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It is entitled:
Anaerobic Biodegradation Patterns for Biodiesel

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ANAEROBIC BIODEGRADATION PATTERNS FOR BIODIESEL

A dissertation submitted to the
Division of Research and Advanced Studies
of the University of Cincinnati
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

In the Department of Biomedical, Chemical, and Environmental Engineering
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ABSTRACT

Biodiesel, a mixture of Fatty Acid Methyl Esters (FAMEs) with or without petrodiesel, is considered a promising alternative liquid fuel and is gaining widespread acceptance. However, the impact and fate of biodiesel when accidentally released in the environment is poorly understood, especially under anaerobic conditions. Studies were designed to investigate the fate of biodiesel in an anaerobic environment and the impact of petrodiesel (petrodiesel) on the biodegradation of biodiesel.

In the first set of experiments, biotransformation of soybean biodiesel and the inhibitory effect of petrodiesel were studied under methanogenic conditions. The kinetics of anaerobic biodegradation of soybean biodiesel B100 (signifying 100% biodiesel) blended with different petrodiesel loads were studied using biomass pre-acclimated to B100 (biodiesel only) and B80 (80% biodiesel and 20% petrodiesel). Results showed that soybean biodiesel was effectively degraded, whereas petrodiesel could not be degraded under methanogenic conditions. The presence of petrodiesel had a greater inhibitory effect on the rate of biodegradation than the biodegradation efficiency (defined as the methane production efficiency). Both the biodegradation rate coefficient and the methane production efficiency increased almost linearly with the increasing fraction of biodiesel.

In a second set of experiments, biotransformation of soybean biodiesel (B100) and its biodiesel/petrodiesel blends were investigated under sulfate-reducing conditions. Three blends of biodiesel, B100, B50, and B0 were compared using cultures pre-acclimated to B100 and
B80. The kinetics of biodegradation of individual FAMEs and the sulfate utilization rate were studied using a serum bottle reactor test. The biodegradation rate for saturated FAMEs decreased with an increase in the carbon chain length. For unsaturated FAMEs, the biodegradation rate increased with an increasing number of double bonds.

In a third set of experiments, the kinetics of biodegradation of flaxseed biodiesel were tested under methanogenic and sulfate-reducing conditions to determine how changes in FAMEs profile affect the biodegradation of biodiesel. Under methanogenic conditions, the presence of moderate amounts of petrodiesel did not exhibit any inhibitory effects on the biodegradation rate of flaxseed biodiesel until the contribution of petrodiesel approached 50% by volume. Petrodiesel caused a greater inhibition effect on soybean biodiesel than flaxseed biodiesel with increasing petrodiesel loads. Under sulfate-reducing conditions, the same trend in biodegradation order occurred but the biodegradation rates were higher compared to soybean biodiesel. This trend was not related to the abundance profiles of FAMEs in the biodiesel, but the physiochemical properties of the individual FAME compounds. The abundance profiles of FAMEs in the biodiesel may affect their degradation rate due to inhibition effect of their hydrolysis products.
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CHAPTER 1

Overview

1.1 Background

Biodiesel, defined as the mono-alkyl esters of long chain fatty acids, has been receiving increased attention as an alternative to fossil fuels. It is made from vegetable oils or animal fats via a transesterification reaction in the presence of an alkaline catalyst (typically sodium methylate, potassium methylate and sodium hydroxide) where the viscous triglycerides are broken into less viscous methyl or ethyl esters depending on the type of alcohol used (Fukuda et al., 2001; Canakci et al., 2001; Saka and Kusdiana, 2001; Demirbas 2003). A major reason for the interest in biodiesel is that the feed stocks for fatty acid methyl esters (FAMEs) of biodiesel are renewable. In contrast, fossil fuels are non-renewable resources and their over-exploitation has caused concern for the depletion of fossil fuels in the near future.

An additional incentive for the use of biodiesel is the potential positive impact on global climate change. Issues that are adverse to the environment and human health, such as acid precipitation, global climate change and increased respiratory diseases, are related to fossil fuel emissions. Compared to fossil fuel, studies have shown that the usage of biodiesel fuel reduces some atmospheric pollutants such as carbon monoxide (CO), particulate matter (PM), hydrocarbons (HCs), and sulfur dioxide (SO$_2$) (Bünger et al., 2012; Anderson 2012). Various greenhouse (GHG) emission models estimate lower CO$_2$ and SO$_2$ emissions for biodiesel
(Ban-Weiss et al., 2008; Cahill et al., 2012). The reduction of carcinogenic and mutagenic polycyclic aromatic hydrocarbons by using biodiesel fuel has also been reported (Turri-Baldassarri et al., 2004). Although several studies confirmed an increase of NOx emissions from biodiesel combustion (Graboski and McCormick, 1998), an EPA report showed that a 20% biodiesel blend cuts PM by about 10%, HCs by about 21%, and CO by about 11% (U.S EPA, 2002). The European Union estimated that the typical GHG emission savings from biodiesel are 36–88%, depending on the feedstock (European Union, 2009). Thus, the usage of biodiesel could help mitigate the global warming challenge. Besides that, since almost no modification is needed for a current petrodiesel engine to run on biodiesel, either in the form of blending with petrodiesel or pure biodiesel, biodiesel is considered a sustainable fuel supply (Kalligeros et al., 2003; Lapuerta et al., 2008).

Currently, the use of biodiesel is often in the form of various blends with petrodiesel containing from 5% to 20% biodiesel. Petrodiesel, which is composed mostly of saturated hydrocarbons and aromatic hydrocarbons, exhibits low chemical reactivity. The different molecular structures of petrodiesel and biodiesel attribute very different physiochemical properties for both fuels. Both petrodiesel and biodiesel have limited solubility in water (Guard et al., 1983; Krop et al., 1997). Biodiesel is slightly more soluble than petrodiesel because of the higher polarity of fatty acid alkyl esters due to the presence of the carboxylate moiety (Knothe et al., 2005). Besides that, biodiesel has been reported to have lower acute toxicity compared to petrodiesel (Khan et al. 2007; Hollebone et al 2008).
While great benefits accrue to using biodiesel as a fuel, its environmental fate and effects need to be evaluated, and the risks associated with their use need to be addressed. The current understanding of the fate and effects of biodiesel and its various blends is inadequate to evaluate environmental risks of its use. Studies have shown that biodiesel exhibited higher degradation rates compared to petrodiesel in an aerobic environment (Prince et al., 2008; Corseuil et al., 2011; Horel et al., 2011; Yassine et al., 2013). A dearth of information exists on the biodegradation of biodiesel and its blends with petrodiesel in an anaerobic environment. Furthermore, biodiesel is made from various sources such as virgin oil feedstock (e.g., soybean oil, rapeseed oil, and flaxseed oil), waste vegetable oil, animal fats and algal lipids, each having different chemical compositions. The physiochemical properties of biodiesel depend on the feedstock type, the FAMEs abundance profile, and structures of its FAMEs (Ramos et al., 2008). This wide variability in biodiesel formulations may result in highly varied toxicological profiles and environmental behaviors depending on the feedstock. Leite et al. (2011) tested the toxicity of the water soluble fractions (WSFs) of various biodiesels and indicated that the highest toxicity of WSFs was castor oil biodiesel, followed by waste oil biodiesel and palm oil biodiesel. Hollebone et al. (2008) found that for all tested biodiesels, soybean biodiesel was most toxic, followed by animal fat biodiesel, and canola biodiesel. However, the variability in biodiesel feedstock type or FAME profile effect on the biodegradation of biodiesel has not been studied.

In the light of the current studies, it is important to conduct research that addresses the issues of biodiesel fuels, their fate in anaerobic environments, and the effects of petrodiesel
petrodiesel-biodiesel blends and feedstock type on biodegradation. A good approach to obtain scientifically sound information on these issues involves the use of Biochemical Methane Potential (BMP) tests of different biodiesels under methanogenic condition and Serum Bottle Reactor (SBR) tests under sulfate-reducing condition.

1.2 Research Objectives

The objective of this research was to achieve a sound understanding of biodiesel biodegradation under anaerobic conditions. This main goal was achieved by pursuing three sub-objectives:

1. To determine the anaerobic biodegradation rate and extent of soybean biodiesel under methanogenic and sulfate-reducing conditions.
2. To investigate the inhibitory effects of petrodiesel on the biodegradation of soybean biodiesel under methanogenic and sulfate-reducing conditions.
3. To compare the results for soybean biodiesel and flaxseed biodiesel to determine how changes in FAMEs profile affect biodegradation.

1.3 Organization of the Dissertation

This dissertation is organized into five chapters. Chapter one presents a brief introductory review of the current status and understanding of biodiesel/petrodiesel blends and defines the specific research objectives undertaken in this study. Chapter two contains original work on the anaerobic biodegradation kinetics of different blends of soybean biodiesel with petrodiesel using pre-acclimated cultures grown on B100 and B80 under methanogenic conditions.
Chapter three contains original work on the anaerobic biodegradation kinetics of soybean–biodiesel/petrodiesel blends (B0, B50, and B100) by cultures previously acclimated on B100 and B80 under sulfate-reducing condition. Chapter four investigates the anaerobic biodegradation kinetics and extent of flaxseed biodiesel under methanogenic and sulfate-reducing conditions. Finally, Chapter five summarizes the conclusions and recommendations derived from this study.

1.4 References


A comprehensive analysis of biodiesel impacts on exhaust emissions; EPA420-P-02-001; United States Environmental Protection Agency: Washington, DC, 2002.


CHAPTER 2

ANAEROBIC BIODEGRADATION OF SOYBEAN BIODIESEL AND PETRODIESEL BLENDS UNDER METHANOGENIC CONDITIONS

ABSTRACT

Biotransformation of soybean biodiesel and the inhibitory effect of petrodiesel were studied under methanogenic conditions. Biodiesel removal efficiency of more than 95% was achieved in a chemostat with influent biodiesel concentrations up to 2.45 g/L. The kinetics of anaerobic biodegradation of soybean biodiesel B100 (biodiesel only) with different petrodiesel loads was studied using biomass pre-acclimated to B100 and B80 (80% biodiesel and 20% petrodiesel). The results indicated that the biodiesel fraction of the blend could be effectively biodegraded, whereas petrodiesel was not biodegraded at all under methanogenic conditions. The presence of petrodiesel in blends with biodiesel had a greater inhibitory effect on the rate of biodegradation than the biodegradation efficiency (defined as the efficiency of methane production). Both the biodegradation rate coefficient and the methane production efficiency increased almost linearly with the increasing fraction of B100. With the increasing fraction of petrodiesel, the biodegradation rate and efficiency were correlated with the concentration of soluble FAMEs in the water.
2.1 Introduction

