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Biodegradation Patterns and Toxicity of the Constituents of Canola Oil

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ABSTRACT

The past decade witnessed a steady growth in the global production of vegetable oil to meet an increased demand that reached more than 110 million metric tons produced in the 2005/06 production year. According to the U.S. Environment Protection Agency (EPA), bulk shipment and storage of non-petroleum oils (e.g. vegetable oils) can result in spills and leaks that have significant impacts on fresh water and marine environments even though such oils may be biodegradable under both aerobic and anaerobic conditions. Among all the vegetable oils commercially available in the market, canola oil is believed to have the "best fatty-acid profile of any edible oil" which makes canola oil one of the most popular oils in the market.

This research was aimed at designing and conducting experiments on five primary component triglycerides of canola oil, three liquids triolein (OOO), trilinolein (LLL), and trilinolenin (LnLnLn) and two solids tripalmitin (PPP) and tristearin (SSS), to investigate their aerobic biodegradability and their toxic byproducts during biodegradation. The rate and extent of triglycerides biodegradation were examined in respirometry flask tests, at the same time toxicity tests were performed on the fatty acid byproducts. Also, a comparative study was performed on commercial canola oil and synthetic canola oil using the same experimental setup and methods to identify and generalize the causes of the observed biodegradability and toxicity.

The performance of the solid triglyceride experiments was largely restricted by their extremely low solubility and low polarity. When mixed with water, PPP and SSS formed irregular lumps, not the uniform and homogeneous suspension required for the lipase activity, rendering them not

available for microbial attack. No substantial mineralization was observed after 30 days of reaction time. In the case of liquid triglycerides, a competition between autoxidation and biodegradation took place due to the presence of the double bonds in their fatty acid chains. The produced hydroperoxides polymerize and become non-biodegradable while the non-oxidized portions readily biodegrade and mostly mineralize.

Microtox[®] Toxicity was only observed at the early stages of biodegradation in the solid phase and was proven to be caused by the degraded FFA (Free Fatty Acids) products. All three unsaturated triglycerides followed the same increasing then gradually decreasing trend during the toxicity studies. Triolein was observed to have the highest toxicity among the five tested triglycerides throughout the 30-day experiment.

Higher toxicity and higher FFA were detected during the commercial canola oil experiments than during the synthetic canola oil ones, possibly due to the presence of additives in the commercial oil intended to prevent breakdown, biodegradation and peroxidation.

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Finally, I dedicate this thesis to my mom. Her unconditional love and selfless devotion to my upbringing and education were all the motivation I needed to overcome any obstacle I encountered in my life.

TO MY MOTHER, JUN

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CHAPTER 1 INTRODUCTION

1.1 Background

1.1.1 Vegetable Oil Consumption and Production Increasing Trend

In the past fifty years, fatty acid (FA) constituents in human diet witnessed a shift away from animal fats coupled with an increase in the consumption of vegetable oils (Hui, 1996a).

According to the United States Department of Agriculture (USDA, 2006), more than 110 million metric tons of vegetable oils were produced in the year 2005/06, a 50% increase compared to 1996/97. Among the numerous applications of vegetable oil other than the traditional culinary uses, such as making cosmetic products, plastic, lubricant and insulator for electrical industry, biofuel is of special importance for its use in the mitigation of greenhouse gas emissions and oil prices. Biofuel production has increased four-folds between 2004 and 2006 to reach 1 billion gallons.

World trade of canola oil has also grown considerably over the last 15 years. Canola oil production currently ranks third behind palm and soybean accounting for over 14% of total vegetable oil production, canola is also the world's second leading source of protein meals (only after soybean meal and containing approximately 36% protein). Since rapeseed yields more oils per seed than soybean and other hard seeds, it can be concluded that canola comes first in terms of production of true oilseeds. World production has been rapidly growing with Food and Agriculture Organization of the United Nations (FAO) reporting that 36 million tons and 46 million tons of rapeseed were produced in the 2003/04 and 2004/05 seasons.

1.1.2 Canola Oil Properties

Canola oil was initially extracted in Canada in the 1970's. It is a generic variation from rapeseed oil and is also known as "LEAR" oil (Low Erucic Acid Rapeseed), containing less than two per cent (%) erucic acid and less than 30 micromoles (μ moles) glucosinolates, both known to be mildly toxic to human bodies. Canola oil is characterized by lower levels of saturated FAs that contribute to high levels of cholesterol in the blood. It also contains a relatively high level of oleic acid (60%) and α -linolenic acid (9.6%), which are necessary for several physiological functions such as cell building and hormone production (Hui, 1996b). Due to the health advantages associated with canola oil, Canada has risen as the leading world exporter of vegetable oils due mainly to the aforementioned increase in the demand for canola oil.

1.1.3 Vegetable Oil Spills Impacts and Biodegradation Pathways

Traditionally regarded as non-toxic consumable goods, vegetable oils may have devastating environmental effects when released in sufficient amounts (USEPA, 1997). Accidental spills occur during transportation, loading and storage of large amounts of vegetable oils (Wincele et al., 2004). Coating birds, mammals, fish food and water surfaces, increasing BOD and depleting dissolved oxygen; and directly poisoning animals are a few of the hazardous effects associated with vegetable oil spills (USEPA, 1997; Mudge, 1995). These effects are similar to those caused by petroleum spills due to common properties such as solubility, specific gravity, and viscosity (Crump-Wiesner et al, 1975). Even though the size and frequency of vegetable oil spills are relatively lower than those of petroleum ones, the effects are still comparable. McKelvey et al. (1980) reported on three small spills of canola oil that caused greater damage to the bird

population in the Vancouver Harbor than 176 spills of petroleum oils over a 5-year period (1974-1978). Some researchers have shown that animals are even more vulnerable to vegetable oils than petroleum oils (Boyd, 1973). Not surprisingly, the size and frequency of canola oil spills in the Vancouver Harbor, the main canola oil exporting port in Canada, increased dramatically since 1974 in conjunction with the demand increase. Consequently, thousands of birds were either killed or poisoned (Bucas & Saliot, 2002). As a result of the devastating environmental impacts of canola oil, it becomes essential to thoroughly evaluate the toxic effects and biodegradability of canola oil and of any intermediates produced during the microbial degradation of its constituents.

The aerobic biodegradation of vegetable oils consists of three major reactions. The process starts with triglycerides (TGCs) undergoing lipolysis (hydrolysis by lipases) and breaking down into glycerol and three long chain fatty acids (LCFA). FAs are then activated by enzyme fatty acyl-CoA-synthetase and are transported into the mitochondria (Riendeau and Meighen, 1985). Once inside the mitochondrial matrix, β -oxidation and thiolysis occur where the FAs are completely mineralized in the tricarboxylic acid cycle.

1.1.4 Regulatory History

The Oil Pollution Act (OPA) was passed by the United States Congress after the Exxon Valdez oil spill in 1989, one of the largest oil spills in history, to prevent further oil spills from occurring in the United States. The act applies to all oils, including petroleum oils, animal fats, vegetable oils, and other non-petroleum oils. Animal fats and vegetable oils have their own unique

properties and legislation, in addition to the ones they share with petroleum-based oils (USEPA, 2000).

In August 12, 1994, a letter titled “Petition for Reconsideration and Stay of Effective Date” was submitted to Environmental Protection Agency (EPA) by seven agricultural organizations including the American Soybean Association and the National Oilseed Processors Association. The purpose of the petition was to exclude the non-petroleum, “non-toxic” oils from the OPA-mandated Facility Response Plan (FRP) final rule that applies to facilities that handle, store, or transport animal fats or vegetable oils.

However due to all the aforementioned vegetable oil spill effects discussed in section 1.1.3, the request to distinguish vegetable oils and animal fat (non-petroleum oils) from petroleum oils with regards to spill cleanups was denied in the 1997 Federal Register Rules and Regulations report (USEPA, 1997). In 2002, the U.S. EPA re-stressed the definition of “oil” and stated that vegetable oils and petroleum oils are equivalent with regards to spill cleanups (USEPA, 2002).

1.2 Research Tools

1.2.1 Toxicity Assay

1.2.1.1 Toxicity Study History

Toxicity is the extent to which a substance produces harm or damage to living organisms. There are typically two types of toxicity: acute and chronic. Acute toxicity refers to the short-term harmful effects through a single dose or multiple exposures, whereas chronic toxicity is the

ability of a substance to cause toxic effect continuously or over an extended period of time, sometimes extending over the entire life of the exposed organism. Toxicity is dose-dependant and recipient-related. In the study of toxicology, it is believed that all substances are poisons; the right dose differentiates a poison from a remedy. Different individuals generally respond differently to the same dose of toxin. LD50 (lethal dose) or LC50 (lethal concentration), a given dose or concentration which causes the death of 50% of the sample population of a specific test-animal in a specified period, is the most common measure for toxicity.

Nowadays, toxicity testing has become a common regulatory requirement to assess potential environmental damage, especially at the “screening” level of an evaluation. Farre and Barcelo (2003) provided a summary of a variety of used bioassays. Fish, such as rainbow trout and fathead minnow exposed to a toxicant to a maximum of 96 hours, are traditionally used for growth-rate tests and lethality tests. They offer good sensitivities and allow real-time analysis. However, standardization of these methods is often hard to achieve and requires operators with adequate training and skills. *Daphnia* is one of the most commonly used invertebrate species due to its high sensitivity and short reproductive cycle. The 21-day test is well-established and standardized as an acute lethality experiment. Toxicity assays based on plant species, like oats (*Avena sativa*) and Chinese cabbage (*Brassica campestris*), have also been developed but seldom used due to the long duration of the tests. Algae are also used as toxicity indicators. The restriction on the algae bioassay is the difficulty in culturing and the lack of reproducibility between individual assays. Bacterial bioassays tend to fall in one of the following five categories: population growth, metabolic heat production, respiration rate, adenosine triphosphate (ATP) luminescence and bioluminescence inhibition assays, (Ricco G. etc, 2004)

with the last one being the most extensively used for evaluating environmental samples.

Numerous researchers have conducted experiments and proved its simplicity, sensitivity, accuracy, reproducibility, low cost, ease of handling and short exposure time.

1.2.1.2 Microtox[®]

Microtox[®] has become a common tool to assess potential environmental toxicity due to the high cost of conventional animal test alternatives. Microtox[®] is believed to offer great sensitivity, reproducibility and precision and is time saving and cost effective (Gitierrez, 2002; Curtix et al., 1982; Dalzell, 2002; Wang, 2002). It is used worldwide especially for assessing aquatic toxicity. Microtox[®] has been adopted by the U.S. Fish and Wildlife Service as a screening test at the National Fisheries Contaminant Research Centre. Microtox[®] is also used in Canada, the United Kingdom, and several European Union countries like Germany, Australia, Sweden and the Netherlands. In 2004, the U.S. EPA considered adding the Microtox[®] Toxicity Test to the approved methods for Whole Effluent Toxicity (WET) testing. If this proposal becomes an accepted standard, testing for National Pollutant Discharge Elimination System (NPDES) permits would be able to utilize the Microtox[®] protocol (Smith, 2005).

