BIOTRANSFORMATION OF POLYCHLORINATED BIPHENYLS BY *PSEUDOMONAS* STRAIN LB400: INFLUENCE OF GROWTH SUBSTRATE AND SURFACTANT SUPPLEMENTATION

by

Kate Billingsley

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ABSTRACT

Studies were undertaken to characterize biotransformation of polychlorinated biphenyls (PCBs) by *Pseudomonas* LB400, a bacterium that was previously isolated from contaminated soil and exhibits a broad specificity towards a range of PCB congeners.

Resting cells of *Pseudomonas* LB400 are known to transform PCBs when the cells are previously grown on biphenyl. In this study, PCB transformation was also observed in resting cells grown on other substrates such as glucose and glycerol. The presence of PCB congeners in the growth medium increased the lag phase for growth of cells on a biphenyl substrate, but not on a glycerol substrate. Supplementation of the degradation medium with biphenyl dramatically decreased the rate of PCB congener transformation while the presence of glycerol or glucose had little or no effect on PCB transformation rates. Removal rates of individual congeners with biphenyl-grown cells were found to vary depending on chlorination pattern of the congener. When compared to cells grown on glucose and glycerol, the relative rates of congener disappearance were not constant. The presence of PCBs adversely affected the viability of biphenyl-grown cells over a 48-h incubation period and may explain the decline observed in PCB conversion capacity over the same incubation period. A major objective of this study was to investigate the significance of using biphenyl as carbon source for growth of *Pseudomonas* LB400 cells capable of PCB transformation. The findings indicate that, whereas higher rates of transformation of PCBs are observed with biphenyl-grown cells, cells grown on other carbon sources retain PCB-transforming enzymes.

When resting cells of *Pseudomonas* LB400 grown on biphenyl were incubated with different Aroclor mixtures, greater degradation of total PCBs was observed in Aroclors containing lower-chlorinated congeners. Cells grown on glucose or glycerol also transformed Aroclors, to lesser extents. Time courses of transformation of individual congeners in the Aroclors were plotted and used to determine the transformation rate constants (k). By analysis of the rate constants, it was concluded that the order of degradation of the different congeners in an Aroclor were similar regardless of growth

substrate. In general, k values for conversion of a particular congener were lower for cells grown on glucose or glycerol compared to cells grown on biphenyl. The data allowed congeners to be grouped according to their relative rates of degradation.

The effectiveness of a variety of commercial surfactants in solubilizing PCBs sorbed to glass was dependent on surfactant concentration and dissolution time. Most surfactants were fully or partially effective as PCB-solubilizing agents at concentrations greater than their critical micelle concentration (CMC) value. Among ethoxylate surfactants tested, those with lower CMCs were more efficient PCB-solubilizing agents. PCBs were not solubilized with a no-surfactant water control, nor with the nonionic block copolymer. At a concentration of 10 g/l, three out of eleven surfactants, a diethanolamide/ethoxylate blend, an alkane sulphonate and a twin alcohol ethoxylate, inhibited bacterial growth. Only two surfactants, Sorbax PMO-20, a fatty acid ethoxylate and Witcomul 3235, an anionic/nonionic blend, supported growth of *Pseudomonas* LB400 as sole carbon sources. In general, at surfactant concentrations above their CMC, anionic surfactants promoted, whereas nonionic surfactants inhibited PCB transformation compared to a water control. Transformation rate constants of each congener in the presence of *Pseudomonas* LB400 and selected surfactants were compared. The inhibitory effect of Igepal CO-630, an alkylphenol ethoxylate, on PCB degradation could be eliminated by diluting the surfactant solution to a lower concentration.

These surfactants were then tested for their abilities to wash PCBs from weathered contaminated soil. While none were effective at solubilizing the PCBs at a surfactant concentration of 1 g/l, six surfactants, at a concentration of 10 g/l, removed greater than 75% of the hexane-extractable PCBs from the soil. The most effective surfactants in soil washing tests were the nonionic alcohol ethoxylate, Bio-Soft EN-600, and Igepal CO-630. The PCB congeners in the soil washings were then transformed by resting cells of *Pseudomonas* LB400, previously grown on biphenyl. Solutions containing the latter efficient soil washing surfactants manifested lower rates of PCB

biodegradation, with only 16 or 32% of congeners transformed over a 48-h incubation period. In contrast, two anionic surfactants, Hostapur SAS 60 and Nansa LSS38/AS, exhibited highest rates and extents of PCB degradation (52-67% of congeners transformed over a 48-h incubation period), although they were less efficient surfactants in the soil washing process.

Cells of *Pseudomonas* LB400, grown on biphenyl, glucose or glycerol, transformed PCB congeners into chlorobenzoic acid (CBA) metabolites. Rates and extents of PCB transformation and metabolite formation were highest with biphenyl-grown cells. Intermediate rates of metabolite production were observed with glycerol-grown cells and lowest rates of production were found with glucose-grown cells. Regardless of carbon source, the rate of degradation of congeners was faster than the rate of production of CBAs. Relative rates of PCB transformation and metabolite production from different congeners with cells grown on a particular substrate followed the same general order, 2,3-CBA (from 2,3-CBP) > 2-CBA (from 2,2'-CBP) > 4-CBA (from 2,5,4'-CBP) >2,4-CBA (from 2,4,2',4'-CBP). *Pseudomonas* LB400 appeared unable to grow on any of the chlorobenzoic acids. However, *Pseudomonas* LB400 cells grown on biphenyl appeared capable of degrading 2-CBA and 2,3-CBA but not 4-CBA nor 2,4-CBA. Cells grown on glycerol appeared unable to metabolize any CBAs.

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When I started this page, it was with the usual "I would like to thank", but somehow this did not convey the depth of my feeling. Then I thought of "I am forever indebted to", but this sounded like I would hand over my first born. Finally, I resorted to the thesaurus (well used in the writing of this document to come up with alternatives to "degradation", I think they are all in here somewhere!). SO...

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We will find a way.

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THESIS INTRODUCTION

Polychlorinated biphenyls (PCBs) are a group of chemicals that have been used in transformers and capacitors due to their excellent dielectric properties and their high chemical and thermal stability. As chlorination of the biphenyl molecule can occur at 10 positions (Figure A.1), there are 209 different isomers or congeners of PCBs possible. Although now banned from production, they were synthesized under the name Aroclor in the U.S.A. These are mixtures of 40-60 congeners, and the associated number refers to the number of carbon atoms (12) and the percent by weight chlorine. For example Aroclor 1254 is 54% by weight chlorine.

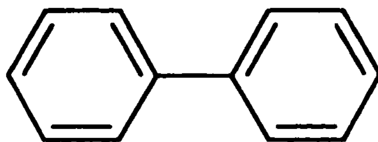

Figure A.1. Molecular structure of biphenyl

PCBs are non-polar and extremely hydrophobic, and therefore tend to partition to non-aqueous phases such as oil, soil, or fat. Thus, if they are released into the environment, they will persist and bioaccumulate. Although they have not been found to be acutely toxic, there are potential chronic effects (Brown 1994). PCBs are listed as priority pollutants by the USEPA. The quantity of PCB-contaminated materials in Canada alone is estimated at 127 000 tonnes (Rowan 1996).

Various methods of disposing of PCBs exist. Although destruction by incineration is possible, and indeed is practised, it is a very expensive solution due to transport costs and the high temperatures required (Rowan 1996). A very common solution ends up being no treatment, so much of the contaminated material remains undisturbed or stored. Extraction with chemical surfactants or solvents can be efficient, however, the PCBs are only physically transferred by this

method, and destruction is still required. Other destructive technologies include thermal/chemical dechlorination, a relatively untried process, and bioremediation. Bioremediation exploits the metabolism of microorganisms to transform xenobiotic chemicals into carbon dioxide and water. It can be very cost-effective if efficient processes are developed.

The choice of remediation strategy will be decided based on a variety of factors including costs and the time scale required for clean-up. In the case of PCBs, the strategy will also depend on the degree of chlorination of the PCBs present, and the difference between the initial and the required final concentration of PCBs. For example, in Canada, environmental quality criteria for PCBs in soil depends on land use. PCB concentrations should be less than 0.5, 5, or 50 ppm for agricultural, residential/parkland, or commercial/industrial land, respectively (CCME 1991). When considering bioremediation, it should be taken into account that it is the lower-chlorinated PCB fraction that is degraded by aerobic bacteria (Abramowicz 1990). Depending on the PCB mixtures present, this fraction could account for most or almost none of the contamination. Thus, bioremediation, using solely aerobic bacteria, would be an effective technology if low PCB levels, or predominantly lighter-chlorinated congeners were present. To use aerobic biodegradation in remediation of some of the higher-chlorinated PCBs, a pretreatment would be required which would dechlorinate these congeners to lower-chlorinated congeners. This dechlorination step may be accomplished biologically using anaerobic bacteria.

Anaerobic dechlorination of PCBs was discovered after it was noticed that the profiles of the PCBs present in anaerobic sediments were changing with time, with a reduction in higher-chlorinated and an increase in lower-chlorinated congeners (Brown et al. 1987). It has been confirmed that anaerobic bacteria can reductively dechlorinate PCBs, replacing chlorine atoms with hydrogens. Anaerobic dechlorination is a slow process, thus it would be fairly inefficient on its own, however, when combined with aerobic degradation, the whole process may be very effective (Evans et al. 1996).

Besides the level of chlorination of the PCBs, there are other factors limiting their degradation by aerobic microorganisms. These include the hydrophobicity of PCBs and their

capacity to tightly adsorb to soil. In addition, PCBs are thought to be metabolized by aerobic bacteria only in the presence of the growth substrate, biphenyl.

Although many aerobic soil microorganisms have been shown to degrade PCBs (Bedard et al. 1986), one organism was used in these investigations, *Pseudomonas* strain LB400. This organism was isolated by researchers at General Electric, and is remarkable due to its broad substrate specificity towards a range of PCB congeners (Bedard et al. 1986).

Previous studies using bacteria to degrade PCBs have generally indicated a requirement for biphenyl to induce the PCB-degrading enzymes (Kohler et al. 1988; Smith and Ratledge 1989). However, biphenyl is an undesirable amendment to soil as it is itself toxic to other organisms and a recognized hazardous compound. Consequently, there is interest in identifying other non-toxic sources of carbon for growth and induction of PCB-degrading enzymes. Some researchers have focussed on using plant compounds which have structures similar to PCBs with encouraging results (Donnelly et al. 1994).

The bioavailability of a compound is the extent to which that molecule in the environment is available to an organism for growth or metabolism. Understanding bioavailability and manipulating the factors influencing bioavailability has been recognized as one of the four main priorities in the study of bioremediation (Alexander 1991). A decrease in availability of a substrate to microorganisms will lead to a decrease in biodegradation rates. PCBs have very low solubilities in water and uptake of PCBs by bacteria is thought to require that the compounds be present in the aqueous phase. Thus if the aqueous solubility of the PCBs can be increased, bacteria may be able to access and metabolize the molecules more readily.

Surfactants have been used to maximize oil recovery for many years (Ganeshalingham et al. 1994), and are now being explored as a means of mobilizing insoluble contaminants (Abdul et al. 1990; Edwards et al. 1991; Viney and Bewley 1990). A surfactant molecule is structured such that it has both a hydrophilic and hydrophobic portion. When the concentration of the surfactant in solution is above its critical micelle concentration (CMC), the surfactant molecules arrange into micelles. Micelles are aggregates of surfactant monomers, with the hydrophilic portions oriented

outwards into the aqueous solution and the hydrophobic portions oriented inwards. The hydrophobic contaminant is taken into the interior of the micelle. Many organisms produce biosurfactants in natural habitats as a means of increasing bioavailability of substrates (Goldman et al. 1982; Cirigliano and Carman 1984). The properties of surfactants may thus be exploited to increase the bioavailability of hydrophobic contaminants to bacteria provided the bacteria can access the contaminants present in the micelle. To date however, research on the use of surfactants in bioremediation processes has produced inconclusive results (Laha et al. 1995), and further research is required to facilitate their consistent and effective exploitation in this area.

Biodegradation is usually monitored by contaminant removal, however, this provides no insight into the mechanisms or pathways of degradation. Detection of intermediate metabolites proves that biological systems are responsible for loss of contaminants, and suggests which pathways are involved (Flanagan and May 1993). Additionally, quantitative mass balance information can be used to determine if the products are further metabolized, or whether other metabolites are produced.

The overall objectives of this thesis are: (a) to characterize the processes for transformation of PCBs and for production of metabolites by *Pseudomonas* LB400 grown on biphenyl and carbon sources which are not structurally related to PCBs, and (b) to examine the influence of chemical surfactants on solubilization and biodegradation of PCBs.

The specific goals of this thesis are as follows:

1) to examine the effect of the carbon source for growth, and the chlorination pattern of congeners on rates of transformation, by comparing transformation of individual congeners after growth of *Pseudomonas* LB400 on different carbon sources. (Chapters 1 & 2)

2) to determine the effect of PCBs on *Pseudomonas* LB400 viability, and the effect of incorporating carbon sources into the transformation medium on the biotransformation process (Chapter 1)

3) to compare the ability of different chemical surfactants to solubilize PCBs from glass and from soil. (Chapters 3 & 4)

4) to examine the effect of the presence of surfactants on biodegradation of PCBs in the resulting solutions. (Chapters 3 & 4)

5) to assess the toxicity of the surfactants to *Pseudomonas* LB400. (Chapter 3)

6) to determine the capacity of *Pseudomonas* LB400 to produce and degrade chlorobenzoic acid metabolites from the transformation of PCBs after growth of the cells on different carbon sources. (Chapter 5)

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CHAPTER 1

Studies on the transformation of selected polychlorinated biphenyl congeners by *Pseudomonas* strain LB400[1]

1.1 Abstract

Resting cells of *Pseudomonas* LB400 are known to transform PCBs when the cells are previously grown on biphenyl. In this study, PCB transformation was also observed in resting cells grown on other substrates such as glucose and glycerol. The presence of PCB congeners in the growth medium increased the lag phase for growth of cells on a biphenyl substrate, but not on a glycerol substrate. Supplementation of the degradation medium with biphenyl dramatically decreased the rate of PCB congener transformation whereas the presence of glycerol or glucose had little or no effect on PCB transformation rates. Initial removal rates with biphenyl-grown cells in the standard degradation medium for 2,4,2',4'-CBP, 2,4,5,2',5'-CBP, and 2,3-CBP were 1.06, 1.66, and 224 μmol/l/h respectively. Relative rates of transformation of 2,3-CBP by biphenyl-, glucose- and glycerol-grown cells were 100:36:36 and were similar to the relative rates of transformation of 2,4,5,2',5'-CBP (100:33:42). Presence of PCBs adversely affected cell viability of biphenyl grown cells over a 48-h incubation period and may explain the decline observed in PCB conversion capacity over the same incubation period. A major objective of this study was to investigate the significance of using biphenyl as carbon source for growth of *Pseudomonas* LB400 cells capable of PCB transformation. Our findings indicate that, whereas higher rates of transformation of PCBs are observed with biphenyl-grown cells, cells grown on other carbon sources retain PCB-transforming enzymes. In addition, it has been demonstrated that biphenyl inhibits transformation of PCBs by the organism whereas glycerol or glucose does not.

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1.2 Introduction

Polychlorinated biphenyls (PCBs), which have excellent dielectric properties and chemical stability, were widely used in industrial applications for about 50 years, especially as dielectric fluids in capacitors and transformers. Over the past 10 years, interest in their degradation through bioremediation has developed due to concerns regarding their persistence in the environment and their characteristic ability to bioaccumulate.

Screening studies describing the capacities of different PCB-degrading isolates to transform PCB congeners have resulted in the identification of a number of strains having superior degradative capacity including *Pseudomonas* strain LB400 (Bedard and Haberl 1990, Bedard et al. 1986, Bopp 1986). This strain can aerobically degrade PCBs with a wide spectrum of chlorination patterns, having up to six chlorines.

The wide range of congeners oxidized by some strains could be explained by the presence of more than one enzyme capable of initiating attack on PCB or to the ability of biphenyl-2,3-dioxygenase to hydroxylate at other positions on the biphenyl molecule (Gibson et al. 1993). Differences in the chlorinated biphenyl degradation specificities of strains LB400 and KF 707 have been attributed to differences in substrate specificity of their biphenyl-2,3-dioxygenases. Biphenyl dioxygenase was resolved into three protein fractions and the non-linear relationship between enzyme activity and protein concentration could be explained by the multi-component nature of the enzyme (Haddock et al. 1993).

Differences in primary degradation of PCB congeners could also reflect permeability differences among strains (Bedard and Haberl 1990). However, parallel results obtained for the transformation of PCB congeners by biphenyl-grown cells and cell extracts suggested that congener diffusion was not responsible for the observed differences between strains LB400 and KF707 (Gibson et al. 1993).

Most studies, including those on *Pseudomonas* LB400 (Bedard et al. 1986, Bopp 1986, Gibson et al. 1993) used resting cells grown on biphenyl for PCB congener degradation. However,

Kohler and coworkers (1988) found that growing cells of *Acinetobacter* with biphenyl as substrate were far superior to resting cells with respect to the range of PCBs transformed. The importance of adding biphenyl to media as an inducer of biphenyl dioxygenase for clean up of contaminated soils has been emphasized (Kohler et al. 1988). Brunner and coworkers (1985) showed that addition of biphenyl had a more pronounced effect on mineralization of Aroclors with indigenous flora than with addition of the bacteria alone. *Pseudomonas* NCIB 10643 could grow on biphenyl or fumarate but lacked the enzymes for degradation of biphenyl when grown on fumarate (Smith and Ratledge 1989).

In this paper, some of the main factors influencing degradation of selected PCB congeners by resting cells of *Pseudomonas* LB400 have been characterized. In particular, the significance of the carbon source used for growth was investigated by comparison of rates of PCB transformation by cells grown on biphenyl and other carbon sources. In addition, the effect of biphenyl added to the PCB transformation medium was evaluated. Biphenyl itself is a recognized hazardous contaminant. Thus, the identification of other carbon substrates to support growth of *Pseudomonas* LB400 increases the potential for use of this strain in large-scale bioremediation processes. Carbon sources such as glucose or glycerol were chosen in this study because of their low cost for field applications.

1.3 Materials and Methods

Chemicals:

Biphenyl was obtained from Sigma Chemical Co. (St. Louis, MO). PCB congeners (2,3-chlorobiphenyl, 2,5,4'-chlorobiphenyl, 2,4,2',4'-chlorobiphenyl, 2,4,5,2',5'-chlorobiphenyl, 2,4,6,2',4'-chlorobiphenyl) in neat form were obtained from Accustandard Ltd. (New Haven, CT). These congeners were chosen due to their chlorination patterns - one with an unchlorinated ring, one with both 2,3 and 3,4 sites open, one with an open 2,3 site, one with an open 3,4 site, and a non-degradable standard, respectively. Purity was 99+%; no biphenyl peak was detected. Stock solutions of individual or mixed congeners were prepared containing 1 mM of each congener in

acetone.

Bacterial Strain:

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, NY) was obtained from the Northern Regional Research Laboratory (Peoria, IL). Cultures were maintained in 50 ml of the standard growth medium in 250 ml Erlenmeyer flasks by storage at 4°C, with monthly subculturing by incubation at 30°C for 24 h. Cultures were streaked onto tryptic soy agar at regular intervals to ensure purity. For long-term storage of cultures, 20% glycerol was added to cell suspensions in the standard growth medium and the mixture was distributed in 1-ml aliquots in 1.5-ml tubes and stored at -20°C.

Standard growth medium:

The growth medium used was the phosphate-buffered mineral salts medium supplemented with yeast extract described by Bedard et al. (1986). This medium contained per litre: 4.4 g K_2HPO_4, 1.7 g KH_2PO_4, 2.1 g NH_4Cl and 50 mg of yeast extract (Becton Dickinson, Cockeysville, MD), 2 g carbon source, and 10 ml of concentrated basal salts solution. Non-volatile carbon sources were added to the bulk medium prior to autoclaving. Basal salts were added after autoclaving. Biphenyl was prepared in a 0.1 g/ml solution in hexane, filter sterilized using a 0.2 µm PTFE syringe filter (Nalge, Rochester, NY) and added to the autoclaved medium. The hexane was allowed to evaporate, leaving crystals suspended in the medium. The concentrated basal salts solution contained $MgSO_4$ (19.5 g/l), $MnSO_4.H_2O$ (5 g/l), $FeSO_4.7H_2O$ (1 g/l), and $CaCl_2.2H_2O$ (0.3 g/l), plus enough H_2SO_4 to prevent precipitation of basic salts. Culture media (50 ml) were prepared in 250-ml Erlenmeyer flasks, and stoppered with foam plugs.

Inoculum Preparation:

Cultures stored at 4°C were incubated on an orbital shaker (Labline Instruments Inc., Melrose Park, IL) at 200 rpm at room temperature until warm. Cells were inoculated into 50 ml

of fresh growth media in a 250-ml Erlenmeyer flask to an OD of 0.1 measured at 615 nm in a 1-cm light path. Unless otherwise stated, cultures were incubated at 30°C on an orbital shaker set at 200 rpm for 22 to 24 h. Cultures containing biphenyl were filtered through sterile glass wool to remove excess crystals. The cell suspension was then centrifuged at 5000 *g* for 15 min, washed twice with 0.05 M sodium phosphate buffer, pH 7.5, and resuspended in the buffer to an OD of 1.0.

Microbial analysis:

Microbial growth was monitored by measurement of absorbance of cultures at 615 nm in a 1-cm light path in a spectrophotometer (Shimadzu, Model UV-120-02, Kyoto, Japan). Measurements were taken when the absorbance readings fell between 0.03 and 0.5 where a linear relationship is observed between absorbance and biomass concentration. Samples with higher cell densities were diluted with distilled water to within the above range and the absorbance was multiplied by the dilution factor.

Microbial counts were measured by determining numbers of colonies of serially diluted cultures on tryptic-soya-agar (TSA) plates, expressed as colony forming units (CFU) using standard methods.

Biotransformation conditions:

One millilitre of bacterial suspension at an OD of 1.0 was added per 8-ml glass vial. Controls were killed by addition of 1 drop of 70% perchloric acid. Congeners were added in the form of a concentrated acetone solution to a final concentration of 10 µM (2-3 ppm) each. Included was a non-degradable internal standard (2,4,6,2',4'-pentachlorobiphenyl) to account for variation in addition, extraction, and injection. Vials were closed with Teflon-lined caps and incubated at 30°C on an orbital shaker set at 200 rpm.

PCB extractions:

Following incubation, reactions were stopped by addition of 1 drop of 70% perchloric acid.

Extraction required the addition of 100 µl of a 10% Triton X-100 (Fisher Scientific, Fair Lawn, NJ) solution to increase PCB solubility, four volumes of hexane, and 0.5 g of sodium sulfate to prevent formation of a stable emulsion. Foil-lined caps were used for this procedure. Samples were shaken horizontally on an orbital shaker at 300 rpm for 20 to 30 min. The hexane layer was transferred to vials for gas chromatography. Storage took place at 4°C until analyses.

Congener analysis:

Extracted samples were analyzed by capillary GC (Hewlett-Packard gas chromatograph, Model HP 5890 series II), equipped with a mass selective detector (Hewlett-Packard, Model HP 5971A) and integrated by Hewlett-Packard MS Chemstation software. Identification and quantitative determinations of the congeners were based on analysis on a fused silica capillary column coated with a film thickness of 0.25 µm of polymethyl (5% phenyl) siloxane, 30 m in length, with a 0.25 mm internal diameter (HP-5MS, Hewlett-Packard). The sample (2 µl) was injected by an autoinjector (Hewlett-Packard, Model HP 7673) in splitless mode with a time delay of 1.25 minutes after injection. The initial oven temperature of 55°C was held for 2 minutes and then programmed to increase by 10°C/min to 100°C, 5°C/min to 215°C, 10°C/min to 285°C and held for 5 minutes. The injector temperature was 250°C and the transfer line was 280°C. The carrier gas was helium with an initial column head pressure of 12 psi. The system was equipped with electronic pressure control (EPC) and operated in constant flow mode (1.66 ml/min). The filament and multiplier were turned on at 8 min. The system was operated in selected ion monitoring (SIM) mode. The ions monitored were 188,190; 222,224; 256,258; 290,292; 326,328. The retention time window for identification was 0.2 min. Standard midmass autotunes using PFTBA were performed before each set of samples was analyzed.

Experimentation and analysis:

The reported PCB congener degradation percentages were calculated by normalization of the parental congener peak to the recovery of the non-degradable internal standard, and

comparison to the killed control. The recovery of PCB congeners from the killed controls was always between 90 and 100%. Preliminary experiments showed that normalization to the internal standard accounted for variations in addition and extraction. Duplicate samples within experiments showed similar results with variations between samples of 5%. Reproducibility between experiments was also very high, with typical variations being ≤ 10%.

1.4 Results

Prior to implementing degradation studies using resting cells, the cells were cultivated in the standard medium containing different carbon sources (2 g/l). Cultures were incubated at 30°C on an orbital shaker at 200 rpm for up to 96 h. Biphenyl, glucose and glycerol supported the highest biomass production and were selected as growth substrates for biodegradation studies.

Resting cells, recovered after 24 h from the culture media containing each of the three carbon sources, were incubated with a mixture of four PCB congeners (10 µM) for 48 h using the standard biotransformation protocol. Three of the four PCB congeners, 2,3-CBP; 2,5,4'-CBP and 2,4,5,2',5'-CBP were completely transformed by cells grown on each of the carbon sources (data not shown). However, complete bioconversion of congener 2,4,2',4'-CBP was only observed with biphenyl-grown cells whereas 18 and 27% congener conversion was observed with cells grown on glucose and glycerol, respectively. The results illustrate that PCB-degrading enzymes are indeed produced on substrates such as glucose and glycerol which, unlike biphenyl, are not related to the structure of PCBs. Congener 2,4,2',4'-CBP, which contains *para*-chlorines, is known to be less efficiently degraded by this organism. The effect of PCB congeners on growth of *Pseudomonas* strain LB400 on biphenyl or glycerol as carbon source was also investigated. The initial concentration of each PCB congener in the growth medium was 10 µM. The time courses for growth of *Pseudomonas* strain LB400 are presented in Figure 1.1. The presence of the PCB congeners increased the lag phase for growth on biphenyl although similar final cell concentrations were obtained. The presence of the PCBs in glycerol-containing medium had no effect on the growth patterns.

