BIODEGRADABILITY OF BIODIESEL IN THE AQUATIC ENVIRONMENT

X. Zhang, C. Peterson, D. Reece, R. Haws, G. Möller

ABSTRACT. *The biodegradability of various biodiesel fuels was examined by the CO_2 evolution method (EPA 560/6-82-003), BOD_5 (EPA 405.1), COD (EPA 410), and gas chromatography (GC) analyses in an aquatic system. The fuels examined included the methyl- and ethyl-esters of rapeseed oil and soybean oil, neat rapeseed oil, neat soybean oil and Phillips 2-D low sulfur, reference petroleum diesel. Blends of biodiesel/petroleum diesel at different volumetric ratios, including 80/20, 50/50, and 20/80, were also examined. The results demonstrate that all the biodiesel fuels are "readily biodegradable". Moreover, in the presence of REE, the degradation rate of petroleum diesel increased to twice that of petroleum diesel alone. The pattern of biodegradation in the blends and reasons why biodiesel is more readily degradable than petroleum diesel are discussed. The biodegradation monitoring results from both CO_2 evolution and GC methods are compared.*
Keywords. *Biodiesel, Biodegradation, BOD, COD.*

Biodiesel fuels developed from vegetable oils offer the promise of a renewable energy source. As these biodiesel fuels are becoming commercialized as complete fuels and as petroleum diesel blends, their fate in the environment is an area of concern. This is a result of petroleum oil spills, which may constitute a major source of contamination of the aquatic and terrestrial ecosystems. In addition, water quality for a variety of uses, including drinking water, can suffer significant degradation as a result of the toxicity and mobility of petroleum hydrocarbon fuels. These properties can be a prime determinant of the potential toxic impact in the event of an environmental release. Although biodiesel primarily consists of fatty acids, persistence in aquatic ecosystems is not desirable. As early as the 1970s, investigators (Novak and Kraus, 1973) reported that long chain fatty acids, either free or combined, comprise about 80% of the grease fraction in sewage. Grease has been found to make up 23 to 52% of the total organic fraction in sewage. Therefore, it is important to examine the biodegradability of biodiesel fuels and their biodegradation rates in natural waters in the event of release into the aquatic environment in the course of their use or disposal.

In general, organic chemicals can be grouped into three categories according to their biodegradability: readily degradable, inherently (potentially) degradable, and persistent or recalcitrant compounds (Pitter and Chudoda, 1990). "Readily biodegradable" is an expression applied to a classification of compounds which, in certain biodegradation test procedures, produce positive results that are unequivocal and which lead to the reasonable assumption that the substance will undergo rapid and ultimate biodegradation in aerobic aquatic environments (EPA, 1992).

There are several test methods for assessing the biodegradability of a compound. Among them, the CO_2 evolution test (shake flask system) indirectly measures the disappearance of a substrate (ultimate degradation or mineralization) (Lyman et al., 1990). It is relatively simple, economical, and pollution preventing since there are no organic solvents used. Under aerobic conditions and nutrient supply (N, P), microorganisms can metabolize a hydrocarbon substance to two final products, CO_2 and water. CO_2 is presumed to be the prevalent indicator of organic substance breakdown. If the substrate is the only carbon source, the amount of CO_2 evolved will be proportional to the carbons consumed by microorganisms from the test substrate. In comparison, gas chromatography (GC) analysis directly detects the disappearance of a substrate (primary degradation), and usually involves the use of an extraction solvent such as methylene chloride or hexane. GC instrumentation is relatively expensive compared to the shake flask and titration equipment used in carbon dioxide evolution methods. This study compares the two methods under the same experimental conditions.

The Biochemical Oxygen Demand test (BOD) is a measure of the amount of oxygen consumed during the microbially mediated assimilation and oxidation of the organic matter present in a substrate/water mixture (Eaton et al., 1995). The five day chemical oxygen demand procedure, BOD_5, used in the study was an empirical bioassay-type procedure wherein the samples were inoculated with a microbial population and incubated for five days using standardized laboratory procedures [EPA (Method 405.1), 1983a]. Similar to BOD_5, the Chemical

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Oxygen Demand (COD) method determines the quantity of oxygen required to chemically oxidize the organic matter in a substrate/water mixture, but the test is completed under specific conditions of an oxidizing agent, temperature and time, and therefore relative to those parameters [EPA (Method 410.1), 1983b]. COD is often used with BOD_5 for determining the relative oxygen requirements of municipal and industrial wastewater. However, the significant variability in values indicates that BOD_5 is not an exact measure of biodegradability of organic substances (Pitter and Chudoba, 1990). The objective of the BOD_5 and COD phase of the present study was to further characterize the biodegradability of biodiesel fuels.

Opportunistic microbial biodegradation typically involves utilization of a substrate for nutrient or energy needs or biochemical detoxification of toxic insult. Co-metabolic biodegradation (co-metabolism) is a term used to describe the process in which microorganisms use a second substrate (readily degradable) as the carbon (energy) source to degrade the first substrate which otherwise is scarcely attacked by the microorganisms when it is the sole carbon source. It is especially significant for degradation of resistant substances. The biodiesel/diesel blend is more commonly used as fuel than is the neat biodiesel (Peterson et al., 1995, 1997). It is therefore of interest to investigate the degradation pattern of the blend.

