Degradation of Poly(ε-caprolactone)-Based 'Green' Plasticizers for Poly(vinyl chloride)

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Abstract

Poly(vinyl chloride) (PVC) requires the addition of large quantities of plasticizers in order to improve the flexibility and processability of the polymer. Since the plasticizer is not bound to the PVC polymer matrix, there is a tendency of the plasticizer to migrate into the surroundings. The most commonly used PVC plasticizer, di-2-ethylhexyl phthalate (DEHP), has become a ubiquitous environmental contaminant. As with other plasticizers, it may either accumulate or lead to the formation of metabolites, which resist further degradation and can themselves be toxic. Accordingly, there is incentive for the development of a 'green' plasticizer as a replacement to DEHP in order to minimize the environmental impact of PVC production and use.

In this study, a method was developed to quantify the degradation of plasticizers used in PVC formulation or proposed as alternatives. The method is based on derivatization in order to lower the boiling points of the compounds and allow for analysis by gas chromatography (GC). Using this developed method, the biodegradation of two different families of potential 'green' plasticizers was considered. The biodegradation was assessed using *Rhodococcus rhodochrous* and evaluated based on the rate and completeness as well as the toxicity and stability of any observed metabolites. It was found that the poly([ε]-caprolactone)-based plasticizers containing octanoate-terminal groups degraded much more rapidly and completely than the benzoate-terminated ones. Furthermore, no metabolites were observed during the degradation of octanoate-terminated plasticizers while the benzoate-terminated plasticizers gave rise to metabolites

which contributed substantial toxicity to the culture media. Under ideal conditions, these metabolites were shown to be biodegraded themselves. The methodology and data presented in this thesis can be used as a tool in selecting a 'green' plasticizer based on the degradation criteria, and aid in the selection of alternative plasticizers to DEHP.

Sommaire

Le polychlorure de vinyle (PVC) requiert l'ajout important de plastifiant afin d'obtenir un polymère flexible et plus facile à mettre en forme. Comme ces plastifiants ne sont pas chimiquement liés à la matrice, ils ont toutefois tendance à migrer dans le milieu environnant. Le phtalate de diéthylhéxyle (DEHP) est le plastifiant le plus couramment utilisé et est maintenant un contaminant omniprésent. Tout comme d'autres plastifiants, il peut s'accumuler dans l'environnement ou se biodégrader et former des métabolites qui peuvent résister à la biodégradation et être potentiellement toxiques. Cette situation inquiétante a susciter l'intérêt envers le développement de plastifiants verts offrant une alternative au DEHP et permettant de minimiser l'impact environnemental de la production et de l'utilisation du PVC.

Dans le cadre de l'étude présentée dans cette thèse, une méthodologie a été développée afin de permettre la quantification et le suivi de la biodégradation de plastifiants utilisé pour le PVC ou proposés comme alternatives. Cette méthode basée sur le dérivatisation permet la diminution du point d'ébullition des composés et l'analyse par chromatographie en phase gazeuse (GC). Cette méthode a été utilisée ici afin d'étudier la biodégradation de deux familles de composés proposés comme plastifiants verts. La biodégradation a été étudiée à l'aide de la bactérie *Rhodococcus rhodochrous* et évaluée en fonction de la cinétique et l'étendue de la dégradation ainsi que selon la formation de métabolites et la toxicité résiduelle. Les résultats ont démontré que les plastifiants

à base de poly([ε]-caprolactone ayant des groupes terminaux octanoïque se biodégradent plus rapidement et que ceux ayant des groupes terminaux benzoïque. De plus, aucun métabolite n'a été détecté lors de la dégradation des plastifiants octanoïque alors que les plastifiants benzoïques ont formés des métabolites et engendré une toxicité résiduelle du milieu de culture. Dans des conditions idéales de biodégradation, les métabolites se sont dégradés. La méthodologie développée et les résultats présentés dans cette thèse seront utiles à la sélection de plastifiants verts offrant une alternative au DEHP.

Acknowledgements

I would like to thank my supervisors Professor David Cooper (retired) and Professor Viviane Yargeau for not only providing me with the opportunity to experience research in the field of chemical engineering at McGill University first-hand, but also for contributing substantial amounts of their time, energy, and expertise in order to make this a reality for me. They continually made working under their supervision a genuine pleasure through their positive encouragement and willingness to share with me the world of academia. I would also like to acknowledge Professor Leask who was the external examiner for this thesis for his valuable comments and suggestions as well as his time.

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Preface

The following thesis complies with McGill University's thesis preparation and submission guidelines. The co-authors Professor Cooper and Professor Yargeau were responsible for supervision of the research project. The data in Figure 25 was acquired and interpreted with the help of technical staff member, Ranjan Roy.

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1. Introduction

1.1. Overview

Poly(vinyl chloride) was discovered in the 19th century when scientists observed the polymer forming as a white solid inside flasks of gaseous vinyl chloride which had been exposed to sunlight. Due to its brittle nature and difficulty processing, PVC had limited commercial use following its discovery. In the 1920's, a more flexible material with better processability was developed by blending PVC with various plasticizing additives (Rahman and Brazel 2004). The plasticized PVC soon achieved widespread industrial and commercial use . However, without the use of plasticizers there would be few uses for PVC plastic in its original form.

Plasticizers are polymer additives which are used to lower the glass transition temperature of the polymer to which they are added. The result is improved flexibility and processability of the polymer to which they are added. Since its introduction in the 1930's, di(2-ethylhexyl) phthalate (DEHP) has been the most widely used plasticizer. Today, it accounts for roughly 47% of the plasticizers produced worldwide (Rahman and Brazel 2004). PVC plasticized with DEHP has uses in the medical industry (bags and tubing), construction industry (flooring, wall coverings, wires and cables), and in countless consumer goods (Schettler 2006).

The main drawback of using plasticizers is their tendency to migrate out of the PVC matrix since they are not chemically bound (Latini 2000). In the environment, these potentially toxic phthalate plasticizers may either accumulate

or lead to the formation of metabolites, which resist further degradation and can themselves be toxic (Nalli, Cooper et al. 2002; Horn, Nalli et al. 2004). Thus, there is an incentive to find a 'green' plasticizer; one which would exhibit minimal leaching, would not be toxic nor have stable and toxic metabolites of degradation, and which would have plasticizing efficiencies comparable to that of DEHP.

1.2. Objectives

The research presented in this paper aims to quantify the degradation of potential 'green' alternative plasticizers by *Rhodococcus rhodochrous*. Benzoate- and octanoate-terminated oligomers of ε-caprolactone of varying chain length were compared. The main objective of the research was to characterize any metabolites of breakdown in terms of their own degradation and toxicity in order to identify potential 'green' plasticizers based on these criteria. Conclusions regarding the effect of their molecular structure on metabolite formation were formulated to suggest favourable structural characteristics of the plasticizer that minimize the build-up of toxic metabolites and/or maximize extent and rapidity of degradation.

As a secondary research objective, it was crucial to first devise a reliable test method to quantify the degradation of plasticizers and their metabolites. To achieve this, a variety of lipid derivatization techniques found in the literature (Stoffel, Chu et al. 1959; Bannon, Breen et al. 1982; Bannon, Craske et al. 1982; Liu 1994) may be used. The selected method, modified from a procedure described by Comeau et al. (Comeau, Hall et al. 1988), used lipid derivatization to

lower the boiling points of the plasticizers to allow for analysis by gas chromatography (GC). The GC analysis method was then used to quantify the degradation of the two different families of potential 'green' plasticizers so that the main research objective could be met.

1.3. Rationale

The ideal 'green' plasticizer for PVC should display the following properties: (1) ready biodegradation; (2) no toxicity of either the plasticizer or the possible metabolites; (3) compatibility with PVC; (4) plasticization efficiencies comparable to DEHP; (5) good processing characteristics and performance in PVC products; (6) high resistance to migration from PVC and (7) relatively low cost.

The incentive of this research, along with the plasticizer leaching, mechanical property testing, and toxicity research done by my colleagues, is to identify a 'green' plasticizer which can be used as an alternative to the environmentally harmful commercial plasticizer, DEHP. With continued increasing use of plasticizers worldwide, the development of a 'green' plasticizer is crucial to the fields of chemical engineering and polymer processing.

2. Background

Poly(vinyl chloride) (PVC) is one of the most common polymers used in consumer products due to its relatively low cost and high versatility. This material is employed in a wide range of applications including construction, biomedical devices, food packaging, cleaning materials, insecticides, and baby-care products (Schettler 2006; Marcilla, Garcia et al. 2008). Typically, large amounts of liquid plasticizers are incorporated into the PVC matrix in order to increase the flexibility and workability of the material (Kraukopf 1988). The most widely used plasticizers in PVC have been the phthalates, with nearly 5 million metric tonnes being used globally in 2006, representing roughly 75% of the plasticizer market (Rahman and Brazel 2004; Markarian 2007). However, since the phthalates are not chemically bound to the polymer they have been shown to migrate from flexible PVC products into the environment (Latini 2000; Lindström and Hakkarainen 2006). In recent years, phthalate plasticizers such as di(ethylhexyl) phthalate (DEHP) have been classified as suspected endocrine disruptors and carcinogenic agents (Swan 2008; Kamrin 2009). Interaction of microbes with phthalate plasticizers leads to the formation of metabolites, which resist further degradation and can themselves be toxic (Nalli, Cooper et al. 2002; Horn, Nalli et al. 2004). These phthalates and their metabolites persist in water, soil, and food, and have been detected in animals and humans (Hakkarainen 2008). This demonstration that human exposure is ubiquitous and the evidence for significant impacts on human health have raised public concerns. As a result, more and more

countries have introduced regulations on the use of these phthalates in toys that are likely to be intended for oral use by children (Fontelles and Clarke 2005).

Due to the wide range of applications for DEHP-plasticized PVC, it is difficult to find an alternative plasticizer capable of completely replacing DEHP. Researchers have been able to test several alternative approaches with some success. However, no material has yet been found that can satisfactorily substitute to create soft PVC, and no low molar mass plasticizers which are not susceptible to migration have been capable of replacing phthalate esters so far. Surface modifications such as radiation cross-linking (Jayakrishnan and Sunny 1996) or poly(ethylene oxide)-based coatings (Messori, Toselli et al. 2004) have been shown to reduce migration but result in deterioration of the physicochemical properties of the resulting PVC products (Jayakrishnan and Lakshmi 1998; Babukutty, Prat et al. 1999). Ionic liquids are one particular alternative whose leaching and migration characteristics are demonstrated to be far better than the plasticizers currently used in medical and commodity plastics (Rahman and Brazel 2006). Though they may provide superior performance in offering flexibility to PVC, while lengthening material lifetime and reducing plasticizer loss by diffusion, these compounds become unstable at elevated temperatures (Rahman and Brazel 2006) and are expensive. Other low molecular weight plasticizers include citrates such as Citroflex® B-6 (Morflex Inc.) and benzoates such as Benzoflex® 2888 (Velsicol Chemical Corporation) have been developed, but the possible toxicity is still largely unknown ((HSDB) ; Gartshore, Cooper et al. 2003).

To achieve complete inhibition of plasticizer migration and minimize the deterioration of physicochemical properties, blending PVC with suitable polymeric plasticizers seems to be one of the better approaches (Choi and Kwak 2007). However, of all the various polymeric plasticizers studied, including polyesters and copolymers, none are capable of giving sufficient flexibility to PVC (Kraukopf 1988; Peña, Hidalgo et al. 2000). In contrast to conventional linear polymers, hyper-branched polymers (HBPs) are known to possess unique physical and chemical properties imparted by both their three-dimensional structure as well as the many possible end group functionalities. In particular, they are known to aid polymer processing when used as an additive; just one example of the potential of HBP application in the conventional polymer industry (Gopala, Wu et al. 1999; Kwak and Ahn 2000). Recently, Lindström and Hakkarainen have been able to advance this idea, showing that branching increases the plasticizing efficiency of a particular hyper-branched polyester plasticizer in the context of a PVC/polyester blend (Lindström and Hakkarainen 2006). At the same time, literature currently exists concerning the employment of hyper-branched poly(ε-caprolactone)s (HPCLs) as plasticizers for flexible PVC (Gopala, Wu et al. 1999; Kwak and Ahn 2000; Choi and Kwak 2004; Choi and Kwak 2007). Choi and Kwak demonstrated that HPCLs with shorter linear segments and larger number of branches impart as high flexibility as di-(ethylhexyl) phthalate (DEHP) and much higher flexibility than their linear analogue, linear poly(ε-caprolactone), while maintaining no plasticizer migration even at very harsh conditions (Choi and Kwak 2007).

Biodegradable poly(lactones), such as US Food and Drug Administration (FDA)-approved poly(glycolide) (PGA), poly(lactide) (PLA), and poly(ε-caprolactone) (PCL), have been widely studied in biomedical applications such as absorbable sutures, artificial organs, controlled drug delivery and tissue regeneration and they have been shown to be biocompatible and easily biodegraded (Domb, Kost et al. ; Nair and Laurencin ; Middleton and Tipton 2000). Accordingly, PCL has been considered an attractive 'green' plasticizer for PVC due to the following: (1) the well-known biodegradation behaviour and non-toxicity of PCL; (2) its low glass transition temperature (T_g) (around -60 °C) which provides flexibility down to low temperatures, and can be further enhanced by using oligo(caprolactones) (Perrin and English 1998; Middleton and Tipton 2000; Nair and Laurencin 2007); (3) the miscibility of PCL with PVC, which facilitates processing and mechanical performance (Hubbell and Cooper 1977; Chiu and Min 2000); (4) the relatively simple synthesis and low cost of PCL make it possible for large-scale production (Shi, Cooper et al. 2011). Moreover, PCL is often used to lower the cost and improve the performance of other polymers by copolymerization (Middleton and Tipton 2000) or by blending (Averous, Moro et al. 2000). Finally, longer chain plasticizers have exhibited greatly reduced migration from PVC (Ha, Kim et al. 1998; Audic, Reyx et al. 2003).

In a previous study by Shi et. al. (Shi, Cooper et al. 2011), preliminary biodegradation studies were done with some of the various octanoate PCLs as well as a few of the benzoate PCLs. The rate of disappearance of these compounds was investigated indirectly using two different methods of

measurement: gel permeation chromatography (GPC, Waters Breeze HPLC) and gravimetric estimates as an alternative. In either case, the concentration of the plasticizers was not monitored. Unfortunately, this methodology provided no way of testing for potential metabolites of biodegradation, or monitoring their rate of disappearance.

3. Materials and methods

3.1. Plasticizers

Six poly([ε]-caprolactone)-based potential plasticizers were considered for degradation in this study. The molecular structures of these are outlined in **Figure 1** to **Figure** 3 and the details are summarized in **Table 1**. These compounds were already available, having been previously synthesized in-house for use in another study which has been recently published (Shi, Cooper et al. 2011). In order for their synthesis, the poly([ε-caprolactone) diols and triols were purchased from Scientific Polymer Products Inc. (Ontario, NY, USA), octanoyl chloride (99%) and benzoyl chloride (99%) were obtained from Sigma-Aldrich, and the ε-caprolactone monomer (99%) was acquired from Acros.

There were two classes of compounds being studied: one having benzoate-terminal groups and the other octanoate-terminal groups. Within both of these classes, there were compounds of varying molecular weights depending on the average number of repeating ε-caprolactone units present on each chain. By comparing the benzoate- and octanoate-terminated plasticizers, the effect of the terminal functional group on degradation rate can be studied in addition to the effect of chain length of the central portion of these plasticizers. While most of these compounds were linear (two-armed), one octanoate-terminated compound, had three arms.

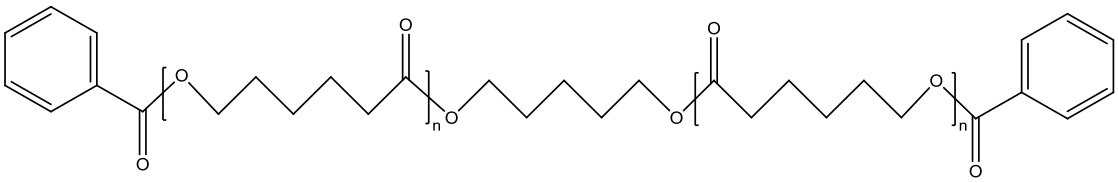


Figure 1. General molecular structure of the two-armed benzoate-terminated poly([ε]-caprolactone)-based plasticizers. Three particular compounds with this structure were considered in this study; these were synthesized with an average number of repeating ε-caprolactone monomer units on each arm of n=1, n=2 and n=3.

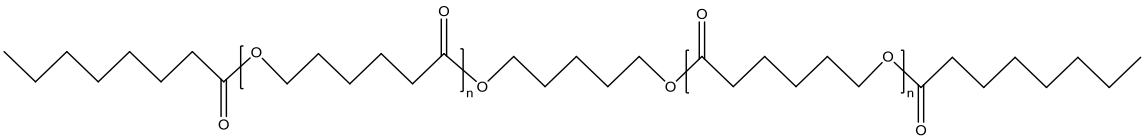


Figure 2. General molecular structure of the two-armed octanoate-terminated poly([ε]-caprolactone)-based plasticizers. Two particular compounds with this structure were considered in this study; these were synthesized with an average number of repeating ε-caprolactone monomer units on each arm of n=1 and n=2.

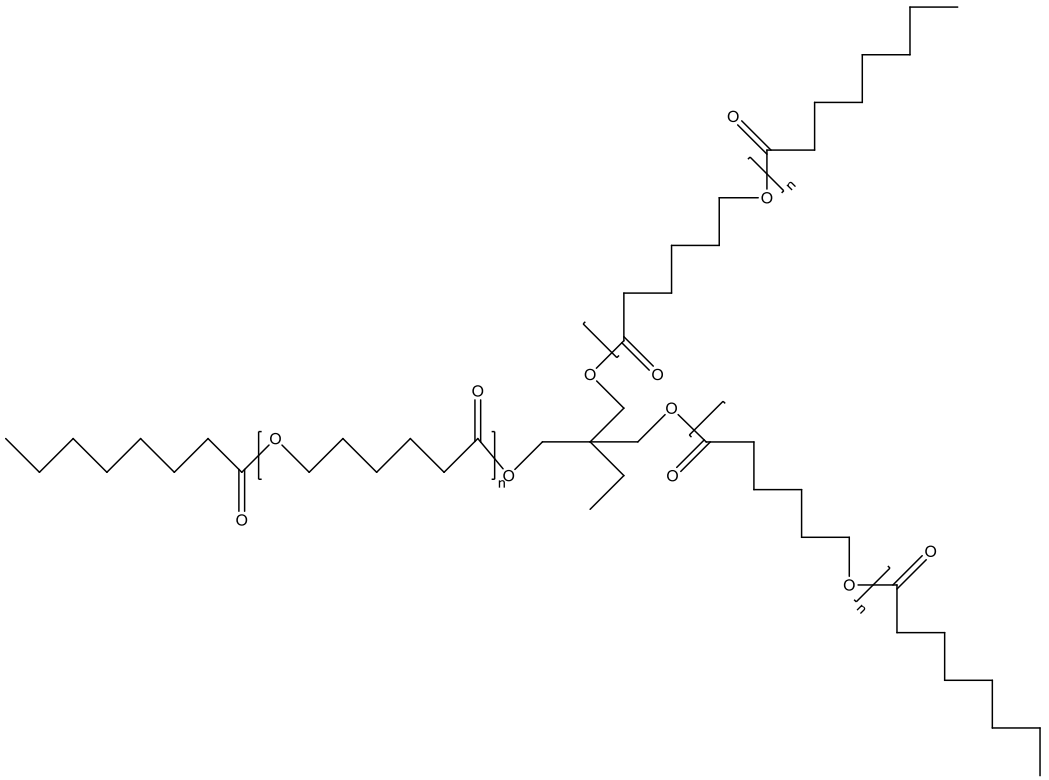
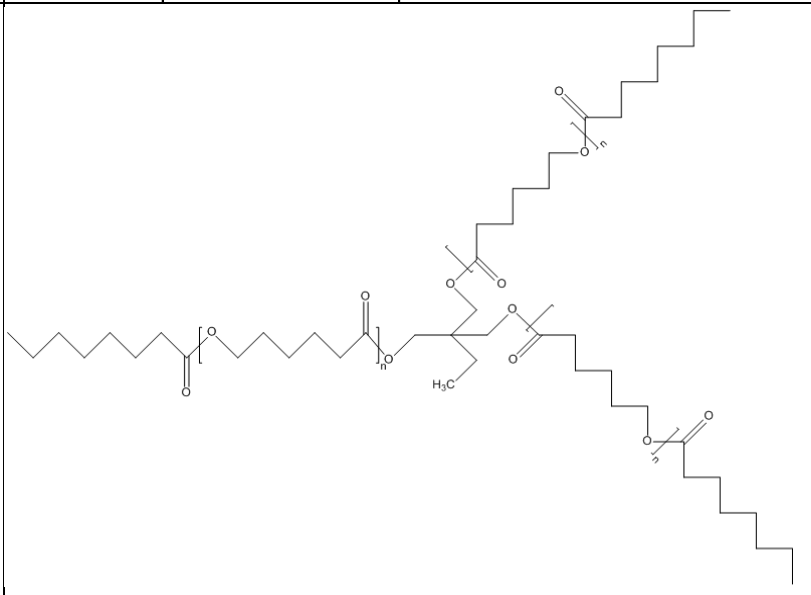


Figure 3. Molecular structure of the three-armed octanoate-terminated poly([ε]-caprolactone)-based plasticizer. Only one compound with this structure was considered in this study; this was synthesized with an average number of repeating ε-caprolactone monomer units on each arm of n=1 (MW=897 g mol^{-1}).