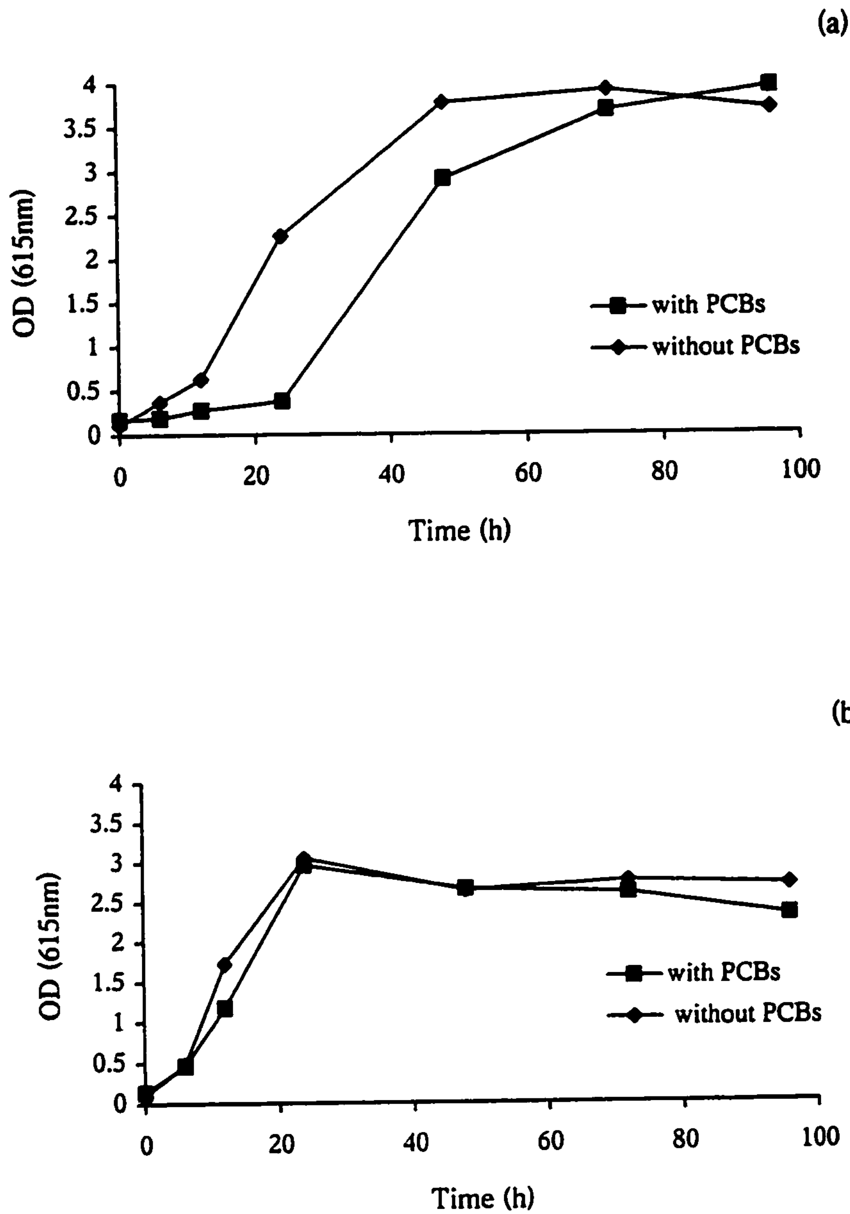

Figure 1.1 Effect of presence of PCBs on growth of *Pseudomonas* LB400 on biphenyl (a) and glycerol (b). A mixture of five PCB congeners at 10 μM each was added to cells at an $OD_{615} = 0.1$ in growth medium with carbon sources present at 2000 ppm.

Biotransformation of a mixture of the five congeners (10 μM each) by cells in the presence and absence of carbon source was investigated using cells previously grown on biphenyl or glycerol. The results are presented in Table 1.1.

The results clearly indicate differential rates of transformation for each of the congeners. In the case of congener 2,3-CBP, 100% removal was observed after 3 h. In general, relative rates of degradation of the other congeners were 2,5,4'-CBP > 2,4,5,2',5'-CBP > 2,4,2',4'-CBP. Supplementation of the biotransformation medium with biphenyl dramatically reduced the rate of PCB congener removal by biphenyl-grown cells. However, the presence of glycerol only slightly decreased the removal rate by glycerol-grown cells. The effect of concentration of carbon source on removal of PCB congener 2,4,5,2',5' -CBP by *Pseudomonas* strain LB 400 grown on biphenyl in the standard transformation medium is illustrated in Figure 1.2. Biphenyl had little effect on congener degradation at concentrations in the range 0 to 20 ppm but had a dramatic effect on the rate of congener conversion at concentrations greater than 200 ppm. Glucose, glycerol, or yeast extract had no effect on conversion by biphenyl-grown cells, even at concentrations of 2000 ppm (data not shown).

The relative rates of transformation of individual congeners by *Pseudomonas* LB400, grown on different carbon sources, was investigated by monitoring the removal of the three selected congeners as a function of time (Figure 1.3). Standard biotransformation conditions were used except that, in the case of congener 2,3-CBP, a cell concentration of absorbance = 0.1 rather than 1.0 was used because of the much higher removal rates observed for this congener. Thus the initial rates for this congener were multiplied by 10 to account for this. Initial rates were determined from the slope of the line over the first time period in which significant removal (>15%) occurred. From these time courses the relative rates of conversion of each congener by cells grown on each carbon substrate were determined. These results are presented in Table 1.2. Relative conversion rates of 2,3-CBP and 2,4,5,2',5'-CBP were similar for glucose- and glycerol-grown cells compared to biphenyl-grown cells. However, relative rates of conversion of congener 2,4,2',4'-CBP were much lower.

Table 1.1 Effect of presence of carbon source on removal of a PCB congener mixture by *Pseudomonas* LB400

Growth substrate	Degradation media	Time (h)	% removal of congener			
			2,3	2,5,4'	2,4,2',4	2,4,5,2',5'
Biphenyl	buffer	3	100	100	24	36
		6	100	100	51	74
		12	100	100	78	91
	mineral medium	3	100	41	5	5
	+	6	100	39	11	6
	2 g/l biphenyl	12	100	53	7	18
Glycerol	buffer	3	100	100	0	15
		6	100	100	7	40
		12	100	100	17	78
	mineral medium	3	100	54	0	11
	+	6	100	100	6	35
	2 g/l glycerol	12	100	100	9	73

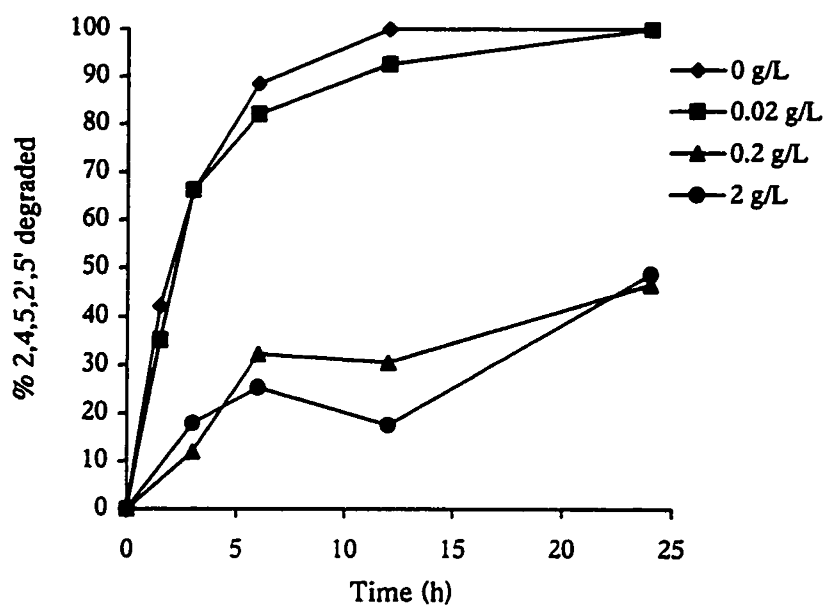

Figure 1.2 Effect of concentration of biphenyl on degradation of 2,4,5,2',5'-CBP.

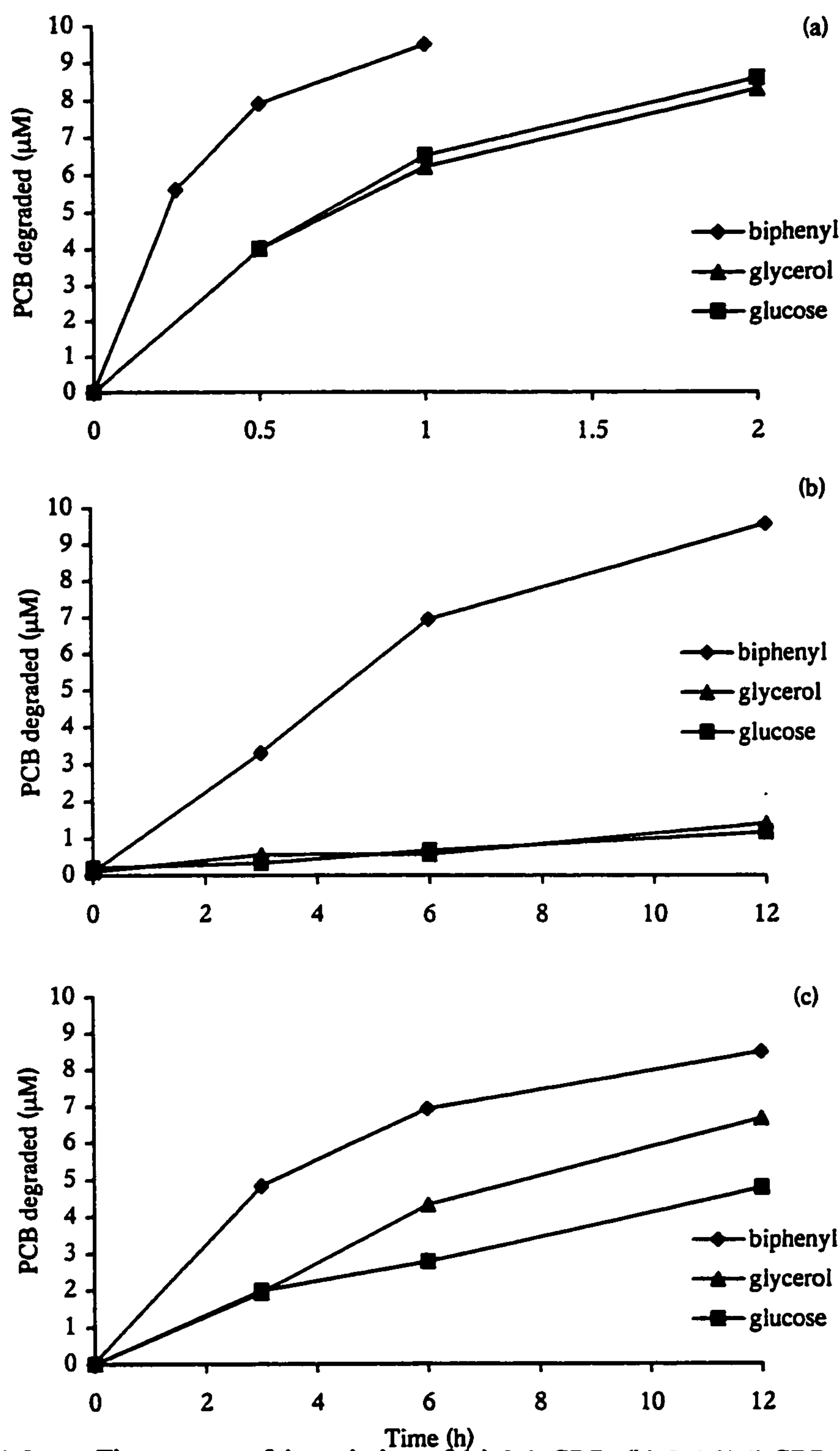

Figure 1.3 Time course of degradation of (a) 2,3-CBP, (b) 2,4,2',4'-CBP, and (c) 2,4,5,2',5'-CBP by LB400 after growth on different carbon sources. Congeners were added as individual solutions to a final concentration of 10 μM to cells suspended in buffer.

Table 1.2 Effect of carbon source on initial rates of removal of individual congeners by *Pseudomonas* LB400

Congener	* initial removal rate (μmol/l/h) after growth on			relative rates		
	biphenyl	glucose	glycerol	biphenyl	glucose	glycerol
2,3	224	80.9	80.9	100	36	36
2,4,2',4'	1.06	0.067	0.1	100	6	9
2,4,5',2',5'	1.66	0.54	0.7	100	33	42

*with cells at OD = 1

The stability of the PCB transformation system containing biphenyl-grown or glucose-grown *Pseudomonas* LB400 cells was also investigated. Bioconversion of a mixture of the four congeners (10 μM each) was monitored over a 48-h incubation period. At this point, a second addition of congeners (10 μM each) was made and congener removal was monitored for a further 48 h. The results are presented in Figure 1.4, and indicate a decline in congener conversion capacity. To determine the viability of resting cells during bioconversion, plate counts were performed with glucose- or biphenyl-grown cells in the presence or absence of PCBs. Figure 1.5a shows that the presence of PCBs adversely affects cell survival quite rapidly for biphenyl-grown cells, whereas the viability of glucose-grown cells in Figure 1.5b is unchanged by the presence of PCBs.

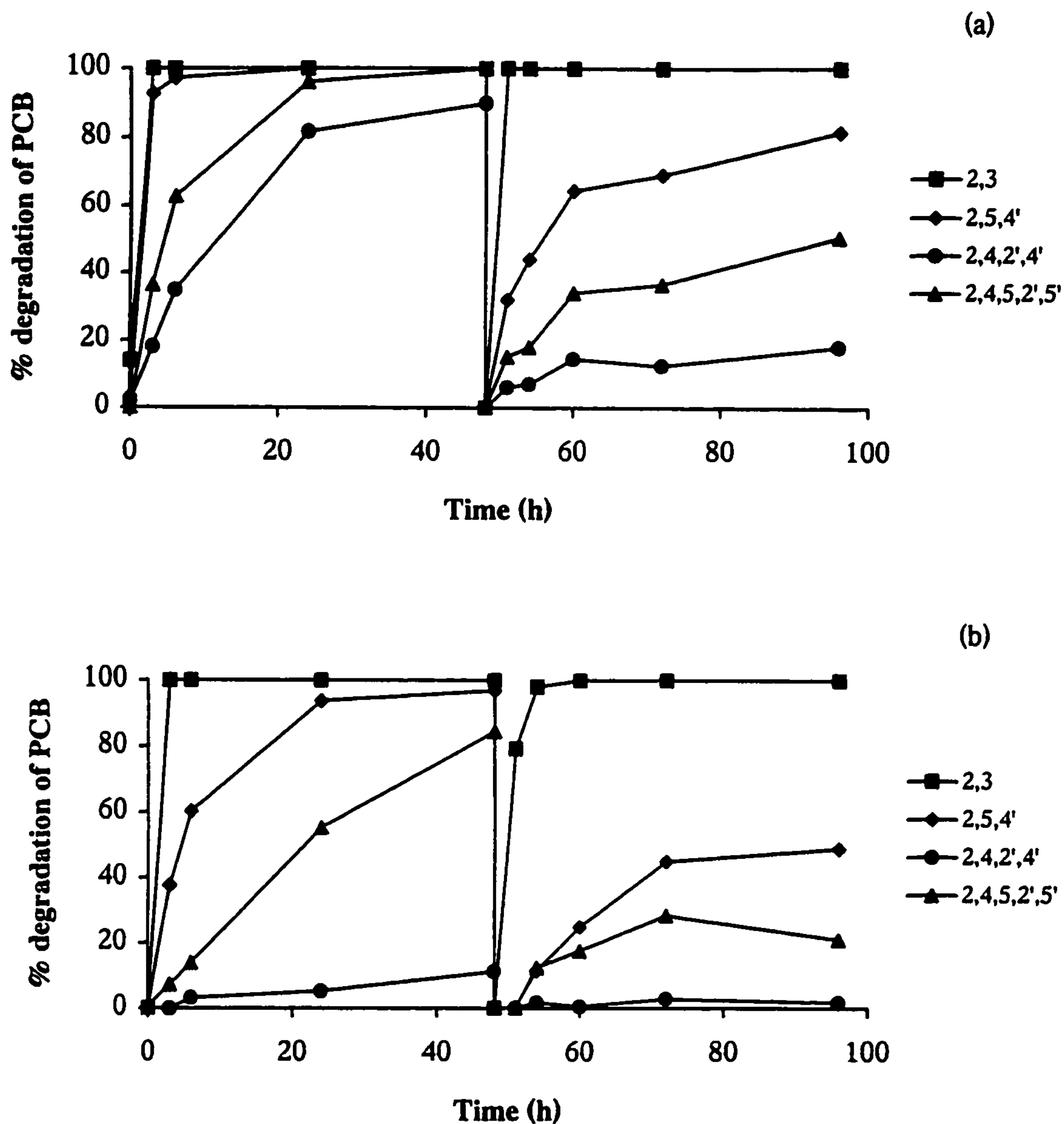

Figure 1.4 Degradation capability of resting cells of LB400 grown on (a) biphenyl and (b) glucose after repeat inoculation of a four congener mixture. Vials were opened at 48 h and the mixture of congeners at 10μM each was added. The calculation of % degradation after 48 h includes residual PCBs from the 0 to 48 h incubation.

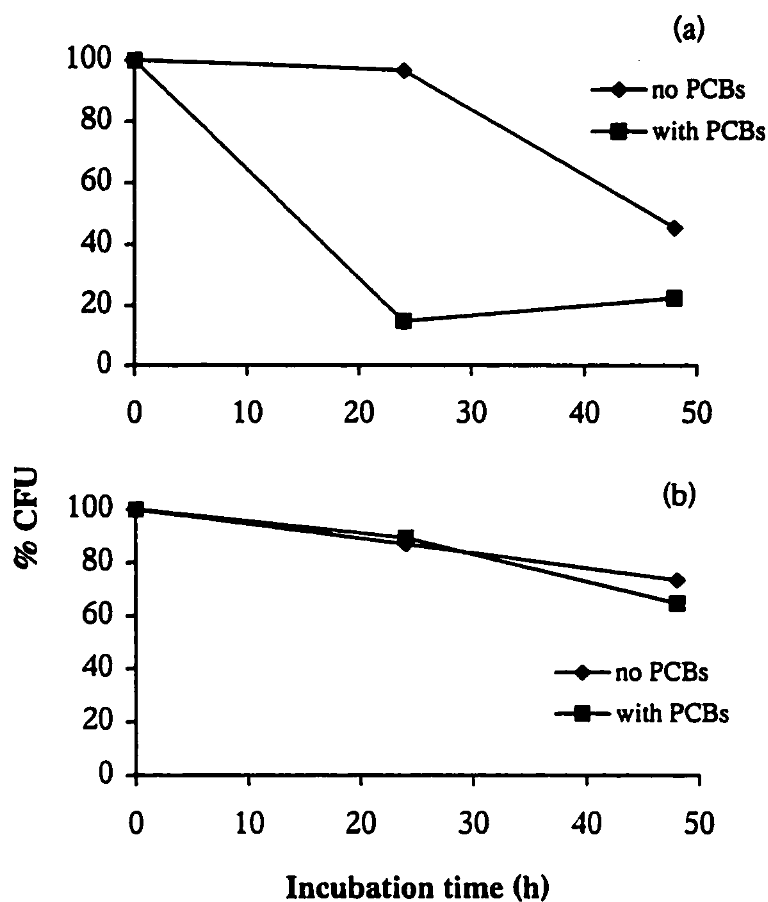

Figure 1.5 Viability of resting cells of LB400 grown on (a) biphenyl and (b) glucose in the presence and absence of PCBs. Incubation started with cells at an $OD_{615} = 1$. Plate counts were carried out in triplicate on TSA plates, and are expressed as average number of CFU at each time period as a percentage of the average at time 0.

1.5 Discussion

The majority of reports (Chapatwala et al. 1992, Kohler et al. 1988, Smith and Ratledge 1989) emphasize the importance of adding biphenyl to the growth medium to induce biphenyl dioxygenase and promote PCB degradation. Walia et al. (1988) observed that high concentrations of glucose, acetate and succinate significantly inhibited the degradation of PCBs. Furukawa et al. (1983) observed that the oxidative enzymes involved in degradation of biphenyl, toluene, salicylate and substituted benzoates by *Pseudomonas paucimobilis* are inducible and inter-related. In contrast, we have shown that the PCB-degrading enzymes of *Pseudomonas* LB400 are produced when the organism is serially subcultured and grown on other carbon sources, namely glucose and glycerol. Bopp (1986) had earlier observed that resting cells of *Pseudomonas* LB400, prepared by growth in Luria broth, retained their capacity to degrade PCBs. Also, Mondello (1989) showed degradation of up to pentachlorinated congeners after growth of *Pseudomonas* LB400 on succinate. *Pseudomonas* NCIB 10643 (Smith and Ratledge 1989) manifested dihydroxybiphenyl-1,2 dioxygenase activity when incubated on fumarate. A *Pseudomonas* strain that could grow on naphthalene but not on biphenyl was, nevertheless, shown to biotransform biphenyl (Kilpi et al. 1988), and it was suggested that the oxygenase enzymes involved in naphthalene degradation may also participate in degradation of biphenyl and chlorobiphenyls. Thus, in a variety of strains, incorporation of biphenyl or chlorobiphenyls into the growth medium is not a requirement for synthesis of biphenyl- or chlorobiphenyl-degrading enzymes.

The initial transformation rates observed in this study for resting cells grown on biphenyl ranged from 1 to 224 μmol/l/h depending on the congener. By comparing initial transformation rates for three congeners by cells grown on different substrates we sought to establish if relative rates of removal of each congener were influenced by growth substrate. For congener 2,3-CBP, relative initial rates of conversion were 100:36:36 for cells grown on biphenyl, glucose and glycerol. For congener 2,4,5,2',5'-CBP, corresponding relative rates were 100:33:42. The similarity of these ratios would be expected if the congeners shared similar initial rate-limiting metabolic conversion steps. In contrast to the latter congeners, relative rates of removal of

2,4,2',4'-CBP by cells grown on glucose or glycerol were much lower. This would be consistent with a situation where a different enzyme set, separately regulated, was involved in the rate-limiting step for conversion of this substrate. The existence of more than one enzyme capable of initiating PCB degradation in *Pseudomonas* LB400 was considered unlikely by Gibson et al. (1993) and all attempts to demonstrate this by mutagenesis and subcloning have been unsuccessful (Mondello 1989). Bedard and Haberl (1990) have concluded that congener specificity between different PCB degrading strains appears to be related to the specificity of the initial dioxygenase and that specificities of enzymes for subsequent degradation appear quite similar in all strains. Strain LB400 and *Alcaligenes eutrophus* H850 degrade an unusually broad range of PCB congeners, exhibiting 2,3- and 3,4-dioxygenase activity. In our studies, the ratios observed were not exactly the same when grown on different substrates and this was to be expected. Further studies are required to explain this.

The presence of PCBs in the growth medium retarded growth of *Pseudomonas* LB400 on biphenyl but did not inhibit growth in glycerol-containing medium. In addition, biphenyl was found to have an inhibitory effect on PCB removal. Taken together, these observations suggest that some kind of competitive enzyme inhibition may be occurring in the presence of the second biphenyl or chlorobiphenyl substrate. For example, if the PCB congeners had a higher affinity for one of the PCB and biphenyl-metabolizing enzymes, this could reduce utilization of biphenyl for bacterial growth.

A decline in resting cell viability and PCB removal was observed over a 48-h incubation period in the transformation medium. The viability experiments without PCBs show that this is probably partly due to a depletion of cell maintenance energy, however, the accelerated decline in biphenyl grown cell numbers in the presence of PCBs suggests that there may be an accumulation of a toxic metabolite. That there was no difference in the viability of glucose-grown cells in the presence or absence of PCBs may indicate that there is no toxic metabolite produced. Further studies are being undertaken to determine if there are differences in degradation metabolites after growth on different carbon sources. Biphenyl oxidation by resting cells of

Acinetobacter decreased in 28 h to less than 8% of the original oxidation rate (Kohler et al. 1988). Therefore, at least in the case where resting cells are used to remediate PCBs, questions regarding their viability and degradation stability need to be addressed.

Our studies with *Pseudomonas* LB400 have shown that resting cells quickly lose their viability and PCB transforming ability, such that, in the absence of a growth substrate, soil would have to be frequently re-inoculated to maintain PCB biodegradation. This was the treatment McDermott and coworkers (1989) used to treat PCB contaminated soils. However, others suggest that it is necessary to add biphenyl or another substrate which induces biphenyl dioxygenase, together with bacterial cell suspensions, to achieve clean up of PCBs in soil (Kohler et al. 1988). Barriault and Sylvestre (1993) showed that there was only slightly more degradation of Aroclor 1242 in soils inoculated once with a PCB-degrading bacterium with subsequent additions of biphenyl, than with repeated inoculations of the bacteria. In general, it is not considered desirable to augment soils with biphenyl to promote PCB degradation as biphenyl is itself a hazardous substance and our studies have shown that, in the case of *Pseudomonas* LB400, biphenyl inhibits PCB bioconversion. The observation that *Pseudomonas* LB400 retains its PCB-transforming activity in the presence of alternative carbon sources, such as glucose or glycerol, suggests that application of this organism to PCB contaminated soils, together with these simple carbon sources, could result in effective PCB bioremediation. However, as these carbon sources eliminate the selection of only PCB-degrading organisms in the soil, a mechanism to retard development of the indigenous microbial population would be required.

Acknowledgement

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CHAPTER 2

Comparison of the degradation patterns of PCB congeners in Aroclors by *Pseudomonas* LB400 after growth on various carbon sources[1]

2.1 Abstract

Resting cells of *Pseudomonas* LB400, grown on biphenyl, transformed 80%, 50% and 17% of Aroclor 1242, 1254 and 1260, respectively. Resting cells grown on glucose or glycerol also transformed these polychlorinated biphenyl (PCB) mixtures to the extent of 60%, 35% and 9% for Aroclors 1242, 1254 and 1260, respectively. Time courses of transformation of the separated individual congeners in the Aroclors were plotted and used to determine the transformation rate constants (k). By analysis of the rate constants, it was concluded that the order of degradation of the different congeners in an Aroclor were similar regardless of growth substrate. In general, k values for conversion of a particular congener were lower for cells grown on glucose or glycerol compared to cells grown on biphenyl. Generally, k values for transformation of the same congener in different Aroclors were not the same: rate constants had highest values for the congener in Aroclor 1242 and lowest values in Aroclor 1260. The data allowed congeners to be grouped according to their relative rates of degradation. The ratio of k values for transformation of individual congeners in Aroclors by cells grown on biphenyl and glucose were not constant.

