Mohamad H Yassine, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Environmental Engineering.

It is entitled:
Aerobic Biodegradation Kinetics, Aquatic Toxicity and Partitioning, and Microbial Community Structures of Petrodiesel/Biodiesel Blends

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Aerobic Biodegradation Kinetics, Aquatic Toxicity and Partitioning, and Microbial Community Structures of Petrodiesel/Biodiesel Blends

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ABSTRACT

For decades, biodiesel which is defined as the mono-alkyl esters of long chain fatty acids has been attracting great interest in the industrial as well as environmental communities. The use of biodiesel as an alternative fuel for petroleum diesel in compression engines presents a viable option in combating fossil fuel dependence and climate change simultaneously. Nevertheless, the impact and fate of biodiesel when accidentally released in the environment, whether blended with petrodiesel or as a stand-alone fuel, are poorly understood. This is partially due to the differences in the bulk and molecular physiochemical properties between petrodiesel and biodiesel, the inherent variability in those properties for both fuels, and the inherent autoxidative instability of biodiesel fuel. In addition, despite major attempts to investigate these interactions, fundamentally, the theoretical understanding of the microbial degradation of those materials in aqueous systems still poses a challenge. This is because of the lack of a sound phenomenological modeling approach that is capable of taking into account the hydrophobic nature of those materials.

To answer these questions, this PhD thesis presents original and fundamental research investigating: 1) the aerobic biodegradation kinetics of petrodiesel/soybean-biodiesel and petrodiesel/flaxseed-biodiesel blends in batch experiments, 2) the partitioning behavior and physical interactions of petrodiesel/biodiesel blends in aquatic systems, 3) the acute toxicity of petrodiesel/biodiesel blends to the aquatic microorganism Vibrio fischeri, and 4) the effect of petrodiesel/biodiesel blends on the community structures of acclimated microbial cultures. In order to better understand the fundamental effect of biodiesel on the microbial utilization of petrodiesel, 5) a mechanistic microbial kinetic model for the degradation of poorly soluble
materials was developed. The assumptions of fast oil dissolution kinetics (i.e. local equilibrium) at the oil/water interface, and that the substrate aqueous concentration remains close to the saturation solubility limit in the bulk aqueous phase, allowed for the derivation of an approximate closed-form solution for the system of equations describing the biodegradation of poorly soluble materials. The results of the biodegradation kinetic study showed that the rate of the primary disappearance of the FAMEs was controlled by abiotic processes, such as autoxidation and polymerization. This abiotic transformation of the FAMEs appeared not to appreciably affect the extent of ultimate mineralization of the fuel. On the other hand, the actual specific microbial utilization rate of the n-alkanes of petrodiesel was significantly enhanced in the presence of biodiesel. Aqueous equilibration experiments of the petrodiesel/biodiesel blends revealed that this enhancement in the n-alkanes’ microbial utilization was chiefly achieved by a co-solubilizing action of the fatty acid methyl esters (FAMEs) to the n-alkanes. While there was no significant difference between the enhancements of the specific utilization rate of the n-alkanes caused by soybean biodiesel or flaxseed biodiesel, in the presence of flaxseed biodiesel, the rate of n-alkanes biodegradation proceeded at a faster total initial rate, possibly due to higher microbial growth yield of the degrading organisms on that feedstock.

The investigation of the partitioning behavior of petrodiesel/soybean-biodiesel blends in water also confirmed the findings that FAMEs are capable of increasing the dissolved concentrations of the n-alkanes. It was also found that the FAMEs are also capable of enhancing the colloidal stability and accommodation of n-alkanes dispersions in water, possibly by forming a surfactant-cosurfactant-like pair of the FAMEs and their autoxidation byproducts. The acute toxicity of the water accommodated fractions (WAFs) of petrodiesel and biodiesel blends was found to be controlled by the toxicity of the aromatic compounds (mono and polycyclic) present.
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The analysis of the microbial community structures of petrodiesel/biodiesel blends
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community profiles of the different cultures were very complex and no obvious trend for the
shifts was deduced.
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Finally and most importantly, I want and share this moment with my precious parents for their continual and unconditional love, support, and sacrifice. I love you, God bless.
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CHAPTER 1: INTRODUCTION
1.1 Background

Biodiesel is defined as the mono-alkyl esters of long chain fatty acids derived from transesterification of vegetable oils and animal fats for use in compression-ignition (diesel) engines. This specification is for pure (100%) biodiesel prior to blending with diesel fuel. Petroleum-derived diesel is composed of about 75 -85% saturated hydrocarbons (primarily normal, branched, and cyclo alkanes), and 15-25% aromatic hydrocarbons (mainly alkylbenzenes). The average chemical formula for common diesel fuel is C_{12}H_{26}, ranging from approximately C_{10}H_{22} to C_{15}H_{32} [1].

A major reason for the renewed interest in fuels made from plant and animal lipids is that feedstocks for fatty-acid methyl esters (FAMEs) of biodiesel are renewable, or can be reclaimed from wastes (e.g., used animal fats, recycled fry oil). In contrast, fossil fuels are non-renewable resources and have increasingly higher costs financially and environmentally. An additional incentive for the use of biodiesel is the potential positive impact on global climate change. Various greenhouse emission models estimate lower net crop-to-combustion carbon and sulfur emissions for biodiesel compared to petroleum diesel [2, 3]. However, several studies confirm a net increase of NOx emissions, an indirect greenhouse gas, from biodiesel combustion [4]. A 2002 EPA report showed that a 20% biodiesel blend boosts NOx emissions by 2%, but cuts particulate matter (PM) by about 10%, hydrocarbons (HC) by about 21%, and carbon monoxide (CO) drops by about 11% [5]. But because of its potential as a renewable energy source and as a method to reduce net greenhouse gas emissions, biodiesel has attracted great interest as an alternative transportation fuel.

While there are great benefits 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. There is a dearth of information on the behavior and partitioning of biodiesel and its blends in the environment, as well as
their toxicological effects on aquatic organisms. The current understanding of the fate and effects of biodiesel and its various blends is inadequate to evaluate environmental risks of its use. Furthermore, unlike petroleum diesel, biodiesel fuels are made from many different sources such as soybean oil, rapeseed oil, flaxseed oil, reclaimed restaurant grease, fish oil, and rendered animal fats…etc, each having different chemical compositions. This wide variability of biodiesel formulations may result in very different toxicological profiles and environmental behaviors depending on the feedstock.

The different molecular structures of petrodiesel and biodiesel attribute very different physiochemical properties for both fuels. Biodiesel has a much higher viscosity, lubricity, and conductivity than petrodiesel [6]. Both petrodiesel and biodiesel have limited solubility in water [7-9]. Though, biodiesel is slightly more soluble than petrodiesel because of the higher polarity of fatty acid alkyl esters due to the presence of the carboxylic group [10, 11]. This property enables the compound to form hydrogen bonds with water at the unsaturated locations. He et al. found that biodiesel absorbs 15-25 times more moisture than petroleum diesel [12], which indicates a higher hydrophilicity for biodiesel.

Biodiesel’s physiochemical properties also depend on the feedstock type, and the profile and structures of its fatty acid alkyl esters. Gerard Knothe showed that the cetane number, heat of combustion, melting point, and viscosity of biodiesel increase with increasing fatty acid carbon chain length, and decrease with increasing unsaturation [13]. He also noted that cold flow properties of biodiesel are markedly improved with the increase of the carbon chain length of the alcohol moiety of the ester.

To add another level of complexity, biodiesel has been found to have appreciable variability in its aquatic solubility even within the same feedstock type. Hollebone et al. measured the water
accommodated fraction (WAF) of different batches and feedstocks of biodiesel and reported that for 4 different batches of soybean-based biodiesel, the WAF concentrations varied from 13 – 104 mg WAF/mL. While for 2 different animal-waste fry oil biodiesels, WAF concentrations were 29 and 60 mg WAF/mL for the same oil load applied [14].

Biodiesel is also inherently prone to hydrolysis in the presence of moisture, as well as to autoxidation due to the presence of double bonds. The autoxidation reaction is catalyzed by a wide range of catalysts such as air, heat, light, trace metals, and peroxides [11]. The rate of biodiesel’s autoxidation increases with the increase of the degree of unsaturation. The relative rates of oxidation for the unsaturated FAMEs given in the literature are 1 for methyl oleate, 41 for methyl linoleate, and 98 for methyl linolenate [15].

1.2 Research Objectives

The objectives of this research are summarized as follows:

1. To determine the aerobic biodegradation kinetics of six soybean-biodiesel/petrodiesel blends (B0, B20, B40, B60, B80, and B100) and two flaxseed-biodiesel/petrodiesel blends (F50 and F100), where B0 is unblended petrodiesel and B100 and F100 are unblended soybean and flaxseed biodiesels, respectively, by acclimated cultures in batch experiments.

2. To investigate the partitioning behavior of petrodiesel/biodiesel blends in aquatic systems.

3. To evaluate the acute toxicity of the water accommodated fractions (WAFs) of the 8 aforementioned blends in 10-fold dilution experiments.
4. To analyze the community structures of the microbial cultures acclimated to the different petrodiesel/biodiesel blends by a Polymerase Chain Reaction–denaturing high performance liquid chromatography (PCR – DHPLC) approach.

1.3 Structure of the Dissertation

This dissertation is organized into nine chapters. Chapter 1 presents a brief introductory review of the current status and understanding of petrodiesel/biodiesel blends, and defines the specific research objectives undertaken in this document. Chapter 2 presents an original microbial kinetic model for the degradation of poorly soluble materials. The presented model was proposed as an attempt to explain the observed kinetic data of petrodiesel/biodiesel biodegradation. In this chapter, a kinetic model is derived, and applied to the data of petrodiesel (B0) and soybean biodiesel (B100) only; and the assumptions made in deriving the model and the parameters obtained from the model are discussed in details. Chapter 3 contains original work on the aerobic biodegradation kinetics and mineralization of soybean – biodiesel/petrodiesel blends (B0, B20, B40, B60, B80, and B100) by acclimated cultures. In this chapter, the microbial kinetic model derived in Chapter 2 was used to analyze the biodegradation kinetics of the soybean biodiesel blends. The mechanisms by which biodiesel enhances the microbial utilization of petrodiesel are completely elucidated in this chapter. Chapter 4 is a continuation for Chapter 3 and contains the application of the kinetic model derived in Chapter 2 to the biodegradation of flaxseed – biodiesel/petrodiesel blends (F50 and F100) by acclimated cultures. Chapter 5 contains an investigation of the partitioning behavior of biodiesel/petrodiesel blends in aquatic systems. In this chapter, a clear distinction is made between dissolved and colloidal suspensions of the fuels in water, and the mechanisms by which biodiesel enhances both (dissolved and colloidal phases) are investigated. Chapter 6 is an investigation of the acute toxicity of the water accommodation fractions (WAFs) of the six soybean – biodiesel/petrodiesel blends by Microtox bioassay. Chapter 7 is a
continuation for Chapter 6 and contains the results of the acute toxicity of the WAFs of flaxseed –
biodiesel/petrodiesel blends by Microtox. Chapter 8 is a study of the microbial community structures of
the cultures acclimated to different petrodiesel/biodiesel blends. In this chapter, the 16S rRNA gene
libraries of the acclimated cultures were analyzed in light of the findings of the previous chapters
regarding the partitioning behavior of those blends during an acclimation period. Comparative
mineralization efficiencies of those cultures were investigated in a 5 X 5 mixed factorial design
experiment in which different combinations of acclimated cultures and fuel blends were experimented.
Chapter 9 summarizes the significance of the findings presented in this thesis, how those findings
advance our understanding of the natural and engineered bio-mediated processes, and identify key
research areas and interests.

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CHAPTER 2: MICROBIAL KINETIC MODEL FOR THE DEGRADATION OF POORLY SOLUBLE MATERIALS
Microbial kinetic model for the degradation of poorly soluble materials

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**ABSTRACT.** A mechanistic model is presented that describes the aerobic biodegradation kinetics of soybean biodiesel and petroleum diesel in batch experiments. The assumptions of fast substrate dissolution kinetics (i.e. local equilibrium) at the oil/water interface, and that the substrate aqueous concentration remains close to the saturation solubility limit in the bulk aqueous phase, allowed for the derivation of an approximate closed-form solution for the system of equations describing the biodegradation of poorly soluble materials. No prior knowledge of the aqueous substrate concentration, $S_w$, and the Monod half-saturation constant, $K_s$, is required, as the term $S_w/(K_s + S_w)$ in the Monod equation remained constant during this phase. The n-alkanes C$_{10}$ – C$_{24}$ of petroleum diesel were all utilized at a relatively constant specific utilization rate of 0.008 – 0.024 mg-alkane/mg-biomass-hr, while the fatty acid methyl esters (FAMEs) of biodiesel were utilized at specific rates that significantly increased with increasing carbon chain length and decreased with increasing number of double bonds. The results were found to be in agreement with reported genetic and metabolic evidence. The genetic evidence pertains to the binding affinity of those molecules to the transmembrane proteins encoded by the *fadL* and *fadD* genes responsible for the transport of large hydrophobic molecules across the cell membrane against a concentration gradient into the cytoplasm. The metabolic evidence involves the fact that unsaturated fatty acids require one to two additional energetic steps per double bond (depending on its location) in the β-oxidation pathway. The estimated microbial specific utilization rate, $kC$, of such materials is regarded to be a better measure of the degradation rate when compared to the maximum specific utilization rate, $k$, which might be orders of magnitude higher than $kC$ and might never be observed in reality. We suggest this model can be applied without major modification to a wide range of poorly soluble materials, ranging from lipids and petroleum products to poorly soluble gases such as methane. The use of this model would provide essential insights into the biodegradation process of those materials and can be used to explain a wide range of degradation behaviors.
2.1 Introduction

Poorly soluble materials such as petroleum products have always presented a serious threat to natural habitats. Petroleum hydrocarbons are considered one of the major surface and subsurface water contaminants [1, 2]. Although many studies investigating the microbial utilization of such materials have been reported in the literature, the majority of these studies usually report their findings using first order decay rates with respect to the total substrate. Theoretical examinations of such mechanisms are scarce and rather outdated [3-9].

Owing to their poor solubility in water (10^{-4} to 10^{-10} g/L for C10 to C24 n-alkanes) [10, 11], many of the kinetic models reported in the literature were built on the assumption that the biological assimilation of poorly soluble materials occurs at the oil/water interface by direct contact between microorganisms and oil droplets [3, 5, 12]. Johnson [12] as well as Aiba et al. [5] independently concluded that microorganisms take up liquid hydrocarbons by direct contact with the oil surface and that dissolved hydrocarbons made negligible contributions to overall growth rates, mainly based on empirical calculations of liquid solubilities and diffusivities of longer n-alkanes. Erickson and Humphrey [3] developed multiple kinetic models that describe the growth of microorganisms on the surface of liquid hydrocarbons and treated the contribution of dissolved substrate to the microbial growth as a secondary mechanism. However, they did not provide any experimental verification for their modeling approach.

In all of the surface attachment models reported in the literature, the microbial growth limiting factor is assumed to be the available interfacial surface area of the oil droplets [3, 6]. Although some microorganisms have been documented to have the ability to attach to the oil surface [13, 14], a larger number of cells often remain unattached to the oil droplets, even when the available surface area of the oil is relatively large compared to the population of cells [4, 15]. Also, mechanisms of substrate
transport from the bulk organic phase directly into the biomass without passing through the aqueous phase are not completely understood [4]. In order to test the surface attachment hypothesis, Yoshida et al. [9] compared the growth rates of *C. tropicalis* on liquid and vapor n-hexadecane in two different fermentors. The specific growth rate of the microorganisms on the vapor hexadecane was found to be the same as that on liquid hexadecane. They concluded that microbial hydrocarbon uptake by direct contact with the liquid hydrocarbon is negligible. Similarly, Blanch and Einsele [7] investigated the growth of *C. tropicalis* on n-hexadecane and found that the growth rate in the exponential growth phase was independent of mixing speed. They concluded that the surface attachment mechanism was not responsible for growth.

In contrast to the surface attachment modeling approach, the biodegradation of poorly soluble materials has also been modeled to occur inside the aqueous phase by coupling the Monod equation [16] with equilibrium substrate partitioning [17] or linear driving force dissolution [18] models, in which the substrate has to dissolve into the aqueous phase prior to microbial utilization. These models often yield complex governing equations that are either solved numerically [19], or by finite [17] or infinite [20] series approximations, or further simplified by imposing constitutive assumptions such as steady state biomass growth or by assuming that the substrate concentration is significantly small compared to the Monod half-saturation constant (i.e. $S_w << K_s$) [18].

The choice of an appropriate degradation kinetics model seems to be influenced by the type of degrading organism as much as the type of material being degraded. However, when the degrading organisms are unknown, which is the case in most environmental applications, we have assumed that microbial growth occurs on the organic matter that is dissolved in the aqueous phase. In this paper, we have developed a simple mechanistic model that adequately explains 90–97% of the aerobic biodegradation kinetics of the fatty acid methyl esters (FAMEs) of soybean biodiesel, and 80–94% of
the biodegradation kinetics of the n-alkanes of petroleum diesel in batch experiments by acclimated cultures. The model was developed based on the assumption that the substrate has to dissolve into the aqueous phase prior to the biological utilization reaction.

2.2 Materials and Methods

**Chemicals.** Low-sulfur petroleum diesel (B0) was purchased from a BP diesel station (Cincinnati, Ohio) with an n-alkanes mole fraction of 0.165. Unblended soybean-methyl ester biodiesel (B100) was purchased from Peter Cramer North America (Cincinnati, Ohio) with FAMEs mole fractions of 0.145 C16:0, 0.055 C18:0, 0.206 C18:1, 0.518 C18:2, and 0.0759 C18:3. Palmitic acid methyl ester (99%) (C16:0-ME), palmitoleic acid methyl ester (99%) (C16:1-ME), stearic acid methyl ester (99%) (C18:0-ME), oleic acid methyl ester (99%) (C18:1-ME), linoleic acid methyl ester (99%) (C18:2-ME), linolenic acid methyl ester (99%) (C18:3-ME), and normal alkanes standard mixture (nC10-nC30), were all purchased from Sigma Aldrich (USA). All other chemicals, if not noted, were purchased from Sigma Aldrich or Fisher Scientific (USA).

**Culture Acclimation.** Bacterial enrichment obtained from the aeration tank of the Mill Creek Wastewater Treatment Plant (Cincinnati, Ohio) was used as starting inocula in two laboratory-scale 6-liter porous pot (PP) chemostats, operated as completely mixed systems with hydraulic retention time of 1-day. Organic feeds of B0 and B100 were delivered to the bioreactors through Hamilton syringe pumps and were increased gradually throughout an acclimation period of six months to final concentrations of 750 mg/L-d oil. Adequate aeration in the bioreactors was maintained throughout the acclimation period by means of continuous air diffusers, operated at a nominal volumetric flow rate of 2–3 L/min. The bioreactors received a separate combined feed of essential nutrients and vitamins minimal medium buffered at pH 7.5 ± 0.1 of the following final concentration: 150 mg/L MgSO$_4$·7H$_2$O, 156 mg/L KNO$_3$, 48.7 mg/L CaCl$_2$·2H$_2$O, 37.3 mg/L FeCl$_2$·4H$_2$O, 1393 mg/L K$_2$HPO$_4$, 1393 mg/L K$_2$HPO$_4$, 1393 mg/L K$_2$HPO$_4$, 1393 mg/L K$_2$HPO$_4$, 1393 mg/L K$_2$HPO$_4$, 1393 mg/L K$_2$HPO$_4$, 1393 mg/L K$_2$HPO$_4$, 1393 mg/L K$_2$HPO$_4$, 1393 mg/L K$_2$HPO$_4$, 1393 mg/L K$_2$HPO$_4$,
544 mg/L KH₂PO₄, 0.08 mg/L CuSO₄·H₂O, 0.15 mg/L Na₂MoO₄·2H₂O, 0.13 mg/L MnSO₄·H₂O, 0.23 mg/L ZnCl₂, 0.42 mg/L CoCl₂·6H₂O, 0.015 mg/L 4-aminobenzoic acid (99%), 0.00585 mg/L biotin, 0.0003 mg/L cyanocabalamin, 0.00585 mg/L folic acid dihydrate (99%), 0.015 mg/L nicotinic acid (98%), 0.015 mg/L pantothenic acid Ca-salt hydrate (98%), 0.03 mg/L pyridoxine hydrochloride (98%), 0.015 mg/L riboflavin (98%), 0.015 mg/L thiamine hydrochloride (99%), 0.015 mg/L thiotic acid (98%). Certain water quality variables, including target analytes of effluent FAMEs and alkanes, total and volatile suspended solids, chemical oxygen demand (COD), pH, and dissolved oxygen (DO), were monitored routinely. When the reactors achieved steady state performance, a portion of each reactor’s culture was collected, washed three times with sterile saline solution, and frozen in 10% w/w glycerol at -80 °C for consistent use in the batch experiments.

**Batch Experiments.** Batch experiments were carried out in sterile and surface-deactivated 160 mL glass serum bottles, in which 50 mL of filter-sterilized nutrient and vitamin medium was added. Respective biomass aliquots of B0 or B100, thawed and washed three times with sterile saline solution, were added to the bottles at an observed solids concentration of 100 ± 15 mg VSS /L. Bottles were sealed with PTFE septa and spiked with 10 µl of either B0 or B100, corresponding to an initial organic substrate concentration of 175 ± 3 mg/L oil. The amount of oxygen available in the headspace was sufficient for the complete oxidation of 1.5X the amount of oil added on the basis of chemical oxygen demand. The bottles were then placed in a rotary tumbler operated at a nominal speed of 20 – 25 rpm. Optimum sampling frequency was determined through range finding experiments that were every 4 hr for the first 24 hr, every 6 hr for the second 24 hr, every 8 hr for the next 24 hr, every 12 hr for the next 24 hr, and every 24 hr thereafter for 168 hr (7 days). Abiotic controls inactivated with sodium azide (0.9% w/v) and biomass controls were also included to quantify abiotic substrate losses and biomass endogenous decay rates, respectively. All experimental work was carried out at 22 ± 2 °C.
**Chemical Analysis.** At every sampling event, triplicate bottles of each treatment were spiked with 50 µl of a standard surrogate solution of C16:1-ME and deuterated nC16 in acetone, completely liquid-liquid extracted twice with 1:1 dichloromethane (DCM), and filtered through anhydrous sodium sulfate and stored at -20 °C until the time of analysis. Extracts were aliquoted in 1 mL volumes in 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 1 mL/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. Detection limits were in the range of 5–25 µg/L for all the compounds.

**2.3 Model Development**

The development of this model was initiated to explain the kinetic data of the biodegradation of n-alkanes and FAMEs in B0 and B100, respectively. The proposed growth kinetics model rests on the following assumptions: 1) good mixing conditions exist in the serum bottles so that both the oil phase and the biomass are uniformly dispersed in the aqueous phase. 2) Substrate utilization takes place in the bulk aqueous phase according to Monod kinetics [16], and all other growth mechanisms are negligible. 3) Oxygen and all essential nutrients and vitamins are in excess and only the organic substrate is rate limiting. 4) The difference in the utilization rate of the different oil constituents is not too great to alter the composition of the oil phase over the course of degradation. Assumption 4 is considered a reasonable one to adopt in the biodegradation of homologous series of long-chain hydrocarbons such as n-alkanes and FAMEs. 5) At the oil/water interface, oil dissolution is assumed to
occur fast enough so that the substrate aqueous phase concentration at the interface can be assumed to remain at the saturation solubility limit. In other words, local partitioning equilibrium conditions exist between the interface aqueous concentration and the oil phase. Assumption 5 has been widely adopted in non-aqueous phase liquids (NAPLs) dissolution studies [18, 20, 21] and was shown to be a good assumption by Seagren et al. [22] and Chu et al. [19]. 6) Diffusion of substrate within the oil phase is not limiting the overall interphase transport, and 7) a mass transfer resistance boundary layer of thickness (l) exists in the aqueous phase. In other words, the interphase substrate transport from the bulk organic phase to the bulk aqueous phase follows the Two-Film theory developed by Lewis and Whiteman [23], in which the substrate has to diffuse from the oil phase to the oil/water interface, and diffuse across an aqueous resistance layer into the bulk aqueous phase. 8) Because of their poor aqueous solubility, once the oil constituents are in the aqueous phase, they become at infinite dilution. And finally, 9) the aqueous solubility of the substrate is assumed to be very low compared to the total non-dissolved substrate in the oil phase. In other words,

\[ S_w << S_{oil} \text{ , and } \]
\[ S_{tot} = S_w + S_{oil} \]  

where \( S_w \) = aqueous phase concentration of the substrate (mg/L), 
\( S_{oil} \) = mass of non-dissolved substrate in the oil phase divided by the total volume of the suspension (mg/L), and 
\( S_{tot} \) = total mass of the substrate in both the dissolved and non-dissolved phases (mg/L).

In our batch experiments, only \( S_{tot} \) for each of the oil constituents was measured over the course of the biodegradation experiment, and it is the only measurement that is used to calibrate the model. The following mathematical treatment is assumed to be applicable to each of the oil constituents.
separately, as the assumption of infinite dilution and assumption 4 imply no solute-solute interaction. Therefore, the mathematical derivation will be similar to that of a single–compound oil phase.

The overall rate of substrate transfer across the concentration boundary layer is given by equation 3.

$$\frac{dM_{oil}}{dt} = -\frac{D_w a}{l}(S_{sat} - S_w)$$  \hspace{1cm} (3)

where $M_{oil}$ = mass of the substrate in the oil phase (mg),

$t$ = time (hr),

$D_w$ = diffusivity of the substrate in water (m²/s),

$a$ = total area of the oil/water interface (m²),

$l$ = average thickness of the concentration boundary layer (m),

$S_{sat}$ = saturation aqueous concentration of the substrate at the oil/water interface (mg/L), and

$S_w$ = substrate concentration in the bulk aqueous phase (mg/L).

Microbial utilization of the substrate occurs in the aqueous phase and follows Monod kinetics. Since both oils are multi-substrate systems, we will assume that every compound in the aqueous phase is degraded by a specific portion of the total biomass. This is done in order to simplify the mathematical derivation and data regression as will be shown later. A general equation for the growth of total biomass is presented in the Results and Analysis section.