Table 1. Nomenclature and parameters of PCL-based plasticizers

Dibenzoate-terminated poly([ε]-caprolactone)s			
Plasticizer Code†	**No. of arms**	**MW**	**n (# of repeating units per arm)**
B_2-540	2	540	1
B_2-769	2	769	2
B_2-997	2	997	3
Dioctanoate-terminated poly([ε]-caprolactone)s			
Plasticizer Code	**No. of arms**	**MW**	**n (# of repeating units per arm)**
O_2-613	2	613	1
O_2-841	2	841	2
Trioctanoate-terminated poly([ε]-caprolactone)			
Plasticizer Code	**No. of arms**	**MW**	**n (# of repeating units per arm)**
O_3-897	3	897	1

† The first letter in the plasticizer code ('B' or 'O') indicates whether the molecule has benzoate or octanoate terminals, the small subscript that follows gives indication of the number of arms (two or three), and the last number designates the molecular weight (from which the number of repeating ε-caprolactone monomers on each arm, 'n', can be inferred).

3.2. Culture of bacterium

The bacterium used in this study was *Rhodococcus rhodochrous* American Type Culture Collection 13808. This common soil bacterium is known to grow on hydrophobic substrates and was previously shown to be degrade the various PCL-

based plasticizers (Shi, Cooper et al. 2011). To preserve the sample, a microcentrifuge tube containing biomass in the exponential growth phase was spun at 14000 x g for a period of 5 min. The supernatant was decanted and, after the addition of 0.5 mL of a sterile solution of 37-g/L Bacto Brain/Heart infusion and 0.5 mL 40% glycerol (Fisher Scientific), the tube was placed in the freezer (-80 °C) until further use. The initial inoculum was prepared by thawing the contents of one tube and transferring this to a 500-mL shaker flask containing sterile growth medium composed of Brain/Heart infusion (37 g/L in 100 mL of reverse osmosis water) and then incubated on a rotary shaker (Infors HT) set at 110 rpm and 30 °C. Every two weeks, a new shaker flask containing 100 mL of 37 g/L Bacto Brain/Heart infusion in distilled water was inoculated with 1 mL of the previous inoculum in order to keep a culture fresh or of constant population age.

3.3. Degradation Experiments

The degradation experiments were carried out in minimum mineral salt medium (MMSM) which consisted of 0.014 g/L Na_2EDTA, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2 \cdot 2H_2O$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 4 g/L NH_4NO_3, 4 g/L KH_2PO_4, and 6 g/L Na_2HPO_4 as well as 0.1 g/L yeast extract (Fisher Scientific) and 0.4 mM plasticizer. Initial studies also included 2 g/L hexadecane (Fisher Scientific) as an additional carbon source. The flasks were autoclaved at 121 °C and 100 kPa for 30 min (Steris Amsco Lab 250), allowed to cool and then inoculated with 1 mL of the aforementioned cell culture in a laminar flow cabinet

(Baker Company, Model VM600). The flasks were then transferred to the incubator-shaker and set to 110 rpm and 30 °C. Eight flasks were prepared for each compound and one flask was extracted using a whole-flask extraction on days 0, 1, 3, 5, 7, 10, 14, and 21. Three additional flasks were prepared without being inoculated in order to serve as abiotic controls for the first, last and an intermediate day.

For the majority of the degradation experiments, two different extractions were carried out. The first extraction was performed at pH 8 using 1 M $NaHCO_3$ (Fisher Scientific), and the second extraction was performed at pH 3-4 using HCl (Fisher Scientific). For each flask, and for both types of extraction, the entire contents were extracted using 25 mL of chloroform (Fisher Scientific) containing 2 g/L of n-pentadecane (A & C American Chemicals; Montréal, QC), which acted as an internal standard for quantification by GC analysis. The organic phase from each extraction was filtered in a separatory funnel using medium porosity filter paper, and then recovered in a glass vial and stored at 4 °C until the sample preparation prior to injection.

Degradation experiments were carried out with each of the different potential plasticizers as well as with ε-caprolactone and 1,6-hexanediol dibenzoate.

3.4. Derivatization

The selected procedure was a modified version of the methyl transesterification technique of Comeau et. al (Comeau, Hall et al. 1988). Briefly, HPLC grade methanol (Fisher Scientific) which had been acidified to 2% sulfuric

acid (Fisher Scientific) by volume was mixed with the chloroform extract from the biodegradation experiment, and the reaction vessel was maintained at a temperature of 85 °C for a duration of 4 hours with constant stirring. The products were washed with sodium bicarbonate to neutralize the acid and remove impurities that may contribute noise to the chromatogram. The organic phase was extracted and mixed with a second rinse (performed using pure chloroform) prior to analysis by GC.

Alternatively, silylation was used with samples from the degradation of B_2-540 in order to study the nature of the metabolites. This was achieved by placing aliquots of the chloroform extracts in 2-mL Eppendorf vials and drying them in a Savant SpeedVac® Concentrator. These dehydrated samples were left to dry overnight in a desiccator. 40 μL of pyridine (Fisher Scientific) and 20 μL of the silylating reagent BSTFA ((N,O-bis(trimethylsilyl)trifluoroacetamide) (SUPELCO) were then added to the dehydrated sample vials. The mixture was left to react for 3 hours at 85 °C using a DigiPREP Jr. Following derivatization, all samples were placed in 1.5-mL GC vials from Fisher Scientific and stored at 4 °C until analysis. Prior to injection into the GC/MS, samples were diluted by a factor of 10 with chloroform.

3.5. Gas Chromatography

Samples were injected into a gas chromatograph (GC) to monitor the degradation of plasticizers or the gas chromatograph/mass spectrophotometer (GC/MS) for analysis of the metabolites formed. The gas chromatograph (Thermo

Scientific Trace GC Ultra) was equipped with a Rxi®-5ms fused silica column (Restek) with an internal diameter of 0.25 mm. The settings of the GC were: injector temperature of 250 °C, initial column temperature of 40 °C, temperature ramp rate of 20 °C/min, final column temperature of 300 °C, detector temperature of 300 °C, ramp hold time of 2.0 min, and final hold time of 5.0 min.

The gas chromatograph/mass spectrophotometer (Thermo Scientific Trace GC Ultra/ITQ 1100) had a Rxi®-5ms fused silica column (Restek) with an internal diameter of 0.25 mm. The settings of the GC/MS were: injector temperature of 250 °C and an initial column temperature of 75 °C. The first ramp had a temperature ramp rate of 3.5 °C/min, reaching a column temperature of 200 °C, with a holding time of 0.5 minutes. The second ramp had a temperature ramp rate of 40 °C/min, reaching a column temperature of 300 °C, with a final hold time of 5.0 minutes. The detector temperature was 300 °C. The mass spectrophotometer settings were 50-250 molecular weights mass spec range, the transfer line was maintained at 300°C and the ion source was maintained at 200 °C.

3.6. Toxicity assessment

Relative changes in toxicity of the degradation broth was evaluated using the Microtox® basic acute toxicity test. Acute Microtox® toxicity was determined using the 5-minute assay with the Model 500 Toxicity Analyzer according to the procedures for the Basic Test recommended by the instrument manufacturer (Azur Environmental, Carlsbad, CA). This involves exposing the bioluminescent

bacterium, *Vibrio fischeri*, to serial dilutions of a sample toxicant in order to measure the reduction in the amount of light emitted for a series of concentration, as normalized against a control. From this, a dose-response curve was generated and the effective concentration which corresponds to a 50% reduction in the amount of light emitted naturally (EC_{50}) is interpolated (or in some cases extrapolated).

For the toxicity studies, degradation experiments were carried out as previously described, but using a smaller media volume of only 40 mL and analyzing the toxicity of the media each day, rather than every few days. The samples consisted of the entire flask volume (40 mL) subjected to centrifugation at 10,000 rpm for 10 minutes (IEC B-22M Programmable Centrifuge) in order to collect the supernatant. The supernatant was then decanted into a 40-mL glass sample vial with a Teflon seal (Fisher Scientific) and stored at 4 °C until a batch of samples was ready for toxicity testing. Prior to toxicity testing, the samples were agitated vigorously to ensure homogeneity of the solution (emulsion).

For the toxicity assay on the Microtox® system, four serial 1:2.1 dilutions of the sample were prepared with diluent (2% NaCl in reverse osmosis water) and allowed to stabilize to 15±0.5 °C. At the same time, 10 µL of bacteria was suspended in 0.5 mL of the diluent and pre-incubated at 15±0.5 °C for 15 minutes. Then, 0.5 mL aliquots of the sample dilutions were added to each bacterial suspension, using duplicates for each sample concentration. The light output of the bacteria was recorded immediately before and 5 minutes after the addition of the sample toxicant to the bacterial suspension. Furthermore, duplicate controls

(without the addition of the degradation broth) were run with each test to account for the natural reduction in light output over the 5-minute timeframe. After normalizing the results to the control, the EC_{50} was reported as the volume fraction of a sample that caused a 50% decrease in light output. In this thesis, toxicity is expressed in toxicity units (TU_{50}) by taking the inverse of the obtained EC_{50} because this was a more intuitive way of evaluating relative toxicity differences (i.e. higher values correspond to a greater toxicity).

4. Results

4.1. Validation of lipid derivatization

One particular family of plasticizers considered in this study was the two-armed, benzoate-terminated PCL-based compounds. Three different compounds with this general structure were investigated, each synthesized by Shi et al. (Shi, Cooper et al. 2011) from commercially available PCL diols of differing molecular weights. **Figure 1** presents the general molecular structure of the two-armed benzoate-terminated poly([ε]-caprolactone)-based plasticizers. The PCL diols were reacted with benzoyl chloride to add the terminal benzoate group. The PCL diols themselves consisted of a central alcohol (1,5-pentanediol) which were connected via ester linkages to two arms of varying chain length, depending on the number of repeating ε-caprolactone monomers. The three resulting compounds had an average of n=1, n=2, and n=3 repeating ε-caprolactone monomer units per arm, resulting in compounds with respective average molecular weights of 540, 769, and 997 g mol^{-1}.

A similar family of two-armed octanoate-terminated PCL-based plasticizers were considered as well, synthesized once again by Shi et al. (Shi, Cooper et al. 2011) but this time using octanoyl chloride. These were also constructed from commercially available PCL diols of differing molecular weights, which had been reacted with octanoyl chloride to give terminal octanoates.

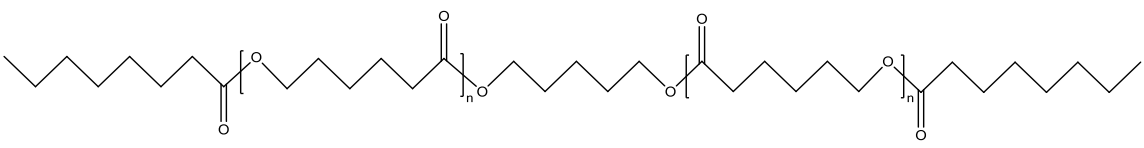


Figure 2 shows the general molecular structure of the two-armed octanoate-terminated poly([ε]-caprolactone)-based plasticizers. In this case, only two of the two-armed octanoates were considered, with an average of n=1 and n=2 repeating ε-caprolactone monomer units on each arm, resulting in compounds with average molecular weights of 613 and 841 g mol^{-1}, respectively.

Another way in which the geometry of the PCL-based plasticizers was altered was by using a PCL triol as the central portion of the molecule, rather than a PCL diol, resulting in a plasticizer with three arms rather than two. Only one such plasticizer was considered in this study, synthesized from a PCL triol with an average of n=1 repeating ε-caprolactone monomer units on each arm. This PCL triol was reacted with octanoyl chloride to yield a three-armed, octanoate-terminated plasticizer with an average molecular weight of 897 g mol^{-1}. **Figure 3** shows the molecular structure of this three-armed octanoate-terminated poly([ε]-caprolactone)-based plasticizer.

The plasticizer molecules had complex IUPAC nomenclature, so a simple coding scheme was devised for naming these oligomers of ε-caprolactone. **Table 1** presents a summary of the nomenclature and various structural similarities and differences for these PCL-based plasticizers. The first letter in the plasticizer code ('B' or 'O') indicates whether the molecule had benzoate or octanoate terminals, the number subscript that follows gives indication of the number of arms (two or three), and the last number designates the molecular weight (from which the number of repeating ε-caprolactone monomers on each arm, 'n', can be inferred).

The plasticizer molecules themselves were comprised of several ester bonds, and were thus readily transesterifiable. Transesterification was carried out for

various concentrations of the smallest benzoate-terminated plasticizer, B_2-540, in order to assess linearity of the method. **Figure 4** shows the amounts of both identified and unidentified fragments of B_2-540 post-transesterification plotted against various initial concentrations of the parent compound. The left axis corresponds to the concentrations of the identified methyl esters (methyl benzoate and methyl 6-hydroxyhexanoate), whereas the right axis reports the area ratios of the larger, unidentified fragments of the plasticizer, referred to by retention times. For each fragment, a linear trend line has been fit relating the detected amount of the identified and unidentified fragments to the initial concentration of the parent compound with the coefficient of determination displayed next to it.

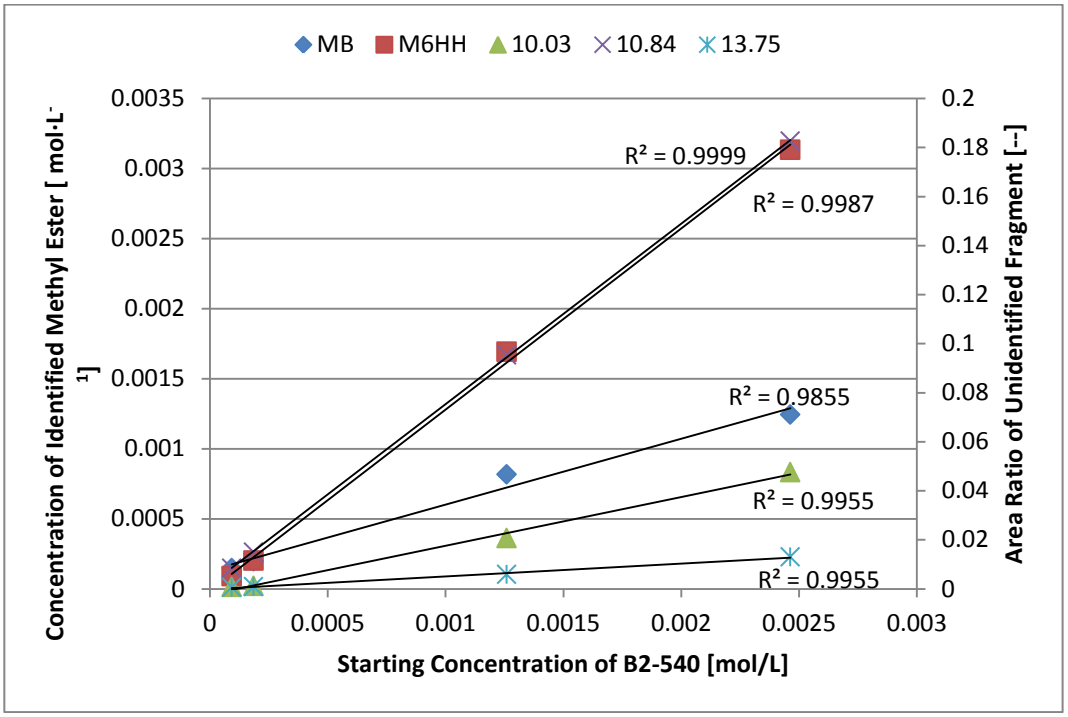


Figure 4. Linearity of methyl transesterification for B_2-540. Shown are the amounts of both identified and unidentified fragments of B_2-540 post-transesterification plotted against various initial concentrations of the parent compound. The left axis corresponds to the concentrations of the identified methyl esters [mol L^{-1}] , whereas the right axis reports the area ratios of the larger, unidentified fragments of the plasticizer [--], referred to by retention times.

4.2. Degradation

Since the plasticizers considered in this study were composed of repeating ε-caprolactone monomers, *R. rhodochrous* was grown in media containing ε-caprolactone either with or without n-hexadecane as an additional carbon source. The concentrations data for these degradation experiments are presented in **Figure 5 (a)** and **(b)**, respectively. New peaks were observed in the chromatograms for both experiments that were not present in any of the abiotic controls and that were not the same for the two experiments. The study with n-hexadecane gave a new peak with a retention time of 8.9 minutes and the study without the additional carbon source gave a new peak with a retention time of 3.5 minutes.

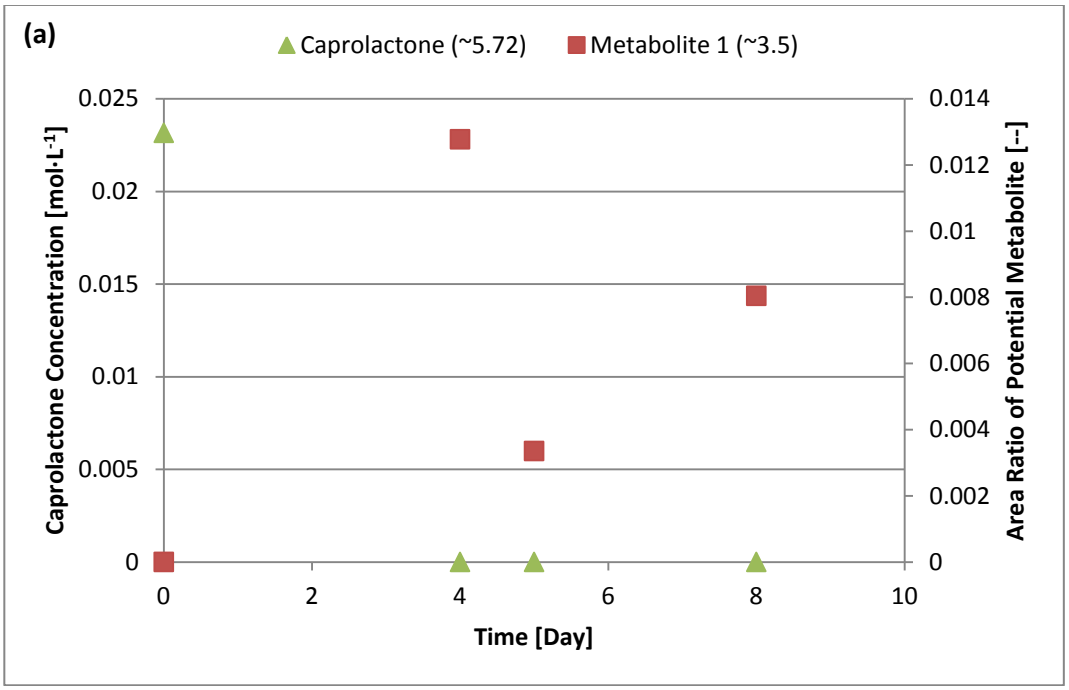


Figure 5. (a) Degradation of ε-caprolactone (retention time 5.72 minutes) in the absence of n-hexadecane. Shown on the left vertical axis is the concentration of ε-caprolactone [mol L^{-1}], and on the right vertical axis is the area ratio of the potential metabolite with a retention time of 3.5 minutes [--].

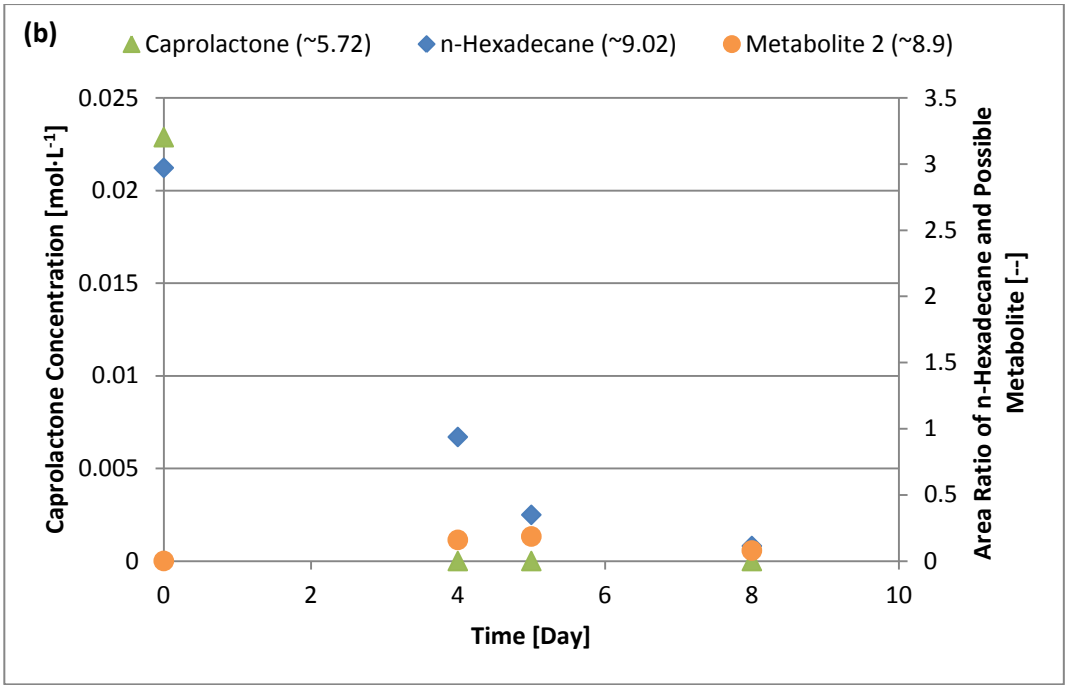


Figure 5. (b) Degradation of ε-caprolactone in the presence of n-hexadecane. Shown on the left vertical axis is the concentration of ε-caprolactone [mol L^{-1}], and on the right vertical axis are the area ratios of n-hexadecane and the potential metabolite with a retention time of 8.9 minutes [--].