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[1] This paper has been accepted to the Canadian Journal of Microbiology. Co-authors are C. Juneson, S.M. Backus and O.P. Ward

2.2 Introduction

Polychlorinated biphenyls (PCBs) were widely used from the 1930s to the 1960s as dielectric fluids in capacitors and transformers and in a variety of other applications which exploited their excellent stability and chemical properties. It was only in the late sixties that the persistence, bioaccumulation and potential toxic effects of PCBs became known and subsequent sampling revealed widespread contamination by PCBs in the environment.

PCBs were prepared industrially by chlorination of biphenyl, producing a complicated mixture of chlorobiphenyls with one to ten chlorine atoms per molecule and different isomers. Thus, Aroclors 1242, 1254 and 1260 contain 42, 54 and 60% of chlorine by weight. Many bacteria appear to be capable of oxidising mono-, di- and trichlorinated biphenyls. The principal pathway in aerobic procaryotes starts with an attack by a dioxygenase at unsubstituted 2,3 (Gibson et al. 1993; Bedard et al. 1987a) or 3,4 positions (Gibson et al. 1993; Ahmed and Focht 1973). In both cases, the organisms appear to require adjacent unchlorinated carbon atoms. However, *Pseudomonas* LB400 has the capacity to degrade an unusually wide range of PCB congeners containing 2 to 6 chlorine atoms including some congeners lacking adjacent unchlorinated carbon atoms (Bedard and Haberl 1990; Bedard et al. 1986; Bopp 1986).

In preparing bacterial cells for PCB-degradation studies, biphenyl has been used as the major carbon source in the medium (Bedard et al. 1986; Bopp 1986; Gibson et al. 1993) to induce PCB-degrading enzymes. It has also been recommended that biphenyl be used as an inducer of biphenyl dioxygenase in the clean up of PCB-contaminated soils (Kohler et al. 1988). For example, biphenyl-degrading enzymes were induced in *Pseudomonas* NCIB 10643 when the organism was grown on biphenyl, but these enzymes were not induced when the organism was grown on fumarate (Smith and Ratledge 1989).

We have shown that the enzymes of *Pseudomonas* LB400, capable of transforming selected congeners of PCBs, were produced when the organism is serially subcultured and grown on carbon sources other than biphenyl, including glucose (Billingsley et al. 1997). In this paper we have compared the degradation patterns of PCB congeners in different Aroclors,

using *Pseudomonas* LB400 grown on biphenyl and glucose. The rate constant for each biotransformation was determined and the data were used to characterize the relative rates of degradation of congeners in Aroclors.

2.3 Materials and Methods

Chemicals:

Biphenyl was obtained from Sigma Chemical Co. (St. Louis, MO). Aroclors 1242, 1254 and 1260 in neat form were obtained from Accustandard Ltd. (New Haven, CT). Stock solutions were prepared containing 1 mg/ml in acetone.

Bacterial Strain:

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, NY) was obtained from the Northern Regional Research Laboratory (Peoria, IL). Cultures were maintained in 50 ml of the standard growth medium in 250 ml Erlenmeyer flasks by storage at 4°C, with monthly subculturing by incubation at 30°C for 24 h. Cultures were streaked onto tryptic soy agar at regular intervals to ensure purity. For long-term storage of cultures, 20% glycerol was added to cell suspensions in the standard growth medium and the mixture was distributed in 1 ml aliquots in 1.5 ml tubes and stored at -20°C.

Standard growth medium:

The growth medium used was the phosphate-buffered mineral salts medium supplemented with yeast extract described by Bedard et al. (1986). This medium contained per litre: 4.4 g K_2HPO_4, 1.7 g KH_2PO_4, 2.1 g NH_4Cl and 50 mg of yeast extract (Becton Dickinson, Cockeysville, MD), 2 g carbon source, and 10 ml of concentrated basal salts solution. Non-volatile carbon sources were added to the bulk medium prior to autoclaving. Basal salts were added after autoclaving. Biphenyl was prepared in a 0.1 g/ml solution in hexane, filter sterilized using a 0.2 μm PTFE syringe filter (Nalge, Rochester, NY) and added

to the autoclaved medium. The hexane was allowed to evaporate, leaving crystals suspended in the medium. The concentrated basal salts solution contained $MgSO_4$ (19.5 g/l), $MnSO_4.H_2O$ (5 g/l), $FeSO_4.7H_2O$ (1 g/l), and $CaCl_2.2H_2O$ (0.3 g/l) plus enough H_2SO_4 to prevent precipitation of basic salts. Culture media (50 ml) were prepared in 250 ml Erlenmeyer flasks, and stoppered with foam plugs.

Inoculum Preparation:

Cultures stored at 4°C were incubated on an orbital shaker (Labline Instruments Inc., Melrose Park, IL) at 200 rpm at room temperature until warm. Cells were inoculated into 50 ml of fresh growth media in a 250-ml Erlenmeyer flask to an OD of 0.1 measured at 615 nm in a 1-cm light path. Unless otherwise stated, cultures were incubated at 30°C on an orbital shaker set at 200 rpm for 22 to 24 h. Cultures containing biphenyl were filtered through sterile glass wool to remove excess crystals. The cell suspension was then centrifuged at 5000 *g* for 15 min, washed twice with 0.05 M sodium phosphate buffer, pH 7.5, and resuspended in the buffer to an OD of 1.0. Microbial growth was monitored by measurement of absorbance of cultures at 615 nm in a 1-cm light path in a spectrophotometer (Shimadzu, Model UV-120-02, Kyoto, Japan). Measurements were taken when the absorbance readings fell between 0.03 and 0.5 where a linear relationship is observed between absorbance and biomass concentration. Samples with higher cell densities were diluted with distilled water to within the above range and the absorbance was multiplied by the dilution factor.

Biotransformation conditions:

One millilitre of bacterial suspension at an OD of 1.0 was inoculated per 8-ml glass vials. Controls were killed by addition of 1 drop of 70% perchloric acid. Aroclors were added in the form of a concentrated acetone solution to a final concentration of 10 ppm. Vials were closed with Teflon-lined caps and incubated at 30°C on an orbital shaker set at 200 rpm.

PCB extractions:

Following incubation, reactions were stopped by addition of 1 drop of 70% perchloric acid. Extraction required the addition of 100 μl of a 10% Triton X-100 (Fisher Scientific, Fair Lawn, NJ) solution to increase PCB solubility, four volumes of hexane, and 0.5 g of sodium sulfate to prevent formation of a stable emulsion. Foil-lined caps were used for this procedure. Samples were shaken horizontally on an orbital shaker at 300 rpm for 20 to 30 min. The hexane layer was transferred to vials for gas chromatography. Storage took place at 4°C until analyses.

Aroclor analysis:

Identification and quantitative determination of the polychlorinated biphenyl (PCB) congeners were based on analysis utilizing a Hewlett-Packard 5890 gas chromatograph equipped with an electron-capture detector (ECD). The column used was a high performance capillary column (HP-5, 30 m long, 0.25 mm in diameter, 0.25 μm film thickness). The sample (2 μl) was injected in splitless mode with a time delay of 1.0 min after injection. The initial oven temperature of 55° was held for 2 min and then programmed to increase by 10°C /min to 90°C; 2.5°C /min to 285°C, and held for a further 10 min. The injector temperature was 250°C and the Ni^{63} EC detector was maintained at 350°C. The carrier gas was hydrogen with a column head pressure of 13 psi and with nitrogen as the make-up gas. The gas flow was maintained at a rate of 30 ml/min with minor adjustment to maximize response, as necessary.

A PCB standard was prepared using purchased Aroclor solutions 1016, 1221, 1242, 1254, and 1262 (Supelco 4-8701;4-8705;4-8706;4-8707;4-4810), reconstituted in hexane at a ratio of 1:1:1:1:1, to a concentration of ~ 2.9 mg/ml and fortified with IUPAC congeners 86 and 209. The quantity of each PCB constituent in the standard was determined using 209 individual PCB standards purchased from AccuStandard (New Haven,CT).

Analytical response factors, chromatographic separation and congener confirmation for the standard were determined using six different chromatographic phases.

2.4 Results

Pseudomonas LB400 was incubated in the phosphate-buffered mineral salts medium supplemented with 0.005% yeast extract and 2000 ppm of each carbon source on an orbital shaker for 24 h. Twice washed cells were resuspended in sodium phosphate buffer to an absorbance of 1.0 and incubated with Aroclors 1242, 1254 and 1260 for up to 96 h. Vials were sacrificed after various incubation times and extracted and analysed for residual PCB content. Each peak in an Aroclor was quantified as described in the Methods. To obtain the total percentage transformed, the sum of all the peaks was normalized to one of the component peaks which was not transformed and compared to a killed control. Time course plots of total percentage congeners transformed by cells grown on each of the carbon sources are presented in Figure 2.1 for Aroclor 1242, 1254 and 1260. *Pseudomonas* LB400 grown on biphenyl was capable of transforming 80% of the total amount of Aroclor 1242 added, while glucose- or glycerol- grown cells transformed up to 60% of the mostly tri- and tetrachlorinated congeners of Aroclor 1242. Cells grown on biphenyl transformed almost 50% of Aroclor 1254 versus 35% after growth on the other carbon sources. Transformation of Aroclor 1260 by cells grown on biphenyl, glucose and glycerol was 17, 9 and 9 % respectively.

The percentage degradation after 48 h of separated PCB congeners in Aroclor 1242, 1254, or 1260 by resting cells grown on biphenyl and glucose are presented in Figure 2.2. Values for glycerol were similar to those for glucose, thus they are not shown. Each peak was normalized to one of the component peaks which was not transformed, and compared to the same peak in a killed control. Where congeners co-elute as one peak, the component congeners are indicated together in the figures. Most of the lightly chlorinated congeners are transformed by cells grown on biphenyl and glucose. The initial and final concentrations of each peak in the Aroclors, before and after incubation with *Pseudomonas* LB400 for 48 h, are presented in Table 2.1.

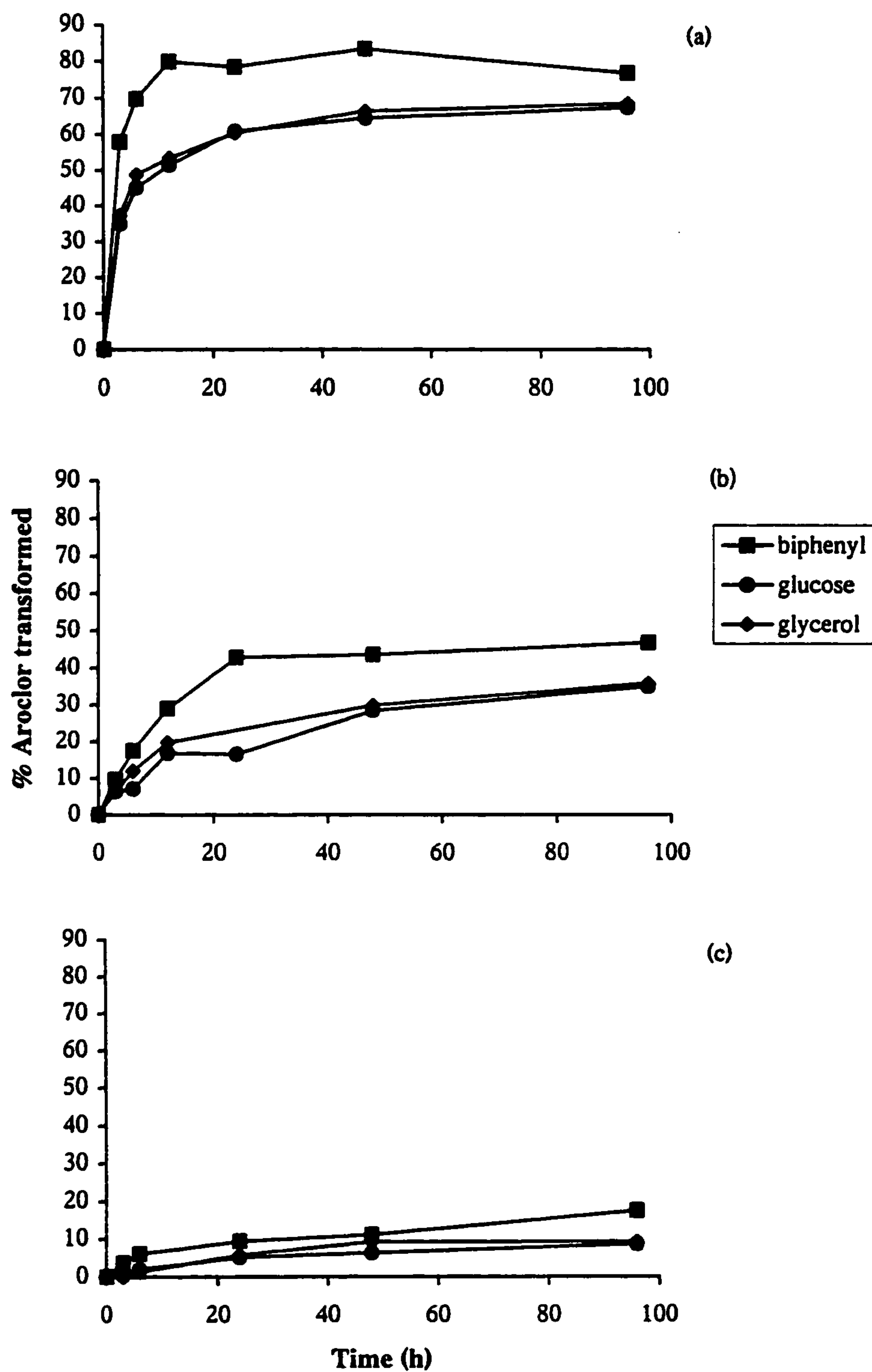

Figure 2.1 Time course of degradation of (a) Aroclor 1242, (b) Aroclor 1254 and (c) Aroclor 1260 by *Pseudomonas* LB 400 grown on different carbon sources.

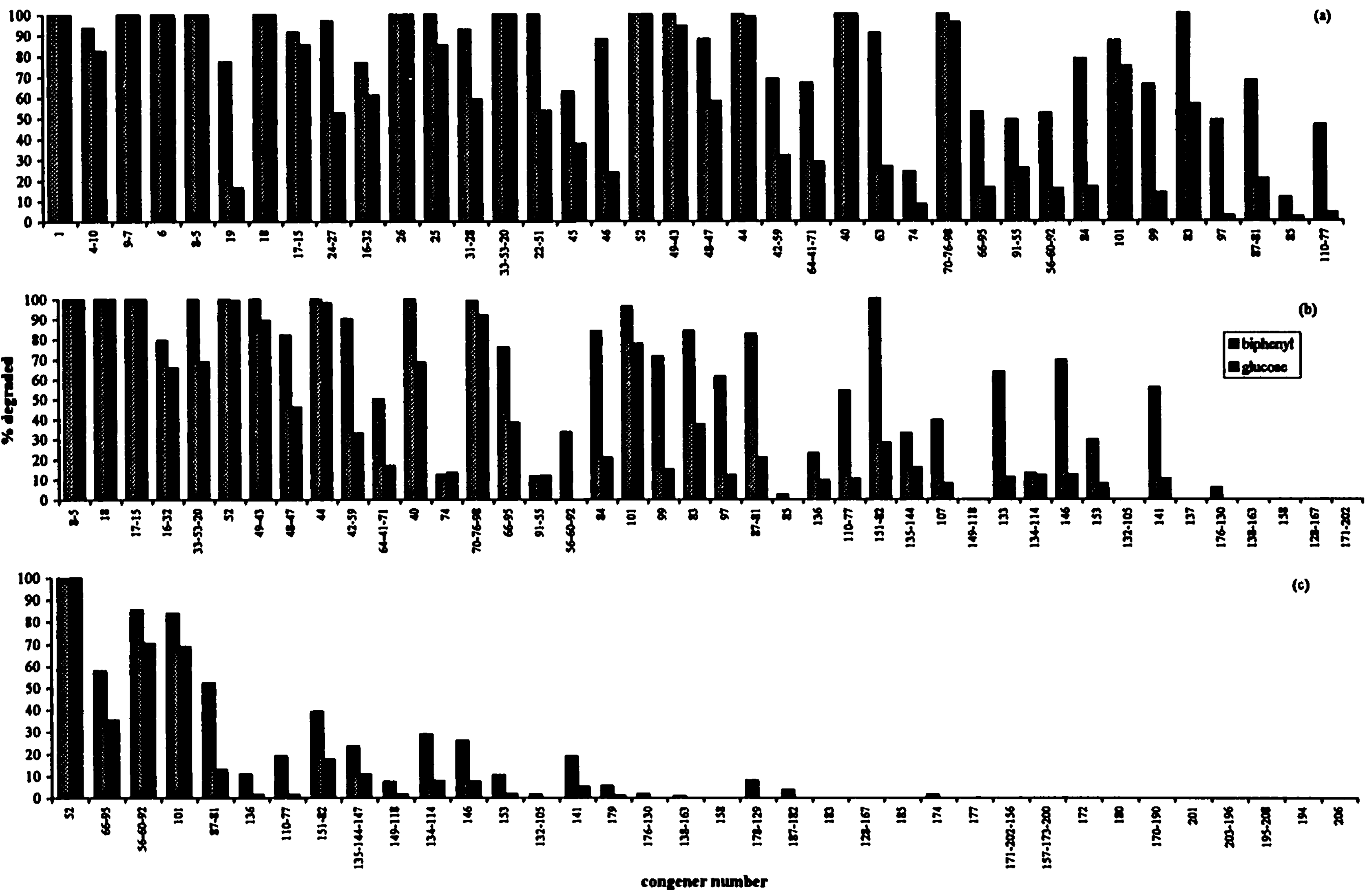

Figure 2.2 Percent degradation of separated congeners of (a) Aroclor 1242, (b) Aroclor 1254 and (c) Aroclor 1260 after 48 h by *Pseudomonas* LB400 cells grown on biphenyl or glucose

Table 2.1 Initial and final congener concentrations (ppb) in degradation tests using biphenyl-grown cells

Congener number	Congener chlorination pattern	Aroclor 1242		Aroclor 1254		Aroclor 1260	
		initial	final	initial	final	initial	final
1	2	75	0				
4-10	2,2' 2,6	417	27				
9-7	2,5 2,4	184	0				
6	2,3'	225	0				
8-5	2,4' 2,3	688	0				
19	2,6,2'	184	39				
18	2,5,2'	985	0				
17-15	2,4,2' 4,4'	833	66				
24-27	2,3,6 2,6,3'	253	8				
16-32	2,3,2' 2,6,4'	997	220				
26	2,5,3'	215	0				
25	2,4,3'	122	0				
31-28	2,5,4' 2,4,4'	1724	115				
33-53-20	3,4,2' 2,5,2',6' 2,3,3'	1030	0	52	0		
22-51	2,3,4' 2,4,2',6'	535	0				
45	2,3,6,2'	229	79				
46	2,3,2',6'	122	14				
52	2,5,2',5'	613	0	911	0	49	6
49-43	2,4,2',5' 2,3,5,2'	532	0	252	0		
48-47	2,4,5,2' 2,4,2',4'	508	56	65	10		
44	2,3,2',5'	630	0	459	0		
42-59	2,3,2',4' 2,3,6,3'	582	167	54	4		
64-41-71	2,3,6,4' 2,3,4,2' 2,6,3',4'	746	229	185	76		
40	2,3,2',3'	176	0	47	0		
63	2,3,5,4'	46	4				
74	2,4,5,4'	370	259	169	121		
70-76-98	2,5,3',4' 3,4,5,2' 2,4,6,2',3'	665	0	641	4		
66-95	2,4,3',4' 2,3,6,2',5'	742	321	1341	265	366	158
91-55	2,3,6,2',4' 2,3,4,3'	200	95	453	348		
56-60-92	2,3,3',4' 2,3,4,4' 2,3,5,2',5'	545	240	84	47	94	25
84	2,3,6,2',3'	106	21	480	65		
101	2,4,5,2',5'	192	23	1367	42	416	150
99	2,4,5,2',4'	137	43	633	155		
83	2,3,5,2',3'	199	0	438	56		
97	2,4,5,2',3'	143	67	615	194		
87-81	2,3,4,2',5' 3,4,5,4'	164	48	988	142	86	42
85	2,3,4,2',4'	102	83	340	276		
136	2,3,6,2',3',6'			167	110	224	192
110-77	2,3,6,3',4' 3,4,3',4'	143	109	1448	552	191	146
151-82	2,3,5,6,2',5' 2,3,4,2',3'			159	0	347	200
135-144-147	2,3,5,2',3',6' 2,3,4,6,2',5' 2,3,5,6,2',4'			357	201	347	250
107	2,3,5,3',4'			111	56		
149-118	2,3,6,2',4',5' 2,4,5,3',4'			1666	1404	896	789
134-114	2,3,5,6,2',3' 2,3,4,5,4'	46	29	157	115	73	49
146	2,3,5,2',4',5'			174	45	185	125
153	2,4,5,2',4',5'			677	400	900	753
132-105	2,3,4,2',3',6' 2,3,4,3',4'	115	104	892	787	311	280
141	2,3,4,5,2',5'			257	96	347	245
179	2,3,5,6,2',3',6'					211	188
137	2,3,4,5,2',4'			127	111		
176-130	2,3,4,6,2',3',6' 2,3,4,2',3',5'			116	92	126	113
138-163	2,3,4,5,2',4' 2,3,5,6,3',4'	42	39	1236	1081	1147	1021
158	2,3,4,6,3',4'			205	192	132	118
178-129	2,3,5,6,2',3',5' 2,3,4,5,2',3'					197	161
175	2,3,4,6,2',3',5'			23	23		
187-182	2,3,5,6,2',4',5' 2,3,4,5,2',4',6'			78	63	705	621
183	2,3,4,6,2',4',5'			53	51	310	283
128-167	2,3,4,2',3',4' 2,4,5,3',4',5'			391	365	89	81
185	2,3,4,5,6,2',5'					78	68
174	2,3,4,5,2',3',6'			121	110	549	493
177	2,3,5,6,2',3',4'			73	67	314	285
171-202-156	2,3,4,6,2',3',4' 2,3,5,6,2',3',5',6' 2,3,4,5,3',4'			296	273	370	335
157-173-200	2,3,4,3',4',5' 2,3,4,5,6,2',3' 2,3,4,6,2',3',5',6'			122	123	152	138
172	2,3,4,5,2',3',5'					113	100
180	2,3,4,5,2',4',5'			155	134	1076	983
170-190	2,3,4,5,2',3',4' 2,3,4,5,6,3',4'			137	127	574	513
201	2,3,4,5,2',3',5',6'					278	245
203-196	2,3,4,5,6,2',4',5' 2,3,4,5,6,2',4',6'					368	325
195-208	2,3,4,5,6,2',3',4' 2,3,4,5,6,2',3',5',6'					162	147
194	2,3,4,5,2',3',4',5'					345	337
205	2,3,4,5,6,3',4',5'					201	195
206	2,3,4,5,6,2',3',4',5'					126	104

In order to determine transformation rate constants of the individual congeners within Aroclors 1242, 1254 and 1260, those congeners which were separated individually in GC chromatograms were plotted as a function of time, for cells grown on biphenyl and glucose (Figure 2.3). A non-linear regression was performed in the form $C = C_0e^{-kt}$, with intercepts forced at C_0 to determine transformation rate constant (k) values. R^2 values for the fit of the data were all greater than 0.75. The rate constants are presented in Table 2.2, in descending order. Some lower chlorinated congeners were completely transformed at the first sampling point (3 h incubation), and thus their rate constants (>2.5) were not quantified. A time course of transformation of some of the slower-degrading PCB congeners in Aroclor 1254 is presented in Figure 2.4. A definite lag phase in degradation of some congeners, for example congener numbers 107, 136, 141 and 146, is revealed, followed by a later increase in transformation rate.

In general, the sequence of degradation of the different congeners in an Aroclor was similar, regardless of growth substrate. However, k values for a particular congener typically were lower for cells grown on glucose compared to the values for cells grown on biphenyl. The k values for the same congeners in different Aroclors were not the same. Generally, rate constants decreased from highest values in Aroclor 1242 to lower values in Aroclor 1260. Based on the k value and its rank within an Aroclor, and the graphs of the time courses of removal, congeners were grouped according to their relative rate of degradation. For congeners which were individually separated on gas chromatograms, relative rates of transformation had the following order (congener numbers given): 1, 6, 18, 26, 25, 40 > 44, 52 > 101, 46, 83, 84 > 19, 45, 63, 97, 99 > 141, 146 > 74, 107, 136, 153. Among trichlorinated compounds, the degradation rate constants had the following order: congener 18 (2,5,2') > 26 (2,5,3'), 25 (2,4,3') > 19 (2,6,2'). Among the tetrachlorinated congeners, the sequence was 40 (2,3,2',3') > 44 (2,3,2',5'), 52 (2,5,2',5') > 46 (2,3,2',6') > 45 (2,3,6,2'), 63 (2,3,5,4') > 74 (2,4,5,4'). Among the pentachlorinated compounds, the order is 101 (2,4,5,2',5'), 83 (2,3,5,2',3'), 84 (2,3,6,2',3') > 99 (2,4,5,2',4'), 97 (2,4,5,2',3') > 107 (2,3,5,3',4'). Removal of hexachlorinated congeners was also observed.

