Towards the Development of Green Plasticizers

By

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Abstract

Research was conducted to investigate the effect of chemical functional groups, including the ether function and alkyl branches, on the biodegradation mechanisms and biodegradation rates of dibenzoate plasticizers. Biodegradation of 1,6-hexandiol dibenzoate, a potential green dibenzoate plasticizer, by *Rhodococcus rhodochrous,* was investigated in the presence of hexadecane as a primary carbon source. The metabolites, produced in the biodegradation process were detected using GC/MS and Fourier transform mass spectroscopy techniques. None of these metabolites were stable, with all tending to biodegrade over the course of the experiments. Biodegradation mechanisms were elucidated for 1,6-hexanediol dibenzoate and two commercial plasticizers, diethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB). Biodegradation of all of these plasticizers was initiated by hydrolysis of one ester bond to release a monobenzoate and benzoic acid. It was demonstrated that the diol fragment of 1,6-hexanediol monobenzoate was processed via a β-oxidation pathway, which was not possible for diethylene glycol monobenzoate (D(EG)MB) and dipropylene glycol monobenzoate (D(PG)MB) due to the presence of an ether function in the diols. Thus, accumulation of D(EG)MB and D(PG)MB was observed in the biodegradation broth.

The biodegradation of commercial plasticizers, D(EG)DB and D(PG)DB and three alternative plasticizers, 1,3-propanediol dibenzoate, 2,2-methyl-propyl-1,3-propanediol dibenzoate and 1,6-hexanediol dibenzoate, were modeled using a Michaelis-Menten/Monod-type kinetic model. Biodegradation was conducted in an aerated bioreactor using resting cells of *Rhodococcus rhodochrous*, which had been grown with hexadecane as the primary substrate. Monobenzoates released from the biodegradation of commercial plasticizers degraded slower than the monobenzoates of alternative plasticizers. The rapid biodegradation of monobenzoates released from microbial hydrolysis of alternative dibenzoate plasticizers was attributed to the lack of an ether bond in these compounds.

The acute toxicities of the above dibenzoate plasticizers and their associated metabolites were examined using the Microtox[TM] toxicity assay. High acute toxicities were observed for D(EG)DB, D(PG)DB and their corresponding hydrolytic metabolites, D(EG)MB and D(PG)MB, in response to Microtox[TM] assay. However, the alternative plasticizers, 1,6-hexanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate, did not exhibit toxicity.

This study represents an important step toward the development of "green plasticizers" of lower toxicity and health impacts and reduced persistence in the environment.

Sommaire

Des recherches ont été réalisées pour étudier l'effet des groupes chimiques fonctionnels, y compris la fonction éther et les branches d'alkyle, sur les mécanismes de biodégradation et les taux de biodégradation des plastifiants dibenzoate. La biodégradation du 1,6-dibenzoate hexanediol, un plastifiant dibenzoate potentiel, par *Rhodochrous rhodococcus*, a été étudiée en présence d'hexadécane comme source de carbone primaire. Les métabolites, produits dans les processus de biodégradation ont été détectés par GC/MS et techniques de spectroscopie de masse à transformée de Fourier. Aucun de ces métabolites ne sont stables, tous avaient une tendance à la dégradation durant les expériences. Les mécanismes de biodégradation ont été élucidés pour le dibenzoate de 1,6-hexanediol et de deux plastifiants commerciaux, le dibenzoate de diéthylène glycol (D(EG)DB) et le dibenzoate dipropylèneglycol (D(PG)DB). La biodégradation de l'ensemble de ces plastifiants a été initié par hydrolyse d'une liaison ester pour libérer un monobenzoate et de l'acide benzoïque. Il a été démontré que le fragment de 1,6-diol monobenzoate hexanediol est généré par une β-oxydation, ce qui n'était pas possible pour le monobenzoate diéthylène glycol (D(EG)MB) et le monobenzoate dipropylèneglycol (D(PG)MB) en raison de la présence d'une fonction éther dans les diols. Ainsi, l'accumulation de D(EG)MB et D(PG)MB a été observée dans le bouillon de biodégradation.

La biodégradation des plastifiants commerciaux, D(EG)DB et D(PG)DB et trois plastifiants de remplacement, le dibenzoate de 1,3-propanediol, le dibenzoate de 2,2-méthyl-propyl-1propanediol et le dibenzoate de 1,6-hexanediol, a été modélisée à l'aide d'un modèle cinétique Michaelis-Menten/Monod-type. La biodégradation a été effectuée dans un bioréacteur aéré à l'aide de cellules au repos *Rhodochrous rhodococcus*, qui avaient été cultivées avec l'hexadécane comme substrat primaire. Les monobenzoates libérés par la biodégradation des plastifiants commerciaux sont dégradés plus lentement que le monobenzoates de plastifiants de remplacement. La biodégradation rapide de monobenzoates libérés

par l'hydrolyse microbienne des plastifiants de remplacement dibenzoate a été attribuée à l'absence d'une liaison éther dans ces composés.

Les toxicités aiguës des plastifiants dibenzoate cités ci-dessus et de leurs métabolites associés ont été examinées en utilisant le test de toxicité MicrotoxTM. Des toxicités aiguës élevées ont été observées pour D(EG)DB, D(PG)DB et de leurs métabolites correspondant hydrolytique, D(EG)MB et D(PG)MB, en réponse au dosage Microtox^{TM}. Toutefois, les plastifiants de remplacement, le dibenzoate de 1,6-hexanediol et le benzoate de 2,2 -méthyl-propyl-1,3-diol ne présentaient pas de toxicité.

Cette étude représente une étape importante vers le développement de «plastifiants verts", de la réduction de leur toxicité de leurseffets sur la santé et de leur persistance dans l'environnement.

"Mind does its fine-tuning, hair-splitting

but no craft or art begins

or can continue without a master

giving wisdom into it"

    Rumi (1207 –1273)

Acknowledgments

Contributions of Authors

This thesis includes chapters containing the manuscripts of three articles, of which two are published and one will be submitted for peer review, as follows:

(1) Azadeh Kermanshahi pour, Orval A. Mamer, David G. Cooper, Milan Maric and Jim A. Nicell (2009) "Metabolites from the biodegradation of 1,6-hexanediol dibenzoate, a potential green plasticizer, by *Rhodococcus rhodochrous*", *Journal of Mass Spectrometry*, 44,662-671.

(2) Azadeh Kermanshahi pour, Orval A. Mamer, David G. Cooper, Milan Maric and Jim A. Nicell (2009) "Mechanism of biodegradation of dibenzoate plasticizers", *Chemosphere*, 77,258-263.

(3) Azadeh Kermanshahi pour, Ranjan Roy, David G. Cooper, Milan Maric and Jim A. Nicell. "Biodegradation kinetics of dibenzoate plasticizers". To be submitted.

A fourth article, as follows, is presented in a chapter of the thesis as a short note and will be submitted for publication following the completion of a series of additional studies involving the collaboration of other students:

(4) Azadeh Kermanshahi pour, David G. Cooper, Milan Maric and Jim A. Nicell. "Preliminary assessment of the toxicities of dibenzoate plasticizers and their metabolites". To be submitted.

The PhD candidate, Azadeh Kermanshahi pour was responsible for designing and performing experiments, analytical method development, data analysis, interpretation of results, and drafting the manuscripts for publication.

Prof. David G. Cooper (Department of Chemical Engineering, McGill University), Prof. Milan Maric (Department of Chemical Engineering, McGill University) and Prof. Jim A. Nicell (Department of Civil Engineering & Applied Mechanics, McGill University) were co-supervisors of the candidate over the course of this project. They collaborated in the definition of the objectives and scope of the project and contributed to supervision of the work, the design of

experiments, aided in the interpretation of results, and assisted in the proofreading, editing and final revisions of the paper manuscripts and thesis.

With respect to the first and second paper, Dr. Orval A. Mamer (Director, Mass Spectrometry Unit, Montreal Genomics and Proteomics Centre, Faculty of Medicine, McGill University) was responsible for GC/MS analyses and contributed to the interpretation of the GC/MS results and drafting of the manuscripts for publication. Interperation of the GC/MS results was done in a collabrative work of Azadeh Kermanshahi pour and Dr. Orval A. Mamer.

With respect to the third paper, Mr. Ranjan Roy (Laboratory Technician, Department of Chemical Engineering, McGill University) contributed to GC/MS analyses for metabolite identification. Azadeh Kermanshai pour was responsible for the interepration of the GC/MS results.

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1. Introduction

The concept of plasticizers was first introduced through application of natural camphor and castor oil for plasticizing celluloid or celluloid lacquers in the late nineteenth century (Gachter and Muller, 1990). Since that time, the market for plasticizers has evolved considerably in response to increasing demands for the use of polymers worldwide. As a result, several classes of plasticizers have been developed for commercial applications ranging from automotive industries to medical devices and consumer products (Rahman and Brazel, 2004). Plasticizers broadly influence polymer properties such as glass transition temperature, tensile strength, melt viscosity and modulus and thus impart flexibility and ease of processing to polymeric materials (Wypych, 2004). These important effects warranted the broad application and high production rate of plasticizers for products in cable production, flooring materials and food packaging films, and they now account for one third of the plastic additives consumed (Lerner, 2003). The global demand for plasticizers was 10.1 billion pounds in 1999 and has been estimated to be growing by approximately 2.8% annually through the early 2000s (Lerner, 2003).

In recent years, criteria used to assess the effectiveness of plasticizers have changed to meet the new challenges in plasticizer markets. The development and adoption of a plasticizer used to be primarily driven by cost and technical performance (Wypych, 2004). However, due to high volume production of plasticizers, their broad applications in industrial and consumer materials (Rahman and Brazel, 2004; Wypych, 2004), and their confirmed ubiquitous presence in the environment (Staples et al., 1997; Horn et al., 2004; Barnabé et al., 2008; Beauchesne et al., 2008), extensive research has been conducted to study the health and environmental impacts of these chemicals.

For example, over the past decade, findings concerning the health and safety implications of some classes of plasticizers including phthalates and some chloroparaffins have resulted in the establishment of environmental regulations

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that govern or limit their use (Health Canada, 2002; European Union, 2005; Hileman, 2007). As a result, criteria used to assess existing plasticizers and develop new ones, must account for their potential short and long-term health and environmental impacts in addition to meeting the cost and technical end-use requirements as functional plasticizers.

Phthalates are the most commonly used plasticizers of the past century, and comprise 92% of the global production of plasticizers. Furthermore, di-(2-ethylhexyl) phthalate (DEHP), the most widely used plasticizer, accounts for 51% of the phthalates used (Murphy, 2001). Human exposure to plasticizers, such as phthalates, occurs due to medical procedures involving the use of intravenous bags and tubing (Tickner et al., 2001), inhalation of indoor air and consumption of plastic wrapped food (Larsen, 2004; Nalli et al., 2006a). Additional concerns associated with these compounds arise from the formation of metabolites of greater toxicity than the parent compound as a result of metabolism in the human body (Albro, 1975; Mitchell et al., 1985; Tickner et al., 2001). For example, mono-2-ethylhexyl phthalate (MEHP), which is a hydrolytic metabolite of di-(2-ethylhexyl) phthalate, is a known endocrine disruptor and peroxisome proliferator (Mitchell et al., 1985; Onorato et al., 2008). 2-ethylhexanoic acid, another metabolite of DEHP, is also a potent peroxisome proliferator (Cornu et al., 1992). These metabolites have been detected in plasma, blood and urine of humans and rats (Albro., 1975; Wahl et al., 2001; Wahl et al., 2004; Sathyanarayana et al., 2008).

The formation and accumulation of metabolites as a result of the interaction of phthalates with common microorganisms were also observed in experiments with pure and mixed microbial culture under both aerobic and anaerobic conditions (Staples et al., 1997; Ejlertsoon et al., 1997; Roslev et al., 1998). The biodegradation of phthalates begins with hydrolysis of an ester bond resulting in the formation of a monoalkyl phthalate and the corresponding alcohol (Staples et al., 1997). The alcohol can also be oxidized to the corresponding carboxylic acid. Phthalates and their related metabolites have also been detected

in various environmental samples such as surface water, groundwater, precipitation, river sediments (Staples et al., 1997; Horn et al., 2004; Barnabé et al., 2008), in treated effluents from sewage treatment plants (Beauchesne et al., 2008), in indoor air (Nalli, 2006a), and in the tissues of living organisms (Staples et al., 1997).

Adipates are another common class of plasticizer of which di(2-ethylhexyl) adipate (DEHA) is the most frequently used plasticizer in this group (Wypych, 2004). Concerns have also been raised recently about these compounds. For example, metabolites originating from the hydrolysis of the ester bond of DEHA were detected in the urine of rats that had been administered DEHA or mono(2-ethylhexyl)adipate (Cornu et al., 1992). Adipic acid, 2-ethylhexanoic acid and 2-ethylhexanol were also identified following metabolism of DEHA (Cornu et al., 1992). 2-ethyl hexanoic acid was detected in the plasma of a human male after administering DEHA orally (Lofus et al., 1993). The metabolism of adipates by soil microorganisms also starts with ester hydrolysis to release monoalkyl adipate and the corresponding alcohol (Nalli et al., 2002). The degradation of DEHP and DEHA by common soil microorganisms and subsequent formation of 2-ethylhexanol and 2-ethylhexanoic acid is shown in Figure 1.1 that illustrates the results of earlier work (Horn et al., 2004).

In response to worldwide concerns about the potential environmental and health implications of phthalates and adipates, alternative plasticizers such as dibenzoates have been proposed (Rahman and Brazel, 2004; Wypych, 2004). For example, the benefits of low toxicity and high rates of biodegradation were reported for a blend of dibenzoate plasticizers, Benzoflex® 2888 (blend of diethylene glycol dibenzoate, triethylene glycol dibenzoate, and dipropylene glycol dibenzoate) (Arendt and Lang, 1998; Lang and Stanhope, 2001). In response to such findings, dibenzoates have been recently approved by the European Chemical Agency as alternatives to phthalates (Deligio, 2009). However, biodegradation of diethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB) by the common soil organism

Rhodotorula rubra resulted in incomplete microbial hydrolysis and the release of the corresponding monoesters di-ethylene glycol monobenzoate (D(EG)MB) and di-propylene glycol monobenzoate (D(PG)MB), respectively (Gartshore et al., 2003). These metabolites were resistant to further degradation and exhibited high acute toxicity as measured using the Microtox^{TM} toxicity assay (Gartshore et al., 2003).

Overall, these results point to the importance of assessing the health and environmental implications associated with alternative plasticizers. In addition, such assessments should not simply focus on evaluating the impacts of the parent plasticizers but those of their metabolites as well. Collectively, this means that if environmentally-benign dibenzoate plasticizers are to be designed, produced and commercialized, it is of great importance to identify the structural features that results in environmental persistence of these chemicals. In other words, the effect of chemical functional groups on biotransformation mechanisms and biodegradation rates should be investigated. To reach this goal, the full spectrum of metabolites created during the biodegradation process should be identified to establish the biodegradation pathways for existing conventional plasticizers and their potential "green" alternatives. Moreover, it is also necessary to study the biodegradation kinetics of alternative plasticizers to make it possible to compare the biodegradation rates of plasticizers and their corresponding metabolites with those of commercially available plasticizers. Such comparisons will provide a basis for choosing amongst potential plasticizers for their potential applications, their eventual large-scale production and commercialization.

Based on the above, the main objective of the research presented here was to develop an understanding of the effect of functional groups, including the ether function and alkyl branches, on the mechanisms and rates of biodegradation of diester plasticizers – in particular, dibenzoate plasticizers. It was hypothesized that these functional groups will influence the stability of the monoester metabolites of dibenzoate plasticizers. To explore this hypothesis, alternative dibenzoate plasticizers were synthesized and the full range of metabolites

produced from their interaction with *Rhodococcus rhodochrous*, a common soil microorganism, was studied and biodegradation mechanisms were established. Biodegradation kinetics was also studied to evaluate the influence of these functional groups on the biodegradation rate. The scope of the research included the following:

(1) The scientific and engineering literature was reviewed to summarize current advances and challenges in the design of biodegradable plasticizers. The requirements to develop environmentally benign plasticizers were also introduced. This review is presented in Chapter 2.

(2) A study was conducted to monitor the biotransformation of 1,6-hexanediol dibenzoate, a potential plasticizer, by *Rhodococcus rhodochrous*, a common soil organism, to identify the full range of metabolites created during biodegradation and to assess the biodegradability of the metabolites. This study is presented in Chapter 3, which was published as: Kermanshahi pour, A., O.A. Mamer, D.G. Cooper, M. Maric, and J.A. Nicell (2009) "Metabolites from the biodegradation of 1,6-hexanediol dibenzoate, a potential green plasticizer, by *Rhodococcus rhodochrous*." *Journal of Mass Spectrometry*. 44, 662-671.

(3) An investigation was done to examine the effect of an ether function on the biodegradation mechanisms of dibenzoate plasticizers. To achieve this, biodegradation mechanisms of the two commercial dibenzoate plasticizers, di-ethylene glycol dibenzoate and di-propylene glycol dibenzoate were compared with that of 1,6-hexanediol dibenzoate, a potential green plasticizer. The results of this investigation are presented in Chapter 4, which was published as: Kermanshahi pour, A., D.G. Cooper, O.A. Mamer, M. Maric, and J.A. Nicell (2009) "Mechanisms of biodegradation of dibenzoate plasticizers". *Chemosphere*. 77, 258-263.

(4) A study was conducted to assess the importance of ether bonds and alkyl branches on the biodegradation rates of plasticizers and their metabolites. This was done by modeling the biodegradation kinetics of these compounds and using the models to compare their biodegradation rates. The investigation involved biodegradation studies of selected dibenzoate plasticizers including two commercial plasticizers, D(EG)DB and D(PG)DB, and three alternative plasticizers; namely, 1,3-propanediol, 2,2-methyl-propyl-1,3-propanediol dibenzoate, and 1,6-hexanediol dibenzoate. The results of this study are presented in Chapter 5.

(5) In addition to the above, a preliminary study was conducted to assess the toxicities of selected dibenzoate plasticizers and their potential metabolites using the Microtox^{TM} toxicity assay. The plasticizers studied included the two commercial plasticizers, D(EG)DB and D(PG)DB, as well as 1,6-hexanediol dibenzoate, 1,3-propanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate. Also included in the study were their potential metabolites including monobenzoates and diols. The results of this preliminary investigation are presented in Chapter 6.
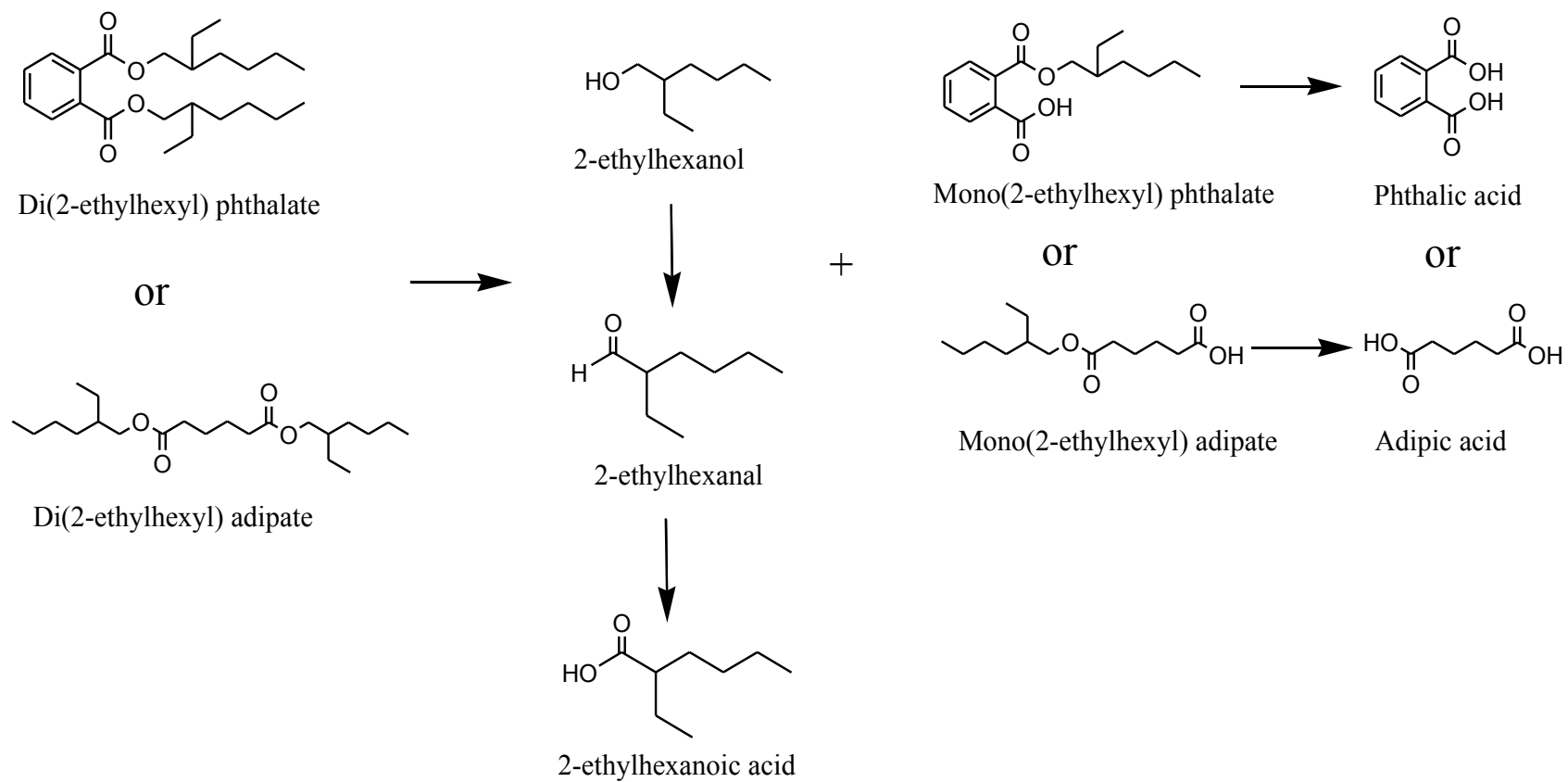
Figure 1.1. Biodegradation pathway of DEHP and DEHA resulting in the release of 2-ethylhexanol and 2-ethylhexanoic acid (Horn et al., 2004).

2. Literature review

2.1. Introduction

Advances in polymer engineering over the course of the past century have made polymers a major component in the products of numerous industries including those of automotive, cable, and medical device manufacturers. Due to their economic advantages, plastic materials have also found broad application in consumer products for replacing paper bags, metal and glass containers in the market (Rahman and Brazel, 2004). The main advantages of plastics over metal or glass include manufacturing cost, reduced weight and ease of processing and design flexibility (Tullo, 2006).

In response to the growing market demand for polymers, a wide variety of polymer additives has been developed to impart the desired characteristics to polymeric materials (Rahman and Brazel, 2004). Most notably, plasticizers account for approximately one third of the overall use of polymer additives and can comprise up to 30 by 40% by weight of the polymers (Tickner et al., 1999)**.** These synthetic additives are incorporated into the amorphous parts of polymers, thereby increasing the free volume of polymer and enhancing the flexibility and workability of the materials (Fedorko et al., 2003). The performance of plasticizers is often characterized in terms of their ability to decrease the glass transition temperature, which results in reducing the modulus and tensile strength of plastics. Other parameters that can be considered are the increase of the elongation at break and processability (Rahman and Brazel, 2004). Plasticizing ability in polymer formulations depends on compatibility with the polymer, resistance to ultraviolet (UV) radiation, leaching, volatility and stability at high and low temperatures (Rahman and Brazel, 2004).

Plasticizers are incorporated into a variety of polymers, including the commonly plasticized polymers poly(vinyl chloride) (PVC), poly(vinyl butyral) (PVB), and poly(vinyl acetate) (PVA). Approximately 80% of all the plasticizers produced are used in the production of PVC materials alone (Stevens, 1999).

Over the past century, the evolution of plasticizers has been extensively influenced by advances in plastic engineering that required higher quality products for a broad range of applications (Rahman and Brazel, 2004). Triphenyl phosphate, tricresyl phosphate, tributyl phosphate and glycerin acetates were amongst the first ester-based plasticizers (Rahman and Brazel, 2004). With the exception of tricresyl phosphate, which is still in use, none of them are used today because of their high volatility (Rahman and Brazel, 2004). Phthalic acid esters have been the most successful type of plasticizers. Excellent compatibility, low volatility, high gelling capacity and low cost led phthalates to be the most widely used class of plasticizers of the past century (Rahman and Brazel, 2004). The phthalate market continued to grow without major competition and one chemical in this class, di (2-ethyl hexyl) phthalate (DEHP), has been ranked as the most widely used plasticizer since the 1930s (Rahman and Brazel, 2004). In 2004, the global production of plasticizers was 6 million tonnes and 92% of the plasticizers produced over the past decade were esters of phthalic acid (Rahman and Brazel, 2004; Firlotte 2009).

Extensive research has been conducted since the mid-1960s to evaluate the health effects of phthalate plasticizers due to their widespread use in intravenous (IV) bags and tubing, food packaging, toys and personal care products (Tickner et al., 2001). There is evidence that the presence of phthalates and their metabolites in rats, mice, human plasma and liver are related to adverse health effects such as endocrine disruption and peroxisome proliferation (Mitchell et al., 1985; Cornu et al., 1992; Onorato et al., 2008). Furthermore, their high volume production and incomplete biodegradation have led to the presence of these compounds and a number of toxic and stable metabolites in the environment (Engelhardt et al, 1977; Staples et al., 1997; Horn et al., 2004). Phthalates and their related metabolites have been detected in surface waters, groundwater, air, soil and tissue of living organisms (Jaeger and Rubin, 1999; Roslev et al., 1998; Staples et al., 1997; Horn et al., 2004; Nalli et al., 2006a; Barnabé et al., 2008; Beauchesne et al., 2008).

Such findings have led to stricter environmental regulations, which consequently affected the plasticizer market. For example, in 2005, the European

Union banned the use of several phthalates in children's toys (European Union, 2005). DEHP is rated as a priority pollutant by U.S. EPA (Keith and Telliard, 1979; Gibbons and Alexander, 1989) and US Food and Drug administration (FDA) recommended labeling DEHP content of certain devices and replacing DEHP with substitute plasticizers, wherever possible (Hileman, 2002). In response to this, alternative plasticizers, including adipates, trimetillates, benzoates and citrates have been commercialized for a broad range of applications (Rahman and Brazel, 2004; Wypych, 2004). Moreover, toxicological research has been prioritized for the development of alternative plasticizers to meet the new and stricter requirement of environmental agencies.

In spite of all the findings mentioned above, phthalates are still in widespread use and their safety is defended by manufacturers, even in sensitive applications such as medical devices (American Chemistry Council, 2009). Phthalates have recently been selected by USEPA for further review (Anonymous, 2009a). The result of this review is expected to lead to action plans with proposed outcomes ranging from the conduct of further research into health and environmental effects to the restriction or complete ban of these chemicals (Anonymous, 2009a). It is reasonable to conclude that the final decision of the USEPA will likely drive intense research in both academia and industry to design alternative plasticizers that are environmentally benign and non-toxic.

Therefore, the objective of this review is to: (1) summarize essential criteria that must be satisfied in order to classify compounds as "green plasticizers"; (2) present the growing body of evidence for the health and environmental impacts associated with plasticizers; (3) discuss commercially-available alternative plasticizers in the market; and (4) briefly review the approaches than can be used in systematic development of green plasticizers focusing on the biodegradability of the parent plasticizers and their metabolites.

2.2. Evaluation of Plasticizers

It is essential that plasticizing compounds meet important functionality and cost-effectiveness requirements in order to be feasible for large-scale

production and use in a variety of commercial applications. In addition, plasticizers must also conform to appropriate environmental and health regulations. Beyond this, a number of other requirements must be satisfied in order to reasonably classify such compounds as "green". The principals of green chemistry provide an important framework within which plasticizers can be evaluated in order to minimize their hazardous effects during production, use and discharge to the environment (Anastas and Warner, 2000). These principles have been formulated in response to growing concerns over the past decades regarding the environmental and health impacts associated with the production and use of numerous chemicals on an industrial scale.

Consistent with this, the major challenges facing the plastics industry in response to growing health and environmental concerns associated with plasticizers will be to minimize the toxicity and persistence of these chemicals while preserving their functionality, as is summarized in the following two of the principals of green chemistry (Anastas and Warner, 2000): "Chemical products should be designed to preserve efficacy of function while reducing toxicity"; and "Chemical products should be designed so that at the end of their function they do not persist in the environment and break down into innocuous degradation products." Health and environmental criteria may differ substantially for each plasticizer application. For medical related applications, where the degree of exposure to humans is high, stricter safety criteria are essential. For example biodegradable plasticizers should be used for biodegradable polymers (e.g. polycaprolactone) in biomedical applications, since these compounds will be released during the normal use of product (Sun, 2004). Therefore, studies of health and safety issues will be a dominant research area in plasticizer development for biodegradable polymers (Rahman and Brazel, 2004). Similarly, as will be described below, research findings concerning the production and accumulation of toxic metabolites as a result of a plasticizer's metabolism in human body and the environment (Staples et al., 1997; Tickler et al., 2001) imply that the greatest challenges in green plasticizer development will likely lie in minimizing the toxicity and environmental persistence of plasticizers and their

metabolites. Therefore, low toxicity and low persistence should be the primary considerations in green plasticizer development particularly in more sensitive applications such as medical devices and for other products for which extensive human contact is either essential or probable.

These functionality, health and environmental considerations will be explored below. Examples will be illustrated primarily using research findings on phthalates, which are currently the most widely used plasticizers and consequently the most researched class with respect to their environmental fate, risk assessment and metabolic pathways.

2.2.1. Functionality considerations

In general, in order to be functional, plasticizing compounds should be compatible with polymers, stable at high and low temperature, resistant to migration (*i.e.*, to liquid, solid or gas phases) and also reasonably stable when subjected to UV radiation such as will occur when plastics are exposed to sunlight (Rahman and Brazel, 2004). It should be noted that technical criteria that define plasticizing abilities may change from one application to another and additional criteria may also need to be satisfied for certain applications. For instance, with plasticizers used in food contact materials, leaching and health hazards arising from the oral exposure to plasticizers are particularly important, as will be discussed below.

The compatibility of plasticizers with polymers is a primary consideration for the development of plasticizers, because this directly impacts the efficiency of the product. Compatibility depends on physical and chemical properties of plasticizers including molecular weight, functional groups and alkyl chain length (Rahman and Brazel, 2004). The development of an understanding of the relationship between these properties and plasticizer effectiveness can open up new routes to allow for the systematic characterization of plasticizer efficiency for a particular polymer and will be particularly useful in supporting the development of alternative plasticizers.

Leaching of a plasticizer from a polymer is a common challenge in plasticizer development and is an important parameter in determining a polymer's shelf life and its effectiveness (Rahman and Brazel, 2004). Leaching also increases the exposure of humans to chemicals with potential adverse health effects, and thus is particularly important in medical devices, food packaging and children's toys. To minimize leaching, one common approach is to modify the polymer surface. Various techniques can be employed to reduce the leaching rate including surface cross-linking, modification of surface hydrophobicity/lipophilicity, and surface coating with non-migrating materials (Krishnan, et al., 1991; Lakshmi et al., 1998). For example, plasma-induced surface cross-linking of PVC film is applied for food packaging applications where plasticizers have the potential to migrate into food (Audic et al., 2001).

Polymers are often exposed to extreme conditions including high and low temperatures and significant UV exposure in applications such as automotive dashboards and medical sheets that need to be autoclaved or preserved at 4°C (Rahman and Brazel, 2004). Thermal degradation of plasticizers and dehydrochlorination of PVC at high temperatures, polymer stiffness at low temperatures (i.e., below the glass transition temperature), and evaporation at high UV levels are major challenges that should be considered in order to ensure plasticizing efficacy under expected conditions (Rahman and Brazel, 2004).

2.2.2. Health implications

Major issues associated with potential health hazards of plasticizers arise from their tendency to leach from the polymer matrix resulting in their ingestion and the subsequent production of intermediates following their metabolism in the body (Tickner et al., 2001). Leaching of DEHP and its deposition in body tissues has been reported since the mid-1960s (Jaeger and Rubin 1970).

Human beings are exposed to plasticizers from a variety of sources including the intake of contaminated food, the inhalation of polluted air in factories, houses and cars, and through blood transfusions (Wams, 1987). The average daily exposure to phthalates in the United States has been estimated to be

0.27 mg/day, excluding workplace and indoor air exposure (Oie et al., 1997; Tickner et al., 2001). DEHP is the most commonly encountered plasticizer in medical procedures involving dialysis patients, hemophiliacs, and neonates and developing fetuses (Tickner, et al., 2001). DEHP can represent as much as 30 to 40% by weight of intravenous bags and up to 80% by weight of tubing [DiGangi, 1999; Tickner et al., 2001). As a result, daily exposure levels may be more than three orders of magnitude higher than average levels for patients exposed to medical components containing phthalates (Tickner et al., 2001). For example, exposure of hemodialysis patients was reported to be 9 to 360 mg/patient during one session (Wahl et al., 2004).

Given the many routes of exposure to phthalates and their widespread use, the toxicity of this class of plasticizers has been studied extensively over many years. For example, in a toxicity study, the oral LD_{50} (median lethal dose) of DEHP was estimated to be 25 g/kg in rats and 30 g/kg in mice (Tickner et al., 1999), which implies a low acute toxicity for this chemical. However, this is a measure of acute toxicity and does not reflect the range and severity of potential impacts to humans and organisms in the environment when they are subjected to exposure to these chemicals in lower doses over extended periods of time. Moreover, identification of a number of metabolites of increased toxicity produced from DEHP metabolism in animals and humans (Mitchell et al., 1985; Pollack et al., 1985; Wahl et al., 2004) has raised significant concerns about the health hazards associated with the ingestion of DEHP.

A proposed pathway for the i*n vivo* metabolism of DEHP is illustrated in Figure 2.1. The metabolites shown have been detected in rats, human plasma and urine (Dirven et al., 1993; Tickner et al., 2001; Wahl et al., 2004) and have potential health consequences. For example, liver, kidney, lungs, pancreas and plasma can hydrolyze DEHP to mono-2-ethylhexyl phthalate (MEHP), a known endocrine disruptor (Mitchell et al., 1985; Onorato et al., 2008) and 2-ethylhexanol (Tickner et al., 2001). MEHP and 2-ethylhexanol can be further transformed into other metabolites in the liver (Tickner et al., 2001). Furthermore,

2-ethylhexanoic acid, which is derived from the oxidation of 2-ethylhexanol, is amongst the most potent peroxisome proliferators (Cornu et al., 1992).

Given the above adverse health effects, especially on sensitive populations such as hemophiliacs, dialysis patients, developing fetuses, and neonates who have been exposed to phthalates through medical procedures, the elimination of DEHP from medical devices is an important goal. Therefore, alternative plasticizers including di-(2-ethylhexyl) adipate (DEHA), diisononyl phthalate (DINP), butyryl trihexyl citrates (BTHC), trioctyl trimellitate (TOTM) (TURI, 2006) and non-PVC materials such as metallocene polyolefin polymers (TURI, 2006), silicones and polyolefins (Tickner, 1999) that do not require plasticizers have been developed and, in certain cases, commercialized as potential alternatives for medical devices. Unfortunately, although the alternative plasticizers mentioned above are, in general, more favorable with respect to carcinogenicity and reproductive toxicity, adverse effects have been reported for some. For instance, while DEHA and BTHC have the important benefits of not inducing genotoxicity of microbial cells and mammals (TURI, 2006). DEHA can be metabolized to 2-ethylhexyl hexanoic acid, a potent peroxisome proliferator, and butyric acid, which is a metabolite of BTHC, can lead to gastrointestinal tract and liver problems (TURI, 2006). At present, limited information is available on the health effects of these alternative plasticizers and their metabolites and, therefore, further toxicological research is required prior to their widespread application.

Another source of concern is the use of plasticizers in toys, where exposure of plasticizers to developing children can occur through direct contact of skin and mouth with toy surfaces (Rahman and Brazel, 2004). The production of children's toys and baby-care products recently accounted for approximately 1% of the phthalates used (Tullo, 2000), with DINP being the most common plasticizer in PVC-based toys. Phthalates and their related metabolites have been detected in the urine of babies exposed to baby-care products containing phthalates (Sathyanarayana et al., 2008). Citrates and dibenzoates are currently used as alternative to phthalates in children's toys (Rahman and Brazel, 2004),

because they show a better toxicity profile compared to phthalates. For instance, citrate-based plasticizers did not induce gene mutation in microbial and mammalian cells (Johnson, 2002). Low toxicities and high biodegradation rates have also been reported for dibenzoate plasticizers (Arendt and Lang, 1998; Lang and Stanhope, 2001).

In response to the adverse health effects summarized above associated with phthalates, new environmental legislation has been introduced to restrict the use of phthalates in sensitive applications. Government regulations of different countries or states within a country vary widely in terms of the use of phthalates. For example, in 2001, a scientific panel convened by the United States Consumer Product Safety Commission (CPSC) concluded that DINP is safe for use in children's toys (Hileman, 2001). In contrast to this, in 2005, the European Union (EU) completely banned the application of DEHP, di-butyl phthalate (DBP) and butyl benzyl phthalate (BBP) in children's toys and further, in 2007, restricted the use of DINP, di-isodecyl phthalate (DIDP) and di-n-octyl phthalate (DnOP) in PVC articles designed to be put in the mouth by children under the age of three (European Union, 2005; Chemsystem, 2008). California law also prohibited the manufacture, sale, and distribution of any toy or child-care products that contain more than 0.1% of DEHP, DBP, or BBP. Toys or children care articles that contain more than 0.1% of DINP, DIDP and DnOP and can be put in the mouth are also banned since January 2009 (Hileman, 2007). Currently, phthalates are amongst the classes of chemicals that are targets of EPA possible action, which might reflect an anticipated change in the regulations governing the production and use of this class of plasticizers (Anonymous, 2009a).

Given the above, toxicity assessment of newly developed plasticizers for sensitive applications such as medical devices and children's toys is very important. Toxicity tests can be done on mammals such as rats and mice (*in vivo*) or can be done with cell cultures of bacteria and mammalian cells. According to the guidelines of the scientific committee on food (EFSA, 2006), the core toxicological tests for substances used in the food contact materials industry must address mutagenicity effects in both mammalian cells and bacteria, as well as

chromosomal aberrations in mammalian cells, *in vivo* oral toxicity, and toxicity to development and reproductive organs. Additional studies are required if these tests indicate some adverse cellular responses such as peroxisome proliferation, neurotoxicity, immunotoxicity or endocrinological effects.

2.2.3. Environmental implications

Plasticizers can impact the environment via two major pathways; namely, environmental partitioning and transformation (Staples et al., 1997). Environmental partitioning is characterized by the tendency of a chemical to physically transfer from one compartment of the environment to another (Staples et al., 1997). Environmental transformations, such as biodegradation, can have a more intense ecological impact than physical partitioning due to the production of byproducts of increased toxicity and persistence (Staples et al., 1997).

Environmental partitioning

Environmental partitioning is often quantitatively expressed by air-water, vapor–aerosol, water-solid partitioning and bioaccumulation factors (Staples et al., 1997). Air-water partitioning characterizes the equilibrium distribution between air and water and is a measure of a chemical's tendency to escape from water into air and is strongly influenced by the volatility of the chemical (Thomas, 1982). Vapor-aerosol partitioning reflects the partitioning of organic chemicals between gas and particles in the air and is influenced by the surface area per unit volume of particles, particle concentration, and vapor pressure of the chemicals. Removal of contaminants from air by rain or snow is also influenced by partitioning behavior (Staples et al., 1997). Field studies have shown that the wash-out ratio, which is defined as the ratio of chemical concentration in a precipitate to that in air, in a range from 1 000 to 100 000 for phthalates (Atlas and Giam, 1981).

Sorption of chemicals to solid particles, such as sediments, or water-solid partitioning is governed by the chemical's hydrophobicity, the type of the solid or sediment, and the presence of soil humic materials (Staples et al., 1997; Carlberg and Martinsen, 1982). Partitioning of phthalates to solids is often reported in

surface water. For example, in the St. Lawrence River near Montreal, Canada, 47% of DEHP detected was in surface water and 53% was in suspended solid particles (Germain and Langlois, 1988). In the same sample, 86% of di-n-butyl phthalate was present in surface water and 14% was bound to solid particles.

Chemicals that are present in the environment can also partition into the tissues of organisms. This phenomenon is quantified by a bioaccumulation factor, which is defined as the ratio of the concentration in the tissue to that in water and generally expressed in terms of milliliters of compound per gram of body weight (Staples et al., 1997). The bioaccumulation factor is greatly influenced by metabolic capabilities of organisms. For instance, aquatic species can hydrolyze a phthalate to release a monoester and the corresponding alcohol (Barron et al., 1995). Metabolism of the alcohol and the alkyl side chain of the monoester can proceed via the β-oxidation pathway (Staples et al., 1997). Bioaccumulation of DEHP and di-butyl phthalate was also observed in terrestrial plants such as barley grown on soil, plants fertilized by contaminated sludges, and corn grown on contaminated soil (Kirchmann, 1991; Shea et al., 1982).