Biodiesel, a mixture of fatty acid methyl esters, with reportedly rapid biodegradation rates, lower acute toxicity (Khan et al., 2007), and a lower carbon dioxide release rate (Hill et al., 2006), is considered a promising alternative to fossil fuels and is gaining widespread acceptance. Annual production of biodiesel has increased from around 25 million gallons in the early 2000s to almost 1.1 billion gallons in 2012 (http://www.biodiesel.org/what-is-biodiesel/biodiesel-basics). At present, it is often used in fuel blends with petrodiesel with the volumetric percentage of biodiesel steadily increasing from 5% to 20%. The industry has established a goal of using 10% biodiesel in blends with petrodiesel by 2022 (http://www.biodiesel.org/what-is-biodiesel/biodiesel-basics). The increased demand for biodiesel, which involves the processing, transportation, storage, and handling of large quantities of this product, often results in high potential for accidental leaks with a majority of those releases occurring in subsurface environments having limited dissolved oxygen concentrations. To this end, it is necessary to understand and evaluate the amenability of biodiesel/petrodiesel blends to bioremediation under anaerobic conditions. Aktas et al. (2010) evaluated anaerobic biodegradation of biodiesel in laboratory serum bottles by five anaerobic inocula and found that biodiesel could be easily hydrolyzed and converted to a variety of fatty acid intermediates. They also found that mineralization occurred within one month. To date, most studies on the biodegradation of biodiesel focused on either the anaerobic biodegradation of glycerol as a by-product of the manufacturing of biodiesel or the aerobic biodegradation of biodiesel/petrodiesel blends. Biodegradation of biodiesel and its blends with petrodiesel under anaerobic conditions has rarely been studied.
Petrodiesel alone is resistant to anaerobic biodegradation because it is composed mostly of saturated and aromatic hydrocarbons that exhibit low chemical reactivity. During the past two decades, anaerobic oxidation of petrodiesel constituents has been reported to occur under strict anoxic conditions (i.e., denitrifying, ferric-iron reducing, and sulfate-reducing processes). A few reports claim observing strict methanogenic biodegradation (Head et al., 2003; Gray et al., 2010). Some studies revealed that adding biodiesel could promote and enhance the biodegradation of petrodiesel by means of co-metabolism or synergistic effects under aerobic conditions. Co-metabolic processes occur when microorganisms use a readily biodegradable co-substrate as the carbon or energy source in the presence of a poorly degradable substrate such as petrodiesel. Miller and Mudge (1997) studied the effect of biodiesel on the rate of removal and weathering characteristics of crude oil in artificial sand columns. They observed that in the absence of biodiesel, crude oil exhibited low mobility and a slow rate of microbial degradation within the sediment. The addition of biodiesel led to a greater recovery of the oil in the un-weathered form. Zhang et al. (1998) claimed that co-metabolism was observed in the biodegradation of the B20 Rapeseed Ethyl Ester (REE)/petrodiesel blends. In the presence of REE, the degradation rate of petrodiesel was twice that observed when petrodiesel was present alone. In addition, the biodegradation rate of the blend increased almost linearly with the increase of REE % in the blend. Pasqualino et al. (2006) found that the overall biodegradability of mixtures of biodiesel and petrodiesel increased with the addition of biodiesel. Owsianiak et al. (2009) studied the primary biodegradation rate of methyl rapeseed biodiesel and petrodiesel blends by a consortium isolated from a crude oil contaminated site. The degradation efficiency was improved by 26%
when the carbon source was changed from pure petrodiesel (B0) to pure biodiesel (B100). Chen et al. (2010) investigated the biodegradability of cottonseed methyl and ethyl esters, and their blends with petrodiesel, and found that at the end of a 28 day test period, the extent of biodegradation of neat cottonseed methyl and ethyl esters (B100’s) exceeded 99%, while the corresponding degrees of removal of 50% blends of the same FAMEs with petrodiesel (B50s) were 93.8% and 95.7%. When the FAMEs were reduced to 20% blends (B20), biodegradation extents declined to 81-82%. The extent of biodegradation of neat petrodiesel was only 49.9%. These reports on the co-metabolism of petrodiesel and biodiesel resulted from studies conducted only under aerobic conditions.

The objective of this study was to investigate the anaerobic biodegradation of soybean biodiesel blends under methanogenic conditions. BMP tests were conducted in serum bottles to determine the anaerobic biodegradation kinetics of biodiesel in the absence and presence of different concentrations of petrodiesel. Furthermore, the possibility of anaerobic biodegradation of petrodiesel and its inhibition effects on the biodegradation of biodiesel was also investigated.

2. 2 Materials and Methods

2.2.1 Chemicals

Unblended soybean biodiesel (B100) was purchased from Peter Cramer North America (Cincinnati, OH) with mole fractions of 0.145 palmitic acid methyl ester (C16:0-ME), 0.055 stearic acid methyl ester (C18:0-ME), 0.206 oleic acid methyl ester (C18:1-ME), 0.518
linoleic acid methyl ester (C18:2-ME), and 0.0759 linolenic acid methyl ester (C18:3-ME). Low-sulfur petrodiesel (B0) was purchased from a local BP petrodiesel station (Cincinnati, OH) with a mole fraction of 0.165 nC10-nC23 n-alkanes. palmitic acid methyl ester (99%), palmitoleic acid methyl ester (99%), stearic acid methyl ester (99%), oleic acid methyl ester (99%), linoleic acid methyl ester (99%), linolenic acid methyl ester (99%), and n-alkanes standard mixture (nC10-nC30), were all purchased from Sigma Aldrich (USA).

### 2.2.2 Culture acclimation

A 12-liter laboratory-scale continuous flow stirred-tank reactor (CSTR) having a 10-liter liquid biomass volume and 2-liter headspace was inoculated with a bacterial culture, obtained and enriched from an anaerobic digester at a local wastewater treatment plant (Cincinnati, Ohio). The bioreactor was operated as a completely mixed system with a solids retention time (SRT) of 40 days. A schematic diagram of the reactor is shown in Figure 2.1. A Hamilton syringe pump was used to feed the biodiesel as the sole organic carbon source to the reactor through a buffer solution feed line. The biodiesel concentration in the feed was gradually increased from 0.52 g/L to 2.45 g/L over 15 months. The pH in the chemostat was maintained at 7.0 ± 0.20. Low pH, nutrient and high pH buffer solutions were provided from separate reservoirs at a total flow rate of 0.25 L/day. The final concentrations of the feed essential nutrients and vitamin minimal medium in the nutrient feed were: 623.25 mg/L NH₄Cl, 196.5 mg/L MgCl₂•6H₂O, 141.662 mg/L CaCl₂•2H₂O, 31.25 mg/L FeCl₂•4H₂O, 416.81 mg/L KH₂PO₄, 9.59 mg/L CuSO₄•5H₂O, 0.32 mg/L Na₂MoO₄•2H₂O, 13 mg/L MnSO₄•H₂O, 10.48 mg/L ZnCl₂, 9.16 mg/L CoCl₂•6H₂O, 0.64 mg/L B(OH)₃, 9.58 mg/L NiCl₂•6H₂O, 0.24
mg/L 4-aminobenzoic acid (99%), 0.096 mg/L biotin, 0.0048 mg/L cyanocabalamin, 0.096 mg/L folic acid dihydrate (99%), 0.24 mg/L nicotinic acid (98%), 0.24 mg/L pantothenic acid Ca-salt hydrate (98%), 0.48 mg/L pyridoxine hydrochloride (98%), 0.24 mg/L riboflavin (98%), 0.24 mg/L thiamine hydrochloride (99%), 0.24 mg/L thioctic acid (98%).

2.2.3 Analytical methods

Flow rate of the nutrient and buffer solutions, effluent pH, and total gas production were monitored on a daily basis. The pH was measured using an Orion Model 720A pH meter (Orion Research, Boston, Massachusetts). COD was measured using Hach Method 8000 on a Hach DR/200 Spectrophotometer (Hach, Loveland, Colorado). Gas composition was analyzed on a weekly basis for CH₄, CO₂, O₂ and N₂. Analysis for gas composition was performed on an HP5890 Series II Gas Chromatograph (GC) (Hewlett Packard, Wilmington, Delaware) equipped with a thermal conductivity detector (TCD) using an HP 10-ft molecular sieve BX-45/60 mesh HP 6 ft HAYESEPQ 80/100column (Supelco, Bellefonte, Pennsylvania). VFAs were measured on an Agilent 6890 II GC equipped with a flame ionization detector (FID) using 2 mm i.d. 1.83 m glass column packed with 4% Carbowax on a 80/120 Carbopack B-DA (Supelco, Bellefonte, Pennsylvania). Liquid samples, collected weekly from the sampling port, were filtered through 0.45 µm filters and analyzed for the target compounds, VFAs, and effluent chemical oxygen demand (COD). Chemical analysis for FAMEs and n-alkanes in the effluent and extracts were determined on an Agilent 6890 II GC equipped with a HP-INNOWAX capillary column (30 m, 0.25 mm i.d, 0.25 um film thickness), and an Agilent 5973 Mass Spectrometer Detector (MSD). The flow rate of the carrier gas helium was 1
ml/min, the temperature of inlet and detector interface was set at 320°C, and the inlet was operated in splitless mode. Oven temperature program was set as follows: hold at 35°C for three minutes, ramp at 5°C/min to 250°C and hold at 250°C for five minutes.

2.2.4 BMP tests

The BMP tests followed the procedure outlined by Gupta, et al. (1996). Once the chemostat reached steady state, i.e., several consecutive measurements revealed consistently low effluent concentrations of the target compounds and effluent COD, and methane gas production was stable and near expected values, biomass was collected from the reactor and used as a source culture for the BMP test. 60-ml serum bottles were sterilized, surface-deactivated and placed in an anaerobic hood. Inside the hood, each bottle was filled with 20 ml of chemostat effluent (which contained the anaerobic biomass), 20 ml of nutrient and buffer solution and 20 ml N\textsubscript{2} headspace. 0.3 mg/L Resazurin was used as an indicator of anaerobic conditions. The nutrient solution was purged for about 5 hours with nitrogen gas prior to placing it in an anaerobic chamber for 3 days to remove any trace oxygen in the solution. After addition of the various solutions, each serum bottle was capped with Teflon (Supelco) and spiked with 15 µl biodiesel containing different volume petrodiesel loads (0 µl, 0.5 µl, 1 µl, 2.5 µl, 5 µl, 7.5 µl, 15 µl, 30 µl and 45 µl). A separate set of bottles was injected with only petrodiesel. Killed control samples were prepared the same way except the effluent was autoclaved at 121°C for 60 minutes. In addition to the biotic and abiotic samples, three blank control samples were prepared with only nutrient and effluent solutions with no injection of any biodiesel and petrodiesel. All experiments were done in triplicate and were performed at room temperature.
A total of 10 biologically active events, three biomass control events (without spiking oil), and three killed control events (abiotic controls) were prepared. Serum bottles were sealed and placed in a tumbler. Sampling was performed at different time intervals, the volume of gas produced was measured, and gas composition of the headspace was analyzed to calculate the volume of methane produced. At the end of the BMP tests, the liquid phase was completely liquid-liquid extracted with dichloromethane. The extracts were filtered through anhydrous sodium sulfate to dewater them and stored at -20°C until analysis on GC-MS.

2.2.5 Partitioning experiment

Super-Q water was purged with nitrogen gas for 5 hours before being placed into the anaerobic chamber and set there for two days to remove any remaining trace concentrations of dissolved oxygen. Each 160 ml serum bottle was filled with 120 ml Super-Q water and then sealed and spiked with 45 µl biodiesel containing different petrodiesel fractions (0 µl, 1.5 µl, 3 µl, 7.5 µl, 15 µl, 22.5 µl, 45 µl, 90 µl and 135 µl). This represented the same oil/water volume ratio as in the BMP batch test. Serum bottles were then placed in a tumbler. After 24 hours mixing, samples were then transferred to 125 ml glass separatory funnels and allowed to settle for 3 hours. 50 ml of the WAFs were withdrawn from the bottom of the funnels and were completely liquid–liquid extracted with 25 ml dichloromethane (DCM). The extracts were filtered through anhydrous sodium sulfate to dewater them and then concentrated 10-fold in a TurboVap II Evaporation System. Samples were analyzed on the GC-MS.
2.3 Results and Discussion

2.3.1 Steady-State

The startup period for the reactor lasted six months and the organic feed concentration was progressively increased from 0.52 g/L to 1.4 g/L. Once the 1.4 g/L feed concentration was reached, reactor operation was continued until conditions stabilized. The feed was then increased to a higher concentration and again allowed to stabilize. This process was repeated until the final feed concentration of 2.45 g/L was reached. Figure 2.2 shows the weekly COD balance for the chemostat after six months of operation with the organic feed ranging from 1.75 g/L to 2.45 g/L. The GC/MS analysis indicated that the extent of FAMEs removal at any influent concentration was more than 95%. The influent COD was in the form of soybean biodiesel B100, and the effluent COD was the soluble COD in the effluent, mainly attributable to the biodegradation intermediate, acetic acid. The difference between the influent COD and the effluent COD + methane COD are the fraction of the COD that is utilized for biomass production, volatized acetic acid and solidified biodiesel. After day 502 of operation, the reactor was fed a blend of biodiesel (80%) and petrodiesel (20%), B80. Effluent chemical analysis revealed that the petrodiesel fraction was not biodegraded and accumulated in the reactor. Methane production decreased to the expected methane production as if biodiesel were the only substrate utilized. The difference between the influent COD and the effluent COD + methane COD was mainly the COD attributable to the undegraded petrodiesel fraction in the blend, the COD that is consumed in biomass production and lost due to volatized acetic acid and solidified biodiesel.
2.3.2 Biodegradation Kinetics

The first BMP test was performed using 15 µl of biodiesel B100 per bottle supplemented with different volumes of petrodiesel. Biomass used for this test was acclimated to soybean biodiesel B100 only. The test was performed to assess the effect of the presence of different volumes of petrodiesel loads on the rate of anaerobic biodegradation of soybean biodiesel. Biodegradation was monitored through methane production, and the cumulative methane production curves obtained for the different petrodiesel treatments are presented in Figure 2.3. In all treatments, methane production curves followed a biodegradation profile of a typical apparent biomass exponential growth approaching a plateau level. The achieved cumulative methane production (Mt) decreased with increasing petrodiesel fractions. In order to quantify the biodegradation of biodiesel through methane production in the biotic microcosms with different petrodiesel loads, first-order biodegradation rate constants were calculated using equation (1):

\[ Mt = Mu(1-e^{-kt}) \]  \hspace{1cm} (1)

where,

- \( Mt \) = cumulative methane produced (ml) at time \( t \),
- \( Mu \) = ultimate methane production (ml),
- \( k \) = first order biodegradation rate constant (d\(^{-1}\)).
- \( T \) = time (d).

