DELIVERY OF HYDROPHOBIC SUBSTRATES TO DEGRADING ORGANISMS IN TWO-PHASE PARTITIONING BIOREACTORS

by

Lars Rehmann

A thesis submitted to the Department of Chemical Engineering in conformity with the requirements for the degree of Doctor of Philosophy

Queen's University

Kingston, Ontario, Canada

August, 2007

Copyright© Lars Rehmann, 2007

Abstract

This thesis examined the use of two-phase partitioning bioreactors (TPPBs) for the biodegradation of poorly water-soluble compounds. TPPBs are stirred tank bioreactors composed of a biocatalyst-containing aqueous phase and an immiscible second phase containing large amounts of poorly water-soluble or toxic substrates. Degradation of the bioavailable substrate in the aqueous phase will result in equilibrium-driven partitioning of additional substrate from the immiscible phase into the aqueous phase, theoretically allowing for complete substrate degradation.

Fundamental work was undertaken with the PCB-degrading organisms *Burkholderia xenovorans* LB400 in liquid-liquid and solid-liquid TPPBs. Initially biphenyl was used as the sole carbon source due to its hydrophobic nature and structural similarity to the environmentally relevant PCBs. The critical $LogK_{O/W}$ (octanol/water partitioning coefficient) of the organism was determined to be 5.5 and its growth kinetics on biphenyl were determined in a liquid-liquid TPPB. A polymer selection strategy for solid-liquid TPPBs was developed in the next chapter, and it was shown in the following chapter that biphenyl degradation in solid-liquid TPPBs was mass transfer limited, as described mathematically utilising the previously estimated microbial kinetics.

The fundamental knowledge gained in the early chapters was then applied to the degradation of PCBs by the same organism. It was shown that the aqueous phase availability of PCBs is the rate-limiting step in biphasic bioreactors, and not the mass transfer rate. The low specific microbial degradation rates, resulting from substrate-limited growth were addressed with increased biomass concentrations; however, it was also found that an additional carbon source was required to maintain microbial activity

over an extended period of time. Pyruvic acid was selected as a carbon source which, once added to actively PCB-degrading cells, maintained the cells' activity towards PCBs and up to 85 % of 100 mg l^{-1} was degraded in 15 h.

It was shown as the final contribution in this thesis that TPPBs can be combined with a PCB soil extraction step as a potential remediation scheme for PCB contaminated soil. PCBs were extracted from soil with polymer beads (up to 75 % removal), followed by biodegradation of the PCBs in a solid-liquid TPPB in which PCBs were delivered to the degrading organism from the same polymer.

Co-Authorship

Chapters 3, 4, 5, 6, 7 and 8 have been submitted to refereed journals and were co-authored by Dr. Andrew J. Daugulis, who provided editorial and technical advice. Mr. Bozhi Sun provided experimental assistance to portions of the work performed in Chapter 4 and is listed as a co-author.

Acknowledgments

First and foremost I express gratitude to my thesis advisor for his constant support throughout the past four years. I have to thank Andrew for accepting me as a Ph.D. student, for the many 'chats' in his office, the fruitful discussions, the editorial advice on almost everything I wrote, and generally for "being the optimistic person he is". Above all, I have to thank him for the annual north campus event in the summer and for the generous supply of firewood in the winter.

I would like to thank Paula Whitley and Allison Rutter at the Analytical Services Unit. I would have not been able to complete this thesis without constantly being able to occupy 'GC-7' on very short notice.

I have to thank the Department of Chemical Engineering, the School of Graduate Studies and Research and the Natural Science and Engineering Research Council of Canada for funding. I also have to thank the members of the Environmental Technologies Program at SAIC Canada for financial support as well as for the many fruitful discussions.

Many thanks to my families in Germany, the Netherlands and India for constant updates on weather on the other side of the Atlantic, and for the many visits that enriched my time tremendously.

The most thanks naturally goes to my wife Parveen without whom I would not have been able to complete this work. I have to thank her for being with me in Kingston, which involved postponing some of her own goals, for supporting me in every aspect of life, and for patiently reading and hearing more about PCBs and TPPBs than any 'normal' human being would ever want to.

Table of Contents

Abstract i
Co-Authorship iii
Table of Contents v
List of Tables viii
List of Figures ix
List of Figures ix
Chapter 1: Introduction 1
1.1 Biphenyl and PCBs 2
1.1.1 PCBs in the Environment and Health Risks 4
1.1.2 Biodegradation of PCBs 6
1.2 Bioavailability of Hydrophobic Compounds 7
1.3 Two-Phase Partitioning Bioreactors 8
1.3.1 Liquid-Liquid TPPBs 11
1.3.2 Solid-Liquid TPPBs 12
1.4 Envisioned Process 14
1.5 Objectives 15
Chapter 2: Biphenyl degradation kinetics by *Burkholderia xenovorans* LB400 in two-phase partitioning bioreactors 17
2.1 Preface to Chapter 2 18
2.2 Abstract 20
2.1 Introduction 21
2.2 Materials and Methods 23
2.2.1 Chemicals 23
2.2.2 Bacterial Strain 23
2.2.3 Standard Growth Medium 24
2.2.4 Inoculum Preparation 24
2.2.5 Solvent Selection 25
2.2.6 Biphenyl Analysis 25
2.2.7 Biomass Analysis 26
2.2.8 Biphenyl Solubility Tests 26
2.3 Results and Discussion 26
2.3.1 Solvent Selection 26
2.3.2 Biphenyl Degradation 30
2.4 Conclusions 36
2.5 Acknowledgements 36
Chapter 3: Polymer Selection for Biphenyl Degradation in a Solid-Liquid Two-Phase Partitioning Bioreactor 37
3.1 Preface to Chapter 3 38
3.2 Abstract 40
3.3 Introduction 41
3.4 Materials and Methods 43
3.4.1 Biocatalyst Selection 43
3.4.2 Delivery Phase Selection 44
3.4.3 Biodegradation of Biphenyl in Solid-Liquid TPPBs – Proof of Concept .. 46
3.5 Results and Discussion 47

3.5.1 Biocatalyst Selection....47
3.5.2 Delivery Phase Selection....47
3.5.3 Biodegradation of Biphenyl in Solid-Liquid TPPBs – Proof of Concept..56
3.6 Conclusions....58
Chapter 4: Biodegradation of Biphenyl in a Solid-Liquid Two-Phase Partitioning....59
4.1 Preface to Chapter 4....60
4.2 Abstract....61
4.3 Introduction....62
4.4 Theory....64
4.5 Materials and Methods....66
4.5.1 Chemicals....66
4.5.2 Bacterial strain....67
4.5.3 Biphenyl uptake....67
4.5.4 Biphenyl release....68
4.5.5 Biphenyl biodegradation....68
4.6 Results and Discussion....69
4.7 Conclusions....78
Chapter 5: Bioavailability of PCBs in Biphasic Bioreactors....80
5.1 Preface to Chapter 5....81
5.2 Abstract....82
5.3 Introduction....83
5.4 Materials and Methods....85
5.4.1 Chemicals.....85
5.4.2 Bacterial Strain.....85
5.4.3 Scintillation Vial Experiments.....85
5.4.4 Bioreactor Experiments.....87
5.4.5 PCB Analysis.....88
5.5 Results and Discussion....89
5.5.1 Scintillation Vial Experiments.....89
5.5.2 Bioreactor Experiments.....93
5.6 Conclusions....101
Chapter 6: Enhancement of PCB degradation by *Burkholderia xenovorans* LB400 in biphasic systems by manipulating culture conditions....102
6.1 Preface to Chapter 6....103
6.2 Abstract....104
6.3 Introduction....105
6.4 Materials and Methods....107
6.4.1 Chemicals....107
6.4.2 Bacterial Strain....107
6.4.3 Inoculum Preparation....108
6.4.4 Cultivation Conditions....108
6.4.5 Enhancement Strategies....108
6.4.6 PCB Extraction and Analysis....110
6.5 Results and Discussion....110
6.5.1 Strategy 1: Amount of Initial Biomass....110
6.5.2 Strategy 2: Addition of Supplementary Aroclor® after Fermentation Stalled 113

6.5.3 Strategy 3: Addition of Supplementary Biomass after Fermentation Stalls 114
6.5.4 Strategy 4: Addition of Co-Substrates after Fermentation Stalls............115
6.5.5 Strategy 5: Addition of Co-Substrate at Outset 119
6.6 Conclusions...122
Chapter 7: Biodegradation of PCBs in Two-Phase Partitioning Bioreactors following Solid Extraction from Soil 123
7.1 Preface to Chapter 7..124
7.2 Abstract 126
7.3 Introduction...127
7.4 Materials and Methods..129
7.4.1 Chemicals..129
7.4.2 Bacterial Strain..130
7.4.3 Uptake of Aroclor® 1242 by Hytrel™ 130
7.4.4 Solid Extraction of Aroclor® 1242 from Soil 131
7.4.5 Biodegradation of Aroclor® 1242 in a Solid Liquid TPPB.....................132
7.4.6 PCB Extraction and Analysis...132
7.5 Results and Discussion 133
7.5.1 Uptake of Aroclor 1242 by Hytrel™ 133
7.5.2 Solid Extraction of Aroclor® 1242 from Soil 135
7.5.3 Biodegradation of Aroclor® 1242 in a Solid Liquid TPPB.....................140
7.6 Conclusions...142
Chapter 8: Summary and Conclusions...143
References...150

List of Tables

Table 1-1 Composition of Aroclor® PCB mixture and selected physical and chemical properties (Cohen, et al. 1993; Guitart, et al. 1993). 4
Table 2-1: Properties of various solvents and the solubility of biphenyl in these solvents at 25 °C. 28
Table 3-1: Properties of solvents typically used in liquid-liquid TPPBs and their biocompatibility with the selected biphenyl degrading consortium. All solvents except dodecane and decane were compatible with *B. xenovorans* LB400. Log$K_{S/W}$ represents the solvent water partitioning coefficient of biphenyl for the respective solvent. 48
Table 3-2: Desirable polymer characteristics for delivery of poorly water-soluble substances to degrading organisms in solid-liquid TPPBs. 48
Table 3-3: Properties of polymers considered as the delivery phase for biphenyl in solid-liquid TPPB 49
Table 3-4: Polymer/water partitioning coefficients of biphenyl 51
Table 5-1: Performance parameters for different PCB degradation conditions. The initial amount of Aroclor® was 10 mg l^{-1} with respect to the aqueous phase volume under all conditions. The vials and Reactor 0 contained no immiscible phase. Reactor 1 contained 1 ml silicone oil with an initial Aroclor® concentration of 10 mg l^{-1}, Reactor 2 contained 1 ml BES with an initial Aroclor® concentration of 10 mg l^{-1} and Reactor 3 10 ml silicone oil with an initial Aroclor® concentration of 1 mg l^{-1}. 96
Table 7-1: Extraction of Aroclor® 1242 from soil using Hytrel™ polymer beads under different initial conditions. The presented values represent the averages of triplicates after 48 h ± 95 % confidence limits. 136

List of Figures

Figure 1-1: General Structure of PCBs ($C_{10}Cl_nH_{(10-n)}$. Each carbon 2-6 and 2'-6' is either substituted with H or Cl. 2
Figure 1-2: Illustration of microbial response to hydrophobic substrate. 8
Figure 1-3: Illustration of a batch TPPB. The two phases are shown as two layers for illustrative purposes only. The immiscible phase can be liquid or solid of various geometries. The right side illustrates the substrate concentration profile where $Q_{S.}$ is the substrate flux during disequilibrium. 9
Figure 1-4: Typical polymer beads as used in soild-liquid TPPBs (ethylene vinyl acetate). Beads leaded with fluranthene (left) and unloaded (right). Scale 1:1 13
Figure 1-5: Remediation process for PCB contaminated soil. PCBs are extracted from soil and the extract can either be treated directly in a TPPB (a) or the PCBs have to be transferred into a suitable delivery phase (b). 15
Figure 2-1: Dioxygenation of biphenyl and ring fission mediated by the *bph* genes (Kimura et al., 1997; Mondello et al., 1997). 22
Figure 2-2: Ability of *B. xenovorans* LB400 to grow in the presence of various solvents with different $LogK_{O/W}$. The relative cell growth is the optical density of the cell suspension in the presence of solvent divided by the optical density in the absence of solvent after incubation of 72 h. The squares represent incubation in TSB medium and triangles show growth on biphenyl as a carbon source in mineral salt medium. 27
Figure 2-3: Biomass formation (circles) and biphenyl degradation (squares) in TPPB by *B. xenovorans* LB400. a) The initial amount of biphenyl was 6.6 g L^{-1} based on the aqueous phase volume. Biphenyl was delivered from octadecene at an initial concentration of 66 g L^{-1}. The solid lines show the Monod simulation and the dashed lines a Monod simulation using the same parameter estimates and assuming a single liquid phase reactor in which biphenyl is provided at an equivalent aqueous phase biphenyl concentration as solid crystals. The dotted line shows the dissolved oxygen concentration. b) The initial amount of biphenyl was 5 g L^{-1} based on the aqueous phase volume. Biphenyl was delivered from BES at initial concentrations of 8 g L^{-1} (open symbols) and 80 g/L (closed symbols). The solid lines show the Monod simulations. The dash-dot line shows the dissolved oxygen concentration in reactor 1 (open symbols) and the dotted line shows the dissolved oxygen concentration in reactor 2 (closed symbols). 34
Figure 3-1: Biphenyl partitioning between water and polymer 50
Figure 3-2: Biphenyl partitioning between methanol and the selected polymers 53
Figure 3-3: Release of biphenyl from polymers loaded in methanol. The upper part of the Figure shows the concentration range of each polymer that results in a biphenyl saturated aqueous phase. The upper part of the Figure does not have a y-axis. The data points are presented on different vertical positions for clarification only 55
Figure 3-4: Biodegradation of biphenyl in solid liquid TPPBs by *B. xenovorans* LB400 (circles) and a microbial consortium (squares). The closed symbols represent the biomass concentration in the aqueous phase (cell dry weight) and the open symbols represent the biphenyl concentration in the solid phase. 57
Figure 4-1: Schematic of solid liquid TPPB; the size of individual polymer beads is not to scale 65

Figure 4-2: Partitioning of biphenyl between Hytrel^{TM} and methanol at 30 ºC. 70
Figure 4-3: Biphenyl release from 1 g Hytrel^{TM} into 50 mL water at different initial biphenyl concentrations in Hytrel^{TM}. 71
Figure 4-4: Normalized biphenyl release rate versus driving force during biphenyl release from 1 g Hytrel^{TM} into 50 mL culture medium at different initial biphenyl concentrations in Hytrel^{TM}. The solid line shows a linear regression of the data and the dashed lines the 95 % confidence limit. 72
Figure 4-5: The log solid liquid partitioning coefficient of biphenyl log $K^{m}_{S/W}$ as a function of biphenyl concentration in the Hytrel^{TM} (circles) and the equilibrium aqueous phase biphenyl concentration $S^{\infty}_{aq.}$ as a function of the initial biphenyl concentration in Hytrel^{TM} $S_{Sol.}$ (squares). The solid line shows an empirical non-linear regression of the data. 74
Figure 4-6: Biodegradation of biphenyl by *B. xenovorans* LB400 delivered from Hytrel^{TM} polymer beads. The circles represents the biomass when biphenyl was delivered from Hytrel^{TM} beads with a specific surface area of 0.079 m^2 dm^{-3} and the squares when biphenyl was delivered from beads with a specific surface area of 0.126 m^2 dm^{-3}. The solid lines represent the simulated biomass formation and the dashed line the biomass formation with no mass transfer limitation. 75
Figure 4-7: Simulated non-equilibrium aqueous phase substrate concentration (solid lines), hypothetical equilibrium substrate concentration (dashed lines) and the mass transfer driving force ΔS (dotted lines) as a function of fermentation time. The simulated values for Hytrel^{TM} beads with a specific surface area of 0.079 m^2 dm^{-3} are shown in plot a) and the simulated values for Hytrel^{TM} beads with a specific surface area of 0.126 m^2 dm^{-3} are shown in plot b). 77
Figure 5-1: Biodegradation of Aroclor® 1242 by *B. xenovorans* LB400 in scintillation vials at an initial biomass concentration of 0.3 g l^{-1} in PAS medium with no biphenyl (squares). The triangles show the Aroclor 1242® in a control experiment with killed biomass. 89
Figure 5-2: Effect of different amount of immiscible phase (square = silicone oil, circles = BES) on the achievable extent of Aroclor® 1242 (10 mg l^{-1}) degradation by *B. xenovorans* LB400. Figures a) and b) are different plots of the same data. Plot a) highlights the direct effect of increased immiscible phase fractions and plot b) highlights the effect of initial PCB concentrations in the immiscible phases, important for PCB degradation process design. 91
Figure 5-3: Time course of Aroclor® 1242 (10 mg l^{-1}) degradation by *B. xenovorans* LB400 (X_0 = 1.2 g l^{-1}) in BioFlo® 1 bioreactors. The effect of different amounts and types of immiscible phase (a), Reactor 0, no second phase (open circles), Reactor 1, 1 ml silicone oil (squares), Reactor 2, 1 ml BES (triangles), Reactor 3, 10 ml silicone oil (circles). Reactors 1 and 2 provided an effective initial Aroclor® concentration in the immiscible phase of 10 g l^{-1} and Reactor 3 of 1 g l^{-1}. The effect of agitation rate (b), 200 rpm (squares), 400 rpm (circles), 600 rpm (triangles). The immiscible phase in all three reactors was 10 ml silicone oil. 94
Figure 5-4: Chromatograms (shown as peak area vs. retention time) of Aroclor® 1242 during biodegradation by *B.* xenovorans LB400 in the presences of BES (Reactor 2), showing the qualitative change in congener distribution. 99

Figure 6-1: The effect of biomass concentration on the degradation of Aroclor® 1242 by B. xenovorans LB400. The initial biomass concentrations were 1 g l^{-1} (squares), 1.2 g l^{-1} (circles) and 3 g l^{-1} (triangles). The initial amount of Aroclor® 1242 was 100 mg dissolved in 10 ml silicone oil. The PCB concentrations are shown as mg Aroclor® per l aqueous medium. 111
Figure 6-2: Effect of additional substrate after stall of degradation activity. The initial biomass concentration was 1 g l-1 and the initial Aroclor® 1242 concentration in the delivery phase (1 ml) was 10 g l-1. After 75 h an additional ml delivery phase containing 10 g l-1 Aroclor® 1242 was added. The PCB concentrations are shown as mg Aroclor® per l aqueous medium. 113
Figure 6-3: Effect of additional biomass after stall of degradation activity. The initial biomass concentration was 0.5 g l^{-1} and the initial Aroclor® 1242 concentration in the delivery phase was 10 g l^{-1}. After 75 h 0.5 g fresh biomass were added in 10 ml culture medium. The PCB concentrations are shown as mg Aroclor® per l aqueous medium. 115
Figure 6-4: Effect of additional carbon sources after stall of degradation activity. The initial biomass concentration was 0.3 g l^{-1} and the initial Aroclor® 1242 concentration in the delivery phase was 10 g l^{-1}. After 40 h and 65 h 0.1 g carbon source were added (triangles = biphenyl, circles = glucose and squares = sodium pyruvate). The filled symbols represent the Aroclor® 1242 concentration (in mg Aroclor® per l aqueous medium) and the open symbols the biomass concentration. 116
Figure 6-5: Degradation of Aroclor® 1242 in TPPB by growing cells of *B. xenovorans* LB400. The initial biomass concentration was 0.3 g l^{-1} (a) and 0.01 g l^{-1} (b). The initial Aroclor® 1242 concentration in the delivery phase was 10 g l^{-1}. The filled symbols represent the Aroclor® 1242 concentration (in mg Aroclor® per l aqueous medium) and the open symbols the biomass concentration. 120
Figure 7-1: Schematic of soil remediation process. PCB contaminated soil is mixed with a solid extraction phase (polymeric material) in the presence of iso-propyl alcohol. PCBs are transferred from the soil to the polymers, the remediated soil is returned to the environment and the polymers are treated in a solid-liquid TPPB for microbial PCB destruction. 129
Figure 7-2: Partitioning of Aroclor® 1242 between methanol and Hytrel™ (triangles) and between water and Hytrel™ (squares). 134
Figure 7-3: Time course of PCB extraction from soil with Hytrel™ polymer beads. The triangles represent the total amount of Aroclor® 1242 recovered, the squares represent the amount in remaining in soil and the circles the amount extracted to the polymer beads. Test series E1 is shown in a), E2 in b), E3 in c) and E4 in d). 138
Figure 7-4: Chromatogram of Aroclor® 1242 extracted from: a) control soil, containing no polymer beads and an initial Aroclor® 1242 concentration of 1000 mg kg^{-1} after 48 h, b) soil containing 10 % (w/w) Hytrel™ and an initial Aroclor® 1242 concentration of 1000 mg kg^{-1} after 48 h, c) Hytrel™ beads, which were in contact with soil from b) after 48 h. No scale is shown on the y-axis to emphasize the similarities in the congener distribution. 139
Figure 7-5: Biodegradation of Aroclor® 1242 by *B. xenovorans* LB400 in a solid liquid TPPB. PCBs are delivered from Hytrel™ polymer beads. 141

Chapter 1: Introduction

A common feature shared by a large number of environmentally relevant contaminants of soil, sediment and water is hydrophobicity and resulting low solubility in water. Such contaminants include polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAH), dioxins, a variety of pesticides and herbicides as well as crude oil and transportation fuels. Most of the compounds listed above pose a severe risk to human health as well as to aquatic and soil ecosystems once released into the environment.

Due to their hydrophobicity such compounds frequently form separate phases in the environment or sorb to soil particles, thereby often being constrained to a relatively small area around their initial point of release. The toxicity of sites contaminated with PCBs or PAHs is therefore usually not of an acute, but of a chronic nature, as humans as well as wildlife are typically exposed only to the low concentrations in water, which however can be maintained over a long period of time in the surroundings of a contaminated site.

Low aqueous phase concentrations of contaminants also result in low contaminant availability to microorganisms with the ability to degrade the contaminants, hence contributing to their persistence in the environment. Engineered bioremediation measures can either attempt to increase the availability *in situ* to accelerate biodegradation, which however might also increase the acute toxicity during the treatment process, or can attempt to selectively remove the contaminants from the contaminated site for *ex situ* treatment.

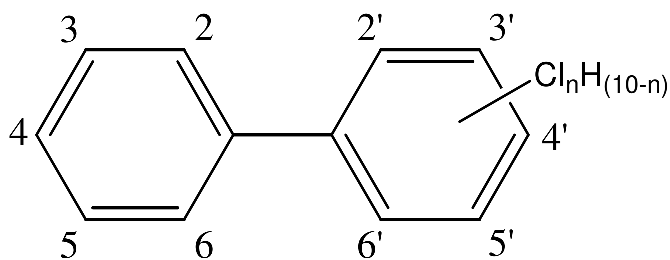

Figure 1-1: General Structure of PCBs ($C_{10}Cl_nH_{(10-n)}$. Each carbon 2-6 and 2'-6' is either substituted with H or Cl.

This thesis investigates the possible use of two-phase partitioning bioreactors as part of an *ex situ* treatment process for PCB contaminated soils.

1.1 Biphenyl and PCBs

PCBs have been chosen as model compounds in this thesis due to their extreme hydrophobicity and high environmental relevance. Decachlorobiphenyl, for example, has an aqueous solubility of 7.43 ng l^{-1} at 25 °C (Yalkowsky and Dannenfelser 1992), which is substantially lower than the solubility of other hydrophobic compounds such as PAHs. However, PCBs typically do not appear as pure compounds, but rather as congener mixtures, and the combined solubility of all congeners in a mixture is higher than the value reported for decachlorobiphenyl.

PCBs are a group of chemical compounds following the general structure presented in Figure 1-1. PCB congeners consist of biphenyl with one or more hydrogen atoms being substituted with chlorine. PCBs are xenobiotics, and were industrially produced through chlorination of biphenyl, allowing theoretically for 209 possible congeners. The biphenyl chlorination process resulted in congener mixtures with different congener distributions, depending on the initial biphenyl to chlorine ratio. PCB mixtures that were sold in North America are named Aroclor®. The name Aroclor® is followed by a four digit number, e.g. 1242, where the first two digits stand for the 12

carbons in biphenyl and the second two digits for the weight percentage of chlorine in the mixture. Aroclor® 1242 therefore has a chlorine content of 42 % (w/w) while the chlorine content of Aroclor® 1260 is 60 % (w/w). Table 1-1 shows the congener distribution of the most common Aroclors; a detailed congener composition of Aroclors can be found elsewhere (Frame, et al. 1996).

Aroclor® mixtures are typically composed of 75-100 individual congeners, which severely complicates quantification of Aroclors in an environmental sample (Erickson 1986). Most congeners can be separated on capillary columns and detected via electron captor detectors (ECD) (Rote and Murphy 1971; Zitko, et al. 1971). Aroclors are often quantified as the sum of their congeners, or based on some indicator congeners, as their general congener distribution often does not change in the environment over long periods of time (Erickson 1986). Analogously, many physical properties of Aroclors are often reported as the sum of the congeners' properties such as the octanol/water partitioning coefficients (Log $K_{O/W}$) shown in Table 1-1. Such simplifications are often required in field studies or applied research on the fate of PCBs in the environment, but have often been criticised (Tulp and Hutzinger 1978). In fundamental research, the different partitioning behaviour (Hawker and Connell 1988; Jabusch and Swackhamer 2005; Rapaport and Eisenreich 1984; Zeng, et al. 2005), congener specific toxicity (Khan and Hansen 2003; Smithwick, et al. 2003) and congener specific biodegradability (Kubatova, et al. 2001; Kuo, et al. 1999; Mukerjee-Dhar, et al. 1998) have to be taken into consideration.

Given the extreme hydrophobicity of PCBs and the difficulties in their quantification, the initial more fundamental investigations regarding substrate availability and reactor configuration (Chapters 2-4) in this thesis were undertaken with biphenyl

Table 1-1 Composition of Aroclor® PCB mixture and selected physical and chemical properties (Cohen, et al. 1993; Guitart, et al. 1993).

Composition and Properties	Aroclor®					
	1221	1232	1242	1248	1254	1260
Biphenyl	11	6	-	-	-	-
Monochlorobiphenyl	51	26	1	-	-	-
Dichlorobiphenyl	32	29	17	1	-	-
Trichlorobiphenyl	4	24	40	23	-	-
Tetrachlorobiphenyl	2	15	32	50	16	-
Pentachlorobiphenyl	0.5	0.5	10	20	60	12
Hexachlorobiphenyl	-	-	0.5	1	23	46
Heptachlorobiphenyl	-	-	-	-	1	36
Octachlorobiphenyl	-	-	-	-	-	6
Specific Gravity at 25°C	1.18	1.27	1.38	1.41	1.5	1.56
Absolute Viscosity at 38° C [cp]	5	8	24	70	700	resin
Solubility at 25°C [µg/L]	200	-	240	54	12	2.7
Vapor Pressure at 25°C [mm Hg]	0.0067	0.0046	0.0004	0.0004	0.00008	0.00004
Log K_{OW}	2.8	3.2	4.1	6.1	6.5	6.9

rather than with PCBs. The second part of this thesis (Chapters 5-7) focuses on the application of TPPBs in a PCB remediation process and therefore uses PCBs rather than biphenyl.

1.1.1 *PCBs in the Environment and Health Risks*

Approximately 635,000 tonnes of PCBs were produced in North America by the single North American manufacturer Monsanto Company in the United States until their production was banned in 1978. Approximately 40,000 tonnes were imported into Canada, of which only 24,000 tonnes have been accounted for as being in use or storage. The remaining 16,000 tonnes are either present in mineral oil at low concentrations, or are assumed to have already entered the environment (Canadian Council of Resource and Environment Ministers 1987). Once released into the

environment, the compositions of commercial PCB mixtures are altered through processes such as volatilization and other kinds of partitioning, chemical or biological transformation, and preferential bioaccumulation (Bamford, et al. 2002; Ghosh, et al. 2000).

The high level of inertness towards chemical and biological processes results in the long term accumulation of PCBs in the environment and in living organisms. This is mainly due to the high hydrophobicity of PCBs. Even though a variety of microorganisms are theoretically capable of degrading PCBs (Arnett, et al. 2000; Evans, et al. 1996; Liu 1981; Maltseva, et al. 1999; Master, et al. 2002), degradation occurs at an extremely slow rate, as the PCBs are adsorbed to the soil matrix (Ghosh, et al. 2000; Ghosh, et al. 2003), and are virtually unavailable to potential PCB-degrading microorganisms, due to their low availability in the aqueous phase.

Human inhabitants near PCB-contaminated sites are exposed to elevated PCB levels despite their inertness and low availability (Alcock, et al. 1998; Bosetti, et al. 2003; Choi, et al. 2006). Most commonly observed effects of PCB exposure to humans are skin disorders, swelling of eyelids, hyper-pigmentation (the darkening of nails, skin and mucous membranes), headaches, vomiting and liver damage. PCB exposure can lead to skin rashes and chloracne, a painful and disfiguring skin condition, similar to adolescent acne (Still, et al. 2003; Yoshimura 2003). These effects and additional visual disturbances and respiratory problems could be observed at the 1968 Yusho incident when 1200 people on a Japanese vessel consumed rice oil heavily contaminated with PCBs over a period of time ranging from 20 to 190 days (Yoshimura 2003). Long term PCB exposure is, among the previously mentioned

symptoms, linked to reproductive disorders (Hauser, et al. 2003) and cancer (Bosetti, et al. 2003).

1.1.2 *Biodegradation of PCBs*

Bacteria have been isolated to degrade PCBs under anaerobic and aerobic conditions. The two routes are fundamentally different since PCBs function as the terminal electron acceptor during anaerobic respiration and as the electron donor under aerobic conditions. Under anaerobic conditions PCBs are not 'degraded', as the biphenyl structure remains intact, instead PCB congeners are dechlorinated via reductive dehalogination, as described in detail elsewhere (Mohn and Tiedje 1992; Tiedje, et al. 1993; Wiegel and Wu 2000). Highly chlorinated congeners are particularly susceptible to reductive dehalogination and are transformed into lower chlorinated congeners by microbial consortia over time periods of months to years (Master, et al. 2002; Wu, et al. 1998).

Aerobic PCB degradation typically follows the biphenyl pathway (*bph*-pathway) and can result in complete mineralization of low-chlorinated PCB congeners (Abramowicz 1990). The pathway is limited to low-chlorinated congeners mainly due to steric hindrance at highly chlorinated congeners and the fact that the initial oxidization of the biphenyl backbone occurs through a dioxygenase and therefore requires two adjacent non-substituted carbons. The *bph*-pathway has been fully resolved and can be found in the literature (Mondello, et al. 1997); the initial pathway by *Burkholderia xenovorans* LB400 is also shown at a later point in this thesis (Figure 3-1). This pathway allows for degradation of up to hexachlorinated biphenyls, as has been reported for the biphenyl degrading organism *B. xenovorans* LB400 (Bopp 1986; Haddock, et al. 1993). This organism is one of the best studied aerobic PCB degraders;

its genome has been sequenced (Chain, et al. 2006) and its metabolic network has been mapped (Denef, et al. 2004; Denef, et al. 2005). Thus, aerobic PCB degradation by *B. xenovorans* LB400 was chosen as the microbial PCB degradation process for this thesis.

1.2 Bioavailability of Hydrophobic Compounds

Hydrophobic compounds such as PCBs are often persistent in the environment despite the presence of microorganisms capable of degrading them (McNamara, et al. 2005). One reason for this is the un-availability of these compounds to degrading organisms. This phenomenon has recently been utilised to reduce toxicity of PCB contaminated sites by reducing PCB availability through the addition of physical adsorbents (Millward, et al. 2005; Zimmerman, et al. 2004). Activated carbon was added to PCB contaminated sediment in these studies, free PCB congener were adsorbed by the carbon, which reduced the aqueous phase PCB concentration and thereby the acute toxicity to aquatic life.

Hydrophobic substances can be accumulated by microbial cells for degradation in different ways, as illustrated in Figure 1-2. Introducing a hydrophobic substance to an aqueous medium above its solubility results in the formation of two separate phases. The non-aqueous phase can consist of the pure target compound or of the target compound dissolved in another hydrophobic substance, such as oil (McNamara, et al. 2005). In both cases there will be a certain concentration in the aqueous phase in equilibrium with the concentration in the non-aqueous phase. In the simplest case this aqueous phase concentration is the only fraction available to degrading microorganisms as shown in Figure 1-2a. Some organisms have the ability to attach to the interface (Macedo, et al. 2005) and take advantage of locally high substrate concentrations as shown in the Figure

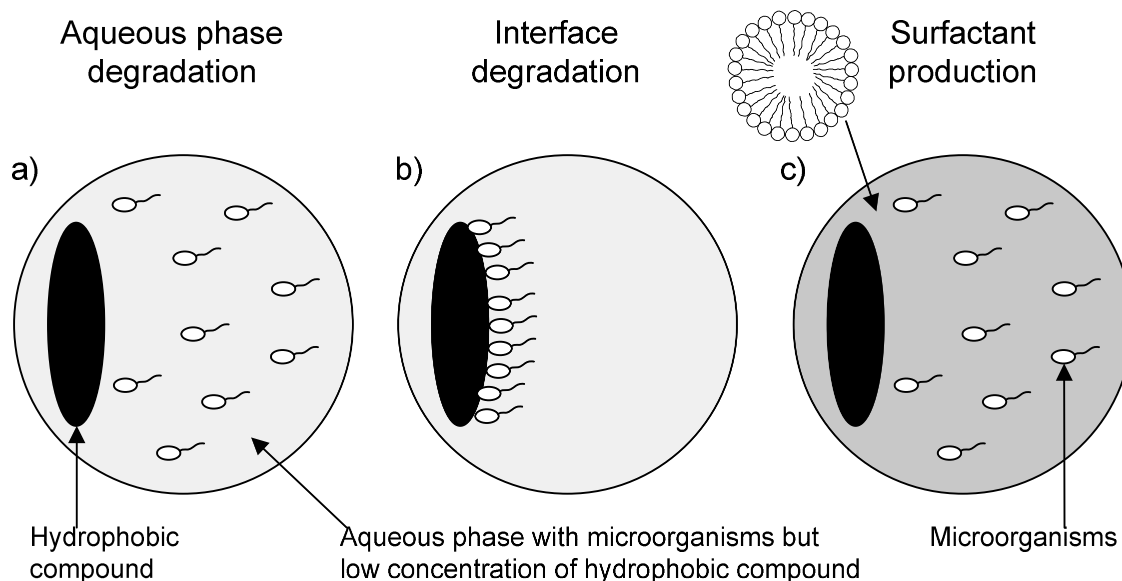

Figure 1-2: Illustration of microbial response to hydrophobic substrate.

1-2b. Other organisms can secrete surfactants which can increase the effective concentration of the hydrophobic compound in the aqueous phase (Chang, et al. 2005), as illustrated in Figure 1-2c. Organisms employing any of the two mechanisms shown in Figure 1-2b and 1-2c might have advantages over other organisms in the environment, but their cultivation adds additional complexity to a reactor system. *B. xenovorans* LB400 falls into the category of organisms shown in Figure 1-2a.

1.3 Two-Phase Partitioning Bioreactors

Two phase partitioning bioreactors (TPPBs) are typically stirred tank bioreactors consisting of a biocatalyst containing aqueous phase and an additional immiscible second phase. The immiscible phase can consist of an organic solvent (Daugulis 2001), an ionic liquid (Baumann, et al. 2005), a surfactant stabilised aqueous phase (Wang 2007) or a solid polymer (Amsden, et al. 2003).

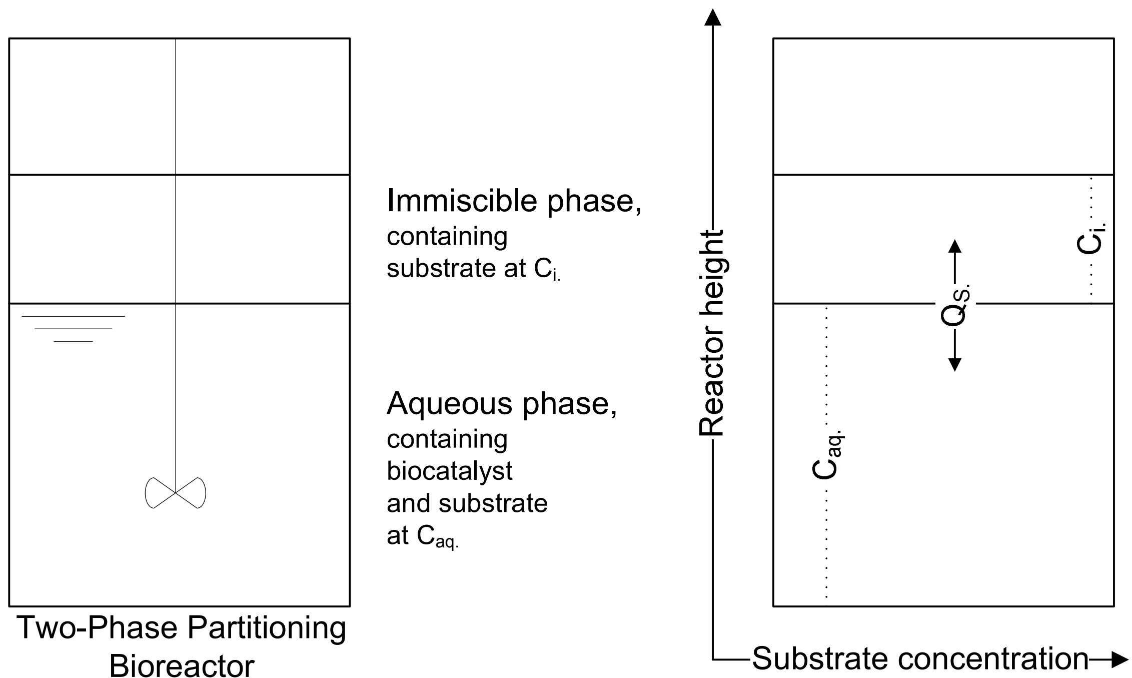

Figure 1-3: Illustration of a batch TPPB. The two phases are shown as two layers for illustrative purposes only. The immiscible phase can be liquid or solid of various geometries. The right side illustrates the substrate concentration profile where $Q_{S.}$ is the substrate flux during disequilibrium.

The second phase usually functions as a substrate reservoir or as a product sink and should therefore have high capacity for either substrate or product. It has to be chosen in order not to interfere with the desired reaction catalyzed by the biocatalyst in the aqueous phase. The topic of this thesis is the delivery of hydrophobic compounds to degrading organisms and this introduction will therefore focus on immiscible phases as substrate reservoirs.

Figure 1-3 shows a schematic diagram of a TPPB. The two immiscible phases are displayed as discrete phases, with the non-aqueous phase above the aqueous phase. This segregation is for illustrative purposes only, as a liquid second phase for example would form a fine dispersion under (agitated) operational conditions. The right side of Figure 1-3 shows the concentration profile of the target compound in the two phases (dotted line). The concentration of the target compound in the immiscible phase can be various orders

of magnitude larger than in the aqueous phase, depending on the properties of the selected phase and the target compound. The equilibrium concentration in the aqueous phase is a function of the concentration in immiscible phase and the partitioning coefficient, and at equilibrium can be written as follows:

$$K_{I/W} = S_{i.}^{eq.} \Big/ S_{aq.}^{eq.} \tag{1-1}$$

where $K_{I/W}$ is the partitioning coefficient, $S_{aq.}^{eq.}$ the equilibrium concentration in the aqueous phase and $S_{i.}^{eq.}$ the equilibrium concentration in the immiscible phase.

The biocatalyst is almost exclusively present in the aqueous phase, at least if whole cell systems are employed. Microbial degradation of the target compound in the aqueous phase results in a temporary reduction of the aqueous phase substrate concentration below $S_{aq.}^{eq.}$, causing thermodynamic disequilibrium. This disequilibrium will induce mass flux ($\vec{Q}_{S.}$) (partitioning) of the target compound from the organic phase into the aqueous phase, where the rate depends on the available surface area, a mass transfer coefficient and a concentration gradient.

The entire substrate dissolved in the immiscible phase can theoretically be transferred into the aqueous phase where biodegradation occurs. The substrate flux is governed by the microbial degradation rate. The system is therefore self-regulating if the mass transfer rate is sufficiently large in comparison to the microbial degradation rate. The ability to dissolve large amounts of poorly water-soluble compounds in a small reactor volume through the addition of a second phase makes TPPBs an excellent technology platform for the degradation of xenobiotics (Daugulis 2001), however to date

no study has investigated the potential use of TPPBs for degradation of poorly soluble substrates such as PCBs.

1.3.1 *Liquid-Liquid TPPBs*

Liquid-liquid TPPBs employing organic solvents as the immiscible phase are the most common biphasic bioreactors found in the literature (Eibes, et al. 2006; Guieysse, et al. 2005; Munoz, et al. 2005; Newman, et al. 2006). The organic solvent has to be selected to be compatible with the selected biocatalyst (neither toxic nor be available as a carbon source) and to meet other process requirements, such as affinity for the target molecule, low costs, high boiling point, etc.; a detailed description of solvent selection for TPPBs can be found elsewhere (Bruce and Daugulis 1991).

The required biocompatibility of the solvent limits liquid-liquid TPPBs essentially to pure cultures or well defined small microbial consortia. TPPBs employing microbial consortia often employ biologically very inert solvents such as silicone oil or the highly branched alkane hepta-methyl-nonane (Al Aalam 1993; Munoz, et al. 2005). Additionally, liquid-liquid TPPBs experience significant operational difficulties when the employed organisms express biosurfactants or tend to degrade hydrophobic compounds at the interface (Munoz, et al. 2005).

The main advantages of organic solvents are the well understood partitioning behaviour in ternary systems and the large available surface area for mass transfer during reactor operation. The solvent solute interaction is well understood and thermodynamic models have been developed to predict partitioning coefficients in tertiary systems under various conditions (Bruce and Daugulis 1991; Ochsner and Sokoloski 1985). This enhanced understanding allows for effective screening of suitable solvents.

An organic solvent will not form a discrete phase under operational conditions (agitated reactor at 500 rpm to 900 rpm) as shown for illustrative purposes in Figure 1-3. The solvent will instead form a fine dispersion, and the average droplet diameter has previously been estimated to be 30 μm (Wang and Ochoa 1972). The small average droplet size results in a high interfacial surface area and high mass transfer rates, which allows the assumption of instantaneous equilibrium formation between the organic and the aqueous phase which is often made in studies describing TPPBs mathematically (Nielsen, et al. 2005).