Environmental transformation

Environmental transformations, such as chemical hydrolysis, photodegradation and biodegradation are important environmental fate processes because transformation might lead to the production of intermediates of greater potency and persistence but their environmental effects might remain undetected for several years (Staples et al., 1997).

The hydrolysis of chemicals with an ester bond is a potential abiotic degradation pathway. Phthalates can undergo hydrolysis in water and release the corresponding monoester and alcohol (Staples et al., 1997). A second hydrolytic step releases phthalic acid and another alcohol (Staples et al., 1997). The hydrolysis half-lives for di-methyl phthalate and di-(2-ethyl hexyl) phthalate in aquatic environments have been estimated to be about 3 years and 2 000 years, respectively (Wolfe et al., 1980). Therefore, abiotic hydrolysis does not seem to be a dominant process with respect to the environmental fate of phthalates (Staples et al., 1997).

Photodegradation can be mediated either through the direct mechanism of ultraviolet (UV) absorption by chemicals or by indirect mechanisms involving UV absorption by water and subsequent formation of singlet oxygen or hydroxyl radicals that can react with chemicals (Staples et al., 1997). Photodegradation is not an important source of phthalic acid esters in aquatic environments (Staples et al., 1997), however, it may be more important in terms of the atmospheric fate of phthalate esters (Staples et al., 1997). Few studies are available regarding the photodegradation of phthalates, but one study reported 5% degradation for a 1 mg/L solution of butylbenzyl phthalate exposed to sunlight for 28 days (Gledhill et al., 1980).

Biodegradation is an important process that influences the toxicity and persistence of chemicals in aquatic and terrestrial ecosystems and numerous studies have shown that biodegradation under aerobic and anaerobic condition is the major route of phthalate removal from soils, sediments, surface waters and wastewaters (Johnson et al., 1984; Wang et al., 1997). Biodegradation processes can be classified into three distinct categories based on the final product of degradation, as follows (Raymod et al., 2001): Complete biodegradation is a term used to described mineralization of organic compounds to inorganic compounds; acceptable biodegradation describes a metabolic process that induces biotransformation in the molecular structure of the organic compounds and results in the production of non-toxic metabolites; and primary biodegradation is biotransformation of the molecular structure of the parent compound that leaves toxic final products in the environment.

Both primary and complete biodegradation of plasticizers by diverse mixed and pure microbial cultures have been reported (Staples et al., 1997; Nalli, 2002, 2006a,b,c; Ejlertsson et al., 1997). The following uptake mechanisms have been proposed for assimilation of phthalates by microorganisms (Gibbons and Alexander, 1989): biotransformation of the compound to solubilized byproducts by extracellular enzymes, direct surface contact of chemical and microorganism, and biosurfactant-enhanced biodegradation or utilization of only the soluble

substrate. Combinations of these mechanisms have also been suggested (Gibbons and Alexander, 1989).

A proposed biodegradation pathway (Staples et al., 1997) for phthalates under aerobic or anaerobic conditions is illustrated in Figure 2.2. For both conditions, biodegradation is initiated by ester hydrolysis to release a monoester and the corresponding alcohol. The monoester is hydrolyzed to form phthalic acid and a second alcohol. Aerobic degradation of phthalic acid proceeds via a 3,5 or 4,5 dihydroxyphthalate pathway to procatechuate. Two pathways of meta and ortho are possible for the ring cleavage of procatechuate. Anaerobic degradation proceeds via the degradation pathway of benzoate to yield acetate and carbon dioxide as final products.

Primary biodegradation of phthalates under aerobic conditions was generally reported to be rapid, with 90% degradation within a week for lower molecular weight phthalates and 90% degradation after 12 days for higher molecular weight phthalates (Staples et al., 1997). The half-lives of DEHP in lagoon sludge and activated sludge was 45.4 days and 28.9 days, respectively. The concentration of DEHP in lagoon sludge was about 29 mg/kg and in activated sludge was about 6.3 mg/kg (Amir et al., 2005).

Under anaerobic conditions, the biodegradation rates of phthalates were lower and more variable compared to aerobic conditions (Gibbons and Alexander, 1989). This variability was attributed to the nature of the test inocula. The difference in biodegradation rate was proposed to be the result of the production of emulsifiers or solubilizers under aerobic condition that increased the bioavailability and subsequently the biodegradation rates of plasticizers. The excretion of emulsifiers apparently does not occur to the same extent as it does under the anaerobic conditions (Gibbons and Alexander, 1989).

The metabolites resulting from primary biodegradation of phthalates have also been detected in the environment (Horn et al., 2004; Otton et al., 2008). Thus, numerous studies have been conducted to assess the potential of indigenous microorganisms to degrade such metabolites. Mono-alkyl phthalate esters (MPE) are the hydrolytic metabolites of phthalate and have been identified as peroxisome

proliferators and endocrine disruptors (Mitchell et al., 1985; Onorato et al., 2008). Therefore, these compounds have been the subject of several biodegradation studies (Otton et al., 2008; Nalli, 2006a,b,c). Biodegradation half-lives of several MPEs in marine and freshwater sediments collected from the Greater Vancouver area, in British Columbia, Canada, ranged from 16 to 39 hours (Otton et al., 2008). Numerous reports of the biodegradation of phthalates and their metabolites are indications of the presence of nonspecific esterase enzymes in naturally-occurring microorganisms in soil, natural, sediments and water at various locations that can act on a wide variety of ester plasticizers (Otton et al., 2008).

2.3. Towards the development of green plasticizers

The demonstrated health and environmental impacts associated with conventional commercial plasticizers coupled with their extensive use, which has resulted in their ubiquitous presence in the environment, must necessarily drive the development of alternative, "green" plasticizers. These plasticizers must meet the important criteria of being technically sound in terms of their plasticizing ability, environmentally benign both in their parent form and following metabolism, non-toxic, and must be produced efficiently in accord with the principles of green chemistry. Some of these alternative plasticizers are described below and additional research that is required to assess these compounds as potential green plasticizers is also briefly discussed.

Isosorbide diesters are bio-based plasticizers produced from the reactions between fatty acids of vegetable origin (e.g., n-octanoate) and isosorbide produced by dehydration of sorbitol, a glucose derivative (Roquette Co.). This class of plasticizers was recently developed by Roquette Corporation in France and is promoted as a non-toxic, biodegradable, bio-based alternative to phthalates for PVC applications. A demonstration unit with a production capacity of 100 tonnes per year of isosorbide diesters was launched in Lestrem, France. The biodegradability of isosorbide di n-octanoate with the trade name of POLYSORB® ID 37 was tested using a CO_2 evolution test (OECD 301 B method) and $ThCO_2$ (mg carbon produced from CO_2 evolution/ mg total organic

carbon as a substrate) was reported to be 83% in 28 days and therefore it was rated as biodegradable (Anonymous, 2009b). However, to the best of our knowledge, the biodegradation pathway has not been established for this class of plasticizers, and, thus, there is no information about potential metabolites. Toxicological information on this plasticizer is limited to acute toxicity and 14-day chronic toxicity assay results (Anonymous, 2009b), while mutagenicity effects and toxicity to development and reproduction organs have not been addressed.

Epoxidized vegetable oils are a bio-based class of plasticizers produced via esterification of vegetable oil with polyols (Anonymous, 2010). A common example of this class of plasticizer is epoxidized soy bean oil, produced via esterifcation of epoxidized soy oil with glycerol and developed through a collaborative venture of the Ohio Soybean Council and Battelle (Anonymous, 2010). The main advantages of this plasticizer are its resistance to migration due to its high molecular weight and bulky structure, its stability at high temperature, and compatibility with PVC (Rahman and Brazel, 2004). Whereas soybean oil-based plasticizers were initially not compatible with PVC as primary plasticizers and their use was limited as secondary plasticizers, the newly developed compounds can be used as primary plasticizers (Anonymous, 2010). The expoxidized diol (such as propylene glycol) fatty acid esters provided the desired functionality needed for the thermal stability and a lower brittleness using a smaller amount of plasticizer (Anonymous, 2010). Toxicity and leaching is indicated to be under investigation for this plasticizer (Rahman and Brazel, 2004).

Di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH) with the trade name of Hexamoll® DINCH is produced from the catalytic hydrogenation of di(isononyl) phthalate (DINP). Hexamoll® DINCH was developed by BASF in Germany, particularly for sensitive applications such as medical devices, food packaging and children's toys (BASF, 2006; Chemsystem, 2008). In 2006, this plasticizer was approved by the European Food Safety Authority for food contact applications such as cling film, tubes or sealants (Chemsystem, 2008). Mechanical properties, including tensile strength, 100% modulus and shore A and D, that

were achieved following the use of this plasticizer in PVC were reported to be comparable with those achieved with DINP and acetyl tributyl citrate. DINCH showed improved low temperature properties in plastisol, better viscosity, and stability compared to DINP (Wadey, 2003). Toxicological tests were conducted based on the Organization for Economic Co-operation and Development (OECD) guidelines and no indication of genotoxicity and toxicity to reproduction was observed (Wadey, 2003). DINCH also showed considerably lower migration rates compared to acetyl tributyl citrate (ATBC) and DEHP (Welle et al., 2005). This plasticizer has also been rated as biodegradable according to the CO_2 evolution test (OECD 301B) (Wadey, 2003). Biodegradation mechanisms and toxicity of potential metabolites must also be investigated in order to fully assess the ecological impacts of this plasticizer.

Citrates, marketed under the trade name of Citroflex®, are based on bio-based citric acid feedstock, which is mainly produced from the fermentation of corn (Vertellus Specialties Inc). Citrates were developed by Morflex Inc. more than 35 years ago as an alternative to phthalates (Chemsystem, 2008). Based on initial toxicity assessments, conducted by the Pfizer Drug Safety Evaluation Department, of acute oral toxicity tests in mice and rabbits, citrates were classified as safe (Rahman and Brazel, 2004). However, more comprehensive toxicity assessments showed adverse effects on blood pressure, calcium metabolism and growth inhibition of cultured mammalian cells (Mochida, 1996; Tickner, 1999). In 2004, acetyl tributyl citrate (ATBC) was approved by the European Union Scientific Toxic Committee for applications in children's toys (Chemsystem, 2008). Citrates are also used in flexible tubing used in medical devices and also in food contact plastics but are not recommended for applications with exposure to high lipid media (Rahman and Brazel, 2004).

The application of *polymeric plasticizers* such as nitrile rubber (NBR), polycaprolactone-polycarbonate (PCL-PC) blends and polycaprolactone-poly(ethylene glycol) multiblock copolymers have been tested for medical devices. The major advantages of polymeric plasticizers are their low volatility and low leachability due to their high molecular weights. A PCL-PC blend of

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molecular weight (M_n) of 32,700 was found to be resistant to extraction by water and phosphate buffer at 37°C for 98 days (Hakkarainen, 2003). However, a trace amount of 6-hydroxyhexanoic acid, a final byproduct of hydrolysis, was detected in water (Hakkarainen, 2003). This indicates that a more precise study is required to monitor the formation of metabolites. Another example of a polymeric plasticizer is a terpolymer of ethylene, vinyl acetate and carbon monoxide (EVACO), which prevented leaching of DEHA in a food-grade PVC into isooctane to a value below the detection limit (Audic et al.,2003).

Dibenzoates, commercially known as Benzoflex ® plasticizers, were developed by Genovique Specialties (formerly Velsicol Chemical) and have been used commercially for more than 40 years (Deligio, 2009). A common example of a dibenzoate plasticizer is Benzoflex ® 2088 (formerly Benzoflex ® 2888), which is a blend of diethylene glycol dibenzoate, triethylene glycol dibenzoate, and dipropylene glycol dibenzoate. An oral LD_{50} of 3 to 5 g/kg in rats has been reported for this blend (Rahman and Brazel, 2004). Initial biodegradation studies also showed high rates of biodegradation (Ardent and Lang, 1998; Lang and Stanhope, 2001). Benzoflex ® 2088 is currently used in the toy industry. Benzoflex ® plasticizers were suggested as alternatives to phthalates by The European Chemical Agency (ECHA) (Deligio, 2009). Pentanediol-based diesters are also included in the dibenzoate class of plasticizers and their applications as plasticizers for vinyl polymers was patented in the 1950s (Hetzel, 1956). 2,2,4-trimethyl-1,3-pentanediol dibenzoate is commercially available for silk-screen ink applications. (Arendt and Beitsch, 1994).

Recent studies have shown that microbial hydrolysis of diethylene glycol dibenzoate and dipropylene glycol dibenzoate by common soil micro-organisms results in the formation of a monobenzoate metabolite (Gartshore et al., 2003; Kermanshahi pour et al., 2009a). These metabolites demonstrated high acute toxicities in response to the Microtox^{TM} toxicity assay (Gartshore et al., 2003; Kermanshahi pour, 2009a). In contrast, biodegradation of two diol-based diesters, 1,5-pentandiol and 1,6-hexanediol dibenzoates, did not result in accumulation of the corresponding monobenzoate (Firlotte et al., 2009; Kermanshahi pour et al.,

2009a,b). Therefore, these two compounds were proposed as potential alternatives to commercial dibenzoate plasticizers (Firlotte et al., 2009; Kermanshahi pour et al., 2009a,b). Common properties such as glass transition temperature and tensile strength of PVC plasticized with 1,5-pentandiol dibenzoate were comparable to those of PVC plasticized with the commercial plasticizers diethylene glycol dibenzoate, dipropylene glycol dibenzoate and DEHP (Firlotte et al., 2009).

As indicated above, while a number of alternative plasticizers are currently available for a variety of applications, further research is required to fully assess them with respect to the principals of green chemistry. Of highest priority, amongst the issues that have yet to be addressed, is the study of the metabolism of these alternative compounds in the human body and the environment. The health and environmental consequences of conventional plasticizers implies that toxicity and biodegradability assessment should not simply be limited to the parent plasticizer but should be extended to their metabolites as well.

Given that biodegradability is amongst the most significant challenges in green plasticizer development, understanding the effect of molecular structure on biotransformation mechanisms and biodegradation rates can lead toward a systematic approach in the development of biodegradable plasticizers. The identification of the persistent structural features will allow for the establishment of a focused strategy to alter chemical structures in order to design greener compounds that undergo complete biodegradation in the environment and do not result in the formation of toxic and persistent metabolites.

Although biodegradability is closely related to the chemical structure of a compound, the effect of environmental variables on biodegradation phenomena in terms of the type and amount of the byproducts produced and their subsequent toxic and environmental impacts should not be ignored. These variables include chemical-physical adsorption, the presence of a co-substrate and sources of nitrogen and phosphorous, type and population of microorganisms, and the extent of microorganism acclimation to the chemicals (Alexander, 1973; Raymond et al., 2001).

To study the relationship between the chemical structure and biodegradability, the full spectrum of metabolites created during the biodegradation process should be identified and the biodegradation pathways for the conventional plasticizers and their potential "green" alternatives must be established. Moreover, it is also essential to study the biodegradation kinetics of alternative plasticizers to determine the biodegradation rates of plasticizers and their corresponding metabolites to compare with those of commercially available plasticizers.

Apart from the effect of the environmental variables on biodegradability, some functional groups such as ether bonds and terminal alkyl branches, especially anteiso branching, have generally been referred to as persistence structures (Schaeffer et al., 1979; White et al., 1996). The persistence of an ether linkage in biodegradation was suggested to be due to the high energy bond of C-O bond compared to that of a C-C bond (White et al., 1996). Persistence of chemicals with terminal alkyl branching is due to the hindrance of the β-oxidation pathway since this pathway, in order to proceed, needs two protons on both the α and β carbons (Alexander, 1979). The ether bond was found to be particularly important to the persistence of monobenzoate metabolites of commercial dibenzoates (Kermanshahi pour et al., 2009a).

Evidence indicates that biodegradation of phthalates and dibenzoate starts with microbial hydrolysis and then proceeds via alkyl branch degradation (Staples et al., 1997; Kermanshahi pour et al., 2009a). Therefore, any structural modification that can facilitate the alkyl branch degradation and/or microbial hydrolysis will likely enhance the biodegradability of these classes of chemicals. For instance, removing the ether linkage from the structure of the commercial plasticizer of diethylene glycol dibenzoate significantly enhanced the biodegradability of the monoester metabolite by providing an alternative β-oxidation pathway for the side-alky branch to be processed (Kermanshahi pour et al., 2009a).

Developing green plasticizers with the desired characteristics solely based on experimental approaches requires tremendous effort and expense in the

synthesis of the new compounds as well as in biodegradation and risk assessment monitoring. Moreover, given the possibility of production of metabolites of higher toxicity than the parent compounds, there is an increasing interest in developing computational methods to predict microbial degradation pathways. Several models have been developed to predict the metabolites resulting from the biodegradation chemicals in the environment. These models are generally based on expert systems, which use biotransformation rules to identify the chemical structures that are susceptible to enzymatic reactions. For instance, the University of Minnesota Pathway Prediction System (UM-PPS) employs the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD), which contains the biodegradation pathways drawn from scientific literature (Ellis et al., 2006). META-CASE is another expert system, originally developed to predict the toxicity of drug compounds using the biotransformation rules for mammalian enzyme (e.g., cytochrome P450s), and then applied for predicting the metabolites of xenobiotics in mammals and aerobic and anaerobic biodegradation (Klopman and Tu, 1997). The application of such "biodegradation pathway prediction" software may be particularly useful in support of green plasticizer development. It would be worthwhile to confirm the accuracy of these methodologies by applying them to plasticizers for which their biodegradation pathways are available.

2.4. Summary

Plastics will likely continue to be the material of choice for many consumer and industrial applications in the foreseeable future. In turn, plasticizers will also be essential additives for the production of a wide variety of products. In response to the urgent need to address the health and environmental consequences of the conventional plasticizers, alternative plasticizers have been developed. Some of these are under evaluation for technical performance and some are at the stage of commercialization or have already been commercialized. While these compounds certainly have potential as alternatives to problematic plasticizers that are currently in widespread use, additional research is required to assess how they conform to the principles of green chemistry. Similarly, the

development of other alternative compounds should be pursued, using the range of tools that are currently available for their assessment. Once chemicals with the desired safety and environmental profiles as well as desired functionality are formulated, a sustainable production technology should be developed, in line with the principles of green chemistry. The development of such plasticizers represents an important challenge, but also an important opportunity for researchers and industries to rise to the challenge and fill market niches that are gradually being created through the restriction of the use of conventional plasticizing compounds.
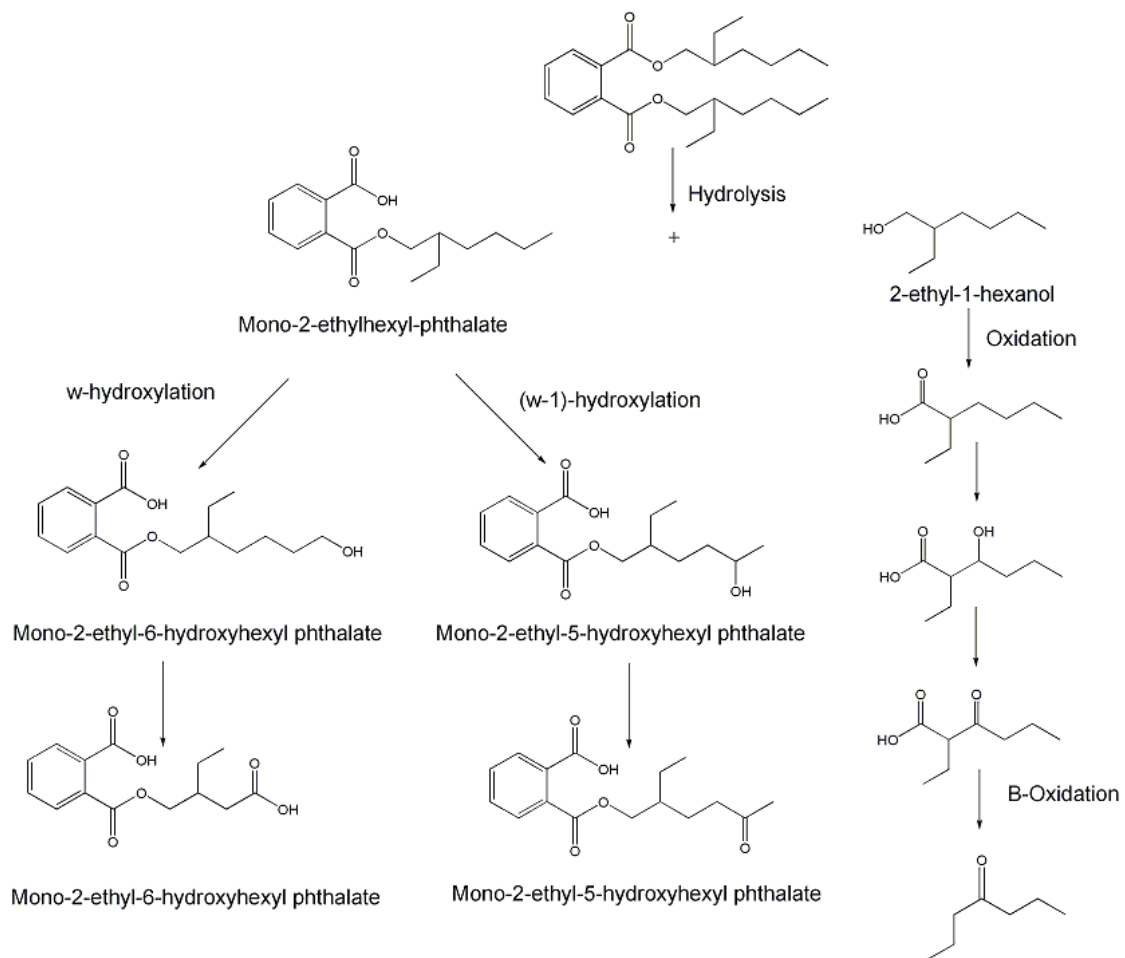
Figure 2.1. Proposed *in vivo* metabolism of DEHP (Reproduced from Dirven et al., 1993; Wahl et al., 2004).
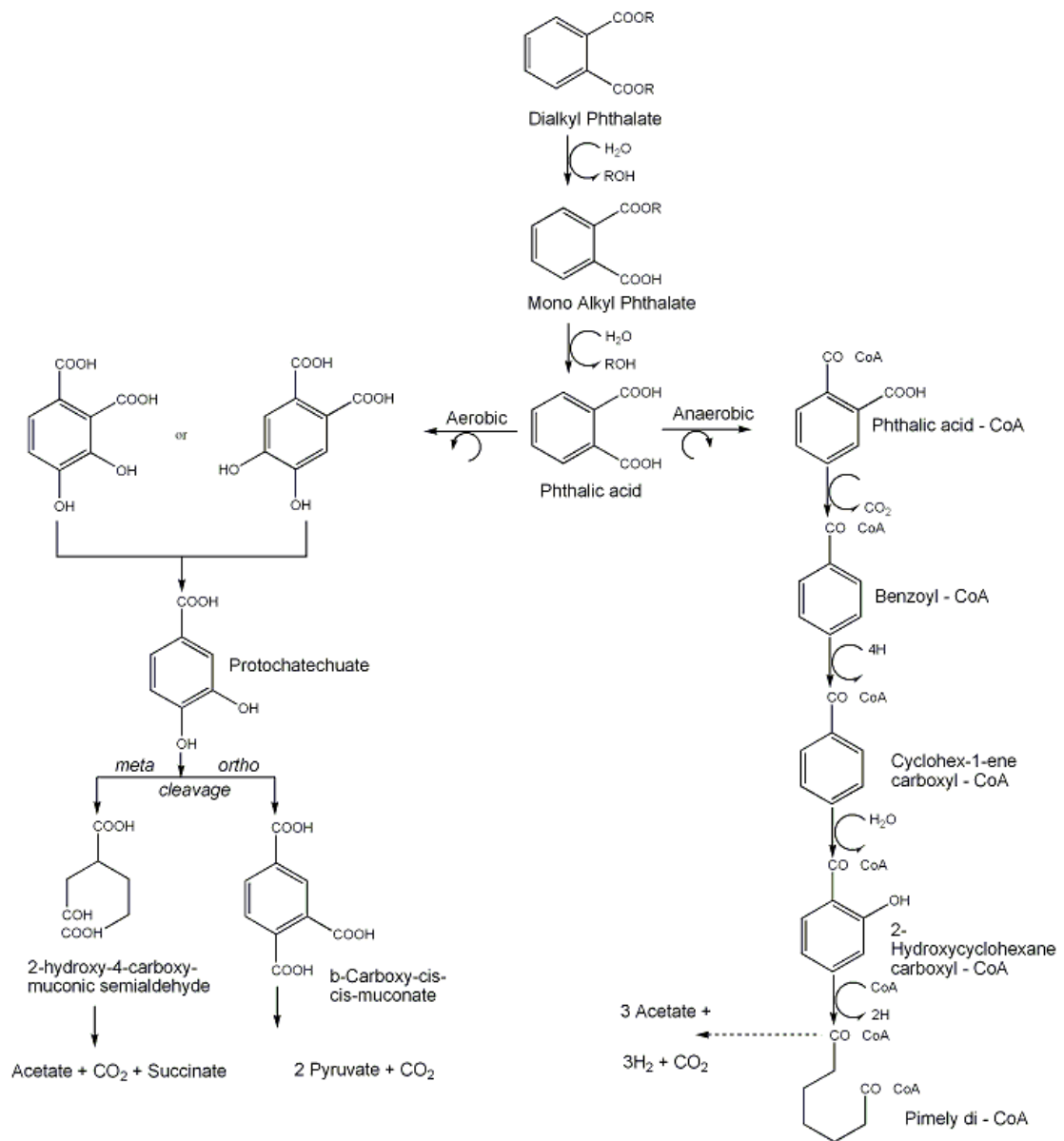
Figure 2.2. Biodegradation pathway of dialkyl phthalate (Reproduced from Staples et al., 1997).

3. Metabolites from the Biodegradation of 1,6-Hexanediol Dibenzoate, a Potential Green Plasticizer, by *Rhodococcus rhodochrous*

Preface

As indicated in the preceding chapter, when developing alternative plasticizers to replace conventional compounds, the potential metabolites produced in their biodegradation should be identified in order to fully assess the environmental impact of these chemicals on the ecosystem. In this chapter, the environmental fate of 1,6-hexanediol dibenzoate, a potential plasticizer, as a result of interaction with *Rhodococcus rhodochrous*, a common soil microorganism in the presence of hexadecane as a primary carbon source is discussed.

All the metabolites produced in the biodegradation process were identified using GC/MS and Fourier transform mass spectroscopy (FTMS). Repeating biodegradation of 1,6-hexanediol di[^{2}H$_5$]benzoate confirmed that all of these metabolites originated from the biodegradation of 1,6-hexanediol di[^{2}H$_5$]benzoate due to the presence of deuterium on the aromatic ring. None of these metabolites were persistent as they degraded over the course of the experiment. The most stable of these metabolites was 1-hexadecyl benzoate, which was proposed to be the result of enzymatic conjugation of benzoate, hydrolyzed from 1,6-hexanediol dibenzoate and hexadecanol, a metabolite of hexadecane. However, this metabolite is not of concern since its formation requires substantial amount of alkane to be present, which is unlikely to be the case in the environment. The results presented here support the consideration of 1,6-heaxendiol dibenzoate as a potential green plasticizer

Metabolites from the Biodegradation of 1,6-Hexanediol Dibenzoate, a Potential Green Plasticizer, by *Rhodococcus rhodochrous*

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3.1. Abstract

Metabolites from the biodegradation of a potential plasticizer, 1,6-hexanediol dibenzoate in the presence of n-hexadecane as a co-substrate by the common soil organism *Rhodococcus rhodochrous* were identified using GC/MS and Fourier transform mass spectrometry (FTMS) techniques. Trimethylsilylation of compounds from the biodegradation broth permitted detection of the following metabolites: 1-hexadecyl benzoate, 6-benzoyloxyhexanoic acid, 4-benzoyloxybutanoic acid, 6-benzoyloxyhexan-1-ol and benzoic acid. The presence of these metabolites was confirmed by repeating the biodegradation with 1,6-hexanediol di[^{2}H$_5$]benzoate, by measurement of their exact masses in FTMS and by comparison with available authentic materials. The results show that

biodegradation of 1,6-hexanediol dibenzoate by *R. rhodochrous* does not lead to the accumulation of persistent metabolites as has been reported for commercial dibenzoate plasticizers.

3.2. Introduction

The interaction of microorganisms with xenobiotic chemicals in the environment is a critical issue that must be studied when assessing their toxic impacts on ecological systems and human health (Gibson, 1968; Peijnenburg, 1994; Raymond et al., 2001; Nalli et al., 2006c). Incomplete biodegradation of the parent compounds in the environment may lead to their accumulation and the production of metabolites of increased mobility, toxicity and persistence (Zink and Lorber, 1995; Nalli, 2002; Horn et al., 2004; Nalli et al., 2006b). Therefore, in order to fully assess the impacts of these xenobiotics in the environment, it is important to identify the full range of compounds that are produced through biodegradation and to assess their toxicity and biodegradability.

In addition, the development of alternative compounds to replace conventional plasticizers requires monitoring of the consequences of their biodegradation to ensure that their metabolites have minimal toxicological impacts in the environment. Plasticizers, which are the most widely used additives in polymer manufacturing (Wypych, 2004), have raised serious health and environmental concerns in recent years (Giam et al., 1984; Roy, 2004). In addition, the concerns raised above about the potential impacts of metabolites have been shown to be the case for a number of widely used commercial plasticizers including phthalates and adipates (Nalli et al., 2006a,b; Nalli et al., 2002; Horn et al., 2004). Studies of the biodegradation of phthalate and adipate plasticizers have demonstrated the production of several different metabolites with greater toxicity than the parent compounds (Nalli et al., 2006b,c; Nalli et al., 2002; Horn et al., 2004).

For example, 2-ethylhexanoic acid, a potent peroxisome proliferator (Cornu et al., 1992; Keith et al., 1999), was identified from the biodegradation of di-2-ethylhexyl adipate, di-2-ethylhexyl phthalate and di-2-ethylhexyl terephthalate (Nalli et al., 2002). Mono-2-ethylhexyl phthalate, a metabolite expected in the biodegradation of di-2-ethylhexyl phthalate (Staples et al., 1997), is classified as an endocrine disruptor (Onorato et al., 2008). Detection of these and related metabolites of phthalates and adipates in mice, rats and human plasma and urine has led to greater concerns and stricter environmental regulations (Mitchell et al., 1985; Wahl et al., 2004; Sathyanarayana et al., 2008).

In recent years, dibenzoate plasticizers such as diethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB) have been proposed as alternatives to the more commonly used compounds because they tend to degrade more rapidly under the action of common microorganisms (Lang and Stanhope, 2001; Peng and Zhang, 2000). While this tendency appears to make them attractive as alternatives to phthalates and adipates, it has been shown that the incomplete microbial hydrolysis of D(EG)DB and D(PG)DB when microorganisms are growing on glucose as a primary co-substrate leads to the accumulation of diethylene glycol monobenzoate and dipropylene glycol monobenzoate, respectively, which exhibit significant toxicity (Gartshore et al., 2003).

However, the rapid degradation of the dibenzoates indicate that a potential route to the development of a "green" plasticizer may be to start with the basic structure of the more easily degraded dibenzoate plasticizer and modify it to reduce the accumulation of metabolites when undergoing biodegradation. Therefore, the aim of this study was to monitor the biotransformation of 1,6-hexanediol dibenzoate, a potential plasticizer, by *Rhodococcus rhodochrous*, a common soil organism in the presence of hexadecane as a primary carbon source and to identify all of the metabolites created during biodegradation. Low resolution GC/MS with electron ionization was used for the identification of the metabolites as their trimethylsilyl (TMS) derivatives. Fourier transform mass

spectroscopy (FTMS) was also used to obtain their underivatized accurate masses with electrospray ionization (ESI).

3.3. Materials and methods

3.3.1. Chemicals and reagents

1,6-Hexanediol 99%, 1-hexadecanol 99%, n-hexadecane 99% and benzoyl chloride 99% were purchased from Sigma-Aldrich (Oakville, ON, Canada). $[^2H_5]$Benzoyl chloride 99.1 atom% D was purchased from CDN isotopes (Montréal, QC, Canada). Bacto^{TM} Brain/Heart infusion and yeast extract were obtained from Difco Microbiology (Montréal, QC, Canada) and Fisher Scientific (Montréal, QC, Canada), respectively. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Chromatographic Specialties (Brockville, ON, Canada). Pentadecane was purchased from A&C American Chemicals (Montréal, QC, Canada). All other chemicals were obtained from Fisher Scientific (Montréal, QC, Canada).

3.3.2. Synthesis of 1,6-hexanediol dibenzoate and 1,6-hexanediol di[$^2H_5$]benzoate

1,6-Hexanediol dibenzoate was synthesized by refluxing 5 grams of 1,6-hexanediol with 20 mL of benzoyl chloride (4 equivalents) under nitrogen in 120 mL of acetone in a round bottom flask for 7 hours. The reaction mixture was cooled to room temperature and then diluted with 100 mL of chloroform. The mixture was washed three times with 100 mL of a saturated sodium bicarbonate solution and concentrated to a yellow oil, which, on standing, yielded an off-white powder. This was re-crystallized from heptane.

The synthesis of 1,6-hexanediol di[$^2H_5$]benzoate was achieved in a similar manner by reacting 1,6-hexanediol (0.39 g) with 1.2 mL of benzoyl-[$^2H_5$]-chloride (3 equivalents) in 11 mL of acetone. The procedure used for work-up and re-crystallization is described above.

The proton NMR spectra of the synthesized 1,6-hexanediol dibenzoate and 1,6-hexanediol di[$^2H_5$]benzoate were consistent with the spectra expected for

these compounds (Figure 3.1). The EI spectrum of the unlabelled diester was virtually identical to the spectrum published in the NIST/EPA/NIH 1998 Mass Spectral Library of the United States Department of Commerce.

3.3.3. Synthesis of 1-hexadecyl benzoate

1-Hexadecyl benzoate was synthesized by reacting 8 grams of 1-hexadecanol with 5 mL of benzoyl chloride (1.3 equivalents) in 120 mL of refluxing acetone in a round bottom flask for 7 hours. The reaction was carried out under nitrogen. The reaction mixture was cooled to room temperature and then diluted with 100 mL of chloroform. The mixture was washed three times with 100 mL of a saturated sodium bicarbonate solution. The solvent was then evaporated and 1-hexadecyl benzoate was crystallized from the residue. The proton NMR spectrum obtained from the synthesized 1-hexadecyl benzoate was in agreement with the spectrum expected for this compound.

3.3.4. Biodegradation Studies

Biodegradation of 1,6-hexanediol dibenzoate or 1,6-hexanediol di[^{2}H$_5$]benzoate was conducted in 500 mL Erlenmeyer flasks with a sponge cap. The medium for the experiments consisted of 100 mL of the sterilized minimum mineral salt medium (MMSM) and 0.1 g/L of yeast extract and 2.5 g/L n-hexadecane. Either 1,6-hexanediol dibenzoate or 1,6-hexanediol di[^{2}H$_5$]benzoate (3 mmol/L) were added to the flasks individually prior to autoclaving. The MMSM contained 4 g/L NH_4NO_3, 4 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2 \cdot 2H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, and 0.014 g/L disodium ethylenediamine-tetraacetic acid.

Rhodococcus rhodochrous ATCC 13808 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and was stored at -70°C in plastic vials containing 20% glycerol and a sterile growth medium of Bacto^{TM} Brain/Heart infusion broth.

To prepare the initial inoculum, the contents of a vial were thawed and transferred to a 500-mL shaker flask containing sterile growth medium composed

of Brain/Heart infusion (30 g/L Brain/Heart infusion broth in 100 mL of distilled water) and then incubated on a rotary shaker (Series 25, New Brunswick Scientific, Edison, NJ, USA) set at 250 RPM and 30ºC. After one day, a new 500 mL shake flask containing 100 mL of 30 g/L sterile growth medium of Brain/Heart infusion in distilled water was inoculated with 1 mL of the initial inoculum.

When exponential growth was reached, this microbial culture was used to inoculate 100 mL of the sterilized MMSM containing 0.1 g/L yeast extract and 2.5 g/L n-hexadecane. This was used to inoculate the shaker flasks containing either 1,6-hexanediol dibenzoate or 1,6-hexanediol di[^{2}H$_5$]benzoate for the biodegradation study. The shaker flasks were incubated for a period of 7 days on a rotary incubator shaker set at 250 RPM and 30ºC.

Abiotic experiments (i.e., without inoculation with the microbial culture) were done in a shake flask containg 100 mL MMSM media, 0.1 g/L yeast extract and 2.5 g/L hexadecane. Plasticizer was added prior to autoclaving.

3.3.5. Sample preparation for GC/MS and GC/FID analyses

Over the course of biodegradation of 1,6-hexanediol dibenzoate, triplicate samples of 3 mL each were taken from the biodegradation broth every day. The samples were adjusted to pH 2 through the addition of sulfuric acid and extracted with 3 mL of chloroform. For GC/MS analysis, the extracts were evaporated to dryness under a dry nitrogen stream and the residues were taken up in 50 µL of anhydrous pyridine. Trimethylsilyl (TMS) derivatives were made by the addition of 50 µL of BSTFA to the pyridine solutions in capped auto injector vials, which were heated in an aluminum block at 60°C for 15 minutes. For GC/FID analysis, chloroform extracts of the samples were used without derivatization.

3.3.6. GC/MS Analyses

Aliquots (1 µL) of the underivatized extracts were analyzed in low resolution GC/MS mode with a GC (time-of-flight) Mass Spectrometer (Micromass, Manchester UK) fitted with a 30 m HP-5 capillary column having a

0.32 mm i.d. and 0.25 μm film thickness. The temperature was programmed from 80°C after 1 min hold to 300°C at 10°C /min followed by a bake-out period of 6 min at 300°C. The injector was operated in 1:100 split mode at 250°C with a constant helium pressure of 70 kPa. The GC re-entrant temperature was 250°C. The EI ion source was operated at 70 eV and 200°C.

TMS-derivatized extracts and the synthesized 1-hexadecyl benzoate were analyzed in GC/MS mode on a 30 m, 0.25 mm i.d. DB-1 column operated as described above. The scan range was m/z 80 to 600 to avoid the intense but uninformative m/z 73 common to TMS derivatives.

3.3.7. FTMS Analyses

High-resolution measurements of the underivatized extracts were made in positive ion electrospray mode with an IonSpec 7.0 tesla FTMS (Lake Forest, CA, USA) calibrated with polyethylene glycol 300. The instrument was equipped with a "Z"-spray source from Waters Corporation (Milford, MA, USA), an accumulation hexapole, a collision cell, a hexapole ion guide, a standard cylindrical ion cyclotron resonance (ICR) cell and Omega 9 software. The analyses employed a direct infusion flow rate of 2 to 3 μL/min in solution with 90:10 vol/vol methanol:water. Formic acid (1%) and sodium iodide were added to enhance cationization and to provide a secondary mass scale calibrating ion (Na$_2$I$^+$, m/z 172.8835). The 'Z'- spray source employed capillary and cone voltages of 3899 and 30 V, respectively. Ions were accumulated in the hexapole for 300 to 1500 ms with a rod voltage of 70 V. For the transfer of ions to the ICR cell through the hexapole ion guide, the low mass range coil with a frequency of 3020 kHz was used along with a voltage of 80 V. For detection, ions were excited through an arbitrary waveform in a range of m/z 100 to 1000 with an amplitude of 135 V(b-p); the ADC rate for the MS was 2 MHz for a scan range of m/z 75-500. Transients were 1M data points long. A waiting time of 5 sec before the detection step was used to allow the pressure in the ICR cell to return to its nominal value of 2×10^{-9} torr.

3.3.8. GC/FID analyses

Aliquots (1 µL) of the chloroform extracts were analyzed in a Varian CP-3800 gas chromatograph equipped with a 30 m × 0.32 mm i.d. fused silica 8CB column (Varian, Montreal, QC, Canada) programmed to 300ºC at 10ºC after a 2 min hold initially at 40 ºC. The injection port and FID were 250ºC and 300 ºC respectively. Helium was used as a carrier gas at a flowrate of 1.5 mL/min. The concentration of the metabolites were estimated by GC/FID.

3.4. Results and Discussion

1,6-Hexanediol dibenzoate was synthesized in an attempt to develop a more environmentally benign version of the standard dibenzoate plasticizers. It has been shown that analogous compounds can be converted to stable, toxic metabolites by microorganisms while growing on glucose (Gartshore et al., 2003). The purpose of this work was to determine whether co-metabolism of 1,6-hexanediol dibenzoate by a typical soil microorganism, *R. rhodochrous* resulted in the production of stable metabolites.

Figure 3.2 shows the total ion current GC of an extract of an experiment using 1,6-hexanediol dibenzoate without derivatization (panel A) and after trimethylsilylation (panel B). Retention times and elution order are different in panel A and panel B due to the use of a HP-5 column and 10ºC program (panel A) *versus* a DB-1 column and a 5ºC temperature program for the derivatized sample (panel B).