The \( k \) and \( Mu \) values were estimated by nonlinear least-squares regression using SigmaPlot 11 (systatsoftware.Inc.,CA) by fitting the experimentally determined cumulative methane
production data to equation (1). The mathematical model fit the experimental data rather well as shown in Figures 2.3 and 2.4, with methane production and first-order rate coefficients listed in Table 2.1. The first-order rate coefficient was $0.15 \pm 0.0088$/d for biodiesel B100 alone. In response to the injection of varying petrodiesel loads, the first-order rate coefficient decreased with the increasing amounts of petrodiesel. The volumes of petrodiesel injected with the 15 µl of B100 were 0, 0.5, 1, 2.5, 5, 7.5 and 15 µl, which resulted in an influent volume fraction of B100 of 1, 0.97, 0.94, 0.86, 0.75, 0.67 and 0.50. The first-order biodegradation rate constant increased with the increasing volume fraction of B100. The first-order rate constants for biodiesel biodegradation are plotted in Figure 2.5 against the corresponding biodiesel volume fraction. The figure shows a good linear correlation between the first-order biodegradation rate constant and the volume fraction of B100, with the linear relationship having a slope of $0.11 \pm 0.001$.

The theoretical methane production of 15 µl biodiesel was calculated by measuring the COD of the biodiesel, which is 2.75g COD/g biodiesel, which corresponds to a methane production of 13.78 ml. The observed methane production in response to the injection of 15 µl biodiesel is that observed from the experiment minus that produced in the biomass controls. The methane produced in response to injection of 15 µl biodiesel is shown in Table 2.3. With the increase in petrodiesel loads, the methane production decreased possibly due to a decrease in the bioavailability of soluble FAMEs in the water and the toxicity of the added petrodiesel. Yassine et al. (2012) examined the microtox toxicity of the water accommodated fraction (WAF) of six soybean biodiesel and petrodiesel blends at different oil loads and observed that
EC 50 for B0 was around 11.52 to 20 at different dilutions from 1:1 to 1:1000. Methane production corresponding to injection of only 15 µl B100 was 91% of the expected value. There was no significant difference (P=0.8) in the ultimate methane produced when lower petrodiesel volume fractions were added, i.e.,≤ 1 µl. The methane production efficiency was greater than 85% of theoretical for the bottles where the added petrodiesel volumes were between 0 µl and 5µl. Over the whole range, the methane production efficiency increased linearly with the fraction of biodiesel as shown in Figure 2.6, and the linear rate of increase was 0.78 ± 0.10. The first order rate constant for the biodegradation of biodiesel in the absence of any petrodiesel was 0.15 ± 0.0088 d⁻¹. The first order rate constant decreased to 0.086 ± 0.008 d⁻¹ when 15 µl of petrodiesel was added to the mix. Hence, in the presence of 15 µl petrodiesel, the biodiesel first order biodegradation rate constant decreased to 59% of the observed value when no petroleum biodiesel was present. The biodegradation efficiency was decreased by a factor of 58% when compared to the case when no petrodiesel was added. The data are summarized in Table 2.3 and Figures 2.5 and 2.6.

No methane production was observed in any of the killed control samples. Small amounts of methane were produced in blank biomass control samples (triangle symbols in part A of Figures 2.3 and 2.4). This methane production was likely due to the biodegradation of residual organic matter in the chemostat effluent. Similar behavior was observed in the petrodiesel alone treatments (open triangle symbols in Figures 2.3 and 2.4). However, in all cases, methane production in the petrodiesel only treatments was lower than that in the biomass blank control samples. Consequently, there was no methane production when
petrodiesel was present alone, suggesting that petrodiesel was not biodegraded under methanogenic conditions. This supports the results that methane produced in the biodiesel and petrodiesel blends was due only to biodiesel biodegradation. The data in Table 2.3 suggest that the final methane produced when 1 µl of petrodiesel was added to 15 µl of biodiesel was slightly greater than that observed for biodiesel alone. Similar behavior was observed in the second BMP test when 0.5, 1, and 2.5 µl of petrodiesel was added. This might be attributed to stress induced by the presence of petrodiesel that may affect the portion of the substrate transformed to biomass.

To further investigate the effect of petrodiesel on the biodegradation of biodiesel and to assess the possibility of co-metabolism, the feed to the chemostat was modified to a volume fraction of 80% biodiesel and 20% petrodiesel. After two SRT turnovers, stable methane production was reestablished. Effluent from this chemostat was used to conduct a second BMP test with petrodiesel volume added similarly to the first BMP test, except in this case two additional petrodiesel volumes of 30 µl and 45 µl were tested, corresponding to volume fractions of B100 of 0.33 and 0.25, respectively. The biodegradation rate coefficients were calculated by fitting the data to the first-order model equation (1). The mathematical model fit the experimental data very well as shown in Figure 2.4, with final methane production and first-order biodegradation rate coefficients listed in Table 2.2. The first-order biodegradation rate coefficients decreased when compared to the results obtained from the test using culture from the chemostat operated on a feed of biodiesel alone. This suggests that the activity of the culture was inhibited by the presence of petrodiesel in the feed. The first-order rate constant
was 0.086 ± 0.012 d\(^{-1}\) when pure 15 µl biodiesel was added compared to 0.15 ± 0.088 d\(^{-1}\) obtained from the BMP test using biomass B100. However, the differences in the value of the first order rate constant for the different petrodiesel added volumes decreased suggesting that the bulk of the inhibitory effect of petrodiesel was absorbed in the biomass that was acclimated to the presence of petrodiesel. The first-order rate constant when 15 µl of petrodiesel added was 0.059 ± 0.009 d\(^{-1}\), which is 68% of the value observed when no petrodiesel was injected, while the biodegradation efficiency was decreased to 85%. When these values are compared to the 59% and 58% obtained from the BMP test using biomass B100, it again supported the conclusion that the presence of petrodiesel in the reactor feed resulted in a culture that was acclimated to the presence of petrodiesel and, therefore, less susceptible to further inhibition. This supports the findings in the first BMP test that the presence of petrodiesel had a greater effect on the rate rather than extent of biodegradation. Observed differences were lower in biodegradation efficiency when the petrodiesel load varied from 0 µl to 2.5 µl, and the ultimate methane production efficiency was greater than 85% for petrodiesel loads up to 15 µl. Similar to the results observed in the first BMP test, both the first-order biodegradation rate constant and the methane production efficiency increased linearly with the increasing volume fraction of B100 as shown in Figure 2.5 and Figure 2.6, the linear rates of increase were 0.78 ± 0.10 and 0.22 ± 0.035, compared to the linear rate 0.11 ± 0.001 and 0.0039 ± 0.009 in the first BMP test.
2.3.2 Biodiesel Bioavailability

In the presence of biodiesel, we expected the readily biodegraded constituents of biodiesel to serve as the primary substrate in the anaerobic co-metabolism of petrodiesel constituents, but the results show that the biodegradation of petrodiesel did not occur under methanogenic conditions. However, the presence of petrodiesel appeared to affect the bioavailability of biodiesel due to the partitioning of biodiesel and petrodiesel in the aqueous phase. The effective oil-oil attraction induces segregation of the bulk oil from water (Chandler, 2005). Figure 2.7 presents the soluble FAME concentrations of biodiesel with different increasing loads of petrodiesel. Compared to 45 µl pure biodiesel in 120 ml water, the partitioning of C16:0-ME, C18:0-ME, C18:1-ME, C18:2-ME and C18:3-ME slightly increased for the blended oil with 1.5 µl petrodiesel added, then decreased. However, when petrodiesel loads increased up to 90 µl, the concentration of soluble FAMEs did not decrease. With further increase in the petrodiesel loads up to 135 µl, the concentration of soluble FAMEs slightly increased. This behavior showed a similar trend as the methane production rate shown in Table 2.1. In the presence of petrodiesel, the partitioning of FAMEs in aquatic systems affects their bioavailability during biodegradation.

2.4 Conclusions

The goal of this study was to evaluate the methanogenic biodegradation rate of soybean biodiesel in the presence of different loads of petrodiesel. Biodiesel was observed to biodegrade to methane even in the presence of elevated concentrations of petrodiesel. Petrodiesel, however, was not biodegraded under methanogenic conditions. As for the effect
of the presence of petrodiesel on the biodegradation of biodiesel, it affected the biodegradation rate due to the bioavailability of biodiesel in the aqueous phase. Both the biodegradation first order rate constant and methane production efficiency increased almost linearly with the increasing fraction of B100. The biodegradation of biodiesel in BMP tests using biomass acclimated to biodiesel only was more sensitive to the presence of petrodiesel than when biomass acclimated to the presence of petrodiesel was used. The presence of petrodiesel has a greater effect on the rate of biodegradation than the biodegradation efficiency.

2.5 References


**Table 2.1** Ultimate methane production of 15 µl soybean biodiesel with different volume loads of petrodiesel and nonlinear regression parameters for methane production curves using culture from reactor B100

<table>
<thead>
<tr>
<th>Added B0(µl)</th>
<th>CH₄ exp (ml)</th>
<th>CH₄ model (ml)</th>
<th>k(d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.70±0.19</td>
<td>15.10±0.25</td>
<td>0.15±0.088</td>
</tr>
<tr>
<td>0.5</td>
<td>16.24±0.14</td>
<td>15.09±0.49</td>
<td>0.14±0.016</td>
</tr>
<tr>
<td>1</td>
<td>15.06±0.53</td>
<td>15.38±0.31</td>
<td>0.14±0.010</td>
</tr>
<tr>
<td>2.5</td>
<td>14.89±0.37</td>
<td>14.40±0.36</td>
<td>0.13±0.009</td>
</tr>
<tr>
<td>5</td>
<td>14.76±0.32</td>
<td>13.90±0.44</td>
<td>0.12±0.012</td>
</tr>
<tr>
<td>7.5</td>
<td>13.09±0.40</td>
<td>13.39±0.47</td>
<td>0.11±0.013</td>
</tr>
<tr>
<td>15</td>
<td>10.46±0.68</td>
<td>10.44±0.32</td>
<td>0.086±0.008</td>
</tr>
</tbody>
</table>

CH₄ exp, experimental methane production ± standard deviation among three replicates; CH₄ model, model methane ultimate production ± standard error; k, first order rate constant ± standard error
Table 2.2 Ultimate methane production of 15 µl soybean biodiesel with different loads of petrodiesel and nonlinear regression parameters for methane production curves using culture from reactor B80

<table>
<thead>
<tr>
<th>Added B0 (µl)</th>
<th>CH₄ exp (ml)</th>
<th>CH₄ model (ml)</th>
<th>k(d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.20±0.41</td>
<td>15.08±0.76</td>
<td>0.086±0.012</td>
</tr>
<tr>
<td>0.5</td>
<td>14.20±0.38</td>
<td>15.12±0.68</td>
<td>0.092±0.012</td>
</tr>
<tr>
<td>1</td>
<td>14.86±0.81</td>
<td>15.96±0.71</td>
<td>0.079±0.009</td>
</tr>
<tr>
<td>2.5</td>
<td>14.44±0.072</td>
<td>15.56±0.77</td>
<td>0.081±0.011</td>
</tr>
<tr>
<td>5</td>
<td>13.79±0.28</td>
<td>14.68±0.65</td>
<td>0.092±0.012</td>
</tr>
<tr>
<td>7.5</td>
<td>13.28±0.62</td>
<td>14.28±0.68</td>
<td>0.077±0.010</td>
</tr>
<tr>
<td>15</td>
<td>13.36±0.48</td>
<td>14.84±0.96</td>
<td>0.059±0.009</td>
</tr>
<tr>
<td>30</td>
<td>12.16±0.59</td>
<td>13.17±0.43</td>
<td>0.060±0.043</td>
</tr>
<tr>
<td>45</td>
<td>11.61±0.18</td>
<td>12.45±0.43</td>
<td>0.063±0.009</td>
</tr>
</tbody>
</table>

CH₄ exp, experimental methane production ± standard deviation among three replicates; CH₄ model, model methane ultimate production ± standard error; k, first order rate constant ± standard error
Table 2.3 15 µl soybean biodiesel B100 methane production efficiency using culture from reactor B100 and B80.