The rate of change in the bulk aqueous-phase concentration due to influx of substrate from the oil phase, and microbial utilization is given by equation 4,

$$\frac{dS_w}{dt} = -\frac{D_w a}{V_w l} (S_{sat} - S_w) - k \frac{S_w}{K_s + S_w} X$$  \hspace{1cm} (4)

where $k$ = maximum specific substrate utilization rate (mg-substrate/mg-biomass-hr),

$K_s$ = substrate half-saturation constant (mg/L),
\( X \) = concentration of biomass fraction (mg/L) that is degrading a specific compound described by equation 4, and

\( V_w \) = volume of the aqueous phase (m³).

Lipids in general, including FAMEs, are inherently prone to a wide range of abiotic transformation pathways, including hydrolysis, autoxidation, rancidity, and polymerization, in the presence of a wide range of catalysts [24, 25]. If the abiotic disappearance of the FAMEs is assumed to occur in the aqueous phase, following first order kinetics, then equation 4 becomes,

\[
\frac{dS_{w}}{dt} = \frac{D_w a (S_{sat} - S_w)}{V_w} - k \frac{S_w}{K_s + S_w} X - k_{ab} S_w
\]

(5)

where \( k_{ab} \) = the FAMEs’ first order abiotic disappearance rate coefficient (hr⁻¹) calculated from the abiotic controls. The coefficient \( k_{ab} \) for the n-alkanes is set to zero since abiotic controls presented in the Results and Analysis section indicate no transformation of these compounds. The growth rate of a specific portion of biomass actively degrading a specific substrate inside the aqueous phase is given by,

\[
\frac{dX}{dt} = Y k \frac{S_w}{K_s + S_w} X - k_d X
\]

(6)

where \( Y \) = biomass yield coefficient on that specific substrate (mg-biomass produced/mg-substrate consumed), and

\( k_d \) = biomass endogenous decay rate (hr⁻¹).

Seagren et al. [18] and others [26], have derived an equation similar to equation 4. To reduce the mathematical complexity of the system of governing equations, Seagren et al. [18] assumed that biomass growth is at steady state \((dX/dt = 0)\), and that \( S_w \ll K_s \) so that they could obtain a closed solution for the biodegradation of NAPLs in the aqueous phase. Seagren et al. [18, 27] as well Chu et al. [19, 20] analyzed NAPLs dissolution kinetics in the presence and absence of biodegradation, and found that the process of NAPLs dissolution was, in fact, enhanced by biodegradation. In other words,
they found that biodegradation increased the driving force of dissolution. Other researchers [28] have also confirmed the possibility of enhancement of dissolution kinetics by biodegradation.

In the absence of biodegradation, and with good mixing conditions, substrate equilibrium saturation concentration in the bulk aqueous phase is achieved in a relatively short time and no net diffusion of substrate from the NAPL phase to the aqueous phase takes place once saturation is attained. When biodegradation takes place, and when the oil phase is still significant compared to the dissolved phase, biodegradation reduces the aqueous concentration of the substrate to below the saturation limit, and, hence, drives the dissolution of oil. Since biodegradation is a slow process when compared to dissolution, it is safe to assume that the aqueous phase concentration remains close to saturation, in other words,

$$\frac{dS_w}{dt} \approx 0, \text{ and}$$

$$S_w \approx S_{sat}$$

Note that $S_w$ is approximately equal to, but remains less than $S_{sat}$ so that the dissolution driving force in equation 4 does not go to zero. With the assumption that $S_w$ is constant and remains close to saturation, equations 3 – 6 can be further simplified. We define the new constant term $C$ as,

$$C = \frac{S_w}{K_s + S_w} \approx \frac{S_{sat}}{K_s + S_{sat}}$$

Substituting the term $C$ in equation 6, one obtains,

$$\frac{dX}{dt} = (Y_kC - k_d)X$$

Equation 10 is a traditional first order growth equation with the constant growth rate coefficient $(Y_kC - k_d)$. With the initial condition of $X = X_0$ at $t = 0$; the solution of equation 10 is given by,

$$X = X_0 e^{(Y_kC - k_d)t}$$
Equations 7–9 & 11 are substituted in equation 5 as follows,

\[ 0 \approx \frac{D_w a}{V_w l} (S_{sat} - S_w) - kCX_0 e^{(YkC-k_d)t} - k_{ab} S_w \]  

(12)

Rearranging equation 12 yields

\[ \frac{D_w a}{V_w l} (S_{sat} - S_w) \approx kCX_0 e^{(YkC-k_d)t} + k_{ab} S_w \]  

(13)

Substituting equation 13 in equation 3, the rate of substrate dissolution from the oil phase is now given by

\[ \frac{1}{V_w} \frac{dM_{oil}}{dt} \approx -kCX_0 e^{(YkC-k_d)t} - k_{ab} S_w \]  

(14)

With \((M_{oil} / V_w = S_{oil})\); equation 14 becomes

\[ \frac{dS_{oil}}{dt} \approx -kCX_0 e^{(YkC-k_d)t} - k_{ab} S_w \]  

(15)

Since \(S_{oil} \gg S_w\),

\[ S_{tot} \approx S_{oil} \]  

(16)

From equations 15 and 16, the governing equation of the biodegradation of total oil is

\[ \frac{dS_{tot}}{dt} \approx -kCX_0 e^{(YkC-k_d)t} - k_{ab} S_w \]  

(17)

Equation 17 can be analytically integrated with the initial condition of \(S_{tot} = S_{tot0} \) at \(t = 0\) to yield the final solution of,

\[ S_{tot} = S_{tot0} - \frac{kCX_0}{(YkC-k_d)} \left( e^{(YkC-k_d)t} - 1 \right) - k_{ab} S_w t \]  

(18)
2.4 Results and Analysis

A nonlinear least squares minimization procedure was adopted to fit the experimental biodegradation data ($S_{tot}$) of each n-alkane and FAME in equation 18. The abiotic disappearance term ($k_{ab} S_w$) in equation 18 for the n-alkanes and FAMEs was calculated from the abiotic controls. No abiotic disappearance of the n-alkanes was observed (i.e. $k_{ab} = 0$); while for the FAMEs, the term ($k_{ab} S_w$) ranged from 0.055 hr$^{-1}$ for C18:0-ME to 0.641 hr$^{-1}$ for C18:2-ME, strongly correlating with the aqueous solubilities of the FAMEs approximated by Raoult’s Law using pure solubilities reported in the literature [29]. The abiotic disappearance rate of the FAMEs was significant, possibly because the presence of metals in the growth medium catalyzes the abiotic transformation of the FAMEs.

Two regression parameters, for each compound, were obtained in fitting the experimental data in equation 18, namely ($kCX_0$) and ($YkC – k_d$). For every compound in B0 and B100, the term ($YkC – k_d$) represents the actual net specific growth rate of the biomass on that compound, while ($kCX_0$) represents the total (as opposed to specific) initial substrate utilization rate of the same compound. No lag phase was observed for all the FAMEs, while a lag phase of 16 hr for nC11–nC13 and 24 hr for nC14–nC24 was observed for the n-alkanes. For the compounds that had a lag phase, that time was excluded from the fit. Figures 2.1 and 2.2 contain the experimental data and model fits predicted using equation 18 for B0 and B100 targeted compounds, respectively. No biomass growth data was fed to the model, and only the observed $S_{tot}$ over time for each compound was used to calibrate the model. Consequently, the initial biomass concentration ($X_0$) degrading each specific compound appeared in the regression coefficient ($kCX_0$). The endogenous decay rates of B0 and B100 cultures, calculated from the biomass controls, were found to be 0.023 and 0.026 day$^{-1}$, respectively. If $Y$ is taken to be the maximum stoichiometric yield coefficient ($Y_{stoic}$) based on bacterial cell composition of $C_{60}H_{87}O_{23}N_{12}P$
and calculated using the balanced anabolic growth reaction shown in equation 19 for an n-decane example substrate,

\[
12\ C_{10}H_{22} + 24\text{NO}_3^- + 2\text{PO}_4^{3-} + 13\ O_2 + 30\ H^+ \rightarrow 2\ C_{60}H_{87}O_{23}N_{12}\ P + 60\ H_2O ,
\]

(19)

then the specific substrate utilization rate \((kC)\) and the initial biomass concentration \((X_0)\) specific for each compound in an oil, according to equation 18, can be calculated by plugging \(Y_{stoic}\) and \(k_d\) in the regression parameters \((YkC – k_d)\) and \((kCX_0)\).

For each compound, the parameter \(kC\) is interpreted as the actual specific microbial utilization rate of a poorly soluble substrate whose dissolution flux is not rate limiting. Similarly, for every compound, the parameter \(X_0\) is interpreted as the initial concentration of actively degrading biomass required to reproduce the observed degradation kinetics of a specific poorly soluble substrate, according to equation 18, assuming a single substrate system. Table S1 in the Supporting Information (SI) section is a compilation of all the calculated parameters of all the n-alkanes and FAMEs. The measured endogenous decay rates of B0 and B100 cultures were found to be only 3–5% of the actual net specific growth rates \((YkC – k_d)\) of the individual compounds shown in Table S1. The use of \(Y_{stoic}\) in calculating \(kC\) and \(X_0\) should not appreciably affect the values of those two parameters. In fact, the use of \(Y_{stoic}\) results in slightly conservative (i.e. slower) estimates for the \(kC\) values of the compounds. This is because any other true or observe yield value is lower than \(Y_{stoic}\), which when substituted in the regression parameter \((YkC – k_d)\), along with the observed \(k_d\), would result in a higher estimate for \(kC\). Similarly, the use of \(Y_{stoic}\) results in a slight over-estimate of the \(X_0\) values, because the regression parameter \((kCX_0)\) is divided by the conservative estimate of the \(kC\). To analyze the behavior of the calculated \(kC\) and \(X_0\) values for each of the compounds, Figures 2.3 and 2.4 contain bar-plots of those parameters for B0 and B100 compounds, respectively. Figures 2.3b and 2.4b also contain overlays of aqueous saturation solubilities \((S_{sat})\) of the targeted compounds approximated using Raoult’s Law in
the form of aqueous concentrations [31-34], as detailed in the SI section. The estimated $S_{sat}$ values were not used in the regression, and are shown in Figures 2.3b and 2.4b to outline general trends.

There were no statistically significant differences in the $kC$ values of the different constituents of B0 ($p > 0.05$). All the n-alkanes were utilized at a specific rate of $0.008 - 0.024$ mg-alkane/mg-biomass-hr (Figure 2.3a). The FAMEs, however, were utilized at rates increasing with increases in carbon chain length and decreasing with increases in the number of double bonds (Figure 2.4a). C16:0-ME was utilized at a specific rate of $0.027$ mg/mg-hr, significantly lower ($p = 0.036$) than C18:0-ME, which was utilized at a specific rate of $0.051$ mg/mg-hr. In terms of degrees of unsaturation, C18:3-ME was utilized at a specific rate of $0.024$ mg/mg-hr, significantly lower than C18:0-ME ($p = 0.030$), and C18:1-ME ($p = 0.047$) which was utilized at a specific rate of $0.044$ mg/mg-hr.

The $X_0$ values of the different oil constituents were found to strongly correlate with the approximated aqueous solubilities of those compounds in their respective oils (Figures 2.3b and 2.4b), possibly because the cultures were pre-acclimated to the substrate in the chemostats. In order to relate the different fractions of biomass ($X_i$’s) degrading the different individual oil constituents ($i$) in B0 and B100 with the total biomass ($X_T$), the following conservation laws can be written,

$$X_T = \sum_{i=1}^{n} X_i$$, or $$\frac{dX_T}{dt} = \sum_{i=1}^{n} \frac{dX_i}{dt}$$ (20) (21)

where $n$ is the total number of constituents in the oil.

If $\frac{dX_i}{dt}$ of a specific oil constituent is given by equation 6, and $\frac{dX_T}{dt}$ is given by,

$$\frac{dX_T}{dt} = \left( \frac{Y_1 k_1 S_{1,w}}{K_{1,s} + S_{1,w}} + \frac{Y_2 k_2 S_{2,w}}{K_{2,s} + S_{2,w}} + ... + \frac{Y_n k_n S_{n,w}}{K_{n,s} + S_{n,w}} - k_d \right) X_T$$ (22)
by applying the constant $S_{i,w}$ assumption (i.e. equation 7) for each component $i$ in equation 22, and substituting equations 22 and equation 10 for each oil component $i$ in equation 21, we obtain,

$$
\left[ \sum_{i=1}^{n} (YkC)_i - k_d \right] X_T = \sum_{i=1}^{n} [(YkC - k_d)_i X_i], \quad \text{or}
$$

$$
X_T = \frac{\sum_{i=1}^{n} [(YkC - k_d)_i X_i]}{\sum_{i=1}^{n} (YkC)_i - k_d}
$$

Had we used equation 22 in the model development, we would have had to fit the data of all the different compounds in each oil simultaneously; which would have resulted in an unnecessary mathematical complexity, especially when various lengths of lag phases are observed for the individual compounds, such as in B0. Using equation 24, $X_{0T}$ for the cultures acclimated to B0 and B100 are found to be 0.81 and 2.41 mg/L, respectively. If $X_{0T}$ was to be calculated by directly summing the individual $X_0$’s for all the compounds shown in Table S1, the corresponding $X_{0T}$ values for B0 and B100 would be 16.27 and 14.2 mg/L, respectively. Note that the mole fraction of the total targeted n-alkanes in B0 is only 0.165, while the mole fraction of the targeted FAMEs make up almost 100% of B100. Yet, both predicted $X_{0T}$ values calculated by equation 24 or by direct summation are significantly less than the measured initial biomass VSS concentrations, which were 93.5 and 116 mg-VSS/L for B0 and B100, respectively. The interpretation of the predicted $X_0$ and $X_{0T}$ values extends well beyond the scope of this paper. They could reflect the fraction of the total biomass to which the substrate is bioavailable inside the aqueous phase. They also could be a function of the active fraction of the bulk biomass and cultures’ viability.
2.5 Discussion

The analytical significance of the proposed model appears to be great. However, we need to be careful in comparing the rates we obtained here with the literature; because different modeling techniques as well as different organisms would indeed result in different calculated rates. Having said that, studies on biodegradation kinetics of n-alkanes have been widely reported in the literature. Mohanty and Mukherji [35] reported no significant difference among the first order biodegradation rates of nC9–nC26 in petroleum diesel by *E. aurantiacum* and *B. cepacia*. On the other hand, Setti et al. [36] found that the first order biodegradation rates of n-alkanes by *Pseudomonas sp*, *Corynebacterium sp*, and *C. lypolitica* inversely correlated with the carbon chain length of the n-alkane. In comparison, Sakai et al. [37] and Yamada et al. [38] observed higher biomass growth of *Acinetobacter sp. M-1* and *P. simplicissimum*, respectively, on the higher molecular weight n-alkanes, but could not explain their results. Blanch and Einsele [7] as well as Yoshida et al. [9] independently investigated the microbial growth of *C. tropicalis* on n-hexadecane as a sole carbon source. Both research groups found the maximum specific growth rate $\mu_{\text{MAX}}$ (where $\mu_{\text{MAX}} = Yk$ using the notations followed in this paper) of the organism to be 0.30 and 0.19 g-biomass/g-alkane-hr, respectively. Those values compare reasonably well with the actual specific growth rates ($YkC$) for the n-alkanes we report in Table S1 (0.019 – 0.038 g-biomass/g-alkane-hr). This gives a rough estimate for the constant $C$ of 0.10 – 0.12, which, using the definition of $C$ from equation 9, results in $S_{\text{sat}} \approx 0.12 K_s$ for the n-alkanes. This justifies the assumption of $S_w << K_s$ adopted by many researchers for poorly soluble materials if a specific amount of error can be tolerated. In our study, the actual specific utilization rate $kC$ for all the n-alkanes was found to be roughly the same. However, the calculated $X_0$’s for those compounds were found to vary significantly, and strongly correlated with the alkanes’ aqueous concentrations approximated by Raoult’s Law (Figure 2.3b). This, in turn, significantly changed the total initial rate...
\( (kCX_0) \) at which the biodegradation of each of the n-alkanes began. In other words, although \( kC \) was relatively constant for the n-alkanes, the total initial utilization rate (\( kCX_0 \)), which appears in equation 18, varied more than three orders of magnitude between nC10 (\( kCX_0 = 0.0158 \text{ mg/L-hr with } X_0 = 1.05 \text{ mg/L} \)) and nC24 (\( kCX_0 = 0.0002 \text{ mg/L-hr with } X_0 = 0.008 \text{ mg/L} \)) due to the difference in their \( X_0 \) values.

A similar behavior in terms of \( kC, X_0, \) and \( kCX_0 \) was observed for the FAMEs in B100. \( kCX_0 \) was highest for C18:2-ME (0.217 mg/L-hr) because its \( X_0 \) value was the highest at 7.90 mg/L, while the lowest \( kCX_0 \) value was for C18:0-ME (0.0106 mg/L-hr) because of its lowest \( X_0 \) value of 0.243 mg/L. However, as mentioned earlier and shown in Figure 2.4a, the specific utilization rate of C18:0-ME was the highest of all the FAMEs and significantly higher than C18:3-ME and C16:0-ME.

Finally, based on the \( kC \) values of the saturated FAMEs, our data suggest that those microorganisms might have a higher affinity toward the compounds with higher molecular weight. In fact, this observation is strongly supported by genetic evidence from studies on the \textit{Escherichia coli} transport membrane-proteins encoded by \textit{fadL} and \textit{fadD} genes, which act on the outer and inner membranes of the cell, respectively [39-42]. The gene \textit{fadL} encodes a membrane protein responsible for the transport of large hydrophobic molecules (including alkanes and fatty acids) from the extracellular matrix into the inter-membranes region [39]. It contains many hydrophobic pockets and binding domains [40], with a substrate binding affinity that was found to increase with the increase in carbon chain length of fatty acids due to the increase of the hydrophobicity of the molecules [41]. Furthermore, the chain length specificity of the acyl-CoA synthetase activity of the inner transport protein encoded by the \textit{fadD} gene was also found to correlate with the uptake affinity of the substrate molecule [42]. In addition to this hydrophobic binding mechanism of the substrate to membrane proteins encoded by \textit{fadL} and \textit{fadD} genes, the decrease in the specific utilization rate of the FAMEs
with the increase in the number of double bonds, as evident in Figure 2.4a, can also be explained by the mechanism by which those molecules are metabolized once they enter the β-oxidation pathway. It is a widely known fact that unsaturated fatty acids require one to two additional energy intensive enzymatic steps per double bond (depending on the location of the bond) in the β-oxidation pathway to isomerize the bond from cis to trans configuration before the reaction can be resumed at the dehydrogenase step [39, 43]. Therefore, it is expected that the more double bonds the molecule contains, the slower its specific utilization rate would be.

The effect of the aqueous solubilities of poorly soluble materials on their biodegradation rates, in our model, seems to be always reflected in their respective $X_0$ concentrations (Figures 2.3b and 2.4b). When dissolution flux is not rate limiting, the higher the aqueous concentration of the compound, the higher is its bioavailability to the degrading populations. However, the specific utilization rate, $kC$, seems to be a measure of the intrinsic biodegradability of that compound. Therefore, although C18:0-ME had the highest specific utilization rate among the FAMEs, this high specific rate was retarded, on the macroscopic level, by its poor aqueous solubility; which in turn translates into significantly lower $X_0$ values, and hence, a lower total initial rate ($kCX_0$) to start the biodegradation with.

APPENDIX: SUPPORTING INFORMATION. All calculated parameters for the FAMEs and n-alkanes (Table S2.1), and Raoult’s Law aqueous solubility approximation procedure.

REFERENCES


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Figure 2.1. Experimental data and model fit of petroleum diesel (B0) n-alkanes. The black symbols are observed residual concentrations in biological treatment and the white symbols are observed residual concentrations in abiotic controls. The errorbars are ±1 standard deviation of triplicate samples. The solid line is model fit by Equation 9 and the dotted line connects abiotic controls.

Figure 2.2. Experimental data and model fit of the soybean biodiesel (B100) fatty acid methyl esters (FAMEs). The black symbols are observed residual concentrations in biological treatment and the white symbols are observed residual concentrations in abiotic controls. The errorbars are ±1 standard deviation of triplicate samples. The solid line is model fit by Equation 9 and the dashed line is the abiotic model fit.
Figure 2.3. (a) Specific utilization rates ($kC'$s) of petroleum diesel (B0) n-alkanes. (b) Initial degrading biomass concentrations ($X_0$) of petroleum diesel n-alkanes. The errorbars are 1 standard error of estimates (bar plot); and the aqueous saturation concentrations ($S_{sat}$) approximated by Raoult’s Law (line plot) using pure solubilities reported by Sutton and Calder [33] and Franks [34].
Figure 2.4. (a) Specific utilization rates ($kC$’s) of soybean biodiesel (B100) FAMEs. (b) Initial degrading biomass concentrations ($X_0$) of soybean biodiesel FAMEs. The errorbars are 1 standard error of estimates (bar plot); and the aqueous saturation concentrations ($S_{sat}$) approximated by Raoult’s Law (line plot) using pure solubilities reported by and Krop et al. [29].
APPENDIX: SUPPORTING INFORMATION

Microbial kinetic model for the degradation of poorly soluble materials

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Table S1. Obtained regression parameters ($Y_{kC-kd}$) and ($kCX_0$) obtained by fitting the experimental data in equation 18; $Y_{stoic}$ values are calculated using equation 19; $kC$ and $X_0$ are calculated from the regression parameters by plugging in the corresponding $Y_{stoic}$ and $k_d^a$ values, and (S.E.) are asymptotic standard errors of the estimates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$[Y_{kC-kd}]^b$, (S.E.), (mg-X/mg-S-hr)</th>
<th>$[kCX_0]^c$, (S.E.), (mg-S/L-hr)</th>
<th>$Y_{stoic}^d$, (S.E.), (mg-X/mg-S)</th>
<th>$[kC]^e$, (S.E.), (mg-S/mg-X-hr)</th>
<th>$[X_0]^f$, (S.E.)$^g$, (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B0 n-alkanes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nC10</td>
<td>0.0233 (0.0039)</td>
<td>0.0158 (0.0024)</td>
<td>1.614</td>
<td>0.015 (0.002)</td>
<td>1.052 (0.232)</td>
</tr>
<tr>
<td>nC11</td>
<td>0.0172 (0.0072)</td>
<td>0.0373 (0.0074)</td>
<td>1.616</td>
<td>0.011 (0.004)</td>
<td>3.317 (1.463)</td>
</tr>
<tr>
<td>nC12</td>
<td>0.0222 (0.0052)</td>
<td>0.0321 (0.0047)</td>
<td>1.618</td>
<td>0.014 (0.003)</td>
<td>2.241 (0.598)</td>
</tr>
<tr>
<td>nC13</td>
<td>0.0290 (0.0048)</td>
<td>0.0217 (0.0031)</td>
<td>1.620</td>
<td>0.018 (0.003)</td>
<td>1.175 (0.250)</td>
</tr>
<tr>
<td>nC14</td>
<td>0.0270 (0.0137)</td>
<td>0.0233 (0.0078)</td>
<td>1.621</td>
<td>0.017 (0.008)</td>
<td>1.353 (0.805)</td>
</tr>
<tr>
<td>nC15</td>
<td>0.0177 (0.0073)</td>
<td>0.0249 (0.0051)</td>
<td>1.622</td>
<td>0.012 (0.004)</td>
<td>2.163 (0.950)</td>
</tr>
<tr>
<td>nC16</td>
<td>0.0123 (0.0028)</td>
<td>0.0225 (0.0022)</td>
<td>1.623</td>
<td>0.008 (0.002)</td>
<td>2.757 (0.643)</td>
</tr>
<tr>
<td>nC17</td>
<td>0.0176 (0.0043)</td>
<td>0.0140 (0.0021)</td>
<td>1.624</td>
<td>0.011 (0.003)</td>
<td>1.224 (0.338)</td>
</tr>
<tr>
<td>nC18</td>
<td>0.0232 (0.0057)</td>
<td>0.0077 (0.0016)</td>
<td>1.624</td>
<td>0.015 (0.004)</td>
<td>0.515 (0.164)</td>
</tr>
<tr>
<td>nC19</td>
<td>0.0346 (0.0049)</td>
<td>0.0032 (0.0006)</td>
<td>1.625</td>
<td>0.022 (0.003)</td>
<td>0.145 (0.034)</td>
</tr>
<tr>
<td>nC20</td>
<td>0.0241 (0.0088)</td>
<td>0.0034 (0.0011)</td>
<td>1.626</td>
<td>0.015 (0.005)</td>
<td>0.221 (0.106)</td>
</tr>
<tr>
<td>nC21</td>
<td>0.0364 (0.0118)</td>
<td>0.0011 (0.0005)</td>
<td>1.626</td>
<td>0.023 (0.007)</td>
<td>0.048 (0.027)</td>
</tr>
<tr>
<td>nC22</td>
<td>0.0387 (0.0111)</td>
<td>0.0006 (0.0003)</td>
<td>1.627</td>
<td>0.024 (0.007)</td>
<td>0.024 (0.013)</td>
</tr>
<tr>
<td>nC23</td>
<td>0.0268 (0.0112)</td>
<td>0.0005 (0.0002)</td>
<td>1.627</td>
<td>0.017 (0.007)</td>
<td>0.029 (0.017)</td>
</tr>
<tr>
<td>nC24</td>
<td>0.0339 (0.0089)</td>
<td>0.0002 (0.0001)</td>
<td>1.628</td>
<td>0.021 (0.005)</td>
<td>0.008 (0.003)</td>
</tr>
<tr>
<td><strong>B100 FAMEs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>0.0374 (0.0072)</td>
<td>0.0674 (0.0193)</td>
<td>1.441</td>
<td>0.027 (0.005)</td>
<td>3.023 (1.154)</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.0737 (0.0122)</td>
<td>0.0106 (0.0049)</td>
<td>1.459</td>
<td>0.051 (0.008)</td>
<td>0.243 (0.121)</td>
</tr>
<tr>
<td>C18:1</td>
<td>0.0629 (0.0077)</td>
<td>0.0439 (0.0162)</td>
<td>1.459</td>
<td>0.044 (0.005)</td>
<td>1.57 (0.421)</td>
</tr>
</tbody>
</table>
Approximation of $S_{\text{Sat}}$ using Raoult’s Law. Raoult’s Law, in the form of aqueous concentrations, has been widely employed to estimate the concentrations of nonpolar solutes in water [1-3], as well as many others. While the application of this law has shortcomings due to the assumption of ideal behavior, it serves the purpose of illustrating the correlation between the $X_0$ values of the different compounds and their saturation solubility limit ($S_{\text{Sat}}$). The equation used for that purpose is shown below,

$$S_{\text{Sat}}^i = S_{\text{Sat}}^0 N_i$$

where $S_{\text{Sat}}^i = \text{the aqueous solubility limit of compound } i \text{ present in the oil (mg/L),}$

$S_{\text{Sat}}^0 = \text{the aqueous solubility limit of pure compounds } i \text{ reported in the literature (mg/L), and}$

$N_i = \text{the mole fraction of compound } i \text{ in the oil.}$

The aqueous solubility limits of the pure FAMEs and n-alkanes ($S_{\text{Sat}}^0$) are adopted from Krop et al [4], and Franks [5] and Sutton and Calder [6], respectively. Some of the pure n-alkanes solubility limits were extrapolated from the reported values.
REFERENCE


CHAPTER 3: AEROBIC BIODEGRADATION KINETICS
AND MINERALIZATION OF SIX
PETRODIESEL/SOYBEAN BIODIESEL BLENDS BY
ACCLIMATED CULTURES
Aerobic biodegradation kinetics and mineralization of six petrodiesel/soybean-biodiesel blends by acclimated cultures

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ABSTRACT. The aerobic biodegradation kinetics and mineralization of six soybean-based biodiesel/petrodiesel blends (B0, B20, B40, B60, B80, and B100), where B100 is 100% unblended biodiesel, were investigated by acclimated cultures. The fatty acid methyl esters (FAMEs) of biodiesel were found to rapidly undergo abiotic transformation in all experiments. In contrast, the C10-C21 n-alkanes of petrodiesel were metabolized at significantly higher microbial specific utilization rates in the presence of biodiesel. The rate of mineralization of the fuel blends was also found to be enhanced in the presence of biodiesel; yet a similar enhancement in the extent of the mineralization was not observed. Further experiments revealed that the FAMEs of biodiesel are capable of co-solubilizing the n-alkanes of petrodiesel, a mechanism that fully explains the faster utilization and mineralization kinetics of petrodiesel in the presence of biodiesel without necessarily enhancing the mineralization efficiency of the blends. The biodegradation of six targeted aromatic compounds in petrodiesel was also influenced by the amount of biodiesel in a blend. While toluene, o-xylene, and tetralin were not degraded in B0 and B20, all the targeted aromatic compounds were degraded to below detection limits in B40 and B80. B60 acclimated biomass, however, was unable to degrade most of the aromatic compounds. These results indicate that the amount of biodiesel in the blend significantly affects the absolute and relative abundance of the dissolved constituents of biodiesel/petrodiesel blends in a way that can considerably alter the biodegrading capacity of microbial cultures.
3.1 Introduction

Biodiesel is defined as the mono-alkyl esters of long chain fatty acids derived from transesterification of vegetable oils and animal lipids for use in compression-ignition (petrodiesel) engines. This specification is for pure (100%) biodiesel prior to blending with petrodiesel fuel. Petroleum-derived diesel is composed of 75–85% saturated hydrocarbons (primarily normal, branched, and cycloalkanes), and 15–25% aromatic hydrocarbons (mainly alkylbenzenes). The average chemical formula for common petrodiesel fuel is C_{12}H_{26}, ranging from approximately C_{10}H_{22} to C_{13}H_{32} [1].