The first objective of this study was to examine the biodegradability of various biodiesel fuels in comparison with diesel fuel in the aquatic environment by CO_2 evolution [EPA (560/6-82-003), 1992], BOD_5, COD, and GC analyses. A second objective was to investigate the biodegradation pattern in biodiesel/diesel blends using the same techniques. This report also discusses why biodiesel is easier to degrade and why the primary degradation rate determined by GC is much faster than the ultimate degradation rate determined by the CO_2 evolution method.

Materials and Methods

The Test Substrates

The test substances included: neat rapeseed oil (NR) and neat soybean oil (NS) as well as their esterified products rapeseed ethyl ester (REE), rapeseed methyl ester (RME), soyate ethyl ester (SEE), and soyate methyl ester (SME), and Phillips 2-D reference petroleum diesel (2-D). The esterified oils were produced by a base catalyzed mixture of the vegetable oil and either methanol (methyl ester) or ethanol (ethyl ester) prepared as reported in Peterson et al. (1991) and Peterson et al. (1997). The test materials were obtained from the University of Idaho Biological and Agricultural Engineering Department Biodiesel Program and from the National Biodiesel Board. The composition and specific properties of biodiesel fuels are listed in table 1 and 2-D in table 2. Fatty acid analysis was conducted by the University of Idaho Plant Science Laboratory using gas chromatography. The tests for specific gravity, viscosity, flash point, and net heat of combustion were performed at the Analytical Lab, Department of Biological and Agricultural Engineering, University of Idaho. The elemental analyses were performed by Phoenix Chemical Labs, Chicago, Illinois. Additional fuel properties of the test materials are contained in Peterson et al. (1997). The characterization of the Phillips 2-D reference diesel was supplied with the fuel.

Table 1. Composition and major physical properties of biodiesel fuels*

	NR	RME	REE	NS	SME	SEE
	Concentration (% by mass)					
Fatty acid components†						
Palmitic (16:0)	2.6	2.2	2.6	10.0	9.9	10.0
Stearic (18:0)	1.0	0.9	0.9	3.8	3.8	3.8
Oleic (18:1)	13.5	12.6	12.8	19.0	19.1	18.9
Linoleic (18:2)	11.7	12.1	11.9	55.7	55.6	55.7
Linolenic (18:3)	7.4	8.0	7.7	10.2	10.2	10.2
Eicosenoic (20:1)	8.5	7.4	7.3	0.2	0.2	0.2
Erucic (22:1)	48.9	49.8	49.5	0.0	0.0	0.0
Major specific properties‡						
Specific gravity, 60/60	0.91	0.88	0.88	0.92	0.89	0.88
Viscosity, cSt @ 40°C	46.7	5.65	6.17	32.5	3.89	4.49
Net heat of combustion (MJ/kg)	40.40	37.77	38.00	39.02	37.04	37.44
Flash point (°C)	274	179	124	257	188	171
Carbon (% by weight)	79.4	78.7	76.8	82.1	82.4	83.4
Hydrogen (% by weight)	11.8	12.66	11.80	N/A	12.9	11.8
Percent esterified	0	98.02	94.75	0	98.1	94.5

* Abbreviations used are neat rapeseed oil (NR), rapeseed methyl ester (RME), neat soy oil (NS), soy methyl ester (SME), soy ethyl ester (SEE).
† Fatty acid analysis was conducted by the University of Idaho Plant Science Laboratory using gas chromatography.
‡ The tests for specific gravity, viscosity, flash point, and net heat of combustion were performed at the Analytical Lab, Department of Biological and Agricultural Engineering, University of Idaho. The elemental analyses were performed by Phoenix Chemical Labs, Chicago, Illinois.

Table 2. Composition and major physical properties of Phillips 2-D low sulfur reference diesel fuel*

Components (Hydrocarbon Type)	Volume (%)
Saturates	66.2
Aromatics	29.9
Olefins	3.9
Physical Properties	
Specific gravity, 60/60	0.8466
Flash point (°C)	74
Viscosity, cSt @ 40°C	2.7
Net heat of combustion (MJ/kg)	42.9
Carbon (% by weight)	86.8
Hydrogen (wt%)	13.2

* Data from fuel supplier.

Table 3. Test system design

System	Substrates*	Concentration (mg carbon/L)	Methods
IA	REE, RME, SME, SEE, NR, NS, 2-D	10	CO_2
IB	REE	10	GC
IIA	Blends REE/diesel (v/v): 80/20, 50/50, 20/80	10	CO_2
IIB	Blends REE/diesel (v/v): 100/0,50/50,0/100	10	GC
III	REE, RME, SME, NR, NS, and 2-D		BOD_5 COD

* Products tested and the designations used in the table are Rapeseed Ethyl Ester (REE), Rapeseed Methyl Ester (RME), Soybean Methyl Ester (SME), Soybean Ethyl Ester (SEE), Neat Rapeseed Oil (NR), Neat Soybean Oil (NS) and Phillips 2-D low sulfur reference diesel fuel (2-D).