The smallest benzoate-terminated poly([ε]-caprolactone)-based plasticizer considered in the degradation studies was the compound designated B_2-540 (see **Table 1** for structure). Each of the two arms on the molecule consisted of an average of n=1 repeating ε-caprolactone monomers. The concentration used was 0.36 mM. For each time point, two chloroform extractions were performed. The first was an alkaline extraction adjusted to pH 8 with 1.0 M sodium bicarbonate, and this was followed by an acidic extraction adjusted to pH 3-4 with hydrochloric acid. Methyl transesterification was carried out for each of these extracts. Typical results for the degradation of B_2-540 are shown in **Figure 6 (a)** (alkaline extracts) and **(b)** (acidic extracts). The concentrations of two of the transmethylation products, methyl benzoate and methyl 6-hydroxyhexanoate,

were plotted over the course of the degradation experiment as well as for abiotic controls.

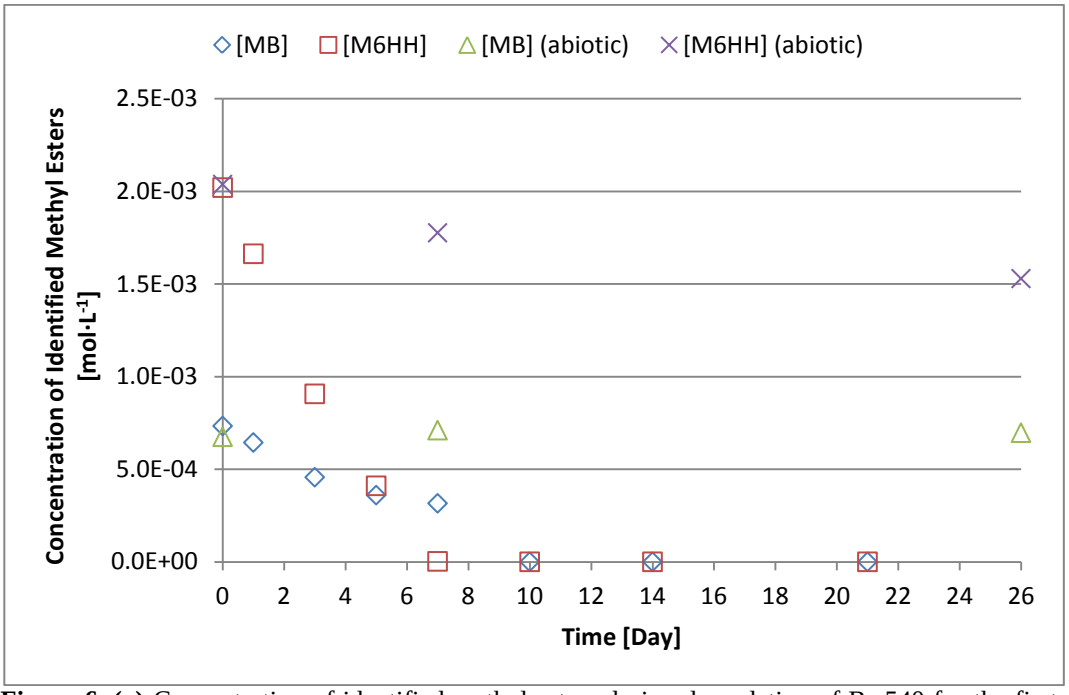


Figure 6. (a) Concentration of identified methyl esters during degradation of B_2-540 for the first (alkaline) extract. Plotted are the concentrations of two of the transmethylation products, methyl benzoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls.

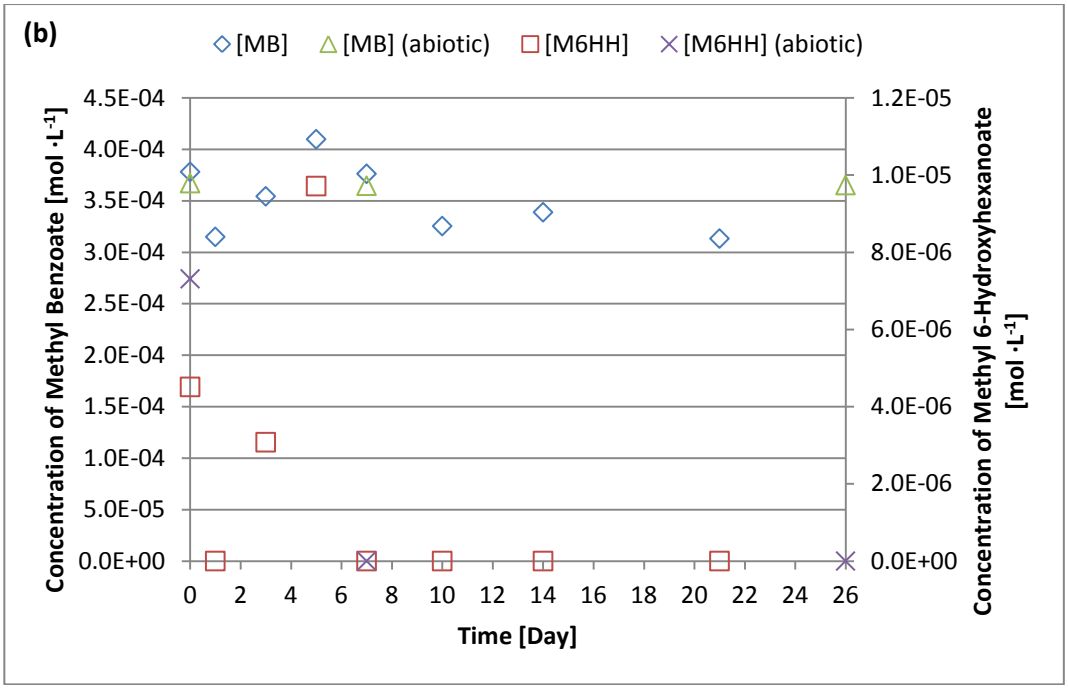


Figure 6. (b) Concentration of identified methyl esters during degradation of B_2-540 for the second (acidic) extract. Plotted are the concentrations of two of the transmethylation products, methyl benzoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls. The concentration of methyl benzoate is represented on the left vertical axis, and the concentration of methyl 6-hydroxyhexanoate is represented on the right vertical axis.

From the same experiment, **Figure 7** and **Figure 7** show the post-transmethylation evolution of the larger, unidentified fragments of B_2-540 over the course of a typical degradation study for this compound. These unidentified compounds represented larger fragments of the parent plasticizer that resulted from incomplete conversion of the molecule to methyl benzoate and methyl 6-hydroxyhexanoate during the transesterification procedure. The amounts of these larger, unidentified fragments were presented as area ratios (relative to the internal standard) and were referred to by their retention times (10.03,10.83, and 13.75 minutes). Note that in **Figure 7 (a)**, the relative amount of compound 10.83 is shown on the left vertical axis, whereas the relative amounts of compound 10.03 and compound 13.75 are shown on the right vertical axis.

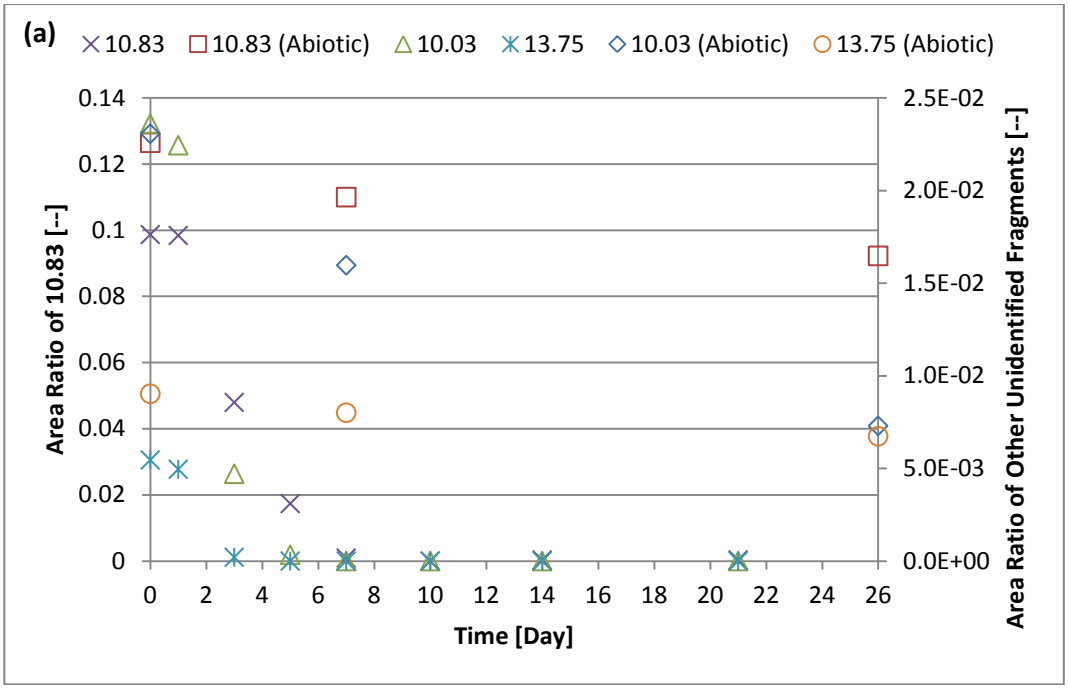


Figure 7. (a) Area ratios of the larger, unidentified fragments of the parent compound B_2-540 for the first (alkaline) extract. Shown here are both data for the degradation experiment as well as for the abiotic controls. These larger, unidentified fragments are referred to by their retention times (10.03,10.83, and 13.75 minutes). The area ratio of compound 10.83 is shown on the left vertical axis, whereas the area ratios of compound 10.03 and compound 13.75 are shown on the right vertical axis.

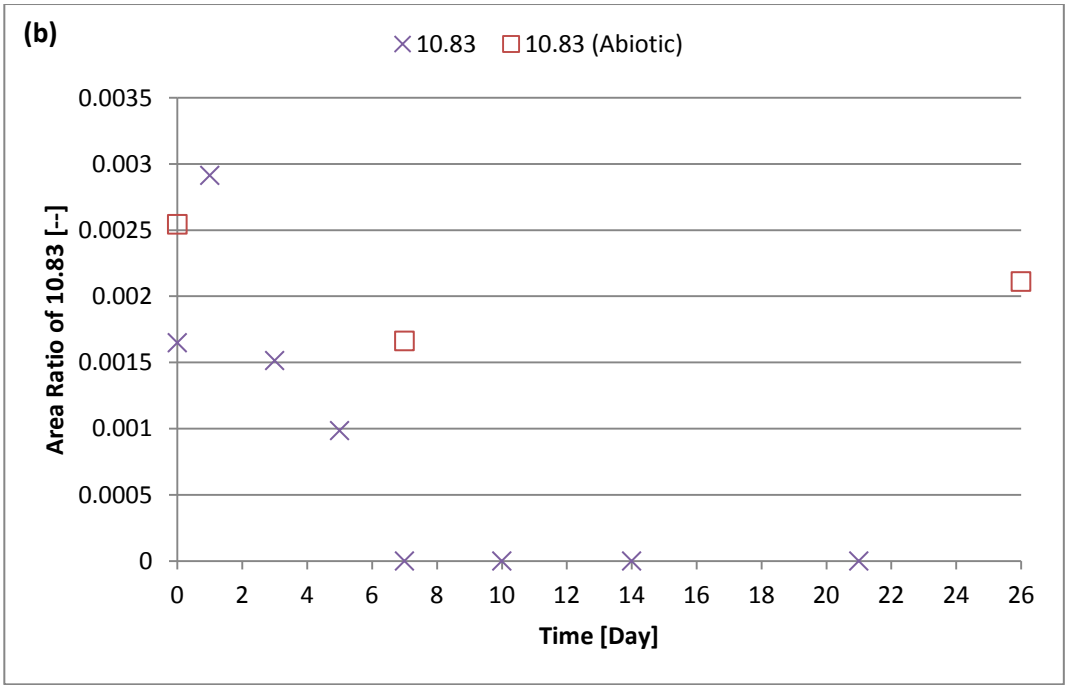


Figure 7. (b) Area ratio of the larger, unidentified fragment with retention time of 10.83 minutes during degradation of B_2-540 for the second (acidic) extract. Data is presented for the degradation experiment as well as for the abiotic controls.

The molecular structure of B_2-769 was nearly identical to that of B_2-540. The only difference was that there were an average of n=2 repeat ε-caprolactone monomers per arm instead of n=1. Results for the degradation of B_2-769 were obtained by subjecting the alkaline and acidic extracts to transmethylation, and are shown in **Figure 8 (a)** and **(b)**. In these figures, the concentrations of methyl benzoate and methyl 6-hydroxyhexanote are plotted against degradation time. The area ratios of the larger, unidentified fragments of B_2-769 that were observed in the post-transmethylation chromatograms are plotted in **Figure 9 (a)** and **(b)**. Once again, these were referred to by their retention times (10.03,10.83, and 13.75 minutes). Data for the abiotic controls have been included alongside the degradation data.

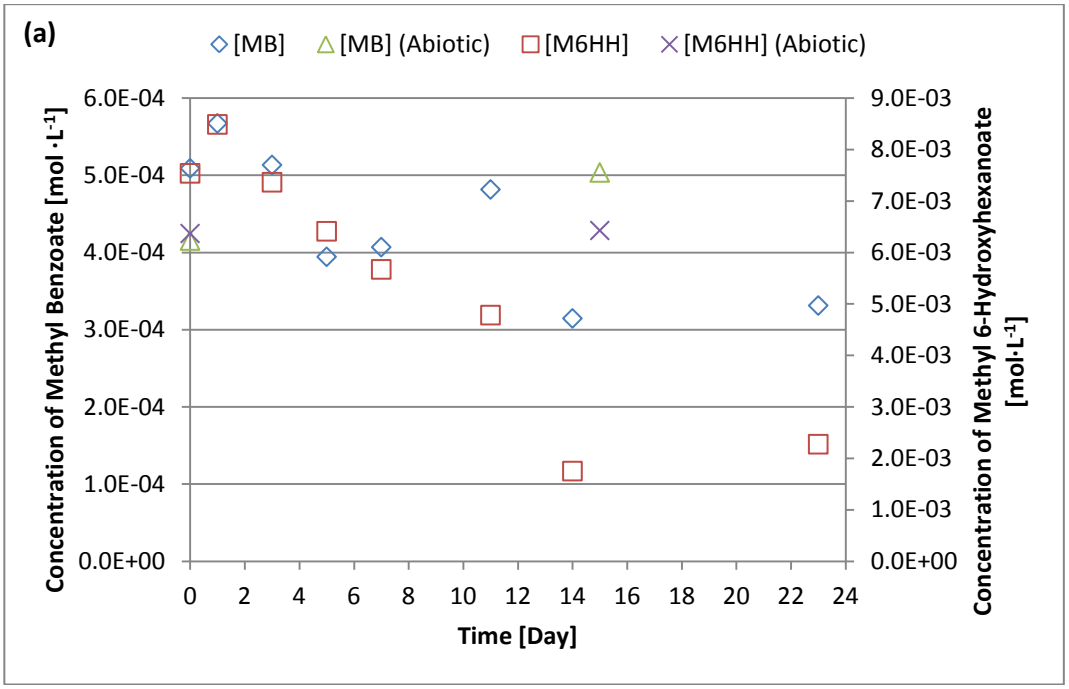


Figure 8. (a) Concentration of identified methyl esters during degradation of B_2-769 for the first (alkaline) extract. Plotted are the concentrations of two of the transmethylation products, methyl benzoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls. The concentration of methyl benzoate is represented on the left vertical axis, and the concentration of methyl 6-hydroxyhexanoate is represented on the right vertical axis.

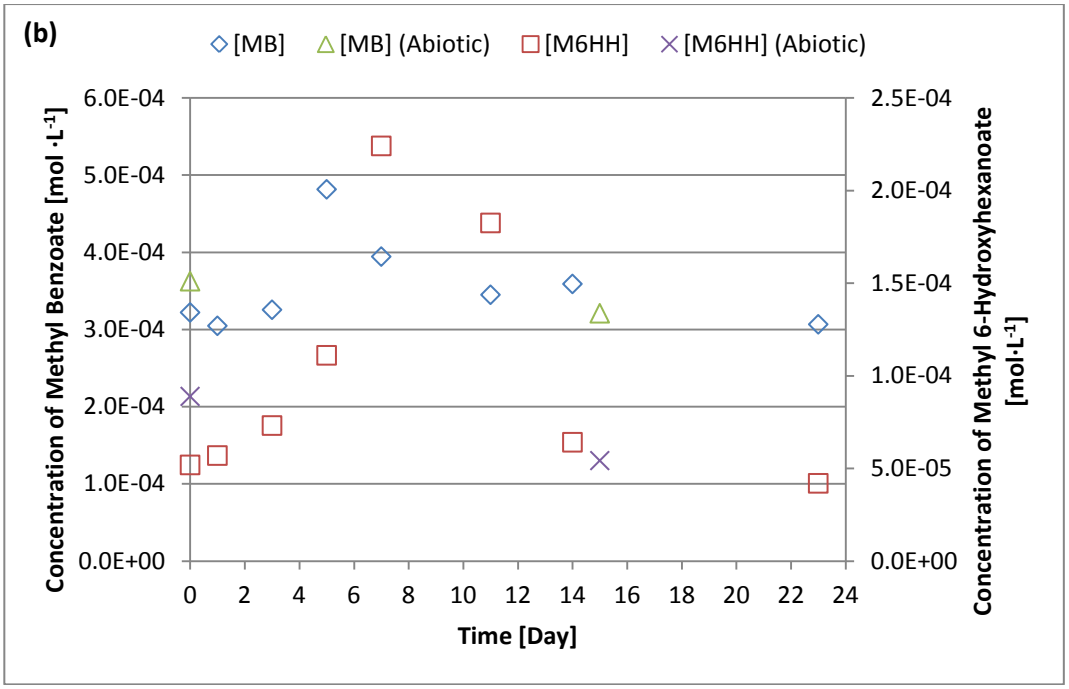


Figure 8. (b) Concentration of identified methyl esters during degradation of B_2-769 for the second (acidic) extract. Plotted are the concentrations of two of the transmethylation products, methyl benzoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls. The concentration of methyl benzoate is represented on the left vertical axis, and the concentration of methyl 6-hydroxyhexanoate is represented on the right vertical axis.

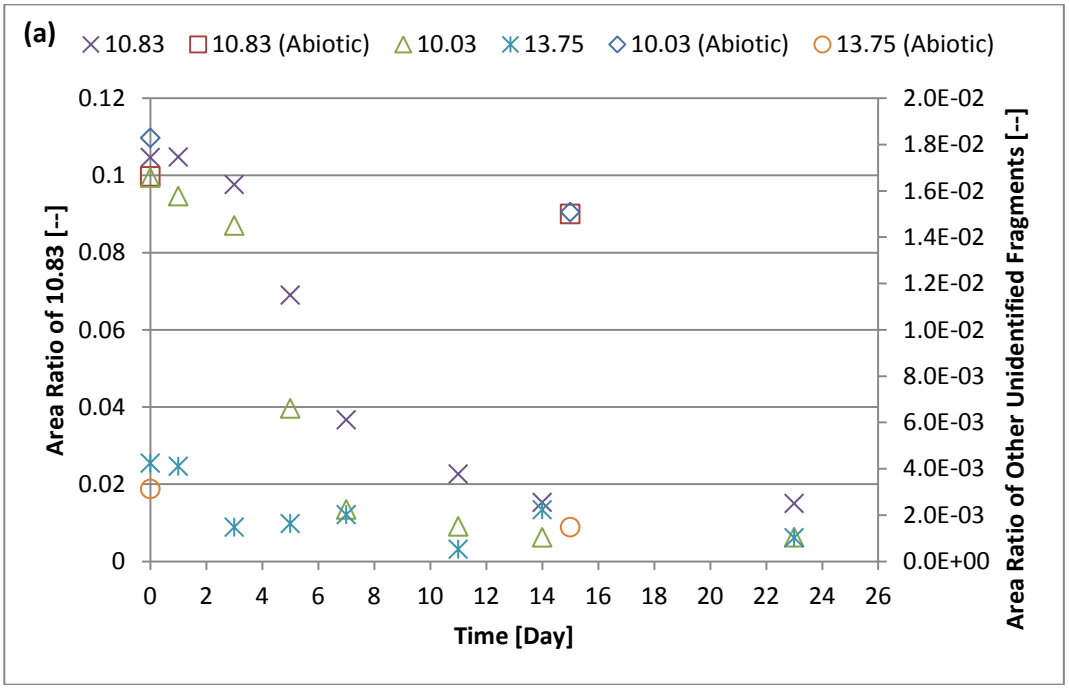


Figure 9. (a) Area ratios of the larger, unidentified fragments of the parent compound B_2-769 for the first (alkaline) extract. Shown here are both data for the degradation experiment as well as for the abiotic controls. These larger, unidentified fragments are referred to by their retention times (10.03,10.83, and 13.75 minutes). The area ratio of compound 10.83 is shown on the left vertical axis, whereas the area ratios of compound 10.03 and compound 13.75 are shown on the right vertical axis.

