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I, Nita Naik, hereby submit this original work as part of the requirements for the degree of:
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It is entitled: Affect of Non-Mixed Condition of Oxygen Depletion and Toxicity in a Water Body Impacted by Canola Oil Spill

Student Signature: Nita Naik

This work and its defense approved by:
Committee Chair: Dr. Makram Suidan
Dr. Albert Venosa
Dr. George Sorial

Approval of the electronic document:
I have reviewed the Thesis/Dissertation in its final electronic format and certify that it is an accurate copy of the document reviewed and approved by the committee.

Committee Chair signature: Makram Suidan
Affect of Non-mixed condition on Oxygen Depletion and Toxicity in a Water Body Impacted by Canola Oil Spill

A Thesis submitted to the
Division of Research and Advanced Studies
of the University of Cincinnati

In partial fulfillment of the
Requirements for the degree of
MASTER OF SCIENCE
in the Department of Civil and Environmental Engineering
of the College of Engineering

by
Nita Naik

Committee
Dr. Makram T. Suidan (Chair)
Dr. Albert Venosa
Dr. George Sorial

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ABSTRACT

Consumption of vegetable oil has increased considerably during the last decade with a commensurate increase in accidental spills. The use of canola oil has also increased significantly. The USEPA has amended regulations governing the cleanup of oil spills to include animal fats and vegetable oils. The effects of oil spills are well known and include the coating of birds and animals, lowering the dissolved oxygen in the impacted water and other toxic effects.

Thus it would be worthwhile to know the effect of a canola oil spill on an unmixed water body and to evaluate these effects relative to oxygen depletion and aqueous toxicity. Experiments were designed to simulate three levels of canola oil spills having magnitudes of 100, 333, and 1000 gal/acre. Respirometers were employed to provide continuous measure of oxygen uptake.

For each experiment all the replicates are expected to behave in a similar manner with regard to oxygen uptake, dissolved oxygen and liquid phase toxicity. It is believed that the toxicity trend comparison to increasing oil load would be useful for further studies.
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Chapter 1: Introduction
1.1 Background:

During the last decade the consumption of vegetable oil has increased considerably and consequently, a commensurate increase in accidental spills has occurred. Along with its use for cooking purposes, Canola oil is also used for the preparation of biodiesel, lubricants etc. On account of this, in 1994, the US Environmental Protection Agency (USEPA), in accordance with the Oil Pollution Act of 1990 (OPA), determined that animal fats and vegetable oils (AFVO) should not be exempted from regulations governing the cleanup of oil spills (USEPA, 1994, cited in Campo et al., 2007).

The behavior and fate of spilled oil depends on its physical characteristics, chemical composition as well as local environmental conditions. Factors such as the extent of dispersion, the influence of temperature and the availability of oxygen alter the fate and impact of spilled oil. These parameters, along with the quantity and rate of the spilled oil determine the thickness of an oil layer on the surface of an affected water body. A thicker oil layer results in a more pronounced decrease in oxygen transfer from air to water, and leads to reduced dissolved oxygen in the water body. This lower dissolved oxygen affects the flora and fauna of the impacted water body. Spilled oils can also coat food and, therefore, cause starvation or poisoning of the fauna through ingestion of polluted food. Oils contaminate the feathers of birds and the furs of mammals, due to their viscous nature, causing hypothermia, loss of buoyancy, and alterations in metabolism (Campo et al., 2007). In addition, the main components of vegetable oil are triacylglycerols (TAGs) which are esters of one molecule of glycerol and three molecules of fatty acids. The biodegradation byproducts of these TAGs and long chain fatty acids have been found to increase the toxicity of the impacted water.
A key question to assess is the maximum quantity of spilled oil that is compliant to bioremediation. Oil spill responders need an answer to this important question to enable proper selection of response techniques.

1.2 Research Objectives:

The overall objective of the project was to quantify the oxygen depletion and ensuing aqueous toxicity in oil impacted water columns that are not mixed (poorly mixed). The reason being depletion of oxygen in such water columns and release of toxic intermediates might lead to severe toxic impacts in the receiving water body.

The overall experimental procedure revolves around:

1. Measuring dissolved oxygen at different levels of the water column; and

2. Determining the toxic effects of canola oil during biodegradation.
Chapter 2: Literature Review
Oil is a complex mixture of many constituents and its fate and behavior in the water body largely depends on its initial properties and composition as well as on specific local environmental conditions. Spreading, dispersion, evaporation, and emulsification can alter oil properties within hours. The same type of oil released under different environmental conditions weathers in dramatically different patterns due to the influence of air and water temperature, wind speed, and the weathering processes. EPA has determined that discharges of oil in quantities that may be harmful to public health or the environment include those that: violate applicable water quality standards; cause a film or "sheen" upon, or discoloration of the surface of the water or adjoining shorelines; cause a sludge or emulsion to be deposited beneath the surface of the water or upon adjoining shorelines.

Even though petroleum oil spills are more common because of the quantities transported, vegetable oil spills have been seen throughout history. Many studies have been conducted related to petroleum oil spills. But there have been large vegetable oil spills reported all over the world. For example, a 400 gallon spill of rapeseed oil occurred in 1989 (Smith & Herunter, 1989) in Vancouver Harbor in Canada. A palm oil spill occurred in the Netherlands (Zoun, Baars, & Boshuizen, 1991), a spill of 10,000 tons of palm and coconut oil occurred in 1975 on Fanning Island in the Pacific Ocean (Russel & Carlson, 1978); 2.5 million gallons of soybean oil were spilled into the Minnesota River and upper Mississippi River, during 1962–1963 (Public Health Service, 1963). Canola oil spills are a major concern in Canada. Spills of canola oil have occurred at a transshipment facility in Vancouver Harbor (Darbi et al., 2005). Vegetable oil that escaped while being transferred through a pumping station to a tanker at Neptune Bulk Terminals reached Vancouver’s Stanley Park and contaminated birds in the harbor (ENS, 2000).
Canola and other vegetable oil spills have resulted in massive losses in marine birds (McKelvey et al., 1980; Anon, 1994).

