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Assessment of Aquatic Toxicity and Oxygen Depletion during Aerobic Biodegradation of Vegetable Oil: Effect of Oil Loading and Mixing Regime

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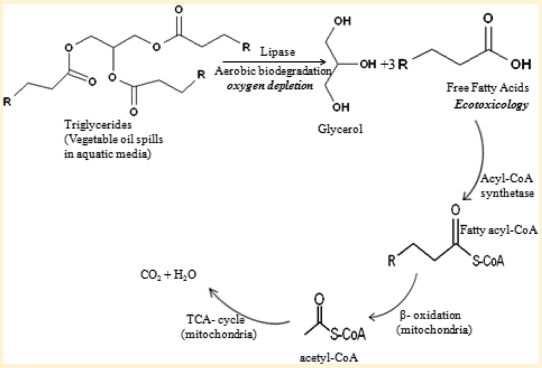
Supporting Information

ABSTRACT: The potential ecological impacts of aerobic biodegradation of vegetable oils on contaminated water columns was investigated in the laboratory at different oil loadings (100, 333, and 1,000 gal acre^{-1}) and mixing regimes (fully, moderately, and nonmixed microcosms). The impacts were estimated by use of the Microtox assay and dissolved oxygen concentration measurements. The results of the Microtox assay showed no major toxicity at the 100 gal acre^{-1} loading. Furthermore, oxygen was not completely depleted from the water column at this oil coverage. At higher oil loadings, oxygen was fully depleted from the mixed and nonmixed water columns. A transient toxicity in the aqueous phase was observed in the case of the moderately mixed microcosms at 333 gal acre^{-1} and was maintained at moderate levels (EC_{50} ~ 30%) in the nonmixed microcosms. A substantial increase in toxicity (EC_{50} ~ 10%) was observed in both mixing conditions when the initial oil loading was increased to 1,000 gal acre^{-1}. At all oil loadings, significant toxicity (EC_{50} < 2%) was found in the solid phase due to the strong partition of lipids to the biomass. Long and medium chains fatty acids associated with the measured toxicity were detected in both liquid and solid phases.



1. INTRODUCTION

The fate, behavior, and environmental impact of spills of vegetable oils in the aquatic environment are not as widely considered as those of mineral oils.[1] Nevertheless, a spill of vegetable oil can be equally problematic.[1,2] Between 1974 and 1978, an estimated 35 barrels from two rapeseed oil spills in the Vancouver Harbor killed about 500 birds, while 176 spills of petroleum oils during the same period resulted in only 50 oiled birds.[2−5]

Most of the deleterious effects from vegetable oil spills are associated with oil floating on the water surface or suspended in the water column.[5−7] Besides their coating properties leading to smothering of surface dwelling organisms,[4] vegetable oils can have significant detrimental effects through oxygen depletion and suffocation.[1,8] This is a result of the reduced oxygen exchange across the air water interface[8] and the high biochemical oxygen demand (BOD) from microbial activity.[4,5,9,10] Oxidative polymerization of unsaturated oils may also lead to anoxic conditions by forming an impermeable cap on the shoreline or over the sediment surface.[1,4,5] Under reduced dissolved oxygen concentration, Lloyd[11] demonstrated that the toxic effect of some poisons to rainbow trout increases as a result of the augmented rate of respiratory flow through the fish gill epithelium.

Toxicity in the aquatic environment is another main concern in the case of vegetable oil spills. Traditionally considered as nontoxic since they are edible products,[2,9] vegetable oils may be directly toxic to aquatic fauna or cause sublethal effects.[4,7,12−15] Salgado[15] studied the sublethal effects of vegetable oils on mussels grown in a flow through seawater system (300 mL/min). He found that mussels exposed over a 4-week period at oil contamination rates as low as 0.3 mL/min had a significantly lower growth rate and higher mortality compared to unexposed controls. Direct toxicity of vegetable oil to benthic organisms was demonstrated by Li et al.[7] in laboratory bioassays using *Hyalella azteca* in sediments amended with canola oil at 17 and 33 g/kg. At both oil concentrations, the authors reported a significant initial reduction in the amphipod survival (35 and 15% survival at oil concentrations of 17 and 33 g/kg, respectively) compared to the unoiled control sediments (97% survival).

Besides their potential direct harmful impact, vegetable oil toxicity is mainly due to the accumulation in the medium of

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long chain fatty acids (LCFAs), the primary hydrolysis products of vegetable oils which are known for their inhibitory effect on microorganisms in both aerobic and anaerobic treatment processes.[6,16−20] Toxicity of LCFAs is mainly related to their adsorption to the microorganisms creating a physical barrier and disrupting the cell membrane transport and protective functions.[7,19] Furthermore, some oil autoxidation byproducts have antibacterial properties that may kill the bacteria or inhibit their activity.[21] For instance, liquid phase toxicity of polyunsaturated triacyglycerols (TAGs) was reported by Campo et al.[8] in abiotic TAGs blanks and was related to the presence of azelaic acid, a known product of lipid autoxidation.

