Environmental Science & Technology

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pubs.acs.org/est

Effect of Butylated Hydroxytoluene (BHT) on the Aerobic Biodegradation of a Model Vegetable Oil in Aquatic Media

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Supporting Information

ABSTRACT: Antioxidants added to vegetable oils to prevent lipid oxidation significantly affect their biodegradation in impacted aquatic environments. In this study, the effect of butylated-hydroxytoluene (BHT) on the biodegradation of glyceryl trilinoleate, a model vegetable oil highly susceptible to autoxidation, was determined. Biodegradation experiments were conducted in respirometric microcosms at an oil loading of 333 gal acre^{-1} (0.31 L m^{-2}) and BHT concentrations ranging from 0 to 800 mg kg^{-1} (0, 50, 100, 200, 400, and 800 mg kg^{-1}). Competition between polymerization and biodegradation of the oil was observed at all BHT concentrations and was significant in the microcosms not supplemented with the antioxidant. In all microcosms, intractable rigid polymers unavailable for bacterial degradation were formed. Infrared analysis evidenced the advanced stages of the oil autoxidation. After 19 weeks of incubation, only about 41% of the oil was mineralized in the microcosms with no BHT. However, mineralization exceeded 67% in the microcosms with added antioxidant and did not significantly increase with increasing BHT concentrations. Biodegradation rate constants were calculated by nonlinear regression and were not significantly different in the microcosms with added BHT (k = 0.001 h^{-1}). Higher k values were measured in the microcosms lacking the antioxidant (k = 0.0023 h^{-1}), most likely due to the increased oxygen consumption associated with the autoxidation process in this case. No toxicity was detected in all biotic microcosms at the end of the incubation period, while high toxicity (EC_{50} = 4.78%) was measured in the abiotic blanks with no antioxidant and was attributed to the accumulation of autoxidation products.

1. INTRODUCTION

The global production of vegetable oils has witnessed a steady growth during the past decade prompted by an ever increasing demand for food and a worldwide increasing production of biodiesel.[1] According to the U.S. Department of Agriculture (USDA) more than 145 million metric tons of vegetable oil were produced in 2009/2010, a 38% increase from the 2000/2001 production year.[2,3] The resulting active exchange of vegetable oil on the world markets involves the transport, handling, and storage of large quantities of these products and has regularly led to spillages during the past 40 years.[4]

Harmful effects of vegetable oil spills in the aquatic environment have been reported extensively in the literature.[4−9] Besides their coating properties leading to smothering of surface dwelling organisms, vegetable oils may provoke significant detrimental effects through oxygen depletion as a result of their high biochemical oxygen demand (BOD),[4,6,10−12] and induce acute and chronic toxicity to the aquatic fauna in the impacted ecosystems.[6] Direct toxicity and sublethal effects of vegetable oils on mussels, rainbow trout, fathead minnows, amphipods, and other benthic organisms have been reported in the literature.[6,13−17] Vegetable oil toxicity is mainly due to the accumulation in the medium of 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.[18−23] Furthermore, some oil autoxidation byproducts have antibacterial properties that may kill the bacteria or inhibit their activity.[24] A thorough discussion of the toxicity of vegetable oil and their deleterious effects in impacted aquatic ecosystems is provided in Salam et al.[25]

The fate and behavior of spilled vegetable oils in the aquatic environment can vary significantly according to their individual characteristics.[4,12] Despite their ready biodegradability, vegetable oils can persist for years in impacted aquatic media as a result of their oxidative polymerization to intractable lumps.[26,27] Polymerized vegetable oil is unavailable for bacterial degrada-

Received: December 26, 2011
Revised: May 24, 2012
Accepted: June 1, 2012
Published: June 1, 2012

tion[7,27] and can bind sediment particles together to form a cap of reduced permeability to water and oxygen.[7]

Oxidative polymerization of vegetable oils involves the intermolecular addition of hydroperoxides, the primary products of the autoxidation of unsaturated triacylglycerols, through either ether or peroxy linkages.[28] Autoxidation is essentially a free radical chain reaction divided into initiation, propagation, branching, and termination steps.[29,30] Free radicals, frequently from unknown origin, initiate the autoxidation process by abstracting the α-methylenic hydrogen atom from the unsaturated lipid.[29,30] As a result, a fatty acid free radical is formed and is susceptible to attack by atmospheric oxygen to produce hydroperoxides.[29,30] Autoxidation of fatty acids is then autocatalytically accelerated by radicals generated from the degradation of hydroperoxides.[30] Termination occurs when two free radicals combine to form a nonradical product.[29,31]