The Microtox[®] Test, developed in 1979, utilizes the concentration of toxicant that causes 50% inhibition of light emission (EC₅₀) of a luminescent gram-negative bacterium (*Photobacterium phosphoreum*, now known as *Vibrio fischeri*) as an indicator of acute toxicity (Bitton & Dutka, 1986). *Vibrio fischeri*, a gram-negative rod-shaped bacterium, is found globally in the marine

environment and emits light naturally due to the presence of a luciferase enzyme. A blue-green light of wavelength of 490 nm is emitted as a result of the following reaction (Inouye, 1994):

* FMNH₂-reduced flavin mononucleotide

R-CHO- aliphatic aldehyde

This light production is directly proportional to the metabolic activity of the bacterial population, hence any inhibition of the enzymatic activity correspondingly causes decrease in bioluminescence.

A luminescence percentage (I%) is the ratio of the response given by a saline control solution to that of the sample.

$$I\% = [1 - (\text{sample light emission}) / (\text{control light emission})] \times 100 \quad [2]$$

Microtox[®] has several applications. It can be used for toxicity measurements of single compounds and mixtures of organic and inorganic compounds. The technique has been used for measuring short and long-term photo-induced toxicity of polynuclear aromatic hydrocarbons (PAHs) (El-Alawi et al., 2002). The bioassay can be utilized for almost all kinds of samples such as complex effluents (Hao, 1996), surface and groundwater (Boyd et al., 1997), municipal waste effluent and sediment (Carlson-Ekvall, 1995; Gutierrez, 2002). Bioluminescence inhibition assays have been used as a sensitive and rapid screening tool for determining the whole effluent toxicity for various industrial effluents such as dye wastewaters from textile

industry (Wang, 2002), white water and effluents from paper mills (Thi Kim Oanh and Bengtsson, 1995), and fuel oils (Lin, 2002).

1.2.2 Respirometry

A respirometric system or respirometer is a device used to obtain estimates of the metabolic rates of living organisms by monitoring respiratory activities, namely oxygen consumption and CO₂ evolution. A respirometer consists of three major components: an air supply, an air-tight closed vessel called the “bioreactor” that contains the living test organisms with tested chemicals, and a measuring device that quantifies the amount of O₂ uptake or CO₂ production. Respirometry is most commonly used for the characterization of wastewater and activated sludge and constituents. In respirometry, the respiration rate is defined as the amount of oxygen per unit of volume and time that is consumed by the microorganisms.

1.3 Research Objectives

The objective of this study is to simulate the condition when vegetable oil is released into the environment, to determine the rate and extent of the TGCs biodegradation, and to monitor the changes in increased toxicity.

Experiments were designed and conducted on five primary canola oil component TGCs; triolein (OOO, 55% w/w), trilinolein (LLL, 26%), trilinolenin (LnLnLn, 18%), tripalmitin (PPP, 4%) and tristearin (SSS, 1.5%); to delineate the cause of the induced toxicity and to investigate the contribution of the various TGCs of vegetable oils to toxicity. Biodegradability and toxicity

studies were also carried out simultaneously on the commercially-available as well as synthetic oils. Accordingly, the objectives of this research encompass the following:

- Design experiments to determine the rate and the extent of TGCs and canola oil biodegradation in respirometry test,
- Monitor changes in toxicity levels during biodegradation,
- Identify the causes of the observed changes in toxicity, and
- Generalize the results to predict toxicity patterns of other commercial oils based on various proportions of the different TGCs investigated in this research.

1.4 Layout of the Thesis

Following the introduction section, chapter two lists the methodology, i.e. material and methods, and experimental plan used in the research. Results of the five TGC respirometry experiments and commercial and synthetic experiments are presented in chapter three, along with their toxicity analyses data. Finally conclusion and discussion are included in chapter four.

CHAPTER 2. METHODOLOGY

2.1 Material and Methods

2.1.1 Chemicals

The five TGCs OOO, LLL, LnLnLn, PPP, and SSS, along with their corresponding FAs (oleic, linoleic, linolenic, palmitic, and stearic) with a minimum purity of 99% were purchased from Sigma–Aldrich (St. Louis, MO, USA). Chemical structures of the five TGCs are shown in Figure 2.1. All glassware used was deactivated with a solution of 5% dimethyldichlorosilane in toluene obtained from Supelco (Bellefonte, PA, USA). All organic solvents were Optima grade and were acquired from Fisher Scientific (Pittsburg, PA, USA). Microtox[®] reagents were obtained from Strategic Diagnostics Inc. (Newark, DE, USA).

Saturated TGCs

Unsaturated TGCs

Figure 2.1 Chemical structures of the five major TGC components of canola oil

2.1.2 Development of a Master Culture for Biodegradation of Triglycerides

Two 6-L continuous flow reactors (No.3 and No.5) were operated for the purpose of growing and harvesting an enriched bacterial culture to be used for the biodegradation experiments of the five TGCs of interest. Activated sludge from a local municipal wastewater treatment plant was used to seed the reactors. Activated sludge was added at a concentration of 100 mg of volatile suspended solids (VSS) per liter of reactor volume. Refined canola oil was fed to the reactors in a continuous manner at a feed rate ranging between 20 and 100 mg/L-d of reactor volume supplemented with a micro- and macronutrients solution feed (Table 2.1). A porous polyethylene pot (20 μm pore size) was used to provide the effective barrier in this reactor to separate the clear effluent from the biomass. The reactor setup is illustrated schematically in Figure 2.2. Ten percent of the reactor volume was withdrawn daily in order to maintain a solids residence time of 10 days. A 40-day operation resulted in the formation of a stable enrichment culture that was used for further study. At the end of these 40 days, the biomass was taken out from each reactor and combined to form an even culture, washed with a sterile saline solution, then frozen at -80 °C for storage. The frozen culture was stored in glycerol in 5-mL Cryo-vials for use throughout the duration of the project.

Table 2.1 Micronutrients, macronutrients and vitamins concentration in respirometry flasks

	Chemical	Concentration, (mg/L)
Micro-nutrients	CuSO ₄ .H ₂ O	0.08
	Na ₂ MoO ₄ .2H ₂ O	0.15
	MnSO ₄ .H ₂ O	0.13
	ZnCl ₂	0.23
	CoCl ₂ .6H ₂ O	0.42
Macro-nutrients	K ₂ HPO ₄	11.25
	KNO ₃	72.17
	FeCl ₂ .4H ₂ O	17.25
	CaCl ₂ .2H ₂ O	22.5
	MgSO ₄ .7H ₂ O	69.6
Vitamins	4-Aminobenzoic acid, 99%	1.5 x 10 ⁻²
	Biotin	5.85 x 10 ⁻³
	Cyanocobalamin (B ₁₂)	3 x 10 ⁻⁴
	Folic acid dihydrate, 99%	5.85 x 10 ⁻³
	Nicotinic acid, 98%	1.5 x 10 ⁻²
	Panthenic acid, Ca salt hydrate	1.5 x 10 ⁻²
	Pyridoxine, hydrochloride, 98%	3 x 10 ⁻²
	(-)-riboflavin, 98%	1.5 x 10 ⁻²
	Thiamine hydrochloride	1.5 x 10 ⁻²
	Thioctic acid, 98%	1.5 x 10 ⁻²

Figure 2.2 Schematic of the porous pot reactor used for microbial culture enrichment

2.1.3 Respirometry Experiments

Respirometry was employed in the biodegradation experiments to measure oxygen (O_2) uptake and carbon dioxide (CO_2) emission of the bacterial culture enriched in the porous pots within a closed experimental system. The rate of microbial respiration within the respirometers is a direct measure of biological activity and therefore provides insight into the extent of canola oil constituents' degradation within the system.

Three computerized respirometers (N-Con Systems Co., Crawford, GA) with a total capacity of 36 reactors were utilized for the biodegradability tests. The oxygen uptake in each reactor was measured continuously and recorded hourly by a computer system. The oxygen demand was measured as a pressure drop within the system caused by the absorption of CO_2 from the headspace by a base such as potassium hydroxide (KOH). The O_2 demand required to metabolize the TGC or oil was extrapolated by subtracting O_2 demand from the total O_2 uptake at each time point (collected hourly) throughout the experiment. The setup of the respirometer with the oxygen supply and computer connection is shown in Figure 2.3 and Figure 2.4.

Each reactor was equipped with a trap containing 0.1 N KOH solution to absorb the evolved CO_2 from the system headspace (Figure 2.5). The amount of CO_2 produced within the system was calculated at discrete points based on the relative drop in pH of the KOH solution. The KOH solution in the traps was replaced when the solution pH dropped from 13 to below approximately 10.5, as indicated by the pH indicator dye Alizarin Red. Curves were fitted to the collected data to approximate the CO_2 profile for each reactor.

Figure 2.3 Respirometer setup

Figure 2.4 Connections between respirometer and each reactor

Figure 2.5 Respirometry reactor setup

Prior to each respirometry experiment, approximately 25 vials (4.5 mL each) of frozen biomass store at -80°C were taken out and allowed to reach room temperature (21°C). The content of the vials were then transferred to a 500 mL flask. A 200 mL volume of saline solution (0.85%) was added in order to dilute the glycerol. The biomass was then centrifuged and the supernatant removed. The biomass was reconstituted with 100 mL of the saline solution. The thirty-two bottles that contained 250 mL of sterilized nutrient and buffer solution were then prepared by adding 275 μL (0.25 triolein) (1g/L), and 2.5 mL of defrosted biomass. The respirometry flasks were daily monitored and sacrificed in conformity with the QA plan. Three flasks were sacrificed at time 0, 1, 2, 4, 8, 12, 16, 24, and 30 days.

Triplicate respirometer reactors were prepared for each experiment. All control reactors were also monitored. For all reactors and controls, 250 mL nutrient solution was dispensed into 500

mL respirometer vessels and sterilized in the autoclave at 121 °C for 20 minutes. To ensure no spores are present in the TGCs or vegetable oil, 20 mL aliquots of TGCs or oil was placed into 40 mL glass vials, capped loosely, and autoclaved at 100 °C for one hour on each of three consecutive days. A sterile pipette was used to dispense 0.25 g of the respective TGC or oil, by weight, to each respirometer vessel. All reactors received 1.0 mL of the culture developed in Section 2.1.2 with the exception of the control flasks that had no microorganisms. Reactors were incubated at 20 °C in N-CON Model 512 Respirometers (N-CON Instruments, Crawford, GA) with automated data recording. Reactor contents were continuously stirred using magnetic stir bars.