While different types of oil behave similarly at the initial stage of a spill, their further evolution depends on various factors including the nature of the oil, the quantity spilled, and the characteristics of the environment.[9] With respect to biodegradation, most studies agree that unsaturated fatty acids are degraded more readily than their less water-soluble saturated counterparts. Campo et al.[8] reported a limited bioavailability of the two solid TAGs, tripalmitin and tristearin, due to their extremely nonpolar nature forming irregular clumps or gumballs once added to water. Furthermore, the inhibitory effect of high concentrations of vegetable oil was reported in the literature. Li et al.[6] observed a strong inhibition of the biotransformation of canola oil in enriched sediments when the initial oil loading was increased from 1.9 to 19 g oil/kg sediment. Characteristics of the impacted aquatic environment greatly influence the biodegradation behavior of spilled oils. In this regard, oxygen availability is critical for rapid biodegradation and is affected by the action of wave and water flow.[22] Low energy environments have been reported to promote anoxic conditions.[23]

Understanding the adverse effects of vegetable oil spills on aquatic environments is crucial for spill impact assessment and response planning. The objectives of the current study were to evaluate the oxygen depletion and toxicity produced by the aerobic biodegradation of vegetable oil in aquatic media. In particular, the impact of oil concentration and mixing condition was investigated. Respirometry experiments were conducted to evaluate the aerobic biodegradation of canola oil at three initial oil loadings (100, 333, and 1,000 gal acre^{-1}, corresponding respectively to 0.09375, 0.3125, and 0.9375 L m^{-2}), which were intended to simulate small, medium, and large oil spills. In each case, fully, moderately, and nonmixed conditions were studied. Canola oil was chosen for its high oleic acid content (C18:1), which is a major intermediate in vegetable oil biodegradation and widely reported for its inhibitory effect on microbial activity.[6,16,18] Aquatic and solid phase toxicity were assessed using the Microtox assay, a commonly used regulatory biotest to evaluate the impact and remediation actions of oil spills.[7] Vertical variation of toxicity and dissolved oxygen (DO) in the water columns was also examined.

2. MATERIALS AND METHODS

2.1. Vegetable Oil Characterization. Refined organic canola oil (Spectrum Naturals) was purchased from a local grocery store. The determination of the peroxide value (PV), tocopherol content, and fatty acids composition of the oil is discussed in the Supporting Information (SI).

2.2. Respirometric Microcosms. Respirometric microcosms were used for canola oil aerobic biodegradation experiments. Fully mixed (FM) microcosms consisted of the standard 500 mL (7 cm i.d. × 17.6 cm) N-Con respirometric flasks filled with 250 mL of mineral medium and equipped with a PTFE encased magnetic stir bar of 1 cm diameter and 6.4 cm length. Two other sets of respirometric microcosms were modified to extend the water column depth to 20 cm equivalent to one liter mineral medium in the flask. One set of the extended microcosms was operated under nonmixed (NM) condition, while the other set was operated under moderately mixed (MM) condition (stir bar dimensions of 0.8 cm diameter and 2.5 cm length). Fully and moderately mixed microcosms were mixed at 300 rpm.

Each of the extended microcosms (MM and NM) was equipped with three sampling ports located 2.5 cm from the flask bottom, in the middle of the water column, and 2.5 cm below the static water level. The ports were used for monitoring of the dissolved oxygen (DO) concentration and for the periodic withdrawal of aqueous samples for the toxicity analysis. Measurements of DO and toxicity were not performed in the FM microcosms. These samples were intended to serve in this study as a standard of comparison of the extent of oil biodegradability in the MM and NM microcosms, where oxygen might be limiting. Each microcosm was equipped with a trap containing 0.1 N potassium hydroxide to capture carbon dioxide (CO_2) produced from the biodegradation activity. A schematic of the different microcosms is shown in Figure S1 (SI) along with a description of the achieved mixing in each case.

2.3. Biodegradation Experiments. Biodegradation experiments were performed in computerized Comput-Ox respirometers, Model OO-244SC (N-Con Systems, Crawford, GA, USA). Three different canola oil loadings of 100, 333, and 1,000 gal acre^{-1} were tested in separate experiments and corresponded respectively to 0.33, 1.1, and 3.3 g of oil per microcosm. For each oil load, three different mixing conditions were simulated in the respirometric microcosms previously described. For each mixing condition, five replicate samples were prepared by filling the respirometric reactors with buffered water supplemented with minimal nutrients, an oil-degrading inoculum added at a volatile suspended solids (VSS) concentration of 250 mg L^{-1}, and an oil slick applied to the surface of the water at the tested amount. The mineral solution composition is presented in Table S1 (SI). A volume of 250 mL of the mineral solution was used for the preparation of the FM microcosms, while 1 L was necessary to create a water depth of 20 cm in the MM and NM extended microcosms. A master culture, developed in the laboratory under continuous flow in a coarse pore membrane bioreactor, was used as inoculum. Activated sludge from a local municipal wastewater treatment plant was originally used as a seed for the reactor. The culture was acclimated to the degradation of canola oil as the sole carbon source over a period of 3 years.

In addition to the real samples, three types of controls were prepared in triplicates. These consisted of three blank flasks with only mineral solution and vegetable oil, three control flasks with only mineral solution and inoculum, and three additional flasks with only mineral solution. This made a total of 42 microcosms per each experiment at a given oil loading. Temperature was maintained at 20 °C for the duration of all experiments. Biodegradation of vegetable oil was monitored through the respirometric oxygen uptake and CO_2 production.