1.3.2 *Solid-Liquid TPPBs*

In recent work it has been shown that the organic solvents typically being used in TPPBs can be replaced by solid polymer beads (Amsden, et al. 2003; Prpich and Daugulis 2005). It was found that small organic molecules partition between solid polymers and water in a similar way as between organic solvents and water. Preliminary work towards the selection of the polymer has been undertaken (Prpich and Daugulis 2004), but no clear protocol describing a systematic selection procedure as known for organic solvents (Bruce and Daugulis 1991) can be found in the literature. Such a selection procedure is suggested in this thesis, however no attempt was made to mechanistically or empirically predict the affinity of a given polymer for a given small molecular substance. Initial attempts have been made to use the group contribution model UNIFAC (Fredenslund, et al. 1977) for the estimation of adhesion enhancement between polymers and mineral surfaces treated with silane coupling agents (Ochsner and Sokoloski 1985), however, none of these models can readily be adopted to describe the interaction between polymers, solute and water, although it is expected that the

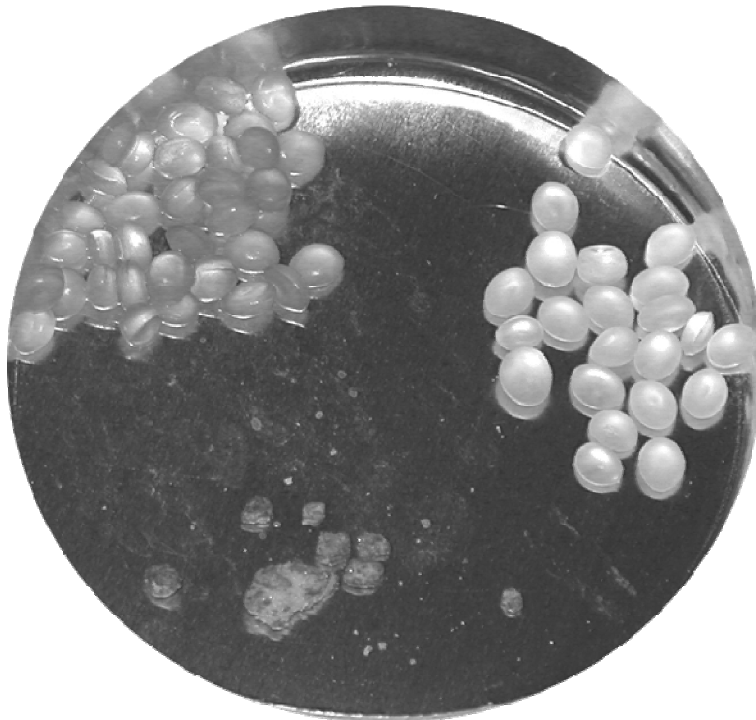

Figure 1-4: Typical polymer beads as used in soild-liquid TPPBs (ethylene vinyl acetate). Beads loaded with fluranthene (left) and unloaded (right). Scale 1:1.

presence/absence of specific functional groups will also be important in the selection of effective polymers for different applications.

Employing solid polymer beads has various advantages over organic solvents. Many polymeric substances are virtually inert to microbial degradation and also express no toxicity. The enhanced biocompatibility allows the use of microbial consortia in solid-liquid TPPBs (Prpich and Daugulis 2005) and even allows employing solid-liquid TPPBs during selective enrichments, which cannot be undertaken with liquid-liquid TPPBs as organic solvents would provide an alternative carbon source, and thereby result in selection pressure towards organisms being able to utilise the solvent as the source of carbon and energy, rather than the target compound dissolved in the organic solvent. Furthermore, organisms which produce biosurfactants can easily be cultivated in solid-liquid TPPBs. Potential biofilm formation on the polymer surface might be of concern in

some applications; however no biofilm formation on polymer beads in solid-liquid TPPBs has been reported in the literature to date.

The polymeric materials that have been employed so far were either shaped as beads or cylinders 4-8 mm in diameter (Figure 1-4). The surface area for mass transfer is hence significantly smaller than in liquid-liquid TPPBs, and mass transfer limitations might occur under some conditions; however no study has investigated possible mass transfer limitations to this date.

1.4 Envisioned Process

A possible application for TPPBs is as part of a remediation scheme for PCB contaminated soil. In order to treat PCBs from soil in TPPBs an initial extraction step is required, as outlined in Figure 1-5. The soil is released after the PCB extraction and the extract is treated in a TPPB. A commercial process which has been approved by the U.S. Environmental Protection Agency uses solvent extraction with iso-propyl-alcohol (IPA) (EPA 1994). The extracted PCBs have to be incinerated in this process, which would require transportation of the extract to an incinerator. In Canada currently only one facility is equipped and licensed to incinerate PCBs. An on-site PCB degradation strategy would therefore be highly beneficial. IPA however is not a suitable solvent for a liquid-liquid TPPB as it is miscible in water. A transfer into a more suitable delivery phase for a TPPB, as outline in Figure 1-5 (route b), would be required in this case. Alternatively the solvent for the extraction could be changed and then directly be used in the TPPB (Figure 1-5, route a). Solvents that can directly be used in a liquid-liquid TPPB have previously been shown to effectively extract PAHs from soil, followed by PAH degradation in TPPBs (Janikowski, et al. 2002). Using solvent to extract contaminants from soil and then using the same solvent in a TPPB however raises some sterility issues. It will be virtually

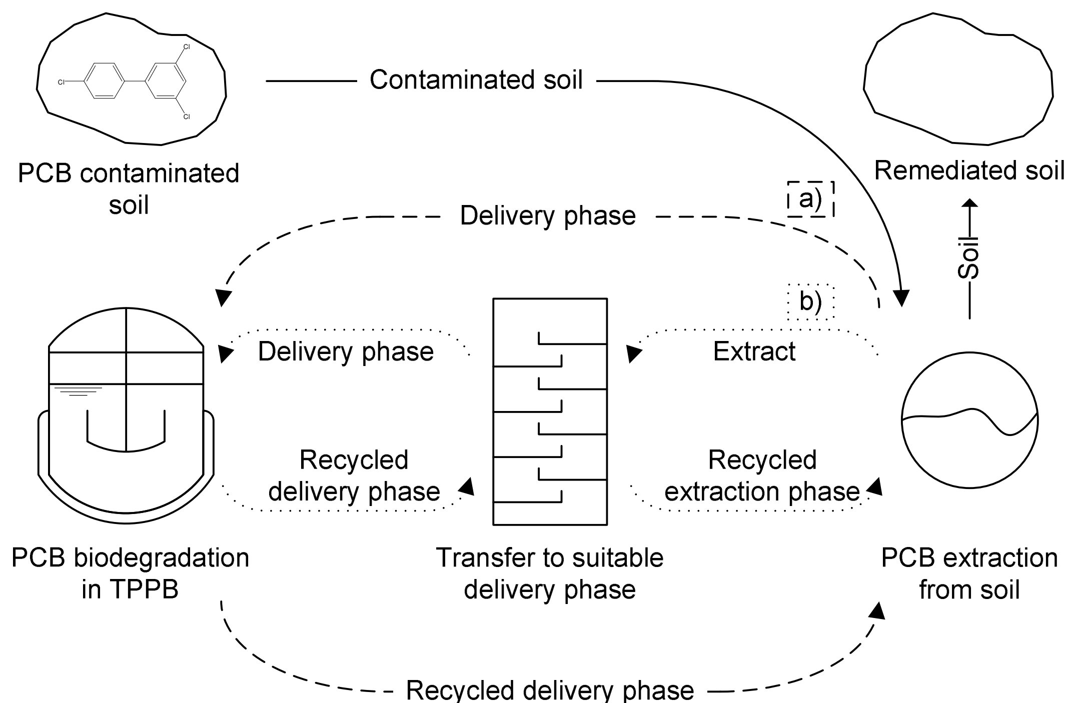

Figure 1-5: Remediation process for PCB contaminated soil. PCBs are extracted from soil and the extract can either be treated directly in a TPPB (a) or the PCBs have to be transferred into a suitable delivery phase (b).

impossible not to introduce some soil microorganism into the reactor, which might be able to degrade the solvent. More recently it was shown that polymer beads could be used to directly extract phenols from soil, followed by phenol degradation in a TPPB (Prpich, et al. 2006). Such a process would, if applicable to PCBs, avoid the sterility requirements of a liquid-liquid system due to the inertness of the polymeric substance. The polymers could be used as the extraction and as the delivery phase as outlined in route a), Figure 1-5.

1.5 Objectives

It is the overall objective of this thesis to evaluate the possible use of liquid-liquid and solid-liquid TPPBs for the biodegradation of highly hydrophobic substrates. The initial objective was to gain an understanding of the fundamental processes involved in both types of reactors with a strong emphasis on the degradation of PCBs, and the final

objective was to then design a possible remediation process involving PCB extraction from soil followed by their biodegradation in a TPPB.

Chapter 2: Biphenyl degradation kinetics by *Burkholderia xenovorans* LB400 in two-phase partitioning bioreactors

Lars Rehmann and Andrew J. Daugulis

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it appears in: *Chemosphere* **63**:972-979 (2006)

2.1 Preface to Chapter 2

TPPBs can enhance the total amount of poorly water-soluble substrates in a given bioreactor system. Although the total amount of substrate being present in the reactor might be high, the fraction of substrate available to the degrading organisms is limited to the substrate dissolved in the aqueous phase. During reactor operation the aqueous phase substrate concentration is at a dynamic steady state where the degradation rate in the aqueous phase equals the mass transfer rate of substrate into the aqueous phase. In liquid-liquid TPPBs the mass transfer rate is large due to the large interfacial surface area allowing the assumption of instantaneous equilibrium formation between the two phases. Under such conditions the equilibrium substrate concentration in the aqueous phase governs the microbial degradation rate, which depends on the available substrate concentration.

Depending on the solvent/water partitioning coefficient of the target compound the aqueous phase substrate concentration can be very low. This can be advantageous and has been employed to circumvent substrate toxicity; however, it might result in substrate-limited cell growth in extremely hydrophobic substrates such as PCBs. The ability of TPPBs to deliver substrates at low concentrations to degrading microorganisms was utilised in this chapter to estimate the dependency of the microbial degradation rate of biphenyl by *B. xenovorans* LB400 using the Monod model. The estimated kinetic parameters can be used to predict reactor performance during biphenyl degradation in TPPBs, and it was shown in this chapter, mathematically and experimentally, how changing process parameters that affect the aqueous phase substrate concentration can be used to influence the reactor performance.

This chapter further provides microbial cell yields of *B. xenovorans* LB400 on biphenyl and a solvent selection step for this organism. It can be anticipated from the results in the chapter that the degradation of PCBs by *B. xenovorans* LB400 in TPPBs will likely be limited by the available aqueous phase substrate concentration as well since the degradation employs the same enzymatic pathway, and since PCBs are significantly more hydrophobic than biphenyl.

2.2 Abstract

Biphenyl could be successfully degraded by *Burkholderia xenovorans* LB400, initially described as *Pseudomonas* sp. LB400, in two-phase partitioning bioreactors (TPPBs). TPPBs are comprised of an aqueous, cell containing phase, and an immiscible, biocompatible organic phase that partitions toxic and/or poorly soluble substrates (in this case biphenyl) based on maintaining a thermodynamic equilibrium. The critical $LogK_{O/W}$ of the organism was found to be approximately 5.5, indicating that solvents with a $LogK_{O/W}$ larger than 5.5 are suitable as delivery phases for *B. xenovorans* LB400. Two solvents selected for the TPPB system were octadecene and bis(2-ethylhexyl)sebacate (BES). In one experiment a total of 6.6 g biphenyl per L aqueous-phase-equivalent (biphenyl delivered in solvent, at an aqueous phase to solvent ratio of 10) could be degraded in 25 h during batch operation with octadecene. The specific growth rate and the half saturation constant of the Monod model were estimated to be μ_{max} = 0.25 h^{-1} and K_S = 0.0001 g L^{-1}, and the yield coefficient was $Y_{X/S}$ = 0.48 g biomass per g biphenyl. These parameter estimates were used to predict the time course of biphenyl degradation at different initial substrate concentrations and with biphenyl delivered from the two solvents with different partitioning behaviour for biphenyl. The predictions were validated by experimental data, confirming the microbial kinetics as well as the expected partitioning effects.

Keywords: Availability, insoluble, Monod, modelling, intermediates

2.1 Introduction

Biphenyl is an aromatic hydrocarbon, comprised of two, six-sided aromatic rings connected at one carbon on each ring. It is used as a fungistat in transportation containers of oranges and other citrus fruits (Ambrose, et al. 1960). Biphenyl is also used as an intermediate for the production of emulsifiers, optical brighteners, plastics, crop protection products and other organic compounds. It is used as a heat transfer medium, as a dyestuff carrier for textiles and copying paper, as a solvent in pharmaceutical production, and it was the parent compound of polychlorinated biphenyls (PCBs) (Weaver, et al. 1979). Biphenyl is considered to be one of the most thermally stable organic compounds (HSDB 1991). Animal studies have indicated that biphenyl exposure results in morphological and histopathological changes in the urinary system and it is considered to be a possible mutagen based on in-vitro studies (Boehncke, et al. 2005).

Various aerobic bacteria are capable of degrading biphenyl via the *bph* encoded pathway (Catelani, et al. 1971; Haddock, et al. 1993), which is also capable of degrading low chlorinated PCBs. *Burkholderia xenovorans* LB400 is the most studied aerobic biphenyl and PCB degrader (Billingsley, et al. 1997; Bopp 1986; Fain and Haddock 2001; Haddock, et al. 1993; Seeger, et al. 1999). A yellow metabolite, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (Bruhlmann and Chen 1999), (Figure 2-1) is often used as an indicator for biphenyl degradation. However, biphenyl, PCBs and high molecular weight polycyclic aromatic hydrocarbons (PAHs) share many physical properties which limit engineered bioremediation processes, and one of these properties is their low solubility and therefore low availability to the degrading microorganisms. Two-phase partitioning bioreactors (TPPBs) have been used in various situations where aqueous solubility is a limiting degradation factor (Daugulis 2001; Janikowski, et al. 2002; Kohler, et al. 1994).

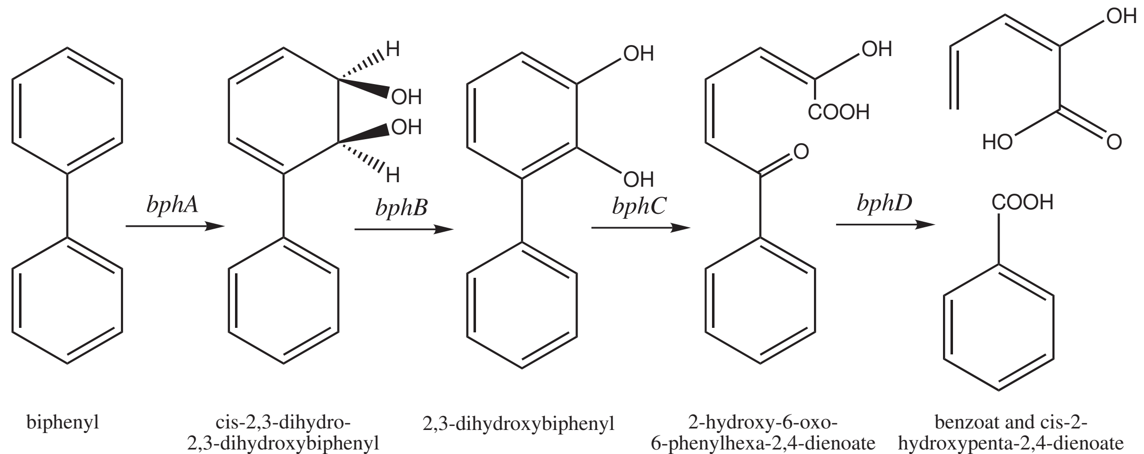

Figure 2-1: Dioxygenation of biphenyl and ring fission mediated by the *bph* genes (Kimura et al., 1997; Mondello et al., 1997).

TPPBs are typically stirred-tank bioreactors consisting of an aqueous phase which contains the required nutrients to support microbial growth and an immiscible organic solvent which can either function as a delivery phase for hydrophobic compounds or can remove toxic products from the aqueous phase (Daugulis 1997). The solvent used in a TPPB should be immiscible with water, non-toxic and non-biodegradable by the employed organism(s), as well as being cheap, non-toxic to humans, and possessing a high boiling point and a low volatility. Another feature of interest in the selection of a suitable TPPB solvent is the partitioning of the compound to be degraded, in this case biphenyl, between the two phases. High affinity of the solvent for the degradable substance is generally an advantage, as it allows high initial substrate loadings. A detailed description of solvent selection for TPPBs can be found in the literature (Collins and Daugulis 1999a).

This work examined the degradation kinetics of biphenyl by *B. xenovorans* LB400 in a TPPB using two delivery solvents. The use of a TPPB allows for continued provision of substrate for microbial degradation over a long period of time and facilitates the study

of microbial degradation kinetics of highly hydrophobic substances. Kinetic studies are otherwise limited by the aqueous solubility of the substrate, which results in very short degradation times at very low substrate concentrations close to the analytical detection limit, and therefore poor estimates of the intrinsic kinetic parameters. A good understanding of the microbial degradation kinetics of hydrophobic compounds such as biphenyl may provide valuable information for engineered bioremediation processes of other poorly soluble contaminants such as PAHs and PCBs.

It was the objective of this work to develop a methodology to estimate kinetic parameters of the biodegradation of poorly water soluble substrates and to use this system to kinetically characterize biphenyl degradation by *B. xenovorans* LB400.

2.2 Materials and Methods

2.2.1 *Chemicals*

All nutrients used in the fermentation media, and solvents, were obtained from either Sigma Aldrich (Canada) or Fisher Scientific (Canada). Biphenyl, 99% (assay) was obtained from Alfa Aesar (USA).

2.2.2 *Bacterial Strain*

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, N.Y.), was obtained from the Northern Regional Research Laboratory (Peoria, IL.). The strain has since been re-classified as *Burkholderia xenovorans* sp. nov. (Goris, et al. 2004). Cultures were maintained on solid mineral salts medium (Bedard et al., 1986), pH 7, with biphenyl as the sole carbon source supplied as vapours from crystals in the lid of the Petri dish. The Petri dishes were incubated at 30°C for 48 h and then stored at 4°C. For long-term storage of cultures, 12% dimethyl-

sulfoxide (DMSO) was added to the liquid cell culture in standard growth medium and 1 mL aliquots of the mixture were stored at -70°C in 1.5 mL vials.

2.2.3 *Standard Growth Medium*

Phosphate buffered mineral salt medium supplemented with yeast extract was used as the standard growth medium (Bedard, et al. 1986). The medium composition per litre was as follows: 75.5 mL stock solution (56.77 g L^{-1} K_2HPO_4, 21.94 g L^{-1} KH_2PO_4 and 27.61 g L^{-1} NH_4Cl), 10 mL trace element solution (19,5 mg L^{-1} $MgSO_4$, 5 g L^{-1} $MnSO_4 \cdot H_2O$, 7 g L^{-1} $FeSO_4 \cdot 7 H_2O$ and 0.2 g L^{-1} $CaCl_2 \cdot 2 H_2O$) and 50 mg yeast extract. The trace element solution was added to the autoclaved medium. Biphenyl was either dissolved in the chosen delivery phase or in acetone, filter sterilized using a 0.22 μm nylon syringe filter (Fisher Scientific (Canada)) and added to the autoclaved medium. Acetone was allowed to evaporate, leaving biphenyl crystals suspended in the medium.

2.2.4 *Inoculum Preparation*

Cells from a Petri dish were inoculated into 50 mL growth medium with biphenyl as the sole carbon source in a 125 mL Erlenmeyer flask and incubated for 22-24 h at 30 °C on an orbital shaker set at 180 rpm. The cell suspension was filtered through sterile glass wool to remove excess biphenyl crystals, centrifuged at 3400 rpm for 20 min, washed twice with 0.05 M sodium phosphate buffer, pH 7.5, and re-suspended in biphenyl-free medium to an OD of 2.0 at 600 nm (OD_{600}), using a 1-cm light path.

2.2.5 *Biphenyl degradation in TPPBs*

Experiments were undertaken in 5-L New Brunswick BioFlo® III bioreactors, agitated with two Rushton turbines at 800 rpm (to avoid mass-transfer limitation) and aerated (sterile air) at 3 L min^{-1}. The aqueous-phase volume was 2800 mL in all

experiments. The solvent in the first experiment was 280 mL octadecene with an initial biphenyl concentration in the solvent of 66 g L^{-1}, corresponding to 6.6 g biphenyl per litre aqueous phase. The solvents in the second experiment were 175 mL and 1750 mL bis(2-ethylhexyl)sebacate (BES), with initial biphenyl concentrations in the solvents of 80 g L^{-1} and 8 g L^{-1}, respectively, corresponding to 5 g biphenyl per litre aqueous phase. The reactors were inoculated with 50 mL inoculum. Conditions were automatically maintained at 30 °C, and at pH 6.9 by adding 3 M KOH. Dissolved oxygen levels were measured with a polarographic-membrane electrode (Broadley and James Corp., USA). All experiments were done in duplicate and replicates were not done simultaneously.

2.2.6 *Solvent Selection*

Bioavailability of selected solvents was assessed by inoculating 50 mL standard growth medium and 5 mL solvent in the absence of any other carbon source with *B. xenovorans* LB400. The OD_{600} of the aqueous phase was measured after 72 h. Biocompatibility was tested by inoculating 50 mL standard growth medium and 5 mL solvent containing biphenyl (50 g/L), and 50 mL tryptic soy broth (TSB) and 5 mL solvent, with *B. xenovorans* LB400. The OD_{600} of the aqueous phase was measured after 72 h.

2.2.7 *Biphenyl Analysis*

The biphenyl concentration in solvent samples was analyzed via a Varian 3400 Gas Chromatograph (GC) (Varian, Inc., CA, USA), equipped with a flame ionization detector (FID) and a 30 m DB5.625 0.25 micron column (J&W Scientific/Agilent Technologies Canada Inc., Mississauga, ON). The following temperature program was used: Initial column temperature 90 °C holding time 2 min, 10 °C min^{-1} to 180°C, 3 °C

min^{-1} to 250 °C followed by a holding time of 60 min. The injector temperature was 150 °C and the detector temperature was 300 °C. Data analysis and peak integration was performed by the software package Millenium[32] (Workstation Version 3.05.01 Waters Corp., USA). The detection limit in the organic phase was 10 μg L^{-1}.

2.2.8 *Biomass Analysis*

Aqueous phase samples were centrifuged at 3400 rpm for 20 min. The pellet was washed twice with buffer, re-suspended to the original volume and diluted to an OD_{600} between 0.1 and 0.6 to ensure that samples were measured in the linear range of the instrument. The OD_{600} was converted to dry cell weight (DCW) using the factor 0.3648 g L^{-1} OD_{600}^{-1} which was determined by filtering and drying cell suspensions and correlating the dry weight to the previously recorded OD_{600} readings.

2.2.9 *Biphenyl Solubility Tests*

Biphenyl solubility in various solvents was tested by adding 5 g biphenyl to 5 mL solvent in 15 mL glass vials closed with foil-lined cap. The vials were placed on a rotary shaker at 25 °C for 48 h. Un-dissolved biphenyl crystals were removed by filtering through glass wool. The filtrate was subsequently analyzed via GC-FID.

2.3 Results and Discussion

2.3.1 *Solvent Selection*

A wide range of possible TPPB solvents were initially considered based on their costs, availability and desirable physical properties (low vapour pressure, high boiling point, etc.), resulting in a short list of solvents to be examined experimentally. The biocompatibility of these selected solvents with *B. xenovorans* LB400 was then tested by comparing microbial growth on biphenyl and TSB in the presence and absence of

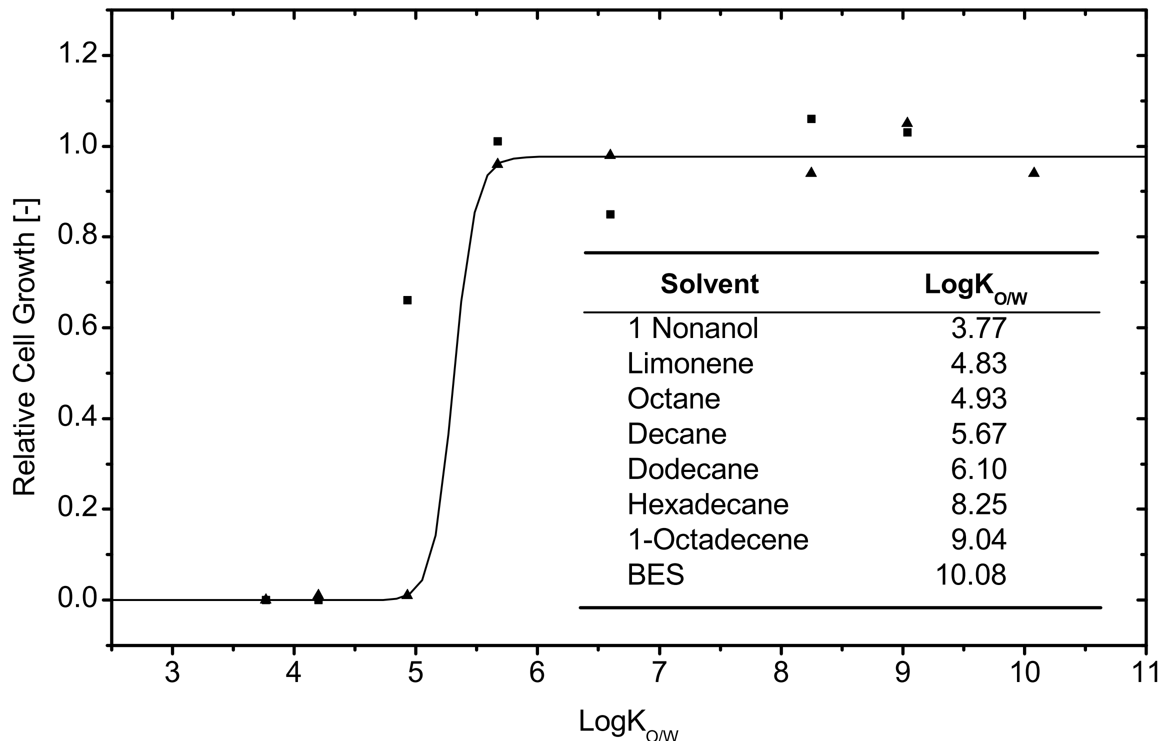

Figure 2-2: Ability of *B. xenovorans* LB400 to grow in the presence of various solvents with different $LogK_{O/W}$. The relative cell growth is the optical density of the cell suspension in the presence of solvent divided by the optical density in the absence of solvent after incubation of 72 h. The squares represent incubation in TSB medium and triangles show growth on biphenyl as a carbon source in mineral salt medium.

solvents. A plot of the relative cell growth as a function of the octanol water partitioning coefficient of the solvents ($LogK_{O/W}$) is shown in Figure 2-2. All solvents shown in this figure were shown not to support cell growth of *B. xenovorans* LB400 within 72 h if provided as the sole carbon source (data not shown) and are therefore considered to be not bioavailable (i.e. not used as a substrate). From Figure 2-2 the critical $LogK_{O/W}$ of *B. xenovorans* LB400 is estimated to be between 5.5 and 6. Solvents with a $LogK_{O/W}$ higher than 6 can therefore be considered as possible delivery phases during biphenyl degradation using *B. xenovorans* LB400 in a TPPB. This critical $LogK_{O/W}$ is within the reported ranges found for bacteria; typical values for gram negative bacteria are between 3 and 5 and for gram positive bacteria between 5 and 7 (Collins and Daugulis 1999a;

Table 2-1: Properties of various solvents and the solubility of biphenyl in these solvents at 25 °C.

Solvent	Boiling Point [°C]	Molar weight [g mol^{-1}]	Density [g · cm^3]	$LogK_{O/W}$ of solvent	$S^*_{sol.}$ [mol · mol^{-1}]	$S^*_{sol.}$ [g · L^{-1}]	$LogK_{S/W}$ of biphenyl
1-Nonanol	215	144.50	0.827	3.77	0.13	117.15	4.23
d-Limonene	176	136.26	0.839	4.20	0.50	471.24	4.84
n-Octane[a]	125	114.23	0.703	4.76	0.15	139.51	4.31
n-Decane[b]	174	142.29	0.730	5.67	0.16	129.44	4.28
n-Dodecane	216	170.34	0.750	6.60	0.18	125.43	4.26
n-Hexadecene[b]	287	226.45	0.773	8.15	0.22	113.23	4.22
n-Octadecene	315	252.48	0.790	9.04	0.21	101.26	4.17
BES	>300	426.70	0.914	10.08	0.50	165.94	4.39
n-Hexane[a]	69	86.18	0.660	3.84	0.12	146.45	4.33
n-Heptane[a]	98	100.20	0.684	4.31	0.13	139.90	4.31
n-Nonane[b]	150	128.26	0.718	5.23	0.16	133.90	4.29
Benzene[a]	80	78.11	0.870	2.04	0.38	654.38	4.98
Toluene[a]	111	92.14	0.865	2.66	0.38	545.79	4.90
Methanol[b]	65	32.04	0.780	-0.66	0.02	69.49	4.01
1-Octanol[b]	196	130.23	0.827	-	0.11	107.43	4.20

[a] Data from Chang (1969), [b] data from De Fina et al. (1999)

Inoue and Horikoshi 1989; Inoue and Horikoshi 1991). The only significant difference in the ability of the organism to grow in the presence of solvents on the two carbon sources employed (biphenyl and TSB) was in the case of octane. Octane is the only solvent that promoted growth on TSB and not on biphenyl. The $LogK_{O/W}$ of octane is 4.9, relatively close to the critical $LogK_{O/W}$ of *B. xenovorans* LB400, and it is speculated that the organism may able to tolerate a larger stress imposed by the presence of a solvent when growing on an easily accessible carbon source (TSB). Two solvents, BES and octadecene were chosen for further consideration as delivery phases for biphenyl degradation due to their high $LogK_{O/W}$ values.

The solubility of biphenyl in the delivery solvent is an additional factor to consider in solvent selection. High biphenyl solubility in the solvent allows high initial substrate loadings, which permits the use of smaller amounts of solvents, thus reducing cost. Although $LogK_{O/W}$ can be used to assess the hydrophobicity and the

biocompatibility of a solvent it cannot be used as an indicator of the solubility of biphenyl in these solvents. Solvents with high $LogK_{O/W}$ value do not necessarily have a stronger affinity for biphenyl than less hydrophobic solvents, as shown in Table 2-1. This table includes not only the tested solvents (upper section), but several additional common solvents for comparison purposes (lower section). The $LogK_{O/W}$ for n-alkanes increases from 3.8 for hexane to 8.15 for hexadecane, as the length of the alkane chain increases. The molar solubility (mol mol^{-1}) of biphenyl in these alkanes increases with the chain length, whereas the mass solubility (g L^{-1}) decreases slightly due to the increased molecular weight of longer alkanes. The solvent/water partitioning coefficient (equation 2-1) of biphenyl, estimated as the solubility of biphenyl in a given solvent divided by its solubility in water, therefore decreases with the length of the alkanes as well.

$$K_{S/W} = \frac{S_{sol.}}{S_{aq.}} \approx \frac{S^*_{sol.}}{S^*_{aq.}} \qquad (2\text{-}1)$$

where $K_{S/W}$ is the equilibrium partitioning coefficient of biphenyl between a given solvent and water, $S_{sol.}$ and $S_{aq.}$ are the concentrations of biphenyl in the given solvent and water, and $S^*_{sol.}$ and $S^*_{aq.}$ are the solubilities in the solvent and in water, respectively.

The highest solubility for biphenyl can be found in substances with aromatic structures, such as benzene and toluene. These solvents, however, do not fulfil the $LogK_{O/W}$ biocompatibility requirements of the selected organism. The selected solvents, BES and octadecene, show high solubility of biphenyl and were therefore both chosen as possible delivery phases for biodegradation of biphenyl.

2.3.2 *Biphenyl Degradation*

B. xenovorans LB400 is the most studied aerobic biphenyl and PCB degrader (Bopp 1986; Fain and Haddock 2001; Haddock, et al. 1993; Seeger, et al. 1999). The degradation pathway has been studied extensively on a molecular and genetic level and a draft of the entire genome is available through the Joint Genome Institute (JGI; http://genome.jgi-psf.org/draft_microbes/burfu/burfu.home.html). However, no extensive kinetic studies on biphenyl degradation in a controlled bioreactor system can be found in the literature, possibly because of operational difficulties with low substrate solubility in conventional (single aqueous phase) bioreactor systems. Degradation of biphenyl by *B. xenovorans* LB400 has been assumed to follow the Monod model (Monod 1949), which is the most frequently used model to describe microbial growth in substrate limited systems. The Monod model in its simplest form describes the microbial growth rate as a function of the concentration of one limiting substrate, in this case biphenyl. In the given system, only biphenyl dissolved in the aqueous phase is considered available to the microorganisms. Biphenyl functions as the sole carbon and energy source and all other nutrients, including oxygen, are in excess. Equations 2-2 and 2-3 describe the biomass and total substrate concentration as a function of time. The total substrate concentration is defined as the total amount of substrate present in both aqueous phase and solvent, normalized by the volume of the aqueous phase (Equation 2-4). Equation 2-1, defined earlier, describes the partitioning of biphenyl between the two phases.

$$\frac{dX}{dt} = X \cdot \frac{\mu_{\max} \cdot S_{aq.}}{K_S + S_{aq.}} \tag{2-2}$$

$$\frac{dS_{tot.}}{dt} = \frac{X}{Y_{X/S}} \frac{\mu_{\max} \cdot S_{aq.}}{K_S + S_{aq.}} \tag{2-3}$$

$$S_{tot.} = \frac{S_{aq.} \cdot V_{aq.} + S_{sol} \cdot V_{sol.}}{V_{aq.}} \tag{2-4}$$

where X is the biomass concentration in the aqueous phase [g L^{-1}], μ_{max} is the maximum specific growth rate [h^{-1}], K_S is the half saturation concentration [g L^{-1}], $Y_{X/S}$ is the yield coefficient [g biomass per g substrate], $S_{aq.}$ and $S_{sol.}$ are the substrate (biphenyl) concentrations in aqueous phase and the solvent [g L^{-1}], respectively. $S_{tot.}$ is the total substrate available in the system normalized to the aqueous phase volume [g L^{-1}] and $V_{aq.}$ and $V_{sol.}$ are the volume of the aqueous phase and the solvent [L], respectively.

This set (equation 2-1 to 2-4) of coupled non-linear ordinary differential equations (ODEs) can be solved in a number of different ways (Mohamed and Hatfield 2005). The most common numerical method to solve stiff systems of ODEs is Gear's Method (Gear 1971); however the finite difference method, which is simpler and faster to compute has been shown to be equally accurate on similar sets of ODEs (Mohamed and Hatfield 2005) and was therefore used here.

Figure 2-3a shows biomass formation and biphenyl degradation in a TPPB by *B. xenovorans* LB400. The delivery phase was octadecene with a Log $K_{S/W}$ = 4.17. Biphenyl degradation was completed after approximately 25 h, and at the same time, no further biomass formation was observed. The solid lines represent the solutions to the previously defined set of equations, and the parameter estimates are μ_{max} = 0.25 h^{-1}, K_S = 0.1 mg L^{-1} and $Y_{X/S}$ = 0.48 g biomass per g biphenyl. These estimated parameters are in the typical range for bacteria (Bailey and Ollis 1986), although the half saturation concentration K_S is at the low end of the spectrum, which can be expected of a hydrophobic substrate. Reichardt, et al. (1981), one of the very few studies on biphenyl degradation kinetics, reported μ_{max} = 0.067 h^{-1} and K_S = 0.00023 mg L^{-1} for a microbial consortium growing

aerobically on biphenyl. Although the maximum specific growth rate of *B. xenovorans* LB400 is significantly higher than that of the consortium, the half saturation constant of the consortium is substantially lower, suggesting that the members of the consortium may have been highly adapted to extremely low substrate concentrations. The half saturation constant found for *B. xenovorans* LB400 indicates that it might show poor degradation performance at low substrate concentrations, compared to the microbial consortium. The initial aqueous phase substrate concentration in the above-discussed experiment was still approximately 50 times higher than the half saturation concentration, but the substrate concentration in the aqueous phase might become the limiting factor in applications with more hydrophobic substrates.

TPPBs will always provide aqueous-phase substrate concentration below the saturation concentration of the specific compound in the aqueous phase, due to thermodynamic equilibrium of the activities of the target compound in both phases. This feature has been proven highly beneficial in cases where the substrate is toxic in high concentration as in the case of phenol (Prpich and Daugulis 2004), however, it might result in substrate-limited systems in the case of batch degradation of highly hydrophobic substances with no or low toxicity to the degrading organisms, as in the case of biphenyl, or high molecular PAHs and PCBs. The dashed lines in Figure 2-3a show simulated biphenyl and biomass concentrations (assuming the same kinetic parameters) for a hypothetical single phase case in which biphenyl is provided at an equivalent aqueous phase biphenyl concentration with the biphenyl provided as solid crystals. In this case, assuming instantaneous dissolution of the solid crystals, the aqueous phase would be saturated with biphenyl over essentially the entire time of the fermentation. The hypothetical saturated aqueous-phase reactor would perform faster than the TPPB only at

the very end of the fermentation. These results confirm that TPPBs can provide a valuable technology for the degradation of highly hydrophobic compounds, as discussed earlier (Daugulis 2001). Large quantities of biphenyl were completely dissolved in the solvent, delivered to the degrading organisms and degraded to completion. The above-mentioned single-phase system would be difficult to examine experimentally, mainly because of difficulties in the analysis of the total substrate concentration.

The parameter estimates were further verified by conducting two experiments in parallel with the same initial amount of biphenyl present in the system based on the volume of the aqueous phase. One reactor contained ten-fold amount of solvent compared to the other reactor. Based on the assumption of a constant partitioning coefficient, an initial ten-fold lower biphenyl concentration in the aqueous phase is expected, which would result in lower specific growth rates and consequently lower degradation rates. The delivery phase was also changed from octadecene ($LogK_{S/W}$ = 4.17) to BES ($LogK_{S/W}$ = 4.39). Figure 2-3b shows the experimental data as well as the predicted time courses. The experimental data follow the model prediction very well, which supports the estimates for the microbial parameters as well as the measured partitioning coefficients ($LogK_{S/W}$ for octadecene and BES). The results show that low concentrations of a target molecule can still be degraded successfully in TPPBs. It also shows that low initial substrate loadings in the organic phase of a TPPB will result in lower microbial growth/degradation rates. For non-toxic substrates, it can be concluded that the most economical way to operate a TPPB is at initial substrate loading close to the substrate solubility in the solvent. This mode of operation will minimize the amount of solvent needed in the reactor and also maximize the degradation rate. It is however necessary that the system be well-mixed. The

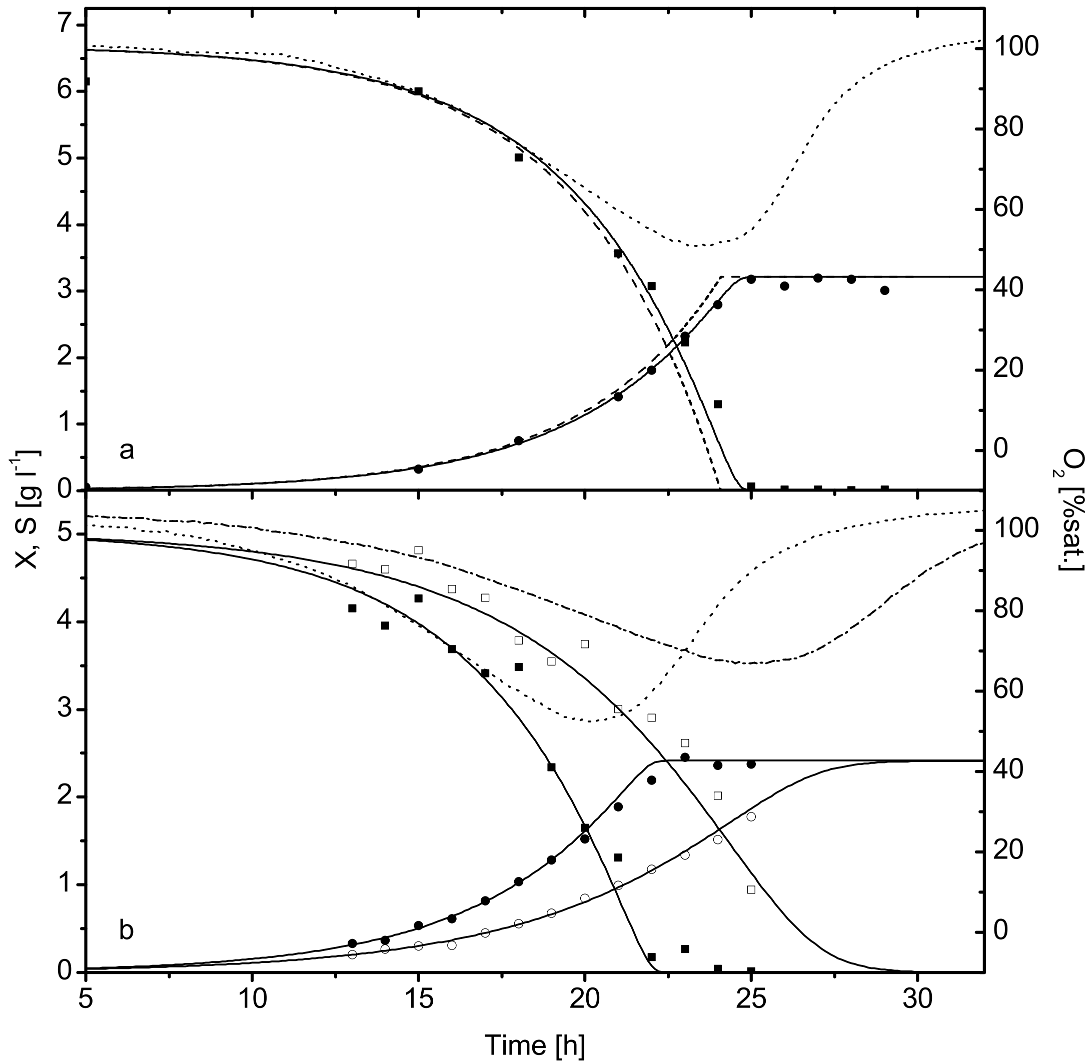

Figure 2-3: Biomass formation (circles) and biphenyl degradation (squares) in TPPB by *B. xenovorans* LB400. a) The initial amount of biphenyl was 6.6 g L^{-1} based on the aqueous phase volume. Biphenyl was delivered from octadecene at an initial concentration of 66 g L^{-1}. The solid lines show the Monod simulation and the dashed lines a Monod simulation using the same parameter estimates and assuming a single liquid phase reactor in which biphenyl is provided at an equivalent aqueous phase biphenyl concentration as solid crystals. The dotted line shows the dissolved oxygen concentration. b) The initial amount of biphenyl was 5 g L^{-1} based on the aqueous phase volume. Biphenyl was delivered from BES at initial concentrations of 8 g L^{-1} (open symbols) and 80 g/L (closed symbols). The solid lines show the Monod simulations. The dash-dot line shows the dissolved oxygen concentration in reactor 1 (open symbols) and the dotted line shows the dissolved oxygen concentration in reactor 2 (closed symbols).

solvent/aqueous phase ratio will become significant if mass transfer from the solvent into the aqueous phase becomes the rate-limiting step.

