Science of the Total Environment 547 (2016) 95–103

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Biodegradation and toxicity of vegetable oils in contaminated aquatic environments: Effect of antioxidants and oil composition

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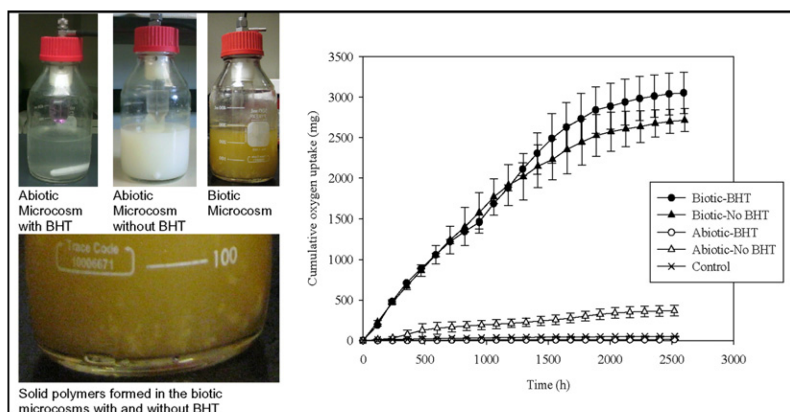
HIGHLIGHTS

- The presence of BHT did not significantly enhance the biodegradation of canola oil.
- Intractable solid polymers were formed in the biotic microcosms.
- Toxicity was observed in the biotic microcosms with and without BHT.
- The potency of an antioxidant is dependent on the oil fatty acid composition.
- The fatty acid composition of spilled vegetable oils defines their fate and impact.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 October 2015
Received in revised form 15 December 2015
Accepted 28 December 2015
Available online 11 January 2016

Editor: D. Barcelo

Keywords:
Aquatic pollution
Vegetable oil spills
Bioremediation
Toxic biodegradation intermediates
Oil polymerization
Autoxidation products

ABSTRACT

Antioxidants may affect the oxidative rate of vegetable oils determining their fate and impact in contaminated aquatic media. In previous studies, we demonstrated the effectiveness of butylated hydroxytoluene (BHT), one of the most used antioxidants in edible oils, in enhancing the biodegradation of glyceryl trilinoleate, a pure triacylglycerol of cis,cis-9,12-octadecadienoic acid (C18:2 delta), through retarding its oxidative polymerization relatively to the oil with no added antioxidant. In this study, the effect of BHT on the biodegradation and toxicity of purified canola oil, a mixed-acid triacylglycerol with high C18:1 content, was investigated in respirometric microcosms and by use of the Microtox® assay. Investigations were carried out in the absence and presence (200 mg kg^{-1}) of the antioxidant, and at an oil loading of 0.31 L m^{-2} (333 gal $acre^{-1}$). Substantial oil mineralization was achieved after 16 weeks of incubation (>77%) and was not significantly different ($p > 0.05$) between the two BHT treatments, demonstrating an important role of the oil fatty acid composition in determining the potency of antioxidants and, consequently, the fate of spilled vegetable oils. Furthermore, for both treatments, toxicity was measured at early stages of the experiments and disappeared at a later stage of incubation. The observed transient toxicity was associated with the combined effect of toxic biodegradation intermediates and autoxidation products. These results were supported by the gradual disappearance of BHT in the microcosms initially supplemented with the antioxidant, reaching negligible amounts after only 2 weeks of incubation.

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http://dx.doi.org/10.1016/j.scitotenv.2015.12.138
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1. Introduction

The world generation of vegetable oils has increased rapidly during the past years driven mainly by a higher consumption of edible oils and an increasing interest in their use as replacements for mineral oil based products (Coyle, 2007). The likelihood of spillages of large volumes of vegetable oils in the aquatic environment, as a result of their dynamic trade on the world's market, is therefore no different from that of mineral oils. Spillages of large volumes of vegetable oils have been reported during the last 40 years and were in some cases more harmful than mineral oil spills (Bucas and Saliot, 2002).

The deleterious effects of vegetable oil spills in impacted aquatic ecosystems include oxygen depletion, smothering of surface dwelling organisms, and toxicity to aquatic fauna. The toxicity of vegetable oils is primarily due to the oil biodegradation intermediates, namely long chain fatty acids (LCFAs), and its autoxidation byproducts. The toxicity of vegetable oils and their detrimental effects in aquatic media are thoroughly reported in the literature (McKelvey et al., 1980; Groenewold et al., 1982; Mudge et al., 1995; Mudge, 1995; Rigger, 1997, Bucas and Saliot, 2002; Pereira et al., 2002; Wincele et al., 2004, Salam et al., 2012a).

The fate and impact of vegetable oils in contaminated aquatic media largely depend on their specific properties (Bucas and Saliot, 2002). Although readily biodegradable, vegetable oils can polymerize to form intractable lumps resistant to many erosional or degradation processes (Mudge et al., 1993; Mudge, 1997). Competition between biodegradation and polymerization was reported by Mudge et al. (1995) in simulated sunflower and linseed oil spills in salt marsh sediments. In the case of sunflower, the oil polymerized after 28 days resulting in the formation of a cap of reduced permeability to oxygen and water, while no polymers were formed in the case of linseed oil. Contrasting results were reported from similar spill simulations conducted by Pereira et al. (1998). In this case, spilled sunflower oil did not polymerize. Rather, it degraded slowly while linseed oil degraded at a faster rate. In a later study, the authors observed that sunflower oil persisted in an unaltered state for six months, whereas 60% of linseed oil was consumed within two months (Pereira et al., 2003a). While based on these studies the authors were not able to make a clear conclusion on what determines the fate of spilled vegetable oils, they suggested that the explanation could be found in the fatty acids composition of the oil and the environmental factors that instigate the oil biodegradation or polymerization pathways (Pereira et al., 2003b).