For a given oil loading, biodegradation experiments were maintained until clear trends in dissolved oxygen levels and toxicity patterns were established. They lasted 56, 91, and 77 days for the 100, 333, and 1,000 gal acre^{-1} oil loadings,

respectively. At the end of the incubation period, all microcosms were sacrificed, and the solid and liquid phases were separated by vacuum filtration. The liquid phase consisted of the aqueous culture medium and was analyzed for free fatty acids (FFAs) separated from water by means of solid phase extraction using octyl C8 (Envi-8, Supelco) stationary phase. The solid phase consisted of the degrading biomass and residual oil which were retained on the filtration beads and filter paper. The solid phase was extracted with 70 mL of methanol followed by two dichloromethane (DCM) extractions of 70 mL each, and the solid phase extracts were analyzed for residual triglycerides and FFAs. Separation of the solid and liquid phases, sample preparation, and chemical analysis followed the procedures and analytical methods fully described by Campo et al.[11]

2.4. Dissolved Oxygen and Toxicity. Dissolved oxygen concentrations were measured twice per week at the three different levels of the extended water columns using a MI-730 Micro-Oxygen electrode (Microeletrodes, Inc., Bedford, NH). Liquid phase toxicity at the three different depths of the microcosms was assessed on a weekly basis using Microtox Analyzer Model 500 (SDI, DE, USA). Solid phase toxicity was measured at the end of the incubation period. Toxicity analysis was performed following the Microtox test, an acute-toxicity bioassay based on suppression of bioluminescence of the marine bacterium *Vibrio fischeri* following exposure to toxicants. Toxicity was expressed as EC_{50} (% sample volume), which is the effective concentration given as percent sample volume that reduces bioluminescence of the marine bacteria by 50%, and was measured at 5 and 15 min exposure time (i.e., the luminescence inhibition of *Vibrio fisheri* was measured after 5 and 15 min of exposure of the test bacterium to the analyzed sample). Additional details on DO and toxicity measurements are provided in the SI.

3. RESULTS AND DISCUSSION

3.1. Canola oil Characteristics. The characteristics of the canola oil used in the current study are presented in Table S2 (SI). Fatty acid composition of the oil showed a high content of oleic acid (56.5% w/w). Furthermore, measured tocopherols level (792 mg/kg) was comparable to concentrations found in finished canola oil and is optimum for providing its intrinsic oxidative stability.[24] Detailed discussion of the oil characteristics is provided in the SI.

3.2. Canola Oil Biodegradation. Vegetable oils are essentially composed of triacylglycerols (98%) which consist of glycerol molecules esterified with three LCFAs.[8] During the aerobic biodegradation of vegetable oils, triacylglycerols are hydrolyzed by extracellular lipases to yield FFAs and glycerol. The FFAs are further metabolized to CO_2 via the β-oxidation pathway and the tricarboxylic acid cycle.[8] The mechanism of vegetable oil biodegradation is detailed in the SI.

Aerobic biodegradation of canola oil was monitored through oxygen uptake and CO_2 production. Figure S2 (SI) shows the achieved oil mineralization at the end of the incubation period for each treatment. While canola oil was not fully mineralized under the experimental biodegradation conditions (Figure S2, SI), biotransformation of triglycerides exceeded 96% in all treatments. No oil polymerization was observed in any of the abiotic blanks as more than 98% of the oil was recovered at the end of the experiments. Furthermore, no oil autoxidation products were detected in these microcosms. However, tocopherol analysis revealed no remaining antioxidant in both biotic and abiotic samples at the end of the incubation period. Phosphorylation, a naturally occurring process in tocopherols in common foods,[25] is expected to have occurred in both biotic and abiotic microcosms in the presence of phosphate buffer (KH_2PO_4). The phosphorylated form of tocopherol is reported to have a potent antioxidant activity as a result of its detergent effect forming a barrier that inhibits the transfer of radicals from one polyunsaturated fatty acid to another.[26] This could explain the inhibition of lipid oxidation in the abiotic microcosms. Additionally, in the biotic microcosms, rapid degradation of tocopherols is anticipated as a result of the combination of free radical oxidation and aerobic biodegradation as demonstrated by Rontani et al.[27] in oxic environments.

3.3. Dissolved Oxygen. Dissolved oxygen concentrations in the MM and NM microcosms at the three different depths of the water columns for all treatments are presented in Figure 1. At the lowest oil load, DO levels fluctuated between 4 and 5 mg L^{-1} in the MM microcosms. These DO values were comparable at the different depths of the water columns ($p > 0.05$) (One Way Anova). Stratification of DO was observed at the same oil loading in the NM water columns with levels decreasing from the surface to the bottom. Average DO values in these microcosms were 2.06, 1.21, and 0.68 mg L^{-1} at the top, middle, and bottom levels, respectively. For the higher two oil loadings (333 and 1,000 gal $acre^{-1}$), oxygen was fully depleted from the MM and NM microcosms. In particular, stagnant water columns (NM) at the highest oil loading (1,000 gal $acre^{-1}$) exhibited zero oxygen levels at an early stage of biodegradation (~10 days). As expected, both biotic and abiotic blanks exhibited relatively high DO concentrations (>5 mg L^{-1}) at all water depths for all treatments.