2.1.4 Chemical Analysis

Funnel filtration was performed to separate the insoluble and soluble components of the respirometry reactors. The filtration setup consisted of a 150-mL fritted glass funnel (Pyrex, Corning, NY) containing a glass fiber filter (0.45 µm define micrometers) (Fisher Scientific, Pittsburgh, PA) covered by a 5-centimeter layer of salinized glass beads. Vacuum was used to accelerate filtration and to ensure the dryness of the filter paper and glass beads prior to extraction. After the separation of the two matrices, the aqueous (liquid) phase was analyzed for FAs while the solid phase was analyzed for TGCs and FAs.

2.1.5 Solid Phase Extraction

Solid-phase extraction (SPE) was used to separate free fatty acids (FFAs) and other byproducts from large quantity of water (approximately 220 mL) after 30 mL of sample collected for aqueous phase toxicity test. A reverse phased octyl-bonded cartridge (ENVI-8; Supelco, Sigma-Aldrich) was used for this purpose.

Free FAs and other by-products were separated from water using SPE techniques after an aqueous sample was collected for toxicity analysis. The stationary phase consisted of 500 mg of octyl C8 (ENVI-8). Cartridges were preconditioned by washing with 10 mL of 40% acetone in DCM followed by 10 mL of methanol and, finally, with 10 mL superQ water (pH $\approx$ 2.0). All samples, blanks, controls, and SPE extraction blanks were spiked with a surrogate compound, Undecanoic Acid (Sigma–Aldrich), for recovery control, and then loaded at a rate of 6 mL/min. After a drying period of approximately 20 minutes, analytes were eluted with 10 mL of acetone/DCM (2:3). Finally, the extracts were transferred to reaction vials and evaporated to dryness under a gentle stream of nitrogen as a preliminary step prior to free fatty acid methyl esters (FAMES) analysis by gas chromatography-mass spectroscopy (GC/MS).

2.1.6 Microtox[®] Bioassay

The following reagents were obtained from Strategic Diagnostic Inc. (SDI, Newark, Delaware) for the Microtox[®] Test: Microtox[®] Reagent (freeze-dried *Vibrio Fishery* bacteria in sealed vials), Microtox[®] Reconstitution solution, Microtox[®] Diluent (2% sodium chloride solution), Microtox[®]

Osmotic Adjusting solution (22% sodium chloride solution), Microtox[®] Disposable Cuvettes.

All the Microtox[®] reagents were stored at –20°C upon receipt.

The Microtox[®] Model 500 Analyzer, shown in Figure 2.6, was utilized for all analyses. The instrument consisted of 30 incubator blocks and one “read” well maintained at 15°C. A reagent well, containing the reconstituted bacteria, was maintained at 5°C.

Figure 2.6 The Microtox[®] Model 500 Analyzer

2.2 Experimental Plan

This section describes the experimental design, operation of the system, performed measurements and sampling plan. The various adopted analytical procedures are shown in the flow chart of Figure 2.7. The sampling strategy and procedures are listed in Tables 2.2 and 2.3, respectively.

Figure 2.7 Flow chart of different analytical procedures

Table 2.2 Sampling Strategy

Sample/Measurement Location	Matrix	Measurement	Frequency	Experimental Quality Control	Total No. Samples
Respirometer flask	aqueous	Microtox [®]	once per flask	triplicate (minimum) flasks per treatment	determined by experimental design
Respirometer flask	organic solvent	Microtox [®]	once per flask	triplicate (minimum) flasks per treatment	determined by experimental design
Respirometer flask	aqueous	FFAs & other degradation products by GC/MS	once per flask	triplicate (minimum) flasks per treatment, surrogate recovery	determined by experimental design
Respirometer flask	organic solvent	TGCs & FFAs by HPLC	once per flask	triplicate (minimum) flasks per treatment	determined by experimental design

Table 2.3 Sampling Procedures

Analysis	Sample Quantity (mL)	Sampling Method	Holding Time (days)	Sample Preservation	Container
Microtox [®]	30/10	Grab	2	Cool at 4 °C	Glass with Teflon [®] lined cap
Solid-phase extraction	220	volume left behind in flask	2	Cool at 4 °C	Bottle with Teflon [®] lined cap
Derivatization	0.5	Whole extracted sample	1	Cool at 4 °C	Glass with Teflon [®] lined cap
GC/MS analysis	1	Whole derivatized sample	1	Cool at 4 °C	Glass with Teflon [®] lined cap
Solid Liquid extraction	60	Whole extracted sample	2	Cool at 4 °C	Glass with Teflon [®] lined cap
HPLC analysis	1	Extracted sample	2	Cool at 4 °C	Glass with Teflon [®] lined cap

2.2.1 Experimental Design: General Description

The overall experimental approach included the following steps:

1. Enrich a hydrocarbon-degrading bacterial culture in porous pot bioreactors for use in respirometry studies;
2. Develop experiments to determine the rate and the extent of TGCs biodegradation in a respirometry flask test;
3. Conduct an analytical investigation of the commercially-available canola oils in order to determine their TGC compositions. The results were then used to reconstitute the oils from the pure TGC constituents according to the predetermined composition;
4. Perform a thorough toxicity analysis on long chain FAs and commercially-available vegetable oils;
5. Perform a comparative analysis of the biodegradation and toxicity of synthetic and refined canola oil; and
6. Provide a criteria and recommendations to generalize the results of the five studied TGCs as well as the synthetic canola oil.

2.2.2 Biodegradability Tests of Five Major Triglyceride Components of Canola Oil

Respirometry experiments were conducted using the five individual canola oil TGCs and refined vegetable oils using inocula from the hydrocarbon-degrading bacterial culture previously enriched in the porous pot bioreactors. The purest available forms of the five TGCs were purchased from a commercial chemicals supplier for that purpose. Triplicate respirometer reactors were prepared for each treatment. Three control flasks containing nutrients and the

respective TGC or oil in addition to three blank flasks containing only nutrients and culture were run as well. For all treatments and controls, a 250-mL nutrient solution was dispensed into a 500-mL respirometer vessel and sterilized at 121°C in an autoclave for 20 minutes. A sterile pipette was used to dispense 0.25 g of the respective TGC or oil to each respirometer vessel, resulting in an initial concentration of 1 g/L. All reactors received 2.5 mL of the mixed culture with the exception of the control flasks that did not include any microorganisms. All reactors were incubated at 20°C in N-CON Model 512 Respirometers with automated data recording (N-CON Systems Co., Crawford, GA). Reactor contents were continuously stirred using magnetic stir bars. Oxygen was supplied continuously and was consumed as needed.

2.2.3 Sampling Schedule

Three respirometry flasks, corresponding to time zero, were immediately sacrificed after the addition of the carbon source, nutrients and inoculums for the analysis of the initial TGC concentration by lowering the pH to 2. The following sampling events were done on days 1, 2, 4, 8, 12, 16, 24 and 30. For each sampling event, three flasks were sacrificed and the contents were filtered in order to separate the solid and aqueous phases.

2.2.4 Toxicity Analysis

The liquid phase of each sacrificed respirometer was analyzed via Microtox[®] immediately in accordance to the Basic Test protocol after raising the sample pH to approximately 7. Bacterial luminescence was measured at time 0 (i.e. prior to the addition of the sample), then after 5 minutes and 15 minutes of contact time. The difference in bioluminescence represented the toxic

effect of the sample. EC₅₀, the concentration causing 50% reduction of the light emission, was read automatically from the Microtox[®] Omni software. Toxicity Unit (TU) calculated using EC₅₀ divided by 100, is also a common measurement for toxicity. Phenol solution, producing an EC₅₀ value ranging between 13 and 26, was used for quality control. As for the solid phase, methylene chloride (DCM) that was previously used for TGC extraction was volatilized. Microtox[®] Basic Test was performed after the TGCs were re-dissolved in ethanol which is relatively nontoxic to *Vibrio fischeri*, the bacterium used as a toxicity indicator. The toxicity of all FA chains starting from formic acid (C1) to palmitic acid (C16), as well as most commercially-available cooking oils, was also measured and used as a reference.

2.2.5 Quantitative Analysis

Following separation by SPE, both solid and liquid phases were analyzed using an HP 6890 Series GC/MS. The SPE filters were also extracted using methanol and DCM then HPLC analysis of the eluents was performed.

2.2.6 Comparative Analysis of Synthetic and Refined Canola Oil Biodegradation

Upon the completion of the experiments involving the TGCs, major biodegradation intermediate FAs and their role in toxicity were identified. The next step was to determine whether the data obtained for individual constituents can be used to predict the behavior of commercially-available refined oil products. The entire experimental procedure was repeated for canola oil synthesized from its major TGC constituents. A similar experiment was run separately using commercially-available refined canola oil.

CHAPTER 3. RESULTS AND DISCUSSIONS

3.1 Bioreactor Performance

Total suspended solids (TSS) and volatile suspended solids were measured on a weekly basis to monitor growth of the microbial culture. Triplicate crucibles containing 0.45-micron glass fiber filters (Millipore AP40 glass fiber filters, Cat. #AP400W405) at the bottom were heated in a muffle oven at 550 °C. The initial weight was measured using an analytical balance (OHAUS analytical plus) after the crucibles cooled down to room temperature inside a desiccator. After the biomass samples were vacuum filtered, the crucibles were kept in an oven at 105 °C for approximately 2 hours and weighed again. The weight difference was calculated to determine the total suspended solids. Following this, the crucibles were put in a muffle oven at 550 °C for another two hours and weighed. The volatile suspended solid portion of the biomass was then calculated. The biomass growth as TSS and VSS in reactors No.3 and No. 5 is illustrated in Figures 3.1 and Figure 3.2, respectively.

Figure 3.1 Biomass growth as VSS and TSS concentrations in Reactor 3

Figure 3.2 Biomass growth as VSS and TSS concentrations in Reactor 5

3.2 Fatty Acids Toxicity

To better understand the toxicity of FAs, the biodegradation byproducts of TGCs and vegetable oil, acute toxicity tests were performed on formic acid (C1) through palmitic acid (C16). The toxicity of different chemicals to the aquatic ecosystem is largely determined by their chemical properties. Among those, solubility is one of the most important factors. The solubility of C1 through C13 is listed in Table 3.1 (Merck, 2001). Solubility decreases as more carbons are linked in the aliphatic chain. LCFAs consisting of more than 10 carbons in their aliphatic chains are generally not soluble in water. The initial concentration in the bulk solution prepared for each FA is also shown in Table 3.1.