Test System Design

A total of three test systems were examined (table 3). System I was to compare the biodegradability of different biodiesel fuels and 2-D by the CO_2 evolution method. System II was to test the biodegradability of blends of REE/2-D at different ratios (v/v) and investigate the

degradation pattern. Finally, system III examined the biodegradability of five biodiesel fuels by BOD_5 and COD analyses. In the CO_2 evolution method, dextrose was used as the reference compound. The method also requires that a growth inhibitor should be established as a control for each substance tested. According to EPA (1992), "The inhibited system must contain the same amount of water, mineral nutrients, inoculum and test substance used in the uninhibited test systems plus 50 mg/L mercuric chloride ($HgCl_2$) to inhibit microbial activity."

CO_2 Evolution Method

The approach in this work followed the U.S. Environmental Protection Agency (EPA) standard method for determining biodegradability of chemical substances (EPA, 1992). A brief review of this method follows.

Shaker Flask System. A 2-L Erlenmeyer flask (fig. 1) contained 900 mL deionized distilled water (DIW), 100 mL of inoculum (acclimation medium), 1 mL of each stock solution (table 4), and 10 mg L^{-1} carbon from the test compound. A reservoir holding 10 mL of barium hydroxide solution was suspended in the flask to trap the CO_2. After inoculation, the test flasks were sparged with CO_2 free air (by passage of compressed air through a series of three 2-L bottles each containing 1000 mL of 5N NaOH) to ensure that the trapped CO_2 came only from the microorganism's metabolizing the test substances. The flasks were sealed and incubated with shaking in a dark room.

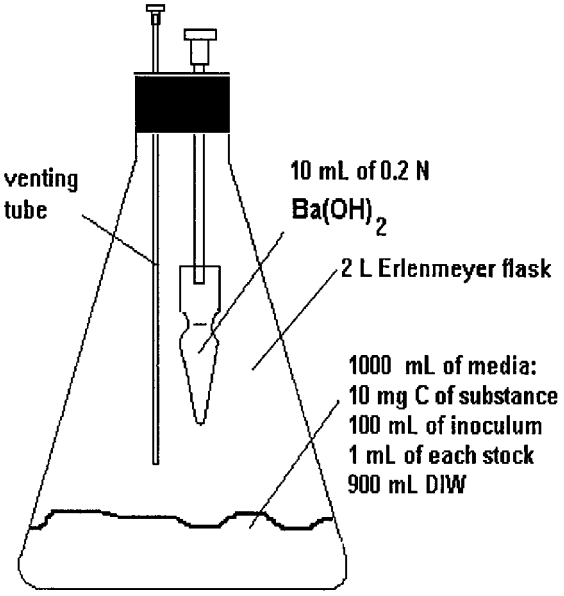

Figure 1–Shake flask system.

Table 4. Medium employed for assay of CO_2 evolution from EPA(1992)

Stock Solution	Compound	Conc. (g/L)
I	NH_4Cl	35
	KNO_3	15
	$K_2HPO_4.3H_2O$	75
	$NaH_2PO_4.H_2O$	25
II*	KCl	10
	$MgSO_4$	20
	$FeSO_4.7H_2O$	1
III	$CaCl_2$	5
	$ZnCl_2$	0.05
	$MnCl_2.4H_2O$	0.5
	$CuCl_2$	0.05
	$CoCl_2$	0.001
	H_3BO_3	0.001
	MoO_3	0.0004

* = Final pH is adjusted to 3.0 with HCL.

Inoculum (Acclimation Medium). To 1000 mL DIW was added one gram of organic matter rich soil, 2 mL of activated (aerated) sewage mixed liquor, 50 mL of raw domestic sewage water, 25 mg/L of Difco vitamin-free casamino acids (Difco Laboratories, Mauston, Wis.), 25 mg/L of yeast extract, and 1 mL of each stock solution I, II, III (table 4). A test substance was added at concentrations equivalent to 4, 8, and 8 mg carbon/L by calculation on days 0, 7, and 11, respectively. At day 14, the inoculum was filtered through glass wool and ready for use.

Measurement of CO_2 Evolution. The quantity of CO_2 evolved was measured by titration of the entire $Ba(OH)_2$ sample (10 mL of $Ba(OH)_2$ plus 10 mL of rinse water) with 0.1 N HCl to the phenolphthalein end point. After sampling, the reservoir was refilled with fresh $Ba(OH)_2$. All the samples were analyzed at least five times in a 28-day period to provide sufficient data to determine biodegradation trend with time. Three mL of 20% H_2SO_4 were added on the day prior to terminating the test. The percent theoretical CO_2 evolved from the test compound was calculated at any sampling time from the formula:

$$\%\ CO_2\ \text{evolution} = \frac{TF - CF}{C} \times 100\% \qquad (1)$$

where TF represents milliliters (mL) of 0.1 N HCl required to neutralize the $Ba(OH)_2$ from the flask with the test substance; CF represents milliliters (mL) of 0.1 N HCl required to neutralize the $Ba(OH)_2$ from the control flask; and C is the theoretical volume of the HCl required to neutralize the CO_2 converted from the carbon. For 10 mg carbon: C = 16.67 mL of 0.1 N HCl.