The degree of autoxidation of vegetable oils is affected, among other factors, by the fatty acid composition, unsaturation level, and the presence of antioxidants.[30] In general, the higher the degree of unsaturation of a vegetable oil, the more susceptible it is to autoxidative deterioration.[29] Most vegetable oils contain antioxidants to retard lipid autoxidation. According to their mechanism of action, these can be classified as primary or secondary antioxidants. Primary antioxidants, mostly phenolic compounds, are free radical acceptors that inhibit the initiation step or interrupt the propagation step of the oil autoxidation.[29,31] Secondary antioxidants, classified as synergists that enhance the antioxidant activity of primary antioxidants, function primarily as oxygen scavengers.[31]

Both synthetic and natural antioxidants are used in food products. Synthetic antioxidants are mostly phenolic and include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroxyquinone (TBHQ), and propyl, octyl, dodecyl gallates.[31] Of the natural antioxidants, vitamin C and tocopherols (vitamin E) are widely used.[32] Residual levels of tocopherol in finished oils appear to be optimum for providing the oxidative stability that is inherent in most vegetable oils.[29] Antioxidants are generally functional at very low concentrations of 0.01% (w/w) or less. When present in excess, they may act as pro-oxidants due to their involvement in the initiation reactions.[31] In general the use of primary antioxidants is limited to 100−200 mg L^{-1} of BHA, BHT, or TBHQ, or 200−500 mg L^{-1} of the gallates for the stabilization of the fats and oils.[32]

While the influence of antioxidants on the oxidative stability of vegetable oils during their storage and heating has been extensively studied in the literature, knowledge of their influence on the fate and impact of vegetable oils in aquatic environments is still lacking. This study aimed at determining the effect of increasing concentrations of BHT, one of the most used synthetic antioxidants in edible oils[32,33] on the biodegradability and toxicity of vegetable oils in contaminated aquatic environments. For this purpose, glyceryl trilinoleate, an ester of *cis*,*cis*-9,12-octadecadienoic acid highly susceptible to autoxidation, was used as a model system of vegetable oils.

2. MATERIALS AND METHODS

2.1. Reagents and Chemicals. Glyceryl trilinoleate and BHT were purchased from Sigma Aldrich (St. Louis, MO). Optima grade organic solvents were purchased from Fisher Scientific (Pittsburgh, PA). Microtox reagents were supplied from Strategic Diagnostics Inc. (Newark, DE). A solution of 5% dimethyldichlorosilane in toluene was used to deactivate glassware[25,34] and was obtained from Supelco (Bellefonte, PA).

2.2. Biodegradation Experiments. Respirometric experiments were performed in computerized Comput-Ox respirometers, model OO-244SC (N-Con Systems, Crawford, GA) in order to determine the effect of increasing concentrations of BHT on the biodegradation patterns of glyceryl trilinoleate. The triglyceride was used at a loading of 333 gal $acre^{-1}$ (equivalent to 0.31 L m^{-2} or 1.1 g per microcosm) and was supplemented with BHT at six different concentrations: 0, 50, 100, 200, 400, and 800 mg kg^{-1}. 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.[34] and Salam et al.[25] 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. The choice of the oil loading used in the current study is further discussed in Supporting Information (SI). Furthermore, the used range of BHT concentrations was selected so that it encompasses the commonly used concentrations of the antioxidant to stabilize vegetable oils, and permits the testing of a potential pro-oxidant effect of high concentrations of BHT (up to 800 mg kg^{-1}).

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, 4.53 g L^{-1}) supplemented with minimal nutrients and autoclaved to ensure initial sterile conditions. The mineral solution composition is presented in SI Table S1. A slick of the oil with the defined BHT concentration was applied to the surface of each microcosm. The oil was loaded under nitrogen to prevent its oxidation during sample preparation. Three biotic samples and three abiotic blanks were prepared per BHT level. An oil-degrading inoculum was added in the case of the biotic samples just before the oil addition, at a volatile suspended solids (VSS) concentration of 250 mg L^{-1}. 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 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.[25,34]

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 39 respirometric microcosms. Each flask was equipped with a trap containing 0.1N potassium hydroxide (KOH) intended to capture carbon dioxide (CO_2) produced from the biodegradation activity. The respirometer temperature was maintained at 20 °C, and the microcosms were stirred at 300 rpm resulting in a full depth vortex and dispersion of the oil in the aqueous phase. This achieved mixing prevents oxygen mass transfer limitation in the biodegradation microcosms as demonstrated in a previous study by Salam et al.[25] Biodegradation of glyceryl trilinoleate was monitored through the respirometric oxygen uptake and CO_2 production. SigmaPlot 11(Systat Software, Inc., CA) was used to perform an analysis of variance (ANOVA) to determine the statistical

significance of any differences in the oil biodegradation observed under the different BHT treatments.

