MICROBIAL DEGRADATION OF POLYCHLORINATED BIPHENYLS

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Dissertation submitted in compliance with the requirements for the Master's Degree in Technology in the Department of Biotechnology, Durban University of Technology, Durban.

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DECLARATON

MICROBIAL DEGRADATION OF POLYCHLORINATED BIPHENYLS

SHUBNUM MUSTAPHA

I hereby declare that the dissertation represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

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2007

APPROVAL

I hereby approve the final submission of the following dissertation.

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DEDICATION

This dissertation is dedicated to my loving family

my Mum, my Dad, my Sisters,

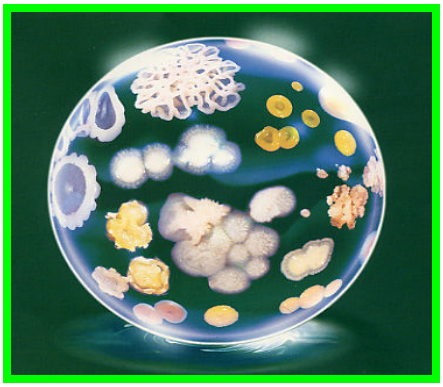
my loving Husband Shaheen

&

In loving memory of

my friend Juju (1979 – 2007).

Her life and her words are an inspiration to me.



"Biotechnology of hazardous compounds can be defined as the development of systems that use biological catalysts (bacteria) to degrade, detoxify or accumulate environmental pollutants"….. (Hardman *et al*., 1993).

2007

ABSTRACT

The aromatic compounds Polychlorinated Biphenyls (PCBs) are one of the largest groups of environmental pollutants. The greatest concern is the release of PCBs in the water systems by industrial effluent, accidental spillages or leaks. PCBs are able to bioaccumulate in the fatty tissues of animals, fish and humans. The impact on human health due to PCBs has prompted interest in their degradation. The application of microbial degradation of PCBs can transform many PCB metabolites. There are a wide variety of microorganisms that can degrade PCBs or utilise them as sole carbon sources.

This study focused on isolating microrganisms from industrial wastewater capable of aerobic degradation of PCBs. The degradation potential of the selected isolates were investigated by using different analytical techniques viz. ultra violet or visible spectrophotometer (UV/Vis), thin layer chromatography (TLC) and gas chromatography electron capture detector (GC-ECD). The strains were purified in the presence of PCBs yielding 25 isolates. There were two unknown organisms selected for further investigations based on their growth on the PCB media and the rate and reproducibility of growth on the media. The isolates were characterised as gram negative short rods. Both the organisms were grown to 10^6cfu/mL (colony forming units per millilitre) and inoculated into 200mL selected-PCB broths in a 1Lt Erlenmeyer flask. The isolates were presumptively identified by a 20E API kit as *Acinetobacter* sp. and *Pseudomonas* sp.

The degradation potential was analysed by comparative GC analysis of selected PCB congeners; 2.3'.4'.5 Tetrachlorobiphenyl (International Union of Pure and Applied Chemistry-IUPAC 70), 2.2'.4.5.5' Pentachlorobiphenyl (IUPAC 101), 2.2'.3.5.5'.6 Hexachlorobiphenyl (IUPAC 151), 2.2'.3.4.4'5.5' Heptachlorobiphenyl (IUPAC 180), 2.2'.3.4.4'.5.6.6' Octachlorobiphenyl (IUPAC 204) and 2.2'.3.3'.4.4'.5.5'.6 Nonachlorobiphenyl (IUPAC 206). The *Acinetobacter* sp. and *Pseudomonas* sp. were unable to transform the higher chlorinated congeners of Aroclor 1254. However the mixed culture was able to transform Aroclor 1254 completely. Aroclor 1260 was transformed by *Acinetobacter* sp. by 80%, *Pseudomonas* sp. by 95% and the mixed

culture by 85%. This was due to the inability of the cultures to transform congener, 2.2'.3.5.5'.6 Hexachlorobiphenyl. The congener was clearly toxic to the cultures and apprehended further transformation of Aroclor 1260. Comparison of Aroclor 1254 and Aroclor 1260 found that in the mixed culture the *Pseudomonas* sp. was influential in the degradation process. This was seen by the higher transformation of Aroclor 1254 and Aroclor 1260 by *Pseudomonas* sp. than the *Acinetobacter* sp.

The complete transformation of Aroclor 1254 and 95% transformation of Aroclor 1260 by the mixed culture can be attributed to the synergistic relationship of *Acinetobacter* sp. and *Pseudomonas* sp. as versatile chlorinated degraders. It was concluded that the mixed culture of *Acinetobacter* sp. and *Pseudomonas* sp. proved efficient to transform both Aroclor 1254 and Aroclor 1260. *Pseudomonas* sp. showed the most efficient transformation of Aroclor 1254 and Aroclor 1260.

PREFACE

Aspects of the work covered in this dissertation can be found in the following publication:

Mustapha S, Khehra MS and Bux F (2007) Degradation of Aroclors by *Pseudomonas aeruginosa* isolated from industrial wastewater. *Water Environment Research*. (In preparation).

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ABBREVIATIONS

cfu/mL	: Colony forming units per milliliter
CLLE	: Continuous Liquid-Liquid Extraction
DDT	: Dichloro-diphenyltrichloroethane
GC	: Gas Chromatography
GC-ECD	: Gas Chromatography Electron Capture Detector
HPLC	: High Performance Liquid Chromatography
IUPAC	: International Union of Pure and Applied Chemistry
LLE	: Liquid-Liquid Extraction
PCBs	: Polychlorinated Biphenyls
sp.	: species
SPE	: Solid Phase Extraction
SWA	: Southern Wastewater Works aerobic zone
SWI	: Southern Wastewater Works influent zone
TLC	: Thin Layer chromatography
TTA	: Amanzimtoti Wastewater Works aerobic zone
TTI	: Amanzimtoti Wastewater Works influent zone
UV/ Vis	: Ultra Violet/ Visible light
WWTP	: Wastewater Treatment Plant

CHAPTER 1: INTRODUCTION

Polychlorinated Biphenyls, commonly known as PCBs, are a family of toxic synthetic organochlorine chemicals. They are a group of 209 PCB forms known as congeners consisting of 1-10 chlorine atoms. In 1930 the compound became a necessity in every household as the perfect insulator in fridges and television sets, to its uses in carbon papers, adhesives, hydraulic fluids and plasticizers (Rahuman *et al*., 2000). Complex mixtures of PCBs are known as Aroclors containing 60-90 PCB congeners (Bernhard and Petron, 2001).

PCBs pose a great toxicological risk to the wildlife, ecosystem and the human food chain because of their stable properties and ubiquitous distribution. Human health effects, on exposure to PCBs are linked with cancers, birth defects, tumours and severe brain abnormalities in newborns and children (Safe, 1991).

Conventional PCB removal methods such as incineration are environmentally unsafe, expensive and ineffective (Costner, 1998). The application of microbial degradation of PCBs began in the early nineties, initially several PCB congeners were degraded and eventually isolates were identified based on their ability to degrade PCBs (Lunt and Evans, 1970).

1.1. Background to Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) have poisoned the world's perception of leading a normal chemical-free life. These "miracle compound" was discovered in the early 18th century and become a global environmental disaster. PCBs were chemically synthesised in 1881. They are composed of 1-10 chlorine atoms attached to various positions on two benzene rings (Pieper, 2004). Complex mixtures of PCBs were fractionally distilled to produce commercial mixtures commonly known as Aroclor (USA and UK), Clophen (Germany) or Kaneclor (Japan) (Bernhard and Petron, 2001). These complex compounds

are industrially used in transformer fluids, flame retardants, solvent extenders and organic diluents (Yadav *et al*., 1995).

After the ban of production in 1980 evidence of bioaccumulation of PCBs in the fatty tissue of fish, marine life and humans were found. Their transport in water has allowed easy access into the human and animal diet. (ATSDR, 2000). PCBs have entered the environment by air, water and soil during manufacturer use and disposal, from spills or leaks, from fires or transport and illegal dumping (Pieper, 2004). The remediation of PCBs by physico-chemical methods are inefficient and toxic to the environment. One notably conventional method of PCB destruction is incineration which releases toxic emissions such as polychlorinated dibenzofuranes (PCDF) and polychlorinated dibenzodioxins (PCDD). Doses with low concentrations between 1 part per billion (ppb) and 5 000ppb can cause damage and death to most living organisms (Robinson, 2006).

Humans have been infected by PCBs via their skin; inhalation or ingestion of PCB contaminated food and vapours in the air. They are promoters of cancer and linked to damage of kidneys, liver and the digestive tract as well as the cause of miscarriage and sterility (Robinson, 2006). Fish and most aquatic organisms and zebra mussels accumulate PCBs in their fatty tissues rapidly (Fisher Environmental, 1997, ATSDR, 2000). The transport through the animal food chain reaches seals, whales and dolphins. These unnatural levels of PCBs have sexually orientated many animals. The polar bears were first found to have haemophrodic sex organs (U.S.A. EPA, 2000b). Today however it has spread to most of the fish in rivers and lakes and is lowering the human male to female ratio as well as decreasing sperm counts (Hauser *et al*., 2005)

There are numerous mechanisms of clean up of PCBs ranging from biochemical to biological degradation. Recent advances have seen modified strains of microorganisms capable of degrading PCBs (Wiegel and Wu, 2000; Pieper, 2004). This has allowed investigations of the chemistry of PCBs as well as the metabolism of the process of degradation of various PCBs.

1.2. Conventional Treatment of PCBs

There are several methods for PCB destruction however they are generally expensive and inefficient. The physico-chemical processes commonly known as the "bury or burn" concept, i.e. landfilling and incineration (Hardman *et al*., 1993).

Landfilling is the disposal of hazardous wastes, such as equipment containing PCBs, into the land. However the problem arises when gases are released into the air and toxic lechates contaminate the soil and groundwater's (Hardman *et al*., 1993). The dumping of wastes is simply moving waste elsewhere. This draws the risk of excavation, handling and transport of hazardous wastes. This system only allows a certain limit of wastes in the area and constant monitoring and maintenance of the area barriers and the remains are essential which is costly (Gray, 2004). Incineration is the destruction of municipal, toxic and clinical wastes. A sufficient contact time and high temperatures are required for efficient combustion and destruction of the compounds. However, this has led to emissions of toxic gases like dioxins, dibenzofuranes and polyaromatics hydrocarbons into the air. Incomplete combustion of the compounds has increased the concentration of heavy metals in the soil due to the dumping of the ashes into the land after incomplete combustion (Costner, 1998). The toxic emission released is exposed to workers and residence and this poses a great risk to human health (ATSDR, 2000). Other methods that are employed to destroy or transform pollutants by chemical decomposition such as base-catalysed dechlorination or Ultra-Violet (UV) oxidation are principally sustainable. However, the high cost for small or large scale treatments and by products released are environmental concerns. The rate of physico-chemical treatment of organic compound is directly related to the energy required. Ultimately the process is inefficient for mineralization of PCBs (Vidali, 2001).

The application of biological agents proved to be inefficient in wastewater treatment plants due to the overload of toxins and the agents restricted ability to only degrade simple carbon and ammonium compounds. The effectiveness of biodegradation is

dependant on the availability of a carbon source and the structure of the compound (Hardman *et al*., 1993).

1.3. Microbial degradation of PCBs

In the last 15 years there have been numerous laboratory breakthroughs for the degradation of PCBs (Wiegel and Wu, 2000; Pieper, 2004). Organic wastes are biologically degraded by naturally occurring microorganisms. The compound is degraded or transformed by the organism's metabolic processes. The microorganism's enzymatically breaks down the PCB into its metabolites (Seeger *et al*., 1997). To enable the microorganism's potential to breakdown the compounds efficiently, the environmental parameters are manipulated allowing the microorganism's to grow faster and efficient microbial biodegradation is achieved. Competent microorganisms are capable of utilising the compound as a carbon source and breakdown the monomers (Pieper, 2004). Microorganisms are versatile and adaptable to an environment that allows a sufficient carbon source for their survival (Vidali, 2001). These microorganisms in the presence of biphenyls are able to utilize the compound as a sole carbon source and metabolize the target compounds to other substrates (Lunt and Evans, 1970; Catelani *et al*., 1970). There are substantial studies concerning the metabolism and microbial degradation of PCBs (Ahmed and Focht, 1973; Aràoz and Viale, 2004; Chàvez *et al*., 2006).

These studies are essential for understanding PCBs in the environment and the microbial responses. The difficulty arises with the complexity of the compounds and the microbial interactions with the PCB. The process is complicated in that different levels of PCBs are degraded at different rates (Kaštànek *et al*., 2004). Microbial degradation is the obvious environmentally friendly approach. The isolation of PCB-degrading bacteria will be a stepping stone for degradation process in South Africa. This would illicit interest in research of microbial degradation of toxic compounds.

1.4. Aim and Objectives

Aim

To investigate the potential of bacteria to degrade polychlorinated biphenyls (PCBs).

Objectives

1. Screening of PCB degrading bacteria from wastewater treatment plants.

2. Enrichment and isolation of PCB degrading bacteria on selective isolation medium using Aroclor 1254 and Aroclor 1260.

3. Purify and characterise the selected bacterial isolates.

4. Quantification of PCB degradation using various analytical techniques.

CHAPTER 2: LITERATURE REVIEW

2.1. Pollution

The quality of life today is inexcusably linked to the quality of our environment. The negligence and carelessness of man has contaminated the earth's water, land and air (Fig. 2.1 and 2.2.). Wastes from industrial activities have accumulated over the globe becoming an environmental hazard. Continual, environmental pollution due to industrial activities and the effect on human and animal health issues has led to international efforts to remedy these concerns (Wikipedia, 2006). Water is essential for sustaining the source of life, development and our environment on this planet. In South Africa and around the world water quality is drastically deteriorating due to contamination by organic substances. The increase in water pollution by these toxic organic compounds is attributed to an increase in the use of chemicals and the advancement of industrial technology.

Figure 2.1.: Water Pollution (Wikipedia, 2006)

Figure 2.2.: Litter in the river bed (Wikipedia, 2006)

2.2. Environmental Water Pollution

Water pollution is the most controversial problem to humankind due to its natural need by all living organisms on earth. Most of the water pollution is caused by adverse human activities. Organic wastes such as sewage and farm wastes impose a high oxygen demand on receiving waters leading to oxygen depletion which impacts on the ecosystem causing eutrophication, an overload of nutrients. Industrial companies discharge a variety of pollutants including heavy metals, organic toxins, oils, nutrients, and solids (Hardman, 1993). Discharges can also have thermal effects, especially those from power stations, and these too reduce the available oxygen. Many municipal water supplies in developed countries can present health risks. Most water pollutants are eventually carried by the rivers into the oceans. In some areas of the world the influence can be traced hundred miles from the source (Abel, 1996).