It has become clear that the extent of vegetable oils autoxidation depends on their degree of unsaturation (Belitz et al., 2009), oils with high content of unsaturated fatty acids being more prone to autoxidation (Sherwin, 1976). Relative oxidation rate of C18 fatty acids is reported to be 1, 100, 1200, and 2500 at 25 °C for stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids respectively (Belitz et al., 2009). Furthermore, the composition of vegetable oils and interaction between their constituent fatty acids significantly affects the rate of the oil oxidation (Frankel, 1980). Oleic acid is reported to initially act as a diluent for the oxidation of linoleic acid, while at advanced stages of oxidation the rate is affected by both propagation and termination reactions (Frankel, 1980). In addition, Neff et al. (1994) reported the effect of fatty acids position in the triacylglycerol (TAG) molecule on the oxidation of purified vegetable oils. The authors found a positive correlation of reduced oxidative stability with increased linoleic acid (C18:2) concentration at carbon 2 of the TAG.

Moreover, the presence of antioxidants may also affect the oxidative rate of vegetable oils (Wanasundara and Shahidi, 2005). In previous experiments conducted by our research group and investigating the effect of antioxidants on the biodegradation of spilled vegetable oils in contaminated aquatic environments (Salam et al., 2012b), we demonstrated the effectiveness of butylated hydroxytoluene (BHT), one of the most used antioxidants in edible oils, in retarding the oxidative polymerization of glyceryl trilinoleate at concentrations as low as 50 mg kg^{-1}. After 19 weeks of incubation, only about 41% of the oil was mineralized in the microcosms with no BHT, while mineralization exceeded 67% in the microcosms with added antioxidant and did not significantly increase with increasing BHT concentrations (50–800 mg kg^{-1}). Glyceryl trilinoleate, a pure triacylglycerol of cis,cis-9,12-octadecadienoic acid (C18:2 delta), was chosen in those experiments as model vegetable oil for its high susceptibility to autoxidation.

In this study, it is our purpose to extend these investigations to commonly used vegetable oils in order to quantitatively evaluate the impact of antioxidants on the biodegradability of mixed-acid triacylglycerols (different fatty acid moieties esterified to the same glycerol molecule) in contaminated aquatic media. Another main objective of this study is to determine the effect of antioxidants on the induced toxicity of spilled vegetable oils. To achieve these aims, respirometric experiments were conducted to evaluate the aerobic biodegradation and toxicity of purified canola oil in the presence and absence of BHT. Canola oil was chosen for its unique composition containing the major saturated and unsaturated LCFAs. In addition, canola oil represents the second largest oil crop globally produced (Ash, 2015) and the preferred feedstock for biodiesel production in Europe (Ajavonic, 2011).

2. Materials and methods

2.1. Canola oil purification

Refined organic canola oil (Spectrum Naturals®) was purchased from a local grocery store and was purified from tocopherols, naturally occurring antioxidants in vegetable oils, by adsorption chromatography using activated alumina (Al_2O_3). Details on canola oil purification are presented in Supporting information (SI). Analysis of the purified oil by high performance liquid chromatography (HPLC) showed no detectable tocopherols and hydroperoxides, and a percent distribution of the triglycerides comparable to the original oil. Fatty acid composition of the purified oil was determined by gas chromatography mass spectroscopy and showed a high content of oleic, linoleic, and linolenic acids (56.5, 22.6, and 12.4% *w*/*w*, respectively). The chemical analysis and characteristics of the purified oil are presented in SI.

2.2. Biodegradation experiments

Biodegradation experiments of purified canola oil with high content of oleic, linoleic, and linolenic acids (56.5, 22.6, and 12.4% *w*/*w*, respectively) were performed in computerized Comput-Ox respirometers, Model OO-244SC (N-Con Systems, Crawford, GA, USA). The biodegradation microcosms consisted of the standard 500 mL (7 cm i.d. × 17.6 cm) N-Con respirometric flasks equipped with a PTFE encased magnetic stir bar of 1 cm diameter and 6.4 cm length. The microcosms were filled with 250 mL mineral medium made of potassium phosphate monobasic buffer (KH_2PO_4) supplemented with minimal nutrients. The mineral solution composition is presented in Table S2 in the Supplementary information (SI).

Two sets of respirometric microcosms were prepared. In one set, a slick of the oil free of antioxidant was applied to the surface of the microcosms, while in the other set the oil was supplemented with 200 mg kg^{-1} of BHT. The tested BHT concentration is commonly used in the food industry to stabilize vegetable oils (Madhavi and Salunkhe, 1995). In addition, previous conducted experiments by Salam et al. (2012b) on the effect of different levels of BHT on the biodegradation of glyceryl trilinoleate in aquatic media demonstrated minimal additional protective effect of the antioxidant above 50 and up to 800 mg kg^{-1}.

In both treatments, the oil was added under nitrogen gas at a loading of 0.31 L m^{-2} (333 gal $acre^{-1}$) corresponding to an amount of 1.1 g per microcosm. The oil loading was selected based on reported amounts of vegetable oil spills in aquatic environments, and considering the results from previous vegetable oil biodegradation experiments conducted by

Campo et al. (2007) and Salam et al. (2012a) at different oil loadings and mixing regimes. Namely, the results from these studies showed no oxygen mass transfer limitation at the used oil loading of 333 gal $acre^{-1}$, and a transient toxicity of the oil within a reasonable incubation period.

For each treatment, 18 biotic samples consisting of the mineral medium supplemented with the oil and an oil degrading inoculum (250 mg VSS L^{-1}), and 18 abiotic blanks with no added inoculum were prepared. The inoculum consisted of a master bacterial culture developed in the laboratory under continuous flow in a coarse pore membrane bioreactor. Activated sludge from a local municipal wastewater treatment plant in Cincinnati, Ohio, was originally used as a seed for the reactor. The culture was acclimated to the degradation of canola oil as the sole carbon source (500 μL day^{-1}) over a period of 3 years and was used in previous vegetable oil biodegradation experiments (Campo et al., 2007; Salam et al., 2012a,b).