Table 3.1 Fatty Acids (C1 through C12) solubility and tested concentrations in liquid phase toxicity studies

CAS Number	Carbon Numbers	Fatty Acids	Solubility (mg/L)	Tested Sample Conc.(mg/L)
64-18-6	1	Formic Acid (C1)	miscible with water	10,736.0
64-19-7	2	Acetic Acid (C2)	miscible with water	10,500.0
79-09-4	3	Propionic Acid (C3)	soluble in water	9,942.0
107-92-6	4	Butyric Acid (C4)	50,000.0	9,600.0
109-52-4	5	Valeric Acid (C5)	40,000.0	2,324.0
142-62-1	6	Hexanoic Acid (C6)	10,820.0	2,325.0
111-14-8	7	Heptanoic Acid (C7)	2,419.0	1,136.0
124-07-2	8	Octanoic Acid (C8)	679.0	682.5
112-05-0	9	Nonanoic Acid (C9)	300.0	227.4
334-48-5	10	Capric Acid (C10)	150.0	15.0
112-37-8	11	Undecanoic Acid (C11)	insoluble in water	25.0
143-07-7	12	Lauric Acid (C12)	insoluble in water	8.0
638-53-9	13	Tridecanoic Acid (C13)	insoluble in water	25.0

In addition to solubility, pH of the solution also plays a significant role in aqueous phase toxicity tests. The 5-minute acute toxicity measured without pH adjustment for C1 through C13 is listed in Table 3.2. In comparison Table 3.3 lists the toxicity observed after the pH was adjusted to a neutral range (6-7). It is evident from Table 3.3 that the toxicity increases as the carbon chain increases. The toxicity value for C13 was not determined due to this LCFA's low solubility in water. The data presented in Table 3.2, however, suggest that the toxicity could have contributed by the acidity of the fatty acid solution to the bacteria. Toxicity data were reported in the literature for several fatty acids and are also summarized in Table 3.2 (Kaiser et al., 1994). The values are comparable to the data obtained without pH adjustment so it is safe to assume that pH was not taken into consideration when those studies were conducted.

Table 3.2 Fatty acids (C1 through C13) liquid phase 5-minute toxicity without pH adjustment as compared to previously reported values

Fatty Acids	Average EC50 – 5-minute (mg/L)	Standard Deviation (STD)(mg/L)	Previously Reported EC50 5- minute (mg/L) (Kaiser et al., 1994)
Formic Acid (C1)	7.18	0.79	7.91±0.22
Acetic Acid (C2)	8.98	0.47	9.24±0.38
Butyric Acid (C4)	23.34	0.57	16.9±0.52
Valeric Acid (C5)	15.22	0.33	NA
Hexanoic Acid (C6)	22.91	4.43	NA
Heptanoic Acid (C7)	21.26	0.67	16.1±0.42
Octanoic Acid (C8)	18.98	6.17	NA
Nonanoic Acid (C9)	24.82	4.68	NA
Capric Acid (C10)	11.85	2.08	11.2±1.27
Undecanoic Acid (C11)	4.72	1.10	NA
Lauric Acid (C12)	3.48	1.35	4.28±0.26
Tridecanoic Acid (C13)	20.59	3.72	4.60±1.69

Table 3.3 Fatty acids (C1 through C13) liquid phase 5-minute and 15-minute toxicity after pH adjustment

Fatty Acids	Average EC50 – 5-minute (mg/L)	STD (mg/L)	Average EC50 – 15-minute (mg/L)	STD (mg/L)
Formic Acid (C1)	1869	336.1	1750	191.4
Acetic Acid (C2)	893.9	42.86	1004	51.74
Propionic Acid (C3)	637.1	25.37	668.6	16.43
Butyric Acid (C4)	457.4	75.05	526.2	52.43
Valeric Acid (C5)	751.5	44.26	796.9	29.56
Hexanoic Acid (C6)	384.3	73.09	476.7	85.57
Heptanoic Acid (C7)	174.4	5.87	181.4	3.61
Octanoic Acid (C8)	89.03	10.24	100.8	12.59
Nonanoic Acid (C9)	46.52	0.80	47.4	7.54
Capric Acid (C10)	16.81	2.05	15.82	1.40
Undecanoic Acid (C11)	6.01	1.18	5.44	1.46
Lauric Acid (C12)	3.74	0.49	3.93	0.51
Tridecanoic Acid (C13)	-	-	-	-

Notes:

- No toxicity data was obtained

Due to their low solubility in water, toxicity data was not successfully estimated for some of the medium and LCFAs using liquid phase assays (C13 through C16). Therefore, solid phase tests were repeated for these acids plus C18 saturated and unsaturated acids by dissolving the compounds of interest in the non-polar solvent denatured ethanol. The 5-minute and 15-minute data are shown in Table 3.4.

Table 3.4 Long chain fatty acids solid-phase 5-minute and 15-minute toxicity

Fatty Acids	Average EC50 – 5-minute (mg/L)	STD (mg/L)	Average EC50 – 15-minute (mg/L)	STD (mg/L)
Capric Acid (C10)	13.3350	0.0495	17.4700	0.4243
Undecanoic Acid (C11)	6.4551	1.1298	6.5762	1.0349
Lauric Acid (C12)	5.2280	0.4665	6.0608	1.6632
Tridecanoic Acid (C13)	12.4250	1.3930	8.7485	0.1464
Myristic Acid (C14)	3.3030	0.7326	2.7990	0.4144
Palmitic Acid (C16)	-	-	-	-
Stearic Acid (C18:0)	-	-	-	-
Oleic Acid (C18:1)	0.2815	0.0215	0.2426	0.0261
Linoleic Acid (C18:2)	0.1922	0.0315	0.2102	0.0137
Linolenic Acid (C18:3)	0.1432	0.0418	0.1729	0.0342

Notes:

- No toxicity data was obtained

Solid-phase FAs toxicities also appear to show the same increasing trend with carbon chain length as in liquid-phase tests except for C13, and C16 and C18:0 whose toxicities could not be calculated using the Microtox[®] software. Although in solid-phase tests FAs were dissolved in ethanol due to their extremely low solubility in water, several dilutions were made during the tests using Microtox[®] diluents, a 2% sodium chloride solution. The dissolved C16 and C18:0 might have been precipitated out of the salinized solutions during the solid-phase tests dilutions.

In addition, solid-phase toxicity tests were performed on two TGCs, tripalmitin and triolein, as well as several common commercially-available vegetable oils, including canola oil, soybean oil and olive oil (Table 3.5). The tested oils appeared to have similar toxicity impact on *Vibrio*

Fisheri, with exception of peanut oil and sunflower oil that showed hormesis effect. Hormesis pertains to biological phenomena that are often adverse or detrimental but become beneficial when applied at lower levels.

Table 3.5 TGC and vegetable oil toxicity in solid-phase tests

Veg. Oil or Triglyceride Tested	Average EC50 - 5 minute (g/L)	STD (g/L)	Average EC50 - 15 minute (g/L)	STD (g/L)
Tripalmitin	0.5210	0.1050	0.5298	0.1121
Triolein	0.1703	0.0013	0.0078	0.0033
Canola Oil	1.4465	0.2199	1.6420	0.4667
Soybean Oil	2.6792	0.3378	3.3223	0.2099
Olive Oil	5.6230	0.5632	5.3340	0.6718
Sesame Oil	1.8017	0.9869	2.0349	1.0257
Walnut Oil	1.0919	0.1411	1.3880	0.3089
Corn Oil	2.017	0.2227	2.2987	0.5826
Peanut Oil	Hormesis	-	Hormesis	-
Sunflower	Hormesis	-	Hormesis	-

Notes:

- No standard deviation was obtained

Hormesis was observed in many Microtox[®] toxicity tests. It is a common and yet controversial phenomenon in the toxicology field. A typical hormetic curve is either U-shaped or has an inverted U-shaped dose–response. In this research, hormesis is considered as a non-toxic/slightly stimulant for the concentration tested, but could be detrimental/highly toxic when a larger dose is applied.

3.3 Respirometry Experiment Toxicity Tests

Respirometry experiments were performed on five major component TGCs (PPP, SSS, OOO, LLL, and LnLnLn) in addition to commercial and synthetic canola oils, which combine the five aforementioned TGCs without any other additives.

Both liquid-phase and solid-phase toxicity tests were performed during the seven respirometry experiments. However, toxicity data from liquid-phase tests were not reproducible throughout the studies due to the large variance between the samples and low solubility of tested TGCs and vegetable oils. Therefore only solid-phase toxicity data are presented and discussed in the following subsections.

3.3.1 Triglycerides Respirometry Experiments

3.3.1.1 Triolein (OOO) Toxicity Test Results

The triolein respirometry experiment was commenced on August 24, 2004 and occurred over a duration of 30 days, sacrificing 3 samples during every sampling event and 3 blank samples on the last sampling event. The 5-minute and 15-minute EC50 data for each sample are listed in Tables 3.6 and 3.7, respectively, along with calculated average and standard deviation EC50 results for each sampling event. The EC50 data are also illustrated in Figures 3.3 through 3.6.

Table 3.6 Triolein respirometry experiment – Solid-phase toxicity (5-minute)

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	6.1250	-	6.6943	0.4973
	O2	6.9145	2.4134		
	O3	7.0435	1.2254		
Day 1	S9	0.9495	-	1.3755	0.3794
	S15	1.5000	-		
	S26	1.6771	-		
Day 2	S2	0.5363	0.4464	0.3799	0.2392
	S13	0.4989	-		
	S24	0.1045	-		
Day 4	S6	0.0929	-	0.1303	0.0528
	S14	-	-		
	S20	0.1676	-		
Day 8	S7	0.3915	-	0.5759	0.1611
	S12	0.6468	0.3610		
	S22	0.6893	0.2077		
Day 12	S11	0.5877	-	0.6572	0.1099
	S23	0.5999	-		
	S25	0.7839	-		
Day 16	S3	0.8820	-	2.0948	1.5316
	S18	1.5867	0.2835		
	S28	3.8160	0.4624		
Day 24	S5	2.1585	0.8789	1.8237	0.3425
	S16	1.8385	0.1450		
	S27	1.4740	-		
Day 30	S1	6.750	-	6.090	2.6391
	S4	8.3370	-		
	S10	3.1840	-		
Blanks	B1	Hormesis	-	1.2189	0.5376
	B2	0.8387	-		
	B3	1.5990	-		

Table 3.7 Trilolein respirometry experiment – Solid-phase toxicity (15-minute)