The observed vertical oxygen profile in the MM and NM water columns is determined by the relative magnitude of oxygen mass transfer, surface reaeration, and biochemical oxygen demand. Surface reaeration was the controlling factor in shaping the oxygen profiles at the lowest oil load resulting in relatively high DO concentrations at all depths of the mixed water columns. For the same oil loading (100 gal $acre^{-1}$), the absence of surface reaeration in the case of nonmixed microcosms sets a DO profile of higher concentration near the surface and lower near the bottom. At higher oil loadings, the decreased oxygen transfer coupled to the increase in BOD values resulted in the complete depletion of oxygen from both MM and NM water columns. Oxygen mass transfer characteristics defined under the different treatments (results not shown) revealed oxygen transfer limitation at the highest oil loading (1,000 gal/acre) at the early stages of incubation, more pronounced in the NM microcosms.

The obtained results suggest that oxygen depletion from vegetable oil spills will mainly depend on the amount of oil spilled and the physical factors governing water reaeration in the impacted water body. While acceptable DO levels to support aquatic life could be maintained in mixed waters polluted with relatively low amounts of oil, low or no DO (hypoxia/anoxia) are expected to be prominent in still water environments and in instances of large spills. Oil spill in St. Helena and Saldanha Bays caused a complete depletion of oxygen from the water column.[9] Hypoxia and anoxia are the most important stressors of estuarine and coastal aquatic biota and are closely associated with hampered aquatic reproduction, declined shell fish production, and massive fish kills as well as major shifts in the kinds of aquatic organisms found in water bodies.[28]

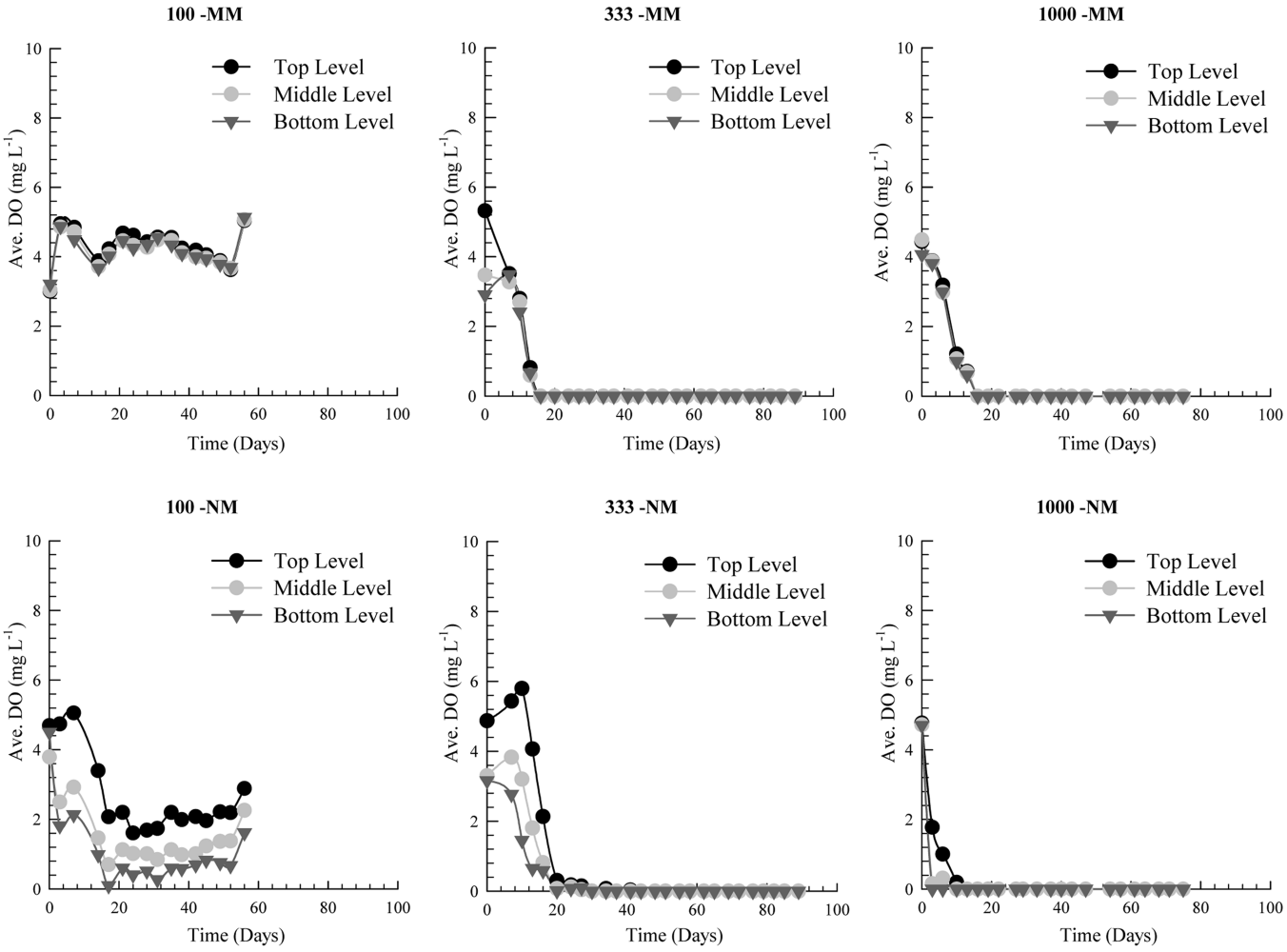

Figure 1. Average dissolved oxygen (Ave.DO) profiles in the moderately mixed (MM) and nonmixed (NM) extended water columns. The numbers 100, 333, and 1,000 represent the different oil loadings in gal acre^{-1}.