2.3. Chemical Analysis. At the end of the incubation period, which lasted 19 weeks, all microcosms were sacrificed, and 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 solid polymers formed by the oil autoxidation, 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 (SPE) were analyzed for residual triglyceride, 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.[34]

Residual BHT concentration in the SP was determined by High Pressure Liquid Chromatography (HPLC) according to the method described by Gratzfeld-HüSgen and Schuster[35] for antioxidant analysis in food products. The analysis was performed on a 1100 series chromatographic system with a UV visible diode array detector (Agilent Technologies, CA) using a C18 column (4.6 × 150 mm, X Terre MS, 5 μm). The sample injection volume was 5 μL. An isocratic elution was carried out using 20% water and 80% acetonitrile at a flow rate of 0.5 mL min^{-1}. The column temperature was maintained at 30 °C, and the antioxidant was detected at 200 nm.

2.4. Toxicity. Toxicity in the sacrificed microcosms at the end of the biodegradation experiments was assessed in both liquid and solid phases. Microtox Analyzer model 500 (SDI, DE) was used for this purpose and samples were analyzed following the Microtox test, an acute-toxicity bioassay based on suppression of bioluminescence of the marine bacterium *Vibrio fischeri* following exposure to toxicants.[36] Liquid phase toxicity analysis was performed according to the basic test protocol with an initial sample volume of 45% or 81.9%.[36] Solid phase toxicity analysis was performed according to the "Protocol for the Basic Test Using Organic Solvent Sample Solubilization".[36] Non-denatured absolute ethanol (Sigma–Aldrich, St. Louis, MO) 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 and were determined 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).

2.5. FTIR. Autoxidative deterioration of glyceryl trilinoleate was examined 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 solid polymers. Each spectrum was obtained by averaging 64 interferograms with a resolution of 4 cm^{-1}.

3. RESULTS AND DISCUSSION

3.1. Biodegradation Experiments. Biodegradation was monitored through oxygen uptake and CO_2 production. The cumulative oxygen uptake curves obtained for the different BHT treatments are presented in Figure 1. In all treatments,

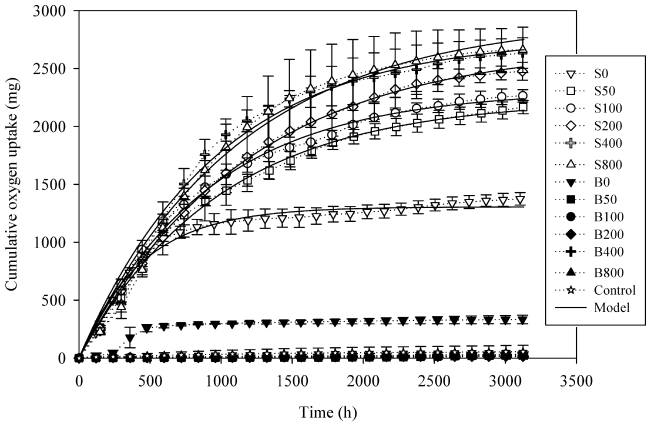

Figure 1. Cumulative oxygen uptake curves for glyceryl trilinoleate biodegradation at different initial BHT concentrations. Points are triplicate experimental data, while lines are curves fit to BOD equation. Error bars indicate standard deviation of triplicate values. The letters S and B in the legend signify samples and blanks, respectively, and the numbers from 0 to 800 indicate the corresponding BHT concentration.

oxygen uptake curves followed the typical biodegradation profile with exponential growth approaching an asymptotic leveling. At the end of the incubation period, the achieved cumulative oxygen uptake (Ou_{Exp}) slightly increased with increasing BHT levels in the biotic microcosms supplemented with the antioxidant (50–800 mg kg^{-1}). Compared to these microcosms, the Ou_{Exp} values measured in the biotic samples with no BHT was significantly lower ($p < 0.001$, Holm-Sidak One Way ANOVA) (Figure 1, Table 1). In all treatments (0–800 mg kg^{-1}), the Ou_{Exp} values were lower than the theoretical oxygen demand (Ou_{Th}; 3170 mg) required for the complete stoichiometric conversion of the added triglyceride (1.1 g/microcosm/BHT level) into CO_2 and water (Table 1). Besides oxygen requirement for biomass growth, these results can be explained by the autoxidation of a fraction of the unsaturated triglyceride in all microcosms reducing the amount of oil available for biodegradation. Autoxidation is supported by the observed rigid composites forming white flakes, and elastic gumballs of yellow color in all biotic microcosms at all initial BHT concentrations (0–800 mg kg^{-1}). These deposits were resistant to dissolution in methanol and DCM during the SP extraction and were also insoluble in a wide range of other organic solvents (hexane, tetrahydrofuran, chloroform, acetone, toluene, dimethylformamide, ethylacetate, and methylisobutyl-ketone). Infrared analysis of these deposits evidenced the advanced stages of the oil autoxidation (Section 3.4).