<table>
<thead>
<tr>
<th>Added B0 (ul)</th>
<th>CH$_4$ production in culture B100 (ml)</th>
<th>CH$_4$ production in culture B80 (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.526</td>
<td>12.599</td>
</tr>
<tr>
<td>0.5</td>
<td>13.065</td>
<td>12.602</td>
</tr>
<tr>
<td>1</td>
<td>12.818</td>
<td>13.265</td>
</tr>
<tr>
<td>2.5</td>
<td>11.719</td>
<td>12.840</td>
</tr>
<tr>
<td>5</td>
<td>11.586</td>
<td>12.194</td>
</tr>
<tr>
<td>7.5</td>
<td>9.915</td>
<td>11.682</td>
</tr>
<tr>
<td>15</td>
<td>7.286</td>
<td>11.761</td>
</tr>
<tr>
<td>30</td>
<td>–</td>
<td>10.556</td>
</tr>
<tr>
<td>45</td>
<td>–</td>
<td>10.011</td>
</tr>
</tbody>
</table>
Figure 2.1 Schematic of Chemostat
Figure 2.2 Weekly COD Balance for Chemostat
Figure 2.3 Cumulative CH₄ production curves for soybean biodegradation with different B₀ loads using culture from reactor B100, points are triplicate experimental data while the solid line is model fit equation.
Figure 2.4 Cumulative CH$_4$ production curves for soybean biodiesel biodegradation with different B0 loads using culture from reactor B80, points are triplicate experimental data while the solid line is model fit equation.
Figure 2.5 First-order rate coefficients in different volume fraction biodiesel
Figure 2.6 Methane production efficiency in different volume fraction biodiesel
Figure 2.7 WAF concentrations of FAMEs in different biodiesel and diesel blends
CHAPTER 3

ANAEROBIC BIODEGRADATION OF SOYBEAN BIODIESEL AND PETRODIESEL BLENDS UNDER SULFATE REDUCING CONDITION

ABSTRACT

Biotransformation of soybean biodiesel and its biodiesel/petrodiesel blends was investigated under sulfate reducing conditions. Three blends of biodiesel, B100, B50, and B0, were treated using microbial cultures pre-acclimated to B100 (biodiesel only) and B80 (80% biodiesel and 20% petrodiesel). Results indicated that the biodiesel could be effectively biodegraded in the presence or absence of petrodiesel whereas petrodiesel could not be biodegraded at all under sulfate-reducing conditions. The kinetics of biodegradation of individual Fatty Acid Methyl Ester (FAME) compounds and their accompanying sulfate reduction rates were studied using a serum bottle test. As for the biodegradation of individual FAME compounds, the biodegradation rates for the saturated FAMEs decreased with increasing carbon chain length. For unsaturated FAMEs, biodegradation rates increased with increasing number of double bonds. The presence of petrodiesel had a greater effect on the rate of biodegradation of biodiesel than on biodegradation efficiency.
3.1 Introduction

The world is confronted with the crises of fossil fuel depletion, global climate change, and environmental pollution and degradation from spills. Biodiesel has been gaining a widespread acceptance because of its potential as a renewable, easily biodegradable energy source that can contribute to decreased greenhouse gas (GHG) emissions. Since the passage of the Energy Policy Act of 2005, biodiesel use has been steadily increasing in the United States. With the increasing use of biodiesel, a thorough understanding of its fate in the environment is needed since it poses similar environmental risks as fossil fuels when accidentally released. The susceptibility of these biofuels to biological degradation is insufficiently understood. Studies reporting the biodegradation of biodiesel have principally focused on aerobic processes (DeMello et al, 2007, Owsianiak et al, 2009, Yassine et al 2013). While anaerobic conditions prevail in subsurface environments where unintended releases frequently occur, few studies have reported on the biological degradation of biodiesel blends in the absence of oxygen. Therefore, it is of great importance to study the biodegradation patterns of biodiesel blends under anaerobic environment. Aktas et al. (2010) evaluated anaerobic biodegradation of biodiesel by five anaerobic inocula and found that biodiesel could be easily hydrolyzed and converted to a variety of fatty acid intermediates within one month. Sørensen et al. (2011) investigated the microbiological stability of biodiesel blends in small-scale microcosms, and their observations confirmed the presence of stable populations of both aerobic and anaerobic microorganisms during incubation. However, petrodiesel, which is composed mostly of saturated and aromatic petroleum hydrocarbons, exhibits low biochemical reactivity and for many decades was thought to undergo biodegradation only in the presence of free oxygen.
Biodegradation of petrodiesel under strictly anoxic conditions has been frequently reported in the past decade (Rueter et al., 1994; Widdel et al., 2001; Wentze et al., 2007). Boopathy et al. (2004) evaluated the biodegradation of petrodiesel under various anaerobic conditions in soil columns and found that the highest petrodiesel biodegradation rate was under mixed electron acceptor conditions followed by sulfate-reducing, nitrate-reducing, and finally methanogenic conditions. However, even under optimum conditions, anaerobic biodegradation was reported to require in excess of 310 days to achieve 81% transformation of petrodiesel fuel. Mukherji et al. (2004) reported that the maximum degradation of petrodiesel oil by a culture isolated from deep-sea sediment was only 18% over 50 days under anoxic nitrate-reducing conditions. It appears that the biodegradation of petrodiesel under anaerobic conditions mostly occurs under sulfate reducing conditions either after long exposure times or when present in low environmental concentrations. Many studies have reported that the biodegradation rate of petrodiesel was improved by the co-metabolism of degradable petrodiesel and biodiesel blends under aerobic conditions (Zhang et al. 1998, Pasqualino et al. 2006, Owsianiak et al. 2009). Information on the anaerobic biodegradation of petrodiesel and biodiesel blends is limited.

I have previously reported the biodegradation of petrodiesel under methanogenic conditions and the inhibitory effect of petrodiesel on that process (Chapter two). When biodiesel was blended with low concentrations of petrodiesel, the petrodiesel did not impact the biodegradation of biodiesel much. Significant effects on the biodegradation rate and extent of transformation started appearing when petrodiesel and biodiesel were present in equal
volumetric proportions (B50). In this study, the biodegradation of pure unblended biodiesel (B100), pure unblended petrodiesel (B0), and B50 was investigated under sulfate-reducing conditions, which is a prevalent condition in anaerobic/anoxic sediments. The objective was to investigate anoxic biodegradation of soybean biodiesel in a sulfate-reducing environment, and compare it to the biotransformation of soybean biodiesel in a methanogenic environment. SBR tests were conducted to determine the anaerobic biodegradation kinetics of individual FAME compounds in biodiesel. Sulfate utilization rate was also investigated. Furthermore, the possibility of biodegradation of petrodiesel under sulfate-reducing conditions and its inhibition effects on the biodegradation of biodiesel was also investigated.

3.2 Materials and Methods

3.2.1 Chemicals

Unblended soybean biodiesel (B100) was purchased from Peter Cramer North America (Cincinnati, OH). The average molecular weight of B100 with FAMEs mole fractions of 0.145 C16:0-ME, 0.055 C18:0-ME, 0.206 C18:1-ME, 0.518 C18:2-ME) and 0.0759 C18:3-ME. Low-sulfur petrodiesel (B0) was purchased from a local BP petrodiesel station (Cincinnati, OH) with a mole fraction of 0.165 nC10-nC23 n-alkanes. B50 fuel blend was blended in our laboratory by volumetric splash mixing. Palmitic acid methyl ester (99%), palmitoleic acid methyl ester (99%), stearic acid methyl ester (99%), oleic acid methyl ester (99%), linoleic acid methyl ester (99%), linolenic acid methyl ester (99%), and n-alkanes standard mixture (nC10-nC30), were all purchased from Sigma Aldrich (USA).
3.2.2 Culture acclimation

Bacterial enrichment, obtained from an anaerobic digester at a local wastewater treatment plant (Cincinnati, Ohio) was inoculated in two 12-liter laboratory-scale continuous flow stirred-tank reactors (CSTR) previously used under methanogenic conditions in our laboratories. One reactor was fed B100 as the sole organic carbon source while the other was fed B80. Reactor construction details, reactor schematic and reactor operation are described in chapter two. At the onset of this study sodium and ammonium sulfate were added to the nutrient solution to convert the redox conditions from methanogenic to sulfate-reducing. Sodium molybdate (Na$_2$MoO$_4$·2H$_2$O), which inhibits sulfate-reducing bacteria (Pareek, S., et al 2000), was not added to the nutrient solution. In a sulfate-reducing environment, sulfide is produced via the reduction of sulfate. High concentrations of sulfide have been reported to inhibit sulfate-reducing microorganisms (Maillacheruvu, et al., 1993). To minimize the potential for inhibition and to avoid sequestration of heavy metals in the nutrient solution via sulfide precipitation, excess ferrous iron in the form of FeCl$_2$·4H$_2$O was added to the nutrient feed. The minimal medium containing essential nutrients and vitamins for the sulfate reducing system were as follows: 1351.88 mg/L Na$_2$SO$_4$, 7691.25 mg/L FeSO$_4$.7H$_2$O, 450.87mg/L NH$_4$SO$_4$, 119.13 mg/L MgSO$_4$·6H$_2$O, 70.75 mg/L CaCl$_2$·2H$_2$O, 2666.25 mg/L FeCl$_2$·4H$_2$O, 208.40 mg/L KH$_2$PO$_4$, 9.59 mg/L CuSO4·5H2O, 13 mg/L MnSO4·H2O, 10.48 mg/L ZnCl2, 9.16 mg/L CoCl2·6H2O, 0.64 mg/L B(OH)$_3$, 9.58 mg/L NiCl$_2$.6H$_2$O, 0.24 mg/L 4-aminobenzoic acid (99%), 0.096 mg/L biotin, 0.0048 mg/L cyanocabalamin, 0.096 mg/L, folic acid dihydrate (99%), 0.24 mg/L nicotinic acid (98%), 0.24 mg/L pantothenic acid Ca-salt hydrate (98%), 0.48 mg/L pyridoxine hydrochloride (98%), 0.24 mg/L riboflavin (98%),
0.24 mg/L thiamine hydrochloride (99%), 0.24 mg/L thioctic acid (98%). The pH in the bioreactor was kept constant at 7.0±0.20. When the bioreactor achieved steady state, the culture was collected for batch experiment.

3.2.3 Analytical methods

The flow rate of the nutrient and buffer solutions was monitored on a daily basis. Certain water quality variables including pH, target analytes, volatile fatty acids (VFAs), influent sulfate, effluent sulfate, and effluent chemical oxygen demand (COD) were monitored twice a week. Gas composition was analyzed weekly to check for leaks. The pH was measured using an Orion Model 720A pH meter (Orion Research, Boston, Massachusetts). COD was measured using Hach Method 8000 on a Hach DR/200 Spectrophotometer (Hach, Loveland, Colorado). Gas composition analysis was performed on an HP5890 Series II Gas Chromatograph (GC) (Hewlett Pachard, Wilmington, Delaware) equipped with a thermal conductivity detector (TCD) using an HP 10 ft molecular sieve (BX-45/60 mesh) and an HP 6 ft HAYESEPQ 80/100 column (Supelco, Bellefonte, Pennsylvania). VFAs were measured on an HP Agilent 6890 II GC equipped with a flame ionization detector (FID) using a 2 mm i.d. 1.83 m glass column packed with 4% Carbowax on an 80/120 Carbopack B-DA (Supelco, Bellefonte, Pennsylvania). Chemical analysis for FAMEs and alkanes in the effluent as well as the batch experiment followed the same procedures. The extracts were aliquoted in 1mL GC vials and spiked with 10 µl of an internal standard solution containing C11:0-ME, C13:0-ME, deuterated nC10, and deuterated nC20 in DCM, and were analyzed on an HP Agilent 5890 II Gas Chromatograph (GC) equipped with an HP-INNOWAX capillary column (30 m
long, 0.25 mm i.d., 0.25 µm film thickness), and an HP Agilent 5971 Mass Spectrometer Detector (MSD). The flow rate of the Helium carrier gas was 1mL/min. Both the inlet and detector interface temperatures were held at 320 °C, and the former was operated in splitless mode. Oven temperature program was as follows: hold at 35 °C for 3 min, ramp at 5 °C/min to 250 °C, and hold at 250 °C for 5 min.