In addition to the biotic samples and abiotic blanks, three control flasks with only mineral solution and inoculum were prepared. This made a total of 75 respirometric microcosms. Each microcosm was equipped with a trap containing 0.1 N potassium hydroxide (KOH) intended to capture carbon dioxide (CO_2) produced from the biodegradation activity. Respirometric experiments were conducted at 20 °C with continuous mixing of the microcosms at 300 rpm resulting in a full depth vortex and the dispersion of the oil in the aqueous phase. Biodegradation of canola oil was monitored through the respirometric oxygen uptake and CO_2 production.

2.3. Sampling and chemical analysis

Six sampling events were conducted during the biodegradation experiments, which lasted 16 weeks. The first sampling event was conducted at time zero (week 0) corresponding to the actual starting point of the respirometric experiments and after an equilibration time of the microcosms of about 3 h. Subsequent samplings were performed after 1, 2, 4, 8, and 16 weeks. In each sampling a total of 12 microcosms were sacrificed. These consisted of 3 biotic samples with BHT, 3 biotic samples without BHT, 3 abiotic blanks with BHT, and 3 abiotic blanks without BHT. Control samples were sacrificed in the last sampling event (week 16).

For all sacrificed microcosms, the solid and liquid phases were separated by vacuum filtration. The liquid phase (LP) consisted of the aqueous culture medium and was analyzed for free fatty acids (FFA). The solid phase (SP) consisted of the degrading biomass, residual oil, and some solid deposits, which were all retained on the filtration beads and filter paper. The SP 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, FFA and BHT. Separation of the solid and liquid phases, sample preparation, and analysis of triglycerides and FFA followed the procedures and analytical methods fully described by Campo et al. (2007). Residual BHT concentration in the SP was determined by HPLC according to the method described by Gratzfeld-HuSgen and Schuster (2001) for antioxidant analysis in food products. The detailed method for BHT analysis is presented in SI.

2.4. Toxicity

Toxicity in the sacrificed microcosms was measured in both liquid and solid phases. Microtox® Analyzer Model 500 (SDI, DE, USA) was used for this purpose, and samples were analyzed following the Microtox® test, which is an acute-toxicity bioassay based on suppression of bioluminescence of the marine bacterium *Vibrio fischeri* following exposure to toxicants (Microbics Corporation, 1994). Liquid phase toxicity analysis was performed according to the basic test protocol with an initial sample volume of 45% or 81.9%. Solid phase toxicity analysis was performed according to the "Protocol for the Basic Test Using Organic Solvent Sample Solubilization". Non-denatured absolute ethanol was used for the sample preparation in this case. 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%. EC_{50} measurements were performed in triplicate analysis and were determined at 5 and 15 min exposure time (i.e., the luminescence inhibition of *V. fischeri* was measured after 5 and 15 min of exposure of the test bacterium to the analyzed sample).

2.5. FTIR

Autoxidation of canola oil was examined throughout the biodegradation experiments by Fourier Transform Infra Red spectroscopy (FTIR). The spectra were collected with a Nicolet Magna IR equipment model 760 (Thermo Fisher Scientific, Walthman, MA) with a ZnSe prism and a deuterated triglycine sulfate detector. The spectra were recorded in the middle infrared region (400–4000 cm^{-1}). Transmittance mode was used to generate the spectra of the solid deposits collected from the biotic samples, and of the solid phase extracts of all microcosms. Each spectrum was obtained by averaging 64 interferograms with a resolution of 4 cm^{-1}.

2.6. Statistical analysis

SigmaPlot® 11(Systat Software, Inc., CA, USA) was used to perform an analysis of variance (ANOVA) to determine the effect of BHT on the extent of oil biodegradation and toxicity. The confidence level was set to 95% (a significance level of 0.05).

3. Results and discussion

3.1. Biodegradation experiments

Biodegradation of canola oil in the absence and presence (200 mg kg^{-1}) of BHT was monitored through the respirometric oxygen uptake and CO_2 production. The cumulative oxygen uptake curves corresponding to the two BHT treatments are shown in Fig. 1.

In both treatments, oxygen uptake increased with time asymptomatically approaching an ultimate value. The achieved cumulative oxygen uptake after 16 weeks of incubation was not significantly different ($p > 0.05$, one way ANOVA) between the two BHT treatments, and was lower than the computed theoretical amount of oxygen required for the full mineralization of the added oil (Fig. 1). Furthermore, intractable solid composites unavailable for bacterial degradation were

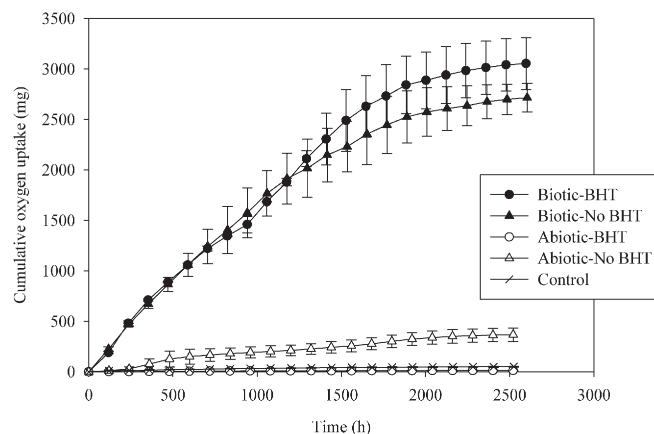

Fig. 1. Cumulative oxygen uptake curves for canola oil biodegradation in the presence and absence of BHT. Biotic-BHT: biotic microcosms supplemented with BHT; Biotic-No BHT: biotic microcosms not supplemented with BHT; Abiotic-BHT: abiotic microcosms supplemented with BHT; and Abiotic-No BHT: abiotic microcosms not supplemented with BHT. Error bars indicate standard deviation of triplicate experimental data. The initial oil loading was 1.1 g/microcosm, and corresponds to a theoretical oxygen uptake of 3308 mg.

formed in the biotic microcosms with and without BHT and were insoluble in methanol and DCM used in the solid phase extraction, as well as in a wide range of other tried organic solvents. The formation of these resistant deposits suggests advanced stages of autoxidation of the unsaturated triglycerides in canola oil. The oxidative deterioration of the oil was further demonstrated through FTIR analysis (Section 3.5).