Triglyceride analysis in all biotic microcosms revealed the disappearance of the oil at all initial BHT treatments (0–800 mg kg^{-1}). However, a full oil mineralization was not achieved in these microcosms under the used biodegradation conditions (Table 1). In the case of the microcosms with no initial antioxidant, only about 41% of the initial oil was mineralized, suggesting that the largest part of the oil was transformed to persistent nonbiodegradable polymers. Mineralization exceeded 67% in the microcosms with added antioxidant and increased with increasing initial BHT concentrations (Table 1). However, in this case, no significant difference ($p > 0.05$, one way

dx.doi.org/10.1021/es2046712 | Environ. Sci. Technol. 2012, 46, 6798–6805

Table 1. Ultimate Oxygen Uptake, % Mineralization, And Nonlinear Regression Parameters for Oxygen Uptake Curves in the Biotic Microcosms[a]

BHT (mg kg^{-1})	Ou_{Exp} (mg)	Ou_m (mg)	mineralization (%)	k (h^{-1})
0	1376 ± 24	1305 ± 1	41.5 ± 0.3	0.0023 ± 9.17 × 10^{-6}
50	2167 ± 57	2233 ± 2	66.8 ± 3.9	0.0010 ± 1.85 × 10^{-6}
100	2264 ± 53	2312 ± 2	68.3 ± 4.9	0.0011 ± 2.50 × 10^{-6}
200	2476 ± 78	2700 ± 2	73.9 ± 2.2	0.0009 ± 1.23 × 10^{-6}
400	2638 ± 127	2772 ± 5	78.1 ± 4.2	0.0010 ± 4.87 × 10^{-6}
800	2661 ± 195	2932 ± 6	78.7 ± 8.4	0.0009 ± 4.40 × 10^{-6}

[a]Initial trilinolein concentration 1.1 g per microcosm. Theoretical oxygen demand, Ou_{Th} = 3170 mg. The Ou_{Th} was calculated from the triglyceride stoichiometric conversion into CO_2 and H_2O ignoring the reduction in oxygen demand due to biomass synthesis, which was estimated to be minimal by Campo et al.[34] under similar experimental conditions used in the current study. Ou_{Exp}, experimental oxygen uptake ± standard deviation among three replicates; Ou_m, model ultimate oxygen uptake ± standard error; % Mineralization ± standard deviation among three replicates, oil mineralization was computed based on CO_2 production with no correction for BHT biodegradation; k, first order rate constant ± standard error.

ANOVA) in the percent oil mineralized was measured among the tested BHT treatments (Table 1), suggesting a minimal additional protective effect of BHT against polymerization above 50 and up to 800 mg kg^{-1}.

The occurrence of autoxidation in the biotic samples supplemented with BHT could be explained by the gradual loss of the protective effect of the antioxidant through its oxidative decomposition and biodegradation. Inui et al.[37] reported the aerobic biodegradation of BHT by activated sludge in aqueous media. The analysis of BHT in the sacrificed microcosms after 19 weeks of incubation, revealed the disappearance of the antioxidant from all biotic samples supporting autoxidation in these microcosms. Nevertheless, the antioxidant effectively enhanced the oil biodegradation by retarding the autoxidation process.

Autoxidation was also supported by the occurrence of oxygen consumption in the abiotic blanks with no BHT (Figure 1). In these microcosms, a lag phase of about 150 h elapsed prior to the onset of oxygen uptake. A steep rise in oxygen consumption was then observed and leveled off shortly. At the same time, rigid composites formed of irregular white flakes similar to those observed in the biotic microcosms were found (SI Figure S1). Triglyceride analysis in these microcosms revealed the complete disappearance of glyceryl trilinoleate. However, no oxygen uptake and solids formation were observed in the blanks supplemented with BHT (Figure 1). In addition, a complete recovery of the oil was achieved in these microcosms (Table 2).

