total	132.1	9				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
B20(Topia)T1 96hrs	5	25	5	14.5
B20(Topia)T2 96hrs	5	27	5.4	18.8

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.4	1	0.4	0.024024	0.880663	5.317655
Within Groups	133.2	8	16.65			
Total	133.6	9				

Diesel 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	3	.8333	.1528	18.3
300*	2	.7500	.0707	9.4
600*	3	.7000	.0000	.0
900*	2	.1000	.0000	.0
1200*	2	.1000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by a t - test with Bonferroni adjustment of alpha level

Minimum detectable difference for
t-tests with Bonferroni adjustment = -.232823
This difference corresponds to -23.28 percent of control

Note - the above value for the minimum detectable difference is approximate as the sample sizes are not the same for all of the concentrations.

Between concentrations
sum of squares = 1.557619 with 5 degrees of freedom.

Error mean square = .006458 with 8 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

Diesel 48hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100*	3	.6667	.2082	31.2
300*	2	.5500	.0707	12.9
600*	3	.4333	.0577	13.3
900*	2	.1000	.0000	.0
1200*	2	.0000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by a t - test with Bonferroni adjustment of alpha level

Minimum detectable difference for
t-tests with Bonferroni adjustment = -.321196
This difference corresponds to -32.12 percent of control

Note - the above value for the minimum detectable difference is approximate as the sample sizes are not the same for all of the concentrations.

Between concentrations
sum of squares = 1.410238 with 5 degrees of freedom.

Error mean square = .012292 with 8 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

Diesel 72hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100*	3	.6000	.1732	28.9
300*	2	.1000	.1414	141.4
600*	3	.3000	.0000	.0
900*	2	.0000	.0000	.0
1200*	2	.0000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by a t - test with Bonferroni adjustment of alpha level

Minimum detectable difference for
t-tests with Bonferroni adjustment = -.289711
This difference corresponds to -28.97 percent of control

Note - the above value for the minimum detectable difference is approximate as the sample sizes are not the same for all of the concentrations.

Between concentrations
sum of squares = 1.655000 with 5 degrees of freedom.

Error mean square = .010000 with 8 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

Diesel 96hrs

Summary Statistics and ANOVA

Transformation = None

Conc. (ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100*	3	.3667	.3786	103.3
300*	2	.1000	.1414	141.4
600*	3	.2667	.0577	21.7
900*	2	.0000	.0000	.0
1200*	2	.0000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by a t - test with Bonferroni adjustment of alpha level

Minimum detectable difference for
t-tests with Bonferroni adjustment = -.573354
This difference corresponds to -57.34 percent of control

Note - the above value for the minimum detectable difference is approximate as the sample sizes are not the same for all of the concentrations.

Between concentrations
sum of squares = 1.435952 with 5 degrees of freedom.

Error mean square = .039167 with 8 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

B100 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc. (ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	.8000	.1414	17.7
300	2	1.0000	.0000	.0
600	3	.8000	.1732	21.7
900	3	.8333	.1155	13.9
1200*	2	.3000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by a t - test with Bonferroni adjustment of alpha level

Minimum detectable difference for
t-tests with Bonferroni adjustment = -.334529
This difference corresponds to -33.45 percent of control

Note - the above value for the minimum detectable difference is approximate as the sample sizes are not the same for all of the concentrations.

Between concentrations
sum of squares = .662619 with 5 degrees of freedom.

Error mean square = .013333 with 8 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

B100 48hrs

Summary Statistics and ANOVA

Transformation = None

Conc. (ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	.8000	.1414	17.7
300	2	1.0000	.0000	.0
600*	3	.4667	.2887	61.9
900	3	.7667	.2309	30.1
1200*	2	.1000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by a t - test with Bonferroni adjustment of alpha level

Minimum detectable difference for
t-tests with Bonferroni adjustment = -.554754
This difference corresponds to -55.48 percent of control

Note - the above value for the minimum detectable difference is approximate as the sample sizes are not the same for all of the concentrations.

Between concentrations
sum of squares = 1.270238 with 5 degrees of freedom.

Error mean square = .036667 with 8 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

B100 72hrs

Summary Statistics and ANOVA

Transformation = None

Conc. (ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	.7000	.2828	40.4
300	2	1.0000	.0000	.0
600*	3	.3333	.4041	121.2
900*	3	.4000	.0000	.0
1200*	2	.1000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by a t - test with Bonferroni adjustment of alpha level

Minimum detectable difference for
t-tests with Bonferroni adjustment = -.653189
This difference corresponds to -65.32 percent of control

Note - the above value for the minimum detectable difference is approximate as the sample sizes are not the same for all of the concentrations.

Between concentrations
sum of squares = 1.467619 with 5 degrees of freedom.