Sampling Event	Sample ID	EC50 15-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	4.5050	-	4.9263	1.5544
	O2	6.6480	0.7594		
	O3	3.6260	-		
Day 1	S9	1.7660	-	1.4107	0.3110
	S15	1.1880	-		
	S26	1.2780	-		
Day 2	S2	0.3594	0.0438	0.2387	0.1606
	S13	0.3002	-		
	S24	0.0564	-		
Day 4	S6	0.0602	-	0.0823	0.0313
	S14	-	-		
	S20	0.1044	0.4226		
Day 8	S7	0.5714	0.2981	0.5664	0.0806
	S12	0.4834	0.0375		
	S22	0.6445	-		
Day 12	S11	0.4082	-	0.4511	0.0461
	S23	0.4454	-		
	S25	0.4998	-		
Day 16	S3	0.8934	0.3140	1.8061	1.2324
	S18	1.3170	0.5289		
	S28	3.2080	0.8528		
Day 24	S5	3.4270	0.5424	2.5442	0.8805
	S16	2.5395	-		
	S27	1.6660	1.4771		
Day 30	S1	5.6115	1.4276	4.948	2.3680
	S4	6.9135	0.9899		
	S10	2.319	-		
Blanks	B1	Hormesis	-	0.9490	0.4851
	B2	0.6059	-		
	B3	1.2920	-

Figure 3.3 Triolein 5-minute solid-phase toxicity– Each sample

Figure 3.4 Triolein 5-minute solid phase toxicity– Each event

Figure 3.5 Triolein 15-minute solid phase toxicity– Each sample

Figure 3.6 Triolein 15-minute solid phase toxicity– Each event

Toxicity was observed to increase rapidly through day 4 followed by a decreasing trend until that continued till the end of the experiment in both EC50 5-minute and 15-minute data. Analytical data including concentrations of myristic acid (C14), oleic acid (C18:1) and triolein in the solid phase of each sample are summarized in Table 3.8 and shown in Figure 3.9. 5-minute EC50 values are also converted to TU by dividing by 100 to demonstrate a positive correlation to toxicity which can be better related to the chemical concentrations. The TU values for each sample are also shown in Table 3.8 and depicted in Figure 3.10. By reviewing Figures 3.9 and 3.10, it is clear that toxicity to the luminescent bacteria is largely contributed by the FAs dissolved in the extraction solution rather than the TGC component. Triolein was degraded to oleic acid and other short or medium chain FAs from the start of the experiment to day 24, when triolein was completely degraded. The production of oleic acid was first observed on day 1 and climaxed on day 4 to subsequently slow down. The same trend was observed also in the TU results throughout the 30 days of the experiment duration.

Table 3.8 Triolein respirometry experiment chemistry analysis results and TU data

Sampling Event	Sample ID	C14 FA (mmole)	C18:1 FA (mmole)	C18:1 TGC (mmole)	TU (%)
Day 0	O1	0.0022	0.0000	0.6245	16.3265
	O2	0.0018	0.0000	0.7016	19.2012
	O3	0.0027	0.0000	0.4749	16.1891
Day 1	S9	0.0026	0.0054	0.4409	105.3186
	S15	0.0022	0.0028	0.5028	66.6667
	S26	0.0020	0.0026	0.5525	59.6303
Day 2	S2	0.0023	0.0120	0.3542	186.4628
	S13	0.0016	0.0106	0.3791	200.4410
	S24	0.0015	0.0468	0.2954	956.9378
Day 4	S6	0.0023	0.0234	0.2685	1076.4263
	S14	0.0012	0.0637	0.1279	-
	S20	0.0106	0.0232	0.1787	596.6587
Day 8	S7	0.0018	0.0072	0.2084	255.4278
	S12	0.0011	0.0167	0.0894	154.6073
	S22	0.0015	0.0142	0.1393	145.0747
Day 12	S11	0.0016	0.0091	0.1112	170.1548
	S23	0.0013	0.0090	0.1298	166.6944
	S25	0.0009	0.0307	0.0326	127.5673
Day 16	S3	0.0008	0.0038	0.0330	113.3787
	S18	0.0028	0.0067	0.0297	72.1501
	S28	0.0015	0.0005	0.0000	40.1768
Day 24	S5	0.0000	0.0056	0.0000	46.3177
	S16	0.0000	0.0111	0.0000	54.3774
	S27	0.0000	0.0229	0.0000	67.8426
Day 30	S1	0.0000	0.0006	0.0000	14.8148
	S4	0.0000	0.0067	0.0000	11.9947
	S10	0.0000	0.0061	0.0000	31.4070
Blanks	B1	0.0000	0.0000	0.4109	-
	B2	0.0000	0.0753	0.3720	119.2321
	B3	0.0000	0.0262	0.3526	62.5391

Figure 3.9 Triolein respirometry experiment chemistry analysis results

Figure 3.10 Triolein respirometry experiment TU results (5-minute)

3.3.1.2 Trilinolein (LLL) Toxicity Test Results

The respirometry experiment for trilinolein was initiated on November 5, 2004 and continued for a total duration of 30 days. The 5-minute and 15-minute EC50 data for each sample, along with calculated average EC50 results and standard deviation for each sampling event, are listed in Tables 3.9 and 3.10 respectively. The EC50 data are also illustrated in Figures 3.11 through 3.14.

Table 3.9 Trilinolein respirometry experiment – Solid-phase toxicity (5-minute)

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	5.0405	1.285	9.7935	4.6164
	O2	14.2600	-		
	O3	10.0800	0.3960		
Day 1	S6	1.6280	0.0679	1.1197	0.4403
	S11	0.8747	0.0400		
	S22	0.8565	0.1432		
Day 2	S1	0.3566	0.1047	0.4482	0.0890
	S12	0.5343	0.2959		
	S20	0.4539	0.2635		
Day 4	S4	0.1024	0.0078	0.1231	0.0205
	S10	0.1235	0.0024		
	S21	0.1434	0.0273		
Day 8	S15	5.4160	1.1264	3.4872	1.8103
	S23	3.2207	0.4660		
	S25	1.8250	0.3055		
Day 12	S19	2.4395	0.8506	2.1693	1.2313
	S24	3.2430	0.3055		
	S26	0.8253	0.2499		
Day 16	S16	2.7120	0.7693	3.5470	1.5275
	S17	2.6190	-		
	S18	5.3100	0.0424		
Day 24	S3	1.4270	0.0396	2.6845	2.1327
	S13	1.4795	0.1747		
	S14	5.1470	0.4398		
Day 30	S2	3.5640	0.0905	4.5587	0.9452
	S7	5.4450	-		
	S9	4.6670	0.4101		

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Blanks	B1	15.0200	2.0365	15.1117	2.6037
	B2	17.7600	0.6223		
	B3	12.5550	1.7890		
Controls	C1	-	-	-	-
	C2	-	-		
	C3	-	-		

Notes:

- No toxicity data or standard deviation was obtained

Table 3.10 Trilinolein respirometry experiment – Solid-phase toxicity (15-minute)

Sampling Event	Sample ID	EC50 15-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O-1	5.8030	-	12.5077	6.1813
	O-2	17.9800	-		
	O-3	13.7400	1.0041		
Day 1	S1-6	1.6505	0.0474	1.2927	0.3510
	S1-11	0.9490	0.0023		
	S1-22	1.2786	0.1761		
Day 2	S2-1	0.3292	0.0264	0.4301	0.1027
	S2-12	0.5346	0.1573		
	S2-20	0.4264	0.3127		
Day 4	S4-4	0.1328	0.0244	0.1321	0.0095
	S4-10	0.1412	0.0160		
	S4-21	0.1223	0.0117		
Day 8	S8-15	6.6370	1.8116	3.7643	2.5917
	S8-23	3.0543	0.2475		
	S8-25	1.6015	0.1648		
Day 12	S12-19	2.1560	0.7962	2.0303	1.2827
	S12-24	3.2455	0.3005		
	S12-26	0.6895	0.1515		
Day 16	s16-16	2.9920	1.0493	3.5357	0.5559
	s16-17	4.1030	0.5360		
	s16-18	3.5120	0.1160		
Day 24	s24-3	1.5265	0.0095	2.8500	2.1995
	s24-13	1.6344	0.1960		
	s24-14	5.3890	0.3352		
Day 30	s30-2	4.3840	0.4243	5.7987	1.410
	s30-7	7.2040	-		
	s30-9	5.8080	0.5261		
Blanks	B1	12.2450	1.8738	12.2633	1.4726
	B2	13.7450	0.1485		
	B3	10.8000	0.7354		

Sampling Event	Sample ID	EC50 15-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Controls	C1	-	-		
	C2	-	-		
	C3	-	-		

Notes:

- No toxicity data was obtained

Figure 3.11 Trilinolein 5-minute solid-phase toxicity– Each sample

Figure 3.12 Trilinolein 5-minute solid-phase toxicity– Each event

Figure 3.13 Trilinolein 15-minute solid-phase toxicity– Each sample

Figure 3.14 Trilinolein 15-minute solid-phase toxicity– Each event

As shown in Figure 3.11, the EC50 value sharply dropped within 1 day from the initiation of the experiment and reached the lowest level on day 4, then gradually increased afterwards. No toxicity was observed in the control samples, hence trilinolein degradation did not occur in the absence of the microbial culture. Compared to the triolein toxicity results, trilinolein demonstrated a similar trend, however it showed slightly lower toxicity levels. It was visually observed and also proven by GC/MS analysis that hydroperoxidation phenomenon occurred in the samples sacrificed on day 0 since the respirometer bottles contained an average of 0.149 mmoles of trilinolein instead of the 0.284 mmoles that were originally injected into each reactor at the beginning of the experiment. Approximately 50% of the TGCs were tied in the cross-linked polymer form instantaneously rendering it not available for microbial attack. This also explains why the toxicities during trilinolein experiments were lower than the results from the triolein experiments, while the individual toxicity of linolenic acid is higher than that of oleic acid.

3.3.1.3 Trilinolenin (LnLnLn) Toxicity Test Results

The trilinolenin respirometry experiment was initiated on March 30, 2005. Due to the high cost of this chemical, only 0.125g (half of the amount used during the other TGCs respirometry experiments) was injected into each respirometry. The 5-minute and 15-minute EC50 data for each sample, along with calculated average and standard deviation EC50 results for each sampling event, are listed in Tables 3.11 and 3.12 respectively. The observed EC50 results are also depicted in Figures 3.15 through 3.18.