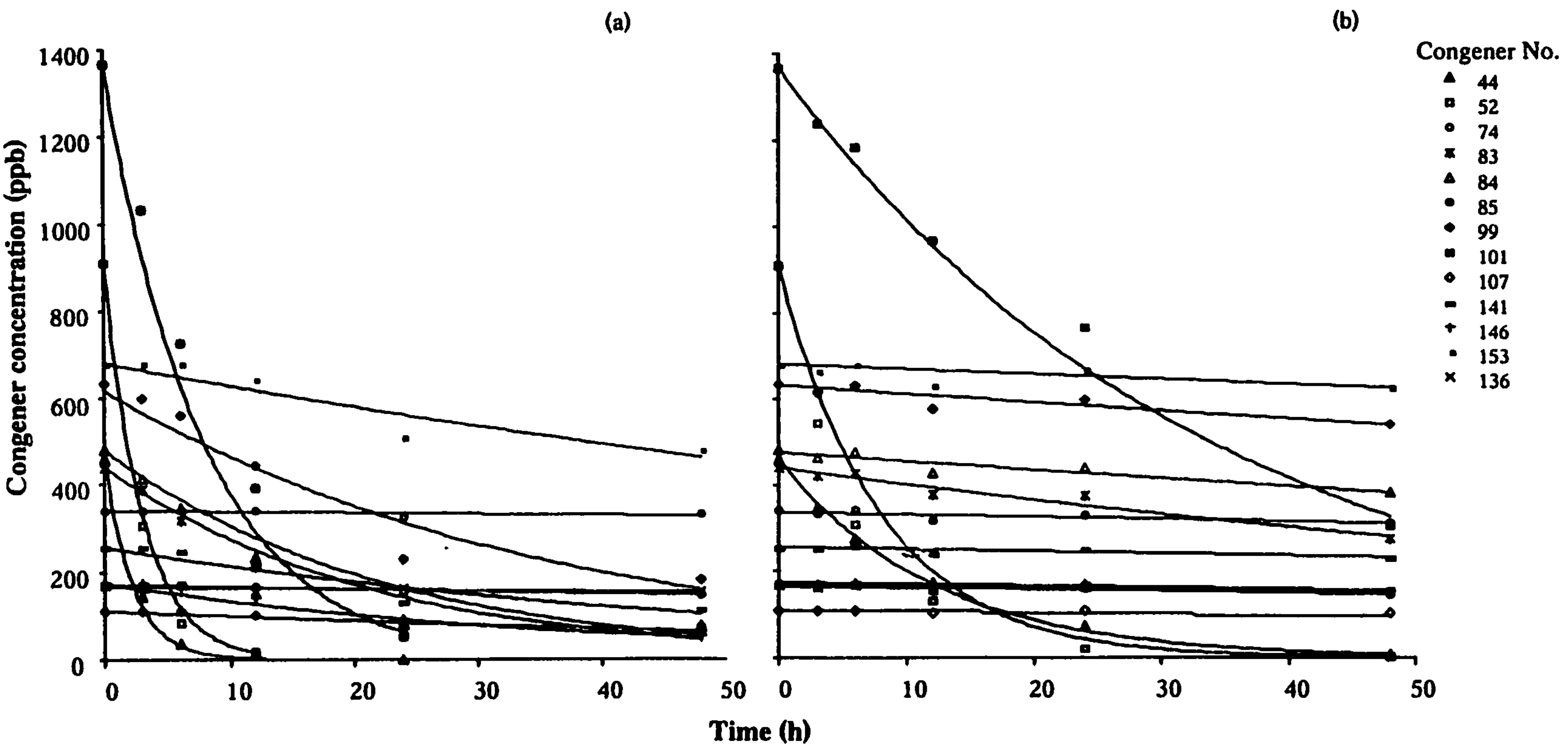

Figure 2.3 Time course of degradation of individual congeners in Aroclor 1254 by *Pseudomonas* LB400 cells grown on (a) biphenyl and (b) glucose

Table 2.2 Rate constants for degradation of individual congeners in Aroclors by cells grown on different carbon sources

Aroclor	Congener	biphenyl-grown		glucose-grown	
1242	number	k (h^{-1})	rank	k (h^{-1})	rank
	52	0.422	1	0.199	1
	44	0.386	2	0.105	2
	101	0.159	3	0.021	3
	46	0.128	4	0.006	6
	83	0.127	5	0.018	4
	84	0.109	6	0.003	10
	45	0.095	7	0.011	5
	19	0.085	8	0.004	8
	99	0.082	9	0.004	9
	97	0.059	10	0.000	12
	63	0.055	11	0.005	7
	74	0.006	12	0.002	11

Aroclor	Congener	biphenyl-grown		glucose-grown	
1254	number	k (h^{-1})	rank	k (h^{-1})	rank
	44	0.448	1	0.084	2
	52	0.232	2	0.126	1
	101	0.129	3	0.026	3
	83	0.047	4	0.008	4
	84	0.046	5	0.005	5
	146	0.019	6	0.003	6
	99	0.019	7	0.003	7
	97	0.014	8	0.003	8
	141	0.013	9	0.002	10
	107	0.008	10	0.002	11
	153	0.006	11	0.001	13
	136	0.004	12	0.002	12
	74	0.002	13	0.003	9

Aroclor	Congener	biphenyl-grown		glucose-grown	
1260	number	k (h^{-1})	rank	k (h^{-1})	rank
	52	0.320	1	0.204	1
	101	0.104	2	0.025	2
	146	0.017	3	0.002	3
	141	0.015	4	0.002	4
	153	0.008	5	0.000	5
	136	0.006	6	0.000	6

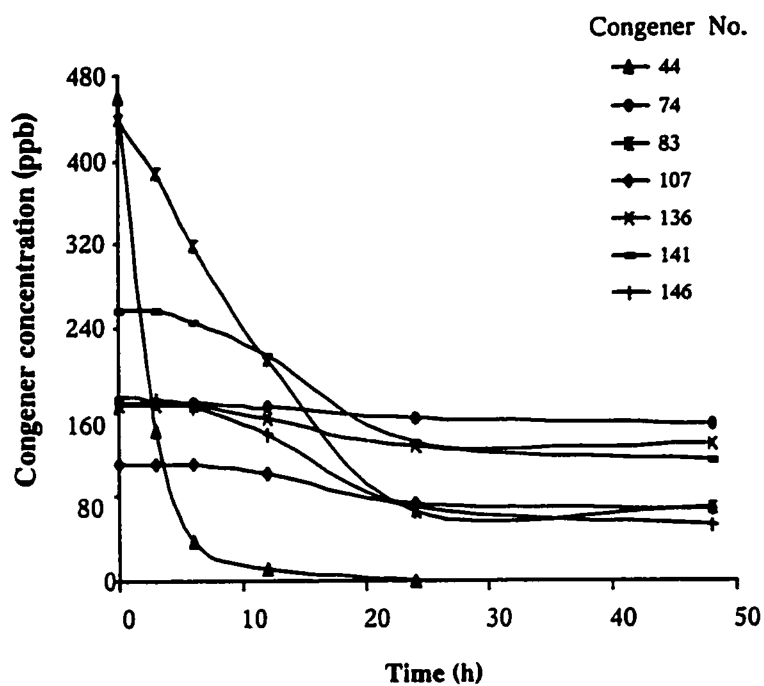

Figure 2.4 Time course of degradation of slower-degrading congeners in Aroclor 1254 by cells grown on biphenyl

2.5 Discussion

While cells of *Pseudomonas* LB400, grown on biphenyl, glucose or glycerol, manifested a capacity to transform Aroclors, highest rates and extents of conversion of PCBs in Aroclors were observed with cells grown on biphenyl. Biphenyl-grown cells were shown to transform 80% and 50% of PCBs in Aroclor 1242 and 1254, respectively. Glucose-grown cells transformed 60% and 35% of Aroclor 1242 and 1254, respectively, under the same conditions. Biphenyl-grown *Alcaligenes eutrophus*, strain H850, has been shown to reduce PCBs in Aroclors 1242 and 1254 by 81% and 35%, respectively (Bedard et al. 1987b). Growing cells of *Acinetobacter* species T6 transformed radio-labelled Aroclor 1254 by 32% whereas biphenyl-grown resting cells exhibited much lower conversions (Kohler et al. 1988). Yadav et al. (1995) reported degradation of 17.6% of Aroclor 1260 by the fungus *Phanerochaete chrysosporium*, claimed to be the first conclusive demonstration of Aroclor 1260 degradation by any aerobic microbial system. Our results indicate that biphenyl-grown *Pseudomonas* LB400 cells are capable of aerobically degrading 17% of PCBs in Aroclor 1260. We have further confirmed that glucose-grown LB400 cells aerobically transformed 9% of congeners present in Aroclor 1260. We have established that the lower chlorinated congeners in this Aroclor are extensively transformed and we have characterized the time courses of degradation of these congeners under aerobic conditions.

In each Aroclor, degradation of individual congeners followed the accepted general pattern, namely, a decrease in rate or extent of degradation with increasing chlorination. This pattern remains virtually the same regardless of the cell growth substrate, biphenyl or glucose. Transformation k values were not the same for the same congener in different Aroclors. This was to be expected, as previous studies (Bedard et al. 1986) have shown that the presence of other congeners affects specific congener degradation. However, our results indicate that the overall order of congener degradation, based on congener k values, is generally the same in the different Aroclors.

We have shown a definite delay or lag in the degradation of some congeners. These

congeners appear to be excluded as substrates for PCB-transforming enzymes until other congeners are transformed, after which rates for their bioconversion increase. A more in depth investigation of the kinetics of conversion of PCB congeners by transforming enzymes, including inhibition studies, is required to more fully explain these observations.

The pattern of transformation among the GC-separated trichlorinated congeners in the Aroclors was determined. The observation that a 2,6-chlorinated biphenyl (congener 19) is inhibitory to degradation whereas a 2,5-chlorination configuration (congeners 18, 26) is beneficial is consistent with observations made by Bedard and co-workers (1987a) using strain *A. eutrophus* H850. Examining the pattern of degradation among separated tetrachlorinated congeners in the Aroclors, the more recalcitrant congener 74 has no open 2,3 or 3,4 sites for degradation on one ring and the other ring has one *para*-chlorine which tends to be inhibitory to degradation (Bedard and Haberl 1990). Congener 63, also having a slower transformation value, has a similar chlorination pattern. Congener 74 was found to be recalcitrant to degradation by *A. eutrophus* H850 (Bedard et al. 1987a). Congeners 45 and 46 have a 2,6-chlorinated ring which, as indicated above, is inhibitory. Among the pentachlorinated PCBs, congener 107 has a similar chlorination pattern to congener 63 with an additional 3'-chlorine producing a 3,4-chlorination pattern which is also hard to degrade (Bopp 1986). Degradation of hexachlorinated congeners in all of the Aroclors was observed. *Pseudomonas* LB400 is one of the only aerobic bacterial strains which degrades hexachlorinated congeners.

The ratio of rate constants of degradation of a particular congener in an Aroclor, for cells were grown on biphenyl or glucose, were found not to be constant for all congeners. This is consistent with results obtained when initial rates of transformation of four individual congeners were determined (Billingsley et al. 1997).

A possible bioremediation system for PCBs includes an anaerobic step to dechlorinate highly chlorinated PCBs followed by an aerobic step to transform low chlorinated PCBs (Abramowicz 1990). The need to use biphenyl, itself a hazardous chemical, as a growth substrate in the aerobic step represented a barrier to implementation of this step. The capacity

of *Pseudomonas* LB400 cells, grown on glucose, to effectively transform lower chlorinated congeners in Aroclors under aerobic conditions may overcome the requirement for the PCB-containing material from the reductive dechlorination step to be amended with biphenyl for the aerobic degradation step.

Acknowledgements

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CHAPTER 3

Effects of surfactants on aqueous solubilization of PCB congeners and on their biodegradation by *Pseudomonas* LB400[1]

3.1 Abstract

The effectiveness of a variety of commercial surfactants in solubilizing PCBs sorbed to glass was dependent on surfactant concentration and dissolution time. Most surfactants were fully or partially effective as PCB solubilizing-agents at concentrations greater than their critical micelle concentration (CMC) value. Among ethoxylate surfactants tested, those with lower CMCs were more efficient PCB-solubilizing agents. PCBs were not solubilized with a no-surfactant water control, nor with the nonionic block copolymer, Pluronic 10R5. At a concentration of 10 g/l, three out of eleven surfactants, a diethanolamide/ethoxylate blend, an alkane sulphonate and a twin alcohol ethoxylate inhibited bacterial growth. Only two surfactants, Sorbax PMO-20, a fatty acid ethoxylate, and Witcomul 3235, an anionic/nonionic blend, supported growth of *Pseudomonas* LB400 as sole carbon sources. In general, at surfactant concentrations above their CMC, anionic surfactants promoted, while nonionic surfactants inhibited, PCB transformation compared to a water control. Transformation rate constants of *Pseudomonas* LB400 for each congener in the presence of selected surfactants were determined and compared. The inhibitory effects of Igepal CO-630, an alkylphenol ethoxylate, on PCB degradation could be eliminated by diluting the surfactant solution to a lower concentration.

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3.2 Introduction

Polychlorinated biphenyls (PCBs) are chlorinated aromatic compounds which were used in capacitors and transformers due to their chemical and thermal stability. Inadvertent contamination of soils and sediments has occurred, and due in part to their hydrophobic nature, PCBs persist in the environment. Bioremediation is a potential treatment for contaminated soils, and biodegradation of PCBs by a range of microorganisms has been demonstrated, using both aerobic and anaerobic procedures (Abramowicz 1990).

The low bioavailability of PCBs is one of the major factors limiting PCB biodegradation (Viney and Bewley 1990; Barriault and Sylvestre 1993). PCBs have very low water solubility and strongly adsorb to soil (Lee 1979). Adsorption of PCBs increases with increasing degree of chlorination (Paya-Perez et al. 1991). Studies have shown that PCBs need to be solubilized in the aqueous phase in order to be assimilated by bacteria (Harkness and Bergeron 1990). Thus, degradation appears to be limited by mass transfer from the solid phase to the liquid phase (Thomas et al. 1986).

Surfactants have been used to increase recovery of oil from oil wells or tar sands and also have the potential for mobilization and/or degradation of hydrocarbon contaminants in the environment (Muller-Hurtig et al. 1993). Indeed, many microorganisms synthesize biosurfactants to facilitate solubilization and degradation of specific hydrophobic contaminants. Petroleum- and vegetable oil-degrading *Pseudomonas*, *Acinetobacter* and other species have been shown to produce biosurfactants when grown on hydrophobic substrates (Goldman et al. 1982; Cirigliano and Carman 1984; Cooper and Paddock 1984; Sim et al. 1997). Above their critical micelle concentrations (CMC), surfactants and detergents induce micellar solubilization of substrates (Bury and Miller 1993). The effectiveness of nonionic surfactants in promoting degradation of hydrocarbons by *Acinetobacter* was influenced by their degree of micelle formation in the aqueous phase (Lupton and Marshall 1978). Ethoxylated alcohols were effective in washing automatic transmission fluid from soil (Abdul et al. 1990), whereas alkylphenol ethoxylates enhanced removal of PAHs from soil (Ganeshalingham et al. 1994). Nonionic surfactants in general and

sodium dodecyl sulphate increased apparent solubilization and concentrations of PAHs in the aqueous phase (Edwards et al. 1991; Volkering et al. 1995).

In some cases surfactants have been found to be non-toxic to the microorganisms (Liu et al. 1995; Volkering et al. 1995) whereas in other cases toxicity has been observed (Rouse et al. 1994; Tiehm 1994). A cationic and an anionic surfactant inhibited growth of *Acinetobacter* on hydrocarbon substrates (Lupton and Marshall 1978). Surfactants may promote or inhibit biodegradation of PAHs (Liu et al. 1995; Thibault et al. 1996). Contaminant degradation is often found to be inhibited at surfactant concentrations greater than the surfactant's CMC (Laha and Luthy 1991). Inhibition of degradation of PAHs was reversed in one experiment by diluting the solution to a surfactant concentration less than the CMC (Laha and Luthy 1991). At surfactant concentrations lower than the CMC, surfactant molecules exist predominantly in the monomeric form. At concentrations greater than the CMC, they exist as micelles.

Very limited work has been carried out on the applications of surfactants to remediate PCBs. Surfactants and biosurfactants enhanced physical removal of PCBs from soil (van Dyke et al. 1993; Abdul and Gibson 1991; Viney and Bewley 1990). Removal efficiency was affected by soil type (van Dyke et al. 1993) and total organic carbon content (Harkness and Bergeron 1990).

This study attempts to address the following questions for a variety of surfactants and a known PCB-degrading organism, *Pseudomonas* LB400: 1) Can the surfactants solubilize PCBs in aqueous solutions? 2) Are the surfactants toxic to the bacterium and can the bacterium utilize the surfactants for growth? 3) Do the surfactants promote or inhibit degradation of PCBs in aqueous medium? 4) How can any inhibitory effects of surfactants be moderated to facilitate biodegradation of PCBs?

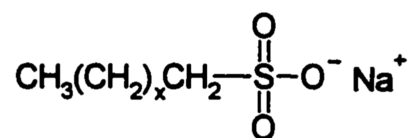
3.3 Materials and Methods

Chemicals:

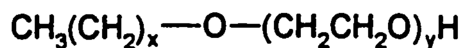
PCB congeners (2,2',5-trichlorobiphenyl, 2,2',4,4'-tetrachlorobiphenyl, 2,2',3,4,5'-pentachlorobiphenyl, 2,2',4,4',5,5'-hexachlorobiphenyl, 2,2',4,4',6-pentachlorobiphenyl and 2,2',4,5,5'-pentachlorobiphenyl) in neat form were obtained from Accustandard Ltd. (New Haven, CT). Purity is 99+%; no biphenyl peak was detected. Stock solutions of individual or mixed congeners were prepared containing 1 mM of each congener in acetone. Biphenyl was obtained from Sigma Chemical Co. (St. Louis, MO).

Surfactants were selected to represent common chemical classes of commercial surfactants such as the nonionic alcohol ethoxylates, alkylphenol ethoxylates, fatty acid ethoxylates, block copolymers and anionic sulphonates, as well as some other types that were specific for hydrocarbon solubilization. Their general structures are illustrated in Figure 3.1. Cationic surfactants were avoided due to their known toxicity to microorganisms (Swisher 1987). Surfactant samples were supplied by their respective manufacturers. These were: Bio-Soft EN-600 (Stepan, Missisauga, Canada), Genapol X-080, Hostapur SAS 60 (Hoechst Celanese, Charlotte, NC), Igepal CO-630, Igepal CO-850 (Rhone-Poulenc, Paris, France), Marlipal O13/120 (Huls, Marl, Germany), Naxchem Dispersant K (Ruetgers-Nease, State College, PA), Nansa LSS38/AS (Albright and Wilson, Richmond, VA), Pluronic 10R5 (BASF, Ludwigshafen, Germany), Sorbax PMO-20 (Chemax, Greenville, SC), Surfynol 485 (Air Products, Allentown, PA), Witcomul 3235 (Witco, New York, NY). The surfactants were used as obtained without further purification. All have 99-100% activity except for Hostapur (60%) and Nansa (38%). Stock solutions of the commercial products (10%, w/v) were made in distilled water and sterilized. Further details describing the surfactants are included in Table 3.6.

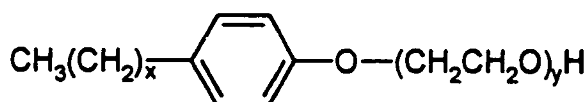
A.



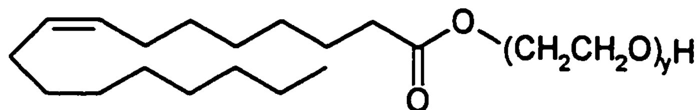
B.



C.



D.



E. Block copolymers are alternating blocks of identical monomer units. Often, one of the monomers is a hydrophobic molecule and the other is hydrophilic. They form very long chains and have high molecular weights.

Figure 3.1 General structures of surfactants.

A. Alkane sulphonate, B. Alcohol ethoxylate, C. Alkylphenol ethoxylate, D. Fatty acid ethoxylate, E. Block copolymer

Determination of CMC:

The critical micelle concentration of each surfactant was determined by the drop volume method (Shaw 1992). In this method a 1-ml pipette having a 0.1-ml increment scale was modified such that the tip was polished level and the mouth connected to a glass syringe. In this way, drops could be slowly released in a controlled manner. The surfactants were diluted with distilled water to a range of concentrations. Using the volumes of the drops, the surface tension of each solution was calculated. The critical micelle concentration for a particular surfactant was taken as the point in the range of concentrations at which the surface tension remained constant (Edwards et al. 1991). The CMC values obtained are included in Table 3.6.

Bacterial Strain:

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, NY) was obtained from the Northern Regional Research Laboratory (Peoria, IL). Cultures were maintained in 50 ml of the standard growth medium in 250-ml Erlenmeyer flasks by storage at 4°C, with monthly subculturing by incubation on an orbital shaker at 200 rpm at 30°C for 24 h. Cultures were streaked onto tryptic soy agar (TSA) at regular intervals to ensure purity. For long-term storage of cultures, 20% glycerol was added to cell suspensions in the standard growth medium and the mixture was distributed in 1-ml aliquots in 1.5-ml tubes and stored at -20°C.

Standard growth medium:

The growth medium used was the phosphate-buffered mineral salts medium supplemented with yeast extract described by Bedard et al. (1986). This medium contained per litre: K_2HPO_4, 4.4 g; KH_2PO_4, 1.7 g; NH_4Cl, 2.1 g; 50 mg of yeast extract (Becton Dickinson, Cockeysville, MD); 2 g biphenyl and 10 ml of concentrated basal salts solution. Basal salts were added after autoclaving. Biphenyl was prepared in a 0.1 g/ml solution in hexane, filter sterilized using a 0.2

μm PTFE syringe filter (Nalge, Rochester, NY) and added to the autoclaved medium. The hexane was allowed to evaporate, leaving crystals suspended in the medium.

The concentrated basal salts solution contained, per liter: $MgSO_4$, 19.5 g; $MnSO_4 .H_2O$, 5 g; $FeSO_4.7H_2O$, 1 g and $CaCl_2 .2H_2O$, 0.3 g; plus enough H_2SO_4 to prevent precipitation of basic salts. Culture medium (50 ml) was prepared in 250-ml Erlenmeyer flasks and stoppered with foam plugs.

Inoculum Preparation:

Cultures stored at 4°C were incubated on an orbital shaker (Labline Instruments Inc., Melrose Park, IL) at 200 rpm at room temperature until warm. Cells were inoculated into 50 ml of fresh growth medium in a 250-ml Erlenmeyer flask to an OD of 0.1 at 615 nm in a 1-cm light path. Unless otherwise stated, cultures were incubated at 30°C on an orbital shaker set at 200 rpm for 22 to 24 h. Cultures were filtered through sterile glass wool to remove excess biphenyl crystals. The cell suspension was then centrifuged at 5000 *g* for 15 min, washed twice with 0.05 M sodium phosphate buffer, pH 7.5, and resuspended in the buffer to an OD of 1.0 at 615 nm, using a 1-cm light path.

Growth on TSA in the presence of surfactants:

Surfactants were added to liquid TSA to obtain a final concentration of 1 or 10 g/l. Following sterilization, the molten agar was poured into petri plates. Cells were prepared as described above under inoculum preparation. Serial dilutions were performed in water, and aliquots were plated in triplicate. The number of CFUs was determined after a 5-day incubation at room temperature.

Growth on surfactants in mineral medium:

Sterile growth medium (45 ml) containing no carbon source was added to sterile 250-ml Erlenmeyer flasks. Autoclaved 100 g/l surfactant aqueous solutions were added to a final

concentration of 2 g/l and a control was prepared by addition of water in place of surfactant solution. The solution was allowed to mix before addition of cells. Cells were grown as in the inoculum preparation, but were washed with growth medium containing no carbon source. Cell suspension at OD=1 (1-cm light path at 615 nm) was added to flasks which were incubated at 30°C and 200 rpm for 48 h. Serial dilutions were prepared in water, and aliquots were plated in triplicate on TSA. The number of CFUs was determined after a 5-day incubation at room temperature.

Solubilization of PCBs from glass:

An aliquot of the mixture of PCBs in acetone was added to 8 ml amber glass vials so that the final concentration in 1 ml of aqueous medium would be 10 μM for each congener. The acetone was allowed to evaporate, leaving the PCBs sorbed to the glass. Surfactant solutions at 1 or 10 g/l were added and the vials were incubated at room temperature and 200 rpm. At each time period, the liquid layer was removed and transferred to clean 8-ml vials which were extracted and analyzed for PCBs.

For dilution experiments, congener 2,2',4,5,5'-pentachlorobiphenyl in acetone was added to 20-ml amber glass vials, so that the final concentration in 10 ml of aqueous medium would be 4 or 40 ppm. After evaporation of the acetone, Igepal CO-630 at 1 g/l concentration was added and the vials were incubated at room temperature and 200 rpm for 24 h. The aqueous solutions were removed, and the solution of 1 g/l surfactant and 40 ppm PCB was diluted 1:10 with distilled water.

Degradation of PCBs sorbed to glass:

An aliquot of a mixture of PCBs in acetone was added to 8-ml amber glass vials so that the final concentration in 1 ml of aqueous medium would be 10 μM for each congener. The acetone was allowed to evaporate, leaving the PCBs sorbed to the glass. One millilitre of inoculum was added, followed by enough surfactant stock solution to obtain a final concentration of 1 or

10 g/l. Controls were killed by addition of 1 drop of 70% perchloric acid. Individual vials were incubated for each surfactant for each time period. Vials were closed with Teflon-lined caps and incubated at 30°C on an orbital shaker set at 200 rpm. Following incubation, reactions were stopped by addition of 1 drop of 70% perchloric acid.

Degradation of PCBs in diluted glass washing solutions:

A 0.5-ml aliquot of glass washing solution was added to 8-ml glass vials, along with 0.5 ml of cell suspension. Cells were prepared as described above for inoculum preparation, however, they were resuspended in 0.1 M sodium phosphate buffer to a final OD of 2.0, 1-cm light path. Controls were killed by addition of 1 drop of 70% perchloric acid. Vials were closed with Teflon-lined caps and incubated at 30°C on an orbital shaker set at 200 rpm.

PCB extraction:

Four millilitres of hexane and 0.5 g of sodium sulfate (to prevent formation of a stable emulsion) were added to the PCB incubations. Foil-lined caps were used for this procedure. Samples were shaken horizontally on an orbital shaker at 300 rpm for 20-30 minutes. A 1-ml aliquot of the hexane layer was transferred to vials for GC/MS analysis.

Analysis:

Extracted samples were analyzed by capillary GC (Model HP 5890 series II, Hewlett-Packard, Meyrin, Switzerland) equipped with a mass selective detector (Model HP 5971A, Hewlett-Packard) and integrated by Hewlett-Packard MS Chemstation software. Identification and quantitative determinations of the congeners were based on analysis on a fused silica capillary column coated with a film thickness of 0.25 µm of polymethyl (5% phenyl) siloxane, 30 m in length, with a 0.25 mm internal diameter (HP-5MS, Hewlett-Packard). The sample (2 µl) was injected by an autoinjector (Model HP 7673, Hewlett-Packard) in splitless mode with a time delay of 1.25 min after injection. The initial oven temperature of 55°C was held for 2 min and then

programmed to increase by 10°C/min to 100°C, 5°C/min to 215°C, 10°C/min to 285°C and held for 5 min. The injector temperature was 250°C and the transfer line was 280°C. The carrier gas was helium with an initial column head pressure of 12 psi. The system was equipped with electronic pressure control and operated in constant flow mode (1.66 ml/min). The filament and multiplier were turned on at 8 min. The system was operated in selected ion monitoring mode. The ions monitored were 188,190; 222,224; 256,258; 290,292; 326,328. Standard midmass autotunes using PFTBA were performed before each set of samples was analyzed.