These results suggest the autoxidative polymerization of the oil in the abiotic blanks lacking the protective effect of the phenolic antioxidant. The initial phase with no oxygen uptake in these blanks most likely corresponds to the induction period during which precursors for the autoxidation reaction are accumulated. The length of the induction period depends, among other factors, on the fatty acid composition of the oil.[30] The more the allyl groups, the shorter the induction period.[30] An induction period of 19 h is reported for linoleic acid at 25 °C.[30]

It is interesting to note the formation of water droplets on the inside wall of the abiotic blanks with no BHT (SI Figure S2), accompanied with a warming of the microcosms and the gradual loss of the purple color of the KOH solution inside the traps turning colorless. These observations followed the steep oxygen uptake by these blanks and were most likely due to a bimolecular degradation mechanism of hydroperoxides accumulated to high concentrations during the autoxidation process. This reaction 1 is exothermic and releases water.[30]

$$2\mathrm{ROOH} \rightarrow \mathrm{RO}_2^{\cdot} + \mathrm{RO}^{\cdot} + \mathrm{H_2O} \quad (1)$$

The absence of autoxidation in the abiotic blanks initially supplemented with BHT is supported by the detection of remaining amounts of the antioxidant in these microcosms after 19 weeks of incubation, regardless of the initial concentration (Table 2). This suggests that the lowest used BHT level (50 mg kg^{-1}) would be sufficient to protect the oil against polymerization under the investigated conditions. The results presented in Table 2 show that increased BHT removal was achieved in the abiotic blanks supplemented with higher initial amounts of the antioxidant, although equal amounts of oil were initially supplied to all microcosms. Doubling the initial BHT concentration in the abiotic microcosms resulted in an increase in BHT removal by a factor of about 1.7. This observation can be explained by the instability of BHT in aqueous solution and its tendency to decompose to a wide range of products. Hence, the measured BHT removal does not account solely for the fraction of antioxidant used for the protection of the oil from autoxidation. Mikami et al.[38] studied the stability of BHT in water under aerobic and no irradiation conditions. After 8 days of incubation, they reported that 60% of the applied antioxidant was recovered as unchanged BHT, while 40% was altered to various degradation products including BHT-OOH, BHT-OH, BHT-CH_2OH, BHT-CHO, BHT-COOH, and unidentified polar compounds. Chang and Maurey[39] also reported the instability of BHT in aqueous solution and identified stilbenequinone and its precursor, a dimer of BHT, as the main degradation products. In this study, the aerobic biodegradation of BHT and its abiotic transformation in aquatic media was tested separately from the vegetable oil under similar experimental conditions described above and at

Table 2. Triglycerides and BHT Recovery in the Abiotic Blanks after 19 Weeks*

initial BHT concentration (mg kg^{-1})	initial BHT amount (ug)	BHT recovery (ug)	BHT removal (ug)	triglycerides recovery (g)
0	0	0	0	0
50	54.9	15.9 ± 1.7	40.0 ± 1.7	1.10 ± 0.01
100	109.9	43.1 ± 1.4	66.7 ± 1.4	1.15 ± 0.01
200	219.7	107.1 ± 5.0	112.7 ± 5.0	1.15 ± 0.05
400	439.4	248.2 ± 9.6	191.3 ± 9.6	1.14 ± 0.02
800	878.9	545.0 ± 27.3	333.9 ± 27.3	1.12 ± 0.04

*Reported amounts are per microcosm.

dx.doi.org/10.1021/es2046712 | Environ. Sci. Technol. 2012, 46, 6798−6805

concentrations ranging from 50 to 3200 mg L^{-1}. After 4 weeks of incubation, no evidence of oil biodegradation was reported in all biotic microcosms as measured by the CO_2 production. In addition, no respirometric oxygen uptake was measured in all abiotic microcosms. More than 80% of the initially added BHT were recovered from the sacrificed microcosms at the end of the experiments while a fraction of the BHT was altered to insoluble byproducts. No further analysis was conducted to identify the insoluble byproducts of BHT or those that could have been formed in the liquid phase. These results were explained by the low solubility of BHT in aquatic media making it unavailable for bacterial degradation. A detailed discussion of these experiments is provided in the SI.

3.2. Biodegradation Kinetics. In order to quantify the biodegradation of glyceryl trilinoleate in the biotic microcosms at the different initial concentrations of BHT, the first order biodegradation rate constants were calculated using the biochemical oxygen demand (BOD) eq 2 [40]

$$O_t = O_u(1 - e^{-kt}) \quad (2)$$

where O_t is the cumulative oxygen uptake (mg) at time t obtained from the respirometric data, O_u is the ultimate oxygen uptake (mg), and k is the first order biodegradation rate constant (h^{-1}). The k and O_u values were estimated by least-squares nonlinear regression using SigmaPlot 11 (Systat Software, Inc., CA) to fit the respirometric cumulative oxygen uptake data to the BOD equation. The best fit parameters are listed in Table 1 and adequately describe the experimental data.