A major reason for the renewed interest in fuels made from plant and animal lipids is that feedstocks for the fatty-acid methyl esters (FAMEs) of biodiesel are renewable or can be reclaimed from wastes (e.g., used animal fats, recycled frying oil). In contrast, fossil fuels are non-renewable resources and have increasingly higher costs both financially and environmentally. An additional incentive for using biodiesel is the potential positive impact on global climate change. Various greenhouse emission models estimate lower net crop-to-combustion carbon and sulfur emissions for biodiesel compared to petrodiesel [2, 3]. However, several studies have confirmed a net increase of NO_{x} emissions, which are considered indirect greenhouse gases, from biodiesel combustion [4]. A 2002 EPA report showed that a 20% biodiesel blend boosted NO_{x} emissions by 2%, but cut particulate matter (PM) by about 10%, hydrocarbons (HC) by about 21%, and carbon monoxide (CO) by about 11% [5]. Because of its potential as a renewable energy source, as well as a method to reduce net greenhouse gas emissions, biodiesel has attracted great interest as an alternative fuel in the transportation industry.

The great benefits of using biodiesel as a fuel demand a more comprehensive understanding of its behavior in the environment. Although numerous reports have confirmed that biodiesel is more
readily biodegradable than petrodiesel [6-8], little is known about how biodiesel blending would affect the biodegradation of petrodiesel. There appears to be an ongoing debate on whether biodiesel would enhance the microbial utilization of petrodiesel or not. And if such enhancement does exist, the mechanisms by which it is achieved are inadequately understood. Zhang et al. [8], Pasqualino et al. [9], and Chen et al. [10] claimed to observe co-metabolic enhancement in the mineralization of petrodiesel by unacclimated cultures when blended with various types of biodiesel feedstocks. Mariano et al. [11] and Owsianiak et al. [12] did not observe such co-metabolic effect in the mineralization of petrodiesel/biodiesel blends by microbial enrichments isolated from contaminated sites. Prince et al. [13] and DeMello et al. [14] found that the biodegradation rate of FAMEs, in petrodiesel/biodiesel blends by unacclimated fresh and seawater microorganisms, respectively, was roughly the same as the biodegradation rate of the n-alkanes. Owsianiak and coworkers [12], in turn, found that for petrodiesel/biodiesel blends of less than 10% biodiesel, the microorganisms preferentially metabolized the FAMEs and slowed the assimilation of the n-alkanes.

Miller and Mudge [15] found that adding biodiesel to a sand column contaminated with crude oil significantly enhanced the mobility of the oil along with the microbial uptake of aliphatic hydrocarbons. They suggested that this apparent enhancement was probably achieved through cosolubilization rather than co-metabolism. In fact, biodiesel’s capacity to mobilize oil and coal tar in sediments was tested in microcosms, mesocosms, and field scale studies [16–18], and was found to significantly enhance the mobility of the contaminants. Taylor and Jones [16] and Alvarez et al. [18] further found that the addition of biodiesel to the contaminated sediments markedly enhanced microbial utilization of the contaminants, compared to adding nutrients or microbial enrichment alone. This study was designed to investigate the impact of soybean biodiesel blending level on the aerobic
biodegradation kinetics and mineralization of the petrodiesel/biodiesel blends B0, B20, B60, B80, and B100 (where B100 is 100% unblended biodiesel) by acclimated cultures.

### 3.2 Materials and Methods

**Chemicals.** Low-sulfur petroleum diesel (B0) was purchased from a BP diesel station (Cincinnati, OH) with an n-alkanes mole fraction of 0.165. Unblended soybean-methyl ester biodiesel (B100) was purchased from Peter Cramer North America (Cincinnati, Ohio) with FAMEs mole fractions of 0.145 C16:0, 0.055 C18:0, 0.206 C18:1, 0.518 C18:2, and 0.0759 C18:3. All other fuel blends (B20, B40, B60, and B80) were blended in our laboratory by volumetric splash mixing. Palmitic acid methyl ester (99%) (C16:0-ME), palmitoleic acid methyl ester (99%) (C16:1-ME), stearic acid methyl ester (99%) (C18:0-ME), oleic acid methyl ester (99%) (C18:1-ME), linoleic acid methyl ester (99%) (C18:2-ME), linolenic acid methyl ester (99%) (C18:3-ME), and normal alkanes standard mixture (nC10-nC30), were all purchased from Sigma Aldrich (USA). All other chemicals, if not noted, were purchased from Sigma Aldrich or Fisher Scientific (USA).

**Culture Acclimation.** Six laboratory-scale, 6-liter porous pot (PP) chemostats were inoculated with a mixture of mixed liquor obtained from the Mill Creek Wastewater Treatment Plant (Cincinnati, OH) and gasoline and triglyceride degrading cultures previously developed in our laboratories at the University of Cincinnati (Cincinnati, OH). The flow-through bioreactors were operated as completely mixed systems with a hydraulic retention time of 1 day. Organic feeds of B0, B20, B40, B60, B80, and B100 were delivered to the bioreactors using Hamilton syringe pumps, and the feed rates were increased gradually throughout an acclimation period of six months to a final organic loading of 750 mg/L-d oil. Adequate aeration in the bioreactors was maintained throughout the operating period by means of continuous air diffusers, operated at nominal volumetric flow rates of 2–3 L/min. The
bioreactors received a combined feed of essential nutrient and vitamin minimal medium buffered at 7.5 ± 0.1 of the following final concentration: 150 mg/L MgSO$_4$·7H$_2$O, 156 mg/L KNO$_3$, 48.7 mg/L CaCl$_2$·2H$_2$O, 37.3 mg/L FeCl$_2$·4H$_2$O, 1393 mg/L K$_2$HPO$_4$, 544 mg/L KH$_2$PO$_4$, 0.08 mg/L CuSO$_4$·H$_2$O, 0.15 mg/L Na$_2$MoO$_4$·2H$_2$O, 0.13 mg/L MnSO$_4$·H$_2$O, 0.23 mg/L ZnCl$_2$, 0.42 mg/L CoCl$_2$·6H$_2$O, 0.015 mg/L 4-aminobenzoic acid (99%), 0.00585 mg/L biotin, 0.0003 mg/L cyanocabalanin, 0.00585 mg/L folic acid dihydrate (99%), 0.015 mg/L nicotinic acid (98%), 0.015 mg/L pantothenic acid Ca-salt hydrate (98%), 0.03 mg/L pyridoxine hydrochloride (98%), 0.015 mg/L riboflavin (98%), 0.015 mg/L thiamine hydrochloride (99%), and 0.015 mg/L thiotic acid (98%). Certain water quality variables including effluent FAMEs and target n-alkane analytes, total and volatile suspended solids (VSS), chemical oxygen demand (COD), pH, and dissolved oxygen (DO) were monitored routinely. When the reactors achieved steady state performance, a portion of each reactor’s culture was collected, washed three times with sterile saline solution, and frozen in 10% w/w glycerol at -80 °C for consistent use in the batch experiments.

**Batch Experiments.** Batch experiments were carried out in sterile and surface-deactivated 160 mL glass serum bottles where 50 mL of filter-sterilized nutrient and vitamin medium was added. A biomass aliquot of each fuel blend, thawed and washed three times with sterile saline solution, was added to the bottles at measured solids concentration of 100 ± 15mg VSS/L. The bottles were sealed with PTFE septa and spiked with 10 µl of their respective fuel blend, corresponding to an initial organic substrate concentration of 175 ± 3 mg/L fuel. The amount of oxygen available in the headspace was sufficient for the complete oxidation of 1.5X the amount of fuel added on a chemical oxygen demand basis. The bottles were then placed in a rotary tumbler operated at a nominal speed of 20–25 rpm. Optimum sampling frequency was determined through range finding experiments that were every 4 hr for the first 24 hr, every 6 hr for the second 24 hr, every 8 hr for the next 24 hr, every 12 hr for the
next 24 hr, and every 24 hr thereafter for 168 hr (7 days). Separate bottles were set for the analysis of CO₂ and volatile suspended solids (VSS) at 7 and 42 days. Abiotic controls inactivated with sodium azide (0.9% w/v) as well as biomass controls were also included to quantify abiotic substrate losses and biomass endogenous decay and respiration rates, respectively. All experiments were conducted at 22 ± 2 °C.

Chemical Analysis. At every sampling event, triplicate bottles of each treatment were spiked with 50 µl of a standard surrogate solution of C16:1-ME and deuterated nC16 in acetone. The bottles were then completely liquid-liquid extracted twice with 1:1 dichloromethane (DCM) and the extract was filtered through anhydrous sodium sulfate and stored at -20 °C until the time of analysis. Extracts were added in 1 mL volumes in 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 Series II gas chromatograph (GC) equipped with an HP-INNOWAX capillary column (30 m long, 0.25 mm i.d., 0.25 um film thickness), and an HP Agilent 5971 Mass Spectrometer Detector (MSD) (HP, Palo Alto, CA). The flow rate of Helium carrier gas was 1 mL/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. Target analytes were the biodiesel FAMEs and the petrodiesel C10-C24 n-alkanes. Six additional peaks eluting within the method range and positively identified as toluene, ethylbenzene, p-xylene, m-xylene, o-xylene, and tetralin were also targeted for analysis. Detection limits were in the range of 5–25 µg/L for all the compounds. Surrogate recoveries of all extractions ranged from 80 to 120%.

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**CO₂ and VSS Analysis.** Two sampling events of six bottles each (biologically active + endogenous respiration controls) per oil treatment were set for the analysis of total CO₂ produced and VSS at 7 and 42 days (ultimate). Just prior to sampling time, 100 µL of HCl was spiked in the bottles through the PTFE septa, delivering a final concentration of 24 mM HCl and dropping the pH < 2. While the bottles were still in the inverted position, the needle of the syringe was slowly pulled out of the bottles to minimize leakage of gas in or out of the bottles. The septa were then wrapped with parafilm, and the bottles were tumbled again for a 24 hr equilibration period. Upon equilibration, a 20 mL gas-tight Popper Micro-mate Syringe (New Hyde Park, NY) was inserted inside the headspace of each bottle, and the gas in the headspace was allowed to expand to equilibrate the pressure inside the bottle with atmospheric pressure. The volume of headspace gas expanded inside the 20 mL syringe was recorded and, while the syringe was still inside the bottle, a 0.5 mL of the headspace gas was withdrawn by a gas-tight Vici Precision Sampling Syringe (Baton Rouge, LA) and analyzed for gas composition using an HP 5890 Series II gas chromatograph (GC) equipped with a dual column HP 10 ft molecular sieve BX-45/60 mesh, HP 6 ft. HAYESEP Q 80/100 mesh, and a Thermal Conductivity Detector (TCD) (HP, Palo Alto, CA). The mass of gas phase CO₂ was calculated by Equation 1.

$$ M_{CO₂-gas} = \left( V_{headspace} + V_{syringe} \right) \times CO₂(\%) \times \frac{44 \text{ g/mole}}{24.2 \text{ L/mole}} $$

(1)

where $M_{CO₂-gas} =$ the mass of CO₂ in the gas phase (mg),

$V_{headspace} =$ the volume of the headspace of the bottle ($V_{headspace} = 110$ mL),

$V_{syringe} =$ the volume of expanded gas measured in the syringe (mL),

CO₂ (%) = the percentage of CO₂ in the headspace measured by the GC-TCD,

44 g/mole = molecular weight of CO₂, and

24.2 L/mole = the molar volume of CO₂ gas at 22 °C and 1 atm.
The mass of aqueous CO$_2$ was calculated using Henry’s Law, assuming negligible ionized carbonate species and equilibrium between the gas and aqueous phase CO$_2$ species according to Equation 2.

$$M_{CO_2-aq} = V_{aq} [CO_2]_{aq} = V_{aq} H [CO_2]_{gas} = V_{aq} H \times CO_2(\%) \times \frac{44 \text{ g/mole}}{24.2 \text{ L/mole}} \quad (2)$$

where $M_{CO_2-aq} =$ total mass of CO$_2$ in the aqueous phase (mg), $[CO_2]_{aq}$ and $[CO_2]_{gas} =$ the concentrations of CO$_2$ in the aqueous and gas phases (mg/mL), respectively, $V_{aq} =$ the volume of aqueous phase ($V_{aq}=$50 mL), and

$H$ is the dimensionless Henry’s constant for CO$_2$ ($H=0.831$) [19].

The total amount of CO$_2$ produced was calculated as the sum of Equations 1 and 2 and was corrected with respect to endogenous respiration in the blanks at every sampling event. The aforementioned procedure was also validated by measuring the dissolved inorganic carbon directly using a Shimadzu TOC-V CSH Analyzer (Tokyo, Japan) according to manufacturer’s procedures and found to be within 3% accuracy. After the CO$_2$ analyses were completed, the bottles were opened, filled completely with sterile saline solution, resealed with the same septa, and placed in an ultrasound sonication bath for 12–16 hr to detach any biomass adhered to the glass surface before measuring the VSS in the bottles, according to EPA Method 160.4. Chemically oxidizable carbon in B0 and B100 was experimentally measured by injecting 10 µL of the fuels in sealed serum bottles containing 20 mL of a 3% potassium dichromate in sulfuric acid (95%) solution and incubated at 150 °C for 24 hr. The bottles were then cooled down to room temperature, and the headspace gas was analyzed as outlined above. The sulfuric acid phase was diluted with deionized water down to 5% and analyzed for inorganic carbon on the Shimadzu TOC analyzer. The amount of chemically oxidizable carbon was found to be 2.346 ± 0.036 and 2.484 ± 0.0315 mg-CO$_2$/µL-oil for B0 and B100, respectively.
3.3 Results and Analysis

**Alkanes.** The results for n-alkanes from the kinetic batch experiments were fit into a mechanistic model, Equation 3, for the biodegradation of poorly soluble materials derived in Chapter 2. The term “substrate” will refer to each individual compound of a given carbon chain length, not the oil in its entirety. Similarly, “biomass” refers to that portion of the total biomass that is actively degrading a given compound with a specific carbon number.

\[
S_{\text{tot}} = S_{\text{tot}0} - \frac{kCX_0}{YkC - K_d} \left( e^{(YkC - k_d)t} - 1 \right)
\]  

(3)

Where \( S_{\text{tot}} = \) the observed total mass of residual substrate (i.e., alkane) divided by the volume of aqueous phase in the bottle (mg/L) at time \( t \) (hr),

\( X_0 = \) the initial concentration of degrading biomass (mg/L),

\( Y = \) the biomass yield coefficient (g-biomass produced/g-substrate consumed),

\( k_d = \) the biomass endogenous decay rate (hr\(^{-1}\)),

\( k = \) the maximum specific substrate utilization rate (mg-substrate/mg-biomass–hr), and

\( C = \) a constant and is equal to \( S_{\text{sat}}/(K_s + S_{\text{sat}}) \), where \( S_{\text{sat}} \) and \( K_s \) are the substrate saturation concentration in the aqueous phase and the Monod half-saturation constant (mg/L), respectively.

A nonlinear least squares minimization procedure was used to fit the experimental data of the n-alkanes into Equation 3 and to estimate the actual net specific growth rate \((YkC - k_d)\) and the total initial utilization rate \((kCX_0)\) for each of the n-alkanes (Figure 3.1). Figure 3.1 shows that n-alkanes utilization rates for B20 and B40 are higher than the other fuel blends. To study this further, the actual microbial specific utilization rate \((kC)\) for each of the n-alkanes was approximated from the regression parameter \((YkC - k_d)\), using the maximum stoichiometric yield coefficient \(Y\) (assuming a bacterial cell formula of \(C_{60}H_{87}O_{23}N_{12}P\) [20]) and measured endogenous decay rate \(k_d\) of 0.020–0.038 d\(^{-1}\) for all the cultures.
The microbial specific utilization rates of n-alkanes in the fuel blends are compiled in Figure 3.2. For almost all the n-alkanes, the microbial specific utilization rates (kC’s) in B20 were significantly higher than those in B0 (p < 0.005) and B80 (p < 0.05). Similarly, the specific utilization rates of the n-alkanes were significantly higher in B40 compared to B0 (p < 0.005) and B80 (p < 0.05). While the differences in the n-alkane specific utilization rates of B20 and B40 were statistically insignificant, as well as the differences in the specific rates of B60 and B80, Figure 3.2 clearly points out a decreasing trend in the n-alkane kC values in the order B20 > B40 > B60 > B80 > B0, which becomes more noticeable for the n-alkanes with longer carbon chains.

FAMES. All the FAMEs in all the fuel blends were found to undergo rapid abiotic transformation in the control treatments (Figure 3.3, dotted lines). FAMEs, and lipids in general, are widely reported to naturally undergo a range of abiotic transformation processes such as hydrolysis, autoxidation, and polymerization in the presence of a wide range of catalysts [21, 22]. The abiotic transformation of the FAMEs was so fast that accurate estimation of biodegradation rates for those compounds was not possible in most of the biological treatments (Figures S3.1 and S3.2 in Supporting Information). Further examination of the FAME abiotic control data (Figure S3.3 in Supporting Information) revealed substantial deviation from the widely adopted first order kinetics for such reactions, especially for the more hydrophobic FAMEs (i.e. C16:0-ME and C18:0-ME). Such a behavior can be accounted for if dynamic equilibrium partitioning of those compounds is assumed to take place between the non-aqueous phase liquid (NAPL) and the aqueous phase, prior to the transformation reaction, which probably takes place in the aqueous phase. The initial rate of abiotic transformation of the FAMEs (Figure S3.3) appears to also be influenced by the amount of petrodiesel in the blend. The FAMEs in B60, followed by B40, seem to have the highest initial slope of abiotic transformation. A wide range of petrodiesel fuel additives have been reported to include metals such as
Ce, Mo, Mn among others, which have been shown to significantly enhance the oxidation kinetics and efficiency of the fuel in internal combustion engines [23, 24], and are also likely to affect the rate of abiotic transformation of the FAMEs in biodiesel/petrodiesel blends. Metal analysis using ICP-MS, however, did not confirm the presence of such a catalyst in B0. There was no significant difference in the metals concentrations between B0 and B100.

**Aromatic Compounds.** Although the targeted aromatic compounds were present in B0 at very low concentrations (0.35-0.70 ppm for toluene, ethylbenzene, and xylene isomers), the analysis of those compounds allowed for a better understanding of how the fraction of biodiesel in the fuel blend could affect the biodegradation capability of microbial cultures for this class of compounds. A substantial enhancement in the biodegradation of aromatic compounds occurred when biodiesel was present in the fuel (Figure S3.4 in Supporting Information). While toluene, o-xylene, and tetralin were not degraded in B0 and B20 treatments, all the aromatic compounds were completely degraded to below detection limits in B40 and B80. In the B60 treatment, however, the cultures were unable to assimilate any of the xylene isomers, toluene, or tetralin during the 7 day sampling period (i.e., only ethylbenzene was degraded). It should also be noted that the biodegradation of o-xylene and tetralin in B40 and B80 was accompanied by the development of an intense yellow coloration of the aqueous phase, most likely due to partial oxidation products of aromatic biodegradation.

**Mineralization.** The mineralization results at 7 and 42 days for all the fuel blends are shown in Figure 3.4. The extent of mineralization of the fuel blends was found to increase linearly with the fraction of biodiesel from 23.08% and 49.17% for B0, to 64.13% and 74.78% for B100, at 7 and 42 days, respectively. Analysis of the mineralization data also indicated that the rates of mineralization of the fuel blends were significantly enhanced in the presence of the biodiesel constituents (p < 0.05). The ratio of carbon mineralized at 7 days to ultimate carbon mineralized, which is an indirect measure of
the mineralization rate, increased from 46.94% for B0, to 67.71%, 63.52%, 76.21%, 71.75%, and 85.76% for B20, B40, B60, B80, and B100, respectively. In order to test for any synergetic enhancement attributable to biodiesel on the mineralization efficiency of fuel blends, the scatter plots in Figure 3.4 were constructed by linearly interpolating the CO2 data of B0 and B100, independently, at each of the other fuel mixtures. The results indicated no statistically significant difference between the actual mineralization and interpolated data. The mineralization of the fuel mixtures (B20, B40, B60, and B80) was found to be equivalent to that of the linearly interpolated data from B0 and B100.

3.4 Discussion

Both the specific microbial utilization rates of the n-alkanes (kC’s) and the rates of mineralization of the fuel blends were significantly enhanced in the presence of biodiesel, but a similar enhancement in the extent of mineralization of those fuel blends was not observed. Specifically, 85.76% of the mineralized carbon in B100 occurred during the first 7 days of the experiment, compared to 46.94% for B0 during the same period. These results suggest that the enhancement of biodegradation kinetics of the n-alkanes was not due to co-metabolism of those compounds since the total amount of CO2 produced in the blends did not increase beyond the amount of CO2 produced either in B0 or B100 separately. In addition, the phenomenon of co-metabolism, in principle, does not apply for the case of FAMEs and n-alkanes. Co-metabolism occurs when a non-growth substrate, a substrate that microorganisms cannot utilize as a sole carbon and/or energy source, is co-oxidized in the presence of a growth substrate [25]. Clearly, both the FAMEs and the n-alkanes are not only growth substrates, but are also metabolized by a metabolically similar mechanism, namely β-oxidation [26, 27].

Since biodiesel is a better growth substrate compared to petrodiesel [28], biodiesel should enhance the rate of microbial degradation of the n-alkanes by increasing the actual growth yield of the biomass. Such enhancement, however, would increase the total degradation rate and would not affect
the specific utilization rate of the substrate as modeled by Monod kinetics [29]. Fundamentally, an increase in the actual specific utilization rate \( kC \) can be attributed to an increase in either the maximum specific utilization rate \( k \), or the substrate aqueous saturation concentration \( S_{sat} \) acting through the constant \( C = \frac{S_{sat}}{K_s + S_{sat}} \). While the intrinsic rate of dissolution of n-alkanes from the NAPL into the aqueous phase is believed to be faster than the intrinsic rate of biological assimilation of the substrate in the aqueous phase [30, 31], and since the value of \( k \) for a biodegradable, poorly soluble material is usually orders of magnitude higher than the value of \( C \) for the same material (i.e. high \( k \) and low \( S_{sat} \)), the actual specific utilization rate of n-alkanes is more likely to be controlled by their poor aqueous solubility rather than their mass transfer rate.