A yellow colorization could be observed in the reactor containing the smaller solvent fraction. This was initially interpreted as a sign of oxygen limitation; however the dissolved oxygen trace, shown as the dotted line in Figure 2-3b, indicates that the oxygen concentration in the aqueous phase never dropped below 50% of the saturation concentration. The yellow colour was observed between 12 h and 22 h of operation, and this is a common phenomenon during biphenyl and PCB degradation (Wesche, et al. 2005). The yellow metabolite has been identified by (Bruhlmann and Chen 1999) as 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, and is the fourth intermediate during biphenyl degradation, as shown in Figure 2-1. Based on the fact that this metabolite only accumulated in the reactor having faster microbial biphenyl degradation, it can be concluded that the breakdown of 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoate, catalyzed by the *bphD* gene product, can become the rate-limiting step in aerobic biphenyl degradation at high substrate concentrations. Kinetic studies of this enzyme can be found in the literature (Speare, et al. 2002), but cannot easily be compared to current findings, as the actual concentration of the intermediate as well as its partitioning behaviour has not been studied, and is beyond the scope of this paper. Moody, et al. (2002) studied the intermediates formed during biphenyl degradation by *Mycobacterium* sp. PYR-1 and could not detect any earlier metabolites other than 2-hydroxy-6-oxo-6-phenylhexa-2, 4-dienoate in the growth medium. The temporary accumulation of this intermediate can easily be avoided by reducing the aqueous phase substrate concentration by increasing the solvent to solute ratio and keeping the total mass of substrate constant. TPPBs can therefore be used to control the metabolic activity of microorganisms, and the degradation rate, and in some cases, the accumulation of metabolites, can be controlled. This can help

reduce accumulation of possibly toxic metabolites, which will, depending on their hydrophobicity, partition into the organic phase, and reduce the toxicity even further.

2.4 Conclusions

Biphenyl could successfully be degraded in TPPBs using two different solvents and different initial substrate concentrations. Microbial growth and biphenyl degradation could be modelled using Monod kinetics and assuming equilibrium partitioning. To the best of our knowledge this was the first attempt to model biphenyl degradation by *B. xenovorans* LB400 using the Monod model.

A method to study microbial kinetics for the degradation of poorly soluble substrate has been developed via the use of a two-phase bioreactor system. The system readily allows the kinetic characterization of microbial biphenyl degradation and is expected to be able to characterize microbial degradation of other hydrophobic compounds. These kinetic constants can be used in more complex models for environmental applications.

Acknowledgements

We are grateful to the Natural Sciences and Engineering Research Council of Canada for financial support, and the Environmental Technologies Program at SAIC Canada.

Chapter 3: Polymer Selection for Biphenyl Degradation in a Solid-Liquid Two-Phase Partitioning Bioreactor

Lars Rehmann, Bozhi Sun, Andrew J. Daugulis

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as is appears in: *Biotechnology Progress* **23**:814-819 (2007)

3.1 Preface to Chapter 3

A major limitation of liquid-liquid TPPBs is the requirement of the non-aqueous phase to be compatible with the employed biocatalyst. The non-aqueous phase has to be non toxic and non bioavailable to the biocatalyst(s), which essentially limits organic solvents to enzymes, pure strains of microorganisms and small defined microbial consortia. Surfactant producing organisms will also cause further operational difficulties in liquid-liquid systems. Environmental applications, such as the extraction of an environmentally significant contaminant from soil followed by its biodegradation in TPPBs, cannot be undertaken under sterile conditions. Foreign microorganisms inevitably introduced to the bioreactor under such conditions might be able to utilise the non-aqueous liquid as a carbon source and interfere with the reactor operation.

Solid-liquid TPPBs can be used to overcome these limitations. In solid-liquid TPPBs the second non-aqueous liquid phase is replaced by a solid polymeric phase. Small organic molecules can partition between various polymers and water in a way analogous to partitioning between an immiscible organic solvent and water. The employed polymers are typically sphere shaped or cylindrical shaped with 2-4 mm diameters and can be visualised as solid solvents from the operational point of a TPPB. The advantage of such polymers over organic solvents is that the polymers are virtually inert to microbial degradation, non-toxic and not affected by the production of biosurfactants, hence allowing the use of a wider range of biocatalyst in TPPBs.

This chapter describes a rational approach to select a suitable polymer for the degradation of biphenyl in solid-liquid TPPBs. The affinity of polymeric substances for biphenyl was determined experimentally, as developing a theoretical framework for the

prediction of the various interactions between the involved compounds would exceed the scope of this thesis.

The selected polymer was used in solid-liquid TPPBs to degrade biphenyl by the previously employed pure strain *B. xenovorans* LB400 and by a microbial consortium isolated from contaminated soil. It shows further that some polymers possess non-linear isotherms for the partitioning of biphenyl between polymer and water, allowing the delivery of biphenyl at almost saturation concentration in the aqueous phase over a wide concentration range in the polymer. This partitioning behaviour is beneficial, as it might reduce substrate limitation due to higher equilibrium concentrations in the aqueous phase. However, the observed growth curves of both microbial systems followed a partly linear trend which indicates that the mass transfer rate from the polymers into the aqueous phase might be limiting under the employed conditions, which was investigated in further detail in Chapter 4.

3.2 Abstract

The commercially available thermoplastic polymer Hytrel^{TM} was selected as the delivery phase for the hydrophobic model compound biphenyl in a solid-liquid two-phase partitioning bioreactor (TPPB), and 2.9 g biphenyl could successfully be degraded in 1 L TPPBs by a pure culture of the biphenyl degrading bacterium *Burkholderia xenovorans* LB400 in 50 h and by a mixed microbial consortium isolated from contaminated soil in 45 h. TPPBs consist of an aqueous, cell containing phase, and an immiscible, second phase that partitions toxic and/or poorly soluble substrates (in this case biphenyl) based on maintaining a thermodynamic equilibrium. This paper illustrates a rational strategy for selecting a suitable solid polymeric substance for the delivery of the poorly water-soluble model compound biphenyl. The partitioning of biphenyl between the selected polymers and water was analogous to partitioning of solutes between two immiscible liquid phases. The partitioning coefficients varied between 180 for Nylon 6.6 and 11,000 for Depmopan®, where the latter numerical value is comparable to biphenyl partitioning coefficients between water and organic solvents. Employing a solid delivery phase enabled the utilization of a surfactant producing microbial mixed culture, which could not be cultivated in liquid-liquid TPPBs and thereby extended the range of biocatalysts that can be employed in TPPBs.

Keywords: Bioremediation, microbial consortia, *Burkholderia xenovornas* LB400, solid phase extraction, xenobiotics

3.3 Introduction

Two-Phase Partitioning Bioreactors (TPPBs) consist of a biocatalyst-containing aqueous phase and an immiscible second phase (Daugulis 1997). The second phase functions typically as either a product sink removing potentially toxic products from the aqueous phase (Newman, et al. 2006), or as a substrate reservoir delivering substrates at low concentrations to the aqueous phase (Daugulis 2001; Nielsen, et al. 2005). The second phase typically has a larger affinity for the target compounds, allowing, in the case of substrate delivery, the loading of large amounts of poorly water-soluble substrate into the second phase, thereby making TPPBs an excellent technology platform for the destruction of hydrophobic xenobiotics (Daugulis 2001). The resulting equilibrium concentration in the aqueous phase is the only substrate available to the biocatalyst and degradation of this available substrate will yield a disequilibrium, which in turns results in partitioning of additional substrate from the second phase into the aqueous phase, ideally allowing complete degradation of all substrate present in the reactor.

The second phase is typically an organic solvent (Guieysse, et al. 2005; Munoz, et al. 2005; Newman, et al. 2006), but examples of aqueous-aqueous two phase systems (Droin and Cooper 1992), a cloud point system (Wang 2007) and TPPBs employing ionic liquids (Baumann, et al. 2005) can also be found in the literature. More recently solid polymers have been used to replace the second liquid phase in TPPBs (Amsden, et al. 2003). It was found that small organic compounds can partition between an aqueous medium and a variety of thermoplastic polymers in the same way as between aqueous medium and immiscible organic solvents, and these polymers can therefore be used to replace organic solvents in TPPBs (Amsden, et al. 2003; Daugulis, et al. 2003; Prpich and Daugulis 2004). The polymeric phase can theoretically be molded into any shape of

interest, but typically cylindrical or spherical beads with diameters of between 2 and 5 mm have been employed to date.

The various operational advantages over organic solvents are mainly due to the polymers' resistance to microbial degradation and their lack of cell toxicity. Furthermore, some organisms capable of degrading hydrophobic compounds tend to secrete biosurfactants to increase the availability of the hydrophobic substrate (Madigan, et al. 2000). In liquid-liquid TPPBs this can result in emulsification of the organic phase causing considerable operational difficulties (Guieysse, et al. 2005), which would not occur in a solid-liquid TPPB. This enhanced compatibility of polymers extends the range of possible biocatalysts that can be used in TPPBs, in particular allowing the use of microbial consortia. This can be of importance for degrading persistent contaminants and mixtures of contaminant such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). Examples of TPPBs employing organic solvents and microbial consortia are rare due to the difficulty of finding solvents that are non biodegradable by all members of the microbial consortium. The choice of the organic phases is therefore essentially limited to a few relatively microbial resistant compounds such as silicone oil (Marcoux, et al. 2000) or the branched alkane 2,2,4,4,6,8,8-heptamethylnonane (HMN) (Al Aalam 1993).

To date various papers have studied the ability to use solid-liquid TPPBs to reduce aqueous phase substrate and product concentrations below inhibitory levels (Prpich and Daugulis 2004; Prpich and Daugulis 2006), however, the target compounds in all reported cases were moderately water soluble substances. To be more generally applicable to the partitioning of a wider range of compounds the performance of a solid-liquid TPPB would need to be confirmed for poorly water-soluble and crystalline substances. This

paper describes the delivery of large quantities of biphenyl to degrading organisms in a solid liquid TPPB. Biphenyl was chosen as a model compound which has successfully been degraded in liquid-liquid TPPBs (Rehmann and Daugulis 2006), and which shares properties such as hydrophobicity with priority contaminants such as PAHs and PCBs.

It was the objective of this study to rationally select a polymer suitable for the degradation of the model compound biphenyl in a TPPB process, and to demonstrate the enhanced spectrum of biocatalyst compatible with solid liquid systems by employing a pure strain as well as a microbial consortium for the degradation of biphenyl.

3.4 Materials and Methods

All nutrients used in the fermentation media, and solvents, were obtained from either Sigma Aldrich (Canada) or Fisher Scientific (Canada). Biphenyl, 99 % (assay) was obtained from Alfa Aesar (USA). The suppliers of the various polymers used in this study are listed in Table 3-3.

3.4.1 *Biocatalyst Selection*

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, N.Y.), was obtained from the Northern Regional Research Laboratory (Peoria, Ill.). The strain has since been re-classified as *Burkholderia xenovorans* sp. nov. (Goris, et al. 2004). Cultivation conditions, medium formulation and biomass analysis can be found elsewhere (Bedard, et al. 1986; Rehmann and Daugulis 2006).

A microbial consortium was isolated via selective enrichment from soil contaminated with a variety of petrochemicals including biphenyl. Mineral salt medium (50 mL) was spiked with 0.5 g L^{-1} biphenyl, inoculated with 1 g soil and incubated on a

rotary shaker (180 rpm) at 30 °C for 72 h. Samples of 0.1 mL were subsequently transferred to fresh medium (total of 10 transfers) to enhance the likelihood that only biphenyl degrading organisms were left in the consortium.

3.4.2 *Delivery Phase Selection*

The biocompatibility of various polymers and organic solvents was tested by inoculating 125 ml shakeflasks containing 50 ml mineral salt medium (Bedard, et al. 1986) and 1 g of polymer or solvent with the degrading organisms in the absence of any additional carbon source. The polymer/solvent was considered bioavailable if the biomass concentration increased relative to a control after 5 days. Similarly shakeflasks were incubated in the presence of 0.1 g biphenyl. Lack of biomass formation after 5 days was attributed to toxicity of the delivery phase.

3.4.2.1 Partitioning of Biphenyl between Water and Solid Phase

The partitioning coefficient of biphenyl between biocompatible polymers and water was characterized to further identify the suitability of the selected polymers as a biphenyl delivery phase. Scintillation vials were filled with 10 ml water and 0.2 g of the respective polymer. Biphenyl was dissolved in methanol at 66 g l^{-1} and 2 µl – 250 µl of this solution were added to the vials containing water and polymers. Biphenyl that is dissolved in methanol forms fine crystals once methanol dissolves in water. The initial amounts of biphenyl in the vials were above the solubility of biphenyl in water, but were reduced below this level after equilibrium partitioning occurred, otherwise the vials were discarded and the polymer was tested with lower initial biphenyl concentrations. It was assumed that the small fractions of methanol did not affect the interaction of biphenyl with either polymer or water. Biphenyl concentrations in the aqueous phase were

measured with an Ultraspec 3000 UV/Visible Spectrophotometer (Biochrom, UK) at λ = 250 nm after agitating the vials 5 days on a rotary shaker at 180 rpm at 30 °C to allow equilibrium to occur. The corresponding equilibrium concentration in the polymer was calculated via mass balance.

3.4.2.2 Loading of Delivery Phase with Biphenyl

In order to effectively transfer large amounts of biphenyl into polymers methanol was used for its ability to dissolve substantial amounts of biphenyl. The partitioning behaviour of biphenyl between methanol and the selected polymers was characterized to allow effective loading of the polymers with biphenyl, and to be able to calculate biphenyl concentrations in polymers after methanol extraction during bioreactor operation. Scintillation vials were filled with 5 ml of methanol containing different initial concentrations of biphenyl and 2 g polymers. The final equilibrium biphenyl concentration in methanol was measured after an incubation period of 5 days on a rotary shaker at 180 rpm and 30 °C, and the amount of biphenyl loaded into the polymer was calculated via mass balance. Control vials with no polymers were treated the same way.

3.4.2.3 Release of Biphenyl into Aqueous Medium

With the aim of determining the extent to which the selected polymer beads would actually release the loaded biphenyl into aqueous medium polymers where loaded with different concentrations of biphenyl as described above (3.4.2.2), followed by equilibration with 10 mL water on a rotary shaker for 5 days and photometric biphenyl determination in the aqueous phase. Methanol remaining on the polymer surface was removed by rinsing with water prior the equilibration. Removing methanol by water washing can be justified by the fact that the polymers had no affinity for methanol and

methanol from the polymer surface could easily be removed with water while removal of poorly water-soluble biphenyl could be neglected.

3.4.3 *Biodegradation of Biphenyl in Solid-Liquid TPPBs – Proof of Concept*

As a final demonstration of the enhanced applicability of solid-liquid TPPBs the biodegradation of a large amount of biphenyl as a poorly water-soluble model compound was shown by a pure microbial strain as well as by a surfactant producing microbial consortium. Biodegradation of biphenyl in solid liquid TPPBs was undertaken in parallel by *B. xenovorans* LB400 and a microbial consortium in two 1.5 l New Brunswick BioFlo® I bioreactors, agitated each with two Rushton turbines at 600 rpm and aerated (sterile air) at 1 L min^{-1}. The pH was maintained at pH = 6.9. The aqueous phase volume was 1 l and the delivery phase consisted of 50 g Hytrel™ polymer beads at an initial biphenyl loading of 58 g kg^{-1}. The beads were loaded with biphenyl from methanol in 1 l shakeflasks and the remaining methanol was removed with water prior the introduction of Hytrel™ to the bioreactors. Inocula for the two reactors were grown for 24 hours on biphenyl crystals as the sole carbon source in mineral salt medium. Biphenyl crystals were removed via filtration through sterile glass wool and the inocula sizes were adjusted in order to obtain initial biomass concentrations in the bioreactors of 10 mg l^{-1}. Samples of the aqueous phase were taken periodically for biomass analysis as were samples of the polymers. Biphenyl concentrations could not be measured directly from the polymers. The concentrations in the polymers were obtained via mass balance after equilibrating approximately 0.1 g polymer with 10 mL methanol and photometric biphenyl analysis in methanol. The polymer methanol partitioning coefficient of biphenyl was obtained before as described in section 3.4.2.2.

3.5 Results and Discussion

3.5.1 *Biocatalyst Selection*

Two different biocatalysts were chosen, one a pure strain and the other a microbial consortium in order to demonstrate that biodegradation of poorly water soluble substrates would be readily achieved with either type of cell system. The biphenyl and PCB degrader *B. xenovorans* LB400, with the ability to degrade biphenyl in liquid-liquid TPPBs has been characterized in previous work (Rehmann and Daugulis 2006), and a biphenyl degrading microbial consortium which was isolated from soil were the selected biocatalysts. The consortium was capable of degrading biphenyl as the sole carbon source if provided as crystals in mineral salt medium. Denaturing gradient gel-electrophoresis (DGGE) revealed that the consortium was composed of at least five different species (data not shown).

3.5.2 *Delivery Phase Selection*

Liquid delivery phases commonly used in TPPBs were also evaluated to establish a benchmark for the polymers. However, silicone oil and HMN were the only delivery phases that were neither toxic nor bioavailable to the microbial consortium, as shown in Table 3-1. The pure strain *B. xenovorans* LB400 is compatible with a variety of organic solvents as reported elsewhere (Rehmann and Daugulis 2006). Biphenyl degradation by the microbial consortium in the presence of silicone oil or HMN was accompanied with strong biosurfactant production, which resulted in emulsification of the delivery phase. Similar problems have also been reported with surfactant producing pure strains (Guieysse, et al. 2005). A simple separation of the two phases of a TPPB is required if recycling of the delivery phase is desired and also for analytical purposes. Liquid-liquid

Table 3-1: Properties of solvents typically used in liquid-liquid TPPBs and their biocompatibility with the selected biphenyl degrading consortium. All solvents except dodecane and decane were compatible with *B. xenovorans* LB400. $LogK_{S/W}$ represents the solvent water partitioning coefficient of biphenyl for the respective solvent.

Solvent	$LogK_{S/W}$	Bioavailable	Toxic	Used in TPPBs
BES	4.39[b]	+	-	(Rehmann and Daugulis 2006)
Octadecene	4.17[b]	+	-	(Rehmann and Daugulis 2006)
Dodecane	4.26[b]	+	-	(Gamerdinger, et al. 1995)
Decane	4.28[a]	-	+	(Gamerdinger, et al. 1995)
HMN	-	-	-	(Marcoux, et al. 2000)
Oleyl alcohol	-	+	-	(Collins and Daugulis 1999b)
Silicone oil	-	-	-	(Eibes, et al. 2006)
Hexandecane	4.22[a]	+	-	(Nielsen, et al. 2005)

[a] (De Fina, et al. 1999)
[b] (Rehmann and Daugulis 2006)

systems were therefore not considered to be suitable for the employed microbial consortium.

As a guide for selecting a suitable delivery phase for a solid-liquid TPPB a list of desirable characteristic of the delivery phase is proposed in Table 3-2 in analogy to previously described desirable solvent characteristics for liquid-liquid TPPBs (Bruce and Daugulis 1991). A shortlist of seven polymers to be considered as delivery phases for

Table 3-2: Desirable polymer characteristics for delivery of poorly water-soluble substances to degrading organisms in solid-liquid TPPBs.

1. Commercially available at a low cost
2. Non hazardous
3. Non toxic to the employed organisms
4. Not available as carbon and energy source or otherwise biodegradable
5. Not promoting biofilm formation under operational conditions
6. Possessing desirable affinity for the target molecule(s)
7. Thermally stable for sterilization purposes
8. Stable in aqueous medium at the pH and electrolyte concentration of the employed culture medium
9. Stable in medium employed to load polymer with target compounds

Table 3-3: Properties of polymers considered as the delivery phase for biphenyl in solid-liquid TPPB

Polymer	Type	Supplier	Costs[a] $ $\mathrm{kg^{-1}}$	Structure
Kraton®	G1657M	Kraton	n/a	Styrene – ethylene/butadiene tribloc co-polymer
Nucrel®	925	DuPont	6.93	Ethylene - methacrylic acid co-polymer
Nylon	6.6	DuPont	3.37-3.70	Polycaprolactam
Silicone Rubber	Mastercraft®	Mastercraft®	12.78-14.08	Polydimethylsiloxane
Desmopan®	DP 9370A	Bayer	4.07-5.61	Polyurethane of poly(oxytetramethylene)glycol and methyldiisocyanate
Elvax®	360	DuPont	1.02-1.14	Poly (ethylene-co-vinyl acetate)
Hytrel™	8206	DuPont	4.07-5.61	Butylene terephthalate - butylene ether glycol terephthalate co-polymer

[a] Average prices from various suppliers as of March 2007 in US$ for same polymeric substances as listed assuming purchase of bulk quantities (Sherman and Schut 2007).

biphenyl degradation in solid-liquid TPPBs was created based on these characteristic and is shown in Table 3-3. All listed polymers fulfill requirements 1-5 and 7-9 (data not shown) and their affinity for the target molecule (requirement 6) will be described in the following sections. The short-listed substances were also chosen to represent a wide class of polymers and further optimization within a selected polymer class might be able to improve the reactor performance, which however is beyond the scope of this study.

3.5.2.1 Partitioning of Biphenyl between Solid Phase and Water

The seven different polymers listed in Table 3-3 were tested for their ability to sorb biphenyl from an aqueous medium. Sorption equilibria for different biphenyl

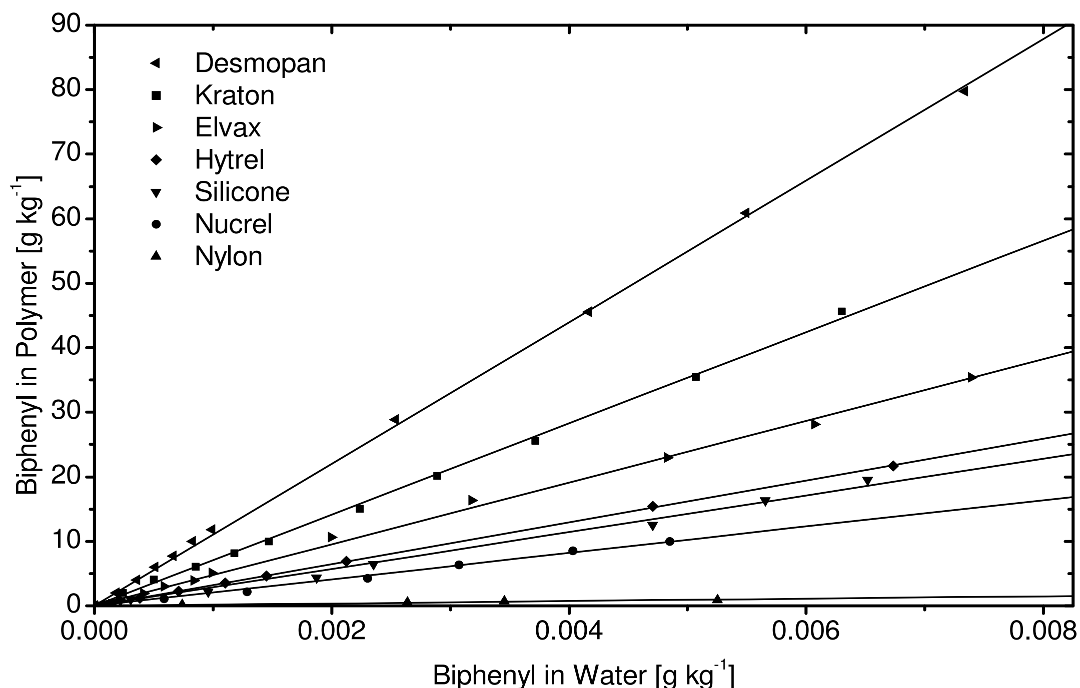

Figure 3-1: Biphenyl partitioning between water and polymer

concentrations are shown in Figure 3-1. It can be seen that all polymers under consideration, with the exception of nylon, show a strong affinity for biphenyl. The equilibrium isotherms follow a linear trend for all polymers over the tested range of concentrations, as similarly observed for phenolic compounds (Prpich, et al. 2006). Linear isotherms indicate that the governing mechanism in these three component systems is equilibrium partitioning analogous to partitioning of solutes between two immiscible liquid phases. The tested polymers show significant differences in their affinity for biphenyl; this can also be seen in their biphenyl partitioning coefficients shown in Table 3-4, which were obtained from linear regression analysis of data presented in Figure 3-1.

Similar to the tested polymers, organic solvents also show differences in their affinity for biphenyl. For example the biphenyl partitioning coefficient between *d*-limonene and water is $K_{S/W} \approx 69{,}000$, which is substantially higher than the biphenyl

Table 3-4: Polymer/water partitioning coefficients of biphenyl

Polymer	Partitioning Coefficient $K_{S/W}$	Error of $K_{S/W}$	$LogK_{S/W}$
Kraton®	7072	56	3.85
Nucrel®	2049	36	3.31
Nylon	184	11	2.27
Silicone Rubber	2850	54	3.45
Desmopan®	10987	56	4.04
Elvax®	4781	50	3.68
Hytrel™	3234	9	3.51

partitioning coefficient between octadecene and water ($K_{S/W} \approx 15,000$) (Rehmann and Daugulis 2006). The solubility of organic compounds in solvents and their partitioning between water and solvents depends on the molecular structure of both, solute and solvent. Group contribution models such as UNIFAC and UNIQAC have been developed based on known properties of functional groups to predict vapour liquid equilibria (Fredenslund, et al. 1977) and have since been extended to liquid-liquid equilibria (Ochsner and Sokoloski 1985) allowing such models to be applied to selected suitable solvents for liquid-liquid TPPBs (Bruce and Daugulis 1991). Initial attempts have been made to use the UNIFAC system for the estimation of adhesion enhancement between polymers and mineral surfaces treated with silane coupling agents (Ochsner and Sokoloski 1985), however, none of these models can readily be adopted to describe the interaction between polymers, solute and water, although it is expected that the presence/absence of specific functional groups will also be important in the selection of effective polymers for different applications.

The partitioning coefficient of biphenyl between the tested polymers and water are very high, as shown in Table 3-4, and are comparable to biphenyl partitioning coefficients between organic solvents and water (Table 3-1). Based on these values all tested polymers, with the exception of nylon, can be considered as potential delivery phases for

TPPBs. The high partitioning coefficients, e.g. $K_{S/W}$ ≈10,000 for Desmopan®, also suggest the possible use of polymers in environmental applications such as in scavenging for low concentrations of hydrophobic toxic contaminates from industrial waste water streams. TPPBs have been discussed in a recent review as a possible technology to bioremediate trace organic compounds found in precious metals refineries' wastewaters (Barbosa, et al. 2007). Extraction of these contaminants with an appropriate polymeric substance followed by biodegradation in a solid liquid TPPB might also be a possible remediation technology.

3.5.2.2 Loading of Delivery Phase with Biphenyl

Biphenyl is crystalline at room temperature and dissolving it in water prior to loading the delivery phase is not effective for generating high biphenyl concentrations in polymers due to the low solubility of biphenyl in water (7 mg kg^{-1} at 30 °C (Bohon and Claussen 1951)). In contrast, the solubility of biphenyl in methanol at 30 °C is 89 g kg^{-1} (De Fina, et al. 1999), which facilitates dissolving large quantities of biphenyl in methanol in order to load the polymers.

Figure 3-2 shows linear equilibrium isotherms of biphenyl between methanol and the tested polymers, similar to what was observed when biphenyl partitioned between polymers and water. These results show again that equilibrium partitioning is the governing process in the observed three component systems. However, comparing Figure 3-2 and Figure 3-1 shows that the maximum achievable concentration in the polymers is much higher in the biphenyl-methanol-polymer system in comparison with the corresponding biphenyl-water-polymer system. This effect is the most pronounced for Hytrel™, which has the fourth highest biphenyl partitioning coefficient between polymer and water but the third highest for biphenyl partitioning between polymer and methanol.

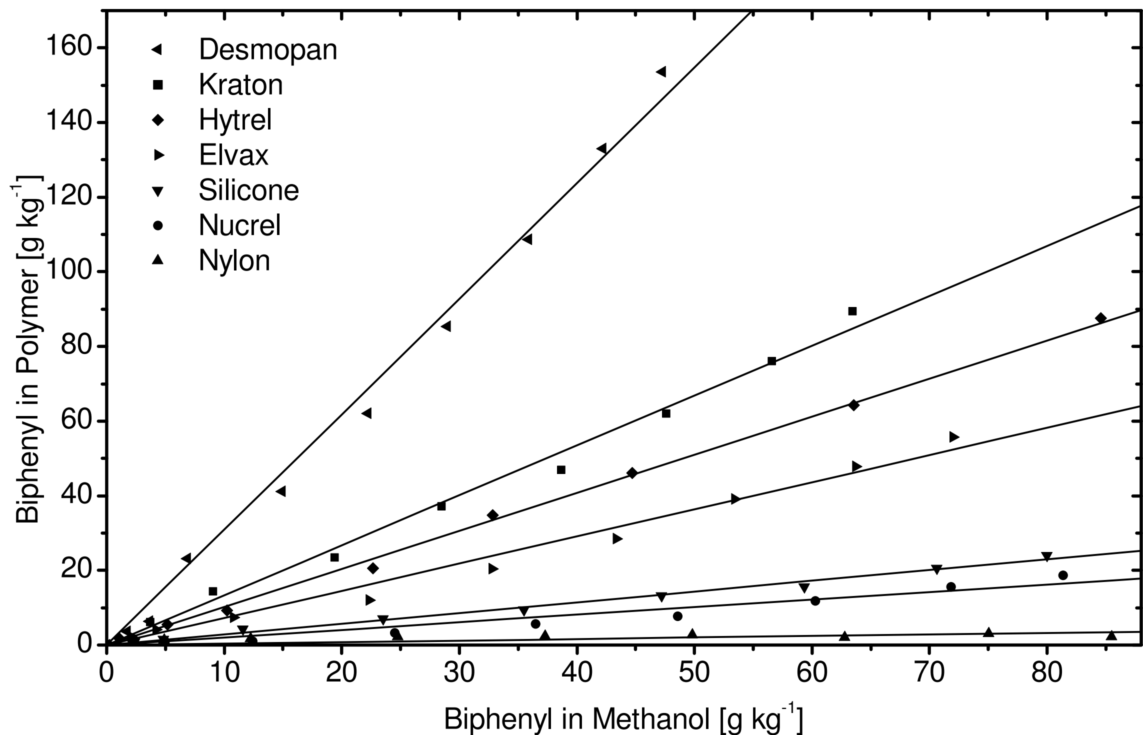

Figure 3-2: Biphenyl partitioning between methanol and the selected polymers

The partitioning coefficients of biphenyl between the three polymers Desmopan®, Kraton®, Hytrel™ and water are > 1, indicating that the relative affinity of these polymers for biphenyl is larger than the biphenyl affinity of methanol. Such high affinity would be of importance if hydrophobic compounds were to be extracted from media such as soil rather than water, followed by biodegradation in a TPPB. In a soil environment organic matter can have high affinity for hydrophobic compounds and the ability of these three polymers to remove biphenyl from organic solvents such as methanol also indicates that polymers might be able to compete effectively for target molecules in soil with high organic content. Prpich et al. (2006) successfully demonstrated the effective extraction of phenol from soil by mixing dry soil with polymer beads (Prpich, et al. 2006), and the subsequent release of the phenol to a microbial consortium in a TPPB. The study was undertaken at laboratory scale and employed polymer beads with average diameters of 4 -

6 mm; for large scale soil remediation other geometrical shapes and sizes of the polymer may be advantageous to simplify the soil polymer separation step.

Changes in the ability of polymers to sorb substances depending on the medium the polymers are immersed in are also known to occur when hydrogels, such as acrylic acid acrylamide co-polymers, absorb water. The addition of 1.2 % of NaCl to distilled water was shown to reduce the water absorption capacity of hydrogels by 50 % (Liu and Rempel 1997). Further, changes of the fluid compositions (e.g. the addition of ethanol and methanol to water) are known to affect the ability of hydrogels to absorb water substantially as first shown by Tanaka (1978) (Tanaka 1978). The opportunity to possibly influence the ability of polymers to sorb hydrophobic compounds by changing the medium conditions is very interesting, which may be a fruitful area of future work.

3.5.2.3 Release of Biphenyl into Aqueous Medium

The polymers loaded with biphenyl from methanol were transferred to aqueous medium after removal of residual methanol. The resulting equilibrium aqueous phase concentrations were measured and are shown in Figure 3-3 as a function of the biphenyl concentration in the polymers. The data shown in Figure 3-3 are similar to the data shown in Figure 3-1 for low biphenyl concentrations. Under these conditions the same equilibria were reached, regardless of whether biphenyl was initially present in the aqueous phase (Figure 3-1) or in the polymer phase (Figure 3-3). At high biphenyl concentrations in the polymers the aqueous phase became saturated with biphenyl. The range of biphenyl concentrations in the polymer phases that results in a biphenyl saturated aqueous phases varied depending on the employed polymer. The upper section of Figure 3-3 shows this constant section for the different polymers.

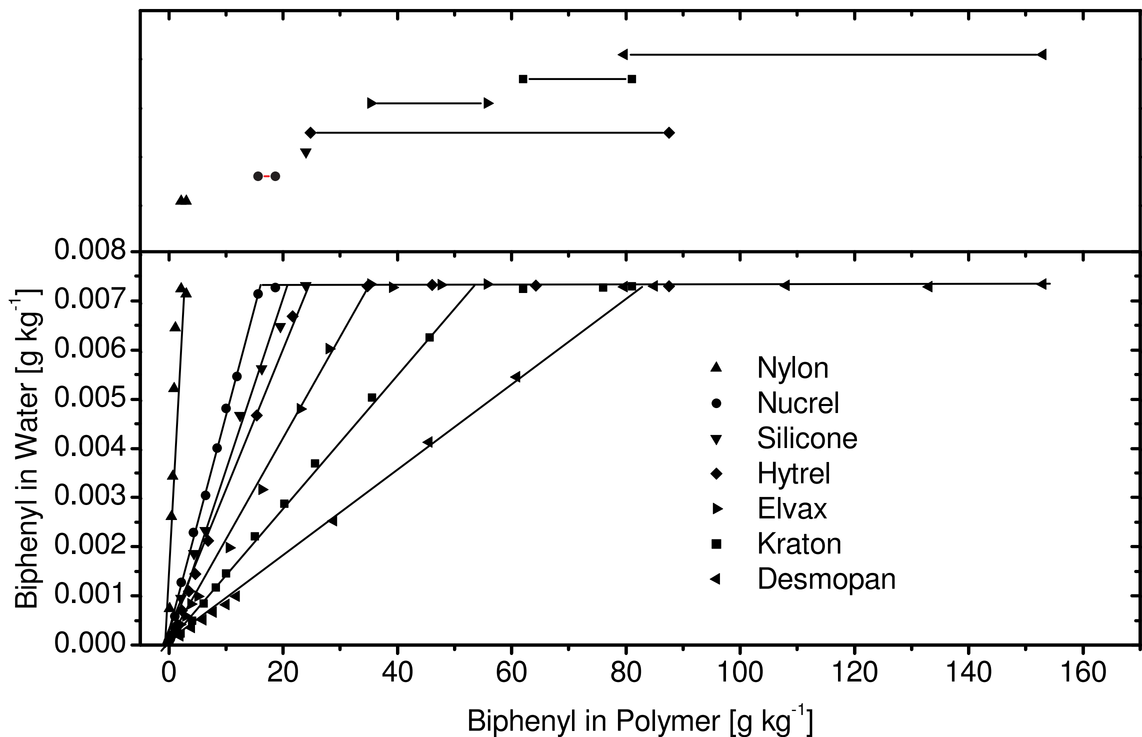

Figure 3-3: Release of biphenyl from polymers loaded in methanol. The upper part of the Figure shows the concentration range of each polymer that results in a biphenyl saturated aqueous phase. The upper part of the Figure does not have a y-axis. The data points are presented on different vertical positions for clarification only.

The ability to load various polymers with large amounts of hydrophobic substances, which can then be released to their saturation concentration in aqueous media provides an interesting delivery system for hydrophobic compounds to degrading organisms. This is of particular interest if bioavailability of the target compounds is the degradation rate limiting step, which can be the case during biphenyl degradation (Rehmann and Daugulis 2006). Under such conditions a polymer such as Hytrel^{TM} which has a very wide range of concentrations resulting in a saturated aqueous phase should be chosen as the delivery phase. Hytrel^{TM} loaded with 60 g kg^{-1} would provide two thirds of the initially available biphenyl at saturated aqueous phase concentrations to degrading organisms, until the concentration in Hytrel^{TM} reaches 20 g kg^{-1}, after which the equilibrium concentration in the aqueous phase would decrease linearly (assuming mass

transfer rates being sufficiently large compared to microbial degradation rates). Desmopan® with a similar initial loading would provide initial aqueous phase concentrations of only ~75 % of the aqueous phase solubility followed by a linear decrease of the equilibrium concentration. Such partitioning behaviours would be of interest if elevated concentrations of the target compound were toxic to the degrading organisms as in the case of phenols (Prpich and Daugulis 2006).

The formation of free biphenyl crystals was not observed under the experimental conditions which resulted in biphenyl saturated aqueous phases, showing that the release of biphenyl from the polymers did not continue once biphenyl saturation in the aqueous phase was reached. However, for the purpose of this study it is important that large quantities of biphenyl could be transferred into polymer beads and subsequently released to aqueous medium. Hytrel™ was chosen for bioreactor experiments as it can provide high aqueous phase biphenyl concentrations over large range of biphenyl concentrations in the polymer.

3.5.3 *Biodegradation of Biphenyl in Solid-Liquid TPPBs – Proof of Concept*

TPPB experiments were conducted to demonstrate the ability of solid-liquid TPPBs to deliver the hydrophobic model compound biphenyl to different degrading organisms which degrade biphenyl to completion. It is evident from the results shown in Figure 3-4 that both microbial systems could readily degrade the provided biphenyl within 50 h. The microbial consortium exhibited a shorter acclimatization phase and also a higher overall biomass yield than the pure culture. Both microbial systems were able to degrade biphenyl to concentrations below the detection limit, showing the suitability of solid liquid TPPBs for complete biphenyl degradation.

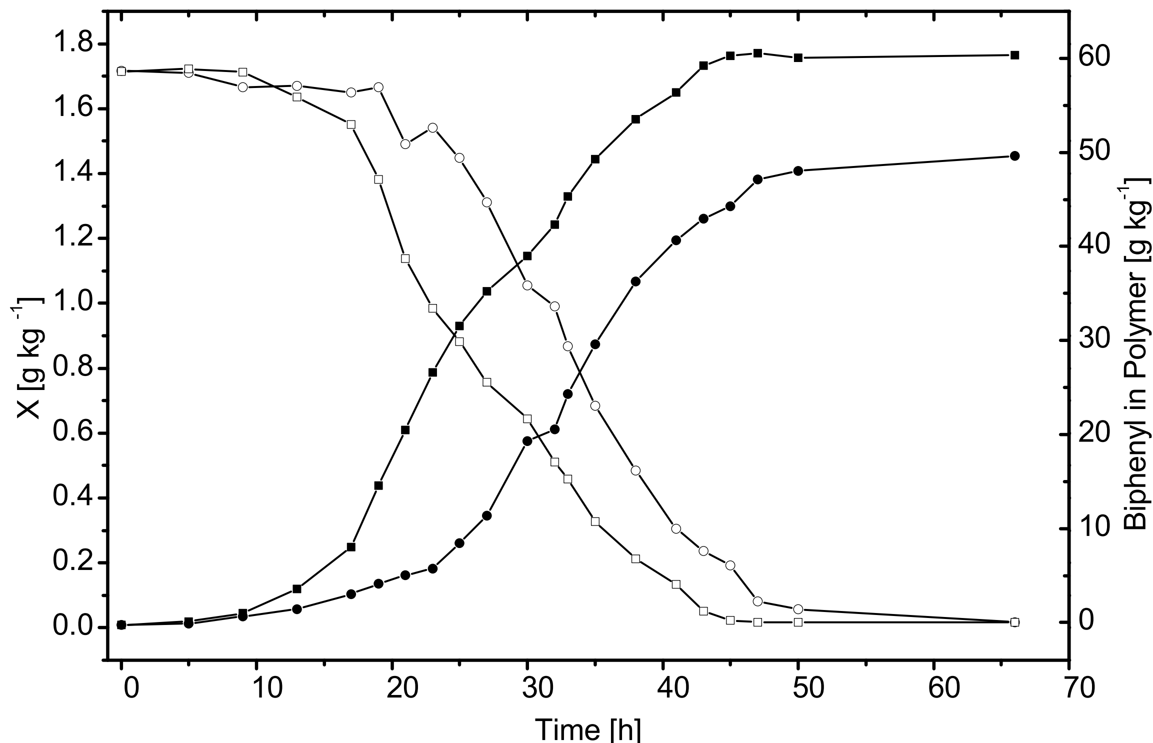

Figure 3-4: Biodegradation of biphenyl in solid liquid TPPBs by *B. xenovorans* LB400 (circles) and a microbial consortium (squares). The closed symbols represent the biomass concentration in the aqueous phase (cell dry weight) and the open symbols represent the biphenyl concentration in the solid phase.

Similar amounts of biphenyl could be degraded by *B. xenovorans* LB400 within 25 h – 30 h in liquid-liquid TPPBs (Rehmann and Daugulis 2006). A possible reason for the slower degradation rates in solid-liquid TPPBs is that the mass transfer rate of biphenyl from the solid phase into the liquid phase might be rate limiting. The available surface area of the employed polymer beads (3-4 mm diameter) is significantly smaller than the surface area available for mass transfer in liquid-liquid system (the estimated average droplet diameter is 30 μm (Wang and Ochoa 1972)). The biomass formation shown in Figure 3-4 also does not seem to follow the typical exponential trend of unrestricted microbial growth but rather a linear trend, a further indication of possible mass transfer limitation.

However, the biodegradation results clearly show that solid-liquid TPPBs can be used to deliver large amounts of poorly water-soluble substances to degrading organisms. The use of a solid delivery phase also allows the combination of TPPBs with large mixed microbial populations and surfactant producing organisms, which expands the spectrum of utilizable biocatalysts and demonstrates that solid liquid TPPBs can be used as a degradation platform for recalcitrant hydrophobic substances.

3.6 Conclusions

Hydrophobic substances such as biphenyl partition between water and selected polymers similarly as between water and organic solvents. The observed partition coefficients are polymer specific and can also reach values similar to those of organic solvents. The biphenyl capacity of the utilized polymers is higher when biphenyl is provided in methanol. Loading polymers such as Hytrel™ with large amounts of biphenyl from methanol and subsequently placing them in aqueous solution results in biphenyl saturation of the aqueous phase. The use of polymer delivery phases permits the use of microbial consortia and surfactant producing bacteria in TPPBs, thereby extending the range of biocatalysts that can be employed relative to two-liquid phase TPPB systems

Acknowledgements

We are grateful to the Natural Sciences and Engineering Research Council of Canada for financial support, and the Environmental Technologies Program at SAIC Canada.

Chapter 4: Biodegradation of Biphenyl in a Solid-Liquid Two-Phase Partitioning Bioreactor

Lars Rehmann, Andrew J. Daugulis

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as is currently in Press in: *Biochemical Engineering Journal* (2007)

4.1 Preface to Chapter 4

The work presented in the previous chapters has shown that biphenyl can readily be degraded by *B. xenovorans* LB400 in liquid-liquid and solid-liquid TPPBs. The microbial degradation rate is not limited by the mass transfer rate of biphenyl between the two phases in liquid-liquid systems. The available surface area for mass transfer in solid-liquid TPPBs however, is several orders of magnitude lower than in liquid-liquid TPPBs, employing similar phase ratios and a typical polymer phase consisting of cylinder with 2-6 mm diameters. This chapter therefore investigates the effect of mass transfer on achievable biodegradation rates in solid-liquid TPPBs.