Figures 3.3A and 3.3B show the EI mass spectra of 1,6-hexanediol dibenzoate and 1,6-hexanediol di[^{2}H$_5$]benzoate, respectively. Weak molecular cations at *m/z* 326 and 336 were observed in their mass spectra, respectively. Fragmentation pathways are proposed in Scheme 3.1. Fragment ions at m/z 204/209 are formed by loss of benzoic acid via a McLafferty mechanism and with a possible subsequent elimination of the elements of formaldehyde to yield ions at m/z 174 and 179. Formaldehyde elimination, as proposed in Scheme 3.1, is not observed in the dibenzoates of 1,5-pentanediol or 1,4-butanediol (data not shown, available in the NIST/EPA/NIH 1998 Mass Spectral Library), and this may be

related to the alkyl chain length. There are three possible precursor ions to m/z 174/179; these are m/z 221/226, 204/209, and the $M^{+\bullet}$. The first would violate the even electron 'rule', and it is not possible to decide between the last two on the basis of the present data. Fragments at m/z 123/128, nominally protonated benzoic acid, may be formed in a 4-centred elimination of a radical olefin. Alternatively, m/z 123/128 may have the Ph-C(OH)$_2^+$ structure. Subsequent loss of water by *m/z* 123/128 results in ions at *m/z* 105/110. Formation of this last ion by a direct C(O)-O fission in the molecular ion cannot be discounted.

Figures 3.4A,B and 3.4C are the mass spectra of 1-hexadecyl benzoate and 1-hexadecyl [^{2}H$_5$]benzoate, respectively, isolated from the broth. The mass spectrum of the synthesized 1-hexadecyl benzoate is shown in Figure 3.3B. The proposed fragmentation scheme for 1-hexadecyl benzoate and 1-hexadecyl [^{2}H$_5$]benzoate in Scheme 3.2 accounts for the formation of fragments at *m/z* 123/128 and 105/110 in a manner parallel to that suggested in Scheme 3.1 for 1,6-hexanediol dibenzoate and 1,6-hexanediol di[^{2}H$_5$]benzoate. Again, as in Scheme 3.1, while m/z 123/128 is drawn as protonated benzoic acid, the ion structure may be that of a α,α-dihydroxybenzyl cation. M/z 105/110 may be formed by H$_2$O loss or directly from the $M^{+\bullet}$. A McLafferty rearrangement in the molecular cations generates *m/z* 224/224, which undergo the sequential loss of 2 ethylene groups.

Figures 3.5A and 3.5B show the mass spectra of the trimethylsilylated metabolites identified as 6-benzoyloxyhexanoic acid and 6-[^{2}H$_5$]benzoyloxyhexanoic acid, respectively. In support of this assignment, Scheme 3.3 accounts for the major ion fragments and deuterium labelling found in Figure 3.5A and B. Loss of methyl radical by the molecular radical cations yields m/z 293 and 298. Subsequent loss of CO$_2$ by m/z 293/298 yields m/z 249/254. M/z 179/184 is formed by the loss of the elements of ε-caprolactone by m/z 293/298, and goes on to lose CO$_2$ to form m/z 135/140 and Si(CH$_3$)$_2$O resulting in m/z 105/110. M/z 105/110 formation directly from the molecular cation cannot be excluded here, nor in the case of Scheme 3.4. M/z 117 is an ion characteristic of TMS derivatives of compounds with carboxy groups, which corresponds to

+COOTMS (Ng and Hupé, 1993). Weak m/z 117 ions were observed in both Figures 3.5A and B.

Figures 3.6A and 3.6B represent the spectra of the TMS derivatives of 6-benzoyloxyhexan-1-ol and 6-[^{2}H$_5$]benzoyloxyhexan-1-ol, which were expected metabolites in the biodegradation of 1,6-hexanediol dibenzoate and 1,6-hexanediol di[^{2}H$_5$]benzoate, respectively, by analogy to the metabolites reported for related commercial plasticizers (Gartshore et al., 2003). Scheme 3.4 proposes fragmentations that account for the major ions in the mass spectra. An interesting Me$_2$Si migration appears to occur in the loss of CO_2 for the transition m/z 179/184 - 135/140. The latter ion is drawn as a silicon analogue of an α,α-dimethylbenzyl cation, but the actual structure is not known. M/z 135 is also an intense fragment in the spectrum of the TMS ester of benzoic acid (NIST/EPA/NIH 1998 Mass Spectral Library) and is similarly formed by loss of CO_2 from m/z 179 produced by methyl radical loss from the molecular cation (m/z 194), both metastable confirmed (data not shown).

The spectra of the TMS derivatives of 4-benzoyloxybutanoic acid and 4-[^{2}H$_5$] benzoyloxybutanoic acids are illustrated in Figures 3.7A and B, and show homology with the spectra of 6-benzoyloxyhexanoic and 6-[^{2}H$_5$]benzoyloxyhexanoic acids (Figure 3.5A and 3.5B). M/z 265 and 270 are formed by the loss of methyl radical by the molecular radical cations (m/z 280 and 285, not detected), and go on to lose CO_2 forming m/z 221 and 226. Ions at m/z 179/184, 135/140 and 105/110 likely have the same structures as those proposed in Scheme 3.3. M/z 117 was also observed in both Figures 3.7A and 3.7B indicating the presence of a carboxyl group.

Figure 3.8A and 3.8B are respectively the mass spectra of the TMS derivatives of benzoic and [^{2}H$_5$]benzoic acids found in the derivatized extracts. The molecular ions at m/z 194 and 199 show fragmentations similar to those found in Figures 3.5, 3.6 and 3.7.

The experimental and calculated exact masses obtained for 1,6-hexanediol dibenzoate and 1,6-hexanediol di[^{2}H$_5$]benzoate and their metabolites are

presented in Table 3.1. The differences between the calculated and experimental masses was 2.3 ppm or better.

None of the above metabolites were observed in abiotic control experiments, eliminating the possibility of the formation of any of these metabolites by chemical hydrolysis or oxidation.

Table 3.2 contains data for the highest observed concentrations and maximum lifetime for each of the metabolites. All of these metabolites eventually disappeared. The most important of these is the monoester, 6-benzoyloxyhexan-1-ol. This compound is analogous to the monoesters produced by co-metabolism of the commercial plasticizers diethylene glycol dibenzoate and dipropylene glycol dibenzoate (Gartshore et al., 2003). However, the monoesters (diethylene glycol monobenzoate and dipropylene glycol monobenzoate) from biodegradation of the commercial plasticizers were not only resistant to further biodegradation but were also shown to exhibit significant toxicity in screening assays (Gartshore et al., 2003). Therefore, the fact that the monoester of 1,6-hexanediol was only observed in small quantities and also degraded rapidly supports the hypothesis that this compound may represent a more environmentally benign dibenzoate plasticizer.

The most long lived of the metabolites in Table 3.2 and one that was observed at an order of magnitude greater concentration than the monoester was 1-hexadecyl benzoate. This is the only metabolite that can not originate directly from the degradation of 1,6-hexanediol dibenzoate. It is hypothesized that the formation of 1-hexadecyl benzoate is the result of an enzymatic conjugation of benzoate hydrolyzed from 1,6-hexanediol dibenzoate or its metabolites to hexadecanol, a metabolite of hexadecane degradation. This is consistent with the fact that biodegradation of 1,6-hexanediol di[^{2}H$_5$]benzoate resulted in formation of 1-hexadecyl [^{2}H$_5$]benzoate. The above metabolites were consistently observed in several biodegradation experiments conducted in this study.

This ester of 1-hexadecanol is not a potential problem. It is an artifact arising from the use of hexadecane as the primary carbon and energy source. Hexadecane was convenient for these experiments because *R. rhodochrous* grows

well on hydrocarbons (Nalli et al., 2006c) and a hydrophobic substrate helps to disperse the water insoluble plasticizer. However, in an environmental situation, this benzoate is unlikely to be formed because there will not be appreciable amounts of alkanes or alcohols present.

3.5. Conclusions

GC/MS and FTMS were used to identify metabolites arising from the biodegradation of 1,6-hexanediol dibenzoate by *Rhodococcus rhodochrous.* All of these metabolites were confirmed by repeating the experiments with deuterium-labelled analogues.

In contrast to commercially-available dibenzoate plasticizers, metabolism of 1,6- hexanediol dibenzoate did not result in accumulation of persistent metabolites. Furthermore the most stable of the metabolites would not be expected to be observed in the environment. These results support the potential to use 1,6-hexanediol dibenozate as a green plasticizer.

3.6. Acknowledgments

Table 3.1. Accurate masses of labelled and unlabelled 1,6-hexanediol dibenzoate and their *Rhodococcus rhodochrous* metabolites measured in positive ion electrospray.

Compound	Ion Composition	Found	Required	Error (ppm)
1,6-Hexanediol dibenzoate	$C_{20}H_{22}O_4Na$	349.1410	349.1410	0.0
	$C_{20}H_{23}O_4$	327.1591	327.1591	0.0
1,6-Hexanediol di[$^2H_{10}$]benzoate	$C_{20}H_{12}\,^2H_{10}\,O_4Na$	359.2037	359.2038	0.3
	$C_{20}H_{13}\,^2H_{10}\,O_4$	337.2222	337.2218	1.1
1-Hexadecyl benzoate	$C_{23}H_{39}O_2$	347.2953	347.2945	2.3
1-Hexadecyl [$^2H_5$]benzoate	$C_{23}H_{34}\,^2H_5O_2$	352.3252	352.3258	1.7
6-Benzoyloxyhexanoic acid	$C_{13}H_{16}O_4Na$	259.0942	259.0941	0.4
6-[$^2H_5$]Benzoyloxyhexanoic acid	$C_{13}H_{11}\,^2H_5O_4Na$	264.1254	264.1255	0.4
6-Benzoyloxyhexan-1-ol	$C_{13}H_{18}O_3Na$	245.1148	245.1148	0.0
6-[$^2H_5$]Benzoyloxyhexan-1-ol	$C_{13}H_{13}\,^2H_5O_3Na$	250.1457	250.1462	2.0

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Table 3.2. Metabolites from the biodegradation of 1,6-hexanediol dibenzoate by *R. rhodochrous*[1]

Metabolites	Highest observed concentration, mmol/L[2]	Time of observation, h	Time of confirmed disappearance, h
6-Benzoyloxyhexan-1-ol	0.03 ± 0.01	40	87
6-Benzoyloxyhexanoic acid	0.30 ± 0.04	40	87
4-Benzoyloxybutanoic acid	0.07 ± 0.01	64	87
Benzoic acid	0.20 ± 0.02	40	87
1-Hexadecyl benzoate	0.31 ± 0.01	40	185

Notes

1. Initial concentration of 1,6-hexanediol dibenzoate was 3 mmol/L
2. Values are the average of triplicate samples. Samples were taken once per day
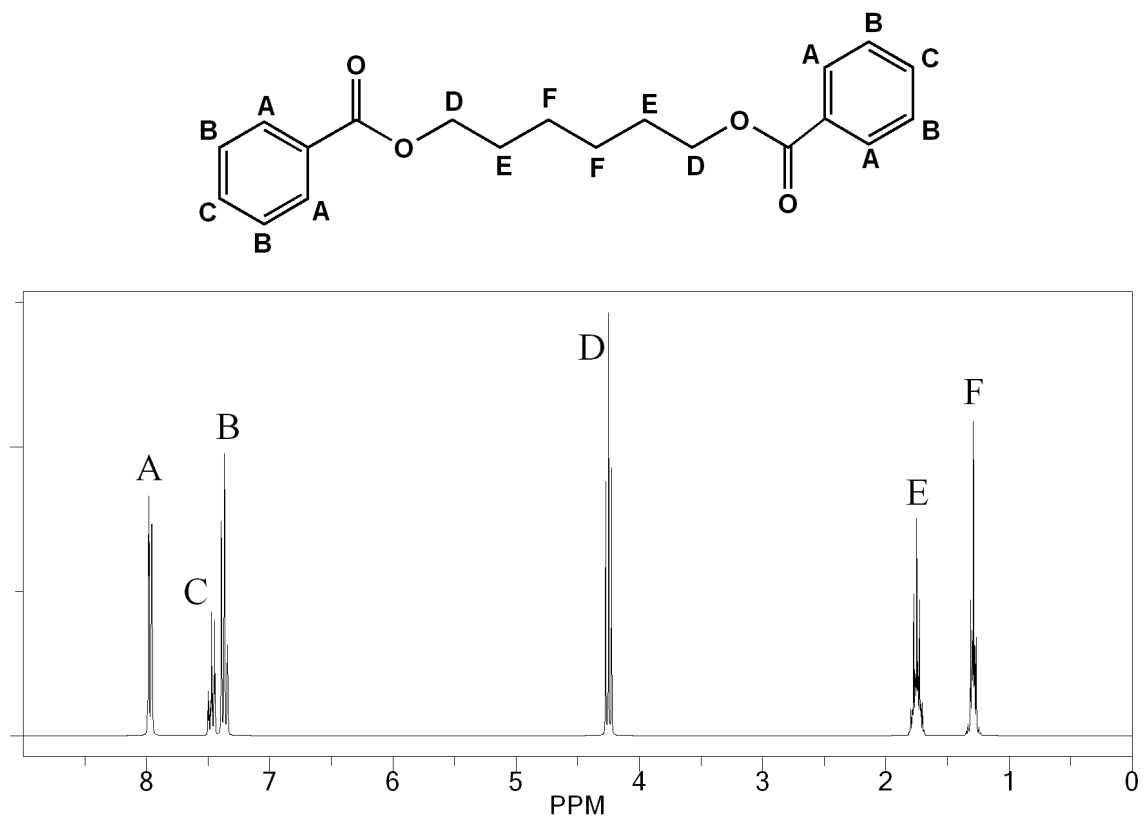
Figure 3.1. NMR spectrum of synthesized 1,6-hexanediol dibenzoate. Relative integration of protons on indicated carbon atom (A:B:C:D:E:F=2:2:1:2:2:2). A,B,C exhibit the expected patterns for a phenyl group. D and F are triplets and E is a multiplet (assumed to be a triplet of triplets) as expected for this part of the structure.
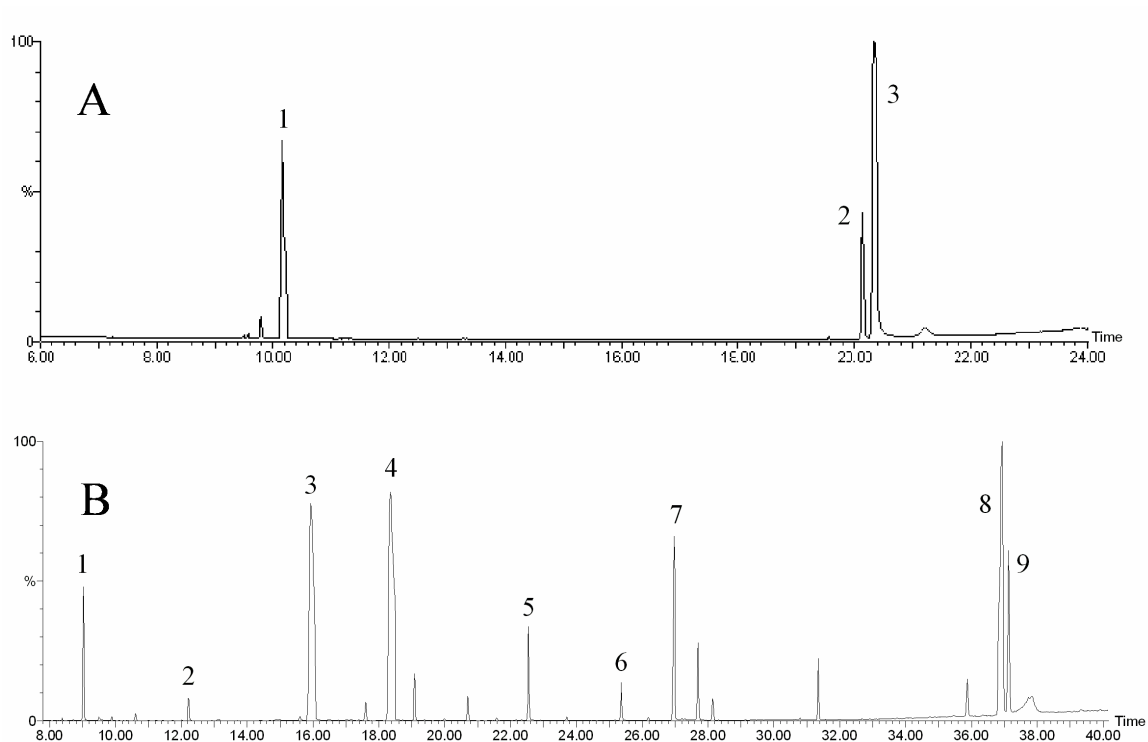
Figure 3.2. Gas chromatograms obtained for the underivatized broth extract (panel A) and for the extract after trimethylsilylation (panel B). In the former, peaks are identified as: 1: hexadecane, 2: 1-hexadecyl benzoate, 3: 1,6-hexanediol dibenzoate. In the latter, peaks are identified as: 1: benzoic acid TMS derivative, 2: 1,6-hexanediol TMS derivative, 3: pentadecane added to the extract as a retention time marker, 4: hexadecane added to the broth as a co-metabolite, 5: 4-benzoyloxybutyric acid TMS derivative, 6: 6-benzoyloxyhexan-1-ol TMS derivative, 7: 6-benzoyloxyhexanoic acid TMS derivative, 8: 1,6-hexanediol dibenzoate, and 9: 1-hexadecyl benzoate. Other eluting peaks in B originate in the solvent and derivatizing reagent. The elution order for 1-hexadecylbenzoate and 1,6-hexanediol dibenzoate is reversed by switching between DB-1 and HP-5 columns.
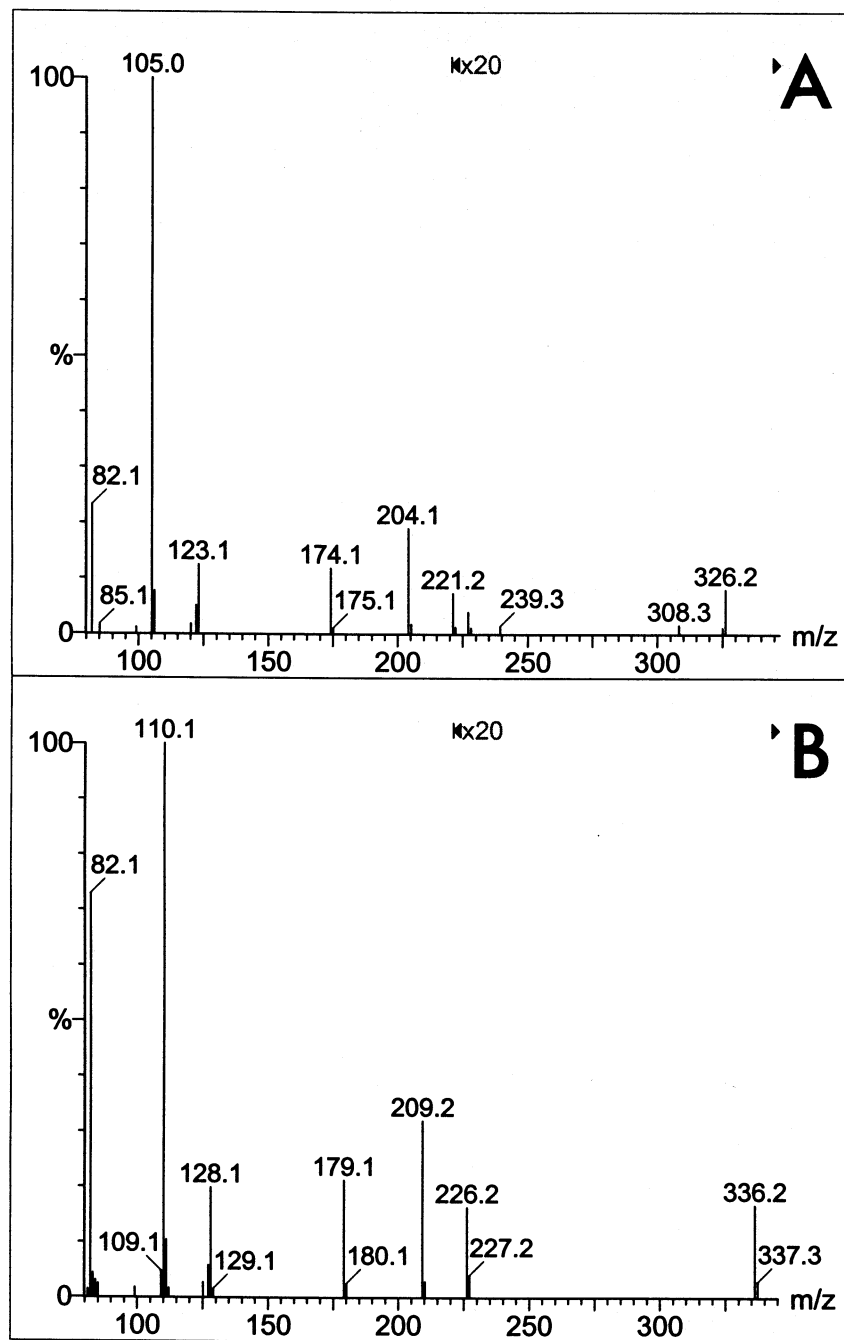
Figure 3.3. Electron ionization mass spectra of (A) 1,6-hexanediol dibenzoate and (B) 1,6-hexanediol di[^{2}H$_5$]benzoate isolated from the incubation mixtures. Their mass spectra and GC retention times are identical to those for the diesters synthesized for this study. The intensities of ions above m/z 220 are multiplied by 20.
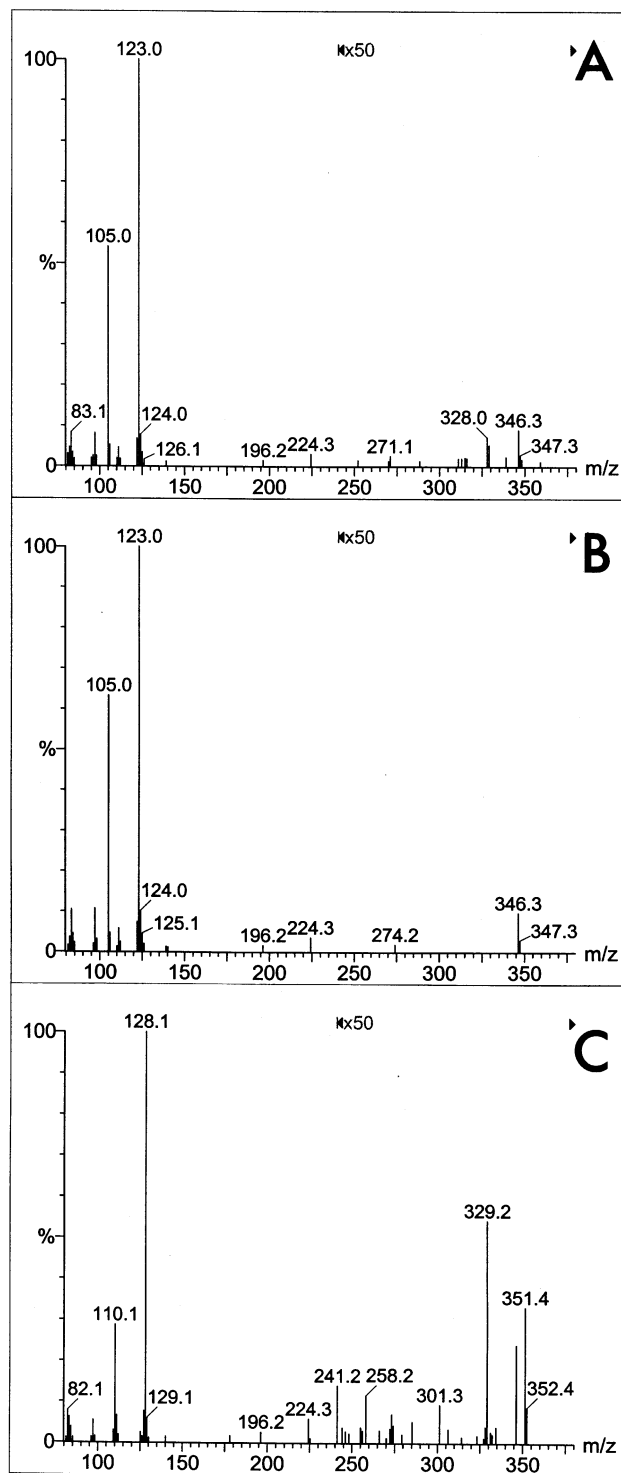
Figure 3.4. Electron ionization mass spectra of (A) 1-hexadecyl benzoate, (B) synthesized 1-hexadecyl benozate and (C) 1-hexadecyl [^{2}H$_5$]benzoate. The intensities of ions above m/z 240 are multiplied by 50.
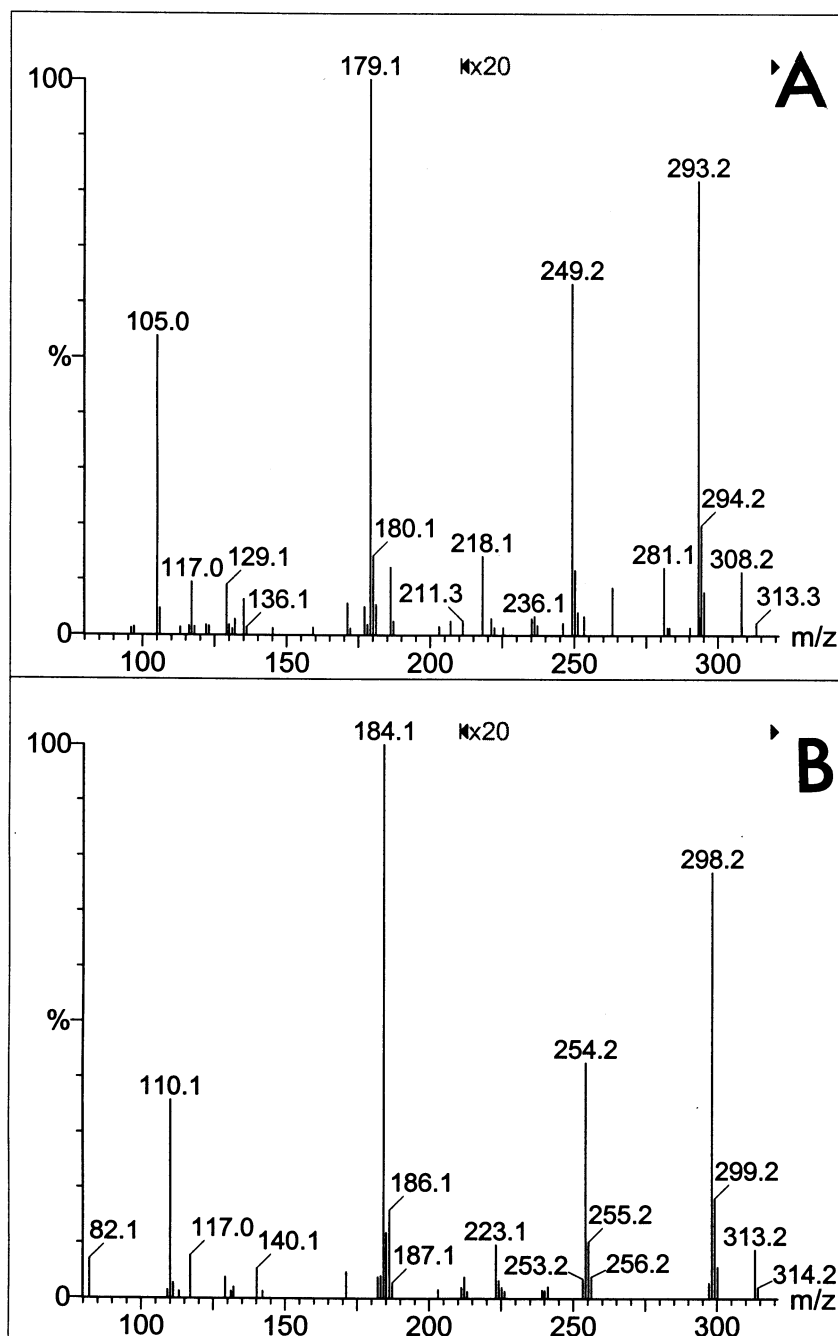
Figure 3.5. Electron ionization mass spectra of the TMS derivatives of (A) 6-benzoyloxy-hexanoic acid and (B) 6-[^{2}H$_5$]benzoyloxyhexanoic acid isolated from the incubation mixtures. The intensities of ions above m/z 210 are multiplied by 20.
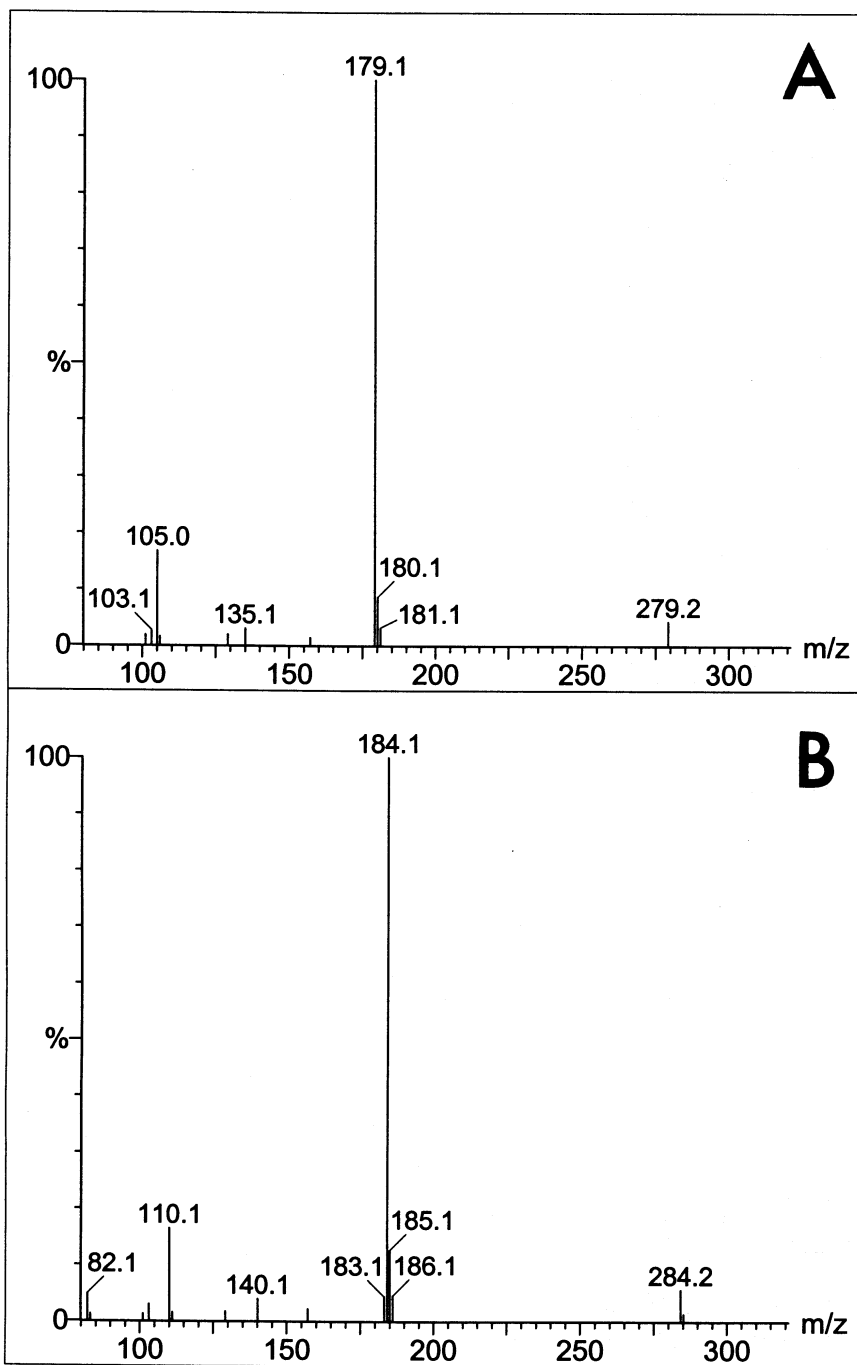
Figure 3.6. Electron ionization mass spectra of the TMS derivatives of (A) 6-benzoyloxyhexan-1-ol and (B) 6-[^{2}H$_5$]benzoyloxyhexan-1-ol isolated from the incubation mixtures.
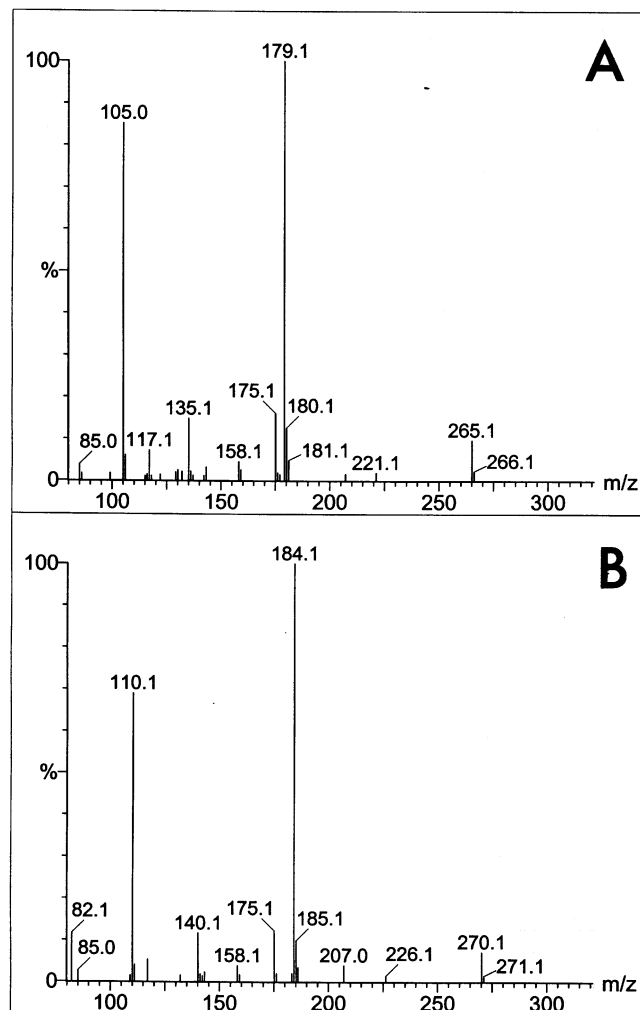
Figure 3.7. Electron ionization mass spectra of the TMS derivatives of (A) 4-benzoyloxybutanoic acid and (B) 4-[^{2}H$_5$] benzoyloxybutanoic acid isolated from the incubation mixtures.
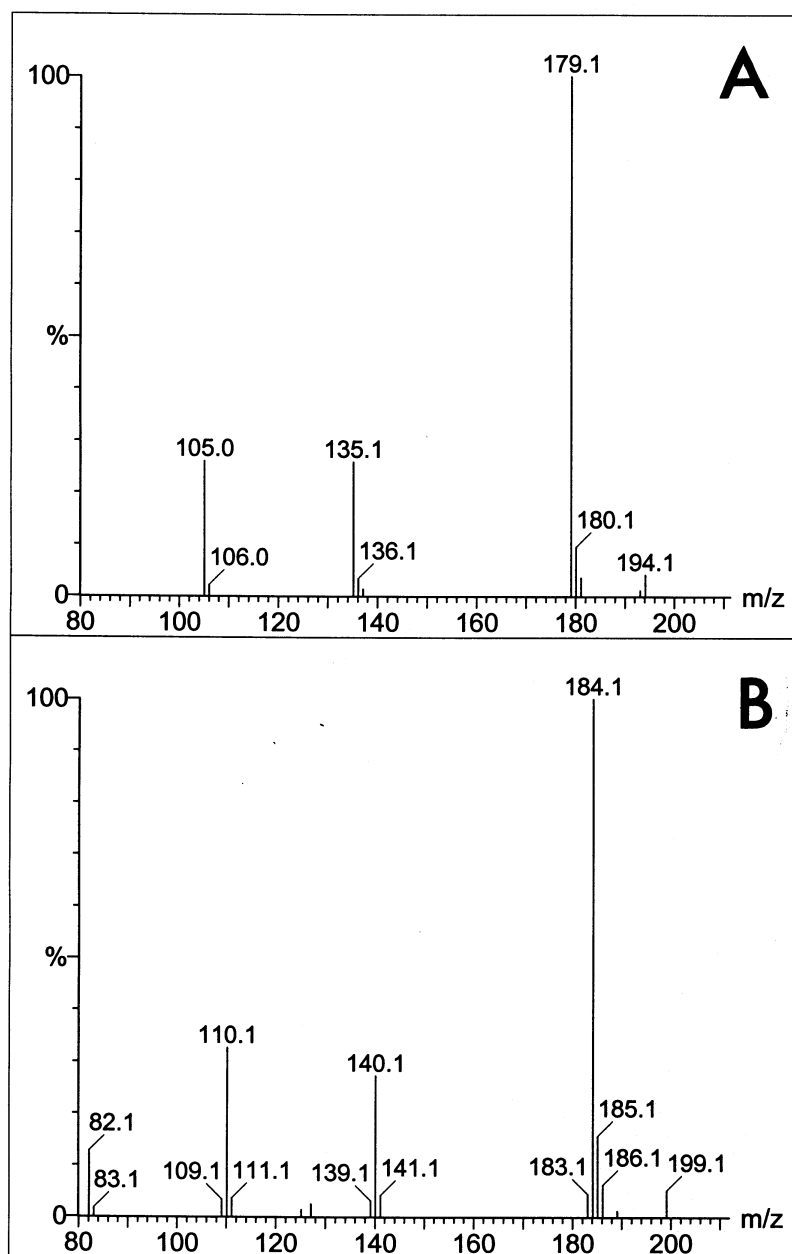
Figure 3.8. Electron ionization mass spectra of the TMS derivatives of (A) benzoic acid and (B) [^{2}H$_5$] benzoic acid isolated from the incubation mixtures.
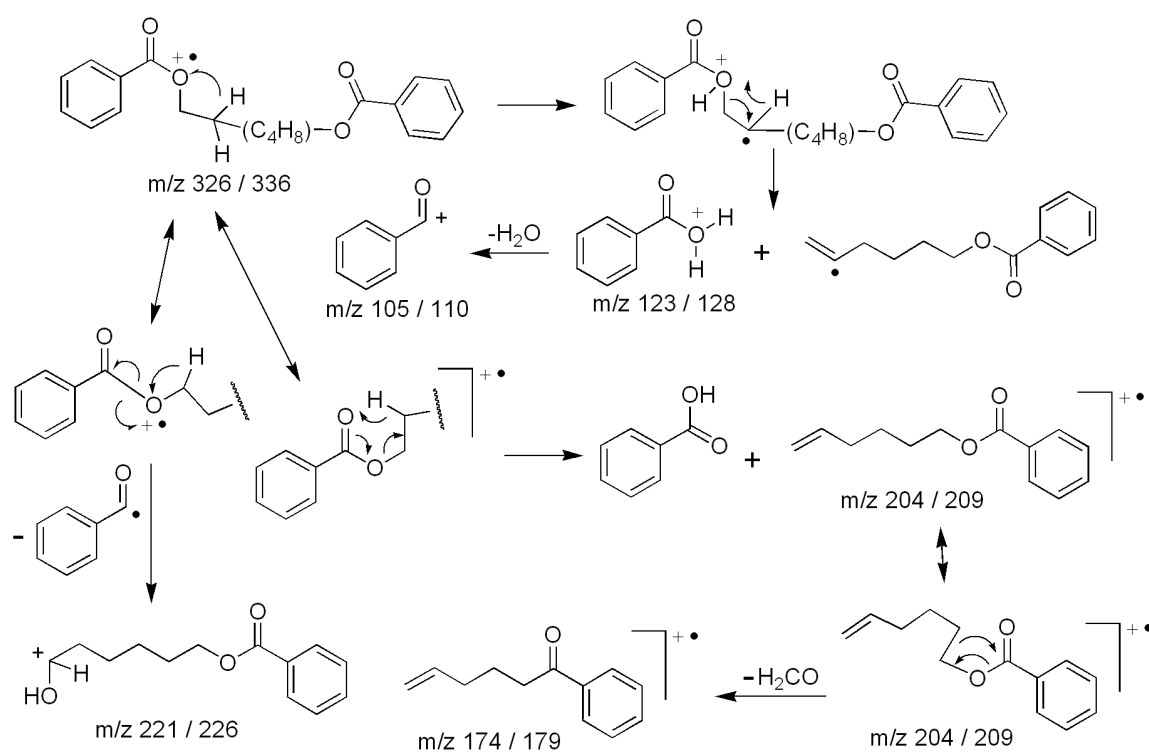
Scheme 3.1. Proposed fragmentation scheme for 1,6-hexanediol dibenzoate and 1,6-hexanediol di[^{2}H$_5$]benzoate. The second m/z value refers to the labelled diester.
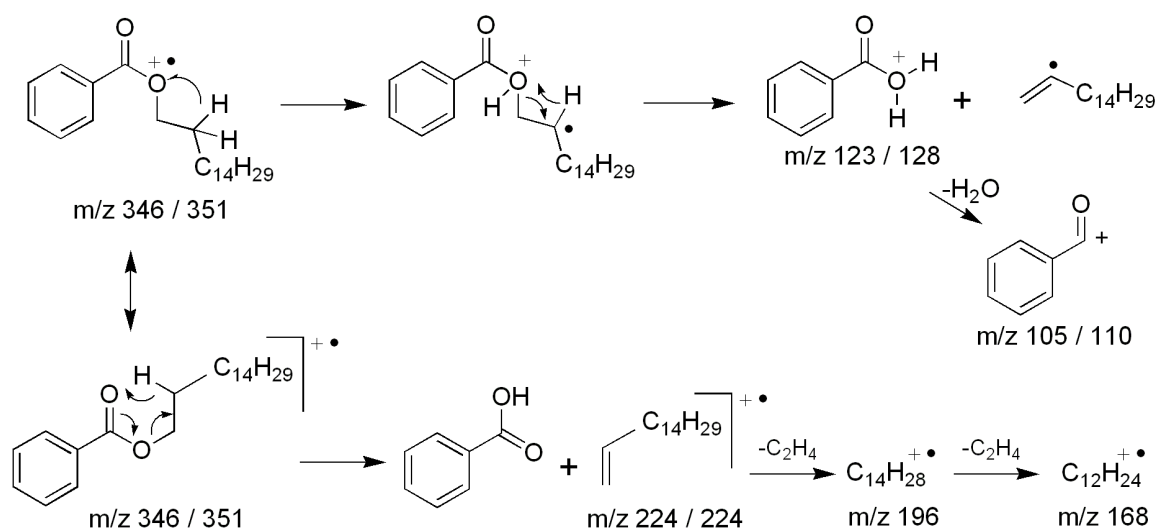
Scheme 3.2. Proposed mass spectrometric fragmentation scheme for 1-hexadecyl benzoate and 1-hexadecyl [^{2}H$_5$]benzoate. The second m/z value refers to the labelled ester.