Pollutants may be synthetic or natural compounds at elevated concentrations which are toxic and capable to disrupting the ecosystem. It has been recorded in history that pollutants of this nature accumulate within the biota and contaminate the human food source. Pesticides, industrial discharges and Dichloro-diphenyltrichloroethane (DDT) are among the low level toxins that accumulate in the environment. Polychlorinated Biphenyls (PCBs) are currently a global concern due to its persistent nature. PCBs have come into contact with humans and this is due to the widespread use and disposal of PCB equipment and supplies (Hardman, 1993).

2.3. Wastewater Treatment

When cities began to grow with industrial developments, water pollution became a concern. Sewers and pipes were initially constructed to redirect polluted water into the adjacent streams and rivers (Hammer, 1977). With the increase discharges and municipal wastes being disposed in rivers and streams, enteric diseases became infectious. Sand

filtration controlled the load and eliminated the diseases however as the city grew a more efficient system was needed (McKinney, 2004).

There are three basic wastewaters to be managed. Storm water from precipitation, domestic sewage wastewater from homes and industrial wastewater from manufacturing of industrial products. Each has a major concern for the world's water treatment systems (Hardman, 1993). The first step was characterizing the wastewater and each of the pollutants and combine physical, chemical and biological process to treat. Wastewater contaminated by PCBs has become a major problem. The sources are currently being studied and cleaned (Kaštànek *et al*., 2004). PCBs are adsorbed by solids and therefore they accumulate in sewage sludge. The high chlorine PCBs are present in sewage sludge, while they amount to just 35% of the total technical PCBs. Recycling of PCBs in the environment is very important and remediation of historical pollution would be necessary if the background levels found are to be reduced. Ultimately all biodegradable organic materials undergo microbial degradation and stabilization in aerobic or anaerobic zones or a combination of both (McKinney, 2004).

2.4. Industrial Wastewater Treatment

The treatment of industrial wastewater is complex due to the different compositions of each industrial manufacturer. However most of the biodegradable organic products are still being discharged to the municipal wastewater treatment plants (WWTP). The wastewater is supplemented with nitrogen and phosphorus, the pH is maintained between 6 and 9 and insoluble oils and greases are removed by pre-treatment. This decreases the toxic levels. At the municipal WWTP a balanced nutrient level is maintained for biotreatment (Hawkes, 1963). This in turn has caused numerous problems for municipal WWTP and industrial manufacturers have had to construct an industrial WWTP which has to abide to strict effluent criteria and process their residual solids. The treatment design is similar to the municipal WWTPs with the exception of specific treatment of the

chemicals and toxins. The two methods of industrial waste biotreatment are aerobic and anaerobic treatment (McKinney, 2004).

2.4.1. Aerobic Biotreatment

Small volumes of industrial wastewaters at high concentrations are aerobically biotreated. This is achieved by microbiological stabilization of nutrients in the presence of excess dissolved oxygen (DO). Protozoa clarify the effluent by consuming dispersed bacteria in a prey-predator relationship. The benefit of this relationship is the increase in settling of biological flocs by reducing the number of bacteria in the wastewater (Hammer, 1977).
Industrial WWTPs utilizes plastic media trickling filters. The advantage of this system is the ability to maximize the surface area to volume ratio which provides a greater microbial growth for organic stabilization. The filter operates as a high synthesis system, enabling soluble organic biodegradable matter to metabolize to bacteria cell mass. The suspenended solid will pass through the plastic media trickling filter (Abel, 1996). The microbial solids produced by metabolism and the suspended solids passing through the trickling filters will have to be removed in the final sedimentation tanks. The final sedimentation tanks settle the sludge solids and are further treated aerobically or anaerobically before being placed on the land (Kaštànek *et al*., 2004).

2.4.1.1. Activated Sludge

Biodegradable industrial wastewaters are treated by activated sludge. Due to the unusual organic compounds a specific microbial load is essential. The high Biological Oxygen Demand (BOD) is diluted in the aeration tank with longer raw retention periods (McKinney, 2004).

2.4.1.2. Stabilisation Ponds

The suspended solids are settled in the deeper ponds to allow more concentration of the suspended solids at the bottom. A multi-cell stabilisation pond is used to accommodate the growing number of industrial plants that require industrial wastewater treatment. In addition nitrogen, phosphorus and trace metals are added to the system for the metabolism of bacteria (Hardman, 1993). As the wastewater is retained in the stabilisation pond for a longer period these nutrients are recycled in the pond. The microbial populations during this treatment step vary due to survival and competition between the bacteria. The predominant bacteria will depend on the most abundant chemical in the industrial wastewater. The most toxic compounds will be metabolised by specific bacteria (Hammer, 1977).

2.4.1.3. Aerated Lagoons

Aerated lagoons were built ahead of the multi-cell stabilisation ponds. The ponds caused obnoxious odours. Floating surface aerators were utilised to accommodate the lack of dissolved oxygen (DO) and odours (Kaštànek *et al*., 2004). The mixing aerated cells allowed the bacteria to stabilise the wastewater and flocculate the suspended solids. The microbial load is removed in the stabilisation pond. This follows another aerated lagoon that converts the wastewater organic mater to an environmentally friendly chemical that is easier to remove (Hardman, 1993).

2.4.2. Anaerobic Biotreatment

Industrial wastewater at high biodegradable concentrations are treated anaerobically. The facultative bacteria metabolize the complex organic compounds to short chain fatty acids that are metabolized by methane forming bacteria to methane gas, carbon dioxide and water (Abel, 1996). Some of the organic compounds will be metabolized by sulphate

reducing bacteria. The metabolism by the sulphate reducing bacteria will reduce the organic matter for methane bacteria to metabolize. If the industrial wastewater contains nitrates or nitrites the facultative bacteria can reduce the organic matter available for methane bacteria. Sulphates in the wastewater stimulates sulphate reducing bacteria. The bacteria work together to maintain an anaerobic environment for wastewater treatment (McKinney, 2004).

2.4.2.1. Anaerobic lagoons

The anaerobic lagoon has been improved to a tank with plastic liners and a plastic cover with a gas collection line. The plastic liner prevents leakage into the ground while the plastic cover prevents obnoxious odours and the gas collector, collects the methane gas for reuse as an energy source (McKinney, 2004). The wastewater is retained for short periods in the tank. The excess suspended solids are removed in the treated effluent. (Hawkes, 1963).

2.5. Polychlorinated Biphenyls (PCBs)

2.5.1. Uncovering the Effects of PCB Exposure

After several years of production of PCBs, in 1933, the first signs of fatal deception occurred. Workers in a PCB production plant in U.S.A. formed acne-like "pustules" on their faces and bodies. In 1936, the women and children of these workers broke out with chloracne, a combination of blackheads and "pustules" (Katers, 2000) as shown in the Fig. 2.3.

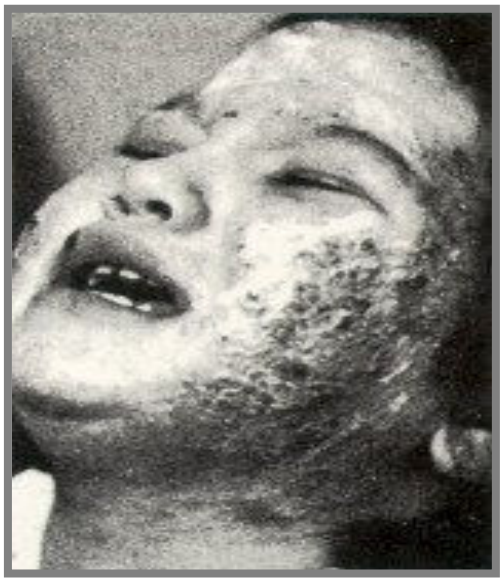

Figure 2.3.: A child in anguish due to the acne-like "pustules" on her face due to the PCB production (Katers, 2000).

PCBs were implemented in electrical equipment with a safety product tag to gain public trust. At the same time an explosion in Monsanto's chemical factory (U.S.A.) rocked the world, in 1949, exposing thousands of workers to 2,4,5-T herbicide contaminated with dioxin. By the late 1949 scientists confirmed the link between dioxin contaminated PCBs and cancers (Katers, 2000; Safe, 1991). In 1954 the greatest source of water to the surrounding areas of America was contaminated. Appleton Paper Company was producing carbon paper with PCBs and discharging the PCB-contaminated effluent into the Fox River (Francis, 1994).

In 1964 Swedish scientists found a recurring contaminant in the samples obtained from human blood. The chemical analysis identified the contaminant as a chlorine-based compound with a similar structure to dichloro-diphenyltrichloroethane or 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT). Similar chemical traces were found in wildlife and fish species, in 1935 (Katers, 2000), years before chlorine-based pesticides were introduced to the environment. Water samples were also contaminated with the same compounds in all parts of Sweden. To substantiate these findings chemicals found in Germany depicting similar chemical patterns to the contaminated samples were analysed. The chemical compositions of both the samples had corresponding gas chromatographic peaks. The chemical found was Polychlorinated Biphenyls (or then known as chlorinated biphenyls). This compound was able to move through air, water and soil and accumulate in living organisms (Cummins, 1998).

Only by 1966 did scientists become aware of the major controversy of PCBs. It was later found that PCBs "bioaccumulate in the food chain"; their biodegradability spanned over many years; they were able to move easily through the lipid portions of cell membranes and they are readily adsorbed into mammalian fat tissue. Over the next decade scientists collected substantial evidence that PCBs disrupted the food web on the planet (Milstein, 1989).

In 1968 the world fell to its knees when 1,300 people of Kyushu, Japan, were poisoned by eating rice-bran oil (Yusho) contaminated by the PCB fluids. The immediate health symptoms were failing vision, severe chloracne and respiratory ailments (Buser, *et al*., 1978). Mothers delivered still born babies and babies that survived acquired PCB diseases including lung and liver cancers, damage to the liver and immune system and abnormal liver functions. Over the next several years the population had decreased by 15 times. The "Yusho Incident" statistically showed an increase in lung cancer, internal tumours, birth defects, and severe chronic effects (Cummins, 1998).

The Japanese outbreak did not hinder the PCB industries as Therminol heat-transfer fluid, which was used inside coils in heat-deep fryers, was the next PCB household seller. This

PCB based product was what poisoned the Japanese and was now being sold in America. Soon people became extremely sick and between 1969 and 1971, nine major food contaminations had occurred across the world (Cummins, 1998). Clearly, the persistent presence of PCBs was a universal affect on the world's environment. This lead to investigations into the PCB chemical industry (Francis, 1994; Katers, 2000).

In 1970 the proposal of banning PCBs was initialised and in 1976 "Toxic Control Act of 1976" was imposed on all PCB manufacturers. It was found that the Sauget plant in Illinois (U.S.A.) contained 74,000 parts per million (ppm) of PCBs. An estimated 13 tons of chemical wastes leached out of the Monsanto company PCB plant into the Mississippi River each year. The incredible task to clean the PCB mess began in 1976. Further scientific investigations revealed that Aroclor 1254 (PCBs at 54% chlorine weight) at 10 ppm caused severe tumours. In 1971 it was discovered that when PCB oil was heated dibenzofurans or furans, were formed which is a highly poisonous compound (Milstein, 1989).

2.5.2. The Ban is imposed but the Damage is revealed

In 1973 laws were regulated to minimise concentrations of PCBs in food, water and dairy products. But in 1975 a small farm in Bloomington was contaminated with 100 tons of PCB sludge. The soil contaminated with 50 ppm of PCBs and the milk from the cows contained 5 ppm of PCBs. In 1979, manufacture, sale and distribution of PCBs were banned. A survey was performed on New York's drinking water and the results confirmed a PCB contamination of 100 ppm (EPA press release, 1979). In 1983 the Federal government evacuated the town of Times Beach in Missouri, because it was highly contaminated with dioxins, PCBs and pesticides. The levels of PCBs in Marine mammal's especially Mediterranean blue-white dolphins contain levels of 833 ppm of PCBs in their blubber (ATSDR, 2000). The PCB effect on humans was now being closely monitored revealing birth defects to be on the increase. Transfer of PCBs into the womb left the babies with loss of muscle tone, weakened reflexes delay in development

at early ages and poor visual and memory systems. A study found that PCBs were linked to problems associated with hormone-like effects and probable disrupters of the human endocrine system (U.S.A. EPA, 2000a).

Research broadened from 1990, and it was found that people living in the arctic who ate fish, seal and whale meat had a higher risk of PCB accumulation. The boys living near rice paddies in Taiwan had smaller penises compared to the rest of the Taiwanese boy's. Women who ate fish gave birth to babies with low immunities, nerve damages to the brain and developing and learning defects. In 1998, four hermaphroditic polar bear cubs were found. For over three years polar bears had been found with both male and female sex organs (Milstein, 1989).

In 2000 the United Nations Environment Program committee called 120 global nations, including South Africa for an international negotiation treaty. The treaty proposed to legally ban and reduce 12 persistent organic pollutants (POPS) in the world, among them PCBs, Dioxins and Furans are listed. Fifty countries signed this treaty on the 22-23 May 2001 (Francis, 1994; Katers, 2000)

2.5.3. The Chemical Structure of PCBs

A synthetic aromatic family of 209 congeners, Polychlorinated biphenyls (PCBs) are synthesised by chlorination of biphenyls using ferric ion or iron-catalysis. PCBs consist of hydrogens, carbons and chlorines, as shown in Fig. 2.4., (Fisher Environmental, 1997). A PCB "congener" is used to define the total number of chlorine substituents and positions, example: 4,4'-Dichlorobiphenyl is a congener consisting of two-chlorine substituents attached to the biphenyl structure (U.S.A. EPA, 2000a). The industrial compounds are composed of two benzene rings linked at the C-1 and C-1', carbons. Other carbons carry from one to ten chlorine or hydrogen atoms (Friend and Franson, 1997; U.S.A. EPA, 2000a; Sierra, *et al*., 2003). The physical structure of a PCB molecule is similar to 1.1'-(2,2,2-trichloroethylidene) bis [4-chloro-benzene] or Dichloro-

diphenyltrichloroethane, notoriously known as DDT. PCBs and DDT are both aromatic hydrocarbons and are referred to as chlorinated hydrocarbons (Friend and Franson, 1997).