3.4. Toxicity. The Microtox test was used to assess the potential ecological impacts of vegetable oil biodegradation in aquatic media. No major toxicity was observed in the aqueous phase at the 100 gal acre^{-1} loading in both MM and NM microcosms (data not shown). This suggests that, at the tested oil amount, potential harmful metabolic intermediates from the oil biodegradation (i.e., FFAs) did not accumulate in the microcosms up to toxic levels at any time during the experiments.

In the case of the medium oil load (333 gal acre^{-1}), the EC_{50} significantly decreased to reach low values (up to 5.4%) after 28 days of incubation in the MM system and then gradually increased to reach medium toxicity levels toward the end of the biodegradation experiments (Figure 2a). The trend of the EC_{50} variation over the duration of the experiments clearly shows that toxic metabolic intermediates (namely LCFAs) were formed at the early stages of the aerobic biodegradation of canola oil and were gradually eliminated through further degradation and mineralization of the oil. A significantly lower toxicity ($p < 0.05$) (Kruskal–Wallis One Way ANOVA on Ranks) was measured in the NM treatment for the same oil loading. In this case, EC_{50} values gradually decreased to reach moderate toxicity levels after 49 days of incubation and were maintained up to 91 days (Figure 2b). Transient toxicity during the aerobic and anaerobic biodegradation of vegetable oils was reported in the literature.[7,8,29] In the current study, the slower biodegradation rates in the NM systems apparently delayed the elimination of the formed toxic FFAs.

A significant ($p < 0.05$) (One Way ANOVA) increase in toxicity (EC_{50} ~10%) was observed when the initial oil loading was increased to 1,000 gal acre^{-1} (Figure 2 c and d), with no significant difference ($p > 0.05$) in measured EC_{50} values in both mixing conditions (Kruskal–Wallis One Way ANOVA on Ranks). In this case, EC_{50} dropped to low values at early stages of the oil biodegradation in both MM and NM microcosms, and toxicity persisted throughout the incubation period, which lasted 77 days. The obtained results suggest that, in instances of large vegetable oil spills, high toxicity would be expected as a result of the heavy accumulation of FFAs generated from the oil biodegradation, with minimal influence of the mixing condition in the impacted water body.

The evaluation of the interactions between time and oil loading across the tested mixing conditions showed a significant difference in the mean EC_{50} values measured throughout the incubation period in the MM microcosms ($p < 0.001$) but not in the NM microcosms ($p = 0.252$), at both the 333 and 1,000 gal acre^{-1}. This is most probably due to the higher degradation rate in the case of the MM microcosms accelerating the appearance and elimination of the oil toxic metabolic intermediates and suggests that the impacts from vegetable oil spills in mixed aquatic environments may be limited.

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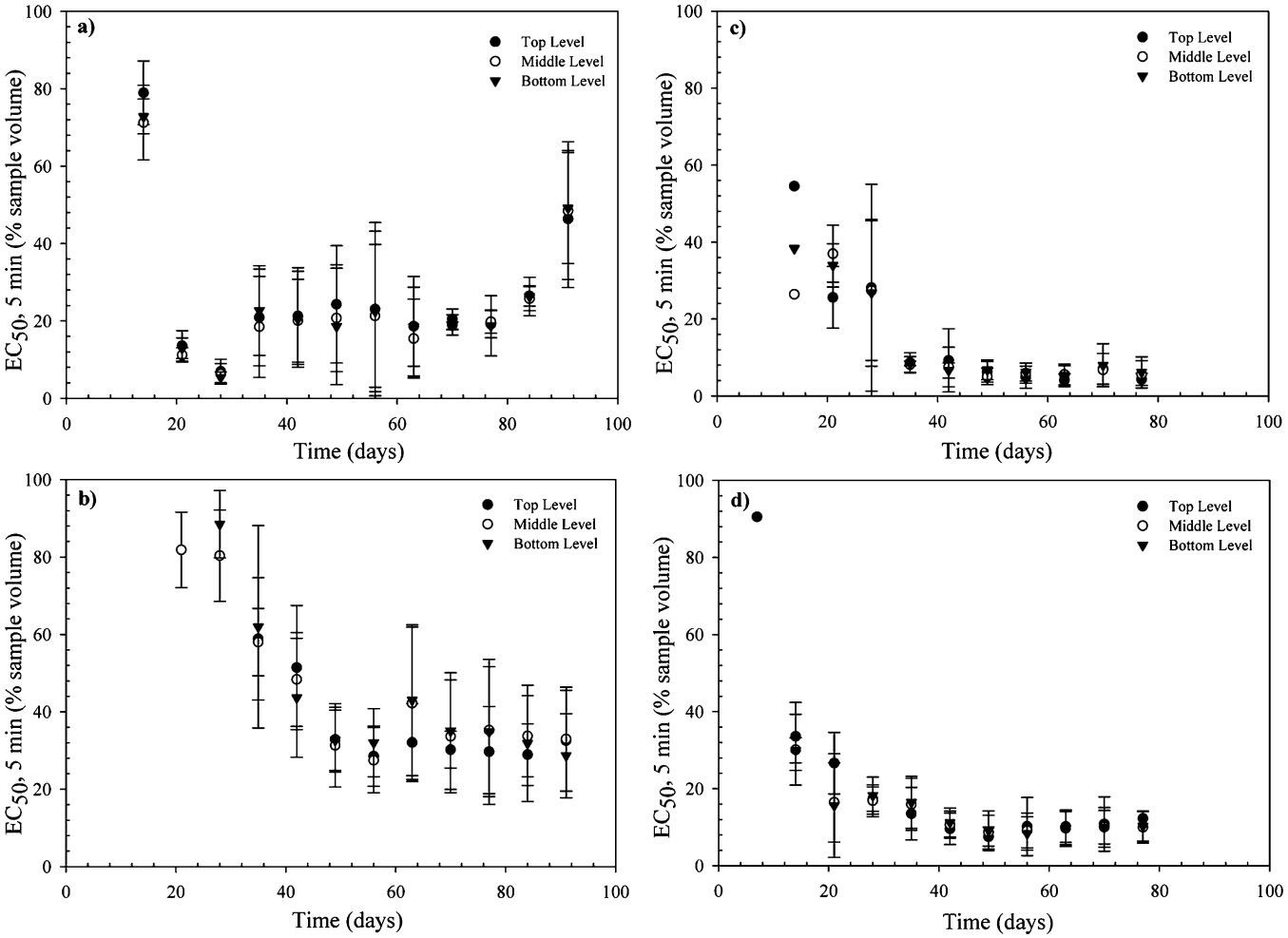