Therefore, we tested the hypothesis that biodiesel is able to co-solubilize the n-alkanes in biodiesel/petrodiesel blends. Miller and Mudge [15] suggested the possibility of co-solubilization of crude oil by biodiesel in sand columns. They did not provide, however, any direct measurement of such co-solubilization. Batch experiments were conducted in which biodiesel/petrodiesel blends were equilibrated with a sterile micronutrients and vitamins containing aqueous phase by vigorous shaking and tumbling for 1 hour in a setup similar to that of the biological batch experiment outlined earlier but without adding biomass. After equilibration, the samples were transferred into separatory funnels and allowed to settle for 1 hour. The supersaturated aqueous phase was withdrawn from the bottom of the funnels and was filtered through fritted glass funnels (6 cm deep, 3 cm i.d., 1 µm pore size) filled with 3-mm surface-deactivated borosilicate glass beads (Chemglass, Vineland NJ) to filter out the particulate oil from the aqueous phase. Filtrates were then extracted with DCM and concentrated 18-fold in a TurboVap II Evaporation System (Biotage, Charlotte NC) according to the manufacturer’s procedure, and analyzed by GC-MS (surrogate recoveries ranged from 84% to 96%). The results from this co-solubilization experiment are summarized in Figure 3.5. Although the total amount of
petrodiesel in the fuel blends decreases as the total amount of biodiesel increases, Figures 3.5 shows that the equilibrium aqueous concentrations of the n-alkanes were significantly higher (p < 0.005) when biodiesel was present in the fuel blends. Figure 3.5 provides clear evidence that the FAMEs of biodiesel are capable of co-solubilizing the n-alkanes of petrodiesel, possibly by reducing the interfacial surface tension between the aqueous phase and the n-alkanes, and hence, increasing the actual specific utilization rate (kC) for those compounds.

Although the FAMEs were found to disappear abiotically in all the petrodiesel/biodiesel blends, this abiotic transformation did not appear to appreciably affect the amount of CO₂ produced at 7 and 42 days (shown in Figure 3.4), which indicates that abiotic transformation byproducts were still amenable to biodegradation. The deviation of the kinetics of abiotic transformation from the first order behavior, especially for C16:0 and C18:0 methyl esters, suggests dynamic partitioning of those compounds between the NAPL and the aqueous phase prior to the abiotic reaction taking place in the aqueous phase. Although metal analysis did not confirm the presence of an assumed catalyst in B0, the findings of the co-solubilization experiment (Figure 3.5) could indeed affect the initial rates of abiotic transformation of the FAMEs. Figure 3.5a shows that the highest co-solubilization of n-alkanes was observed in B60 followed by B40, and Figure S3.3 shows that the highest initial slope of abiotic transformation of the FAMEs was also observed in B60 followed by B40. Yet, the aqueous equilibrium concentration of the FAMEs in the petrodiesel/biodiesel blends (Figure 3.5b) did not show preferential co-solubilization of the FAMEs in B60 and B40. However, polymerized deposits of the autoxidation the FAMEs were visible in the co-solubilization experiment. This observation indicates that the measured equilibrium concentrations of the FAMEs were not necessarily the true solubility limit of the FAMEs, but were most likely reduced by autoxidation and polymerization.
Monitoring the aromatic compounds during the batch experiments provided a different aspect of the effect of biodiesel/petrodiesel blending on the cultures’ acclimation and biodegradation capabilities. It is unclear why most of the aromatics in the B60 blend resisted biodegradation while they were utilized in the other blends. Complete degradation of all aromatic compounds to below detection limits occurred in the B40 and B80 blends, and toluene, o-xylene, and tetralin in B0 and B20 resisted biodegradation. This suggests a direct effect of the type of fuel blend on the development and acclimation of the degrading culture. In other words, it is reasonable to conclude that these observations are due to the fraction of the substrate bioavailable to the biomass in the dissolved phase. This means that both the absolute and relative concentrations of the dissolved constituents significantly varied in a nonlinear fashion in the petrodiesel/biodiesel blends (Figure 3.5). These variances in dissolved constituents among the different blends may have significantly shifted the composition of the degrading microbial communities to metabolize the oils as a function of the compounds that co-solubilized into the dissolved phase in preferential order. Finally, the bright yellow metabolite observed in the B40 and B80 treatments and not B0 or B20 indicates that this metabolite could be a product of the oxidation of either o-xylene or tetralin. In fact, a similar colored metabolite was observed in the biodegradation of crude oil in the presence of biodiesel, but not in the biodegradation of crude oil alone, according to Mudge and Pereira [32]. The authors, however, failed to associate this metabolite with a specific group of parent compounds. Wrenn and Venosa [33] associated similar colored metabolites with the accumulation of partial oxidation products of polycyclic aromatic hydrocarbons (PAHs). Similarly, other researchers [34-36] reported that during microbial degradation of PAHs, yellow-brown colored products are synthesized that have quinone-like structures from the condensation of phenolic compounds in mineral media. Further research investigating the effect of
biodiesel on the microbial metabolism of aromatic compounds is desirable, as those compounds may pose greater risks in the environment.

**APPENDIX: SUPPORTING INFORMATION.** Figure S3.1. FAME biological degradation data with model regression, Figure S3.2. Microbial utilization rates of FAMEs, Figure S3.3. Deviation of abiotic transformation kinetics of the FAMEs from first order behavior, and Figure S3.4. Biodegradation of targeted aromatic compounds.

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Figure 3.1. nC10–nC21 alkanes biological degradation data in B0 (○), B20 (Δ), B40 (□), B60 (◊), and B80 (▽). Errorbars are ±1 standard deviation of triplicate samples. Solid lines are regression fits by Equation 3.
Figure 3.2. Actual microbial utilization rates (kC’s) of the n-alkanes in B0, B20, B40, B60, and B80 as color coded in figure key. Errorbars are ±1 standard error of the estimates.
Figure 3.3. FAMEs biological degradation data in all the petrodiesel/biodiesel blends (solid symbols), and abiotic controls (hollow symbols). C16:0-ME (● and ○), C18:0-ME (▼ and △), C18:1-ME ( ■ and □), C18:2-ME (◆ and ◊), C18:3-ME (▲ and △). Errorbars are ±1 standard deviation of triplicate samples, and dotted lines connect abiotic controls.
Figure 3. 4. 7-days (black) and ultimate (grey) mineralization efficiency of the petrodiesel/biodiesel blends. Bar plot is the observed experimental results (errorbars are ±1 standard deviation of triplicate samples); scatter plot is linear interpolation from B0 and B100 experimental data (errorbars are error-propagated), and dashed lines connect scatter plot.
Figure 3.5. Results of the co-solubilization experiment. Equilibrium aqueous concentrations of the dissolved phase n-alkanes (a), FAMEs (b), and aromatic compounds (c) for B0, B20, B40, B60, B80, and B100, as color coded in the figures keys. Errorbars are ±1 standard deviation of triplicate samples.
APPENDIX: SUPPORTING INFORMATION

Aerobic biodegradation kinetics and mineralization of six petrodiesel/soybean-biodiesel blends by acclimated cultures

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**FAMEs Biodegradation and Abiotic Transformation.** The biodegradation rates of the FAMEs were estimated according to equation (ES.1) derived in Chapter 2 for the biodegradation of poorly soluble materials. Equation ES.1 treats the abiotic transformation reaction as a first order reaction with respect to the aqueous substrate concentration, which is also assumed to remain approximately constant and close to the saturation level \( S_{sat} \) as long as its value is very low compared to the total amount of substrate available in the bottle, and the substrate mass transfer is not limiting.

\[
S_{tot} = S_{tot0} - \frac{kCX_0}{(YkC - K_d)} \left(e^{(YkC - K_d)t} - 1\right) - k_{ab}S_{sat}t \tag{ES. 1}
\]

Where \( k_{ab}S_{sat} \) is the slope of the linear part of abiotic controls data of the FAMEs (mg/L-hr), and all the other parameters are the same as defined for Equation 3 in the main text.

Figure S3.1 shows the FAMEs biological and abiotic controls data of batch experiments. The linear regression of the abiotic controls data in \( S_{tot} = S_{tot0} - k_{ab}S_{sat}t \), as well as the nonlinear fit of the biological data in equation (ES.1), are also superimposed in Figure S3.1. Only the FAMEs, which abiotic transformation was slow enough to allow accurate estimation of the microbial specific utilization rate \( kC \) in equation (ES.1), are fitted and plotted in Figure S3.1. Figure S3.2 complies with the microbial specific utilization rates \( kC's \) for those FAMEs. Unreported FAMEs \( kC \) values in Figure S3.2 (such as all the FAMEs of B60, and the saturated FAMEs in B20, B40, and B80) could not be fitted in equation (ES.1) because their degradation was governed by the abiotic transformation reaction, which rendered the contribution of the biological part in equation ES.1 very minimal and inaccurate. Only in the case of B100, accurate estimation of the FAMEs microbial specific utilization rates \( kC \) was possible. The very high standard errors observed in the \( kC \) estimates for some of the FAMEs (e.g. C18:3-ME in B80) in Figure S3.2 were due to the lack of enough biological data points in the period between 24–42 hours, the period in which the biological behavior started accelerating and became controlling.

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Figure S3.3 is a plot of the abiotic controls, in a semi-log scale, along with a nonlinear first order fit of equation EQ.2.

\[ S_{tot} = S_{tot0} e^{-\kappa_{tot} t} \]  

(ES. 2)

Aromatic Compounds. Figure S3.4 shows the biodegradation behavior of the aromatic compounds in the different petrodiesel/biodiesel blends. Toluene, o-xylene, and tetralin were not degraded in B0 and B20, while all the target analytes were degraded to below detection limits in B40 and B80. B60 biomass, however, was able to degrade only ethylbenzene during the course of the experiment.
Figure S3.2. Actual microbial utilization rates ($kC$'s) of the FAMEs in B0, B20, B40, B60, and B80 as color coded in figure key. Errorbars are ±1 standard error of the estimates. Unreported data were not able to estimate properly because of the abiotic transformation.

Figure S3.3. Deviation of the kinetics of abiotic transformation of the FAMEs from the first order kinetics. Scatter plots are FAMEs abiotic controls experimental data in B20 (△), B40 (○), B60 (□),
B80 (▼), and B100 (○). Errorbars are ±1 standard deviation of triplicate samples and dotted lines connect experimental data. Solid line is model nonlinear fit by equation ES.2.

Figure S3.4. Biodegradation of the targeted aromatic compounds in the petrodiesel/biodiesel blends. Tetralin (□), m-xylene (Δ), o-xylene (■), p-xylene (▼), toluene (●), and ethylbenzene (○). Errorbars are ±1 standard deviation of triplicate samples.
CHAPTER 4: AEROBIC BIODEGRADATION KINETICS AND MINERALIZATION OF FLAXSEED B100 AND B50, AND COMPARISON WITH THAT OF SOYBEAN B100, B60, AND B40 BIODIESEL BLENDS
Aerobic biodegradation kinetics and mineralization of flaxseed B100 and B50 biodiesels, and comparison with that of soybean B100, B60, and B40 biodiesel blends

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ABSTRACT. The aerobic biodegradation kinetics and mineralization of flaxseed biodiesel (Flax-B100) and a 50% flaxseed-biodiesel/petrodiesel blend (Flax-B50) by acclimated cultures were investigated in batch experiments. The results were analyzed using the kinetic model developed in Chapter 2, and were compared with the kinetic data of the biodegradation of soybean-biodiesel/petrodiesel blends obtained in Chapter 3. The abiotic disappearance of the fatty acid methyl esters (FAMEs) in Flax-B100 and Flax-B50 was so rapid that no biological rates were obtained for these compounds. The microbial specific utilization rates \( kC' \)s of the n-alkanes in Flax-B50 were very similar to the specific utilization rates observed in the Soy-B40 and Soy-B60 fuels, which were significantly higher than the specific utilization rates observed in unblended petrodiesel (B0). However, the biodegradation of n-alkanes in Flax-B50 proceeded at a faster total rate \( kCX \), because, according to our model, the initial concentration of the degrading biomass \( X_0 \) was higher in Flax-B50 compared to Soy-B40 and Soy-B60, even though the measured initial volatile suspended solids (VSS) in all the experiments were relatively the same (~100 mg-VSS/L). In addition, the extent of mineralization of flaxseed biodiesel blends was markedly higher for Flax-B50 compared to soybean biodiesel blends and pure petrodiesel.
4.1 Introduction

In Chapter 3, we investigated the aerobic biodegradation kinetics and mineralization of soybean-biodiesel/petrodiesel blends (B0, Soy-B20, Soy-B40, Soy-B60, Soy-B80, and Soy-B100, where B0 and Soy-B100 are unblended petrodiesel and unblended soybean biodiesel, respectively) by acclimated cultures. The use of the microbial kinetic model for the degradation of poorly soluble materials developed in Chapter 2 and shown in equation 1, allowed us to experimentally confirm that the biodiesel’s fatty acid methyl esters (FAMEs) are capable of enhancing the microbial specific utilization rate \((kC)\) of the n-alkanes by increasing the alkanes’ aqueous saturation concentration \(S_{sat}\).

\[
S_{tot} = S_{sat0} - \frac{kCX_0}{(YkC - K_d)}(e^{(YkC - K_d)t} - 1)
\]

Where \(S_{tot}\) = the observed total mass of residual substrate (i.e., alkane) divided by the volume of aqueous phase in the bottle (mg/L) at time \(t\) (hr),

\(X_0\) = initial concentration of degrading biomass (mg/L),

\(Y\) = biomass yield coefficient (g-biomass produced/g-substrate consumed),

\(k_d\) = biomass endogenous decay rate (hr\(^{-1}\)),

\(k\) = maximum specific substrate utilization rate (mg-substrate/mg-biomass–hr), and

\(C\) = constant, and is approximately equal \(S_{sat}/(K_s + S_{sat})\), where \(S_{sat}\) and \(K_s\) are the substrate saturation concentration in the aqueous phase and the Monod half-saturation constant (mg/L), respectively.

In this study, we investigated the aerobic biodegradation kinetics and mineralization of flaxseed biodiesel (Flax-B100) and its 50% blend with petrodiesel (Flax-B50) by acclimated cultures using the same methods previously developed in Chapter 3.
4.2 Materials and Methods

All the materials and methods for cultures acclimation, batch experiments, and liquid and gas phase chemical analyses were detailed in Chapter 3. Flaxseed virgin vegetable oil with fatty acids profile (% mass) of 5.5% C16:0, 5.5% C18:0, 16% C18:1, 15.75 C18:2, and 57.25% C18:3 was purchased from Botanic Oil Innovations Inc (Spooner, WI) and was transesterified in our laboratory. The transesterification reaction was carried out at a temperature of 60 – 70 °C for 35 minutes using 0.75% (w/w) sodium methanoxide as a catalyst dissolved in twice the required stoichiometric amount of methanol. Glycerol, a byproduct of the transesterification reaction, was separated in a separatory funnel for 1 hour, and the biodiesel was re-transesterified with 25% of the stoichiometric amounts of methanol to insure complete conversion. The mixture was separated again and was washed thoroughly with warm water (41 °C) until no methanol was detected in the wash water. A 1% (w/w) magnesium silicate was added in the biodiesel, and was mixed at 35 °C for 45 minutes to absorb un-reacted glycerides from the biodiesel [1]. A 15% (w/w) anhydrous sodium sulfate was added to the biodiesel, and was mixed at room temperature for 2 hours to absorb residual wash water [2]. The magnesium silicate and sodium sulfate were allowed to settle out overnight, and the biodiesel phase was filtered through glass filter paper (1 micron pore size).

In brief, the microbial cultures were acclimated in chemostats to 750 mg/L-d of each of the fuel blends for a period six months, after which biomass were harvested and frozen at –80 °C. Batch experiments were carried out in serum bottles at initial fuel and biomass concentrations of ~175 and ~100 mg/L, respectively. Chemical analysis consisted of sacrificial solvent extraction with dichloromethane (DCM) and Gas Chromatograph – Mass Spectrometer (GC–MS) analysis. Total CO₂ generated in the bottles was measured at 7- and 42-days (ultimate) by a GC system equipped with a Total Conductivity Detector (TCD).
4.3 Results and Analysis

FAMEs and Alkanes. All the FAMEs in the batch experiments were found to disappear very rapidly in the abiotic controls. Therefore, no microbial specific utilization rates for the FAMEs were obtained. The results of the C10 – C21 n-alkanes biodegradation in the Flax-B50 treatment are overlaid, in Figure 4.1, with the biodegradation data of the n-alkanes in petrodiesel (B0), Soy-B40, and Soy-B60 (from Chapter 3). The solid lines in Figure 4.1 are nonlinear fits for the experimental data of the different fuel treatments in equation 1. Figure 4.1 shows that the n-alkanes biodegradation in Flax-B50 was faster than B0, Soy-B40 and Soy-B60. The actual microbial net specific growth rate \( (YkC-k_d) \) and the initial total degradation rate \( (kCX_0) \) for each of the n-alkanes were estimated by nonlinear least squares regression. The microbial specific utilization rate \( (kC) \) for each of the n-alkanes was calculated from the regression parameter \( (YkC-k_d) \) using the maximum stoichiometric yield coefficient \( Y \) (using a bacterial cell formula of \( C_{60}H_{87}O_{23}N_{12}P \)) and measured endogenous decay rate \( k_d \) of 0.013–0.038 d\(^{-1}\) for all the cultures. The concentration of the initial biomass degrading a specific n-alkane \( (X_0) \) in equation 1 was subsequently calculated by dividing the estimated total initial rate \( (kCX_0) \) by actual specific utilization rate \( (kC) \) calculated from the growth rate \( (YkC-k_d) \). Figure 4.2a summarizes the actual specific utilization rates \( (kC) \) of the n-alkanes in B0, Flax-B50, Soy-B40, and Soy-B60, and Figure 4.2b presents the estimated values of \( X_0 \)’s for the n-alkanes in the fuel blends.

Figure 4.2a shows that the actual specific utilization rates \( (kC) \) of the n-alkanes in Soy-B40, Soy-B60, and Flax-B50 were significantly \( (p < 0.005) \) higher than that in B0. Figure 4.2a also shows that actual specific utilization rates \( (kC) \) of the n-alkanes in Soy-B40, Soy-B60, and Flax-B50 were roughly the same. Figure 4.2b shows that the calculated \( X_0 \) values for the n-alkanes were highest in the B0 treatment followed by Flax-B50, while \( X_0 \)’s of the individual n-alkanes in Soy-B40 and Soy-B60 treatments were roughly the same. Even though the \( kC \) values of the n-alkanes in Soy-B40, Soy-B60,
and Flax-B50 were relatively the same (Figure 4.2a), Figure 4.1 shows that the biodegradation of the n-alkanes was faster for Flax-B50 compared to Soy-B40 and Soy-B60. This can be explained by the higher $X_0$ values associated with the Flax-B50 treatment (Figure 4.2b), which caused the biodegradation of the n-alkanes to start at a higher total initial rate ($kCX_0$) according to equation 1. The same logic can be applied to the B0 treatment. Although the values of $X_0$ in B0 treatment were the highest (Figure 4.2b), the low $kC$ values associated with B0 n-alkanes (Figure 4.2a) rendered the biodegradation of the n-alkanes to proceed at a slower total initial rate ($kCX_0$) and slower specific growth rate ($Y_{kC - k_d}$) as well in the exponential term of equation 1. The mineralization efficiencies at 7- and 42- days of the Flax-B50 and Flax-B100 are overlaid with those of B0, Soy-B40, Soy-B60, and Soy-B100 in Figure 4.3. The mineralization of Flax-B50 was significantly ($p < 0.05$) higher than that of Soy-B40 and Soy-B60. However, the difference between the mineralization of Flax-B100 and Soy-B100 was statistically insignificant.

REFERENCES

Figure 4.1. Experimental data of C10 – C21 n-alkanes biological degradation in B0 (○), Soy-B40 (□), Soy-B60 (◊), and Flax-B50 (●). Errorbars are ±1 standard deviation of triplicate samples. Solid lines are regression fits by Equation 1.
Figure 4.2. Comparison of the parameters obtained by Equation 1 for the degradation of n-alkanes in B0, Soy-B40, Soy-B60, and Flax-B50: a) Actual specific microbial utilization rates ($k_C$) of the n-
alkanes in the different blends. b) Calculated initial degrading biomass concentrations ($X_0$) of the n-alkanes in the same fuel blends. d) The initial total degradation rates ($kCX_0$) of those n-alkanes in the same fuel blends. Errorbars are ±1 standard error of the estimates.

Figure 4. Comparison of the mineralization efficiencies (% CO$_2$) of the different petrodiesel/biodiesel blends by acclimated cultures after 7-day (black) and 42-day (grey) incubation periods. Bar-plots are experimental data and scatter-plots are linearly extrapolated from the experimental data of the unblended fuels. Errorbars are ±1 standard deviation of triplicate samples.
CHAPTER 5: PARTITIONING BEHAVIOR OF
PETRODIESEL/BIODIESEL BLENDS IN WATER
Partitioning behavior of petrodiesel/biodiesel blends in water

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ABSTRACT. The partitioning behavior of six petrodiesel/soybean-biodiesel blends (B0, B20, B40, B60, B80, and B100, where B100 is 100% unblended biodiesel) in water was investigated at various oil loads by the 10-fold dilution method. Five fatty acid methyl esters (FAMEs), C10 – C20 n-alkanes, and four aromatic compounds were targeted for analysis. Only the aromatic compounds were partitioned according to Raoult’s Law at all oil loads. The partitioning of the FAMEs and n-alkanes at higher oil loads was found to be orders of magnitude higher than the reported aqueous solubilities of those compounds, and directly correlated with the amount of oil load applied. Depth filtration of the water-accommodated fractions (WAFs) significantly reduced the observed concentrations of the FAMEs and n-alkanes, but did not appreciably affect the aromatic compounds. The FAMEs and n-alkanes concentrations in the filtered WAFs agreed with the aqueous solubilities of those compounds reported in the literature, but the n-alkanes showed progressive deviations from those solubilities with the increase of amount of biodiesel in the oil blends. Further dilution experiments on pure n-hexadecane confirmed the presence of a metastable colloidal phase that seems to be controlled by hydrophobic interactions and surface phenomena. The addition of biodiesel to the oil blend appeared to have a positive impact on the dissolved concentrations and the colloidal accommodation of the n-alkanes. Autoxidation of the biodiesel constituents was found to be significant, and increased with increasing oil load. Chemical products such as hexanal, butyl acetate, diethylene glycol monobutyl ether, and diethylene glycol monobutyl ether acetate were positively identified among the FAMEs autoxidation byproducts. Our data suggest a positive enhancement for biodiesel on the formation of the oil in water colloidal phase, possibly by forming a surfactant-cosurfactant-like pair of the FAMEs and their autoxidation byproducts.
5.1 Introduction

Biodiesel has been attracting special attention in the sustainable energy arena because of its potential in transforming the automotive and transportation industries. The renewable and versatile feedstock sources as well as the positive impact on reducing global emissions make biodiesel a very attractive investment for policymakers, market developers, and researchers. The need to understand the behavior and physical interactions of petrodiesel/biodiesel blends in the aquatic environment cannot be overemphasized. Recent attempts to investigate physical interactions and behavior of petrodiesel/biodiesel blends in the aquatic environment have been very modest [1, 2]. In contrast, physical interactions and rheological behavior of petrodiesel/biodiesel blends in combustion engines have been heavily investigated [3, 4].

The fundamentally different molecular structures of petrodiesel and biodiesel result in very different macroscopic physiochemical properties for both fuels. Biodiesel primarily consists of C16 to C22 fatty acid methyl esters (FAMEs) with various degrees of unsaturation [5]. Petrodiesel, on the other hand, is composed of 80% – 90% saturated hydrocarbons (primarily normal, branched, and cycloalkanes), 10 – 20% aromatic hydrocarbons (mainly alkylbenzenes), and <1% olefins [6]. Macroscopically, biodiesel is known to have a considerably higher viscosity and surface tension compared to petrodiesel [3, 7]. Both petrodiesel and biodiesel have limited solubility in water [8-10]. Yet FAMEs are slightly more soluble than n-alkanes because of the higher polarity of FAME molecules due to the presence of the carboxylic acid ester group as well as the double bonds [11, 12]. Those properties enable the FAME molecules to form hydrogen bonds with water at those moieties. The presence of double bonds on the FAME molecules also contributes to the autoxidative instability of the FAMEs [12].
When petrodiesel and biodiesel are blended together, the physiochemical properties of the oil mixtures can exhibit high uncertainty, and can be different than those of the pure parent oils. The higher aqueous solubility, viscosity, and different surface properties of biodiesel compared to petrodiesel, along with biodiesel’s autoxidative susceptibility, make the behavior and impact of petrodiesel/biodiesel spill in aquatic environments very hard to predict. This study investigates the partitioning behavior of low-sulfur petrodiesel/soybean-biodiesel blends (B0, B20, B40, B60, B80, and B100, where B100 is unblended biodiesel) in water at varying oil loads using the 10-fold dilution method.

5.2 Materials and Methods

Chemicals. Low-sulfur petroleum diesel (B0) was purchased from a BP diesel station (Cincinnati, OH). The average molecular weight of B0 was estimated by the Total Petroleum Hydrocarbon (TPH) centroid-of-chromatogram method developed by Gerhon [13] and adopted by Lee et al. [14], and was found to be ~195 g/mole with a 0.165 mole fraction of C10–C24 n-alkanes. Unblended soybean-methyl ester biodiesel (B100) was purchased from Peter Cramer North America (Cincinnati, OH). The average molecular weight of B100 was ~291.5 g/mole, with mole fractions of 0.145, 0.055, 0.206, 0.518, and 0.0759 for C16:0, C18:0, C18:1, C18:2, and C18:3 methyl esters, respectively. No synthetic antioxidants were added to B100. All the other fuel blends (B20, B40, B60, and B80) were volumetrically blended in the laboratory by splash mixing. Water used for the dilution experiments was of Super-Q grade, purified in our laboratory using Corning Mega Pure Water Purification System (Corning, NY). Palmitic acid methyl ester (99%) (C16:0-ME), palmitoleic acid methyl ester (99%) (C16:1-ME), stearic acid methyl ester (99%) (C18:0-ME), oleic acid methyl ester (99%) (C18:1-ME), linoleic acid methyl ester (99%) (C18:2-ME), linolenic acid methyl ester (99%) (C18:3-ME), and normal alkanes standard mixture (nC10-nC30), were all purchased from Sigma.
Aldrich (USA). All other chemicals, if not noted, were purchased from Sigma Aldrich or Fisher Scientific (USA).