GC Analysis

The GC method involved extraction of the samples with hexane and injection of a portion of the extract into a gas chromatograph. Quantitation was accomplished by using internal and external standards. In the extraction, the sample was first acidified to a pH of 2.0 or lower by adding 5 mL hydrochloric acid (1:1). One milliliter of internal standard was then added to the sample and shaken to mix well. Finally, 30 mL of hexane was added and the mixture was vigorously shaken for one minute. The layers were allowed to separate. The solvent layer was passed through a funnel containing sodium sulfate. The extract was transferred to a vial, sealed, and kept in a refrigerator (4°C) prior to the GC analysis.

The parameters of the GC analysis for biodiesel were: methyl ester of heptadecanoic acid C17:0 (methyl 17:0) was the internal standard with the concentration at 0.003 g mL^{-1} and hexane was the solvent. The instrument used was an HP 5890 series II GC equipped with a flame ionization detector (FID) and a capillary fused silica DB-23 column (30 m × 0.25 mm I.D. 0.25 μm film thickness; J&W Scientific, Folsom, Calif.) The temperatures were: injection port 250°C, detector 300°C, oven initial 215°C

for 3 min, and final 230°C at a rate of 3°C/min. Five microliters of extract were injected at 50:1 split ratio.

The parameters of GC analysis for 2-D were: 2-fluorobiphenyl was the internal standard and methylene chloride was the extraction solvent. The instrument used was an HP 5890 series II gas chromatograph equipped with a capillary fused silica DB-5MS column (25 m × 0.21 mm × 0.33 μm; J&W Scientific, Folsom, Calif.) and MS interface. The interface temperature was set at 280°C. The MS detector was set initially at 250°C. A linear gradient of the oven temperature from 100°C to 320°C at 20°C/min was used.

The percentage of primary degradation was determined by subtracting the substrate concentration at sampling time from the initial substrate concentration, and the difference was divided by the initial concentration.

BOD_5 and COD Analyses

BOD_5. The method consisted of overfilling an airtight 300 mL BOD bottle with a sample, to remove the headspace, and incubating it for 5 days at 20°C in the dark, EPA Method 405.1 (EPA, 1983a) Samples were prepared using a non-nutrient buffer and appropriate dilution. Dissolved oxygen was measured initially and following incubation. BOD_5 was computed from the difference between the initial and final dissolved oxygen (DO). Replicate analyses were performed in triplicate (the method specifies duplicate samples be used), therefore n = 6. Reference samples of glucose/glutamic acid solutions and WasteWatR™ (Environmental Resource Associates, Arvada, Colo.) were also tested in duplicate. Concentrations of the water accommodated fraction (WAF) are presented in table 5 for all substances tested. All substances were analyzed at or below the WAF for BOD_5. WAF values were converted to pure substances for statistical comparisons.

COD. The COD procedure, EPA Method 410.1 (EPA, 1983b), entailed the potassium dichromate oxidation of organic and oxidizable inorganic substances in a 50% sulfuric acid solution at reflux temperature. The test was performed using commercially prepared vials which contained a potassium dichromate solution in sulfuric acid. The sample was introduced into these vials which were then sealed, mixed, and heated. In this method, any "organic" material present reduced the dichromate to chromium ion and was detected spectrophotometrically at 600 nm. A standard solution of potassium biphthalate is used for standard curve concentrations, and WasteWatR (Environmental Resource Associates, Arvada, Colo.) is used as a reference sample. Fuels were tested at their appropriate WAF and these values were converted to pure substances for statistical comparison.

Table 5. Water Accomodated Fraction (WAF) values for test and reference substances*

Test Substance	WAF (mg/L)
REE	26.3
RME	25.7
NR	83.7
SME	12.9
NS	63.3
2-D	3.8

* Products tested and the designations used in the table are Rapeseed Ethyl Ester (REE), Rapeseed Methyl Ester (RME), Soybean Methyl Ester (SME), Neat Rapeseed Oil (NR), Neat Soybean Oil (NS), and Phillips 2-D reference diesel fuel (2-D).

Results

Biodegradability of Biodiesel Fuels (System I)

The average cumulative percent theoretical CO_2 evolution from six biodiesel fuels NR, NS, REE, RME, SEE, and SME and 2-D, tested in triplicate as required by the method, in 28 days is shown in figure 2 . The maximum CO_2 evolution values from REE, RME, SEE, SME were between 84.4 and 88.7% and were similar to that for dextrose. The statistical analysis indicates there is no difference in their biodegradability ($p \leq 0.05$.) The maximum percent CO_2 evolution values from NR and NS were 78.4 and 75.9%, respectively, which are slightly lower than their modified products. The CO_2 evolution value from 2-D was 18.2%. As illustrated in figure 3, the disappearance of REE and 2-D as measured by the GC shows that within one day the REE fatty acids in the sample were reduced by 63.8% while the reduction in 2-D was 27.0%. At day 2, no fatty acids were detectable. This suggests that the rate of primary biodegradation was much faster than that of mineralization (as measured by the CO_2 evolution method).