Oxygen consumption was also observed in the abiotic blanks with no BHT (Fig. 1). In these microcosms, a rise in oxygen uptake followed a lag phase of about 250 h, and leveled off shortly thereafter. A clear change in the oil emulsion was observed in these blanks, which turned completely white after 4 weeks of incubation. These results indicate the autoxidation of the oil in the absence of BHT. The lag phase preceding the oxygen uptake could be explained by the initial accumulation of the precursors of the autoxidation reaction (induction period). No oxygen uptake occurred in the abiotic microcosms supplemented with BHT (Fig. 1) demonstrating the protective effect of the antioxidant against oil autoxidation in these microcosms.

The presence of solid deposits in the biotic microcosms and not in the abiotic blanks with no BHT suggests that their formation results from an interaction between the oil and the degrading biomass rather than through crosslinking reactions of the fatty acid hydroperoxides generated by oil autoxidation. Infrared analysis of these deposits showed specific bands associated with the oil autoxidation products, as well as characteristic bacterial spectral features (Section 3.5). It is suggested that the radicals generated from the hydroperoxides (RO•) during the oil autoxidation might have reacted with the bacterial proteins (PH) by the abstraction of a hydrogen atom, leading to the formation of protein radicals (P•) as described by Belitz et al. (2009) (Reaction 1). Protein radicals can combine with each other resulting in the formation of a protein network of low solubility (Reaction 2) (Belitz et al., 2009)

$$RO^{\bullet} + PH \rightarrow P^{\bullet} + ROH \quad (1)$$

$$2\ P^{\bullet} \rightarrow PP \quad (2)$$

Another pathway for the formation of the observed solid deposits in the biotic microcosms might be related to a reaction between aldehydic compounds formed at advanced stages of the oil autoxidation and bacterial proteins. Aldehydes readily condense with free-NH_2 groups

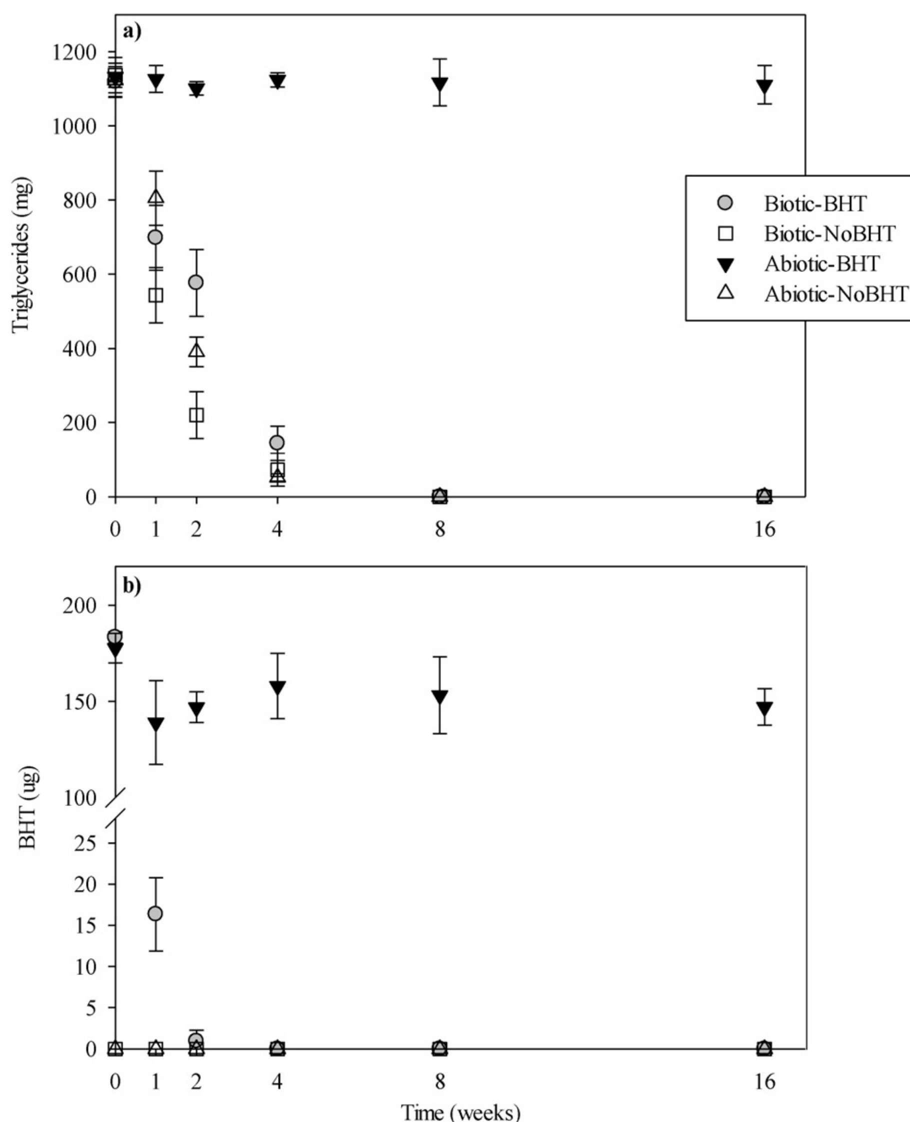

Fig. 2. Fate of triglycerides (a) and BHT (b) throughout the biodegradation experiments. Biotic-BHT: biotic microcosms supplemented with BHT; Biotic-NoBHT: biotic microcosms not supplemented with BHT; Abiotic-BHT: abiotic microcosms supplemented with BHT; and Abiotic-No BHT: abiotic microcosms not supplemented with BHT. Error bars indicate standard deviation of triplicate values. The initial oil loading was 1.1 g/microcosm. The initial BHT amount in the microcosms supplemented with BHT was 219.72 μg. Data points at week 0 of the experiments represent results after 3 h incubation period.

of proteins forming polymers by repeated aldol condensations (Belitz et al., 2009).