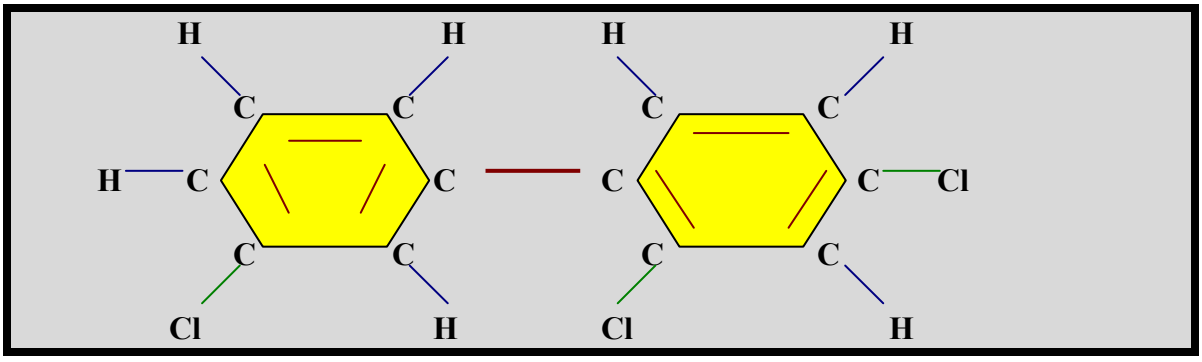

Figure 2.4.: Example of a PCB molecule (Fisher Environmental, 1997)

2.5.4. Physical and Chemical Identification

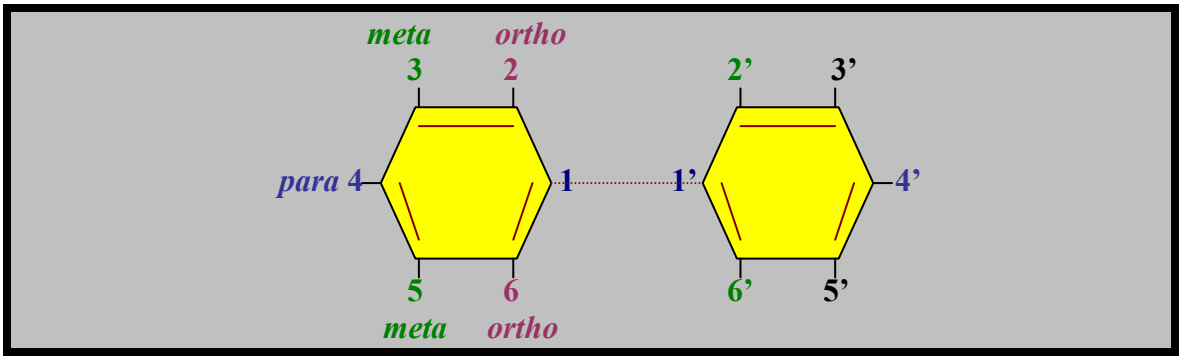

Figure 2.5.: Nomenclature for PCB (Wiegel and Wu, 2000)

The addition of chlorine (Cl_2) to a biphenyl ($C_{12}H_{10}$) forms a dual-ring with two 6-carbon benzene rings linked by a single carbon-to-carbon bond as seen in Fig. 2.4. This versatile aromatic ring allows various attachments and substitutions. Reference to the various PCBs are made in terms with the number of chlorines: a single chlorine attachment is referred to as a Monochlorobiphenyl or Chlorobiphenyl, two chlorine attachment is

referred to as a Dichlorobiphenyl, thereafter three to ten attachments are referred to as Tri…., Tetra… to Deca… (U.S.A. EPA, 2000a). The degree of chlorination brought the term "homolog" which is used to refer to a group of PCBs with a equal number of chlorines, example: Trichlorobiphenyls or Tri-PCBs or Tri-CBs or Tris, all have three chlorine substituents that are arranged at different positions on or around the benzene ring. PCB molecules with different substitution positions of chlorines or hydrogens are called isomers (e.g. dichlorophenyl homolog contains 12 isomers), (Barbalace, 2000).

The nomenclature for a PCBs molecule is shown in Fig. 2.5. The positions of the chlorines are expressed by the relationship to the carbon-to-carbon bond between the aromatic rings (Barbalace, 2000). This is how the two side chains or substitutions are named: positions 2, 2', 6, and 6' are called *ortho* positions, positions 3, 3', 5, and 5' are called *meta* positions, and positions 4 and 4' are called *para* positions (ATSDR, 2000).

The chlorine position determines the degree of toxicity of a PCB. If there are two chlorines, both in the *ortho* positions, the PCB congener is less toxic. However a congener with six chlorines and no *ortho*-chlorines allows the PCB congener a higher toxicity and the ability to bioaccumulate in species. The toxic "dioxin-like" compounds are formed when the chlorines are positioned on the *para* positions and two chlorines on the *meta* positions (Wiegel and Wu, 2000).

2.5.5. Physical Attributions

Polychlorinated biphenyls were manufactured as mixtures of 60 to 90 different congeners to create the "inevitable" indestructible properties. The PCB mixtures range from light coloured liquids, amber, yellow and black, with varying viscosities, some are oily molasses, others are sticky, brittle and still others exist as vapours in the air. PCBs have a variety of "suitable" properties. They are fire-resistant, thermo-stable, do not conduct electricity and have a low volatility below 40ºC. However they are very volatile when an electrical equipment containing PCBs is overheated. PCBs are also soluble in most

organic solvents but are nearly insoluble in water (Fisher Environmental, 1997; Barbalace, 2000). Their insolubility in water decreases with the increase in numbers of chlorines present on the biphenyl ring. However, a chlorobiphenyl containing 10 chlorines Decachlorobiphenyl is readily soluble in water due to the positions of the chlorines on the biphenyl ring. Largely it is known that solubility of PCBs depends on the environment. The aquatic and terrestrial environments play an important role in determining the fate and transport of PCBs in the environment. The fate PCBs is dependent on the temperature, pH and atmospheric pressure of a habitat (Nagpal, 1992).

2.5.6. PCB Mixtures: Aroclors

The industrial manufacturers combined mixtures of congeners and experimented with batch chlorination of biphenyls to achieve a target percentage and weight of chlorine to form Aroclors (U.S.A. EPA, 2000a). The numerous Aroclors were identified according to the number of carbons and chlorines attached to the Aroclor (PCB mixture), example Aroclor 1254 has 12 carbons (6 in each phenyl ring) and the last two digits indicate the average weight of the chlorine substituents i.e. 54%. Others include Aroclor 1221, 1232, 1242, 1248, 1260, 1262, 1268 (Safe, 1991; Barbalace, 2000). This study focused on two of the Aroclors i.e. 1254 and 1260, due to their high toxicity to humans and the high concentrations of both these Aroclors in industrial wastewater.

2.5.6.1. Aroclor 1254 and Aroclor 1260

These Aroclors were used in hydraulic fluid, rubber-plasticizers, synthetic resin, adhesives, inks, etc. although production was banned in 1979 most Aroclors are still used in electric capacitors, transformers, vacuum pumps and gas-transmission turbines (Chemical Fact Sheet, 2000).

Two common industrial, toxic PCB compounds which are the focus of the study are Aroclors 1254 and Aroclor 1260. Aroclor 1254 is a mixture containing approximately 21% $C_{12}H_6Cl_4$, 48% $C_{12}H_5Cl_5$, 23% $C_{12}H_4Cl_6$, and 6% $C_{12}H_3Cl_7$ with an average chlorine content of 54%, and 1260 is a mixture containing approximately 38% $C_{12}H_4Cl_6$, 41% $C_{12}H_3Cl_7$, 8% $C_{12}H_2Cl_8$, and 12% $C_{12}H_5Cl_5$ with an average chlorine content of 60%, (USAF, 1989). These are just two of the highly chlorinated PCBs which are persistent and toxic to mankind (Pieper, 2004; Yadav *et al*., 1995). Aroclor 1254 and 1260 are mixtures of chlorobiphenyl congeners and are mainly found in the soil and the water environments. Their current release into the environment is from PCB waste materials and product landfills, incineration of municipal refuse and sewage sludge. The experimental screening process of Aroclor 1254 and 1260 show a high resistance to biodegradation with an extremely slow-to-no biodegradation in the environment (Gray, 2004). They have a high affinity to soil particles and adsorb to the deeper soil sediment levels. However, due to the stability of Aroclor 1254 and 1260 and loss of chlorine weight by volatilisation the Aroclor eventually leaches into the water. Aroclor 1254 and 1260 are persistent in water matrixes (Sinkkonen and Paàsivirta, 2000). This poses a huge threat to aquatic organisms as Aroclor 1254 and 1260 bioconcentrates at high levels in most fish (Chemical Fact Sheet, 2000).

Aroclor 1254 and 1260 are chemically stable and physical removal from the air is achieved by a wet and dry deposition. In water, Aroclor 1254 and 1260 are biodegraded by reductive dechlorination process using anaerobic microorganisms isolated from aquatic sediments at a very slow rate (Gray, 2004).

2.5.7. Transport of PCBs in the Environment

During 1929 and 1978 the United States, Japan and Europe manufactured 635 million kg of polychlorinated biphenyl each. It was estimated that 31% was released into the environment (Rodrigues *et al.*, 2006).

Environmental PCB contamination tends to be around industrialized areas, like the Great Lakes. PCBs enter the general environment mainly through waterways, by leakage of supposedly closed systems, from landfill sites, incineration of waste, agricultural lands, industrial discharges and sewage effluents. PCBs are also widely dispersed in the atmosphere where they are transported by winds and fall to the surface in precipitation. Approximately 98%, of the PCBs entering the ocean are currently from the atmosphere. Factors such as air temperature, wind speed, storm frequency, rainfall rates and the volatility of individual PCB isomers influence the pattern and rates of PCB movement in the atmosphere (Jones *et al*., 1991). The principal transport route for PCBs through aquatic systems is from waste streams into receiving waters, with further downstream movement occurring by solution and re-adsorption onto particles as well as by the movement of sediments. This leaves the marine environment as one of the final sinks for PCBs. This entry can be from direct pollution such as a company dumping waste into a river or indirect through air pollution. From the air, they can settle onto grasslands, farms, and backyards. Eventually the PCBs then find their way into waterways and to the oceans where the compounds are transported around the planet by ocean currents and in the tissues of migrating fish.

The Inuit Eskimos have been known to carry some of the highest PCB concentrations in their bodies due to their high fish diet. Although not water soluble, PCBs do dissolve in oil and fats making marine mammals a high-risk group. Since they cannot detoxify the chemical from their bodies, mammals store it in their fat and blubber (ATSDR, 2000).

2.5.7.1. Aquatic Systems

PCBs are found in higher concentrations in the sediments of aquatic systems due to their chemical and physical properties, which cause high sorption reactions. Sorption increases with chlorine content, surface area and with the organic content of the sorbent. Therefore, PCBs adsorb onto falling sediments that eventually end up as bottom sediments. PCBs are associated particularly with suspended sediments of a diameter less than 0.15mm (U.S.A. EPA, 2000a). The release of PCBs from sediments to overlying waters can occur by slow desorption, especially when PCB concentrations are high or when sudden hydrographic activity like flooding or dredging causes sediments to be re-suspended and redistributed. Translocation can also occur through biological activity. Desorption of PCBs from particulate is more likely to occur from lower chlorinated, more water-soluble PCB congeners (Jones *et al*., 1991).

2.5.7.2. Air Systems

Airborne contamination has been recognized as a significant source of PCB contamination in the Great Lakes Basin since the mid-1980. This is especially true for Lake Superior, in USA, which is still largely isolated from industrial and municipal sources (U.S.A. EPA, 2000a). An estimated 90% of the PCBs in Lake Superior come from that source. Many of the sources of airborne PCBs to the Great Lakes Basin, especially those with unusually high concentrations, tend to originate from the southern U.S., Mexico, and Latin America (Jones *et al*., 1991).

2.5.7.3. Soil Systems

Sorption reactions also affect transport in soils. PCBs that are adsorbed by soils, especially highly chlorinated ones, remain significantly immobile against leaching. They are also unlikely to be taken up by plants and therefore are not readily mobile in soil

systems. However, because PCBs have a moderate vapour pressure, vapour phase transport may allow for redistribution or migration through the saturated soil pores (Fitzpatrick, 2006).

2.5.8. The Effect of PCBs on Human and Wildlife

The composition of chlorinated hydrocarbons in PCBs allows their bioaccumulation in the fat of animals. This lipophillic ability of PCBs allows a high accumulation in animals on the higher level of the food chain (Friend and Franson, 1997).

2.5.8.1. Species Affected

Mammals are more susceptible to direct toxicity of PCBs than birds and invertebrates. However seabirds have been affected dramatically as their food source is predominately from the ocean. These fish-eating birds use the inland water supplies, which imposes a direct threat of PCB levels. The PCB levels induce the liver enzyme system that increases the metabolism of hormones. This has caused the thinning of eggshells of white pelicans and double-crested cormonts. Unfortunately no direct link of PCBs to these problems has been made however seabirds, chickens and doves have all some link to PCB effect on their reproduction and behaviour, lack of locomotion and muscle co-ordination (ATSDR, 2000).

To humans PCBs pose a huge threat such as enlarged livers and kidneys, spleen defects and heart problems. They have been known to interfere with tissue permeability and cardiac functions. Most disturbingly is the residues of chlorinated hydrocarbons found in the brain tissue of humans exposed to PCBs. Diagnostics have reported poor reproductive performance and immunosuppression in humans and animals at low levels over a long period of exposure to PCBs (Friend and Franson, 1997). Further implications of exposure

were linked to breast cancer, brain cancer and malignant effects to the organs in the human body (Francis, 1994).

Initially PCBs first manifested itself in humans by skin conditions such as acne and rashes by skin contact or breathing. Further effects were seen by irritation to the nose and the lungs, gastrointestinal discomfort and changes in the blood and liver (ATSDR, 2000). Later discoveries revealed the ability of PCBs to mimic natural hormones and disrupt the endocrine system (Soontornchat *et al*., 1994). This adversely resulted in thyroid inflammation, birth defects and sterility. The transfer of PCBs from a mother to its unborn child manifested when the child was born with defects in developmental growth, learning disorders and a low immune system which was linked from the transfer of breast milk to a baby. Breast milk contains a large amount of fat and PCB contamination of breast milk is estimated to be liable from over 65 varieties of PCBs (Korrick, 1998).

More experimentation revealed that mothers who ate fish from rivers contaminated with PCBs produced babies that had lower birth weights, smaller head circumferences and neonatal jaundice. The children were further scrutinised and analyses revealed low memory levels, poor eyesight, slower development rates to normal children and slower locomotive and reflex responses (Safe, 1991).

2.5.9. Conventional Treatment of PCBs

The method of incineration has caused more release of PCBs in vapour form and accumulation in sediments. Incineration was achieved at 1 200ºC to remove 99.999% of PCBs. However hazardous waste incineration causes exposure to toxic emissions and the incineration process is very expensive and time consuming (Mikszewski, 2004).

Landfilling remediation of PCBs was a mechanism that could not eliminate the possibility of environmental contamination. The apparent danger of landfilling included PCB volatilisation and lechate from the landfills into surrounding sediments and water

bodies. This process left the aquatic life contaminated with PCBs and impurities. The process to clean-up the mess was called dredging (Katers, 2000). Unfortunately, the process involves stirring of the sediment causing the resurface of PCBs and impurities increasing the level of PCBs on surface waters. The combination of dredging and incineration is proven to be effective however it will cost half a million dollars to construct an adequate size for remediation of PCBs. Other methods such as low temperature thermal desorption, extraction, chemical dehalogenation or bioremediation have been used for PCB removal (Rahuman *et al*., 2000).