Experimentation and analysis:

The reported degradation percentages were calculated by normalization of the parental congener peak to the recovery of a non-degradable internal standard (2,2',4,4',6-CBP), and comparison to the killed control. The recovery of PCB congeners from the killed controls was always between 85 and 100%, thus loss of less than 15% was not considered significant. Preliminary experiments showed that normalization to the internal standard accounted for variations in addition and extraction. Duplicate samples within experiments showed very similar results, with variations between samples averaging 5%. Reproducibility between experiments was also very high, with typical variations being <10%.

3.4 Results

Can surfactants solubilize PCBs adsorbed to glass?

The capacity of aqueous solutions of surfactants to solubilize PCBs was examined by testing their abilities to remove and solubilize a mixture of five PCB congeners adsorbed to glass. Surfactant solutions were added to vials containing sorbed PCBs, and allowed to mix for up to 6 h on an orbital shaker set at 200 rpm. The aqueous phase was removed after different periods of mixing, and the amount of each PCB congener present in the aqueous phase was expressed as a percentage of the starting amount of each congener (Table 3.1). For many of the surfactants at a concentration of 10 g/l, complete PCB solubilization was observed after 1 h. Nansa LSS38/AS

and Igepal CO-850 solubilized between 40-86% of the PCBs. Surfynol 485 solubilized ≤ 20% of PCBs, while no solubilization was observed with the nonionic block copolymer, Pluronic 10R5, nor with water without surfactant. The latter four surfactants with poorer PCB-solubilizing properties had CMC values of ≥0.15 g/l with the exception of the nonionic block copolymer, Pluronic 10R5 (CMC = 0.047 g/l). The surfactants manifesting 100% solubilization of PCBs under these conditions had CMC values ≤ 0.11 g/l with the exception of the undefined mixture Witcomul 3235 and the anionic Hostapur SAS 60 (CMC = 0.42 g/l).

At the surfactant concentration of 1 g/l, some surfactants were still capable of totally solubilizing PCBs in less than 1 h. However, Marlipal O13/120, Witcomul 3235, Sorbax PMO-20 and Igepal CO-850 exhibited a reduced PCB solubilizing capacity, whereas Nansa LSS38/AS and Surfynol 485 lost their solubilizing capacity at this concentration. Surfactants that exhibited incomplete PCB solubilization showed that individual congeners were solubilized to different extents. The higher chlorinated congeners were, in general, solubilized to a lesser extent than the lower chlorinated congeners.

The effect of lower surfactant concentrations of Igepal CO-630 on solubilization of PCBs was investigated. At a surfactant concentration of 0.01 g/l, which is below its CMC, there was no solubilization of any of the congeners over 6 h. In contrast, a 0.1 g/l surfactant solution, which is slightly higher than the CMC value, solubilized 50-70% of the congeners in 1 h, which increased to 90% after 6 h. This illustrates that solubilization of the PCBs sorbed to glass only occurs at surfactant concentrations above the CMC value, where micelles exist.

Table 3.1 Effect of surfactants on aqueous dissolution of PCB congeners adsorbed to glass

Surfactant			% dissolution of congener				
Concentration (g/l)	Name	Dissolution time (h)	2,5,2′	2,4,2′,4′	2,3,5,2′,5′	2,4,6,2′,4′	2,4,5,2′,4′,5′
10	Hostapur, Bio-Soft, Genapol, Marlipal, Igepal 630, Sorbax, Naxchem, Witcomul	1	100	100	100	100	100
10	Igepal 850	1	88	77	72	74	61
	Nansa		88	81	73	74	55
	Surfynol		25	16	10	11	4
	Pluronic, Water		0	0	0	0	0
1	Hostapur, Bio-Soft,Genapol, Igepal 630, Naxchem	1	100	100	100	100	100
1	Sorbax	1	100	100	81	75	56
	Witcomul		72	60	49	49	36
	Igepal 850		71	52	46	44	26
	Marlipal		38	33	29	30	31
	Nansa, Surfynol, Pluronic, Water		0	0	0	0	0
0.1	Igepal 630	1	84	83	86	81	87
		3	93	94	94	95	100
		6	99	100	100	100	100
0.01	Igepal 630	1	48	52	70	57	76
		3	76	68	70	76	86
		6	90	88	86	86	86
0.001	Igepal 630	1	0	0	0	0	0
		3	8	3	4	3	0
		6	4	0	0	0	0

Are the surfactants toxic to the bacterium?

To determine the toxicity of surfactants to *Pseudomonas* LB400, TSA plates were prepared containing 10 g/l and 1 g/l surfactant. Serial dilutions of log phase *Pseudomonas* LB400 were plated and examined for growth after an incubation period of 5 days at room temperature. The same number of colonies was observed on the control plates with no surfactant added as on plates containing surfactant at 10 g/l for all surfactants except for the alkane sulphonate, Hostapur SAS 60, the diethanolamide/alkylphenol ethoxylate blend, Naxchem Dispersant K, and the twin alcohol ethoxylate, Surfynol 485 (Table 3.2, second column). Inhibition of growth was observed in the presence of the latter three surfactants. At a concentration of 1 g/l surfactant, colony counts were similar to the control for all surfactants on TSA plates, except on plates containing Hostapur SAS 60, where growth was inhibited (Table 3.2, third column).

Does the bacterium utilize the surfactants for growth?

A standard inoculum was added to Erlenmeyer flasks containing growth medium without a carbon source. Surfactants were diluted in buffer and added to the medium to a final concentration of 2 g/l. Flasks were incubated for 48 hours, then culture samples were serially diluted and plated on TSA to determine the number of colonies.

Media containing the fatty acid ethoxylate, Sorbax PMO-20, and the unknown mixture, Witcomul 3235, showed significantly more colonies than the control medium containing no surfactant after the 5 day incubation (Table 3.2, fourth column), suggesting that *Pseudomonas* LB400 used these surfactants as carbon sources for growth. Media supplemented with the rest of the surfactants showed similar counts to the medium containing no surfactant, suggesting that these surfactants did not support growth of *Pseudomonas* LB400.

Table 3.2 Effect of surfactants on growth of *Pseudomonas* LB400

Surfactant	Inhibition of growth on TSA at surfactant concentration		CFUs x 10^7 in mineral medium + surfactant after 48 h
	10 g/l	1 g/l	
None	no	no	6
Hostapur SAS 60	yes	yes	3
Nansa LSS38/AS	no	no	4
Bio-Soft EN-600	no	no	10
Genapol X-080	no	no	7
Marlipal O13/120	no	no	4
Igepal CO-630	no	no	8
Igepal CO-850	no	no	8
Sorbax PMO-20	no	no	240
Naxchem Dispersant K	yes	no	15
Witcomul 3235	no	no	77
Surfynol 485	yes	no	11

Do the surfactants promote or inhibit degradation of PCBs in aqueous medium?

From the initial solubilization experiment, it was shown that the PCBs sorbed to glass are essentially insoluble in water. A mixture of five PCB congeners was applied to glass vials which were then inoculated with *Pseudomonas* LB400 in buffer. Surfactant solutions were added to obtain final surfactant concentrations of 10 or 1 g/l. The PCB content of the vials was monitored in time course studies over a 48-h period. The results after a 48-h incubation with surfactants at concentrations of 1 g/l are presented in Table 3.3. In the absence of surfactant, the extent of degradation of the tri-, tetra-, penta- and hexachlorinated congeners after 48 h was 100, 92, 81 and 27%, respectively. Compared to the control with no surfactant, the presence of nonionic surfactants did not have a beneficial effect on the degradation of PCBs sorbed to glass, and some surfactants inhibited PCB degradation. However, the anionic surfactants Hostapur SAS 60, a good PCB-solubilizing surfactant, and Nansa LSS38/AS, a poor PCB-solubilizing surfactant, increased the extent of PCB degradation compared to the control. The nonionic surfactants which were most effective at solubilization, inhibited degradation of PCBs the most.

The time course of degradation of PCBs in the control with no surfactant present, and in the presence of representative surfactants Hostapur SAS 60, Surfynol 485 and Igepal CO-630 at 10 and 1 g/l is shown in Figure 3.2. Compared to the control, Hostapur SAS 60 at a concentration of 1 g/l (Figure 3.2E) showed increased rates and extents of degradation of PCB congeners, while at a surfactant concentration of 10 g/l (Figure 3.2D), rates and extents of degradation were similar to the control. The presence of Surfynol 485 exhibited little or no effect on degradation at a concentration of 1 or 10 g/l. The presence of Igepal CO-630 at both concentrations inhibited PCB degradation.

For representative surfactants, the time-course data for congener degradation were used to determine the transformation rate constants. A non-linear regression was performed in the form $C = C_o e^{-kt}$, with intercepts forced at C_o to determine transformation rate constant (k) values. Rate constants for transformation of congeners that were deemed not to follow first order kinetics were not determined. R^2 values for the fit of the data were all greater than 0.9. In Table 3.4, the

transformation rate constants obtained in the presence of surfactants are compared to the corresponding transformation rate constants observed for a control containing no surfactant. The presence of 1 g/l of Hostapur SAS 60 increased transformation rates for all congeners compared to the control with no surfactant. Overall, the lower chlorinated congeners were degraded the fastest, with the hexachlorinated biphenyl degraded slowly or not at all.

How can any inhibitory effects of surfactants be moderated to facilitate biodegradation of PCBs?

As there is no inhibition of PCB degradation in the presence of nonionic surfactants at concentrations below their CMC (i.e. in the absence of micelles), the inhibition of PCB biodegradation may be due to solubilization of PCBs in micellar form, whereby the PCB in the micelle is unavailable to *Pseudomonas* LB400. Therefore, if the surfactant concentration of the latter solution is diluted to bring the surfactant concentration near or below its CMC value, the micelles should disassociate, possibly making the PCBs bioavailable. An experiment was designed to test this hypothesis. Igepal CO-630 at 1 g/l was used to solubilize, in micellar form, 4 and 40 ppm of congener 2,2',4,5,5'-pentachlorobiphenyl from glass. The 40 ppm PCB solution was then diluted with distilled water to obtain a solution of 4 ppm PCBs and 0.1 g/l surfactant concentration. All solutions were then mixed with an inoculum of *Pseudomonas* LB400 in buffer and incubated under standard biodegradation conditions. The results are presented in Table 3.5. Solutions of surfactant diluted to 0.1 g/l (around the CMC) showed similar degradation patterns to the control with no surfactant, whereas those at 1 g/l (> CMC) showed inhibition of degradation. This indicates that for one surfactant, micellar solubilization of PCBs inhibits degradation, and that inhibition of degradation can be reversed by diluting the solution to near or below the CMC of the surfactant.

A summary of the main physical properties of the surfactants tested and their effects on PCB solubilization and transformation are presented in Table 3.6.

Table 3.3 Effect of 1 g/l aqueous surfactant solution on degradation of PCB congeners sorbed to glass by *Pseudomonas* LB400 after a 48 h incubation

Surfactant	% degradation of congener*			
	2,5,2′	2,4,2′4′	2,3,5,2′,5′	2,4,5,2′,4′,5′
Control (no surfactant)	100	92	81	27
Hostapur SAS 60	100	100	100	63
Nansa LSS38/AS	100	97	93	43
Bio-Soft EN 600	98	14	15	4
Genapol X-080	99	24	20	0
Marlipal O13/120	100	34	30	6
Igepal CO-630	100	40	26	17
Igepal CO-850	100	65	52	15
Sorbax PMO-20	100	35	36	12
Naxchem Dispersant K	100	33	31	15
Witcomul 3235	100	78	79	9
Pluronic 10R5	100	93	83	26
Surfynol 485	100	90	80	35

*Assuming full solubilization in the aqueous solution, the concentration of each congener would be 10 μM.

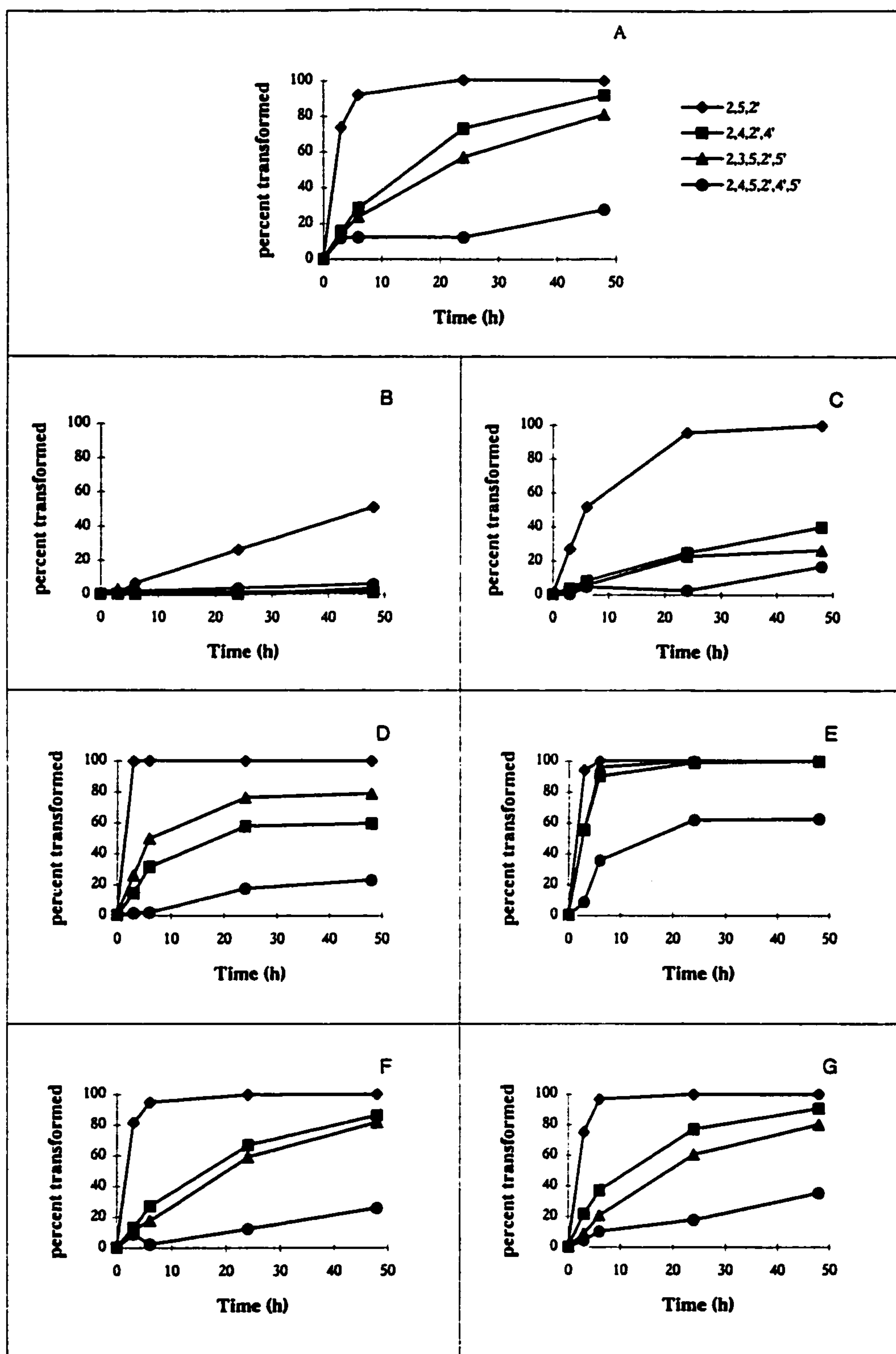


Figure 3.2 Time course of degradation of congeners in the presence or absence of selected surfactants.

A. No surfactant; B,C. Igepal CO-630 at 10, 1g/l; D,E. Hostapur at 10, 1g/l; F,G. Surfynol at 10, 1g/l.

Table 3.4 Rate constants for transformation of PCB congeners by resting cells of *Pseudomonas* LB400 in the presence and absence of surfactants

Surfactant Name	Surfactant concentration (g/l)	Rate constant for transformation of congener*			
		2,5,2′	2,4,2′4′	2,3,5,2′5′	2,4,5,2′,4′,5′
Control (no surfactant)	0	0.420	0.053	0.035	0.007
Hostapur SAS 60	10	>1.80	0.038	0.064	0.006
	1	1.300	0.360	0.480	0.042
Genapol X-080	10	ND	0.000	0.000	0.000
	1	0.120	ND	ND	0.000
Igepal CO-630	10	ND	0.000	0.000	0.000
	1	0.120	ND	ND	0.000
Naxchem Dispersant K	10	0.044	0.000	0.000	0.000
	1	0.290	ND	ND	0.000
Surfynol 485	10	0.500	0.042	0.035	ND
	1	0.540	0.052	0.034	ND

* units = h^{-1}

ND= not determined

Table 3.5 Effect of surfactant concentration on congener degradation

Initial concentration		Concentration for degradation		% 2,4,5,2′,5′-CBP degraded after time (h)	
Igepal CO-630 (g/l)	Congener (ppm)	Igepal CO-630 (g/l)	Congener (ppm)	24	48
0	4	0	4	97	99
1	40	0.1	4	89	99
1	4	1	4	33	58

Table 3.6 Summary of surfactant properties and effectiveness in PCB solubilization and biodegradation

Surfactant	Chemical class	Length of chain	Charge	HLB[a]	CMC[b] (g/l)	PCB solubilizing ability[c]	Surfactant toxicity to *Pseudomonas* LB400[d]	Surfactants used as carbon source for growth by *Pseudomonas* LB400[e]	Relative effect of surfactants on PCB transformation[f]
Hostapur SAS 60	secondary sodium alkane sulphonate	[g]Alk=12-15	anionic	NA[i]	0.42	1 (highest)	+,+	-	+2
Nansa LSS38/AS	sodium alkene sulphonate	Alk=12	anionic	NA	0.8	6	-	-	+1
Bio-Soft EN-600	alcohol ethoxylate	Alk=14 [h]EO=9	nonionic	12.2	0.086	1 (highest)	-	-	-5
Genapol X-080	alcohol ethoxylate	Alk=13 EO=8	nonionic	13	0.083	1 (highest)	-	-	-4
Marlipal O13/120	alcohol ethoxylate	Alk=13-15 EO=7	nonionic	14.5	0.11	4	-	-	-3
Igepal CO-630	alkylphenol ethoxylate	Alk=9 EO=9	nonionic	13	0.074	1 (highest)	-	-	-3
Igepal CO-850	alkylphenol ethoxylate	Alk=9 EO=20	nonionic	16	0.15	5	-	-	-2
Sorbax PMO-20	fatty acid ethoxylate	EO=20	nonionic	15	0.092	2	-	+	-3
Naxchem Dispersant K	blend- diethanolamide, alkylphenol ethoxylate		nonionic	NA	0.094	1 (highest)	+	-	-3
Witcomul 3235	mixture		anionic/nonionic blend	NA	0.42	3	-	+	-1
Pluronic 10R5	block copolymer	EO=30	nonionic	12-18	0.047	8	-	-	0
Surfynol 485	twin alcohol ethoxylate		nonionic	17	3.8	7	+	-	0
Control (none)						8			0

[a]HLB= Hydrophile-lipophile balance, data from supplier
[b]Calculated by drop method
[c]1-highest, 100% PCB solubilization at 1 g/l surfactant; 8-lowest, 0% solubilization at 10 g/l surfactant
[d]+,+ : toxic at 10 g/l surfactant, and at 1 g/l surfactant; + : toxic at 10 g/l surfactant, not at 1 g/l surfactant; - : non-toxic at 10 g/l surfactant
[e]Growth on surfactant: - none, + growth
[f]0-rate of transformation without surfactant; - values to + values: order of increasing rates of PCB transformation
[g]Number of carbons in alkyl chain; [h]Number of ethoxylate units
[i]Not available

3.5 Discussion

Solubilization and washing effects of surfactants.

The main findings of this study are summarized in Table 3.6. At surfactant concentrations above the CMC value, most surfactants promoted some solubilization of PCBs sorbed to glass into the aqueous phase. A notable exception was Pluronic 10R5, which has a low CMC value but did not solubilize PCBs. Although block copolymers form micelles (Piirma 1992), their characteristics may be different due to their large molecular structure, which could affect solubilization. Among the ethoxylate surfactants, those with the lower CMC values (0.083 to 0.086) exhibited better PCB-solubilizing ability than those with the higher CMC values (0.092 to 0.15). The better PCB-solubilizing ethoxylates also had lower hydrophile-lipophile balance (HLB) numbers, meaning they were more lipophilic, which leads to greater solubilization of hydrophobic compounds. In general, solubilization of contaminants by nonionic surfactants appears to increase at surfactant values greater than the CMC value (Edwards et al. 1991).

Surfactant toxicity.

All of the nonionic alcohol, alkylphenol and fatty acid ethoxylate surfactants, along with Nansa LSS38/AS, Witcomul 3235 and Pluronic 10R5, at supra-CMC concentrations, allowed growth of *Pseudomonas* LB400 on TSA over 5 days. Naxchem Dispersant K, Hostapur SAS 60 and Surfynol 485, at a concentration of 10 g/l, totally inhibited growth. Normal growth was observed on TSA plates containing 1 g/l Naxchem Dispersant K and Surfynol 485 but colony growth on TSA plates containing 1 g/l Hostapur SAS 60 was inhibited.

Anionic surfactants are known to be more toxic to bacteria than nonionic surfactants (Swisher 1987). Nansa LSS38/AS may not have exhibited the same toxicity as Hostapur SAS 60 because it only contains 38% active agent, vs. 60% for Hostapur. Volkering et al. (1995) observed that growth of pure *Pseudomonas* strains on succinate was not affected by the presence of up to 10 g/l alkylphenol ethoxylate surfactants. However, the nonionic surfactant Triton X-100 (an isooctyl phenol ethoxylate) which was very effective at desorbing PCB congeners from sand, at

a surfactant concentration of 5 g/l inhibited growth of *Pseudomonas* LB400 on biphenyl (Viney and Bewley 1990). Toxicity may be related to surfactant capacity to disrupt lipid and protein components in the membrane (Rouse et al. 1994). Nonionic ethoxylate surfactants with ethoxylate chain lengths of 9 to12 units did not allow growth of *Mycobacterium* species on acetate or PAHs, whereas those with ethoxylate chains of 15 to 30 permitted growth. In our experiments however, surfactants with ethoxylate chains of 7 to 9 were not toxic. The growth of *Escherichia coli* or a PAH-degrading mixed culture on glucose in the presence of 1 g/l nonionic ethoxylated surfactants was not inhibited, nor was mineralization of glucose by a PAH-degrading mixed culture (Liu et al. 1995).

Microbial growth on surfactants.

Sorbax PMO-20 and Witcomul 3235 supported growth of *Pseudomonas* LB400 when incorporated into media as sole carbon source. Sorbax PMO-20 is a fatty acid ethoxylate. *Pseudomonas* LB400 probably hydrolyses the internal ether linkage and utilizes the fatty acid by β-oxidation (Balson and Felix 1995). No insight into the mechanism of degradation of Witcomul 3235 is possible as its composition is not known. This bacterium does not appear to be able to use the alcohol or alkylphenol ethoxylates for growth, unlike some other bacteria (Balson and Felix 1995). Alkylphenols tend to be resistant to attack, often due to the branched hydrophobic chain and the presence of the phenol group (van Ginkel 1996).

In some cases, utilization of the surfactants by bacteria may promote degradation of contaminants. If a contaminant within a surfactant micelle is not bioavailable, contaminant biodegradation may be facilitated if the microorganisms first biodegrade the surfactant. Also, in a cometabolic system such as PCB degradation, use of the surfactant as a growth substrate will also increase bacterial cell numbers, which may result in greater PCB degradation. However the extent of degradation in the presence of Sorbax PMO-20 and Witcomul 3235 did not increase. Surfactants are seldom completely mineralized by a pure culture, however, but typically require

a mixed bacterial population (van Ginkel 1996). Such degradation is desirable to ensure that they do not persist in the environment.

Although *Pseudomonas* LB400 was not able to grow on TSA in the presence of Hostapur SAS 60, the presence of this surfactant did not actually kill the organism as cell numbers were maintained in mineral medium compared to the control with no surfactant over 48 h. This may be explained by the fact that organisms are more vulnerable to surfactants during active metabolism on glucose than when resting (Komor et al. 1979). This was also demonstrated by *Corynebacterium* sp. MB1, which maintained PCB-degradative capacity in the presence of Triton X-100 despite inhibition of growth on biphenyl (Viney and Bewley 1990). Therefore, Hostapur SAS 60 may be described as being bacteriostatic, rather than toxic.

Surfactant effects on contaminant degradation.

It was observed that, in general, anionic surfactants promoted whereas nonionic surfactants, at concentrations greater than the CMC value, inhibited degradation of PCBs by *Pseudomonas* LB400. Inhibition of degradation may be due to several factors. Surfactants may reduce the actual concentration of the contaminant substrate in the aqueous phase by concentrating the contaminant in micelles (Rouse et al. 1994). The exit rates of PAHs from micelles are supposed to be very fast, as micelles are continually breaking and forming (Tiehm 1994; Laha et al. 1995). Nevertheless, PAH substrates in nonionic surfactant micelles have previously been shown to be not bioavailable (Volkering et al. 1995). Some degradation of the less chlorinated congeners continued, perhaps because they are more water-soluble than the higher chlorinated ones which may be more tightly held inside the micelles, and thus less bioavailable. Degradation of PCBs by *Pseudomonas* LB400 was inhibited in the presence of the nonionic alkylphenol ethoxylate Triton X-100 (Viney and Bewley 1990).

Inhibition of contaminant transformation by nonionic surfactants has also been hypothesized to be due to micellar toxicity to bacteria, or preferential use of surfactant as growth substrate (Laha and Luthy 1991). However, we have shown that cell numbers are maintained in

the presence of micelles, and as PCBs are transformed by cometabolism, the presence of alternate carbon sources likely will not affect PCB utilization.

Anionic surfactants may promote increased degradation as they resist aggregation due to repulsion of charges, which may make the micelles more unstable and allow access of the PCBs to bacteria. Also, PCBs have lower affinity for the interior of anionic than for nonionic micelles (Kile and Chiou 1989), which may allow them to be more available to bacteria.