Vegetable oil is more biodegradable than the petroleum products increasing the demand for oxygen and thus causing depletion of dissolved oxygen in the water column. Thus the dissolved oxygen would rapidly drop to zero, which can cause the death of flora and fauna in the water column. The petroleum spill in the Vancouver Harbor in Canada in 1970 caused oiling of about 50 birds. The oiling of birds is the main problem occurring from an oil spill. But about 500 birds were found oiled due to the canola oil spill in the same Vancouver Harbor. This indicates that the effects of a vegetable oil spills should be studied in detail and given equal priority as petroleum oil spills.

On April 2, 1999, a railroad tank car carrying 22,000 gallons of crude soybean oil derailed, spilling nearly 9,000 gallons. The spill presented a threat to both Buffalo Creek and to the Coggon Impoundment, a downstream resource used for recreational fishing and swimming. Local responders worked quickly to dig ditches and contain the spill on the night of the accident. During the first 4 days of the response, contractors collected 3,500 gallons of spilled oil. Despite the quick response, oil reached the surface water through an underground drainage system. Oil spilled into a corn field that had a drainage system of underground pipes that drained into the tributary of Buffalo Creek. Long-term measures to address the spill included booming areas around the points where the drainage system emptied into the stream, installing valves on the affected outlets to control the flow, monitoring the area, and recovering the oil
with sorbent pads. The spill caused a substantial damage to farmland but no negative impact on fish and wood duck habitats were detected. (EPA oil spill program update).

In a study conducted by Al-Darbi et al. (2005), the biodegradability of various vegetable and animal oils in natural seawater, seawater inoculated with wastewater, and seawater enriched with nutrients was investigated. Spills of animal and vegetable oils are a major concern because of their serious effects on marine life and their economic impact on coastal activities. Natural marine microorganisms have the potential to degrade different oil contaminants. The addition of any of the oils to each environment led to an increase in bacteria numbers. Environmental Scanning Electron Microscope and Energy Dispersive X-ray analysis and latroscan techniques were used to study and analyze the different oil contaminated samples. Results showed that both natural remediation (oxidation) and biodegradation took place in the oil-contaminated samples. The addition of nutrients and the elevated numbers of bacteria in wastewater highly increased the degradation process. Canola oil exhibited the highest degree of biodegradation, followed by mustard and cod liver oil, with the slowest being olive oil.

Many oil products are highly viscous. In particular, crude oils and heavy fuel oils can be deposited on shorelines and shoreline resources in thick, sticky layers that may either disrupt or completely prevent normal biological processes within the environment. Even if an oil product is not especially toxic, when oil physically covers plants and animals, they may die from suffocation, starvation, or other physical interferences with normal physiological function. (Oil spills in mangroves, NOAA, Jan 02). Seawater contains various microorganisms that can partially or completely degrade oil to water-soluble compounds and eventually to carbon dioxide
and water and the byproducts of the biodegradation could be toxic in nature (Dean-Raymond and Bartha, 1975; Tango and Islam, 2002).

The main constituents of vegetable oil are triacylglycerols (TAGs), which are esters of one glycerol molecule and three molecules of fatty acids. Canola oil has 94.4% to 99.1% triacylglycerols. (Shahadi and Fereidoon; 2005). The three most abundant fatty acids are unsaturated; oleic (C18:1), linoleic (C18:2) and linolenic (C18:3). Campo et al., 2007 conducted a respirometry experiment to determine biodegradation kinetics of the five dominant TAGs and long chain fatty acids (LCFAs) present in vegetable oil. They observed that liquid TAGs followed typical biodegradation oxygen uptake rates. After 30 days particles in suspension coagulated, resulting in decreased surface area and limiting bioavailability and degradation.

It is worthwhile to mention that linoleic acid showed the highest oxygen uptake, lowest hydrogen peroxide formation resulting in lower polymers and highest toxicity in solid phase. Microtox analysis suggested that as the length of the alkyl chain increases, acids having above 12 carbon atoms did not show any toxicity. For saturated fatty acids, at pH 4-5, EC50 ranging from 3.5 to 24.8% was found, indicating toxicity in the liquid phase. But no EC50 values were measureable for any TAG due to insolubility in the water column. But TAGs are present in the solid phase and were found to have EC50 values of 2.67 to 3.77% indicating high toxicity. The general toxicity trend showed an increase in toxicity during early stages of biodegradation followed by a decrease in that toxicity after approximately 96 hours. In the control flasks no toxicity was observed in both liquid and solid phases. It is worthwhile to mention that the rate of degradation of LCFAs decreases with increasing chain length and unsaturated LCFAs are more rapidly degraded than corresponding saturated acids (Loehr and Roth, 1968).
When a spill occurs, oil floating on the water surface or suspended in the water column has detrimental effects. In response, sedimentation followed by anaerobic biodegradation had been proposed as an effective countermeasure. But under anaerobic conditions, vegetable oil may produce inhibitory intermediates. Vegetable oil also has been reported to be phytotoxic (Foster, 1992; Sampedro et al. 2005). The environmental acceptability of sinking floating vegetable-oil spills depends on the fate of the oil in the sediments. Potential toxic effects of vegetable oil to contaminated sediments led to the research conducted by Li et al. (2004). Canola oil amended microcosms were studied along with unoiled controls. The purpose of this comparison was to determine whether canola oil or any of its biodegradation products are inhibitory in a standard microbial toxicity test. Microtox Solid Phase Toxicity analysis was used to conduct the toxicity tests. Toxicity was measured at various points in accordance with the oil mineralization process to determine whether changes in sediment toxicity were correlated with the extent of oil mineralization. The study was conducted for around 125 days. After 5 days of incubation, the toxicity of sediments from microcosms to which oil was added was greater than from unoiled microcosms, but the EC50 increased (i.e., toxicity decreased) between 5 and 48 days in most oiled microcosms. Average EC50 values of 25.5±1.8 mg/ml indicated no toxicity in the controls. Microcosms with 17g canola oil per kg of sediment showed decreased EC50 by 15 ± 6%, 42 ±14% relative to controls after incubation of zero to two weeks respectively. The values observed were 22.5 mg/ml for time zero, 15mg/ml for 2 weeks and 30mg/ml for 8 weeks measurements. The EC50 value for the 17g oil load after 8 weeks (30mg/ml) was higher than the control (25.5mg/ml) which was assumed due to stimulation of test organisms by the metabolites of the anaerobic biodegradation which served as an energy source. With higher load of 33g
canola oil per kg sediments, EC50 reduced by 43±4% and 89±0.5% to values of 14.9mg/ml and 2.7 mg/ml after zero and two weeks of incubation. But after 8 weeks, the EC50 increased suggesting the anaerobic degradation resulted in decreased toxicity. Thus as the oil mineralization took place completely after around 125 days, the toxicity in the sediments decreased. Thus it can be summarized that toxicity of oil contaminated sediments decreased as oil was degraded under anaerobic conditions over the time and the transitional toxicity was observed due to some biodegradation intermediates. According to Canadian regulatory compliance the threshold toxicity concentration for sediment contamination is 1mg/ml, established in the Ocean-Dumping Guidelines of Environmental Canada. (Tay et al., 1997)