Polychlorinated Biphenyls are worldwide persistent pollutants. The conventional methods such as incineration and landfilling are often prohibitively expensive. The alternative strategy of PCB removal is biodegradation by microorganisms that are capable of metabolising PCBs (Leigh *et al*., 2006).

2.6. Microbial Biodegradation

Biodegradation began in 1973 by Ahmed and Focht using *Achromobacte*r species from sewage, which could biodegrade several PCB isomers. Other microorganisms include: *Norcardia*, *Pseudomonas*, *Alcaligenes*, *Acinetobacter* and mixed anaerobic and aerobic microorganisms. One extensively studied organism that is able to effectively biodegrade PCBs is the *Rhodoccus globerulus*, isolated in 1976 and properly identified in 1993 (Waterloo, 1995). Some microorganisms are unable to utilize the carbon or energy from degrading of low weight-chlorine PCBs. They are limited to the bioavailability of PCBs to the microbes resulting in PCB sorption to organic compounds in soils and sediments. This causes unfavourable temperatures and moisture conditions and the inability of anaerobic bacteria to completely dechlorinate PCB molecules (Wiegel and Wu, 2000).

Degradation of PCBs in aquatic systems varies with salinity. PCBs readily biodegrade in fresh waters while the process is considerably slower is saltwater. Both aerobic and anaerobic biodegradation have been demonstrated to result in dechlorination of PCB

congeners. Biological dechlorination rates vary due to individual PCB congeners. An estimated aerobic biodegradation half lives of di-, tri-, and tetrachlorobiphenyls (the more readily biodegradable PCB congeners) to be six, seven, and 38 months respectively (Rahuman *et al*., 2000).

Since 1980 many microorganisms have been isolated and identified to degrade mono-, di- and tri-, chlorobiphenyls (Friend and Franson, 1997). The next discovery was the ability of co-metabolism of microorganisms to degrade PCBs. The major breakthrough was discovered in deeper parts of the sediments in the Hudson River projects. There were a few microorganisms that were able to biodegrade PCBs by reductive dechlorination in the anaerobic zones of the sediment. The degradation was specific to the *meta* and *para* bonds of highly chlorinated congeners. However different sediment samples produced different dechlorination patterns and this brought up the dependence of environmental factors contributing to biodegradation (Waterloo, 1995; Katers, 2000). Eventual isolation identified the microorganisms to be a mixed population of the anaerobic level. The by-products of the anaerobic biodegradation form stable and soluble metabolites that are more susceptible to aerobic biodegradation. Theoretically this would enable highly chlorinated biphenyls to be degraded under anaerobic conditions and the soluble products can be further degraded aerobically (Waterloo, 1995).

With further investigations it was discovered that Ortho-dechlorination of Aroclor 1260 was achieved by "spiking" methogenic sediments with brominated biphenyls (Pieper, 2004). With the aid of certain chemicals in the microbial degradation process more soluble metabolites have been formed. The emulsification of PCBs with sodium lignosulfonate enhances microbial biodegradation of Aroclor 1254 to Aroclor 1221 (a lower chlorinated biphenyl), (Nagpal, 1992).

2.6.1. Aerobic degradation

The process involves oxidative degradation of the PCB molecule via a series of intermediates. This is achieved by synergistic, aerobic microorganisms. However this process is used for the low-weighted chlorine PCBs (1-5 chlorine substituents) (Abramowicz, 1990). The aerobic biodegradation of PCBs is an enzymatic process commonly referred to as the 2,3 dioxygenase pathway. The process is initiated by the addition of oxygen atoms at the carbon 2 and 3 positions (by the enzyme biphenyl dioxygenase referred to as 2,3-dioxygenase). The result is the formation of a chlorinated 2,3-dihydrodiol that is converted by dihydrodiol dehydrogenase into a chlorinated 2,3 dihydroxybiphenyl. This compound is then transformed by the enzyme 2,3-dihydroxybiphenyl dioxygenase into a chlorinated 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid via a meta-cleavage reaction. The hydrolayse catalaysed reaction converts this compound to a chlorinated benzoic acid and a five-carbon compound (Mondello, 2002).

During lab scale investigations aerobic PCB degrading microorganisms are capable of oxidising chlorinated biphenyl rings to lesser chlorinated rings, yielding benzonate and pentonic acid derivatives. These products are often degradable by other bacteria (Rodrigues *et al*., 2006).

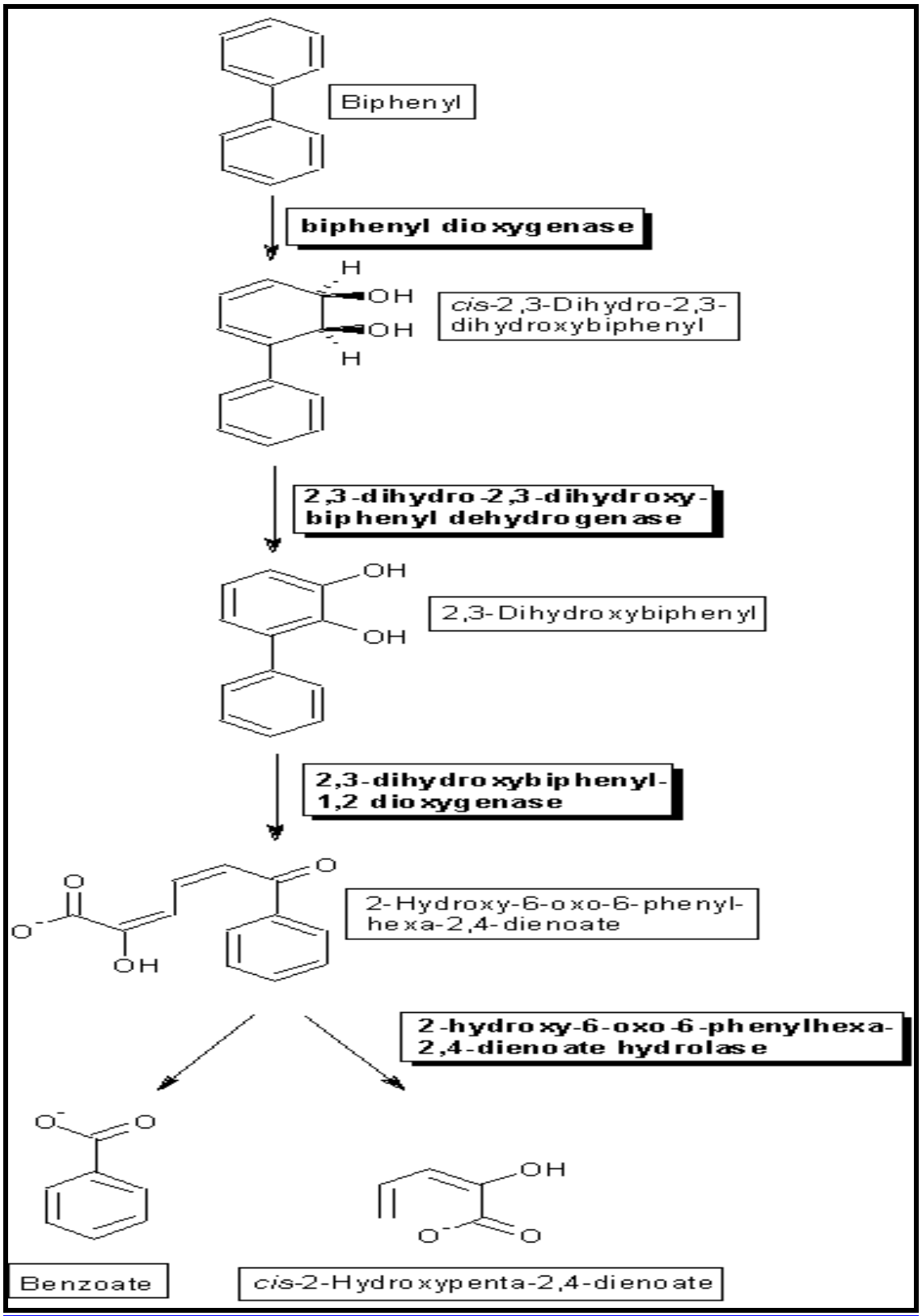

Figure 2.6.: The upper pathway for metabolism of biphenyl by *Pseudomonas* sp. strain LB400 (Mondello, 2002).

2.6.2. Anaerobic Reductive Dechlorination

This process involves the removal of the chlorine group in the form of electrons and protons. The process decreases the toxicity and increases degradability by reduction of chlorines (Abramowicz, 1990). However this process depends on temperature, pH, partial pressure of H_2 and the presence or absence of utilisable carbon sources, electron donors and acceptors as observed in the equation below. Anaerobic bacterial degradation of PCBs is not understood by a carbon source, but rather the transfer of electron acceptors. This is the chemical reaction of anaerobic PCB degradation is as follows:

$$\{CH_2O\} + H_2O + 2\ Cl^{-}\ PCB \longrightarrow CO_2 + 2\ H^{+} + 2\ Cl^{-} + 2\ H^{-}\ PCB$$

The equation is interpreted by the final PCB product with a monochlorinated biphenyl (**PCB**). If oxygen is supplied with the addition of water as seen in the equation above, the final product is a dechlorinated hydrocarbon (**2 H^{-} PCB**), (Mondello, 2002).

2.7. Analytical Methods for Detection of PCBs

Chlorinated organics constitute half of the organic pollutant problems on earth (Tiedje *et al*, 1993). The environmental methods have been modified with time to ensure efficient detection limits, precision and accuracy. There are many federal agencies that have developed these techniques based on three decades of experimentation. These Federal associations are the Environmental Protection Association (EPA), The National Institute for Occupational Safety and Health (NIOSH), The Association Official Analytical Chemists (AOAC) and The American Public Health Association (APHA) (ATSDR, 2000; U.S.A. EPA, 2000b).

There are several steps to ensure approximate precision for PCB detection, but the main steps are sample collection and storage, extraction, cleanup and determination. Sample collection has to be maintained according to regulation to prevent contamination of the

sample from impurities that are able to interfere with the accuracy of detection. Therefore all equipment has to be contaminant free (U.S.A. EPA, 2000b).

Separation of PCBs from the sample can be performed by a solid-phase extraction (SPE), separatory funnel extraction, continuous liquid/liquid extraction (CLLE), Soxhelt extraction or Soxhlet/Dean-Stark extraction (Bernhard and Petron, 2001). However due to the volatility and varying solubility of PCBs, problems occur during extractions whereby the sample evaporates or is contaminated and a great concentration is lost. This is where the cleanup step is vital. A compound that is present in the sample and has the potential to interfere with the analysis is eliminated with chromatography using techniques such as gel-permeation, silica gel, activated carbon or high-performance liquid chromatography (HPLC), (EPA, 1998a). Cleanup also fractionates the PCB groups into their non-*ortho* and mono-*ortho* Chlorobiphenyls. Gas Chromatography has been successfully used to identify and quantify PCBs. The capillary or high-resolution gas chromatography (HRGC) has been modified to separate many PCB congeners. The Gas Chromatography/Electron Capture Detector (GC/ECD) has accomplished the separation of PCBs based on the Gas Chromatography/ Mass Spectrometer (GC/MS) experimentation (ATSDR, 2000).

For Aroclors a packed column chromatography was developed with the Webb-McCall technique to identify each Aroclor. This technique was based on weight percent and homolog identification. Methods such as the comparative chlorobiphenyl environmental patterns are still used today (Zaater *et al*., 2005). However the technical standard mixtures may vary as different sources, diffusion rates and formulations do not resemble the original. Generally the maximum detection limit for Aroclor in water is between 0.054-0.90µg/L and Aroclor in soil ranges between 57-70µg/kg. Another method of determination is congener-specific analysis by the combination of HRGC and High Resolution Mass Spectrometry (HRMS). This method accurate calculates the PCB toxic equivalency (TEQ) regulated in the EPA method 1668 (Revision A), (EPA, 1998a). This method has successfully been tested for PCB samples in soil, water and tissue. It was

used to test the level of PCBs bioaccumulated in the fish in the American Columbia River Basin, in 1996 (Zaater *et al*., 2005).

The method discussed is one of the many methods regulated for environmental samples in the EPA Method 1668 (Revision A), (EPA, 1998a). There are methods that just detect the validity of PCBs present and some methods that detect specific PCB congeners. The EPA's methods of PCBs span over a variety of techniques and protocols specific for each environmental sample analysis (EPA, 1998c).

2.8. Extraction of Polychlorinated Biphenyls

The appropriately prepared sample can be extracted by any one of a number of techniques. The main points to consider are to allow adequate time of exposure of the solvent system in the sample matrix and to limit sample handing steps, i.e. avoid filtration steps by using Soxhlet or semi-automated systems (e.g. pressurized fluid extractors, EPA method 3545A). Extractions can also be accelerated by the use of ultrasonication. Cross contamination from residues left behind by high levels of POPs in other samples is a concern at this stage and equipment must be thoroughly cleaned and checked from batch to batch (EPA, 1998b).

Purity of extraction solvents is also a major consideration. Only high purity glass distilled solvents should be used. Separation of the PCB can be relatively straightforward generally small Silica gel or Florisil columns (Zaater *et al*., 2005); (either prepared in the lab or pre-purchased) will suffice. The purpose of this step is to remove co-extractive pigments and to separate non-polar PCB from more polar POPs (HCH, most chlordanes, dieldrin/endrin). This is achieved by applying the extract in a small volume of non-polar solvent and fractionating by eluting with hexane followed by one or two other elutions of increasing polarity (EPA, 1998b).

2.9. Quantification of PCBs by Gas Chromatography/Electron Capture Detector (GC-ECD)

There are numerous chromatographic techniques and corresponding instruments. Gas chromatography (GC) is one of these techniques. Gas chromatography (GC) is the separation of a mixture of compounds (solutes) into separate components. By separating the sample into individual components, it is easier to identify (qualitative) and measure the amount (quantitative) of the various sample components. It is estimated that 10-20% of the known compounds can be analyzed by GC (Zaater *et al*., 2005). To be suitable for GC analysis, a compound must have sufficient volatility and thermal stability. If all or some of the compounds are in the gas or vapour phase at 400 - 450 ºC or below, they do not decompose at these temperatures. However the compound can still be analyzed by the GC. The introduction of chemically bonded fused silica capillary columns was a very recent innovation in gas chromatography, more commonly referred to high resolution gas chromatography (HRGC). Hybrid techniques, particularly using a mass spectrometer as a detector, GC-MS, have added a further dimension to GC analyses enabling separated compounds to be readily identified. GC is a well developed technique that is acclaimed world-wide. In the early 1980's the capillary column was developed. This lead to a significant increase in the separating power of gas chromatography (Zlatkis and Poole, 1981).