In the case of the biotic microcosms supplemented with BHT, no significant difference ($p > 0.05$, One Way ANOVA) in the calculated k values was observed among the different BHT treatments (50−800 mg kg^{-1}). This suggests no effect of the initial BHT concentration above 50 and up to 800 mg kg^{-1} on the biodegradation kinetics of glyceryl trilinoleate under the investigated conditions. The k value was almost double (k = 0.0023 h^{-1}) in the case of the microcosms with no BHT. The higher k value obtained in this case may be attributed to the higher oxygen consumption associated with the autoxidation process in the absence of any antioxidant protective effect, significantly contributing to the overall oxygen uptake during the initial stages of the biodegradation process. Oxygen consumption of about 14.5% of the total measured uptake in the microcosms with no added BHT was associated with the autoxidation process. Hence, the computed k value might not adequately reflect the biodegradation kinetics in these microcosms.

3.3. Toxicity. After 19 weeks of incubation, no toxicity was detected in the liquid and solid phases of all microcosms except for the abiotic blanks with no antioxidant. In the latter case, toxicity was found in the LP, and the average EC_{50} values at 5 and 15 min exposure were 6.39 ± 1.06 and 4.78 ± 0.68% sample volume, respectively. This toxicity is most likely the result of the accumulation of the autoxidation products in these abiotic microcosms with no BHT. Liquid phase analysis revealed the presence of relatively high concentrations (191 ug ±23 ug) of methyl octanoate (caprylic acid methyl ester, C8:0) in these microcosms. Methyl octanoate has been reported in the oxidation of oleate, linoleate, and linolenate, and is produced by carbon−carbon cleavage of the corresponding fatty acid hydroperoxides.[41]

The absence of toxicity in the biotic samples after 19 weeks does not infer that toxicity did not occur at anytime in these microcosms during the incubation period. Rather, combined autoxidation and biodegradation byproducts of glyceryl trilinoleate, namely LCFAs, are expected to have induced toxicity during the biodegradation experiments. This toxicity had most probably decreased over time as the oil was degraded and potential transient toxic biodegradation and autoxidation intermediates were eliminated. In a similar study investigating the effect of BHT (0 and 200 mg kg^{-1}) on the biodegradation and toxicity of purified canola oil (333 gal $acre^{-1}$), toxicity was measured in the biotic microcosms 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. Transient toxicity was also reported during the aerobic biodegradation of commercial canola oil (333 gal $acre^{-1}$) and was attributed to the accumulation in the medium of LCFAs.[25] Transient toxicity during the aerobic and anaerobic biodegradation of vegetable oils was reported in the literature[20,34,42]

Negligible amounts of free fatty acids were found in both liquid and solid phases of the biotic and abiotic microcosms after 19 weeks of incubation.

3.4. FTIR. Oxidative degradation of glyceryl trilinoleate under the investigated biodegradation conditions was examined by FTIR. The infrared spectroscopy affords information on the functional groups of a sample and was used to identify the chemical nature of the solid and elastic polymers formed in the microcosms.

3.4.1. Polymers Formed in the Abiotic Microcosms. Intractable solid polymers were formed in the abiotic blanks with no BHT. The spectrum of these polymers (Figure 2b) was compared to the spectrum of the unaltered triglyceride, glyceryl trilinoleate, presented in Figure 2a. The triglyceride spectrum shows the functional groups associated with the major observed bands. The frequencies of these bands, and their assigned functional groups and mode of vibration are also given in SI Table S2 and were adapted from Guillen and Cabo.[43] Major differences between the spectra of the original oil (Figure 2a) and solid polymers (Figure 2b) can be discussed.

The small band near 3471 cm^{-1} in the original oil (Figure 2a) associated with the overtone of the triglyceride ester carbonyl (-C═O) absorption, became broad and more intense in the blanks with no BHT (Figure 2b). The frequency of this band was about 3469.7 cm^{-1} after 19 weeks of incubation (Figure 2b). The broadening of the ester carbonyl overtone band was observed by Guillen and Cabo,[44] and Guillen et al.[45] during the thermal oxidation of edible oils. The authors reported an initial decrease in the frequency of this band to about 3440 cm^{-1} due to overlapping new absorptions of generated hydroperoxides, followed by a shift in the frequency back to its original value near 3470 cm^{-1} associated with the accumulation of significant proportions of alcohols at a later stage of the oxidation process. This suggests that advanced stages of the oil oxidation were attained in the blanks with no antioxidant after 19 weeks under the investigated conditions.