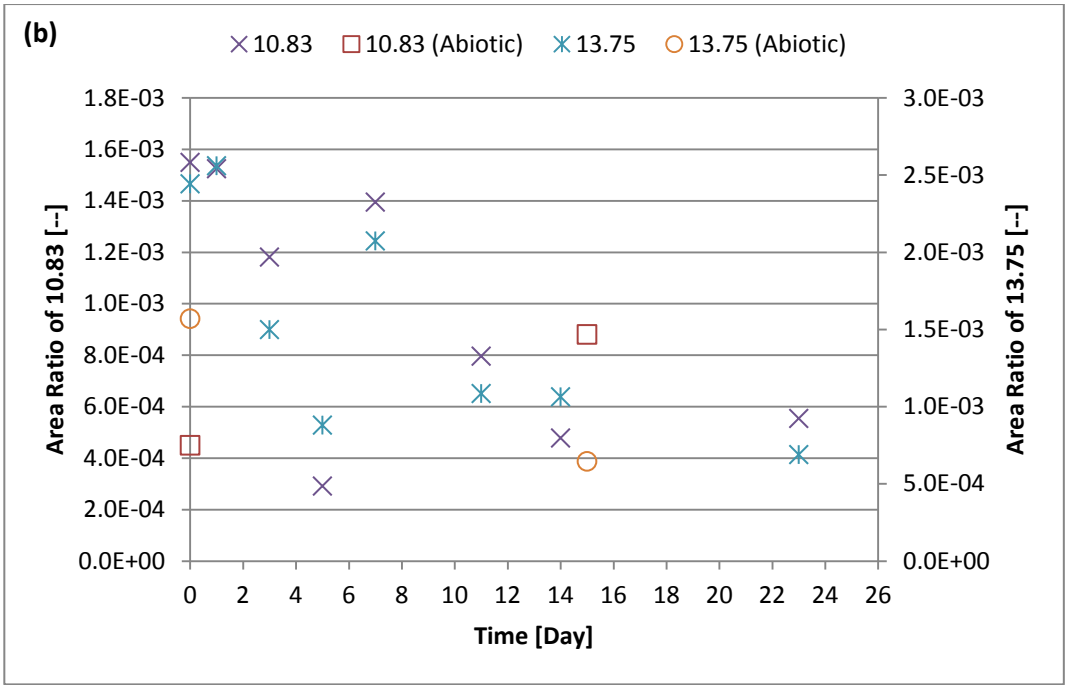


Figure 9. (b) Area ratios of the larger, unidentified fragments with retention times of 10.83 and 13.75 minutes during degradation of B_2-769 for the second (acidic) extract. Data is presented for the degradation experiment as well as for the abiotic controls. The area ratio of compound 10.83 is shown on the left vertical axis, whereas the area ratios of compound 13.75 is shown on the right vertical axis.

The largest of the benzoate-terminated plasticizers considered for degradation was the compound designated B_2-997, which had an average of n=3 repeating ε-caprolactone monomers per arm. The concentrations of methyl benzoate and methyl 6-hydroxyhexanoate observed for alkaline and acidic extracts are plotted against degradation time in **Figure 10 (a)** and **(b)** for degraded samples of B_2-997 as well as for abiotic controls. Evolution of the larger, unidentified fragments that occurred from transmethylation are shown in **Figure 11 (a)** and **(b)** for these degradation experiments as well as for the abiotic controls, and are referred to by retention times.

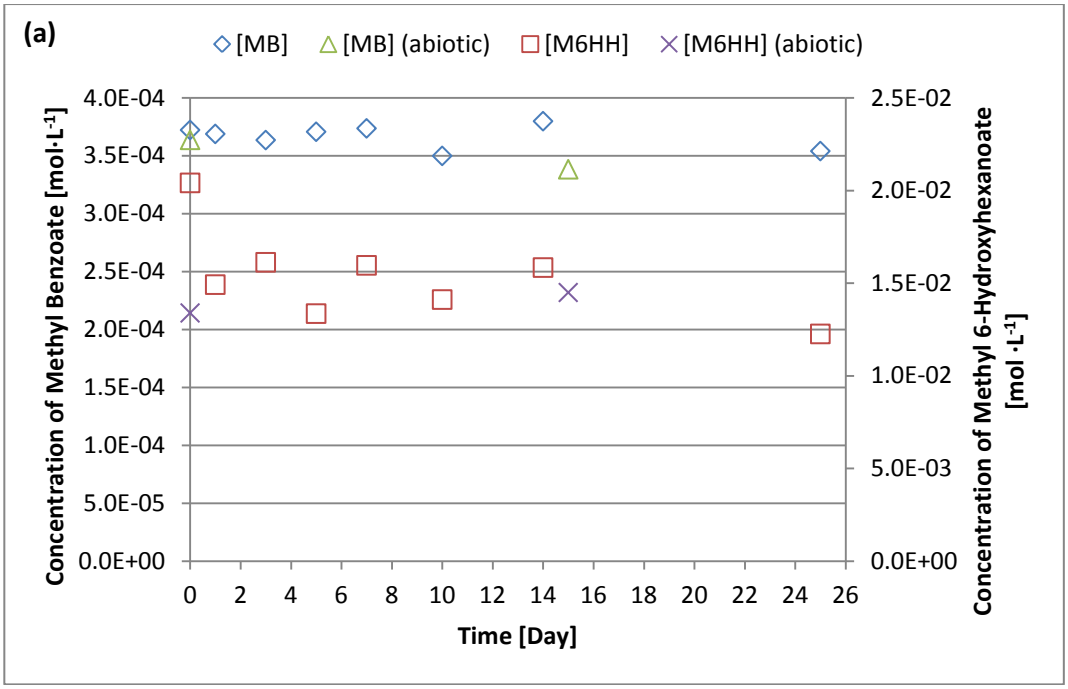


Figure 10. (a) Concentration of identified methyl esters during degradation of B_2-997 for the first (alkaline) extract. Plotted are the concentrations of two of the transmethylation products, methyl benzoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls. The concentration of methyl benzoate is represented on the left vertical axis, and the concentration of methyl 6-hydroxyhexanoate is represented on the right vertical axis.

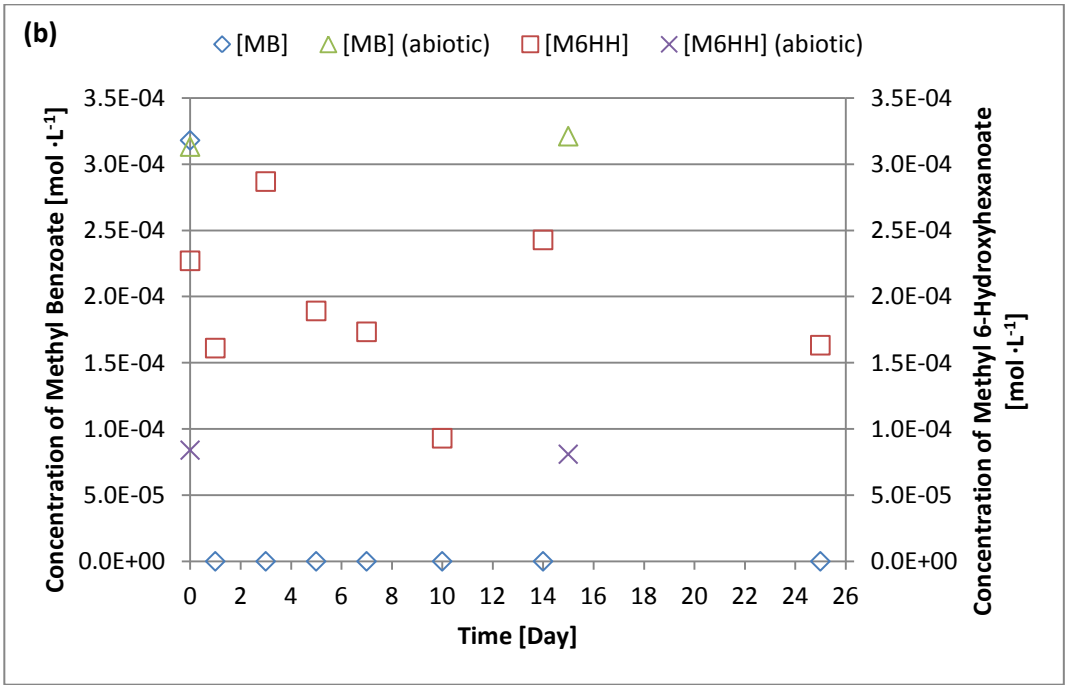


Figure 10. (b) Concentration of identified methyl esters during degradation of B_2-997 for the second (acidic) extract. Plotted are the concentrations of two of the transmethylation products, methyl benzoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls. The concentration of methyl benzoate is represented on the left vertical axis, and the concentration of methyl 6-hydroxyhexanoate is represented on the right vertical axis.

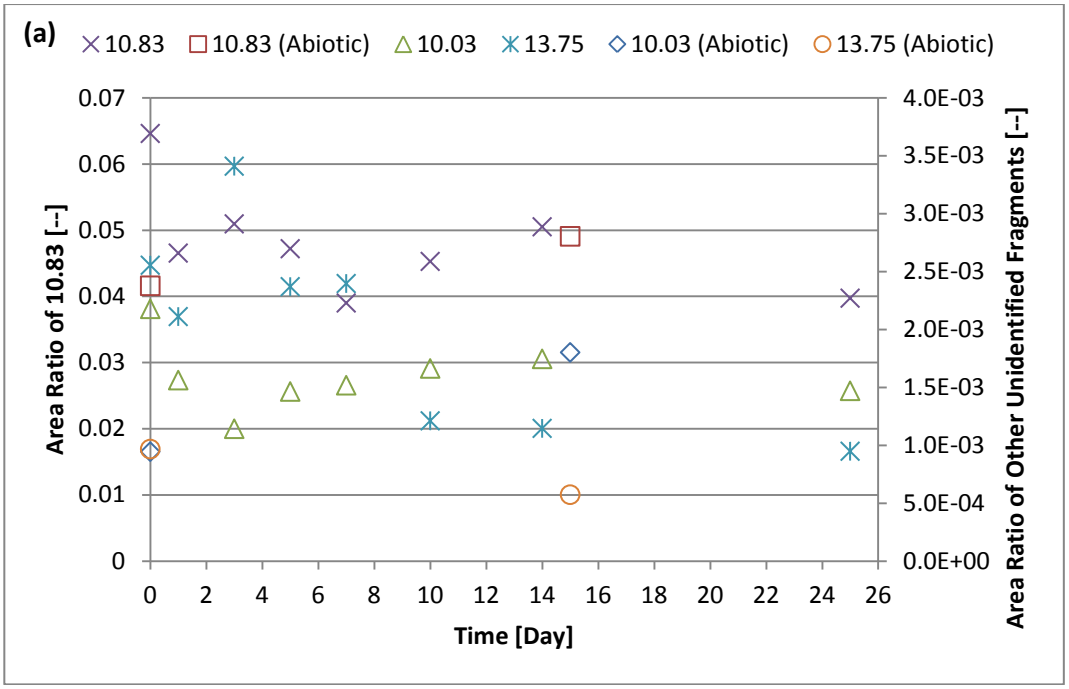


Figure 11. (a) Area ratios of the larger, unidentified fragments of the parent compound B_2-997 for the first (alkaline) extract. Shown here are both data for the degradation experiment as well as for the abiotic controls. These larger, unidentified fragments are referred to by their retention times (10.03,10.83, and 13.75 minutes). The area ratio of compound 10.83 is shown on the left vertical axis, whereas the area ratios of compounds 10.03 and 13.75 are shown on the right vertical axis.

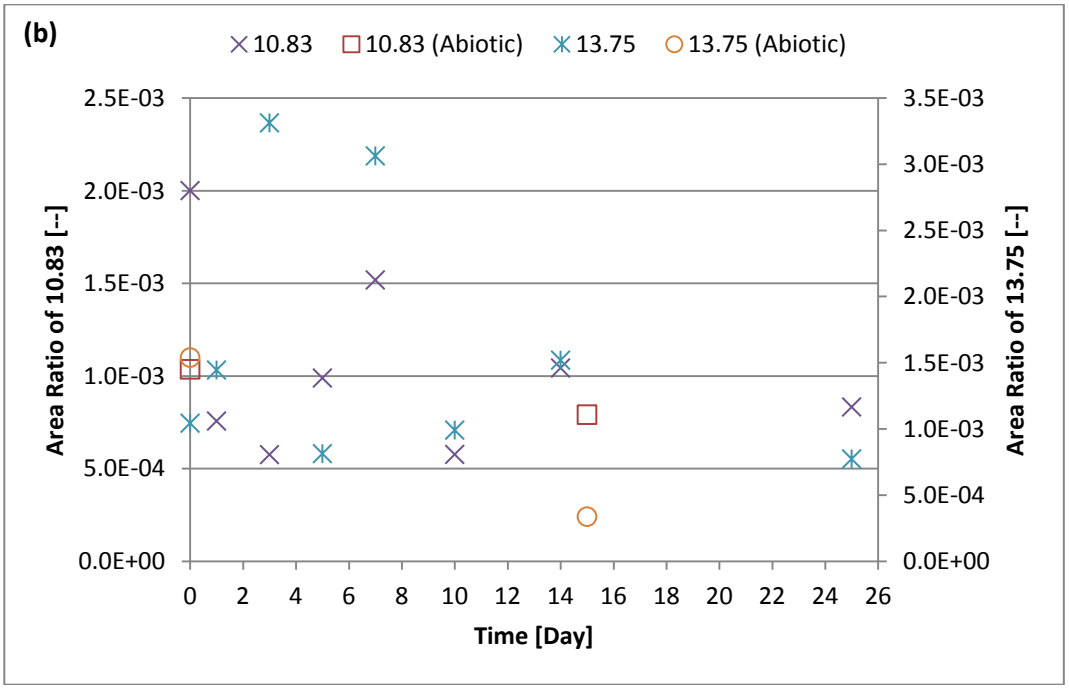


Figure 11. (b) Area ratios of the larger, unidentified fragments with retention times of 10.83 and 13.75 minutes during degradation of B_2-997 for the second (acidic) extract. Data is presented for the degradation experiment as well as for the abiotic controls. The area ratio of compound 10.83 is shown on the left vertical axis, whereas the area ratios of compound 13.75 is shown on the right vertical axis.

The smallest octanoate-terminated PCL-based plasticizer considered for degradation was the compound designated O_2-613. The concentration used was 0.23 mM per flask. Each of the two arms of this molecule consisted of an average of n=1 repeating ε-caprolactone monomers. The dual extraction procedure was carried out as described for the benzoate-terminated PCL-based plasticizers. This was followed by methyl transesterification for each of the extracts. The results for the degradation of O_2-613 are in shown in **Figure 12 (a)** (alkaline extracts) and **(b)** (acidic extracts). In these figures, the concentrations of two of the transmethylation products, methyl octanoate and methyl 6-hydroxyhexanoate, were plotted over the course of the degradation experiment as well as for abiotic controls.

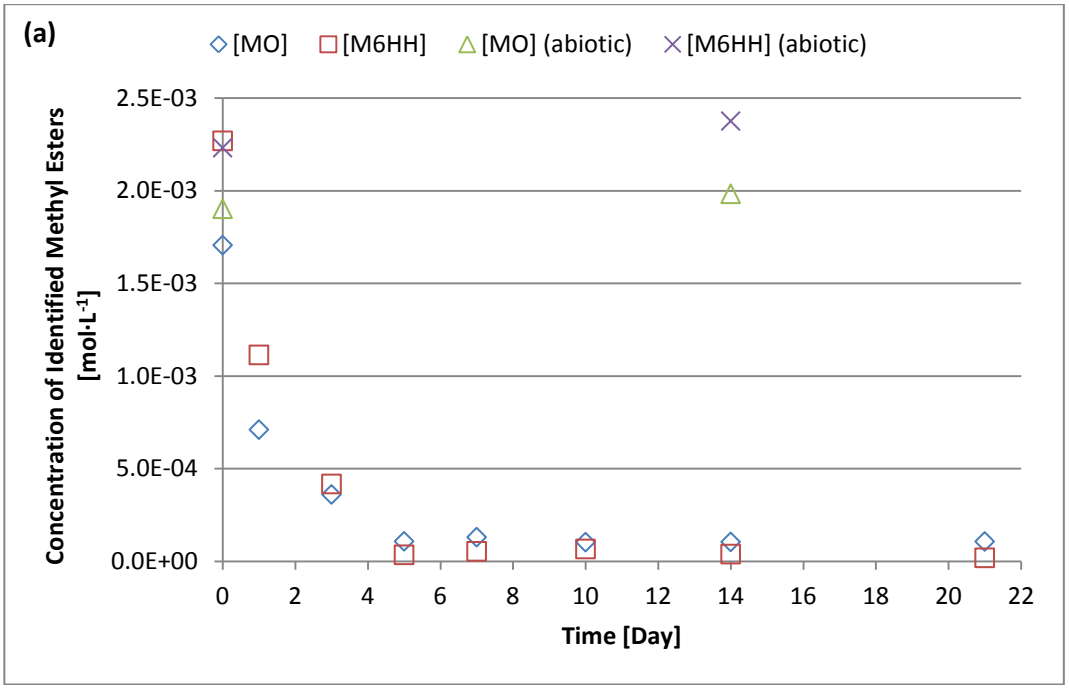


Figure 12. (a) Concentration of identified methyl esters during degradation of O_2-613 for the first (alkaline) extract. The concentrations of two of the transmethylation products, methyl octanoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls are represented on the vertical axis.

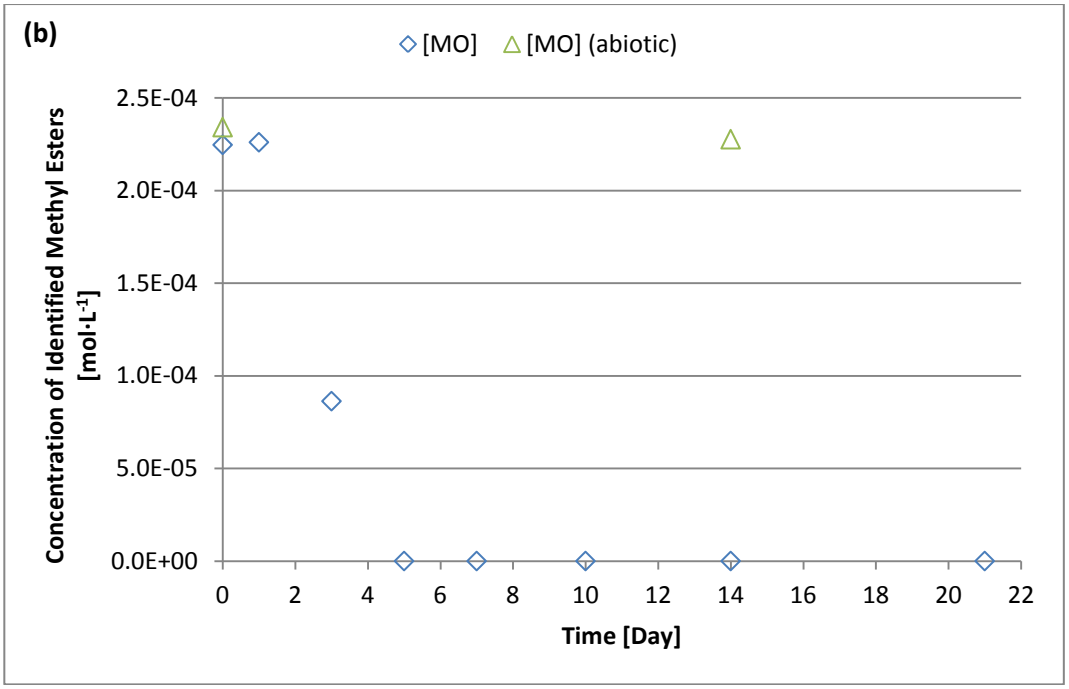


Figure 12. (b) Concentrations of identified methyl esters during degradation of O_2-613 for the second (acidic) extract. The concentration of the transmethylation product, methyl octanoate, for the degradation experiment as well as the abiotic controls is represent by the vertical axis. Note that no methyl 6-hydroxyhexanoate was detected in the acidic extract.

The molecular structure of O_2-841 differed from that of O_2-613 only in that there were an average of n=2 repeat ε-caprolactone monomers per arm instead of n=1. The concentrations of transmethylated products from the alkaline and acidic extractions taken during the degradation of O_2-841 are shown in **Figure 13 (a)** and **(b)**. Included in these figures are data for the abiotic controls. At first glance, the data for the acidic extracts appears to have indicated that small amounts of methyl octanoate and methyl 6-hydroxyhexanoate remained at the end of the experiment. However, the concentrations of these methyl esters in the acidic extracts were negligible overall, compared to the initial concentrations detected.