The capillary column used for this project is the Perkin Elmer OV 17 it is a proprietary bonded and crosslinked phase (Spark, 2006). The column is specifically designed for analysing chlorinated pesticides and PCBs abiding by the EPA 508, 608 and 8080 methods (EPA, 1998c). The Perkin Elmer OV17 is composed of phenyl and methyl compounds. These phases have been specifically designed for the analysis of volatile organic compounds and pesticides, as specified in many EPA Methods including 502.1, 502.2, 503.1, 524.1, 524.2, 601, 602, 608, 624, 8010, 8011, 8015, 8020, 8240, and 8260 (EPA, 1998c).

2.10. Persistent Organic Pollutants (POPs) in South Africa

South Africa is among the world's top ten "Toxic Hot Spots". The global effect of Persistent Organic Pollutants (POPs) was discussed in Johannesburg, South Africa, on the 22nd of May 2001 at the United Nations Convention on Persistent Organic Pollutants. South Africa was among the countries that signed the United Nations Convention (GroundWork, 2001).

South Africa produces an estimated 2 million tons of waste a year and imports toxic waste from abroad. The waste is disposed on land with 95% disposal in Gauteng. Most of the wastes in South Africa are incinerated releasing over 200 toxic substances among them are furans and dioxin into the atmosphere. These emissions are released into the air and contaminating the land and water (GroundWork, 2001). These emissions have, also been found at high concentrations in Durban, South Africa. However a few companies have cancelled the incinerator plans to a cleaner technology to prevent further pollution of the environment (GAIA, 2002).

2.11. Conclusion

Wastewater treatment plants are a good source of indigenous bacterial isolates that are applicable for cost-effective and efficient degradation of contaminates in the environment. Currently there has been no notable research, with regard to microbial degradation of PCBs in S.A. There are many advantages of using microorganisms and their enzymes to catalyse, dehalogenate and degrade sites and waters polluted with toxic chlorinated hydrocarbon compounds.

Microbial degradation of PCBs has been extensively investigated around the world (Ahmed and Focht, 1973; Abramowicz, 1990; Wiegel and Wu, 2000). However the complexity of the metabolic pathways have prevented or apprehended the optimization of bacterial species for effective microbial transformation of PCBs. Although various

approaches have been taken to optimize degradation of PCBs it is limiting. PCBs in the environment are complex mixtures and the catabolic enzyme for each congener is different. However it has become apparent that microorganisms are the focus of PCB biodegradation. The complex congener mixtures would require a PCB-utilising population with their complex metabolic interactions. This has to begin with the study of a single organism to understand the entire community (Bokvajová *et al*., 1994).

Thus this study aimed to isolate potential PCB degraders from the environment and investigated the potential to degrade PCBs.

CHAPTER 3: ENRICHMENT, ISOLATION AND SCREENING

3.1. Introduction

The interaction of microorganisms with the environment has allowed their constant change to their environment. The environmental factors exerted on microorganisms influence a selective population in a specific environment. This adaptive response has been harnessed by environmental studies (Wainwright, 1999). Natural selection of microorganisms with a particular characteristic is enriched and screened with the required selective conditions. This favours natural evolution with the desired traits. The microorganisms are adaptive and tend to be metabolically versatile. Their enzymes and metabolic transport system have a high affinity to constant change to favour the microorganisms, survival. The aim is to manipulate the microorganism's environment to select and isolate the most effective organism within a population for the most effective trait (Hardman *et al*., 1993).

To achieve isolation of PCB degrading microorganism's enrichment is employed. Enrichment is essential for PCB-degrading communities as the densities would be too low for effective isolation (Wagner-Döbler *et al*., 1998). Purification is the next step of isolation which entails the sub-culturing of the bacteria to a specific media allowing the specific traits to be highlighted. The Gram stain is routinely used as an initial procedure in the identification of an unknown bacterial species. It is used to classify bacteria on the basis of their forms, sizes, cellular morphologies, and gram reaction. It is a critical test for rapid presumptive diagnosis. (Prescott *et al*., 1996). Bacteria bear a slight net negative charge and usually bind positively charged dyes such as methylene blue and crystal violet. A species can be classified as Gram positive, Gram negative, or Gram variable depending on the ability of cells to retain the blue dye. Gram negative bacteria do not retain the dark blue colour, but can be counterstained a light red so that they can be seen in bright field microscopy. Since two dyes are used to distinguish types of bacteria, Gram staining is called a differential staining method (Benson, 1998).

Selection is based on the organism's physiological ability in the environmental sample. Therefore the microorganism's characteristics are investigated for proper enrichment conditions. The selection procedures of the microorganisms are subjected to different concentration (Hardman *et al*., 1993). This study utilizes Aroclor 1254 and Aroclor 1260 as carbon and energy sources at different concentrations to select PCB-degraders.

3.2. Methodology

3.2.1. Chemicals

Pure Aroclor 1260 (98% purity), Aroclor 1254 (98% purity) and biphenyl were obtained from Supelco® (Durban, S.A.) and Chem Services, Separations (Gauteng, S.A.). The media components, chemical components and HPLC grade n-hexane were purchased from Merck Supplies (Durban, S.A.). Stock solutions for the Aroclors and biphenyl and were prepared in HPLC grade n-hexane and refrigerated.

3.2.2. Stock Solution of Aroclor 1254 and Aroclor 1260

The Aroclor 1254 and Aroclor 1260 were purchased as a 50mg (dehydrated form) each. The dehydrated Aroclors were made-up to 10mL HPLC grade n-hexane. The stock of Aroclor 1254 and Aroclor 1260 was therefore 1mL stock solution from 50mg in 10mL of HPLC grade n-hexane.

3.2.3. Sample Collection and Culture Purification

The samples were collected in a hexane-rinsed (to ensure no other compounds remained to interfere with the sample) amber 500mL Schott glass bottles from the aerobic and influent zones

of two industrial wastewater treatment plants around Durban, South Africa, i.e. Aerobic zone Southern Wastewater Works (SWA), Aerobic zone Amanzitotti Wastewater Works (TTA), Influent zone Southern Wastewater Works (SWI), Influent zone Amanzitotti Wastewater Works (TTI). The samples were stored at 4^{o}C in the dark until required. The samples were serially diluted and spread plates were performed on nutrient agar with 0.2% glucose (Appendix A). The plates were incubated at 30^{o}C for 72hrs.

3.2.4. Morphological Analysis

The bacterial colonies that formed from the nutrient agar with 0.2% glucose were inoculated onto selective PCB media (Appendix B) and incubated at 30^{o}C for 72hrs. The colonies that appeared after 72hrs were morphologically analysed based on their gram reaction, shape and appearance of the colony on the selective polychlorinated biphenyl media. The bacterial colonies were continuously sub-cultured on fresh selective PCB media plates to obtain pure cultures. Purity was confirmed with regular gram staining. This procedure is based upon the Hucker modification of the original Gram's stain method (Appendix C) and microscopic examination using a Nikon Eclipse 80i microscope fitted with a digital Nikon camera and the ACT2U software.

3.2.5. Enrichment and Isolation

The bacterial colonies were inoculated on selective PCB media (Appendix B) and incubated at 30^{o}C for 72hrs. The colonies that formed were inoculated into an enriched nutrient broth supplemented with 0.2% glucose (Appendix A) for activation of bacterial growth. The 24hr culture was centrifuged, the supernatant discarded and the pellet was inoculated into 50mL selective PCB broth in a 200mL erlenmeyer flask. The bacteria were grown for four days in aerobic conditions at 30^{o}C in a shaker incubator at 140rpm. The activated cell suspension were serially diluted and spread onto nutrient agar without the additional 0.2% glucose (Appendix A)

and selective polychlorinated biphenyl media agar (Appendix B) containing 5mgmL^{-1} of Aroclor 1254 or 5mgmL^{-1} of Aroclor 1260.

3.2.6. Screening and Maintenance

The organisms were screened on the selective polychlorinated biphenyl media agar (Appendix B) containing 5mgmL^{-1}, 10mgmL^{-1} and 30mgmL^{-1} of Aroclor 1254 or 1260. The concentrations were made-up by 1mL of Aroclor 1254 and Aroclor 1260 stock solution (50mg/10mL in HPLC grade n-hexane). There were 25 bacterial isolates obtained from the enrichment process in this study. Each of these isolates were inoculated on the different PCB concentrations and observed for growth rate.

The 10 isolates which were selected based on their efficiency on the different concentrations of PCBs were qualitatively analysed, for the most efficient potential of PCB-degradation by spectrophotometric analysis using a UV/Vis Spectrophotometer (Pharmacia Biotech, Ultrospec 2000). The control was the selective PCB media either with Aroclor 1254 or Aroclor 1260 depending on the sample analysis without inoculum. The samples were initially tested for the best absorbance to achieve a standard absorbance curve for the Aroclors at the appropriate wavelength. A wavelength range from 200nm to 600nm were tested on Aroclor 1254 and Aroclor 1260. The wavelength was selected based on maximum absorbance. The wavelength selected was 300nm as the maximum absorbance (Wilson and Walker, 2000) for both Aroclor 1254 and Aroclor 1260 shown in Table 3.5. and Fig. 3.1. Aromatic compounds such as PCBs are efficiently analysed by UV/Vis Spectrophotometer (Carr *et al*., 1982).

The isolates were maintained and stored on nutrient media with 0.2% glucose slants (Appendix B) and selective media slants, refrigerated at 10^{o}C for future use.

3.3. Results

3.3.1. Morphological Analysis

After 72hrs a variety of microorganisms appeared including yeast and fungi. Only bacterial colonies that appeared on the selective media were morphologically characterised as shown in Table 3.1. The gram reaction, colony appearance and shape was investigated. The majority of isolates were morphologically identified as gram negative short-rod bacteria. There were six gram positive rods and seven gram positive cocci isolated. The isolates appeared translucent, creamy or white dot like. The isolates in Table 3.1. were categorised according to the wastewater plant (WWTP) zone names and the sample collection location it was isolated from.

Zone names and location of the sample collection

1. Southern Wastewater Works aerobic zone (SWA)
2. Amanzitotti Wastewater Works aerobic zone (TTA)
3. Southern Wastewater Works influent zone (SWI)
4. Amanzitotti Wastewater Works influent zone (TTI)

The focus of this aspect of research was to screen, select and isolate efficient PCB degrading organisms. Identification of the isolates using molecular methods will be the subject of future research.

Table 3.1: Gram staining and plate appearance of the isolates

No. of Organisms according to Zone	Gram Reaction	Plate appearance
SWA		
1.1.	+ cocci	Translucent, creamy
1.2.	+ rods	white-dot-like growth, clearing on plate
1.3.	+ cocci	Translucent, creamy
1.4.	- rods	Translucent, white
1.5.	- rods	Translucent, creamy
1.6.	- rod	Translucent, white-creamish
1.7.	- rod	Translucent, white
TTA		
2.1.	- rods	Translucent, creamy, clearing on plate
2.2.	- rods	white-dot-like growth
2.3.	+ rods	Translucent, clearing on plate
2.4.	- rod	White dot-like growth
SWI		
3.1.	+ cocci	Translucent, creamy
3.2.	+ rod	white-dot-like growth
3.3.	- rods	Translucent, creamy
3.4.	- rods	Translucent, white, clearing on plate
3.5.	+ cocci	Translucent, creamy
3.6.	+ cocci	Translucent, creamy
3.7.	- rod	white-dot-like growth
3.8.	+ cocci	Translucent, white
TTI		
4.1.	+ rod	Translucent, creamy
4.2.	+ rod	Translucent, creamy, clearing on plate
4.3.	+ cocci	white-dot-like growth
4.4.	+ rod	Translucent, creamy
4.5.	- rod	Translucent, white-creamish
4.6.	- rod	Translucent, white

A gram positive is represented by a (+) and a gram negative is represented by a (-).

3.3.2. Enrichment and Isolation

Table 3.2: Growth rate of isolates on Aroclor 1254 and Aroclor 1260

No. of Organisms according to Zone	Growth rate Aroclor 1254 $5mgmL^{-1}$	Growth rate Aroclor 1260 $5mgmL^{-1}$
SWA		
1.	++	++
2.	++	+++
3.	+	+
4.	++++	++++
5.	++++	++++
6.	++	+++
7.	++	++
TTA		
1.	++	++
2.	+++	+++
3.	+++	+++
4.	+	+
SWI		
1.	++	++
2.	++	++
3.	++	++
4.	+++	+++
5.	+++	+++
6.	++	++
7.	++	++
8.	+++	+++
TTI		
1.	++	++
2.	+++	+++
3.	+++	+++
4.	+++	+++
5.	++	++
6.	++	++

(+) low growth, (++) good growth, (+++) very good growth, (++++) best growth.

The isolates shown in Table 3.2. were isolated from the zones indicated. The 25 organisms were isolated based on their growth on 5mgmL^{-1} of Aroclor 1254 and 5mgmL^{-1} Aroclor 1260 selective

PCB media. Organism SWA 2 and SWA 6 grew better in Aroclor 1260 than Aroclor 1254. Organisms SWA 1 and SWA 7 had moderate growth on both Aroclors while SWA 3 had very

poor growth on both the Aroclors. The TTA 1 in zone TTA grew moderately compared to the very poor growth of TTA 4. However TTA 2 and TTA 3 had a good growth on both the Aroclors. Organisms SWI 1, SWI 2, SWI 3, SWI 6 and SWI 7 had an average similar growth on both Aroclor 1254 and Aroclor 1260. while organisms TTA 4 and TTA 5 had good growth for both the Aroclors. The TTI zone had similar average growth for SWI 1, SWI 5 and TTI 6, although SWI 2 SWI 3 and SWI 4 had good growth on both the Aroclors. Organisms SWA 4 and SWA 5 had an overall efficient growth on both Aroclor 1254 and Aroclor 1260. Their ability to grow in the presence of both the Aroclors indicated that these organisms were capable of efficient degradation of PCBs. The following 10 organisms: SWA 4, SWA 5, TTA 2, TTA 3, SWI 4, SWI 5, SWI 8, TTI 2, TTI 3 and TTI 4, were selected from a total of 25 initial isolates for further investigations. The 10 isolates were selected based on their growth rate and maintenance on the selective PCB-media investigated in Table 3.2. The remaining 15 microorganisms were stored and maintained in nutrient agar with 0.2% glucose media (Appendix A), at 10^{o}C, for further studies.