3.2.4 SBR tests

The SBR tests followed the procedure outlined by Gupta et al. (1996). Each sterilized and surface-deactivated 60-ml serum bottle was filled with 25 ml of the effluent from the reactor (which contained the anaerobic biomass), 25 ml of deoxygenated nutrient and buffer solution, and 10 ml N2 headspace. After addition of the various solutions, each serum bottle was spiked with 15 µl of 1 of 3 different blended fractions of petrodiesel and biodiesel. In all cases, the sulfate concentration in the solution was sufficient to complete the biodegradation of approximately twice the amount of oil added. In the case of B100 treatment, 11 sampling events took place at 0, 1, 2, 3, 4, 5, 7, 9, 12, 15, and 19 days. In the case of B50 treatment, 12 sampling events took place at 0, 1, 2, 3, 4, 5, 7, 10, 14, 19, 24, and 30 days. In the case of B0 treatment, 9 sampling events took place at 0, 1, 3, 6, 10, 14, 19, 24, and 30 days. In the case of killed controls (KCs) treatment, triplicates of controls were sampled at 9 and 19 days for B100 treatment, 15 and 30 days for B50 and B0 treatments. All serum bottles were sealed with Teflon-lined septa, and were placed in a tumbler. At a given sampling event, triplicate serum bottles of each treatment were randomly selected and sacrificed. at each sampling event, 1 ml of the bottle contents was withdrawn using a 3 ml syringe and filtered through a
0.45 µl filter for sulfate analysis on IC, and the remaining contents were completely liquid-liquid extracted with dichloromethane. The extracts were filtered through anhydrous sodium sulfate to dewater them and stored at -20°C until analysis on GC-MS.

3.3 Results and Discussion

3.3.1 Sulfate Utilization

Sulfate was added to the serum bottles in excess of what was theoretically needed to ensure complete degradation of the substrate, as shown in Figure 3.1 and Figure 3.2. For the B100 and B50 treatments with culture pre-acclimated to B100 (Culture B100), the first-order rate coefficients for sulfate utilization were calculated by nonlinear regression and were 0.18 ± 0.028 d⁻¹ for B100 (open circle symbols in Figure 3.1) and 0.20 ± 0.036 d⁻¹ for B50 (open triangle symbols in Figure 3.1). The first-order rate coefficient was slightly higher for B50, although the initial concentration of FAMEs in B100 was twice the concentration in B50. When the total mass and rate of sulfate utilized was compared to the total mass and rate of FAMEs degraded for B100, the ratio of sulfate to FAMEs was found to be 4.30 mg sulfate/mg FAMEs. Another series of SBR tests were run with biomass pre-acclimated to B80. For the treatment of B50 and B100, the first-order rate coefficients of sulfate utilization were respectively 0.064 ± 0.023 d⁻¹ (open circle symbols in Figure 3.2) and 0.085 ± 0.027 d⁻¹ (open triangle symbols in Figure 3.2). These results show that when using culture B80, the sulfate utilization rate in the B100 and B50 substrate treatments decreased by an order of magnitude compared to the B100 culture (36% for the B100 substrate treatment and 43% for the B50 treatment). This decrease in sulfate utilization rates when changing the biomass pre-
acclimation from B100 to B80 was greater than the 20% difference in biodiesel in the reactor feed to the two chemostats, which suggests that the activity of the culture could have been inhibited by the presence of petrodiesel in the feed. Yassine et al. (2012) examined the Microtox™ toxicity of the water accommodated fraction (WAF) of six soybean biodiesel and petrodiesel blends at different oil loads and observed that at dilutions of 1:1 to 1:100, WAFs of B80 and B100 had similar EC50s. However, when dilutions exceeded 1:1000, the toxicity of the WAF was decreased when the fraction of biodiesel increased in the blends, and hence B80 becoming more toxic than B100. In fact, analysis of the petrodiesel fraction in the effluent showed that it did not degrade and instead accumulated in the chemostat. Consequently, the microorganisms in the reactor fed on B80 were subjected to the toxicity of petrodiesel in B80 blends and seemed to have resulted in lower microbial activity. There was no decline in sulfate concentration in the B0 substrate treatment using culture B100, culture B80, and KCs indicating that no petrodiesel was degraded within the 30-day period of observation in all SBR tests. Co-metabolism did not appear to occur after acclimating the culture to petrodiesel. This supports the observation that any sulfate concentration decrease in the B50 treatment with both cultures was probably due to the biodegradation of the biodiesel fraction, the FAMEs compound in the oil blends.

3.3.2 FAMEs Degradation

The basic steps involved in the overall anaerobic degradation of waste are: (1) hydrolysis, (2) acidogenesis and (3) final anaerobic oxidation. For biodiesel, which is composed of mixed FAMEs, the first step is ester hydrolysis to their corresponding long chain fatty acids
(LCFAs) and methanol. C18:1-ME and C18:2-ME, the two highest concentration compounds in soybean biodiesel, are hydrolyzed to oleic acid and linoleic acid. The initial concentrations of C18:1-ME and C18:2-ME in B100 treatment were 48.41 mg/l and 119.68 mg/l. When B100 was biodegraded using the culture pre-acclimated to B100 (circle symbols in Figure 3.3), a 1-day lag phase was observed for C16:0-ME, C18:0-ME and C18:1-ME. The reported solubility for C16:0-ME, C18:0-ME, and C18:1-ME is 4 µg/l, 0.2 ug/l, and 3.7 µg/l, respectively (Krop HB et al., 1997). Because of their low solubility in water, their microbial bioavailability is low. Many studies have shown that high concentrations of LCFAs could cause microbial inhibition (Zonta et al., 2013). Lalman et al. (2000, 2001) reported that oleic acid and linoleic acid concentrations above 30 mg/l inhibited the biodegradation of acetate, and linoleic acid above 100 mg/l significantly inhibited butyrate degradation (Lalman et al., 2002). Sousa et al. (2013) found that unsaturated C18:1 had a more inhibitory effect on methanogens than saturated C16:0-ME and C18:0-ME. Adsorption of LCFAs onto granular biomass has been considered to be the main mechanism for their toxicity due to physical interactions with the cell wall, affecting its transport and protective function (Pereira et al., 2005). This protective functions likely the reason for the observed lag phase at the beginning of the biodegradation process. For those compounds having a lag phase, that initial time was excluded from the fitting of the data to the first order kinetic model. The first-order rate coefficients for each individual FAME compound are shown in Table 3.1. The first-order rate coefficient for the biodegradation of C18:1 was significantly lower than for the other compounds. The extent of biodegradation for all the FAME compounds was above 98% at the end of the experiment (day 19) as shown in Table 3.2.
Similar findings were observed for the biodegradation of B100 using culture B80 (triangle symbols in Figure 3.3), and, in this case, the lag-phase lasted two days for C16:0-ME, C18:0-ME and C18:1-ME. The first-order biodegradation rate coefficient was determined for each compound after excluding the lag-phase, and the resulting values are shown in Table 3.1. Compared to the same oil treated using culture B100, the first-order biodegradation rate coefficients for each FAME compound decreased by 46% for C16:0-ME, 45% for C18:0-ME, 43% for C18:1-ME, 49% for C18:2-ME, and 47% for C18:3-ME. However, the extent of biodegradation decreased to approximately 91% for all the FAMEs at the end of the experiment, compared to 98% when the culture B100 was used. Similar results were obtained in a previous study on biodiesel biodegradation under methanogenic conditions.

When B50 was biodegraded using culture B100 (circle symbols in Figure 3.4), no lag phase was observed for all compounds probably because the initial concentration of the FAMEs in B50 was half the concentration in B100. The initial concentration of C18:1-ME and C18:2 ME were 23.36 mg/l and 60.23 mg/l, and the inhibition effect from their hydrolyzed LCFAs was reduced compared to B100 treatment when using the same culture. The microbial activity was not inhibited at the initial time when compared to the treatment of B50 using culture B80. The first-order biodegradation rate coefficients for the individual FAMEs calculated by nonlinear regression are shown in the 4th column of Table 3.2. Rate coefficients were found significantly different (p = 0.003) for the different FAME compounds. For saturated FAMEs, the degradation rate of C16:0-ME was slightly higher than that of C18:0-ME. As for the unsaturated FAMEs, the first-order biodegradation rate coefficient was highest for C18:3-ME.
and the biodegradation rate increased as the number of double bonds increased. The extent of biodegradation for each FAME at the end of the experiment (day 30) is shown in Table 3.2. For C16:0-ME, C18:0-ME and C18:1-ME, the removal efficiency was above 95% and over 97% for C18:2-ME and C18:3-ME, respectively. Although the initial concentration of FAMEs in B50 was lower than that in B100, after 19 days of incubations, the removal of FAMEs in B50 were 93.49% for C16:0-ME, 93.54% for C18:0-ME, 93.58% of C18:1-ME, 95.08% for C18:2-ME and 97.44% for C18:3-ME. In comparison, all the FAMEs in B100 were depleted by more than 98% after the same period of incubation. The decrease of the removal efficiency of FAMEs in the B50 treatment compared to B100 treatment by the same culture was probably due to the inhibition effect from the petrodiesel fraction of B50 blends.

The biodegradation of B50 using culture B80 (triangle symbols in Figure 3.4) revealed a 3-day lag phase for C18:0 and a 3-day slow degradation phase for all the other FAMEs. This may be attributed to the fact that the culture, in this case, was exposed to elevated levels of petrodiesel. When compared to the same oil blends treatment by culture B100, the first-order biodegradation rate for each FAME in B50 treatment by culture B80 were 54% for C16:0-ME, 50% for C18:0-ME, 62% for C18:1-ME, 61% for C18:2-ME and 52% for C18:3-ME (Table 3.1). In terms of the biodegradation efficiency, the FAME biodegradation extent was decreased to around 80% of that by culture B100.