Biodegradation of Biodiesel/diesel Blends (System II)

The percentage of CO_2 evolution, averaged for three replicates, from REE/2-D blends, as shown in figure 4, increased in a linear fashion with the increase of REE concentration in the blend. The higher the volume of REE

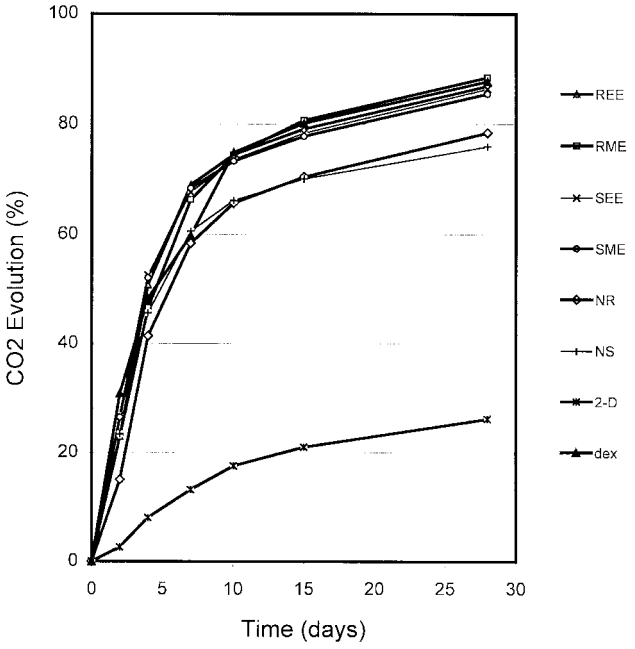

Figure 2–CO_2 evolution from test substance in 28 days. Products tested and the designations used in the chart are Rapeseed Ethyl Ester (REE), Rapeseed Methyl Ester (RME), Soybean Methyl Ester (SME), Soybean Ethyl Ester (SEE), Neat Rapeseed Oil (NR), Neat Soybean Oil (NS), Phillips 2-D reference diesel fuel (2-D), and dextrose (dex). Each point is the average of three replicates. All test substances have CO_2 evolution rates significantly higher than the 2-D control ($p < 0.05$). NS and NR are also significantly different ($p < 0.05$) than REE, RME, SEE, and SME.

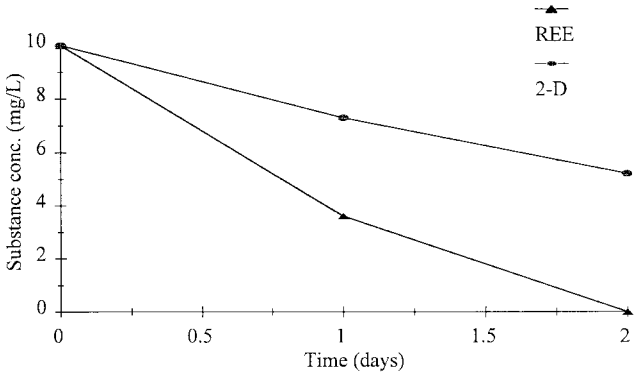

Figure 3–Disappearance of rapeseed ethyl ester (REE) and Phillips 2-D reference diesel fuel (2-D) in two days as indicated by GC analysis. Each point is the average of three replicates.

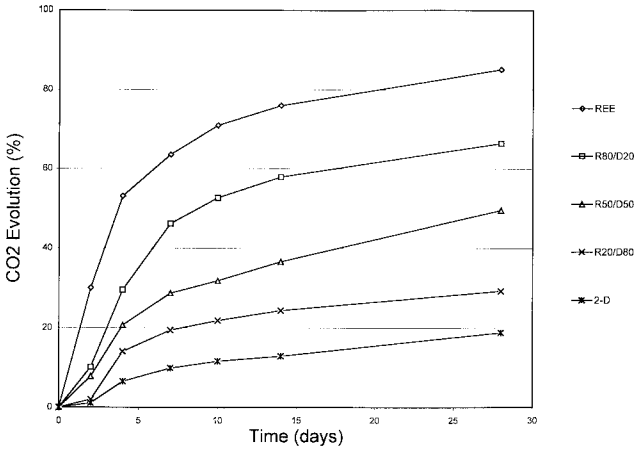

Figure 4–CO_2 evolution (%) from REE/2-D blends in 28 days. The products tested were Rapeseed Ethyl Ester (REE), Phillips 2-D reference diesel fuel (2-D) and blends of REE/2-D (v/v) designated as R80/D20 (80/20 REE/2-D), R50/D50 (50/50 REE/2-d), and R20/D80 (20/80 REE/2-d). Each point is the average of three replicates.

in the blend, the higher the percentage of CO_2 evolution. The relation was described by a linear equation $Y = 0.629 X + 20.16$ with R^2 of 0.992, where X = the percent concentration of REE in the blend and Y = the cumulative percent CO_2 evolved in 28 days.