Advanced stages of oxidation were evidenced also by major modifications in the unsaturation bands of the original oil. First, the band at 3009.8 cm^{-1} due to the stretching vibration of *cis*-CH olefinic groups (═C—H) in the original oil (Figure 2a), disappeared in the blanks with no BHT (Figure 2b). In addition, the extinction of the band at about 1655 cm^{-1} associated with the stretching vibration of the carbon−carbon double bonds of *cis* olefins (C═C), was also noticed (Figure

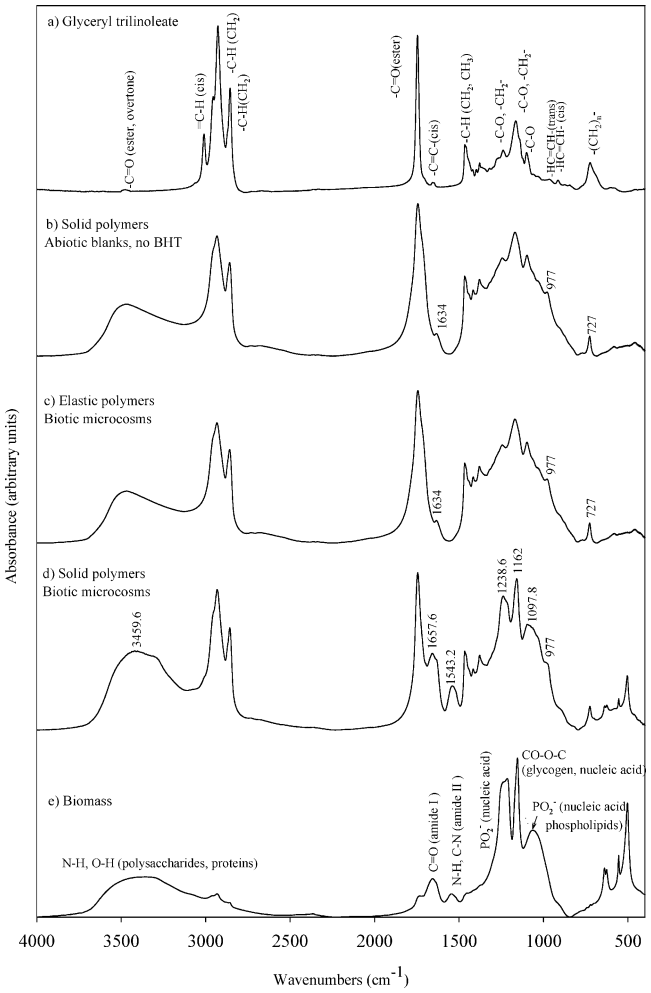

Figure 2. Fourier transform infrared spectra of (a) glyceryl linoleate (unaltered oil), (b) solid polymers from the abiotic microcosms with no BHT, (c) Elastic polymers from the biotic microcosms, (d) Solid polymers from the biotic microcosms (e) biomass. The infrared spectra of the elastic (c) and solid (d) polymers presented herein belong to the biotic microcosms with an initial BHT concentration in the oil of 200 ppm. Identical spectra were recorded for the polymers collected from all other biotic microcosms with the different initial BHT treatments.

2b). Triglycerides oxidation involves the isomerization of the *cis* double bonds to *trans* groups alongside with hydroperoxide generation, and their breakdown to secondary oxidation products at advanced oxidation stages.[30]

Furthermore, the band at 1746 cm^{-1} due to the ester carbonyl group (—C═O) of the triglyceride in the original oil (Figure 2a), shifted to about 1744 cm^{-1} and became broader at the end of the incubation period (Figure 2b). This change has been associated with the appearance of secondary autoxidation products, namely, saturated aldehydes and ketones[46] causing new carbonyl absorptions that overlaps with the band of the ester functional group. A wide range of volatile and nonvolatile secondary products are formed during advanced lipid oxidation.[41] Hexanal, methyl octanoate, 2,4-decadienal, methyl 9-oxononanoate, 3-nonenal, and pentane have been reported in the oxidation of glyceryl trilinoleate.[30,41] These oil oxidation products are produced mainly from the β-scission of the conjugated 9- and 13-hydroperoxides (9-hydroperoxioctadeca-10, 12-dienoic acid, and 13-hydroperoxioctadeca-9, 11-dienoic acid) formed at the initial stages of the oxidation process.[30,41] In aqueous systems such as in this study, hexanal, a major product in the volatile fraction, is preferentially formed by heterolytic cleavage of hydroperoxides, which involves the simultaneous formation of oxo-acids. Volatile products from the oxidation of glyceryl trilinoleate were most probably responsible for the strong oxidized odors detected in some microcosms during the biodegradation experiments and at the end of the incubation period, namely, in the blanks with no BHT.