The dissolved oxygen measurement is a critical measurement considering toxicity issues, either by the by-products of vegetable oil spill or by any residual toxicity present in the impacted water body. Lloyd, (1961) conducted a test for studying toxicity of several poisons like ammonia to rainbow trout by varying the dissolved oxygen concentration in a simple flow respirometers at 17.5 °C. It was observed that as the dissolved oxygen decreased, carbon dioxide production by the rainbow trout decreased and the pH increased near the gill surface and ammonia at high pH was present in its toxic form. This was considered the reason for increase in the toxicity.

In a study conducted by Downing (1953), fish were experimented with potassium cyanide solution and regulating dissolved oxygen. Seven tests were conducted to determine the effect of concentration of oxygen and cyanide on the period of survival of the fish. In most tests every fish overturned that is laid without making movements for 5 seconds. In water containing 0.105ppm cyanide and 7.37ppm oxygen, one fish remained after 22 hours but in the same concentration of
cyanide and 8.92ppm of oxygen, 3 fish remained after 39 hours. In control tests without cyanide, all fish survived in 3.66ppm dissolved oxygen for 48 hours. But with dissolved oxygen 1.11ppm, all the fish overturned in 18 minutes. Thus it can be concluded that dissolved oxygen is important for the survival of the fish even when the toxic impact remains the same.

**Comparison of biodegradation kinetics and toxicity of vegetable oil under aerobic conditions and anaerobic conditions in freshwater sediments:**

Vegetable oil biodegradation can occur aerobically or anaerobically. Aerobic degradation is considered to proceed more rapidly and efficiently in the environment (Brock and Madigan, 1991). The basis for aerobic biodegradation is that ample supply of molecular oxygen is needed for biodegradation. Oxygen plays an important role in aerobic biodegradation and thus regular monitoring of cumulative oxygen consumption and corresponding carbon dioxide production is essential. Anaerobic biodegradation takes place at a slower pace than aerobic degradation, but it has been found to play a role when oxygen supply is limited and fast kinetics are not essential (Rittmann and McCarty, 2001). Some anaerobic bacteria use nitrate, sulfate, iron, manganese, and carbon dioxide as their electron acceptors, and break down organic chemicals into smaller compounds, often producing carbon dioxide and methane as the final products (Brock and Madigan, 1991).

The activity of aerobic microorganisms can be measured by the amount of oxygen they consume or the amount of carbon dioxide they produce. Biodegradation by anaerobic microorganisms can be measured by the amount of methane that they may be able to produce. Anaerobic biodegradation is essential phenomenon to be studied in sediment contamination by oil spills.
If the substitute electron acceptors are limited, vegetable oil and other lipids can be transformed to methane and carbon dioxide in freshwater sediments. This transformation is made by means of complex microbial consortia involving several physiologically distinct groups of microorganisms. In usual circumstances, glycerol is fermented to volatile fatty acids and alcohols. Long chain fatty acids are oxidized to hydrogen, acetate, and shorter chain length fatty acids by hydrogen producing acetogens (Mackie et al. 1991). LCFA’s have been confirmed to be inhibitory to wide range of microorganisms as aerobic heterotrophs, hydrogen producing acetogens, and acetoclastic methanogens.

The objective of a study conducted by Li, Z, Wrenn, B.A., 2005, was to explore the fatty acid sensitivity of anaerobic freshwater sediment microbial consortia that were enriched on a non-inhibitory concentration of vegetable oil under methanogenic or iron reducing condition. Freshwater sediment microbial communities were enriched on canola oil under methanogenic and iron reducing conditions and the kinetics of methane production from canola oil and mixtures of acetate and oleic acid were compared. Anaerobic sediment microcosms were provided with 1.9 g oil/ kg sediment or 19 g oil/kg sediment with canola oil to study the biodegradation kinetics of canola oil

Low concentrations of canola oil (1.9g oil/kg sediment) were rapidly and completely biotransformed to methane. But meager biotransformation occurred with higher concentrations of oil (19g oil /kg sediment), even when incubated for more than a year. In the microcosms which had 1.9 g oil/kg of sediment, the mineralization of oil was almost complete in about 25
days. Regardless the sediment-enrichment condition, consistency was seen in the results. The difference in sediment enrichment by addition of Fe was more prominent in the higher oil load of 19g oil/kg sediment. The reactions almost reached steady state after about 4-6 weeks. It was observed that during the period of methane increase, rapid acetate consumption took place which suggests that degradation of LCFAs by hydrogen-producing acetogens was inhibited for longer period of time in microcosms constructed with sediments enriched under methanogenic conditions. It was also observed that even after more than one year of incubation, less than 30% of the Canola oil had been mineralized. Sediments enriched under iron reducing conditions completely transformed canola oil to methane regardless of the oil load.