3.2. Triglycerides and BHT

Triglycerides analysis (Fig. 2a) showed the gradual disappearance of the oil in the biotic microcosms and in the abiotic blanks with no BHT. After 8 weeks of incubation, no triglycerides were found in these microcosms. However, a complete recovery of the oil was achieved in the blanks supplemented with BHT (Fig. 2a).

The disappearance of triglycerides in the abiotic blanks with no BHT is consistent with the autoxidation of the oil in these microcosms in the absence of antioxidant protective effect. In the biotic microcosms, triglycerides disappearance is the result of the simultaneous biodegradation and autoxidation of the oil. Mineralized oil fraction as determined from the cumulative CO_2 produced after 16 weeks of incubation, was 81.33 ± 3.88 and $77.86 \pm 4.96\%$ in the biotic microcosms with and without added BHT, respectively, and was not statistically different (($p > 0.05$, one way ANOVA).

The occurrence of autoxidation in the biotic samples supplemented with BHT is supported by the disappearance of the antioxidant in these microcosms at early stages of the biodegradation experiments (Fig. 2b). This can be explained by the simultaneous oxidative decomposition and biodegradation of the antioxidant. Inui et al. (1979) reported the aerobic biodegradation of BHT by activated sludge in aqueous media. Mikami et al. (1979) studied the stability of BHT in water under aerobic and no irradiation conditions. They recovered 60% of the applied antioxidant as unchanged BHT after 8 days of incubation, while 40% were altered to various degradation products. Oxidative degradation of BHT in aquatic media also explains its decline in the abiotic blanks initially supplemented with the antioxidant (Fig. 2b). About 20% decrease in the initial BHT concentration was measured in these blanks at time zero (week 0) of the experiments, and subsequent BHT measurements showed minimal further BHT removal. Remaining

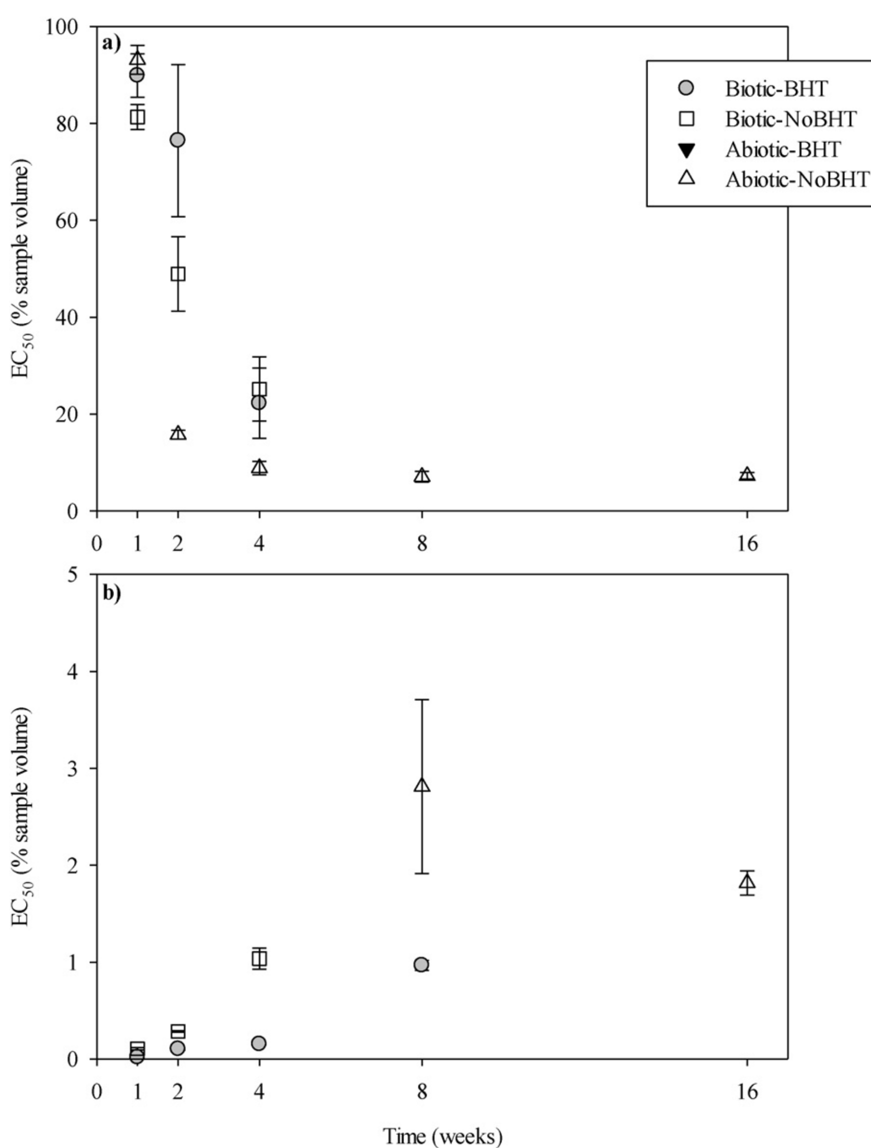

Fig. 3. Microtox toxicity in the different microcosms. a) Liquid phase (LP) toxicity, b) Solid phase (SP) toxicity. Biotic-BHT: biotic microcosms supplemented with BHT; Biotic-NoBHT: biotic microcosms not supplemented with BHT; Abiotic-BHT: abiotic microcosms supplemented with BHT; and Abiotic-No BHT: abiotic microcosms not supplemented with BHT. Error bars indicate standard deviation of triplicate values. The 5 min exposure EC_{50} values are presented. No toxicity was measured in the abiotic microcosms supplemented with BHT in both LP & SP. No EC_{50} values were measured in the LP of the biotic microcosms (Biotic-BHT and Biotic-NoBHT) at weeks 8 and 16 indicating no toxicity at these time periods. Similarly, EC50 were not measured in the SP of the biotic microcosms at weeks 8 (Biotic-NoBHT) and 16 (Biotic-BHT and Biotic-NoBHT). Note that the absence of EC_{50} data points at week zero of the experiments also indicates the lack of toxicity at this time period.

amounts of BHT in these blanks were sufficient to protect the oil against autoxidation up to 16 weeks of incubation.