Overall, the data collected in this study suggest that the first-order rate constant for the biodegradation of FAME decreased with increases in the carbon chain length for saturated
FAMEs and increased with the increasing number of double bonds for unsaturated FAMEs. The long-chain hydrophobic hydrocarbons have low solubilities in water. However, the presence of the carboxylate ester group and the double bonds enable the FAME molecules to form hydrogen bonds with water, which makes them more bioavailable. For the same carbon chain length, the solubility increased with the increase in number of double bonds. The substrate bioavailable for microorganisms refers to the soluble substrate in the water. The reported solubility limits for the saturated C16:0-ME and C18:0-ME are 4 µg/l and 0.2 µg/l, and unsaturated C18:1-ME, C18:2-ME and C18:3-ME are 3.7 µg/l, 21 ug/l and 91 µg/l (Krop HB et al., 1997). For unsaturated FAMEs, the biodegradation rate increased with increases in the number of double bonds. This correlates well with their aqueous solubility due to the bioavailability of the substrate. FAMEs partitioning test data from chapter two revealed that the soluble concentrations of FAMEs in water were 25.49 µg/l for C16:0-ME, 7.89 µg/l for C18:0-ME, 42.07 µg/l for C18:1-ME, 83.65 ug/l for C18:2-ME, and 6.69 µg/l for C18:3-ME. All the FAMEs except C18:3-ME showed a similar trend as reported. C18:3-ME with three double bonds is mostly unstable and easier to be autoxidized (Ullrich, F., et al 1988, Hamilton et al 1997). This may have occurred during the partitioning test where, after sacrificing a set of samples, they were allowed to settle for 3 hours under ambient conditions before the samples were further processed. Considering the mass transfer through the cell membrane, the flux of soluble substrate though the cell membrane is given by Fick’s First Law of Diffusion:

\[ J = - \frac{D(C_0-C_i)}{\Delta x} \]  

(2)
Where,

\[ J = \text{the flux of substrate } M, \text{L}^2\text{T}^{-1}; \]

\[ D = \text{the substrate diffusion coefficient } \text{L}^2\text{T}^{-1}; \]

\[ \Delta x = \text{the thickness of the diffusion path, } \text{L}; \]

\[ C_0 = \text{the substrate concentration outside the cell membrane, } \text{ML}^{-3}; \]

\[ C_i = \text{the substrate concentration inside the cell membrane, } \text{ML}; \]

Then the unsaturated FAMEs, such as C18:2-ME, which have the highest bioavailability, would have a higher diffusion driving force across the cell membrane compared to the other FAMEs. However, once they are inside the cell, these unsaturated FAMEs would require more energy-intensive enzymatic steps per double bond to complete their metabolism. Therefore, the faster mass transfer kinetics across the cell membrane might be countered by slower enzymatic metabolism inside the cell to break down the double bonds. Aktas et al. (2010) studied the intermediates of anaerobic biodegradation of biodiesel and found that the anaerobic biodegradation of saturated FAMEs undergo a \( \beta \)-oxidation pathway in which the fatty acid intermediates are shortened by removal of two carbon fragments from the carboxyl end of the molecule to form a series of smaller molecular-weight compounds. The saturated C18:0-ME is first hydrolyzed to its fatty acid product octadecanoic acid (C18:0), then degraded to hexadecanoic acid (C16:0) by beta-oxidation. The saturated C16:0-ME, which has two fewer carbons in its carbon chain, is first hydrolyzed to hexadecanoic acid (C16:0) directly.
3.3.3 Alkane Degradation

No biodegradation of total alkanes (nC10-nC22) in B0 and B50 was observed within the 30 days of observation, as shown in Figure 3.5 and Figure 3.6. It was confirmed that the decrease in sulfate in B50 blends occurred as a result of FAMEs degradation only, not hydrocarbon degradation. The total alkane concentration in the B80 culture treatment was higher than that in B100, likely because the alkanes did not degrade and accumulated in the reactor B80. Alkanes, as a group of low chemical reactivity compounds, are only degraded aerobically by certain microbial cultures, and their metabolism requires specific enzymes and a sufficient exposure time to become a microbial substrate.

3.4 Conclusions

The findings from this study indicate that biodiesel was effectively biodegraded whereas petrodiesel resisted biodegradation under sulfate-reducing condition. We initially expected that easily biodegraded biodiesel would act as the primary substrate in the anaerobic co-metabolism of petrodiesel constituents using a biomass enriched on biodiesel with petrodiesel subsequently introduced as a co-substrate at increasingly higher concentrations. However, for total alkanes (nC10-nC22) in B0 and B50, no biodegradation was observed within the 30 days of the experiments. After comparing the FAMEs biodegradation in B100 and B50 treated by cultures pre-acclimated to B100 and B80, we found that when blended with petrodiesel, the biodegradation of FAMEs in biodiesel was affected by: (1) the solubility of the specific FAME compounds, which affected their bioavailability when the substrate was transferred into the cell membrane; (2) the lag-phase shown in high concentration FAMEs in B100
treated by culture B100 was attributed to the hydrolysis product of fatty acids; (3) inhibition came from previous exposure to petrodiesel for treatment with B80 culture and the injected petrodiesel fraction for B50 treatment due to the toxicity of petrodiesel. The findings from this study further suggest that the first-order rate coefficients for the biodegradation of FAMEs decreased with increases in the carbon chain length of saturated FAMEs and increased with the increasing number of double bonds for unsaturated FAMEs. In the case of the inhibition effect of petrodiesel on the biodegradation of biodiesel, the presence of petrodiesel has a greater effect on the rate of biodegradation than the biodegradation efficiency, which is consistent with the findings reported in Chapter two for methanogenic biodegradation of biodiesel.

3.5 References


unsaturated and saturated long-chain fatty acids. Applied and Environmental Microbiology 79 (14), 4239-4245.

### Table 3.1 First-order degradation rate for soybean biodiesel FAMEs biodegradation (d⁻¹)

<table>
<thead>
<tr>
<th></th>
<th>FAMEs in B100</th>
<th></th>
<th>FAMEs in B50</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture B100</td>
<td>Culture B80</td>
<td>Culture B100</td>
<td>Culture B80</td>
</tr>
<tr>
<td>C16:0-ME</td>
<td>0.215±0.017</td>
<td>0.100±0.038</td>
<td>0.246±0.039</td>
<td>0.134±0.023</td>
</tr>
<tr>
<td>C18:0-ME</td>
<td>0.214±0.018</td>
<td>0.097±0.038</td>
<td>0.227±0.035</td>
<td>0.113±0.020</td>
</tr>
<tr>
<td>C18:1-ME</td>
<td>0.189±0.020</td>
<td>0.082±0.029</td>
<td>0.170±0.026</td>
<td>0.105±0.021</td>
</tr>
<tr>
<td>C18:2-ME</td>
<td>0.215±0.021</td>
<td>0.105±0.032</td>
<td>0.319±0.060</td>
<td>0.196±0.037</td>
</tr>
<tr>
<td>C18:3-ME</td>
<td>0.230±0.021</td>
<td>0.109±0.028</td>
<td>0.427±0.064</td>
<td>0.224±0.051</td>
</tr>
</tbody>
</table>
Table 3.2 Removal extent of soybean biodiesel FAMEs in B100 by day 19 and B50 by day 30 using culture from reactor B100 and B80 (%)

<table>
<thead>
<tr>
<th></th>
<th>Culture B100</th>
<th>Culture B80</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B100</td>
<td>B50</td>
</tr>
<tr>
<td>C16:0-ME</td>
<td>98.73</td>
<td>95.58</td>
</tr>
<tr>
<td>C18:0-ME</td>
<td>98.62</td>
<td>95.36</td>
</tr>
<tr>
<td>C18:1-ME</td>
<td>98.74</td>
<td>95.83</td>
</tr>
<tr>
<td>C18:2-ME</td>
<td>99.12</td>
<td>97.12</td>
</tr>
<tr>
<td>C18:3-ME</td>
<td>99.13</td>
<td>98.53</td>
</tr>
</tbody>
</table>
**Figure 3.1** Sulfate utilization with the culture from reactor B100
Figure 3.2 Sulfate utilization with the culture from reactor B80
Figure 3.3 Biodegradation of soybean biodiesel FAMEs in B100 treatment. The circle symbols are for the culture from reactor B100. The triangle symbols are for the culture from reactor B80. The blank symbols are KCs.
Figure 3.4 Biodegradation of soybean biodiesel FAMEs in B50 treatment. The circle symbols are for the culture from reactor B100. The triangle symbols are for the culture from reactor B80. The blank symbols are KCs.
Figure 3.5 Total alkanes (nC10 to nC22) degradation with the culture from reactor B100
**Figure 3.6** Total alkanes (nC10 to nC22) degradation with the culture from and reactor B80
CHAPTER 4

ANAEROBIC BIODEGRADATION PATTERNS OF FLAXSEED BIODIESEL AND PETRODIESEL BLENDS

ABSTRACT

Anaerobic biodegradation of flaxseed biodiesel was investigated under methanogenic and sulfate-reducing conditions using the BMP test and the primary disappearance rate of target compounds. The results indicated that flaxseed biodiesel could be effectively degraded under both conditions. The kinetics of methanogenic biodegradation of unblended flaxseed biodiesel, B100, with different amounts of petroleum diesel did not exhibit any inhibitory effects on the biodegradation rate of biodiesel until the contribution of petroleum diesel approached 50% of the volume of organic substrate. The methane production efficiency, defined as actual volume of methane produced normalized to the expected methane production, increased almost linearly with the increasing fraction of B100. Under sulfate-reducing condition, the rate of biodegradation of the individual FAMEs compounds and the utilization rate of sulfate ions were studied using the SBR test. The biodegradation rate for the saturated FAMEs decreased with increasing carbon chain length, and for unsaturated FAMEs, the biodegradation rate increased with increasing number of double bonds. The presence of 50% petroleum diesel in the substrate showed inhibitory effect on the biodegradation efficiency of FAMEs under sulfate reducing conditions.
4.1 Introduction

The depletion of the non-renewable fossil fuel resources and the pollution generated from their burning have prompted a race for the development of alternative energy sources, including biodiesel. Biodiesel, which is biodegradable, less toxic, and has more favorable emissions profiles when compared to petroleum diesel (Ma, F. et al., 1999), consists of fatty acid methyl esters. With its increased use in blended diesel, biodiesel can pose detrimental risks and threats to the environmental similar to those caused by petroleum diesel. Several researchers have demonstrated the biodegradability of biodiesel and petroleum diesel blends under aerobic condition (DeMello, J. A., 2007; Owsianiak, M. 2009; Yassine, M.H, 2013), while limited research has been performed to investigate the anaerobic biodegradation of biodiesel (Aktas, 2010; Sørensen, 2011). As many oil spills occur underground, it is therefore necessary to evaluate and understand the amenability of biodiesel/diesel blends to bioremediation under anaerobic conditions.

Biodiesel is derived from renewable sources such as virgin oil feedstock, waste vegetable oil, animal fats and algal lipids, via transesterification with methanol in the presence of a catalyst. Because it is made from a diverse mix of raw materials, the FAMEs profile of biodiesel varies with the raw material used. The five most common FAMEs in biodiesel are: palmitic acid methyl ester (C16:0-ME), stearic acid methyl ester (C18:0-ME), oleic acid methyl ester (C18:1-ME), linoleic acid methyl ester (C18:2-ME) and linolenic acid methyl ester (C18:3-ME). Four oil crops dominate the feedstock market used for worldwide biodiesel production: soybean, rapeseed, palm and sunflower. Biodiesels made from rapeseed and palm oils have
similar profiles of FAMEs with C18:1-ME being the highest in abundance. Biodiesels manufactured from sunflower and soybean oil have similar profiles of FAMEs with C18:2-ME the highest in abundance. Numerous algal strains have been investigated as potential sources for biodiesel production due to their rapid growth rates, high lipid contents and tolerance for poor quality water (Hoekman, et al., 2012). Some algal lipids have the profile of FAMEs with C18:3-ME being the highest in abundance. Vegetable oils such as camelina and flaxseed also have the highest abundance of C18:3-ME. Different sources of biodiesel were found to have different physiochemical properties and toxicological profiles (Ramos, et al., 2008; Leite, et al. 2011). Hollebone (2008) found that petroleum diesel was most toxic followed by soybean biodiesel, animal fat biodiesel and canola biodiesel.

In chapter two and three I have presented the anaerobic biodegradation pattern of soybean biodiesel. The aim of this study is to investigate the anaerobic biodegradation of flaxseed biodiesel under methanogenic and sulfate-reducing conditions, and to compare the behavior of this biodiesel to that of soybean biodiesel under the same conditions. Flaxseed oil is, in fact, not a commercially viable feedstock for biodiesel production, because it has high nutritional value (Oomah 2001; Jhala et al., 2010; Singh et al., 2011) and a relatively lower autoxidative stability than other oils (Bozan et al., 2008; Chander 2010). The reason we chose the flaxseed feedstock in this study is that flaxseed vegetable oil is composed of more than 50% linolenic acid (C18:3), while soybean vegetable oil is composed of more than 50% linoleic acid (C18:2). The objective is to determine how changes in FAMEs profile affect biodegradation. BMP tests were conducted to determine the anaerobic biodegradation kinetics.
of flaxseed biodiesel in the presence of different petroleum diesel concentration and to investigate any inhibitory effect of diesel on the biodegradation of flaxseed biodiesel. SBR tests were conducted to determine the kinetics of sulfate utilization and the biodegradation of the individual FAMEs compounds in flaxseed biodiesel under sulfate reducing conditions.