55
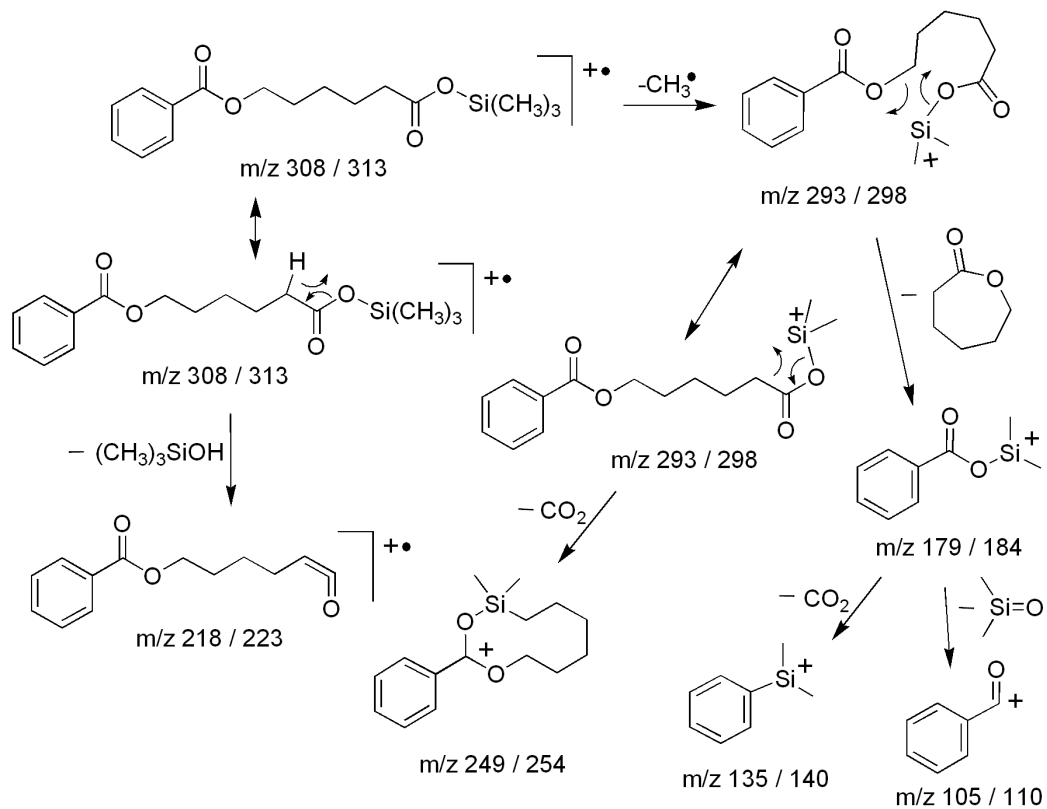
Scheme 3.3. Proposed mass spectrometric fragmentation scheme for the TMS derivatives of 6-benzoyloxyhexanoic and 6-[^{2}H$_5$]benzoyloxyhexanoic acids. The second m/z value refers to the labelled TMS ester.
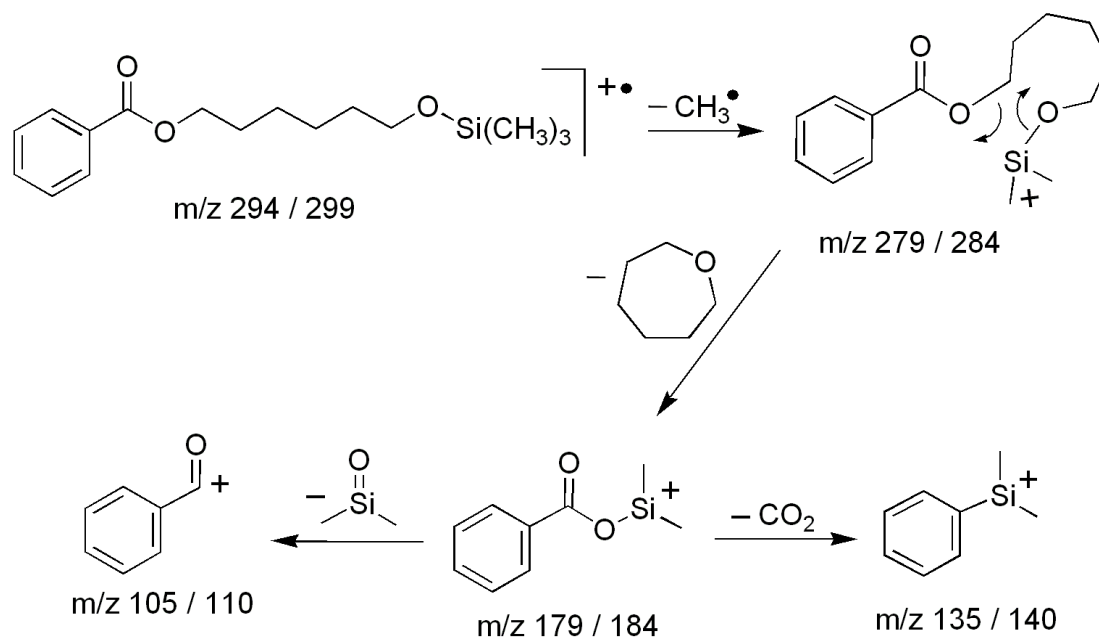
Scheme 3.4. Proposed mass spectrometric fragmentation scheme for the TMS derivatives of 6-benzoyloxyhexan-1-ol. The second m/z value refers to the labelled TMS ester.

4. Mechanisms of Biodegradation of Dibenzoate Plasticizers

Preface

The previous chapter demonstrated that biotransformation of 1,6-hexanediol dibenzoate did not lead to the formation of stable metabolites. Furthermore, an in-depth study was essential to establish the biodegradation mechanisms for this compound and its related metabolites. In the following chapter, biodegradation of 1,6-hexanediol dibenzoate and two commercial plasticizers, diethylene glycol dibenzoate (D(EG)DB), dipropylene glycol dibenzoate (D(PG)DB) were studied and their biodegradation mechanisms were elucidated. Biodegradation of 1,6-hexanediol dibenzoate was done by *Rhodococcus rhodochrous* in the presence of hexadecane or $[^2H_{30}]$tetradecane. Characterization of the metabolites produced during the biodegradation process provided a basis for a proposed biodegradation pathway for 1,6-hexanediol dibenzoate, D(EG)DB and D(PG)DB. It was concluded that biodegradation of all of these dibenzoate plasticizers was initiated with ester hydrolysis and the release of the corresponding monoester. However, biodegradation of the monoester released from biodegradation of 1,6-hexanediol dibenzoate, namely 1,6-hexanediol monobenzoate, proceeded by oxidation of the alcohol group to generate 6-(benzoyloxy) hexanoic acid, followed by progressive β-oxidation steps. Therefore this monoester metabolite was quickly degraded. This pathway was blocked for the monoesters of commercial plasticizers, D(EG)MB and D(PG)MB, by the presence of an ether function. Furthermore, the use of a $[^2H_{30}]$tetradecane as a co-substrate in biodegradation of 1,6-hexanediol dibenzoate resulted in the formation of 1-$[^2H_{29}]$ tetradecyl benzoate. Detection of this metabolite confirmed that production of esters of the alcohols was a result of enzymatic esterification reaction between the benzoate hydrolyzed from the esterification reaction and 1-$[^2H_{29}]$tetradecanol, an oxidative metabolite of $[^2H_{30}]$tetradecane.

Mechanisms of Biodegradation of Dibenzoate Plasticizers

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4.1. Abstract

Biodegradation mechanisms were elucidated for three dibenzoate plasticizers: di-ethylene glycol dibenzoate (D(EG)DB), dipropylene glycol dibenzoate (D(PG)DB), both of which are commercially available, and 1,6-hexanediol dibenzoate, a potential green plasticizer. Degradation studies were done using *Rhodococcus rhodochrous* in the presence of pure alkanes as a co-substrate. As expected, the first degradation step for all of these systems was the hydrolysis of one ester bond with the release of benzoic acid and a monoester. Subsequent biodegradation of the monobenzoates of diethylene glycol (D(EG)MB) and dipropylene glycol (D(PG)MB) was very slow, leading to significant accumulation of these monoesters. In contrast, 1,6-hexanediol monobenzoate was quickly degraded and characterization of the metabolites indicated that the

biodegradation proceeded by way of the oxidation of the alcohol group to generate 6-(benzoyloxy) hexanoic acid followed by β-oxidation steps. This pathway was blocked for D(EG)MB and D(PG)MB by the presence of an ether function.

The use of a pure hydrocarbon as a co-substrate resulted in the formation of another class of metabolites; namely the esters of the alcohols formed by the oxidation of the alkanes and the benzoic acid released by hydrolysis of the original diesters. These metabolites were biodegraded without the accumulation of any intermediates.

Key words: Biodegradation, plasticizers, 1,6-hexanediol dibenzoate, metabolites

4.2. Introduction

To improve the flexibility and workability of plastic resins, it is often necessary to incorporate plasticizers into polymeric matrices (Sears and Darby, 1982). This has resulted in the broad application of plasticizers in industries for the production of electrical cables, paints, wall papers and other construction materials and, to a lesser extent, food packaging films and medical products (Staples et al., 1997). The global demand for plasticizers was approximately 5×10^9 kg in 1999 and has been estimated to be growing by approximately 2.8% annually (Lerner, 2003).

Due to their widespread use in such large quantities, extensive research has been conducted to investigate the impacts associated with the release of plasticizers into the environment during manufacturing, while they are being used, and following their disposal (Cadogan et al., 1993; Staples et al., 1997; Bauer and Herrmann, 1997). The most commonly used class of plasticizers is the phthalates (Rahman and Brazel, 2004), for which significant concerns have been raised about the health and environmental consequences associated with their use (Wams, 1987; Staples et al., 1987; Scholz et al., 1997; Tickner et al., 2001). For example, phthalates and their intermediary metabolites have been detected in aquatic and terrestrial environmental samples (Roslev et al., 1998; Cartwright et

al., 2000, Horn et al. 2004, Otton et al., 2004), as well as in human plasma and urine (Wahl et al., 2001; Wahl et al., 2004; Sathyanarayana et al., 2008). Moreover, the findings of numerous toxicological studies have resulted in several phthalates and their metabolites being placed in the list of priority pollutants of the United States Environmental Protection Agency (Keith and Telliard, 1979). Our earlier work identified toxic metabolites from the degradation of phthalates and adipates and elucidated the breakdown mechanism of their biodegradation by a common soil microorganism, *Rhodococcus rhodochrous* (Nalli et al., 2002; Nalli et al., 2006b,c).

The health and environmental implications of phthalates and increasingly strict environmental legislation has led to their partial replacement in a number of plastics applications with dibenzoate plasticizers (Wypych, 2004; Rahman and Brazel, 2004). This is due to the higher biodegradation rates and lower toxicity of the dibenzoates (Arendt and Lang, 1998; Lang and Stanhope, 2001). The European Chemical Agency has recently approved dibenzoates as alternatives to phthalates (Deligio, 2009). However, earlier studies have shown that the interaction of *Rhodotorula rubra* with dibenzoate-based plasticizers resulted in incomplete microbial degradation leading to the accumulation of monobenzoates, which had significantly higher toxicity than the original plasticizers (Gartshore et al., 2003).

Consequently, it is of considerable importance to identify the functional groups that influence the biodegradation pathways of these dibenzoate plasticizers. This will provide insight that can be used to design alternative plasticizers that do not result in the accumulation of toxic intermediates when interacting with common microorganisms.

The two most important commercial dibenzoate plasticizers, di-ethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB), both contain ether functions. The objective of this study was to investigate the effect of the ether function on the biodegradation mechanisms of dibenzoate plasticizers. This was done by comparing the biodegradation mechanisms of the

two commercial dibenzoate plasticizers with that of 1,6-hexanediol dibenzoate, a potential green plasticizer.

4.3. Materials and Methods

4.3.1. Chemicals and reagents

1,6-Hexanediol (99%), n-hexadecane (99%), benzoyl chloride (99%), D(EG)DB (96%) and D(PG)DB (98%) were purchased from Sigma-Aldrich (Oakville, ON). BactoTM Brain/Heart infusion was obtained from Difco Microbiology (Montréal, QC). $[^2H_{30}]$Tetradecane (98 atom % D) was purchased from CDN isotopes (Montréal, QC). Pentadecane (99%) was purchased from A&C American Chemicals (Montréal, QC), bis(trimethylsilyl) trifluoroacetamide (BSTFA) was purchased from Chromatographic Specialties (Brockville, ON), and all other chemicals were purchased from Fisher Scientific (Montréal, QC). 1,6-Hexanediol dibenzoate was synthesized as described previously (Kermanshahi pour et al., 2009).

4.3.2. Microorganism and growth conditions

R. rhodochrous ATCC 13808 (ATCC, Manassas, VA, USA) was maintained at -70°C in plastic vials containing 20% glycerol and the optimal growth medium of Bacto Brain/Heart infusion broth, as recommended by the ATCC. The contents of the vials were used to grow inocula in sterile Brain/Heart infusion broth. One mL of this inoculum was transferred to shake flasks containing 100 mL of the sterilized minimum mineral salt medium (MMSM), 0.1 g/L yeast extract and 2.5 g/L of one of n-hexadecane, n-tetradecane or $[^2H_{30}]$tetradecane. The concentration of 2.5 g/L of the hydrocarbon was chosen to ensure that growth of bacteria was not carbon-limited.

The MMSM consisted of 4 NH_4NO_3, 4 KH_2PO_4, 6 Na_2HPO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 0.01 $CaCl_2 \cdot 2H_2O$, 0.01 $FeSO_4 \cdot 7H_2O$, and 0.014 disodium ethylenediaminetetraacetic acid (in g/L). When the stationary phase was reached, 2 mL of this microbial culture was used to inoculate shake flasks containing 200 mL of sterile MMSM, 2.5 g/L of the appropriate hydrocarbon, and either 1.6 g/L

(5.1 mmol/L) D(EG)DB or 1.3 g/L (3.8 mmol/L) D(PG)DB or 1.5 g/L (4.6 mmol/L) 1,6-hexanediol dibenzoate. These shake flasks were incubated on a rotary incubator shaker (Series 25, New Brunswick Scientific, Edison, NJ, USA) set at 250 rpm and 30°C.

4.3.3. Sample preparation for GC analyses

Triplicate samples of 3 mL were taken from the shake flasks, usually once per day. The pH was reduced to approximately 2 by the addition of sulfuric acid and the sample was then extracted with 3 mL of chloroform containing 1.5 g/L pentadecane as an internal standard. The mixture was stirred vigorously for 1 min and then the organic phase was transferred to a glass vial using a glass syringe. The samples were stored at 4°C until analysis by GC.

4.3.4. GC analyses

The concentrations of the plasticizers and the metabolites were determined by GC/FID. Aliquots (1 µL) of the chloroform extracts were analyzed using a Varian CP-3800 GC equipped with a FID detector and a fused silica 8CB column (Varian, Montreal, QC) with a length of 30 m and an inner diameter of 0.32 mm. The chromatographic conditions were as follows: injection port temperature of 250°C; initial column temperature of 40°C; initial time of 2 min; heating rate of 10°C min^{-1}; final temperature of 300°C; and detector temperature of 300°C. Helium was used as the carrier gas at a flowrate of 1.5 mL/min.

4.3.5. Sample preparation for GC/MS Analyses

Samples for GC/MS analyses were treated in the same manner as for GC/FID analysis except that the chloroform used for extraction did not contain pentadecane. The solvent was removed with a dry nitrogen stream and the residues were dissolved in 50 µL of anhydrous pyridine. Trimethylsilyl (TMS) derivatives were made by the addition of 50 µL of BSTFA to the pyridine solutions in capped auto injector vials and these were heated in an aluminum block at 60°C for 15 min.

4.3.6. GC/MS analyses

Aliquots (1 µL) of the TMS derivatized extracts were analyzed in low resolution GC/MS mode with a GC (time-of-flight) mass spectrometer (Micromass, Manchester UK) fitted with a 30 m DB-1 capillary column having a 0.25-mm inner diameter and 0.25-µm film thickness. The temperature varied from 80°C after a 1 min holding time to 300°C at 10°C min^{-1} under controlled conditions followed by a bake-out period of 6 min at 300°C. The injector was operated in a 1:100 split mode at 250°C with a constant helium pressure of 70 kPa. The GC re-entrant temperature was 250°C. The ion source was operated in election ionization mode at 70 eV and 200°C.

Un-derivatized extracts were also analyzed in GC/MS mode on a 30 m long, 0.32-mm inner diameter HP-5 column programmed at 10 °C min^{-1}, with all other parameters remaining the same as those above.

4.4. Results

The metabolites generated from the biodegradation experiments of 1,6-hexanediol dibenzoate or D(EG)DB by *R. rhodochrous* growing on various hydrocarbons as a co-substrate are presented in Table 4.1. Many of these compounds have been identified previously in experiments using glucose or hexadecane as the co-substrate (Gartshore et al., 2003; Kermanshahi pour et al., 2009). The new compounds identified here were 6-(benzoyloxy)-3-hydroxy butanoic acid, 4-(benzoyloxy)-3-hydroxy butanoic acid, 1-[^{2}H$_{29}$]tetradecyl benzoate and 2-[2-(benzoyloxy)ethoxy] acetic acid. These were all identified by GC/MS using the molecular weight of the parent ions (Table 4.1) and by comparison with the fragmentation patterns of similar compounds reported previously in Chapter 3 (Kermanshahi pour et al., 2009b).

Figure 4.1a demonstrates a typical degradation of D(EG)DB by *R. rhodochrous* in a medium containing hexadecane. There was a significant accumulation of di-ethylene glycol monobenzoate accounting for 70% of the initial D(EG)DB. During the same time period there was considerable

accumulation of benzoic acid. Both of these metabolites were eventually metabolized further.

Figure 4.1b shows data for the degradation of D(PG)DB. This compound has three different possible isomers depending on the positions of the methyl branches on the central propylene glycol dimer and all of these seem to degrade at the same rate. The monoester metabolite D(PG)MB also has isomers but only two peaks were seen in significant concentrations. These both had similar patterns of appearance and degradation. The concentrations increased to maximum values at about the time that the D(PG)DB disappeared and then two monobenzoates slowly degraded. A small amount of benzoic acid was also observed early in the experiment, but this quickly disappeared.

The results of biodegradation of 1,6-hexanediol dibenzoate by *R. rhodochrous* are presented in Figures 4.2a and 4.2b with either hexadecane or tetradecane, respectively, as the co-substrate carbon source. In both cases, the dibenzoate was degraded and benzoic acid was produced and then degraded. The other metabolites are more interesting. 4-Benzoyloxy-butanoic acid was observed in both sets of experiments but, while it was the major metabolite when tetradecane was a co-substrate (Figure 4.2b), it was only observed in trace amounts when hexadecane was used (Table 4.1). The metabolite 1-hexadecyl benzoate was only observed when hexadecane was used. In all of these experiments, the other metabolites listed in Table 4.1 were either not observed or only seen in small amounts and had degraded by the end of the experiments.

The same type of experiment was repeated in the presence of $[^2H_{30}]$tetradecane as a co-substrate and the same major metabolites seen in Figure 4.2b, 4-benzoyloxybutanoic acid and benzoic acid, were observed to behave in a similar fashion. The only deuterium-containing metabolite observed was 1-$[^2H_{29}]$tetradecyl benzoate and this was only detected in trace amounts (Table 4.1).

In all of the above experiments, the co-substrate (i.e., hexadecane or $[^2H_{30}]$tetradecane) biodegraded and supported the growth of bacteria. As an example, biodegradation of hexadecane during the biodegration of 1,6-hexanediol dibenzoate is shown in Figure A1-1 in Appendix 1.

Each of the above experiments were repeated either in duplicate or triplicate and in all of these, the trend of the biodegradation of the parent compound and the formation of the metabolites were reproducible and consistent with the results shown in Figure 4.1 and 4.2.

4.5. Discussion

The biodegradation of the commercial plasticizers D(EG)DB and D(PG)DB by the yeast, *R. rubra* resulted in the formation of substantial amounts of the metabolites diethylene glycol monobenzoate (D(EG)MB) or dipropylene glycol monobenzoate (D(PG)MB) (Gartshore et al., 2003). These monoesters were shown to be toxic and, especially in the case of D(PG)MB, resistant to further degradation. In the current study, using a common soil bacterium, *R. rhodochrous*, it was shown that while the monoesters were again generated, this bacterium was more effective at degrading these metabolites.

The compounds containing dipropylene glycol (both the diester and the monoester) exist as isomers because there are several possible variations depending on the position of the methyl groups. Each of the two moieties of propylene glycol has a single methyl substituent. Both methyl groups can be on the carbon atoms adjacent to the central ether function; both can be on the carbon atoms adjacent to the alcohol functions; or one moiety can have a methyl group adjacent to the ether and the other a methyl group adjacent to the alcohol. It was possible to differentiate among some of these with the GC column being used and it is clear that the presence of these methyl branches can slow down the rate of hydrolysis of the monoesters. In the results presented here, both the diester and monoester of diethylene glycol were biodegraded more quickly than those of the dipropylene glycol. However, there was no evidence of a difference in stability of the isomers of either the D(PG)DB or D(PG)MB compounds. Therefore, the actual placement of the methyl groups on the propylene glycol fragment is not a significant factor in the biodegradability of these compounds. In the cases of commercial plasticizers, biodegradation led to significant accumulation of

D(EG)MB and D(PG)MB accounting for up to 70% and 80% of the initial molar concentration of D(EG)DB and D(PG)DB, respectively (Figures 1a and b). These metabolites were stable over the course of the experiment, even though they did eventually biodegrade.

To develop a green plasticizer analogue of this class of compounds, it would be essential to ensure that there was not a build-up of a toxic monoester, or any other toxic metabolite, during interaction with microorganisms. While the removal of the methyl groups did not eliminate the production of a monoester, replacing the ether function of D(EG)DB with an ethylenic group achieved this goal. Biodegradation of the new compound, 1,6-hexanediol dibenzoate, by *R. rhodochrous* resulted in only trace amounts of the corresponding monoester, 1,6-hexanediol monobenzoate, and this was quickly degraded.

Several other metabolites were produced, but most of these were detected in only trace amounts (Table 4.1). The most noticeable exception was benzoic acid, which was observed as a metabolite for the biodegradation of all of the dibenzoates, using any of the co-substrates (Table 4.1). While benzoic acid was observed in the studies with the potential green plasticizer, it was not observed in concentrations as high as those observed with the two commercial plasticizers and benzoic acid was not resistant to further degradation. Even if small amounts of benzoic acid are released by biodegradation of this green plasticizer, benzoic acid is not a source of significant environmental concern and, in fact, is approved for use as a food preservative (US FDA, 1973).

The presence of significant amounts of benzoic acid early on in all of the biodegradation experiments is consistent with the hydrolysis of one ester bond being the first step in the biodegradation of all of the dibenzoate plasticizers. The hydrolysis of the second ester bond seems to be much slower, at least in the cases of D(EG)MB and D(PG)MB, and it is possible that the free hydroxyl function on the monoester inhibits the enzyme activity.

The pattern for the biodegradation of 1,6-hexanediol dibenzoate (Figure 2a) is significantly different to that of the two commercial plasticizers. In particular, there were high concentrations of 1-hexadecyl benzoate. It was

previously suggested that this was formed by the esterification reaction between benzoic acid, released by hydrolysis of the plasticizer, and hexadecanol, a metabolite of hexadecane (see Scheme 4.1) (Kermanshahi pour et al., 2009b). This hypothesis is now confirmed. The data in Table 4.1 show that the nature of the benzoate ester formed was dependent on the co-substrate. Thus, substitution of hexadecane by deuterated tetradecane resulted in the side product being 1-[^{2}H$_{29}$]tetradecyl benzoate, which could only come from the deuterated co-substrate. This indicates that the creation of this type of intermediate should not be considered an environmental risk. Its presence is an artifact of working with a pure culture growing on a sufficient amount of an easily oxidized alkane to generate excess amounts of the alcohols faster than the microorganism is metabolizing these alcohols. It is unlikely that there would be appreciable amounts of these alkanes in most environmental sites so the formation of this type of intermediate is not an issue.

However, the presence of an alkyl benzoate does create a problem in the interpretation of the data and the elucidation of the mechanism of biodegradation of dibenzoates. As mentioned above, the biodegradation of 1,6-hexanediol dibenzoate does not generate a stable monoester, but the data in Table 4.1 show the presence of three metabolites, (benzoyloxy)hexanoic acid, 6-(benzoyloxy)-3-hydroxy hexanoic acid and 4-(benzoyloxy) butanoic acid, which all could have originated from the progressive β-oxidation of 1-hexadecyl benzoate (Scheme 4.1). However, when the alkane used as a co-substrate was [^{2}H$_{30}$]tetradecane, the same, non-deuterated, oxidation products were observed. Thus, these must have originated from the monoester, not 1-hexadecyl benzoate. The elimination of the pathway in Scheme 4.1 leads to the pathway shown in Scheme 4.2. This mechanism was developed after identifying the various metabolites shown in Table 4.1.

There are actually two possible pathways for the monoester derived from 1,6-hexanediol dibenzoate shown in Scheme 4.2 and both are probably operating. The monoester could undergo a second hydrolysis to release the diol and a second molecule of benzoic acid. It is important to note that this pathway is the only

possibility available for the monoesters D(EG)MB and D(PG)MB because of the presence of the ether bond in the diol fragment. If the ether bond is removed as in 1,6-hexanediol benzoate, the other pathway becomes an option and the monoester can be oxidized to 6-(benzoyl)hexanoic acid followed by β-oxidation to generate 6-(benzoyl)-3-hydroxy hexanoic acid and then 4-(benzoyloxy)butanoic acid.

The biodegradation of D(EG)DB leads to small amounts of a compound [2-(benzoyloxy)ethoxy]acetic acid, which must be the oxidation product of the monoester. However, there is no evidence of the products from β-oxidation of this compound, thereby confirming that this pathway is not possible if the ether bond is present. Overall, it seems likely that the only pathway for the biodegradation of the monoesters from D(EG)MB or D(PG)MB is the relatively slow hydrolysis of the second ester bond.

All of these considerations lead to the conclusion that a stable monoester will be generated if the β-oxidation pathway was limited by the presence of an internal ether bond. This implies that the monoester is resistant to hydrolysis of the second ester bond but that this mechanism does slowly break down this toxic intermediate. In the case of the monoester from 1,6-hexanediol dibenzoate, the β-oxidation pathway is very efficient but this implies that, again, the monoester is resistant to hydrolysis of the second ester bond. It seems reasonable to conclude that the free hydroxyl function is somehow interfering with the action of the esterase enzymes on the monoesters.

4.6. Conclusions

In this study, it has been demonstrated that biotransformation of two commercial dibenzoate plasticizers, D(PG)DB and D(EG)DB, by *R. rhodochrous* results in the accumulation of monoester metabolites. In contrast, the biodegradation of 1,6-hexanediol dibenzoate, a potential green plasticizer analogue to D(EG)DB, did not result in the accumulation of the corresponding monoester, 1,6-hexanediol monobenzoate. The biodegradation pathway established for 1,6-hexanediol dibenzoate shows that 1,6-hexanediol monobenzoate degraded via oxidation and β-oxidation.

Biodegradation mechanisms established for the dibenzoate plasticizers demonstrate that the presence of the ether function leads to significant quantities of toxic metabolites. This, in turn, can be seen to be an important consideration in the design of green plasticizers.

4.7. Acknowledgements

Table 4.1. Metabolites from the biodegradation of dibenzoates by *R. rhodochrous* in the presence of a primary carbon source.

Metabolites	Molecular cation [$M^{+\cdot}$], (m/z)	Metabolites detected [1]			
		1,6-Hexanediol dibenzoate [2]			Diethylene glycol dibenzoate[2]
		Hexadecane[3]	Tetradecane[3]	[^{2}H30]Tetradecane[3]	Hexadecane[3]
1,6-Hexanediol monobenzoate	294[4,5]	0.02±0.01	ND	0.03±0.005	ND
6-(Benzoyloxy)hexanoic acid	308[4]	0.02±0.004	ND	Trace	ND
4-(Benzoyloxy)butanoic acid	280[4,5]	Trace	0.90±0.08	0.85±0.04	ND
6-(Benzoyloxy)-3-hydroxyhexanoic acid	396[4,5]	Trace	ND	Trace	ND
4-(Benzoyloxy)-3-hydroxybutanoic acid	368[4,5]	ND	0.04±0.08	0.03±0.004	ND
1,6-Hexanediol	262[4]	Trace	ND	ND	ND
1-[^{2}H$_{29}$]Tetradecyl benzoate	347	ND	ND	Trace	ND
Benzoic acid	194[4]	1.31±0.08	0.25±0.01	0.05±0.01	3.34±0.23
1-Hexadecyl benzoate	346	0.44±0.08	ND	ND	Trace
Diethylene glycol monobenzoate	282[4]	ND	ND	ND	3.68±0.48
2-[2-(Benzoyloxy)ethoxy] acetic acid	296[4]	ND	ND	ND	0.32±0.002

Note:

1. All concentrations are in units of mM. Concentrations are the average of triplicate sampling and correspond to the highest concentration observed. ND: not detected.
2. Dibenzoate plasticizer.
3. Hydrocarbon primary substrate.
4. [$M^{+\cdot}$] of the trimethylsilyl derivative.
5. [$M^{+\cdot} - CH^{\cdot}_3$] was detected in the mass spectrum.
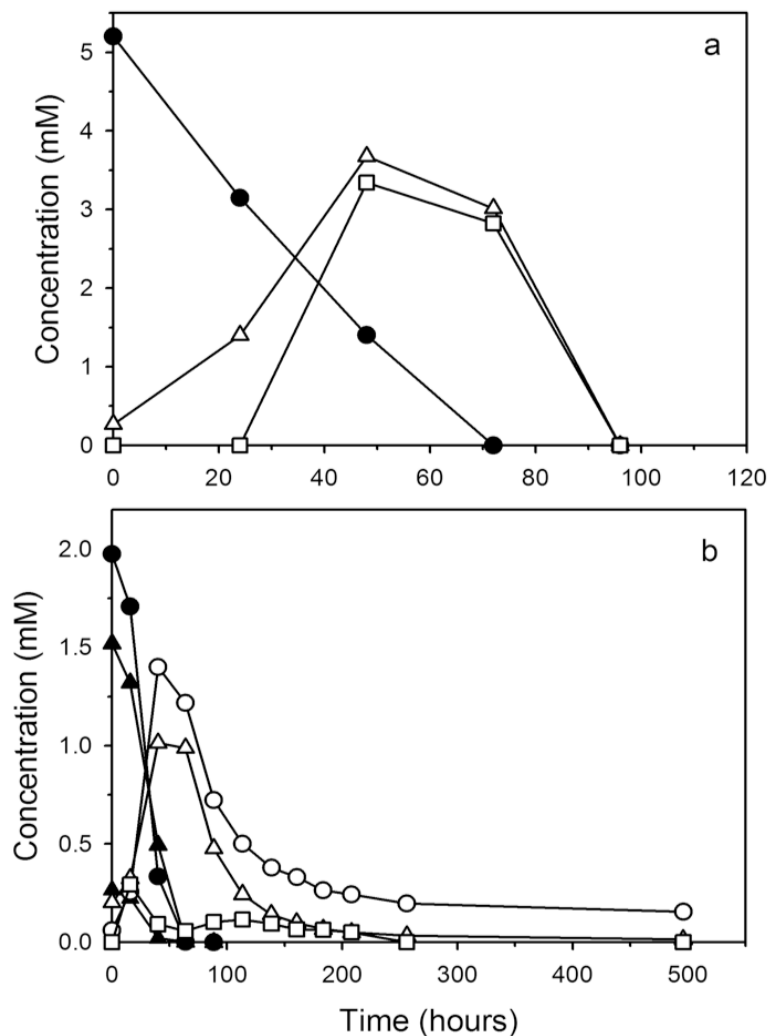
Figure 4.1a. Biodegradation of D(EG)DB (●) by *Rhodococcus rhodochrous* ATCC 13808 and the corresponding accumulation of D(EG)MB (Δ) and benzoic acid (□). The initial concentration of D(EG)DB was 5.2 mM and the growth medium contained 2.5 g/L of hexadecane. b. Biodegradation of D(PG)DB by *Rhodococcus rhodochrous* ATCC 13808. The three isomers of D(PG)DB (●, ▲ and ■), the two major isomers of D(PG)MB (○ and Δ) and benzoic acid (□) are all indicated. The initial concentration of D(PG)DB was 3.8 mM and the growth medium contained 2.5 g/L of hexadecane.
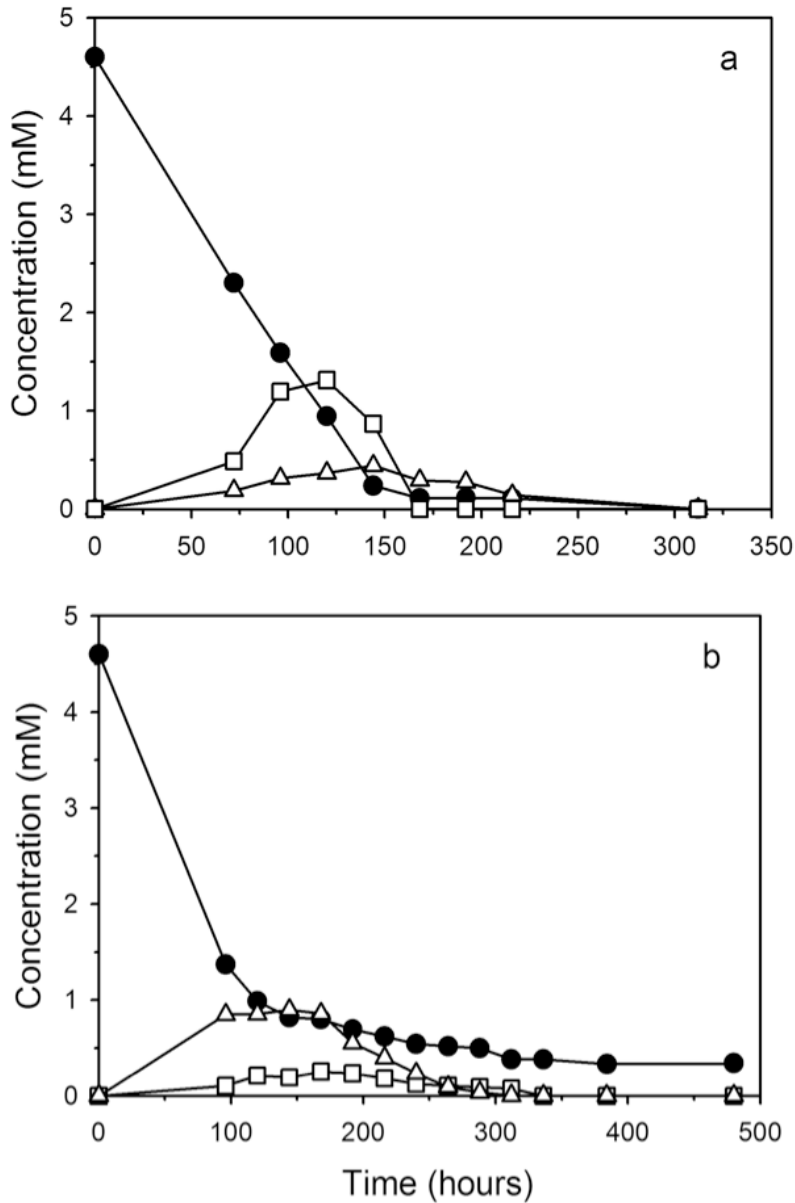
Figure 4.2a. Biodegradation of 1,6-hexanediol dibenzoate (●) by *Rhodococcus rhodochrous* ATCC 13808 in the presence of hexadecane and the corresponding accumulation of 1-hexadecyl benzoate (Δ) and benzoic acid (□). The initial concentration of 1,6-hexanediol dibenzoate was 4.6 mM and the growth medium contained 2.5 g/L of hexadecane. b. Biodegradation of 1,6-hexanediol dibenzoate (●) by *Rhodococcus rhodochrous* ATCC 13808 in the presence of tetradecane and the corresponding accumulation of 4-benzoyloxybutanoic acid (Δ) and benzoic acid (□). The initial concentration of 1,6-hexanediol dibenzoate was 4.6 mM and the growth medium contained 2.5 g/L of tetradecane.
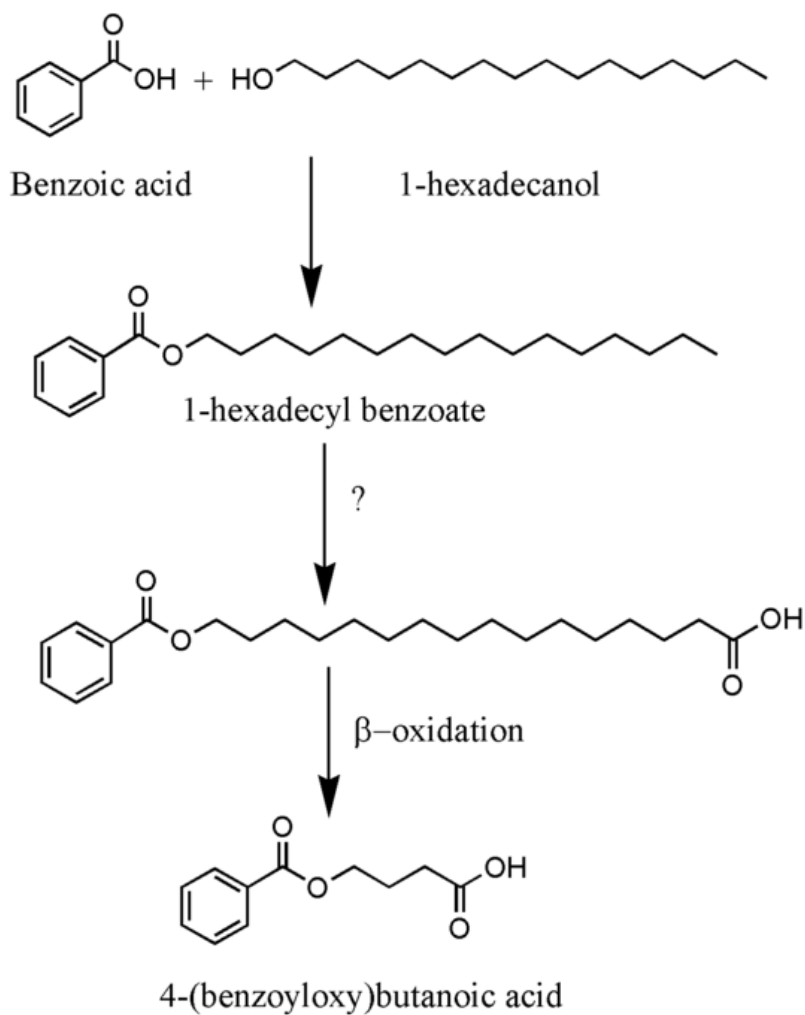
Scheme 4.1. Proposed mechanism for the formation of 1-hexadecyl benzoate arising from the biodegradation of 1,6-hexanediol dibenzoate by *Rhodococcus rhodochrous* in the presence of hexadecane as a co- substrate, including a possible mechanism for the formation of 4-(benzoyloxy)butanoic acid.
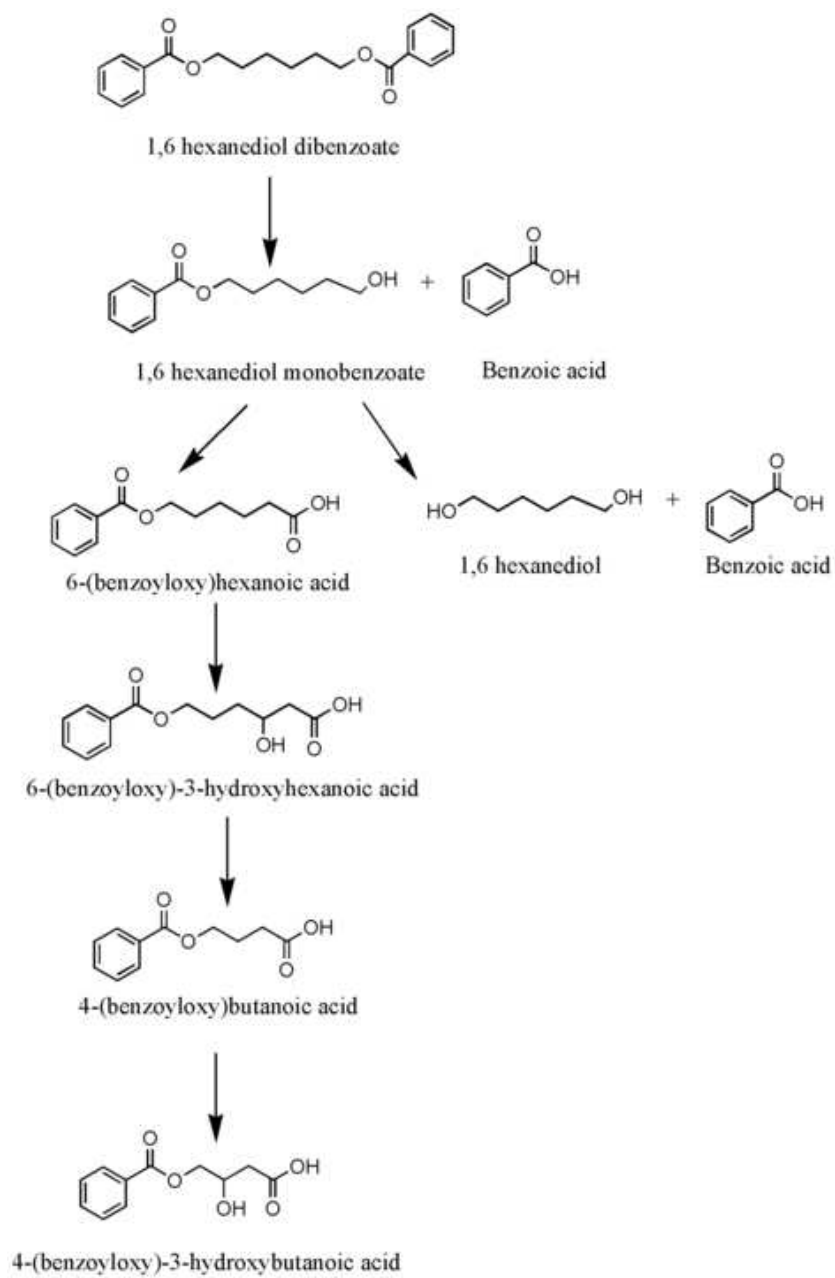
Scheme 4.2. Proposed biodegradation pathway of 1,6-hexanediol dibenzoate by *Rhodococcus rhodochrous*.