In anaerobic treatment instability due to self inhibition by Long chain fatty acids is a chronic problem. Aceticlastic methanogens have been shown to be particularly sensitive to fatty acid inhibition in anaerobic digesters. Hanaki et al. (1981) observed that presence of LCFA caused prolonged lag phases before biodegradation of acetate and butyrate in batch cultures of anaerobic sludge digesters.

In another study conducted by Li et al. (Water research 2004), canola oil with 18.6 g oil/ kg sediment load was added to the sediment microcosms. The purpose was to investigate the potential of ferric hydroxide to affect the rate and extent of vegetable oil mineralization in anaerobic freshwater sediments through treatments of clay and calcium and study the effects of this on toxicity of the oil contaminated sediments. It was concluded that the conversion of canola oil to methane was self inhibitory at high oil concentrations but with addition of ferric hydroxide this inhibition could be calmed. It was observed that addition of ferric hydroxide could reduce
the toxicity of Long chain fatty acids by reducing fatty acid bioavailability or by selecting microorganisms that are less sensitive to inhibition by long chain fatty acids.

The sediment microcosms were constructed in an anaerobic chamber with sediments (50g) obtained from Horseshoe Lake, Illinois and 50ml of mineral salts nutrient solution. The production of methane from canola oil in the anaerobic sediment microcosms was affected by all the treatments. The more prevalent effect was due to ferric hydroxide which reduced the methane production by 10%. It was thought that the ability of ferric hydroxide to prevent the inhibition of microorganisms by toxic intermediates was caused by segregation of fatty acids which reduced bioavailability or selection for microorganisms that were inherently less sensitive to fatty acid inhibition.

The toxicity of sediments from oil amended microcosms was compared to that of unoiled control samples using microtox solid phase test. Also the comparison between different treatment factors (ferric hydroxide clay and calcium) was made using the solid phase microtox test. Main effect was due to oil which increased the toxicity after 5 days of incubation. This showed that canola oil or an intermediate rapidly formed from its metabolism was toxic to the Vibrio Fisheri bacteria in the microtox test. The toxicity of oiled sediments decreased over time due to biodegradation of toxic intermediates. This suggested that fatty acids are the toxic intermediates of canola oil’s anaerobic biodegradation which can be metabolized to nontoxic products. Among the other treatments, clay showed toxic effect on sediments. It showed toxicity after 48 days and did not decrease over time suggesting that the toxic substance formed in clay remained stable. It seemed the toxicity was due to production of sulfide by the added contaminant in clay, sulfate.
On the basis of comparison between the anaerobic biodegradation in sediments and the aerobic biodegradation in water column, information obtained from research conducted by Li et al and Campo et al shows that the toxicity increased after a specific time and then decreased as the test duration proceeded. For the anaerobic biodegradation, the toxicity decreased after 2 weeks of the experiment duration to 15mg/ml from 22.5 mg/ml and 30mg/ml was measured for the eighth week, for the 17g oil load experiment. For the aerobic biodegradation the toxicity decreased to lowest values of EC50 after 4 days, and decreased successively till 4 weeks. The duration of the experiments varied in both the cases but more rapid biodegradation and change in sample toxicity was observed in the aerobic experiment.
Chapter 3: Materials and Methods
Three experiments were conducted where the quantity of oil spilled was varied:

1. 100 gallons/acre spill.
2. 333.33 gallons/acre spill
3. 1000 gallons/acre spill.

To simulate these oil loads, and based on the microcosm’s geometry, 0.36, 1.2 and 3.6 milliliters of canola oil were added to the microcosms in each experimental condition respectively.

### 3.1 Experimental Design:

Respirometry tests were used to conduct the experiments. Respirometry tests were conducted in three computerized respirometers, Comput-Ox WB Series (N-Con Systems, Crawford, GA, USA) with a total capacity of 36 flasks. The oxygen uptake in each flask was recorded on an hourly basis by the computers throughout the experimental runs. Each flask was equipped with a trap containing 0.1N potassium hydroxide to remove the carbon dioxide that would be produced from the microcosms if oxygen is consumed. The amount of CO$_2$ produced within the system was calculated based on the relative decrease in pH of the KOH solution and the relevant temperature, as indicated by the color change of the pH indicator dye Alizarin Red. The respirometers supply pure oxygen when a pressure drop is created by oxygen consumption by microorganisms. Calibrated valves meter oxygen into the flasks to balance pressure against temperature-controlled reference cells. Each respirometers is equipped with a temperature-controlled water bath and magnetic stirrers.

Dissolved oxygen was measured twice per week and toxicity in the aqueous phase was also monitored weekly using Microtox assay. In each experiment control samples of abiotic and
biotic blanks were run. Abiotic blanks include only mineral solution and mineral solution with oil. Biotic blanks include mineral solution and biomass. At least one of each type of blanks was run in each of the experiment.

A bacterial culture developed in the laboratory under continuous flow using a coarse pore membrane bioreactor was used in this study. A good description of an innate biodegradability test is one where conditions for acclimation have been relaxed, (Painter et al., 1995). Thus pre-exposure of the innoculum increases chance of biodegradation occurrence and resulting in correct testing methods. The original seed was obtained from a local activated sludge municipal wastewater treatment plant. The flow rate of canola oil feed for the reactor was 0.48ml/day, the buffer flow rate was 0.4 L/day and nutrient flow rate was 0.2 L/day.

For every experiment, the biomass was collected, treated and frozen by the following procedure:

1. Around 5 liters of biomass from the bioreactor was collected.
2. It was centrifuged in 500ml centrifuge bottles for 20 minutes, at 10,000 rpm and 4\textdegree{} C.
3. The supernatant was discarded and around 500ml of saline solution (8.5g/L of sodium chloride) was added and centrifuged again. This was repeated 3 times to clean the biomass and use it for the experiments in the future.
4. The final pellet was re-dissolved in saline solution and 10\% by weight of glycerin was added and mixed well.
5. The final 500ml was transferred to cryogenic vials of 5ml capacity and 4.5ml was added to each vial to account for expansion.
6. The vials were stored at -70° C and required number of vials was used for subsequent experiments. The biomass was again re-suspended in saline solution by cleaning and centrifuging.