Table 3.11 Trilinolenin respirometry experiment – Solid-phase toxicity (5-minute)

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	4.6960	0.4879	4.5317	0.1451
	O2	4.4777	0.1323		
	O3	4.4213	0.4600		
Day 1	S2	1.0080	0.2669	1.2066	0.3616
	S15	0.9877	0.1472		
	S24	1.6240	0.0215		
Day 2	S4	0.8915	0.3494	1.3307	0.6212
	S11	1.7700	0.3557		
Day 4	S9	4.3040	0.5379	3.8496	0.4557
	S13	3.3927	0.9386		
	S19	3.8520	0.5063		
Day 8	S3	5.3340	0.4857	5.2629	4.5041
	S17	0.7237	0.1121		
	S18	9.7310	0.1640		
Day 12	S5	13.8800	1.9959	9.3720	3.9174
	S12	7.4413	0.6721		
	S21	6.7947	0.6317		
Day 16	S6	8.8927	0.4950	7.6951	1.0484
	S10	7.2500	0.5240		
	S20	6.9427	1.336		
Day 24	S8	7.2887	0.3976	7.1842	0.7813
	S16	7.9080	0.4809		
	S23	6.3560	0.2178		
Day 30	S1	7.7787	0.3344	7.1068	0.9501
	S7	6.4350	0.3315		
	S14	-	-		
Blanks	B1	8.1823	0.1516	9.6545	1.2951
	B2	10.6185	1.713		
	B3	10.1627	2.675		

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Controls	C1	-	-		
	C2	-	-	-	-
	C3	-	-		

Notes:

- No toxicity data was obtained

Table 3.12 Trilinolenin respirometry experiment – Solid-phase toxicity (15-minute)

Sampling Event	Sample ID	EC50 15-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	5.1163	0.6218	5.0668	0.0487
	O2	5.0190	0.5851		
	O3	5.0650	0.4592		
Day 1	S2	1.1560	0.3383	1.4205	0.4737
	S15	1.1380	0.0708		
	S24	1.9673	0.0229		
Day 2	S4	1.0162	0.3446	2.5110	1.8591
	S11	1.9240	0.1142		
Day 4	S9	4.5927	0.4477	4.1882	0.4482
	S13	3.7063	0.8078		
	S19	4.2655	0.5254		
Day 8	S3	6.8500	0.2890	6.6446	5.6942
	S17	0.8505	0.0882		
	S18	12.2333	2.1139		
Day 12	S5	14.7200	-	10.7120	3.4858
	S12	8.3877	1.1021		
	S21	9.0283	1.4954		
Day 16	S6	11.0900	0.6930	9.6182	1.3175
	S10	8.5487	0.4794		
	S20	9.2160	0.9792		
Day 24	S8	7.4670	0.7261	8.0303	0.9749
	S16	9.1560	0.2705		
	S23	7.4680	0.2119		
Day 30	S1	9.6493	0.3805	8.660	1.515
	S7	6.9153	0.8740		
	S14	9.4145	0.5395		
Blanks	B1	19.3840	-	21.4420	2.9105
	B2	-	-		
	B3	23.5000	-		

Sampling Event	Sample ID	EC50 15-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Controls	C1	-	-		
	C2	-		-	
	C3	-			

Notes:

- No toxicity data or standard deviation was obtained

Figure 3.15 Trilinolenin 5-minute solid-phase toxicity– Each sample

Figure 3.16 Trilinolenin 5-minute solid-phase toxicity– Each event

Figure 3.17 Trilinolenin 15-minute solid-phase toxicity– Each sample

Figure 3.18 Trilinolenin 15-minute solid-phase toxicity– Each event

Unlike the findings of the triolein and trilinolein experiments, high toxicity levels were observed in the day 0 samples although as mentioned earlier they contained only half of the TGC amounts as the triolein and trilinolein respirometer reactors. This can be traced to the instability and extremely high reaction rate of the three double bonds trilinolenin has in its structure. Toxicity increased to a maximum by day 1 then gradually dropped until it reached a plateau on day 12. This observation was supported by the measured solid-phase FA concentrations. An average of 0.00202 mmoles and 0.00148 mmoles of linolenic acid was detected in the day 2 and day 4 samples, respectively. Neither TGC nor FFA (C18:3) was detected in the liquid and solid phases on day 4 or beyond.

The average final values for oxygen uptake and CO₂ release were respectively 0.091 mmoles and 0.113 mmoles, which resulted from the mineralization of 0.001 to 0.002 mmoles of trilinolein. This is only a small fraction of what was detected at time zero, 0.110 mmoles, and the originally injected amount, 0.143 mmoles.

Both toxicity and chemical analysis results indicated that a competition between polymerization and degradation occurred during the first stages of the experiment. The trilinolenin fraction that did not form hydroperoxides was completely mineralized. Trilinolein was not detected in any of the blank samples, which all contained a solid precipitate. The observed solid palletes were robust and insoluble in several organic solvents such as DCM, acetone, methanol and tetrahydrofuran.

□

Polymerization is a well-known phenomenon observed in the aftermath of many offshore vegetable oil spills (Mudge et al., 1993). Polymerized vegetable oils are resistant to erosion and degradation processes, with some portions of concrete-like aggregates persisting in the environment for approximately six years after the spill accident (Mudge, 1997). Due to their exceptional stability and resistance to degradation, polymerized vegetable oils have been used for centuries in manufacturing protective coating, paints, varnishes and enamel (Shogren, 2004).

The polymerization process, also known as peroxidation when referring to polyunsaturated FAs, can occur in reactions triggered by free radical species such as oxyl, peroxy, and hydroxyl radicals. As shown in Figure 3.19 below, lipid peroxidation in the presence of reduced oxygen may originate with the reduction or dismutation of metabolically or photochemically generated O_2^- to H_2O_2 . The produced $HO\cdot$ can prompt chain peroxidation by abstracting allylic hydrogens from proximal unsaturated lipid (LHs). Rapid addition of 3O_2 to the resulting lipid peroxy radicals ($L\cdot$) drives the reaction via peroxy radical lipid hydroperoxide (LOOH) species (Girotti, 1998).

Figure 3.19 Hydroperoxidation reaction

The lipid peroxy radical is the main component of the lipid peroxidation chain reaction. This radical abstracts a bis-allylic hydrogen from an adjacent FA to form a lipid hydroperoxide and a second lipid radical (Equation 1), which will react with oxygen to regenerate a peroxy radical (Equation 2).

Reactions 1 and 2 are the chain-propagation steps of lipid peroxidation with reaction 1 being the rate limiting step; therefore the reaction rate is proportional to the concentration of lipid peroxy radicals. Consequently, any reaction that changes the concentrations of peroxy radicals will influence the rate of lipid peroxidation (Hogg, 1999). Comparing the three polyunsaturated

LCFAs, trilinolein contains three double bonds in each of its FA branches, resulting in the most rapid reaction rate and highest ratio of produced peroxide during its respirometry experiments.

3.3.1.4 Tripalmitin (PPP) Toxicity Results

Unlike triolein, trilinolein and trilinolenin, the TGCs discussed earlier, tripalmitin is a saturated TGC occurring in solid form and characterized by an extremely low solubility in water. The powdered chemical tends to coagulate and form solid pellets, therefore its distribution in solution is even more difficult than the liquid form TGCs. The respirometry experiment involving this TGC was commenced on April 14, 2004 and continued for 30 days. Since it was the first respirometry experiment conducted in the research discussed in this thesis, only liquid-phase toxicity tests were performed on the sacrificed samples. Toxicity studies were not reproducible in the liquid-phase samples due to the above mentioned solubility and distribution characteristics. Therefore, the toxicity results are not presented here for comparison purposes.

3.3.1.5 Tristearin (SSS) Toxicity Results

Similar to tripalmitin, tristearin is a saturated TGC in a solid state. With 2 more carbon atoms in each of its 3 FA constituents, tristearin has a higher molecular weight and melting point. Its carboxyl group end is hydrophilic, while the rest of the molecule is highly hydrophobic.

The respirometry experiment was initiated on January 25, 2005 and lasted 40 days. Ten extra days were added to the original schedule to allow for complete biodegradation of the carbon source. Oxygen uptake and carbon dioxide release curves did not reach a plateau even after 40

days of experimentation (Figures 3.20 and 3.21) indicating the continuation of tristearin daughter FA products biodegradation.

The stoichiometric reaction for the total mineralization of the tristearin (SSS) is given in Equation 3.

According to this reaction, the total mineralization of the initial 0.280 mmoles of SSS requires 22.82 mmoles of oxygen. The measured oxygen uptake at day 40 yielded an average value of 5.22 mmoles, which implies the mineralization of only 0.064 mmoles of SSS. The average amount of TGC measured after 40 days by GC/MS analysis was 0.173 mmoles of SSS. The analysis of the samples at time zero gave an initial concentration of 0.237 mmoles as SSS. The percent removal after 40 days was 27% based on induced value from the stoichiometric reaction.

Figure 3.20 Tristearin experiment oxygen uptake

Figure 3.21 Tristearin experiment CO₂ release

Solid-phase toxicity results obtained after 5 minutes and 15 minutes of contact time for each sample are listed in Tables 3.13 and 3.14, respectively. No apparent trend was observed throughout the 40-day experiment due to the unique properties of the tested compound, mainly its high hydrophobicity, low solubility, and low bioavailability. Also demonstrated by chemical analysis, no formation and accumulation of FFAs or other by-products which are the sources for toxicity in poly-unsaturated TGC experiments was detected.

Table 3.13 Tristearin respirometry experiment – Solid-phase toxicity (5-minute)

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	13.3000	1.3435	12.6713	4.6947
	O2	17.0200	-		
	O3	7.6940	-		
Day 1	S6	29.8300	-	17.2910	11.0874
	S7	13.2600	2.2910		
	S22	8.7830	2.2161		
Day 2	S2	-	-	8.8490	-
	S10	-	-		
	S16	8.8490	0.8146		
Day 4	S11	-	-	4.1645	-
	S17	4.1645	0.3048		
	S18	-	-		
Day 8	S5	22.8400	-	22.8400	-
	S12	-	-		
	S19	-	-		
Day 12	S13	14.6600	-	10.6560	3.5062
	S14	8.1350	0.0000		
	S15	9.1730	-		
Day 23	S4	19.3045	4.8760	21.6423	3.3061
	S21	23.9800	3.6120		
	S24	-	-		
Day 30	S1	10.4600	2.9755	14.5003	5.8836
	S20	11.7950	1.1809		
	S23	21.2500	-		
Day 40	S3	10.3275	0.6258	12.0888	2.4908
	S8	13.8500	-		
	S9	-	-		
Blanks	B1	-	-		
	B2	-	-		
	B3	-	-		

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Controls	C1	-	-		
	C2	12.5700		12.5700	
	C3	-			

Notes:

- No toxicity data was obtained

Table 3.14 Tristearin respirometry experiment – Solid-phase toxicity (15-minute)

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	18.0500	5.3457	15.7662	6.0766
	O2	20.3700	-		
	O3	8.8785	3.4669		
Day 1	S6	-	-	12.6800	0.0707
	S7	12.6300	1.7395		
	S22	12.7300	2.2203		
Day 2	S2	14.1600	-	13.1525	1.4248
	S10	-	-		
	S16	12.1450	2.2840		
Day 4	S11	-	-	2.5130	-
	S17	2.5130	0.2249		
	S18	-	-		
Day 8	S5	14.4500	-	14.4500	-
	S12	-	-		
	S19	-	-		
Day 12	S13	-	-	23.0270	18.7850
	S14	9.7440	-		
	S15	36.3100	-		
Day 23	S4	28.4700	3.2710	28.8900	0.5940
	S21	29.3100	2.8930		
	S24	-	-		
Day 30	S1	11.4965	5.1527	16.8283	7.5402
	S20	-	-		
	S23	22.1600	8.9237		
Day 40	S3	11.4050	0.4455	14.3825	4.2108
	S8	17.3600	-		
	S9	-	-		
Blanks	B1	-	-		
	B2	-	-		
	B3	-	-		

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Controls	C1	-	-		
	C2	14.9205	7.6219	10.8478	5.7597
	C3	6.7750	-		

Notes:

- No toxicity data or standard deviation was obtained

3.3.2 Vegetable Oil Respirometry Experiments

After the conclusion of respirometry and toxicity experiments on the five major TGC components of canola oil, it was essential to perform identical experiments on canola oil itself. Commercial canola oil and synthetic canola oil, prepared by combining the 5 major TGC components according to their weight percentage in commercial canola oil, were tested by applying the same procedures used for the constituent TGCs. The main objective of these experiments was to investigate the biodegradability and toxicity of these oils and to provide insight into the potential effects of the minor TGC components and other canola oil additives.