Again, GC analysis showed much faster degradation in the REE/2-D 50/50 blend, 63.62% and 95.6% in day 1 and 2, respectively. The 2-D in the blend was degraded twice as fast as the 2-D alone; 62.8% versus 27.0% at day 1 as shown in figure 5. This observation may suggest that in the presence of REE, microorganisms used the fatty acids as an energy source to promote the degradation of 2-D. A comparison of GC identification in these two cases is shown in figure 6. One can see that when 2-D alone was the substrate, the peak counts of 2-D (top part), have no significant change between time 0 and day one. However, the 2-D peak counts decreased significantly within one day when REE was present (bottom part of fig. 6). At day one, only the internal standard, retention time = 7.072 min., remains the same, which indicates the same extraction efficiency.

The pattern of biodegradation of the diesel in the R50/D50 blend can also be found from the data. The

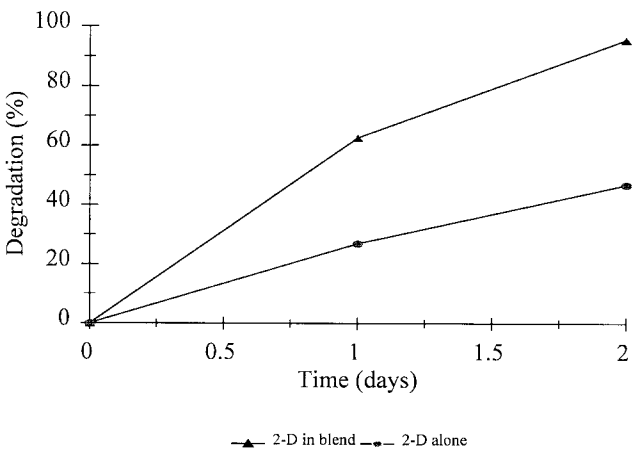

Figure 5–Degradation of Phillips 2-D reference diesel (2-D) in the blend of rapeseed ethyl ester (REE) and 2-D (REE/2-D 50/50 v/v) and 2-D alone as the substrate in two days. Each point is the average of three replicates.

degradation for the R50/D50 blend and the 2-D in the blend at day one are almost the same, 63.6% versus 62.8%. For day 2, the degradation of the R50/D50 blend and the 2-D in the blend were both 95.6%. The reduction of the area counts for both 2-D and REE were of the same proportion as shown in figure 6. The microorganisms apparently attack the fatty acids in the biodiesel and the aliphatic chains in the 2-D at the same time and same rates instead of favoring the fatty acids only.

Results of BOD_5 and COD (System III)

The BOD_5 analyses are shown in figure 7 for all test substances. The BOD_5 value for 2-D was significantly ($p < 0.01$) lower than all other test substances at 3.97×10^5 mg L^{-1}. Mean BOD_5 values for REE and RME were slightly lower than for that of NR. Replicate analyses of soybean oil products were very similar to those for the rapeseed products. Comparison of SME and REE values were not significantly different at $p > 0.20$. However, a difference was noted for comparison of REE to RME and SME to RME at $p < 0.05$.

Replicate mean COD values are presented in figure 8. Mean COD values for replicate analyses of REE and RME averaged 2.53 mg/L, 23% lower than NR. Replicate analyses of soybean oil products yielded mean COD values of 2.63×10^6 mg/L only 3% lower than NS. The 2-D had a mean COD value about 11% lower than the average of the soy and rape esters. No significant difference, at $p > 0.20$, was seen for comparison of any test substances to 2-D.

Discussion

The results show that the biodegradation of biodiesel is much easier and faster than that of 2-D. There may be several reasons for this. First, the rate of a catalyzed reaction may be regulated by the amount of catalyzing enzymes that are present in the cell. In other words, for a biochemical process to occur rapidly, appropriate enzymes must be available. Biodiesel consists of pure fatty acids; the enzymes responsible for their breakdown naturally exist. All the fatty acids are even hydrocarbon chains in ester form with two oxygen atoms attached, which makes them