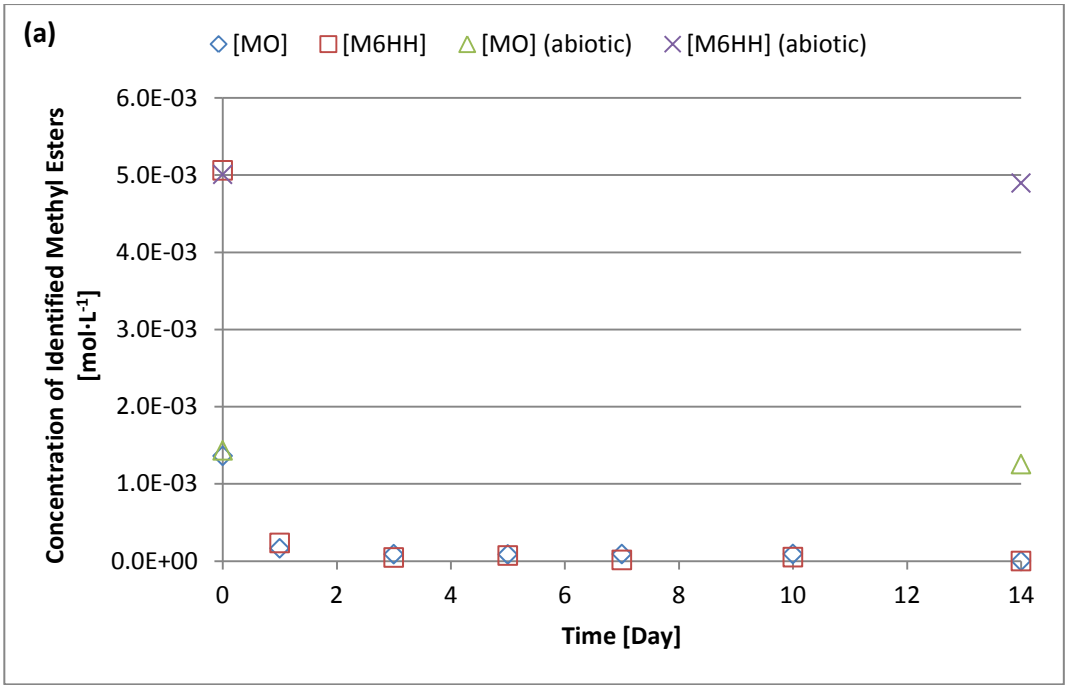


Figure 13. (a) Concentrations of identified methyl esters during degradation of O_2-841 for the first (alkaline) extract. The concentrations of two of the transmethylation products, methyl octanoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls are represented on the vertical axis.

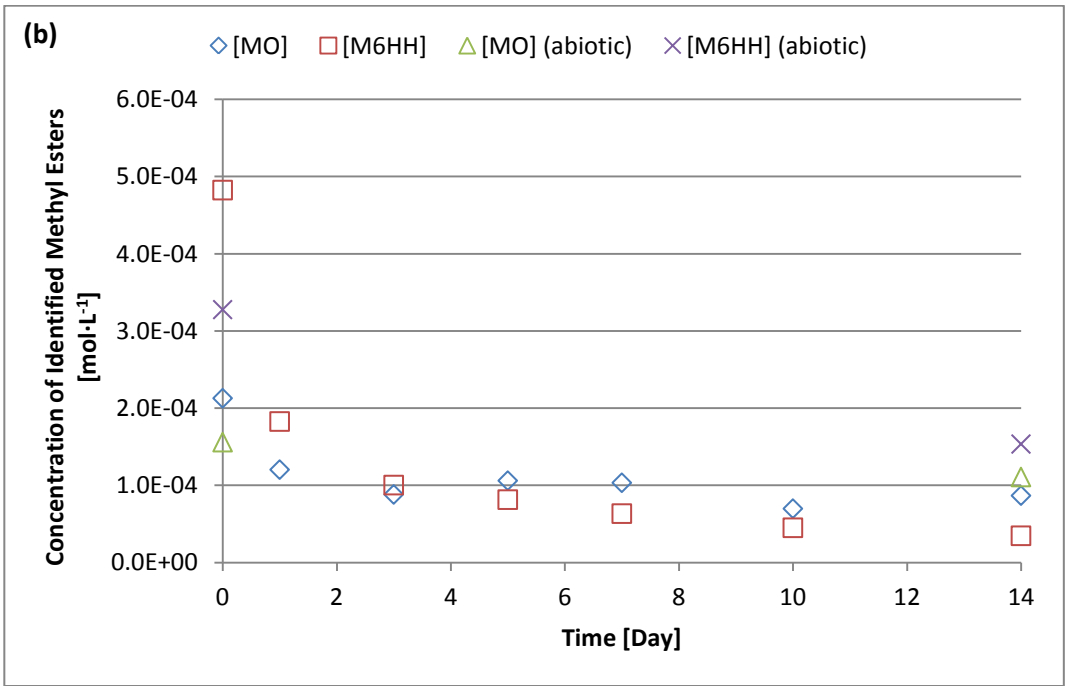


Figure 13. (b) Concentrations of identified methyl esters during degradation of O_2-841 for the second (acidic) extract. The concentrations of two of the transmethylation products, methyl octanoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls are represented on the vertical axis.

The only three-armed plasticizer to be considered in this study was O_3-897, which had an average of n=1 repeating ε-caprolactone monomers per arm. As the name indicates, the arms themselves were terminated by ester linkages to octanoate groups. The concentrations of methyl octanoate and methyl 6-hydroxyhexanoate observed for both alkaline and acidic extracts are plotted against degradation time in **Figure 14 (a)** and **(b)** for degraded samples of O_3-897 as well as for abiotic controls.

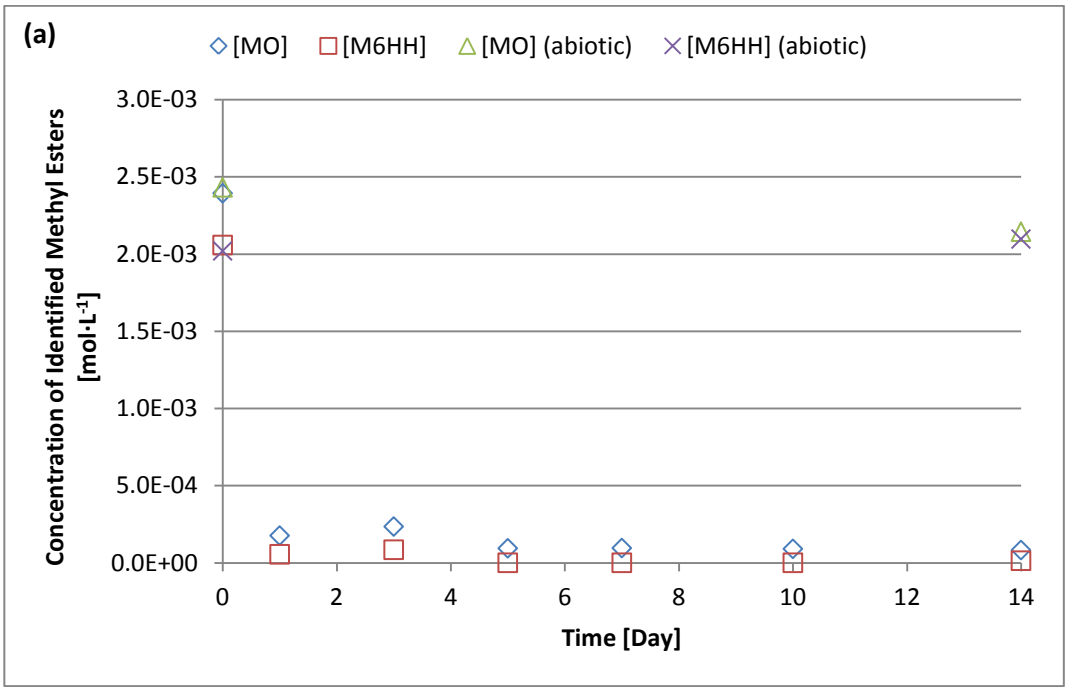


Figure 14. (a) Concentrations of identified methyl esters during degradation of O_3-897 for the first (alkaline) extract. The concentrations of two of the transmethylation products, methyl octanoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls are represented on the vertical axis.

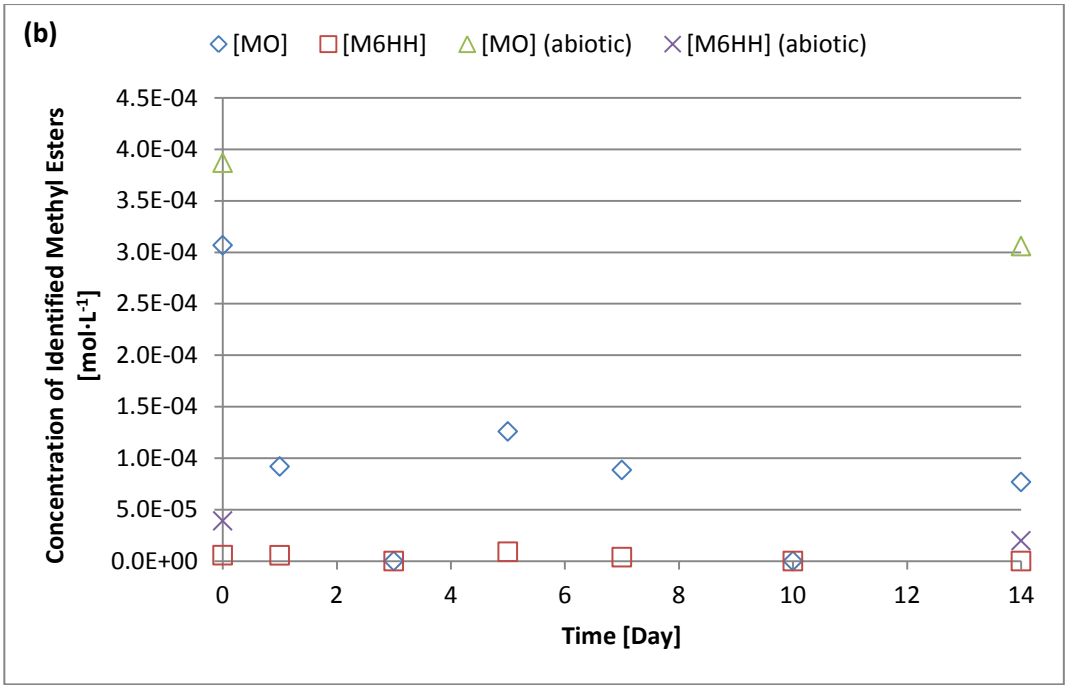


Figure 14. (b) Concentrations of identified methyl esters during degradation of O_3-897 for the second (acidic) extract. The concentrations of two of the transmethylation products, methyl octanoate and methyl 6-hydroxyhexanoate, for the degradation experiment as well as the abiotic controls are represented on the vertical axis.

Additional long-term experiments for the degradation of B_2-997 were carried out at three different concentration levels (0.21, 1.0 and 2.1 mM). For these experiments, the samples were extracted strictly under acidic conditions, using hydrochloric acid to adjust the pH to a value of 3-4. **Figure 15 (a)**,**Figure** 16 **(a)**, and **Figure** 17 **(a)** show the concentrations of methyl 6-hydroxyhexanoate and methyl benzoate plotted against biodegradation time for the three distinct experiments involving differing concentrations of B_2-997 (0.21, 1.0 and 2.1 mM, respectively). For these three experiments, the area ratios of the larger, unidentified compounds with retention times of 10.83 and 13.75 minutes were also plotted in **Figure 15 (b)**,**Figure** 16 **(b)**, and **Figure** 17 **(b)**. Note that compound 10.03 was not detected in any of the samples for all three of these

experiments because the extractions were carried out at acidic pH and compound 10.03 was only ever observed in alkaline extracts.

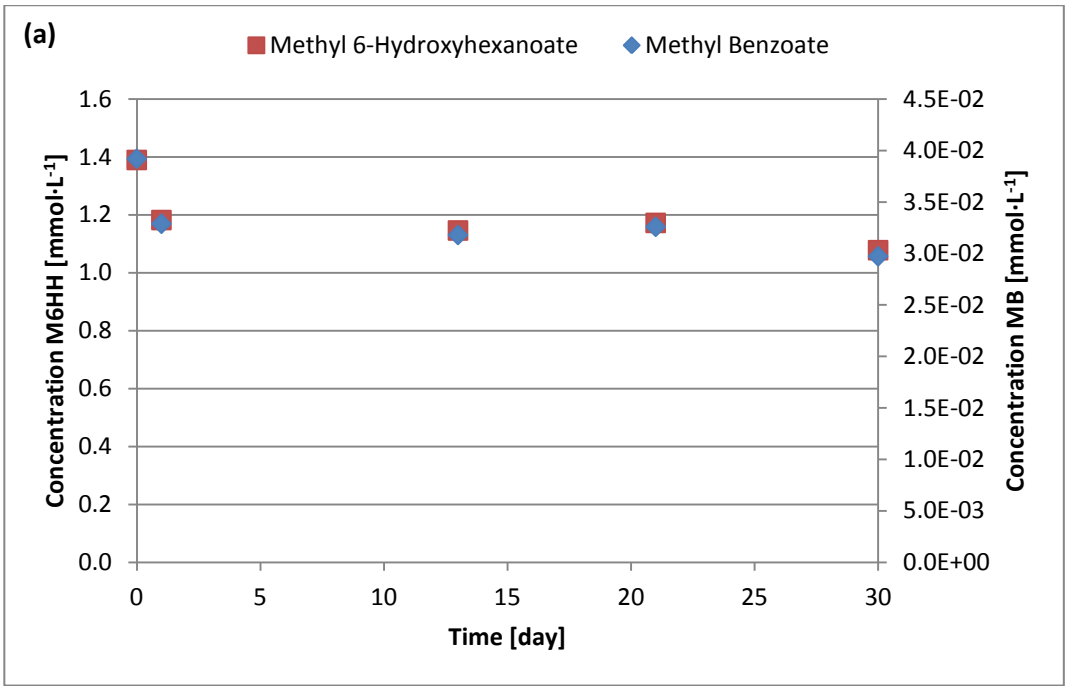


Figure 15. (a) Concentrations of methyl 6-hydroxyhexanoate (left vertical axis) and methyl benzoate (right vertical axis) during long-term biodegradation of B_2-997; lowest concentration level (0.21 mM).

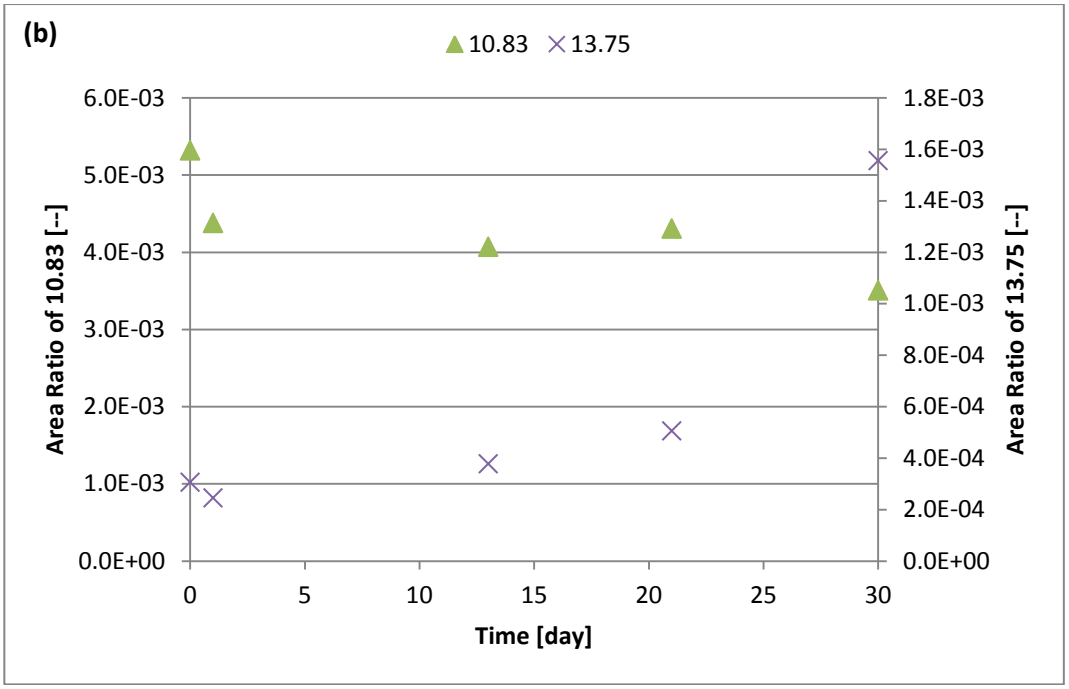


Figure 15. (b) Area ratios of the larger, unidentified fragments with retention times of 10.83 (left vertical axis) and 13.75 minutes (right vertical axis) during long-term biodegradation of B_2-997; lowest concentration level (0.21 mM).

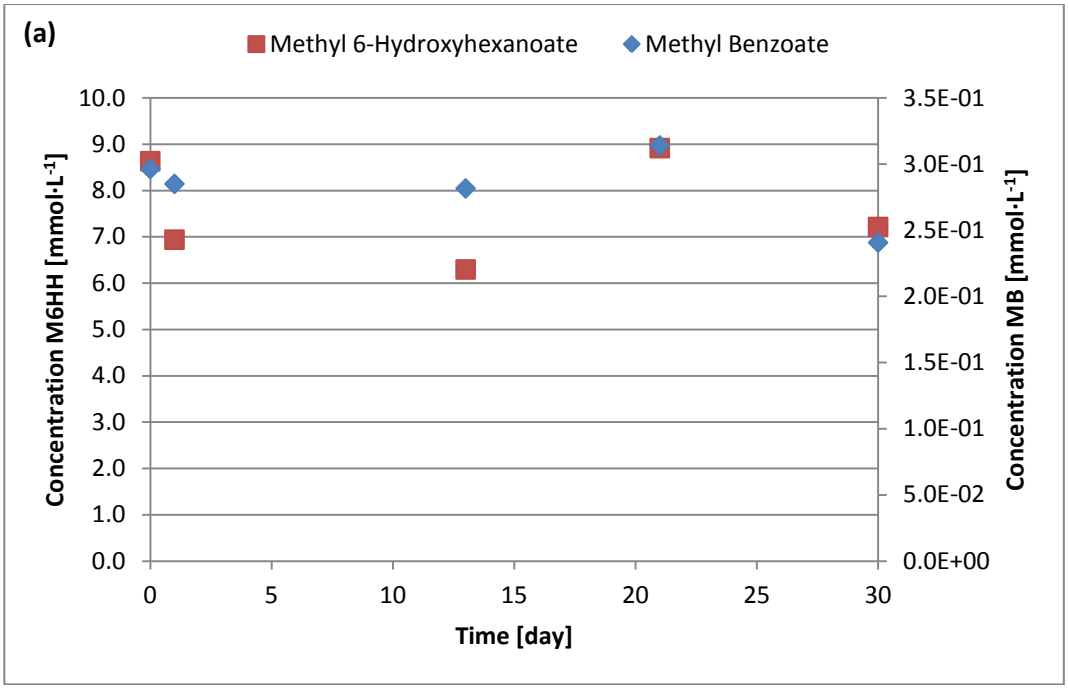


Figure 16. (a) Concentrations of methyl 6-hydroxyhexanoate (left vertical axis) and methyl benzoate (right vertical axis) during long-term biodegradation of B_2-997; medium concentration level (1.0 mM).

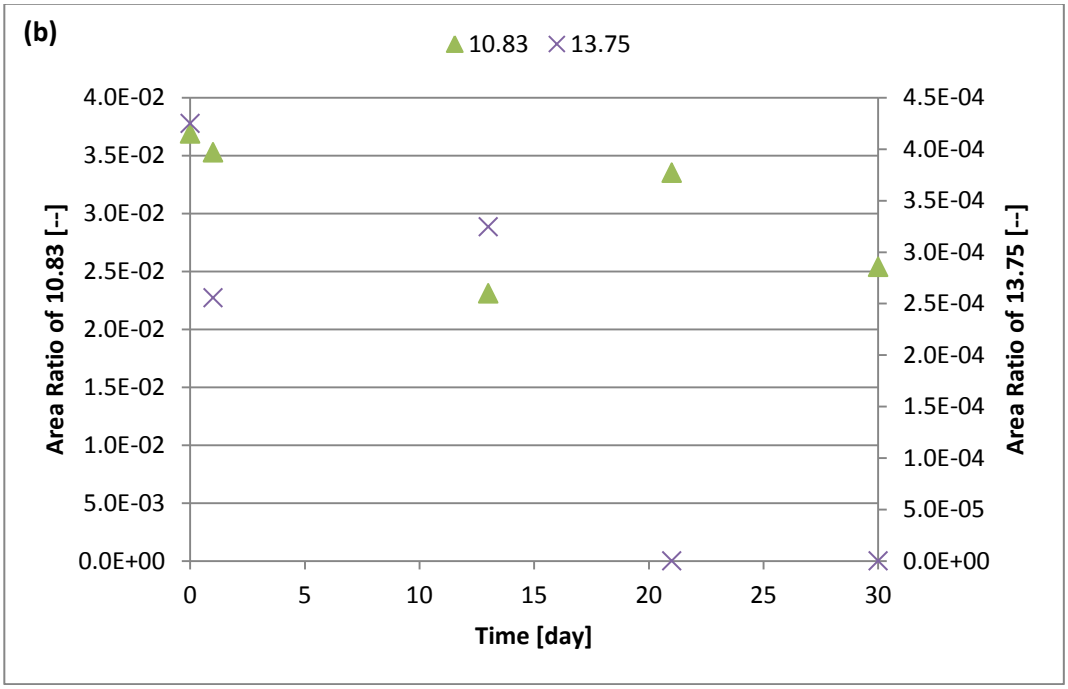


Figure 16. (b) Area ratios of the larger, unidentified fragments with retention times of 10.83 (left vertical axis) and 13.75 minutes (right vertical axis) during long-term biodegradation of B_2-997; medium concentration level (1.0 mM).