**Dilution Experiments.** All the glassware was pre-cleaned and surface-deactivated with Sylon CT (Sigma), and baked at 280 °C. Dilution experiments were carried out in 500 mL glass Pyrex bottles. In triplicate samples, 400 mL Super-Q water (or 200 mL for the highest oil load) was poured in the bottles. Two-inch, pre-cleaned magnetic stir bars (Fisherbrand, USA) were inserted in the bottles and aliquots of B0, B20, B40, B60, B80, or B100 oils were gently introduced on the surface of the water in volumetric ratios of 1:1, 1:10, 1:100, and 1:1000 (oil:water) corresponding to oil loads of approximately 875, 87.5, 8.75, and 0.875 g-oil/L-water, respectively. The bottles were then capped with polyethylene caps and were stirred in the dark for a 24 hours period using Bell-Enmmium Digital Magnetic Stirrers (Bellco, USA). The stirring speed was gradually increased from 150, 200, 250, to 330 rpm with decreasing oil loads to maintain a constant vortex depth under the oil layer of 20 – 30% of the total depth of the water column as per the Chemical Response to Oil Spills: Ecological Effects Research Forum (CROSERF) [15] guidelines for the preparation of physically enhanced water accommodated fractions (WAFs). After equilibration, the samples were gently transferred to 500 mL glass seperatory funnels and were allowed to settle for 2 hours in the dark. Non-aqueous phase liquid (NAPL) was visible on the surface of the WAFs in all of the treatments at the end of the equilibration period.

**Chemical Analysis.** 150 mL of the WAFs were withdrawn from the bottom of the funnels. No oil droplets were visible in the withdrawn samples. A subsample of 20 mL was analyzed for total organic carbon (TOC) using a Shimadzu TOC-V CSH Analyzer (Tokyo, Japan) according to the manufacturer’s procedures. 90 mL of the remaining WAFs were spiked with 25 µl of a standard surrogate solution of C16:1-ME and deuterated nC16 in acetone, and were transferred to clean
seperatory funnels. The WAFs were then completely liquid-liquid extracted with 30 mL dichloromethane (DCM) twice, and the extracts were filtered through anhydrous sodium sulfate. Extracts were prepared in 1 mL volumes in GC vials and were 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 Series 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) (HP, Palo Alto, CA). The flow rate of the Helium carrier gas was 1 mL/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. Target analytes were the biodiesel FAMEs, the petrodiesel C10 – C20 n-alkanes and the 4 aromatic compounds ethylbenzene, p-xylene, m-xylene, and o-xylene. The detection limits were in the range of 5–10 µg/L for all the compounds. Surrogate recoveries of all extractions ranged from 80 to 120%. Periodically, extracts from different dilution treatments were analyzed on the GC-MS in scan mode to identify possible FAMEs autoxidation byproducts.

For the lowest oil load (0.875 g/L), another 90 mL of the WAF was filtered through fritted glass funnels (6 cm deep, 3 cm i.d., 1 µm pore size) filled with 3-mm surface-deactivated borosilicate glass beads (Chemglass, Vineland NJ) to filter out any particulate oil present in the WAFs. Filtrates were then extracted with DCM and concentrated 16-fold in a TurboVap II Evaporation System (Biotage, Charlotte NC) according to the manufacturer’s procedure, and analyzed on the GC-MS. Dilution experiments with pure n-hexadecane (99%) (Arcros Organics, USA) were subsequently conducted with and without depth filtration following the same experimental protocols outlined above.
5.3 Results and Analysis

Target Analytes. The results of the dilution experiments of petrodiesel/biodiesel blends in water can be conceptualized as a 3-D graph in which the x-axis is the percent of biodiesel in the blend, the y-axis is the dilution level or oil load, and the z-axis is WAF concentration. Figures 5.1, 5.2, and 5.3 present 2-D visualizations of the WAFs concentrations vs. dilution levels of the n-alkanes, FAMEs, and aromatic compounds, respectively. The WAFs concentrations vs. percent biodiesel of the n-alkanes, FAMEs, and aromatic compounds are shown in Figures S5.1, S5.2, and S5.3, respectively, in the Supporting Information (SI) section. Figure S5.4 in the SI section shows the calculated Log oil/water partitioning coefficient (Log[Koil/water]) vs. the measured aqueous concentrations of the targeted n-alkanes, aromatic compounds, and FAMEs in B0 and B100 treatments. Aqueous solubilities reported in the literature for C10 – C16 even number n-alkanes [16], FAMEs [9], and the aromatic compounds [17] in deionized water are overlaid on Figures 5.(1–3), and Figures S5.(1–3). Raoult’s Law, in the form of aqueous concentrations, was employed for calculating the aqueous solubilities of the target analytes in the WAFs according to equation 1,

\[ S_{\text{aq,WAF}}^{\text{sat}} = S_{\text{sat}}^0 X \]  

(1)

where

- \( S_{\text{aq,WAF}}^{\text{sat}} \) = aqueous solubility of the compound in the WAF (mg/L),
- \( S_{\text{sat}}^0 \) = reported aqueous solubility of the pure compound in deionized water at room temperature (mg/L), and
- \( X \) = mole fraction of the compound in the petrodiesel/biodiesel blend.

Examination of Figure 5.1 reveals that the partitioning of the n-alkanes in B0 and B20 in the filtered WAFs was very similar to the solubilities calculated by Raoult’s Law using the aqueous
solubilities reported by Franks [16]. However, the concentrations of n-alkanes in the filtered WAFs of B40, B60, and B80 were found to progressively deviate from the values reported by Franks [16] with the increase of the percent of biodiesel in the blends. Another important observation in Figure 5.1 is the water accommodation capacity of the n-alkanes at higher oil loads in the presence and absence of biodiesel. Figure 5.1 clearly shows that the accommodation level of the n-alkanes in B0 at 1:1 dilution was significantly (p < 0.0005) higher than that of 1:10 dilution, which accommodated n-alkanes at levels very close to those of 1:100 and 1:1000 dilutions. On the other hand, the accommodation of n-alkanes in B20, B40, B60, and B80 at the 1:1 and 1:10 dilutions were statistically insignificant, but were significantly higher (p<0.05) than the accommodation of n-alkanes at 1:100 and 1:1000 dilutions.

The partitioning of the FAMEs (Figure 5.2) showed a somewhat similar behavior to that of the n-alkanes. The partitioning of the C16:0, C18:1, C18:2, and C18:3 methyl esters at 1:1000 dilutions and in the filtered WAFs were in fair agreement with the values reported by Krop et al. [9] in all the petrodiesel/biodiesel blends. C18:0 methyl ester, however, seemed to always accommodate at levels higher than the value reported by Krop et al.[9] (~0.3 μg/L). The accommodation of the FAMEs at the higher oil loads was also found to exhibit a behavior similar to that of the n-alkanes. The accommodation of the FAMEs in B100 at 1:1 dilution was significantly higher (p<0.05) than that at 1:10. But the accommodation of the FAMEs in B20, B40, B60, and B80 at 1:10 dilution was equivalent to that of the 1:1 dilution.

The partitioning of the aromatic compounds in water (Figure 5.3) showed a different behavior than that of the n-alkanes and FAMEs. The partitioning of the aromatic compounds showed very weak dependence on the amount of applied oil at 1:1, 1:10, and 1:100 dilutions (Figures 5.3 and S5.3). Figure S5.3 also shows that Raoult’s Law is able to accurately describe the partitioning behavior of the aromatic compounds as a function of their mole fraction in the oil blends. In comparison, the n-alkanes
and FAMEs partitioning showed little to no dependence on their mole fraction in the oil blends at higher oil loads (Figures S5.1 and S5.2, respectively). In other words, Figures S5.1 and S5.2 show that for a given oil load, the partitioning of the n-alkanes and the FAMEs, respectively, was not a strong function of the volume fraction (and hence the mole fraction) of biodiesel in the oil blends, whereas the partitioning of the aromatic compounds decreased linearly with the decrease of petrodiesel in the oil blends from B0 to B80, as it would be predicted by Raoult’s Law (Figure S5.3).

**Pure n-hexadecane.** In order to investigate the presence of colloidal oil in the water phase, the 10-fold dilution experiment was carried out on pure n-hexadecane. The WAFs were solvent extracted with and without depth filtration and the results are compiled in Figure 5.4. The unfiltered WAFs showed strong dependence on the amount of n-hexadecane applied. The concentrations of n-hexadecane in the unfiltered WAFs ranged from 9.8 μg/L for the lowest oil load up to 2200 μg/L at the highest oil load. The concentrations of n-hexadecane in filtered WAFs, however, were relatively constant regardless of the magnitude of the oil load. The aqueous solubility of n-hexadecane in deionized water was, therefore, calculated to be 3.37 ± 0.97 μg/L (n = 12). Franks[16] reported a value of n-hexadecane solubility 6.3 ± 0.76 μg/L. Sutton and Calder [18] reported a solubility limit of n-hexadecane of 0.90 ± 0.14 μg/L. The absolute discrepancies in those values are negligible given the stochastic nature of colloidal accommodation and its sensitivity to trace impurities possibly present in the “pure n-hexadecane” among other factors [10, 19, 20].

**Autoxidation of FAMEs.** Total organic carbon (TOC) was measured in all the WAFs of petrodiesel/biodiesel blends and the results are compiled in Figure 5.5. The accommodation of TOC at the higher oil loads was found to linearly increase with the increase of the percent of biodiesel in the blends. At 1:1 dilution, the TOC accommodation increased from 50.7 mg-TOC/L for B0 to 95.4 mg-TOC/L for B100. Similarly at 1:10 dilution, the TOC accommodation increased from 10 mg-TOC/L
for B0 to 15.77 mg-TOC/L for B100. Interestingly, the accommodation of TOC at the lower oil loads (1:100 and 1:1000 dilutions) decreased linearly with the increase in percent of biodiesel in the blends. At 1:100 dilution, TOC accommodation decreased from 4.3 mg-TOC/L for B0 down to 2.8 mg/L for B100. Similarly, at 1:1000 dilution, the TOC accommodation decreased from 3.74 mg-TOC/L for B0 down to 1.4 mg-TOC/L for B100. It is important to point out that those TOC values are considerably higher than the carbon equivalents of the accommodated n-alkanes, FAMEs, and aromatic compounds at the same dilution levels. For example, the sum of the FAMEs carbon equivalents in the B100 WAF at the 1:1 and 1:10 dilutions are ~7.40 and 1.11 mg-carbon/L, respectively. The measured TOC values for the same WAFs, however, were found to be 95.64 and 15.77 mg-TOC/L, respectively. Clearly, the FAMEs carbon equivalents are not the only form of organic carbon present in their WAFs. Chromatographic GC-MS scans of the WAF extracts revealed numerous small peaks and a few massive ones that were not present in the parent oils. We were able to identify some of the largest peaks as butyl acetate, hexanal, diethylene glycol monobutyl ether, and diethylene glycol monobutyl ether acetate by matching the ion-chromatographs against a database. The identities of those peaks were confirmed by the standard addition of the pure chemicals. In fact, those chemical species have been reported amongst the possible products of lipids oxidation [21-24].

5.4 Discussion

The aqueous solubility of paraffin hydrocarbons has always been the subject of intensive research. McAuliffe [25, 26] was among the first investigators who pointed out a discontinuity in the aqueous solubility behavior of n-alkanes at the C11 chain length, where a change from true solubility to a state of aggregate accommodation appears to occur. Baker [27] attributed this higher accommodation to intermolecular association between the long chain n-alkane molecules, which, according to him, gives rise to clusters of n-alkanes comparable to micelles formed in dilute solutions
of colloidal electrolytes. It was made clear by McAuliffe [26], Baker [27], and Franks [16] that the aqueous solubility of C12 – C36 n-alkanes is considerably greater than what would be predicted from the shorter n-alkanes. Hildebrand [28] warned against the use of the term “hydrophobicity” in describing the apparent antipathy between nonpolar hydrocarbons and water by showing that the molecular forces between those molecules are actually attractive and not repulsive, and are of the same magnitude as of the hydrocarbon-hydrocarbon attractive forces [29]. Many other researchers confirmed those finding using different approaches [30-32]. Tanford [29] showed that the unfavorable free energy associated with this “hydrophobicity” per unit area of contact between bulk hydrocarbon and water, is about 3-folds larger than a similar figure derived from solubility data per unit area between a single dissolved hydrocarbon molecule and water. This discrepancy signifies the importance of macroscopic properties such as interfacial surface tension on the aqueous solubility of hydrocarbons at the molecular level.

More recently, Chandler and co-workers [32-34] proposed a theory that describes the formation of micelles (or aggregation) of nonpolar molecules that combines hydrophobic interactions and surface properties as the main driving mechanism. Chandler proposed that the apparent effective oil-oil attraction that drives aggregation is actually a result of the strong water-water hydrogen bond attractive forces, which induces segregation of the oil from water. According to Chandler [32], the free energy of solvating small hydrophobic species scales linearly with the volume of the solute. This is attuned with what was already proposed by other authors [16, 26, 27]. But when the size of the hydrophobic solute becomes large enough compared to the size of the solvent, Chandler reasoned that the free energy of solvating the solute becomes a linear function of the area of the interface between the hydrophobic solute and water. According to Chandler’s calculations [33], the surface area at which the crossover of the regimes occurs is ~1 nm², after which the accommodation of the hydrophobic species would
require the breakage, instead of re-distribution, of water-water hydrogen bonds in the vicinity of the solute. Therefore, the free energy required to solvate well separated hydrophobic molecules can exceed the free energy required to solvate large clusters or micelles of the same species [34], which in turn serves as the driving force in the formation of the micelles.

The findings we presented in this paper are in agreement with the reported aqueous solubility of the target analytes as well as the current understanding of micelles formation driven by the hydrophobic effects [34]. The measured aqueous solubility of the n-alkanes in B0 filtered WAF is in good agreement with the values reported by Franks [16]. On the other hand, the observed aqueous solubility of the n-alkanes in the filtered WAFs of B20, B40, B60, and B80 was found to progressively increase with the increase of percent biodiesel, compared to the solubility of those compounds in B0 WAF and reported by Franks (Figure 5.1). It appears that the presence of biodiesel in the fuel blends has a positive effect on the dissolved concentrations of n-alkanes. The same was not true for the FAMEs. The observed aqueous solubility of the FAMEs in the filtered WAFs, which were also in fair agreement with the values reported by Krop et al. [9], were neither enhanced nor suppressed in the presence of petrodiesel (Figure 5.2). The aromatic compounds, on the other hand, were found to closely obey Raoult’s Law at all the oil loads (Figures 5.3 and S5.3), and their concentrations in the WAFs were not appreciably affected by filtration.

The formation of colloids of n-alkanes and FAMEs at higher oil load was found to directly correlate with amount of oil load applied (Figures 5.1 and 5.2), but did not for the aromatic compounds (Figures 5.3 and S5.3). Further dilutions experiments on pure n-hexadecane revealed that the saturation concentrations of n-hexadecane in the filtered WAFs were relatively constant regardless of the oil load (Figure 5.4). The presence of biodiesel in the oil blends had a positive impact on the formation and stabilization of oil micelles in water. The water accommodated fractions of n-alkanes and FAMEs were
significantly enhanced at the 1:10 dilutions in B20, B40, B60, and B80 compared to B0 and B100, respectively. The excessive autoxidation of the FAMEs, revealed by TOC analysis (Figure 5.5) and confirmed by GC-MS scans, could indeed contribute to the colloidal stability of the oils. In fact, the chemical structures of the identified autoxidation byproducts of the ethoxylated hydrocarbons and aldehydes, among others, have been shown to possess favorable surface active properties [35-38], while the FAMEs themselves have been demonstrated to have surfactant properties in crude oil and coal tar mobilization studies [39-41]. Therefore, we find the FAMEs partitioning in water and their autoxidation byproducts are able of enhancing the colloidal stability of petrodiesel/biodiesel blends in water, possibly by lowering the interfacial surface tension due to the adsorption of the surface active autoxidation byproducts on the oil/water interface, while the FAMEs molecules, having an amphiphilic character [30], can further enhance the colloidal stability of the oil by forming more stable reversed micelles of the oil in water.

**APPENDIX: Supporting Information**

Figure S5.1. n-alkanes WAF concentrations vs. percent biodiesel in the blend, Figure S5.2. FAMEs WAF concentrations vs. percent biodiesel in the blend, and Figure S5.3. aromatic compounds WAF concentrations vs. percent biodiesel in the blend.
REFERENCES


Figure 5.1. n-alkanes WAF concentrations at dilution levels 1:1 (●), 1:10 (∇), 1:10² (■), 1:10³ (◇), and 1:10³ filtered (▲); $S_{aq,WAF}^{sat}$ n-alkanes concentrations calculated by equation 1 using solubilities reported by Franks [Ref. 16] (√) in: (a) B0, (b) B20, (c) B40, (d) B60, and (e) B80. Means and standard deviations are calculated geometrically (n=3). Error bars are ± 1 geometric standard deviation.
Figure 5.2. FAMEs WAF concentrations at dilution levels 1:1 (●), 1:10 (▼), 1:10² (■), 1:10³ (◇), and 1:10³ filtered (▲); $S_{aq,WAF}^{sat}$ FAMEs concentrations calculated by equation 1 using solubilities reported by Krop et al. [Ref. 9] (◇) in: (a) B20, (b) B40, (c) B60, (d) B80, and (e) B100. Means
and standard deviations are calculated geometrically (n=3). Error bars are ± 1 geometric standard deviation.

Figure 5.3. Target aromatic compounds WAF concentrations at dilution levels 1:1 (●), 1:10 (▽), 1:10² (■), 1:10³ (◇), and 1:10³ filtered (▲); $S_{aq,WAF}$ aromatic compounds concentrations calculated by equation 1 using solubilities reported by Sanemasa et al. [Ref. 17] (▴) in: (a) B0, (b) B20, (c)
B40, (d) B60, and (e) B80. Means and standard deviations are calculated arithmetically (n=3). Error bars are ± 1 arithmetic standard deviation.

Figure 5.4. WAFs of n-hexadecane dilution experiments before depth filtration (solid) and after filtration (hatched). Means and standard deviations are calculated geometrically (n=3). Error bars are ± 1 geometric standard deviation.

Figure 5.5. Total organic carbon (TOC) WAF concentration as a function of oil blend at 1:1 (☉), 1:10 (▼), 1:10² (□), and 1:10³ (◇) dilution levels. Means and standard deviations are calculated arithmetically (n=3). Error bars are ± 1 arithmetic standard deviation.
APPENDIX: SUPPORTING INFORMATION

Partitioning behavior of petrodiesel/biodiesel blends in water

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Figure S5. 1. n-alkanes WAF concentrations in B0 (■), B20 (○), B40 (△), B60 (□), B80 (▲), and sat 
WAFaqS n-alkanes concentrations calculated by equation 1 using solubilities reported by Franks
[Ref. 16] (●) at dilution levels: (a) 1:1, (b) 1:10, (c) 1:10², (d) 1:10³, (e) and 1:10³ filtered.
Means and standard deviations are calculated geometrically (n=3). Error bars are ± 1 geometric
standard deviation
Figure S5. 2. FAMEs WAF concentrations in B20 (■), B40 (■), B60 (■), B80 (■), B100 (■), and $S_{\text{aq,WAF}}^{\text{sat}}$ is FAMEs concentrations calculated by equation 1 using solubilities reported by Krop et al. [Ref. 9] (■) at dilution levels: (a) 1:1, (b) 1:10, (c) 1:10^2, (d) 1:10^3, (e) and 1:10^3 filtered. Means and standard deviations are calculated geometrically (n=3). Error bars are ± 1 geometric standard deviation.
Figure S5.3. Target aromatic compounds WAF concentrations in B0 ( ), B20 ( ), B40 ( ), B60 ( ), B80 ( ), and $S_{eq,WAF}^{sat}$ is aromatic compounds concentrations calculated by equation 1 using solubilities reported by Sanemasa et al. [Ref. 17] ( ) at dilution levels: (a) 1:1, (b) 1:10, (c)
1:10², (d) 1:10³, (e) and 1:10³ filtered. Means and standard deviations are calculated arithmetically (n=3). Error bars are ± 1 arithmetic standard deviation.

Figure S5. 4. Calculated oil/water partitioning coefficients vs. measured aqueous concentrations (mole/L) (in log-log scale) of the n-alkanes and aromatic compounds in B0 treatment, and the FAMEs in B100 treatment. Error-bars are ± 1 log standard deviation of triplicate samples.
CHAPTER 6: ACUTE TOXICITY OF THE WATER ACCOMODATED FRACTIONS (WAFS) OF SIX PETRODIESEL/SOYBEAN-BIODIESEL BLENDS USING MICROTOX BIOASSAY
Acute toxicity of the water accommodated fractions (WAFs) of six petrodiesel/soybean-biodiesel blends using Microtox bioassay

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ABSTRACT. The acute toxicity of the water accommodated fraction (WAF) of commercial soybean biodiesel/petrodiesel blends (B0, B20, B40, B60, B80, and B100, where B100 is 100% biodiesel) were investigated at different oil loads using the Microtox bioassay and the bacterium *Vibrio fischeri* as a test species. Five fatty acid methyl esters (FAMEs), C10 – C24 n-alkanes, four aromatic compounds, methanol, and the total organic carbon (TOC) content were all targeted for the chemical analysis. At high oil loads (87.5 – 875 g/L), the acute toxicity of the WAFs was significantly higher for the fuel blends containing biodiesel (i.e. B20 – B100); while at the lowest oil load (~0.875 g/L) the acute toxicity of the WAFs appeared to increase linearly with increasing petrodiesel in the blend. At an oil load of 8.75 g/L, the WAFs of all the blends appeared to have a comparable toxicity. At the high oil loads, chemical analysis of the WAFs confirmed the presence of high concentrations of FAMEs’ autoxidation byproducts predominated by the chemical species 2-(2-butoxyethoxy) ethanol, 2-(2-butoxyethoxy) ethanol acetate, and n-butyl acetate, each having a 15-minute EC50 of 865, 29, and 55.8 mg/L, respectively. The pure compounds of the unsaturated FAMEs (C18:1, C18:2, and C18:3) as well as C10 and C16 n-alkanes were found to be nontoxic to *V. fischeri* when present in deionized water at their reported solubility limits. However, the equilibrated WAFs of C18:1 and C18:3 pure FAMEs, which contained high amounts of TOC, were found to be highly toxic. Meanwhile the WAF of C18:2 pure FAME, which contained low amount of TOC, did not show any toxic effects to the test species. It was concluded that at high oil loads, the acute toxicity of the WAFs of petrodiesel/biodiesel blends was caused by the autoxidation byproducts of the FAMEs; while at low oil loads, the toxicity of the WAFs appeared to be caused primarily by the aromatic compounds present in the petrodiesel. The addition of 225 ppm of butylated hydroxytoluene (BHT) antioxidant to B100 did not appear to affect the concentration of the autoxidation byproducts in the WAF measured as TOC, but resulted in a slight decrease in its toxicity. It was also found that the major autoxidation byproducts identified in the
WAFs of the commercial biodiesel fuel blends were not present in the WAFs of the pure unsaturated FAMEs. Additionally, the toxicity of the WAF of another batch of soybean biodiesel that was transesterified in our laboratory did not exhibit any toxicity to *V. fischeri*, and elicited a negligible amount of TOC in water. We concluded that the transesterification and clean-up processes of biodiesel might be a more critical factor in determining the aquatic toxicity of biodiesel blends compared to the source of feedstock itself.

### 6.1 Introduction

The invention of the diesel engine in the 1890s placed the humans’ social and technological development on an accelerated track. The industrial boom of the 20th century resulted in the introduction of large amounts of foreign chemicals into the environment. Emissions of greenhouse gases from burning fuels and release of hazardous chemicals into land and aquatic environments are major mechanisms by which human actions are degrading natural environments. The introduction of biodiesel, as an alternative fuel, is viewed by technology experts as a successful strategy in combating climate change and depletion of natural resources simultaneously. Such an introduction requires a thorough understanding of biodiesel’s fate in the environment as well as its effects on living systems.

Commercial production methods of biodiesel typically involve the transesterification of plants and animals fats with methanol in the presence of a catalyst to produce fatty acid methyl esters (FAMEs) and a glycerol byproduct. A clean-up procedure is employed after the transesterification reaction to remove the glycerol, excess methanol, and the catalyst from the biodiesel. Different sources of biodiesel feedstocks were found to result in different physiochemical properties and toxicological profiles for the fuels [1, 2]. Toxicological studies on petrodiesel/biodiesel blends have produced mixed
results for different toxicity tests, test organisms, and biodiesel feedstocks [2-6]. Leite et al. [2] tested the toxicity of the water soluble fractions (WSFs) of castor oil, palm oil, and waste cooking oil biodiesels using Tetraselmis chuii (microalgae) and Echinometra lucunter (sea urchin) for culture growth inhibition and early life stage toxicities, respectively. The highest toxicity of the WSFs was found to be of castor oil biodiesel, followed by waste oil and palm oil biodiesels in both toxicity assays. The authors found their toxicity results to correlate with aqueous phase methanol detected in the WSFs, which probably resulted from the hydrolysis of the methyl esters in water. They concluded that the toxicity of the WSF was probably caused by the methanol. Hollebone et al. [3] investigated the toxicity of the water accommodated fractions (WAFs) of petroleum diesel and different biodiesels produced from soybean oil, canola oil, and waste fry oil to Daphnia magna (water flea), Oncorhynchus mykiss (rainbow trout), and the bioluminescent bacterium Vibrio fischeri. The sensitivity of the test species to the WAFs significantly varied from one organism to another, but the order of the toxicities of WAFs of the different oils was generally the same for all the organisms. The results of Hollebone et al. [3] indicated that for all the tested organisms, petroleum diesel was the most toxic, followed by soybean biodiesel, animal fat biodiesel, and then canola biodiesel being the least toxic. Khan et al. [4] studied the toxicity of recycled cooking oil biodiesel/petrodiesel blends using D. magna and O. mykiss. They also concluded that biodiesel is considerably less toxic than petroleum diesel in the tested organisms.