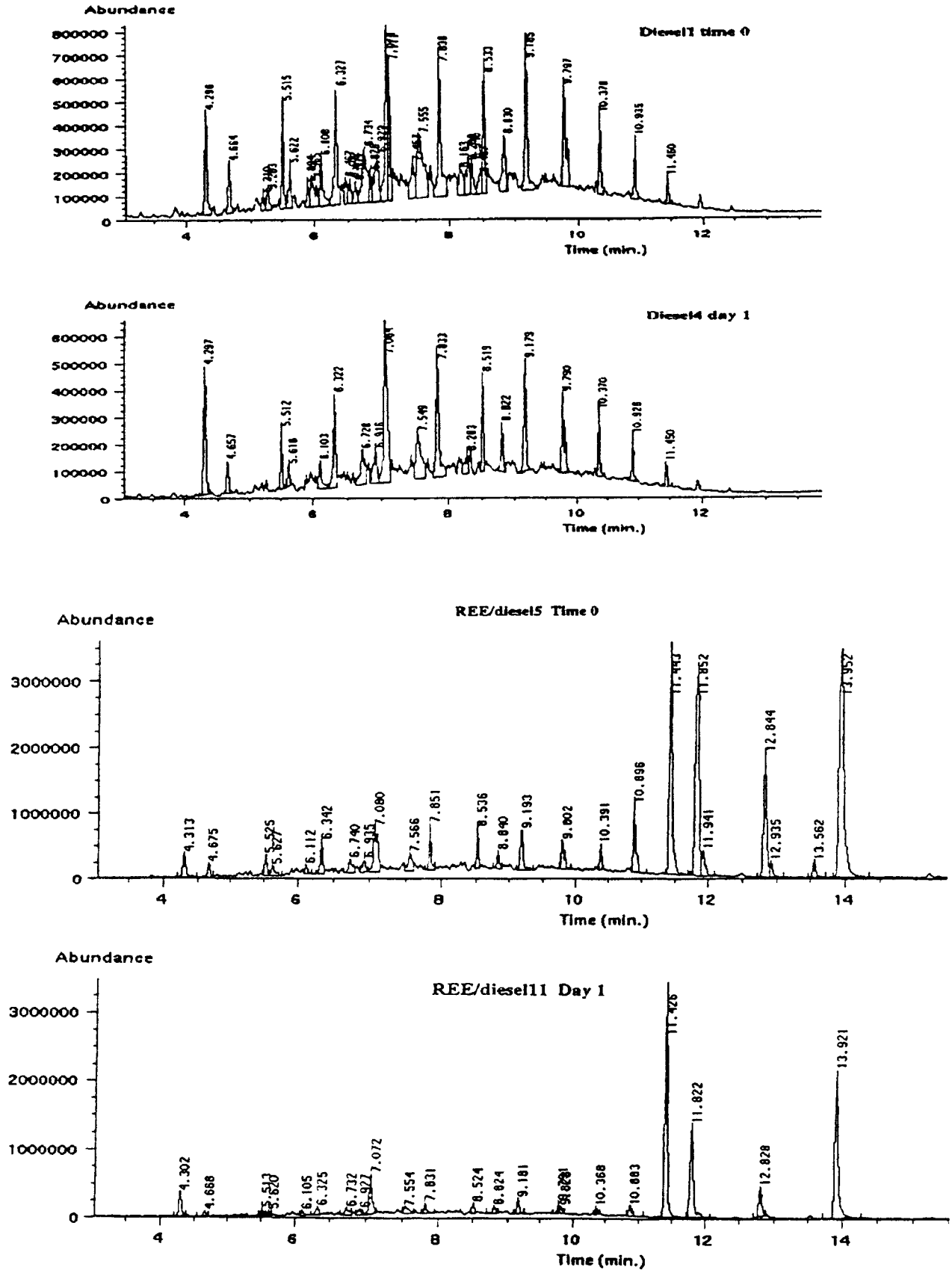

Figure 6–The GC analysis of the disappearance of the Phillips 2-D reference diesel fuel (2-D) in two different media at time 0 and day 1. The top part is the case of 2-D alone as the sole carbon source and shows almost no difference in the area counts between time 0 and day 1. The bottom part demonstrates the significant change of the area counts of rapeseed ethyl ester (REE)/2-D blend (50/50) between time 0 and day 1 with the larger peaks on the right being REE. In both cases, the concentration of the 2-D was 10 mg/L (or 10 ppm).

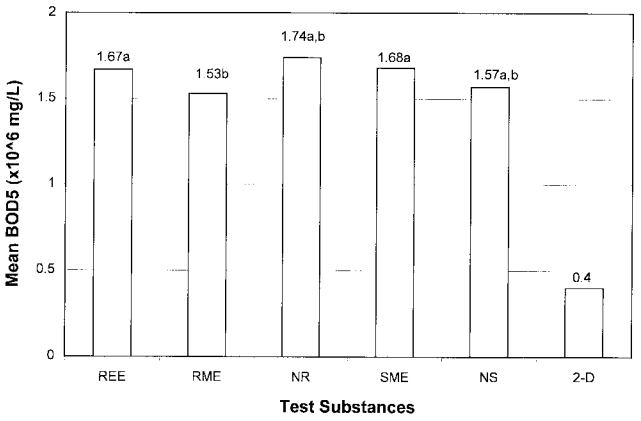

Figure 7–Mean BOD_5 values for test substances, n = 3. Products tested and the designations used in the chart are Rapeseed Ethyl Ester (REE), Rapeseed Methyl Ester (RME), Neat Rapeseed Oil (NR), Soybean Methyl Ester (SME), Neat Soybean Oil (NS), and Phillips 2-D reference diesel fuel (2-D). Each point is the average of six replicates. Test substances with the same letter are not significantly different, $p \leq 0.05$.