For each experiment the microcosms were prepared by combining the nutrient and buffer solution in the ratio 1:2 and inoculum (10 ml) and canola oil depending on the oil load. Temperature was maintained at 20 °C for all experiments. Once started, the test were continued until either at least 80% of the theoretical oxygen demand has been satisfied or the oxygen uptake curve starts leveling off suggesting that some of the oil constituents have been transformed to bioreistant polymers unavailable for further biodegradation, which can be concluded by the respirometry oxygen uptake curves.

The respirometers were calibrated every 4 months. Calibration is achieved by using the calibration function in the software. When a pressure sensor detects a difference between the reactor flask and the reference cell, it causes the valve to open and deliver oxygen into the flask. It continues to do this until pressures are balanced. The respirometer bath must be at ambient temperature. A burette closed from both sides with stop corks is used to simulate the demand of oxygen from the reactor flask demand on the pressure sensor by allowing water to drip from the stopcock. When the software indicates, the volume of water displaced from the burette is read, entered, and a coefficient is calculated.

The pH electrode and meter used for test media pH measurement was calibrated following the instructions given by the manufacturer, using ph 7.0 and 10.0 Standards, prior to each
measurement event. The standard of 7.0 was checked after every 10 samples and at the end of the event and the meter was recalibrated if the value is not within 0.2 units of the check standard

3.2. Design of the microcosms:

The respirometry microcosms were fabricated to represent water column of minimum depth of 8 inches (70 mm internal diameter × 326 mm). Each of the extended microcosms was equipped with at least three sampling ports located at three different sampling depths. The ports were used for monitoring of dissolved oxygen and for the periodic withdrawal (once per week) of aqueous samples for Microtox (toxicity) analysis as well. The desired locations of the sampling ports were at the bottom, one in the middle of the water column, and one approximately an inch below the static water.

The respirometry flasks were cleaned and silanized and sterilized by autoclaving. Following was the composition of the nutrients and the buffer:
**Buffer:** 6.8 g/l \( \text{KH}_2\text{PO}_4 \)

**Table (3-1): Nutrients Composition**

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<thead>
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<tr>
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<tr>
<td>Cyanocobalamin(B(_{12}))</td>
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<tr>
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<td>Panthotenic acid, Ca salt hydrate</td>
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<tr>
<td>Pyridoxine, hydrochloride, 98%</td>
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<td>(-)-Riboavin, 98%</td>
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<td>Thiamine hydrochloride</td>
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<tr>
<td>Thioctic acid, 98%</td>
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**3.3 Glassware and analytical instruments:**

The microcosms are fabricated according to the specification as mentioned in part 4.2 by Glassblowers manufacturing company. The instruments used for measurement are oxygen electrode by Microelectrodes Inc, (MI-730 Micro-Oxygen electrode) (Microelectrodes, Inc., Bedford, NH), Microtox Toxicity Model 500 Analyzer (Azur Environmental / Strategic Diagnostics).
Chapter 4: Sampling and Analysis
Following measurements were made during the experimental runs:

1. Cumulative oxygen uptake throughout the run
2. Carbon dioxide production
3. Dissolved oxygen measurement - twice per week
4. Toxicity analysis using Microtox - once a week.

Cumulative oxygen uptake was recorded continuously by the Computox software. Carbon dioxide production was evaluated by measuring the pH of the KOH solution withdrawn from the traps, when it needed to be changed as indicated by the emergence of a pink color. The initial normality of the KOH solution, the room temperature and the pH of the exhausted KOH solution are plugged into the software (developed by Dan Moxny) designed to compute the concentration of inorganic carbon in the solution.

Dissolved oxygen was measured by dissolved oxygen measuring electrodes (MI-730 Micro-Oxygen electrode) (Microelectrodes, Inc., Bedford, NH). This electrode allows measuring oxygen concentration in water with a response range between 0 and 100% saturation. Its depth of immersion is 0.1 mm with a response time of 20 seconds. The dissolved oxygen was measured from all the three sampling ports twice per week.

The MI-730 Electrode Kit contains one Oxygen Electrode with membrane housing, bottle of electrolyte. Following is the procedure of preparing the oxygen electrode:

1. Remove the acrylic housing from the Oxygen electrode by unscrewing it from the electrode body.
2. Add oxygen electrolyte solution to the acrylic housing.

3. Insert the housing on to the Oxygen electrode, being careful not to trap any air bubbles near the electrode tip.

The electrode was calibrated before start of every experiment using the following procedure:

1. Immerse the tip of the electrode into the 0% standard (distilled water bubbled with nitrogen for at least 30 minutes) and adjust the zero of the meter after a stable reading is obtained.

2. Remove the electrode from the first standard and place it into the second standard (distilled water bubbled with air for at least 30 minutes). Adjust the calibration control to the value of the second standard (21%). The table and formula for conversion of measured percent oxygen to solubility is provided in Appendix A.

When necessary, the membrane of the electrode could be replaced by following the assembly procedure mentioned above. When removing and replacing a membrane as well as when calibrating or making measurements, care had to be taken not to apply pressure against the internal electrode. Any excessive pressure against the internal electrode can cause the electrode to crack rendering it useless and un-repairable.
Sampling for microtox was done once a week and the samples were analyzed for toxicity using Microtox Toxicity Model M500 Analyzer (Azur Environmental / Strategic Diagnostics) and following the ASTM (1997) procedure. The samples were filtered and stored in the refrigerator for analysis throughout the week. The toxicity is expressed as EC50 value which is a measure of the reduction in light output in the analyzer by the test bacteria relative to the control bacteria. Higher EC50 values indicate lower toxicity. The procedure for the microtox analysis using the 81.9% Basic Test is provided in Appendix A.
Chapter 5: Results and Discussion
All the replicates in the experiments were expected to behave in a similar way with regards to their oxygen uptake, dissolved oxygen and liquid phase toxicity. Toxicity was expected to increase with the increasing oil load. Also, as the oil load increases, the content of dissolved oxygen measured in the water column was expected to decrease. This could then be correlated to the increased toxicity.