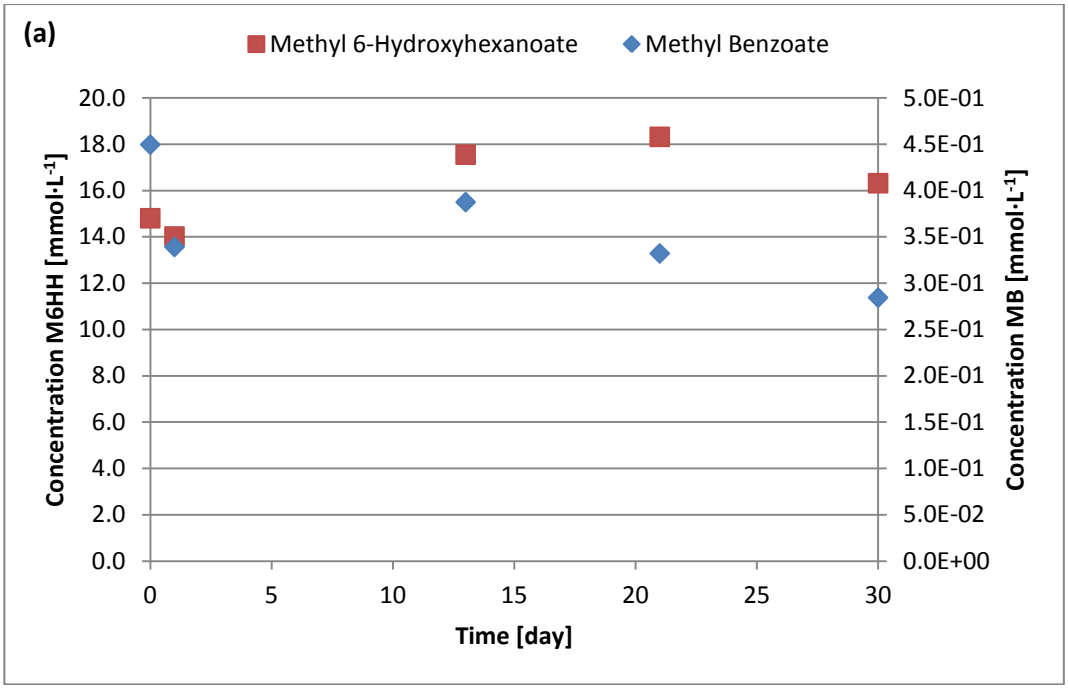


Figure 17. (a) Concentrations of methyl 6-hydroxyhexanoate (left vertical axis) and methyl benzoate (right vertical axis) during long-term biodegradation of B_2-997; highest concentration level (2.1 mM).

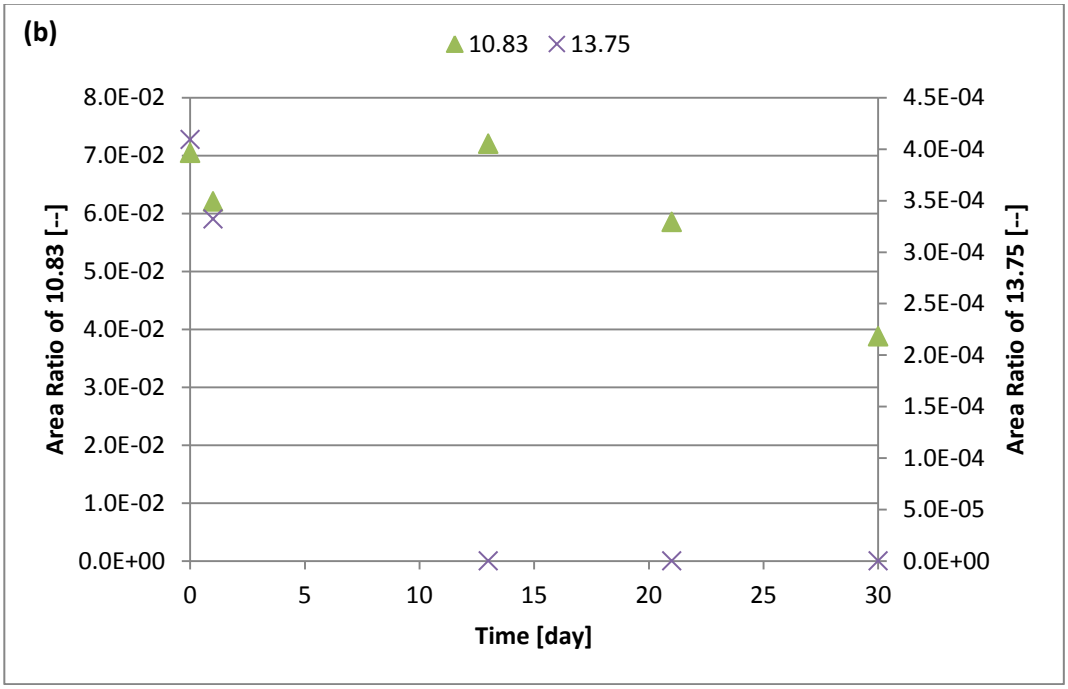


Figure 17. (b) Area ratios of the larger, unidentified fragments with retention times of 10.83 (left vertical axis) and 13.75 minutes (right vertical axis) during long-term biodegradation of B_2-997; highest concentration level (2.1 mM).

The concentration data for the three experiments with differing levels of concentration were consolidated and have been summarized in **Figure 18**, with the normalized amounts of identified methyl esters indicated on the left vertical axis. Cumulative area ratios of the two larger, unidentified fragments are indicated on the right vertical axis. Important results for these experiments are summarized numerically in **Table 2**.

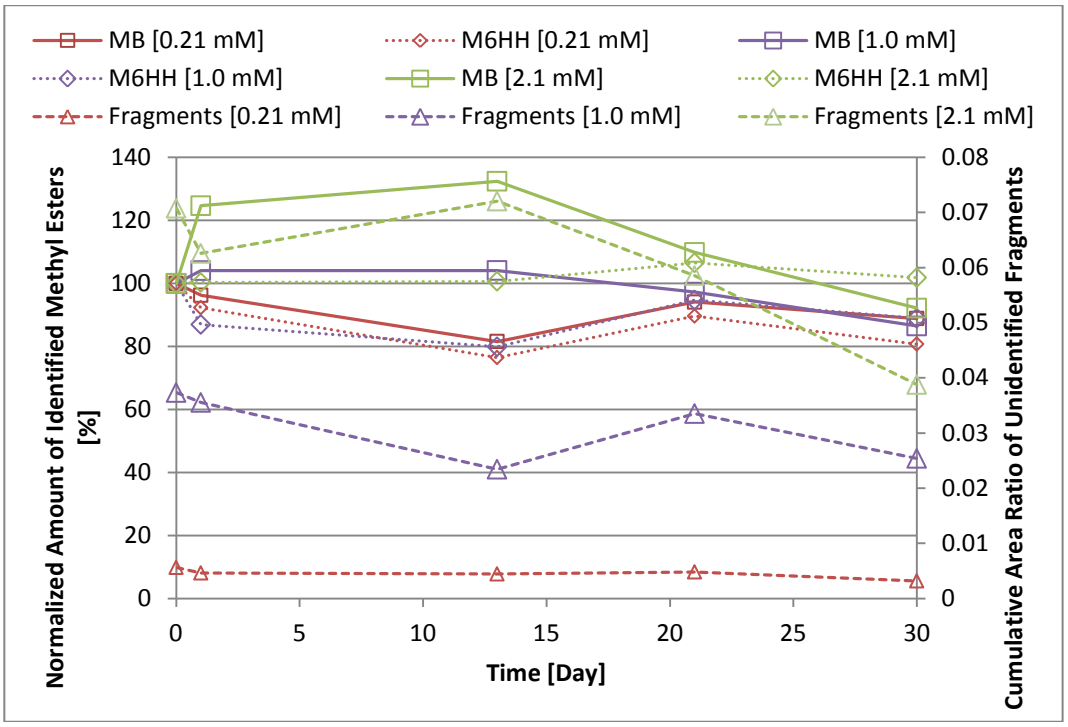


Figure 18. Long-term degradation experiments for B2-997 at three different concentration levels (0.21, 1.0, and 2.1 mM). The left vertical axis represents the percentage of the initial amounts of methyl benzoate and methyl 6-hydroxyhexanoate obtained by normalizing the detected concentrations to an average concentration value obtained for all of the abiotic controls for each experiment . The right vertical axis represents the cumulative area ratios of the larger, unidentified fragments (compounds 10.83 and 13.75) for each experiment. Data from alkaline and acidic extracts have been combined.

Table 2. Percent degradation of B_2-997 for long-term experiments

Initial concentration of B_2-997 ($mmol \cdot L^{-1}$)	Degradation of methyl benzoate (%)	Degradation of methyl 6-hydroxyhexanoate (%)	Degradation of larger, unidentified fragments (%)
0.21	11	19	43
1.0	10	11	32
2.1	8	0	45

During the degradation studies of the various PCL-based plasticizers, the concentrations and area ratios for the abiotic controls may appear to have changed slightly over time. However, this was attributed to human error in physically measuring out a consistent mass of plasticizer as there was simply variability in the amount of plasticizer present in the media from one abiotic control to the next. Since this type of error is virtually impossible to eliminate (although it can be minimized), the artifact revealed the benefit of normalizing any measurements against the known amount of plasticizer initially added.

By consolidating the experimental data from both alkaline and acidic extracts, as well as by amassing the area ratios for any larger, unidentified fragments, **Figure 19** compares the data for the degradation of all three benzoate-terminated PCL plasticizers designated B_2-540, B_2-769, and B_2-997. In this figure, the normalized amount of the identified methyl esters (methyl benzoate and methyl 6-hydroxyhexanoate) are indicated by the left vertical axis. On the right vertical axis, the cumulative amounts of the larger, unidentified fragments of the parent molecules are displayed in terms of area ratio. Important results of these experiments (namely, the percent degradation of the various fragments for each plasticizer) are summarized in **Table 3**.

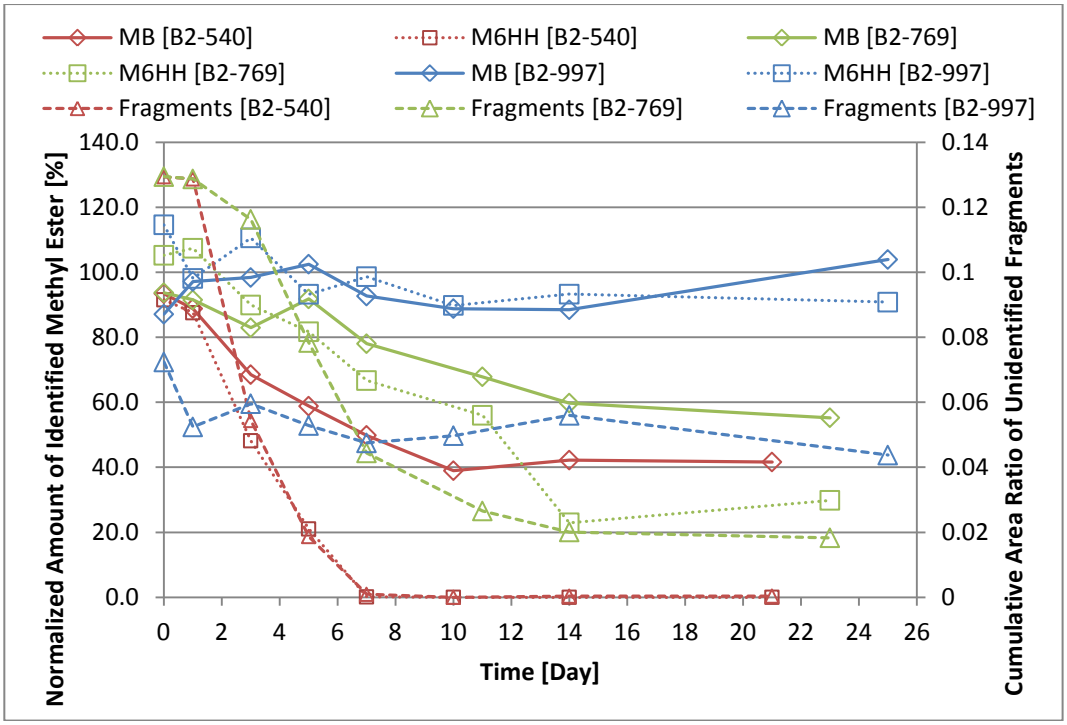


Figure 19. Comparison of the degradation of B_2-540, B_2-769, and B_2-997. The left vertical axis represents the percentage of the initial amounts of methyl benzoate and methyl 6-hydroxyhexanoate obtained by normalizing the detected concentrations to an average concentration value obtained for all of the abiotic controls for each parent compound. The right vertical axis indicates the cumulative area ratios of the larger, unidentified fragments (compounds 10.03, 10.83, and 13.75) for each parent compound. Data from alkaline and acidic extracts have been combined.

Table 3. Percent degradation and resulting metabolites for benzoate-terminated PCL plasticizers

Plasticizer Designation	Initial concentration ($mmol \cdot L^{-1}$)	Degradation of methyl benzoate (%)	Degradation of methyl 6-hydroxyhexanoate (%)	Degradation of larger, unidentified fragments (%)	Maximum area ratio of metabolites	
					Metabolite #1	Metabolite #2
B_2-540	0.36	55	100	100	0.0343	0.0045
B_2-769	0.37	41	72	86	0.0124	0.0008
B_2-997	0.33	11	20	40	-	-

While the concentration of methyl 6-hydroxyhexanoate decreased to zero for the compound B_2-540, the concentration of methyl benzoate plateaued after day 10 at roughly 40% of the initial concentration. A similar plateau occurred during the degradation of B_2-769, in this case for both of the identified methyl esters as well as the larger, unidentified fragments beginning around day 14. It was not

clear whether or not such a plateau occurred in systems containing B_2-997 since the rate of degradation was so slow.

Figure 20 compares the degradation data for systems containing the octanoate-terminated PCL plasticizers designated O_2-613, O_2-841, and O_3-897. The normalized amount of identified methyl esters (this time methyl octanoate, and again methyl 6-hydroxyhexanoate) are plotted against time. The relevant results are summarized in **Table 4**.

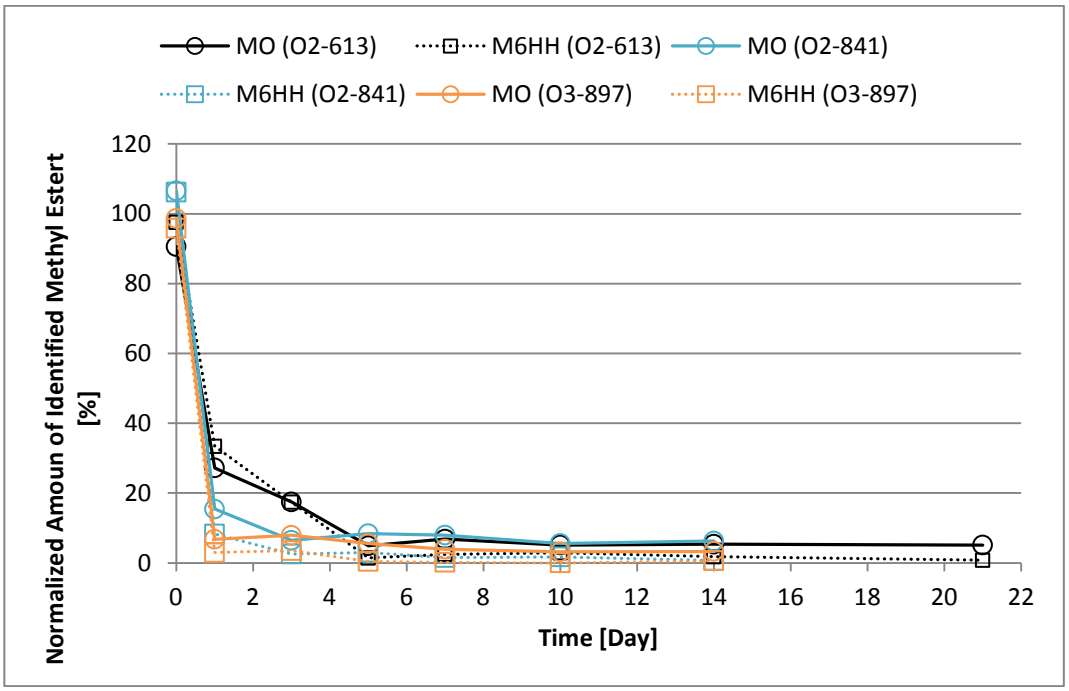


Figure 20. Comparison of degradation of the three octanoate-terminated PCL plasticizers: O_2-613, O_2-841, and O_3-897. The vertical axis represents the percentage of the initial amounts of methyl octanoate and methyl 6-hydroxyhexanoate normalized to values obtained for the abiotic controls for each parent compound. NOTE: no additional fragments were detectable.

Table 4. Percent degradation for octanoate-terminated PCL plasticizers

Plasticizer Designation	Initial concentration ($mmol \cdot L^{-1}$)	Degradation of methyl octanoate (%)	Degradation of methyl 6-hydroxyhexanoate (%)
O_2-613	0.23	96	98
O_2-841	0.23	94	99
O_3-897	0.22	97	99

4.3. Metabolites

Over the course of the degradation of the plasticizer with the designation B_2-540, the appearance of two new peaks was observed, indicating breakdown of the parent compound into metabolites. In **Figure 21**, The combined area ratios for both alkaline and acidic extractions for the compound with a retention time of 9.6 minutes (metabolite #1) are indicated by the right vertical axis. Likewise, the cumulative area ratios of both extractions for the compound with a retention time of 8.9 minutes (metabolite #2) are displayed by the left vertical axis. Neither of these metabolites was extracted to an appreciable extent during the alkaline extraction. It should be noted that these metabolites were also observed during the degradation of B_2-769 (as shown in **Figure 22**), but not during degradation of the largest benzoate-terminated PCL plasticizer, B_2-997. Moreover, no observable metabolites were detected during the degradation studies of any of the octanoate-terminated PCL plasticizers.

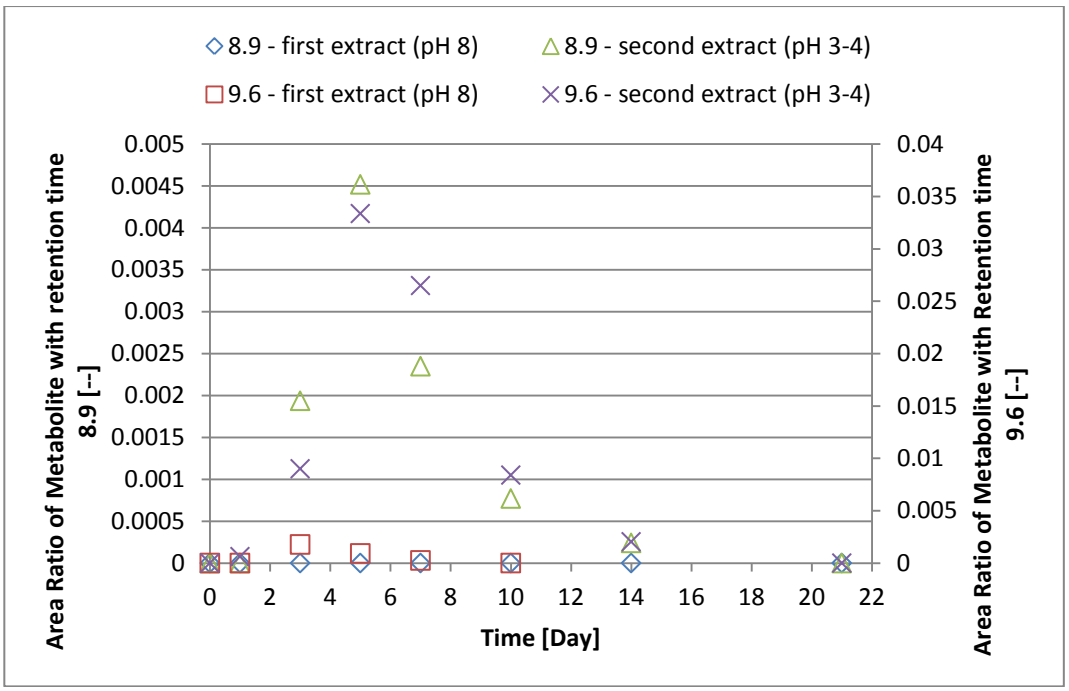


Figure 21. Evolution of metabolites during biodegradation of B_2-540 for both alkaline and acidic extracts. The left vertical axis indicates the area ratio of compound 8.9 and the right vertical axis indicates the area ratio of compound 9.6.