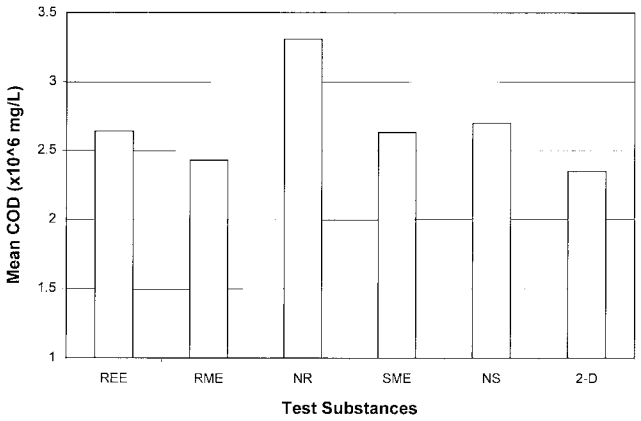

Figure 8–Mean COD values for test substances. Products tested and the designations used in the chart are Rapeseed Ethyl Ester (REE), Rapeseed Methyl Ester (RME), Neat Rapeseed Oil (NR), Soybean Methyl Ester (SME), Neat Soybean Oil (NS), and Phillips 2-D reference diesel fuel (2-D). Each point is the average of three replicates. No significant difference ($p > 0.20$) was found for any of the test substances compared to the 2-D control.

very biologically active. Enzymes such as acetyl-CoA dehydrogenase can recognize and attack them immediately.

It is reported that in the process of degradation, fatty acids are oxidized at the β carbon (hence β-oxidation) and degraded to acetic acid and a fatty acid with two fewer carbons (Zubay, 1993). On the other hand, the alkanes and alkenes (with hydrocarbon chains from C10-C20) in 2-D have no oxygen attached; therefore, they are not readily biologically degraded. However, bacteria populations have very strong mutability and adaptability; therefore, mutants may be present in the population which can produce new enzymes that can utilize new substrates (Pitter and Chudoba, 1990).

According to Pitter and Chudoba, (1990), initial 4-oxidation of the terminal CH_3 group to the carboxylic group is the main metabolic pathway during the biochemical oxidation of alkanes (monoterminal oxidation). This is then followed by β-oxidation of the aliphatic chain.

Finally, the composition of 2-D is chemically more complicated than biodiesel. In addition to alkanes and alkenes, it has a high aromatic content (about 30%) and is thus more toxic than some crude oils (Wilson and Bradley, 1996) and more resistant to degradation than aliphatic hydrocarbon chains.

Another theoretical problem to be addressed is why the rate and extent of mineralization determined by the CO_2 evolution method is lower than the primary degradation determined by GC analysis. First of all, microbial breakdown of fatty acids to CO_2 and H_2O is a complex process and consists of a series of reactions. During degradation the original form of the test substance may disappear from the medium. For example, Pitter and Chudoba (1990) suggest that degradation occurs by β-oxidation with two carbon atoms less in each degradation cycle. The carbon is converted to cell structure and CO_2 which is released to the atmosphere. The remaining carbon remains in the medium but is now present in a different form. It takes some time for the cells to digest 16-22 carbon chains (Loehr and Roth, 1968). The GC method detects the substrate disappearance while the CO_2 method examines respiration.

Secondly, a portion of the carbon from the substrates will be assembled into cell structure instead of being converted to CO_2. The higher the substrate concentration, the more significant the cell growth and, therefore, less CO_2 is detected (Pitter and Chudoba, 1990).

Finally, it is possible that some fatty acids are broken down to intermediates accumulated in the medium which are no longer detected by GC, but the digestion of these intermediates continues by the mixture of microorganisms. Hejzlar and Chudoba (1986) found that the organic substances excreted by activated sludge microorganisms into the cultivation medium can be both end products and intermediates, whereas intermediates are readily re-assimilated during the growth of a mixed culture. Why are intermediates accumulated? According to Hejzlar and Chudoba (1986), some bacteria, e.g., *Pseudomonas aeruginosa*, can metabolize glucose in two ways. At low concentrations glucose is completely oxidized inside the cells whereas at high concentrations it is instead oxidized via the extracellular pathway (by periplasic enzymes) to gluconic acid which is released from the cells.

CONCLUSION

1. The cumulative percent theoretical CO_2 evolution over 28 days from each biodiesel is within the range of 77 to 89% while 2-D was only 18%. This indicates that the biodiesel fuels are “readily biodegradable” according to the EPA definition and will be biodegraded in natural aerobic freshwater environments.
2. The cumulative percent theoretical CO_2 evolution over 28 days from the REE/2-D blends increases linearly with increasing concentration of biodiesel in the blend. The more biodiesel present in a blend, the higher the percentage of CO_2 detected. Thus, a blend with 63% REE and 37% 2-D will yield 60% CO_2 over a 28-day period.

3. The rates and extent of primary degradation determined by GC analysis for both the biodiesel and diesel are much higher than those of mineralization by the CO_2 evolution, 100% in two days versus 84% in 28 days for REE and 48% versus 18% for diesel, respectively.
4. Based on the GC analyses, the biodiesel in the blend appeared to promote and increase the rate biodegradation of petroleum diesel.
5. The BOD_5 values of all biodiesel fuels are significantly higher than that of diesel. However, no significant difference was noted for COD values between the biodiesel fuels and 2-D.

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