5. Biodegradation Kinetics of Dibenzoate Plasticizers

Preface

In the preceding chapter, it was shown that the metabolism of 1,6-hexanediol dibenzoate is characteristic of hydrolytic, oxidative and β-oxidative processes. However, the β-oxidation pathway is blocked for the analogous monoester metabolites of commercial dibenzoate plasticizers. In this chapter, the extent of the effect of the ether bond and alkyl branch on persistence of the dibenzoate plasticizers and their metabolites is investigated. The biodegradation of selected dibenzoate plasticizers was conducted by resting cells of *Rhodococcus rhodochrous* in an aerated bioreactor. *R. rhodochrous* had been grown with hexadecane as the primary substrate prior to the biodegradation study. The selected dibenzoate plasticizers consisted of two commercial plasticizers, diethylene glycol dibenzoate and dipropylene glycol dibenzoate, and three alternative plasticizers: 1,3-propanediol dibenzoate, 2,2-methyl-propyl-1,3-propanediol dibenzoate and 1,6-hexanediol dibenzoate. Experiments were conducted over a range of plasticizer concentrations to observe the effect of initial concentration on biodegradation rates. Biodegradation of plasticizers and their related metabolites were modeled using a Michaelis-Menten/Monod-type kinetic model. The first step in the biodegradation of all of these dibenzoate plasticizers was always the hydrolysis of an ester bond, releasing the corresponding monobenzoate and benzoic acid. The metabolism of the monobenzoate released from alternative plasticizers proceeded via hydrolysis, oxidation and β-oxidation processes. However, the β-oxidation pathway was not available for monobenzoate metabolites of commercial plasticizers. In the development of the mathematical models, all of these possible pathways based on the experimental observations were taken into account. It was concluded that removing the ether bond significantly enhanced the biodegradation rate of the monobenzoates and the

effect of alkyl branches was much less important on the rate of biodegradation of the monobenzoates.

Biodegradation Kinetics of Dibenzoate Plasticizers

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5.1. Abstract

The kinetics of the biodegradation of two commercial plasticizers, diethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB), as well as three alternative plasticizers, 1,3-propanediol dibenzoate, 2,2-methyl-propyl-1,3-propanediol dibenzoate and 1,6-hexanediol dibenzoate, were investigated in an aerated bioreactor. The experiments were conducted with resting cells of *Rhodococcus rhodochrous*, which had been grown with hexadecane as the primary substrate. The first step in the biodegradation was always the hydrolysis of an ester bond, releasing the corresponding monobenzoate and benzoic acid. Biodegradation of plasticizers and their associated metabolites were modeled using a Michaelis-Menten/Monod-type kinetic model. Significant differences between the biodegradation of commercial and alternative plasticizers were observed both in the biodegradation pathway and the biodegradation rates of monobenzoate metabolites. Diethylene glycol monobenzoate, produced from biodegradation of D(EG)DB, was completely

oxidized to 2-[2-(benzoyloxy)ethoxy]acetic acid. At a selected concentration of 0.4 g/L, the monobenzoates released from the biodegradation of 1,3-propanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate were degraded 13 and 4 times faster than the monobenzoate released from the biodegradation of D(PG)DB, respectively. 1,6-hexanediol monobenzoate was not detected during the biodegradation of 1,6-hexanediol dibenzoate, indicating that the degradation of the monobenzoate of 1,6-hexanediol was very rapid. The rapid biodegradation of monobenzoates released from microbial hydrolysis of alternative dibenzoate plasticizers was attributed to the lack of an ether bond in these compounds.

Key words: Biodegradation, plasticizers, dibenzoate, metabolites, kinetics

5.2. Introduction

Biodegradation is an important process that must be examined when assessing the potential impacts of xenobiotics on natural ecosystems and human health (Gibson, 1968; Peijnenburg, 1994). Microbial degradation of these chemicals may lead to production of metabolites and, thus, it is imperative to consider the range of metabolites produced and the persistence and toxicity of these metabolites (Peijnenburg 1994, Zink and Lorber, 1995; Staples et al., 1997). The full spectrum of metabolites produced from microbial degradation must be identified and these can be used to establish the biodegradation pathways and biodegradation kinetics of the parent compound as well as of any metabolites. This not only improves our understanding about the behavior of anthropogenic compounds in the environment, but more importantly it can lead to the insight required to develop environmentally benign alternatives.

Phthalates, which are the most widely used plasticizers, have been shown to biodegrade into toxic and persistent intermediates (Wypych, 2004; Staples et al., 1997; Nalli et al., 2002, 2006b,c). Mono-2-ethylhexyl phthalate (MEHP), a hydrolytic metabolite of di-2-ethylhexyl phthalate (DEHP), is a known endocrine disruptor and peroxisome proliferator (Mitchell et al., 1985, Onorato et al., 2008). Another metabolite from DEHP that is a potent peroxisome proliferator is 2-

ethylhexanoic acid (Cornu et al., 1992; Keith et al., 1992). In response to growing concerns associated with common commercial plasticizers such as phthalates, alternative diester plasticizers are now being used (Arendt and Lang, 1998; Rahman and Brazel, 2004, Wypych, 2004). For example, dibenzoates including dipropylene glycol dibenzoate (D(PG)DB) and diethylene glycol dibenzoate (D(EG)DB) were developed as more environmentally benign plasticizers (Deligio, 2009). However, biodegradation of these two plasticizers by *Rhodotorula rubra* and *Rhodococcus rhodochrous* leads to the formation and accumulation of monobenzoate metabolites (Gartshore et al., 2003; Kermanshahi pour et al., 2009a). The monobenzoates exhibited high acute toxicity in response to a Microtox™ assay (Gartshore et al., 2003). 1,5-Pentandiol and 1,6-hexanediol dibenzoates were reported to produce less stable metabolites and have also been tested as potential alternatives to commercial dibenzoate plasticizers (Firlotte, et al., 2009; Kermanshahi pour, 2009a,b).

Commercial dibenzoates have been recently approved by the European Chemical Agency as phthalate alternatives (Deligio, 2009). Therefore, higher volume production and greater application of dibenzoates can be expected in the future. Given the importance of the potential for the formation of persistent and/or toxic metabolites, research is required to improve our understanding of their biodegradation pathways and their metabolites. In response to this, the present study compares the biodegradation rates of selected dibenzoate plasticizers including two commercial plasticizers, D(EG)DB and D(PG)DB, and three alternative plasticizers; namely, 1,3-propanediol, 2,2-methyl-propyl-1,3-propanediol dibenzoate, and 1,6-hexanediol dibenzoate.

5.3. Materials and methods

5.3.1. Chemical and reagents

1,3-Propanediol (98%), 2-methyl-2-propyl-1,3-propanediol (98%), dipropylene glycol (99%), diethylene glycol (99%), benzoyl chloride (99%), D(EG)DB (96%) and D(PG)DB (98%) were purchased from Sigma-Aldrich (Oakville, ON, Canada). 1,6-Hexanediol dibenzoate was synthesized as described

previously (Kermanshahi pour et al., 2009b). Bis(trimethylsilyl) trifluoroacetamide (BSTFA) was obtained from Chromatographic Specialties (Brockville, ON, Canada). Pentadecane was purchased from A&C American Chemicals (Montreal, QC, Canada). Bacto Brain/Heart infusion and yeast extract were obtained from Difco Microbiology (Montreal, QC, Canada) and Fisher Scientific (Montreal, QC, Canada), respectively. Silica gel was obtained from Sigma-Aldrich (Oakville, ON, Canada). All other chemicals were obtained from Fisher Scientific (Montreal, QC, Canada).

5.3.2. Syntheses

Synthesis of 1,3-propanediol dibenzoate

1,3-propanediol dibenzoate was synthesized by refluxing 4 g (52.6 mmol) of 1,3-propanediol with 15 mL (129 mmol) benzoyl chloride (i.e., 2.4 equivalents) under nitrogen in 100 mL of acetone in a round bottom flask for 8 hours. The reaction mixture was diluted with 100 mL of chloroform and then washed three times with 100 mL of a saturated sodium bicarbonate solution and concentrated to a yellow oil using a rotary evaporator. 1,3-propanediol was recrystallized from a 20:80 vol/vol mixture of chloroform/heptane. The NMR spectrum of the purified compound (see Figure A2.1 in Appendix 2) was consistent with the expected theoretical spectrum.

Synthesis of 2,2-methyl-propyl-1,3-propanediol dibenzoate

2,2-methyl-propyl-1,3-propanediol dibenzoate was synthesized by refluxing 5.1 g (38.6 mmol) diol and 15 mL (129 mmol) benzoyl chloride (i.e., 3.5 equivalents) under nitrogen in 100 mL of acetone in round bottom flask for 8 hours. The reaction mixture was diluted with 100 mL of chloroform, prior to washing with 100 mL of saturated sodium bicarbonate. The reaction mixture was concentrated to a yellow oil in rotary evaporator and purified using column chromatography with silica gel as the stationary phase and with a 20:80 vol/vol mixture of hexane/methylene chloride as the mobile phase. The NMR of this compound is shown in Figure A2.2 (Appendix 2).

Synthesis of diethylene glycol monobenzoate

Diethylene glycol monobenzoate was synthesized by refluxing 5.90 g (55.7 mmol) diol and 4.80 mL (41.5 mmol) benzoyl chloride (i.e., 0.74 equivalents) under nitrogen in 100 mL of acetone in a round bottom flask for 8 hours. The reaction mixture was diluted with 100 mL of chloroform prior to washing three times with 100 mL of saturated sodium bicarbonate. The reaction mixture was then concentrated to a yellow oil in a rotary evaporator and was further purified using column chromatography with silica gel as the stationary phase and a 30:70 vol/vol mixture of acetone/hexane as the mobile phase. The NMR of this compound is shown in Figure A2.3 (Appendix 2).

5.3.3. Microorganisms and growth conditions

R. rhodochrous ATCC 13808 (ATCC, Manassas, VA, USA) was first grown in 100 mL sterile Brain/Heart infusion broth and incubated on a rotary incubator shaker (Series 25, New Brunswick Scientific, Edison, NJ, USA) set at 250 RPM and 30°C. 1 mL samples were transferred to shake flasks containing 100 mL of the sterilized minimum mineral salt medium (MMSM), 0.1 g/L yeast extract and 1 g/L of n-hexadecane. The MMSM consisted of 4 g/L NH_4NO_3, 4 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2 \cdot 2H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, and 0.014 g/L disodium ethylene diaminetetraacetic acid. The microbial culture was incubated on a rotary incubator shaker until the stationary phase was reached and hexadecane was completely degraded. Biodegradation experiments were subsequently conducted using this microbial culture as described below.

5.3.4. Biodegradation experiments

The system used for the biodegradation study consisted of a glass bioreactor, a centrifugal pump, a glass tube heat exchanger, and a recirculating water bath to maintain the temperature at 30°C. The centrifugal pump provided mixing by a 1/55-horsepower March pump, which was connected to an

autoclavable detachable plastic casing (Miller Plastics, Montreal, QC). The plastic casing was connected to the reactor by latex tubing (VWR Canlab, Mississauga, ON). Air was filter-sterilized (Millipore Millex-FG50, 0.2 μm) before entering the bioreactor. Another air filter was placed in the bioreactor outlet. The bioreactor, detachable plastic casing of the pump, glass tube heat exchanger, and the latex tubing that connected these pieces were steam sterilized for two hours.

1.2 L of MMSM media containing 0.1 g/L yeast extract in a 10 L polypropylene carboy was autoclaved and added aseptically to the sterile bioreactor. Hexadecane was autoclaved and added to the reactor using a sterile plastic syringe. The initial concentration of hexadecane in the reactor was between 1 and 1.5 g/L. Ten mL of the microbial culture of *R. rhodochrous* were injected to the reactor using a sterile plastic syringe. When hexadecane had been completely degraded and bacteria reached the stationary phase, sterilized samples of dibenzoate plasticizers or monobenzoates were introduced into the bioreactor.

5.3.5. GC/FID analyses

Duplicate samples of 4 mL were taken aseptically from the bioreactor. The pH was reduced to approximately 2 by the addition of sulfuric acid and the sample was then extracted with 4 mL of chloroform containing 0.5 g/L pentadecane as an internal standard. The mixture was stirred vigorously for 1 min and then the organic phase was transferred to a glass vial using a glass syringe and analyzed using GC/FID right away.

Aliquots (1 μL) of the chloroform extracts were analyzed using a Varian CP-3800 GC equipped with a FID detector and RTX column (Varian, Montreal, QC) with a length of 30 m and an inner diameter of 0.32 mm. The chromatographic conditions were as follows: injection port temperature of 250°C; initial column temperature of 40°C; initial time of 2 min; heating rate of 10°C/min; final temperature of 300°C; and detector temperature of 300°C. Helium was used as the carrier gas at a flow rate of 1.5 mL/min.

5.3.6. GC/MS analyses

Samples were prepared for GC/MS analysis in the same manner as for GC/FID analysis except that the chloroform was removed with a dry argon stream and the residues were dissolved in 50 µL of anhydrous pyridine. Trimethylsilyl (TMS) derivatives were made by the addition of 50 µL of BSTFA to the pyridine solutions in capped auto injector vials and these were heated in an aluminum block at 50°C for 15 min.

Aliquots (1 µL) of the TMS derivatized extracts were analyzed using a Thermo Finnigan Polaris Q GC/MS (West Palm Beach, Florida, US) fitted with a 30-m Restek Rtx®-5MS column (Restek, Bellefonte, PA) having a 0.25 mm inner diameter and 0.25-µm film thickness. The column temperature varied from 60 to 275°C at 5°C/min under controlled conditions followed by a bake-out period of 1 min at 275°C. The injector was operated in a 1:100 split mode at 200°C with a constant helium flow of 0.7 mL/min. The EI ion source was operated at 70 eV and 200°C. The scan range was m/z 75-400.

5.3.7. Biomass measurement

Duplicate samples of 10 mL were taken aseptically from the bioreactor. The samples were centrigued (IEC, Model B-22M) at 10,000 RPM at room temperature for 10 minutes. The supernatant was discarded and the pellet was rinsed with 10 mL of MMSM media and centrifuged. The centrifugation and rinsing steps were repeated twice and then the final pellet was resuspended in distilled water and placed in pre-weighed aluminum dish. The dishes were placed in an oven (Fisher Isotemp Oven 100 series, model 126G) at 105 °C for 48 hours. The dishes were cooled and the mass was obtained using an analytical balance (Mettler, model AE160).

5.3.8. Surface tension measurement

The surface tension was measured using the Wilhelmy plate method (Kruss Tensiometer K12).

5.4. Experimental Results

5.4.1. Biodegradation of plasticizers

Many of the metabolites detected in the present study have been previously identified and reported in earlier works (Kermanshahi pour et al., 2009a,b). In addition to those metabolites, new metabolites were identified in this study and are summarized in Table 5.1. These metabolites were all identified by GC/MS using the molecular weights of the parent ions (Table 5.1) and by interpreting their fragmentation patterns as described previously (Kermanshahi pour et al., 2009a,b).

The microbial hydrolysis of D(EG)DB by *R. rhodochrous,* which was previously grown on hexadecane, at three different initial concentrations and the accompanying formation of D(EG)MB and benzoic acid is demonstrated in Figure 5.1. These results show that D(EG)DB was hydrolyzed to D(EG)MB and D(EG)MB was completely converted to the corresponding carboxylic acid, 2-[2-(benzoyloxy)ethoxy]acetic acid. The observed increase in biomass concentration was attributed to the mineralization of benzoic acid; a metabolite from the initial hydrolysis of D(EG)DB. It can be seen in Figure 5.1 that once the benzoic acid that had been released from the initial hydrolysis of D(EG)DB was degraded, no increase in biomass concentration was observed thereafter.

Biodegradation studies of D(EG)MB by *R. rhodochrous* were conducted at three different initial concentrations and the results are presented in Figure 5.2. Complete biotransformation of D(EG)MB to 2-[2-(benzoyloxy)ethoxy]acetic acid was always observed in these experiments and the biomass concentrations did not change over the course of the experiments.

Figure 5.3 illustrates the results of biodegradation of D(PG)DB at two different concentrations. D(PG)DB was also hydrolyzed to benzoic acid and the corresponding monoester, D(PG)MB. However, unlike D(EG)MB, D(PG)MB was hydrolyzed and only trace amounts of oxidized D(PG)MB were detected. This metabolite was identified using GC/MS and the *m/z* of major ion peaks are shown in Table 5.1.

Biodegradation of 1,3-propanediol dibenzoate also begins with ester hydrolysis to release the monobenzoate, 1,3-propanediol monobenzoate (Table 5.1). It can be seen in Figures 5.4a and 5.4b that the monobenzoate was only observed at low concentrations due to its rapid conversion by further hydrolysis to benzoic acid and/or β-oxidation and oxidation to 3-(benzoyloxy)propanoic acid (Table 5.1). The increase in biomass was attributed to mineralization of benzoic acid released from hydrolysis of both dibenzoate and monobenzoate and also diols.

2,2–methyl-propyl-1,3-propanediol dibenzoate was degraded to monobenzoate and benzoic acid but degradation of benzoic acid seemed to be rapid and, thus, only trace amounts were detected (Figure 5.5). The first hydrolysis step was also much slower compared to that of 1,3-propanediol dibenzoate and the two of commercial plasticizers.

Biodegradation of 1,6-hexanediol dibenzoate is shown in Figure 5.6. Inconsistencies in concentration measurements, as indicated by the error bars, are most likely due to the fact that this compound is solid and insoluble in water, and thus sampling was not representative. A monobenzote metabolite was not detected in this experiment but the metabolite of monobenozoate due to β-oxidation, 4-(benzoyloxy)butanic acid, was detected. Trace amounts of benzoic acid were also detected.

5.5. Model Development and Calibration

Kinetic models were developed in order to describe the biodegradation of a selection of dibenzoate plasticizers and to evaluate their relative biodegradability. The system used for this kinetic study consisted of resting cells of *R. rhodochrous,* previously grown on hexadecane in a cyclone bioreactor.

Hydrolysis of one ester bond to release the corresponding monobenzoate and benzoic acid as metabolites was the first step in all of the mechanisms studied. The biodegradation rates of dibenzoates and their corresponding metabolites were described using the typical Michaelis-Menten/Monod-type kinetic expression for substrate degradation and modified, as needed, based on

experimental observations for particular substrates (e.g., incorporating the effect of substrate inhibition due to high substrate concentration).

The Michaelis-Menten/Monod equation, as described by equation 5.1 below, is commonly used for interpretation of biodegradation rates by relating the specific substrate depletion rate and microbial growth.

$$\frac{dC_s}{dt} = \frac{kC_S C_B}{(K_s + C_s)} \tag{5.1}$$

where k represents the maximum biodegradation rate, K_s is the half saturation constant of substrate, C_s is the substrate concentration, and C_B is the biomass concentration.

The following assumptions were used in the development of the original Monod equation: (1) the organic molecule (S) is water soluble, non-toxic and is the limiting reactant; and (2) inorganic growth requirements are present in excess (Alexander, 1999). However, the model has been further modified and extended to analyze the kinetic data obtained under substrate and product inhibition and reactant (e.g. O_2 and NAD(P)H) limitation conditions (Alvarez-Cohen and McCarty, 1991; Chang and Alvarez-Cohen, 1995). For instance, at high substrate concentration, when an increase in substrate concentration can cause a decrease in biodegradation rate, the Michaelis-Menten/Monod-type approach was modified to model the kinetic data of substrate-inhibited reactions (Bailey and Ollis, 1986). Several mathematical models such as Andrew's inhibition model, shown in equation 5.2 below, have been developed to quantify the effect of substrate inhibition on biodegradation rate (Andrews, 1968):

$$\frac{dC_s}{dt} = \frac{kC_S C_B}{(K_s + C_S + \frac{C_s^2}{KI})} \tag{5.2}$$

where KI is the substrate inhibition constant. The inhibitory mechanism assumed here is binding of the excess substrate to the enzyme resulting in the formation of

an unreactive intermediate and subsequent slower biodegradation rate (Bailey and Ollis, 1986).

The Monod equation has also been modified to describe the biodegradation kinetics under reactant-limiting conditions (Chang and Alvarez-Cohen, 1995). That is, when required reactants (e.g. O_2 or NAD(P)H) for oxidation of the substrate are not present in excess, the rate can be expressed as a function of each of these rate limiting reactants, as follows :

$$\frac{dC_s}{dt} = \frac{kC_S C_B}{(K_s + C_s)} \times (\frac{R}{K_R + R})$$ (5.3)

where R is the concentration of the limiting reactant and K_R is the half-saturation constant of the limiting reactant.

A reducing energy substrate (e.g. NADPH) is a required reactant in biodegradation processes including co-metabolism (Chang and Alvarez-Cohen, 1995). Co-metabolism is defined as biotransformation of a non-growth substrate, by growing cells in the presence of a growth substrate (e.g., hexadecane) or by resting cells in the absence of growth substrate (Criddle, 1993). The growth substrate is the electron donor that is readily oxidized and provides reducing energy for growth and energy maintenance of the cells (Criddle, 1993). However, co-metabolism in the absence of a growth substrate may encounter depletion of reducing energy substrate. These phenomena are depicted in Figure 5.7. Figure 5.7a shows a typical enzymatic reaction in the presence of a growth substrate and oxygen. Oxidation of growth substrate results in production of metabolites that act as an electron donor for regeneration of NAD(P)H. Figure 5.7b depicts the co-metabolic reaction in the absence of growth substrate. If the co-metabolic substrate produces toxic metabolites, the energy reducing substrate cannot be regenerated and leads to enzyme and/or cell damage (Ciddle, 1993). However, if the co-metabolic substrate produces some readily biodegradable metabolites that can act as an electron donor (reactant) to provide energy for cell maintenance, the biodegradation of the parent compound of interest will continue but will depend

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on the concentration of the electron donor. Equation 5.3 can describe this enzymatic reaction under reactant-limiting conditions.

An important consideration in model development is to understand the mechanism of uptake of chemicals by microorganisms to determine the rate-limiting step in a biodegradation process. It was originally believed that an uptake mechanism for water insoluble hydrocarbons was based on passive transport of solubilized hydrocarbons (Britton, 1984; Whyte et al., 1999). Later, it was observed that some water insoluble compounds have higher biodegradation rates than their dissolution rate, which led to a proposal for a second alternative uptake mechanism (Thomas et al., 1986, Leahy and Colwell, 1990); that is, many bacteria, including members of the genus *Rhodococcus,* produce surface active agents that increase the surface area of the insoluble compounds and enhance the bioavailability of hydrophobic compounds (Lang and philp, 1998).

A third possible uptake mechanism by microorganisms is the direct assimilation of the organic molecule by adhesion of microorganisms to the organic phase at the aqueous-organic interface (Britton, 1984, Whyte et al., 1999). Microorganisms are also capable of enhancing the cell surface hydrophobicity by changing the cell surface components (Watkinson and Morgan, 1990). This phenomenon was also observed in the assimilation of phthalates in the presence of hexadecane by *R. rhodochrous*. It was shown that when the cells were grown with hexadecane, the cell wall hydrophobicity was increased and this led to a better contact between the plasticizers and the cells (Nalli, 2006c).

In the present study, *R. rhodochrous* was grown to the stationary phase in the presence of hexadecane as a primary carbon source. When hexadecane was completely degraded, the surface tension of the biodegradation broth was found to be between 47 and 50 mN·m^{-1}. Separating the cell debris did not change the surface tension of the supernatant, indicating that the decrease in surface tension from 70 mN·m^{-1} (surface tension of distilled water) to 50 mN·m^{-1} resulted from the presence of emulsifiers. These emulsifiers increase the dispersion and bioavailability of the organic compounds for further assimilation by bacteria. All the dibenzoate plasticizers studied in this work have low aqueous solubility and

their microbial uptake mechanism is hypothesized to be the result of direct cellular contact with plasticizer droplets. Therefore, the mass transfer of plasticizer from organic to the aqueous phase was not incorporated into the models developed below and it was assumed that the reactions are kinetically controlled

To develop the kinetic model for the metabolites, a mass balance was first performed on the metabolites based on the possible pathways for their formation and further degradation. The Monod-type kinetic model was used to describe the rate of formation and disappearance of the metabolites. Matlab^{TM} was used to simultaneously solve the set of differential equations for each compound. Optimal values for the biokinetic coefficients were determined by minimizing the least squares of the differences between experimental and numerical values.

The kinetics of the biodegradation of the five dibenzoate plasticizers and their metabolites are described below.

5.5.1. Biodegradation kinetics of diethylene glycol dibenzoate

The initial concentrations of D(EG)DB in the experiments were relatively high (i.e., between 0.64 g/L and 1.8 g/L) as shown in Figure 5.1 and substrate inhibition was observed since the biodegradation rate decreased with increasing the concentration of D(EG)DB. Therefore, the hydrolysis of D(EG)DB was described using the Andrew's inhibition model, as follows:

$$\frac{dC_{DB}}{dt} = \frac{k_{DB} C_B C_{DB}}{K_{DB} + C_{DB} + \frac{C_{DB}^2}{KI_{DB}}} \qquad (5.4)$$

where k_{DB} represents the maximum biodegradation rate of dibenzoate, C_B is the biomass concentration, C_{DB} is the concentration of D(EG)DB, K_{DB} is the half-saturation constant of D(EG)DB, and KI_{DB} is the inhibition constant. The rate constants k_{DB}, K_{DB} and KI_{DB} were determined by fitting the experimental data from three sets of experiments into the model. The obtained values of the calibrated constants are presented in Table 5.2.

D(EG)MB was formed from the hydrolysis of D(EG)DB and, as shown in Figure 5.1, was completely oxidized to 2-[2-(benzoyloxy)ethoxy]acetic acid. The formation of D(EG)MB and its subsequent oxidation, as described by the Monod equation, is represented by the following equation:

$$\frac{dC_{MB}}{dt} = \frac{-dC_{DB}}{dt} \times \frac{MW_{MB}}{MW_{DB}} - \frac{k_{MB-OM}C_B C_{MB}}{(K_{MB-OM} + C_{MB})} \tag{5.5}$$

where k_{MB-OM} is the maximum biotransformation rate of D(EG)MB to 2-[2-(benzoyloxy)ethoxy]acetic acid, K_{MB-OM} is the half-saturation constant, and C_{MB} is the concentration of D(EG)MB. The biokinetic rate constants k_{MB-OM} and K_{MB-OM} were obtained by fitting D(EG)MB concentrations from the three sets of experiments into equation 5.5. Values of the calibrated rate constants are presented in Table 5.1.

Experimental data in Figure 5.1 shows that D(EG)MB was completely converted to 2-[2-(benzoyloxy)ethoxy]acetic acid, which was a stable metabolite and did not degrade within the period of the experiment. Therefore, the rate of formation of 2-[2-(benzoyloxy)ethoxy]acetic acid is equal to the rate of degradation of D(EG)MB, as follows:

$$\frac{dC_{OM}}{dt} = \frac{k_{MB-OM}C_B C_{MB}}{(K_{MB-OM} + C_{MB})} \times \frac{MW_{OM}}{MW_{MB}} \tag{5.6}$$

where MW_{MB} and MW_{OM} are the molecular weights of D(EG)MB and 2-[2-(benzoyloxy)ethoxy]acetic acid, respectively.

Benzoic acid was released only from the hydrolysis of D(EG)DB and was further mineralized, as described in equation 5.7 below:

$$\frac{dC_{BA}}{dt} = \frac{-dC_{DB}}{dt} \times \frac{MW_{BA}}{MW_{DB}} - \frac{k_{BA}C_B C_{BA}}{K_{BA} + C_{BA}} \tag{5.7}$$

where C_{BA} is the concentration of benzoic acid, MW_{BA} is the molecular weight of benzoic acid, MW_{DB} is the molecular weight of D(EG)DB, k_{BA} is the maximum mineralization rate of benzoic acid, and K_{BA} is half-saturation constant of benzoic

acid. The calibrated values of the rate constants k_{BA} and K_{BA} are presented in Table 5.2.

Benzoic acid is the only metabolite that is mineralized and results in the formation of new biomass. The relationship between the degradation of benzoic acid and formation of biomass is presented by a Monod-type equation, expressed by equation 5.8.

$$\frac{dC_B}{dt} = Y_{BA} \times \frac{k_{BA}C_BC_{BA}}{K_{BA} + C_{BA}} \tag{5.8}$$

where Y_{BA} is the yield coefficient, which for the three sets of experiments shown in Figure 5.1 was calculated to be 0.77 g of biomass/g of benzoic acid consumed.

5.5.2. Biodegradation kinetics of diethylene glycol monobenzoate

The biodegradation of pure D(EG)MB was studied at three different initial concentrations to compare the biodegradation rates of pure D(EG)MB and D(EG)MB that was produced as a metabolite of D(EG)DB. As shown in Figure 5.2, as with the biodegradation of D(EG)MB when it was present as a metabolite, complete biotransformation of pure D(EG)MB to 2-[2-(benzoyloxy)ethoxy]acetic acid was observed in these experiments. The rate of degradation of the monobenzoate was described as follows

$$\frac{dC_{MB}}{dt} = \frac{k_{MB-OM}C_BC_{MB}}{K_{MB-OM} + C_{MB}} \tag{5.9}$$

where k_{MB-OM} is the maximum biotransformation rate of D(EG)MB to 2-[2-(benzoyloxy)ethoxy]acetic acid and K_{MB-OM} is the half-saturation constant. The rate constants k_{MB-OM} and K_{MB-OM} were calibrated using the data in Figure 5.2 and the values are shown in Table 5.2.

5.5.3. Biodegradation kinetics of dipropylene glycol dibenzoate

The degradation of D(PG)DB with initial concentrations of 0.32 and 0.64 g/L could be modeled using Monod-type kinetics, as per the following:

$$\frac{dC_{DB}}{dt} = \frac{-k_{DB}C_{DB}C_B}{K_{DB} + C_{DB}} \tag{5.10}$$

where C_{DB} is the concentration of D(PG)DB, k_{DB} is the maximum biodegradation rate of D(PG)DB, and K_{DB} is the half saturation constant of D(PG)DB.

D(PG)DB was hydrolyzed to D(PG)MB, which was further hydrolyzed to benzoic acid, according to equation 5.11.

$$\frac{dC_{MB}}{dt} = \frac{-dC_{DB}}{dt} \times \frac{MW_{MB}}{MW_{DB}} - \frac{k_{MB-BA}C_{MB}C_B}{K_{MB-BA} + C_{MB}} \times \frac{C_{BA}}{KI_{BA} + C_{BA}} \tag{5.11}$$

where C_{DB} is the concentration of D(PG)DB, C_{MB} is the concentration of D(PG)MB, C_{BA} is concentration of benzoic acid, MW_{DB} is the molecular weight of D(DG)DB, MW_{MB} is the molecular weight of D(PG)MB, k_{MB-BA} is the maximum biotransformation rate of D(PG)MB to benzoic acid, K_{MB-BA} is the half saturation constant of D(PG)MB and KI_{BA} is the half saturation constant of benzoic acid.

In the above expression, a modified Monod equation under reactant-limiting condition with a general form of equation 5.3 was used to model the degradation of D(PG)MB. It was assumed that the hydrolysis of D(EG)MB was limited by the presence of benzoic acid, as an electron donor to provide reducing energy for cell growth and maintenance. Therefore, the effect of benzoic acid as a limiting reactant is incorporated in the hydrolysis rate expression of D(PG)MB, as described by the second term in the equation. The supporting evidence for this assumption was that the hydrolysis of D(PG)MB at the beginning of the experiment was observed to be faster than that at end (see Figure 5.3). The faster rate of hydrolysis of D(PG)MB at the beginning of the experiment was attributed

to the initial rapid release of benzoic acid due to the quick hydrolysis of D(PG)DB.

Benzoic acid is formed as a result of hydrolysis of both D(PG)DB and D(PG)MB, as described in the first and second terms of equation 5.12 below and mineralization of benzoic acid was modeled using a Monod type equation, as indicated in the third term in equation 5.12.

$$\frac{dC_{BA}}{dt} = \frac{-dC_{DB}}{dt} \times \frac{MW_{BA}}{MW_{DB}} + (\frac{k_{MB-BA}C_{MB}C_B}{K_{MB-BA}+C_{MB}}) \times (\frac{C_{BA}}{KI_{BA}+C_{BA}}) \times \frac{MW_{BA}}{MW_{MB}} - \frac{k_{BA}C_{BA}C_B}{K_{BA}+C_{BA}} \qquad (5.12)$$

where k_{BA} and K_{BA} are the maximum biodegradation rate constant and half-saturation constant of benzoic, respectively.

The mineralization of benzoic acid results in the formation of new biomass. The relationship between the degradation of benzoic acid and formation of biomass is expressed by equation 5.13.

$$\frac{dC_B}{dt} = Y_{BA} \times \frac{k_{BA}C_{BA}C_B}{K_{BA}+C_{BA}} \qquad (5.13)$$

where Y_{BA} is the yield coefficient, which for the two sets of experiments of D(PG)DB shown in Figure 5.3 was calculated to be 0.85 g of biomass/g of benzoic acid consumed.

5.5.4. Biodegradation kinetics of 1,3-propanediol dibenzoate

The rate of biodegradation of 1,3-propanediol dibenzoate was described by a Monod-type equation, as follows:

$$\frac{dC_{DB}}{dt} = -\frac{k_{DB}C_B C_{DB}}{K_{DB}+C_{DB}} \qquad (5.14)$$

where C_{DB} is the concentration of 1,3-propanediol dibenzoate, k_{DB} is the maximum biodegradation rate of 1,3-propanediol dibenzoate, and K_{DB} is the half-saturation constant of the dibenzoate. The values of k_{DB} and K_{DB} were calibrated using the

two sets of biodegradation experiments shown in Figure 5.4 and are presented in Table 5.2.

1,3-propanediol monobenzoate was formed from the hydrolysis of the dibenzoate (i.e., the first term in equation 5.15) and degraded via two pathways of hydrolysis/β-oxidation to benzoic acid and oxidation to 3-(benzoyloxy) propanoic acid. Hydrolysis/β-oxidation of 1,3-propanediol monobenzoate to benzoic acid (i.e., the second term in equation 5.15) was modeled using a first order equation and oxidation to the corresponding carboxylic acid, 3-(benzoyloxy) propanoic acid (i.e., the third term in equation 5.15) was modeled using the Monod equation, as follows:

$$\frac{dC_{MB}}{dt} = -\frac{dC_{DB}}{dt} \times \frac{MW_{MB}}{MW_{DB}} - k_{MB-BA} C_B C_{MB} - \frac{k_{MB-OM} C_B C_{MB}}{K_{MB-OM} + C_{MB}} \tag{5.15}$$

where MW_{MB} is the molecular weight of 1,3-propanediol monobenzoate, MW_{DB} is the molecular weight of 1,3-propanediol dibenzoate, k_{MB-BA} is the maximum biotransformation rate of 1,3-propanediol monobenzoate to benzoic acid, C_{MB} is the concentration of 1,3-propanediol monobenzoate, K_{MB-OM} is the maximum biotransformation rate of 1,3-propanediol monobenzoate to 3-(benzoyloxy) propanoic acid (oxidized monobenzoate), k_{MB-OM} is the maximum biotransformation rate of 1,3-propanediol dibenzoate to 3-(benzoyloxy) propanoic acid, and K_{MB-OM} is the half-saturation constant of 1,3-propanediol monobenzoate. The calibrated values for the rate constants $k_{MB-BA,}$ $K_{MB-OM,}$ and k_{MB-OM} are presented in Table 5.2.

As described by equation 5.16 below, 3-(benzoyloxy) propanoic acid, which was formed as a result of oxidation of 1,3-propanediol monobenzoate (i.e., first term in equation 5.16), degraded via progressive β-oxidation to benzoic acid (i.e., second term in equation 5.16). The degradation to benzoic acid was modeled using a first order equation.

$$\frac{dC_{OM}}{dt} = \frac{k_{MB-OM} C_B C_{MB}}{K_{MB-OM} + C_{MB}} \times \frac{MW_{OM}}{MW_{MB}} - k_{OM-BA} C_B C_{OM} \tag{5.16}$$

where C_{OM} is the concentration of 3-(benzoyloxy) propanoic acid, MW_{OM} is the molecular weight of 3-(benzoyloxy) propanoic acid, k_{MB-BA} is the maximum biotransformation rate of 1,3-propanediol monobenzoate to benzoic acid, and K_{OM-BA} is the maximum biotransformation rate of 3-(benzoyloxy) propanoic acid to benzoic acid. The calibrated rate constant of k_{OM-BA} are presented in Table 5.2.

As described in equation 5.17 below, benzoic acid, which was formed from the hydrolysis of dibenzoate (i.e., the first term in the equation), monobenzoate (i.e., the second term), and β-oxidation of oxidized monobenzoate (i.e., the third term) was subsequently mineralized (i.e., fourth term). Mineralization was described by a Monod-type equation.

$$\frac{dC_{BA}}{dt} = -\frac{dC_{DB}}{dt} \times \frac{MW_{BA}}{MW_{DB}} + k_{MB-BA} C_B C_{MB} \times \frac{MW_{BA}}{MW_{MB}} + k_{OM-BA} C_B C_{OM} \times \frac{MW_{BA}}{MW_{OM}} - \frac{k_{BA} C_B C_{BA}}{K_{BA} + C_{BA}} \qquad (5.17)$$

where C_{BA} is the concentration of benzoic acid, MW_{BA} is the molecular weight of benzoic acid. k_{BA} is the maximum biodegradation rate of benzoic acid and K_{BA} is half saturation constant of benzoic acid. k_{BA} and K_{BA} were calibrated using the experimental data shown in Figure 5.4. The resulting rate constants values are presented in Table 5.2.