3.3. Effect of oil composition

Previous experiments investigating the effect of BHT on the biodegradability of glyceryl trilinoleate in aquatic media (Salam et al., 2012b) showed the polymerization of a major fraction of the oil in the biotic microcosms with no added BHT, which significantly reduced the extent of the oil mineralization compared to the microcosms initially supplemented with the antioxidant. This study demonstrated similar behavior of the oil supplemented or not with BHT with substantial oil mineralization being achieved in both treatments. This suggests that the potency of an antioxidant is highly dependent on the fatty acid composition of the oil, which seems to have the principal influence in determining its fate and behavior in aquatic media. Rosas Romero and Morton (1975) investigated the kinetics of the autoxidation of oleic (C18:1)-linoleic (C18:2) acid mixtures and found that the reaction rates decrease with decreasing linoleic acid molar fraction. In addition, the authors noticed a slight antioxidant effect for mixtures containing less than 25% oleic acid when the reaction enter the period of bimolecular decomposition of hydroperoxides. In this study, oleic acid (C18:1) might have initially acted as a diluent for the oxidation of both linoleic (C18:2) and linolenic (C18:3) acids in the microcosms not supplemented with BHT. Simultaneously, the biodegradation progress would have reduced the amounts of these more readily biodegradable polyunsaturated fatty acids rendering the mixture much more stable against autoxidation. As a consequence, an enhancement of the oil biodegradation was observed and was not significantly different from the microcosms initially supplemented with BHT. Indeed, the early disappearance of antioxidant in these latter microcosms after only two weeks of the experiments further converged the behavior of oil in both BHT treatments.

3.4. Toxicity

Microtox® toxicity in the sacrificed microcosms was measured in both liquid and solid phases (Fig. 3).

3.4.1. Liquid phase toxicity

Liquid phase toxicity (Fig. 3a) was detected in the biotic microcosms after one week of incubation and increased (EC_{50} decreased) with time to reach high levels ($EC_{50} < 25\%$) after 4 weeks. No toxicity was found in all biotic microcosms at weeks 8 and 16 of incubation. These results suggest that toxic metabolic intermediates were formed at early stages of the aerobic biodegradation of canola oil and were eliminated at a later time of the experiments.

Analysis of the liquid phase in the biotic microcosms (Fig. 4a, b) showed increasing concentrations of fatty acids up to week 4 of incubation. Notably, higher concentrations of caprylic (C8:0), pelargonic (C9:0), palmitic (C16:0), palmitoleic (C16:1), oleic (C18:1), and linoleic (C18:2) acids, were measured at weeks 1, 2, and 4 of incubation. Medium and long chain fatty acids have been widely reported for their inhibitory effects on microorganisms in both aerobic and anaerobic treatment processes (Becker et al., 1999; Lalman and Bagley, 2000, 2001; Shin et al., 2003; Pereira et al., 2005; Li et al., 2005; Campo et al., 2007; Salam et al., 2012a).