The mass transfer rate of biphenyl between the polymer Hytrel™ and water was characterised as a function of the concentration gradient and the interfacial surface area. This could be combined with the kinetic parameters estimated in Chapter 3 to predict the dynamic substrate concentration in the aqueous phase during reactor operation and hence the microbial degradation rate. Different geometries of the polymer phase were employed and it was shown that the available surface area limits the mass transfer rate and thereby the microbial degradation rate. This could be shown both experimentally and mathematically.

The implications of the results in this chapter are that instantaneous equilibrium formation cannot be assumed when mathematically describing solid-liquid TPPBs. It was further shown that solid-liquid TPPBs might theoretically be able to overcome low aqueous phase substrate concentrations by favourable partitioning, but can still result in low aqueous phase substrate concentrations due to low mass transfer rates.

4.2 Abstract

Biphenyl was successfully degraded by *Burkholderia xenovorans* LB400, initially described as *Pseudomonas* sp. LB400, in a solid-liquid two-phase partitioning bioreactor (TPPB). Solid-liquid TPPBs are comprised of an aqueous, cell containing phase, and a solid polymeric phase that partitions toxic and/or poorly soluble substrates (in this case biphenyl) based on maintaining a thermodynamic equilibrium. The employed polymer was Hytrel™, a thermoplastic polyester elastomer. The surface area available for mass transfer of biphenyl was limiting and resulted in mass transfer limited growth, as demonstrated experimentally by employing two different geometric shapes (cylinders with different aspect ratios) of the polymer phase with different specific surface area, while keeping all other parameters constant. The linear microbial growth rates were substantially higher when more polymer surface area was provided.

The mass transfer coefficient of biphenyl from Hytrel™ to water was measured under experimental conditions, which allowed predicting the release rate based on the biphenyl concentration gradient. The partitioning behaviour of biphenyl between Hytrel™ and culture medium was measured as well, which allowed the development of a simple mechanistic model describing microbial growth based on known microbial properties in combination with substrate delivery from the solid polymer phase. The model was capable of describing the experimental data well and can be used to predict degradation rates for other geometric shapes of a solid delivery phase such as sheets or rods, which might be of operational advantage in various applications of TPPBs to the controlled uptake and release of other recalcitrant molecules.

Keywords: Availability, mass transfer, biokinetics, modelling, multiphase bioreactors, *Burkholderia xenovorans* LB400.

4.3 Introduction

Biphenyl is an aromatic hydrocarbon, comprised of two, six-sided aromatic rings connected at one carbon on each ring. It was mainly used as a precursor for polychlorinated biphenyls (PCBs), but is also industrially used as a dyestuff carrier for textiles and copying paper, as a solvent in pharmaceutical production and as a fungistat in transportation containers of oranges and other citrus fruits (Ambrose, et al. 1960; Weaver, et al. 1979). Biphenyl can be degraded aerobically by a variety of soil bacteria which are often also capable of degrading low chlorinated PCBs (Catelani, et al. 1971; Haddock, et al. 1993). Biphenyl has low solubility in water and high solubility in organic solvents suggesting the use of two-phase partitioning bioreactors (TPPBs) for microbial biphenyl degradation. Kinetic parameters for the strain *Burkholderia xenovorans* LB400 and the Monod model were previously estimated in liquid-liquid TPPBs (Rehmann and Daugulis 2006). TPPBs are typically stirred tank bioreactors containing two immiscible phases, an aqueous phase containing the biocatalyst (bacteria, yeasts, other fungi or mammalian cells) and a second phase functioning either as a substrate reservoir or a product sink (Daugulis 1997). Depending on the process, the second phase has to be carefully chosen to show high affinity for the desired product or the substrate, without interfering with the microbial system. TPPBs can be used to deliver large amounts of hydrophobic substrates to degrading organisms. Large amounts of substrates can be dissolved in the second phase, resulting in substrate partitioning to a reduced equilibrium concentration in the aqueous phase. Only substrate in the aqueous phase is available to the biocatalysts and degradation will result in a disequilibrium, which in turn will result in partitioning of additional substrate from the second phase into the aqueous phase. The substrate delivery into the aqueous phase is controlled by the microbial degradation rate if the mass transfer rate is

significantly larger than the microbial consumption rate. Substances for the second phases in TPPBs have traditionally been hydrophobic organic solvents such as octyl-alcohol or octadecene (Inoue and Horikoshi 1991). Aqueous-aqueous two phase systems have been employed (Droin and Cooper 1992) and more recently solid polymers have been used (Daugulis, et al. 2003). It was found that some thermoplastics have strong affinities for hydrophobic organic molecules and show partitioning behaviour similar to organic solvents.

Solid-liquid TPPBs have several advantages over liquid-liquid TPPBs in terms of biological compatibility of the second phase. Solid polymeric substances which are typically used in TPPBs are biologically inert, being neither toxic to the organism of choice nor can they substitute as an alternative carbon source and divert biological activity away from the target substrate (Prpich and Daugulis 2004).

The organic phase in liquid-liquid TPPBs has to be selected carefully to also fulfill these requirements, which often limits the choice of the delivery phase, and generally limits liquid-liquid TPPBs to pure strains or small well defined microbial consortia (Bruce and Daugulis 1991). The advantages of liquid-liquid TPPBs are the physical properties of the delivery phases. The fundamental principles of solvent extraction and the involved chemical and physical interactions are well understood, allowing systematic screening for organic solvents with high capacities for most target molecules (Malinowski 1999). A further advantage is the large interfacial area available for mass-transfer between aqueous and delivery phase under conditions of high mixing intensity. The two phases form a fine emulsion under operational conditions in a stirred tank bioreactor (n = 600rpm, Re>10,000). The average droplet diameter can be estimated to 30 µm, resulting in a total specific interfacial area of 8.7 $m^2 \cdot dm^{-3}$ (m^2 interfacial area

per dm^3 fermentation broth at 50 mL organic phase per L aqueous phase) (Wang and Ochoa 1972). Such conditions can allow the assumption of instantaneous equilibrium formation of a given substance partitioning between the two liquid phases (Nielsen, et al. 2005; Rehmann and Daugulis 2006)h. The available surface area for mass-transfer in solid-liquid TPPBs is determined by the size and shape of the employed solid phase. Typically employed delivery phases are spherical or cylindrical beads with diameter of 2 – 4 mm resulting in a total specific interfacial area of 0.07 $m^2 \cdot dm^{-3}$ (m^2 interfacial area per dm^3 fermentation broth at 50g solid phase per L aqueous phase). Other geometrical shapes with even smaller surface to mass ratios such as sheets or rods might hold operational advantages, but mass transfer limitations are likely to occur under such conditions.

The objective of this study was to determine whether solid-liquid TPPBs are suitable for biphenyl degradation by *B. xenovorans* LB400, which occurs at high microbial rates and to gain an understanding of the mass transfer processes involved in this system. Mass transfer limited cell growth has been shown to occur when insoluble substrates are delivered as solid crystal (Volkering, et al. 1992) and liquid-liquid TPPBs have been shown to overcome these limitation in some instances (Eibes, et al. 2006), whereas mass transfer rates are found limiting in others (Wang and Ochoa 1972). This is the first study to investigate the effects of mass transfer rates on microbial growth in solid-liquid TPPBs.

4.4 Theory

A schematic diagram of a solid-liquid TPPB is shown in Figure 4-1. A simple model for microbial growth on a single substrate delivered from a solid phase can be described as follows:

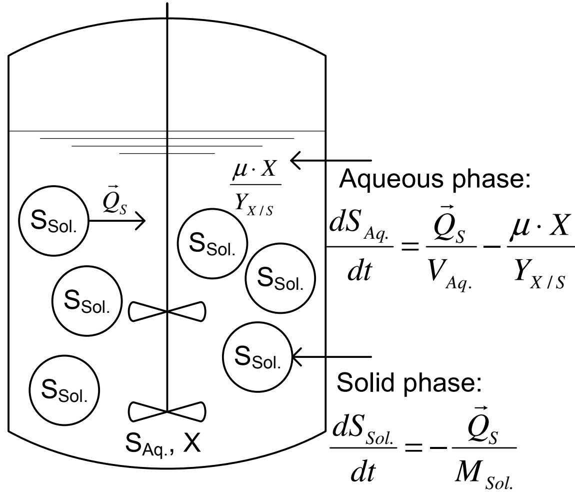

Figure 4-1: Schematic of solid liquid TPPB; the size of individual polymer beads is not to scale.

$$\frac{dX}{dt} = \mu \cdot X \tag{4-1}$$

where X is the biomass [g · m^{-3}], μ is the specific growth rate [h^{-1}] and t is time [h]. The change of substrate concentration in the aqueous phase consists of two terms, microbial degradation, which is linked to biomass formation via a yield coefficient (Tempest 1969) and the substrate flux from the solid phase $\vec{Q}_S$.

$$\frac{dS_{aq.}}{dt} = \frac{1}{V_{aq.}} \cdot \vec{Q}_S - \mu \cdot X \cdot \frac{1}{Y_{X/S}} \tag{4-2}$$

where $S_{Aq.}$ is the aqueous phase substrate concentration [g · m^{-3}], $\vec{Q}_S$ is the substrate flux from the solid phase [g · h^{-1}] and $V_{Aq.}$ is the volume of the aqueous phase [m^3]. The biomass yield coefficient has previously been estimated to be $Y_{X/S}$ = 0.48 g · g^{-1} (Rehmann and Daugulis 2006). The substrate concentration in the solid phase changes according to:

$$\frac{dS_{sol.}}{dt} = -\frac{1}{M_{sol.}} \cdot \vec{Q}_S \tag{4-3}$$

where $S_{sol.}$ is the concentration in the solid phase [g · kg^{-1}] and $M_{sol.}$ is the mass of the solid phase [kg]. The specific growth μ [h^{-1}] is dependent on the substrate concentration in the aqueous phase and can be modeled via the Monod model.

$$\mu = \frac{\mu_{\max} \cdot S_{aq.}}{K_S + S_{aq.}} \tag{4-4}$$

where μ_{max} is the maximum specific growth rate, previously estimated to μ_{max} =0.25 h^{-1} and K_S is the half saturation constant K_S=0.1 g · m^{-3} (Rehmann and Daugulis 2006). The flux from the solid phase to the liquid phase can be adopted from models describing flux from solid substrate crystal into solution (Perry and Perry 1963):

$$\vec{Q}_S = K_t \cdot A \cdot \left(S_{aq.}^{eq.} - S_{aq.}^{t}\right) \tag{4-5}$$

where K_t is a constant [m · h^{-1}], A is the interfacial surface area [m^2], $S_{aq.}^{eq.}$ is the substrate concentration in the aqueous phase in equilibrium to the substrate concentration in the solid phase [g · m^{-3}] and $S_{aq.}^{t}$ is the substrate concentration in the aqueous phase at the time t [g · m^{-3}].

A correlation between $S_{aq.}^{t}$ and $S_{sol.}$ as well as the mass-transfer coefficient K_t have to be determined experimentally.

4.5 Materials and Methods

4.5.1 *Chemicals*

All nutrients used in the fermentation media, and solvents, were obtained from either Sigma Aldrich (Canada) or Fisher Scientific (Canada). Biphenyl, 99 % (assay) was obtained from Alfa Aesar (USA). Hytrel™ (Hytrel™ is a registered trademark of E. I. du Pont de Nemours and Company) is a thermoplastic polyester elastomer with a density of 1.17 g · cm^{-3}. The polymer chain contains approximately 50% poly-butylene

terephthalate (PBT) and 50% butylene ether glycol terephthalate. It was found not to be available as a carbon source to the employed microorganism and no biofilm formation on the polymer surface has been observed (data not shown). Hytrel^{TM} polymer beads were obtained from DuPont Canada, in cylindrical shapes with a specific surface are of 1.49 $m^2 \cdot kg^{-1}$ (m^2 polymer surface per kg polymer). A fraction of these polymer beads was reduced in size to smaller cylinders with a higher specific surface area of 2.38 $m^2 \cdot kg^{-1}$. The reported surface area is the average of the mechanically measured surface areas (based on assumption of cylindrical shape) of 20 beads.

4.5.2 *Bacterial strain*

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, N.Y.), was obtained from the Northern Regional Research Laboratory (Peoria, Ill.). The strain has since been re-classified as *Burkholderia xenovorans* sp. nov. (Goris, et al. 2004). Cultivation conditions, medium formulation and biomass analysis can be founds elsewhere (Bedard, et al. 1986; Rehmann and Daugulis 2006).

4.5.3 *Biphenyl uptake*

Hytrel^{TM} beads (1 g) were added to scintillation vials containing 10 mL methanol and varying amounts of biphenyl. The system was allowed to equilibrate by placing it on a rotary shaker at 30 ºC for 48 hours. A control vial containing methanol and biphenyl but no Hytrel^{TM} was added for every vial containing Hytrel^{TM}. Biphenyl concentrations were measured with an Ultraspec 3000 UV/Visible Spectrophotometer (Biochrom, UK) at λ = 250 nm. The biphenyl concentration in methanol was measured after equilibration and the difference in concentration between the vials containing beads and the control vials was

attributed to partitioning into the Hytrel™ beads. The concentration in beads was calculated via mass balance and was considered to be in equilibrium with the final concentration remaining in methanol.

4.5.4 *Biphenyl release*

Hytrel™ polymer beads (1 g) were loaded with different amounts of biphenyl from methanol as described above. Methanol was removed by washing of the beads with water for 3 minutes. This step removed methanol from the bead surface and did not significantly reduce the amount of biphenyl present in the beads (confirmed by re-equilibrating the washed beads with methanol and measuring biphenyl concentration in methanol, data not shown). The methanol free beads were added to 50 mL cell-free culture medium and agitated at 600 rpm. Aqueous phase samples were periodically analyzed spectroscopically for their biphenyl concentration.

4.5.5 *Biphenyl biodegradation*

Experiments were undertaken in parallel in two 5-L New Brunswick BioFlo® III bioreactors, agitated each with two Rushton turbines at 600 rpm and aerated (sterile air) at 3 L min^{-1}. The aqueous-phase volume was 2000 mL and the mass of Hytrel™ beads was 105 g. Conditions were automatically maintained at 30°C, and at pH 6.9 by adding 3M KOH. A 50 mL inoculum was grown in a 100 mL shake flask on biphenyl for 24 hours, split into two equal volumes and inoculated into the two reactors. The first reactor contained Hytrel™ beads with a specific surface area of 0.079 m^2 dm^{-3} and the second reactor the same amount of beads with a specific surface area of 0.126 m^2 dm^{-3}. The beads were previously equilibrated with 200 mL methanol and 20 g biphenyl on a rotary shaker for 48 h at 30ºC and washed with sterile water, resulting in beads containing 78 g

biphenyl per kg beads. Biomass concentration in the reactors was periodically measured spectroscopically as described elsewhere (Rehmann and Daugulis 2006).

4.6 Results and Discussion

The hydrophobic substrate biphenyl is a solid crystal at ambient conditions. It has to be transferred into the designated solid phase of a TPPB in order to be delivered to the degrading organisms. This substrate uptake was mediated by dissolving biphenyl initially in methanol. Different amounts of biphenyl were dissolved in methanol, and Hytrel™ was added as a solid phase. Hytrel™ has no affinity for methanol (data not shown) and methanol physically attached to the polymer surface can easily be removed with water, prior to the release experiments. The amounts of biphenyl removed during this washing are insignificant due to the low solubility of biphenyl in water.

The calculated biphenyl concentration in the solid phase (based on mass balance) can be plotted against the equilibrium concentration in methanol to determine the partitioning behaviour. Biphenyl partitions between Hytrel™ and methanol with a constant partitioning coefficient in the measured range, as shown in Figure 4-2. Similar linear behaviour has been found for the partitioning of phenols between aqueous medium and Hytrel™ (Prpich and Daugulis 2006). The partitioning coefficient for biphenyl concentrations below 45 $g \cdot kg^{-1}$ in methanol was found to be $K_{S/L}^{m} = 1.019 \pm 0.021\ g \cdot g^{-1}$, showing that the relative affinities of Hytrel™ and methanol for biphenyl are almost identical. The constant partitioning coefficient allows calculation of the equilibrium biphenyl concentration in Hytrel™ according to equation 4-6.

$$S_{Sol.} = \frac{M_{BP.}}{M_{Sol.} + M_{liq} / K_{S/L}^{m}} \quad (4\text{-}6)$$

where $M_{BP.}$ is the mass of biphenyl in the system [g] and $M_{liq.}$ is the mass of methanol [kg].

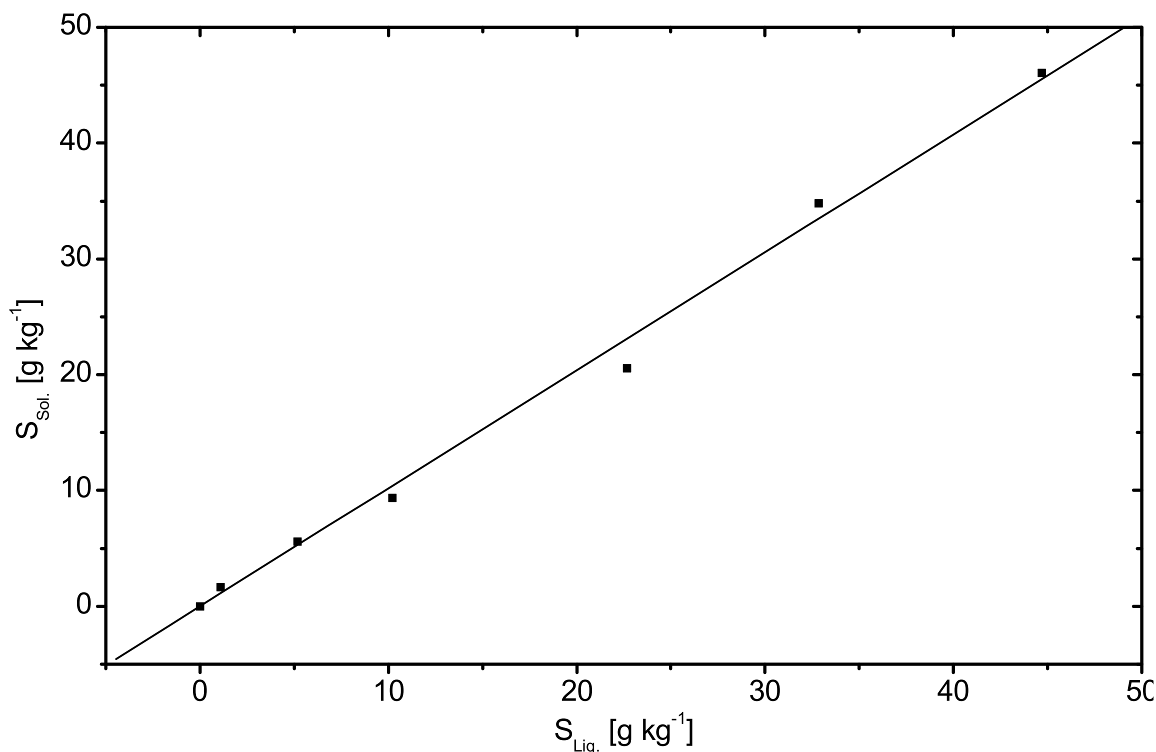

Figure 4-2: Partitioning of biphenyl between Hytrel™ and methanol at 30 ºC.

The dynamic release of biphenyl from Hytrel™ polymers into cell-free microbial culture medium was investigated under reactor conditions. Figure 4-3 shows the biphenyl concentration in the aqueous phase as a function of time for different initial biphenyl concentrations in Hytrel™. It can be seen that both the rate and extent of the release vary substantially with the initial biphenyl concentration in Hytrel™. The extent of the release is expected to be the concentration in the aqueous phase in equilibrium with the biphenyl content in the polymer. However, initial biphenyl concentration in the solid phase > 35 g · kg^{-1} released biphenyl at the same rate and to the same final concentration in the aqueous phase. The highest achieved aqueous phase concentration was 0.00692 g · dm^{-3}, which is the solubility of biphenyl in the employed medium at 30 ºC. The solubility of biphenyl has been reported to be 0.00608 g · dm^{-3} at 25 ºC for aqueous phase solutions with similar salt contents (Bohon and Claussen 1951), which is very similar to the value found here.

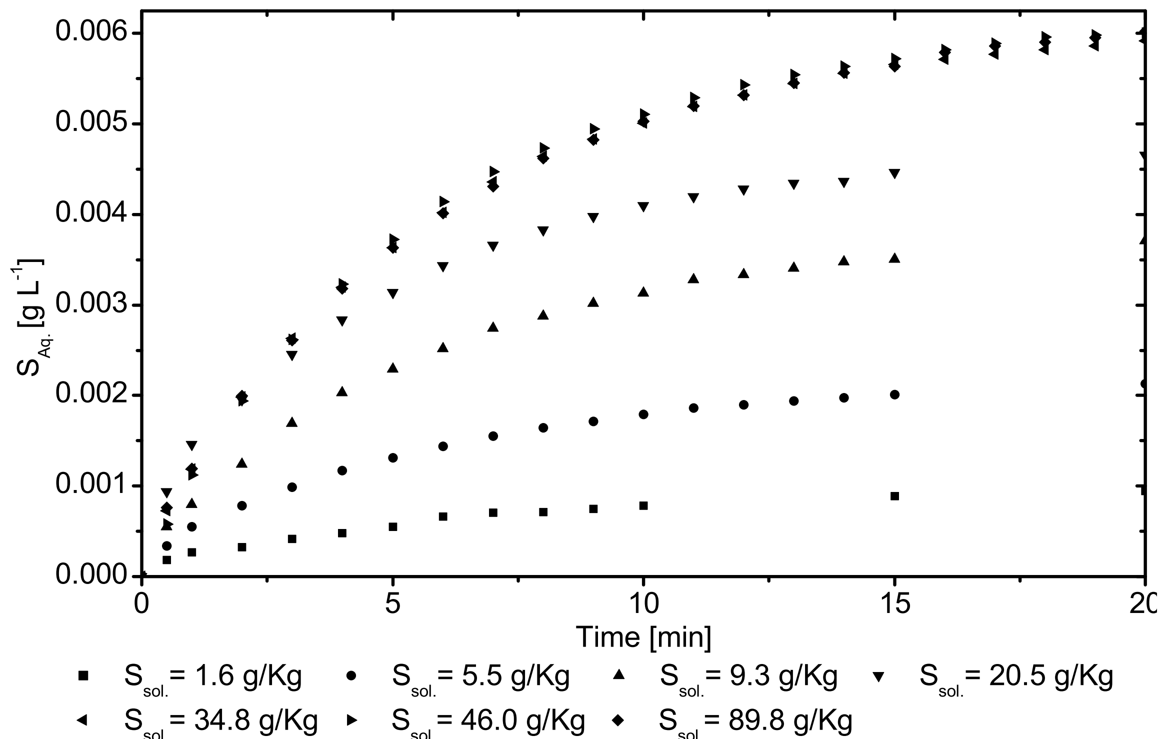

Figure 4-3: Biphenyl release from 1 g Hytrel™ into 50 mL water at different initial biphenyl concentrations in Hytrel™.

The observed release rates are very rapid if compared to solubilization rates of other hydrophobic compounds such as PAHs. Viamajala et al. (2007) achieved constant concentrations of phenanthrene, fluorene and fluoranthene in aqueous solution after 20 h to 40 h during solubilization experiments. Delivery of biphenyl from Hytrel™ beads achieved saturation/equilibrium concentration within 20 to 40 min. The differences in the rates might be due to differences in aqueous phase solubility of the PAHs compared to biphenyl (the solubility of the above mentioned PAHs is approximately one order of magnitude lower than the solubility of biphenyl) and the lower agitation rates employed by Viamajala et al (2007).

The biphenyl concentration in the solid phase remains essentially constant over the time of the release experiments due to the high initial concentrations in the polymer and the low solubility of biphenyl in the aqueous phase. The total change in concentration

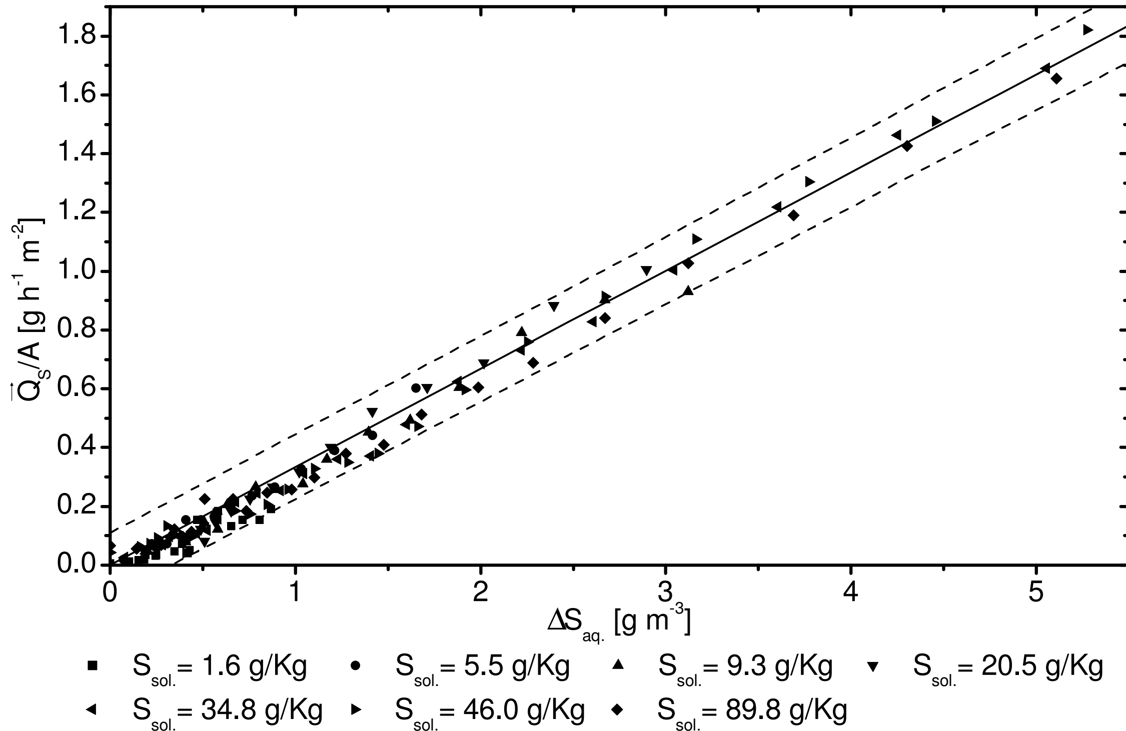

Figure 4-4: Normalized biphenyl release rate (estimated via centered leap-frog estimation) versus driving force during biphenyl release from 1 g Hytrel^{TM} into 50 mL culture medium at different initial biphenyl concentrations in Hytrel^{TM}. The solid line shows a linear regression of the data and the dashed lines the 95 % confidence limit.

in the polymer phase under experimental conditions is between 0.3 % and 1.7 %, depending on the initial concentration of biphenyl in Hytrel^{TM}.

Neglecting the change of biphenyl concentration in Hytrel^{TM} and assuming no biphenyl consumption or loss in the aqueous phase allows simplifying equation 4-5 to:

$$\vec{Q}_S = \frac{dS_{aq.}}{dt} \cdot V_{aq.} = K_t \cdot A \cdot \left(S_{aq.}^{eq.} - S_{aq.}^{t}\right) \tag{4-7}$$

where the equilibrium biphenyl concentration in the aqueous phase $S_{aq.}^{eq.}$ equals the final biphenyl concentrations $S_{aq.}^{\infty}$ in the release experiments, which allows the estimation of K_t by plotting $\frac{\vec{Q}_S}{A}$ versus $S_{aq.}^{\infty} - S_{aq.}^{t}$, as shown in Figure 4-4.

The same linear relationship between release rate and the driving force

$\left(S_{aq.}^{\infty} - S_{aq.}^{t}\right)$ can be seen for all experimental conditions. K_t was estimated by least square regression to be $K_t = 0.33397 \pm 0.00533$ m · h^{-1}. The rate at which biphenyl is released from Hytrel^{TM} at any given time therefore depends on the concentration gradient, the mass transfer coefficient, and the available surface area, with the latter two expected to remain constant during the course of a fermentation.

Predicting release rates during bioreactor operation requires a relationship between the concentrations in the solid and aqueous phases. From the initial Hytrel^{TM} loadings and the equilibrium aqueous concentrations presented in Figure 4-3 it can be deduced that there is no linear relationship between the equilibrium concentration in the aqueous phase and the initial (and final) concentration in the solid phase.

Figure 4-5 shows the relationship between the equilibrium biphenyl concentration in the aqueous phase and in the solid phase. The data clearly show that the partitioning coefficient is not constant over the employed range of concentrations. A constant partitioning coefficient of approximately log $K_{S/W}^{m}$ =3.5 can be found for $S_{Sol.}$ <25 g · kg^{-1}. This value is similar in magnitude to the octanol water partitioning coefficient of biphenyl, log $K_{O/W}^{m}$= 3.89 (Rapaport and Eisenreich 1984). The partitioning coefficient increases for biphenyl concentration in the solid phase > 25 g · kg^{-1}. An empirical asymptotic equation was used to predict the aqueous phase equilibrium concentration as a function of the biphenyl concentration in the solid phase over the entire concentration range, as shown by the solid line in Figure 4-5. The data could be described by:

$$S_{aq.}^{\infty} = 7.05417 \cdot 10^{-3}\left(1 - 0.92902^{S_{sol.}}\right) \qquad (4\text{-}8)$$

Various methods to describe solid-liquid equilibria thermodynamically can be found in the literature (Costa, et al. 2006; Ioannidis and Anderko 2000; Slaughter and

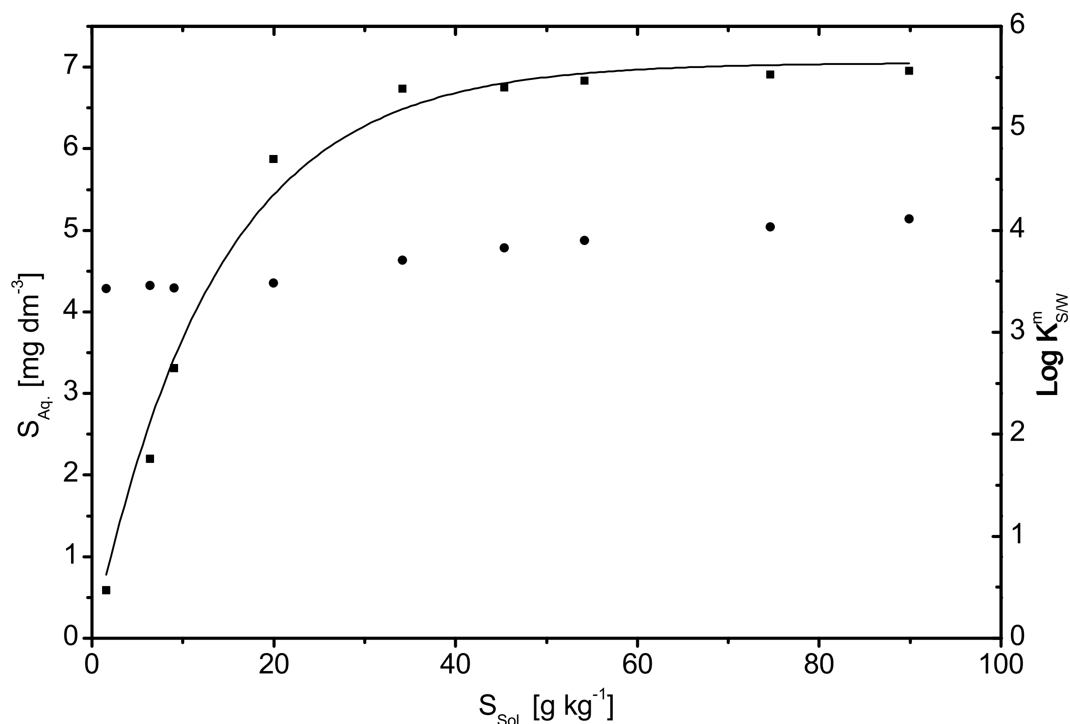

Figure 4-5: The log solid liquid partitioning coefficient of biphenyl log $K^{m}_{S/W}$ as a function of biphenyl concentration in the Hytrel^{TM} (circles) and the equilibrium aqueous phase biphenyl concentration $S^{\infty}_{aq.}$ as a function of the initial biphenyl concentration in Hytrel^{TM} $S_{Sol.}$ (squares). The solid line shows an empirical non-linear regression of the data.

Doherty 1995). Different methods have been applied, ranging from predicting activity coefficients of the target compound in both phases via the UNIFAC model (Gmehling, et al. 1993), to treating the solid phase containing the target molecule as a new compound (Slaughter and Doherty 1995), or to describing the interaction between the solid phase and target compound as surface adsorption (Ioannidis and Anderko 2000). Some of these methodologies might be applicable to the situation in this study, however, a fundamental thermodynamic description of the solute polymer interaction is beyond the scope of this study and also is not required to describe the effects of mass transfer limitations on microbial performance in TPPBs.

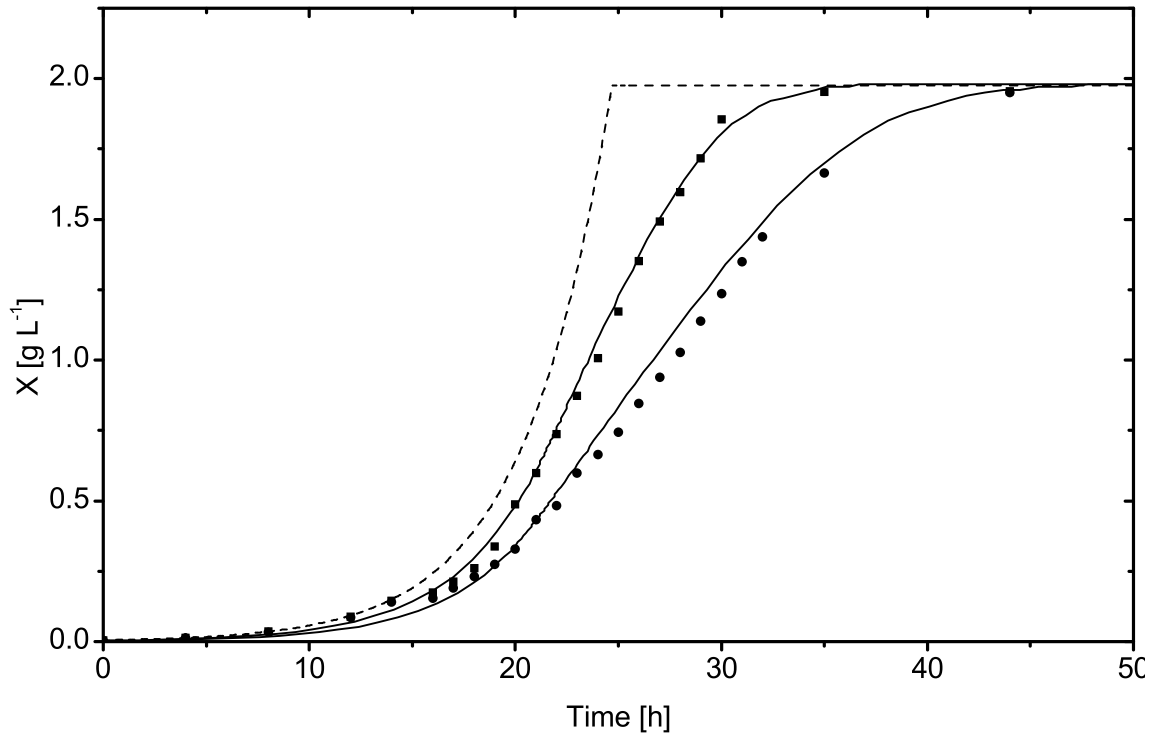

Figure 4-6: Biodegradation of biphenyl by *B. xenovorans* LB400 delivered from Hytrel™ polymer beads. The circles represents the biomass when biphenyl was delivered from Hytrel™ beads with a specific surface area of 0.079 m^2 dm^{-3} and the squares when biphenyl was delivered from beads with a specific surface area of 0.126 m^2 dm^{-3}. The solid lines represent the simulated biomass formation and the dashed line the biomass formation with no mass transfer limitation.

Using the estimated parameters for K_t, μ_{max} and K_S and the empirical equation 4-8 allows simulating microbial biphenyl degradation in a solid-liquid TPPB by solving equations 4-1 to 4-5. These equations were solved numerically using the ordinary differential equation solver of Matlab (ODE23s). Figure 4-6 shows the biomass formation during biphenyl degradation by *B. xenovorans* LB400. The circles show the biomass formation and the corresponding line the simulated values. It is evident that the model describes the experimental data very well. Initially, biomass formation follows the typical exponential growth curve. After approximately 20 h biomass seems to increase linearly rather than exponentially, a clear indication of a mass transfer limited system, as captured by the model. The dashed line shows the expected course of the fermentation assuming

instantaneous equilibrium formation (e.g. no mass transfer limitation), as observed in liquid-liquid TPPBs (Rehmann and Daugulis 2006). The effect of the mass transfer limitation is vast; the total fermentation time increases from 25 h to 44 h. The squares in Figure 4-6 represent the same fermentation with Hytrel^{TM} beads of a smaller diameter. The total amount of Hytrel^{TM} and the initial biphenyl concentration in the solid phase were identical, but the specific surface area was increased by approximately a factor of 1.6. It can also be seen that the overall rate increases significantly over the rate observed with the larger beads and the total fermentation time decreases to approximately 35 h, whereas the time course also contains the characteristic linear growth kinetics. The corresponding solid line shows the solution of equations 4-1 to 4-5 and 4-8 with the same parameters as before and the increased surface area A.

The simulated and the experimental data fit very well, showing that the model describes the system within the range of operating conditions and can also potentially describe delivery phases with different geometries. Using geometries with larger specific surface areas (e.g. smaller beads) would result in curves more similar to the ones observed in liquid-liquid systems, whereas geometries with smaller specific surface areas (sheets or rods) would yield curves with linear segments with a smaller slope. Such geometries are of interest for bioremediation applications. Prpich et al. (2006) recently applied polymer beads to extract phenol contaminations from soil followed by ex situ biodegradation of phenol in a solid liquid TPPB. Sheets or rods would provide operational advantages during the soil extraction step, and the results of this study can help predict the effects of these geometries on substrate release to the degrading organisms.

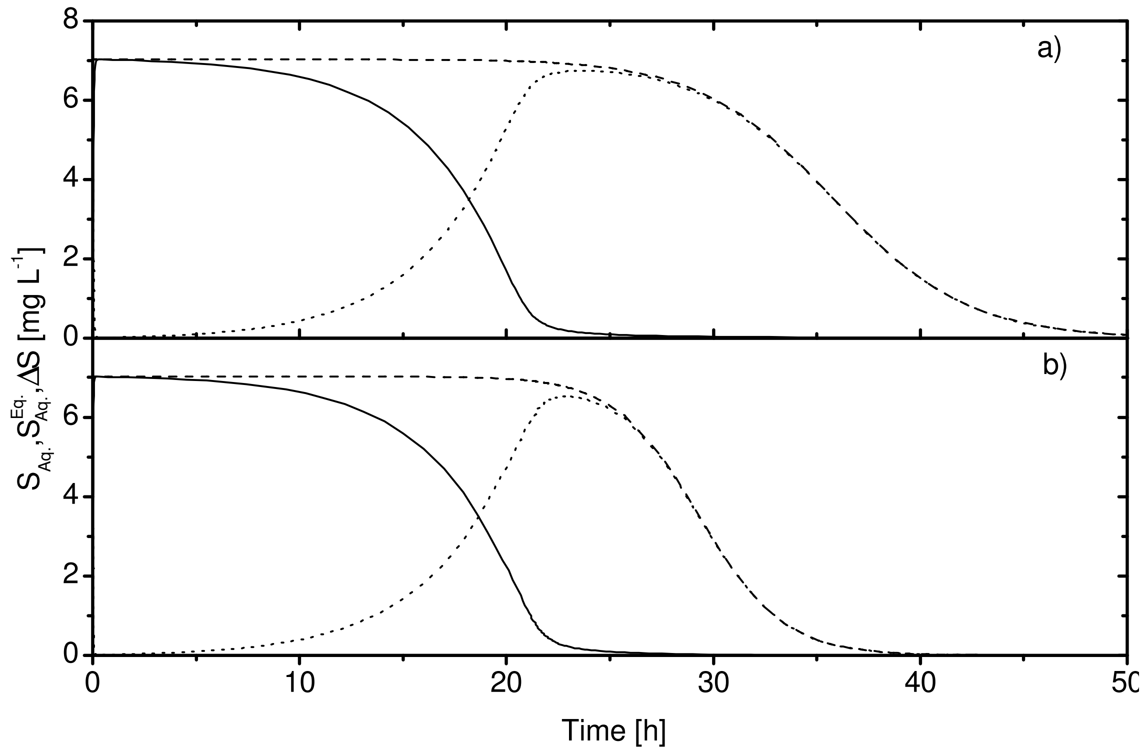

Figure 4-7: Simulated non-equilibrium aqueous phase substrate concentration (solid lines), hypothetical equilibrium substrate concentration (dashed lines) and the mass transfer driving force ΔS (dotted lines) as a function of fermentation time. The simulated values for Hytrel^{TM} beads with a specific surface area of 0.079 m^2 dm^{-3} are shown in plot a) and the simulated values for Hytrel^{TM} beads with a specific surface area of 0.126 m^2 dm^{-3} are shown in plot b).

The effect of mass transfer limitation is shown more dramatically in simulations plotted in Figure 4-7. Taking into account the rate of microbial uptake of biphenyl, as well as its rate of release from the polymer, allows predicting the non-equilibrium aqueous phase biphenyl concentration by solving equations 4-1 to 4-5 and 4-8 for $S_{Aq.}$. The non-equilibrium biphenyl concentration in the aqueous phase declines at almost similar rates for both delivery phase geometries and falls below 0.1 mg · dm^{-3} after approximately 25 h. At this time the biomass concentration is X = 0.84 g · dm^{-3} for the larger beads and X = 1.23 g · dm^{-3} for the smaller beads as shown in Figure 4-6, indicating that 50 % more biphenyl has been consumed in the case of the smaller beads. This can also been seen through the larger predicted equilibrium concentration displayed as the

dashed lines in Figure 4-7. This aqueous biphenyl concentration would be achieved if phase equilibrium was established due to partitioning without simultaneous microbial uptake, but is never reached in the observed system as the microbial biphenyl consumption is faster than the mass transfer rate of biphenyl from the Hytrel^{TM} beads into the aqueous phase. The difference between the actual biphenyl concentration and the equilibrium concentration is the mass transfer driving force $\Delta S = S_{aq.}^{\infty} - S_{aq.}^{t}$, represented by the dotted lines in Figure 4-7. The driving force is low during the initial period of both fermentations, indicating that the mass transfer rate is sufficiently large compared to the microbial substrate consumption rate, allowing the biphenyl concentration to be almost at equilibrium in both phases (solid and aqueous phase). The driving force reaches a maximum between 25 and 30 hours and decreases towards the end of the fermentation. The reactor providing more specific surface area (Figure 4-7b) operates at aqueous phase biphenyl concentrations far below the equilibrium concentration only for the last 15 hours of the fermentation (seen by the maximum in the driving force), whereas the reactor providing less surface area Figure 4-7) operates under these conditions for the last 30 hours of the fermentation. The reactor using the larger bead size operates under mass transfer limited conditions for a longer period of time than the reactor providing more surface area, which limits the bioavailable amount of substrate and results in lower microbial degradation rates and longer fermentation times.