3.3.1.6 Commercial Canola Oil

Respirometry experiment using commercial canola oil (Wesson[®] brand, purchased from a local grocery store) was initiated on October 6, 2005 with a total duration of 30 days.

Solid-phase toxicity results after 5-minute and 15-minute reaction durations are summarized in Tables 3.15 and 3.16, respectively. The presented toxicity data for each sample and each sampling event are also graphically illustrated in Figures 3.22 through 3.25.

Table 3.15 Commercial canola oil respirometry experiment – Solid-phase toxicity (5-minute)

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	11.0400	-	11.9093	2.7895
	O2	9.6580	-		
	O3	15.0300	-		
Day 1	S2	0.3491	0.0430	1.682	1.759
	S12	3.6760	-		
	S21	1.0217	0.2748		
Day 2	S1	0.2712	0.0208	0.3443	0.3090
	S7	0.6833	0.0354		
	S22	0.0784	0.0100		
Day 4	S13	0.2719	0.0442	0.1737	0.1335
	S14	0.2277	0.0262		
	S23	0.0217	0.0088		
Day 8	S17	0.0275	0.0093	0.0800	0.0859
	S18	0.1791	0.0049		
	S19	0.0334	0.0023		
Day 12	S10	0.5052	0.0045	0.2054	0.2598
	S16	0.0437	0.0071		
	S20	0.0675	0.0104		
Day 16	S11	0.3406	0.0559	0.4930	0.3172
	S15	0.2809	0.0582		
	S25	0.8577	0.0220		
Day 24	S3	0.4101	0.0874	0.5862	0.3606
	S5	0.3474	0.0491		
	S24	1.0010	0.0976		
Day 30	S4	0.5431	0.1145	0.5782	0.1629
	S6	0.6250	0.1377		
	S8	0.5665	0.1225		
	S9	0.8966	0.0771		

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Blanks	B1	0.0320	0.0128	0.3869	0.5638
	B2	0.0918	0.0253		
	B3	1.0370	0.1811		
Controls	C1	-	-	-	
	C2	-	-		
	C3	-	-		

Notes:

- No toxicity data or standard deviation was obtained

Table 3.16 Commercial canola oil respirometry experiment –Solid-phase toxicity (15-minute)

Sampling Event	Sample ID	EC50 15-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	12.5000	-	12.2023	2.9179
	O2	9.1470	-		
	O3	14.9600	-		
Day 1	S2	0.2943	0.0969	1.1939	0.8900
	S12	2.0740	-		
	S21	1.2135	0.1138		
Day 2	S1	0.2774	0.0320	0.2669	0.1858
	S7	0.4472	0.1869		
	S22	0.0761	0.0063		
Day 4	S13	0.2618	0.0357	0.1685	0.1297
	S14	0.2235	0.0355		
	S23	0.0204	0.0115		
Day 8	S17	0.0272	0.0097	0.0777	0.0828
	S18	0.1733	0.0050		
	S19	0.0327	0.0054		
Day 12	S10	0.5019	0.0129	0.2026	0.2594
	S16	0.0431	0.0054		
	S20	0.0628	0.0118		
Day 16	S11	0.3394	0.0494	0.4366	0.2282
	S15	0.2731	0.0534		
	S25	0.6974	0.1020		
Day 24	S3	0.4278	0.0483	0.5310	0.2591
	S5	0.3394	0.0462		
	S24	0.8258	0.3072		
Day 30	S4	0.5475	0.0972	0.6673	0.1805
	S6	0.6246	0.1666		
	S8	0.5636	0.1137		
	S9	0.9334	0.1295		

Sampling Event	Sample ID	EC50 15-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Blanks	B1	0.0289	0.0070	0.3910	0.6053
	B2	0.0543	0.0290		
	B3	1.0897	0.3314		
Controls	C1	-	-		
	C2	-	-		
	C3	-	-		

Notes:

- No toxicity data or standard deviation was obtained

Figure 3.22 Commercial canola oil 5-minute solid-phase toxicity– Each sample

Figure 3.23 Commercial canola oil 5-minute solid-phase toxicity– Each event

Figure 3.24 Commercial canola oil 15-minute solid-phase toxicity– Each sample

Figure 3.25 Commercial canola oil 15-minute solid-phase toxicity– Each event

Relatively low toxicity levels were observed for event 0 samples, sacrificed only one hour after the chemicals and biomass were added to the sterilized nutrient and buffer solutions. This was followed by a rapid increase in toxicity starting on day 1 and continuing until the end of the experiment. The 5-minute toxicity unit data along with the FA concentration results for each sample are depicted in Figure 3.26. A similar toxicity trend was observed as that of FFA concentrations except S23, sacrificed during event 4 which confirms the hypothesis that the observed toxicities were caused by the FAs generated from the breakdown of TGCs.

According to the chemical analysis, C18:1 (oleic acid) was detected at the highest concentration throughout the experiment as compared to the four other FAs analyzed, which is consistent with the composition of commercial canola oil. An increasing trend in the FA concentrations was observed from day 0, reaching a maximum on day 12 and followed by a gradually decreasing trend. By day 30, only a trace amount (an average of 0.005 mmoles) was detected indicating an almost complete degradation. The FFA concentrations analyzed for each respirometry sample are presented in Table 3.17.

Table 3.17 Free fatty acids concentration in commercial canola oil experiment

Sampling Event	Sample ID	Free Fatty Acid Concentrations (mmole)			
		C18:2	C16:0	C18:1	C18:0
Day 0	O1	0	0	0	0
	O2	0	0	0	0
	O3	0	0	0	0
Day 1	S2	0.0015	0	0.0077	0.0009
	S12	0	0	0.0034	0.0007
	S21	0	0	0	0
Day 2	S1	0.0009	0.0008	0.0066	0.0009
	S7	0.0012	0.0007	0.0070	0.0011
	S22	0.0089	0.0032	0.0276	0.0017
Day 4	S13	0.0014	0.0008	0.0077	0.0011
	S14	0.0017	0.0011	0.0096	0.0012
	S23	0.0142	0.0049	0.0617	0.0033
Day 8	S17	0.0112	0.0042	0.0419	0.0027
	S18	0.0020	0.0012	0.0088	0.0013
	S19	0.0377	0.0137	0.1381	0.0095
Day 12	S10	0.0011	0.0011	0.0043	0.0020
	S16	0.0204	0.0082	0.0862	0.0097
	S20	0.0197	0.0088	0.0847	0.0085
Day 16	S11	0.0029	0.0027	0.0122	0.0047
	S15	0.0064	0	0.0218	0.0089
	S25	0.0015	0	0.0052	0.0047
Day 24	S3	0.0024	0.0017	0.0070	0.0027
	S5	0.0041	0.0054	0.0159	0.0035
	S24	0.0008	0.0012	0.0038	0.0019
Day 30	S6	0.0021	0.0017	0.0066	0.0014
	S8	0.0015	0.0008	0.0041	0.0010
	S9	0	0.0007	0.0024	0.0014
	S4	0.0020	0.0015	0.0079	0.0017
Blanks	B1	0.0119	0.0063	0.0847	0.0059
	B2	0.0147	0.0036	0.0456	0.0013
	B3	NA	NA	NA	NA
Controls	C1	0	0	0	0
	C2	0	0	0	0
	C3	0	0	0	0

Figure 3.26 Commercial canola oil respirometry experiment– FFA concentrations vs. TU results

3.3.1.7 Synthetic Canola Oil

The initiation of the respirometry experiment on synthetic canola oil, on January 24, 2006, occurred following the conclusion of the commercial canola oil experiment. Solid-phase toxicity results after 5-minute and 15-minute reaction durations are summarized in Tables 3.18 and 3.19, respectively. The presented toxicity data for each sample and each sampling event are also graphically illustrated in Figures 3.27 through 3.30.

Table 3.18 Synthetic canola oil respirometry experiment – Solid-phase toxicity (5 -minute)

Sampling Event	Sample ID	EC50 5-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	1.0411	0.2318	1.9819	0.8334
	O2	2.2770	0.9178		
	O3	2.6275	0.9228		
Day 1	S2	0.4991	0.0238	0.5088	0.0341
	S17	0.5467	0.0993		
	S19	0.4807	0.0430		
Day 2	S3	0.1639	0.0800	0.2169	0.1494
	S5	0.1013	0.0502		
	S10	0.3856	0.1292		
Day 4	S9	0.0528	0.0212	0.0737	0.0520
	S12	0.1334	0.0442		
	S18	0.0500	0.0206		
Day 8	S14	0.3783	-	0.2331	0.2054
	S15	0.0879	0.0317		
Day 12	S7	0.2216	0.1126	0.1637	0.0819
	S11	0.1058	0.0059		
Day 16	S8	0.2419	0.0326	0.2873	0.0642
	S4	0.3327	0.0672		
Day 24	S13	0.2205	0.0594	0.2692	0.1348
	S16	0.1655	0.0508		
	S21	0.4216	0.1056		
Day 30	S20	0.7377	-	0.7377	
Blanks	B1	-	-	0.2625	
	B2	0.2625	-		
	B3	-	-		
Controls	C1	2.2650	-	2.2650	
	C2	-	-		
	C3	-	-		

Notes:

- No toxicity or standard deviation was observed.