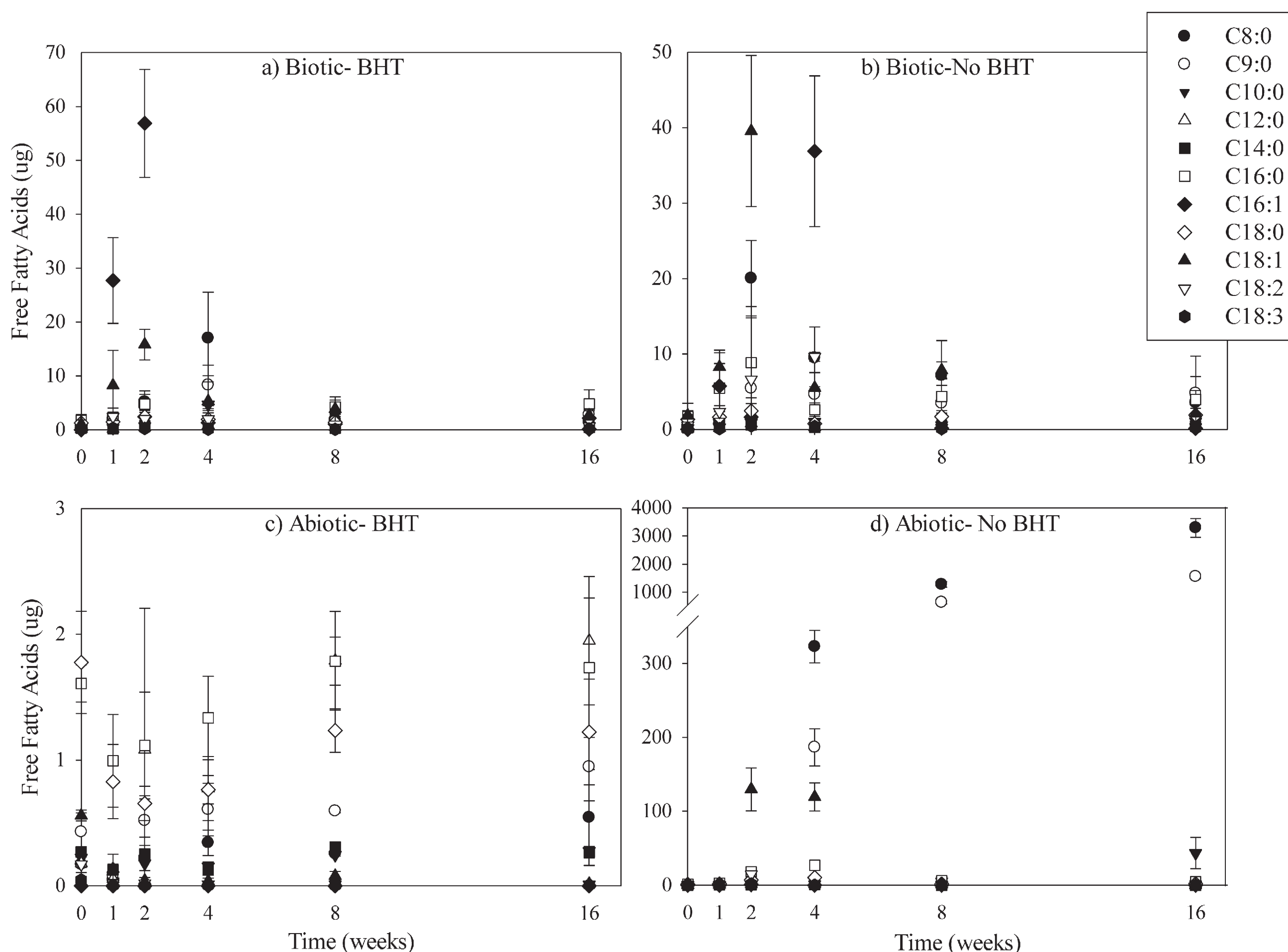

Fig. 4. Free fatty acids profile in the liquid phase, a) Biotic-BHT: biotic microcosms supplemented with BHT; b) Biotic-NoBHT: biotic microcosms not supplemented with BHT; c) Abiotic-BHT: abiotic microcosms supplemented with BHT; and d) Abiotic-No BHT: abiotic microcosms not supplemented with BHT. Error bars indicate standard deviation of triplicate values.

The higher toxicity ($p < 0.05$, one way ANOVA) observed in the biotic microcosms not supplemented with BHT during the first and second weeks of incubation (Fig. 3a) is most likely attributed to the combined effect of biodegradation intermediates and autoxidation products, namely hydroperoxides formed at early stages of the experiments. In the biotic microcosms initially supplemented with BHT, remaining amounts of the antioxidant detected up to the second week of incubation (Fig. 2b) most likely delayed the formation of toxic autoxidation products resulting in higher EC50 values.

In the abiotic blanks with no BHT, EC_{50} values of about 15% were measured after only 2 weeks of incubation (Fig. 3a). Higher toxicity levels ($EC_{50} < 9\%$) were attained at later stages of the experiments and persisted throughout the incubation period. Liquid phase analysis showed exponentially increasing concentrations of FFA, namely caprylic and pelargonic acids (Fig. 4d). Methyl octanoate (caprylic acid methyl ester) has been reported in the oxidation of oleate, linoleate, and linolenate, and is produced by carbon-carbon cleavage of the corresponding fatty acid hydroperoxides (Frankel, 1980). However, the occurrence of high concentrations of pelargonic acid can be explained by the oxidative cleavage of the double bond of oleic acid at the C9 position. This is supported by the concomitant decreasing concentrations of oleic acid observed throughout the incubation period. The oxidative cleavage of oleic acid by hydroxy radicals resulting in the formation of nonenal and pelargonic acid has been reported by Kawamura and Gagosian (1987).

The inhibitory effect of medium chain fatty acids (C6 to C11) was demonstrated by Fay and Farias (1975), C9:0 being the most effective. Other autoxidation products of canola oil, namely carbonyl compounds formed at advanced stages of oxidation as evidenced by the infrared analysis, might have accumulated to toxic levels in the blanks with no BHT contributing to the overall observed toxicity to *V. fischeri*, the Microtox® test organism. No toxicity was found in the abiotic blanks supplemented with BHT at any time of the incubation period. Negligible amounts of FFA (< 3 μg) were measured in these microcosms at all times of the experiments (Fig. 4c).

3.4.2. Solid phase toxicity

High toxicity levels (<2%) were found in the solid phase of the biotic microcosms for both BHT treatments (0 and 200 mg kg^{-1}) (Fig. 3b). In these microcosms, toxicity gradually decreased with time and disappeared at a later period of incubation. Detected toxicity can be attributed to the high amounts of long chain fatty acids found in the solid phase (Fig. 5a, b). In particular, high levels of oleic acid were measured after one week of incubation and were gradually eliminated during biodegradation. The lower amounts of oleic acid measured in the microcosms with no BHT are likely attributed to its simultaneous biodegradation and autoxidation during early stages of the experiments, and explains the lower toxicity values ($p < 0.05$, one way ANOVA) measured in these microcosms compared to the ones initially supplemented with the antioxidant.

No solid phase toxicity was found in the abiotic microcosms supplemented with BHT at any time of the experiments (Fig. 3b). Furthermore, negligible amounts of FFA were detected in the solid phase of these microcosms (Fig. 5c). However, solid phase toxicity was found in the abiotic blanks with no BHT at later stages of the experiments (weeks 8 and 16) (Fig. 3b) and was associated with the appearance of FFA,