Figure 2. EC_{50} values (% sample volume) measured at 5 min at different levels of the microcosms' water columns: (a) 333 gal $acre^{-1}$, moderately mixed; (b) 333 gal $acre^{-1}$, nonmixed; (c) 1,000 gal $acre^{-1}$, moderately mixed; (d) 1,000 gal $acre^{-1}$, nonmixed. Error bars indicate standard deviation of five replicate samples. The absence of data points at time zero of the experiments and after 7 or 14 days of incubation indicates the lack of toxicity at these times of the experiments.

With regards to toxicity variation with depth, no significant differences in EC_{50} values were observed for all treatments ($p > 0.05$) (Kruskal–Wallis One Way ANOVA on Ranks). While mixing ensures an even distribution of the oil toxic byproducts in the MM microcosms, diffusion of these intermediates throughout the water columns is expected to have homogenized the NM microcosms, thereby minimizing toxicity differences along the water depth. Furthermore, no aqueous toxicity was measured in the water columns of the biotic and abiotic blanks at any time during the experiments.

High toxicity (<2%) was observed in the solid phase for all treatments regardless of the oil load and mixing regime (Table 1). This is likely a result of the strong partitioning of LCFAs to the biomass as evidenced by their occurrence at relatively high amounts in the solid phase. No solid phase toxicity was measured in all biotic and abiotic microcosms at any time during the incubation period.

It is important to note that toxicity measurement was conducted on reaerated aliquots withdrawn from the different microcosms. Indeed, the Microtox test method entails the preparation of serial dilutions (1:2) of the test sample and mixing after each dilution, which ensures the reaeration of the analyzed sample. Furthermore, the small volume of the samples (1 mL), and the fact that they are kept open to air during the Microtox test, results in further reaeration. Therefore, the measured Microtox toxicity is primarily due to the oil biodegradation intermediates (i.e., FFAs) present in the tested samples. Regression analysis showed no correlation between toxicity and dissolved oxygen data in all treatments (Figure S3 in the SI).

3.5. Fatty Acids. Liquid and solid phase analysis at the end of the biodegradation experiments confirmed the presence of LCFAs. Shorter hydrophilic fatty acids were also detected in the liquid phase. Tables 1 and 2 show the FFAs distribution in the solid and liquid phases, respectively. The amount of FFAs in the microcosms varied among treatments as a result of the different exhibited biodegradation rates in each case, as determined by the kinetics of the oil hydrolysis, FFAs β-oxidation, inhibition by accumulated LCFAs, and oxygen mass transfer limitation, which could have varied under the different tested oil loadings and mixing conditions.

The most prominent intermediate of the oil biodegradation found in both liquid and solid phases was oleic acid (C18:1), the major constituent of canola oil. Besides oleic acid, palmitic acid (C16:0) was also prevailing in the solid phase and exhibited concentrations exceeding its proportion in the oil. This increase in palmitic acid is most likely the result of its accumulation following β-oxidation of C18:0. A modification of the initial composition of the oil marked by an increase in