5.1 Oxygen Uptake and Carbon Dioxide Results and Discussion:

The overall objective of this research revolves around the phenomenon of biodegradation. To define the extent of biodegradation in the case of aerobic degradation, the key points of interest are the oxygen consumed by the biomass and the carbon dioxide produced. The oxygen uptake in all the three experiments was plotted against time. Measuring the carbon dioxide production was a very good indicator for monitoring the system. The average oxygen uptake was lower than the theoretical oxygen required for total degradation of the oil because the overall process was governed by the lower oxygen condition in the non-mixed system. It was observed that the carbon dioxide production levels were lower than expected theoretical values were calculated from fatty acid consumption of oxygen due to the fraction of carbon needed for formation of biomass. This was also reported by Campo, P et al., 2007. The theoretical ratio of carbon dioxide produced over the oxygen consumed during canola oil biodegradation is around 0.7.

5.1.1 100 gal/acre oil load:

In the 100 gal/acre spill experiment, the best three reproducible replicates were considered and the final average oxygen uptake was 441 mg/l with a standard deviation of 18.41. The typical
uptake rate was 0.3 mg/L-Hr. The theoretical oxygen required for oil load corresponding to 100 gal/acre spill condition was 521.5 mg assuming total conversion of all the oil to carbon dioxide and water.

The carbon dioxide production, measured by the pH of the KOH solution, correlated well with the oxygen production. The theoretical carbon dioxide production was 509.97 mg for this experiment. The ratio of CO₂/O₂ was checked every week and it was found out to be around 0.7 for each sample. This value was equal to the theoretical CO₂/O₂ ratio which indicated that the reported oxygen uptake was consumed for canola oil degradation and the system was leak proof.

5.1.2 333gal/acre Oil Load:

In the 333 gal/acre spill experiment, 5 replicates were run. The experiments were conducted with two blanks of biotic, abiotic and mineral solution to quantify any excess oxygen uptake, which could be accounted for as oxygen required for biodegradation of the oil. Considering the best 3 replicates, the total average cumulative oxygen uptake was 781.1 mg/L with a standard deviation of 30. The uptake rates were 0.3 and 0.6 mg/L-Hr. The experiment was run for 2200 hours (i.e.13 weeks). The blanks with oil and mineral solution had no uptake throughout the experiment, as expected. The blanks with oil had a small initial uptake, which is believed to be the oxygen required for formation of hydrogen peroxide subsequently forming polymers, Campo, P et al., 2007. The theoretical oxygen required for this experiment was calculated to be 1738 mg, considering total conversion of oil to carbon dioxide and water.
The theoretical carbon dioxide produced for the oil load of 333 gal/acre is 1699.91 mg. The ratio of carbon dioxide over oxygen was around 0.7 for all the replicates indicating good reproducibility. As with the previous experiment, this value correlates well with the theoretical ratio.

5.1.3. 1000 gal/acre Oil Load:

The experiment having highest oil load of 1000 gal/acre canola oil was conducted with 5 replicates and a biotic and abiotic blank with oil. Considering the 3 lower replicates as the best possible fit, the average final oxygen uptake was 1062 mg/L with a standard deviation of 18. The oxygen uptake rates were around 0.3, 0.6 or 1 mg/L-Hr. The biotic blanks in the experiment had an intermittent uptake of 0.3 mg/L-Hr. The theoretical value of oxygen uptake for this experiment is 5251 mg considering complete transformation of oil to carbon dioxide and water.

The carbon dioxide production, measured by the pH of the potassium hydroxide solution, correlates well with the oxygen production. The theoretical carbon dioxide production was 5099.74 mg for 1000 gal/acre oil load.

Following figures represent the cumulative oxygen uptakes and the cumulative carbon dioxide production for all the three experiments respectively.
Figure 5-1: Cumulative Oxygen Uptake Curve for 100 gal/acre Oil Spill Load.

Figure 5-2. Cumulative Oxygen Uptake for 333 gal/acre Oil Load
Figure 5-3. Cumulative oxygen uptake for 1000 gal/acre oil load.
Figure 5-4: Cumulative CO$_2$ production for the 100 gal/acre oil load experiment

Figure 5-5: Cumulative CO$_2$ production for the 333 gal/acre oil load experiment
Figure 5-6: Cumulative CO₂ production for the 1000 gal/acre oil load experiment
5.2 Discussion on dissolved oxygen for 100, 333 and 1000 gal/acre spill experiments:

5.2.1 100 gal/acre oil load:

For the 100 gal/acre spill experiment, the average dissolved oxygen in the highest port was around 5 to 6 mg/L and it decreased to around 1 mg/L after about 25 days. For the middle port, the initial average dissolved oxygen measured was 3.5 mg/L which decreased to 0.7 mg/L after 17 days. The initial average dissolved oxygen at the lowest level was 3.2 mg/L, which subsequently reached zero after around 15 days. Thus we can infer that the dissolved oxygen varied considerably throughout the water column in the non-mixed regime, the lowest being at the bottom.

5.2.3 333 gal/acre oil load:

For the experiment of 333 gal/acre spill load, in the highest port, the average initial dissolved oxygen measured was 5.3 mg/L. It reduced to zero after around 25 days and stayed zero throughout the rest of the experiment. The average initial dissolved oxygen in the middle port was 3.3 mg/L and in the lowest port was 3 mg/L. Subsequent measurements showed it decreased to zero in both ports after around 25 days and remained zero throughout. The biomass present in the samples also had turned black due to lack of dissolved oxygen. The blanks with mineral solution had initial dissolved oxygen of 8 mg/L but gradually oxygen rose to a maximum reading of 25 mg/L. At this point the samples were oversaturated. The biotic blanks had initial dissolved oxygen of around 5 mg/L, which increased to 8 mg/L and plateaued for all the ports.
It is worthwhile to mention that the dissolved oxygen decreased to zero throughout the water column around the same time at all the levels.