The increase in biomass concentration as a result of benzoic acid utilization is described by equation 5.18.

$$\frac{dC_B}{dt} = Y_{BA} \times \frac{k_{BA} \times C_{BA} \times C_B}{K_{BA} + C_{BA}} \qquad (5.18)$$

The yield coefficient, Y_{BA}, was calculated to be 0.75 (g increase in biomass/g benzoic acid consumed), as shown in Table 5.2.

5.5.5. Biodegradation kinetics of 2,2-methyl-propyl-1,3-propanediol dibenzoate

The biodegradation of 2,2-methyl-propyl-1,3-propanediol dibenzoate (see Figure 5.5) was described by the Andrew's inhibition model since a decrease in

biodegradation rate was observed with increasing the initial concentration of the compound.

$$\frac{dC_{DB}}{dt} = \frac{k_{DB}C_B C_{DB}}{K_{DB} + C_{DB} + \frac{C_{DB}^2}{KI_{DB}}}$$ (5.19)

where C_{DB} is the concentration of 2,2-methyl-propyl-1,3-propanediol dibenzoate, k_{DB} is the maximum biodegradation rate of the dibenzoate, K_{DB} is the half saturation constant of the dibenzoate, and KI_{DB} is the inhibition constant.

The formation of monobenzoate and its subsequent hydrolysis to benzoic acid is described in the first and second terms, respectively, of equation 5.20 below

$$\frac{dC_{MB}}{dt} = -\frac{dC_{DB}}{dt} \times \frac{MW_{MB}}{MW_{DB}} - \frac{k_{MB-BA}C_{MB}C_B}{K_{MB-BA} + C_{MB}}$$ (5.20)

where MW_{MB} is the molecular weight of 2,2-methyl-propyl-1,3-propanediol monobenzoate, MW_{DB} is the molecular weight of 2,2-methyl-propyl-1,3-propanediol dibenzoate, k_{MB-BA} is the maximum biotransformation rate of monobenzoate to benzoic acid, K_{MB-BA} is the half saturation constant of the monobenzoate, and C_{MB} is the concentration of monobenzoate.

The increase in biomass concentration is due to decrease in dibenzoate concentration, as described by equation 5.21.

$$\frac{dC_B}{dt} = -Y_{DB} \times \frac{dC_{DB}}{dt} = Y_{DB} \times \frac{k_{DB}C_B C_{DB}}{K_{DB} + C_{DB} + \frac{C_{DB}^2}{KI_{DB}}}$$ (5.21)

The yield coefficient, Y_{DB}, above was calculated based on the decrease of dibenzoate concentration in contrast with other compounds, for which yield was calculated based on the decrease in concentration of benzoic acid. The reason for this is that the increase in biomass could not be correlated to benzoic acid degradation since benzoic acid was detected in only trace amounts. Furthermore, dibenzoate was mineralized and directly contributed to the increase in biomass

concentration. Therefore, it was reasonable to calculate the yield coefficient (Y_{DB}) based on the decrease in dibenzoate concentration.

5.5.6. Biodegradation of 1,6-hexanediol dibenzoate

1,6-Hexanediol dibenzoate was hydrolyzed to 1,6-hexanediol monobenzoate. This monobenzoate metabolite was not detected, presumably due to its rapid β-oxidation to 4-benzoyloxy butanoic acid. 4-benzoyloxy butanoic acid was further degraded to release benzoic acid, which was detected in trace amounts (Figure 5.6). Duplicate samples demonstrated inconsistencies in sampling due to the presence of solid particles of 1,6-hexanediol dibenzoate (Figure 5.6). This inconsistency became more obvious at the higher initial 1,6-hexanediol dibenzoate concentration of 1.3 mM. It is possible that mass transfer from the organic phase to the aqueous phase and/or penetration of the compound into the cell membrane becomes important and controls the rate of reaction.

Within the first 20 hours of the experiments, approximately 83% and 62% of 1,6 hexanediol dibenzoate degraded for initial concentrations of 0.56 and 1.33 mM, respectively. The biodegradation rate of 1,6 hexanediol dibenzoate could not be determined, nor modeled, due to the inconsistencies in the measured concentrations.

5.6. Discussion

Previous studies have shown that the biodegradation of D(EG)DB and D(PG)DB by yeast, *R. rubra* and a common soil bacterium, *R. rhodochrous* growing on either glucose or hexadecane as co-substrates resulted in the release of substantial amounts of the corresponding monoesters (Gartshore et al., 2003, Kermanshahi pour et al., 2009a). *R. rhodochrous* was shown to be more efficient than *R. rubra* in degrading the monoester metabolites. Biodegradation of D(EG)DB by resting cells of *R. rhodochrous,* that had been grown on hexadecane in a bioreactor, show a different pattern of metabolite formation (Figure 5.1) compared to biodegradation experiments conducted in the presence of hexadecane (Kermanshahi pour et al., 2009a). Although, substantial amounts of D(EG)MB

were reported to be released during biodegradation of D(EG)DB by *R. rhodochrous* in the presence of hexadecane (Kermanshahi pour et al., 2009a), the monobenzoate metabolites of D(EG)DB and D(PG)DB eventually degraded and only trace amounts of monobenzoate were converted to the corresponding carboxylic acid, 2-[2-(benzoyloxy)ethoxy]acetic acid (Kermanshahi pour et al., 2009a). However, in the experiments with resting cells presented here, 2-[2-(benzoyloxy)ethoxy]acetic acid was observed in substantial amounts (Figure 5.1). The same result was obtained for the biodegradation of pure D(EG)MB and this compound was completely converted to the corresponding carboxylic acid (Figure 5.2).

The major difference between the previously reported biodegradation experiments conducted in shake flasks and the current experiments in which a bioreactor was used is the absence of hexadecane in the latter experiments. When the growth substrate (i.e., hexadecane) is present, it is oxidized and the metabolites produced act as an electron donor that provide energy for cell maintenance. In the absence of hexadecane, the lack of an easily-degradable substrate may lead to production of toxic metabolites that cause enzyme or cell damage (Chang and Alvarez-Cohen, 1995). The effect of the presence of growth substrate is depicted in Figure 5.7. When biodegradation of D(EG)DB was conducted in the presence of hexadecane in the bioreactor, D(EG)MB was completely degraded and 2-[2-(benzoyloxy)ethoxy]acetic acid was not detected (data not shown). Thus, the reason that biodegradation of 2-[2-(benzoyloxy)ethoxy]acetic acid ceased and led to a significant accumulation of this metabolite may be attributed to the lack of a readily degradable substrate that could provide energy to further hydrolyze or oxidize this metabolite.

The main purpose of the biodegradation studies was to compare the biodegradation rates of the commercial and potential green plasticizers and their related metabolites, thereby providing a basis for assessing the influence of the functional groups on biodegradation rates. The accuracy of estimated rates depends on the abilities of the models to reflect the biodegradation behavior of these compounds over time. In general, as can be seen in Figures 5.1 to 5.5, the

models were able to successfully describe the experimental concentration-time profiles of the parent compounds, their metabolites and biomass.

In modeling the hydrolysis of D(EG)DB and 2,2-methyl-propyl-1,3-propanediol dibenzoate, a decrease in hydrolysis rate with increasing initial substrate concentration was observed. This trend was successfully captured by Andrew's inhibition model, which is a common substrate inhibition model (Figures 5.1 and 5.5).

Hydrolysis of D(PG)DB and 1,3-propanediol was described by a Monod-type equation. In both cases, as shown in Figures 5.3 and 5.4, there is a very good agreement between the experimental data and the model.

Biodegradation of the monobenzoates of the commercial and alternative plasticizers proceeded via different pathways. Oxidation of D(EG)MB to 2-[2-(benzoyloxy)ethoxy]acetic acid, when present as a metabolite (Figure 5.1) or added initially as a pure compound (Figure 5.2), was described very well by the Monod equation. The rate constants obtained for the degradation of D(EG)MB as a metabolite and as a pure compound were 0.0013 and 0.0018 min^{-1}, respectively (Table 5.2); i.e., a 30% difference between the estimated rate constants for this compound between experiments is not significant given the complexities of biological systems, in general, and the difference in the nature of initial substrates introduced into these two particular systems.

 Biodegradation of D(PG)MB proceeded via hydrolysis, which was described by a Monod-type equation that was modified for a reactant-limiting condition (equation 5.11). Biodegradation of the monobenzoate of propanediol was described by two pathways of oxidation/β-oxidation and hydrolysis (equation 5.15). In both systems of D(PG)DB and 1,3-propanediol dibenzoate, the pattern of formation and degradation of monobenzoate metabolites showed good agreement with the model results (Figures 5.3 and 5.4).

Mineralization of benzoic acid was described using a Monod equation and in all cases, except for that of 2,2-methyl-propyl-1,3-propanediol dibenzoate, correlated very well with the change in biomass concentration (equations 5.8, 5.13, and 5.18). In the biodegradation study of 2,2-methyl-propyl-1,3-propanediol

dibenzoate, biodegradation of dibenzoate was correlated with the change in biomass due to the very rapid mineralization rate of benzoic acid (equation 5.21).

Generally, the developed models in conjunction with the calibrated rate constants were able to simultaneously describe the patterns of dibenzoate degradation, metabolite formation and degradation, and also increase in biomass concentrations. However, the rate constants obtained from the models could not be compared directly in order to rank compounds in terms of their biodegradability. This was due to the variety of compounds involved in biodegradation processes and consequently, different kinetic models (e.g. Andrew's inhibition and Monod) that were used to capture their biodegradation behavior. The dependency of the biodegradation rates on the concentrations and different biodegradation pathways available for the biodegradation of monobenzoate metabolites (i.e., oxidation and hydrolysis) were other complexities that did not allow a direct comparison of rate constants.

Therefore, in order to compare the biodegradation rates of the parent compounds and their related metabolites, biodegradation rates were estimated using the models at fixed concentrations of 0.4 and 0.1 g/L, which were in the range of the concentrations of plasticizers and metabolites used in the experimental systems. The calculated biodegradation rates of parent compounds and their associated metabolites at these fixed concentrations are presented in Table 5.3. In the discussion below, only the relative rates at a concentration of 0.4 g/L are compared to discuss the effect of the functional groups. Trends in relative rates are similar at 0.1 g/L and support the same conclusions made in the discussion below.

A comparison of the biodegradation rates of D(EG)DB and D(PG)DB shows that the presence of alkyl branches does not have a significant influence on the rate of hydrolysis of the dibenzoates. The hydrolysis of D(PG)DB was only 1.5 times slower than the hydrolysis of D(EG)DB at a fixed concentration of 0.4 g/L (see Table 5.3).

D(EG)MB and D(PG)MB were degraded via two different mechanisms. D(EG)MB was oxidized to the corresponding carboxylic acid (Figure 5.1),

whereas D(PG)MB was hydrolyzed to benzoic acid (Figure 5.2). The hydrolysis of the second ester bond is generally slower than the first hydrolysis step as was shown qualitatively in previous studies (Kermanshahi pour et al., 2009a). For instance, D(PG)MB is estimated to be hydrolyzed 11 times more slowly than D(PG)DB at a concentration of 0.4 g/L (Table 5.3). The reason for complete oxidation of D(EG)MB to the corresponding carboxylic acid might be due to faster oxidation of D(EG)MB than the competing hydrolysis reaction and formation of stable metabolite of 2-[2-(benzoyloxy)ethoxy]acetic acid.

Only trace amounts of the oxidized monobenzoate was detected during the biodegradation of D(PG)DB, which can be attributed to the presence of methyl branches that inhibit the oxidation of the terminal carbon. In this case, the only possible pathway for degradation of this monobenzoate is a slow hydrolysis to yield benzoic acid and dipropylene glycol.

1,3-propanediol dibenzoate, an alternative plasticizer, does not have the ether bond that is present in the commercial plasticizers. This facilitates the biodegradation of its monoester, 1,3-propanediol monobenzoate. Hydrolysis of this alternative plasticizer was approximately 3 times slower that the rate observed for the commercial plasticizer, D(EG)DB at the concentration of 0.4 g/L (Table 5.3). Biodegradation of 1,3-propanediol monobenzoate proceeded via the two mechanisms of hydrolysis and oxidation. At a concentration of 0.4 g/L, the rate of degradation of 1,3-propanediol monobenzoate was about 13 times faster than that of D(PG)MB (Table 5.3). More importantly, unlike 2-[2-(benzoyloxy)ethoxy]acetic acid, 3-(benzoyloxy) propanoic acid was not stable.

The estimated rate of hydrolysis of 2,2-methyl propyl-1,3-propanediol dibenzoate was approximately 10 times slower than the rate for its non-branched analogue-1,3-propanediol dibenzoate at a concentration of 0.4 g/L (see Table 5.3). It is reasonable to hypothesize that a short chain of the aliphatic portion of propanediol and the presence of alkyl branches in the structure of this dibenzoate causes steric hinderance and significantly slows hydrolysis. An oxidized monobenzoate was not detected in this case. At a concentration of 0.4 g/L, the biodegradation rate of the monobenzoate was about 3 times slower than that of the

monobenzoate of 1,3-propanediol and approximately 4 times faster than the rate of D(PG)MB biodegradation.

Benzoic acid was detected during the biodegradation of all dibenzoate plasticizers but was not always degraded at the same rate in every case. This difference might be attributed to the presence of other components including diols and β-oxidation metabolites that interfered with the benzoic acid degradation in the cases of D(PG)DB and 1,3-propanediol dibenzoate. However, in the case of D(EG)DB, benzoic acid is the only metabolite that is mineralized. Benzoic acid was detected in trace amounts in the case of 2,2-methyl-propyl-1,3-propanediol dibenzoate and 1,6 hexanediol dibenzoate, possibly due to slow hydrolysis of the compounds and consequently slow release of benzoic acid. This demonstrates that the rate of mineralization was higher than the rate of formation of benzoic acid in these cases.

The rate of biodegradation could not be determined for 1,6 hexanediol dibenzoate (Figure 5.6) due to the fact that mass transfer was possibly rate limiting in this case. However, the important result of these experiments was the pattern of metabolite formation. A monobenzoate metabolite was not detected, which is most likely due to rapid β-oxidation to 4-(benzoyloxy) butanoic acid. This is another example demonstrating the importance of removing the ether function in the aliphatic segment as a step toward enhancing the biodegradability of monobenzoate metabolites.

5.7. Conclusions

A study of the kinetics of the biodegradation of dibenzoate plasticizers demonstrated the importance of ether bonds and alkyl branches on the rate of biodegradation of plasticizers and their metabolites. Removing the ether bond significantly enhanced the biodegradation rate of the monobenzoates. The effect of alkyl branches was much less important on the rate of biodegradation of the monobenzoates. Monobenzoates of commercial plasticizers have been shown to exhibit significant acute toxicity and therefore, increasing the degradation rate of these monoesters may be a useful approach toward developing green plasticizers.

5.8. Acknowledgments

5.9. Nomenclature

C_s: Growth limiting substrate concentration (g/L)

C_B: Biomass concentration (g/L)

C_{DB}: Concentration of dibenzoate (g/L)

C_{MB}: Concentration of monobenzoate (g/L)

C_{BA}: Concentration of benzoic acid (g/L)

C_{OM}: Concentration of oxidized monobenzoate (g/L)

k : Maximum specific growth rate (1/min)

k_{DB}: Specific degradation rate of dibenzoate plasticizer (1/min)

k_{BA}: Maximum specific rate of mineralization of benzoic acid (1/min)

k_{MB-OM}: Maximum specific biotransformation rate of monobenzoate (MB) to oxidized monobenzoate (OM) (1/min)

k_{MB-BA}: Maximum specific rate of hydrolysis of monobenzoate to benzoic acid(1/min or L/g.min)

k_{OM-BA}: Maximum specific rate of hydrolysis of oxidized monobenzoate (OM) to benzoic acid (BA) (L/g.min)

K_s: Saturation constant

K_I: Inhibition constant

K_R: Half-saturation constant of the limiting reactant.

K_{DB}: Saturation constant of dibenzoate plasticizer

K_{BA}: Half saturation constant of mineralization of benzoic acid (g/L)

$K_{MB\text{-}OM}$: Half saturation constant of biotransformation of monobenzoate to oxidized monobenzoate (g/L)

$K_{MB\text{-}BA}$: Half saturation constant of hydrolysis of monobenzoate to benzoic acid

KI_{DB}: Inhibition constant of dibenzoate

KI_{BA}: the half-saturation constant of the limiting reactant, benzoic acid

MW_{DB}: Molecular weight of dibenzoate

MW_{MB}: Molecular weight of monobenzoate

MW_{OM}: Molecular weight of oxidized monobenzoate

MW_{BA}: molecular weight of benzoic acid

R: Concentration of the limiting reactant (g/L)

Y: Yield coefficient (g biomass/g substrate)

Y_{DB}: Yield coefficient (g biomass/g dibenzoate mineralized)

Y_{BA}: Yield coefficient (g biomass/g benzoic acid mineralized)

Table 5.1. Metabolites from the biodegradation of dibenzoates by *R rhodochrous*.

Compound	Molecular Cation [M$^{+\bullet}$], (m/z)[1]	Retention Time (min)	*m/z* of major ion peaks of trimethylsilyl derivative of metabolites (%)
2-[2-(benzoyloxy)propoxy]propanoic acid	324	28.66	105(60), 135(6), 179(37), 309(weak), 324 (not detected)
1,3propanediol monobenzoate	252	23.16	105(100), 135 (7.3), 179(14), 237 (6), 252 (weak)
3-(benzoyloxy) propanoic acid	266	24.81	105 (100) 135 (15) 179 (66) 251 (15) 266 (weak)

Note:
 1. [M$^{+\bullet}$] of the trimethylsilyl derivative

Table 5.2. Summary of rate constants for kinetic models of the degradation of dibenzoate plasticizers. Rate constants were calibrated from data presented in Figures 5.1 to 5.5.

Rate constants	Initial compound biodegraded				
	Diethylene glycol dibenzoate	Diethylene glycol Monobenzoate	Dipropylene glycol dibenzoate	1,3-Propanediol dibenzoate	2,2-Methyl propyl-1,3-propanediol dibenzoate
From equations	4-8	9	10-13	14-18	19-21
Calibrated from data in figures	1	2	3	4	5
k_{DB}	0.0520 min^{-1}	-	0.010 min^{-1}	0.0078 min^{-1}	0.0014 min^{-1}
K_{DB}	0.2943 g·L^{-1}	-	0.012 g·L^{-1}	0.4500 g·L^{-1}	0.1429 g·L^{-1}
KI_{DB}	0.3997 g·L^{-1}	-	-	-	0.1527 g·L^{-1}
k_{MB-OM}	0.0013 min^{-1}	0.0018 min^{-1}	-	0.0018 min^{-1}	-
K_{MB-OM}	0.0074 g·L^{-1}	0.0074 g·L^{-1}	-	0.0478 g·L^{-1}	-
k_{BA}	0.0061 min^{-1}	-	0.0023 min^{-1}	0.0018 min^{-1}	-
K_{BA}	0.3586 g·L^{-1}	-	0.0390 g·L^{-1}	0.0056 L·g^{-1}·min^{-1}	-
k_{MB-BA}	-	-	0.0008 min^{-1}	0.0206 L·g^{-1}·min^{-1}	0.0074 min^{-1}
K_{MB-BA}	-	-	0.0170 g·L^{-1}	-	-
k_{OM-BA}	-	-	-	0.0007 L·g^{-1}·min^{-1}	-
KI_{BA}	-	-	0.0054 g·L^{-1}	-	-
Yield	0.77	-	0.85	0.75	0.67

Table 5.3. Comparison of biodegradation rates of dibenzoates and their metabolites estimated from kinetic models at the selected concentrations of 0.1 and 0.4 g/L of plasticizers and metabolites.

Initial compound biodegraded	Concentration at which rate is estimated (g/L)	Biodegradation rate (min^{-1})			
		Initial compound	Metabolites		
			Benzoic acid	Monobenzoate	Oxidized monobenzoate
Diethylene glycol dibenzoate	0.1	0.0081	0.0013	0.0012	Stable - did not degrade
	0.4	0.0122	0.0032	0.0013	
Diethylene glycol monobenzoate	0.1	0.0017	Trace amount detected	Not applicable	Stable - did not degrade
	0.4	0.0018			
Dipropylene glycol dibenzoate	0.1	0.0050	0.0017	0.0006	Rapid - trace amount detected
	0.4	0.0081	0.0021	0.0007	
1,3-Propanediol dibenzoate	0.1	0.0014	0.0017	0.0032	0.0001
	0.4	0.0036	0.0018	0.0098	0.0003
2,2-Methyl propyl-1,3-propanediol dibenzoate	0.1	0.0004	Trace amount detected	00007	Not detected
	0.4	0.0003		0.0030	
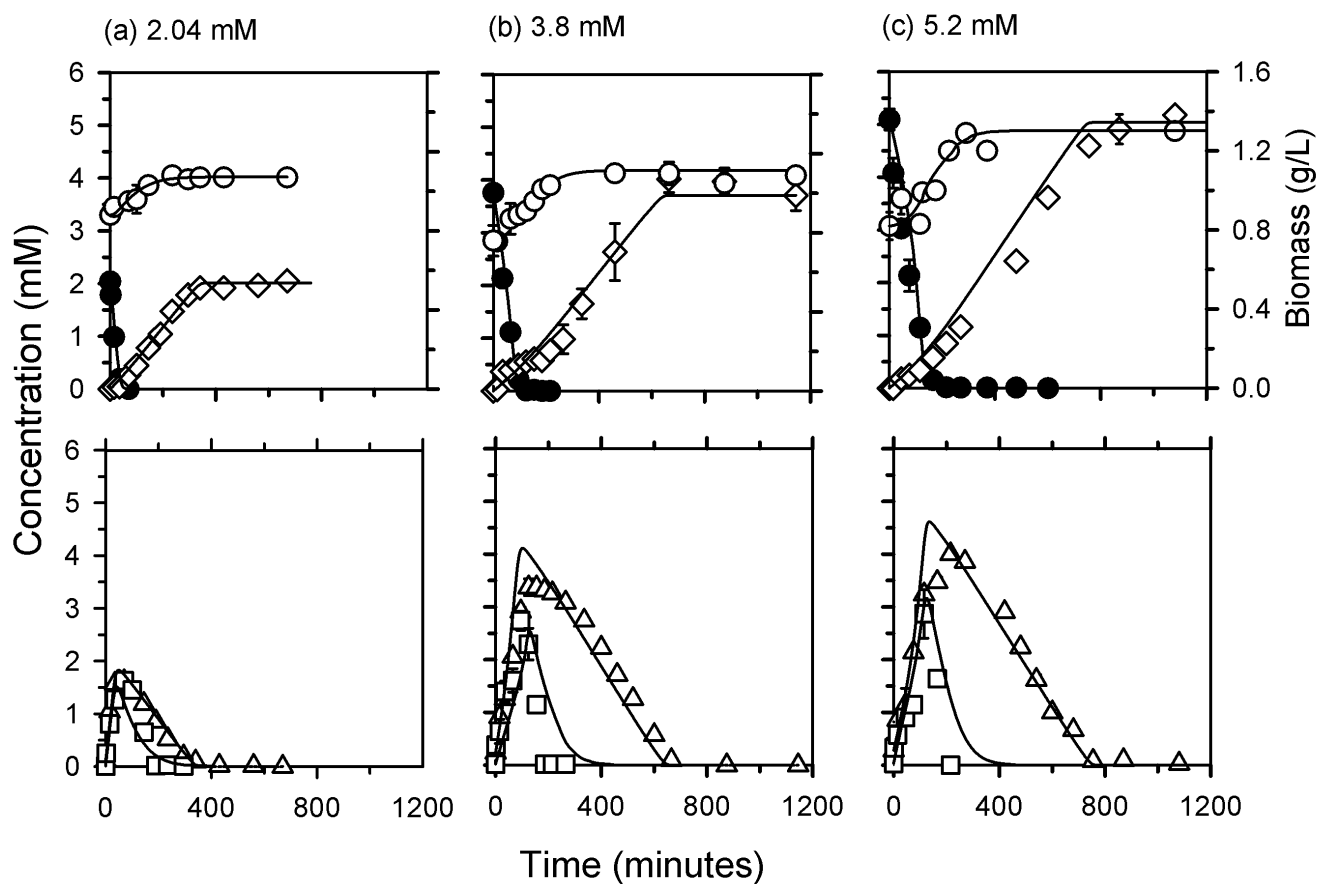
Figure 5.1. Biodegradation of D(EG)DB (●) by *R. rhodochrous* at respective initial concentrations of (a) 2.04 mM (b) 3.8 mM and (c) 5.2 mM and corresponding formation of 2-[2-(benzoyloxy)ethoxy]acetic-acid (◊), biomass (○), D(EG)MB (Δ) and benzoic acid (□). Symbols are the experimental data and lines are model results. Concentrations are the average of duplicate samples and error bars indicate the range of two measurements (Note: bars are only visible when they are larger than the symbols).
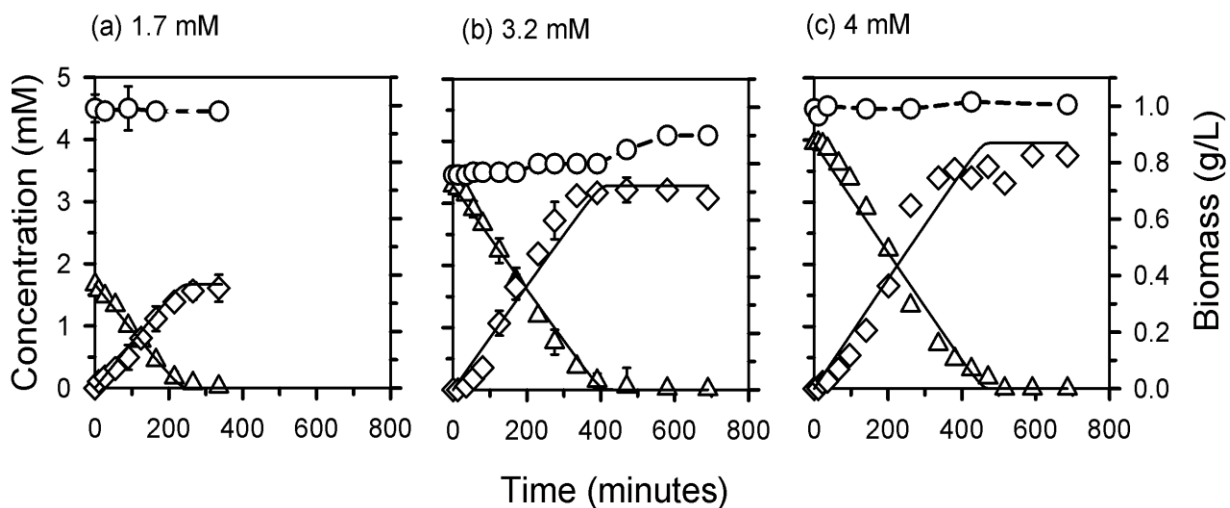
Figure 5.2. Biodegradation of D(EG)MB (Δ) by *R. rhodochrous* at respective initial concentrations of (a) 1.7 mM (b) 3.2 mM and (c) 4.0 mM and corresponding biomass concentrations ($\circ$) and formation of 2-[2-(benzoyloxy)ethoxy]acetic-acid ($\Diamond$). Symbols are the experimental data and lines are model results. Concentrations are the average of duplicate samples and error bars indicate the range of two measurements (Note: bars are only visible when they are larger than the symbols).
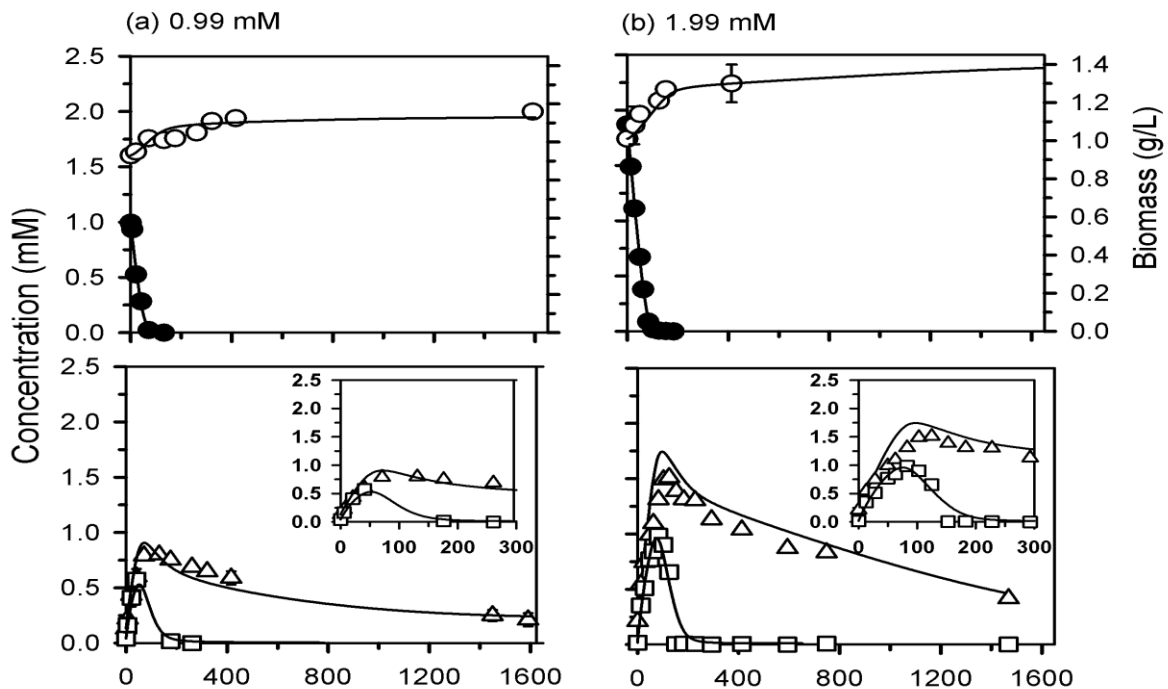
Figure 5.3. Biodegradation of D(PG)DB (●) by *R. rhodochrous* at respective initial concentrations of (a) 0.99 mM and (b) 1.99 mM and corresponding formation of D(PG)MB (Δ), biomass (○) and benzoic acid (□). Symbols are the experimental data and lines are model results. Concentrations are the average of duplicate samples and error bars indicate the range of two measurements (Note: bars are only visible when they are larger than the symbols). The lower figures contain magnified representations of D(EG)MB and benzoic acid data to show trends in the first 300 minutes.
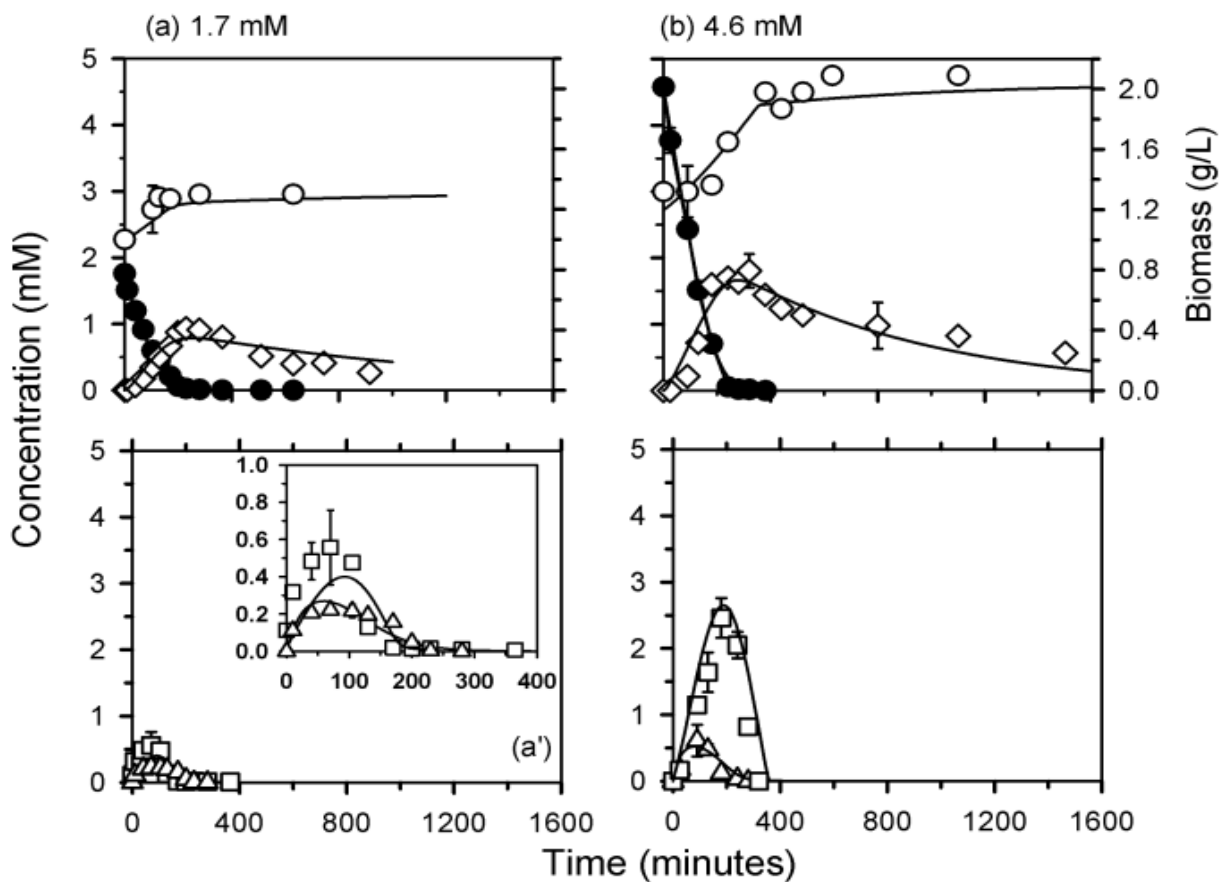
Figure 5.4. Biodegradation of 1,3-propanediol dibenzoate (●) by *R. rhodochrous* at respective initial concentrations of (a) 1.7 mM and (b) 4.6 mM and corresponding formation of 3-(benzoyloxy)propanoic acid (◊), biomass (○), 1,3-propanedol monobenzoate (Δ) and benzoic acid (□). Symbols are the experimental data and lines are model results. Concentrations are the average of duplicate samples and error bars indicate the range of two measurements (Note: bars are only visible when they are larger than the symbols). The lower figure includes a magnified representation of 1,3-propanediol monobenzoate and benzoic acid data to show trends in the first 400 minutes.

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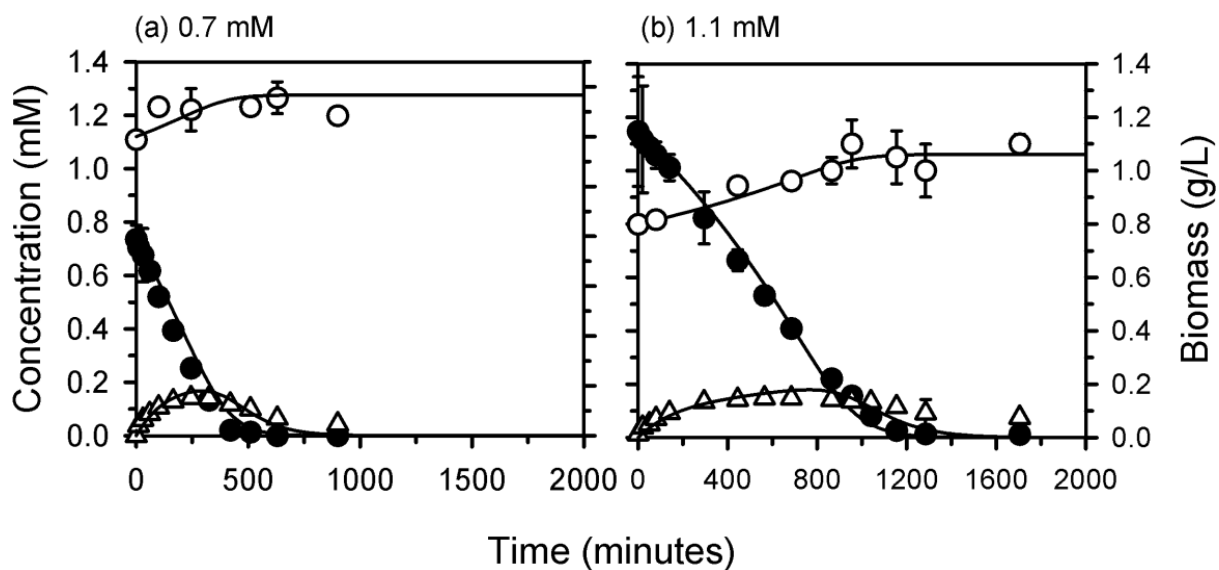
Figure 5.5. Biodegradation of 2,2-methyl-propyl-1,3-propanediol dibenzoate (●) by *R. rhodochrous* at respective initial concentrations of (a) 0.7 mM and (b) 1.1 mM and corresponding formation of 2,2-methyl-propyl-1,3-propanediol monobenzoate (Δ) and biomass (○). Symbols are the experimental data and lines are model results. Concentrations are the average of duplicate samples and error bars indicate the range of two measurements (Note: bars are only visible when they are larger than the symbols).
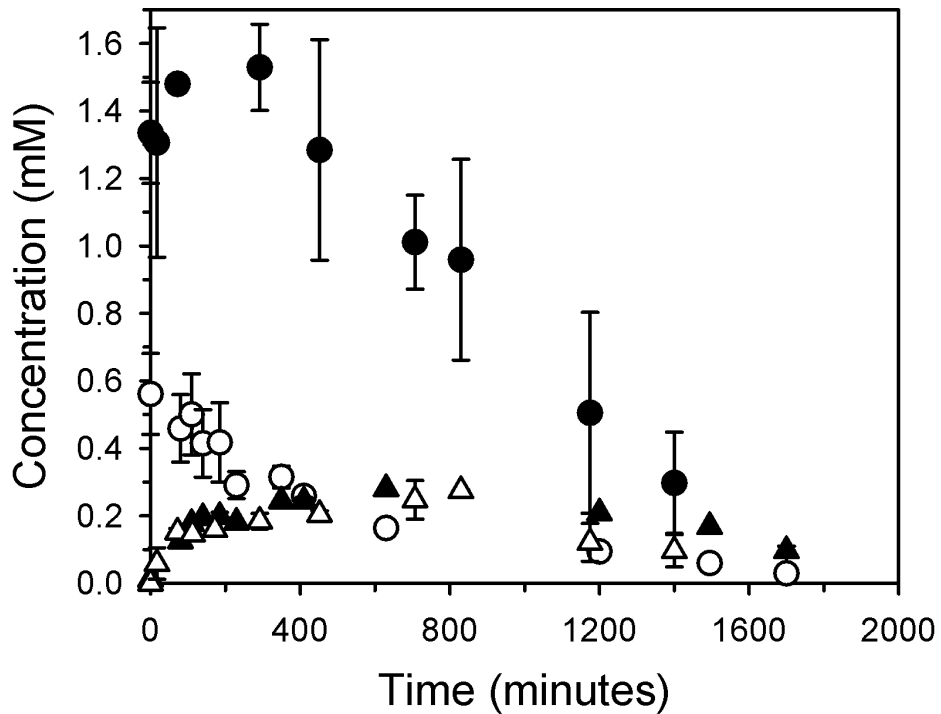
Figure 5.6. Biodegradation of 1,6-hexanediol dibenzoate at initial concentrations of 1.3 mM (●) and 0.56 mM (○) by *R. rhodochrous* and corresponding formation of 4-(benzoyloxy)butanoic acid (▲, △). Concentrations are the average of triplicate samples and error bars indicate standard deviations (Note: bars are only visible when they are larger than the symbols).
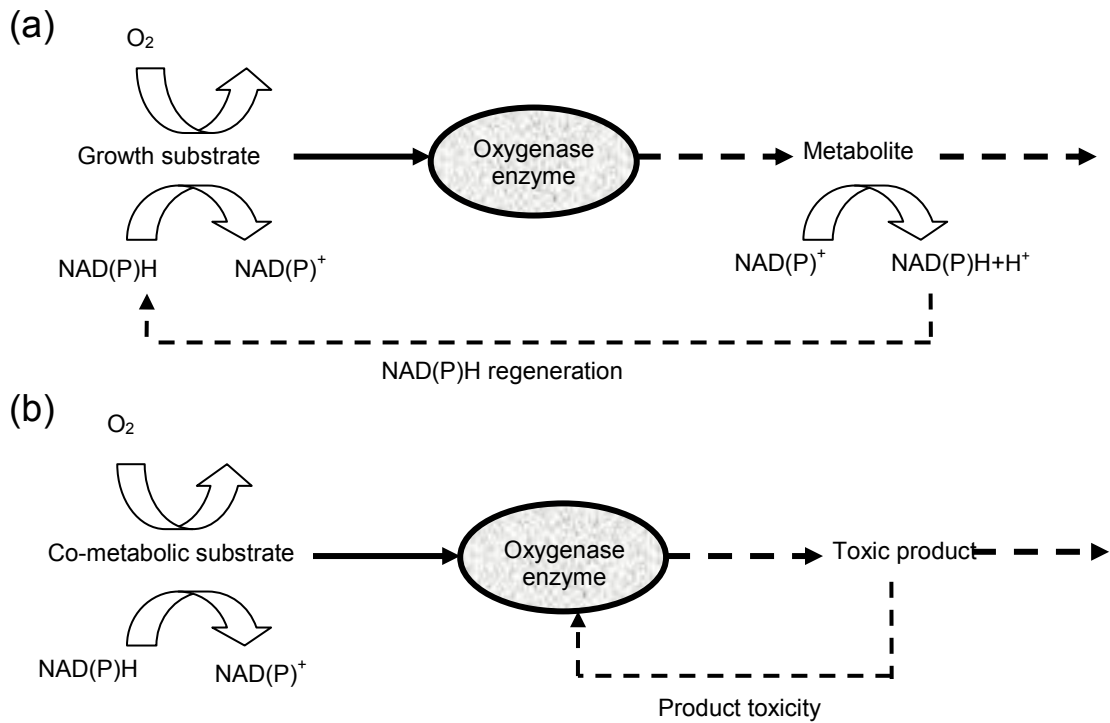
Figure 5.7. Effect of growth substrate on regeneration of NAD(P)H: (a) oxygen is the electron acceptor and NAD(P)H is electron donor, and metabolites of growth substrate are degraded and regenerate NAD(P)H; (b) metabolites of co-mtabolic substrate do not regenerate NAD(P)H and may lead to cell damage. Reproduced from Chang and Alvarez-Cohen, 1995.