The results indicate both the positive and negative effects of surfactants as potential agents for environmental remediation. While the anionic surfactant, Hostapur SAS 60 manifested high PCB-solubilizing activity and promoted PCB degradation by resting cells of *Pseudomonas* LB400, it was inhibitory to growth of this organism. The other anionic surfactant showed poorer PCB-solubilizing activity. Selected nonionic surfactants, at a concentration greater than their CMC values, can be exploited to solubilize PCBs. However, at surfactant concentrations effective for PCB solubilization, degradation of PCBs is inhibited. Our results also show that if the surfactant-PCB extract is diluted to bring the surfactant concentration near or below the CMC value, the PCBs are more readily biodegraded. This was also found to occur for solubilized PAHs (Laha and Luthy 1991; Laha et al. 1995). This suggests that it is the presence of micelles that is inhibiting degradation and not the surfactant itself. Thus a practical approach to soil PCB bioremediation is to utilize nonionic surfactants to solubilize PCBs in a soil washing step followed by dilution of the resulting extract to promote PCB biodegradation in the aqueous phase.

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CHAPTER 4

Studies on the effectiveness of chemical surfactants in washing PCBs from weathered contaminated soil and on subsequent biodegradation of contaminants in the wash by *Pseudomonas* LB400[1]

4.1 Abstract

Eleven commercial nonionic or anionic surfactants were tested for their abilities to wash polychlorinated biphenyls (PCBs) from weathered contaminated soil. While none was effective at solubilizing the PCBs at a surfactant concentration of 1 g/l, six surfactants, at a concentration of 10 g/l, removed greater than 75% of the hexane-extractable PCBs from the soil. The most effective surfactants in soil washing tests were the nonionic alcohol ethoxylate, Bio-Soft EN-600, and the alkylphenol ethoxylate, Igepal CO-630. The PCB congeners in the soil washings were then transformed by resting cells of *Pseudomonas* LB400, previously grown on biphenyl. Solutions containing the latter efficient soil washing surfactants and the bacterium manifested low rates of PCB biodegradation, with only 16 or 32% of congeners transformed over a 48-h incubation period. Conversely, in PCB-washing solutions of two anionic surfactants, Hostapur SAS 60 and Nansa LSS38/AS, the bacterium exhibited highest rates and extents of PCB degradation (52-67% of congeners transformed over a 48-h incubation period), although these surfactants were less efficient in the soil washing process.

The results suggest that washing of contaminants from soil can be achieved by micellar solubilization using nonionic surfactants at concentrations above their critical micelle concentration (CMC), but that the presence of the micelles retards the transformation of the contaminants. Such nonionic surfactants may be used in a combined soil wash/waste water treatment train provided the washings are diluted to bring the surfactant concentration below the CMC value which dissociates the micelles and makes the PCBs more bioavailable.

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4.2 Introduction

Although hydrophobic contaminants can often be metabolized by microorganisms, they are not readily bioavailable because of low aqueous solubility or their tendency to absorb tightly to soil. Surfactants have the potential to induce micellar solubilization of hydrophobic substrates (Bury and Miller 1993), thereby increasing their apparent solubility in the aqueous phase. Use of surfactants in this manner also facilitates removal of the contaminants by aqueous soil washing, which is an attractive option if there is a requirement to quickly remediate the soil. A soil washing approach offers potential to use an aqueous phase reactor as a biodegradation system to treat the contaminants removed in the soil washes.

Despite the demonstrated capacity of surfactants to desorb hydrophobic contaminants from soil into an aqueous phase, use of surfactants in bioremediation systems has met with mixed success. Surfactants may promote or inhibit biodegradation of PAHs (Liu et al. 1995; Thibault et al. 1996). Inhibition of contaminant degradation has often been observed at surfactant concentrations greater than the critical micelle concentration (CMC), although these surfactant concentrations are required for contaminant solubilization (Laha and Luthy 1991).

Polychlorinated biphenyls (PCBs), for years incorporated into capacitors and transformers, have been found to persist in the environment because of their hydrophobic nature and chemical stability. Some studies with surfactants and biosurfactants have shown that these compounds can promote enhanced removal of PCBs from soil (Abdul and Gibson 1991; van Dyke et al. 1993), but further treatment is required to dispose of the PCBs in the resulting solution.

Pseudomonas LB400 manifests a relatively broad specificity towards a range of PCBs and has been shown to transform the PCB congeners containing up to 6 chlorine atoms. PCBs are typically biodegraded by cometabolism and, in the case of *Pseudomonas* LB400, the highest rate of PCB degradation is achieved with cells previously grown on biphenyl (Billingsley et al. 1997a,b). Consequently, use of a reactor-based system inoculated with biphenyl-grown cells of *Pseudomonas* LB400, represents a practical option for bioremediation of PCBs in aqueous-surfactant soil washings. The effectiveness of a variety of chemical surfactants in solubilizing four

selected PCB congeners adsorbed to glass and in promoting their biodegradation has previously been examined (Billingsley 1997). In this paper, experiments are described on the use of the same surfactants to wash weathered soils contaminated with a mixture of Aroclors, together with tests on the ability of *Pseudomonas* LB400 to degrade the PCBs in the aqueous surfactant washings.

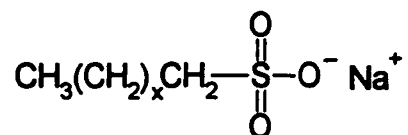
4.3 Materials and Methods

Chemicals:

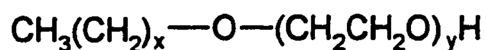
PCB congeners in neat form were obtained from Accustandard Ltd. (New Haven, CT). Purity is 99+%; no biphenyl peak was detected. Stock solutions of individual congeners were prepared in acetone. Biphenyl was obtained from Sigma Chemical Co. (St. Louis, MO).

Surfactants were selected to represent common chemical classes of commercial surfactants such as the nonionic alcohol ethoxylates, alkylphenol ethoxylates, fatty acid ethoxylates, block copolymers and the anionic sulphonates, as well as some other types that were specific for hydrocarbon solubilization. Their general structures are illustrated in Figure 4.1. Cationic surfactants were avoided due to potential toxicity to microorganisms (Swisher 1987). Surfactant samples were supplied by their respective manufacturers. These were: Bio-Soft EN-600 (Stepan, Missisauga, Canada), Genapol X-080, Hostapur SAS 60 (Hoechst Celanese, Charlotte, NC) Igepal CO-630, Igepal CO-850 (Rhone-Poulenc, Paris, France), Marlipal O13/120 (Huls, Marl, Germany), Naxchem Dispersant K (Ruetgers-Nease, State College, PA), Nansa LSS38/AS (Albright and Wilson, Richmond, VA), Pluronic 10R5 (BASF, Ludwigshafen, Germany), Sorbax PMO-20 (Chemax, Greenville, SC), Surfynol 485 (Air Products, Allentown, PA), Witcomul 3235 (Witco, New York, NY). The surfactants were used as obtained without further purification. All have 99-100% active agent except for Hostapur (60%) and Nansa (38%). Stock solutions of the commercial products (10% w/v) were made in distilled water and sterilized. Further details describing the surfactants are included in Table 4.1.

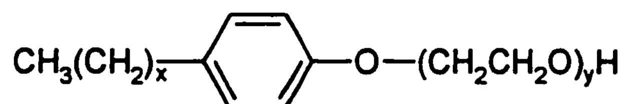
A.



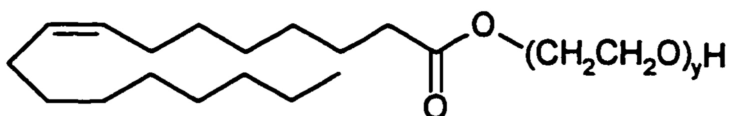
B.



C.



D.



E. Block copolymers are alternating blocks of identical monomer units. Usually one of the monomers is a hydrophobic molecule and the other is hydrophilic. They form very long polymer chains and have high molecular weights.

Figure 4.1 General structures of surfactants.
A. Alkane sulphonate, B. Alcohol ethoxylate, C. Alkylphenol ethoxylate, D. Fatty acid ethoxylate, E. Block copolymer

Table 4.1 Properties of Surfactants

Surfactant	Chemical Class	Length of chain	Charge	HLB[a]	CMC[b] (g/l)
Hostapur SAS 60	secondary sodium alkane sulphonate	Alk=12-15	anionic	NA	0.42
Nansa LSS 38/AS	sodium alkene sulphonate	Alk=12	anionic	NA	0.8
Bio-Soft EN 600	alcohol ethoxylate	Alk=14 EO=9	nonionic	12.2	0.086
Genapol X-080	alcohol ethoxylate	Alk=13 EO=8	nonionic	13	0.083
Marlipal O13/120	alcohol ethoxylate	Alk=13-15 EO=7	nonionic	14.5	0.11
Igepal CO-630	alkyl phenol ethoxylate	Alk=9 EO=9	nonionic	13	0.074
Igepal CO-850	alkyl phenol ethoxylate	Alk=9 EO=20	nonionic	16	0.150
Sorbax PMO-20	fatty acid ethoxylate	EO=20	nonionic	15	0.092
Naxchem Dispersant K	Blend - diethanolamide, alkyl phenol ethoxylate		unknown	NA	0.094
Witcomul 3235	mixture		anionic/nonionic blend	NA	0.42
Pluronic 10R5	block copolymer		nonionic	12-18	0.047
Surfynol 485	twin alcohol ethoxylate	EO=30	nonionic	17	3.8

a= Hydrophile-lipophile balance, data from supplier
b = calculated by drop method (Billingsley 1997)

Soil:

Soil obtained from a PCB-contaminated site near London, Ontario, was air dried and passed through a 2-mm sieve. It had a moisture content of 0.8% and an organic fraction of 1.5%. The soil was classified as Sandy Loam, and was composed of 6% clay, 38% silt and 56% sand. The soil contained approximately 15 ppm of a mixture of Aroclors 1242 and 1260. The soil was also spiked with a mixture of 5 congeners: 2,2',5-trichlorobiphenyl, 2,2',4,4'-tetrachlorobiphenyl, 2,2',3,4,5'-pentachlorobiphenyl, 2,2',4,4',5,5'-hexachlorobiphenyl, and 2,2',4,4',6-pentachlorobiphenyl, dissolved in 50 ml of acetone and poured over 150 g soil in a glass beaker to achieve a final concentration for each congener of approximately 8 μg/g soil. The soil was mixed with a glass rod, and the acetone was left to evaporate. The spiking was carried out to observe any differences in extraction efficiency of spiked versus weathered PCBs.

Bacterial Strain:

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, NY) was obtained from the Northern Regional Research Laboratory (Peoria, IL). Cultures were maintained in 50 ml of the standard growth medium in 250-ml Erlenmeyer flasks by storage at 4°C, with monthly subculturing by incubation on an orbital shaker at 200 rpm at 30°C for 24 h. Cultures were streaked onto tryptic soy agar (TSA) at regular intervals to ensure purity. For long-term storage of cultures, 20% glycerol was added to cell suspensions in the standard growth medium and the mixture was distributed in 1-ml aliquots in 1.5-ml tubes and stored at -20°C.

Standard growth medium:

The growth medium used was the phosphate-buffered mineral salts medium supplemented with yeast extract described by Bedard et al. (1986). This medium contained per litre: 4.4 g K_2HPO_4, 1.7 g KH_2PO_4, 2.1 g NH_4Cl, 50 mg of yeast extract (Becton Dickinson, Cockeysville, MD), 2 g biphenyl, and 10 ml of concentrated basal salts solution. Basal salts were added after

autoclaving. Biphenyl was prepared in a 0.1 g/ml solution in hexane, filter sterilized using a 0.2 μm PTFE syringe filter (Nalge, Rochester, NY) and added to the autoclaved medium. The hexane was allowed to evaporate, leaving crystals suspended in the medium.

The concentrated basal salts solution contained per litre: 19.5 g $MgSO_4$, 5 g $MnSO_4.H_2O$, 1 g $FeSO_4.7H_2O$, and 0.3 g $CaCl_2.2H_2O$, plus enough H_2SO_4 to prevent precipitation of basic salts. Culture medium (50 ml) was prepared in 250-ml Erlenmeyer flasks, and stoppered with foam plugs.

Inoculum Preparation:

Cultures stored at 4°C were incubated on an orbital shaker (Labline Instruments Inc., Melrose Park, IL) at 200 rpm at room temperature until warm. Cells were inoculated into 50 ml of fresh growth medium in a 250 ml-Erlenmeyer flask to an OD of 0.1 measured at 615 nm in a 1-cm light path. Unless otherwise stated, cultures were incubated at 30°C on an orbital shaker set at 200 rpm for 22 to 24 h. Cultures were filtered through sterile glass wool to remove excess biphenyl crystals. The cell suspension was then centrifuged at 5000 *g* for 15 min, washed twice with 0.1 M sodium phosphate buffer, pH 7.5, and resuspended in the buffer to an OD of 2.0 measured at 615 nm, using a 1-cm light path.

Soil washing experiment:

Five grams of spiked soil was weighed into 50-ml hypovials. A 10 g/l surfactant solution (20 ml) was added and incubated on an orbital shaker at room temperature at 200 rpm for 24 h. Samples were left to settle, so that as much as possible of the liquid layer could be removed and transferred to a glass centrifuge tube. These were centrifuged at 12000 *g* for 10 min. The liquid was removed and transferred to a pre-weighed hypovial to determine recovery.

Degradation of PCBs in washing solutions:

A 0.5-ml sample of soil washing solution was added per 8 ml glass vial, along with 0.5 ml

of cell suspension. Controls were killed by addition of 1 drop of 70% perchloric acid. Vials were closed with Teflon-lined caps and incubated at 30°C on an orbital shaker set at 200 rpm. Following incubation, reactions were stopped by addition of 1 drop of 70% perchloric acid.

PCB Extraction:

From soil washings: A 5-g sample of liquid was transferred to a 50-ml hypovial, and after addition of 20 ml of hexane, was shaken horizontally for 20 min at 300 rpm. A 1-ml aliquot of the hexane layer was removed for analysis by GC/ECD. The remaining wash solution was stored at 4°C for the degradation experiments.

From soil: The extraction solvent, consisting of 25 ml of a mixture of acetone:hexane (2:3) was added to the soil pellets in the glass centrifuge tubes, and the resulting slurry was transferred back to the original hypovial. Samples were sonicated for 30 min and the soil allowed to settle. The liquid layer was removed and transferred to another hypovial where it was re-extracted with 8 ml of water for 15 min at 300 rpm. The hexane layer was removed and filtered through sodium sulfate. The soil extraction was repeated two more times with 15 min of sonication each time. The combined hexane layers were evaporated with 5 ml of pentane added, to a final volume of approximately 5 ml. Evaporation was continued under nitrogen to a final volume of 1.5 ml. The concentrate was added to a florisil column and eluted with pentane. A 1-ml sample was removed for analysis by GC/ECD. The amount extracted using this method was compared to a Soxhlet-extracted sample and were found to be very similar.

Analysis:

Identification and quantitative determination of the PCB congeners were based on analysis utilizing a gas chromatograph (Model HP 5890, Hewlett-Packard, Meyrin, Switzerland) equipped with an electron-capture detector (ECD). The column used was a high performance capillary column (HP-5, length 30 m, internal diameter 0.25 mm, film thickness 0.25 µm, Hewlett Packard). The sample (2 µl) was injected in splitless mode with a time delay of 1.0 min after injection. The

initial oven temperature of 55°C was held for 2 min and then programmed to increase by 10°C /min to 90°C; 2.5°C /min to 285°C, and held for a further 10 min. The injector temperature was 250°C and the Ni^{63} EC detector was maintained at 350°C. The carrier gas was hydrogen with a column head pressure of 13 psi and with nitrogen as the make-up gas. The gas flow was maintained at a rate of 30 ml/min with minor adjustment to maximize response, as necessary.

A PCB standard was prepared using purchased Aroclor solutions 1016, 1221, 1242, 1254, and 1262 (Supelco, Bellefonte, PA, 4-8701;4-8705;4-8706;4-8707;4-4810), reconstituted in hexane at a ratio of 1:1:1:1:1, to a concentration of ~ 2.9 mg/ml and fortified with IUPAC congeners 86 and 209. The quantity of each PCB constituent in the standard was determined using 209 individual PCB standards purchased from Accustandard (New Haven, CT).

Analytical response factors, chromatographic separation and congener confirmation for the standard were determined using six different chromatographic phases.

Experimentation and analysis:

The reported degradation percentages were calculated by normalization of each peak to the recovery of a non-degradable internal standard (2,2',4,4',6-CBP), and comparison to the killed control. Preliminary experiments showed that normalization to the internal standard accounted for variations in extraction. Duplicate samples within experiments showed very similar results, with variations between samples averaging 5%.

4.4 Results

The surfactants were tested for their abilities to remove PCBs from soil previously contaminated with PCBs (weathered soil), spiked with five additional congeners to a final concentration of each congener of 8 ppm. When this soil (5 g) was washed with aqueous solutions (20 ml) containing 1 g/l of surfactant, no removal of PCBs was achieved (data not shown). In similar washings with aqueous solutions containing 10 g/l surfactant, PCB contaminants were successfully released from the soil. The percentages of solvent-extractable

PCBs washed off the soil, together with the percentages of solvent-extractable PCBs remaining in the soil following washing are illustrated in Figure 4.2. Six of the surfactants removed greater than 75% of the solvent-extractable PCBs from the soil. They included the alcohol ethoxylates, Bio-Soft EN-600 and Marlipal O13/120, the alkylphenol ethoxylates Igepal CO-630 and Igepal CO-850, the fatty acid ethoyxlate Sorbax PMO-20 and the alkylphenol ethoxylate/diethanolamide blend, Naxchem Dispersant K. The alcohol ethoxylate Genapol X-080 and the anionic sulphonate surfactants, Hostapur SAS 60 and Nansa LSS38/AS manifested slightly lower efficiency, while Witcomul 3235 removed 35% of the PCBs. The twin alcohol ethoxylate, Surfynol 485, the block copolymer, Pluronic 10R5, and water, were unable to remove any PCBs from soil. Analysis indicated that the soil accounted for the rest of the PCBs in most cases. Mass balances varied somewhat, however they were being compared to the solvent-extractable PCBs and solvent extraction efficiency from soil may be affected by other variables including surfactant type. The results for Bio-Soft EN-600 were over 100% due to extra peaks on the baseline of the chromatogram which added to the total area count. It is possible that compounds other than PCBs that co-elute with the PCBs on the GC were desorbed by Bio-Soft EN-600. Alternatively, this surfactant may increase the efficiency of the solvent extraction.

An analysis of the individual PCB congeners in the soil before and after soil washing with some of the most interesting surfactants is presented in Table 4.2. We have combined data for 31 congeners which exhibited contaminant levels of less than 50 ppb per congener in the prewashed soil under "others". The table also highlights the five spiked congeners, numbers 18, 47, 87, 100 and 153. We examined the relative efficiencies of the selected surfactants to remove spiked congeners versus the total of the other native congeners originally present in the weathered contaminated soil. Table 4.3 shows the percentages of the remaining congeners in the soil (spiked ones and the total of the native congeners) following surfactant soil washing.

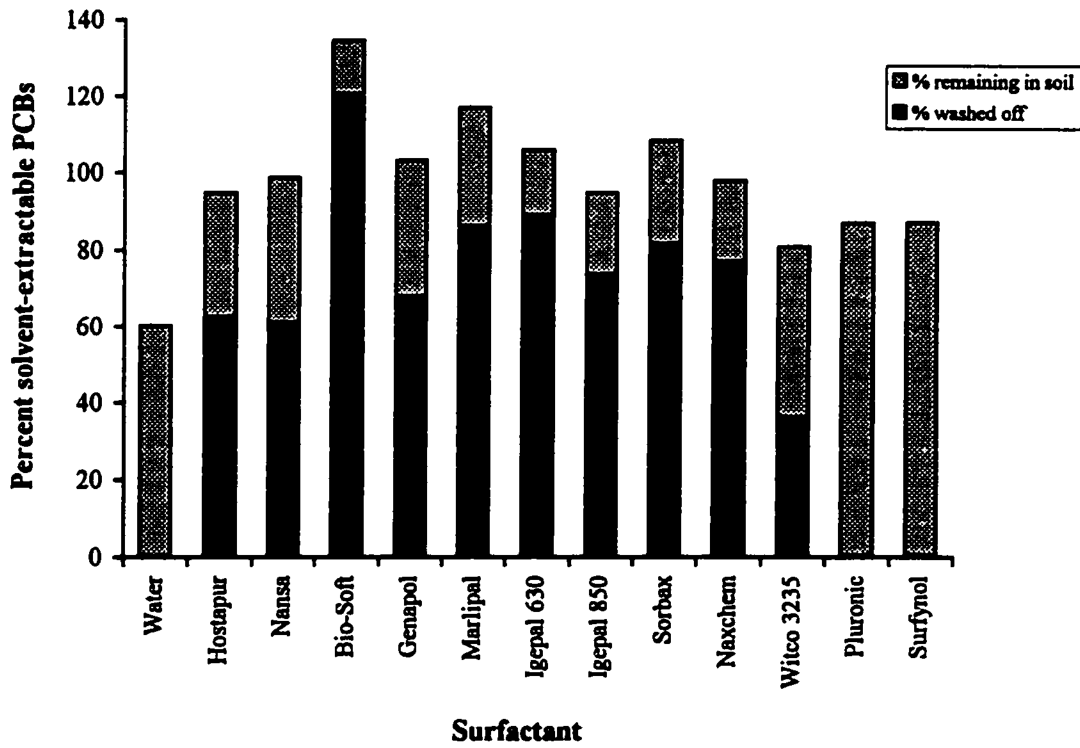


Figure 4.2 Efficiency of 10 g/l surfactant solutions to solubilize total PCBs from soil.

Table 4.2 PCBs remaining in soil after washing with 10 g/l surfactant solutions

Congener	Congener chlorination pattern	Starting spiked soil	Amount remaining in soil (ppb) after washing with surfactant				
			Bio-Soft	Igepal 630	Sorbax	Hostapur	Nansa
1	2	125	11	0	14	84	56
3	4	71	10	0	0	28	26
4, 10	2,2' 2,6	1122	120	91	237	160	134
9, 7	2,5 2,4	62	8	6	11	60	64
6	2,3'	129	17	14	28	51	57
8, 5	2,4' 2,3	439	63	53	97	139	164
19	2,6,2'	827	86	81	182	80	74
18[a], 17, 15	2,5,2' 2,4,2' 4,4'	7967	1094	1107	1974	1912	2306
24, 27	2,3,6 2,6,3'	666	80	82	155	155	179
16, 32	2,3,2' 2,6,4'	1565	196	207	384	356	403
26	2,5,3'	525	75	81	127	336	437
25	2,4,3'	336	46	48	76	185	244
31, 28	2,5,4' 2,4,4'	1833	298	315	458	1018	1316
33, 53, 20	3,4,2' 2,5,2',6' 2,3,3'	318	0	0	0	31	41
22, 51	2,3,4' 2,4,2',6'	229	31	33	55	191	212
45	2,3,6,2'	200	24	27	48	29	34
46	2,3,2',6'	73	8	10	18	8	9
52	2,5,2',5'	730	117	136	206	428	542
49, 43	2,4,2',5' 2,3,5,2'	858	131	155	239	442	566
48, 47[a]	2,4,5,2' 2,4,2',4'	8049	1222	1491	2303	2627	2850
44	2,3,2',5'	501	72	84	136	201	247
42, 59	2,3,2',4' 2,3,6,3'	572	82	95	147	135	164
64, 41, 71	2,3,6,4' 2,3,4,2' 2,6,3',4'	974	129	156	258	273	317
40	2,3,2',3'	149	10	13	41	2	1
100[a], 67	2,4,6,2',4' 2,4,5,3'	4111	599	778	1167	1578	1707
74	2,4,5,4'	316	51	61	83	204	273
70, 76, 98	2,5,3',4' 3,4,5,2' 2,4,6,2',3'	490	78	93	131	288	379
91, 55	2,3,6,2',4' 2,3,4,3'	318	42	53	87	118	150
56, 60	2,3,3',4' 2,3,4,4'	227	32	38	59	38	45
92	2,3,5,2',5'	139	22	27	40	115	150
84	2,3,6,2',3'	162	22	27	47	0	0
101	2,4,5,2',5'	376	60	76	109	228	302
99	2,4,5,2',4'	273	41	54	77	164	225
83	2,3,5,2',3'	382	57	75	117	195	350
97	2,4,5,2',3'	155	23	29	43	61	83
87[a], 81	2,3,4,2',5' 3,4,5,4'	6489	1164	1521	2331	2081	2244
85	2,3,4,2',4'	99	16	20	30	48	60
136	2,3,6,2',3',6'	62	10	12	19	33	43
110, 77	2,3,6,3',4' 3,4,3',4'	583	80	103	166	166	211
82	2,3,4,2',3'	55	6	8	16	3	4
151	2,3,5,6,2',5'	82	13	17	25	45	61
135, 144, 147	2,3,5,2',3',6' 2,3,4,6,2',5' 2,3,5,6,2',4'	112	19	24	36	67	89
149, 118	2,3,6,2',4',5' 2,4,5,3',4'	252	38	49	75	133	172
131, 134	2,3,4,6,2',3' 2,3,5,6,2',3'	55	0	8	12	20	28
146	2,3,5,2',4',5'	89	13	18	26	52	73
153[a], 132, 105	2,4,5,2',4',5' 2,3,4,2',3',6' 2,3,4,3',4'	8343	938	1327	1860	1928	2082
176, 130, 137	2,3,4,6,2',3',6' 2,3,4,2',3',5' 2,3,4,5,2',4'	296	3	4	5	6	7
138, 163	2,3,4,5,2',4' 2,3,5,6,3',4'	369	55	74	109	157	213
187, 182	2,3,5,6,2',4',5' 2,3,4,5,2',4',6'	135	20	29	41	73	98
128	2,3,4,2',3',4'	51	7	10	16	6	7
174	2,3,4,5,2',3',6'	72	11	15	23	35	48
177	2,3,5,6,2',3',4'	54	8	11	17	14	19
171, 202, 156	2,3,4,6,2',3',4' 2,3,5,6,2',3',5',6' 2,3,4,5,3',4'	78	12	17	25	20	22
180	2,3,4,5,2',4',5'	154	22	33	47	74	104
170, 190	2,3,4,5,2',3',4' 2,3,4,5,6,3',4'	88	13	19	28	21	30
201	2,3,4,5,2',3',5',6'	50	7	11	17	23	31
203, 196	2,3,4,5,6,2',4',5' 2,3,4,5,6,2',4',6'	64	9	14	21	28	38
others[b]		639	99	134	205	277	389

a - congeners that were spiked in soil

b - includes congeners 12, 13, 54, 29, 63, 119, 107, 133, 114, 141, 179, 158, 178, 129, 175, 183, 185, 172, 197, 191, 199, 198, 167, 189, 195, 208, 207, 194, 205, 206, 209

Table 4.3 Percentage of native congeners and spiked congeners remaining in soil after soil washing

Congener number	Surfactant used				
	Bio-Soft	Igepal 630	Sorbax	Hostapur	Nansa
	Percentage of congeners left in soil after washing				
Native congeners					
total	13	15	25	38	48
Spiked congeners					
18	13	14	25	34	41
47	15	19	29	33	35
87	18	23	36	32	35
100	15	19	28	38	42
153	11	16	22	23	25
average	14	18	28	32	36

We found no difference in extraction efficiency except with the anionic surfactants Hostapur SAS 60 and Nansa LSS38/AS, which removed more of the spiked congeners than the native ones. We also examined the relative efficiency of an individual surfactant to extract different congeners from the soil. There was a much greater variation in extraction efficiency of individual congeners by Hostapur SAS 60 and Nansa LSS38/AS than for the nonionic surfactants. However, there were no common chlorination patterns among the congeners that were more efficiently extracted. Among the nonionic surfactants, variations in extraction efficiency were small.