5.2.3. 1000gal/acre oil load:

For the experiment of 1000 gal/acre spill, the decrease in the dissolved oxygen was rapid. In the highest port, the initial average dissolved oxygen was measured to be 4.77 mg/L, which dropped to zero in about 10 days. For the middle level and lowest level, the dissolved oxygen initially had an average value of 4.66 mg/L which fell to zero in just 3 days with a consistent reading of zero for the rest of the experiment.

In each experiment for all the dissolved oxygen measurements, at least one sample was an outlier and not considered in calculation of the average stated above. The variation in these samples is believed to be due to problems with the respirometer valves supplying oxygen or the high buffering capacity for oxygen of the samples.

The following plots show the average dissolved oxygen plotted against time for the three levels and the figures provide better understanding of the variation of dissolved oxygen with time and varying oil load.
Figure 5-7: Average DO vs Time for all the experiments (Highest Level)

Figure 5-8: Average DO vs Time for all the experiments (Middle Level)
Figure 5-9: Average DO vs Time for all the experiments (Lowest Level)
5.3 Toxicity analysis results and discussion:

Toxicity in the liquid phase was analyzed for samples collected from all the three ports of the microcosms every week. In the experiments of 333gal/acre and 1000 gal/acre the toxicity was observed after 2 weeks of canola oil biodegradation. Similar trends in toxicity were seen in case of anaerobic canola oil biodegradation (Li, et al., 2001). In the study conducted by Li et al. in 2004 different treatments were applied to study biodegradation anaerobic microcosms contaminated with canola oil and it was observed that time was an important factor which reduced the toxicity of the oiled sediments. This phenomenon was observed in the aerobic experiments of canola oil biodegradation. The toxicity of the microcosms was seen to reduce in the later stages of the experiments.

5.3.1 100 gal/acre oil load:

In the experiment of 100 gal/acre canola oil load, toxicity was not observed in any of the sample analysis. The lowest EC50 value observed was 44% which cannot be defined as toxic. It should be noted that this experiment had the lowest oxygen uptake and the highest dissolved oxygen. The lack of toxicity is believed to be due to the absence of by-products of biodegradation of the canola oil, and complete mineralization of canola oil.

5.3.2. 333 gal/acre oil load:

The duration of the 333 gal/acre oil load experiment was 13 weeks. In general the replicates showed reduction in EC50, implying an increase in toxicity after 7 weeks of experimentation.
Eventually the toxicity decreased slightly at near end. The average EC50 value after 7 weeks was 30%. The EC50 values remained constant for most of the replicates till the end of 13 weeks. Out of the 5 replicates, one sample was outlier. It had also recorded high dissolved oxygen and was not found to be toxic till 9 weeks.

The toxicity remained constant throughout the depth of the water column, not complying with the hypothesis that higher toxicity would be observed at the lowest port. This can be correlated with the fact that the dissolved oxygen was constant throughout the whole water column.

The following plots show the variation of toxicity with time in weeks for three replicates. All the values are for EC50 of 5 minutes data.

Figure 5-10: EC50 vs Time for 333 gal/acre spill experiment (Highest Level)
Figure 5-11: EC50 vs Time for 333 gal/acre spill experiment (Middle Level)

Figure 5-12: EC50 vs Time for 333 gal/acre spill experiment (Lowest Level)
5.3.4 1000 gal/acre oil load:

The effect of toxicity was seen prominently in the 1000 gal/acre oil load experiment. Toxicity was observed at all levels of the samples right from the second week. The average initial EC50 value was 30% which decreased to almost 3% at the end of 10 weeks. It was observed that the toxicity was higher at lower depths of the water column.

It was found that the EC50 was lowest at 7 and 8 weeks. Then there was a slight increase in the EC50 at end of the 10th week. Thus it is clear that the toxicity increased till 8 weeks and then started to drop. But the original EC50 values were not reached, which indicated that the toxicity was still prevalent. The toxic byproducts seemed to persist in the water column even after 10 weeks of the experiment run.

The following plots show the EC50 values for the highest, middle and lowest port of the microcosm. The sampling was stopped for the highest port after 7 weeks because the water level dropped below the port level. All the values are measured by the 5 minute test of microtox.
Figure 5-13: EC50 vs Time for 1000 gal/acre spill experiment (Highest Level)

Figure 5-14: EC50 vs Time for 1000 gal/acre spill experiment (Middle Level)
Figure 5-15: EC50 vs Time for 1000 gal/acre spill experiment (Lowest Level)
Chapter 6: Comparison
6.1 Oxygen uptake

It was observed that as the oil load increased, the total amount of oxygen uptake increased. However it was not commensurate with the ratio of the oil loads, i.e. when the oil load was increased ten fold from 100 to 1000 gal/acre, the total oxygen uptake increased by only 2 times. The cause of this disproportionate oxygen uptake is believed to be the non-mixing state and reduced bioavailability of the canola oil dispersed on the surface.

The trend in oxygen consumption in aerobic system correlates with the observed trend in methane production by the anaerobic micro-organisms. The increase in methane production in anaerobic microcosms corresponding to the applied oil load increases over time, as observed by Li et al. (2006). In case of aerobic system the carbon dioxide production corresponding to the applied load also goes up.
Figure 6-1: Cumulative oxygen uptake comparison for the 3 oil loads
6.2 Carbon dioxide production

Comparison of carbon dioxide production between the three experiments is shown in figure (6-2). As seen from the figure, the trends in CO$_2$ production are similar to those described for oxygen uptake above. It is observed that as the oil load increases, the total amount of carbon dioxide production increases. The carbon dioxide production increased only 2 times when the oil load was increased ten times which is similar to the oxygen uptake described above.

Figure 6-2: Carbon dioxide production for the 3 oil loads
6.3 Toxicity variability with Dissolved Oxygen:

The EC50 values obtained from microtox results can be correlated with the dissolved oxygen measurements over the time. Lower dissolved oxygen has been one of the reasons for increase in toxicity in the water column as suggested in chapter 2. It can be inferred that decreased oxygen availability due to non-mixed condition precludes complete mineralization of canola oil, which in turn leads to the production of toxic intermediates. It is interesting to estimate the variability of toxicity with the dissolved oxygen, measured at all the levels in the water column.