6. Preliminary Assessment of the Toxicities of Dibenzoate Plasticizers and their Metabolites

Preface

In the studies presented in the previous chapters, the effect of functional groups on the biodegradability of dibenzoate plasticizers and related metabolites was discussed. In addition to identifying the functional groups that lead to the persisitent metabolites, in order to design safer chemicals, another important goal of this study was to assess the toxicity of the metabolites that had been previously identified. In this chapter, the toxicity of selected commercial dibenzoate plasticizers (i.e., diethylene glycol dibenzoate and dipropylene glycol dibenzoate), alternative plasticizers (i.e., 1,6-hexanediol dibenzoate, 1,3-propanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate) and some of their related metabolites (i.e., monobenzoates and diols) were examined using the Microtox^{TM} toxicity assay. The toxicities of these compounds to algae and daphnia were also predicted using predictive software, ECOSAR of the United States Environmental Protection Agency (USEPA). High acute toxicities for commercial plasticizers and their monobenzoate metabolites were observed in response to the Microtox^{TM} assay. However, toxicity according to the Microtox^{TM} assay, was not detected for the two alternative plasticizers of 1,6-hexanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate. High acute toxicities observed for the monobenzoates of both commercial and alternative plasticizers highlights the importance of efforts detailed in earlier chapters to increase the biodegradability of monobenzoate metabolites via modification of the chemical structure of dibenzoates. These results point to the necessity of conducting more detailed and comprehensive toxicity assessments of all the above compounds using higher organisms and other toxicity assays.

Preliminary Assessment of the Toxicities of Dibenzoate Plasticizers and their Metabolites

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6.1. Abstract

Toxicity assessments of selected dibenzoate plasticizers and related metabolites produced during their biodegradation were performed using the Microtox^{TM} toxicity assay. These measurements were also compared to the toxicities to algae and daphnia predicted by the ECOSAR software of the United States Environmental Protection Agency. Phenol was used as a standard to provide a comparative measure of toxicity, expressed in terms of an EC_{50}, which is the effective concentration of the toxicant that causes a 50% reduction in light output from the bacteria. The commercial plasticizers di-ethylene glycol dibenzoate and di-propylene glycol dibenzoate exhibited higher acute toxicities than phenol. However, the new alternative plasticizers, 1,6-hexanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate did not exhibit toxicity. The highest toxicity amongst all the compounds was observed for 1,3-propanediol dibenzoate. Monobenzoates, which are potential hydrolytic metabolites of both commercial and alternative plasticizers, showed comparable toxicities to that of phenol. Diols of both alternative and commercial plasticizers showed very low acute toxicity.

6.2. Introduction

Plasticizers are amongst the most widely used polymer additives (Wypych, 2004) and their high volume production, broad application and incomplete biodegradation have led to their widespread presence in the environment (Staples et al., 1997; Horn et al., 2004). Concerns associated with heavily used diester plasticizers such as phthalates and adipates include the fact that their biodegradation leads to metabolites with higher toxicity than the parent compound (Albro, 1975; Mitchell et al., 1985; Horn et al., 2004). Metabolism of phthalates and adipates was shown to begin with hydrolysis of an ester bond, resulting in the formation of the corresponding monoester and alcohol (Staples et al., 1997; Horn et al., 2004). Mono-2-ethylhexyl phthalate, the hydrolytic metabolite of di-2-ethylhexyl phthalate, is classified as an endocrine disruptor (Onorato et al., 2008) and 2-ethylhexanoic acid, another metabolite identified in the metabolism of di-2-ethylhexyl phthalate and di-2-ethylhexyl adipate, is a known potent peroxisome proliferator (Cornu et al., 1992; Keith et al., 1999; Horn et al., 2004).

In response to worldwide concern over the environmental and health implications of phthalates, alternative plasticizers such as dibenzoates have been proposed to replace phthalates in the case of critical applications such as children's toys (Deligio, 2009). Most notably, low toxicity and high biodegradation rates were reported for a blend of dibenzoate plasticizers sold under the commercial name of Benzoflex® 2888; i.e., a blend of diethylene glycol dibenzoate, triethylene glycol dibenzoate, and dipropylene glycol dibenzoate (Arendt and Lang, 1998; Lang and Stanhope, 2001). In addition, an oral LD_{50} of 3-5g/Kg in rats was observed (Rahman and Brazel, 2004). However, more recently, biodegradation studies of dibenzoate plasticizers demonstrated the formation of some stable monoester metabolites; a result of incomplete hydrolysis of the parent dibenzoate plasticizers (Gartshore et al., 2003, Kermanshahi pour et al., 2009a). Such findings could undermine the acceptability of dibenzoate plasticizers as alternatives to the problematic phthalates. Therefore, it is necessary to study the toxicity of the metabolites of dibenzoates.

The objective of this study was to obtain preliminary information on the toxicities of dibenzoate plasticizers. The Microtox[TM] toxicity assay was used as a screening tool for toxicity assessment. The study encompassed toxicity measurements of two commercial dibenzoate plasticizers, diethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB), as well as three potential alternative plasticizers, 1,6-hexanediol dibenzoate, 1,3-propanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate. The toxicities of their potential hydrolytic metabolites, including their respective monobenzoates and diols, were also measured. In addition, the predictive software ECOSAR of the United States Environmental Protection Agency (USEPA) was used to predict the respective toxicities of these compounds to algae and daphnia based on their chemical structures.

6.3. Materials and methods

6.3.1. Chemicals and reagents

D(EG)DB (96%), D(PG)DB (98%), 1,6-hexanediol (99%), 1,3-propanediol (98%), 2-methyl-2-propyl-1,3-propanediol (98%), dipropylene glycol (99%), diethylene glycol (99%) and phenol (99%) were purchased from Sigma-Aldrich (Oakville, ON). The synthesis of 1,6-hexanediol dibenzoate, 1,3-propanediol dibenzoate, di-ethylene glycol monobenzoate, 2,2-methyl-propyl-1,3-propanediol dibenzoate have been described elsewhere (see Chapters 3 and 5). Microtox[TM] assay reagents (i.e., diluent, reconstitution solution, osmotic adjustment solution, freeze-dried *Vibrio fischeri*) were obtained from Strategic Diagnostic Inc. (Newark, DE).

6.3.2. Syntheses

1,6-Hexanediol monobenzoate

1,6- Hexanediol monobenzoate was synthesized by refluxing 9.9 g of 1,6-hexanediol with 5.9 g benzoyl chloride (0.5 equivalents) under nitrogen in 120 mL of acetone in round bottom flask for 7 hours. 1,6-Hexanediol monobenzoate was purified using column chromatography. Silica gel was used as the stationary phase and a solution of acetone and hexane (30:70 vol:vol) was used as the mobile phase. The proton nuclear magnetic resonance (NMR) spectra of the synthesized 1,6-hexanediol monobenzoate was consistent with the spectrum expected for this structure (see Figure 6.1).

2,2-Diethy-1,3-propanediol monobenzoate

2,2-diethy-1,3-propanediol monobenzoate was synthesized by refluxing 6.8 g of 2,2-diethy-1,3-propanediol with 18 g benzoyl chloride (2.5 equivalents) under nitrogen in 120 mL of acetone in round bottom flask for 7 hours. The reaction mixture was purified employing column chromatography with silica gel as the stationary phase and hexane/methylene chloride (20:80 vol:vol) as the mobile phase. The NMR spectrum of this compound is shown in Figure 6.2.

6.3.3. Microtox toxicity assay

The Microtox assay is based on exposure of a bioluminescence marine bacterium, _Vibrio fischeri,_ to a series of different concentrations of the test compound in an aqueous sample. Toxic contaminants interfere with the biochemical pathway of light production and cause a reduction in light output by this bacterium. This biochemical reaction of flavinmononucleotide, oxygen and a long chain aldehyde catalyzed by the luciferase enzyme is shown in equation 6.1 (Ghioureliotis, 1997).

$$FMNH_2 + O_2 + RCHO \xrightarrow{\quad Luciferase \quad} FMN + H_2O + RCOOH + light \qquad (6.1)$$

Microtox toxicity is typically expressed in terms of the effective concentration of the toxicant that causes a 50% reduction in light output from the

bacteria (*i.e.*, EC_{50}). The Microtox EC_{50} has been found to correlate well with LD_{50} values, which is the median lethal dose needed to kill half the members of a tested population, for rats and mice (Kaiser et al., 1994).

In the present study, serial dilutions of dibenzoates were prepared in 200 mL of distilled water. 0.5% by volume HPLC-grade methanol was used to solubilize the plasticizer in water. Testing with methanol in water showed that up to 1% by volume of methanol does not cause any toxicity to the cells and, therefore, does not interfere with the toxicity assay. All samples were osmotically adjusted to 2% NaCl using an osmotic adjustment solution prior to analysis. Samples of pure monobenzoate and diols were prepared in 15 mL of diluent (i.e., 2% NaCl in reagent water). All samples were prepared at concentrations below the solubility level of the substance being assayed.

The light output of the bacteria was monitored immediately before and after 15 minutes of exposure of bacteria to serial dilutions of the samples using a Model 500 Toxicity Analyzer (Strategic Diagnostic Inc. Newark, DE). EC_{50} was converted to concentration units by multiplying the EC_{50} expressed as a volume fraction by the sample concentration. A control was run simultaneously with each test to account for the normal drop of the light output of the bacteria. For this purpose, 0.1 g/L phenol solution was used as a control to ensure the accuracy of the technique. The EC_{50} of phenol was measured to be 0.018 ± 0.002 g/L in this study, which is within the recommended range of the manufacturer of the Microtox system (i.e., 0.013 - 0.027 g/L).

6.4. Results and discussion

The Microtox EC_{50} values of the two commercial dibenzoate plasticizers, D(EG)DB and D(PG)DB, and three alternative plasticizers, 1,6-hexanediol dibenzoate, 1,3-propanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate, and a series of monobenzoates and diols are presented in Table 6.1.

The predicted toxic response of green algae (96 hr) and Daphnid (48 hr) to the dibenzoate plasticizers and related metabolites were obtained using ECOSAR software of USEPA. The predicted toxicity results are presented in Table 6.2 and

6.3. The solubilities of the above compounds of interest and their respective metabolites were also predicted by the ECOSAR software and are presented in Table 6.4. Information on solubility is important in toxicity measurement by Microtox[TM] since the toxicant concentration should be below the solubility level. Solubility determination is also important in order to verify, whether there is a relationship between the solubilities and toxicities of similar classes of compounds.

The results of the Microtox assay in Table 6.1 show that the commercial dibenzoates, D(EG)DB and D(PG)DB, with EC_{50}s of 4 and 10 mg/L, respectively, were more toxic than phenol, with an EC_{50} of 18 mg/L. No toxicity was detected for 1,6-hexanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate (Table 6.1). The most toxic compound amongst those studied was 1,3-propanediol dibenzoate, which had a measured EC_{50} of 0.1 ± 0.02 mg/L. 1,3-propanediol dibenzoate has similar functional groups compared to 1,6-hexanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate and, therefore, the high toxicity of 1,3-propanediol dibenzoate compared to the other two plasticizers was unexpected. However, the toxicities predicted using ECOSAR for 1,3-propanediol dibenzoate exposed to algae (for 96 hr) and Daphnia (for 48 hr) were comparable to those of the other plasticizers (Tables 6.2 and 6.3).

The toxicities of the monobenzoate metabolites was generally lower than the corresponding dibenzoates for both the experimentally-measured EC_{50}'s using the Microtox assay (Table 6.1) and the predicted EC_{50}'s and LC_{50}'s of algae and daphnia (Tables 6.2 and 6.3). For example, diethylene glycol monobenzoate and 2-[2-(benzoyloxy)ethoxy]acetic acid had higher EC_{50}'s (i.e., lower toxicities) compared to their corresponding dibenzoates (Table 6.1). However, 1,6-hexanediol monobenzoate showed higher toxicity compared to the corresponding dibenzoate (Table 6.1). It should also be noted that, due to the low solubility of 1,6-hexanediol dibenzoate, lower initial concentrations were prepared for use in the Microtox assay and, in that concentration range, toxicity was not detected. Therefore, the absence of detected toxicity does not mean that this is a completely non-toxic compound. On the other hand, 1,6-hexanediol monobenzoate had much

higher solubility according to the ECOSAR solubility prediction as presented in Table 6.4 and, therefore, higher concentrations of 1,6-hexanediol monobenzoate could be prepared in order to detect EC_{50}. 2,2-Diethyl-1,3-propanediol monobenzoate, for which its dibenzoate analogue was not available for toxicity measurement, had also comparable toxicity to that of the other monobenzoates (Table 6.1).

Diols have the highest solubilities amongst the compounds studied here according to the ECOSAR predictions, presented in Table 6.4. As indicated in Table 6.4, solubilities of diols are several orders of magnitude higher than the solubilities of their respective monobenzoate and dibenzoate. However, diols had the lowest toxicities amongst the metabolites (Tables 6.1, 6.2 and 6.3).

Generally, the toxicities of the monobenzoates were comparable to that of phenol, which implies a high acute toxicity for this class of chemicals. Toxicity assessment of monobenzoates of commercial plasticizers is more crucial than that of the alternative plasticizers because the former monobenzoates were more stable when undergoing biodegradation by soil bacterium (Gartshore et al., 2003; Kermanshahi pour et al., 2009a). In contrast, the monobenzoates of alternative plasticizers were either not detected or were degraded very rapidly (Firlotte et al., 2009; Kermanshahi pour et al., 2009a,b).

6.5. Conclusions

D(EG)DB, D(PG)DB and their associated monobenzoate metabolites exhibited high acute toxicities in response to the Microtox[TM] toxicity assay. The EC_{50}'s of the two alternative plasticizers, 1,6-hexanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate, could not be detected, which implies a low acute toxicity. These preliminary toxicity results are promising and support the use of alternative plasticizers as potential safe compounds to replace the commercial plasticizers. However, a comprehensive study to characterize the toxicities of the alternative plasticizers and their associated metabolites is essential prior to making definitive conclusions about the potential health and environmental consequences of these chemicals.

6.6. Acknowledgments

Table 6.1. Toxicities of commercial and alternative dibenzoate plasticizers and their potential metabolites, including monobenozates and diols, assessed using the Microtox[TM] acute toxicity assay.

Compound	Dibenzoate	Monobenzoate[1]	Diol[1]	Oxidized monobenzoate[1,2]
	EC_{50}[3] mg/L(mM)	EC_{50}[3] mg/L (mM)	EC_{50}[3] mg/L (mM)	EC_{50}[3] mg/L (mM)
Diethylene glycol dibenzoate	4.4±1.3 (0.0013±0.003)	34.5±4.9 (0.16±0.02)	Not detected[7]	20.3±8.4 (0.9±0.035)
Dipropylene glycol dibenzoate	10.7 ±1.2 (0.029 ±0.0029)	-[6]	2100±50 (15.67±0.37)	-
1,6-Hexanediol dibenzoate	Not detected[4]	1.2±0.3 (0.004±0.001)	530±10 (4.49±0.08)	-
1,3-Propanediol dibenzoate	0.1±0.02 (0.0004±0.00007)	-	Not detected[8]	-
2,2-Methyl propyl-1,3-propanediol dibenzoate	Not detected[5]	-	340±20 (2.6±0.15)	-
2,2-Diethyl-1,3-propanediol dibenzoate	-	12.7±3.1 (0.05±0.01)	-	-

Note:
1. Monobenzoates, diols and oxidized monobenzoates are the potential metabolites of the corresponding dibenzoate.
2. The oxidized monobenzoate of diethylene glycol is 2-[2-(benzoyloxy)ethoxy]acetic acid.
3. EC_{50} is reported in units of mg/L and mM. Values are the average of EC_{50} of three solutions with different initial concentrations.
4. Initial concentration of 1,6-hexanediol dibenzoate was 5 mg/L (0.017 mM/L)
5. Initial concentration of 2,2-methyl-propyl-1,3-propanediol dibenzoate was 5 mg/L (0.034 mM).
6. The compound (-) was not available for test.
7. Initial concentration of di-ethylene glycol was 2500 mg/L (23.6 mM).
8. Initial concentration of 1,3-propanediol was 2900 mg/L (38 mM).

Table 6.2. Predicted toxicities using ECOSAR software of commercial and alternative dibenzoate plasticizers and their potential metabolites, when exposed to algae for 96 hrs.

Compound	Dibenzoate	Monobenzoate[1]	Diol[1]
	EC_{50} [2] mg/L (mM)	EC_{50} [2] mg/L (mM)	EC_{50} [2] mg/L (mM)
Diethylene glycol dibenzoate	7.0 (2.3×10^{-2})	2.3×10^{2} (1.1)	3.3×10^{3} (3.1×10)
Dipropylene glycol dibenzoate	2.0 (6.0×10^{-3})	7.0×10 (3.0×10^{-2})	1.2×10^{3} (9.3)
1,6-Hexanediol dibenzoate	2.0 (7.0×10^{-3})	7.0 (3.3×10^{-2})	1.4×10^{2} (1.2)
1,3-Propanediol dibenzoate	2.0 (7.0×10^{-3})	6.0×10 (3.3×10)	7.8×10^{2} (1.0×10)
2,2-Methyl propyl-1,3-propanediol dibenzoate	Prediction above the solubility limit	4.0 (1.8×10^{-2})	9.3 (7.1×10^{-1})
2,2-Diethyl-1,3-propanediol dibenzoate	Prediction above the solubility limit	4.0 (1.8×10^{-2})	9.3 (7.1×10^{-1})

Note:
1. Monobenzoates, diols and oxidized monobenzoates are the potential metabolites of the corresponding dibenzoate.
2. All EC_{50} values are in units of mg/L and mM. The values in parentheses correspond to mM.

Table 6.3. Predicted toxicities using ECOSAR software of commercial and alternative dibenzoate plasticizers and their potential metabolites, when exposed to daphnia for 48 hr.

Compound	Dibenzoate	Monobenzoate[1]	Diol[1]
	LC_{50} [2] mg/L (mM)	LC_{50} [2] mg/L (mM)	LC_{50} [2] mg/L (mM)
Diethylene glycol dibenzoate	1.9×10 (6.2×10^{-2})	4.6×10^2 (2.2)	3.3×10^3 (3.0×10^2)
Dipropylene glycol dibenzoate	6.0 (1.8×10^{-2})	1.5×10^2 (6.5×10^{-1})	8.3×10^3 (6.2×10)
1,6-Hexanediol dibenzoate	Prediction above the solubility limit	1.9 (8.5×10^{-2})	5.2×10^2 (4.4)
1,3-Propanediol dibenzoate	6.0 (2.0×10^{-2})	1.3×10^2 (7.2×10^{-1})	5.3×10^3 (7.0×10)
2,2-Methyl propyl-1,3-propanediol dibenzoate	Prediction above the solubility	1.1×10 (5×10^{-2})	2.9×10^2 (2.2)
2,2-Diethyl-1,3-propanediol monobenzoate	Prediction above the solubility limit	1.1×10 (5×10^{-2})	2.9×10^2 (2.2)

Note:
1. Monobenzoates, diols and oxidized monobenzoates are the potential metabolites of the corresponding dibenzoate.
2. LC_{50} is the median lethal concentration and is defined as the concentration required to kill half of the tested population. All LC_{50} values are expressed in units of mg/L and mM. The values in parentheses correspond to mM.

Table 6.4. Predicted solubilities using ECOSAR software of dibenzoates, and their potential metabolites including monobenzoates and diols.

Compound	Dibenzoate	Monobenzoate[1]	Diol[1]
	Solubility[2] mg/L (mM)	Solubility[2] mg/L (mM)	Solubility[2] mg/L (mM)
Diethylene glycol dibenzoate	2.5×10 (0.08)	1.3×10^4 (6.1×10)	1.0×10^6 (9.4×10^3)
Dipropylene glycol dibenzoate	4.0 (1×10^{-2})	5.5×10^3 (2.4×10)	1.0×10^6 (7.4×10^3)
1,6-Hexanediol dibenzoate	5.0 (2.6×10^{-1})	4.2×10 (1.9)	2.2×10^4 (1.9×10^2)
1,3-Propanediol dibenzoate	8.0 (3.0×10^{-2})	1.0×10^4 (5.5×10)	1.0×10^6 (13158)
2,2-Methyl propyl-1,3-propanediol dibenzoate	5.0 (1×10^{-1})	1.7×10^2 (7.1×10^{-1})	9.0×10^3 (7.2×10)
2,2-Diethyl-1,3-propanediol dibenzoate	5.0 (1×10^{-1})	1.7×10^2 (7.1×10^{-1})	2.0×10^5 (1.5×10^3)

Note:
1. Monobenzoates, diols and oxidized monobenzoates are the potential metabolites of the corresponding dibenzoate.
2. All solubility values are in units of mg/L and mM. The values in parentheses correspond to mM.

Figure 6.1. NMR spectrum (CDCl₃, 300 MHz) of synthesized 1,6-hexanediol monobenzoate. Relative integration of protons on indicated carbon atom (A:B:C:D:E:F:G:H:I= 2:2:1:2:2:1:2:2:4). A, B and C are the expected pattern for a phenyl group. D and E are triplets and G and H are multiplets, as expected for this part of the structure, and F is a singlet.
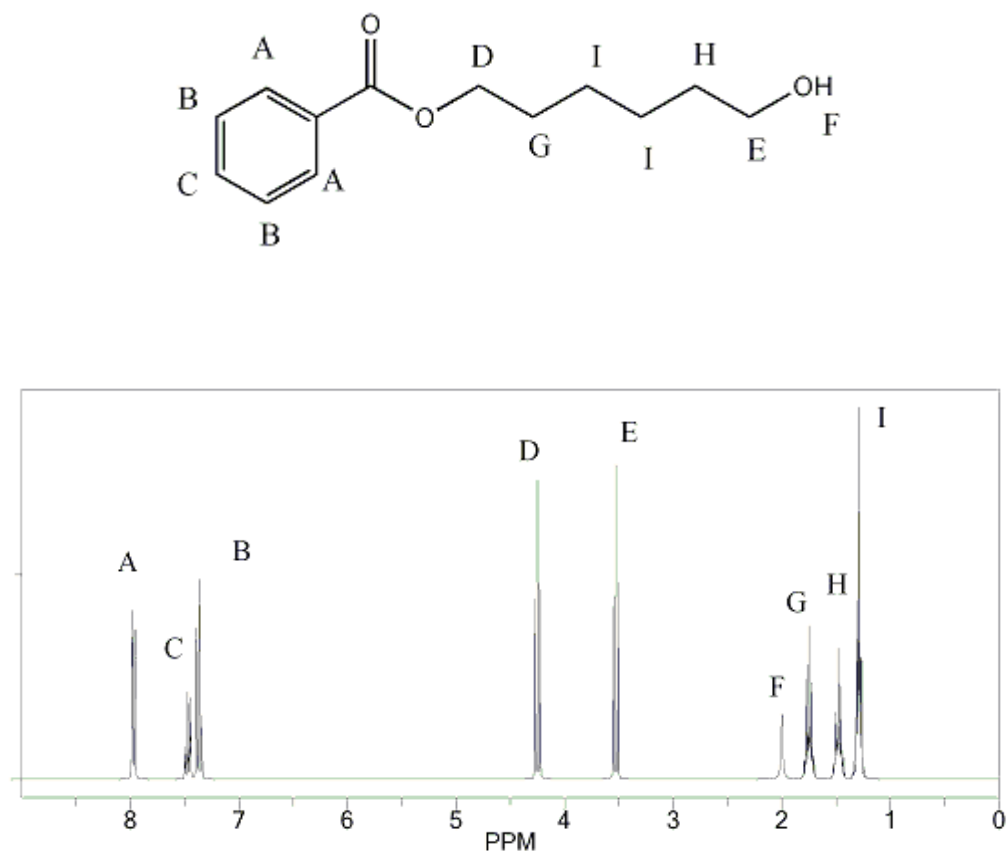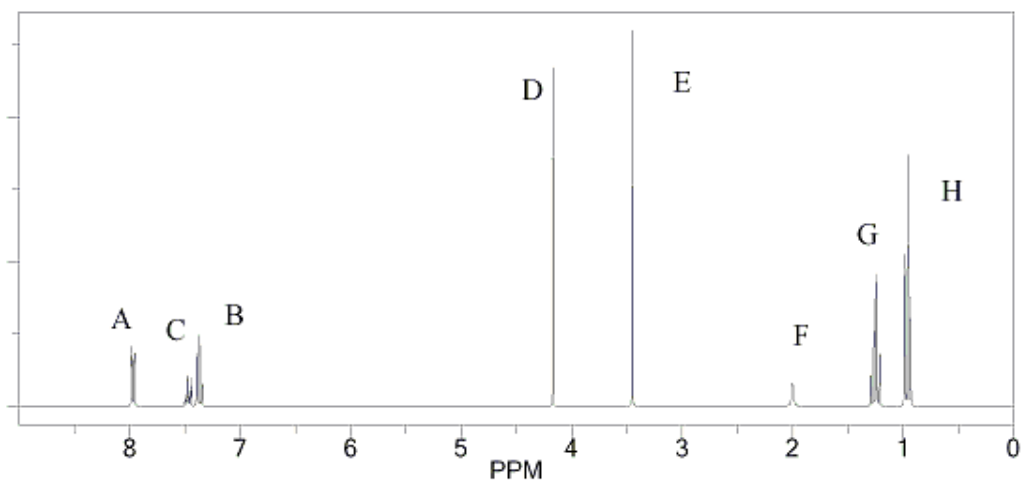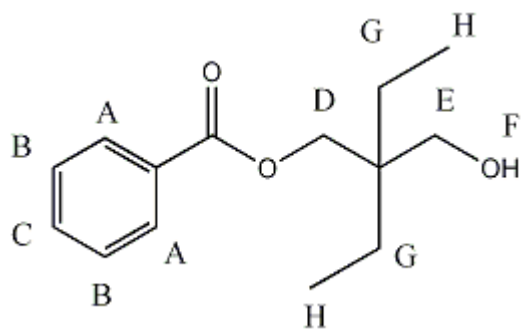
Figure 6.2. NMR spectrum (CDCl$_3$, 300 MHz) of synthesized 2,2-di-ethyl-1,3-propanediol monobenzoate. Relative integration of protons on indicated carbon atoms (A:B:C:D:E:F:G:H= 2:2:1:2:2:1:4:6). A, B and C exhibit the expected pattern for a phenyl group. D, E and F are singlets, G is a quadruplet and H is a triplet.

7. Summary and Conclusions

In this thesis, the potential environmental implications of commercially available dibenzoate plasticizers and persistent metabolites arising from their incomplete microbial hydrolysis were discussed. Alternative plasticizers have been synthesized and their potential as replacements for commercial dibenzoate plasticizers has been explored by studying their biodegradation and identifying their related metabolites. The most important criterion used to assess a compound as a potential alternative to conventional plasticizers was complete biotransformation of the parent plasticizer to non-toxic and biodegradable metabolites. Through the research presented in this thesis, an understanding has been developed with respect to the effect of functional groups (i.e., ether function and alkyl branches) on the mechanisms and rate of biodegradation of dibenzoate plasticizers and their associated metabolites. This understanding has shown that the presence of the ether function leads to the incomplete hydrolysis of dibenzoate plasticizers. Following these findings, environmentally benign versions of dibenzoate plasticizers were developed that undergo complete biodegradation and do not result in the formation of stable metabolites.

More specifically, in this thesis, the potential health and environmental implications of conventional plasticizers are reviewed, the current advances and challenges in the design of biodegradable plasticizers were introduced, and the requirements to develop environmentally benign plasticizers were briefly discussed. The review (Chapter 2) concludes that the greatest challenge in green plasticizer development will likely lie in minimizing the toxicity and environmental persistence of plasticizers and their metabolites. Moreover, health and environmental impact assessments should not be limited to the parent plasticizer, but should also be extended to potential metabolites that will likely arise from their biotransformation in the environment.

Consistent with the above conclusion about the potential impact of the persistent metabolites on the environment and based on the previously reported formation of toxic and persistent metabolites from incomplete microbial

hydrolysis of dibenzoate plasticizers, an alternative plasticizer, 1,6 hexanediol dibenzoate was synthesized. Biodegradation of 1,6-hexanediol dibenzoate by a common soil microorganism, *Rhodococcus rhodochrous,* in the presence of hexadecane as a primary carbon source, was investigated. The metabolites produced from the biodegradation of this potential plasticizer were not persistent but were completely degraded over the course of the experiment. These metabolites, identified using GC/MS and FTMS techniques were as follows: 1-hexadecyl benzoate, 6-benzoyloxyhexanoic acid, 4-benzoyloxybutanoic acid, 1,6-hexanediol monobenzoate and benzoic acid. It was proposed that the formation of 1-hexadecyl benzoate resulted from an enzymatic esterification reaction between the benzoate hydrolyzed from 1,6-hexanediol dibenzoate and hexadecanol, a metabolite of hexadecane. The presence of these metabolites was confirmed by repeating the biodegradation with 1,6-hexanediol di[^{2}H$_5$]benzoate and characterization of the metabolites that contained deuterium on the aromatic ring. This study was presented in Chapter 3.

The research was followed by biodegradation studies of the commercial dibenzoate plasticizers, diethylene glycol dibenzoate (D(EG)DB) and dipropylene glycol dibenzoate (D(PG)DB), as well as 1,6-hexanediol dibenzoate by *Rhodococcus rhodochrous*. Biodegradation was done in the presence of either [^{2}H$_{30}$]tetradecane or hexadecane as co-substrates. Biodegradation of 1,6-hexanediol dibenzoate in the presence of [^{2}H$_{30}$]tetradecane resulted in the formation of 1-[^{2}H$_{29}$]tetradecyl benzoate, which confirmed the hypothesis of enzymatic conjugation, proposed in the previous chapter. The aliphatic chain of all the other metabolites (i.e., 6-benzoyloxyhexanoic acid and 4-benzoyloxybutanoic acid) did not contain any deuturium, when [^{2}H$_{30}$]tetradecane was used as a co-substrate indicating that these metabolites originated from the biodegradation of 1,6-hexanediol dibenzoate as opposed to β-oxidation of [^{2}H$_{30}$]tetradecane. Characterization of the metabolites permitted the determination of the biodegradation pathway of 1,6-hexanediol dibenzoate and the metabolism was found to be characteristic of hydrolytic, oxidative and β-oxidative processes. None of the metabolites were persistent as they degraded over the course of the

experiment. On the other hand, biodegradation of D(EG)DB and D(PG)DB, led to the accumulation of the monobenzoates, D(EG)MB and D(PG)MB, respectively, as a result of slow hydrolysis. These metabolites were eventually degraded. Biodegradation mechanisms that were elucidated for these compounds show that the diol fragment of 1,6-hexanediol monobenzoate was processed by a β-oxidation pathway, which was not possible for D(EG)MB and D(PG)MB due to the presence of an ether function in the diols. These results were presented in Chapter 4.

The biodegradation kinetics of commercial dibenzoate plasticizers, D(EG)DB, D(PG)DB and alternative plasticizers, 1,6-hexanediol dibenzoate, 1,3-propanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate were studied to determine the biodegradation rates of the above compounds and the related metabolites (see Chapter 5). The system used consisted of resting cells of *Rhodococcus rhodochrous* that had previously been grown on the primary carbon source of hexadecane. Biodegradation of plasticizers and their associated metabolites were modeled using a Michaelis-Menten/Monod-type kinetic model. Experiments were performed over a range of initial concentrations of plasticizers and the rate constants were calibrated by fitting the concentration versus time data to the respective models. Biodegradation rates determined from the kinetic models allowed the comparison of the biodegradability of commercial and alternative plasticizers. It was concluded that removing the ether bond significantly enhances the biodegradation of the monobenzoate metabolites and the effect of alkyl branches on biodegradation of the monobenzoates is not as important as the ether bond.

Toxicities of selected dibenzoate plasticizers and related metabolites were tested using the Microtox^{TM} toxicity assay (see Chapter 6). The toxicities of these same compounds to algae and daphnia were predicted using the ECOSAR software of the United States Environmental Protection Agency. Commercial dibenzoates, D(EG)DB and D(PG)DB exhibited significant acute toxicity in Microtox^{TM} assay. However, the alternative plasticizers 1,6-hexanediol dibenzoate and 2,2-methyl-propyl-1,3-propanediol dibenzoate did not exhibit

toxicity. Monobenzoate metabolites, produced by incomplete hydrolysis of the parent commercial and alternative plasticizers, also exhibited high acute toxicities. The high acute toxicities of monobenzoates imply that enhancing the biodegradation rate of these compounds by removing the ether bond from the chemical structure of dibenzoate plasticizers is of great importance in order to develop safer and more environmentally benign plasticizers.

In the approach undertaken in this research, the biodegradation mechanisms were established for commercial and alternative plasticizers, which not only led to the identification of the full spectrum of the metabolites for further biodegradability and toxicity assessment but also helped to identify the functional groups that influence the biodegradation mechanisms. Collectively, this approach establishes a strategy to alter the chemical structures of conventional plasticizers towards the development of greener analogues with lower toxicities and health impacts and reduced persistence in the environment. Overall, these results point to the importance of investigating the biodegradability, toxicity and health impacts of not only the parent compounds but also the metabolites produced during biodegradation. Such an approach is advisable not only for plasticizers but for any chemical product that would be in widespread use with significant potential for release into the environment or exposure to organisms.

8. Statement of Original Contributions to Knowledge

A significant original contribution of this research has been the demonstration of the effect of the ether function, present in the chemical structure of commercial dibenzoate plasticizers, on the mechanisms and rates of biodegradation of the parent plasticizers and their associated metabolites. The discovery that this ether function was responsible for the persistence of the metabolites of dibenzoate plasticizers was the basis for another important contribution; namely, the development of environmentally benign versions of these compounds that do not lead to the formation of persistent metabolites.

Specifically, the following original contributions were made over the course of this project:

(1) The effect of ether function and alkyl branches on the biodegradability of dibenzoate plasticizers were explored and new dibenzoate plasticizers that lack the ether bond on the aliphatic moiety were synthesized including: 1,6-hexanediol dibenzoate, 1,3-propanediol dibenzoate, 2,2-methyl-propyl-1,3-propanediol dibenzoate.

(2) Metabolites created during the biodegradation of 1,6-hexanediol dibenzoate were fully characterized using GC/MS and Fourier transform mass spectroscopy (FTMS) techniques in order to elucidate a mechanism for the environmental biotransformation of this new plasticizer. Biodegradation experiments were conducted using a common soil microorganism, *Rhodococcus rhodochrous*, in the presence of hexadecane as a co-substrate. The proposed mechanism was confirmed by repeating the experiments with 1,6-hexanediol di[^{2}H$_5$]benzoate, which resulted in the detection of deuterium labeled metabolites.

(3) A key outcome of the biodegradation studies was the demonstration that biodegradation of 1,6-hexanediol dibenzoate by *R. rhodochrous* did not

lead to the accumulation of persistent metabolites. These findings support the application of 1,6-hexanediol dibenzoate as a green plasticizer.

(4) It was shown that the formation of 1-hexadecyl benzoate, a metabolite identified in the biodegradation of 1,6-hexanediol dibenzoate in the presence of hexadecane, resulted from an enzymatic esterification reaction between the benzoate hydrolyzed from 1,6-hexanediol dibenzoate and hexadecanol, a metabolite of hexadecane. This was confirmed by repetition of the biodegradation experiments of 1,6-hexanediol dibenzoate using $[^2H_{30}]$tetradecane as a co-substrate leading to the formation of 1-$[^2H_{29}]$tetradecyl benzoate as a metabolite.

(5) It was confirmed that the first step in the metabolism of 1,6-hexanediol dibenzoate was hydrolysis, resulting in the release of 1,6 hexanediol monobenzoate. It was also shown that all of the other metabolites such as 6-(benzoyloxy)hexanoic acid, 4-(benzoyloxy)butanoic acid, 6-(benzoyloxy)-3-hydroxy hexanoic acid and 4-(benzoyloxy)-3-hydroxybutanoic that were detected in the biodegradation study, originated from biodegradation of 1,6-hexanediol monobenzoate, not from progressive β-oxidation of 1-hexadecyl benzoate. This was done by following the biodegradation of 1,6-hexanediol dibenzoate in the presence of $[^2H_{30}]$tetradecane as a co-substrate, which resulted in non-deuterated versions of the above metabolites.

(6) Biodegradation mechanisms were elucidated for two commercial plasticizers, D(EG)DB and D(PG)DB, and the proposed alternative plasticizer, 1,6-hexanediol dibenzoate, by conducting biodegradation studies using a pure microbial culture of *R. rhodochrous* with hexadecane as a co-substrate. Characterization of the metabolites generated during the biodegradation process illustrated that biodegradation of all of these compounds was initiated by hydrolysis of the first ester bond and the diol fragment of 1,6-hexanediol monobenzoate. The diol fragment was converted by a β-oxidation

pathway, which was not possible for the monoesters, D(EG)MB and D(PG)MB, due to the presence of an ether function in the diols. It was shown that the only biodegradation pathway available for the monobenzoate metabolites was slow hydrolysis of the second ester bond in these two metabolites. Therefore, the slow degradation of D(EG)MB and D(PG)MB was attributed to the presence of ether functions in their aliphatic structures.

(7)   It was demonstrated that the presence of a readily-biodegradable substrate such as hexadecane can greatly influence the biodegradation mechanism of the metabolites of dibenzoate plasticizers. The biodegradation of commercial and alternative plasticizers by resting cells of *R. rhodocrous*, which had previously been grown on hexadecane, was also initiated by hydrolysis of the first ester bond to release the corresponding monobenzoate. Biodegradation of D(PG)MB proceeded by slow hydrolysis of the second ester bond. However D(EG)MB, released from hydrolysis of D(EG)DB, was not hydrolyzed and was completely oxidized to 2-[2-(benzoyloxy)ethoxy]acetic acid.

(8)   Kinetic models that were developed to describe the biodegradation rates of selected commercial and alternative dibenzoates were able to simultaneously describe the patterns of dibenzoate degradation, metabolite formation and degradation, and also increase in biomass. The dibenzoates that were studied included two commercial plasticizers, D(EG)DB, D(PG)DB and three alternative plasticizers, 1,3-propanediol dibenzoate, 2,2-methyl-propyl-1,3-propanediol dibenzoate, and 1,6-hexanediol dibenzoate. The rate constants were calibrated by fitting the experimental data, measured in studies conducted with a range of initial concentrations of plasticizers, with the appropriate model.

(9)   Biodegradation rates, determined from the kinetic models showed that removing the ether bond significantly enhanced the biodegradation rates of the monobenzoates. The effect of alkyl branches on biodegradation rates of the monobenzoates was less important.

(10) Preliminary toxicity assessments showed that the commercial plasticizers, D(PG)DB and D(EG)DB, exhibited significant toxicity in the Microtox assay. Monobenzoates of both commercial and alternative plasticizers also showed significant acute toxicity. These findings highlight the importance of enhancing the biodegradation rates of the monoester metabolites by removing the ether bond.

This research was conducted in response to concerns associated with the health and environmental consequences of commercial plasticizers. Accordingly, the long term goal is the design of alternative plasticizers with the desired safety and environmental profiles to replace conventional plasticizers. This study has developed an understanding of the relationship between the chemical structure and biodegradability for dibenzoate plasticizers, which provides an important basis for developing alternative green plasticizers.

9. References

Albro, P. W., 1975. The metabolism of 2-ethylhexanol in rats. *Xenobiotica*. 15, 625-636.