Error mean square = .050833 with 8 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

B100 96hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	.7000	.2828	40.4
300	2	1.0000	.0000	.0
600*	3	.2333	.2309	99.0
900*	3	.1667	.0577	34.6
1200*	2	.0000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by a t - test with Bonferroni adjustment of alpha level

Minimum detectable difference for
t-tests with Bonferroni adjustment = -.450374
This difference corresponds to -45.04 percent of control

Note - the above value for the minimum detectable difference is approximate as the sample sizes are not the same for all of the concentrations.

Between concentrations
sum of squares = 2.115238 with 5 degrees of freedom.

Error mean square = .024167 with 8 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

B50 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	1.0000	.0000	.0
300	2	1.0000	.0000	.0
600*	2	.6000	.0000	.0
900*	2	.3000	.0000	.0
1200	2	1.0000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = .000000
This difference corresponds to .00 percent of control

Between concentrations
sum of squares = .896667 with 5 degrees of freedom.

Error mean square = .000000 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

B50 48hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	1.0000	.0000	.0
300	2	.8000	.2828	35.4
600*	2	.3000	.0000	.0
900*	2	.3000	.0000	.0
1200*	2	.1000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.326780
This difference corresponds to -32.68 percent of control

Between concentrations
sum of squares = 1.576667 with 5 degrees of freedom.

Error mean square = .013333 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero

B50 72hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	.9000	.0000	.0
300	2	.7000	.1414	20.2
600*	2	.1500	.2121	141.4
900*	2	.0000	.0000	.0
1200*	2	.0500	.0707	141.4

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.305675
This difference corresponds to -30.57 percent of control

Between concentrations
sum of squares = 2.036667 with 5 degrees of freedom.

Error mean square = .011667 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed
as 1 or more of the variances is zero.

B50 96hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	.9000	.0000	.0
300*	2	.5500	.3536	64.3
600*	2	.0000	.0000	.0
900*	2	.0000	.0000	.0
1200*	2	.0000	.0000	.0

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.408475
This difference corresponds to -40.85 percent of control

Between concentrations
sum of squares = 2.224167 with 5 degrees of freedom.

Error mean square = .020833 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed
as 1 or more of the variances is zero.

B5 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	1.0000	.0000	.0
300	2	.9500	.0707	7.4
600	2	1.0000	.0000	.0
900*	2	.2000	.0000	.0
1200*	2	.1000	.0000	.0

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.081695
This difference corresponds to -8.17 percent of control

Between concentrations
sum of squares = 1.884167 with 5 degrees of freedom.

Error mean square = .000833 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed
as 1 or more of the variances is zero.

B5 48hrs

Summary Statistics and ANOVA

Transformation = None

Conc. (ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	.4500	.6364	141.4
300	2	.7000	.0000	.0
600	2	.5000	.0000	.0
900*	2	.2000	.0000	.0
1200*	2	.0500	.0707	141.4

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.739780
This difference corresponds to -73.98 percent of control

Between concentrations
sum of squares = 1.166667 with 5 degrees of freedom.

Error mean square = .068333 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

B5 72hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	.9000	.0000	.0
300*	2	.2000	.0000	.0
600*	2	.3500	.2121	60.6
900*	2	.0000	.0000	.0
1200*	2	.0500	.0707	141.4

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.258342
This difference corresponds to -25.83 percent of control

Between concentrations
sum of squares = 1.866667 with 5 degrees of freedom.

Error mean square = .008333 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed
as 1 or more of the variances is zero.

B5 96hrs

Summary Statistics and ANOVA

Transformation = None

Conc.(ppm)	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
100	2	.6000	.4243	70.7
300*	2	.1000	.1414	141.4
600*	2	.1000	.1414	141.4
900*	2	.0000	.0000	.0
1200*	2	.0500	.0707	141.4

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.548027
This difference corresponds to -54.80 percent of control

Between concentrations
sum of squares = 1.624167 with 5 degrees of freedom.

Error mean square = .037500 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

B20 (Topia) 24hrs

Summary Statistics and ANOVA

Transformation = None

Conc.	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
2	2	1.0000	.0000	.0
3	2	1.0000	.0000	.0
4	2	.9000	.0000	.0
5*	2	.7000	.1414	20.2
6*	2	.4500	.0707	15.7

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.182676
This difference corresponds to -18.27 percent of control

Between concentrations
sum of squares = .504167 with 5 degrees of freedom.

Error mean square = .004167 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed
as 1 or more of the variances is zero.

B20 (Topia) 48hrs

Summary Statistics and ANOVA

Transformation = None

Conc.	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
2	2	1.0000	.0000	.0
3	2	1.0000	.0000	.0
4*	2	.6000	.1414	23.6
5*	2	.5000	.0000	.0
6*	2	.2500	.0707	28.3

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.182676
This difference corresponds to -18.27 percent of control

Between concentrations
sum of squares = 1.037500 with 5 degrees of freedom.