3.3.3. Screening of isolates

3.3.3.1. Screening based on growth rate on different concentrations of Aroclor 1254 and Aroclor 1260

Table 3.3: Growth rate of isolates in 5mgmL^{-1}, 10mgmL^{-1} and 30mgmL^{-1} concentration of Aroclor 1254

Organism	5mgmL^{-1}	10mgmL^{-1}	30mgmL^{-1}
SWA 4	++++	++++	+++
SWA 5	++++	++++	+++
TTA 2	+++	+	+
TTA 3	+++	++	+
SWI 4	+++	+	+
SWI 5	+++	++	+
SWI 8	+++	++	+
TTI 2	+++	++	+
TTI 3	+++	++	++
TTI 4	+++	+	+

(+) low growth, (++) good growth, (+++) very good growth, (++++) best growth.

Table 3.4: Growth rate of isolates in 5mgmL^{-1}, 10mgmL^{-1} and 30mgmL^{-1} concentration of Aroclor 1260

Organism	5mgmL^{-1}	10mgmL^{-1}	30mgmL^{-1}
SWA 4	++++	++++	+++
SWA 5	++++	++++	+++
TTA 2	+++	+	+
TTA 3	+++	++	+
SWI 4	+++	+	+
SWI 5	+++	++	+
SWI 8	+++	++	+
TTI 2	+++	++	+
TTI 3	+++	++	++
TTI 4	+++	+	+

(+) low growth, (++) good growth, (+++) very good growth, (++++) best growth.

SWA = Southern Wastewater Works Aerobic zone

TTA = Amanzitotti Wastewater Works Aerobic zone

SWI = Southern Wastewater Works Influent zone

TTI = Amanzitotti Wastewater Works Influent zone

The 10 efficient selected isolates (SWA 4, SWA 5, TTA 2, TTA 3, SWI 4, SWI 5, SWI 8, TTI 2, TTI 3 and TTI 4) as shown in tables 3.3. and 3.4. The isolates were screened by exposure to Aroclor 1254 and Aroclor 1260 at different concentrations of $5mgmL^{-1}$, $10mgmL^{-1}$ and $30mgmL^{-1}$. All the isolates maintained growth on different concentrations of Arolor 1254 and Aroclor 1260. The isolates TTA 2 to TTI 4 shown in Tables 3.3. and 3.4. had good growth on exposure to $5mgmL^{-1}$ Aroclor 1254 and $5mgmL^{-1}$ Aroclor 1260. However the increase PCB concentration of $10mgmL^{-1}$ and $30mgmL^{-1}$ decreased the growth of organisms TTA 2 to TTI 4. The two isolates SWA 4 and SWA 5 had a high growth on exposure to the different Aroclor concentrations. Compared to the other isolates SWA 4 and SWA 5 had a higher growth with the increased concentration of Aroclor 1254 and Aroclor 1260. Therefore isolates SWA 4 and SWA 5 were selected for GC-ECD analysis to further evaluate their degradation potential.

3.3.3.2. Screening by Spectrophotometric Analysis

The 10 efficient selected organisms (SWA 4, SWA 5, TTA 2, TTA 3, SWI 4, SWI 5, SWI 8, TTI 2, TTI 3 and TTI 4) were spectrophotometrically analysed for evaluation of their utilisation of the Aroclors by comparison of the absorbance as seen in Fig. 3.2. A wavelength range from 200nm to 600nm was tested on Aroclor 1254 and Aroclor 1260. The wavelength was selected based on absorbance maximum which is known as the Lambda maximum (λ max), (Wilson and Walker, 2000). The wavelength selected was 300nm as the maximum absorbance for both Aroclor 1254 and Aroclor 1260 shown in Table 3.5. and Figure 3.1. The Aroclor 1254 control had an absorbance of 0.250 and Aroclor 1260 control had an absorbance of 0.270 at the wavelength 300nm. The degree of absorbance is directly proportional to the degree of PCB concentration. Organisms SWA 4 and SWA 5 showed the best potential in degrading the PCBs as they were able to utilise both Aroclor 1254 and Aroclor 1260 efficiently. The isolates had a high affinity for Aroclor 1260 especially SWA 5 with an absorbance of 0.0003 compared to SWA 4 with an absorbance of 0.0020. The comparison with SWA 4 and SWA 5 affinity to Aroclor 1254 had a slightly higher absorbance. Organism SWA 5 had an absorbance of

0.0030 and SWA 4 with an absorbance of 0.0180 for Aroclor 1254. Organisms TTA 2 and SWI 5 did show PCB potential degradation for Aroclor 1254, however the Aroclor 1260 absorbance was much too high. In comparison to organisms TTI 2 which had a very good absorbance for Aroclor 1260 with an absorbance of 0.0023 but Aroclor 1260. Clearly SWA 4 and SWA 5, had to be selected for further PCB-degradation evaluation.

Table 3.5: Standard Curve Absorbance of Aroclor 1254 and Aroclor 1260

Wavelength	**Aroclor 1254**	**Aroclor 1260**
200	0.1830	0.1040
250	0.1950	0.1320
300	**0.2500**	**0.2700**
350	0.1750	0.2040
400	0.1250	0.1270
450	0.0230	0.1050
500	0.0080	0.0310
550	0.0050	0.0650
600	0.0031	0.0020

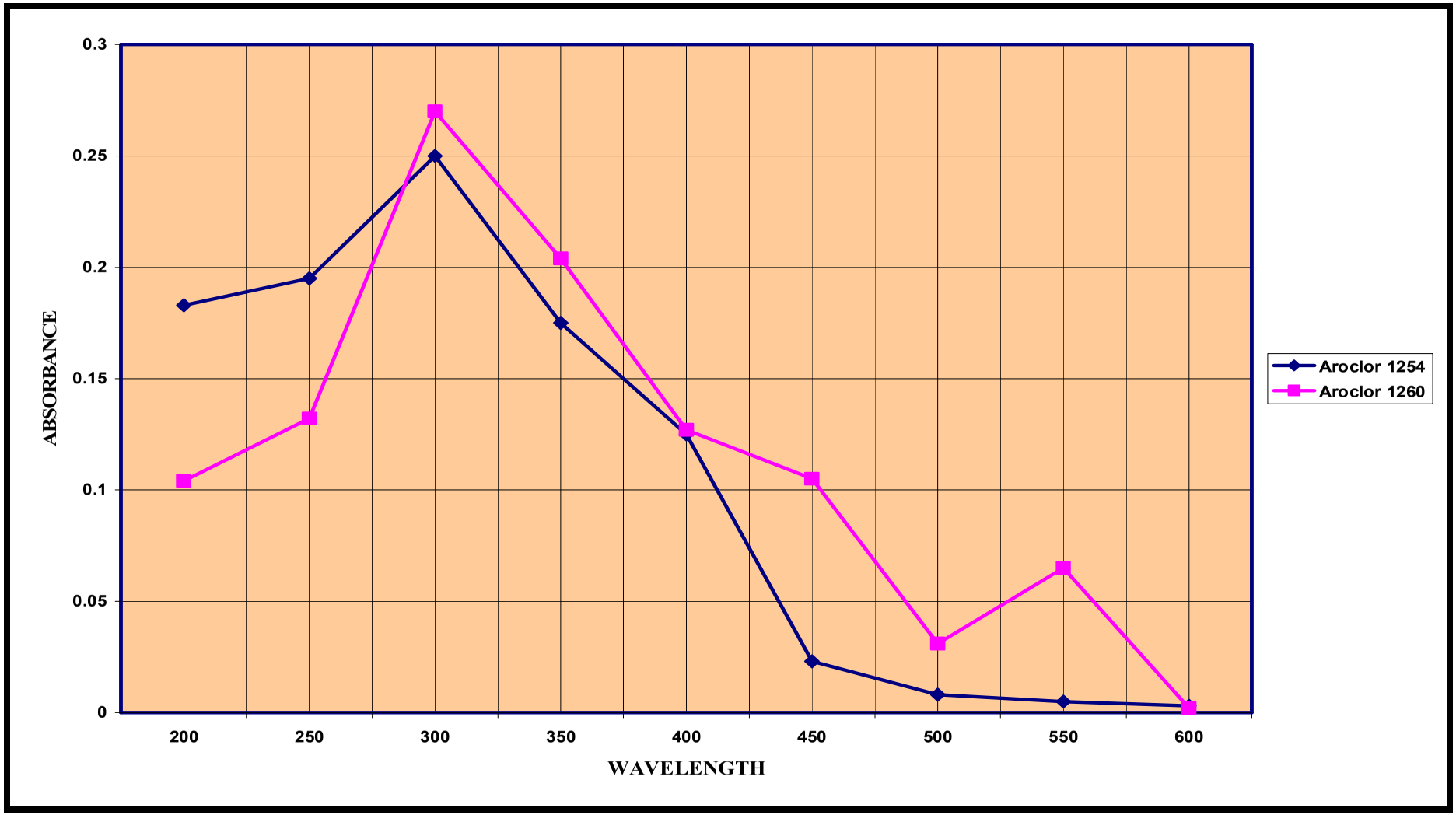

Figure 3.1: Standard Curve of Aroclor 1254 and Aroclor 1260

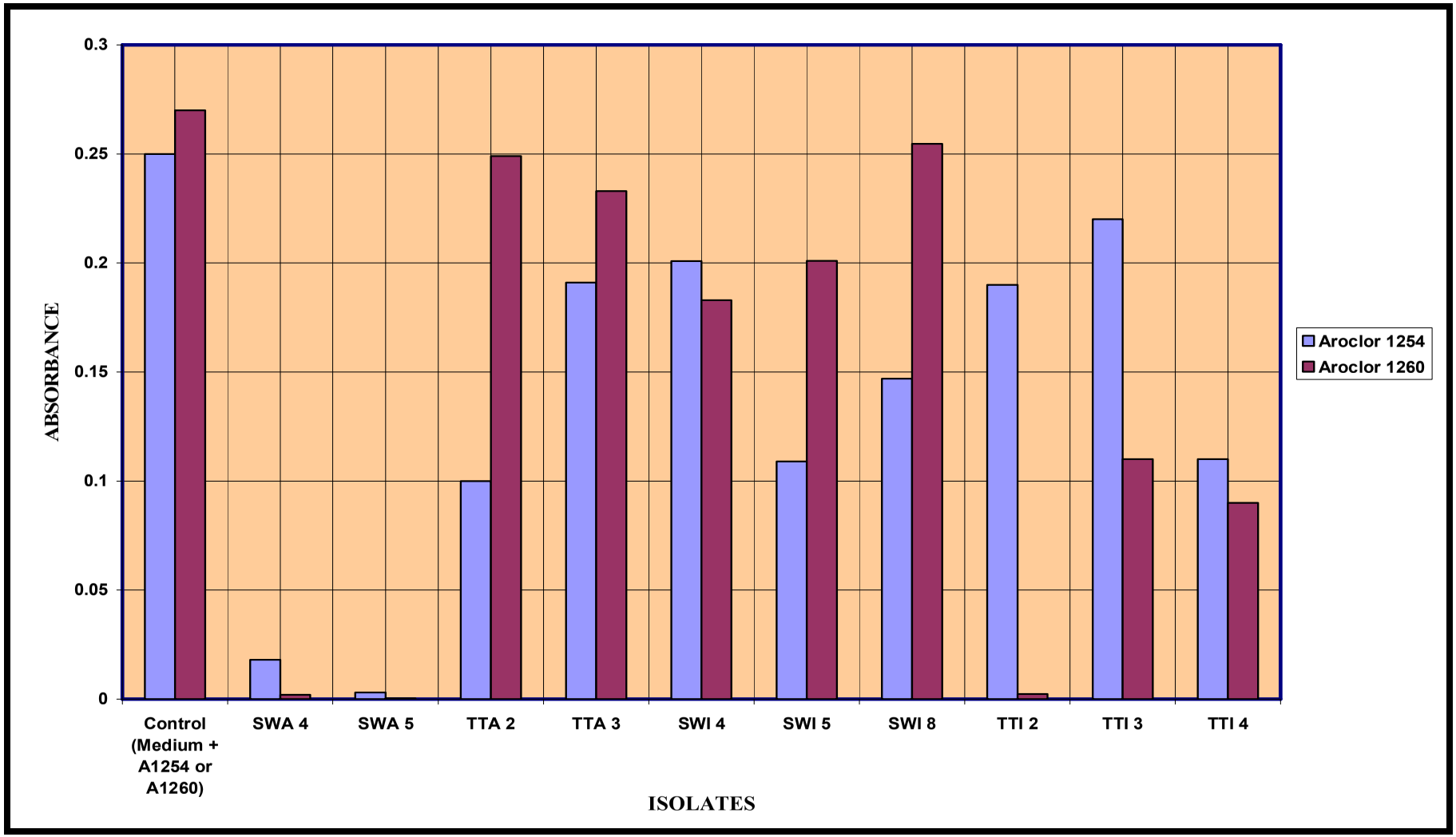

Figure 3.2: Absorbance of Aroclor 1254 & Aroclor 1260 for the ten selected isolates at 300nm

3.3. Discussion

An abundant number of microorganisms were initially able to grow on the PCB media, including yeasts and fungi. The bacteria found in wastewater are able to degrade pesticides and organic pollutants due to the number of organic pollutants entering the wastewater on a regular basis (Yadav *et al*., 1995; Geng *et al*., 2006). This has allowed the bacteria to utilise the organic pollutants as a food source to survive (Abel, 1996).

The bacteria from the spread plates were selected based on their morphology i.e. the gram reaction and colony morphology on nutrient agar plates. There were 25 bacteria selected and purified as seen in Table 3.1. There were 12 isolates that were morphologically identified as gram negative short-rod bacteria while six were gram positive rods and seven were gram positive cocci (Table 3.1.). Most PCB-degrading isolates are characterized as Gram-negative bacteria (Maeda *et al*., 1998). The studies found compared to the isolates found in literature clearly establish that the selected isolates are potential PCB-degraders (Wagner-Döbler *et al*., 1998).

The 25 organisms were exposed to 5mgmL^{-1} Aroclor 1254 and 5mgmL^{-1} Aroclor 1260. Organisms SWA 4, SWA 5, TTA 2, TTA 3, SWI 4, SWI 5, SWI 8, TTI 2, TTI 3 and TTI 4 had good growth on both Aroclor 1254 and Aroclor 1260 compared to the remaining isolates in Table 3.2. The growth can be related to the stress proteins produced by one stimulus that can induce other stresses to the microorganisms during laboratory exposure (Wainwright, 1999). The 10 isolates listed above were analysed with 5mgmL^{-1}, 10mgmL^{-1} and 30mgmL^{-1} Aroclor 1254 or Aroclor 1260 shown in Tables 3.3. and 3.4. Isolates SWA 4 and SWA 5 were able to utilise all three concentrations of Aroclor 1254 and Aroclor 1260. However the other 8 organisms were excluded from further research within the current investigation due to comparatively poorer growth patterns. This can be attributed to the toxicity of the Aroclor or the ability of PCBs to accumulate in the cytoplasmic membrane and disrupt function (Lunt and Evans, 1970). The growth of SWA 4 and SWA 5 can be related to the oxidative stress exerted by the PCB which is related to

the degradation pathway of PCBs (Lunt and Evans, 1970). The oxidation reaction catalysed by the biphenyl dioxygenase, Fig. 2.6., is a critical component in the aerobic

degradation pathway (Mondello, 2002). The general response of the organisms to the supply of Aroclor 1254 and Aroclor 1260 confirmed that these industrial wastewater bacteria have the ability to utilise even toxic pollutants to survive. This ability has allowed microorganisms to survive the increasing complex natural environment (Chàvez *et al*., 2006).