4.7 Conclusions

Hydrophobic compounds such as biphenyl can be delivered to degrading organisms from Hytrel^{TM} polymers is solid-liquid TPPBs. The mass transfer rate of the substrate into the aqueous phase is lower than in liquid-liquid two-phase systems

(Rehmann and Daugulis 2006), but higher than in aqueous systems containing pure substrate crystals (Viamajala, et al. 2007). The mass transfer rate can be lower than the microbial degradation rate resulting is substrate limited growth, which could be described successfully with a simple mechanistic model. The framework of this model can potentially be extended to other solid phase geometries which might be advantageous as easily removable contaminant absorbents in bioremediation applications (Prpich, et al. 2006).

Acknowledgements

We are grateful to the Natural Sciences and Engineering Research Council of Canada for financial support, and the Environmental Technologies Program at SAIC Canada.

Chapter 5: Bioavailability of PCBs in Biphasic Bioreactors

Lars Rehmann, Andrew J. Daugulis

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as is currently in Press in: *Biochemical Engineering Journal* (2007)

5.1 Preface to Chapter 5

The previous three chapters investigated various aspects of substrate availability of biphenyl in TPPBs. Biphenyl was used as it is an excellent model compound for hydrophobic substrates and its degradation pathway is the same as for the environmentally relevant PCBs. The chlorine atoms on PCBs prove an additional challenge during biodegradation. The results from the previous chapters therefore cannot be directly applied to PCBs, but can qualitatively show where PCB degradation in TPPBs might be limited.

This chapter shows the effect of substrate limitation of PCBs during biodegradation in liquid-liquid TPPB systems by *B. xenovorans* LB400. It was shown experimentally that low equilibrium concentrations of PCBs in the aqueous phase were rate-limiting, while the mass transfer rate of PCBs from the non-aqueous phase into aqueous medium did not pose a limitation. These findings were in accordance with results found during biphenyl degradation, showing that biphenyl can function as a model compound to identify possible limitations during biodegradation of PCBs.

The results presented in this chapter further show that the initial PCB concentrations in the non-aqueous phase should be maximized to achieve high degradation rates, which has implications for remediation processes involving PCB extraction from soil followed by biodegradation in TPPBs. It was further shown that a high initial biomass concentration can result in high volumetric degradation rates, while the specific degradation rates (rates normalized to the biomass concentration) were low. The total amount of PCBs degraded per reactor volume was 10 mg l^{-1}, which was extended to larger amounts as shown in Chapter 6.

5.2 Abstract

Polychlorinated biphenyls (PCBs) are xenobiotic and toxic contaminants of soil and sediment. A possible remediation scheme involves solvent extraction followed by microbial biodegradation in biphasic bioreactors. This study examined the effect of two water immiscible liquid phases on the extent and rate of aerobic Aroclor® 1242 biodegradation by *Burkholderia xenovorans* LB400 under controlled conditions. The immiscible phases were neither toxic nor biodegradable by the employed organism and it was found that reduced aqueous phase availability, caused by the immiscible phases, had a negative effect on the extent and rate of degradation. The initial PCB concentration in the immiscible phase and the nature of the immiscible phase was shown to influence biodegradation. It was further found that under the employed conditions, providing intensive mixing, the microbial degradation rate and not the mass transfer of PCBs from the immiscible phase into the aqueous phase was limiting the degradation process. Despite reduced specific microbial degradation rates in the presence of a water immiscible phase a maximum volumetric degradation rate of 0.44 mg l^{-1} h^{-1} in the presence of 0.1 % silicone oil could be achieved. The findings in the study show that microbial degradation of PCBs in biphasic bioreactors might be a suitable technology for the treatment of solvent extracts of contaminated soils.

KEYWORDS: Aroclor® 1242, availability, *Burkholderia xenovorans* LB400, sequestration, equilibrium partitioning

5.3 Introduction

Polychlorinated biphenyls (PCBs) are toxic xenobiotics, manufactured until the late 1970s, and mixtures were commercially sold in North America under the trade name Aroclor®. PCBs had a wide industrial use prior to their ban in 1978 and are now widely distributed contaminants of soil and sediment (Abramowicz 1990; Robinson and Lenn 1994). PCBs are subject to microbial degradation under anaerobic conditions via reductive dehalogination (Mohn and Tiedje 1992; Tiedje, et al. 1993) and aerobic conditions via the biphenyl pathway (*bph*-pathway). One of the best studied aerobic PCB degraders is *Burkholderia xenovorans* LB400, which is known to be able to degrade up to hexachlorinated biphenyls (Bopp 1986; Fain and Haddock 2001; Hofer, et al. 1994) and was therefore used in this study.

Rates and extents of natural PCB degradation at different sites vary considerably (Young and Cerniglia 1995) and the extent and rate of degradation depends on the congener composition of the contaminating PCBs (Aroclor® 1242 consists of mainly tri- and tetrachlorobiphenyl and is mainly subject to aerobic degradation, whereas Aroclor® 1260 consists of predominantly hexa- and heptachlorobiphenyl, making it mainly subject to reductive dechlorination), on the physical parameters at the contaminated site, and on the presence of organisms capable of PCB degradation (Abramowicz 1990; Amend and Lederman 1992). Another critical parameter is the presence of residual petroleum products which are, due to their widespread use in industrial applications, often found at PCB contaminated sites (Young and Cerniglia 1995). These hydrocarbons can form separate phases in soil or sediments and PCBs can dissolve in these non aqueous phase liquids (NAPL), changing the soil -or sediment- water distribution coefficient of PCBs (Luthy, et al. 1997; McNamara, et al. 2005; Zwiernik, et al. 1999).

The negative effect of co-contaminates at PCB contaminated sites has often been attributed to physiological and physiochemical factors. Short chain aliphatic hydrocarbons are significantly more soluble in water than PCB congeners and can solvate the lipids in bacterial cell membranes and thereby alter the permeability of the membrane or destroy its structure (Bruce and Daugulis 1991). Hydrocarbons also provide a major source of carbon and therefore a selective advantage for hydrocarbon utilizing bacteria, often resulting in an increase of less diverse microorganisms (Thomas and Ward 1992).

More recently it has been shown that the aqueous phase PCB concentration might be controlled by equilibrium partitioning of PCBs between oil and water (Ghosh, et al. 2000; McNamara, et al. 2005). It could further be shown that aqueous phase PCB concentration increased over time in sediments in which the degradation rate of NAPL was faster than the degradation rate of PCBs, thus resulting in an increase of PCB availability (Ghosh, et al. 2000). Increased PCB availability might be beneficial for biodegradation, but it also results in increased toxicity at the PCB contaminated site, increased bioaccumulation of PCBs in wildlife, as well as increased PCB mobility (Millward, et al. 2005). Some current studies have therefore focused on reducing PCB availability by adding various sorbents to sediments, reducing the overall toxicity (Ghosh, et al. 2003; Millward, et al. 2005; Zimmerman, et al. 2004).

A possible process to remediate PCB contaminated soils is solvent extraction followed by biodegradation in a biphasic reactor environment (Amend and Lederman 1992). Such a reactor would operate according to the two-phase partitioning bioreactor concept, where large amounts of hydrophobic and/or toxic compounds are dissolved in a water-immiscible phase before being introduced to a bioreactor, where microbial degradation of these compounds could take place in the aqueous phase (Daugulis 2001).

Solvent extraction of soil contaminants followed by biodegradation in TPPBs has been demonstrated successfully for polyaromatic hydrocarbons (PAHs) (Janikowski, et al. 2002). Sequestration of PCBs between the aqueous phase and the immiscible phase might however reduce the availability to the degrading organism in a similar way as NAPL co-contamination do in soil environments. It is therefore the objective of this study to investigate the effect of hydrophobic water immiscible phases on the degradability of PCBs under controlled conditions.

5.4 Materials and Methods

5.4.1 *Chemicals.*

All chemicals used in the fermentation media and the solvents were obtained from either Sigma Aldrich (Canada) or Fisher Scientific (Canada). Biphenyl, 99 % (assay) was obtained from Alfa Aesar (USA) and Aroclor® 1242 (CAS Number: 53469-21-9) was obtained from Chromatographic Specialties Inc. (Brockville, Ontario).

5.4.2 *Bacterial Strain.*

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, N.Y.) (Bopp 1986), was obtained from the Northern Regional Research Laboratory (Peoria, Ill.). The strain has since been re-classified as *Burkholderia xenovorans* sp. nov. (Goris, et al. 2004). Cultivation conditions and maintenance were described previously (Rehmann and Daugulis 2006).

5.4.3 *Scintillation Vial Experiments.*

A time course of PCB degradation in the absence of an immiscible phase was generated and experiments to investigate the effect of a wide range of immiscible to aqueous phase ratios where conducted in scintillation vials.

5.4.3.1 Time Course of Neat Aroclor® 1242 Degradation.

Sterile 20 ml scintillation vials were filled with 0.5 ml sterile medium (mineral salt medium (PAS) (Bopp 1986)). Neat Aroclor® 1242 was dissolved in acetone, filter-sterilized (0.22 μm nylon syringe filter (Fisher Scientific, Canada)) and added to the medium to a desired total concentration of 20 mg l^{-1} Aroclor® 1242. The vials were kept open in the fume hood for 48 hours to allow the acetone to evaporate. *B. xenovorans* LB400 was grown for 24 hours at 30 °C on an orbital shaker set at 180 rpm on biphenyl in PAS medium, filtered through sterile glass wool to remove excess biphenyl crystals, centrifuged at 3000 rpm for 20 min at 4°C, washed twice with 0.05 M sodium phosphate buffer, pH 7.5, and re-suspended in PAS medium to a concentration of 0.6 g l^{-1}. 0.5 ml of this cell suspension was added to each of the vials containing medium and Aroclor® 1242, resulting in final biomass concentrations of 0.3 g l^{-1} and Aroclor® 1242 concentrations of 10 mg l^{-1}. The vials were closed with Teflon-lined caps and incubated for up to 5 days at 30 °C on an orbital shaker set at 180 rpm. Vials were periodically sacrificed and the cells were killed by addition of 10 μL 10 N HCl and the remaining PCBs were extracted three times with 2 ml hexane. Controls vials with no biomass were treated identically.

5.4.3.2 Effect of Presence of an Immiscible Phase on the Extent of PCB Degradation.

Vials were prepared as described above. In addition to Aroclor® 1242 Bis(2-ethylhexyl)sebacate (BES) and low viscosity silicone oil (5 cSt) dissolved and filter-sterilized in acetone were added to achieve final equivalent immiscible phase to aqueous phase concentrations between 1 mg l^{-1} and 10,000 mg l^{-1}. All vials were incubated on a rotary shaker at 180 rpm for 5 days, prior PCB extraction and analysis. Experiments were undertaken in duplicate and numbers are reported as the average values.

5.4.4 *Bioreactor Experiments.*

To ensure no physical limitations (e.g. mixing, pH, dissolved oxygen) bioreactors rather than scintillation vials were employed to investigate the effect of the presence of an immiscible phase on PCB degradation rates.

5.4.4.1 Inoculum Preparation for Bioreactors.

The inoculum was prepared in a single fermentation vessel to generate enough biomass to operate three PCB degradation reactors concurrently with different amounts and types of immiscible phase but otherwise under similar conditions, including identical inocula. *B. xenovorans* LB400 was cultivated in a 5-L New Brunswick BioFlo® III bioreactor at 30 °C, agitated with two Rushton turbines at 500 rpm and aerated (sterile air) at 3 l min^{-1}. Biphenyl was delivered from Hytrel™ polymer beads, which were pre-loaded from biphenyl saturated methanol, as the sole carbon source. Cultivating *B. xenovorans* in solid-liquid TPPBs allows the generation of high inoculum concentrations as well as subsequent complete biphenyl removal before use; the rationale for, and details of, this procedure are described in detail elsewhere (Rehmann and Daugulis 2007b). Cells were harvested in mid exponential phase and the solid immiscible phase was removed by filtration through sterile glass wool. Biomass was removed from culture medium via centrifugation at 3000 rpm for 20 minutes at 4 °C and re-suspended in fresh medium to the desired biomass concentration and distributed to three 1 l BioFlo® I bioreactors.

5.4.4.2 Effect of Presence of an Immiscible Phase on the Rate of PCB Degradation.

Rate investigations were conducted in three parallel bioreactors. Aroclor® 1242 (10 mg) was dissolved in 1 ml silicone oil (Reactor 1), 1 ml BES (Reactor 2) and 10 ml silicone oil (Reactor 3). Bioreactor experiments were undertaken in 1 l BioFlo® I bioreactors at 30 °C, agitated with two Rushton turbines at 500 rpm and aerated (sterile

air) at 1 l min^{-1}. The aqueous-phase volume was 1000 ml (PAS medium with 1.2 g l^{-1} *B. xenovorans* LB400) and the initial amount of PCBs was 10 mg neat Aroclor® 1242 in each reactor. The pH was maintained at 6.9. Samples were taken periodically for PCB and biomass analysis. The agitation rate was increased to 900 rpm prior to sampling to ensure that samples contained representative fractions of both liquid phases. Samples were extracted three times with 2 ml hexane and analyzed via GC-ECD

5.4.4.3 *Effect of Agitation rate in the Presence of an Immiscible Phase on the Rate and Extent of PCB Degradation.*

The effect of the agitation rate on microbial degradation was investigated in three parallel bioreactors. Aroclor® 1242 (10 mg) was dissolved in 10 ml silicone oil each and the agitation rate in the individual reactors were n = 200 rpm, n = 400 rpm and n = 600 rpm. The reactors were operated as described above.

5.4.5 *PCB Analysis.*

Samples were extracted three times with 2 ml hexane. The hexane extract was analyzed with an Agilent 6890 gas chromatograph (Agilent Technologies Canada Inc., Mississauga, Ontario) equipped with a fused silica cap capillary column (Supelco SPB-1, Sigma-Aldrich Corp. St. Louis, MO, USA), an electron capture detector (ECD) (280 °C) and split injector (250 °C). The temperature program was as follows: 100 °C for 4 min, 100 °C to 180 °C at 10 °C min^{-1}, 180 °C for 1 min, 180 °C to 240 °C at 1.5 °C min^{-1}, 240°C for 1 min, 240 °C to 300 °C at 20 °C min^{-1}, 300 °C for 10 min. Aroclor® standards were run for every analysis and blank hexane was run after every four samples. Aroclor® was quantified by using the summed peak area according to EPA Method 304 h.

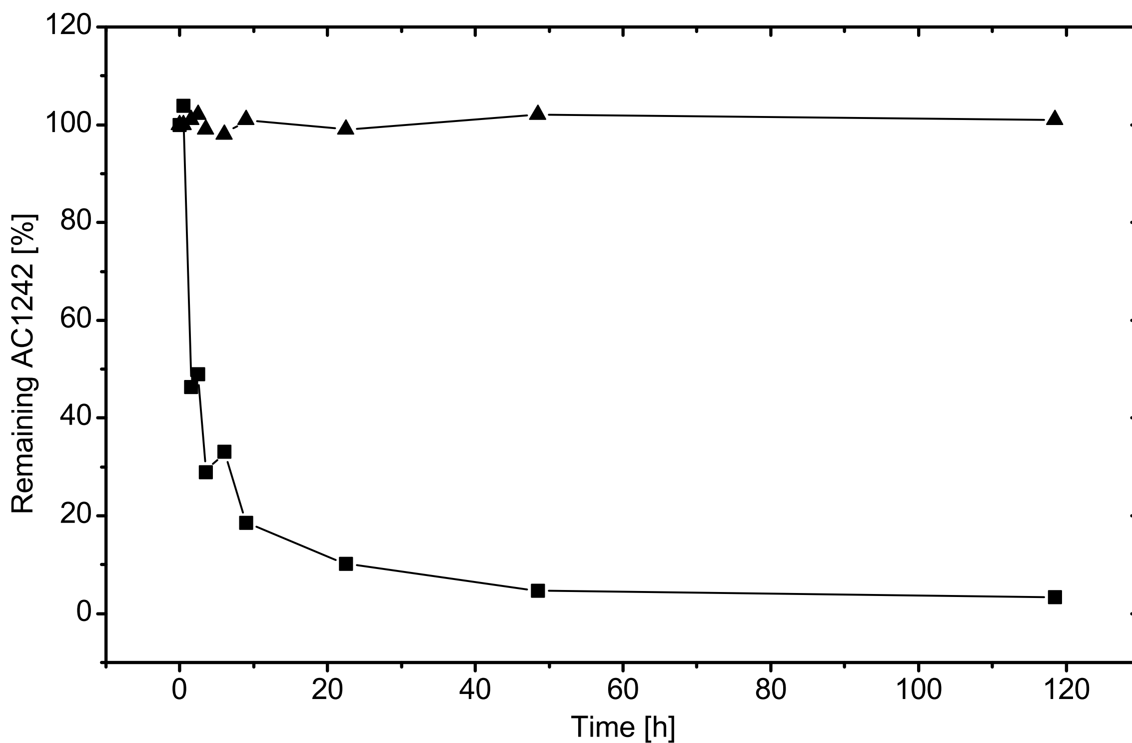

Figure 5-1: Biodegradation of Aroclor® 1242 by *B. xenovorans* LB400 in scintillation vials at an initial biomass concentration of 0.3 g l^{-1} in PAS medium with no biphenyl (squares). The triangles show the Aroclor 1242® in a control experiment with killed biomass.

5.5 Results and Discussion

5.5.1 *Scintillation Vial Experiments.*

5.5.1.1 Time Course of Neat Aroclor® 1242 Degradation.

The time course for the degradation of neat Aroclor® 1242 in scintillation vials was determined to establish a benchmark degradation time to subsequently study the effect of the presence of an immiscible organic phase on the extent of Aroclor® 1242 degradation. The time course is shown in Figure 5-1, and the achieved extent and rate are similar to degradation data reported elsewhere (~80 % of Aroclor 1242 at 10 mg l^{-1} after 48 h) (Billingsley, et al. 1997). It can be seen that the initial degradation was rapid and no further degradation occurred after 48 hours. The reduction of detectable Aroclor® is due to biodegradation and not due to reduced extractability or sorption to the biomass, as can

be seen by the constant PCB levels in the presence of deactivated biomass (triangle in Figure 5-1).

5.5.1.2 Effect of Presence of an Immiscible Phase on the Extent of PCB Degradation.

The applicability of the selected solvents for a possible soil extraction step of a PCB remediation process train was not a criterion during solvent selection for this experiment. Low viscosity, easy recovery and high affinity for the target compounds, among others, would be desired characteristics for such use. This experiment however investigates the effect of the affinity of the selected solvent towards the target molecules during the biodegradation step in a TPPB. Therefore two immiscible phases were chosen based on their different affinities for PCBs and on their compatibility with the employed organism; the organic solvent BES, a di-ester which is characterised by a very high octanol/water partitioning coefficient ($LogK_{O/W} = 10.08$) (Rehmann and Daugulis 2006) and silicone oil, which is expected to have a significantly lower affinity for PCB congeners than most hydrophobic organic solvents (Zeng, et al. 2005). Both immiscible phases are neither toxic to *B. xenovorans* LB400 nor can they be used as a carbon and energy source (data not shown). BES has previously been used as the delivery phase for rapid degradation of large amounts of biphenyl to *B. xenovorans* LB400 in two-phase partitioning bioreactors (Rehmann and Daugulis 2006).

Different amounts of both immiscible phases were added to vials containing Aroclor® 1242 prior to the addition of the degrading organism. It was expected that Aroclor® 1242 would sequester between the two phases and that the equilibrium concentration in the aqueous phase would be affected by both the amount and nature of the immiscible phase. The aqueous phase PCB fraction is the only fraction available to the degrading organism and PCB degradation in the aqueous phase will result in

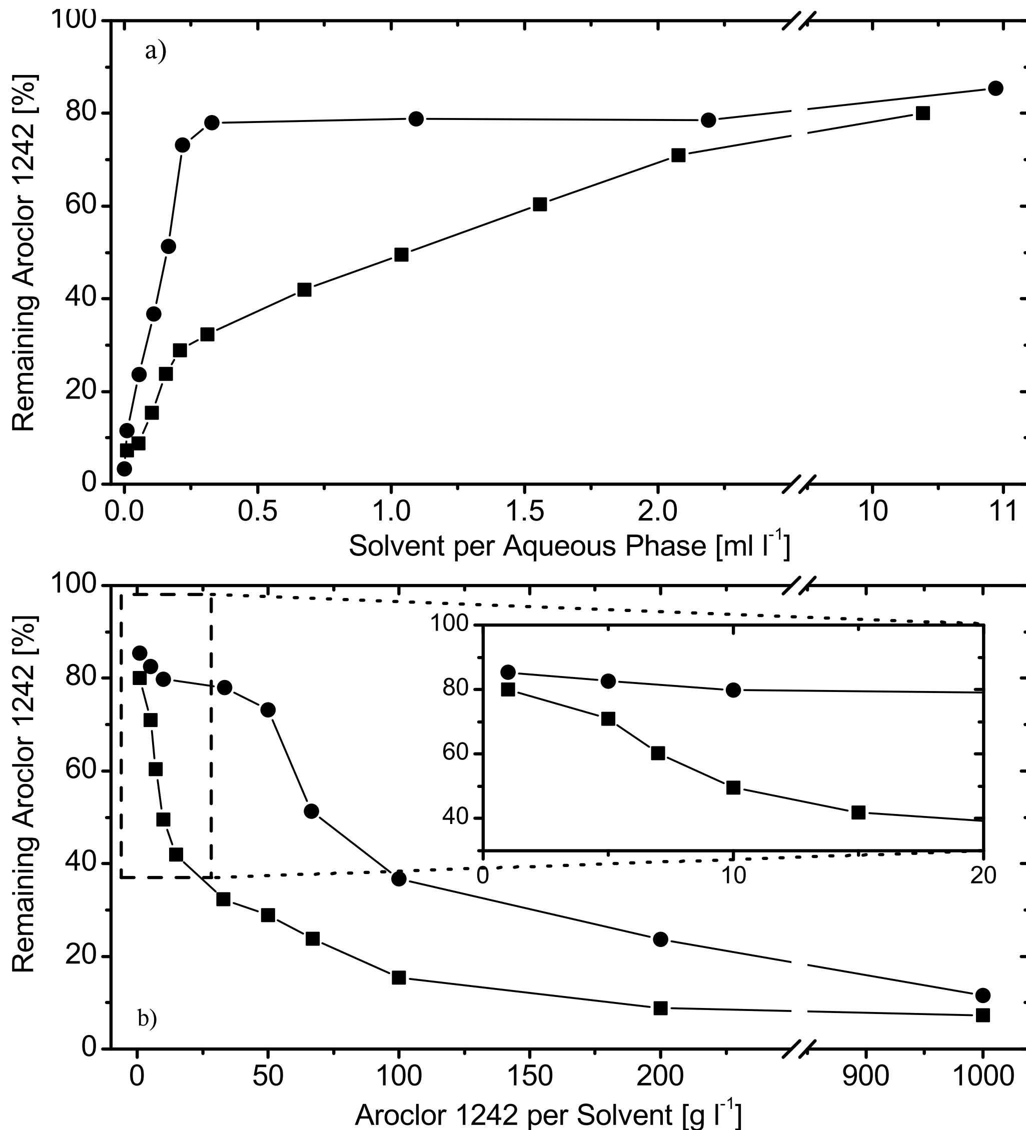

Figure 5-2: Effect of different amount of immiscible phase (square = silicone oil, circles = BES) on the achievable extent of Aroclor® 1242 (10 mg l^{-1}) degradation by *B. xenovorans* LB400. Figures a) and b) are different plots of the same data. Plot a) highlights the direct effect of increased immiscible phase fractions and plot b) highlights the effect of initial PCB concentrations in the immiscible phases, important for PCB degradation process design.

partitioning of fresh PCBs from the immiscible phase into the aqueous phase to an extent which is dependent on the amount and type of the immiscible phase.

Figure 2 shows the effect of increased immiscible phase fraction on the extent of degradation after 5 days. While under the given conditions 10 mg l^{-1} PCBs were degraded

almost to completion if provided directly in the aqueous phase (Figure 5-1), increases in the fraction of the immiscible phases resulted in increased residual PCB concentrations, as shown in Figure 5-2a. The addition of BES had a stronger effect than the addition of silicone oil due to the higher affinity of BES for PCBs and the corresponding lower PCB concentration in the aqueous phase, which has been shown to singularly govern the maximum dechlorination rate of Aroclor® 1242 under anaerobic conditions (Zwiernik, et al. 1999). The aqueous phase biphenyl concentration and the possibility of influencing it with different volumes of an immiscible phase has also been shown to determine the degradation rate of biphenyl by *B. xenovorans* LB400 (Rehmann and Daugulis 2006). The aqueous phase PCB concentration has not been measured directly because of experimental difficulties as described by Chou and Griffin (Chou and Griffin 1986), however Luthy *et al.* (Luthy, et al. 1997) have shown that the aqueous phase Aroclor® 1242 concentration is reduced to approximately 20 % of its original value if the hydraulic oil Fryquel 220 is present at an Aroclor® to Fryquel ratio of 50 g l^{-1}.

A linear relationship between the amount of BES present in the system and the achieved Aroclor® 1242 degradation can be seen in Figure 5-2a until the BES fraction reaches 0.3 ml l^{-1}. In the presence of larger amounts of solvent, only 15-20 % of the initially available Aroclor® 1242 congeners were degraded. The effect of silicone oil on the extent of PCB degradation was substantially weaker than the effect of BES. At high silicone oil concentrations only 15-20 % of the initially available Aroclor® 1242 congeners were degraded, similar to the results with BES. However, more than 20 % degradation could be observed for silicone oil contents < 2.1 ml l^{-1} compared to similar degradation extents for BES contents < 0.3 mg l^{-1}.

Plotting the same data in a different way (Figure 5-2b) clearly shows that high initial PCB concentrations in the given immiscible phases are required for extensive biodegradation to occur. At low initial concentrations only 15-20 % of the initially available Aroclor® 1242 congeners were degraded. Initial concentrations in BES have to be approximately 10 times higher than the initial concentrations in silicone oil to achieve similar degrees of degradation; for example the initial Aroclor® 1242 concentration of 7 g l^{-1} in silicone and 66 g l^{-1} in BES both result in approximately 50 % degradation. This relationship can be seen only if the immiscible phase fraction is large enough to dissolve the available Aroclor®. At higher equivalent Aroclor® to immiscible phase ratios (> 100 g l^{-1}) both aqueous phase and solvent are saturated with PCBs and a free Aroclor® phase is present. Under these conditions the type of immiscible phase does not have a strong effect.

The absolute numbers presented in Figure 5-2 represent only the extent of degradation achieved under the given conditions (i.e. closed scintillation vials). The data presented in Figure 5-2 show that under the given conditions even small fraction of the immiscible phase will severely limit the extent of PCB degradation. It was hypothesized that the limited extent of degradation in these closed systems was due to low degradation rates caused by low substrate levels, which was further investigated in bioreactor experiments.

5.5.2 *Bioreactor Experiments.*

5.5.2.1 Effect of Presence of an Immiscible Phase on the Rate of PCB Degradation.

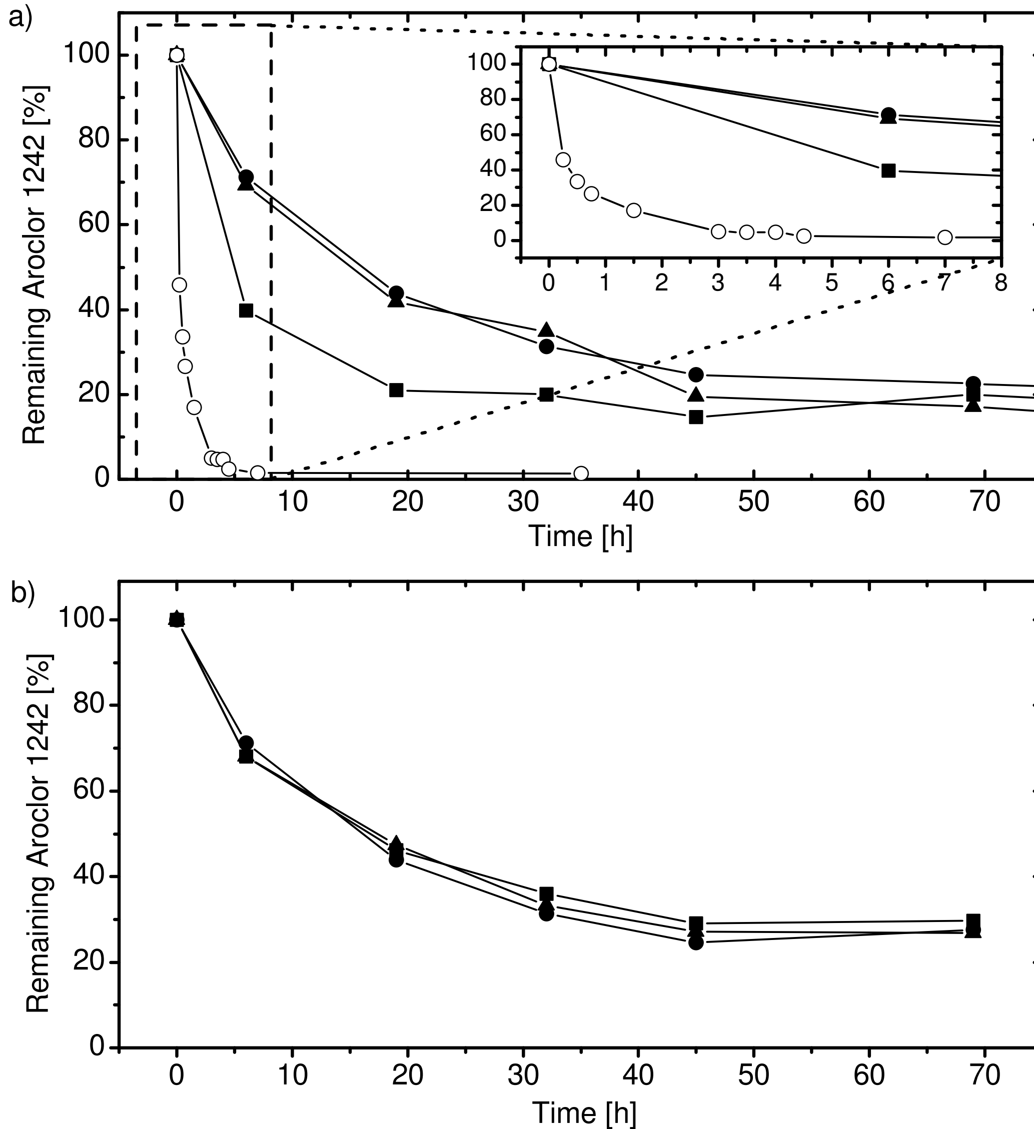

Figure 5-3: Time course of Aroclor® 1242 (10 mg l^{-1}) degradation by *B. xenovorans* LB400 ($X_0 = 1.2$ g l^{-1}) in BioFlo® 1 bioreactors. The effect of different amounts and types of immiscible phase (a), Reactor 0, no second phase (open circles), Reactor 1, 1 ml silicone oil (squares), Reactor 2, 1 ml BES (triangles), Reactor 3, 10 ml silicone oil (circles). Reactors 1 and 2 provided an effective initial Aroclor® concentration in the immiscible phase of 10 g l^{-1} and Reactor 3 of 1 g l^{-1}. The effect of agitation rate (b), 200 rpm (squares), 400 rpm (circles), 600 rpm (triangles). The immiscible phase in all three reactors was 10 ml silicone oil.

The data presented in Figure 5-2 do not give any information about degradation rates. To obtain degradation rates, bioreactors rather than scintillation vials were employed. As noted, bioreactors are expected to provide enhanced degradation conditions

compared to the previously discussed scintillation vial experiments. The initial amount of Aroclor® 1242 per volume aqueous phase was maintained at 10 mg l^{-1}, while the initial biomass concentration was increased from 0.3 g l^{-1} to 1.2 g l^{-1} in comparison to the scintillation vial experiments. Figure 3a shows the time course of PCB degradation in bioreactors in the presence of different amounts of immiscible phases (BES or silicone oil). Maintaining the total amount of PCBs at 10 mg l^{-1} and the initial PCB concentration in the solvents high, as suggested by the results shown in Figure 5-2, resulted in solvent:aqueous phase ratio of 1:100 and 1:1000, while typical ratios employed in TPPBs are 1:10 -1:4 (Daugulis 2001). Increasing the solvent:aqueous phase ratio while maintaining the PCB concentration in the immiscible phase constant would substantially increase the total amount of PCBs in the system, and exceed the scope of this study. The three reactors containing a second phase were operated concurrently and the initial biomass for the three reactors was grown in the same seed reactor, while the control reactor containing no solvent was inoculated separately. The biomass concentration did not change during the course of the experiment due to the small amount of available carbon source (data not shown).

The extent of degradation in all three reactors substantially surpassed the extent that was achieved at similar initial Aroclor® 1242 concentration in the immiscible phase in scintillation vials (Figure 5-2). Reactors 1 and 2 provided an effective initial Aroclor® concentration in the immiscible phase of 10 g l^{-1} and Reactor 3 of 1 g l^{-1}. Similar initial PCB concentrations in the immiscible phase in scintillation vials as in Reactor 1 resulted in approximately 60% degradation, and initial PCB concentrations in the immiscible phase similar to those in Reactor 2 and 3 resulted in only 20% degradation. The rate and extent of degradation in the absence of an immiscible phase (Reactor 0) is substantially

Table 5-1: Performance parameters for different PCB degradation conditions. The initial amount of Aroclor® was 10 mg l^{-1} with respect to the aqueous phase volume under all conditions. The vials and Reactor 0 contained no immiscible phase. Reactor 1 contained 1 ml silicone oil with an initial Aroclor® concentration of 10 mg l^{-1}, Reactor 2 contained 1 ml BES with an initial Aroclor® concentration of 10 mg l^{-1} and Reactor 3 10 ml silicone oil with an initial Aroclor® concentration of 1 mg l^{-1}.

Reactor	X_0 [g l^{-1}]	Volumetric degradation rate [mg l^{-1} h^{-1}] [a]	Specific degradation rate [mg g^{-1} h^{-1}] [a]	Relative specific rate [%] [b]	Extent [%]	Total time [h]
Vials	0.3	0.48	1.60	100	96	20
Reactor 0	1.2	1.96	1.63	102.1	98	5
Reactor 1	1.2	0.44	0.36	22.7	87	20
Reactor 2	1.2	0.17	0.14	**8.8**	81	48
Reactor 3	1.2	0.18	0.15	**9.3**	86	48

[a] Based on extent and time shown in this table
[b] Percentage of the specific rate in scintillation vials

higher than in reactors containing any of the tested immiscible phase fractions. The rate is also higher than in the scintillation vial experiments (Figure 5-1), mainly due to the above mentioned increase of biomass.

To better compare reactor performances degradation rates were estimated and are presented in Table 5-1. Volumetric rates were calculated as the amount of PCB degraded per reactor volume and time required until no further degradation occurred [mg l^{-1} h^{-1}]. These volumetric rates were also normalized with respect to the initial biomass concentration to obtain specific rates [mg g^{-1} h^{-1}]. The volumetric rates are commonly used to assess the reactor performance, whereas the specific rates reflect on the performance of the catalyst/biomass. The volumetric degradation rates are very high, and in the case of Reactor 1 the volumetric rate of 0.44 mg l^{-1} h^{-1} is similar to the rate of 0.48 mg l^{-1} h^{-1} that was achieved in scintillation vials with pure Aroclor® 1242 (Figure 5-1), however it is substantially lower than volumetric rate of 1.96 mg l^{-1} h^{-1} that was achieved under similar conditions in a bioreactor in the absence of a immiscible phase (Reactor 0). The specific rate in the reactor however is only 23 % of the specific rate in the

scintillation vials (Table 5-1), while the specific rates in scintillation vials and Reactor 0 are identical. The similarity of the volumetric rates in Reactor 1 and in the scintillation vials shows that high biomass concentration can compensate for the reduced substrate availability arising from the presence of an immiscible phase, which is the reason for the low specific rates. The fact that the specific substrate degradation rates (σ) of the bioreactor containing no immiscible phase (Reactor 0, σ = 1.63 mg g^{-1} h^{-1}) and the scintillation vials (σ = 1.60 mg g^{-1} h^{-1}) are within 3 % of each other shows further that enhanced bioreactor aeration, agitation and pH control did not have a significant effect on the degradation of small amounts of PCBs. The fact that the biocatalyst performed identically in both system (vials and bioreactor) also allows direct comparison of results achieved in scintillation vials and bioreactors, under consideration of the different amounts of biomass. The observed lower extents of degradation in vials containing similar amounts of immiscible phase as Reactors 1-3 can therefore be contributed to the smaller initial amount of biomass.

The volumetric rates in Reactor 2 and 3 are lower than in Reactor 1, as expected. Reactor 2 was expected to result in a lower aqueous phase PCB concentration than Reactor 1 due to the higher PCB affinity of BES compared to silicone oil. Reactor 3 provides a lower aqueous phase PCB concentration due to the larger volume of the immiscible phase. The conditions provided in Reactors 2 and 3 still allow the degradation of > 80 % of the initially available Aroclor® 1242 congeners within 48 hours; however the specific degradation rate drops to less than 10 % of the rate achieved in the two single phase system in scintillation vials. The specific microbial degradation rate is a direct function of the aqueous phase substrate concentration, which is determined by the type

and amount of immiscible phase that is present. In a related study it was previously shown that the presence of an immiscible phase can be used to influence the aqueous phase biphenyl concentration and the resulting specific microbial degradation rate of *B. xenovorans* LB400 (Rehmann and Daugulis 2006). The low specific rates however do not obviate the possibility of degrading PCBs in the presence of an immiscible phase as seen in the extents of degradation and in the volumetric rates at high biomass concentrations.

5.5.2.2 Effect of Agitation rate in the Presence of an Immiscible Phase on the Rate and Extent of PCB Degradation.

The observed decrease of volumetric and specific degradation rates when the amount of immiscible phase was increased shows that, under the given conditions, it is not the dissolution rates of Aroclor® 1242 but slow microbial kinetics caused by low aqueous phase substrate concentration that are rate limiting. This was also confirmed by varying the agitation rate in the bioreactors from 200 rpm to 600 rpm while keeping all other parameters identical (Figure 5-3b). It can clearly be seen that moderate variations of the agitation rate have no effect on the rate and extent of microbial PCB degradation, showing that the microbial rate and not the mass transfer rate is limiting the degradation process under the employed reactor conditions. Similar results were found by Zwiernik *et al.* for achievable PCB dechlorination rates under anaerobic conditions (Zwiernik, et al. 1999). In contrast Kose *et al.* (2003) showed that the dissolution rate of PAHs from a NAPL and not the aqueous phase concentration can be the rate limiting step under conditions simulating oil spills on tidal flats. Those conditions did, due the objective of their study, not provide extensive mixing of the two phases. The intensive mixing in the bioreactors employed in this study results in the formation of a fine emulsion with large surface area for mass-transfer to occur. It has been shown previously that under these

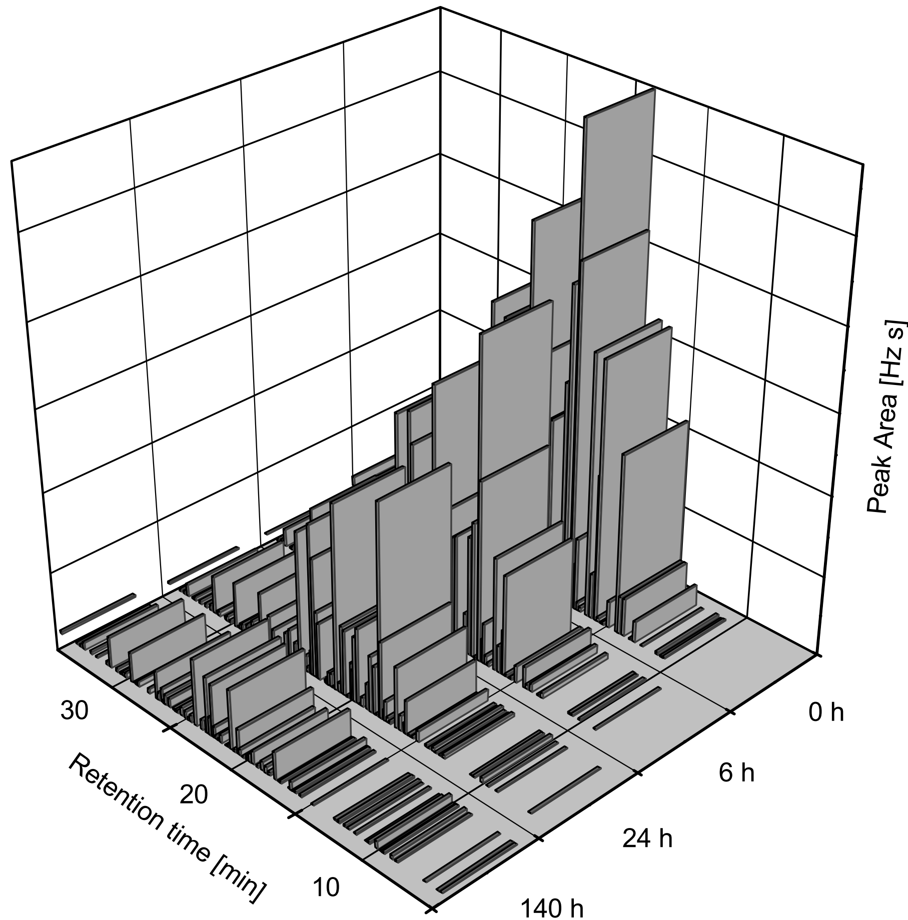

Figure 5-4: Chromatograms (shown as peak area vs. retention time) of Aroclor® 1242 during biodegradation by *B.* xenovorans LB400 in the presences of BES (Reactor 2), showing the qualitative change in congener distribution.

circumstances instantaneous equilibrium conditions exist for a substance partitioning between an immiscible phase and aqueous media (Rehmann and Daugulis 2006), which is substantially different from environmental condition as simulated by Kose *et. al.* (2003). It can therefore be concluded that conditions created in biphasic bioreactors can be used to overcome mass transfer limitations during PCB biodegradation. The fact that Reactor 3 yields lower volumetric degradation rates than Reactor 1, despite its larger immiscible phase volume and hence more surface area for mass transfer, shows that biphasic bioreactors can be operated without mass transfer limitations during PCB biodegradation, as confirmed by the lack of effect of varying agitation rates on the degradation rate. Low aqueous phase PCB

concentrations however can limit the microbial degradation rate and high initial PCB concentrations as well as high initial biomass concentrations can be used to overcome this.