Other observed spectral changes in the original oil, including changes in the fingerprint region, attested for the advanced stages of the oil autoxidation. Although the assignment of the bands in the fingerprint region is difficult because of the occurrence of more than one single functional group,[47] some observed changes in this region might be due to the stretching vibrations from ether C—O—C intermolecular linkages in the solid polymers and are further discussed in the SI.

3.4.2. Polymers Formed in the Biotic Microcosms. Elastic and solid polymers were formed in all biotic microcosms (0–800 mg kg^{-1}). Elastic polymers were prevailing in the ones with no BHT. Solid polymers were associated with the degrading biomass, which was retained on the filter paper at the moment of the separation of the solid and liquid phases of the microcosms. Hence, the spectra of the solid polymers included characteristic bacterial spectral features and were identical independently of the initial BHT concentration in the oil (0–800 mg kg^{-1}). However, the elastic polymers were free of biomass, and all presented identical spectra to those collected for the solid polymers from the blanks with no BHT. Figure 2 shows the infrared spectra of the two types of polymers (Figure 2c, d), and the spectrum of the degrading biomass collected from the biotic blanks (Figure 2e).

Compared to the original oil (Figure 2a), major spectral modifications were observed in the case of the elastic deposits (Figure 2c) and were identical to the changes discussed in the case of the solid deposits from the abiotic blanks with no BHT (Figure 2b). This suggests similar chemical composition of the two types of deposits and indicates comparable oxidation mechanisms in both the biotic and abiotic microcosms.

Similar infrared spectra were obtained in the case of the solid deposits collected from the biotic microcosms with different initial BHT concentrations (0–800 mg kg^{-1}) (Figure 2d). These spectra were practically identical to those previously discussed (spectra b and c) but were altered by the interference from the absorption bands associated with some bacterial structural features. The assignments of these bands are presented on the biomass spectrum in Figure 2e and in SI Table S3 and were adapted from Garip et al.[48] Infrared spectra of the solid deposits from the biotic microcosms are further discussed in the SI.

4. RESEARCH SIGNIFICANCE

Antioxidants present in vegetable oils play a major role in defining the oil fate and behavior in impacted aquatic environments. The effectiveness of BHT in retarding the oxidative polymerization of triglycerides in aquatic media, thus substantially improving the extent of the oil mineralization, was demonstrated in this study for glyceryl trilinoleate at BHT concentrations as low as 50 mg kg^{-1}. However, in another laboratory study investigating the effect of BHT on the biodegradation and toxicity of purified canola oil at oil loading of 333 gal $acre^{-1}$ (0.31 L m^{-2}), the authors reported no

dx.doi.org/10.1021/es2046712 | Environ. Sci. Technol. 2012, 46, 6798–6805

significant difference ($p > 0.05$) in the oil mineralization between the microcosms with no BHT and those with an initial BHT concentration in the oil of 200 mg kg^{-1}. A substantial oil mineralization (>77%) was measured after 16 weeks of incubation in both BHT treatments. These studies suggest that the potency of an antioxidant in retarding lipids oxidation might be highly dependent on the fatty acid composition of the oil, which seems to have a major influence in determining the oil fate and behavior in aquatic media. This has significant implications in understanding the persistence of vegetable oils in contaminated aquatic environments and in defining suitable remediation strategies for vegetable oil spills according to the oil individual characteristics and antioxidant content. Antioxidant free vegetable oils and unsaturated triglycerides highly susceptible to autoxidation are expected to undergo polymerization once spilled in aquatic media forming solid lumps unavailable for bacterial degradation. In such case, the most appropriate response technique is that of containment and recovery. Floating lumps should be removed from the water surface before they are able to reach the shoreline or bind sediment particles forming an impermeable cap of reduced permeability to oxygen and water.

■ ASSOCIATED CONTENT

Supporting Information

Details on the selection of oil loading, mineral medium composition in the respirometric microcosms, polymers formed by glyceryl trilinoleate autoxidation, BHT biodegradation experiments, FTIR band assignments, and infrared spectra of the polymers formed in the abiotic and biotic microcosms. 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.

■ ACKNOWLEDGMENTS

This research was funded by the National Risk Management Research Laboratory of the US Environmental Protection Agency.

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