4.2 Materials and Methods

4.2.1 Chemicals

Low-sulfur petroleum diesel (B0) was purchased from a local BP diesel station (Cincinnati, OH) with a mole fraction of 0.165 nC10-nC23 n-alkanes. Flaxseed biodiesel was transesterified from flaxseed vegetable oil in our laboratory via base-catalyzed transesterification. The feedstock was heated to a temperature of 60 to 70°C for 35 minutes. Sodium methanoxide 0.75% (w/w) was used as a catalyst, and was dissolved in twice the required stoichiometric amount of methanol. Glycerol was separated in a separatory funnel for one hour, after which the biodiesel was re-transesterified with 25% of the stoichiometric amounts of methanol to insure complete conversion. The mixture was then separated again and washed thoroughly with warm water (41°C) until no methanol was detected in the wash water. Magnesium silicate, 1% (w/w), was added in the biodiesel and was mixed at 35°C for 45 min to absorb unreacted glycerides from the biodiesel. Anhydrous sodium sulfate, 15% (w/w), was added to the biodiesel and was mixed at room temperature for two hours to absorb the residual water. The magnesium silicate and sodium sulfate were allowed to settle out overnight, and the biodiesel phase was filtered through glass fiber filter (1 µm pore size). The average molecular weight of flaxseed biodiesel was 286.9 g/mole with FAMEs representing
the following mole weight fraction of 0.0538 C16:0-ME, 0.0442 C18:0-ME, 0.234 C18:1-ME, 0.157 C18:2-ME and 0.514 C18:3-ME.

4.2.2 Culture Acclimation

Bacterial enrichment was inoculated in two 12-liter laboratory-scale continuous flow stirred chemostats previously operated under methanogenic condition and sulfate-reducing condition, respectively with soybean biodiesel as feed as described in chapters two and three. Detailed schematic of the reactor and operating protocols were described in chapter two. Organic feed to these reactors was switched to flaxseed biodiesel, which was delivered to the chemostats through Hamilton syringe pumps with a concentration of 2.1 g/L.d oil in the influent. The final concentration of the feed essential nutrients and vitamins minimal medium in the nutrient reservoirs were the same as described in chapter two and three. The pH in the chemostats was maintained at 7.0 ± 0.20. Headspace gas composition was analyzed once a week and certain water quality variables including pH, target analytes, volatile fatty acids (VFAs), sulfate concentration and effluent chemical oxygen demand (COD) were monitored routinely. After two turnovers of sludge retention time, the biomass was acclimated to flaxseed biodiesel and steady-state operation was attained (i.e. several consecutive measurements revealed consistently low effluent concentrations of the target compounds and COD, and methane gas production was stable and near expected values), the culture from the reactors was collected for the batch experiment.
4.2.3 Batch Experiments

The biodegradation of flaxseed biodiesel under methanogenic condition was monitored using the BMP test as chapter two. Sterile and surface-deactivated 60 ml serum bottles were filled with 20mL of the effluent from the reactor, 20 ml of deoxygenated nutrient and buffer solution and 20 ml headspace in an anaerobic chamber. After addition of the various solutions, serum bottles were sealed and spiked with 15 µl flaxseed biodiesel containing different petroleum diesel loads (0 - 45 µl). A separate set of bottles was injected with only petroleum diesel and no biodiesel. Killed control samples were prepared the same way except the effluent was autoclaved at 121°C for 60 minutes to kill the activity of the biomass. Blank control samples were also prepared the same way as the biotic samples but with no oil injection. The bottles were then placed in a rotary tumbler operated at a nominal speed of 25 rpm and sampling was performed at different time intervals. The volume and composition of gas produced in the headspace of the bottles were analyzed to calculate the volume of methane produced.

The biodegradation of flaxseed biodiesel under sulfate-reducing condition was monitored by SBR test as chapter three. Sterile and surface-deactivated 60 ml serum bottles were filled with 25 ml of the effluent from the reactor, 25 ml of deoxygenated nutrient and buffer solution and 10 ml headspace. After addition of the various solutions, serum bottles were sealed and spiked with 15 µl flaxseed biodiesel B100 and B50 in an anaerobic chamber. Killed control samples were prepared the same way except the effluent was autoclaved at 121 °C for 60 minutes to
kill the activity of the biomass. The bottles were then placed in a rotary tumbler operated at a nominal speed of 25 rpm. At a given sampling event, triplicate serum bottles of each treatment were randomly selected and sacrificed. One ml of sample was withdrawn using a 3 ml syringe and filtered through a 0.45 µm filter for sulfate analysis in an Ion Chromatograph (IC). Subsequently, the remaining contents were completely liquid-liquid extracted with dichloromethane. The extracts were filtered through anhydrous sodium sulfate to dewater them and stored at -20 °C until analysis on GC-MS.

4.2.4 Analytical Methods

Gas composition analysis was performed on a HP5890 Series II Gas Chromatograph (GC) (Hewlett Pachard, Wilmington, Delaware) equipped with a thermal conductivity detector (TCD) using an HP 10 ft molecular sieve BX-45/60 mesh HP 6ft HAYESEPQ 80/100 column (Supelco, Bellefonte, Pennsylvania). The sulfate concentration in the aqueous phase was analyzed on a Dionex LC20 Ion Exchange Chromatograph. The chemical analysis for FAMEs and alkanes in the effluent as well as the batch experiment followed the same procedures (Yassine M.H. et al, 2012). The extracts were aliquoted in 1mL GC vials, and spiked with 10 µl of an internal standard solution containing C11:0-ME, C13:0-ME, deuterated nC10 and deuterated nC20 in DCM, and were analyzed on an HP Agilent 5890 II Gas Chromatograph (GC) equipped with an HP-INNOWAX capillary column (30 m long, 0.25 mm i.d., 0.25 µm film thickness), and an HP Agilent 5971 Mass Spectrometer Detector (MSD). The flow rate of the Helium carrier gas was 1mL/min. Both the inlet and detector interface temperatures were held at 320 °C and the former was operated in splitless mode. The oven temperature
program was as follows: hold at 35 °C for 3 min, ramp at 5 °C/min to 250 °C and hold at 250 °C for 5 min.

4.3 Results and Discussion

4.3.1 Toxicity of Flaxseed Biodiesel

The preparation of the water-accommodated fraction (WAF) was according to the same procedure presented in the paper (Yassine et al, 2012). The acute microtox toxicity of the WAF of flaxseed biodiesel was investigated at different oil loads. The data in Figure 4.1 show a comparison of the toxicity of petrodiesel, soybean biodiesel and flaxseed biodiesel. Flaxseed biodiesel exhibited the lowest toxicity at all dilution levels. At higher oil loads (1:1 and 1:10), soybean biodiesel was more toxic than diesel. At the lowest oil loads (1:1000), it showed the same toxicity trend as in the literature (Hollebone, 2008). Our previous study on the soybean biodiesel toxicity indicated that the aquatic toxicity of biodiesel was not caused by the FAMEs, but their autoxidation byproducts because FAMEs compounds were found to be nontoxic (Yassine et al, 2012).

4.3.2 Methanogenic Biodegradation

To assess the effect of the amount of petroleum diesel on the rate of biodegradation of flaxseed biodiesel under methanogenic condition, the BMP test was performed using 15 µl of flaxseed biodiesel B100 per bottle supplemented with different volumes of petroleum diesel (0–45 µl). Biomass used for this test was acclimated to flaxseed biodiesel B100. Biodegradation was monitored through methane production. The cumulative methane
production curves obtained for 15 µl flaxseed biodiesel with different volumes of petroleum diesel loads are presented in Figure 4.2. The shape of the curves followed the typical biodegradation profile corresponding to first-order biodegradation kinetics. In order to quantify the biodegradation of flaxseed biodiesel in the biotic microcosms with the different petroleum diesel loads, the first-order biodegradation rate constants were estimated using equation (1):

\[ M_t = M_u (1 - e^{-kt}) \]  

(1)

Where,

\( M_t \) = cumulative methane produced (ml) at time \( t \) obtained from the BMP test data

\( M_u \) = ultimate methane production (ml)

\( k \) = first order biodegradation rate constant (d\(^{-1}\))

\( t \) = time (d)

The \( k \) and \( M_u \) values were estimated by fitting Equation 1 to the experimentally determined data of cumulative methane production using least-squares nonlinear regression (SigmaPlot 11, Systat Software. Inc., CA). The best-fit parameters are listed in Table 4.1, and Equation (1) was found to adequately describe the experimental data as shown in Figure 4.2. The first order rate constant for the biodegradation of biodiesel in the absence of any petroleum diesel was 0.092 ± 0.009 d\(^{-1}\). In response to the injection of varying volumes of petroleum diesel, the
first-order rate coefficient increased with the increasing amounts of diesel when smaller volumes of petroleum diesel were added (1 µl to 2.5 µl). The rate constant subsequently decreased with the increasing volumes of petroleum diesel when the volume of diesel ranged from 5 µl to 45 µl. With petroleum diesel loads up to 7.5 µl, the first-order rate coefficients were all greater than those when no petroleum diesel was present except for the petroleum diesel load 0.5 µl. The first order rate constant decreased to 0.075 ± 0.008 d⁻¹ when 15 µl of petroleum diesel was added to the mix. Hence, in the presence of 15 µl diesel, the biodiesel first order biodegradation rate constant decreased to 81% of the observed value when no petroleum biodiesel was present. However, when increasing the petroleum diesel loads to 30 µl and 45 µl, the biodegradation rate constant did not change very much. This was in agreement to what was observed for the biodegradation efficiency.

The observed methane production in response to an injection of 15 µl biodiesel was that which was observed from the experimental treatment minus that produced in the blank controls. The methane produced in response to injection of 15 µl biodiesel at the end of the experiment and the corresponding production efficiency is shown in Table 4.2 and 4.3. In all treatments (0 µl -45 µl), the Mu/exp values for 15 µl flaxseed biodiesel at the end of the experiment were lower than the theoretical expected methane production (14.03 ml) if 15 µl of biodiesel was completely converted to methane. But methane production corresponding to injection of only 15 µl B100 was 94% of the expected value. Some of the biodiesel was consumed to support growth of the bacteria, and some of the produced methane was dissolved
in aquatic phase. With the increase in diesel loads, the methane production decreased due to the toxicity of diesel. There was no significant difference (p=0.86) in the ultimate methane produced when lower diesel volume fractions were added. The methane production efficiency was greater than 84.9% when petroleum diesel volumes between 0 µl and 7.5 µl were added. Over the whole range, the methane production efficiency increased linearly with the added fraction of biodiesel as shown in Figure 4.3, and the linear rate of that increase was 0.22 ± 0.034. When 15 µl of petroleum diesel was added to the mix, the biodegradation efficiency decreased to 77.96% of the biodiesel theoretical methane. This amount is 83% lower than the amount of methane produced when no petroleum diesel was added. When 30 µl and 45 µl petroleum diesel was added to the mix, the biodegradation efficiency decreased to 78.93% and 75.22%, respectively. There was no significant difference (p=0.54) in biodegradation efficiency for petroleum diesel loads 15 µl to 45 µl.

No methane production was observed in any of the killed control samples. Small amounts of methane were produced in blank control samples (triangle symbols in Figure 4.2A). This methane production is due to the biodegradation of residual organic matter in the chemostat effluent. Similar behavior was observed in the diesel only injection sets (open triangle symbols in Figure 4.2). However, in all cases, methane production in the diesel only injection sets was lower than that in the blank control samples. Consequently, there was no methane production when petroleum diesel was present alone, suggesting that diesel was not biodegraded under methanogenic condition. This supports the hypothesis that methane produced in the flaxseed biodiesel and diesel blends was due only to flaxseed biodiesel
biodegradation.

The biodegradation rate for pure soybean biodiesel was higher than that for flaxseed biodiesel. However, when the linear rates of methane production efficiency are compared, this rate was $0.217 \pm 0.034$ for flaxseed biodiesel and $0.781 \pm 0.104$ for soybean biodiesel. This indicated that with the increase of petroleum diesel loads, diesel showed greater inhibition effect on soybean biodiesel than flaxseed biodiesel.