6.3.1 100 gal/acre Oil load:

For the experiment of 100 gal/acre canola oil load, the toxicity results indicate almost no toxicity in any of the replicates. The plots of EC50 variation with dissolved oxygen are shown in appendix (B). The plots are provided only for the highest port of 3 representative samples. It can be observed that lower EC50 values are obtained when the dissolved oxygen decreased during the experiment run.

6.3.2 333 gal/acre Oil load:

For the experiment of 333 gal/acre oil load, a lag phase can be clearly seen before the samples turn toxic. As the dissolved oxygen went down to zero in all the levels, the EC50 started to decrease as well. Toxicity was observed from the 4th week and the dissolved oxygen reduced to zero from the third week onwards. We can infer that the toxicity is related to the dissolved oxygen deviation. Representative plots of one sample for this experiment are provided in appendix B.
6.3.3 1000 gal/acre oil load:

In the experiment of 1000 gal/acre oil load, EC50 and dissolved oxygen started decreasing simultaneously. The lack of oxygen results in incomplete mineralization of the canola oil leading to the formation of toxic intermediates. The impact of high oil load was manifest from the second week itself, the dissolved oxygen went down to zero and the samples became toxic. This is clearly seen in the plots provided in the Appendix B. Plots are provided for 3 representative samples for all the 3 levels.
Chapter 7: Conclusions and Future Work
Respirometry tests were conducted to simulate the conditions of Canola oil spill on a poorly mixed water body. Oxygen depletion in the water column was observed for all the three oil loads and it has been correlated with the subsequent toxicity measured at the three levels of the water column. In all the three experiments, considerable reproducibility was obtained between the different microcosms. Also it could be seen that as the oil load increases, the cumulative oxygen uptake increased, the dissolved oxygen decreased and the corresponding toxicity increased. As the oil load increases, thicker oil layer limits the oxygen transfer through the water column, decreasing the dissolved oxygen. Thus we infer that the quantity of oil spilled is a very important criterion in selecting a quick and effective spill response technique.

On the basis of comparison between previous studies on anaerobic and aerobic biodegradation of oil in the water column, it can be concluded that the toxicity increased after a short duration and then decreased as the test proceeded. The biodegradation byproducts as defined by previous studies seem to be the cause of toxicity.

Also, along with studying the dissolved oxygen variation and toxicity issues, it is important to know the composition of Canola oil and study its extent of biodegradation in the water. The extent of bioavailability and stability of the byproducts, especially the long chain fatty acids, can form the basis of oil spill bioremediation techniques. Focus should be on several other factors such as the type of oil, the nature of biomass and temperature of the water column.
REFERENCES:


CO2 Version 1.1 Calculation of CO2 Absorption in an Aqueous KOH Reservoir.

Biosystems Branch-RREL US Environmental Protection Agency Cincinnati Ohio
Appendix A:
Output Conversion

Formula for conversion of percent oxygen to solubility in moles/liter:

\[ S = \frac{a}{22.414} \times \frac{(760-p)}{760} \times \frac{r\%}{100} \]

- **S** = solubility of gas in moles per liter
- **a** = absorption coefficient of gas at temperature
- **P** = vapor pressure of water at temperature
- **r\%** = actual reading in percent Oxygen

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**Procedure for Microtox analysis using the 81.9% Basic test:**

**Chemicals:** Filtered samples, Microtox acute reagent (Vibrio Fisheri Bacteria), Microtox® Diluent, Microtox Reconstituion solution, Osmotic adjusting solution

**Materials:** Microtox Model M500 Analyzer, glass cuvettes, pipettes Pipettors: 100-1000 μL pipettor and tips.

**Test Preparation:**

1. Place cuvettes in wells A1 to A5 till D1 to D5 and F3 and Reagent Well.
2. Add 1000μL Microtox Reconstitution solution to cuvette in the Reagent Well.
3. Add 1500μL Microtox Diluent to cuvette F3.
4. Add 1000μL Microtox Diluent to cuvette A1to A5 and C1 to C4.
5. Add 2500μL Sample to B5 and 250μL Microtox® OAS and mix well using the pipette.
6. Wait 5 minutes.
7. Reconstitute a vial of Microtox Reagent
8. Add 150μL reconstituted Reagent into cuvette F3.
9. Add from F3 to
10. Wait 15 minutes and then place B1 into Read Well and press the Set button.
11. Touch the computer <space bar> key.
12. Read the Io light levels as prompted by the computer.
13. Immediately transfer 900μL from A1 to B1, A2 to B2….C1 to C2, C5 to D5.
16. When timer sounds, READ light levels as prompted by the computer monitor: B1 to B5 and D1 to D5.

The test gives the results for the 5 minutes analysis and also for 15 min analysis. These results are directly provided by the software as EC50 values in percent volume.
Appendix B:

Figures for EC50 vs DO for the three experiments:

Figure (B-1) EC50 vs DO for 100 gal/acre oil load experiment
Figure (B-2) EC50 vs DO for 100 gal/acre oil load experiment

Figure (B-3) EC50 vs DO for 100 gal/acre oil load experiment
Figure (B-4) EC50 vs DO for 333 gal/acre oil load experiment

Figure (B-5) EC50 vs DO for 333 gal/acre oil load experiment
Figure (B-5) EC50 vs DO for 333 gal/acre oil load experiment

Figure (B-6) EC50 vs DO for 1000 gal/acre oil load experiment
Figure (B-7) EC50 vs DO for 1000 gal/acre oil load experiment

Figure (B-7) EC50 vs DO for 1000 gal/acre oil load experiment
Figure (B-8) EC50 vs DO for 1000 gal/acre oil load experiment

Figure (B-9) EC50 vs DO for 1000 gal/acre oil load experiment
Figure (B-10) EC50 vs DO for 1000 gal/acre oil load experiment

Figure (B-11) EC50 vs DO for 1000 gal/acre oil load experiment
Figure (B-12) EC50 vs DO for 1000 gal/acre oil load experiment