The observed PCB disappearance is due to microbial activity, as control experiments in which the biomass was inactivated with hydrochloric acid prior to the incubation with Aroclor® in scintillation vials showed no significant degradation (Figure 5-1). Figure 5-4 shows chromatograms of samples at different incubation times from Reactor 2. It can clearly be seen that the congener distribution changes over the fermentation time, with faster degradation of lower chlorinated congeners (lower chlorinated congeners have typically lower retention times (Erickson 1986)), a sign of biodegradation (Abramowicz 1990). Low chlorinated congeners are more susceptible to aerobic biodegradation than highly chlorinated congeners due to less steric hindrance by the chlorines. Also, low chlorinated congeners are less hydrophobic as shown in lower octanol water partitioning coefficients (Hawker and Connell 1988), which results in higher availability of those congeners in the aqueous phase.

The fact that Aroclor® 1242 could be degraded, if provided at initial concentrations of 1 g l^{-1} (Reactor 3) in silicone oil as the immiscible phase is important for possible engineered PCB biodegradation schemes, as concentrations of 1 g l^{-1} in the extract can be achieved via solvent extraction (Amend and Lederman 1992). The results presented in this study show that the choice of this PCB-containing water immiscible liquid can have a profound impact on substrate availability to degrading organisms in the aqueous phase. For example a ten-fold lower initial PCB concentration in silicone oil was sufficient to achieve similar rates and extents of degradation compared to using BES (Table 5-1). In practice, this would mean that while choosing a solvent for PCB soil extraction or a solvent to reduce viscosity of a soil extract, the effect of this solvent on the

aqueous phase concentration during biodegradation in a biphasic reactor environment should be taken into consideration. Solvents which result in high extraction efficiency during soil extraction may result in low aqueous phase concentrations during a possible subsequent biodegradation step. However, high initial PCB concentrations in the immiscible phase and high initial biomass concentrations can counteract the effect of low aqueous phase availability on volumetric biodegradation rates to some extent, as shown in this study.

5.6 Conclusions

The aqueous phase PCB concentration is responsible for the rate and extent of degradation under laboratory conditions in the presence of an immiscible organic phase. The aqueous phase concentration is dependent on the nature and volume of the immiscible phase. The presence of an immiscible organic phase can be rate limiting during aerobic PCB degradation via the *bph*-pathway (this study), similar to what was found for PCBs in the presence of NAPL in a soil environment during anaerobic PCB dechlorination (Zwiernik, et al. 1999). The rate of PCB release from the immiscible phase into the aqueous phase was, under the conditions in this study, not limiting the degradation rate. However, it could be limiting under conditions present in a soil NAPL environment. Low chlorinated PCB mixtures such as Aroclor® 1242 can be degraded in a biphasic reactor environment if provided at sufficiently high initial concentrations in the immiscible phase. This finding shows that TPPBs might be a suitable technology for the treatment of PCBs extracted from soil with organic solvents.

Acknowledgements

We are grateful to the Natural Sciences and Engineering Research Council of Canada and the Environmental Technologies Program at SAIC Canada, for financial support.

Chapter 6: Enhancement of PCB degradation by *Burkholderia xenovorans* LB400 in biphasic systems by manipulating culture conditions

Lars Rehmann, Andrew J. Daugulis

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as is currently in Press in: *Biotechnology and Bioengineering* (2007)

6.1 Preface to Chapter 6

It is essential to demonstrate the degradation of large amounts of PCBs in a small reactor volume in order to consider TPPBs as a viable option for the biological destruction of PCBs. The amount of PCBs degraded in the study presented in Chapter 5 (10 mg l^{-1}) was too low, but the results from biphenyl degradation as presented in Chapters 2-4 showed that large amounts of poorly soluble substrate (8 g l^{-1}) can readily be degraded in TPPBs. It was found that microbial activity towards PCBs diminished after approximately 40-50 h of operation, against the initial expectation of ready use of high PCB concentrations. It was therefore the objective of this chapter to investigate the reasons for the observed loss of microbial activity.

It was found through a series of experiments that the degrading organisms lost activity due the lack of a carbon and energy source; PCBs did not fulfill this role as biphenyl did, most likely due to the low available amounts. The vast amount of literature describing the metabolic flux of the employed organism (*B. xenovorans* LB400) was used as a guide during the selection of a co-carbon source that would not result in deregulation of microbial activity towards PCBs, not compete for PCB-degrading enzymes, but that would provide *B. xenovorans* LB400 with carbon and energy. Pyruvate was found to fulfill these requirements and the addition of pyruvate to the reactor medium was shown to be a simple and effective method to maintain the activity of *B. xenovorans* LB400 against PCBs. The amounts of PCBs degraded could be increased by a factor of 10 compared to the amounts degraded in the experiments presented in Chapter 5.

6.2 Abstract

Two-phase partitioning bioreactor (TPPBs) can be used to biodegrade environmental contaminants after their extraction from soil. TPPBs are typically stirred tank bioreactors containing an aqueous phase hosting the degrading microorganisms and an immiscible, non-toxic and non bioavailable organic phase functioning as a reservoir for hydrophobic compounds. Biodegradation of these compounds in the aqueous phase results in thermodynamic disequilibrium and partitioning of additional compounds from the organic phase into the aqueous phase. This self regulated process can allow the delivery of large amounts of hydrophobic substances to degrading micro organisms. This paper explores the reactor conditions under which the poly-chlorinated biphenyl (PCB) degrader *Burkholderia xenovorans* LB400 can degrade significant amounts of the PCB mixture Aroclor® 1242. Aroclor® degradation was found to stall after approximately 40 h if no carbon source other than PCBs was available in the reactor. Sodium pyruvate was found to be a suitable carbon source to maintain microbial activity against PCBs and to function as a substrate for additional cell growth. Both biphenyl (while required during the inoculum preparation) and glucose had a negative effect during the Aroclor® degradation phase. Initial Aroclor® 1242 degradation rates in the presence of pyruvate were high (6.2 mg l^{-1} h^{-1}) and 85% of an equivalent concentration of 100 mg Aroclor® 1242 per l aqueous phase could be degraded in 48 h, which suggest that solvent extraction of PCBs from soil followed by their biodegradation in TPPBs might be a feasible remediation option.

6.3 Introduction

Polychlorinated biphenyls (PCBs) are toxic xenobiotics, manufactured during the midtwentieth century as congener mixtures. PCB mixtures were commercially available in North America under the trade name Aroclor® and found industrial applications in hydraulic and heat-transfer systems, inks, lubricants, paints and adhesives. Prior to their ban in 1977 570 million kg were sold in the Unites States, 75 million kg are estimated to have entered the environment, and 400 million kg are still in use and potential sources of future contamination (Cohen, et al. 1993).

PCBs were designed to be non-flammable as well as chemically and thermally stable which results in their persistence in the environment. Magar (2003) concluded in a recent editorial that the challenge of PCBs in the environment today is virtually the same as it was 20 years ago (Magar 2003). Transport of PCBs and their fate in the environment are well understood (Dercova, et al. 1999; Pier, et al. 2003; Platonow, et al. 1971; Thomann and St John 1979; Weber, et al. 1991) and precise analytical techniques have been developed for their enumeration (Cochran and Frame 1999; Erickson 1986; Frame, et al. 1996; Rapaprot and Eisenreich 1984; Rote and Murphy 1971). A large number of PCB degrading organisms have been isolated (Bedard, et al. 1987; Bopp 1986; Commandeur, et al. 1995; Seto, et al. 1995; Sierra, et al. 2003) and, especially in the case of the aerobic PCB degrader *Burkholderia xenovorans* LB400, well characterized on a genomic and metabolic level (Chain, et al. 2006; Denef, et al. 2004; Denef, et al. 2005). Despite these advances there is still a scarcity of treatment options for soils and sediments, with very few alternatives to in situ treatment (Magar 2003).

This study examines factors affecting the second step of a proposed two stage process consisting of solvent extraction of PCBs from soil followed by microbial

degradation in two-phase partitioning bioreactors (TPPBs). TPPBs are typically stirred tank bioreactors containing an aqueous phase in which microbial transformation can take place and an immiscible organic solvent phase acting as a reservoir for the target substrate(s) (Daugulis 2001). Hydrophobic compounds such as polycyclic aromatic hydrocarbons (PAHs) or PCBs can be dissolved at high concentrations in the organic delivery phase and will, driven by thermodynamic phase equilibrium, partition at low concentrations into the aqueous phase where biodegradation occurs. Previously in an analogous strategy PAHs were extracted from soil using dodecane and ethanol and subsequently degraded by a *Sphingomonas* sp. in TPPBs (Janikowski, et al. 2002), and high concentrations of biphenyl (equivalent to 5 g l^{-1} aqueous phase) could also be degraded by *B. xenovornans* LB400 in TPPBs (Rehmann and Daugulis 2006). PCBs are substantially more hydrophobic than biphenyl and low bioavailability in TPPBs might reduce degradation rates. Recent studies showed that sequestration of PCBs between water and oil in the environment have a rate reducing effect on PCB degradation (McNamara, et al. 2005; Zwiernik, et al. 1999).

Notwithstanding the success of degrading PAHs and biphenyl at high rates in TPPBs, preliminary experiments with resting cells (no carbon source other than PCBs present) could only achieve degradation of up to 90 % of the initially available Aroclor® in a TPPB with low initial Aroclor® 1242 concentration equivalents of 10 mg l^{-1}. In these systems microbial degradation stopped, however, after approximately 40 h and resulted in significant PCB residues, depending on the operating conditions of the bioreactor. This limited time of degradative activity combined with low degradation rates therefore limits the amount of PCB that can be degraded per unit time and reactor volume resulting in inefficient PCB degradation. PCB degradation by growing cells of *B. xenovorans* LB400

in TPPBs containing large amounts of biphenyl (equivalent to 5 g per l in the aqueous phase) in the delivery phase (bis(2-ethylhexyl)sebacate) and Aroclor® 1242 concentration between 10 mg l^{-1} and 1000 mg l^{-1} could not be achieved, despite significant biomass formation (data not shown).

Arrested degradation with resting cells, and minimal degradation with growing cells (with biphenyl as the carbon source) are clear limitations to rapid and near complete degradation of PCBs in TPPBs. It was therefore the objective of this study to manipulate microbial cultivation condition in TPPBs to enhance degradation rates and to prolong microbial activity to biodegrade the majority of PCB congeners in 100 mg Aroclor® 1242 per l aqueous phase.

6.4 Materials and Methods

6.4.1 *Chemicals*

All chemicals used in the fermentation media and the solvents were obtained from either Sigma Aldrich (Canada) or Fisher Scientific (Canada). Biphenyl, 99 % (assay) was obtained from Alfa Aesar (USA) and Aroclor® 1242 (CAS Number: 53469-21-9) was obtained from Chromatographic Specialties Inc. (Brockville, Ontario).

6.4.2 *Bacterial Strain*

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, N.Y.) (Bopp 1986), was obtained from the Northern Regional Research Laboratory (Peoria, Ill.). The strain has since been re-classified as *Burkholderia xenovorans* sp. nov. (Goris, et al. 2004). Cultivation conditions, maintenance and biomass determination were described previously (Rehmann and Daugulis 2006).

6.4.3 *Inoculum Preparation*

B. xenovorans LB400 was cultivated in 5-L New Brunswick BioFlo® III bioreactors at 30 °C, agitated with two Rushton turbines at 500 rpm and aerated (sterile air) at 3 l min^{-1}. Biphenyl was delivered from Hytrel™ polymer beads, which were pre-loaded from biphenyl saturated methanol, as the sole carbon source. Cultivating *B. xenovorans* in solid-liquid TPPBs allows the generation of high inoculum concentrations as well as subsequent complete biphenyl removal before use and is described in detail elsewhere (Rehmann and Daugulis 2007b). Cells were harvested in mid exponential phase and the solid delivery phase was removed by filtration through sterile glass wool. Biomass was removed from culture medium via centrifugation at 3000 rpm for 20 minutes at 4 °C and re-suspended in fresh medium to the desired biomass concentration and distributed to three 1 l BioFlo® I bioreactors.

6.4.4 *Cultivation Conditions*

All PCB degradation experiments were undertaken in 1 l BioFlo® I bioreactors at 30 °C, agitated with two Rushton turbines at 500 rpm and aerated (sterile air) at 1 l min^{-1}. The pH was maintained at 6.9. Samples were taken periodically for PCB and biomass analysis. The agitation rate was increase to 900 rpm prior to sampling to ensure that samples contained representative fractions of both liquid phases.

6.4.5 *Enhancement Strategies*

To examine the effect of varying a number of operating conditions to enhance PCB degradation in TPPBs, a variety of strategies were employed.

6.4.5.1 Strategy 1: Variation of Initial Biomass Concentration

Neat Aroclor® 1242 (100 mg) was dissolved in 10 ml silicone oil and added to three bioreactors. Biomass from a single inoculum preparation reactor was distributed

unevenly over the three reactors to initial concentrations of 1 g l^{-1}, 1.2 g l^{-1} and 3 g l^{-1}. The reactors were operated concurrently, under otherwise identical conditions.

6.4.5.2 Strategy 2: Addition of Supplementary Aroclor® after Fermentation Stalls

Neat Aroclor® 1242 (10 mg) was dissolved in 1 ml silicone oil and added to a bioreactor containing an initial biomass concentration of X = 1 g l^{-1}. After 75 h of operation an additional 1 ml of silicone oil containing 10 mg neat Aroclor® 1242 was added to the reactor.

6.4.5.3 Strategy 3: Addition of Supplementary Biomass after Fermentation Stalls

Neat Aroclor® 1242 (100 mg) was dissolved in 10 ml silicone oil and added to a bioreactor containing an initial biomass concentration of X = 0.5 g l^{-1}. After 75 h of operation an additional 10 ml of growth medium containing 0.5 g fresh biomass generated as described above were added to the reactor.

6.4.5.4 Strategy 4: Addition of Co-Substrates after Fermentation Stalls

Neat Aroclor® 1242 (100 mg) was dissolved in 10 ml silicone oil and added to three bioreactors. Biomass from the inoculum preparation reactor was distributed evenly to achieve initial concentrations of 0.3 g l^{-1}in all three reactors. The reactors were operated concurrently. After 40 h and 65 h 0.1 g of biphenyl, glucose or sodium pyruvate were added to the reactors.

6.4.5.5 Strategy 5: Addition of Co-Substrate at Outset

Neat Aroclor® 1242 (100 mg) was dissolved in 10 ml silicone oil and added to two bioreactors. The biomass from the inoculum preparation reactor was distributed unevenly to achieve initial concentrations of 0.01 g l^{-1} and 0.3 g l^{-1}. The initial medium was supplemented with 10 g and 7.5 g sodium pyruvate, respectively; providing growth

substrate with the intention to investigate the PCB degradation ability of biomass growing on pyruvate from a small inoculum. The reactors were operated concurrently.

6.4.6 *PCB Extraction and Analysis*

Fermentation samples of 5 ml were extracted three times with 2 ml hexane (4 h of rotary shaker at 180 rpm and 30 °C). At the time of extraction samples contained both aqueous phase and silicone oil. Biomass was not removed prior to extraction in order to extract PCBs that might physically be associated with the biomass. Control experiments with initial biomass concentration of 1 g l^{-1} which had been inactivated via autoclaving showed no decrease of extractable PCBs over 80 hours with a recovery of > 95% of the initially added Aroclor® (data not shown). The hexane extract was analyzed with an Agilent 6890 gas chromatograph (Agilent Technologies Canada Inc., Mississauga, Ontario) equipped with a fused silica cap capillary column (Supelco SPB-1, Sigma-Aldrich Corp. St. Louis, MO, USA), an electron capture detector (ECD) (280 °C) and split injector (250 °C). The temperature program was as follows: 100 °C for 4 min, 100 °C to 180 °C at 10 °C min^{-1}, 180 °C for 1 min, 180 °C to 240 °C at 1.5 °C min^{-1}, 240°C for 1 min, 240 °C to 300 °C at 20 °C min^{-1}, 300 °C for 10 min. Aroclor® standards were run for every analysis and blank hexane was run after every four samples. Aroclor® was quantified by using the summed peak area according to EPA Method 304 h.

6.5 Results and Discussion

6.5.1 *Strategy 1: Amount of Initial Biomass*

Three TPPBs with different initial amounts of the degrading organism, grown in the same seed fermentor, were run in parallel to observe the effect of initial active biomass concentration on the rate and extent of degradation. The conditions in all three reactors were identical except for the different amounts of biomass. The rate and extent of

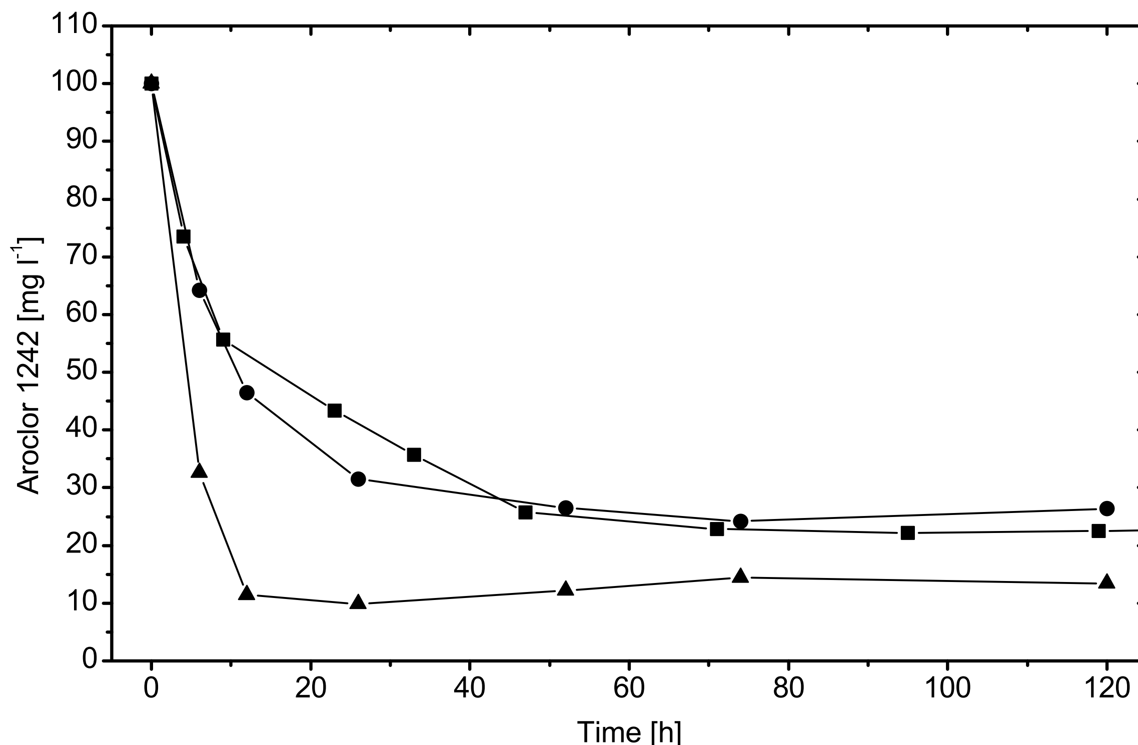

Figure 6-1: The effect of biomass concentration on the degradation of Aroclor® 1242 by B. xenovorans LB400. The initial biomass concentrations were 1 g l^{-1} (squares), 1.2 g l^{-1} (circles) and 3 g l^{-1} (triangles). The initial amount of Aroclor® 1242 was 100 mg dissolved in 10 ml silicone oil. The PCB concentrations are shown as mg Aroclor® per l aqueous medium.

the degradation were both affected by the amount of biomass being present in the system, as shown in Figure 6-1. The amount of Aroclor® 1242 in the reactors is reported as the total amount of PCBs extracted from samples containing representative fractions of aqueous phase, biomass and silicone oil, normalised by the total volume of aqueous phase for comparison purposes. This method of PCB extraction was chosen to ensure that the reported disappearance of PCB is due to biodegradation and not biosorption.The total amount of biomass did not change over the course of the fermentation (data not shown), indicating that no significant fraction of the degraded PCB congeners was used as a carbon and energy source by the organism. Most aerobic PCB degraders utilize only monochlorinated congeners for cell growth (Ahmed and Focht 1973), even though some

have the ability to degrade congeners containing up to six chlorines, but presumably not to obtain energy for growth (Bopp 1986). The volumetric rate at which Aroclor® 1242 was degraded, the slope in Figure 6-1, increased with an increase in initial biomass present. The effect of biomass concentration on the volumetric rate suggests that the mass transfer rate of PCBs from the delivery phase into the aqueous phase is not the rate limiting step under the employed conditions. Mass transfer effects have been examined in a related study by varying the agitation rate under otherwise similar conditions (Aroclor® 1242 degradation in biphasic bioreactors) and it was found that the mass transfer rate was not limiting (Rehmann and Daugulis 2007a). Mass transfer rates can become rate limiting in biphasic systems (Kose, et al. 2003), however, the reactor system employed in this study is well mixed. The fact that the degradation rate does depend on the initial biomass concentration shows that the microbial degradation rate and not the mass transfer rate is the rate limiting step under the employed conditions.

The degradation extent also varied depending on the initial biomass concentration as shown in Figure 6-1, with PCB degradation "stalling" after approximately 20 – 50 h. Kohler et al. also observed a decrease of biphenyl oxidation activity to less than 8 % of its original value after 28 h when exposing resting cells of *Acinetobacter* sp. strain P6 and B1B to Aroclor® 1254 (Kohler, et al. 1988). It can be concluded from the data in Figure 6-1 that the initial amount of active biomass dictates the rate and extent of degradation in the employed system. Each unit of biomass seems to have a limited ability to perform PCB transformation which is lost after a certain period of time. Possible reasons for the loss of activity towards PCBs include the depletion of degradable congeners, the formation of toxic by-products and the lack of a carbon and energy source required to maintain microbial activity.

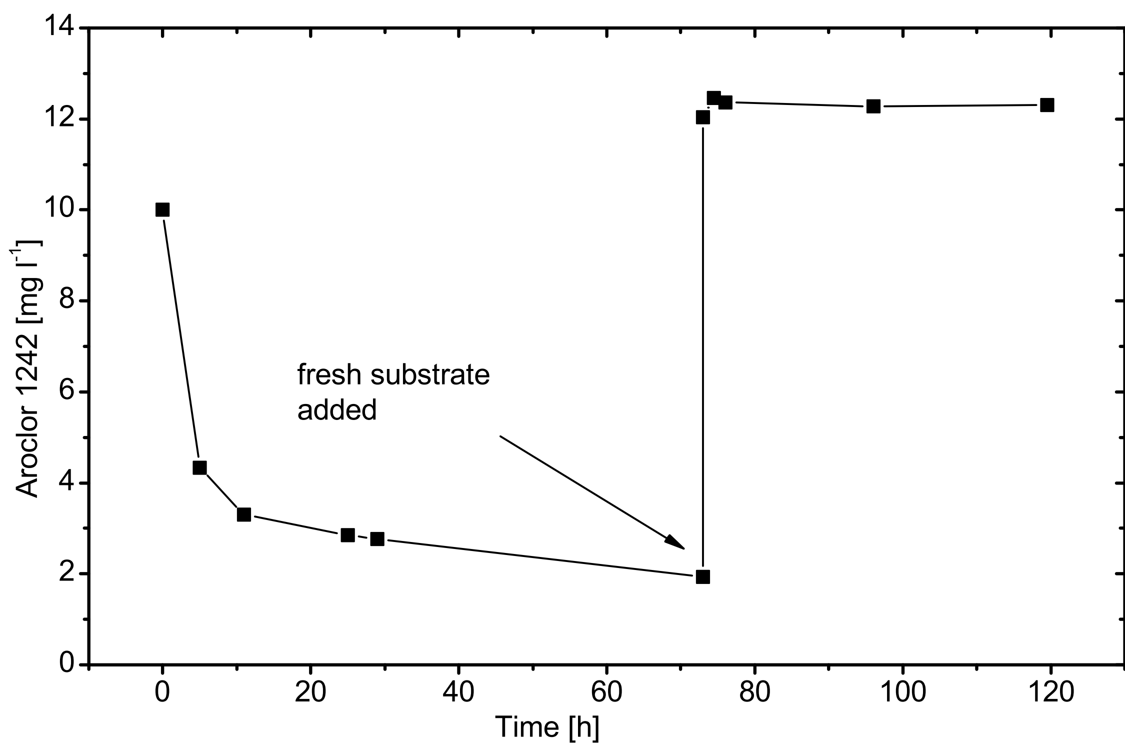

Figure 6-2: Effect of additional substrate after stall of degradation activity. The initial biomass concentration was 1 g l^{-1} and the initial Aroclor® 1242 concentration in the delivery phase (1 ml) was 10 g l^{-1}. After 75 h an additional ml delivery phase containing 10 g l^{-1} Aroclor® 1242 was added. The PCB concentrations are shown as mg Aroclor® per l aqueous medium.

6.5.2 *Strategy 2: Addition of Supplementary Aroclor® after Fermentation Stalled*

The observed PCB residuals after 50 h (seen in Figure 6-1) could have been due to the presence of a group of (highly chlorinated) congeners which cannot be degraded by the employed pure species. This however would suggest that activity towards other congeners may still be present. Figure 6-2 shows the time course of the degradation of 10 mg l^{-1} Aroclor® 1242. 80% of the initially available PCBs were rapidly degraded within 20 h. The same amount of PCBs was added after 75 h, and no subsequent degradation occurred, despite the fact that a large number of low chlorinated congeners were reintroduced to the system. Degradation intermediates such as chlorobenzoic acids have been observed to accumulate with inhibitory effects on PCB degrading organisms

(Seeger, et al. 1999) and have therefore often been addressed by co-cultivating known benzoate degraders (Fava 1996). Chlorobenzoate formations was not monitored, but it is unlikely that inhibitory levels were reached as significantly larger amount of Aroclor® 1242 were degraded under similar conditions as seen in Figure 6-1. To verify the suitability of the reactor environment for further PCB degradation after extensive incubation time fresh biomass was added to the reactor in Strategy 3.

6.5.3 *Strategy 3: Addition of Supplementary Biomass after Fermentation Stalls*

The suitability of the reactor environment for further PCB degradation after extensive incubation time was verified by the addition of fresh biomass after the degradation catalyzed by the initial biomass had stopped. A TPPB was inoculated with a relatively small initial amount of biomass (0.5 g l^{-1}). Based on the results shown in Figure 6-1 it was expected that high amounts of PCBs would remain in the reactor after the fermentation stalls, as expected and seen in Figure 6-3. No further degradation occured after approximately 40 h when the remaining amount of Aroclor® 1242 was approximately 50 mg l^{-1}. The addition of fresh biomass after 75 h resulted in new degradation activity, which lasted for approximately 40 h (similarly to the activity of the initial biomass) and reduced the Aroclor® 1242 concentration to approximately 25 mg l^{-1}. The fact that fresh biomass showed activity towards the residual PCBs suggests that no inhibitory intermediates accumulated in the reactor medium and shows further that the remaining congeners can be degraded by active cells of *B. xenovorans* LB400, if present in the reactor. The cells present initially remained viable, which was confirmed by streaking out fermentation broth onto agar Petri dishes with biphenyl as the sole carbon source. No significant decrease in the number of colonies from samples during the entire fermentation was observed after 48 hours of incubation (data not shown), confirming that

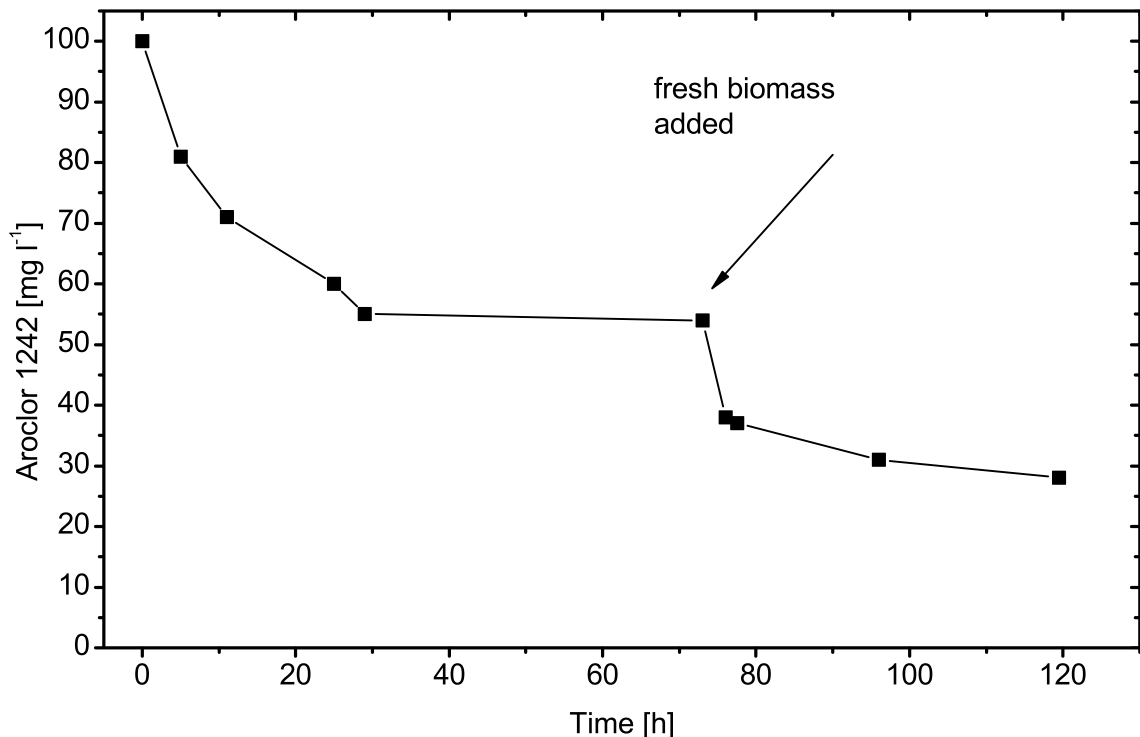

Figure 6-3: Effect of additional biomass after stall of degradation activity. The initial biomass concentration was 0.5 g l^{-1} and the initial Aroclor® 1242 concentration in the delivery phase was 10 g l^{-1}. After 75 h 0.5 g fresh biomass were added in 10 ml culture medium. The PCB concentrations are shown as mg Aroclor® per l aqueous medium.

viable cells were constantly present in the reactor. The observed inability of the cells to degrade PCBs for an extended period of time might therefore be due to the lack an available carbon and energy source. It can be speculated that in the absence of utilizable carbon source the cells metabolism slows down and PCB degrading enzymes or co-factors might not be re-generated.

6.5.4 *Strategy 4: Addition of Co-Substrates after Fermentation Stalls*

All reactors described above employed resting cell with no observed cell growth. Resting cell assays are typically used to study pathways and the formation of intermediates, however for degradation applications growing cells are generally more suitable (Kohler, et al. 1988). Actively growing cells might be able to maintain PCB degradation abilities over extended time periods; for example Lambo and Patel (2006)

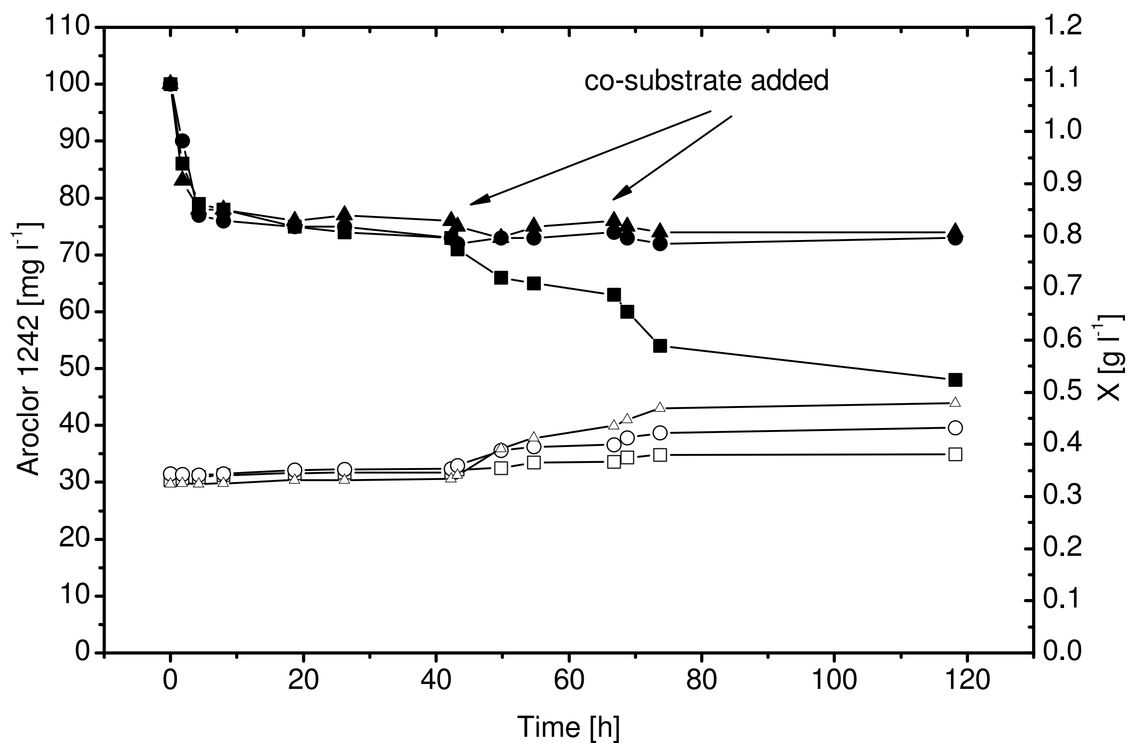

Figure 6-4: Effect of additional carbon sources after stall of degradation activity. The initial biomass concentration was 0.3 g l^{-1} and the initial Aroclor® 1242 concentration in the delivery phase was 10 g l^{-1}. After 40 h and 65 h 0.1 g carbon source were added (triangles = biphenyl, circles = glucose and squares = sodium pyruvate). The filled symbols represent the Aroclor® 1242 concentration (in mg Aroclor® per l aqueous medium) and the open symbols the biomass concentration.

recently showed that the bacterium *Hydrogenophaga* sp. IA3-A maintained biphenyl dioxygenases activity when growing on tryptic soy broth or succinic acid (Lambo and Patel 2006). The effect of additional carbon sources was investigated by adding 100 mg of three additional carbon source (biphenyl, glucose and sodium pyruvate) to the reactors after 40 h and 65 h of operation. The initial biomass concentrations were 0.3 g l^{-1} in each case which ensured that the degradation would stall while PCB concentrations were still reasonably high. Figure 6-4 shows that Aroclor® 1242 concentrations were reduced to approximately 75 mg l^{-1} in all three reactors before substrate addition. The addition of biphenyl and glucose could not reinitiate PCB degradation, even though an increase in biomass was observed, most dominantly in the case of added biphenyl. The immediate

increase of biomass concentration after the addition of a suitable growth substrate is also an indication of the general viability of the organisms present.

The inability of biphenyl to reinvigorate PCB degradation is surprising as biphenyl naturally possess stronger inducing activity for the biphenyl degradation pathway (*bph*-pathway) than later intermediates (Denef, et al. 2005). The fact that the cells are actively growing, as seen by the formation of new biomass, also shows that the *bph*-pathway is active. The lack of resulting PCB degradation might in this case be due to competition between PCB congeners and biphenyl for available enzymes. Biphenyl is approximately 30 times more soluble in water than Aroclor® 1242 (Chou and Griffin 1986; Paul 1952), and the concentration difference between biphenyl and PCB congeners in the aqueous phase becomes further enhanced in a TPPB. The octanol/water partitioning coefficient of biphenyl ($LogK_{O/W} = 3.79$ (Chou and Griffin 1986)) is substantially lower than the $LogK_{O/W}$ of Aroclor® 1242 congeners, which are reported to be in the range $LogK_{O/W} = 4.4$ and $LogK_{O/W} = 7$ depending on the level of chlorination of the specific congener (Hawker and Connell 1988; Rapaport and Eisenreich 1984). The biphenyl concentration in the aqueous phase of a biphasic octanol/water system would therefore be significantly larger than the aqueous phase Aroclor® 1242 concentration in an octanol/water system and by analogy it is likely that the aqueous phase availability of biphenyl in the current system is orders of magnitude higher than PCB availability. With this higher concentration of biphenyl in the aqueous phase the *bph* enzymes might be likely to preferably degrade biphenyl over PCB congeners.

The addition of sodium pyruvate also resulted in a moderate increase of biomass, but additionally stimulated PCB degradation. The Aroclor® 1242 concentration was reduced to approximately 60 mg l^{-1} after the first sodium pyruvate addition and then

further reduced to approximately 50 mg l^{-1} after the second addition. Pyruvate was chosen as a carbon source because it is a key downstream intermediate in the PCB degradation pathway of *B. xenovorans* LB400 (Seeger, et al. 1995; Seeger, et al. 1997). *B. xenovorans* LB400 forms pyruvate from 4-hydroxy-2-oxovalerate by the *bphI* gene-product after 6 enzymatic conversions of biphenyl (Denef, et al. 2004). Pyruvate does therefore not compete with PCBs for the early enzymes of the *bph*-pathway as biphenyl did. The biphenyl pathway in *B. xenovorans* LB400 is known to be regulated by at least two mechanisms, up-regulation by growth on biphenyl and down-regulation by growth on succinate (and probably other carbon sources) (Beltrametti, et al. 2001). The expression of the *bph*-genes was initiated during the cell growth on biphenyl, and might be continuously up-regulated, or not down regulated, by the presence of PCBs, while the decrease of degradative activity is most likely due to the fact that the cells have no available carbon or energy source, for example for the production of co-factors. Providing glucose might have had a down-regulating effect of the *bph*-genes as suggested by Beltrametti et al. (Beltrametti, et al. 2001), resulting in cell growth, but no further PCB degradation as shown in Figure 6-4. Pyruvate does not seem to have this down-regulating effect and it can be speculated that this is due to its occurrence downstream in the *bph* pathway. It has previously been shown by Master and Mohn that *B. xenovorans* LB400 expresses the same levels of the *bphA* gene product in the presence of pyruvate as it expresses in the presence of biphenyl (Master and Mohn 2001), which is consistent with the findings reported in this study. Another example of pyruvate having a similar effect is the biodegradation of naphthalene by *Pseudomonas putida* G7. Lee *et al.* showed in a chemostat study that the degradation rates of naphthalene by *P. putida* G7 were enhanced in the presence of pyruvate, while no diauxic growth was found (Lee, et al. 2003).

Pyruvate did not have the expected inhibitory effect on naphthalene degradation by *P. putida* G7 or on PCB degradation by *B. xenovorans* LB400, as shown here, which suggest that it might be of possible advantage in other cases of recalcitrant pollutants.

6.5.5 *Strategy 5: Addition of Co-Substrate at Outset*

The amount of initial biomass required to degrade 100 mg l^{-1} Aroclor® 1242 with resting cells of *B. xenovorans* LB400 to an appreciable extent is very high (Figure 6-1). Growing cells of *Arthrobacter* sp. have been shown to be superior over resting cells in aqueous phase PCB degradation (Kohler, et al. 1988). The fact that pyruvate was able to reinitiate PCB degradation by *B. xenovorans* LB400 indicates that it might function as a non-competitive growth substrate during PCB degradation of growing cells, which could allow using smaller inocula sizes. Figure 6-5 a shows the time course of PCB degradation by a large initial biomass concentration (0.3 g l^{-1}) in the presence of 7.5 g l^{-1} pyruvate. Pyruvate is consumed at a much faster rate than PCBs are degraded and therefore this high amount of pyruvate was chosen to provide the cells with carbon and energy over an extended period of time. The concentration of Arcolor 1242 was reduced to 25 mg l^{-1} after 10 h and to a final concentration of 15 mg l^{-1} after 70 hours. Both rate and extent exceed the performance achieved with a similar initial amount of resting cells (Figure 6-3), and the residual of PCBs might be an accumulation of highly chlorinated congeners, or due to low bioavailability at these low PCB concentrations. However, the cells were growing during the time PCB degradation occurred, and the cell growth has to be attributed to pyruvate and not to PCBs, as cell concentrations remained constant when degrading similar amounts of PCBs in the absence of pyruvate (Figure 6-3).

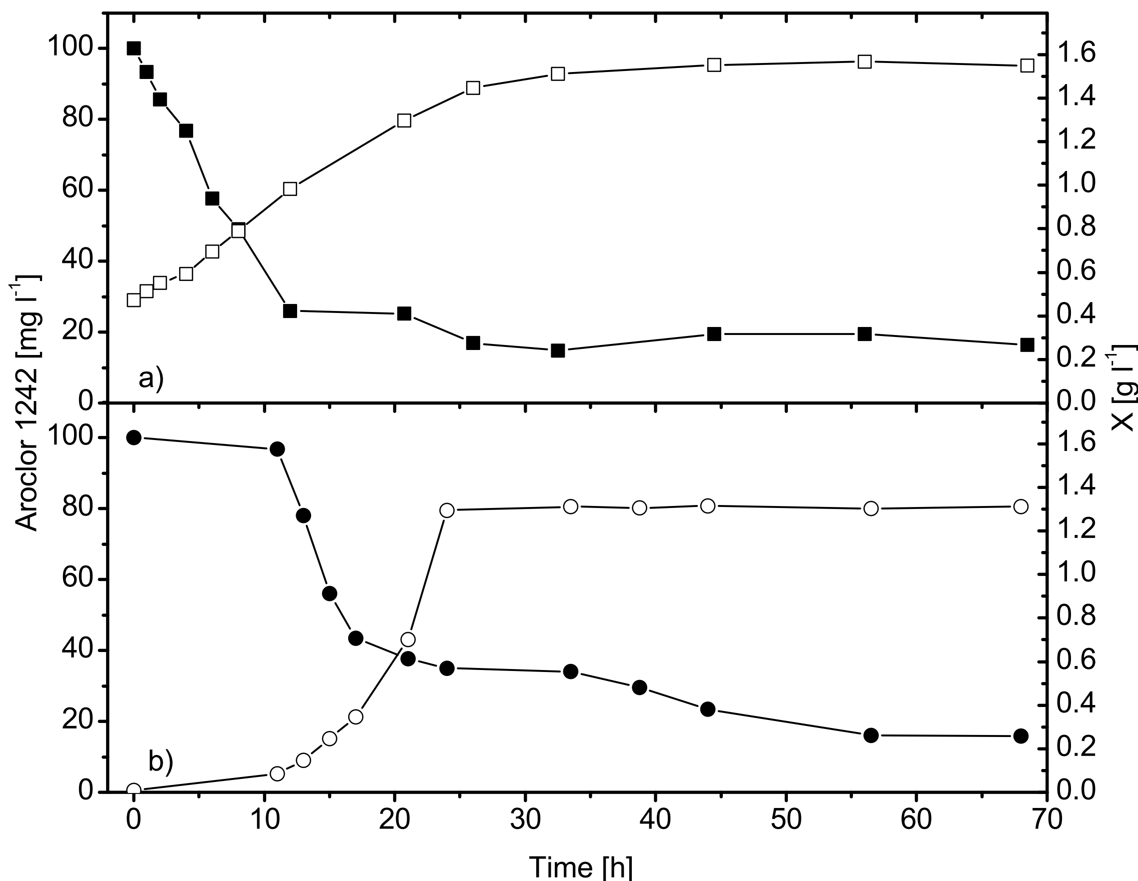

Figure 6-5: Degradation of Aroclor® 1242 in TPPB by growing cells of *B. xenovorans* LB400. The initial biomass concentration was 0.3 g l^{-1} (a) and 0.01 g l^{-1} (b). The initial Aroclor® 1242 concentration in the delivery phase was 10 g l^{-1}. The filled symbols represent the Aroclor® 1242 concentration (in mg Aroclor® per l aqueous medium) and the open symbols the biomass concentration.