Error mean square = .004167 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

B20 (Topia) 72hrs

Summary Statistics and ANOVA

Transformation = None

Conc.	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
2	2	.9000	.1414	15.7
3	2	.9500	.0707	7.4
4*	2	.5000	.0000	.0
5*	2	.3000	.0000	.0
6*	2	.0000	.0000	.0

*) the mean for this conc. is significantly less than
the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.182676
This difference corresponds to -18.27 percent of control

Between concentrations
sum of squares = 1.664167 with 5 degrees of freedom.

Error mean square = .004167 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed
as 1 or more of the variances is zero.

B20 (Topia) 96hrs

Summary Statistics and ANOVA

Transformation = None

Conc.	n	Mean	s.d.	cv%
1 = control	2	1.0000	.0000	.0
2	2	.9000	.1414	15.7
3	2	.8500	.2121	25.0
4*	2	.4500	.0707	15.7
5*	2	.2000	.1414	70.7
6*	2	.0000	.0000	.0

*) the mean for this conc. is significantly less than the control mean at alpha = 0.05 (1-sided) by Dunnett's test

Minimum detectable difference for Dunnett's test = -.346603
This difference corresponds to -34.66 percent of control

Between concentrations
sum of squares = 1.696667 with 5 degrees of freedom.

Error mean square = .015000 with 6 degrees of freedom.

Note - the test for equality of variances could not be computed as 1 or more of the variances is zero.

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 6-12-2004
TEST NUMBER: T1 & T2
CHEMICAL: DIESEL
SPECIES: O.MYKISS

RAW DATA:

CONCENTRATION (PPM)	100.00	300.00	600.00	900.00	1200.00
NUMBER EXPOSED:	30	20	30	20	20

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	% TRIM
24	578.13	421.28	793.37	16.67
48	350.38	173.04	709.47	33.33
72	133.52	88.94	200.45	40.00
96	NC	NC	NC	NC

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 6-12-2004
TEST NUMBER: (T1 & T2)
CHEMICAL: B100
SPECIES: O.MYKISS

RAW DATA:

CONCENTRATION(PPM)	100.00	300.00	600.00	900.00	1200.00
NUMBER EXPOSED:	20	20	30	30	20

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	% TRIM
24	1073.54	994.69	1158.63	30.00
48	756.68	646.56	885.56	10.00
72	555.19	462.94	665.82	15.00
96	455.28	391.30	529.73	15.00

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 6-12-2004
TEST NUMBER: (T1 & T2)
CHEMICAL: B50
SPECIES: O.MYKISS

RAW DATA:

CONCENTRATION (PPM)	100.00	300.00	600.00	900.00	1200.00
NUMBER EXPOSED:	20	20	20	20	20

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	% TRIM
24	NC	NC	NC	NC
48	491.11	386.78	623.59	10.00
72	348.32	267.24	453.99	10.00
96	276.71	212.79	359.82	10.00

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 6-12-2004
TEST NUMBER: (T1 & T2)
CHEMICAL: B20
SPECIES: O.MYKISS

RAW DATA:

CONCENTRATION (PPM)	100.00	300.00	600.00	900.00	1200.00
NUMBER EXPOSED:	20	20	30	20	20

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	% TRIM
24	1074.31	752.15	1534.46	45.00
48	659.02	566.51	766.64	10.00
72	541.27	427.62	685.13	10.00
96	497.60	421.00	588.15	17.50

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 6-12-2004
TEST NUMBER: (T1 & T2)
CHEMICAL: B5
SPECIES: O.MYKISS

RAW DATA:

CONCENTRATION (PPM)	100.00	300.00	600.00	900.00	1200.00
NUMBER EXPOSED:	20	20	20	20	20

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	% TRIM
24	780.67	718.61	848.09	10.00
48	463.30	343.07	625.65	10.00
72	234.47	177.93	308.97	10.00
96	124.57	84.38	183.92	40.00

TRIMMED SPEARMAN-KARBER METHOD. MONTANA STATE UNIV

FOR REFERENCE, CITE:
HAMILTON, M.A., R.C. RUSSO, AND R.V. THURSTON, 1977.
TRIMMED SPEARMAN-KARBER METHOD FOR ESTIMATING MEDIAN
LETHAL CONCENTRATIONS IN TOXICITY BIOASSAYS.
ENVIRON. SCI. TECHNOL. 11(7): 714-719;
CORRECTION 12(4):417 (1978).

DATE: 2-10-2005
TEST NUMBER: T1+T2
CHEMICAL: B20 TOPIA
SPECIES: O.MYKISS

RAW DATA:

CONCENTRATION (PPM)	100.00	300.00	600.00	900.00	1200.00
NUMBER EXPOSED:	20	20	20	20	20

DURATION (HOURS)	LC50	LOWER 95% LIMIT	UPPER 95% LIMIT	%TRIM
24	1132.91	918.15	1397.89	45.00
48	790.87	619.02	1010.43	25.00
72	606.21	510.08	720.46	7.50
96	527.96	406.28	686.07	10.00

BL-74-136