The capacity of *Pseudomonas* LB400 to degrade PCBs in the surfactant solutions from the soil washing experiment was also investigated. The washings were incubated at 30°C with a resting cell suspension of *Pseudomonas* LB400, previously grown in medium containing biphenyl, using standard degradation conditions. The starting PCB concentrations varied, due to the differences in efficiency of the different surfactants for PCB removal from soil. The total amount of PCBs degraded in a 48 h incubation are presented in Table 4.4. PCBs solubilized in Nansa LSS38/AS were degraded the most over a 48-h time period, with Hostapur SAS 60 and Sorbax PMO-20 also allowing good degradation. In solutions of other surfactants, concentrations of PCBs ranging from 1334 to 2063 ppb were degraded, except for Naxchem Dispersant K and Witcomul 3235 where the amount of PCBs degraded was less than 1000 ppb. Table 4.4 also indicates the percentages of PCBs in the soil washings which were transformed. By combining data on the percentage of PCBs washed from soil and the percentage transformed in the washing, the percentage of native soil PCBs transformed was also calculated to evaluate the overall efficiency of the washing and biodegradation steps. The most effective surfactant overall appeared to be Nansa LSS38/AS, followed by Hostapur SAS 60 and Sorbax PMO-20.

Table 4.4 Degradation of total PCBs in washings from soils extracted with aqueous surfactant solutions using *Pseudomonas* LB400

Surfactant	Starting concentration (ppb)	Amount degraded (ppb) in time (h)				Percentage degradation at 48 h	
		6	12	24	48	of PCBs in wash	of PCBs in original soil
Hostapur SAS 60	4242	1172	1406	2019	2235	52	33
Nansa LSS38/AS	4139	1799	2086	2737	2775	67	41
Bio-Soft EN-600	8176	266	306	1128	1334	16	20
Genapol X-080	4601	396	612	1342	1733	38	26
Marlipal O13/120	5813	337	548	1350	1720	30	25
Igepal CO-630	6030	892	1322	1624	2063	32	30
Igepal CO-850	5000	936	1253	1471	1652	33	24
Sorbax PMO-20	5538	910	1216	2028	2285	41	34
Naxchem Dispersant K	5220	312	609	593	814	16	12
Witcomul 3235	2456	226	438	754	921	38	14

The concentrations of individual congeners in the washes from five of the surfactants before and after a 48 h incubation with *Pseudomonas* LB400 are presented in Table 4.5. Despite residual soil results which show complete removal of the lowest chlorinated congeners (congener numbers 1, 3, 9, 7), these congeners do not always appear in the soil washing results. This may be due to physical losses of these more volatile PCBs. As expected from previous experiments (Billingsley et al. 1997a), the lower chlorinated congeners get degraded to the greatest extent, with very little if any degradation of congeners with more than six chlorines.

Table 4.5 Analysis of PCB concentrations in soil washing after incubation with *Pseudomonas* LB400

Congener	Concentration at 0 and 48 hours of incubation of PCBs in soil washing (ppb) using surfactant									
	Bio-Soft		Igepal 630		Sorbax		Hostapur		Nansa	
	0	48	0	48	0	48	0	48	0	48
1	0	0	0	0	0	0	22	0	38	9
3	0	0	0	0	0	0	0	0	0	0
4, 10	322	119	142	17	117	22	139	34	141	22
9, 7	12	0	0	0	4	0	8	0	8	0
6	79	0	11	0	10	0	7	0	9	0
8, 5	77	0	43	0	38	0	26	0	30	0
19	175	166	108	76	95	81	27	37	63	20
18[a],17,15	1389	761	1160	153	1037	76	708	38	680	34
24, 27	142	101	71	29	62	29	39	0	48	0
16, 32	204	200	175	132	156	134	97	57	113	49
26	69	39	55	10	48	5	33	2	37	0
25	51	34	32	0	27	0	17	0	20	0
31, 28	217	174	178	78	159	77	102	20	114	8
33, 53, 20	0	33	38	0	0	0	4	0	5	0
22, 51	33	29	20	0	18	0	39	28	42	29
45	34	33	22	16	21	15	10	8	12	7
46	0	0	0	0	0	0	4	1	5	0
52	132	102	97	57	84	43	56	1	63	1
49, 43	137	108	107	56	97	34	60	1	67	0
48, 47[a]	1318	1323	1150	815	1054	752	645	283	739	200
44	90	63	60	24	53	7	32	3	38	0
42, 59	97	88	60	36	54	31	29	6	34	6
64, 41, 71	203	253	107	81	95	81	60	31	68	29
40	35	33	17	0	16	0	6	0	7	1
100[a], 67	339	351	295	300	548	510	446	436	491	448
74	54	55	32	28	29	28	20	15	22	14
70, 76, 98	72	64	52	28	47	23	29	0	31	0
91, 55	58	60	37	31	32	29	19	16	21	17
56, 60	117	109	22	17	20	18	11	6	12	6
92	71	66	17	13	15	12	10	3	10	3
84	53	46	20	14	17	13	9	4	10	4
101	76	62	48	36	42	40	29	12	30	11
99	58	30	32	25	28	26	19	12	20	11
83	66	47	37	32	72	31	24	20	27	17
97	29	20	17	14	52	14	9	6	10	6
87[a], 81	1074	1085	953	784	980	756	620	232	678	207
85	16	14	11	9	19	10	6	10	0	10
136	9	7	7	6	13	6	6	6	4	7
110, 77	74	73	63	52	61	52	38	27	40	27
82	19	19	6	5	5	5	3	2	3	2
151	19	18	9	8	8	7	6	4	6	4
135, 144, 147	22	22	13	11	11	11	18	13	18	15
149, 118	38	39	32	27	28	27	19	17	21	18
131, 134	0	0	0	0	0	0	0	0	0	0
146	67	70	10	9	8	8	7	5	7	5
153[a], 132, 105	1016	1052	837	942	1022	936	615	511	615	485
176, 130, 137	0	0	0	0	0	0	1	0	0	0
138, 163	39	41	37	30	32	30	22	18	23	17
187, 182	39	40	15	13	14	12	11	9	10	8
128	14	12	6	5	4	3	3	2	3	3
174	7	12	8	7	7	7	5	4	4	0
177	7	12	5	5	5	5	4	3	3	3
171, 202, 156	0	0	0	0	0	0	2	2	1	1
180	25	64	21	14	11	12	10	8	8	7
170, 190	27	29	16	14	7	7	20	11	8	11
201	35	37	11	9	0	0	3	3	3	3
203, 196	23	24	10	8	0	0	4	3	3	3
others[b]	475	547	115	81	150	76	19	7	21	8

a - congeners that were spiked in soil

b - includes congeners 12, 13, 54, 29, 63, 119, 107, 133, 114, 141, 179, 158, 178, 129, 175, 183, 185, 172, 197, 191, 199, 198, 167, 189, 195, 208, 207, 194, 205, 206, 209

4.5 Discussion

Surfactant solutions at concentrations of 1 g/l were unsuccessful at solubilizing complex mixtures of PCBs in this soil. In contrast, I have previously shown that with many surfactants, concentrations above the CMC value are effective in desorption and solubilization of selected PCB congeners from glass (Billingsley 1997). As 1 g/l is above the CMC for all of the surfactants except for Surfynol 485, there may have been a decrease in surfactant concentration to below the CMC probably due to some sorption of surfactants to soil. Laha and Luthy (1991) also noticed this phenomenon for solubilization of PAHs from soil. Also, PCBs may interact more strongly with soil as compared to glass and thus may be more difficult to solubilize. Interaction of PCBs and glass is only a surface phenomenon, whereas with soil, PCBs can enter the porous organic matrix and associate with hydrophobic compounds such as humic acids (Paya-Perez et al. 1991).

At a concentration of 10 g/l, six of the eleven surfactants tested removed greater than 75% of hexane-extractable PCBs from the soil. A lower CMC did not necessarily mean greater solubilization of PCBs. The surfactant with the lowest CMC, Pluronic 10R5 (a block copolymer), did not solubilize any PCBs. Although block copolymers form micelles (Piirma 1992), their characteristics may be different due to their large molecular structure, which could affect solubilization. The best PCB-solubilizing ethoxylate surfactant, Bio-Soft EN-600, had the lowest hydrophile-lipophile balance (HLB) number, meaning it was more lipophilic, which can lead to greater solubilization of hydrophobic compounds. PCBs have previously been shown to be effectively washed from sand using 10 g/l surfactant solutions of alcohol ethoxylates (Abdul and Gibson 1991; Viney and Bewley 1990).

Factors which may affect the capacity of surfactants to wash contaminants from soil are the organic, ionic, and clay contents of the soil (Jafvert and Heath 1991). Surfactants have been found to bind to organics or clays, thereby decreasing the concentration of micelles and thus the extent of solubilization of the contaminants (Brownawell et al. 1997). The organic content of this soil was 1.5%, with 4620 ppm of Ca^{2+}, and 273 ppm of Mg^{2+}. Cations, especially calcium, can cause precipitation of anionic surfactants, likewise decreasing effective surfactant concentration.

The dissolved concentrations of the anionic surfactants Hostapur SAS 60 and Nansa LSS38/AS, and the anionic/nonionic blend Witcomul surfactant may have been affected by the very high Ca^{2+} levels in the PCB-contaminated soil, resulting in fewer micelles and thus removing lower amounts during washing. Also, the inner core of anionic surfactant micelles is not as accessible as nonionic micelles to PCBs due to greater hydration of the hydrophilic portion, thus decreased partitioning may result (Kile and Chiou 1989). This may explain the increased solubilization of spiked congeners, which sometimes have been shown to be more easily removed than weathered congeners.

The anionic surfactants Hostapur SAS 60 and Nansa LSS38/AS allowed the most degradation of the PCBs in the washing solutions. Anionic surfactants may allow more degradation than nonionic surfactants because they resist aggregation due to repulsion of charges. Thus there may be faster dissociation of micelles releasing the PCBs to the aqueous phase. PCBs have lower affinity for the interior of anionic than for nonionic micelles with a similar nonpolar chain length (Kile and Chiou 1989), and this may also promote release of the PCBs from the micelles and bring them into contact with the microorganisms. Greater loss of PCBs in the washing with Sorbax PMO-20 may be linked to its use as a growth substrate by *Pseudomonas* LB400 (Billingsley 1997), which may allow more degradation due to an increase in the number of cells, or a decrease in concentration of surfactant leading to dissociation of micelles which makes the PCBs bioavailable.

In conclusion, nonionic surfactants were more efficient than anionic surfactants in washing PCBs from soil while greater PCB biodegradation was observed in the undiluted anionic surfactant washings. An overall analysis of each surfactant's performance showed that despite lower washing efficiency, anionic surfactants allowed the most degradation of the initial PCBs in the soil. However, it is also known from earlier work (Billingsley 1997) that degradation of PCBs in nonionic surfactant solutions occurs if the surfactant is diluted below its CMC value. Therefore, another soil washing/biodegradation treatment process for PCB removal is likely to involve use of a nonionic surfactant at a concentration high enough to generate micelles in soil, even allowing

for some surfactant sorption to the soil, followed by dilution of the washings to dissociate the micelles and make the PCBs bioavailable.

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CHAPTER 5

Production of metabolites from chlorobiphenyls by resting cells of *Pseudomonas* strain LB400, after growth on different carbon sources[1]

5.1 Abstract

Cells of *Pseudomonas* LB400, grown on biphenyl, glucose or glycerol, transformed polychlorinated biphenyl (PCB) congeners into chlorobenzoic acid (CBA) metabolites. Transformation of the PCB congeners, 2,3-chlorobiphenyl (CBP), 2,2'-CBP, 2,5,4'-CBP and 2,4,2',4'-CBP produced the metabolites, 2,3-CBA, 2-CBA, 4-CBA and 2,4-CBA, respectively. Rates and extents of PCB transformation and metabolite formation were highest with biphenyl-grown cells. Intermediate rates of metabolite production were observed with glycerol-grown cells and lowest rates of production were found with glucose-grown cells. Regardless of carbon source, the rate of degradation of congeners was faster than the rate of production of CBAs. Relative rates of PCB transformation and metabolite production from different congeners with cells grown on a particular substrate followed the same general order, 2,3-CBA (from 2,3-CBP) > 2-CBA (from 2,2'-CBP) > 4-CBA (from 2,5,4'-CBP) >2,4-CBA (from 2,4,2',4'-CBP). *Pseudomonas* LB400 appeared unable to grow on any of the chlorobenzoic acids. However, *Pseudomonas* LB400 cells grown on biphenyl appeared capable of degrading 2-CBA and 2,3-CBA but not 4-CBA nor 2,4-CBA. Cells grown on glycerol appeared unable to metabolize any CBAs.

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5.2 Introduction

Polychlorinated biphenyls (PCBs) are a family of congeners consisting of a biphenyl nucleus containing 1 to 10 chlorine atoms. These stable, hydrophobic compounds were manufactured and extensively used in electrical transformers and as hydraulic fluids and plasticisers and, as a result, have become widely dispersed in the environment. A proposed system for clean up of PCBs involves combined use of an anaerobic reductive dehalogenation of highly chlorinated congeners followed by aerobic degradation of more moderately chlorinated PCBs, mediated by oxidative ring attack (Abramowicz 1990).

The majority of the work on aerobic degradation of chlorobiphenyls has utilized biphenyl-grown cells (Bopp 1986; Bedard et al. 1986; Gibson et al. 1993). *Pseudomonas* strain LB400 grown on biphenyl contains a multicomponent enzyme system, biphenyl 2,3-dioxygenase, which incorporates both atoms of molecular oxygen into biphenyl producing cis-biphenyl 2,3-dihydrodiol (Haddock et al. 1993). Following dehydrogenation and meta cleavage, hydrolysis results in the production of benzoate and a 5 carbon molecule (2-oxopent-4-enoate) (Furukawa and Miyazaki 1986; Omori et al. 1986).

Recombinant strains that contained and expressed the genes for the PCB-degrading enzymes were isolated, several of which had all the enzymes to metabolize a variety of PCBs to the corresponding chlorobenzoic acids (CBA) (Mondello 1989). In this way, it was conclusively demonstrated that the pathway consisted of four key enzymes, biphenyl dioxygenase, biphenyl dihydrodiol dehydrogenase, biphenyl catechol oxygenase, and hydrolase.

Some PCB-transforming isolates have been reported which can utilize monochlorobiphenyls in addition to biphenyl as growth substrates. Many isolates can utilize 4-chlorobiphenyl (Ahmad et al. 1990; Ahmed and Focht 1973; Furukawa et al. 1978; Furukawa and Miyazaki 1986; Masse et al. 1984; Mondello 1989) and also 2- and 3-chlorobiphenyl (Bedard et al. 1986; Parsons et al. 1988). However, aerobic bacteria can only cometabolize PCB congeners containing two or more chlorines (Bedard et al. 1986; Furukawa et al. 1979), which has been claimed to require biphenyl as a carbon source and inducer of the chlorobiphenyl-transforming

enzymes (Kohler et al. 1988).

Strategies for demonstration of PCB contaminant degradation generally involve monitoring of PCB removal. The production of intermediate metabolites, which are structurally related to the PCB parent substrates, provides strong evidence that PCB biodegradation or biotransformation is occurring (Flanagan and May 1993). Such PCB metabolites as chlorobenzoic acids have been detected as evidence of naturally occurring aerobic PCB biodegradation in Hudson River sediments (Flanagan and May 1993). In addition to confirming PCB depletion, metabolite formation in experimental work can unequivocally demonstrate that bacterial oxidation is occurring (Unterman et al. 1988) and also provides valuable information on the nature of the metabolic pathway. Quantitative mass balance information, relating substrate utilized to product formed, can suggest whether the product itself is further metabolized or if other intermediates may be accumulated.

Bopp (1986) reported the appearance of metabolites using HPLC to provide evidence that *Pseudomonas* LB400 aerobically metabolized representatives of all major structural classes of PCBs, including several congeners which lacked adjacent unchlorinated carbon atoms. Chlorinated dead-end metabolites, usually CBAs, often accumulate during biotransformation of PCBs (Furukawa et al. 1979). The intermediary metabolites produced by *Pseudomonas* LB400, as well as metabolic pathway elucidation has been confirmed by cloning genes encoding for the enzymes of the biphenyl locus (*bph*) into *Escherichia coli* in a manner which facilitates characterization of the individual enzymes and identification of the metabolites (Seeger et al. 1995).

It has been demonstrated that resting cells of *Pseudomonas* LB400, grown on glycerol or glucose, could also transform purified PCB congeners and mixtures of PCBs in Aroclors, although transformation rates were less than those observed with biphenyl-grown cells (Billingsley et al. 1997a, b). In this paper, we have investigated production of CBAs from different PCB congeners in time course studies when incubated with resting cells of *Pseudomonas* LB400 grown on biphenyl, glucose or glycerol.

5.3 Materials and Methods

Chemicals:

PCB congeners (2,3-dichlorobiphenyl, 2,2′-dichlorobiphenyl, 2,5,4′-trichlorobiphenyl and 2,4,2′,4′-tetrachlorobiphenyl) in neat form were obtained from Accustandard Ltd. (Newhaven, CT). Purity was 99+%; no biphenyl peak was detected. Stock solutions of individual congeners were prepared containing 2 or 2.5 mg/ml of the congener in acetone. Biphenyl, chlorobenzoic acids (2-chlorobenzoic acid; 4-chlorobenzoic acid; 2,3-dichlorobenzoic acid and 2,4-dichlorobenzoic acid), and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) 99:1, were obtained from Sigma-Aldrich. (St. Louis, MO).

Bacterial strain:

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, NY) was obtained from the Northern Regional Research Laboratory (Peoria, IL). Cultures were maintained in 50 ml of the standard growth medium in 250-ml Erlenmeyer flasks by storage at 4°C, with monthly subculturing by incubation on an orbital shaker at 200 rpm at 30°C for 24 h. Cultures were streaked onto tryptic soy agar (TSA) at regular intervals to ensure purity. For long-term storage of cultures, 20% glycerol was added to cell suspensions in the standard growth medium and the mixture was distributed in 1-ml aliquots in 1.5-ml tubes and stored at -20° C.

Standard growth medium:

The growth medium used was the phosphate-buffered mineral salts medium supplemented with yeast extract described by Bedard et al. (1986). This medium contained per litre: 4.4 g K_2HPO_4, 1.7 g KH_2PO_4, 2.1 g NH_4Cl and 50 mg of yeast extract (Becton Dickinson, Cockeysville, MD), 2 g carbon source, and 10 ml of concentrated basal salts solution. Non-volatile carbon sources were added to the bulk medium prior to autoclaving. Basal salts were added after autoclaving. Biphenyl was prepared in a 0.1 g/ml solution in hexane, filter sterilized using a 0.2

μm PTFE syringe filter (Nalge, Rochester, NY) and added to the autoclaved medium. The hexane was allowed to evaporate, leaving crystals suspended in the medium.

The concentrated basal salts solution contained per litre 19.5 g $MgSO_4$, 5 g $MnSO_4 .H_2O$, 1 g $FeSO_4.7H_2O$, and 0.3 g $CaCl_2 .2H_2O$, plus enough H_2SO_4 to prevent precipitation of basic salts. Culture media (50 ml) were prepared in 250-ml Erlenmeyer flasks, and stoppered with foam plugs.

Inoculum preparation:

Cultures stored at 4°C were incubated on an orbital shaker (Labline Instruments Inc., Melrose Park, IL) at 200 rpm at room temperature until warm. Cells were inoculated into 50 ml of fresh growth media in a 250-ml Erlenmeyer flask to an OD of 0.1 measured at 615 nm in a 1-cm light path. Unless otherwise stated, cultures were incubated at 30°C on an orbital shaker set at 200 rpm for 22-24 h. Cultures containing biphenyl were filtered through sterile glass wool to remove excess crystals. The cell suspension was then centrifuged at 5000 *g* for 15 minutes, washed twice with 0.05 M sodium phosphate buffer, pH 7.5, and resuspended in the buffer to an OD of 1.0 measured at 615 nm, using a 1-cm light path.

PCB biotransformation conditions:

One millilitre of bacterial suspension at an OD of 1.0 was added per 8-ml amber glass vial. Controls were killed by addition of 1 drop of 70% perchloric acid. Individual PCB congeners were added in the form of a concentrated acetone solution to a final concentration of 20 or 25 ppm. Vials were closed with Teflon-lined caps and incubated at 30°C on an orbital shaker set at 200 rpm.

PCB extraction:

Reactions were stopped with 1 drop of perchloric acid. One percent Triton X-100, 4 ml of hexane and 0.5 g of sodium sulfate (to prevent formation of a stable emulsion) were added to

the vials. Foil-lined caps were used for this procedure. Samples were shaken horizontally on an orbital shaker at 300 rpm for 20 to 30 minutes. A 1-ml aliquot of the hexane layer was transferred to vials for GC/MS analysis.

Metabolite Extraction:

Cell suspensions were acidified with 1 N HCl to obtain a pH of 3, and extracted with 2 ml of ethyl acetate for 20 min at room temperature on an orbital shaker set at 300 rpm. The organic layer was removed and passed over sodium sulfate. The extraction was repeated, and the organic layers were evaporated under nitrogen to dryness. The samples were derivatized with 100 µl BSTFA + TMCS, 99:1, and 100 µl hexane at 60°C for 15 min. Samples were made up to 1 ml with hexane before analyses by GC/MS.

PCB congener analysis:

Extracted samples were analyzed by capillary GC (Model HP 5890 series II, Hewlett-Packard, Meyrin, Switzerland), equipped with a mass selective detector (Model HP 5971A, Hewlett-Packard) and integrated by Hewlett-Packard MS Chemstation software. Identification and quantitative determinations of the congeners were based on analysis on a fused silica capillary column coated with a film thickness of 0.25 µm of polymethyl (5% phenyl) siloxane, 30 m in length, with a 0.25 mm internal diameter (HP-5MS, Hewlett-Packard). The sample (2 µl) was injected by an autoinjector (Model HP 7673, Hewlett-Packard) in splitless mode with a time delay of 1.25 min after injection. The initial oven temperature of 55°C was held for 2 min and then programmed to increase by 10°C/min to 100°C, 5°C/min to 215°C, 10°C/min to 285°C and held for 5 min. The injector temperature was 250°C and the transfer line was 280°C. The carrier gas was helium with an initial column head pressure of 12 psi. The system was equipped with electronic pressure control and operated in constant flow mode (1.66 ml/min). The filament and multiplier were turned on at 8 min. The system was operated in selected ion monitoring mode. The ions monitored were 188,190; 222,224; 256,258; 290,292. Standard midmass autotunes using

PFTBA were performed before each set of samples was analyzed.

Metabolite analysis:

Metabolites were analyzed under the same conditions as the PCB congeners, except that the system was operated in full scan mode from 50 - 550 amu.

5.4 Results

Identification of reaction products

Resting cells of *Pseudomonas* LB400, grown on biphenyl, glucose or glycerol, were incubated with single PCB congeners at a concentration of 20 or 25 ppm for time periods of 0 to 100 h. The contents of the vials were extracted with hexane for residual PCB congener determination by GC/MS. Products of PCB metabolism were recovered by extraction of replicate vials with ethyl acetate followed by derivatization with BSTFA and analysis by GC/MS. Figure 5.1 is an example of the mass spectrum of the metabolite produced by transformation of 2,3-CBP. The major metabolite produced from each congener was the same, regardless of whether the cells were grown on biphenyl, glucose or glycerol as sole carbon source. 2,3-CBA, 2-CBA, 4-CBA and 2,4-CBA were produced from the PCB congeners, 2,3-CBP, 2,2′-CBP, 2,5,4′-CBP and 2,4,2′,4′-CBP, respectively. Compounds were identified by comparison of the mass spectra and the GC retention time to authentic preparations of CBAs. No other metabolites were detected at acidic or neutral pH in any of the extracts. However, the supernatants of the incubations of 2,5,4′-CBP and 2,4,2′,4′-CBP were quite yellow, which suggests the presence of chlorinated 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoates (CHOPDAs), yellow compounds, which are metabolites of PCB breakdown (Seeger et al. 1995).