The acute toxicity of rapeseed biodiesel and petroleum diesel were tested in an extensive list of aquatic plants and animals by Birchall et al. [5]. Tests on D. magna, O. mykiss, Gammarus pulex (water louse), and Lymnaea peregra (water snail) resulted in markedly higher mortality rates on petrodiesel compared to biodiesel. Similarly, Lemna minuta (least duckweed), Myriophyllum spicatum (water milfoil), and five green and blue-green microalgae species were more severely affected by petroleum diesel compared to biodiesel. Lemna minor (common duckweed) was found to be affected by both fuels.
equally. According to the same authors, biodiesel appeared to severely affect the growth of *Elodea canadensis* (North American pondweed) at low oil loads (0.0125 – 1.25 g/L), but did not produce such an effect at higher oil loads (12.5 – 125 g/L). In general, the results of Birchall et al. [5] indicate that the toxicity of the WAF significantly varies when the oil load is increased or decreased in large increments. In this paper, we investigated the acute aquatic toxicity of the WAFs of petrodiesel/soybean biodiesel blends (B0, B20, B40, B60, B80, and B100, where B100 is 100% biodiesel) in an oil loading range of 0.875 – 875 g-oil/L-water, by the Microtox bioassay using the bacterium *Vibrio fischeri* as test species. Comprehensive chemical analysis of the WAFs was conducted to identify chemical groups responsible of the toxic effects.

### 6.2 Materials and Methods

**Chemicals.** Low-sulfur petroleum diesel (B0) was purchased from a BP diesel station (Cincinnati, OH) with a mole fraction of 0.165 of C10 – C24 n-alkanes. Unblended soybean-methyl ester biodiesel (B100) was purchased from Peter Cramer North America (Cincinnati, OH) with mole fractions of 0.145, 0.055, 0.206, 0.518, and 0.0759 for C16:0, C18:0, C18:1, C18:2, and C18:3 methyl esters, respectively. No synthetic antioxidants were added to B100 initially; but when noted, 225 ppm butylated hydroxytoluene (BHT) (99%) antioxidant was added in B100 to test its effect. All the other fuel blends (B20, B40, B60, and B80) were volumetrically blended in the laboratory by splash mixing. The deionized water used for the preparation of the WAFs was of Super-Q grade purified in our laboratory using a Corning Mega Pure Water Purification System (Corning, NY). Palmitic acid methyl ester (99%) (C16:0-ME), palmitoleic acid methyl ester (99%) (C16:1-ME), stearic acid methyl ester (99%) (C18:0-ME), oleic acid methyl ester (99%) (C18:1-ME), linoleic acid methyl ester (99%) (C18:2-ME), linolenic acid methyl ester (99%) (C18:3-ME), n-decane (99%), n-hexadecane (99%), 2-(2-butoxyethoxy) ethanol (99.2%), 2-(2-butoxyethoxy) ethanol acetate (99.2%), n-butyl acetate (98%),
n-hexanal (98%), n-hexanoic acid (98%), as well as an n-alkanes calibration standard mixture (C10 – C30), were all purchased from Sigma Aldrich (USA). Methanol (Optima Grade) was purchased from Fisher Scientific (USA). All other chemicals, if not noted, were either purchased from Sigma Aldrich or Fisher Scientific (USA).

**Preparation of the Water Accommodated Fractions (WAFs).** All of the glassware was pre-cleaned and baked at 450 °C, surface-treated with Sylon CT (Sigma), and dried at 280 °C. In triplicate samples, 400 mL Super-Q water (or 200 mL for the highest oil load) was poured in 500 mL glass Pyrex bottles. Two-inch, pre-cleaned magnetic stir bars (Fisherbrand, USA) were inserted in the bottles, and aliquots of B0, B20, B40, B60, B80, or B100 oils were gently introduced on the surface of the water in volumetric ratios of 1:1, 1:10, 1:100, and 1:1000 (oil:water) corresponding to oil loads of approximately 875, 87.5, 8.75, and 0.875 g-oil/L-water, respectively. The bottles were capped with polyethylene caps and were stirred in the dark for 24 hours using Bell-Ennium Digital Magnetic Stirrers (Bellco, USA). The speed of the stirrers was steadily increased from 150 rpm at the 1:1 dilution to 330 rpm at the 1:1000 dilution to maintain a constant vortex depth of 20 – 30% of the total depth of the water column under the oil/water interface as prescribed by the guidelines of the Chemical Response to Oil Spills: Ecological Effects Research Forum (CROSERF) [7] for the preparation of physically enhanced water accommodated fractions (WAFs). After equilibration, the samples were gently transferred to 500 mL glass separatory funnels and were allowed to settle in the dark for 2 hours.

**Chemical Analysis.** Volumes of 150 mL of the WAFs were withdrawn from the bottom of the funnels. A subsample of 20 mL was taken for the Microtox test. Another subsample of 20 mL was analyzed for total organic carbon (TOC) using a Shimadzu TOC-V CSH Analyzer (Tokyo, Japan) according to the manufacturer’s procedures. A third subsample of 1 mL was spiked with an ethanol internal standard and was analyzed for aqueous phase methanol using an HP Agilent 6890 Plus Gas
Chromatograph (GC) (Palo Alto, CA) equipped with a flame ionization detector (FID) and an 80/120 Carbopack B-DA/4% Carbowax 20M packed column (Supelco), with N₂ carrier gas at a flow rate of 15 mL/min. The inlet was operated in splitless mode at a temperature of 200 °C, and the oven temperature program was the following: hold at 35 °C for 0 min, ramp at 4 °C/min to 55 °C, ramp at 10 °C/min to 155 °C, and hold at 155 °C for 5 min. The methanol detection limit was ~ 0.25 mg/L.

A final subsample of 90 mL of the remaining WAF was spiked with 25 µl of a standard surrogate solution of C16:1-ME and deuterated n-C16 in acetone, and was transferred into a clean separatory funnel. The transferred WAF was then completely liquid-liquid extracted with 30 mL dichloromethane (DCM) twice, and the extract was filtered through anhydrous sodium sulfate. DCM extracts were prepared in volumes of 1 mL in GC vials and were spiked with 10 µl of an internal standard solution containing C11:0-ME, C13:0-ME, deuterated n-C10, and deuterated n-C20 in DCM, and were analyzed on an HP Agilent 5890 Series 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) (Palo Alto, CA). Both the inlet and the detector interface temperatures were held at 320 °C, and the inlet was operated in splitless mode. The flow rate of the Helium carrier gas was held at 1 mL/min and 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. Target analytes were the biodiesel FAMEs (C16:0-ME, C18:0-ME, C18:1-ME, C18:2-ME, and C18:3-ME), the petrodiesel C10 – C24 n-alkanes, and the 4 aromatic compounds ethylbenzene, p-xylene, m-xylene, and o-xylene. The detection limits were in the range of 5–10 µg/L for all of the compounds. Surrogate recoveries of all extractions ranged from 80 to 120%.

The DCM extracts were also analyzed on the GC-MS in scan mode to identify possible FAMEs autoxidation byproducts. Ion chromatograms of the detected peaks were matched against the HP
Agilent EnviroQuant Chemstation (version A.03.00) standard database, and the identities of those peaks were confirmed by standard chemical addition of the pure compounds. The concentrations of the positively identified autoxidation byproducts in the WAFs were roughly estimated by external calibration of those compounds on the GC-MS scan mode. The partitioning of the autoxidation byproducts between the DCM solvent and the aqueous phase was also estimated by DCM extraction of standard solutions 5 or 10 mg/L of the pure chemicals in water, following the same extraction procedure outlined above.

**Microtox Acute Toxicity Test.** In the Microtox acute toxicity test, the gram-negative bioluminescent seawater bacterium *Vibrio fischeri* NRRL B-11177 (formerly known as *Photobacterium phosphoreum*) is exposed to serial dilutions of the contaminant, and the reduction in luminescence emission is measured in a luminometer [8]. The toxicity endpoint of the test was the 15-min EC$_{50}$ (%), which is defined as the effective concentration of the toxicant at which luminescence emission is reduced by 50% compared to a negative control, after 15 minutes of exposure time.

Lyophilized *V. fischeri* standard cultures were purchased from SDI Strategic Diagnostics Inc. (Newark, DE), and were kept at -21 °C until the time of analysis. Standard reconstitution solution, and standard diluents and osmotic adjustment solutions were also purchased from the same vendor. The test was carried out on a Microtox Analyzer Model 500 (Azur Environmental), following the manufacturer’s standard protocols. The pH of the samples was adjusted to 6 – 8 using NaOH or HCl as per the manufacturer’s standard procedures to phase out any pH induced toxicity. The data was collected by the computer software MicrotoxOmni version 1.18 (Azur Environmental) which calculated the 5 and 15 minute EC$_{50}$’s automatically. Phenol (99%) positive reference toxicant controls and dilution water negative controls were included every other run to sustain optimal and stable conditions.
6.3 Results and Analysis

The results of the chemical analysis and acute toxicity of the WAFs are compiled in Figure 1. The sum of the C10 – C24 n-alkanes, the sum of the targeted aromatic compounds, the sum of the FAMEs, methanol, and the concentration of total organic carbon (TOC) (all as scatter plots) are overlaid with the measured EC$_{50}$’s of the WAFs (bar plots). Figure 1 a and b show that at high oil loads (1:1 and 1:10 dilutions), the WAFs of B20, B40, B60, B80, and B100, which had very similar EC$_{50}$’s, were significantly (p<0.005) more toxic than the WAF of B0. At the dilution of 1:100 (Figure 1c), the differences in the EC$_{50}$’s of all WAFs were statistically insignificant (p > 0.05). However at 1:1000 dilution (Figure 1d), the toxicity of the WAFs appeared to increase with higher percentages of petrodiesel in the blends. The concentration of methanol in the WAFs of the 1:1 dilution was found to increase almost linearly from 2.04 mg/L in the WAF of B20 up to 13.49 mg/L in the WAF of B100. In the 1:10 dilution, the concentration of methanol was below the detection limit in the WAF of B20 (~0.25 mg/L), but also increased steadily to 1.31 mg/L in the WAF of B100. No methanol was detected in the WAFs of 1:100 and 1:1000 dilutions.

The toxicity of the WAFs seemed to correlate fairly well with the measured TOC values, but neither the n-alkanes nor the FAMEs correlated with the EC$_{50}$’s of the WAFs. On the other hand, the aromatic compounds were found to correlate with the EC$_{50}$ and TOC of the WAFs at the lowest oil load (1:1000 dilution). Figure 1 also revealed that the measured TOC concentrations in the WAFs (mg-TOC/L), and especially for the 1:1 and 1:10 dilutions, were markedly higher than the sum of the concentrations of methanol, n-alkanes, FAMEs, and aromatic compounds together expressed in mg-carbon/L. Untargeted higher molecular weight compounds present in the petrodiesel are not expected to partition in such high concentrations in the aqueous phase, while the 5 targeted FAMEs account for about 95% of the mass of the biodiesel.
Spectroscopic GC-MS scans of the WAFs revealed a large number of peaks with varying sizes present in the DCM extracts of the fuels contained biodiesel. The identities of the largest peaks were confirmed by standard chemical addition to correspond to diethylene glycol monobutyl ether (IUPAC nomenclature: 2-(2-butoxyethoxy) ethanol, DEGBE thereafter), diethylene glycol monobutyl ether acetate (IUPAC nomenclature: 2-(2-butoxyethoxy) ethanol acetate, DEGBE-acetate thereafter), and n-butyl acetate. Another two smaller peaks corresponded to n-hexanal and n-hexanoic acid. A medium-size peak identified by the computer software as 9 oxo-nonanoic acid methyl ester (92% match) did not match the peak of the pure compound when spiked in the WAF sample.

The 15-min EC$_{50}$’s of the pure DEGBE, DEGBE-acetate, and butyl acetate measured using Microtox were found to be 865, 29, and 55.8 mg/L, respectively. Further analysis on the toxicity of the DEGBE and DEGBE-acetate pure compounds showed non-additive effects for their EC$_{50}$’s. For example, the EC$_{50}$ of a solution of 2.5 g/L DEGBE was 38.55% and the EC$_{50}$ of a solution of 100 mg/L DEGBE-acetate was 27.1%. However, the EC$_{50}$ of a solution of 2.5 g/L DEGBE and 100 mg/L DEGBE-acetate was 25.36%. The toxicity of methanol to *V. fischeri* at the concentrations measured in the WAFs was found to be negligible. No toxic effect was measured for methanol at concentrations up to 10,000 mg/L (EC$_{50}$ > 100%).

The concentration of the identified autoxidation byproducts in WAFs was roughly estimated by running an external calibration curve for those compounds on the GC-MS in scan mode. The partitioning of those compounds between the DCM solvent phase and the aqueous phase was also estimated by standard spikes of the pure compounds in deionized water followed by liquid-liquid extraction with DCM. The DCM/water partitioning coefficients (Log P$_{DCM/water}$) of the autoxidation byproducts were found to be -0.004, 0.56, and 0.48 for DEGBE, DEGBE-acetate, and butyl acetate, respectively. Values reported in the literature for the octanol/water partitioning coefficients (Log
Poct/water) for those compounds are found to reasonably agree with values reported in this study for Log $P_{DCM/water}$ [9, 10]. This means that 51%, 77%, and 75% of the total DEGBE, DEGBE-acetate, and butyl acetate, respectively, partitioned to the DCM phase during solvent extraction. The estimated concentrations of the autoxidation byproducts in the WAFs are summarized in Table 1. Significant amounts of DEGBE, DEGBE-acetate, and butyl acetate were detected at the 1:1 and 1:10 dilutions. But at the 1:100 and 1:1000 dilutions, negligible to non-detectable amounts of the autoxidation byproducts were found in the WAFs. At the 1:100 dilution, only B80 and B100 contained 0.74 – 0.87 mg/L DEGBE, 0.04 – 0.05 mg/L DEGBE-acetate, and 0.36 – 0.46 mg/L butyl acetate.

The toxicities of the pure unsaturated FAMEs were investigated in greater detail. We measured the EC$_{50}$'s of standard solutions of pure C18:1-ME, C18:2-ME, or C18:3-ME dissolved in deionized water at their reported solubility limits (3.7, 21, and 91 $\mu$g/L for C18:1, C18:2, and C18:3 methyl esters, respectively) [11]. None of the unsaturated FAMEs were found toxic to the test organism when presented at the solubility limit. On the contrary, when the FAMEs were present at the solubility limit, they had a positive effect (i.e. hormesis) on the metabolism (i.e. light emission) of *V. fischeri*. Similarly, the pure compounds of C10 and C16 n-alkanes dissolved at their solubility limits (~19.75 and ~6.3 $\mu$g/L for n-C10 and n-C16, respectively) [12] did not produce any toxic effects.

Further, WAF experiments on the pure unsaturated FAMEs were prepared at 1:100 dilution level following the same experimental procedures detailed in the Materials and Methods section. The WAFs were analyzed for acute toxicity and chemical composition before and after depth filtration. Depth filtration was carried out by passing the WAF samples though fritted glass funnels (6 cm deep, 3 cm i.d., 1 $\mu$m pore size) filled with 3-mm surface-deactivated borosilicate glass beads (Chemglass, Vineland NJ), and was conducted to determine the toxicity the dissolved fraction of the WAFs. Table 2 summarizes the results of the WAF experiments of the pure unsaturated FAMEs. Of the three tested
FAMEs, the WAF of C18:1-ME was highly toxic ($EC_{50} = 2.8\%$) followed by the WAF of C18:3-ME ($EC_{50} = 12.78\%$). The WAF of the C18:2-ME was not toxic at all ($EC_{50} > 100\%$). The concentration of the FAMEs in their WAFs was markedly reduced after depth filtration to values even lower than the ones reported by Krop et al. [11]. This was probably due to further autoxidation of the FAMEs during the depth filtration. However, depth filtration and the removal of the FAMEs from the WAFs did not affect the $EC_{50}$ or the TOC values of the WAFs. Table 2 shows that there was no difference in the $EC_{50}$’s nor TOCs of the WAFs before and after depth filtration, even though the FAMEs in the WAFs were almost completely removed, possibly because the concentration of FAMEs in the WAFs was negligible compared to the measured TOC concentrations.

Interestingly, the spectroscopic GC-MS scans of the WAFs of the pure FAME compounds did not show any of the peaks that corresponded to DEGBE or DEGBE-acetate identified in the WAFs of the commercial biodiesel blends. The largest characteristic peak found in the WAFs of C18:1-ME and C18:3-ME, but not found in the WAF of C18:2-ME, was the peak that corresponded to 9-oxo-nonanoic acid methyl ester that did not match with the identity of the pure compound. In fact, two different batches of the purchased 9-oxo-nonanoic acid methyl ester (Sigma) dissolved very poorly in water and DCM, and showed at least three distinct peaks in the GC-MS scan run. This indicates that these stocks of the 9-oxo-nonanoic acid methyl ester, which is a highly reactive chemical species, might have transformed during chemical synthesis or handling and formed autoxidation and polymerization byproducts. Still, these stocks of 9-oxo-nonanoic acid methyl esters when suspended and homogenized in de-ionized water at a nominal concentration of 283.3 mg/L showed highly toxic effects in the test species ($EC_{50} = 3.8\%$).

A second batch of soybean biodiesel was purchased from the same vendor and WAF experiments at the 1:100 dilution were carried out with and without adding of 225 ppm butylated
hydrotoluene (BHT) antioxidant in B100. The EC$_{50}$ (%) of the WAFs of B100 with and without BHT were $10.72 \pm 0.6 \%$ and $8.43 \pm 0.88 \%$, respectively. The WAF of B100 with BHT was slightly less toxic than that without the BHT ($p = 0.02$). However, the TOC of the WAFs with BHT ($9.74 \pm 0.46$ mg/L) and without BHT ($9.62 \pm 0.04$ mg/L) did not show a significant difference.

A third batch of soybean vegetable oil was purchased from Kroger (Cincinnati, OH) and was transesterified in our laboratory. The vegetable oil was transesterified at a temperature of 60 – 70 °C for a 35 minute reaction time, using 0.75% (w/w) sodium methanoxide as a catalyst dissolved in twice the required stoichiometric amount of methanol. Glycerol was separated in a separatory funnel for 1 hour, and the biodiesel was re-transesterified with 25% of the stoichiometric amounts of methanol to insure complete conversion. The mixture was separated again and was washed thoroughly with warm water (41 °C) until no methanol was detected in the wash water. A 1% (w/w) magnesium silicate was added in the biodiesel, and was mixed at 35 °C for 45 minutes to absorb un-reacted glycerides from the biodiesel [13]. A 15% (w/w) anhydrous sodium sulfate was added to the biodiesel, and was mixed at room temperature for 2 hours to absorb residual wash water [14]. The magnesium silicate and sodium sulfate were allowed to settle out overnight, and the biodiesel phase was filtered through glass filter paper (1 micron pore size). WAF experiment at the 1:100 dilution with this batch of B100 was found to be nontoxic (EC$_{50}$ > 100%), and elicited TOC level in the WAF of less than 1 mg-TOC/L.

6.4 Discussion

The experimental results presented in this paper suggest that the aquatic toxicity of petrodiesel/biodiesel blends is not caused by the FAMEs or n-alkanes molecules. This conclusion is not a surprising one. The long chain FAMEs, as well as long chain fatty acids, are biological molecules which all organisms and tissues shed all the time in their surround environments. The fact that those long chain hydrocarbons have very low solubility in water, due to their hydrophobic nature, does not
allow them to be naturally bioavailable at concentrations that would be deleterious to aquatic organisms. This explanation is found to be elegantly supported by the work of Vermue and coworkers [15] on the aquatic toxicity of homologous series of organic solvents to four different strains of bacteria. The authors have found that the transition between toxic and nontoxic solvents generally occur between $\log_{10}P_{oct/water}$ of 3 and 5, depending on the homologous series and the type of bacteria (i.e. gram positive vs. gram negative). The authors also found that when dissolved at their solubility limits in water, the C5 – C18 n-alkanes were nontoxic, while the 1-alkanol homologous series became nontoxic at carbon chain lengths of C10 or longer. It is interesting to note that Vermue and coworkers showed that for the homologous series of C3 – C6 alkyl-ethers and C2 – C5 alkyl-acetates, the aquatic toxicity increases in a dose-response manner even well above the solubility limit of the solvents. They have associated this behavior with what they have termed as “phase toxicity”, as opposed to “molecular toxicity”, which, according to the authors, is caused by the non-dissolved phase of the solvent primarily by the extraction of essential compounds from the cell envelope [15]. While assessing such “phase toxicity” for the FAMEs was not possible because of their high susceptibility to autoxidation, we have tested the “phase toxicity” of the WAF of pure n-hexadecane at 1:1 and 1:10 dilutions by measuring the $EC_{50}$’s of the WAFs before and after depth filtration. No toxicity was found in the WAFs of n-hexadecane before or after filtration.

The hydrolysis of the methyl esters into fatty acids and methanol appeared to occur at a slower rate than the autoxidation of the FAMEs. This conclusion is based on the fact that in the WAFs, the measured amount of methanol was well below that of the autoxidation byproducts. In the case where the autoxidation of biodiesel is not significant, the aromatic compounds present in the petrodiesel, which are more soluble and toxic than the n-alkanes [16], appeared to generally be the cause of the aquatic toxicity of the petrodiesel/biodiesel blends. The extent or susceptibility of biodiesel to
autoxidation, however, appears to be affected by many factors. Higher oil loads clearly shift the
autoxidation reaction to the formation of the byproducts and increase their concentrations in the WAF.
But the mechanism of lipids autoxidation itself is a fairly complex one. The autoxidation of biodiesel is
known to occur in the presence of a wide range of different types of catalysts [17, 18], and it is
generally accepted that the autoxidation process consists of three distinct steps: initiation, propagation,
and termination reactions. The most critical step is the initiation reaction, which requires an activation
catalyst [19].

The transesterification and clean-up processes of biodiesel appear to play a major role in
affecting its susceptibility to autoxidation. It is unclear whether this is a result of the thermal
degradation of natural antioxidants present in the parent oil during the transesterification reaction [20],
or due to the failure to efficiently remove the impurities form biodiesel after the transesterification
reaction. The addition of BHT to the commercial soybean biodiesel did not reduce the amount of TOC
elicited in the WAF. Furthermore, we measured the natural antioxidant as ($\Sigma$ tocopherol) in both the
commercial and lab-transesterified soybean B100 fuels using an HPLC method outlined in Abidi [21],
and did not find a significant difference in measured values (2 – 6 ppm in both fuels).

Polyethylene glycols, such as the ones identified in the WAFs of the commercial biodiesel,
have been documented to occur as undesirable byproducts in the ethoxylation reaction of FAMEs in
the presence of an alkaline (such as NaOH) or an acid catalyst [22]. This suggests that inefficient
removal of the catalyst after the transesterification or some other contamination during the process may
have catalyzed the formation of DEGBE and DEGBE-acetate in the WAFs. The formation of the
diethylene glycols in the WAF of commercial soybean biodiesel, but not in the lab-transesterified one
and nor in the pure FAMEs, suggests the presence of a putative catalyst in the former. However, those
diethylene glycols as well as butyl acetate are not expected to pose a serious long term threat in aquatic
systems as they have been shown to be readily biodegradable [9, 23]. Whatever the cause of excessive autoxidation may be, it does not appear to be influenced by the source of the biodiesel feedstock itself because the FAME molecules were determined to be nontoxic, and only the autoxidation byproducts of C18:1-ME and C18:3 were. Yet, the unidentified autoxidation byproduct, which structure resembled the structure of 9-oxo-nonanoic acid methyl ester and which was detected in the WAFs of the C18:1-ME and C18:3-ME (but not C18:2-ME), as well as in the WAFs of the commercial biodiesel blends, appears to be responsible for some of the observed toxicity as well.

REFERENCES


Figure 6. 1. The 15-minute EC₅₀ (%) of the WAFs of the petrodiesel/biodiesel blends ( ), and measured TOC ( ), methanol ( ), ∑ₙ-alkanes ( ), ∑FAMEs ( ), and ∑aromatic compounds ( ) (all in mg/L) in the WAFs for the: a- 1:1 dilution, b- 1:10 dilution, c- 1:100 dilution, and d- 1:1000 dilution. Error bars are ± 1 standard deviation of 3 replicate samples.
Table 6. 1. Average concentration (mg/L) of the identified autoxidation byproducts in the WAFs of petrodiesel/biodiesel blends (average ± standard deviation of 3 replicates).

<table>
<thead>
<tr>
<th></th>
<th>B100</th>
<th>B80</th>
<th>B60</th>
<th>B40</th>
<th>B20</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DILUTION 1:1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEGBE\textsuperscript{a}*</td>
<td>114.42 ± 29</td>
<td>104.3 ± 27.76</td>
<td>76.4 ± 18.06</td>
<td>55 ± 13.32</td>
<td>1.89 ± 0.52</td>
</tr>
<tr>
<td>DEGBE-acetate\textsuperscript{b}*</td>
<td>1.05 ± 0.27</td>
<td>1 ± 0.27</td>
<td>0.76 ± 0.20</td>
<td>0.65 ± 0.17</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>Butyl acetate*</td>
<td>1.64 ± 0.27</td>
<td>1.41 ± 0.37</td>
<td>1.04 ± 0.21</td>
<td>0.79 ± 0.19</td>
<td>0.33 ± 0.07</td>
</tr>
<tr>
<td><strong>DILUTION 1:10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEGBE</td>
<td>13.28 ± 2.14</td>
<td>10.28 ± 2.04</td>
<td>7.24 ± 1.20</td>
<td>4.6 ± 0.52</td>
<td>1.96 ± 0.44</td>
</tr>
<tr>
<td>DEGBE-acetate</td>
<td>0.59 ± 0.12</td>
<td>0.47 ± 0.08</td>
<td>0.33 ± 0.05</td>
<td>0.2 ± 0.04</td>
<td>0.17 ± 0.11</td>
</tr>
<tr>
<td>Butyl acetate</td>
<td>1.49 ± 0.28</td>
<td>1.2 ± 0.2</td>
<td>0.87 ± 0.12</td>
<td>0.59 ± 0.07</td>
<td>0.33 ± 0.04</td>
</tr>
</tbody>
</table>

\textsuperscript{a} DEGBE = diethylene glycol monobutyl ether.