The spectrophotometric analysis of the 10 isolates (SWA 4, SWA 5, TTA 2, TTA 3, SWI 4, SWI 5, SWI 8, TTI 2, TTI 3 and TTI 4) as seen in Fig. 3.2. and the comparative evaluation was able to confirm that SWA 4 and SWA 5 were the most efficient potential PCB degraders. Aromatic compounds such as PCBs are easily detected by a UV/Vis Spectrophotometer (Wilson and Walker, 2000). The different concentrations of Aroclors used and the spectrophotometric measurements were able to screen the most efficient potential PCB-degraders. However, isolates SWA 4 and SWA 5 were selected for GC-ECD analysis, not only for their efficiency as potential PCB degraders, but also to minimise the work load required for each isolate. This was decided as the study is a preliminary investigation of potential PCB isolates. The isolates SWA 4 and SWA 5 were further characterised by their growth kinetics and identified for future studies. The remaining 23 isolates will be used for future PCB studies.

CHAPTER 4: CHARACTERISATION OF EFFICIENT DEGRADERS

4.1. Introduction

Microorganisms exhibit diverse metabolic activities which make them ideal for transformation of a wide variety of pollutants (Wainwright, 1999). Bacterial characteristics are essential to establish the strains ability to utilise a specific carbon source. The rate of growth in a specific environment indicates the bacterial strains requirements of growth. This is required to manipulate the microorganism's desired traits to utilise or transform the specific compound (Prescott, 1996). The microorganisms are adaptive and tend to be metabolically versatile. Their enzymes and metabolic transport system have a high affinity to constant change to favour the microorganism's survival. The aim is to manipulate the microorganism's environment to select and isolate the most effective organism within a population for the most effective trait (Hardman *et al*., 1993). The evaluation is based on the response the microorganisms have to the desired compounds. The most efficient utiliser of PCBs would be easier to expose to the polluted environment for effective transformation (Chàvez *et al*., 2006).

The bacterial taxonomy is organized in groups of genera. To utilise PCBs each genera has a specific pathway to metabolise the PCB. The true bacteria are classified primarily on the basis of their morphology and physiology. The specific tests that are most useful will differ depending upon the groups investigated, but tests which are frequently useful are the catalase, oxidase, urease, and deaminase/decarboxylase reactions, as well as tests for fermentation products and nitrate reduction (Benson, 1998), these tests are made simpler by the use of API kits. Recently, molecular methods have been employed to phylogenetically classify organisms due its high degree of accuracy (El-Fantroussi, 2000).

4.2. Methodology

4.2.1. Growth Curve Analysis

The growth curve analysis was performed using the Live/Dead stain technique (Appendix D) optimized to count the selected bacteria. This method was selected based on the elimination of various growth curve techniques. The haemocytometer-microscope method was not effective as it was unable to visualize the PCB degrading bacteria due to their size. The UV Spectrophotometer method had allowed dead bacterial cells to be counted as the spectrophotometer measures turbidity and not the actual amount of viable bacterial cells.

Invitrogen LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit is a molecular probe which is universally used as a direct-count viability assay. Compared to the conventional methods of cell count such as Haemocytometer counter, Spectrophotometer and plate counts these methods abide by the metabolic characteristics and membrane integrity. The assay is sensitive to growth and staining conditions. This is due to the bacterial morphology, cytology and physiology. The Live/Dead stain allows easy, reliable and quantitative live and dead bacteria in minutes (Molecular Probes, 2001).

The Live/Dead probe utilizes a mixture of SYTO® 9 green fluorescent nucleic acid stain and red-fluorescent nucleic acid stain, propidium iodide. The appropriate mixture of SYTO® 9 and propidium iodide is essential (Appendix D) to allow the bacteria's cell membranes to remain intact for live cells (Molecular Probes, 2001). The stain fluoresces green as seen in photo-micrograph Fig. 4.1, when the cells are alive, whereas bacteria with damaged membranes, i.e. dead cells, stain fluorescent red as seen in photo-micrograph Fig. 4.2.

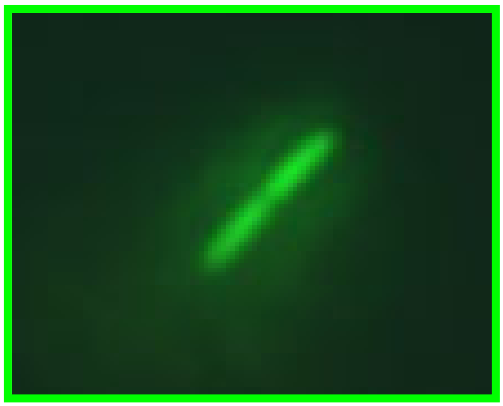

Figure 4.1: Photo-micrograph: green bacteria representing live bacteria

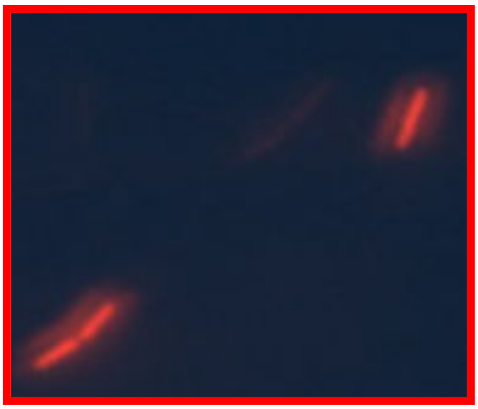

Figure 4.2: Photo-micrograph: red bacteria representing dead bacteria

The cells were grown in 250mL flasks containing the selective PCB media. Hourly counts were performed on the samples following the Live/Dead probe technique (Appendix D). A Carl (Germany) Zeiss, Axiolab microscope, fitted with epifluorescence and a Zeiss filter set. The fluorescein filter (Code: 09, BP 546 FT 580, LP 590) allows the live bacteria to appear green and the rhodamine filter (Code: 02, G 365, FT 395, LP 420), allows the dead cells to appear red. An approximate fifteen fields from each slide was counted at each hour using a KS 300 Vol. 30 imaging system software. A maximum growth of a million (10^6cfu/mL) colony forming units per millilitre (cfu/mL) was achieved at approximately 9hrs for both organism SWA 4 and SWA 5.

4.2.2. Spectrophotometric Analysis

The selected isolates, SWA 4, SWA 5 and the mixture of both the cultures were analysed by a UV/Vis spectrophotometer (Pharmacia Biotech, Ultrospec 2000) at 300nm. The investigation compared the pure cultures and the mixed cultures ability and efficiency to degrade PCBs. The control consisted of the selective PCB media with Aroclor 1254 or Aroclor 1260 (30mgmL^{-1}) depending on the sample.

4.2.3. Identification of isolates

An API 20E kit was used to identify the selected isolates (Appendix E). The system identifies Enterobacteriaceae and other non-fastidious Gram-negative rods, (Appendix E). The strip contained 20 microtubes as seen in Fig. 4.2. containing dehydrated substrates. The tests were inoculated with SWA 4 and SWA 5 bacterial suspension which reconstitutes the media. During incubation, colour changes occur spontaneously or after additions of reagents. The reactions are read of the reading table (Table Appendix E.2.) and the identification is obtained by using the approved API identification software data base. The results obtained code a specific profiling code which is compared to the API data base and a strain is selected for each of the codes provided (BioMérieux® SA 2002).

Organism SWA 4 and SWA 5 were grown to their maximum growth of nine hours as seen by the growth curve analysis (Fig. 4.1.). The bacterial suspensions of SWA 4 and SWA 5 were pipetted into wells shown in Fig. 4.3. The reagents were added to specific wells (Appendix E) after incubation. The results were tabulated and the code, identified specific strains using the API identification software.

Figure 4.3: Example of an API microtube

4.3. Results

4.3.1. Growth Curve Analysis

The growth curve analysis was performed on isolates SWA 4 and SWA 5 using the Live/Dead Stain technique (Appendix D). This was due to the size of the organisms; therefore the haemocytometer technique was inefficient while the unstable behaviour of the PCBs proved difficult for turbidity examinations by a spectrophotometer. Both SWA 4 and SWA 5's as seen in Fig. 4.4. maximum growth was at nine hours. The growth increased after six hours thereafter the bacterial cells began doubling indicating the initial exponential phase. Organism SWA 4 showed a very steady growth rate from six hours (4hr – 9hr) but plummeted after nine hours to the death phase. Organism SWA 5 maintained a steady stationary plateau for five hours (9hr – 13 hr) before the death phase begins at the 14^{th} hour. The data for the bacterial cell count is recorded in the Raw Data Table 1. for SWA 4 and Table 2. for SWA 5.

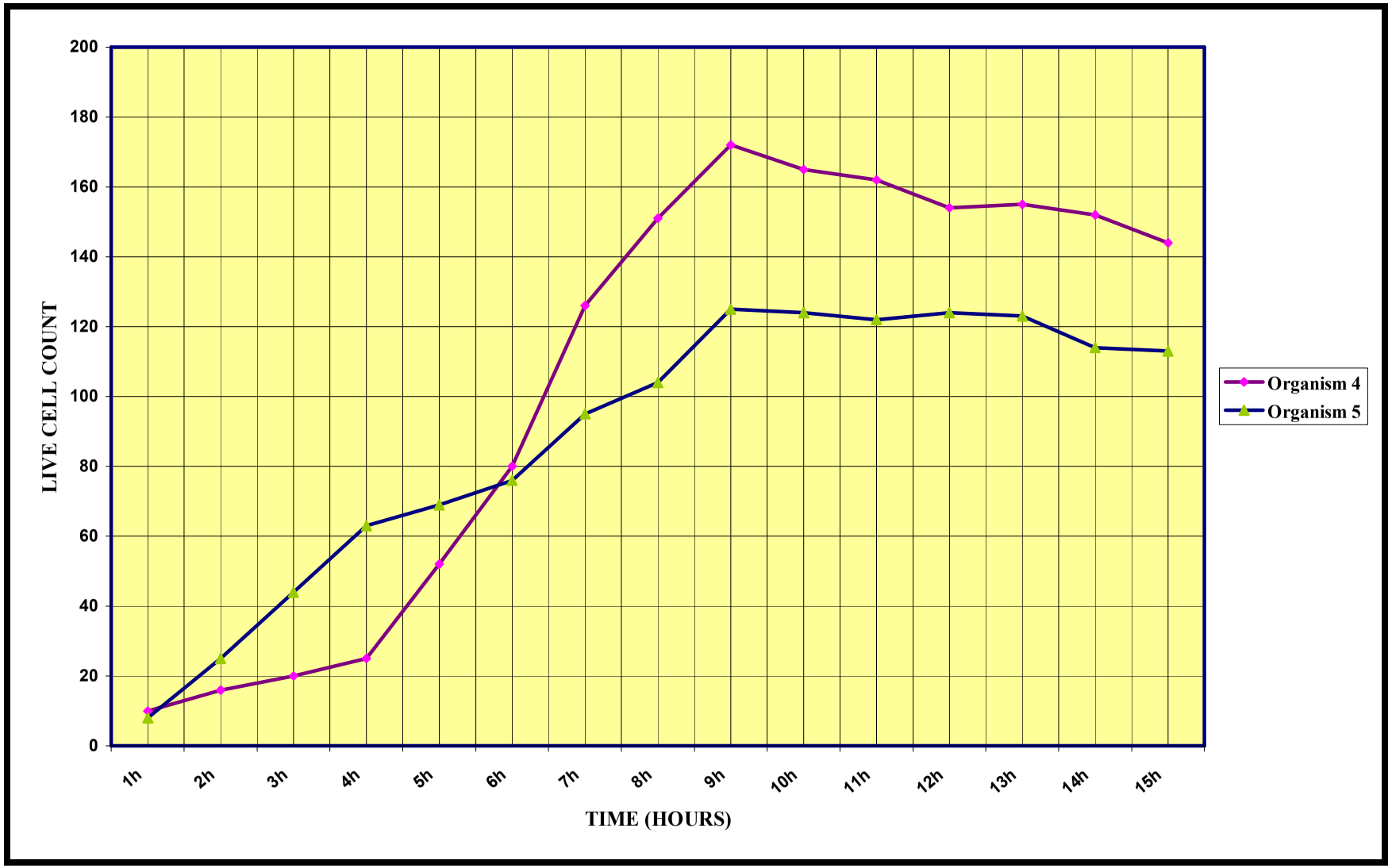

Figure 4.4: Growth Curve Analysis of Organisms SWA 4 & SWA 5

4.3.2. Spectrophotometer Analysis

The mixed culture and pure SWA 4 and SWA 5 cultures were analysed by UV/Vis Spectrophotometer at 300nm to compare the efficiencies in degradation between a pure culture and a mixed culture, shown in Fig. 4.5. The degree of absorbance is directly proportional to the degree of PCB concentration (Carr *et al*., 1982). The mixed culture had a high absorbance of 0.0030 indicating its ability to utilise Aroclor 1254 more efficiently than Aroclor 1260. The isolate SWA 4 had a higher absorbance, 0.1000 for Aroclor 1260. Organism SWA5 had an absorbance of 0.0090 for Aroclor 1254 and 0.0030 absorbance for Aroclor 1260. SWA 5 showed a good potential degradation for Aroclor 1254 and Aroclor 1260.