4.3.3 Sulfate-Reducing Biodegradation

To determine the rate of biodegradation of flaxseed biodiesel under sulfate-reducing condition, the SBR test was performed using 15 µl of flaxseed biodiesel B100 and B50 biodiesel/diesel blends. Biomass used for this test was acclimated to flaxseed biodiesel B100. The total FAMEs disappearance in B100 (circle symbols in Figure 4.4) and B50 (triangle symbols of Figure 4.4) was monitored along with the utilization of sulfate. The first-order rate constants for sulfate utilization were calculated by nonlinear regression and the first-order rate coefficients were $0.22 \pm 0.033$ d$^{-1}$ for B100 and $0.15 \pm 0.022$ d$^{-1}$ for B50. In the case of the biodegradation of individual FAMEs in flaxseed biodiesel, no lag phase was observed for all compounds both in B100 and B50. The shape of biodegradation curves followed the typical biodegradation profile with exponential decay as shown in Figures 4.5 and 4.6. The first-order biodegradation rate coefficients for the individual FAMEs were estimated using nonlinear regression and are presented in Table 4.3. In the case of B100, the different FAME compounds were found to have significantly different rate coefficients ($p=0.002$). For the
saturated FAMEs, the degradation rate of C16:0-ME (0.37 ± 0.021 d\(^{-1}\)) was slightly higher than that of C18:0-ME (0.37 ± 0.017 d\(^{-1}\)), indicating slower biodegradation for longer carbon chains. Among unsaturated FAMEs compounds, the first order rate coefficient was 0.31 ± 0.023 d\(^{-1}\) for C18:1-ME, 0.50 ± 0.027 d\(^{-1}\) for C18:2-ME, and 0.65 ± 0.053 d\(^{-1}\) for C18:3-ME, indicating faster biodegradation kinetics corresponding to the higher unsaturated levels of the carbon chain of the FAME. For FAMEs biodegradation in B50, there were also significant differences in the rate coefficients among different FAMEs compounds (p=0.004). In general, the biodegradation rate coefficients of the FAMEs in B50 was about half of those in B100, closely following the relative initial concentration of the FAME compounds in the fuel blends.

Similar to FAMEs biodegradation in B100, the biodegradation rate decreased with the increasing carbon chain length for saturated FAMEs, and increased with the increasing number of double bonds for unsaturated FAMEs, which, in fact, correlated very well with their solubility limit trend. This is identical to findings from the soybean biodiesel experiment in chapter three.

The biodegradation efficiency of the FAME compounds in B100 after 7 days of biodegradation was 94% for C16:0-ME, 93% for C18:0-ME, 92% for C18:1-ME, 96% for C18:2-ME and 98% for C18:3-ME. On day 9, C18:2-ME and C18:3-ME were non-detectable, and the rest of the compounds were below detection limits on day 12. On the other hand, the degradation efficiency of the FAMEs in B50 treatment at 7 days was 79% for C16:0-ME, 77% for C18:0-ME, 75% for C18:1-ME, 86% for C18:2-ME and 92% for C18:3-ME. C18:3-ME was below detection limits by day 19 and C18:2-ME was non-detected on day 24. At the
end of the experiment (day 30), there was a residual of 2% of C16:0-ME and C18:1-ME, and 4% of C18:0-ME. The lower extent of removal and the longer time needed for complete removal may be attributable to inhibition effects of petroleum diesel in B50 biodiesel/diesel blends. No biodegradation of petroleum diesel was observed in B50 as the petroleum diesel could not be degraded under sulfate-reducing condition in our study.

Comparing the methane production rate for 15ul flaxseed biodiesel (0.092 ± 0.009 d⁻¹) under methanogenic condition and the sulfate utilization rate (0.22 ± 0.033 d⁻¹) for 15 µl flaxseed biodiesel under sulfate-reducing condition, the sulfate-reducing culture exhibited higher rates for biodiesel degradation. The biodegradation rate for pure soybean biodiesel was higher than that for flaxseed biodiesel under methanogenic conditions. However, when the linear rates of methane production efficiency are compared, this rate was 0.22 ± 0.034 for flaxseed biodiesel and 0.78 ± 0.10 for soybean biodiesel. This indicated that with the increase in petrodiesel loads, petrodiesel showed greater inhibition effect on soybean biodiesel than flaxseed biodiesel. Comparing the biodegradation of flaxseed biodiesel and soybean biodiesel under sulfate-reducing condition, although the abundance of the FAMEs and their profile changed, the degradation order remained the same, which emphasizes the importance of the physical and chemical properties of the FAMEs compounds. However, the degradation rate was also affected by the hydrolysis of long chain fatty acid. The FAMEs in flaxseed biodiesel degraded much faster than FAMEs in soybean biodiesel for B100 treatment under sulfate-reducing conditions. Because flaxseed biodiesel was less toxic than soybean biodiesel, and as discussed in our previous study, soybean biodiesel had a higher abundance of C18:2-ME, and its
corresponding hydrolysis product linoleic acid exhibited greater inhibition effect on the biodegradation of biodiesel.

4.4 Conclusions

Flaxseed biodiesel was effectively degraded under methanogenic and sulfate-reducing conditions. Under methanogenic conditions, no inhibition was observed at petrodiesel loads 1 -7.5 µl. Inhibition effect began to prevail when the petrodiesel volume was 15 µl. The biodegradation of the individual FAMEs compounds under sulfate-reducing conditions decreased with the increase in the carbon chain length for saturated FAMEs and increased with the increased number of double bonds for unsaturated FAMEs. This degradation order trend was not related to the abundance of FAME compound in the biodiesel, but rather to the physiochemical properties of the individual FAME compounds. The long chain fatty acids showed different inhibition effects. Lalman et al. (2000, 2001) reported that oleic acid and linoleic acid concentrations above 30 mg/l inhibited the biodegradation of acetate, and linoleic acid above 100 mg/l significantly inhibited butyrate degradation (Lalman et al., 2002). The initial concentration of C18:1-ME and C18:2-ME biodiesel was 50.75 mg/l and 33.98 mg/l in B100 flaxseed SBR test. All the FAMEs compounds exhibited high biodegradation rates and no inhibition effect was observed in B50 and B100. The abundance of FAMEs in the biodiesel appears to affect their degradation rate due to the inhibitory effect of their hydrolysis products. Petrodiesel exhibited a greater inhibition effect on soybean biodiesel than flaxseed biodiesel with increases in the petrodiesel loads.
4.5 References


Table 4.1 Ultimate methane production of 15ul flaxseed biodiesel with different loads of petrodiesel and nonlinear regression parameters for methane production curves using culture from reactor B100

<table>
<thead>
<tr>
<th>Added B0(ul)</th>
<th>CH₄exp (ml)</th>
<th>CH₄ model (ml)</th>
<th>k(d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.755±1.217</td>
<td>15.348±0.516</td>
<td>0.092±0.009</td>
</tr>
<tr>
<td>0.5</td>
<td>14.849±1.314</td>
<td>15.510±0.579</td>
<td>0.090±0.012</td>
</tr>
<tr>
<td>1</td>
<td>13.606±0.210</td>
<td>13.808±0.592</td>
<td>0.099±0.013</td>
</tr>
<tr>
<td>2.5</td>
<td>13.831±0.611</td>
<td>13.860±0.341</td>
<td>0.115±0.010</td>
</tr>
<tr>
<td>5</td>
<td>13.576±0.680</td>
<td>13.469±0.311</td>
<td>0.104±0.008</td>
</tr>
<tr>
<td>7.5</td>
<td>13.463±0.322</td>
<td>13.337±0.291</td>
<td>0.101±0.007</td>
</tr>
<tr>
<td>15</td>
<td>12.488±0.161</td>
<td>12.916±0.299</td>
<td>0.075±0.005</td>
</tr>
<tr>
<td>30</td>
<td>12.625±0.043</td>
<td>12.887±0.384</td>
<td>0.070±0.005</td>
</tr>
<tr>
<td>45</td>
<td>12.103±0.371</td>
<td>12.829±0.269</td>
<td>0.069±0.004</td>
</tr>
</tbody>
</table>

CH₄ exp, experimental methane production ± standard deviation among three replicates; CH₄ model, model methane ultimate production ±standard error; k, first order rate constant ±standard error
Table 4.2 15ul flaxseed biodiesel B100 methane production and production efficiency

<table>
<thead>
<tr>
<th>Added B0 (ul)</th>
<th>CH₄ production (ml)</th>
<th>CH₄ production efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.206</td>
<td>94.13</td>
</tr>
<tr>
<td>1</td>
<td>13.299</td>
<td>94.79</td>
</tr>
<tr>
<td>1</td>
<td>12.057</td>
<td>85.94</td>
</tr>
<tr>
<td>3</td>
<td>12.281</td>
<td>87.54</td>
</tr>
<tr>
<td>5</td>
<td>12.027</td>
<td>85.72</td>
</tr>
<tr>
<td>7.5</td>
<td>11.914</td>
<td>84.92</td>
</tr>
<tr>
<td>15</td>
<td>10.938</td>
<td>77.96</td>
</tr>
<tr>
<td>30</td>
<td>11.075</td>
<td>78.93</td>
</tr>
<tr>
<td>45</td>
<td>10.553</td>
<td>75.21</td>
</tr>
</tbody>
</table>
Table 4.3 First-order degradation rate for FAMEs in flaxseed biodiesel (d\(^{-1}\))

<table>
<thead>
<tr>
<th></th>
<th>B100</th>
<th>B50</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0-ME</td>
<td>0.373±0.021</td>
<td>0.203±0.011</td>
</tr>
<tr>
<td>C18:0-ME</td>
<td>0.366±0.017</td>
<td>0.187±0.011</td>
</tr>
<tr>
<td>C18:1-ME</td>
<td>0.311±0.023</td>
<td>0.161±0.009</td>
</tr>
<tr>
<td>C18:2-ME</td>
<td>0.503±0.027</td>
<td>0.277±0.016</td>
</tr>
<tr>
<td>C18:3-ME</td>
<td>0.649±0.053</td>
<td>0.390±0.025</td>
</tr>
</tbody>
</table>
Figure 4.1 Comparison of 15-minutes median effective concentration (EC50 %) of WAFs of the petrodiesel, soybean biodiesel and flaxseed biodiesel at different dilutions
Figure 4.2 Cumulative CH₄ production curves for flaxseed biodegradation with different B0 loads in biomass B100. Points are triplicate experimental data while the solid line is the model fit equation.
Figure 4.3 Methane production efficiency in different volume fraction biodiesel
Figure 4.4 Sulfate utilization with the culture from reactor flaxseed B100
Figure 4.5 Biodegradation of flaxseed biodiesel FAMEs in B100 treatment. The open symbols are biotic samples. The blank symbols are KCs.
Figure 4.6 Biodegradation of flaxseed biodiesel FAMEs in B50 treatment. The open symbols are biotic samples. The blank symbols are KCs.
CHAPTER 5

SUMMARY, CONCLUSIONS, AND FUTURE WORK

The data presented in this thesis indicated that both soybean biodiesel and flaxseed biodiesel were readily biodegradable under methanogenic and sulfate-reducing conditions while petrodiesel, either alone or in blends with biodiesel, was not biodegraded. As for the effect of the presence of petrodiesel on the biodegradation of biodiesel under methanogenic condition, both the first order biodegradation rate constant and methane production efficiency decreased linearly with the increasing fraction of B0 for soybean biodiesel. However, the presence of moderate amounts of petrodiesel did not exhibit any inhibitory effects on the biodegradation rate of biodiesel until the contribution of petrodiesel approached 50% by volume for flaxseed biodiesel. The presence of petrodiesel has a greater effect on the biodegradation rate than the biodegradation efficiency for both biodiesels. And petrodiesel exhibited a greater inhibition effect on soybean biodiesel biodegradation than flaxseed biodiesel with increases in petrodiesel loads.

In terms of biodegradation of the individual FAME compounds under sulfate-reducing conditions, the degradation rate decreased with increases in the carbon chain length for saturated FAMEs and increased with increasing number of double bonds for unsaturated FAMEs for both biodiesels. This trend in the degradation order was not related to the abundance profiles of FAMEs in the biodiesel, but related to the physiochemical properties of the individual FAME compound. The biodegradation of FAMEs in biodiesel/petrodiesel blends is affected by (1) the solubility of the specific FAMEs compound, (2) the inhibition
effect from the hydrolysis byproduct, and (3) the inhibition effect from the petrodiesel. The research presented in this thesis is of significant importance in understanding the behavior of biodiesel/petrodiesel blends when spilled in anaerobic aquatic environments. The identified inhibition effects of long chain fatty acids are shown in the literature (Lalman et al., 2000; Mykhaylov et al., 2005; Palatsi et al., 2012; Sousa et al., 2013). It is recommended to investigate the different hydrolysis byproducts and biodegradation byproducts of the biodiesel and the assessment of their apparent toxicity in further work.