Alexander, M., 1973. Biotechnology report: Nonbiodegradable and other recalcitrant recalcitrant molecules. *Biotechnology and Bioengineering*.15, 611-647

Alvarez-Cohen, L., McCarty, P.L., 1991. Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene transformation by methanotrophic resting cells. *Applied and environmental microbiology*. 57, 1031-1037.

American Chemistry Council (ACC), 2009. ACC ansewres phthalate questions, http://www.americanchemistry.com/s_phthalate/sec.asp?CID=1907&DID= 10417

Amir, S., Hafidi, M., Merlina, G., Hamdi, H., Jouraiphy, A., Gharous, M.EI, Revel, J.C., 2005. Fate of phthalic acid esters during composting of both lagooning and activated sludges. *Process Biochemistry*. 40, 2183-2190.

Anastas, P. T., Warner, J.C., 2000. *Green Chemistry Theory and Practices*. Oxford University Press Inc., New York.

Andrews, J. F., 1968. A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrate. *Biotechnology and Bioengineering*. 10, 707-723

Anonymous, 2009a. EPA targets half-dozen substances for action plans. *Chemical Engineering News (C&EN)*, 87, October 19, 28- 29.

Anonymous, 2009b. Safety data sheet of POLYSORB® ID 37, http://www.specialchem4polymers.com/tc/dei-plasticizer/download-brochure.aspx

Anonymous, 2010. Biodegradable plasticizers. Battelle enterprise. Available at http://www.battelle.org/ASSETS/E0733573EAE34395B1148F6C2E69FE9C/Bio degradable%20Plasticizers.pdf.

Arendt, W.D., Lang, J., 1998. New benzoate plasticizers for polyvinyl chloride: Introduction and performance example. *Journal of Vinyl & Additive Technology*. 4, 184-188.

Arendt, W. D., Beitsch, H.O. 1994. Trimethylpentanediol dibenzoate: New plasticizer for plastisol screen ink. *Journal of Vinyl Additive Technology*. 16, 52-56.

Audic, J. L., Reyx, D., Brosse, J.C. 2003. Migration of additives from food grade poly(vinyl chloride) films: effect of plasticization by polymeric modifiers instead of conventional plasticizers. *Journal of Applied Polymer Science*. 89, 1291-1299.

Atlas, E., Giam, C.S. 1981. Global transport of organic pollutants: ambient concentrations in remote marine atmosphere. *Science*. 211, 163-165.

Audic, J. L., Epaillard, F.P., Reyx, D., Brosse, J.C. 2001. Cold plasma surface Modification of conventionally and nonconventionally plasticized poly(vinyl chloride)-based flexible films: global and specific migration of additives into isooctane. *Journal of Applied polymer Science*. 79, 1284.

Bailey, J.E., Ollis, D.F., 1977. *Biocehmical Engineering Fundamentals*, *2nd ed*. McGraw-Hill Inc., New York.

BASF Press Release, 2006. Hexamoll® DINCH – the success story continues. http://www.basf.com/group/pressrelease/P-06-436.

Barron, M. G., Albro, P.W., Hayton, W.L. 1995. Biotransformation of di(2-ethylhexyl)phthalate by rainbow trout. *Environmental Toxicology Chemistry*. 14, 873-876.

Barnabé, S., Beauchesne, I., Cooper, D. G., Nicell, J.A., 2008. Plasticizers and their degradation products in the process streams of a large urban physicochemical sewage treatment plant. *Water research*. 42, 153-162.

Bauer, M.J., Herrmann, R. 1997. Estimation of the environmental contamination by phthalic acid esters leaching from household wastes. *Science of the Total Environment*. 208, 49-57.

Beauchesne, I., Barnabé, S., Cooper, D.G., Nicell., J. A., 2008. Plasticizers and related toxic degradation products in wastewater sludges. *Water science and technology*. 57, 367-374.

Britton, L.N., 1984. Microbial degradation of aliphatic hydrocarbons. In *Microbial degradation of organic compounds*, Gibson, D.T., (ed.), Marcel Dekker Inc., New York, 89–129.

Cadogan, D.F., Papez, M., Poppe, A.C., Scheubel, J. 1993. An assessment of the release, occurrence and possible effect of plasticizers in the environment. *Progress in Rubber and Plastics Technology*. 10, 1-19.

Carlberg, G. E., Martinsen, K., 1982. Adsorption/complexation of organic micropollutants to aquatic humus. *Science of Total Environment*. 25, 245-254.

Cartwright, C.D., Owen, S.A., Thompson, I.P., Burns, R.G. 2000. Biodegradation of diethyl phthalate in soil by a novel pathway. *FEMS microbiology letters*. 186, 27-34.

Chang, H.L., Alvarez-Cohen, L., 1995. Model for the cometabolic biodegradation of chlorinated organics. *Environmental Science and Technology*. 29, 2357-2367.

Chemsystem, 2008. Development in non-phthalate plasticizers. PERP07/08S4. http://www.chemsystems.com/about/cs/news/items/PERP%200708S4_Plasticizer s.cfm

Cornu, M.C., Lhuguenot, J.C., Brady, A.M., Moore R., Elcombe, M.C., 1992. Identification of the proximate peroxisome proliferator(s) derived from di(2-ethylhexyl) adipates and species differences in response. *Biochemical Pharmacology*. 43, 2129-2134.

Criddle, C.S., 1992. The kinetics of cometabolism. *Biotechnology and Bioengineering*. 41, 1048-1056.

Deligio, T., 2009. Phthalate Alternative Recognized By ECHA. http://www. plasticstoday.com/articles/phthalate-alternative-recognized-echa.

Di Gangi, J. 1999. Phthalates in vinyl medical products, Washington DC: Greenpeace USA. http://archive.greenpeace.org/toxics/reports/medprods.pdf.

Dirven, H. A. A. M., Van den broek, P.H.H., Jongeneelen, F.J. 1993. Determination of four metabolites of the plasticizer di(2-ethylhexyl)phthalate in human urine samples. *Archives of Occupational and Environmental Health*. 64, 555-560.

EFSA (European Food Safety Authority), 2006. Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request related to a 12th list of substances for food contact materials. *The EFSA Journal*. 395-401, 1-21, http://www.plasticizers.basf.com/icms/stream er?fid=214733.

Ejlertsson, J., Alnervik, M., Jonsson, S., Svensson, B., 1997. Influence of water solubility, side chain degradability, and side chain structure on the degradation of phthalic acid estesr under methanogenic conditions. *Enviornmental Science and technology*, 31, 2761-2764.

Ellis, L. B. M., Roe, D., Wackett, L.P. 2006. The University of Minnesota Biocatalysis /Biodegradation Database: the first decade. *Nucleic Acids Research*. 34, 517-521.

Engelhardt, G., Tillmanns, G., Wallnofer, P.R., Hutzinger, O., 1977. Biodegradation of di-iso-butyl phthalate and related dialkyl phthalates by *Penicillium lilacinum*. *Chemosphere*, 6,347-354.

European Union, 2005. Directive 2005/84/EC of the European Parliament and of the Council of of 14 December 2005. *Offical Journal of the European Union*. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:344:0040:004 3:en:PDF

Fedorko, P., Djurado, D. Trznadel, M., Dufour, B., Rannou, P., Travers, J.P., 2003. Insulator-metal transition in polyaniline induced by plasticizers. *Synthetic Metals*. 135-136, 327-138.

Firlotte, N., Cooper, D.G., Maric,M., Nicell, J.A. 2009. Characterization of 1,5-pentanediol dibenzoate as a potential "green" plasticizer for poly(vinyl chloride. *Journal of vinyl & additive technology*. 15; 99-107.

Gachter, R., Muller, H., 1990. Plastics additives handbook. 3rd edition. Gachter, R., Muller, H., (ed.). Carl Hanser, New York.

Gartshore J., Cooper D.G., Nicell J.A., 2003. Biodegradation of plasticizers by *Rhodotorula Rubra*. *Environmental Toxicology and Chemistry*. 22, 1244-1251.

Germain, A., Langlois, C., 1988. Contamination des eaus et des sédiments du fleuve Saint-Laurent par les pesticides organochlorés, les biphényles polychlorés et d'autres Contaminants organiques prioritaires. *Water Pollution Research Journal of Canada*. 23, 602-614.

Ghioureliotis, M., 1997. Assessment of residual by-products from the enzyme-catalyzed polymerization of aqueous phenolic compounds. Department of Civil Engineering and Applied Mechanics. Montreal, McGill University.

Giam C.S., Atlas E., Powers Jr M.A., Leonard J.E., 1984. Phthalic acid esters. In: Hutzinger, O. (Ed.), *The Handbook of Environmental Chemistry: Anthropogenic Substances*. Springer-Verlag, Berlin, 3, 67-142.

Gibson D.T., 1968. Microbial degradation of aromatic compounds. *Science*, 161, 1093–1097.

Gibbons, J. A., Alexander, M., 1989. Microbial degradation of sparingly soluble organic chemicals: phthalate esters. *Environmental Toxicology and Chemistry*, 8, 283-291.

Gledhill, W.E., Kaley, R.G., Adams, W.J., Hicks, O., Michael, P.R., Saeger, V.W., LeBlanc, G.A., 1980. An environmental safety assessment of butyl benzyl phthalate. *Environmental Science and Technology*. 14, 301-305.

Hetzel, S. J. 1956. *Esters of pentanediols and their use as plasticizers for vinyl-type polymers*. U.S. patent 2, 757, 157.

Hakkarainen, M., 2003. New PVC materials for medical applications the release profile of PVC/polycaprolactone-polycarbonate aged. *Polymer Degradation and Stability*. 80, 451-458.

Health Canada, 2002. Health Canada expert advisory panel on DEHP in medical devices.

Hileman, B., 2001. Panel concludes phthalate is safe. *Chemical Engineering News* (*C&EN*). 79, 12.

Hileman, B., 2002. FDA suggest replacing DEHP in plastics. *Chemical Engineering News* (*C&EN*). 80, 6.

Hileman, B., 2007. California bans phthalates in toys for children. *Chemical Engineering News* (*C&EN*). 22, 12.

Horn O., Nalli S., Cooper D.G., Nicell J.A., 2004. Plasticizer metabolites in the environment. *Water Research*. 38, 3693-3698.

Intertek labtest sparkle bullitin, 2005. EU Phthalates Directive 2005/84/EC. Available at http://www.intertek-labtest.com/bulletins/359989.

Jaeger, R. J., Rubin, R.J., 1970. Plasticizers from plastic devices: extraction, metabolism and accumulation by biological systems. *Science*. 170, 460.

Johnson, B.T., Heitkamp, M.A., Jones, J.R., 1984. Environmental and chemical factors influencing the biodegrdation of phthalic acid esters in freshwater. *Environmental pollution*. 8, 101-118.

Johnson, W., 2002. Final report on the safety assessment of acetyl triethyl citrate, acetyl tributyl citrate, acetyl trihexyl citrate, and acetyl trioctyl citrate. *International Journal of Toxicology*. 21(5), 1-17.

Kaiser, K. L. E., McKinnon, M.B., Fort, F.L., 1994. Interspecies toxicity correlations of rat, mouse and Photobacterium phosphoreum. *Environmental Toxicology Chemistry*. 13, 1599-1606.

Keith, L.H., Telliard, W.A., 1979. Special report: Priority pollutants − I. A perspective review. *Environmental Science and Technology*. 13, 416-423.

Keith Y., Cornu M.C., Canning P.M., Foster J., Lhuguenot J.C., Elcombe, C.R., 1999. Peroxisome proliferation due to di(2-ethylhexyl) adipate, 2-ethylhexanol and 2-ethylhexanoic acid. *Archives of Toxicology*. 66, 321-326.

Kermanshahi pour, A., Cooper, D.G., Mamer, O.A., Maric, M., Nicell, J.A., 2009a. Mechanism of biodegradation of dibenzoate plasticizers. *Chemosphere*. 77, 258-263.

Kermanshahi pour, A., Mamer, O.A., Cooper, D.G., Maric, M., Nicell, J.A., 2009b. Metabolites from the biodegradation of 1,6-hexanediol dibenzoate, a potential green plasticizer, by *Rhodococcus rhodochrous*. *Journal of Mass Spectrometry*. 44, 662-671.

Kirchmann, H. A. T., A., 1991. Organic pollutants in sewage sludge. *Swedish journal of agricultural research*. 21, 115-119.

Klopman, G., Tu, M. 1997. Structure-biodegradability study and computer-automated prediction of aerobic biodegradation of chemicals. *Environmental Toxicoloy and Chemistry*. 16,1829-1835.

Krishnan, V. K., Jayakrishnan, A., Francis, J.D., 1991. Radiation grafting of hydrophilic monomers on to plasticized poly(vinyl chloride) sheets: II. migration behaviour of the plasticizer from *N*-vinyl pyrrolidone grafted sheets. *Biomaterials*. 12, 489-492.

Lakshmi, S., Jayakrishnan, A., 1998. Migration resistant, blood-compatible plasticized Polyvinyl chloride for medical and related application. *Artif Organs*. 22. 222-229.

Lang, J., Stanhope, B.E., 2001. Benzoate plasticizer for flexible PVC injection molded toy applications. *Plastics Additives and Compounding*. 3, 30-33.

Lang, S., Philp, J.C., 1998. Surface-active lipids in rhodococci. *Antonie van Leeuwenhoek*. 74: 59-70.

Larsen, S. T. , 2004. Health and safety issues with plasticizers and plasticized materials. In *The Handbook of Plasticizers*. Wypych, G. (ed). ChemTec Laboratories Inc., Toronto, 536-586

Leahy, J.G., Colwell, R.R., 1999. Microbial degradation of hydrocarbons in the environment. *Microbial molecular Biology Reviews*. 54, 305-315.

Lerner, I., 2003. Plasticizers to reach $8 billion by 2004. Look Smart, Ltd; http://www.findarticles.com/cf_dls/m0FVP/9_258/65462810/p1/article.jhtml

Loftus, N. J., Laird, W.J. Steel, G.T., Wilks, M.F., Woollen, B.H., 1993. Metabolism and pharmacokinetics of deuterium-labelled di-2-(ethylhexyl) adipate (DEHA) in humans. *Food and Chemical Toxicology* 31, 609-614.

Mochida, K., Gomyoda, M., Fujita, T., 1996. Acetyl tributyl citrate and dibutyl sebacate inhibit the growth of cultured mammalian cells. *Bulletin of Environmental Contamination and Toxicology*. 56, 635-637.

Mitchell, A.M., Lhuguenot, J.C., Bridges, J.W., Elcombe, C.R., 1985. Identification of the peroximate peroxisome proliferator(s) derived from di(2-ethylhexyl) phthalate. *Toxicology and Applied Pharmacology*. 80, 23-32.

Murphy, J., 2001. *Additives for plastics handbook, 2nd ed*. Elsevier, New York.

Nalli S., Cooper D.G., Nicell J.A., 2002. Biodegradation of plasticizers by *Rhodococcus rhodochrous*. *Biodegradation*. 13, 343-352.

Nalli, S., Horn, O.J., Grochowalski, A.R., Cooper, D.G., Nicell, J.A. 2006a. Origin of 2-ethylhexanol as a VOC. *Environmental pollution*, 140, 181-185.

Nalli A., Cooper D.G., Nicell J.A., 2006b. Metabolites from the biodegradation of di-ester plasticizers by *Rhodococcus rhodochrous*. *Science of the Total Environment*. 366, 286-294.

Nalli A., Cooper D.G., Nicell J.A., 2006c. Interaction of metabolites with *R. rhodochrous* during the biodegradation of di-ester plasticizers. *Chemosphere*. 65, 1510-1517.

Ng, L.K., Hupé M., 1993. Simple gas chromatographic method for the assay of salts of carboxylic acids as their trimethylsilyl derivatives. *Journal of Chromatography*. 637, 104-108.

Oie, L., Hersoug, L., Madsen, J. 1997. Residential exposure to plasticizers and its possible role in the pathogenesis of asthma. *Environmental health pespectives*. 105, 964-971.

Onorato T.M., Brown P.W., Morris P., 2008. Mono-(2-ethylhexyl)phthalate increase spermatocyte mitochondrial peroxiredoxin 3 and cyclooxygenase 2. *Journal of Andrology*. 29, 293-303

Otton, S. V., Sura, S., Blair, J., Ikonomou, M.G., Gobas, F.A.P.C., 2008. Biodegradation of mono-alkyl phthalate esters in natural sediments. *Chemosphere*. 71, 2011-2016.

Peijnenburg W.J.G.M., 1994. Structure-activity relationship for biodegradation: A critical review. *Pure and Applied Chemistry*. 66, 1931-1941.

Pollack, G., Buchanan, J., Slaughter, R., Kohll, R., Shen, D. 1985. Circulating concentration of di(2-ethylhexyl) phthalate and its deesterified phthalic acid products following plasticizer exposure in patients receiving hemodialysis. *Toxicology Applied Pharmacology*. 79, 257-267.

Peng X., Zhang X., 2000. Application of a new plasticizer—Diethylene glycol dibenzoate (DEDB) in poly (vinyl chloride) products. *Zhongguo Suliao*. 14, 64-67.

Rahman, M., Brazel, C.S., 2004. The plasticizer market: An assessment of traditional plasticizers and research trends to meet new challenges. *Progress in Polymer Science*. 29, 1223-1248.

Raymond J.W., Rogers T.N., Shonnard D.R., Kline A.A., 2001. A review of structure-based biodegradation estimation methods. *Journal of Hazardous Material*. 84, 189-215.

Roquette Co. First pilot production of isosorbide diesters POLYSORB ID[®]. http://www.roquette.com/delia-CMS/t1/article_id-4979/topic_id-1291/roquette-first-pilot-production-of-isosorbide-diesters-polysorb-id-r-.html.

Roslev, P., Madsen, P.L., Thyme, J.B., Henriksen, K., 1998. Degradation of phthalate and di-(2-ethylhexyl)phthalate by indigenous and inoculated microorganisms in sludge-amended soil. *Applied and Environmental Microbiology*. 64, 4711-4719.

Roy W.R., 2004. The environmental fate of plasticizer. In: *The Handbook of Plasticizers,* Wypych, G., (ed.), *ChemTec Laboratories Inc*., Toronto, 591-613.

Sathyanarayana, S., Karr, C.J., Lozano, P., Brown, E., Calafat, A.M., Liu, F., Swan, S.H., 2008. Baby care products: Possible sources of infant phthalate exposure. *Pediatrics*. 121, 260-268.

Schaeffer, T. L., Cantwell, S.G., Brown, J.L., Watt, D.S., Fall, R.R. 1979. Microbial growth on hydrocarbons: Terminal branching inhibits biodegradation. *Applied and Environmental Microbiology*. 38, 742-746.

Scholz, N., Diefenbach, R., Rademacher, I., Linnemann, D., 1997. Biodegradation of DEHP, DBP, and DINP: poorly water soluble and widely used phthalate plasticizers. *Bulletin of Environmental Contamination and Toxicology*. 58, 527-534.

Sears, J.K., Darby, J.R., 1982. The Technology of Plasticizers. John Wiley & Sons Inc, New York.

Shea, P. J., Weber, J.B., Overcash, M.R. 1982. Uptake and phytotoxicity of di-n-butyl phthalate in corn *zea mays*. *Bulletin of Environmental Contamination Toxicology*. 24. 153-158

Stevens, M., 1999. *Polymer Chemistry: An Introduction*, 3rd ed. Oxford University Press, New York.

Sun, X. S., 2004. Biodegradable plastics from wheat starch and polylactic acid (PLA), Grain. *Science and Industry*. http://www.mindfully.org/ Plastic/Biodegra dable-Wheat-Starch-PLA.htm.

Staples, C.A., Peterson, D.R., Parkerton, T.F., Adams, W.J., 1997. The environmental fate of phthalate esters: A literature review. *Chemosphere*. 35, 667-749.

Thomas, J. M., Yordy, J.R., Amador, J.A., Alexander, M., 1986. Rates of dissolution and biodegrdation of water-insoluble organic compounds. *Applied and Environmental Microbiology*.52, 290-296.

Tickner, J.A., Rossi, M., Haiama, N., Lappe, M., Hunt, P. 1999. The use of di(2-diethylhexyl) phthalate in PVC medical devices: exposure toxicity and alternative Center for Sustainable Production, Lowell, MA. http://www.sustainableproductio n.org/downloads/DEHP%20Full%20Text.pdf

Tickner, J. A., Schettler, T., Guidotti, T., McCally, M., Rossi, M., 2001. Health risks posed by use of di-2-ethylhexyl phthalate (DEHP) in PVC medical devices: a critical review. *American Journal of Industrial Medicine*. 39, 100-111.

Tullo, A. H., 2006. High-performance plastics are finding increased relevance as a way to make cars less costly to manufacture − and fill up at the gas pump. *Chemical Engineering News. (C&EN)*. 84, 12-18.

TURI (Toxics use reduction institute University of Massachusetts Lowell), 2006. Five chemical alternative assessment study. Vertellus Specialties Inc. http://www.vertellus. com/Market.aspx?MarketID=1&ProductFamilyID=17.

US FDA. 1973. Evaluation of the Health Aspects of Benzoic Acid and Sodium Benzoate as Food Ingredients. DHEW, Washington, DC. Report No. SCOGS-7. NTIS PB-223837/6.

Wadey, B. L. 2003. An innovative plasticizer for sensetive application. *Journal of Vinyl and Additive Technology*. 4, 172-176.

Wahl, H.G., Hong, Q., Stube, D., Maier, M.E., Haring, H.U., Liebich, H.M., 2001. Simultaneous analysis of the di(2-ethylhexyl)phthalate metabolites 2-ethylhexanoic acid, 2-ethyl-3-hydroxyhexanoic acid and 2-ethyl-3-oxohexanoic acid in urine by gas chromatography–mass spectrometry. *Journal of Chromatography*. B 758, 213-219.

Wahl H.G., Hong Q., Hildenbrand S., Risler T., Luft D., Liebich H., 2004. 4-Heptanone is a metabolite of the plasticizer di(2-ethylhexyl) phthalate (DEHP) in haemodialysis patients. *Nephrology Dialysis Transplantation*. 19, 2576-2583.

Wams, T.J. 1987. Diethylhexylphthalate as an environmental contaminant: A review. *Science of the Total Environment*. 66, 1-16.

Wang, J., Liu, P., Shi, H., Qian, Y., 1997. Kinetics of phthalic acid ester degradation by acclimated activated sludge. *Process Biochemistry*, 32, 567-571.

Welle, F., Wolz, G., Franz, R., 2005. Migration of plasticizers from PVC tubes into enteral feeding solutions. *Pharma International*. 3, 17-21.

Watkinson, R., Morgan, P., 1990. Physiology of aliphatic hydrocarbon degrading microorganisms. *Biodegradation*. 1, 79-92.

White, G. F., Russell, N.J., Tidswell, E.C. 1996. Bacterial scission of ether bonds. *Microbiology Reviews*. 60, 216-232.

Whyte, L. G., Slagman, S.J., Pietrantonio, F., Bourbonniere,L., Koval, S.F., Lawrence, J.R., Inniss, W.E., Greer, C.W., 1999. Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus* sp. strain Q15. *Applied and Environmental Microbiology*. 65, 2961–2968.

Wolfe, N. L., Burns, L.A., Burns, W.C., 1980. Phthalate ester hydrolysis: Linear free energy relationships. *Chemosphere*. 9, 403-408.

Wypych, G., 2004. Plasticizer Types. In: *The Handbook of Plasticizers,* Wypych, G. (ed). ChemTec Laboratories Inc., Toronto, 7-10.

Zink, G., Lorber K.E., 1995. Mass spectral identification of metabolites formed by microbial degradation of polycyclic aromatic hydrocarbons (PAH). *Chemosphere*. 31, 4077-4084.

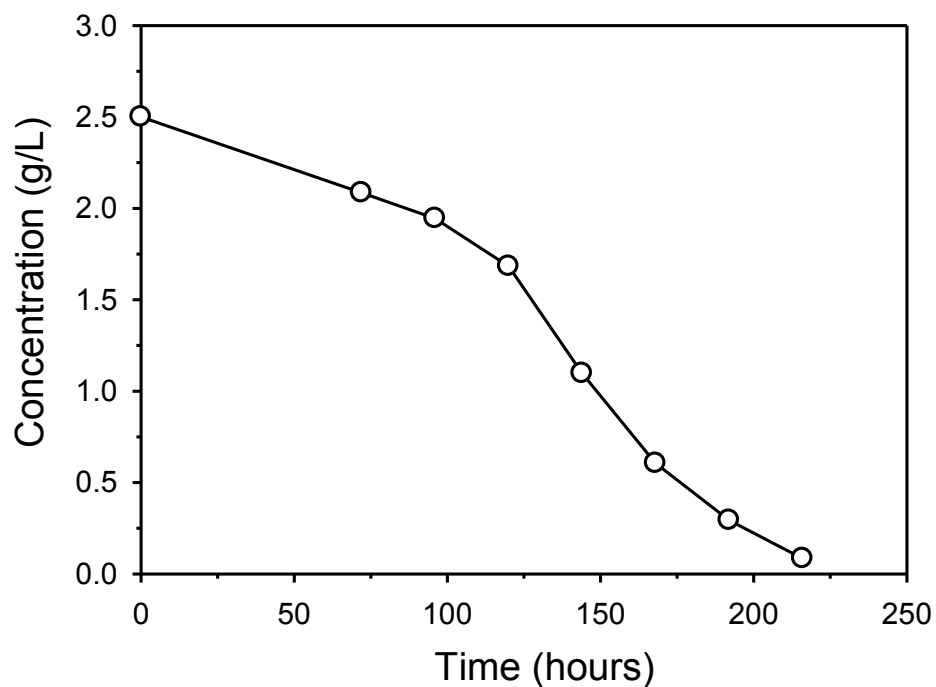# Appendix 1: Biodegradation of hexadecane

Figure A1.1. Biodegradation of hexadecane (○) as a co-substarte by *Rhodococcus rhodochrous* ATCC 13808 in the presence of 1,6-hexanediol dibenzate. The initial concentration of hexadecane was 2.5 g/L.
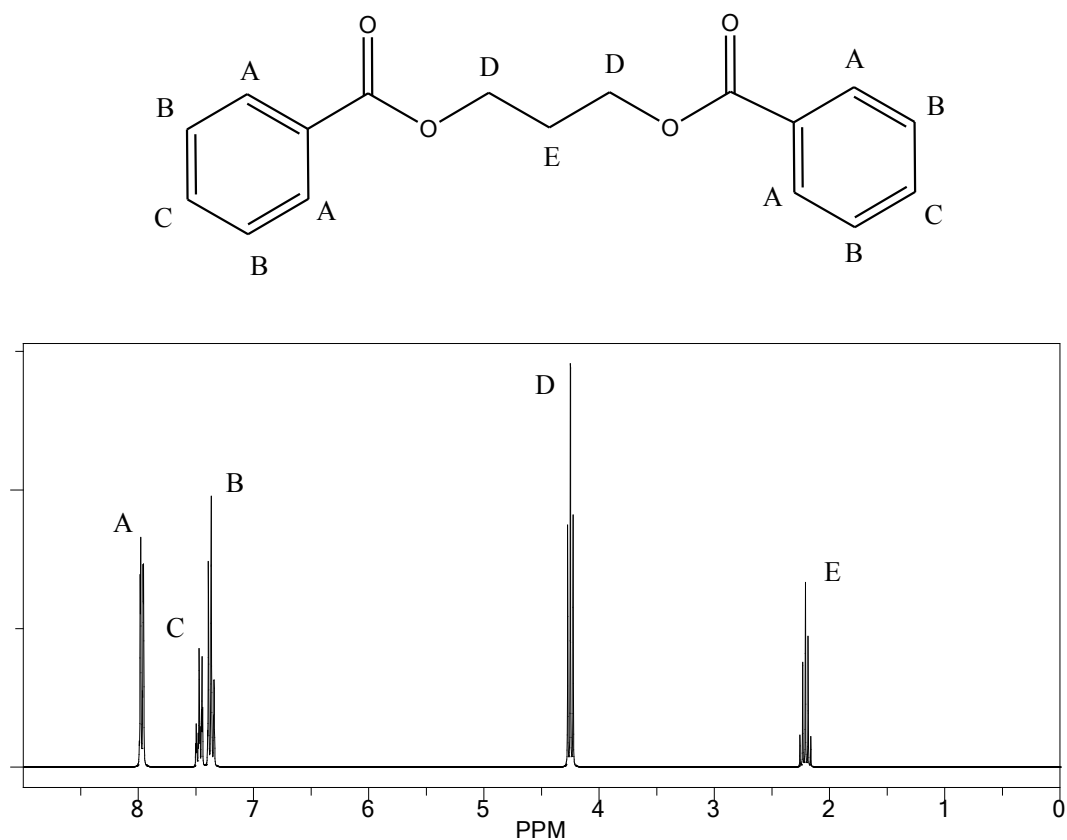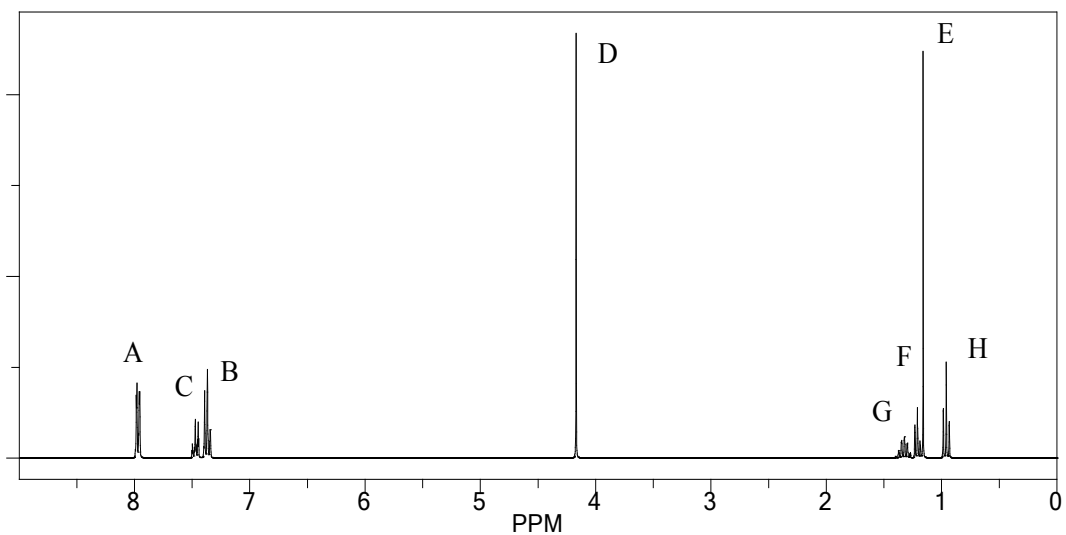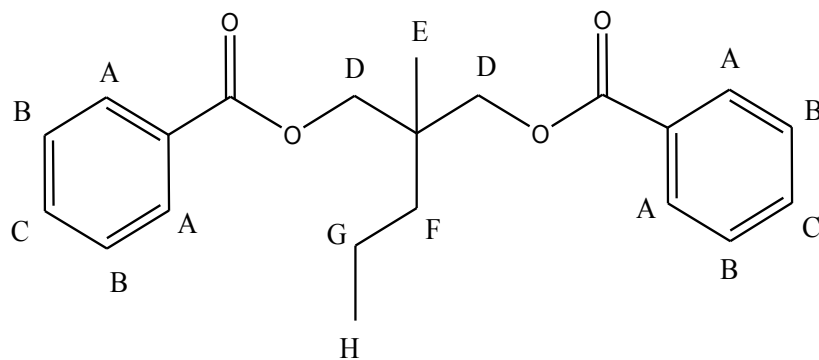
Appendix 2: NMR Spectra

Figure A2.1. NMR spectrum of synthesized 1,3-propanediol dibenzoate. Relative integration of protons on indicated carbon atom (A: B: C: D: E = 2: 2: 1: 2: 1). A, B, C exhibit the expected patterns for a phenyl group. D is triplets and E is a multiplet (assumed to be a triplet of triplets) as expected for this part of the structure.

Figure A2.2. NMR spectrum of synthesized 2,2-methyl-propyl-1,3-propanediol dibenzoate. Relative integration of protons on indicated carbon atom (A : B : C : D : E : F : G : H = 4 : 4 : 2 : 4 : 3 : 2 : 2 : 3). A, B, C exhibit the expected patterns for a phenyl group. E and D are singlet. F, G and H are multiplet.
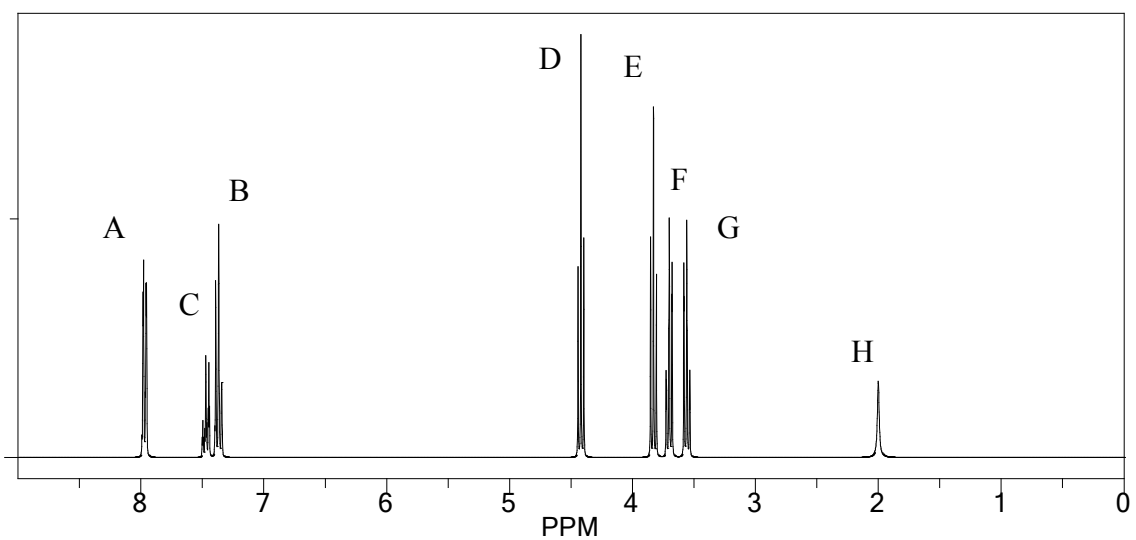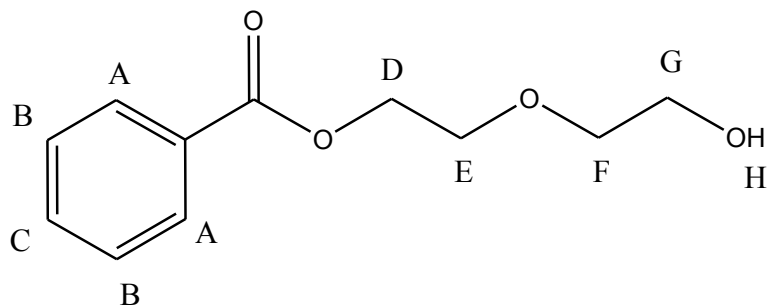
Figure A2.3. NMR spectrum of synthesized diethylene glycol monobenzoate. Relative integration of protons on indicated carbon atom (A : B : C : D : E : F : G : H : I = 2 : 2 : 1 : 2 : 2 : 2 : 2 : 1). A, B, C exhibit the expected patterns for a phenyl group. D, E, F and G are multiplet and H is singlet.
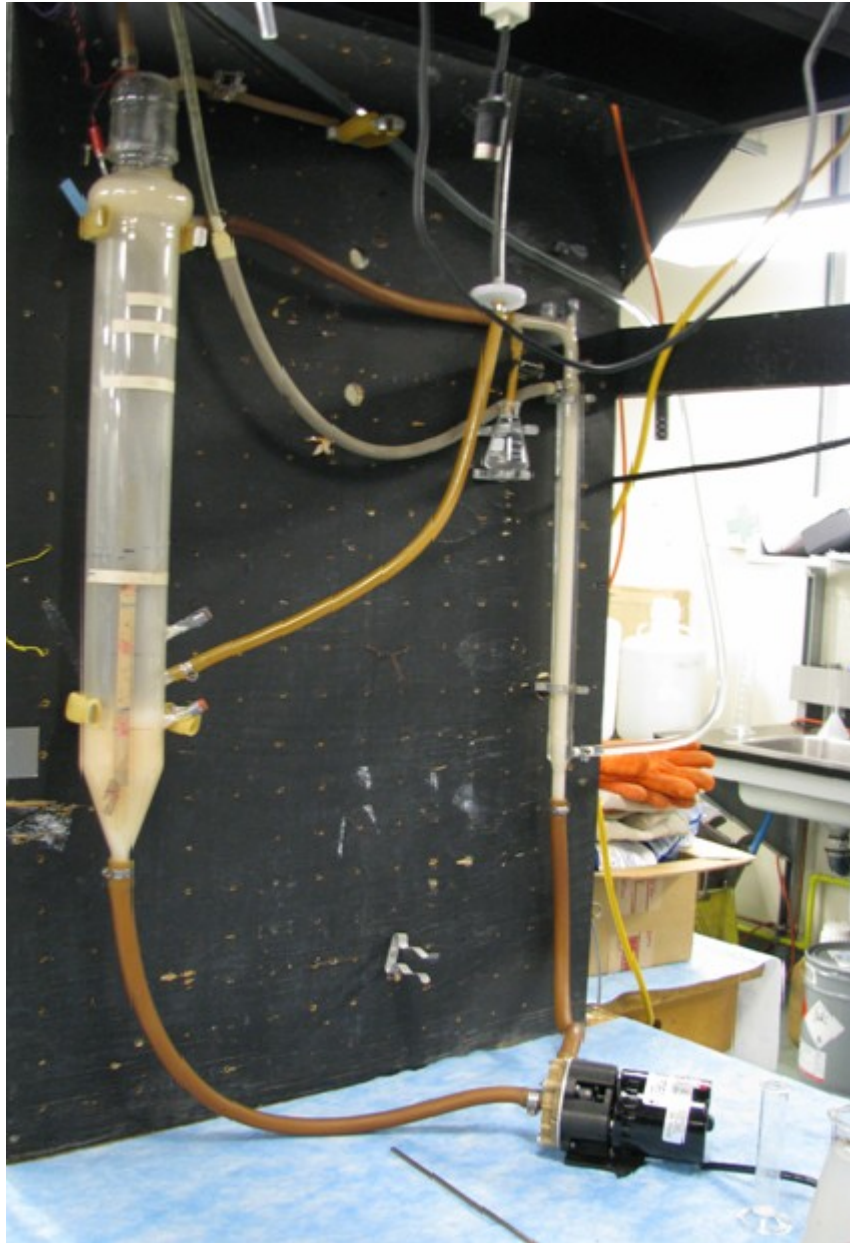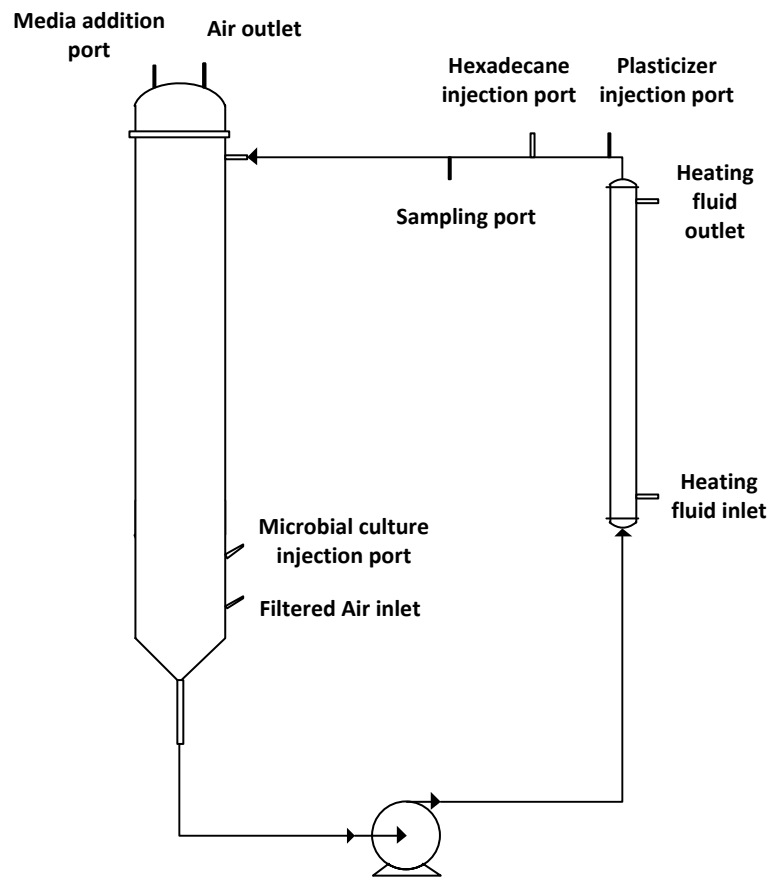
Appendix 3: Bioreactor set-up

Figure A3.1. Photograph of the bioractor

Figure A3.2. Schematic of the bioractor