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Table 1. FFAs Amount and Microtox Toxicity in the Solid Phase[b]

	free fatty acids (μg)											toxicity (% sample volume)	
treatment[a]	C8:0	C9:0	C10:0	C11:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	5 min	15 min
100 FM	0	0	0	0	0	32.6 ± 4.14	432.7 ± 89.54	57.9 ± 4.97	329.7 ± 70.08	3126.7 ± 328.45	626.1 ± 173.88	73.8 ± 24.09	0.75±0.43	0.70±0.32
100MM	0	0	0	0	0	52.9 ± 26.73	2995.2 ± 285.20	68.4 ± 14.65	1135.6±238.82	15359.8 ± 794.87	2993.8 ± 353.62	305.4 ± 87.64	0.12±0.08	0.11±0.07
100NM	0	0	0	0	0	55.5 ± 16.57	4851.8 ± 520.56	191.7 ± 44.42	1925 ± 302.03	35735.7±2668.35	3955.7 ± 582.47	584.1 ± 76.21	0.11±0.07	0.09±0.06
333 FM	0	0	0	0	0	103.7 ± 24.37	901.5 ± 84.50	22.8 ± 10.92	1348.3±140.90	4663.8 ± 831.84	139.1 ± 34.10	25.6 ± 17.3	0.91±0.43	0.87±0.36
333 MM	0	0	0	0	0	654.54±325.75	98377.6 ± 5400.7	194.1 ± 41.53	8502.2±922.42	38003.4±2342.64	4806.9±1044.91	741.2±186.42	0.11±0.07	0.10±0.06
333 NM	0	0	0	0	0	1524.1±118.46	32349.9±1512.34	564.6±265.38	5274 ± 847.31	103357±6922.55	5937 ± 1314.32	681.8 ± 81.63	0.04±0.02	0.03±0.02
1000 FM	0	0	0	0	0	1163.2±231.51	40914.6±2376.69	925 ± 110.44	4753.1±314.25	36679.5±3337.15	8319.7 ± 730.94	1382.9±89.75	0.18±0.13	0.16±0.11
1000 MM	0	0	0	0	0	56.47 ± 32.56	9872.3 ± 1255.17	292.1 ± 47.14	1887.5±157.34	43118.4±2246.32	279.2 ± 46.96	22.35 ± 13.54	0.14±0.06	0.10±0.05
1000 MM	0	0	0	0	0	34 ± 15.93	3838.5 ± 900.17	212.9±109.86	1391.5±215.60	30960.7±1880.34	829 ± 110.03	35.76 ± 18.30	0.39±0.25	0.32±0.24

[a]The treatments FM, MM, and NM represent, respectively, fully, moderately, and nonmixed conditions, while the numbers 100, 333, and 1000 represent the oil loading in gal acre^{-1}. [b]Results are the average of 5 replicate samples ± standard deviation.

saturated FFAs C16:0 and C18:0 was reported by Pereira et al.[30] following aerobic and anaerobic biodegradation of linseed oil. The occurrence of relatively high amounts of LCFAs, namely C18:1 and C16:0, supports the reported high toxicity in the solid phase (<2%).

The toxicity of oleic acid has been widely reported.[16,18,31−35] Becker et al.[16] observed a severe growth inhibition of the degrading biomass during the aerobic degradation of olive oil as a result of oleic acid accumulation at concentrations of 100−150 mg L^{-1}. Lalman and Bagley[18] reported the methanogenic inhibitory effect of oleic acid at concentrations above 30 mg L^{-1}. Furthermore, the accumulation and inhibitory effect of palmitic acid were extensively stated. A transient accumulation of palmitic and myristic (C14:0) acids was reported by Lalman and Bagley[18] during oleic acid degradation, and these fatty acids were found to be inhibitory to their own subsequent degradation during linoleic acid biodegradation.[17] A significant accumulation of palmitic acid (>80% of the total LCFAs), mainly adsorbed to the anaerobic sludge, was reported by Pereira et al.[19,36] under continuous feeding with oleic acid and induced an inhibition of the microbial activity. The accumulation of palmitic acid in the current study suggests that its further biodegradation might have been a difficult step at the relatively high occurring concentrations of oleic acid.

Other detected toxic LCFAs in both liquid and solid phases included stearic (C18:0), linoleic (C18:2), linolenic (C18:3), palmitoleic (C16:1), and myristic (C14:0) acids. While C14:0 and the three C18 fatty acids are expected intermediates from canola oil biodegradation, the detection of palmitoleic acid (C16:1) indicates that direct β-oxidation of oleic acid (C18:1) with no prior saturation to stearic acid (C18:0) occurred during the oil degradation. Two pathways of the oil biodegradation are thus proposed. In the first one, unsaturated C18 fatty acids in canola oil (C18:1, C18:2, and C18, 3) are first converted to stearic acid (C18:0) which is further degraded via the β-oxidation pathway. In the second pathway, oleic acid (C18:1) originally present in the oil or produced by hydrogenation of linoleic (C18:2) and linolenic (C18:3) acids undergoes a direct β-oxidation to form palmitoleic acid (C16:1) which is further converted to palmitic acid (C16:0) and degraded inside the cells. The detection of palmitoleic acid (C16:1) as a product of oleic (C18:1) and linoleic (C18:2) acids biodegradation has been observed previously.[17,18,37]

Medium chain length fatty acids (MCFAs) were detected in the liquid phase in addition to LCFAs and included caprylic (C8:0), pelargonic (C9:0), capric (C10:0), and lauric (C12:0) acids. Trace amounts of C8:0, C10:0, and C12:0 have been observed as byproducts from the biodegradation of linoleic, oleic, stearic, palmitic, and myristic acids.[17] However, C9:0, a non-native β-oxidation intermediate, is most likely the result of the breakdown of C18:1. Kawamura and Gagosian[38] demonstrated that under certain oxidative conditions, pelargonic acid (C9:0) can be produced as a result of the oxidative cleavage of the double bond at the C9 position of the monounsaturated C18:1 fatty acid. The inhibitory effect of MCFAs (C6 to C11) to the growth of *Escherichia coli* at 0.4% (w/v) was demonstrated by Fay and Farias,[39] C9:0 and C10:0 being the most effective inhibitors. In the current study, the observed liquid phase toxicity to *Vibrio fisheri*, the Microtox test organism, is most likely the result of the combined effect of long and medium chain fatty acids at the relatively low detected amounts.