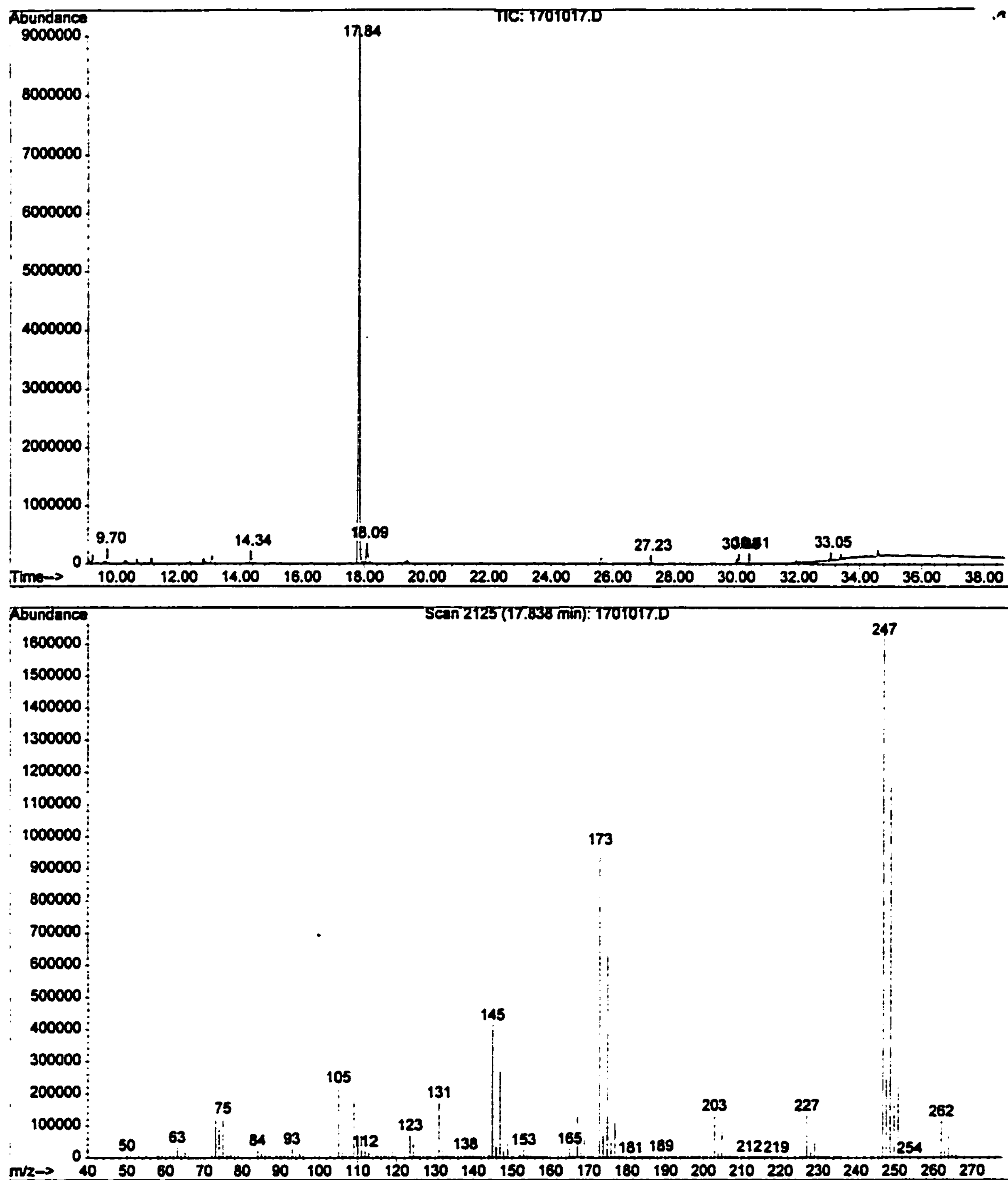

Figure 5.1 Mass spectrum of GC/MS analysis of metabolites of transformation of 2,3-CBP by *Pseudomonas* LB400 grown on biphenyl. Peak at 17.84 min corresponds to a dichlorobenzoic acid.

Identification of the CBAs produced gives an indication of which biphenyl rings were attacked. 2,3-CBP, which was substituted on only one ring, was found to be attacked at the non-chlorinated ring. 2,5,4′-CBP was found to be oxidized on the 2′-chlorinated ring exclusively. The other congeners had identical substitutions on both rings, so there was no preferred ring of attack.

Effect of growth substrate on production of metabolites from four PCB congeners

The time courses of metabolism of CBPs and production of CBAs by *Pseudomonas* LB400 cells, grown on biphenyl, glucose or glycerol, are presented in Figure 5.2. The CBPs and CBAs were quantified by comparison with standard curves of authentic preparations of these compounds. From the time course data, the initial rate of transformation of each congener and of production of the resulting chlorobenzoic acid was determined by measuring the initial slope of the line (Table 5.1). R^2 values for the fit of the lines were greater than 0.85. The maximum amount of CBA produced in each incubation is expressed as a percentage of the amount that could have theoretically been produced from the maximum amount of PCB transformed, in Figure 5.3.

Transformation of the dichlorinated congeners resulted in the highest rates and greatest extents of chlorobenzoic acid production. Cells grown on biphenyl rapidly metabolized both 2,3-CBP and 2,2′-CBP and produced the corresponding chlorobenzoic acids in less than 15 min. In the case of 2,3-CBA, a concentration of 8 ppm was observed after 15 min, after which a slight decrease in 2,3-CBA occurred. A maximum concentration of 2-CBA produced from 2,2′-CBP of 6 ppm was observed after a 0.5 h incubation. A maximum concentration of 4-CBA, produced from 2,5,4′-CBP of 0.4 ppm was observed after a 24 h incubation with biphenyl grown cells. The concentration of 2,4-CBA produced by transformation of 2,4,2′,4′-CBP with biphenyl grown cells increased to 0.6 ppm over a 72-h incubation period.

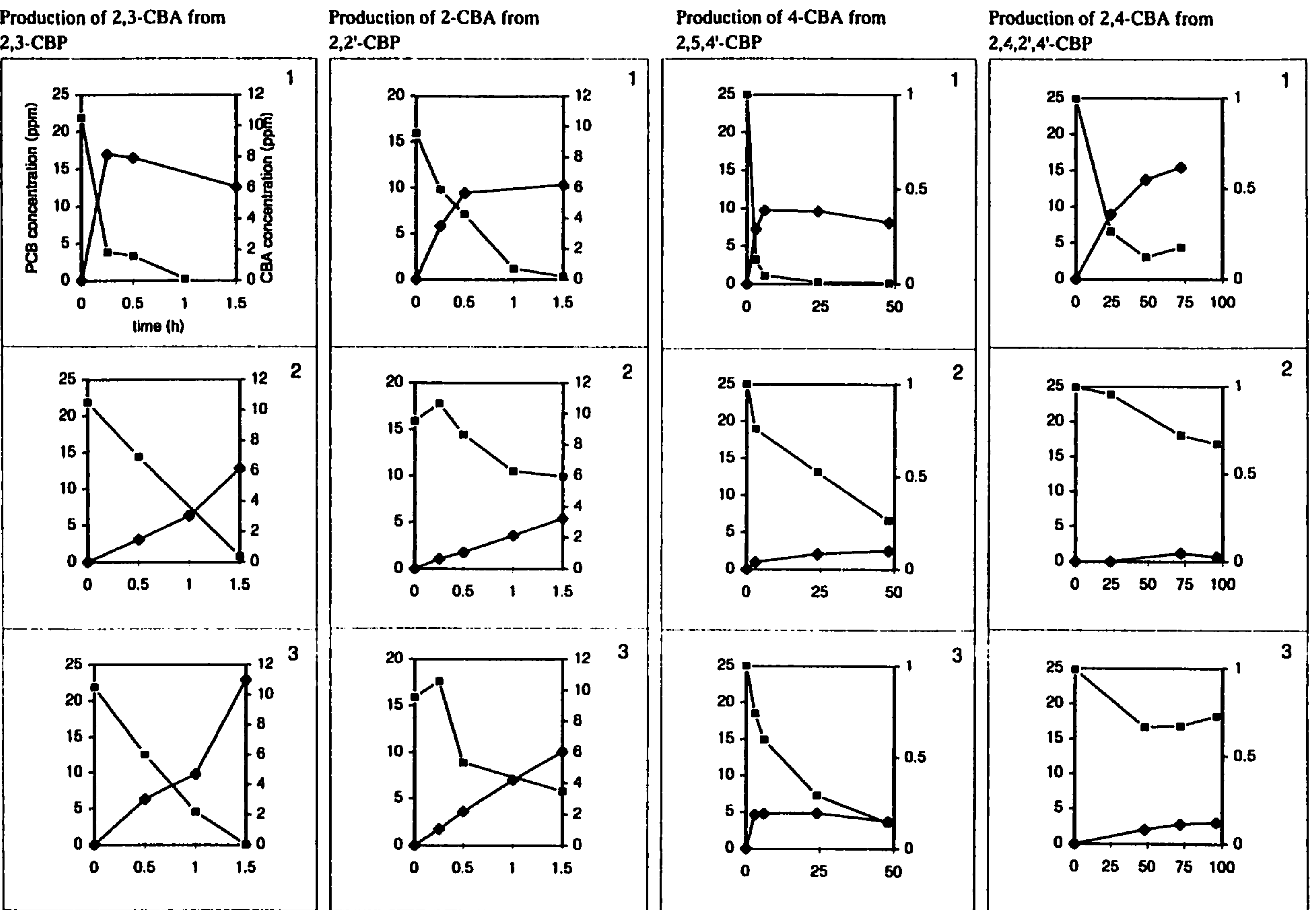

Figure 5.2 Time course of degradation of PCB congeners (■) and production of chlorobenzoic acids (◆) by *Pseudomonas* LB400 grown on different carbon sources. 1- biphenyl, 2- glucose, 3- glycerol.

Table 5.1 Rates of degradation of PCB congeners and production of chlorobenzoic acids by *Pseudomonas* LB400 after growth on different carbon sources

Carbon source for growth	Rate of change of concentration of CBP or CBA*							
	2,3-CBP	2,3-CBA	2,2'-CBP	2-CBA	2,5,4'-CBP	4-CBA	2,4,2',4'-CBP	2,4-CBA
Biphenyl	-72	33	-19	12	-7.3	0.07	-0.77	0.010
Glycerol	-16	6.5	-6.8	4.1	-1.8	0.06	-0.09	0.000
Glucose	-14	3.7	-5.0	2.1	-1.0	0.01	-0.17	0.002

* units are μg/ml/h

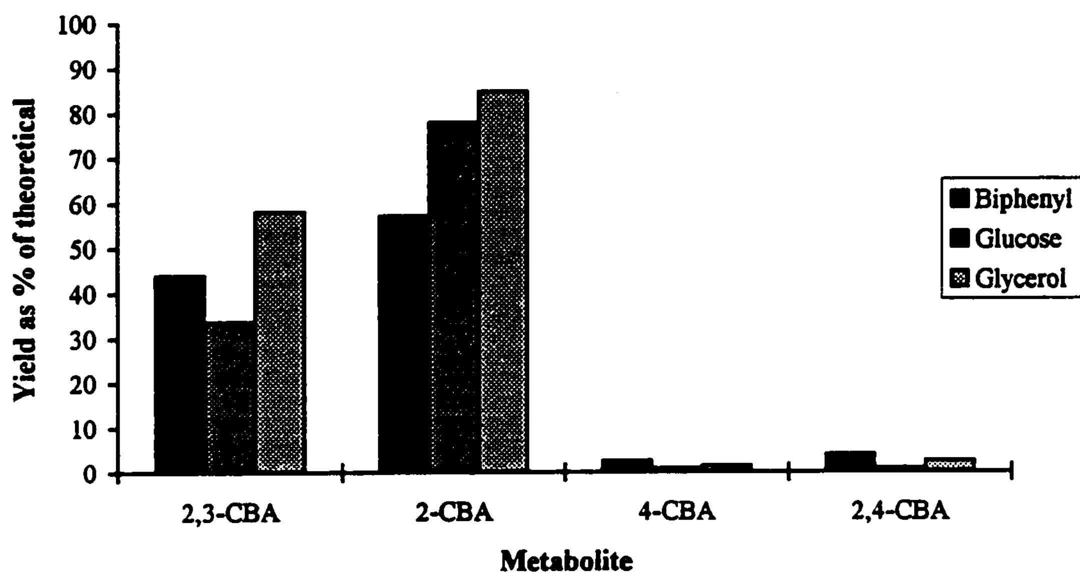

Figure 5.3 Quantitative yields of CBAs produced from PCB congener metabolism. The maximum amount of CBA produced is expressed as a percentage of the amount that could have theoretically been produced from the maximum amount of PCB transformed.

Resting cells of *Pseudomonas* LB400, grown on glycerol or glucose, produced CBAs to lesser extents and at slower rates than those observed for biphenyl-grown cells. However, the rates and extents of degradation of the PCBs were also lower. For all carbon sources, the rate of degradation of the dichlorinated PCBs was approximately twice the rate of CBA production. Rates of production of CBAs from the tri- and tetrachlorinated biphenyls were negligible. The relative rates and extents of metabolite formation always followed the same sequence, namely: 2,3-CBA (from 2,3-CBP) > 2-CBA (from 2,2′-CBP) > 4-CBA (from 2,5,4′-CBP) > 2,4-CBA (from 2,4,2′,4′-CBP).

Quantitative yields of CBAs did not result from the metabolism of any congener for cells grown on any of the carbon sources. However, in the case of the dichlorinated congeners, the time period of the incubations was quite short, and the time courses indicated that CBA production was increasing. A higher yield may have been obtained in a longer incubation. Other reasons for the low yields will be discussed below.

Metabolism of CBAs

It was noted that CBAs were not formed in quantitative amounts, and one of the possible explanations is that they were further metabolized. Initial experiments showed that *Pseudomonas* LB400 could not grow on any of these chlorobenzoic acids (data not shown). However, like CBPs, it is possible that CBAs could be transformed by cometabolism. Biphenyl- and glycerol-grown cells were incubated with individual CBAs at a concentration of 10 ppm for 24 h in order to determine their abilities to transform the CBAs. The results are presented in Table 5.2, and are averages of three samples. Cells grown on biphenyl were capable of degrading 2- and 2,3-CBA, but not 4- or 2,4-CBA. Cells grown on glycerol were not able to metabolize any CBAs.

Table 5.2 Degradation of CBAs by resting cells of *Pseudomonas* LB400

Carbon source for growth	percent degraded after 24 h			
	2-CBA	4-CBA	2,3-CBA	2,4-CBA
Biphenyl	89	0	20	0
Glycerol	0	0	0	0

5.5 Discussion

Production of chlorobenzoic acids from PCBs by cells grown on glucose or glycerol, in addition to biphenyl, confirms that all of the initial enzymes involved in PCB metabolism are indeed produced after growth on carbon sources which are not structurally similar to PCBs. To the best of our knowledge, this is the first time products of PCB metabolism have been shown for cells of *Pseudomonas* LB400 grown on glucose or glycerol.

Although no other metabolites were detected using the extraction, derivatization, and GC/MS procedure described above, visual examination of the supernatants suggested that the yellow-coloured *meta* cleavage products, CHOPDAs, may be produced. Ahmad et al. (1991) indicated that this metabolite is unstable in aqueous solutions, and may be converted into a molecular species that is not detected by GC/MS. Other researchers have detected transitory hydroxylated metabolites by using higher PCB substrate concentrations and by purifying and concentrating the reaction mixture extracts by HPLC (Haddock et al. 1995; Arensdorf and Focht 1995; Ahmad et al. 1991; Bopp 1986; Masse et al. 1989).

Rates of 2,3- and 2-CBA production ranged from 26 to 63% of rates of depletion of their respective substrates, 2,3- and 2,2'-CBP, and conversion yields of substrate to CBA ranged from 34 to 85%. In contrast, rates of 4-CBA and 2,4-CBA production ranged from 0 to 3.3% of the rates of depletion of their respective substrates, and conversion yields of substrate to CBA were similarly low, ranging from 1 to 4%. The location of the initial oxidative attack of the congeners with differing chlorination patterns on both rings was on the unchlorinated or 2-chlorinated ring.

Seeger et al. (1995) found that the rates of formation of CHOPDAs by *E. coli* cells containing the first three genes for biphenyl metabolism from *Pseudomonas* LB400 (*bph ABC*) were high for the PCB congeners 2,3- and 2,2′-CBP whereas a moderate rate of formation of CHOPDA was observed for 2,5,4′-CBP. In experiments with *E. coli* cells containing the enzymes capable of converting PCB to CBA (*bph ABCD*) from *Pseudomonas* LB400, almost quantitative yields of CBA were produced from 2,3-CBP and 2,2'-CBP whereas no CBAs from 2,5,4'-CBP were detected.

The non-quantitative yields of 4-CBA from 2,5,4'-CBP could be due to the fact that 2,5-CBA is also produced but used instantaneously by the cells, or that 4-CBA is also used quickly. However, externally added 4-CBA was not transformed by the cells. 2,5-CBA is probably not the product, as *Pseudomonas* LB400 is not known to metabolize *para*-chlorinated rings (Bedard and Haberl 1990). This means that the 2,5-substituted ring is being attacked, and as there are no free 2,3 sites on this ring, the attack must be either at the 3,4 position, or dechlorination of the 2 position chlorine molecule occurs during oxidation. Both of these scenarios have been described previously with this organism (Bedard 1990). In fact, experiments with purified 2,3-dioxygenase from *Pseudomonas* LB400 showed 90% production of a 2,3-catechol from 2,2'-CBP and only 10% production of a 5,6-dihydrodiol, suggesting that the dechlorination attack is preferred. It is thought that the second enzyme in the pathway, dihydrodiol dehydrogenase cannot act on 3,4-dihydrodihydroxybiphenyl and thus degradation of 2,5,4'-CBP would stop here. However, the observed yellow colour suggested that one of the biphenyl rings was cleaved producing the *meta*-cleavage compound, CHOPDA, from 2,5,4'-CBP, but was not further metabolized. This suggests that it is the hydrolase that will not act on this chlorinated metabolite. Accumulation of a yellow compound also in the supernatant of the 2,4,2',4'-CBP incubations suggests that the pathway may be blocked at the hydrolase so that very little CBA is produced from these congeners.

2- and 2,3-CBA added directly to cultures of biphenyl-grown cells appeared to be metabolized by *Pseudomonas* LB400. This would explain why the concentrations of 2- and 2,3-CBA produced by degradation of PCBs by biphenyl-grown cells, increased and then decreased or leveled off without reaching quantitative yields. No further metabolites were detected, so the products of this transformation are not known. *Pseudomonas cepacia* P166 was able to metabolize all monochlorinated CBPs to the corresponding CBAs and then to chlorocatechols (CCs) by *meta* cleavage (Arensdorf and Focht 1994, 1995). One of these chlorocatechols, 3-CC, was found to be toxic to the cells probably due to irreversible binding to 2,3-dihydroxybiphenyl-dioxygenase. If *Pseudomonas* LB400 cleaves CBAs at the *meta* position, similar toxic metabolites could be produced, however, no other metabolites were detected. 4- and 2,4-CBA were not

metabolized by biphenyl-grown cells, suggesting that the CBA-metabolizing enzymes could not utilize these compounds as substrates.

Sondossi et al. (1992) found that CBA transformation was controlled by biphenyl-induced oxygenases in *Pseudomonas testosteroni*. In *Alcaligenes* JB1, benzoate dioxygenase activity was induced by growth on benzoate but not citrate (Commandeur et al. 1996). We have shown that, unlike biphenyl-grown cells of *Pseudomonas* LB400, glucose- or glycerol-grown cells were not able to use any CBAs, indicating that biphenyl may be required to stimulate production of CBA-degrading enzymes.

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THESIS CONCLUSIONS

This thesis has described studies investigating biotransformation of PCBs by *Pseudomonas* LB400. The main findings are as follows:

1) Cells grown on carbon sources which are not structurally related to PCBs (glucose and glycerol) retain the ability to transform PCBs.
2) Cells grown on biphenyl manifest higher PCB degradation rates than cells grown on glucose or glycerol, and regardless of the carbon source, degradation rates depend on the chlorination pattern of the congener.
3) The presence of biphenyl inhibits transformation of PCBs by *Pseudomonas* LB400.
4) Incubation of *Pseudomonas* LB400 with PCBs leads to a decrease in viability and degradation capacity of the cells over time
5) In general, nonionic surfactants are capable of solubilizing PCBs sorbed to glass at concentrations above their CMC value, but higher concentrations are required to wash PCBs from soil. Anionic surfactants were less effective at solubilizing PCBs from soil.
6) Toxicity of surfactants to the bacterium depended on the surfactant, and the surfactant concentration. In general, most surfactants were non-toxic.
7) At concentrations of nonionic surfactants greater than the CMC value, PCB degradation was inhibited, however, at supra-CMC concentrations, anionic surfactants increased PCB degradation relative to a no-surfactant control.
8) Dilution of a nonionic surfactant-PCB mixture to a concentration near the CMC value eliminated the inhibition of degradation of PCBs.
9) Cells grown on glucose or glycerol contain the enzymes to convert PCB congeners into the corresponding CBAs.
10) Extents of CBA production from PCBs were lower than the extents of PCB removal indicating formation of undetected intermediates, or further metabolism of CBAs.
11) Further metabolism of CBAs by cells grown on biphenyl depended on the chlorination

pattern. However cells grown on glucose and glycerol were unable to transform any CBAs.

Several important observations have been presented in this thesis. Firstly, biphenyl, a hazardous contaminant itself, is not required to induce PCB-degrading enzymes in *Pseudomonas* LB400. This means that application of this organism to PCB-contaminated soils, together with simple carbon sources, could result in effective PCB bioremediation. Current research is focussing on the combination of anaerobic dechlorination of PCBs to congeners with fewer chlorines, followed by aerobic degradation (Abramowicz 1990). *Pseudomonas* LB400 grown on glycerol or glucose would be able to metabolize these lower chlorinated congeners. Another important observation was that cells grown on glucose or glycerol were capable of degrading PCBs to CBAs. Mineralization of the CBAs could then be achieved by adding known CBA-degrading bacteria (Hickey et al. 1993). Although *Pseudomonas* LB400 grown on biphenyl can degrade some CBAs, they may generate toxic metabolites by *meta*-cleavage. Cells grown on glucose or glycerol do not metabolize CBAs further, which would leave these compounds as substrates for other bacteria.

Anionic surfactants have tended to be avoided for the purpose of solubilizing contaminants for degradation because of their potential toxicity and their soil-complexation capacity (Jafvert and Heath 1991; Swisher 1987). However, the acceleration of PCB degradation in the presence of these kinds of surfactants was demonstrated in this thesis, and suggests that the use of anionic surfactants for this purpose should be examined further. While nonionic surfactants were very successful at solubilizing PCBs, comprehensive data have been provided which illustrate the inhibitory effects of these surfactants on PCB degradation at concentrations above the CMC value. The results provided in this thesis show that these surfactants can be used effectively to wash PCBs from soil. Then if surfactant-PCB aqueous solutions are diluted to near or below the CMC value, it has been demonstrated that these surfactants do not impede PCB degradation.

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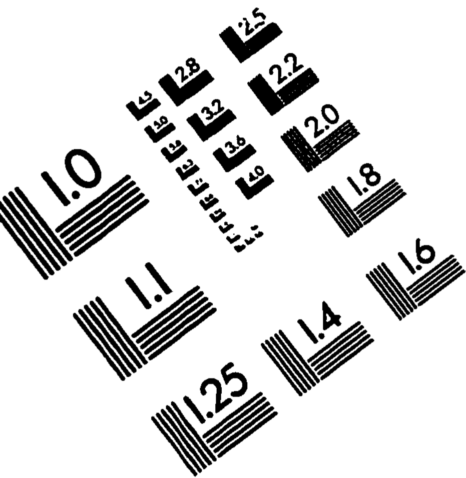
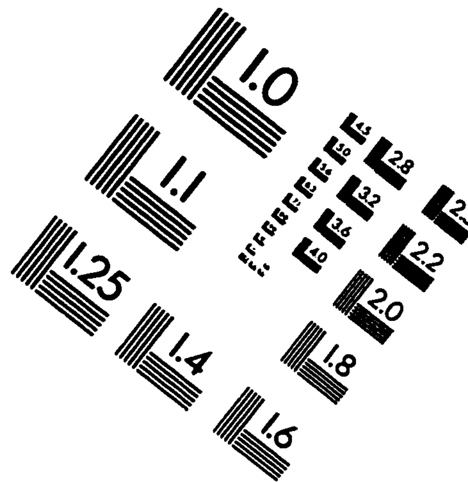
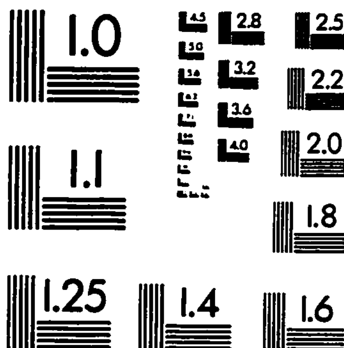
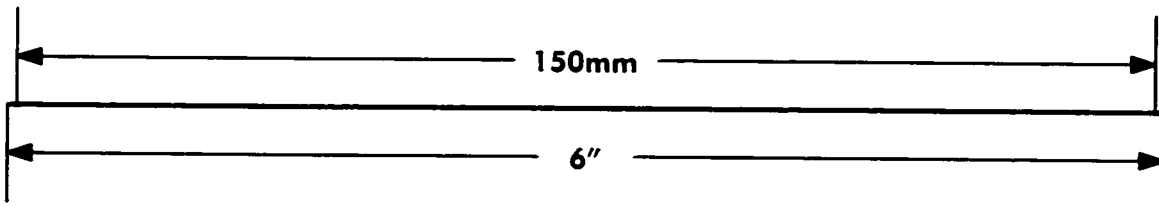
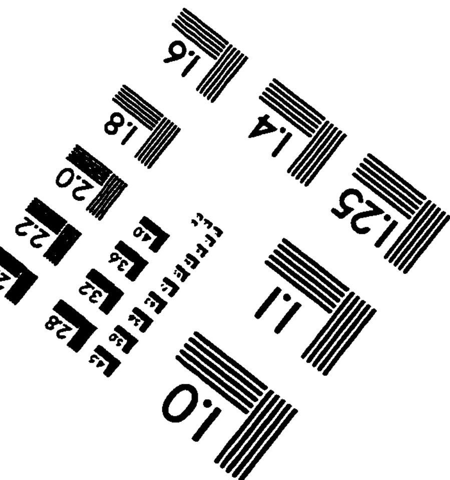
IMAGE EVALUATION TEST TARGET (QA-3)

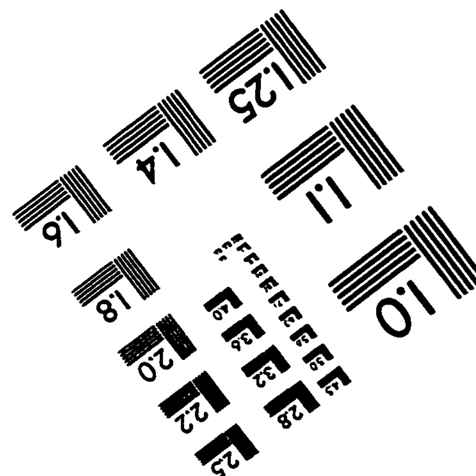












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1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
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