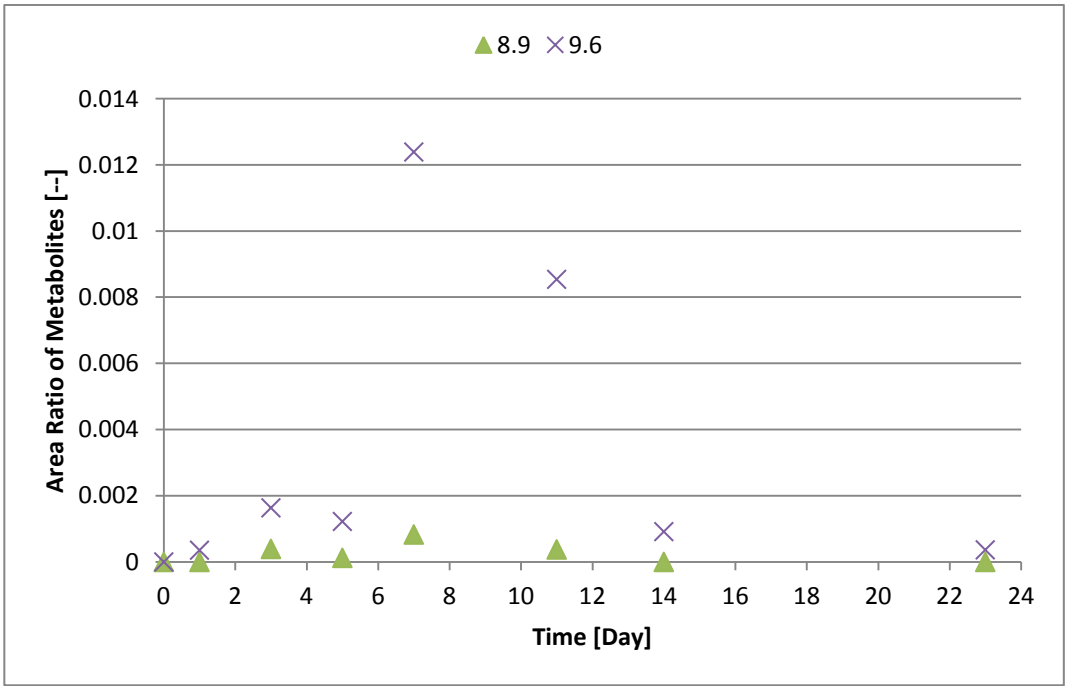


Figure 22. Evolution of metabolites during biodegradation of B_2-769 for acidic extracts. The area ratios of both compound 8.9 and 9.6 are indicated by the vertical axis. Note that there were no detectable metabolites in the alkaline extract.

The retention times of various likely candidates for metabolites were obtained on the GC and tabulated in **Table 5**.

Table 5. Gas chromatography retention times of various relevant compounds

Retention Time	Compound
4.32	1,5-Pentanediol
5.32	Methyl benzoate
5.57	Methyl octanoate
5.72	Glutaric acid
5.72	ε-caprolactone
6.15	Methyl 6-hydroxyhexanoate
6.58	Adipic acid
8.9	Hexyl benzoate
8.9	Metabolite 2 (Lesser)
9.6	Metabolite 1 (Greater)
10.03	Lesser unidentified fragment (from Benzoates)
10.61	1,6-Hexanediol monobenzoate
10.83	Greater unidentified fragment (from Benzoates)
12.75	1,3-Propanediol monobenzoate
13.75	Moderate unidentified fragment (from benzoates)

Degradation experiments were also carried out for 1,6-hexanediol dibenzoate owing to its similar molecular structure to that of the benzoate-containing PCL plasticizers. These experiments were analyzed both without and then with the methyl transesterification of samples prior to injection into the GC. The non-transesterified data of **Figure 23** shows the detection of a new peak that was not present in the abiotic controls with a retention time of 9.9 minutes. When samples were transesterified prior to injection (**Figure 24**), this retention time shifted to 9.6 minutes, the same as that of metabolite #1 observed during the breakdown of B_2-540 and B_2-769.

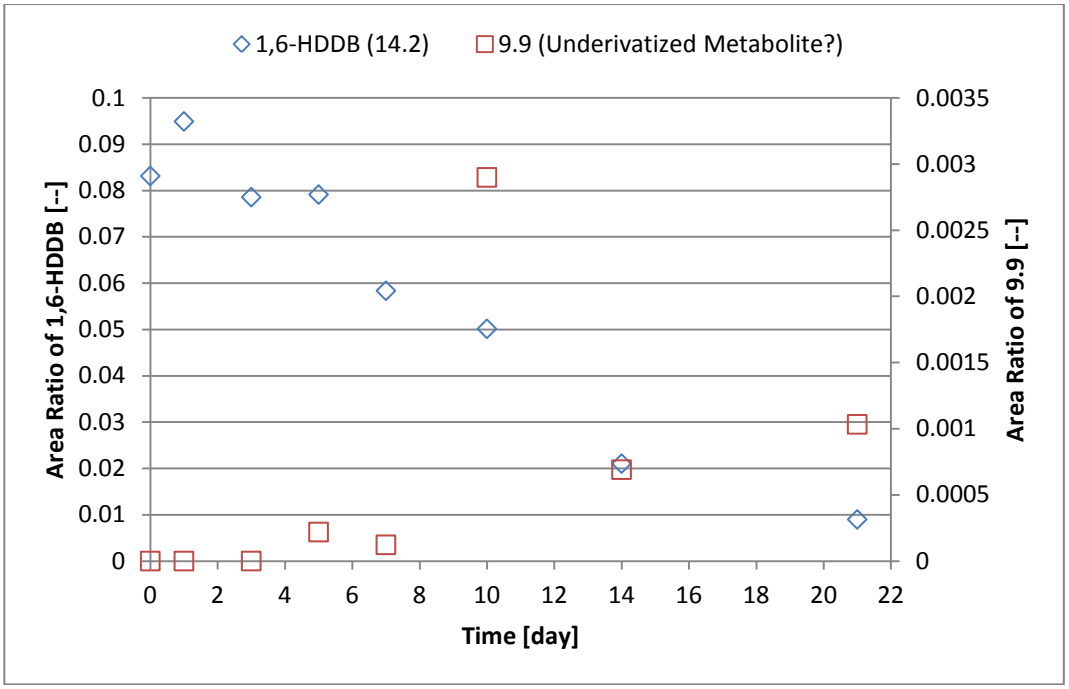


Figure 23. Data for the degradation of 1,6-hexanediol dibenzoate (analyzed without methyl transesterification). The area ratio of 1,6-hexanediol dibenzoate (retention time: 14.2 minutes) is represented by the left vertical axis. The area ratio of the metabolite with a retention time of 9.9 minutes is indicated by the right vertical axis.

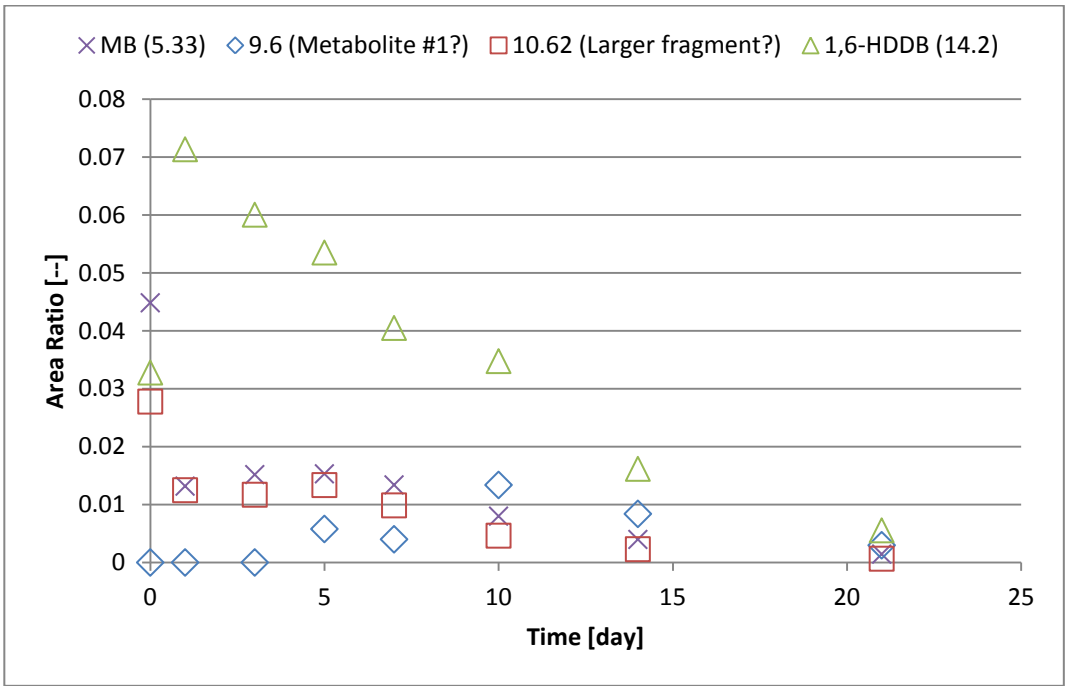


Figure 24. Data for the degradation of 1,6-hexanediol dibenzoate (analyzed post-methyl transesterification). The vertical axis indicates the area ratio of the detected compounds: methyl benzoate (retention time: 5.33 minutes); the derivatized metabolite (9.6 minutes); a larger, unidentified fragment of the parent compound (10.62 minutes); and the parent compound 1,6-hexanediol dibenzoate (14.2 minutes).

Transmethylated samples from the breakdown of B_2-540 were analyzed by GC/MS. In addition, extracts from the same degradation experiments were silylated as an alternative method of derivatization. **Figure 25** shows the annotated mass spectrum of one particular compound which is believed to be a metabolite that has been silylated.

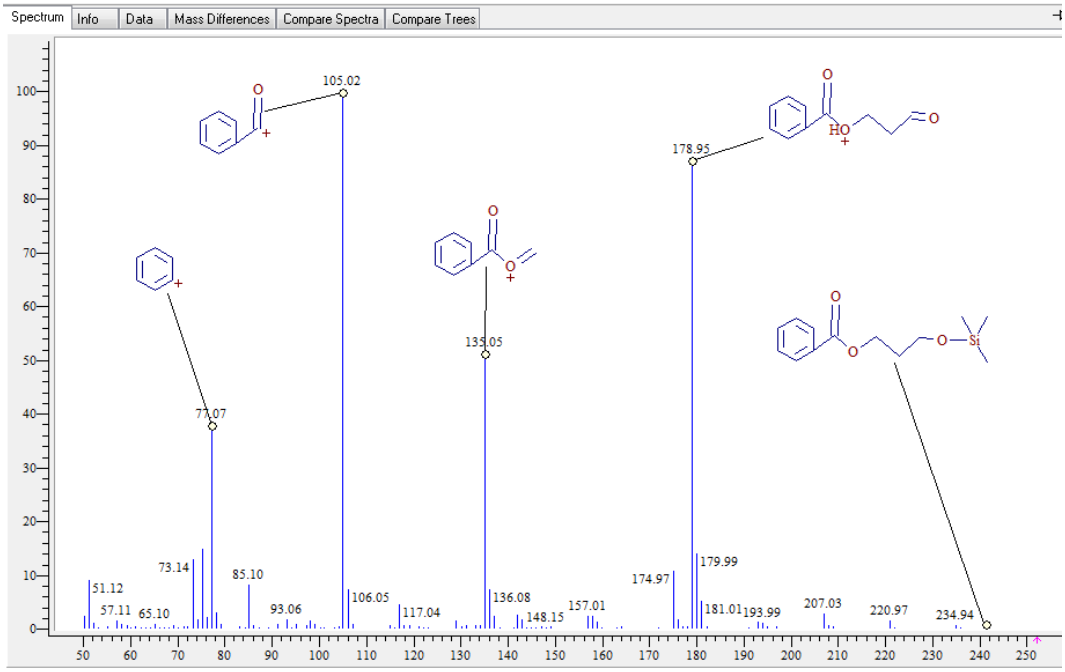


Figure 25. Mass spectrum of silylated metabolite obtained from silylated extract of degraded B_2-540. Characteristic mass/charge ratios have been annotated with the corresponding fragments according to the breakdown pattern of 3-((trimethylsilyl)oxy)propyl benzoate.

4.4. Toxicity

Changes in broth toxicity were monitored during degradation of B_2-540 using the Microtox® acute toxicity test. **Figure 26** summarizes this the changes in relative toxicity alongside the degradation of B_2-540. Broth toxicity is indicated by the left vertical axis in **Figure 26 (a).** Here, toxicity was presented as the inverse of the Microtox® EC_{50} because this was a more intuitive way of

evaluating relative toxicity differences (i.e. higher values correspond to a greater toxicity). The area ratios of metabolite #1 (retention time: 9.6 minutes) and metabolite #2 (retention time: 8.9 minutes) are indicated by the right vertical axis. The left vertical axis of **Figure 26 (b)** indicates the normalized concentrations of the identified methyl esters, and the cumulative area ratio of the larger, unidentified fragments are indicated on the right.

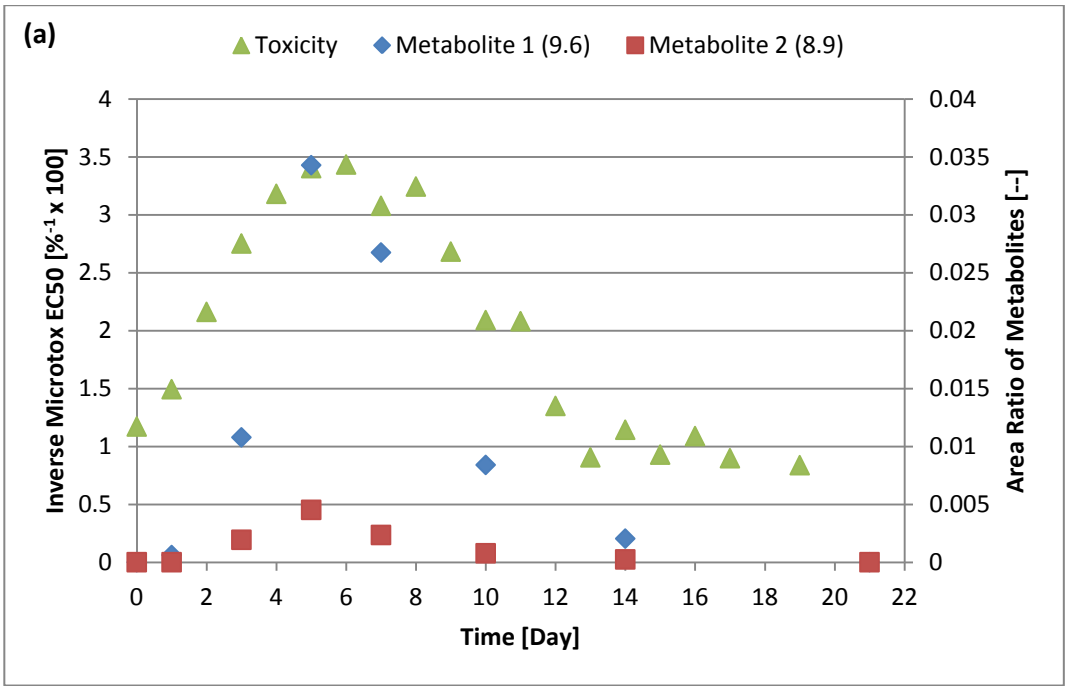


Figure 26. (a) Broth toxicity (as indicated by the inverse of the Microtox® EC_{50}) during the degradation of B_2-540 is represented by the left vertical axis. The right vertical axis indicates the area ratios of metabolite #1 and metabolite #2 over the course of the same experiment; data for alkaline and acidic extracts have been combined.

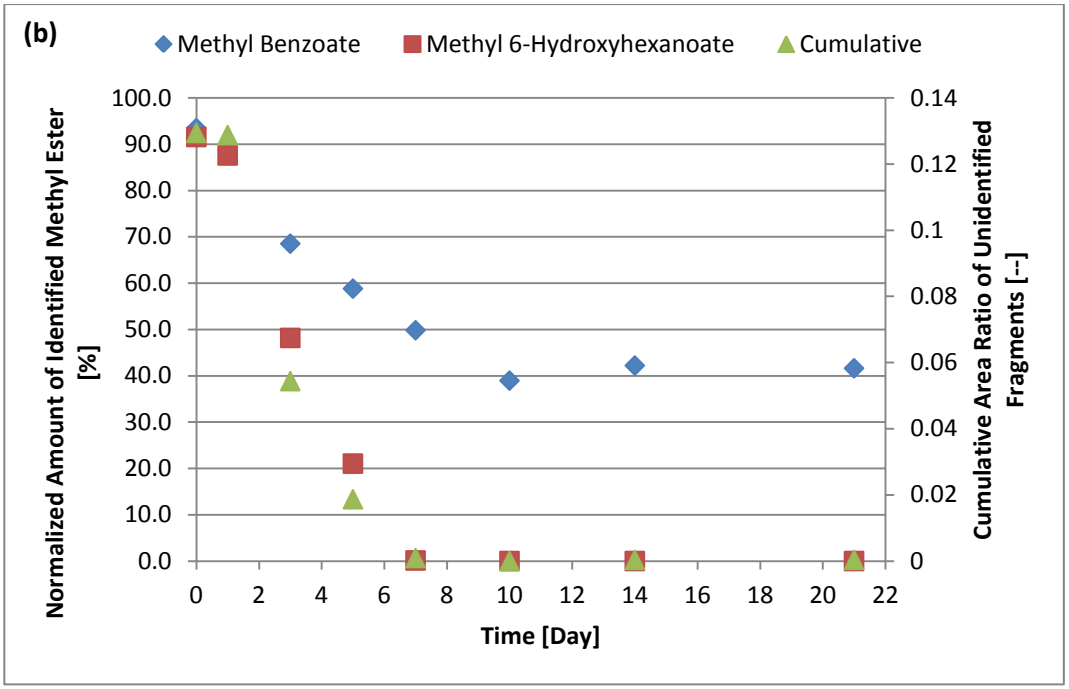


Figure 26. (b) Combined acidic and alkaline degradation data gathered during the toxicity studies for B_2-540. The left vertical axis represents the percentage of the initial amounts of methyl benzoate and methyl 6-hydroxyhexanoate normalized to values obtained for the abiotic controls. The right vertical axis indicates the cumulative area ratios of the larger, unidentified fragments (compounds 10.03, 10.83, and 13.75).

5. Discussion

5.1. Validation of lipid derivatization

While the relatively small compound, ε-caprolactone, could be quantified by direct injection into a GC, the boiling points of the poly([ε]-caprolactone)-based plasticizers are quite high due to their larger molecular weights and this made the use of gas chromatography to measure their concentrations problematic. The problem associated with the analysis of long-chain fatty acid components is frequently overcome by derivatization. In its simplest form, this technique changes the functional groups of a molecule to lower its boiling point. With compounds containing internal ester bonds, certain derivatization procedures can also break these internal bonds resulting in smaller, more volatile, fragments.

Numerous lipid derivatization techniques can be found in the literature (Stoffel, Chu et al. 1959; Bannon, Breen et al. 1982; Bannon, Craske et al. 1982; Liu 1994) as outlined in the introduction. A modified procedure for the methyl transesterification of the plasticizer molecules was developed, based on a method described by Comeau, et. al. (Comeau, Hall et al. 1988). The approach was selected for various reasons. The structures of the target plasticizer molecules made them suitable for transesterification. Each plasticizer molecule contains a minimum of four ester bonds, which represents a minimum of four distinct sites at which the molecule can be cleaved. Furthermore, methyl transesterification is one of the best understood, and is one of the safest to use, of the potential approaches. Most importantly, initial laboratory assessments of the technique gave reasonable and reproducible results.

During preliminary analyses in which B_2-540 was subjected to methyl transesterification and subsequently analyzed by GC, observable fragments included compounds with retention times matching those of methyl benzoate as well as methyl 6-hydroxyhexanoate. The detection of methyl benzoate indicated that the selected derivatization method was successful with regards to methyl transesterification of the ester bond linking the terminal benzoate group to the rest of the molecule. The presence of methyl-6-hydroxyhexanoate not only proved that this fragment could be transmethylated but, more importantly it proved that the ester bonds linking the ε-caprolactone monomer to the central diol were broken by the transesterification procedure.

At the same time, other fragments were observed, which were larger and unidentified. These resulted from the incomplete conversion of the parent compound by the described transesterification mechanism.

When a given amount of the plasticizer B_2-540 was subjected to the transmethylation reaction, a predictable amount of each of the product fragments were formed, and this resulted in an acceptable linear fit over a range of concentrations (see **Figure 4**). This is of particular importance considering that the conversion of the parent plasticizer compounds through the transmethylation procedure was demonstrated to be incomplete. While the linear fits were not forced through zero, each of the trend lines passed very close to the origin. Interestingly, the slope for each linear fit varied from one compound to another, which may explain some of the discrepancies observed in comparing the normalized concentrations of identified methyl esters between parent plasticizers of a given family. Regardless, **Figure 4** proved that all of these fragments,

identified and unidentified, were useful in monitoring changes in concentration for the breakdown of B_2-540 as well as the other larger plasticizer compounds.

5.2. Alkaline and acidic extractions

For the majority of the degradation experiments two different extractions were carried out on each of the samples, the first one under alkaline conditions and the second one under acidic conditions. Since the alkaline extractions were carried out first, almost all of the compounds detected were generally present in much greater abundance than for those of the acidic extractions. For example, during the degradation of B_2-540 the concentration of methyl benzoate in the alkaline extract (**Figure 6 (a)**) was nearly twice that of the acidic extract (**Figure 6 (b)**). Moreover, the same figures reveal concentrations of methyl 6-hydroxyhexanoate in the alkaline extract that were two orders of magnitude higher than for the acidic extracts.

One consequence of this was that there appeared to be more scatter in the concentration data for methyl 6-hydroxyhexanoate from the acidic extractions. Upon closer inspection, these fluctuations were small in relation to the overall concentration. The fact that negligible amounts of methyl 6-hydroxyhexanoate were detected in the acidic extract for the degradation of B_2-540 may indicate one of two things. Either the initial compound that contained the ε-caprolactone monomer (either the parent molecule or one of its breakdown products) was completely extracted during the alkaline extract, or this compound preferred the aqueous phase when there was a high proton concentration.