\textsuperscript{b} DEGBE-acetate = diethylene glycol monobutyl ether acetate.

\* Values are estimated by external calibration of the GC-MS on scan mode and are corrected by Log[P\textsubscript{DCM/water}] of -0.004, 0.56, and, 0.48 for DEGBE, DEGBE-acetate, and butyl acetate, respectively.
Table 6. 2. 1:100 dilution experiments of the unsaturated FAMEs pure compounds. Values are the average ± standard deviation of 3 replicates.

<table>
<thead>
<tr>
<th>Pure FAME</th>
<th>WAF FAME Concentration (mg/L)</th>
<th>WAF TOC Concentration (mg/L)</th>
<th>WAF 15-min EC&lt;sub&gt;50&lt;/sub&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Filtration</td>
<td>After Filtration</td>
<td>Before Filtration</td>
</tr>
<tr>
<td>C18:1</td>
<td>0.050 ± 0.003</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.08 ± 0.17</td>
</tr>
<tr>
<td>C18:2</td>
<td>0.111 ± 0.052</td>
<td>ND</td>
<td>1.37 ± 0.04</td>
</tr>
<tr>
<td>C18:3</td>
<td>0.190 ± 0.017</td>
<td>0.006 ± 0.001</td>
<td>12.78 ± 0.41</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND = not detected.

<sup>b</sup> NT = not toxic (i.e. EC<sub>50</sub> > 100%).
CHAPTER 7: ACUTE TOXICITY OF THE WATER ACCOMODATED FRACTIONS (WAFS) OF FOUR PETRODIESEL/FLAXSEED-BIODIESEL BLENDS USING MICROTOX BIOASSAY
Acute toxicity of the water accommodated fractions (WAFs) of four petrodiesel/flaxseed-biodiesel blends using Microtox bioassay

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ABSTRACT. The acute toxicity of the water accommodated fractions (WAFs) of four flaxseed biodiesel/petrodiesel blends (B0, Flax-B40, Flax-B60, and Flax-B100, where Flax-B100 is 100% flaxseed-biodiesel) were investigated at different oil loads using the Microtox bioassay and the bacterium Vibrio fischeri as a test species. The results of this study were compared with those of soybean biodiesel/petrodiesel blends presented in Chapter 6. The toxicity of the WAFs of different flaxseed biodiesel/petrodiesel blends was always higher for fractions of petrodiesel in the blend, regardless of the oil load applied. Furthermore, the WAFs of pure Flax-B100 were significantly less toxic than those of pure Soy-B100 at all the oil loads tested. Spectroscopic GC-MS scans of the WAFs containing flaxseed biodiesel revealed the presences of the chemical species 9-oxo nonanoic acid methyl ester in a dose dependent manner, but the other autoxidation byproducts which were found in soybean biodiesel WAFs (i.e. hexanal, butyl acetate, diethylene glycol mono-butyl ether, and its acetate ester) were not detected in the WAFs of flaxseed biodiesel. Spectroscopic scans of two different batches of the pure chemical 9-oxo nonanoic acid methyl ester showed at least 2 – 3 peaks of different sizes, all of which did not match the identity of the compound detected in the WAFs. We think this chemical species may in fact be 9-oxo nonanoic acid methyl ester or any other chemical with a similar structure as those compounds are widely reported in the literature as byproducts of the autoxidation of triglycerides and fatty acid methyl esters (FAMEs) [1-3]. This chemical species could have been poorly synthesized and handled by the commercial vendor as this compound has very a poor autoxidative stability.
7.1 Introduction

In Chapter 6, we investigated the acute toxicity of the water accommodated fractions (WAFs) of soybean biodiesel/petrodiesel blends (B0, Soy-B20, Soy-B40, Soy-B60, Soy-B80, and Soy-B100) using the Microtox bioassay. We found that at high oil loads, the toxicity of the WAFs increased as the fraction of soybean biodiesel in the oil blend was increased; but at low oil loads, the toxicity of the WAFs was found to increase as the amount of petrodiesel in the oil blend was increased. Further analysis revealed that the toxicity of the WAFs of soybean biodiesel was due to the toxicity of the autoxidation byproducts of the fatty acid methyl esters (FAMEs) and not due the FAMEs molecules themselves. Similarly, the toxicity of the WAFs of petrodiesel (B0) was not due to the toxicity of the n-alkanes, but due to the toxicity of the aromatic compounds (mono and polycyclic) present in petrodiesel. More investigations showed that the autooxidative stability of biodiesel can be largely affected by the transesterification and subsequent purification methods employed [4].

In this study, we investigated the acute toxicity of flaxseed biodiesel/petrodiesel blends (B0, Flax-B40, Flax-B60, and Flax-B100) using the same experimental approach employed in Chapter 6. Flaxseed oil is, in fact, not a commercially viable feedstock for biodiesel production, because it has high nutritional value [5], and a relatively lower autooxidative stability than other oils [6, 7]. The reason we chose the flaxseed feedstock in this study is because flaxseed vegetable oil is composed of more than 50% (w/w) linolenic acid (C18:3), while soybean vegetable oil is composed of more than 50% (w/w) linoleic acid (C18:2). The objective was to test the effect of higher level of unsaturation on the aquatic toxicity of the fuels.


7.2 Materials and Methods

All the materials and methods of the WAFs preparation, chemical and toxicity analyses are similar to the ones detailed in Chapter 6. Flaxseed virgin vegetable oil with fatty acids profile (% mass) of 5.5% C16:0, 5.5% C18:0, 16% C18:1, 15.75 C18:2, and 57.25% C18:3 was purchased from Botanic Oil Innovations Inc (Spooner, WI), and was transesterified in our laboratory at the University of Cincinnati (Cincinnati, OH). The transesterification reaction was carried out at a temperature of 60 – 70 °C for 35 minutes using 0.75% (w/w) sodium methanoxide as a catalyst dissolved in twice the required stoichiometric amount of methanol. Glycerol was separated in a separatory funnel for 1 hour, and the biodiesel was re-transesterified with 25% of the stoichiometric amounts of methanol to insure complete conversion. The mixture was separated again and was washed thoroughly with warm water (41 °C) until no methanol was detected in the wash water. A 1% (w/w) magnesium silicate was added to the biodiesel, and was mixed at 35 °C for 45 minutes to absorb un-reacted glycerides from the biodiesel [8]. A 15% (w/w) anhydrous sodium sulfate was added to the biodiesel, and was mixed at room temperature for 2 hours to absorb residual wash water [4]. The magnesium silicate and sodium sulfate were allowed to settle out overnight, and the biodiesel phase was filtered through glass filter paper (1 micron pore size).

7.3 Results and Discussion

The results of chemical analysis and toxicity test of the WAFs of flaxseed biodiesel/petrodiesel blends (B0, Flax-B40, Flax-B60, and Flax-B100) at volumetric dilution levels 1:1 – 1:1000 are shown in Figure 7.1. The bar-plots in Figure 7.1 correspond to the measure EC50’s of the WAFs, and the scatter-plots correspond to the measured total organic carbon (TOC), sum of the C10 – C24 n-alkanes, sum of the FAMEs, and the sum of the targeted aromatic compounds, as detailed in the figure caption.
Figure 7.2 contains the results of flaxseed biodiesel/petrodiesel blends shown in Figure 7.1, as well as the results of soybean biodiesel/petrodiesel blends (B0, Soy-B40, Soy-B60, and Soy-B100) obtained in Chapter 6. Figure 7.2 shows that the EC$_{50}$’s of WAFs of flaxseed biodiesel blends (hollow hatched bar-plots) are significantly higher (i.e. less toxic) than those of soybean biodiesel blends. In fact, Figure 7.2d shows that at dilution level of 1:1000, the WAF of Flax-B100 was nontoxic (EC$_{50}$> 100%), while the EC$_{50}$ of the WAF of Soy-B100 at the same dilution level was ~75%. It is evident from Figure 7.2 that the WAFs of soybean biodiesel, in general, are more toxic than the WAFs of flaxseed biodiesel blends, although the measured concentrations of the target analytes (i.e. n-alkanes, FAMEs, and aromatic compounds) were reasonably the same in the WAFs of both feedstocks.

Spectroscopic GC-MS scans of the WAFs of flaxseed biodiesel blends revealed the presence of 9-oxo-nonanoic acid methyl ester (92% match) in a dose dependant manner. The same compound was also detected in the WAFs of soybean biodiesel, and in the WAFs of C18:1 and C18:3 methyl esters. Further, this compound is widely reported, among others, as a byproduct in the autoxidation of FAMEs and triglycerides [1, 2]. However, spectroscopic GC-MS scans of two different batches of the 9-oxo-nonanoic acid methyl ester pure compound purchased from Sigma-Aldrich always contained 2 – 3 peaks of different sizes, none of which agreed with the peak detected in the WAFs. This indicates that the purchased pure 9-oxo-nonanoic acid methyl ester may be either contaminated with other chemicals, or has been autoxidized during chemical synthesis or handling and shipping. Whatever is the case, this 9-oxo nonionic acid methyl ester peak was the only major and common unidentified peak present in the WAFs of flaxseed biodiesel, the WAFs of soybean biodiesel, and the WAFs of C18:1 and C18:3 pure methyl esters, all of which were toxic. This peak was not detected in the WAF of pure C18:2 methyl ester, which was not toxic.
The findings of this study support the results obtained in Chapter 6 concerning the aquatic toxicity of biodiesel not being caused by the FAMEs molecules themselves, but primarily caused by the autoxidation byproducts. Although flaxseed biodiesel is composed of more than 50% C18:3-ME, which was shown in Chapter 6 (Table 6.2) to produce high concentrations of toxic autoxidation byproducts in its WAF, and soybean biodiesel is composed of more than 50% C18:2-ME, which was shown in Table 6.2 to produce nontoxic WAFs, the toxicity of the WAFs of flaxseed biodiesel was lower and contained less autoxidation byproducts compared to that of soybean biodiesel.

The findings of this study and the study presented in Chapter 6 leave no doubt that the aquatic toxicity observed for biodiesel is chiefly determined by the fuel’s autoxidative stability, which appears not to be a function of the source of the feedstock itself, but is determined by the transesterification and purification methods used in the fuel’s production [4]. The soybean biodiesel used in Chapter 6 was purchased from a commercial vendor (Peter Cremer North America, Cincinnati OH) who is a BQ – 9000 certified biodiesel producer and marketer. This vendor would not reveal to us the details of his biodiesel transestrification process. It appears that more regulations in biodiesel transestrification methods are required to insure better biodiesel’s autoxidative stability.
REFERENCES


Figure 7. The 15-minute EC50 (%) of the WAFs of the petrodiesel/flax-biodiesel blends ( ), and measured TOC ( ), \( \sum n \)-alkanes ( ), \( \sum FAMEs \) ( ), and \( \sum \) aromatic compounds ( ) (all in mg/L) in the WAFs for the: a- 1:1 dilution, b- 1:10 dilution, c- 1:100 dilution, and d- 1:1000 dilution. Error bars are ± 1 standard deviation of 3 replicate samples.
Figure 7.2. EC$_{50}$ (%) of the WAFs of the petrodiesel/biodiesel blends (bar-plots): soybean blends (solid grey) and flaxseed blends (hollow cross-hatched). Measured TOC (diamond symbol), $\Sigma n$-alkanes (circle symbols), $\Sigma$FAMEs (square symbols), and $\Sigma$aromatic compounds (inverted triangles) (all in mg/L): soybean blends (solid grey symbols) and flaxseed blends (hollow symbols) in the WAFs for the: a- 1:1 dilution, b- 1:10 dilution, c- 1:100 dilution, and d- 1:1000 dilution. Error bars are ± 1 standard deviation of 3 replicate samples.
CHAPTER 8: MICROBIAL COMMUNITY STRUCTURES
OF PETRODIESEL/BIODIESEL BLENDS
ACCLIMATED CULTURES
Microbial community structures of petrodiesel/biodiesel blends acclimated cultures

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ABSTRACT. The Microbial community structures of 8 cultures acclimated to the petrodiesel/biodiesel blends (B0, Soy-B20, Soy-B40, Soy-B60, Soy-B80, Soy-B100, Flax-B50, and Flax-B100, where B0 is unblended petrodiesel, Soy-B100 is unblended soybean biodiesel, and Flax-B100 is unblended flaxseed biodiesel) were investigated by a polymerase chain reaction – denaturing high performance liquid chromatography (PCR – DHPLC) approach. In a previous work (Chapter 3), we have shown that different petrodiesel/soybean-biodiesel blends produced oddly variable profiles of dissolved n-alkanes, fatty acid methyl esters (FAMEs), and aromatic compounds in the aqueous phase. The objective in this study was to examine if such a solubilization behavior of petrodiesel/biodiesel blends would result in different microbial community profiles for acclimated degrading cultures. A cloning step was employed to separate the different detected strain, as the DHPLC was unable to separate the individual peaks efficiently. We also conducted a 5 X 5 factorial design mineralization experiment using the cultures acclimated to B0, Soy-B40, Soy-B100, Flax-B50, and Flax-B100, in which different combinations of acclimated cultures and fuel blends were experimented. The results of the mineralization experiment indicated that the cultures acclimated to flaxseed biodiesel blends were significantly better at mineralizing the fuels compared to those acclimated to petrodiesel and soybean biodiesel blends, regardless of the type of the fuel blend being mineralized. The PCR–DHPLC analysis of the cultures allowed for the identification of 16 different microbial strains in the 8 cultures belonging to 13 different species. The microbial community profiles of the different cultures were very complex and no obvious trend for the shifts in the microbial community profiles was identified.
8.1 Introduction

In Chapter 3, we have shown that different blends of soybean-biodiesel/petrodiesel fuels produce oddly different and complex profiles of dissolved fuel constituents in the aqueous phase. We speculated that such a complex solubilization behavior may result in different microbial community profiles after an acclimation period, due to different selection pressures applied in the dissolved (or bioavailable) phase. The objective of this study was to investigate the validity of proposed hypothesis.

In principle, if the phenomenon that fatty acid methyl esters (FAMEs) are capable of enhancing the bioavailability of long chain n-alkanes is true, the relative abundances and bioavailability of the different chemicals in the water column will be altered and can affect the microbial ecology of the acclimated cultures. This is because the cultures will thrive on different concentrations of different chemical species. However, the literature available on similar types of studies is really sparse. Cyplik et al.[1] Investigated the bacterial community dynamics during the biodegradation of petrodiesel, biodiesel, and a blend of B20, under different reducing conditions using the quantitative q-PCR approach. They found that the biodegradation rates increased with the increase of biodiesel in the substrate under aerobic conditions, but were markedly reduced under nitrate reducing conditions. Regardless of the different degradation rates amongst the different treatments, the authors found that the relative magnitude of microbial populations did not vary more than one order of magnitude. Sorensen et al. [2] and Schleicher et al. [3] independently studied the microbial growth and stability on petrodiesel/biodiesel blends. The former research group observed higher microbial growth on fuels containing more biodiesel using q-PCR analysis; while the latter observed higher microbial counts on the fuels containing more petrodiesel. Hawrot-Paw and Coworkers [4, 5] investigated the influence of petrodiesel, biodiesel, and a 5% biodiesel blend (B5) on the microbial growth and dehydrogenase activity in soil biomass. A slight stimulation of the microbial growth and dehydrogenase activity was
observed at a biodiesel oil load of 1%, but a general decrease of the microbial growth and dehydrogenase activity was observed in all treatments in a dose-response manner. Hamamura et al. [6] examined the microbial population dynamics of different types of soils during the biodegradation of crude oil. They used a combination of radio-labeled substrate mineralization experiments and denaturing gradient gel electrophoresis (DGGE) profiling of the 16S rRNA genes. After a 50-day incubation period, all the soils achieved a comparable mineralization of the crude oil (> 80%), but the initial rates of mineralization varied up to 10-folds among the different soils. The authors identified the most genetically diverse species among the different soils as *Nocardiodes albus*, *Collimonas sp.*, and *Rhodococcus coprophilus*, which are known hydrocarbon degrading organisms. Kleinsteuber et al. [7] analyzed the dynamics of microbial populations at water salinities in the range of 0 – 20% NaCl during the biodegradation of petrodiesel fuel. They employed a respirometric experimental approach along with 16S rRNA – single strand conformation polymorphism (SSCP) profiling. At reduced salinity, the microbial cultures were dominated by *Sphingomonas spp.*, while the elevated salinity favored the growth of *Ralstonia spp.*, *Halomonas spp.*, *Dietzia spp.*, and *Alcanivorax spp*. Margesin et al. [8] investigated the effect of petrodiesel oil load, the addition of fertilizers (source of macronutrients), and incubation time on the removal efficiency of petrodiesel, lipase activity, and microbial phospholipid fatty acids in bench-scale soil studies. The addition of fertilizers significantly enhanced hydrocarbon removal and lipase activity, irrespective of the type of fertilizer used. Phospholipid fatty acids were found to be markedly influenced by the initial amount of petrodiesel applied followed by fertilization, but incubation time had negligible effect on the phospholipids. Gram-staining showed significantly higher gram-negative populations at higher oil loads and with soil fertilization, while gram-positive populations were not appreciably influenced by those factors. Bundy et al. [9] used basal respiration assay and microbial phospholipids profiling to study the effect of adding petrodiesel fuel on the
microbial communities of three different soils. Based on their results, the authors claimed that the microbial communities in different soils might not converge back to their initial population after soils decontamination.

The application of denaturing high performance liquid chromatography (DHPLC) in environmental microbial ecology is still in its infancy, but the technique has been widely exploited in clinical investigations and mutation detection [10, 11]. Studies which have successfully used DHPLC in environmental microbial ecology have pointed out the superiority and robustness of this technique compared to DGGE [12, 13]; but they often encountered problems with the separation and isolation of the different DNA fragments [14]. A cloning step with the DHPLC profiling appears to be eminent for the separation, and subsequently sequencing, of DNA fragments in complex environmental samples. In this study, we developed a simple analytical tool to profile, separate, and sequence the microbial communities of 8 microbial cultures acclimated to different blends and feedstocks of petrodiesel/biodiesel fuels. We also investigated the mineralization efficiencies of those cultures in a 5 x 5 factorial design mineralization experiment.

8.2 Materials and Methods

Chemicals. Low-sulfur petroleum diesel (B0) was purchased from a BP diesel station (Cincinnati, OH) with an n-alkanes mole fraction of 0.165. Unblended soybean-methyl ester biodiesel (Soy-B100) was purchased from Peter Cramer North America (Cincinnati, Ohio) with FAMEs mole fractions of 0.145 C16:0, 0.055 C18:0, 0.206 C18:1, 0.518 C18:2, and 0.0759 C18:3. Flaxseed virgin vegetable oil with fatty acids profile (% mass) of 5.5% C16:0, 5.5% C18:0, 16% C18:1, 15.75 C18:2, and 57.25% C18:3 was purchased from Botanic Oil Innovations Inc (Spooner, WI) and was transesterified in our laboratory. The transesterification reaction was carried out at a temperature of 60 – 70 °C for 35 minutes using 0.75% (w/w) sodium methanoxide as a catalyst dissolved in twice the
required stoichiometric amount of methanol. Glycerol was separated in a separatory funnel for 1 hour, and the biodiesel was re-transesterified with 25% of the stoichiometric amounts of methanol to insure complete conversion. The mixture was separated again and was washed thoroughly with warm water (41 °C) until no methanol was detected in the wash water. A 1% (w/w) magnesium silicate was added in the biodiesel, and was mixed at 35 °C for 45 minutes to absorb un-reacted glycerides from the biodiesel [15]. A 15% (w/w) anhydrous sodium sulfate was added to the biodiesel, and was mixed at room temperature for 2 hours to absorb residual wash water [16]. The magnesium silicate and sodium sulfate were settled out, and the biodiesel phase was filtered through glass filter paper (1 micron pore size). All other fuel blends (Soy-B20, Soy-B40, Soy-B60, Soy-B80, and Flax-B50) were blended in our laboratory by volumetric splash mixing.

**Cultures Cultivation.** Eight laboratory-scale, 6-liter porous pot (PP) chemostats were inoculated with a mixture of mixed liquor obtained from the aeration tank of Mill Creek Wastewater Treatment Plant (Cincinnati, OH), and gasoline and triglyceride degrading cultures previously developed in our laboratories at the University of Cincinnati (Cincinnati, OH). The flow-through bioreactors were operated as completely mixed systems with a hydraulic retention time of 1 day. Organic feeds of B0, Soy-B20, Soy-B40, Soy-B60, Soy-B80, Soy-B100, Flax-B50, and Flax-B100 were delivered to the bioreactors using Hamilton syringe pumps, and the feed rates were increased gradually throughout an acclimation period of six months to a final organic loading of 750 mg/L-d oil. Adequate aeration in the bioreactors was maintained throughout the operating period by means of continuous air diffusers, operated at nominal volumetric flow rates of 2–3 L/min. The bioreactors received a combined feed of essential nutrient and vitamin minimal medium buffered at pH 7.5 ± 0.1 of the following final concentration: 150 mg/L MgSO₄·7H₂O, 156 mg/L KNO₃, 48.7 mg/L CaCl₂·2H₂O, 37.3 mg/L FeCl₂·4H₂O, 1393 mg/L K₂HPO₄, 544 mg/L KH₂PO₄, 0.08 mg/L CuSO₄·H₂O, 0.15 mg/L

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Na$_2$MoO$_4$·2H$_2$O, 0.13 mg/L MnSO$_4$·H$_2$O, 0.23 mg/L ZnCl$_2$, 0.42 mg/L CoCl$_2$·6H$_2$O, 0.015 mg/L 4-aminobenzoic acid (99%), 0.00585 mg/L biotin, 0.0003 mg/L cyanocabalam, 0.00585 mg/L folic acid dihydrate (99%), 0.015 mg/L nicotinic acid (98%), 0.015 mg/L pantothenic acid Ca-salt hydrate (98%), 0.03 mg/L pyridoxine hydrochloride (98%), 0.015 mg/L riboflavin (98%), 0.015 mg/L thiamine hydrochloride (99%), and 0.015 mg/L thiotic acid (98%). Certain water quality variables including effluent FAMEs and target n-alkane analytes, total and volatile suspended solids (VSS), chemical oxygen demand (COD), pH, and dissolved oxygen (DO) were monitored routinely. When the reactors achieved steady state performance, a portion of each reactor’s culture was collected, washed three times with sterile saline solution, and frozen in 10% w/w glycerol at -80 °C for consistent use in the batch experiments.

**Factorial Mineralization Experiment.** Batch mineralization experiments were carried out in sterile and surface-deactivated 160 mL glass serum bottles where 50 mL of filter-sterilized nutrients and vitamins medium was added. Biomass aliquots acclimated to B0, Soy-B40, Soy-B100, Flax-B50, and Flax-B100, thawed and washed three times with 0.85% (w/w) sterile saline solution, were added to the bottles at measured solids concentration of 100 ± 15 mg VSS/L. The bottles were sealed with PTFE septa and spiked with 10 µl of B0, Soy-B40, Soy-B100, Flax-B50, or Flax-B100 in a 5 X 5 mixed factorial design experiment, in which different combinations of acclimated cultures and oil blends were batched together. This was done to test the intrinsic effect of the oil blend and type on the development of the mineralization capability of the microbial cultures. The initial concentration of the organic phase was ~175 mg/L oil, and the amount of oxygen available in the headspace was sufficient for the complete oxidation of 1.5X the amount of oil added on a chemical oxygen demand basis. The bottles were then placed in a rotary tumbler operated at a nominal speed of 20–25 rpm. Two sampling events of six bottles each (biologically active + endogenous respiration controls) per oil treatment were set for
the analysis of total CO$_2$ produced at 7 and 42 days (ultimate). Just prior to sampling time, 100 µL of HCl was spiked in the bottles through the PTFE septa, delivering a final concentration of 24 mM HCl and dropping the pH < 2. While the bottles were still in the inverted position, the needle of the syringe was slowly pulled out of the bottles to minimize leakage of gas in or out of the bottles. The septa were then wrapped with parafilm, and the bottles were tumbled again for a 24 hr equilibration period. Upon equilibration, a 20 mL gas-tight Popper Micro-mate Syringe (New Hyde Park, NY) was inserted inside the headspace of each bottle, and the gas in the headspace was allowed to expand to equilibrate the pressure inside the bottle with atmospheric pressure. The volume of headspace gas expanded inside the 20 mL syringe was recorded and, while the syringe was still inside the bottle, a 0.5 mL of the headspace gas was withdrawn by a gas-tight Vici Precision Sampling Syringe (Baton Rouge, LA) and analyzed for gas composition using an HP 5890 Series II gas chromatograph (GC) equipped with a dual column HP 10 ft molecular sieve BX-45/60 mesh, HP 6 ft. HAYESEP Q 80/100 mesh, and a Thermal Conductivity Detector (TCD) (HP, Palo Alto, CA). The mass of gas phase CO$_2$ was calculated by equation 1.

\[
M_{CO_2-gas} = \left(V_{\text{headspace}} + V_{\text{syringe}}\right) \times CO_2(\%) \times \frac{44 \text{ g/mole}}{24.2 \text{ L/mole}}
\]

where \(M_{CO_2-gas}\) = the mass of CO$_2$ in the gas phase (mg),

\(V_{\text{headspace}}\) = the volume of the headspace of the bottle \((V_{\text{headspace}}=110 \text{ mL})\),

\(V_{\text{syringe}}\) = the volume of expanded gas measured in the syringe (mL),

\(CO_2(\%)\) = the percentage of CO$_2$ in the headspace measured by the GC-TCD,

44 g/mole = molecular weight of CO$_2$, and

24.2 L/mole = the molar volume of CO$_2$ gas at room temperature and 1 atm.
The mass of aqueous CO$_2$ was calculated using Henry’s Law, assuming negligible carbonic acid species and equilibrium between the gas and aqueous phase CO$_2$ species according to equation 2.

\[
M_{CO_2-aq} = V_{aq} [CO_2]_{aq} = V_{aq} H [CO_2]_{gas} = V_{aq} H \times CO_2(\%) \times \frac{44 \text{ g/mole}}{24.2 \text{ L/mole}}
\]  

(2)

where \( M_{CO_2-aq} \) = total mass of CO$_2$ in the aqueous phase (mg), \([CO_2]_{aq}\) and \([CO_2]_{gas}\) = the concentrations of CO$_2$ in the aqueous and gas phases (mg/mL), respectively, \( V_{aq} \) = the volume of aqueous phase (\(V_{aq}=50\) mL), and 
\( H \) is the dimensionless Henry’s constant for CO$_2$ \((H=0.831)\) [17].