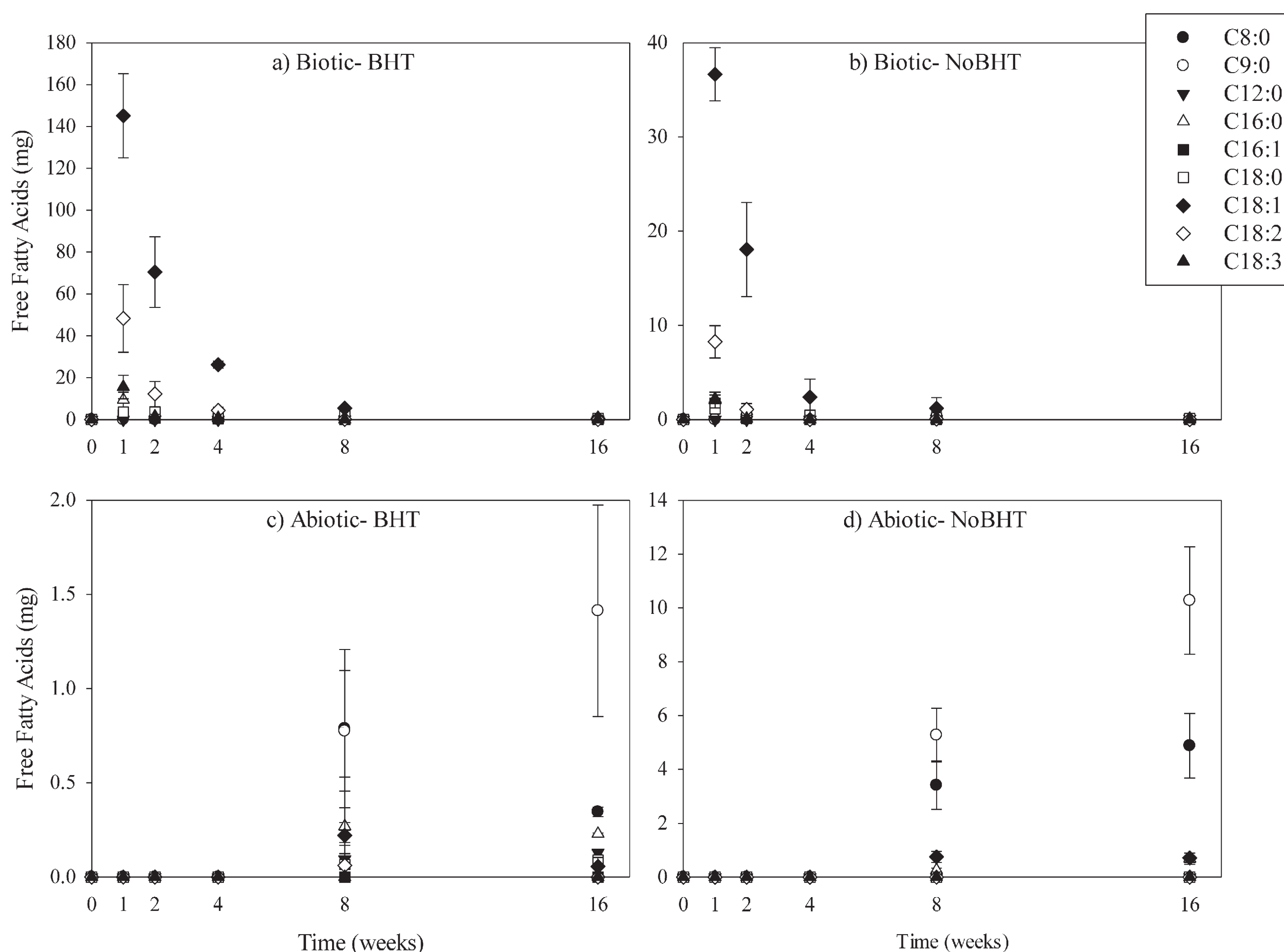

Fig. 5. Free fatty acids profile in the solid phase, a) Biotic-BHT: biotic microcosms supplemented with BHT; b) Biotic-NoBHT: biotic microcosms not supplemented with BHT; c) Abiotic-BHT: abiotic microcosms supplemented with BHT; and d) Abiotic-No BHT: abiotic microcosms not supplemented with BHT. Error bars indicate standard deviation of triplicate values.

particularly caprylic and pelargonic acids resulting from canola oil oxidation (Fig. 5d).

3.5. FTIR

Autoxidative deterioration of canola oil throughout the biodegradation experiments was examined by FTIR. Significant spectral changes in the original oil were identified in the abiotic blanks with no BHT at early stages of the experiments and evolved with the incubation period attesting to the advanced stages of the oil oxidation after 16 weeks. The early manifestation of the autoxidation process in these blanks through the formation of hydroperoxides and the persistence of secondary autoxidation products until late stages of incubation as evidenced by the infrared spectra, coincide well with the early toxicity observed in these microcosms and its persistence after 16 weeks. The evolution of the oxidative degradation of canola oil in the abiotic blanks with no BHT is presented in SI Figure S1 along with a detailed discussion of the observed spectral changes. No spectral changes were observed in the oil extracted from the abiotic microcosms supplemented with BHT, demonstrating the protective effect of the phenolic antioxidant in these microcosms at the tested concentration of 200 mg kg^{-1} and under the investigated conditions.

Infrared spectra of the oil extracts (Fig. S2, SI) and solid deposits (Fig. S3, SI) formed in the biotic microcosms, supplemented or not with BHT, also confirmed advanced stages of oil autoxidation in both BHT treatments. However, the frequencies of some bands associated with the oil autoxidation were altered due to the interference of some adsorption bands attributed to bacterial structural components. This hindered a clear distinction in the time evolution of the autoxidation process between the two types of microcosms.

4. Conclusion

The biodegradability and toxicity of purified canola oil, a mixed-acid triacylglycerol with high C18:1 content, were studied in the presence and absence of BHT. The results showed similar behavior of the oil supplemented or not with the antioxidant, with substantial oil mineralization being achieved in both treatments. In addition, transient toxicity was observed in the biotic microcosms in the absence and presence of BHT and was associated to the oil biodegradation intermediates and autoxidation products, while toxicity was persistent in the case of abiotic blanks with no BHT and was associated with high concentrations of caprylic and pelargonic acids formed during the oil autoxidation. Previous experiments investigating the effect of BHT on the biodegradability of glyceryl trilinoleate, a pure triacylglycerol of cis,cis-9,12-octadecadienoic acid (C18:2 delta), in aquatic media, showed the polymerization of a major fraction of the oil in the microcosms with no added BHT, which significantly reduced the extent of the oil mineralization compared to the microcosms initially supplemented with the antioxidant. Hence, the findings from this study demonstrate that the oil fatty acid composition plays a major role in defining the behavior and impact of spilled vegetable oils in contaminated aquatic environments, and significantly influences the protective effect of antioxidants against the oxidative deterioration and polymerization of the oil.

Acknowledgments

This research was funded by the National Risk Management Research Laboratory of the US Environmental Protection Agency (EP-C-11-006).

Appendix A. Supplementary data

Details on the used reagents and chemicals, canola oil purification, chemical analysis and characteristics of purified canola oil, mineral medium composition in the respirometric microcosms, BHT analysis, FTIR spectra, are available in supplementary information. Supplementary data to this article can be found online at doi:http://dx.doi.org/10.1016/j.scitotenv.2015.12.138.

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