For the degradation of B_2-769, roughly an equal amount of methyl benzoate originated from both alkaline and acidic extracts, whereas the alkaline extract gave more methyl 6-hydroxyhexanoate than did the acidic extract (**Figure 8 (a)** and **(b)**). One unexpected result was that the amount of methyl 6-hydroxyhexanoate in the acidic extract increased at first (**Figure 8 (a)**). This initial increase could be explained if the original source of this particular methyl ester had changed over time as the result of degradation into a similar compound that was extracted at acidic pH more readily. Alternatively, partial breakdown of the molecule could have allowed for greater transmethylation, which resulted in an increasing amount of methyl 6-hydroxyhexanoate at first. In any case, the amount of methyl 6-hydroxyhexanoate began to decrease again after about one week.

Another point of interest is that one of the larger, unidentified fragments (retention time of 10.83 minutes) was the only one of the larger, unidentified fragments that was observed in both the alkaline and acidic extracts during the degradation of B_2-540 (**Figure 7 (a)** and **(b)**). The other two fragments were not detectable whatsoever in the acidic extract, and as a result were not shown. Contrarily, another of the larger, unidentified compounds (retention time 13.75 minutes) was detected in both alkaline and acidic samples obtained during the degradation of B_2-769 (**Figure 9 (a)** and **(b)**). The same result was obtained for the degradation of B_2-997 (**Figure 11 (a)** and **(b)**). This was interesting because this compound was not observed in the acidic extracts during the degradation of B_2-540. This likely arose as a consequence of the abundance of compound 13.75, as it appeared in greater quantities during the degradation of B_2-769 and even

more so during the degradation of B_2-997. Accordingly, this fragment was considered to be related to the ε-caprolactone arm, whose average length for B_2-769 is twice that of B_2-540 and thrice that of B_2-540 for B_2-997.

For degradation studies of the compound designated O_2-613, there was no detectable methyl 6-hydroxyhexanoate in any of the acidic extracts (**Figure 12 (b)**). In contrast to this, an appreciable amount of methyl 6-hydroxyhexanoate was detected in acidic extracts during the breakdown of O_2-841 (**Figure 13 (b)**). The discrepancy may be attributed to the increased amount of ε-caprolactone present in the compound O_2-841, which was twice that of O_2-613. Alternatively, this could be explained if the extraction efficiency of O_2-841 was significantly lower than that of O_2-613. At the same time, trace amounts of methyl 6-hydroxyhexanoate were detected in acidic extracts for degradation studies of the compound O_3-897 (**Figure 14 (b)**). This compound contained ε-caprolactone in quantities greater than O_2-613 yet less than O_2-841. Accordingly, the observations indicated that the detection of methyl 6-hydroxyhexanoate in the acidic extracts was dependent on the abundance of ε-caprolactone in the parent plasticizer compound.

In general, the majority of the observed compounds were detected in both extracts, alkaline and acidic. Sometimes, a particular compound was only extracted during the acidic extraction and this must be related to the structure of the compound. However, in most cases, the important numbers were the sums of the concentrations from the two extractions. These data were used to determine the degree of degradation and the appearance of metabolites.

5.3. Degradation of ε-caprolactone

Samples from the degradation of the simple compound, ε-caprolactone, by *R. rhodochrous* were extracted solely at pH 3-4 (using HCl to adjust) and were analyzed without the use of the transmethylation procedure prior to injection. It was clear from **Figure 5 (a)** and **(b)** that ε-caprolactone was rapidly degraded by *R. rhodochrous*, both with and without n-hexadecane as the additional carbon source, as indicated by the absence of detectable ε-caprolactone by the fourth day in both experiments. The apparent ease and rapidity with which ε-caprolactone was degraded is in agreement with the findings of other studies which demonstrated poly(lactones) to be readily biodegraded (Domb, Kost et al. ; Middleton and Tipton 2000; Nair and Laurencin 2007).

Both figures emphasize a crucial point, that *R. rhodochrous* was capable of completely degrading ε-caprolactone without the need for a secondary carbon source. The bacterium preferentially degraded ε-caprolactone, as indicated by **Figure 5 (b)** in which all of the ε-caprolactone was degraded before all of the n-hexadecane. Accordingly, all degradation experiments for the poly([ε]-caprolactone)-based plasticizers were carried out without the use of a secondary carbon source.

The abiotic controls for this compound and all of the other compounds showed that all of the degradation was due to the presence of *R. rhodochrous*.

5.4. Degradation of benzoate-terminated PCL-based plasticizers

Samples for degradation of the smallest benzoate-terminated PCL-based plasticizer, B_2-540, were analyzed by first subjecting them to transmethylation prior to injection. The data from **Figure 6 (a)** and **(b)** indicates that the interior of the plasticizer molecule (as represented by the presence of methyl 6-hydroxyhexanoate) was fully degraded.

Figure 6 (b) shows that a considerable and consistent amount of methyl benzoate was detectable, even at the end of the experiment, in the acidic extract. At the same time, **Figure 6 (a)** shows that the amount of methyl benzoate in the alkaline extract had disappeared completely by about day 10. It was clear that the stable remnant of the plasticizer molecule that contributed the methyl benzoate after transmethylation was extracted exclusively at low pH . This implied that the structure of the stable remnant was either quite small, or contained a functional group capable of becoming protonated – probably a carboxylic acid.

The larger B_2-769 had an average of n=2 repeating ε-caprolactone units per arm compared to the average of n=1 for B_2-540. The concentrations of methyl benzoate and methyl 6-hydroxyhexanoate obtained from alkaline and acidic extracts are shown in **Figure 8 (a)** and **(b)**, for the degradation of B_2-769 as well as for the abiotic controls. The data for the alkaline extracts indicated a steady reduction in the concentrations of both methyl esters until 14 days, after which no more observable degradation occurred. In contrast, all of the data points from the acidic extracts contained a consistent amount of methyl benzoate.

The largest benzoate-terminated plasticizer, B_2-997, contained an average of n=3 repeating ε-caprolactone units per arm. **Figure 10 (a)** and **(b)** show the alkaline and acidic extract concentrations of methyl benzoate and methyl 6-hydroxyhexanoate as the degradation experiment proceeded as well as for abiotic controls. The relatively consistent concentrations of methyl benzoate and methyl 6-hydroxyhexanoate for each of the time points suggested that very little degradation took place, if any at all. The concentration data for methyl benzoate and methyl 6-hydroxyhexanoate alone made it difficult to conclude whether or not degradation of B_2-997 had taken place.

Along with the recalcitrance of B_2-997, the detection of a constant amount of methyl benzoate in the acidic extracts for each of the time points during degradation studies of B_2-540 and B_2-769 signified that the bacteria were unable to fully mineralize the benzoate-terminated plasticizers. Furthermore, a metabolite from this degradation was resistant to degradation and this had to be an ester of benzoic acid in order to be transesterified to produce methyl benzoate.

Interestingly, during degradation studies for B_2-540 the concentration of methyl benzoate in the alkaline extract was reduced to zero by about day 10. This indicated that the stable remnant of the original plasticizer could only be extracted under acidic conditions. In a similar manner, the concentration of methyl benzoate in the alkaline extract for degradation studies of B_2-769 decreased considerably while the concentration in the acidic extract remained stable throughout. In the case of the degradation of B_2-540, methyl 6-hydroxyhexanoate was completely degraded even though methyl benzoate was still detectable in appreciable

amounts by the end of the experiment. This suggested that the resistance to degradation was in fact contributed by the benzoate groups of the molecule.

Figure 7 (a) and **(b)** show the evolution of the larger, unidentified fragments of the plasticizer designated B_2-540 over the course of a typical degradation study. These larger, unidentified fragments arose as a result of the methylation reaction. The data shown in **Figure 7 (a)** and **(b)** correspond to alkaline and acidic extracts, respectively. In both of these figures it is clear that the area ratios of all the larger, unidentified fragments diminished until these fragments were no longer detectable after a time period of about one week. The disappearance of these larger, unidentified fragments was especially significant because it proved that all of the parent compound was being metabolized.

Over the course of the degradation studies for B_2-769, **Figure 9 (a)** and **(b)** show the evolution of the three largest, unidentified fragments from the alkaline and acidic extracts. As was the case with the identified methyl esters observed during this experiment (**Figure 8 (a)**), the amounts of all three larger, unidentified fragments present in the alkaline extracts decreased dramatically until about day 14 followed by no further degradation. The amounts of these larger, unidentified fragments found in the acidic extracts, while somewhat lower than those in the alkaline extracts, also decreased considerably but each was still detectable by the end of the experiment. Overall, the prevalence of these larger, unidentified fragments showed that the degradation of B_2-769 was incomplete.

Figure 11 (a) and **(b)** show the area ratios of the larger, unidentified fragments for degradation experiments of B_2-997 as well as for abiotic controls (alkaline and acidic extracts, respectively). The area ratios for all of the larger,

unidentified fragments (10.03, 10.83, and 13.75) diminished a small but noticeable amount over time in the alkaline extracts. This was important because, even though the trend was subtle, it showed that degradation of the parent compound B_2-997 had, in fact, taken place to some extent.

5.5. Degradation of 1,6-hexanediol dibenzoate

Degradation experiments were carried out for 1,6-hexanediol dibenzoate since it had a similar molecular structure to that of the benzoate-containing PCL plasticizers which exhibited resistance to degradation. The results were analyzed first without and then with the use of methyl transesterification, prior to injection into the GC (see **Figure 23** and **Figure 24**, respectively). From **Figure 23**, it is clear that 1,6-hexanediol dibenzoate was nearly completely degraded over the experimental timeframe, as indicated by the diminishing area ratio.

5.6. Degradation of octanoate-terminated PCL-based plasticizers

Samples for degradation of the octanoate-terminated PCL-based plasticizers were analyzed by first subjecting them to transmethylation prior to injection. **Figure 12 (a)** and **(b)** show that O_2-613 was essentially completed degraded, as indicated by the reduction in the concentrations of methyl octanoate and methyl 6-hydroxyhexanoate to almost zero after roughly five days.

The data for the alkaline extracts during degradation of the larger O_2-769 (**Figure 13 (a)**) indicated a rapid reduction in the concentrations of both methyl esters to near zero after only about three days. In contrast, the concentrations of

the identified methyl esters in the acidic extracts (**Figure 13 (b)**) appeared to decrease more slowly over the entire course of the experimental timeframe.

During degradation of the three-armed, octanoate-terminated PCL-based plasticizer, O_3-897, the concentrations of both methyl octanoate and methyl 6-hydroxyhexanoate decreased to nearly zero after only one day. This was observed for alkaline as well as acidic extracts (**Figure 14 (a)** and **(b)**). This indicated that degradation was very rapid and nearly complete. Very low, albeit consistent, quantities of methyl octanoate were detected thereafter while only trace amounts of methyl 6-hydroxyhexanoate could be detected after the first day.

Interestingly, there were no larger, unidentified fragments detected for any of the octanoate-terminated PCL-based plasticizers, even for the abiotic controls.

Most importantly, it was demonstrated that the octanoate-terminated PCL-based plasticizers were much easier to degrade than those containing the benzoate groups. The experiments showed that all three compounds from the octanoate-terminated family were fully degraded within one week.

5.7. Long-term degradation of B_2-997

Because appreciable amounts of B_2-997 remained at the end of the original experiments, longer degradations of 30 days were carried out. Even at the lowest concentration, appreciable amounts of the compound were still present after 30 days. The decreases are small but it did seem that there was a slightly larger amount of the target compound removed by the longer time period. However, the overall conclusion is that B_2-997 is resistant to biodegradation.

5.8. Metabolites

During the degradation of ε-caprolactone, distinct, new peaks were observed in the chromatograms that were not observed in those of the abiotic controls. One new peak was observed in samples that did not include n-hexadecane as the additional carbon source. Accordingly, this was considered to represent a metabolite resulting from the breakdown of ε-caprolactone. This newly observed peak (retention time: 3.5 minutes) was only detectable in trace amounts and as a result there was apparent scatter in the concentration data for this metabolite. A different new peak was observed in studies that included n-hexadecane. This new peak (retention time: 8.9 minutes) was present in a much higher amount and appeared to be mostly degraded within 8 days. Thus, the experiments revealed that while some new compounds were formed during the breakdown of ε-caprolactone, they did not accumulate.

During the degradation of B_2-540, two new peaks were observed on the gas chromatograms which were not detected in the abiotic controls. Correspondingly, it follows that these newly observed peaks represent metabolites resulting from degradation by *R. rhodochrous*. The metabolite with a retention time of 9.6 minutes was detected in much greater quantities than that with a retention time of 8.9 minutes (the area ratio is nearly an order of magnitude greater). Accordingly, the compound corresponding to 9.6 minutes will be referred to as metabolite #1 and that to 8.9 minutes will be referred to as metabolite #2. Most importantly,

despite their emergence, the metabolites themselves were completely degraded in turn.

Metabolites #1 and #2 were also both observed in the degradation studies done with B_2-769, albeit they were present in lower quantities (by area ratio) compared to those measured for B_2-540. The metabolites that were observed during the breakdown of B_2-769 were also completely degraded by the end of the experiment. For systems with B_2-997, virtually no metabolites could be detected, which may be a direct consequence of the slow rate at which this compound was degraded. In other words, the rate of breakdown of any intermediates was slower than the rate of degradation of the parent compound.

Metabolite #1 (9.6 minutes) was only observable in the first, alkaline extract in trace amounts. However, appreciable amounts were seen in the second, acidic extraction. Reversing the extraction order had no impact on this result. This suggested that the metabolite #1 contained functional groups capable of becoming protonated, such as a carboxylic acid. Upon ionization with mass spectrometry, metabolite #1 gave a fragmentation pattern that contained characteristic mass-to-charge ratios indicative of the aromatic benzene ring.

During the degradation of 1,6-hexanediol dibenzoate, a new compound, which was not present in any of the abiotic controls was detected with a retention time of 9.9 minutes. When subjected to the methyl transesterification reaction, the retention time of this metabolite shifted to 9.6 minutes, which is the same as that of metabolite #1 observed during the breakdown of B_2-540 and B_2-769. Thus, they may be the same compound.

Metabolite #2 was not detected in the first, alkaline extracts for any of the experiments. This observation signified that the structure of the metabolite contained a functional group capable of becoming protonated. Upon ionization with mass spectrometry, metabolite #2 resulted in a fragmentation pattern that contained predominant mass-to-charge ratios characteristic of an ester of benzoic acid (e.g. m/z: 135, 105, 77). These samples will need to be analyzed by GC/MS for further clarification.

While these new compounds may have appeared in the gas chromatogram in the form of methyl esters occurring from the derivatization reaction, it is still entirely possible that these newly observed compounds were actually the metabolites themselves in their unaltered state. In any case, both metabolites #1 and #2 exhibited degradation that was slow enough for them to accumulate at first, but complete enough that they were ultimately degraded in the end. Further analysis with GC/MS will be required in order to completely identify metabolites #1 and #2 with acceptable certainty.

In order to gather more information about these metabolites, samples taken from the degradation of B_2-540 were subjected to the alternate derivatization technique of silylation prior to analysis with GC/MS. Using this method, only one metabolite was detected in any appreciable amount. Based on the mass spectrum, this trimethylsilylated compound was believed to be 3-((trimethylsilyl)oxy)propyl benzoate (see **Figure 25**), indicating that one of the metabolites of the degradation of B_2-540 was 3-hydroxypropyl benzoate.

Degradation experiments involving the octanoate-terminated PCL plasticizers yielded no observable metabolites. Any intermediary metabolites that resulted

from the degradation of the octanoate-terminated plasticizers were likely further metabolized much too rapidly to be detected. This made sense in light of the completeness and rapidity with which the identified methyl esters disappeared over the course of degradation.

In contrast, B_2-997 gave no detectable metabolites likely due to the recalcitrance of the compound.

5.9. Comparison of the degradation of PCL-based plasticizers

Figure 19 compares typical degradation experiments for the three benzoate-terminated PCL plasticizers designated B_2-540, B_2-769, and B_2-997. The consolidated data for the concentrations of the methyl esters (from both alkaline and acidic extracts) have been expressed as percentages of the expected amount (based on initial amount of plasticizer added). Since the percentages of the expected amount obtained were low due to incomplete conversion of the parent compound, they were normalized to average values obtained for the abiotic controls. Since values obtained for the abiotic controls were in some cases moderately variable, the normalized amounts of a given compound presented for the day zero time point were in some cases slightly above or below 100%. Furthermore, the area ratios of the larger, unidentified fragments were combined into one cumulative area ratio for each sample. The most important results of the degradation experiments for the three benzoate-terminated PCL plasticizers shown in **Figure 19** are also summarized in **Table 3**.

For all three of the benzoate containing compounds, the degree of degradation was greater for the internal portion of the molecule (represented by methyl 6-hydroxyhexanoate) compared to that of the terminal benzoate portions (represented by methyl benzoate). The benzoate-terminated plasticizer that exhibited the most rapid and complete degradation, B_2-540, was also the smallest. In contrast, the larger B_2-769 was slower to degrade and the extent of degradation observed over the experimental timeframe was considerably less than that of B_2-540. It is evident that B_2-997 was even more recalcitrant than B_2-769, with very small amounts of degradation taking place over a long period of time. Overall, the general trend emerged that as the chain length of the molecule was increased, the more difficult the plasticizer was for *R. rhodochrous* to degrade.

Figure 20 compares typical degradation data for systems containing the three octanoate-terminated PCL plasticizers designated O_2-613, O_2-841, and O_3-897. For all three compounds, nearly 80% of the original methyl octanoate and methyl 6-hydroxyhexanoate had disappeared by the third day. By day seven, they were all virtually completely degraded. These findings agree with those determined experimentally by Shi et al. (Shi, Cooper et al. 2011), in which degradation of the octanoate-terminated PCL-based plasticizers were demonstrated to occur several times more rapidly than for the benzoate-terminated PCL-based plasticizers.

It is not surprising that there were no stable metabolites observed for these compounds. This is consistent with the rapid biodegradation. The key observation was that the octanoate-terminated PCL-based plasticizers were degraded completely, compared to the benzoate-terminated plasticizers which exhibited recalcitrance. A simple substitution of the benzoate terminal with an octanoate

group resulted in complete degradation of the plasticizer compound. While there was significant degradation of the benzoate-terminated PCL-based plasticizers, this stills resulted in appreciable amounts of stable metabolites which means that the benzoate terminals would not be good choices as green plasticizers.

5.10. Toxicity

Previous studies had shown that the degradation of the commercial plasticizer, bis 2-ethylhexyl adipate, led to the formation of stable metabolites which contributed significant toxicity to the degradation broth (Nalli, Cooper et al. 2002). A similar study was done with B2-540. It is clear that the fermentation broth initially exhibited minimal toxicity as indicated by the inverse of the EC_{50}. However, as degradation proceeded, there was an increase in toxicity up to a maximum, which was consistent with the rising metabolite area ratios. The toxicity of the broth then dropped at the same time that the concentrations of the metabolites began to decrease. It is clear that these metabolites contributed considerable toxicity However, during the later part of the experiment, the concentrations of the metabolites decreased and this decrease was accompanied with a decrease in the toxicity. In fact, the final level of toxicity was less than the initial value at day-zero.

While the slow biodegradation of the benzoate plasticizers is a problem, at least with respect to the toxicity test used here, it did not have any toxic affect. While it did slowly produce more toxic metabolites, these were seen to be biodegradable in a relatively short period of time.

6. Conclusion

The defining criteria of a green plasticizer are that it is not toxic and does not degrade into stable and toxic by-products.

The derivatization method developed in this thesis made it possible to easily monitor the concentrations of otherwise difficult to assess potential plasticizers. This, in turn, resulted in measurements that demonstrated trends in biodegradation of the parent compound as well as metabolite degradation.

The octanoate-terminated PCL plasticizers were degraded rapidly and completely with no stable metabolites being detected, making them ideal candidates for a 'green' PVC plasticizer. Not only did the benzoate-terminated PCL-based plasticizers degrade more slowly than those that were octanoate-terminated, their degradation also led to the formation of transient metabolites which contributed some toxicity to the broth in comparison to the parent compound.

7. Limitations of the study and consideration for future work

The research presented in this thesis only considered concentration data post-transmethylation. The transmethylation reaction was necessary to allow an indirect quantitative analysis of the residual concentration of plasticizers by GC. Accordingly, this approach does not provide any information about the origin of the compounds that were monitored, it was only concerned with their fate. Furthermore, it was impossible to discern whether the observed metabolites were unadulterated or if they were structurally altered by transmethylation. It would be interesting to determine the nature of these metabolites with a great level of certainty. I have begun preliminary investigations using gas chromatography/mass spectrometry, some of which are ongoing and could not be included in this thesis. These investigations should be continued in order to confirm the nature of these unknown metabolites.

The extraction efficiency was another limitation which effected the interpretation of the data. For example, the identified methyl esters and the larger, unidentified fragments of the parent compounds were often detected in both the alkaline and acidic extracts. This confounded the interpretation of results since it was unclear whether or not their detection in both extracts was due to poor extraction efficiency. It could also have been due to the evolution of different products of degradation in the media which were extracted at different levels of acidity but contributed the same methylation products post-transesterification.

While some data on the mechanical properties and biodegradation of these potential 'green' plasticizers has already been published, toxicology data has yet to

be studied. It would be of interest to examine the potential toxic effects of the best candidate plasticizers on mammalian cell lines, for example. Another aspect that has yet to be researched is the rheological data of the PVC/plasticizer melts. These measurements are necessary for optimizing the processing of plasticizer and PVC blends and estimating the retention of plasticizing properties over the material's working life span. Accordingly, it would be of interest to evaluate the viscoelastic response to stress and strain of the best candidates identified as potential 'green' alternatives to DEHP, namely the octanoate-terminated PCL-based compounds.

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