Table 3.19 Synthetic canola oil respirometry experiment –Solid-phase toxicity (15-minute)

Sampling Event	Sample ID	EC50 15-minute (%)	STD Each Sample (%)	Average EC50 Each Sampling Event (%)	STD Each Sampling Event (%)
Day 0	O1	1.2765	0.2298	1.9327	0.7179
	O2	1.8220	0.5954		
	O3	2.6995	1.7572		
Day 1	S2	0.4892	0.0274	0.5131	0.0402
	S17	0.5595	0.0868		
	S19	0.4907	0.0660		
Day 2	S3	0.1555	0.0632	0.2159	0.1449
	S5	0.1109	0.0359		
	S10	0.3813	0.1094		
Day 4	S9	0.2966	0.3591	0.1594	0.1265
	S12	0.1344	0.0488		
	S18	0.0474	0.0196		
Day 8	S14	0.3924	-	0.2467	0.2061
	S15	0.1010	0.0255		
Day 12	S7	0.2001	0.0838	0.1571	0.0608
	S11	0.1141	0.0043		
Day 16	S8	0.2404	0.0457	0.2806	0.0569
	S4	0.3208	0.0480		
Day 24	S13	0.2370	0.0638	0.2723	0.1299
	S16	0.1637	0.0442		
	S21	0.4162	0.0848		
Day 30	S20	0.6742	-	0.6742	-
Blanks	B1	-	-	0.2556	-
	B2	0.2556	-		
	B3	-	-		
Controls	C1	1.9010	-	1.9010	-
	C2	-	-		
	C3	-	-		

Notes:

- No toxicity or standard deviation was observed.

Figure 3.27 Synthetic canola oil 5-minute solid-phase toxicity– Each sample

Figure 3.28 Synthetic canola oil 5-minute solid-phase toxicity– Each event

Figure 3.29 Synthetic canola oil 15-minute solid-phase toxicity– Each sample

Figure 3.30 Synthetic canola oil 15-minute solid-phase toxicity– Each event

The most significant variation between the commercial and synthetic canola oil toxicity data occurred on day 0 when synthetic canola oil showed a significantly higher toxicity than its commercial counterpart. This is possibly due to the additives introduced to the commercial oil mix during its production, which possibly prohibited its bio-availability to the microbial culture and prevented it from being biodegraded and oxidized. The high toxicity was confirmed by the detection of FFA on day 0. Also, the highest FFA concentration was detected on day 4 rather than day 8 as in the commercial canola oil experiment, which is possibly due to the time delay needed for the oil to break down to its TGC components.

Quantitatively, less FFA was detected in the synthetic canola oil experiment although the same amount was injected into the reactors during the commercial oil experiment. Significantly higher amounts of FFA were observed in the blank samples of the commercial canola oil experiment which contained only nutrient and buffer solution with injected oil. Again, this is possibly due to the presence of additives in the commercial canola oil, which hinders its breakdown, biodegradation, and peroxidation. The FFA concentrations analyzed for each sample of the synthetic canola oil experiment are listed in Table 3.20. The correlation between the FFA concentration and the Toxicity Unit are shown in Figure 3.31.

Table 3.20 Free fatty acids concentration in synthetic canola oil experiment

Sampling Event	Sample ID	Free Fatty Acid Concentrations (mmole)				
		C18:3	C18:2	C18:1	C18:0	C16:0
Day 0	O1		0.0007	0.0015	0.0009	0.0029
	O2	0.0006	0.0007	0.0017	0.0009	0.0030
	O3		0.0008	0.0012	0.0010	0.0026
Day 1	S2	0.0013	0.0038	0.0101	0.0017	0.0025
	S17	0.0007	0.0024	0.0057	0.0009	0.0027
	S19	0.0010	0.0041	0.0108	0.0009	0.0025
Day 2	S3	0.0017	0.0122	0.0222	0.0009	0.0025
	S5	0.0017	0.0144	0.0273	0.0009	0.0025
	S10	0.0007	0.0037	0.0094	0.0008	0.0021
Day 4	S9	0.0011	0.0083	0.0349	0	0.0008
	S12	0.0018	0.0103	0.0233	0.0005	0.0013
	S18	0.0045	0.0273	0.0775	0.0009	0.0023
Day 8	S14	0	0.0021	0.0079	0.0010	0.0027
	S15	0	0.0060	0.0235	0.0010	0
Day 12	S17	0	0.0008	0.0288	0.0010	0
	S11	0	0.0028	0.0189	0.0007	0.0017
Day 16	S14	0	0	0.0102	0.0008	0.0020
	S18	0	0.0008	0.0162	0.0008	0.0022
Day 24	S13	0	0.0010	0.0118	0.0008	0.0024
	S16	0	0	0.0197	0.0008	0.0019
	S21	0	0	0.0062	0.0006	0.0019
Day 30	S1	0	0	0.0017	0	0.0024
	S6	0	0	0.0014	0	0.0023
	S20	0	0	0	0	0
Blanks	B1	0	0.0006	0.0080	0.0009	0.0022
	B2	0	0.0008	0.0125	0.0012	0.0027
Controls	C1	0	0	0	0.0009	0.0024
	C2	0	0	0	0	0
	C3	0	0	0	0.0011	0.0033

Figure 3.31 Synthetic canola oil respirometry experiment– FFA concentrations vs. TU results

CHAPTER 4. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

4.1 Conclusions

Based on these respirometry experiments and Microtox[®] toxicity tests conducted in part of this research, the following findings were concluded:

- During the three liquid, unsaturated, TGCs experiments, competition was observed between biodegradation and autoxidation due to the presence of double-bonds in the TGC's chemical structures. Theoretical oxygen uptake for the three TGCs was not reached due to the formation of the hydroperoxides and their subsequent polymerization. Nevertheless, the non-oxidized TGC portions were readily biodegradable and were mostly mineralized.
- During the two solid or saturated TGC experiments, low solubility and high hydrophobicity have limited the degradation process. The observed oxygen uptake was even lower than that of the unsaturated ones.
- Toxicities for individual fatty acids were observed to be directly correlated to the carbon number in the aliphatic chain. EC50 values decreased as the carbon chain lengths increase. Thus, the larger the fatty acid molecule, the higher the toxicity. The EC50 values of the commercially available vegetable oils were observed to be very high, hence these oils are considered not toxic.

- Toxicity was only observed at early biodegradation stages in the solid phase and was proven to originate from the degraded FFA products. All three unsaturated triglycerides followed the same increasing then gradually decreasing trend during the toxicity studies. Triolein was observed to have the highest toxicity levels among the five tested triglycerides throughout the 30-day experiment.
- Higher toxicity and higher FFA were detected during the commercial canola oil experiment than during the synthetic canola oil one, possibly due to the presence of additives in the commercial oil used to prevent breakdown, biodegradation and peroxidation.

4.2 Future work

Experiments need to be designed and performed to investigate the effect of surfactants and antioxidants on the degradation process. Higher interfacial area between the oil and the aqueous phase is expected to enhance the biodegradation rate and extent. Different oxygen consumption rates are also anticipated following surfactant addition. The toxicity and biodegradability of edible canola oil and palm oil should be studied and compared. In addition, in-depth analysis of the polymerization phenomenon should be conducted in part of future work to provide more insight into the causes and effects of this phenomenon.

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APPENDIX A

Toxicity analysis of solid phase by Microtox[®] Assay

Sediments which may have organic toxicants strongly bound to the constituent particles, or be too coarse to be amenable to the Solid Phase Test protocol, can be extracted. This procedure is especially suited to petroleum hydrocarbons such as crude oil, or other relatively heavy oils. The extraction is performed with methylene chloride, which is toxic to *Vibrio fischeri*, so it is exchanged for ethanol. The ethanol extract is then tested according to the Microtox[®] “Protocol for the Basic Test Using Organic Solvent Sample Solubilization” (Microbics Corporation, 1994). This involves the use of a 1% (so that the ethanol is not eliciting a toxic response) ethanol-diluent solution (20 ppt salinity) to make the test concentrations in a manner similar to the Microtox[®] Basic Test.

Equipment

Microtox[®] Analyzer: Azur Environmental, Series 500. Instrument Control and Data Collection/Reduction Software: Azur Environmental, Microtox[®] Omni Software, #50H070, as well as compatible computer, cables, printer, and monitor.

Incubation Bath with Digital Display: Azur Environmental 220V, 50/60Hz or its equivalent, with the capability to incubate the SPT tubes at 15°C.

Cuvettes and Pipettes:

- Disposable Glass Cuvettes, Azur Environmental (All Azur distributed under SDI).
- 50 ml Disposable Polypropylene Beaker.
- 1-5 ml Adjustable Volume Pipette.
- 1-5 ml Pipette Tips.
- 100-1000 µL Micropipetter.
- 100-1000 µL Pipette Tips.
- 10-100 µL Fisher/Wheaton Step-Pette Repetitive Dispenser.

0.75 ml Disposable Syringes, Fisher/Wheaton.

Chemicals

Ethanol, 100% non-denatured

1% ethanol-diluent (20 ppt) made using Microtox[®] Diluent and the 100% ethanol

Methylene chloride (Fisher Scientific D150-4, HPLC-GC/MS Grade, UV cutoff 232 nm)

Microtox[®] Diluent

Microtox[®] Acute Reagent (freeze-dried bacteria, *V. fischeri*)

Nitrogen (99.999%)

Procedure

1. Ten milliliters of solvent sample is transferred to a 15 mL graduated centrifuge tube using a 9 inch Pasteur pipette.
2. The sample is further evaporated to 0.5 mL a stream of nitrogen in an N-Evap analytical evaporator. The water bath is set at $50 \pm 5^{\circ}\text{C}$.
3. The methylene chloride extract is quantitative transferred to a 2 mL reaction vial, using a 9 inch Pasteur pipette. Afterward, the methylene chloride extract is evaporated to dryness and 1 mL with 100% ethanol is added.
4. 1000 μL of 1% ethanol-diluent is added to cuvette A5 in the Microtox[®] M500 analyzer. To this added 990 μL diluent (without ethanol) and a 10 μL aliquot of the extract in ethanol, which keeps the ethanol concentration at 1%. Mix with the pipette.
5. The dilution series in cuvettes A1-A5 (dilution factor = 0.5) is made as in the SOP using 1% ethanol-diluent mixture.
6. Set up the Microtox[®] Omni Software for the Basic Test.
7. Reconstitute a vial of Microtox[®] Acute Reagent.
8. After 5 minutes, the cuvettes B1-B5 are inoculated with 10 μL of Microtox[®] reconstituted bacterial reagent.
9. After 15 minutes, press the set button on the M500 and read the initial light output of the cuvettes.

10. Immediately, transfer 500 μ L from cuvettes A1-A5 to B1-B5, mixing 3 times upon each transfer.
11. After 15 minutes of incubation, measure the photoluminescence of each cuvette on the M500 Analyzer.
12. Data is analyzed and reported using the Microtox[®] Omni software.

Reference:

Microbics Corporation 1994. Microtox[®] M500 Manual, *A Toxicity Testing Handbook*, ver.3, Microbics Corporation, pp.4-31 to 4-34.