The addition of pyruvate to a reactor with a relatively high initial biomass concentration may have provided the cells initially present with sufficient energy to allow for extensive PCB degradation. However, it is not clear that the cells resulting from growth on pyruvate would possess *bph* activity. Accordingly, a modified experiment was conducted in which cells growing from an initial biomass concentration of 10 mg l^{-1} were still able to degrade the majority of 100 mg l^{-1} Aroclor® 1242 to a similar final concentration when growing on pyruvate as shown in Figure 6-5 b. The measured PCB concentrations did not change significantly over the first 10 hours due to the small

amount of active biomass and decreased rapidly once sufficient microbial activity was present in the reactor. The observed activity towards PCBs under these conditions also shows that the newly formed cells which grew on pyruvate as the carbon source still maintained their activity towards PCBs and still expressed the *bph*-genes. This metabolic state might either be inherited from the original inoculum or the upper *bph*-genes are constantly up-regulated by the PCBs present.

PCB degradation under both conditions shown in Figure 6-5 occurred at very high rates. The initial volumetric degradation rate under the conditions employed in Figure 6-5 a can be estimated via linear regression analysis of the first seven data points to be 6.23 g l^{-1} h^{-1} [5.65, 6.81], the values in the brackets giving the upper and lower 95% confidence limits. A process with a similar scope applying soil washing with surfactant solutions followed by PCB biodegradation with *B. xenovorans* LB400 achieved up to 69 % degradation after 24 h of the extracted PCBs, which was a mixture of Aroclor® 1242 and the highly chlorinated Aroclor® 1260 (Billingsley, et al. 2002). The total amount of PCBs which can be introduced in a surfactant system is limited, however with the initial PCB concentrations in surfactant solutions employed by Billingsley et al. being below 6 mg l^{-1}, while the biphasic system employed in this study could achieve initial aqueous phase equivalent concentrations of 100 mg l^{-1}. This value could easily be increased by employing a larger solvent fraction or high initial PCB-in-solvent loadings. The degradation rates achieved in this study are higher than the ones achieved by Billingsley et al., but the comparison is inadequate as this study did not employ any Aroclor® 1260. The high Aroclor® 1242 degradation rates achieved in this study suggest that solvent extraction of PCBs followed by biodegradation in TPPBs might be a viable option to remediate PCB contaminated soils under appropriate cultivating conditions.

6.6 Conclusions

Under appropriate conditions Arcolor 1242 can be degraded by *B. xenovorans* LB400 in TPPBs by either resting or growing cells. Resting cells will lose their ability to degrade PCBs, and the addition of fresh PCB congeners cannot revert this, whereas the generation of active biomass can. A simple and effective method to generate active biomass is to provide a suitable carbon source during PCB degradation after initial inoclum growth on biphenyl. A growth substrate has to be chosen to avoid competition with PCBs for degrading enzymes and to avoid down-regulation of the *bph*-pathway; pyruvate was found to fulfill these requirements. High initial PCB concentrations (100 mg l^{-1}) were degraded to 15% of the initial concentration in less than 48 h at initial rates of 6.2 mg l^{-1} h^{-1}, which makes TPPBs following solvent soil extraction of PCBs an attractive option for PCB soil bioremediation.

Acknowledgements

We are grateful to the Natural Sciences and Engineering Research Council of Canada for financial support, and the Environmental Technologies Program at SAIC Canada.

Chapter 7: Biodegradation of PCBs in Two-Phase Partitioning Bioreactors following Solid Extraction from Soil

Lars Rehmann, Andrew J. Daugulis

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it has been submitted to: *Environmental Science and Technology* (2007)

7.1 Preface to Chapter 7

It was one of the overall aims of this thesis to develop a process to remediate PCB-contaminated soil. The previous chapters systematically evaluated the possible use and the limitations of TPPBs for the actual PCB degradation step, employing either organic solvents or polymer beads as the substrate delivery phase. This final chapter of the experimental section combines the knowledge gained in the previous chapters and attempts to develop a full PCB remediation strategy that includes PCB extraction from contaminated soil using polymer beads followed by biodegradation of the extracted PCBs in a solid-liquid TPPB.

The process discussed in this chapter utilises the polymer Hytrel^{TM}, which has been selected from a wide number of polymeric substances for the biodegradation of biphenyl in Chapter 3. The polymer beads were directly used to extract Aroclor® 1242 from artificially contaminated soil under varying conditions, resulting in a significant decrease of PCB levels in the soil. It was further shown that Aroclor® 1242 could be degraded under aerobic conditions by *B. xenovorans* LB400 in a solid-liquid TPPB when delivered from Hytrel^{TM} polymer beads. The reactor was configured and operated based on the conditions identified in Chapters 5 and 6 and an equivalent of 70 mg Aroclor® 1242 could be degraded per l aqueous medium in 80 h.

Polymer beads were chosen over organic solvents in this final demonstration of a remediation process due to operational advantages during the biodegradation step. Liquid-liquid TPPBs would require sterile operation, in order to prevent contamination of the reactor with organisms capable of degrading the organic phase, which is difficult to realise during soil extraction and would therefore require an additional sterilization step. The inertness of polymers allows for abandoning of such a step and it could be shown in

this chapter that soil extraction and PCB degradation can be realized using polymer beads.

7.2 Abstract

This paper demonstrates the feasibility of a novel process concept for the remediation of PCB contaminated soil. The proposed process consists of PCB extraction from soil using solid polymer beads, followed by biodegradation of the extracted PCBs in a solid-liquid two-phase partitioning bioreactor (TPPB), where PCBs are delivered from the polymer beads to the degrading organisms. The commercially available thermoplastic polymer Hytrel^{TM} was used to extract Aroclor® 1242 from contaminated artificial soil in bench scale experiments. Initial PCB contamination levels of 100 mg kg^{-1} and 1000 mg kg^{-1} could be reduced to 32 % ± 1 to 41 % ± 7 of the initial value after 48 h mixing in the presence of a mobilizing agent at polymer-to-soil ratios of 1 % (w/w) and 10 % (w/w). The decrease of detectable PCBs in the soil was consistent with an increase of PCBs in the polymer beads. It was further shown that Aroclor® 1242 could be delivered to the PCB degrading organism *Burkholderia xenovorans* LB400 in a solid liquid TPPB via Hytrel^{TM} beads. A total of 70 mg Aroclor® 1242 could be degraded in a 1 l solid-liquid TPPB within 80 h of operation.

KEYWORDS: Polychlorinated biphenyls, Aroclor® 1242, bioremediation, brownfields, availability, *Burkholderia xenovorans* LB400, sequestration, equilibrium partitioning

7.3 Introduction

Polychlorinated biphenyls (PCBs) are toxic xenobiotics, manufactured in North America until the late 1970s as congener mixtures under the trade name Aroclor®. PCBs had broad industrial use prior to their ban in 1978 and are now widely distributed contaminants of soil and sediment (Abramowicz 1990; Robinson and Lenn 1994). PCBs are subject to limited microbial degradation under anaerobic conditions via reductive dehalogenation (Mohn and Tiedje 1992; Tiedje, et al. 1993) and aerobic conditions via the biphenyl pathway (*bph*-pathway). The bacterium *Burkholderia xenovorans* LB400 is one of the best studied aerobic PCB degraders, known to be able to degrade up to hexachlorinated biphenyls (Bopp 1986; Fain and Haddock 2001; Hofer, et al. 1994), and was therefore used in this study.

Natural biodegradation of PCBs in contaminated soils and sediments occurs at low rates (Young and Cerniglia 1995), and various attempts to accelerate biodegradation have been undertaken (Fava, et al. 2003; Manzano, et al. 2003; Singer, et al. 2003). *In-situ* and *ex-situ* processes have been applied. Phytoremediation is a promising technique for *in-situ* soil remediation (Whitfield Aslund, et al. 2007), and recently the *bph*-genes of *B. xenovorans* LB400 have been cloned into tobacco plants to increase PCB degradation (Mohammadi, et al. 2007). Other *in-situ* strategies focus on reducing PCB availability and toxicity by adding sorption material such as activated carbon to sediments (Millward, et al. 2005; Werner, et al. 2006; Werner, et al. 2005; Zimmerman, et al. 2004). This method however does not degrade PCBs. Recently iron nano-particles have been shown to dechlorinate PCBs when added to soil (Varanasi, et al. 2007).

Ex-situ methods include excavation of soil followed by incineration (Magar 2003), the application of soil-slurry bioreactors (Fava, et al. 2000), and various extraction

techniques, such as microwave assisted steam extraction (Di, et al. 2002), solvent extraction (EPA 1994; Jakher, et al. 2007), supercritical fluid extraction (Anitescu and Tavlarides 2002; Wu and Marshall 2001), and surfactant soil washing (Berselli, et al. 2006; Billingsley, et al. 1999; Billingsley, et al. 2002). Prpich *et al*. recently demonstrated that polymers can be used to extract phenols from contaminated soil by mixing solid polymer beads with dry soil followed by phenol degradation in a solid-liquid two-phase partitioning bioreactor (TPPB) (Prpich, et al. 2006).

TPPBs are typically comprised of an aqueous phase containing a biocatalyst, and a water-immiscible phase containing large amounts of hydrophobic and/or toxic substrate (Daugulis 2001). The substrate partitions, at low concentrations, from the water-immiscible phase into the aqueous phase, where degradation occurs. TPPBs employing organic solvents (Rehmann and Daugulis 2006) or solid polymer beads (Rehmann and Daugulis 2007b) as water-immiscible phases have been shown to be effective systems for the degradation of biphenyl by *B. xenovorans* LB400.

TPPBs can be combined with PCB extraction from soil to form a soil remediation process. However solvents which are used during soil extraction typically have low viscosity and low boiling points, such as methanol, acetone or iso-propyl alcohol (EPA 1994; Jakher, et al. 2007) and do not possess the desired characteristics of solvents to be used in TPPBs, including immiscibility (Bruce and Daugulis 1991). This limitation does not occur with solid polymers and Figure 7-1 illustrates a possible process of polymer bead PCB extraction followed by PCB degradation in a solid-liquid TPPB.

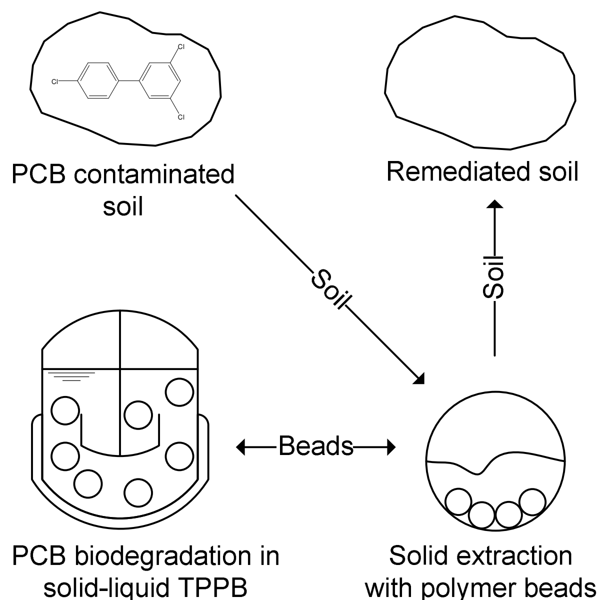

Figure 7-1: Schematic of soil remediation process. PCB contaminated soil is mixed with a solid extraction phase (polymeric material) in the presence of iso-propyl alcohol PCBs are transferred from the soil to the polymers, the remediated soil is returned to the environment and the polymers are treated in a solid-liquid TPPB for microbial PCB destruction.

It was the objective of this study to demonstrate the feasibility of the process shown in Figure 7-1 in bench scale studies. The soil extraction step was demonstrated by contaminating model soils with Aroclor® 1242 followed by its extraction with polymer beads, while the feasibility of the TPPB step was shown by degrading an equivalent of 100 mg Aroclor® 1242 per l aqueous phase in a solid-liquid TPPB.

7.4 Materials and Methods

7.4.1 *Chemicals*

All chemicals used in the fermentation media and the solvents were obtained from either Sigma Aldrich (Canada) or Fisher Scientific (Canada). Biphenyl, 99 % (assay) was obtained from Alfa Aesar (USA) and Aroclor® 1242 (CAS Number: 53469-21-9) was obtained from Chromatographic Specialties Inc. (Brockville, Ontario). Hytrel™ (Hytrel™ is a registered trademark of E. I. du Pont de Nemours and Company) is a

thermoplastic polyester elastomer with a density of 1.17 g cm^{-3}. The polymer chain contains approximately 50% poly-butylene terephthalate (PBT) and 50% butylene ether glycol terephthalate. It was found not to be available as a carbon source to *B. xenovorans* LB400 and no biofilm formation on the polymer surface has been observed (Rehmann and Daugulis 2007b). Hytrel™ polymer beads were obtained from DuPont Canada, in cylindrical shapes with a specific surface are of 1.49 m^2 kg^{-1} (m^2 polymer surface per kg polymer).

7.4.2 *Bacterial Strain*

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, N.Y.) (Bopp 1986), was obtained from the Northern Regional Research Laboratory (Peoria, Ill.). The strain has since been re-classified as *Burkholderia xenovorans* sp. nov. (Goris, et al. 2004). Cultivation conditions, maintenance and biomass determination were described previously (Rehmann and Daugulis 2006).

7.4.3 *Uptake of Aroclor® 1242 by Hytrel™*

The affinity of Hytrel™ for Aroclor® 1242 was demonstrated by measuring the partitioning coefficients of Aroclor® 1242 between Hytrel™ and water and between Hytrel™ and methanol. Methanol solutions containing 3-9 g kg^{-1} Aroclor® 1242 were prepared and 1 g of each solution was equilibrated with 0.1 g Hytrel™ in 10 ml glass scintillation vials sealed with an aluminum capped lid and agitated for 48 h on a rotary shaker at 180 rpm and 30 °C. The remaining Aroclor® 1242 was measured in methanol and compared to control vials containing no Hytrel™. The concentration in the polymer was calculated via mass balance and was assumed to be in equilibrium with the final

concentration in methanol. The Hytrel™ water partitioning coefficient was estimated by equilibrating the Hytrel™ previously loaded with PCBs from methanol with 10 l water (48 h). The remaining PCB concentration in the beads was calculated via mass balance after re-equilibrating the Hytrel™ with methanol, which then allowed estimating the amount released to the water.

7.4.4 *Solid Extraction of Aroclor® 1242 from Soil*

All experiments were undertaken with artificial soil composed of 10 % organics (peat), 20 % clay and 70 % industrial sand at pH 6 as outlined in OECD method 207. Soil was contaminated in 10 ml glass scintillation vials (aluminum sealed lid) with Aroclor® 1242 dissolved in IPA to final PCB in soil concentrations of either 100 mg kg^{-1} or 1000 mg kg^{-1}, followed by 48 h mixing (rotary shaker at 240 rpm and 22 °C), to ensure distribution of the PCBs throughout the soil.

Hytrel™ polymer beads and 15 % IPA (mobilizing agent) were subsequently added to the contaminated soil in the scintillation vials to form four extraction series with different initial conditions: 0.1 g beads were each added to 12 vials containing 1 g of soil contaminated with 100 mg kg^{-1} Aroclor® 1242 (extraction series E1) and to 12 vials containing 1 g soil contaminated with 1000 mg kg^{-1} Aroclor® 1242 (extraction series E2), 0.03 g beads were added to vials containing 3 g of soil contaminated with 100 mg kg^{-1} Aroclor® 1242 (extraction series E3) and to 12 vials containing 3 g soil contaminated with 1000 mg kg^{-1} Aroclor® 1242 (extraction series E4). Extraction series E1 and E2 employed a polymer to soil ratio of 10 % (w/w) and an initial PCB level of 100 mg kg^{-1} and 1000 mg kg^{-1} respectively, while E3 and E4 employed a polymer to soil ratio of 1 % (w/w) at similar initial contamination levels. All vials were incubated on a rotary shaker at 240 rpm and 22 °C. Three vials of each extraction series and one control vial per

extraction series containing no beads were removed from the shaker after 6 h, 12 h, 24 h and 48 h. Polymers and soil were separated and PCBs were extracted from soil and polymer as described below.

7.4.5 *Biodegradation of Aroclor® 1242 in a Solid Liquid TPPB*

Hytrel™ polymer beads (2.5 g) were loaded with Aroclor® 1242 by equilibrating them with 3.5 g methanol containing 160 mg Aroclor® 1242. The final Aroclor® 1242 concentration in the polymer was 48 mg kg^{-1}. The polymers were added to a 1 l BioFlo® I bioreactor at 30 °C, agitated with two Rushton turbines at 500 rpm and aerated (sterile air) at 1 l min^{-1}. The pH was maintained at 6.9. Samples of the polymer beads were taken periodically for PCB analysis as described below and of the aqueous phase for biomass analysis as described elsewhere (Rehmann and Daugulis 2006). The reactor was inoculated with PAS medium (Bopp 1986) containing *B. xenovorans* LB400 at initial concentrations of 2 g l^{-1}. The biomass for the inoculum was grown in a solid-liquid TPPB with biphenyl as the sole carbon source as described elsewhere (Rehmann and Daugulis 2007b). Sodium pyruvate was added at the outset of the fermentation (6 g) and after 48 h (3 g) to provide the degrading organism with a carbon and energy source that promotes cell activity and PCB degradation (Rehmann and Daugulis 2007c).

7.4.6 *PCB Extraction and Analysis*

PCBs were extracted from soil samples of 1 g or 3 g with 10 ml hexane and from Hytrel™ (0.03 g or 0.1 g) with 5 ml methanol (24 h on a rotary shaker at 180 rpm and 30 °C). The extract was analyzed with an Agilent 6890 gas chromatograph (Agilent Technologies Canada Inc., Mississauga, Ontario) equipped with a fused silica cap capillary column (Supelco SPB-1, Sigma-Aldrich Corp. St. Louis, MO, USA), an electron

capture detector (ECD) (280 °C) and split injector (250 °C). The temperature program was as follows: 100 °C for 4 min, 100 °C to 180 °C at 10 °C min^{-1}, 180 °C for 1 min, 180 °C to 240 °C at 1.5 °C min^{-1}, 240°C for 1 min, 240 °C to 300 °C at 20 °C min^{-1}, 300 °C for 10 min. Aroclor® standards in hexane and methanol were run for every analysis and blank hexane/methanol was run after every four samples. Aroclor® was quantified by using the summed peak area according to EPA Method 304 h.

7.5 Results and Discussion

7.5.1 *Uptake of Aroclor 1242 by Hytrel™*

Hytrel™ had been selected from a variety of polymeric substances as a suitable delivery phase for biphenyl in solid-liquid TPPBs in a previous study (Rehmann, et al. 2007). It was found that Hytrel™ had a suitable polymer/water partitioning coefficient for biphenyl of $LogK_{S/W}$ = 3.51, and it was also shown that Hytrel™ had the ability to extract biphenyl from methanol or water and subsequently release it into aqueous medium where biodegradation by *B. xenovorans* LB400 took place (Rehmann and Daugulis 2007b). The ability of Hytrel™ to sorb large amounts of PCBs, which are environmentally more significant than biphenyl, from methanol was evaluated, and the equilibrium data are shown in Figure 7-2. The equilibrium isotherm follows a linear trend over the observed range of concentrations, and it can be seen that Aroclor® 1242 partitions preferentially into Hytrel™. Similar partitioning behavior had also been found for biphenyl, where the Hytrel™ methanol partitioning coefficient was $K_{S/M}$ ~ 1.3 (Rehmann and Daugulis 2007b). Aroclor® 1242 partitions between Hytrel™ and methanol with a partitioning coefficient of $K_{S/M}$ ~ 4.2.

The fact that Hytrel™ has an approximately four times higher affinity for PCBs than organic solvents such as methanol indicates the possibility of using a material such

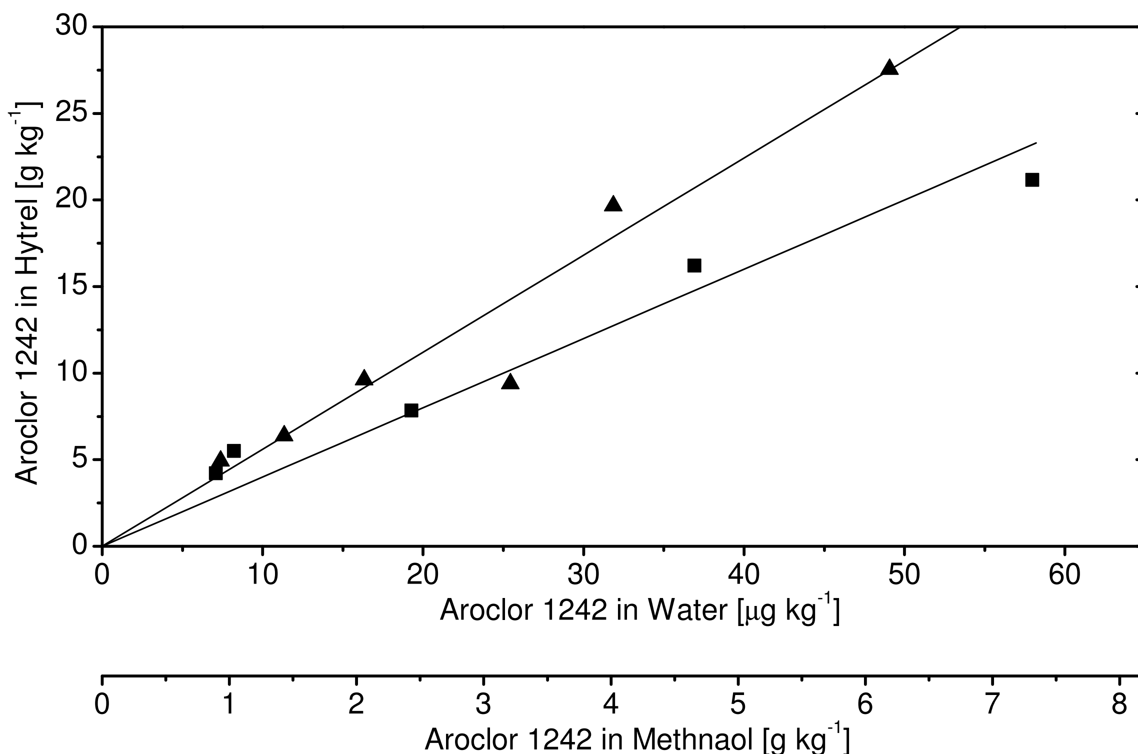

Figure 7-2: Partitioning of Aroclor® 1242 between methanol and Hytrel™ (triangles) and between water and Hytrel™ (squares).

as Hytrel™ in PCB remediation schemes. However, if the remediation scheme requires subsequent biodegradation of PCBs in aqueous medium, then reversible release of PCBs from the polymeric carrier material is required. The PCB-loaded Hytrel™ was therefore equilibrated with water and the equilibrium isotherm is also shown in Figure 7-2. Linear partitioning of Aroclor® 1242 could be found over the observed range of concentrations, with a high Hytrel™ /water partitioning coefficient of $LogK_{S/W} = 5.52$. The observed partitioning coefficient is significantly larger than the octanol/water partitioning coefficient of Aroclor 1242 of $LogK_{O/W} = 4.2$ (Cohen, et al. 1993), again showing the high affinity of the polymeric substance for PCBs.

7.5.2 *Solid Extraction of Aroclor® 1242 from Soil*

Extraction of Aroclor® 1242 from soil was evaluated in four different extraction series employing different initial contamination levels and initial Hytrel^{TM} to soil ratios. Isopropyl alcohol (IPA) was added as a mobilizing agent to facilitate mass transfer of PCBs from soil particles into the polymer beads. Mobilizing agents have been shown to significantly enhance PCB extractability in soil (Berselli, et al. 2006). IPA was chosen as it has been approved by the US environmental protection agency in a commercial soil extraction process (EPA 1994). Table 7-1 shows the initial contamination levels, Hytrel^{TM} to soil ratios and PCB distribution after 48 h for the four different extraction series. The PCB concentration in the soil was reduced to 30 % - 40 % of its original value after mixing for 48 h, while between 50 % and 70 % could be recovered in the polymer. Comparing the results of extraction series E1 and E3 shows that the amount of Aroclor® 1242 in soil contaminated at initial concentrations of approximately 100 mg kg^{-1} could be reduced to 37 and 35 mg kg^{-1} using ether 10 % (w/w) or 1 % (w/w) of Hytrel^{TM}. The ten fold smaller amount of Hytrel^{TM} in E3 in comparison to E1 did not result in the removal of a smaller PCB fraction, showing the efficiency of the extraction process. The lower bead-to-soil ration resulted in a substantially increased PCB loading in the polymer. The final PCB concentration in the polymer was > 4,000 mg kg^{-1} in the case of E3, which is lower than the range of concentrations shown in Figure 7-2, indicating that a further reduction of the Hytrel^{TM} to soil ratio might be possible without losing performance.

Even though the final PCB removal in E1 and E3 was similar, as shown in Table 7-1, the rate at which the extraction occurred was significantly higher in E1, as can be seen in Figure 7-3a and 3c. In the case of E1 the majority of PCBs was extracted within the initial 12 h, whereas it took 48 h to reach similar levels in E3. The difference can

Table 7-1: Extraction of Aroclor® 1242 from soil using Hytrel™ polymer beads under different initial conditions. The presented values represent the averages of triplicates after 48 h ± 95 % confidence limits.

Test series	Initial PCB in soil [mg kg $^{-1}$]	Bead/soil ratio [g g $^{-1}$]	Final PCB in soil [mg kg $^{-1}$]	Final PCB in polymer [mg kg $^{-1}$]	Residual in soil [%]	Amount extracted in polymer [%]
E1	100	0.1	37 ± 3	621 ± 124	34 ± 3	57 ± 11
E2	1000	0.1	271 ± 2	5,653 ± 641	32 ± 1	67 ± 8
E3	100	0.01	35 ± 6	4,039 ± 337	42 ± 7	48 ± 4
E4	1000	0.01	334 ± 27	68,036 ± 7,746	33 ± 3	68 ± 8

readily be explained by the tenfold higher available surface area in E1, as a result of the higher polymer to soil ratio. The mass transfer rate of biphenyl from Hytrel™ to water has been shown to be directly proportional to the available surface area (Rehmann and Daugulis 2007b). The surface to volume ratio of the Hytrel™ beads employed in this study was high, as the average bead diameter was 4 – 6 mm. Such geometries are suitable for fundamental lab-scale studies, however for field applications it would not be suitable to separate rice grain sized polymer beads from soil. The performance of different geometries such as polymer sheets or rods will have to be evaluated for large scale applications, which however is beyond the scope of this study. In a related study Hytrel™ sheets have been used for the *in-situ* removal of 3-methylcatechol from a fermentation vessel, and the performance of the sheets was comparable to the performance of beads of similar mass as far as the extent of removal was concerned, however the removal rate was lower due to the decrease in surface area (Prpich and Daugulis 2007).

Due to the high affinity of the selected polymer for PCBs a ratio of only 1 % (w/w) was enough to reduce the concentration of Aroclor® 1242 in soil to below 50 mg kg $^{-1}$ (E3), which is the concentration in soil set by the US code of federal regulation (40 CFR

761.65) above which PCB spills have to be reported to the authorities for further actions. This key value was not achieved if the initial PCB concentration in the soil was 1000 mg kg^{-1} (E2 and E4), however the PCB concentration in soil was reduced significantly in both extraction series and the possibility of two or sequential extraction will be investigated in future research.

The conditions in E1 and E2 were similar except that the total amount of PCBs present in the soil was increased from 100 mg kg^{-1} to 1000 mg kg^{-1}. The residual amount of PCBs after 48 h increased from 37 mg kg^{-1} to 271 mg kg^{-1}, however, there is no significant difference in the final % distribution of PCBs between soil and polymer (Table 7-1). The time course of E2 resembles the time course of E1 more than the time course of E3 (Figure 7-3). E2 and E1 share the same polymer to soil ratio, while E2 and E3 share the same polymer to PCB ratio. It can be seen in Figure 7-3 that the bead to soil ratio seems to govern the rate at which PCBs are removed from soil. The final concentration of Aroclor® 1242 in Hytrel™ is higher in E2 (5,653 ± 641 mg kg^{-1}) than in E3 (4,039 ± 337 mg kg^{-1}), suggesting that more PCBs could have been extracted in E3 given additional extraction time. Figure 7-3c seems to confirm this as it appears that the PCB concentration in both phases did not reach a constant level after 48 h. This is most likely due to the hydrophobic nature of PCBs, which can associate with soil particles (Ehlers and Luthy 2003) resulting in slow transfer of PCBs from soil to the polymer despite the addition of IPA as a mobilizing agent. More hydrophilic substances have been shown to transfer from soil into polymers at a much faster rate. Pripch *et al.* could reduce the amount of phenol in soil from 2,300 mg kg^{-1} to approximately 100 mg kg^{-1} in 24 h under otherwise similar conditions to the ones employed in this study (Prpich, et al. 2006).

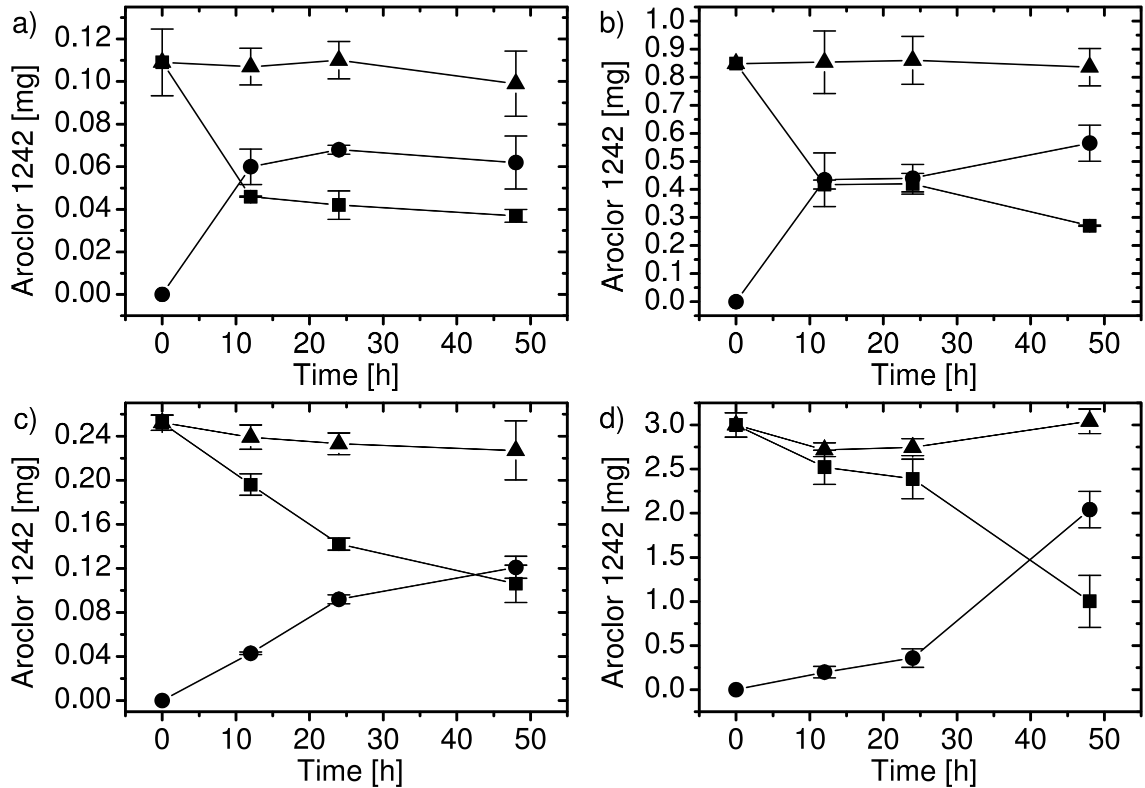

Figure 7-3: Time course of PCB extraction from soil with Hytrel^{TM} polymer beads. The triangles represent the total amount of Aroclor® 1242 recovered, the squares represent the amount in remaining in soil and the circles the amount extracted to the polymer beads. Test series E1 is shown in a), E2 in b), E3 in c) and E4 in d).

In series E4 the initial PCB concentration was 1000 mg kg^{-1}, as in series E2, and the bead to soil ratio was 1 % (w/w) as in series E3. The final concentration remaining in the soil is only slightly higher than in E2 (334 mg kg^{-1} compared to 271 mg kg^{-1}), despite the tenfold decrease of the amount of extractant. The time course of the extraction generally follows the same trend as E3 (Figure 7-3), confirming that the extraction rate is strongly affected by the polymer to soil ratio. The total amount of PCBs extracted in series E4 is substantially higher than in the other extraction series. The final Aroclor® 1242 concentration in Hytrel^{TM} was 68,000 mg kg^{-1}, showing the strong PCB sorption capacity of Hytrel^{TM}. These findings are significant for possible PCB remediation strategies even if no biodegradation of the extracted PCBs follows, as the amount of contaminated

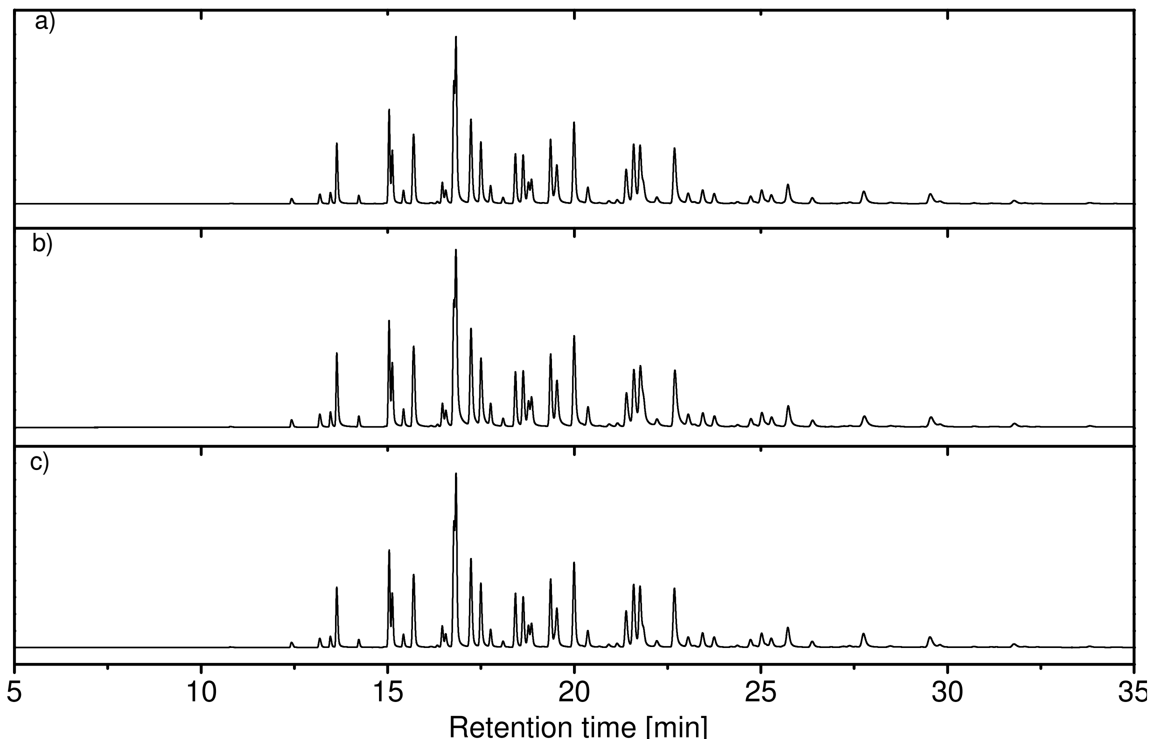

Figure 7-4: Chromatogram of Aroclor® 1242 extracted from: a) control soil, containing no polymer beads and an initial Aroclor® 1242 concentration of 1000 mg kg^{-1} after 48 h, b) soil containing 10 % (w/w) Hytrel^{TM} and an initial Aroclor® 1242 concentration of 1000 mg kg^{-1} after 48 h, c) Hytrel^{TM} beads, which were in contact with soil from b) after 48 h. No scale is shown on the y-axis to emphasize the similarities in the congener distribution.

material can be reduced by orders of magnitude if PCBs are extracted from soil using 1 % (w/w) of extractant. Even though soil extraction was shown only for Aroclor® 1242 and not for higher chlorinated PCB mixtures such as Aroclor® 1260, it is expected that that the methodology can be extended towards other Aroclors. Figure 7-4 shows chromatograms of PCB extracts from control soil, and from the soil and Hytrel^{TM} fraction of extraction series E2. Each peak represents one or more congeners, and it can clearly be seen that the congener distribution in the treated soil (Figure 7-4b) is not significantly altered from non treated soil (Figure 7-4a). The extracted PCB fraction consequently follows the same distribution (Figure 7-4c). The fact that highly chlorinated congener (high retention times)

are extracted to the same extent as low chlorinated (short retention times) suggests that other Aroclors can be extracted the same way as Aroclor® 1242.

7.5.3 *Biodegradation of Aroclor® 1242 in a Solid Liquid TPPB*

In order to recycle the polymer beads in a possible soil remediation scheme it would be necessary to remove PCBs from the polymer followed by PCB degradation, as suggested in Figure 7-1. This can be achieved in solid liquid TPPBs, which have previously been demonstrated to be an effective technology platform for biodegradation of phenols (Prpich and Daugulis 2006) and biphenyl (Rehmann and Daugulis 2007b). Introducing polymer beads containing large amounts of PCBs into a bioreactor will result in equilibrium partitioning of PCBs into the aqueous medium (as shown in Figure 7-2), where they can be degraded by appropriate microorganisms. Hydrophobic substances such as PCBs will result in low aqueous phase PCB concentrations and it can be inferred from Figure 7-2 that the PCB concentrations in the aqueous phase delivered from Hytrel™ beads will be below 100 $\mu g\ kg^{-1}$. This low PCB level is the only bioavailable fraction in TPPBs and studies in liquid-liquid TPPBs have shown that substrate availability of PCBs can be the rate limiting factor in biphasic systems during aerobic PCB degradation by *B. xenovorans* LB400 (Rehmann and Daugulis 2007a).

It has previously been shown that the specific microbial PCB degradation rate in the presence of an immiscible liquid phase was reduced to less than 10 % of its value in a single phase system, and that the resulting low degradation rates can be partly circumvented by employing large amounts of biomass (Rehmann and Daugulis 2007a). It was further shown that cells of *B. xenovorans* LB400 lost their *bph*-activity after approximately 40 h when degrading PCBs in the absence of a metabolizable carbon

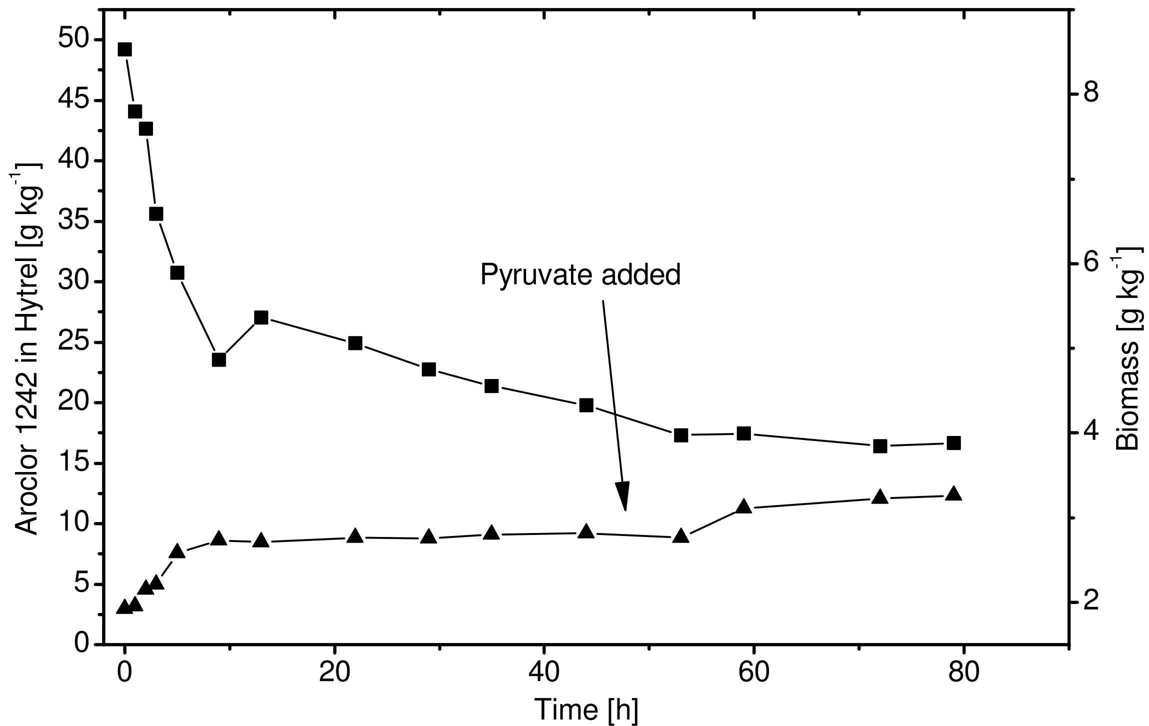

Figure 7-5: Biodegradation of Aroclor® 1242 by *B. xenovorans* LB400 in a solid liquid TPPB. PCBs are delivered from Hytrel™ polymer beads.

source, and that the addition of sodium pyruvate could provide carbon and energy to the organism while sustaining its activity towards PCBs (Rehmann and Daugulis 2007c). In anticipation of similar limitations in solid-liquid TPPBs, the initial amount of biomass was therefore chosen to be 2 g kg^{-1} and sodium pyruvate was added at the outset of the fermentation and after 48 h. The initial PCB loading in the polymer beads was chosen to be 50 g kg^{-1} based the concentrations achieved during soil extraction (Table 7-1, E4).

Figure 7-5 shows the time course of Aroclor® 1242 degradation in a solid liquid TPPB. The initial Aroclor® 1242 concentration of 50 g kg^{1} in the solid phase was reduced to 16 g kg^{-1} over a total fermentation time of 80 h. Biomass formation due to growth on pyruvate was observed during the first 10 hours after inoculation and after the addition of supplementary pyruvate after 48 h. The fact that new biomass was formed

after the second addition of pyruvate shows that the cells were viable. The minimal effect on PCB degradation at this point might be due to low PCB availability. However, the general performance achieved in this solid-liquid TPPB is comparable to the performance of liquid-liquid TPPBs employing silicone oil as a water-immiscible phase (Rehmann and Daugulis 2007c). Approximately 70 % of the initially present PCBs, an equivalent of 70 mg PCB per l aqueous phase, was degraded within 80 h.

7.6 Conclusions

The individual steps constituting a PCB soil remediation process comprised of soil extraction with polymers followed by PCB degradation in a solid liquid TPPB were demonstrated in this paper. It can be concluded that the volume of PCB contaminated material can be reduced significantly if PCBs are extracted from soil into polymers, and that low chlorinated PCB mixtures can subsequently be degraded aerobically in a solid liquid TPPB. Future work will investigate the use of weathered soil and the possibility of treating higher chlorinated Aroclors.