The total amount of CO$_2$ produced was calculated as the sum of equations 1 and 2 and was corrected with respect to endogenous respiration in the blanks at every sampling event. The aforementioned procedure was also validated by measuring the dissolved inorganic carbon directly using a Shimadzu TOC-V CSH Analyzer (Tokyo, Japan) according to manufacturer’s procedures and found to be within 3% accuracy. Chemically oxidizable carbon in B0, SB100, and FB100 was experimentally measured by injecting 10 µL of the fuels in sealed serum bottles containing 20 mL of a 3% potassium dichromate in sulfuric acid (95%) solution and incubated at 150 °C for 24 hr. The bottles were then cooled down to room temperature, and the headspace gas was analyzed as outlined above. The sulfuric acid phase was diluted with deionized water down to 5% and analyzed for inorganic carbon on the Shimadzu TOC analyzer. The amount of chemically oxidizable carbon was found to be 2.346 ± 0.036, 2.484 ± 0.032, and 2.508 ± 0.066 mg-CO$_2$/µL-oil for B0, SB100, and FB100, respectively.

**Extraction of Genomic DNA.** The genomic DNA (gDNA) extraction protocol employed in this study was modified based on the commercially available FastDNA Spin kit (MP Biomedicals, USA) and DNeasy Blood and Tissue kit (Qiagen, USA) to yield higher purity gDNA extracts. The
biomasses frozen earlier were thawed, washed and centrifuged three times with sterile saline solution. In the final wash, the biomasses were centrifuged at 10,000 rpm, for 20 min, and the supernatants were decanted. The biomasses were then weighed in 200 mg (wet mass) in Lysing Matrix A tubes (FastDNA Spin kit, MP Biomedicals) and were resuspended in 200 µL sterile saline solution. A negative control containing only the saline solution was carried throughout the extraction. Volumes of 1 mL of ALT buffer (DNeasy Blood and Tissue kit, Qiagen) were added to the tubes, and the tubes were homogenized in a FastPrep Instrument (MP Biomedicals) for 40 seconds at a standard speeding of 6. The tubes were then centrifuged at 14,000 x g for 10 minutes to pellet the debris. Of the supernatants in the tubes, 360 µL was transferred into clean 0.5 mL centrifuge tubes. To the 0.5 mL tubes, 40 µL of proteinase K enzyme (Qiagen) was added, and the tubes were vortexed and incubated at 56 °C overnight to lyse the cells completely. The contents of the tubes were then transferred into 1.5 mL centrifuge tubes, and the extraction and purification of the gDNA’s were completed in accordance to the DNeasy Blood and Tissue spin-column protocol (Qiagen). The yield and purity gDNA extracts were quantified using a Nanodrop 3300 Spectrophotometer (Thermo Scientific, USA) by UV absorbance at 260 and 280 nm, respectively, and were stored in aliquots of 10 µL at -21°C.

**Polymerase Chain Reaction (PCR).** The gDNA extracts of the different cultures were diluted with dH2O 30 folds to reduce the effect of PCR inhibitors present in some of the extracts. The PCR reaction was performed using the commercially available Taq PCR Master Mix kit (Qiagen) according to the manufacturer’s instruction manual. A 204 bp of the V3 variable region of the 16S rRNA genes were amplified in two different reactions using the primer pairs 341F/518R and 341F-GC/518R developed by Muyzer et al. [18]. The sequences of the primers and the GC-clamp are shown in Table 8.1. The incorporation of the GC-clamp in the 5’-prime-end of the forward primer was required to yield higher detection sensitivity in the DHPLC as demonstrated by Barlaan et al. [13], while the PCR
products without the GC-clamp were used in the subsequent cloning reaction to isolate the different peaks detected. In 100 µL PCR reaction tubes (Thermo Scientific, USA), 25 µL of the Taq PCR Master Mix was added to 25 µL dH₂O containing 10 ng of the gDNA template and 20 pg of each primer. A touchdown PCR cycling program (shown in Table 8.1) which was developed by Schafer and Muyzer [19] for the pair of primers was adopted using an Applied Biosystems Model 2720 Thermal Cycler. After the reaction was completed, the PCR products were purified using a QIAquick PCR Purification kit (Qiagen) according to manufacturer’s protocol, and were quantified using the Nanodrop and stored at -21 °C in aliquots of 10 µL.

**DHPLC Analysis.** A Transgenomic (Omaha, NE) WAVE 3500 Nucleic Acid Fragment Analysis System equipped with an oven, a DNA-Sep cartridge, a UV detector, and a fragment collector was used in this study. The separation of DNA sequences was achieved by reverse-phase ion-pairing (RP-IP) of the PCR products in an alkylated C18 stationary phase bound to the DNA-Sep cartridge in the presence of a mobile phase that consists of triethylammonium acetate (TEAA) and acetonitrile. TEAA acts as an ion-pairing agent in which the ammonium positive charge interacts ionically with the negatively charged phosphate backbone of the DNA molecule. The TEAA-DNA complex subsequently interacts hydrophobically through the triethyl moiety with the stationary phase in the cartridge. The extent of TEAA – DNA interaction is determined by the helicity of the DNA molecule, which is a function of the DNA sequence, and the denaturation extent of the DNA controlled by the temperature at which the analysis is conducted. After the binding of DNA on the stationary phase, an analytical gradient of acetonitrile is applied by the instrument to disrupt the hydrophobic interactions between the TEAA – DNA and elute the DNA molecules. Detection of the DNA fragments was done by measuring the UV absorbance during elution using a synchronous UV detector connected downstream of the cartridge.
Calibration of the DHPLC was done using a DNA sizing standard, a low range mutation standard, and a high range mutation standard, all purchased from Transgenomic, in accordance to the user’s manual. The buffers used for the analysis and for cleaning the instrument were purchased from the same vendor as well. The optimum conditions and temperature of the analysis were determined by trial and error. Initially, the flow rate was fixed at 0.9 ml/min, and different temperatures were experimented. After determining the optimum temperature for partially denatured analysis (65.5 °C), the flow rate and elution gradient were varied systematically to arrive at the optimal instrumental conditions which are shown in Table 8.2.

Approximately 50 ng of the 16S rRNA PCR products of the acclimated cultures were loaded and analyzed on the DHPLC as outlined above. Collection of the detected peaks using the WAVE fragment collector produced very unsatisfactory fragment separation, possibility due to the complexity of the chromatograms. Therefore, a cloning approach was employed to isolate the detected peaks as detailed in the following section.

Cloning of PCR Products. After analyzing each of the PCR products of the 8 cultures separately on the DHPLC, 5 µL of each of the PCR products were mixed together forming what we will call mixed PCR product with GC-clamp. This mixed PCR product was loaded and analyzed on the DHPLC. Because the GC-clamp would result in poor ligation and unsuccessful cells transformation in the cloning reaction, the PCR products (without the GC-clamp) of the 8 cultures were thawed on ice, and were mixed together to form what we will call mixed PCR product without GC-clamp, and were concentrated using a Qiagen MiniElute kit. This mixed PCR product without GC-clamp was cloned using a PCR Plus Cloning kit (Qiagen) according to the manufacturer’s protocol. In brief, 26 ng of the mixed PCR product (10-times molar excess) was ligated with 50 ng of pDrive cloning vector using the ligation master mix provided with the kit. After the ligation reaction was completed, the cloning vector
was used to transform Qiagen QZ competent cells by heat shock. After the transformation reaction, 250 µL of SOC medium was added to the tube, and 100 µL of the transformation mixture were plated on an LB agar plate containing ampicillin and IPTG/X–GAL solution (Fisher BioReagents). The agar plate was then incubated at room temperature for 15 minutes until the mixture has absorbed into the agar, and was inverted and incubated at 37 °C for 12 hours. A negative control cloning reactions was included in the transformation.

**Colonies Screening and Whole–Cell Nested–PCR.** The agar plate was taken out of the 37 °C incubator, and was incubated at 4 °C for 15 minutes to help developing the dark-/light- blue colonies for screening. A total of 96 white and light-blue colonies were selected out of the plate using sterile pipette tips, and were directly pipetted into a 96-well plate containing 25 µL PCR reaction mixture and 0.4 µM of M13- forward and reverse universal primers (shown in Table 8.1) to amplify the cloned fragments out of the plasmids. For this round of PCR, the PCR cycler program was similar to the one outlined in Table 8.1 with but the addition of an extra 10 minutes initial cell-walls denaturation and DNase deactivation period at 94°C at the beginning of the program. After the reaction was completed, another round of 25 µL PCR reactions in a 96-well plate were setup using the 341F-GC/518R primer pair, and 2 µL of the first PCR reaction contents was used as a template. Negative controls were included with all the reactions. The second round PCR was required to remove the M13 primers out of the DNA fragments, and to add to them the GC-clamp so that matching the cloned peaks with the original chromatograms is possible. The second round PCR program was the same as the one shown in Table 8.1. After the reaction was completed, the 96 samples were cleaned up using QIAquick PCR Purification kit (Qiagen) according to the manufacturer’s protocol. The 96 samples were then analyzed on the DHPLC, and the separated single peaks were match against the peaks in the original chromatograms of the non-mixed cultures based on retention times. A total of 16 unique peaks were
successfully isolated using this procedure. The isolated peaks were then prepared in 10 ng PCR product + 7 pmole of each of the 341F/518R primers in volumes of 12 uL dH₂O. The products were sequenced at the University of Cincinnati Childern’s Hospital Core Facility (Cincinnati, OH) by the Sanger Chain-Termination Method. The obtained sequences were searched against the GenBank database using the BLAST search algorithm.

### 8.3 RESULTS AND DISCUSSION.

**Mineralization Experiment.** The results of the 5 x 5 factorial mineralization experiment are shown in Table 8.3. The results in Table 8.3 indicate that acclimation of microbial cultures to a specific fuel blend does not necessarily result in a higher mineralization for that fuel blend. For example, the cultures acclimated to B0, Soy-B40, and Soy-B100 mineralized only 49.17%, 40.89%, and 46.17% of B0 carbon after 42 days, respectively. Meanwhile, the cultures acclimated to Flax-B50 and Flax-B100 were able to mineralize 86.58% and 64.28% of B0, respectively. Similarly, the culture acclimated to B0 was able to mineralize 68.14% and 80.88% of Flax-B50 and Flax-B100, respectively; while the cultures acclimated to Flax-B50 and Flax-B100, respectively, mineralized 86.25% and 85.39% of Flax-B50, and 84.92% and 76.51% of Flax-B100 after a 42-day incubation period. From Table 8.3, it appears that the cultures that were acclimated to flaxseed biodiesel blends were significantly better in mineralizing the different petrodiesel/biodiesel fuels, regardless of the type and blend of the fuel being degraded.

It is known that the morphology and physiology of bacterial cultures, specifically the fluidity and permeability of the cell’s membrane, can be chiefly altered through the biochemical processes taking place within the cell [20, 21]. For example, heat shock tolerance and acid resistance are affected by the unsaturation level of fatty acids substrates [22, 23]. In our case, more than 50% of the flaxseed biodiesel consists of C18:3 FAME, while more than 50% of soybean biodiesel is made of C18:2
FAME. Petrodiesel, on the other hand, is characterized by 75 – 85% saturated hydrocarbon, 10 – 15% aromatic hydrocarbons, and less than 1% alkenes [24]. Higher unsaturated fatty acids (i.e. C18:3) are shown to increase the fluidity and permeability of cells’ membranes [25, 26]. This means higher C18:3 FAME in the fuel, as in flaxseed biodiesel, could possibly alter the structure and permeability of the cell membrane, and may increase the solubility of the substrates in the membrane [26-28].

**Microbial Communities.** Figure 8.1 contains the UV-absorbance chromatograms of the 8 aforementioned cultures acclimated to the different biodiesel/petrodiesel blends. Figure 8.2 shows the chromatograms of the mixed PCR product (Figure 8.2a) and the individually isolated clones in the clone reaction (Figure 8.2b). The successfully identified peaks are numbered in Figure 8.1 as P1 – P16. Table 8.4 summarizes the identities of the sequenced clones, and provides a summary of the information available in the literature on those species.

Each peak in Figure 8.1, in theory, corresponds to a unique strain. There was no obvious trend governing the shifts of the microbial communities in Figure 8.1. The peaks recognized as P6 and P10 were identified as two different strains of the species *Enterobacter hormaechei*, while P7, P11, and P13 were identified as three different strains belonging to *Zhihengliuella sp.* The cultures of Flax-B50 and Flax-B100, which showed the best mineralization capabilities, appear to be dominated by *Enterobacter gergoviae* (P2), *Enterobacter hormaechei* (P6 and P10), and *Acidovorax avenae* subsp. (P9). *E. gergoviae* (P2) has been shown to possess esterase hydrolytic activity [15, 16], while *A. avenae subsp.* (P9) has been shown to possess oxidase activity [28]. *E. hormaechei* (P6 and P10), on the other hand, is a widely reported species that seems to be capable to thrive under a wide range of environmental conditions [23, 24]. P9 also appears to be a dominant species in the Soy-B100 culture, while Soy-B40 and B0 cultures were dominated by a wide range of different organisms. In conclusion,
whatever factors govern the shifts in the microbial community structures of the different cultures, they appear to be complex ones.

REFERENCES


40. Mechichi, T.; Stackebrandt, E.; Gad'on, N.; Fuchs, G., Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of Thauera phenylacetica sp. nov., Thauera aminoaromatica sp. nov., and Azoarcus buckelii sp. nov. Archives of Microbiology 2002, 178, (1), 26-35.


In *Bergey’s Manual® of Systematic Bacteriology*


Table 8. Sequences of PCR primers used and PCR thermal cycler program.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>341F</td>
<td>5’- CCT ACG GGA GGC AGC AG - 3’</td>
<td>Ref.[29]</td>
</tr>
<tr>
<td>518R</td>
<td>5’- ATT ACC GCG GCT GCT GG - 3’</td>
<td>Ref.[29]</td>
</tr>
<tr>
<td>GC-Clamp</td>
<td>5’- CGC CCG CCG CGC GCG GCG GGC GGG GCG GCA CGG GGG G - 3’</td>
<td>Ref.[29]</td>
</tr>
<tr>
<td>M13F</td>
<td>5’- GTA AAA CGA CGG CCA G - 3’</td>
<td>Ref.[30]</td>
</tr>
<tr>
<td>M13R</td>
<td>5’- CAG GAA ACA GCT ATG AC - 3’</td>
<td>Ref.[30]</td>
</tr>
</tbody>
</table>

**Touchdown PCR Cycling Program**

5 min at 94°C initial, 20 cycles of: 1 min at 94°C, 1 min at 65 ...55°C (touchdown -0.5°C C/cycle); followed by 15 cycles of: 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C, followed by a final extension period of 7 min at 72°C. Ref.[19]
Table 8.2. DHPLC method conditions.

<table>
<thead>
<tr>
<th>Analytical Step</th>
<th>Time (min)</th>
<th>% Buffer A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Buffer B&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Loading</td>
<td>0</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Start Gradient</td>
<td>0.5</td>
<td>56</td>
<td>44</td>
</tr>
<tr>
<td>Stop Gradient</td>
<td>28.5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Start Clean</td>
<td>28.7</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Stop Clean</td>
<td>30.5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Start Equilibrate</td>
<td>30.6</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>Stop Equilibrate</td>
<td>32.5</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

Buffer B Slope = 2% per min.

<sup>a</sup> Buffer A is an aqueous solution of 0.1 M TEAA, pH 7.

<sup>b</sup> Buffer B is similar to Buffer A with 25% (v/v) acetonitrile.

- Flow rate = 0.3 mL/min; oven temperature = 65.5 °C.
Table 8. 3. Results of the 5 X 5 factorial design mineralization experiments using the cultures acclimated to the five petrodiesel/biodiesel blends (B0, Soy-B40, Soy-B100, Flax-B50, and Flax-B100) after 7-day and 42-day incubation periods.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>B0</th>
<th>Soy B40</th>
<th>Soy B100</th>
<th>Flax B50</th>
<th>Flax B100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acclimated To</td>
<td>7-day [CO₂%]</td>
<td>42-day [CO₂%]</td>
<td>7-day [CO₂%]</td>
<td>42-day [CO₂%]</td>
<td>7-day [CO₂%]</td>
</tr>
<tr>
<td>B0</td>
<td>23.08±2.11</td>
<td>49.17±.63</td>
<td>42.68±0.78</td>
<td>64.32±2.33</td>
<td>58.0 ±1.95</td>
</tr>
<tr>
<td>Soy- B40</td>
<td>22.54±0.5</td>
<td>40.89±2.46</td>
<td>41.47±1.48</td>
<td>65.29±1.38</td>
<td>53.9± 1.04</td>
</tr>
<tr>
<td>Soy- B100</td>
<td>31.29±0.82</td>
<td>46.1±0.91</td>
<td>45.04±1.47</td>
<td>65.94±1.88</td>
<td>64.13±1.05</td>
</tr>
<tr>
<td>Flax-B50</td>
<td>48.28±2.57</td>
<td>86.58±1.33</td>
<td>63.67±2.72</td>
<td>86.88±5.68</td>
<td>70.27±0.95</td>
</tr>
<tr>
<td>Flax- B100</td>
<td>37.32 ±0.61</td>
<td>64.28±8.62</td>
<td>51.88±1.23</td>
<td>79.78±1.96</td>
<td>58.62± 1.1</td>
</tr>
</tbody>
</table>
Figure 8.1. UV absorbance chromatograms using DHPLC of the 16S rRNA PCR products of eight microbial cultures acclimated to: a) B0, b) Soy-B20, c) Soy-B40, d) Soy-B60, e) Soy-B80, f) Soy-B100, g) Flax-B50, and h) Flax-B100. The peaks P1 – P16 are detailed in Table 8.4.
Figure 8.2. UV absorbance using DHPLC of the: a) mixed PCR product of the eight cultures together, b) unique isolated peaks via cloning of the Mixed PCR product.
Table 8.4. Summary of sequenced clones P1 – P16.

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Identity Match</th>
<th>Closest Relative</th>
<th>Family</th>
<th>Order</th>
<th>Accession Number</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>98%</td>
<td><em>Rhodobacter changlensis</em></td>
<td><em>Rhodobacteraceae</em></td>
<td><em>Rhodobacterales</em></td>
<td>AM399030</td>
<td>Facultative organoautotroph, gram-negative, oval to rod shaped. [31, 32]</td>
</tr>
<tr>
<td>P2</td>
<td>98%</td>
<td><em>Enterobacter gergoviae</em></td>
<td><em>Enterobacteriaceae</em></td>
<td><em>Enterobacteriales</em></td>
<td>AB004748</td>
<td>Aerobic, gram-negative, rod shaped esterase hydrolytic activity. [33, 34]</td>
</tr>
<tr>
<td>P3</td>
<td>99%</td>
<td><em>Sneathiella chinensis</em></td>
<td><em>Sneathiellaceae</em></td>
<td><em>Sneathiellales</em></td>
<td>NR043652</td>
<td>Strictly aerobic, halotolerant, chemoheterotroph, gram-negative, activated sludge organism with catalyase and oxidase activities. [35-37]</td>
</tr>
<tr>
<td>P4</td>
<td>98%</td>
<td><em>Thauera butanivorans</em></td>
<td><em>Rhodocyclaceae</em></td>
<td><em>Rhodocyclales</em></td>
<td>NR040797</td>
<td>Formerly known as &quot;Pseudomonas butanovora&quot;. Facultative, chemoheterotroph, gram-negative, alkanes' oxidizing but not aromatic hydrocarbons. [38]</td>
</tr>
<tr>
<td>P5</td>
<td>99%</td>
<td><em>Thauera aromatica</em></td>
<td><em>Rhodocyclaceae</em></td>
<td><em>Rhodocyclales</em></td>
<td>NR026153</td>
<td>Formerly classified as a &quot;Psseudomonad sp.&quot;, facultative, gram-negative, aromatic hydrocarbons degrading organism. [39, 40]</td>
</tr>
<tr>
<td>Clone Number</td>
<td>Identity Match</td>
<td>Closest Relative</td>
<td>Family</td>
<td>Order</td>
<td>Accession Number</td>
<td>Remarks</td>
</tr>
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</tr>
<tr>
<td>P6</td>
<td>97%</td>
<td>Enterobacter hormaechei</td>
<td>Enterobacteriaceae</td>
<td>Enterobacterales</td>
<td>NR042154</td>
<td>Aerobic, gram-negative, chemolithotrophic organism; widely reported in different environments. [41-43]</td>
</tr>
<tr>
<td>P7</td>
<td>98%</td>
<td>Zhihengliuella sp.</td>
<td>Micrococcineae</td>
<td>Actinomycetales</td>
<td>EU847536</td>
<td>Aerobic, gram-positive, rod shaped actinobacterium. [44]</td>
</tr>
<tr>
<td>P8</td>
<td>99%</td>
<td>Mycobacterium rutilum</td>
<td>Corynebacterineae</td>
<td>Actinomycetales</td>
<td>NR043761</td>
<td>Strictly aerobic, gram-positive PAH degrading organism. [45]</td>
</tr>
<tr>
<td>P9</td>
<td>98%</td>
<td>Acidovorax avenae subsp.</td>
<td>Comamonadaceae</td>
<td>Burkholderiales</td>
<td>AF137504</td>
<td>Strictly aerobic, gram-negative, motile, chemooorganotrophic organism with oxidase activity. [46]</td>
</tr>
<tr>
<td>P11</td>
<td>100%</td>
<td>Zhihengliuella sp.</td>
<td>Micrococcineae</td>
<td>Actinomycetales</td>
<td>NR044575</td>
<td>See P7.</td>
</tr>
<tr>
<td>P12</td>
<td>99%</td>
<td>Algoriphagus hitonicola</td>
<td>Cyclobacteriaceae</td>
<td>Cytophagales</td>
<td>NR044249</td>
<td>Aerobic, gram-negative, heterotrophic organism isolated from lagoons and coastal sediments. [47, 48]</td>
</tr>
<tr>
<td>P13</td>
<td>98%</td>
<td>Micrococcineae</td>
<td>Actinomycetales</td>
<td></td>
<td>EU847536</td>
<td>See P7.</td>
</tr>
<tr>
<td>Clone Number</td>
<td>Identity Match</td>
<td>Closest Relative</td>
<td>Family</td>
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<tr>
<td>P14</td>
<td>98%</td>
<td><em>Ralstonia sp.</em></td>
<td><em>Alcaligenaceae</em></td>
<td><em>Burkholderiales</em></td>
<td>EF016361</td>
<td>Aerobic, gram-negative, motile organism reported to be able to degrade a wide range of substitute aromatic and aliphatic hydrocarbons.[49-51]</td>
</tr>
<tr>
<td>P15</td>
<td>100%</td>
<td><em>Hyphomicrobium zavarzinii</em></td>
<td><em>Hyphomicrobiaceae</em></td>
<td><em>Rhizobiales</em></td>
<td>Y14305</td>
<td>Morphologically, ecologically, and metabolically diverse genus.[52-54]</td>
</tr>
<tr>
<td>P16</td>
<td>91%</td>
<td><em>Pseudaminobacter defluvii</em></td>
<td><em>Phyllobacteriaceae</em></td>
<td><em>Rhizobiales</em></td>
<td>NR036945</td>
<td>Gram-negative, obligate aerobic heterotrophs able to grow on substituted aromatics and naphthalenes. [55, 56]</td>
</tr>
</tbody>
</table>
CHAPTER 9: CONCLUDING REMARKS
SIGNIFICANCE OF THIS WORK

The research presented in this thesis is of significant importance in understanding the behavior and bio-physical interactions of poorly soluble materials in general, and biodiesel/petrodiesel blends in specific, in aquatic environments. The development of the microbial kinetic model for the degradation of poorly soluble materials provided a valuable framework in analyzing the biodegradation data of petrodiesel/biodiesel blends, and allowed us to determine the mechanism by which biodiesel enhances the microbial utilization of petrodiesel. In fact, this model is expected to serve as a critical tool to better understand the biotransformation data of similar low solubility compounds in environmental systems as well as controlled bio-mediated synthesis of high-end chemicals.

The findings presented in this work outlines the positive impact of biodiesel in the bioremediation of petroleum fuels by enhancing the bioavailability of hydrophobic hydrocarbons by mechanisms such as increasing the dissolved solubility as well as the colloidal dispersibility of nonaqueous-phase liquids (NAPLs). Another mechanism by which biodiesel enhances the bioremediation of petroleum NAPLs is by increasing the actual growth yield of the degrading organisms.
KEY RESEARCH AREAS

There are some key problems associated with the use of biodiesel that need to be investigated in further details. Specifically, the autoxidative instability of biodiesel can cause acute ecological hazards at an incident of a spill; while in the chronic-effect timeframe, the identified autoxidation byproducts are shown in the literature to be readily biodegradable. The factors that affect the autoxidative instability of biodiesel need to be further examined. The effect of different transesterification and purification processes on the variability of biodiesel’s physiochemical properties and autoxidative instability are essential information that need to be researched in further details. Also, the effect of the addition of antioxidant to biodiesel needs to be thoroughly investigated. From the findings presented in this thesis, it appears that the autoxidation reaction occurs mainly in the aqueous phase. Therefore, the addition of combination hydrophilic and hydrophobic antioxidants in biodiesel might mitigate the risks associated with the acute toxicity of its autoxidation byproducts.