Table 2. FFAs Amount in the Liquid Phase[a]

	free fatty acids (μg)										
treatment[b]	C8:0	C9:0	C10:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
100 FM	0.34 ± 0.11	0.96 ± 1.08	0.23 ± 0.16	0.25 ± 1.15	0.82 ± 0.38	9.40 ± 6.45	1.38 ± 0.57	4.25 ± 2.10	43.65 ± 12.61	7.87 ± 2.69	1.49 ± 0.6
100MM	0.16 ± 0.10	0.65 ± 0.34	0.19 ± 0.09	0.22 ± 0.17	0.67 ± 0.57	2.65 ± 2.03	0.38 ± 1.00	1.20 ± 0.42	5.64 ± 2.54	1.52 ± 0.23	0.50 ± 0.65
100NM	0.70 ± 0.33	2.92 ± 1.61	0.63 ± 0.25	0.29 ± 0.06	0.49 ± 0.16	2.15 ± 1.28	1.63 ± 1.64	0.85 ± 0.54	6.55 ± 3.01	1.18 ± 1.59	0.35 ± 0.42
333 FM	21.87 ± 8.49	12.65 ± 5.48	0.67 ± 0.22	0.25 ± 0.15	0.55 ± 0.26	10.03 ± 2.28	3.81 ± 0.04	1.15 ± 0.43	19.36 ± 12.33	10.16 ± 3.95	0.85 ± 0.80
333 MM	2.64 ± 1.23	4.91 ± 1.78	2.55 ± 1.29	2.08 ± 1.34	3.35 ± 1.99	20.61 ± 5.30	3.44 ± 2.08	3.28 ± 1.77	34.20 ± 9.12	5.78 ± 3.58	0.59 ± 0.27
333 NM	87.45 ± 13.33	86.14 ± 15.96	15.77 ± 4.27	1.78 ± 1.07	1.41 ± 0.62	10.71 ± 4.95	26.78 ± 6.02	1.53 ± 0.71	19.45 ± 6.52	7.07 ± 4.67	1.17 ± 0.50
1000 FM	0.88 ± 0.56	1.77 ± 1.02	0.45 ± 0.23	0.30 ± 0.20	0.46 ± 0.22	3.09 ± 1.52	2.66 ± 1.65	1.04 ± 0.48	10.25 ± 6.13	1.77 ± 2.48	0.38 ± 0.12
1000 MM	26.35 ± 14.50	62.76 ± 19.46	12.95 ± 3.03	1.32 ± 0.79	1.23 ± 0.49	18.46 ± 4.02	5.36 ± 1.16	5.11 ± 1.50	92.71 ± 16.78	1.96 ± 1.63	0.13 ± 0.07
1000 MM	36.12 ± 9.88	121.72 ± 24.56	23.90 ± 7.93	1.21 ± 0.43	1.47 ± 0.34	16.56 ± 8.4	4.21 ± 1.23	6.25 ± 4.29	82.68 ± 14.08	4.71 ± 2.62	0.40 ± 0.12

[a]Results are the average of 5 replicate samples ± standard deviation. Undecanoic acid (C11:0) was used as internal standard and is not included in the results. [b]The treatments FM, MM, and NM represent, respectively, fully, moderately, and nonmixed conditions, while the numbers 100, 333, and 1000 represent the oil loading in gal acre^{-1}.

Furthermore, potential toxic intermediates from the biodegradation of the phenolic antioxidants (tocopherols) might have been formed contributing to the overall liquid toxicity. Rontani et al.[27] reported that the aerobic metabolism of tocopherol by marine bacteria is the result of a simultaneous involvement of biodegradation and autoxidation, which leads to the formation of a numerous intermediates. The authors did not, however, discuss the toxicity of these intermediates. Nevertheless, some studies reported the inhibiting effect of antioxidants on biodegradation. In their investigations of the effect of the common phenolic antioxidants on the biodegradation of palm oil, Aluyor et al.[40] reported a reduction of oil biodegradation with increasing concentrations of the antioxidant tertiary butyl hydroquinone. Olive mill wastewaters are reported to have poor biodegradability and high phytotoxicity due in particular to their phenolic content.[41] Further research is needed to evaluate the toxicity of tocopherol degradation intermediates.

■ ASSOCIATED CONTENT

Supporting Information

Details on oil characterization, biodegradation microcosms and achieved mixing, mineral medium composition, dissolved oxygen and toxicity measurements, characteristics of canola oil, mechanism of vegetable oil biodegradation, oil mineralization, and regression analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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