Acknowledgements

We are grateful to the Natural Sciences and Engineering Research Council of Canada for financial support, and the Environmental Technologies Program at SAIC Canada.

Chapter 8: Summary and Conclusions

It was the objective of this thesis to thoroughly investigate the possible use and the limitations of TPPBs for the degradation of hydrophobic compounds. Both liquid-liquid and solid-liquid TPPBs were considered throughout this work and both reactor types were described experimentally and mathematically for the biodegradation of biphenyl. It was found that the mass transfer rate was the rate-limiting step in solid-liquid TPPBs but not in liquid-liquid TPPBs. Solid-liquid TPPBs however could provide higher equilibrium substrate concentrations in the aqueous phase and allowed a microbial consortium to be employed in the reactors. Finally it could be shown that solid-liquid TPPBs could be utilised in a soil remediation scheme, in which PCBs were extracted from soil using polymer beads, and later be delivered to degrading microorganisms from the polymer beads in a solid-liquid TPPB.

The initial significant contribution derived from this thesis was the developement of a method to estimate the microbial kinetic parameters for a poorly water-soluble substrate (biphenyl). The Monod parameters for the degradation of biphenyl by *B. xenovorans* LB400 could be estimated in a liquid-liquid TPPB, following the selection of an appropriate solvent and the determination of the partitioning coefficient of biphenyl between the selected solvents and water. The use of a TPPB was essential for estimating the microbial parameters, as data at very low aqueous phase substrate concentrations were required. The use of a TPPB provided aqueous phase biphenyl concentrations below 0.2 mg L^{-1} for more than 10 h, which is otherwise not possible. The applied methodology can be applied to estimate kinetic parameters of microbial degradation of other hydrophobic compounds. The estimated half saturation constant of the Monod model of K_S = 0.1 mg

L^{-1} also suggested that PCB degradation by the same organism will most likely be substrate limited, as the solubility of PCBs, which are degraded via the same pathway as biphenyl, is below the estimated half saturation constant.

In the next step biphenyl degradation in solid-liquid TPPBs was investigated. A polymer selection strategy analogous to solvent selection for liquid-liquid TPPBs was developed and the commercially available polymer Hytrel^{TM} was chosen for the biphenyl degradation process. It was found that Hytrel^{TM}, amongst other polymers, expressed linear partitioning for biphenyl between polymer and water as well as between polymer and methanol. However, equilibrating the polymers with high biphenyl concentrations in methanol resulted in biphenyl concentration above the maximum level that could be achieved in polymer-water systems. Equilibrating these highly loaded polymers with aqueous medium resulted in biphenyl saturated water. Similar effects are known in drug delivery applications, and the possiblility that such highly loaded beads could maintain a substrate saturated aqueous phase was considered highly beneficial for the biphenyl/PCB degradation process which might be limited by the aqueous phase substrate concentration. It was further shown that a biphenyl-degrading consortium could be employed in a solid-liquid TPPB. This consortium could not be used in a liquid-liquid TPPB as at least one of the members produced a biosurfactant. The biphenyl degradation rates of *B. xenovorans* LB400 and the microbial consortium were comparable and the growth curve appeared to follow a linear rather than an exponential trend, which leads to the notion that the process might be mass transfer limited, which was further investigated in the next chapter.

A mathematical model combining mass transfer from the immiscible phase to the aqueous phase, and microbial biphenyl degradation kinetics in the aqueous phase, was developed to investigate possible mass transfer limitations during biphenyl degradation in

solid-liquid TPPBs. It was found that the release rate of biphenyl from Hytrel^{TM} into the aqueous phase is proportional to the available polymer surface area and the difference between the actual aqueous phase biphenyl concentration and the aqueous phase biphenyl concentration in thermodynamic equilibrium with the concentration in the polymer. The microbial degradation kinetics and their dependency on the aqueous phase biphenyl concentration were known from previous chapters. The predictions made by the model showed the same linear trend and followed the experimental data well. The model was verified by employing polymer beads with a different geometry and a larger surface to volume ratio. The reactor employing the smaller beads showed enhanced performance and faster degradation rates, as predicted by the model. It was concluded from this chapter that solid-liquid TPPBs might be able to provide higher equilibrium concentrations than liquid-liquid TPPBs in the aqueous phase, but that the rate at which they provide the substrate can be limiting.

The following three chapters applied the findings from the previous three chapters to the degradation of PCBs in TPPBs. At first the effect of PCB availability in the aqueous phase was evaluated. This was done by adding small fractions of two different immiscible phases to aqueous medium containing PCBs and *B. xenovorans* LB400. The two solvents BES and silicone oil were used. Both were neither toxic nor bioavailable to the employed organism, and BES had successfully been used as a delivery phase for biphenyl in previous chapters. The two solvents were chosen because of their dissimilar affinity for PCBs. BES was expected to have a higher affinity than silicone oil and hence it was expected that adding BES to PCB-containing aqueous medium would reduce the aqueous phase PCB concentration and therefore the microbial degradation rate more than adding the same amount of silicone oil would. It was found that the addition of small

fractions of either BES or silicone oil significantly decreased the amount of PCBs degraded over an incubation period of 5 days. The observed effect was stronger for BES than it was for silicone oil, as previously anticipated. The rates were then investigated in bioreactors containing different amounts of either BES or silicone oil and it was found that approximately ten times the amount of immiscible phase in silicone oil reactors resuled in similar degradation rates as BES reactors. A control reactor containing no solvent resulted in substantially higher degradations rates. The specific degradation rates in the presence of 10 mL silicone oil and 1 mL BES per L aqueous phase was reduced to less than 10 % of the value achieved in the reactor containing no solvent, and the total fermentation time was increased from 5 h to 48 h. It was further shown that the PCB degradation was not mass transfer limited, as bioreactors operated with different agitation rates resulted in the same PCB degradation rates. However it was found that no further PCB degradation occurred after approximately 40 h to 50 h despite the presence of degradable congeners. Possible reasons for this were investigated in the following chapter.

A variety of different strategies were employed to investigate the reasons for the apparent loss of PCB degrading activity of *B. xenovorans* LB400 after 40 to 50 h incubation time. It was found that the addition of fresh PCB congeners after 50 h did not stimulate further degradation, whereas the addition of fresh biomass did. It was therefore concluded that the cells lost their activity towards PCBs rather than running out of degradable congeners. The cells were found to be viable when incubated on a different carbon source and it was therefore speculated that the cells might not be able to utilise sufficient amounts of carbon and energy from PCBs which were the only source of carbon and energy in the reactors. Different carbon sources were investigated as an additional substrate to reinitiate PCB degradation. The addition of glucose and biphenyl

resulted in the formation of new biomass, but could not reinitiate PCB degradation. It was concluded that the presence of a readily accessible carbon source such as glucose resulted in preferred glucose consumption and a reduction in *bph*-activity. Biphenyl was not expected to cause a similar effect as it gets degraded via the same pathway as PCBs (*bph*-pathway). However biphenyl is significantly more soluble in water than PCBs and it was speculated that PCB congeners and biphenyl compete for the same enzymes and that the higher aqueous phase solubility of biphenyl combined with more favourable partitioning into the aqueous phase resulted in preferred biphenyl degradation. Pyruvate was chosen as it is a downstream intermediate in the biphenyl pathway and therefore does not compete for the early enzymes and might further not result in down-regulation of the pathway. It was then shown that pyruvate could re-initiate PCB degradation activity if added to inactive cells. Further cells initially cultivated on biphenyl and subsequently growing of pyruvate were able to degrade PCBs. The addition of pyruvate was found to be a simple and effective method to increase PCB degradation activity of *B. xenovorans* LB400 in biphasic reactor systems.

The final contribution of this thesis suggested a possible remediation process for PCB-contaminated soil consisting of PCB extraction from soil using Hytrel^{TM} polymer beads followed by PCB degradation in a solid-liquid TPPB. All steps required in such a process were demonstrated and it was shown the PCB concentrations in soil of up to 1000 mg kg^{-1} could be reduced to 30 % - 40 % of their initial value in a single extraction stage (48 h) employing a bead to soil ratio of 10 % (w/w). Decreasing the bead to soil ratio to 1 % (w/w) resulted in the same overall PCB reduction, however the rate at which PCBs were extracted from soil was lower due to the smaller amount of available surface area. It was further shown that PCBs could be degraded by *B. xenovornas* LB400 in a solid-liquid

TPPB employing Hytrel™ polymer beads. An equivalent of 70 mg Aroclor® 1242 per L aqueous phase was degraded in the presence of pyruvate as an additional carbon source. Polymer beads had various advantages over organic solvents in this process, as the recovery from soil is less challenging than it would be for organic solvents and the system is more robust towards possible soil organisms that will inevitably be introduced to the reactor and might be able to utilise the immiscible phase as a carbon source. This final contribution built on the knowledge gained in all previous chapters of this thesis and showed that TPPBs can be utilised for the remediation of contaminated soils, even for poorly water-soluble and persistent substances such as PCBs.

8.1 Future work

The results presented in this thesis indicated that the extraction of priority environmental contaminants from soil followed by their biodegradation in TPPBs is a possible treatment option for contaminated soils. In the specific case of PCBs future work would include PCB extraction from environmental samples. Weathered soil that has been contaminated with PCBs for a long period of time might be more difficult to treat than artificially contaminated well-defined soil in bench scale experiments. The ability to target and degrade higher chlorinated PCB congeners should also be investigated in future work. Utilizing solid-liquid TPPBs would further allow the use of microbial consortia, which might result in larger extents of degradation. Aroclor® mixtures with higher chlorine contents than the Aroclor®1242 which was used in this study cannot be degraded aerobically to an appreciable extent. Such Aroclors would either require an anaerobic pre-treatment to dechlorinate the PCB congeners to a level suitable for aerobic degradation, or an abiotic process for PCB destruction. The volume reduction of

contaminated material if soil can be extracted with 1 % (w/w) polymer alone might be of economical interest, which would have to be evaluated in future work.

A scale-up of the extraction technology, possibly employing different geometries of the polymeric phase, would also be required for this technology to be applied in field scale applications. Polymer sheets or rods would significantly simplify the polymer recovery from soil and the extraction as well as release kinetics from such geometries would have to be studied. The mathematical framework presented in Chapter 4 could be used as a basis for models describing the release from sheets or rods.

The solid phase extraction technology is currently being investigated for PAHs and diesel fuels. Both projects are currently under investigation and preliminary results show that these compounds can be removed by selected polymeric materials. The polymers that were selected for PAHs and diesel fuel are different from the polymers utilized in this thesis, and a better understanding of the interaction between polymers and environmental contaminants is required to provide a rational polymer selection methodology.

References

Abramowicz DA. 1990. Aerobic and anaerobic biodegradation of PCBs: A Review. Crit Rev Biotechnol 10:241-300.

Ahmed M, Focht DD. 1973. Degradation of polychlorinated biphenyls by two species of Achromobacter. Can J Microbiol 19:47-52.

Al Aalam S. 1993. High efficiency styrene biodegradation in a biphasic organic/water continuous reactor. Appl Microbiol Biotechnol 39:696-699.

Alcock RE, Behnisch PA, Jones KC, Hagenmaier H. 1998. Dioxin-like PCBs in the environment-human exposure and the significance of sources. Chemosphere 37:1457-1472.

Ambrose AM, Booth AN, Deeds F, Cox AJJ. 1960. A toxicological study of biphenyl, a citrus fungistat. F Res 25:328-336.

Amend LJ, Lederman PB. 1992. Critical evaluation of PCB remediation technologies. Environ Prog 11:173-177.

Amsden BG, Bochanysz J, Daugulis AJ. 2003. Degradation of xenobiotics in a partitioning bioreactor in which the partitioning phase is a polymer. Biotechnol Bioeng 84:399-405.

Anitescu G, Tavlarides LL. 2002. Methanol as a cosolvent and rate-enhancer for the oxidation kinetics of 3,3 ',4,4 '-tetrachlorobiphenyl decomposition in supercritical water. Ind Eng Chem Res 41:9-21.

Arnett CM, Parales JV, Haddock JD. 2000. Influence of chlorine substituents on rates of oxidation of chlorinated biphenyls by the biphenyl dioxygenase of *Burkholderia* sp. strain LB400. Appl Environ Microbiol 66:2928-2933.

Bailey JE, Ollis DF. 1986. Biochemical Engineering Fundamentals. New York:McGraw-Hill.

Bamford HA, Poster DL, Huie RE, Baker JE. 2002. Using extrathermodynamic relationships to model the temperature dependence of Henry's law constants of 209 PCB congeners. Environ Sci Technol 36:4395-4402.

Barbosa VL, Tandlich R, Burgess JE. 2007. Bioremediation of trace organic compounds found in precious metals refineries' wastewaters: A review of potential options. Chemosphere: In Press

Baumann MD, Daugulis AJ, Jessop PG. 2005. Phosphonium ionic liquids for degradation of phenol in a two-phase partitioning bioreactor. Appl Microbiol Biotechnol 67:131-137.

Bedard DL, Unterman R, Bopp LH, Brennan MJ, Haberl ML, Johnson C. 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl Environ Microbiol 51:761-768.

Bedard DL, Wagner RE, Brennan MJ, Haberl ML, Brown JF,Jr. 1987. Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl Environ Microbiol 53:1094-1102.

Beltrametti F, Reniero D, Backhaus S, Hofer B. 2001. Analysis of transcription of the bph locus of *Burkholderia* sp. strain LB400 and evidence that the ORF0 gene product acts as a regulator of the bphA1 promoter. Microbiology 147:2169-2182.

Berselli S, Benitez E, Fedi S, Zannoni D, Medici A, Marchetti L, Fava F. 2006. Development and assessment of an innovative soil-washing process based on the use of cholic acid-derivatives as pollutant-mobilizing agents. Biotechnol Bioeng 93:761-770.

Billingsley KA, Backus SM, Juneson C, Ward OP. 1997. Comparison of the degradation patterns of polychlorinated biphenyl congeners in Aroclors by *Pseudomonas* strain LB400 after growth on various carbon sources. Can J Microbiol 43:1172-1179.

Billingsley KA, Backus SM, Ward OP. 1999. Effect of surfactant solubilization on biodegradation of polychlorinated biphenyl congeners by *Pseudomonas* LB400. Appl Microbiol Biotechnol 52:255-260.

Billingsley KA, Backus SM, Wilson A, Singh A, Ward OP. 2002. Remediation of PCBs in soil by surfactant washing and biodegradation in the wash by *Pseudomonas* sp. LB400. Biotechnol Lett 1827-1832.

Boehncke A, Koennecker G, Mangelsdorf.I, Wibbertmann A. 2005. Biphenyl. Concise international chemical assessment document no. 6. Geneva:World Health Organization. p 1-31.

Bohon RL, Claussen WF. 1951. The Solubility of Aromatic Hydrocarbons in Water. J Am Chem Soc 73:1571-1578.

Bopp LH. 1986. Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400. J Ind Microbiol 23-29.

Bosetti C, Negri E, Fattore E, La Vecchia C. 2003. Occupational exposure to polychlorinated biphenyls and cancer risk. Eur J Cancer Prev 12:251-255.

Bruce LJ, Daugulis AJ. 1991. Solvent Selection Strategies for Extractive Biocatalysis. Biotechnol Prog 7:116-124.

Bruhlmann F, Chen W. 1999. Tuning biphenyl dioxygenase for extended substrate specificity. Biotechnology and Bioengineering 63:544-551.

Canadian Council of Resource and Environment Ministers. 1987. The PCB Story.

Catelani D, Sorlini C, Treccani V. 1971. The Metabolism of Biphenyl by *Pseudomonas-Putida*. Experientia 27:1173-1174.

Chain PS, Denef VJ, Konstantinidis KT, Vergez LM, Agullo L, Reyes VL, Hauser L, Cordova M, Gomez L, Gonzalez M, Land M, Lao V, Larimer F, LiPuma JJ, Mahenthiralingam E, Malfatti SA, Marx CJ, Parnell JJ, Ramette A, Richardson P, Seeger M, Smith D, Spilker T, Sul WJ, Tsoi TV, Ulrich LE, Zhulin IB, Tiedje JM. 2006. *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.73-Mbp genome shaped for versatility. Proc Natl Acad Sci U S A 103:15280-15287.

Chang JS, Chou CL, Lin GH, Sheu SY, Chen WM. 2005. *Pseudoxanthomonas kaohsiungensis*, sp. nov., a novel bacterium isolated from oil-polluted site produces extracellular surface activity. Syst Appl Microbiol 28:137-144.

Choi AL, Levy JI, Dockery DW, Ryan LM, Tolbert PE, Altshul LM, Korrick SA. 2006. Does living near a Superfund site contribute to higher polychlorinated biphenyl (PCB) exposure? Environ Health Perspect 114:1092-1098.

Chou, S. F. J., Griffin RA. 1986. Solubility and soil mobility of polychlorinated biphenyls. In: Waid JS, editor. PCBs and the environment. Boca Raton, FL.:CIC Press, Inc. p 101-120.

Cochran JW, Frame GM. 1999. Recent developments in the high-resolution gas chromatography of polychlorinated biphenyls. J Chromatogr A 843:323-368.

Cohen RM, Mercer JW, Matthews J. 1993. DNAPL site evaluation. Boca Raton, Fla.:C.K. Smoley.

Collins LD, Daugulis AJ. 1999a. Benzene/toluene/p-xylene degradation. Part I. Solvent selection and toluene degradation in a two-phase partitioning bioreactor. Appl Microbiol Biotechnol 52:354-359.

Collins LD, Daugulis AJ. 1999b. Simultaneous biodegradation of benzene, toluene, and p-xylene in a two-phase partitioning bioreactor: concept demonstration and practical application. Biotechnol Prog 15:74-80.

Commandeur LCM, Vaneyseren HE, Opmeer MR, Govers HAJ, Parsons JR. 1995. Biodegradation kinetics of highly chlorinated biphenyls by *Alcaligenes* Sp Jb1 in an aerobic continuous-culture system. Environ Sci Technol 29:3038-3043.

Costa MC, Rolemberg MP, Boros LAD, Krahenbuhl MA, deOliveira MG, Meirelles AJA. 2006. Solid-liquid equilibrium of binary fatty acid mixtures. J Chem Eng Data: In Press

Daugulis AJ. 2001. Two-phase partitioning bioreactors: a new technology platform for destroying xenobiotics. Trends Biotechnol 19:457-462.

Daugulis AJ. 1997. Partitioning Bioreactors. Curr Opin Biotechnol 8:169-174.

Daugulis AJ, Amsden BG, Bochanysz J, Kayssi A. 2003. Delivery of benzene to *Alcaligenes xylosoxidans* by solid polymers in a two-phase partitioning bioreactor. Biotechnol Lett 25:1203-1207.

De Fina KM, Sharp TL, Acree WE. 1999. Solubility of biphenyl in organic nonelectrolyte solvents. Comparison of observed versus predicted values based upon Mobile Order theory. Can J Chem 77:1589-1593.

Denef VJ, Park J, Tsoi TV, Rouillard JM, Zhang H, Wibbenmeyer JA, Verstraete W, Gulari E, Hashsham SA, Tiedje JM. 2004. Biphenyl and benzoate metabolism in a genomic context: outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. Appl Environ Microbiol 70:4961-4970.

Denef VJ, Patrauchan MA, Florizone C, Park J, Tsoi TV, Verstraete W, Tiedje JM, Eltis LD. 2005. Growth substrate- and phase-specific expression of biphenyl, benzoate, and C1 metabolic pathways in *Burkholderia xenovorans* LB400. J Bacteriol 187:7996-8005.

Dercova K, Vrana B, Balaz S. 1999. A kinetic distribution model of evaporation, biosorption and biodegradation of polychlorinated biphenyls (PCBs) in the suspension of *Pseudomonas stutzeri*. Chemosphere 38:1391-1400.

Di P, Chang DP, Dwyer HA. 2002. Modeling of polychlorinated biphenyl removal from contaminated soil using steam. Environ Sci Technol 36:1845-1850.

Droin CM, Cooper DG. 1992. Biosurfactants and aqueous two-phase fermentation . Biotechnol Bioeng 40:86-90.

Ehlers LJ, Luthy RG. 2003. Contaminant bioavailability in soil and sediment. Environ Sci Technol 37:295A-302A.

Eibes G, Moreira MT, Feijoo G, Daugulis AJ, Lema JM. 2006. Operation of a two-phase partitioning bioreactor for the oxidation of anthracene by the enzyme manganese peroxidase. Chemosphere 66:1744-1751.

EPA. 1994. Innovative technology evaluation report: Terra-Kleen Response Group, Inc. Solvent extraction technology. 540R94521:1-68.

Erickson MD. 1986. Analytical chemistry of PCBs. Boston:Butterworth Publishers. 508 p.

Evans BS, Dudley CA, Klasson KT. 1996. Sequential anaerobic-aerobic biodegradation of PCBs in soil slurry microcosms. Appl Biochem Biotechnol 57-58:885-894.

Fain MG, Haddock JD. 2001. Phenotypic and phylogenetic characterization of *Burkholderia (Pseudomonas)* sp. strain LB400. Curr Microbiol 42:269-275.

Fava F. 1996. Aroclor 1221 aerobic dechlorination by a bacterial co-culture: role of chlorobenzoic acid degrading bacteria in the process. Chemosphere 32:1477-1483.

Fava F, Bertin L, Fedi S, Zannoni D. 2003. Methyl-beta-cyclodextrin-enhanced solubilization and aerobic biodegradation of polychlorinated biphenyls in two aged-contaminated soils. Biotechnol Bioeng 81:381-390.

Fava F, Di Gioia D, Marchetti L. 2000. Role of the reactor configuration in the biological detoxification of a dump site-polychlorobiphenyl-contaminated soil in lab-scale slurry phase conditions. Appl Microbiol Biotechnol 53:243-248.

Frame GM, Cochran JW, Boewadt SS. 1996. Complete PCB congener distributions for 17 Aroclor mixtures determined by 3 HRGC systems optimized for comprehensive, quantitative, congener-specific analysis. J High Res Chrom 19:657-688.

Fredenslund A, Gmehling J, Rasmussen P. 1977. Vapor-liquid equilibria using UNIFAC : a group-contribution method. Amsterdam ; New York; New York:Elsevier Scientific Pub. Co.; distributors for the U.S. and Canada, Elsevier North-Holland. 380 p.

Gamerdinger AP, Achin RS, Traxler RW. 1995. Effect of aliphatic nonaqueous phase liquids on naphthalene biodegradation in multiphase systems. J Environ Qual 24:1150-1156.

Gear CW. 1971. Numerical Initial Value Problems in Ordinary Differential Equations. Engliwood Cliffs, NJ.:Pretince-Hall.

Ghosh U, Weber AS, Jensen JN, Smith JR. 2000. Relationship between PCB desorption equilibrium, kinetics, and availability during land biotreatment. Environ Sci Technol 34:2542-2548.

Ghosh U, Zimmerman JR, Luthy RG. 2003. PCB and PAH speciation among particle types in contaminated harbor sediments and effects on PAH bioavailability. Environ Sci Technol 37:2209-2217.

Gmehling J, Li J, Schiller M. 1993. A modified UNIFAC model. 2. Present parameter matrix and results for different thermodynamic properties. Ind Eng Chem Res 32:178-193.

Goris J, De Vos P, Caballero-Mellado J, Park J, Falsen E, Quensen JF,III, Tiedje JM, Vandamme P. 2004. Classification of the biphenyl- and polychlorinated biphenyl-degrading strain LB400T and relatives as *Burkholderia Xenovorans* sp. nov. Int J Syst Evol Microbiol 54:1677-1681.

Guieysse B, Autem Y, Soares A. 2005. Biodegradation of phenol at low temperature using two-phase partitioning bioreactors. Water Sci Technol 52:97-105.

Guitart R, Puig P, omez-Cata J. 1993. Requirement for a standardized nomenclature criterium for PCBs: Computer assisted assignment of correct congener denomination and number. Chemosphere 27:1451-1458.

Haddock JD, Nadim LM, Gibson DT. 1993. Oxidation of biphenyl by a multicomponent enzyme system from *Pseudomonas* sp. Strain LB400. J Bacteriol 175:395-400.

Hauser R, Chen Z, Pothier L, Ryan L, Altshul L. 2003. The relationship between human semen parameters and environmental exposure to polychlorinated biphenyls and p,p'-DDE. Environ Health Perspect 111:1505-1511.

Hawker DW, Connell DW. 1988. Octanol-water partition coefficients of polychlorinated biphenyl congeners. Environ Sci Technol 22:382-387.

Hofer B, Backhaus S, Timmis KN. 1994. The biphenyl/polychlorinated biphenyl-degradation locus (bph) of *Pseudomonas* sp. LB400 encodes four additional metabolic enzymes. Gene 144:9-16.

HSDB. 1991. Information from the Hazardous Substance Databank. Toxicology Data Network System.

Inoue A, Horikoshi K. 1991. Estimation of solvent-tolerance of bacteria by the solvent Pparameter Log-P. J Ferment Bioeng 71:194-196.

Inoue A, Horikoshi K. 1989. A *Pseudomonas* thrives in high-concentrations of toluene. Nature 338:264-266.

Ioannidis S, Anderko A. 2000. Thermodynamic modeling of molecular adsorption using parameters derived from binary surface excess data. Ind Eng Chem Res 39:1095-1105.

Jabusch TW, Swackhamer DL. 2005. Partitioning of polychlorinated biphenyls in octanol/water, triolein/water, and membrane/water systems. Chemosphere 60:1270-1278.

Jakher A, Achari G, Langford CH. 2007. An investigation into solvent extraction of PCBs from weathered soils. Environ Technol 28:49-57.

Janikowski TB, Velicogna D, Punt M, Daugulis AJ. 2002. Use of a two-phase partitioning bioreactor for degrading polycyclic aromatic hydrocarbons by a *Sphingomonas* sp. Appl Microbiol Biotechnol 59:368-376.

Khan MA, Hansen LG. 2003. ortho-Substituted polychlorinated biphenyl (PCB) congeners (95 or 101) decrease pituitary response to thyrotropin releasing hormone. Toxicol Lett 144:173-182.

Kohler A, Schuttoff M, Bryniok D, Knackmuss HJ. 1994. Enhanced biodegradation of phenanthrene in a biphasic culture system. Biodegradation 5:93-103.

Kohler HP, Kohler-Staub D, Focht DD. 1988. Cometabolism of polychlorinated biphenyls: enhanced transformation of Aroclor 1254 by growing bacterial cells. Appl Environ Microbiol 54:1940-1945.

Kose T, Mukai T, Takimoto K, Okada M. 2003. Effect of non-aqueous phase liquid on biodegradation of PAHs in spilled oil on tidal flat. Wat Res 37:1729-1736.

Kubatova A, Erbanova P, Eichlerova I, Homolka L, Nerud F, Sasek V. 2001. PCB congener selective biodegradation by the white rot fungus *Pleurotus ostreatus* in contaminated soil. Chemosphere 43:207-215.

Kuo CE, Liu SM, Liu C. 1999. Biodegradation of coplanar polychlorinated biphenyls by anaerobic microorganisms from estuarine sediments. Chemosphere 39:1445-1458.

Lambo AJ, Patel TR. 2006. Cometabolic degradation of polychlorinated biphenyls at low temperature by psychrotolerant bacterium *Hydrogenophaga* sp. IA3-A. Curr Microbiol V53:48-52.

Lee K, Park J, Ahn I. 2003. Effect of additional carbon source on naphthalene biodegradation by *Pseudomonas putida* G7. J Hazard Mater 105:157-167.

Liu D. 1981. Biodegradation of Aroclor 1221 type PCBs in sewage wastewater. Bull Environ Contam Toxicol 27:695-703.

Liu ZS, Rempel GL. 1997. Preparation of superabsorbent polymers by crosslinking acrylic acid and acrylamide copolymers. J Appl Polym Sci 64:1345-1353.

Luthy RG, Dzombark DA, Shannon MJR, Unterman R, Smith JR. 1997. Dissolution of PCB congeners from an Aroclor and an Aroclor/hydraulic oil mixture. Water Res 31:561-573.

Macedo AJ, Kuhlicke U, Neu TR, Timmis KN, Abraham WR. 2005. Three stages of a biofilm community developing at the liquid-liquid interface between polychlorinated biphenyls and water. Appl Environ Microbiol 71:7301-7309.

Madigan, Martinko, Parker. 2000. Brock Biology of Microorganisms. Upper Saddle River:Prentice Hall.

Magar VS. 2003. PCB treatment alternatives and research directions. J Envir Engrg 129:961-965.

Malinowski JJ. 1999. Evaluation of liquid extraction potentials for downstream separation of 1,3-Propanediol. Biotechnol Tech V13:127-130.

Maltseva OV, Tsoi TV, Quensen JF,III, Fukuda M, Tiedje JM. 1999. Degradation of anaerobic reductive dechlorination products of Aroclor 1242 by four aerobic bacteria. Biodegradation 10:363-371.

Manzano MA, Perales JA, Sales D, Quiroga JM. 2003. Enhancement of aerobic microbial degradation of polychlorinated biphenyl in soil microcosms. Environ Toxicol Chem 22:699-705.

Marcoux J, Deziel E, Villemur R, Lepine F, Bisaillon JG, Beaudet R. 2000. Optimization of high-molecular-weight polycyclic aromatic hydrocarbons' degradation in a two-liquid-phase bioreactor. J Appl Microbiol 88:655-662.

Master ER, Mohn WW. 2001. Induction of bphA, encoding biphenyl dioxygenase, in two polychlorinated biphenyl-degrading bacteria, psychrotolerant *Pseudomonas* strain Cam-1 and mesophilic *Burkholderia* strain LB400. Appl Environ Microbiol 67:2669-2676.

Master ER, Lai VW, Kuipers B, Cullen WR, Mohn WW. 2002. Sequential anaerobic-aerobic treatment of soil contaminated with weathered Aroclor 1260. Environ Sci Technol 36:100-103.

McNamara SW, Ghosh U, Dzombak DA, Weber AS, Smith JR, Luthy RG. 2005. Effect of oil on polychlorinated biphenyl phase partitioning during land biotreatment of impacted sediment. J Envir Engrg 131:278-286.

Millward RN, Bridges TS, Ghosh U, Zimmerman JR, Luthy RG. 2005. Addition of activated carbon to sediments to reduce PCB bioaccumulation by a polychaete (*Neanthes arenaceodentata*) and an amphipod (*Leptocheirus plumulosus*). Environ Sci Technol 39:2880-2887.

Mohamed MM, Hatfield K. 2005. Modeling microbial-mediated reduction in batch reactors. Chemosphere 59:1207-1217.

Mohammadi M, Chalavi V, Novakova-Sura M, Laliberté J, Sylvestre M. 2007. Expression of bacterial biphenyl-chlorobiphenyl dioxygenase genes in tobacco plants. Biotechnol Bioeng 97:496-505.

Mohn WW, Tiedje JM. 1992. Microbial reductive dehalogenation. Microbiol Rev 56:482-507.

Mondello FJ, Turcich MP, Lobos JH, Erickson BD. 1997. Identification and modification of biphenyl dioxygenase sequences that determine the specificity of polychlorinated biphenyl degradation. Appl Environ Microbiol 63:3096-3103.

Monod J. 1949. The growth of bacterial cultures. Annual Reviews of Microbiology 3:371-383.

Moody JD, Doerge DR, Freeman JP, Cerniglia CE. 2002. Degradation of biphenyl by *Mycobacterium* sp strain PYR-1. Appl Microbiol Biotechnol 58:364-369.

Mukerjee-Dhar G, Hatta T, Shimura M, Kimbara K. 1998. Analysis of changes in congener selectivity during PCB degradation by *Burkholderia* sp. strain TSN101

with increasing concentrations of PCB and characterization of the bphBCD genes and gene products. Arch Microbiol 169:61-70.

Munoz R, Rolvering C, Guieysse B, Mattiasson B. 2005. Aerobic phenanthrene biodegradation in a two-phase partitioning bioreactor. Water Sci Technol 52:265-271.

Newman JD, Marshall J, Chang M, Nowroozi F, Paradise E, Pitera D, Newman KL, Keasling JD. 2006. High-level production of amorpha-4,11-diene in a two-phase partitioning bioreactor of metabolically engineered *Escherichia coli*. Biotechnol Bioeng 95:684-691.

Nielsen DR, Daugulis AJ, McLellan PJ. 2005. Transient performance of a two-phase partitioning bioscrubber treating a benzene-contaminated gas stream. Environ Sci Technol 39:8971-8977.

Ochsner AB, Sokoloski TD. 1985. Prediction of solubility in nonideal multicomponent systems using the UNIFAC group contribution model. J Pharm Sci 74:634-637.

Paul MA. 1952. The Solubilities of naphthalene and biphenyl in aqueous solutions of Eelectrolytes. J Am Chem Soc 74:5274-5277.

Perry RH, Perry JH. 1963. Chemical Engineers' Handbook. New York:McGraw-Hill.

Pier MD, Betts-Piper AA, Knowlton CC, Zeeb BA, Reimer KJ. 2003. Redistribution of polychlorinated biphenyls from a local point source: Terrestrial soil, freshwater sediment, and vascular plants as indicators of the halo effect. Arct , Antarc , Alp Res 35:349-360.

Platonow NS, Funnell HS, Bullock DH, Arnott DR, Saschenbrecker PW, Grieve DG. 1971. Fate of polychlorinated biphenyls in dairy products processed from the milk of exposed cows. J Dairy Sci 54:1305-1308.

Prpich GP, Adams RL, Daugulis AJ. 2006. Ex situ bioremediation of phenol contaminated soil using polymer beads. Biotechnol Lett 28:2027-2031.

Prpich GP, Daugulis AJ. 2006. Biodegradation of a phenolic mixture in a solid–liquid two-phase partitioning bioreactor. Appl Microbiol Biotechnol V72:607-615.

Prpich GP, Daugulis AJ. 2005. Enhanced biodegradation of phenol by a microbial consortium in a solid-liquid two phase partitioning bioreactor. Biodegradation 16:329-339.

Prpich GP, Daugulis AJ. 2004. Polymer development for enhanced delivery of phenol in a solid-liquid two-phase partitioning bioreactor. Biotechnol Prog 20:1725-1732.

Prpich GP, Daugulis AJ. 2007. A novel solid-liquid two-phase partitioning bioreactor for the enhanced bioproduction of 3-methylcatechol. Biotechnol Bioeng: In Press

Rapaport RA, Eisenreich SJ. 1984. Chromatographic determination of octanol-water partition coefficients (Kow's) for 58 PCB polychlorinated biphenyl congeners. Environ Sci Technol 18:163-170.

Rehmann L, Daugulis AJ. 2007a. Bioavailability of PCBs in biphasic bioreactors. Biochem Eng J: In Press

Rehmann L, Daugulis AJ. 2007b. Biodegradation of biphenyl in a solid-liquid two-phase partitioning bioreactor. Biochem Eng J: In Press

Rehmann L, Daugulis AJ. 2007c. Enhancement of PCB degradation by *Burkholderia xenovorans* LB400 in biphasic systems by manipulating culture conditions. Biotechnol Bioeng: In Press

Rehmann L, Daugulis AJ. 2006. Biphenyl degradation kinetics by *Burkholderia xenovorans* LB400 in two-phase partitioning bioreactors. Chemosphere 63:972-979.

Rehmann L, Sun B, Daugulis AJ. 2007. Polymer selection for biphenyl degradation in a solid-liquid two-phase partitioning bioreactor. Biotechnol Prog 23:814-819.

Reichardt PB, Chadwick BL, Cole MA, Robertson BR, Button DK. 1981. Kinetic-study of the biodegradation of biphenyl and its monochlorinated analogs by a mixed marine microbial community. Environ Sci Technol 15:75-79.

Robinson GK, Lenn MJ. 1994. The bioremediation of polychlorinated biphenyls (PCBs): problems and perspectives. Biotechnol Genet Eng Rev 12:139-188.

Rote JW, Murphy PG. 1971. A method for the quantitation of polychlorinated biphenyl (PCB) isomers. Bull Environ Contam Toxicol 6:377-384.

Seeger M, Timmis KN, Hofer B. 1995. Conversion of chlorobiphenyls into phenylhexadienoates and benzoates by the enzymes of the upper pathway for polychlorobiphenyl degradation encoded by the bph locus of *Pseudomonas* sp. strain LB400. Appl Environ Microbiol 61:2654-2658.

Seeger M, Zielinski M, Timmis KN, Hofer B. 1999. Regiospecificity of dioxygenation of di- to pentachlorobiphenyls and their degradation to chlorobenzoates by the bph-encoded catabolic pathway of *Burkholderia* sp. strain LB400. Appl Environ Microbiol 65:3614-3621.

Seeger M, Timmis KN, Hofer B. 1997. Bacterial pathways for the degradation of polychlorinated biphenyls. Mar Chem 58:327-333.

Seto M, Kimbara K, Shimura M, Hatta T, Fukuda M, Yano K. 1995. A novel transformation of polychlorinated biphenyls by *Rhodococcus* sp. strain RHA1. Appl Environ Microbiol 61:3353-3358.

Sherman LM, Schut JH. 2007. Your business pricing update, March 2007, polyolefin, PVC Prices Mmoving up. Plast Technol 20.03.2007.

Sierra I, Valera JL, Marina ML, Laborda F. 2003. Study of the biodegradation process of polychlorinated biphenyls in liquid medium and soil by a new isolated aerobic bacterium (*Janibacter* sp.). Chemosphere 53:609-618.

Singer AC, Smith D, Jury WA, Hathuc K, Crowley DE. 2003. Impact of the plant rhizosphere and augmentation on remediation of polychlorinated biphenyl contaminated soil. Environ Toxicol Chem 22:1998-2004.

Slaughter DW, Doherty MF. 1995. Calculation of solid-liquid equilibrium and crystallization paths for melt crystallization processes. Chem Eng Sci 50:1679-1694.

Smithwick LA, Smith A, Quensen JF,III, Stack A, London L, Morris PJ. 2003. Inhibition of LPS-induced splenocyte proliferation by ortho-substituted polychlorinated biphenyl congeners. Toxicology 188:319-333.

Speare DM, Olf P, Bugg TDH. 2002. Hammett analysis of a C-C hydrolase-catalysed reaction using synthetic 6-aryl-2-hydroxy-6-ketohexa-2,4-dienoic acid substrates. Chemical Communications 2304-2305.

Still KR, Arfsten DP, Jederberg WW, Kane LV, Larcom BJ. 2003. Estimation of the health risks associated with polychlorinated biphenyl (PCB) concentrations found onboard older U.S. Navy vessels. Appl Occup Environ Hyg 18:737-758.

Tanaka T. 1978. Collapse of gels and the critical endpoint. Phys Rev Lett 40:820-823.

Tempest DW. 1969. The continuous cultivation of microorganisms. I. Theory of the chemostat. Methods Microbiol 2:260-276.

Thomann RV, St John JP. 1979. The fate of PCBs in the Hudson River ecosystem. Ann N Y Acad Sci 320:610-629.

Thomas JM, Ward CH. 1992. Subsurface microbial ecology and bioremediation. Journal of Hazardous Materials 32:179-194.

Tiedje JM, Quensen JF,III, Chee-Sanford J, Schimel JP, Boyd SA. 1993. Microbial reductive dechlorination of PCBs. Biodegradation 4:231-240.

Tulp MTM, Hutzinger O. 1978. Some thoughts on aqueous solubilities and partition coefficients of PCB, and the mathematical correlation between bioaccumulation and physico-chemical properties. Chemosphere 7:849-860.

Varanasi P, Fullana A, Sidhu S. 2007. Remediation of PCB contaminated soils using iron nano-particles. Chemosphere 66:1031-1038.

Viamajala S, Peyton BM, Richards LA, Petersen JN. 2007. Solubilization, solution equilibria, and biodegradation of PAH's under thermophilic conditions. Chemosphere 66:1094-1106.

Volkering F, Breure AM, Sterkenburg A, Andel JG. 1992. Microbial degradation of polycyclic aromatic hydrocarbons: Effect of substrate availability on bacterial growth kinetics. Appl Microbiol Biotechnol V36:548-552.

Wang DI, Ochoa A. 1972. Measurements on the interfacial areas of hydrocarbon in yeast fermentations and relationships to specific growth rates. Biotechnol Bioeng 14:345-360.

Wang Z. 2007. The potential of cloud point system as a novel two-phase partitioning system for biotransformation. Appl Microbiol Biotechnol: In Press

Weaver WC, Simmons PB, Thompson QE. 1979. Diphenyl and terphenyls. In: Grayson M, editor. Kirk-Othmer encyclopedia of chemical technology. New York:Wiley. p 782-793.

Weber J,Walter J., McGinley PM, Katz LE. 1991. Sorption phenomena in subsurface systems: Concepts, models and effects on contaminant fate and transport. Wat Res 25:499-528.

Werner D, Ghosh U, Luthy RG. 2006. Modeling polychlorinated biphenyl mass transfer after amendment of contaminated sediment with activated carbon. Environ Sci Technol 40:4211-4218.

Werner D, Higgins CP, Luthy RG. 2005. The sequestration of PCBs in Lake Hartwell sediment with activated carbon. Water Res 39:2105-2113.

Wesche J, Hammer E, Becher D, Burchhardt G, Schauer F. 2005. The bphC gene-encoded 2,3-dihydroxybiphenyl-1,2-dioxygenase is involved in complete degradation of dibenzofuran by the biphenyl-degrading bacterium *Ralstonia* sp. SBUG 290. J Appl Microbiol 98:635-645.

Whitfield Aslund ML, Zeeb BA, Rutter A, Reimer KJ. 2007. In situ phytoextraction of polychlorinated biphenyl - (PCB) contaminated soil. Sci Total Environ 374:1-12.

Wiegel J, Wu Q. 2000. Microbial reductive dehalogenation of polychlorinated biphenyls. FEMS Microb Ecol 32:1-15.

Wu Q, Marshall WD. 2001. Approaches to the remediation of a polychlorinated biphenyl (PCB) contaminated soil - a laboratory study. J Environ Monit 3:281-287.

Wu Q, Sowers KR, May HD. 1998. Microbial reductive dechlorination of Aroclor 1260 in anaerobic slurries of estuarine sediments. Applied and Environmental Microbiology 64:1052-1058.

Yalkowsky SH, Dannenfelser RM. 1992. Aquasol database of aqueous solubility.

Yoshimura T. 2003. Yusho in Japan. Ind Health 41:139-148.

Young LY, Cerniglia C. 1995. Microbial transformation and degradation of toxic organic chemicals. New York:Wiley-Liss. 654 p.

Zeng EY, Tsukada D, Noblet JA, Peng J. 2005. Determination of polydimethylsiloxane-seawater distribution coefficients for polychlorinated biphenyls and chlorinated pesticides by solid-phase microextraction and gas chromatography-mass spectrometry. J Chromatogr A 1066:165-175.

Zimmerman JR, Ghosh U, Millward RN, Bridges TS, Luthy RG. 2004. Addition of carbon sorbents to reduce PCB and PAH bioavailability in marine sediments: Physicochemical tests. Environ Sci Technol 38:5458-5464.

Zitko V, Hutzinger O, Safe S. 1971. Retention times and electron-capture detector responses of some individual chlorobiphenyls. Bull Environ Contam Toxicol 6:160-163.

Zwiernik MJ, Quensen JF, Boyd SA. 1999. Residual petroleum in sediments reduces the bioavailability and rate of reductive dechlorination of Aroclor 1242. Environ Sci Technol 33:3574-3578.