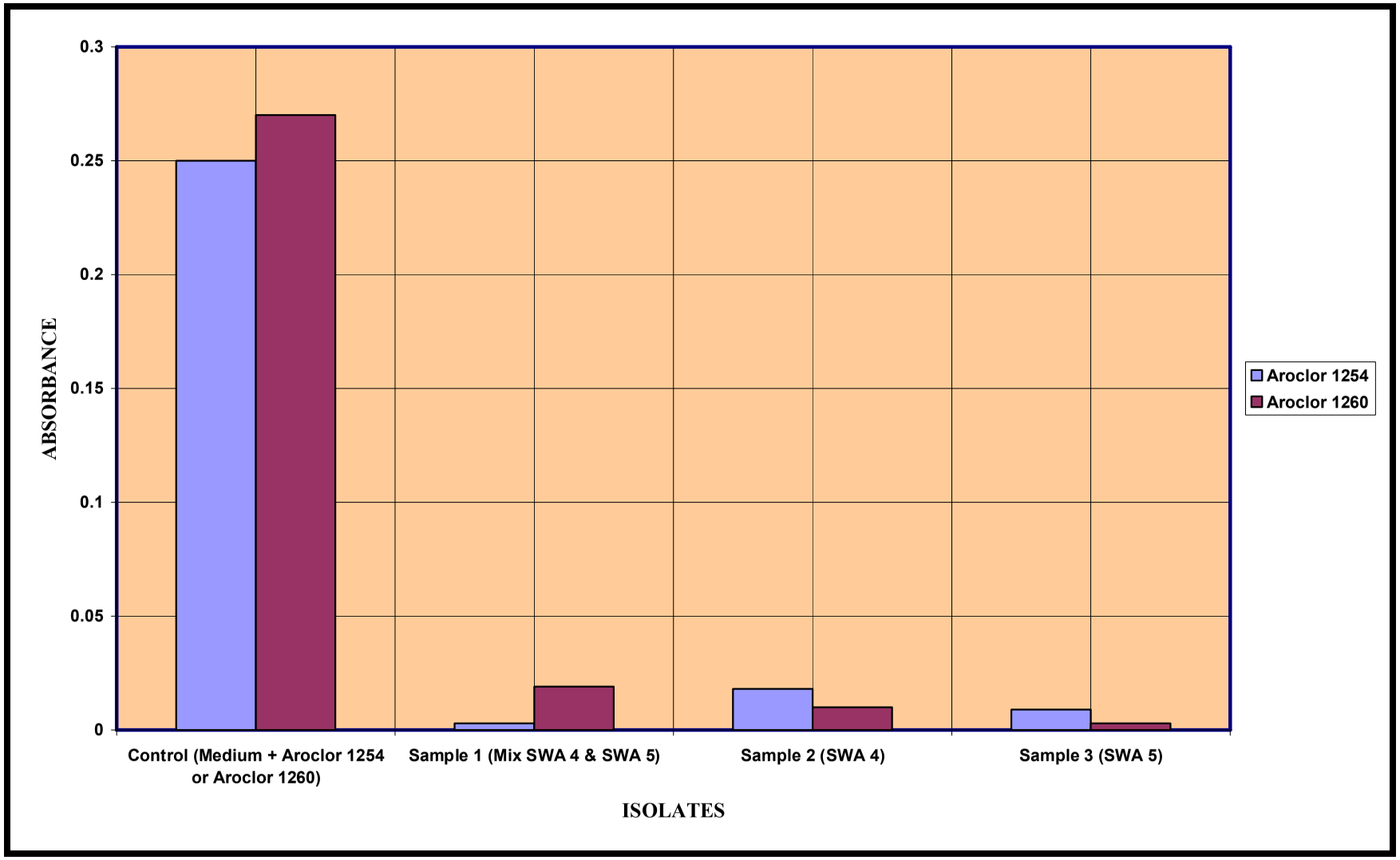

Figure 4.5: Absorbance of Aroclor 1254 & Aroclor 1260 for Organism's SWA 4 and SWA 5 at 300nm

4.3.3. Identification of Isolates

The API 20E bacterial identification kit tested various carbohydrates and biochemical properties for isolates SWA 4 and SWA 5. The API test results are tabulated in Table 4.1. The Ortho-nitrophenyl-β-D-galactopyranosidase (ONPG) reaction tests for the presence of the enzyme β-galactosidase, an enzyme involved in lactose catabolism, *Acinetobacter* sp. was positive for lactose breakdown. The next three reactions (in order, arginine - ADH, lysine - LDH and ornithine - ODH) test for amino acid decarboxylation. Decarboxylation is shown by an alkaline reaction (red colour of the particular pH indicator used). *Acinetobacter* sp. and *Pseudomonas* sp. decarboxylased both Arginine and lysine but no reaction was observed for ornithine. The Citrate utilisation tests for the ability of bacteria to convert citrate into oxaloacetate. It also tests the bacteria's ability to utilise citrate as a carbon source. Both *Acinetobacter* sp. and *Pseudomonas* sp. had a blue colour reaction indicating citrate utilisation. The Hydrogen Sulphide (H_2S) production investigates fermentation of glucose, lactose, and sucrose and tests hydrogen sulphide production. The H_2S reacted with the ferrous sulphate added to the tube. The presence of a black colour indicated that H_2S was produced. There was no reaction for both the organisms.

The Urea test investigates break down of urea to ammonia. The change in pH is indicated by a pH indicator called phenol red. A colour change from yellow to bright pinkish-red is positive as ammonia is a base, increasing the pH, which was observed for both *Acinetobacter* sp. and *Pseudomonas* sp. The TDA reaction tested Tryptophane deaminase and correlates with amino acid breakdown in the ADH, LDC and ODC tests. The Indole production investigates the breakdown of amino acid tryptophan into indole. The addition of Kovac's reagent formed a red/pink colour, which was a positive result. Both *Acinetobacter* sp. and *Pseudomonas* sp. were negative for indole production. The Gelatinase investigates the bacteria's ability to digest gelatin. There was a black formation in both the microtubes of *Acinetobacter* sp. or *Pseudomonas* sp. Therefore both the bacteria are capable of breaking down gelatin.

There were nine carbohydrates tested for fermentation, glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. Fermentation was observed by a yellow color indicating acid reaction. *Acinetobacter* sp. was able to utilise the glucose, mannitol, melibiose and arabinose sugars indicating acid production compared to the *Pseudomonas* sp. which could only utilise glucose and melibiose. *Acinetobacter* sp. was able to utilise most of the carbohydrates with the exception of Innositol and Saccharose. Both the organisms were positive for Arginine, Ornathine, Citrate, Urease, Tryptophane, Gelatinase Glucose and Melibiose. Most of the reactions were positive for *Acinetobacter* sp. The isolates were presumptively (98% accurate) identified as *Acinetobacter* sp. for isolate SWA 4 and *Pseudomonas* sp. for isolate SWA 5 shown in Fig. 4.6. and Fig. 4.7.

Table 4.1: API 20E kit for SWA 4 and SWA 5

TEST	REACTION/ENZYME	PRINCIPLE	SWA 4	SWA 5
ONPG	Ortho-nitrophenyl-β-D-galactopyranosidase (β-galactosidase)	Lactose breakdown	+	-
ADH	Arginine Dihydrolylase	Amino acid decarboxylation	+	+
LDC	Lysine Decarboxylase	Amino acid decarboxylation	-	-
ODC	Ornathine Decarboxylase	Amino acid decarboxylation	+	+
CIT	Citrate utilisaton	Citrate converts to oxaloacetate	+	+
H_2S	H_2S production	Fermentation of glucose, sucrose or lactose	-	-
URE	Urease	Urea breakdown to ammonia	+	+
TDA	Tryptophane Deaminase	Tryptophane Deaminase	-	-
IND	Indole production	Tryptophan converts to indole	-	-
VP	Voges Proskauer (acetoin production)	Glucose breakdown to acetoin	+	+
GEL	Gelatinase	Digestion of gelatinase	+	+
GLU	Glucose (fermentation/oxidation)	Carbohydrate fermentation	+	+
MAN	Mannitol (fermentation/oxidation)	Carbohydrate fermentation	+	-
INO	Inositol (fermentation/oxidation)	Carbohydrate fermentation	-	-
SOR	Sorbitol (fermentation/oxidation)	Carbohydrate fermentation	+	-
RHA	Rhamnose (fermentation/oxidation)	Carbohydrate fermentation	+	-
SAC	Sacchrose (fermentation/oxidation)	Carbohydrate fermentation	-	-
MEL	Melibiose (fermentation/oxidation)	Carbohydrate fermentation	+	+
AMY	Amygdalin (fermentation/oxidation)	Carbohydrate fermentation	+	-
ARA	Arabinose (fermentation/oxidation)	Carbohydrate fermentation	+	-

Colour changes in the API kit shown in Fig. 4.6. and Fig. 4.7. indicated the reactions labelled below each microtube. This was recorded in Table 4.1.

Figure 4.6: Organism SWA 4 – *Acinetobacter* sp. colour reactions in the API 20E kit

Figure 4.7: Organism SWA 5 – *Pseudomonas* sp. colour reactions in the API 20E kit

4.4. Discussion

The spectrophotometric analysis obtained indicated that the mixed culture had a greater potential of efficient degradation of Aroclor 1254 as seen in Fig. 4.5. While the pure cultures had a greater potential of efficient degradation of Aroclor 1260, especially *Acinetobacter* sp. (SWA 4). This organism is diverse in nature and has degraded oil pollutants (Jirasripongpun, 2002) and phenols (Geng *et al*., 2006), which are predominantly aromatic compounds. The pure isolates *Pseudomonas* sp. and *Acinetobacter* sp. had a greater efficiency for Aroclor 1260 which can be attributed to that most pure bacteria are able to transform most of the PCB, this is shown by the high absorbance (Pazlarovà *et al*., 1997).

Acinetobacter sp. was able to utilise the glucose, mannitol, melibiose and arabinose sugars. The genus *Acinetobacter* is capable of utilizing a very diverse range of compounds as sources of carbon and energy, and of growing on relatively simple media. This robust metabolism gives them a high capacity for adaptation, which explains why these bacteria are found in very diverse environments like their cousins from the genus *Pseudomonas* sp. For this reason they have aroused growing interest because of possible biotechnological and environmental applications such as PCB bioremediation (Abd El-Haleem, 2003). The carbohydrates also indicate acid production and *Acinetobacter* sp. has been consistent within its classification. The API kit was able to identify most of the substrates which agree to the genus classifications of *Acinetobacter* sp. and *Pseudomonas* sp. The *Acinetobacter* sp. is able to utilise all sugars and produces acid which is indicated by the yellow colour observed in Fig. 4.6. While *Pseudomonas* sp. could only utilise glucose and melibiose. The *Pseudomonas* sp. are classified by their low utilisation of specific sugars (Geng *et al*., 2006). Most *Pseudomonas* sp. are classified by their selective utilisation of carbohydrates (Sakai *et al*., 2005).

The substrates in the API kit are designed to differentiate the genus and species within the Enterobacteriaceae family. Most PCB-degrading microorganisms have been identified as a *Pseudomonas* sp. or an *Acinetobacter* sp. (Chàvez *et al*., 2006). The mixed

culture and the pure cultures *Pseudomonas* sp. and *Acinetobacter* sp., were used in the GC-ECD evaluation for validation of degradation.

The biochemical properties of the isolates, acquired in this aspect of research will aid in improving the nutrient requirements of the potential PCB-degraders. The specific carbon utilisations of both the isolates will be used in supplementing the specific nutrient requirements for optimisation of growth. The optimisation of the isolates conditions will improve PCB removal. The main objectives for the development and the optimisation of PCB degradation studies are the understanding of the potential PCB-degraders environment by assessing their physiological and biochemical functions.

CHAPTER 5: QUANTIFICATION OF PCB DEGRADATION

5.1. Introduction

Metabolic breakdown of PCBs by microorganisms is one of the major treatments for the widespread of pollutants. There are a number of microorganisms able to degrade PCBs (Bokvajová, 1994). At aerated conditions microorganisms are capable of metabolising PCBs to less chlorinated aromatic compounds. The industrial PCB mixtures (Aroclors) are complex and proper investigations require specific congeners for each Aroclor (Pazlarovà, 1997). The Environmental Protection Agency (EPA) recommends that the total PCBs evaluated should be expressed by indicator congeners specified by the International Union of Pure and Applied Chemistry (IUPAC), (EPA, 1998c). The recommended peaks were too varied and thus six specific congeners were selected based on the differentiation between Aroclor 1254 and Aroclor 1260 as well as the indication of which chlorine derivative the specific microorganism is able to utilise.

Sample extraction and cleanup is vital for any analytical experimentation. The appropriate method depends on the compounds physical state at room temperature, i.e. solid, liquid or gas. The appropriately prepared sample can be extracted by any one of a number of techniques. The main points to consider are to allow adequate time of exposure of the solvent system in the sample matrix (EPA, 1998c). This extraction utilizes an immiscible liquid e.g. hexane in which the sample component (PCB) will dissolve without extracting other materials. This extraction is regarded as the batch or continuous liquid extraction due to continuous shaking of the two liquids in a separatory funnel. The solvent extraction is the isolation of single chemical species. The technique is comprehensive for purely organic compounds from the starting point of experimentation (Budhiraja, 2004). The volume collected is distilled to purify and concentrate the specific compound which is ready to be investigated (Fritz, 1999).

PCBs have been found in water, soil, and other environmental matrices, and in foods. Concerns over their persistence in the environment prompted the US Environmental

Protection Agency (US EPA) to issue guidelines limiting PCB levels in various matrices and to promote methods for PCBs analysis. A typical EPA method includes sample work-up, clean-up procedures to minimize interference, and analysis by gas chromatography using an electron capture detector (GC-ECD) (EPA, 1998c).

5.2. Methodology

5.2.1 Chemicals

The HPLC grade solvents; n-hexane, chloroform, methanol and sodium sulphate was obtained from Merck Supplies (Durban, S.A.). The Thin Layer Chromatography (TLC) plates used were silica gel HF^{254} which was also purchased from Merck Supplies (Durban, S.A.). The PCB congeners; 2.3'.4'.5 Tetrachlorobiphenyl (IUPAC 70), 2.2'.4.5.5' Pentachlorobiphenyl (IUPAC 101), 2.2'.3.5.5'.6 Hexachlorobiphenyl (IUPAC 151), 2.2'.3.4.4'5.5' Heptachlorobiphenyl (IUPAC 180), 2.2'.3.4.4'.5.6.6' Octachlorobiphenyl (IUPAC 204) and 2.2'.3.3'.4.4'.5.5'.6 Nonachlorobiphenyl (IUPAC 206), (98% purity) were purchased from Separations (Gauteng, S.A.). Stock solutions of the congeners were prepared with HPLC grade n-hexane and refrigerated.

5.2.2. Degradation Assay Media

The organisms were inoculated into 50ml of nutrient broth with 0.2% glucose (Appendix A) in 200mL erlenmeyer flasks and grown for 24hrs in a shaker incubator at 30^0C in aerobic conditions at 140rpm. Erlenmeyer flasks, were setup containing either Aroclor 1254 or Aroclor 1260 with a concentration of $30mgmL^{-1}$ each, with the selective PCB media (Appendix B) and the isolate. A flask was set up as the control containing Aroclor 1254 or Aroclor 1260, the selective media without the inoculation of an organism. The cells were washed with distilled waster in a centrifuge and the pellet was added into

250mL selective media in a 1Lt erlenmeyer flask. The flasks were placed on a shaker incubator at 30^0C in aerobic conditions shaking at 140rpm.

5.2.3. Extraction and Concentration of Samples

After two weeks the PCB samples were removed and extracted by Liquid-Liquid Extraction (LLE) hexane (EPA, 1998b) and concentrated by simple distillation at 70^oC, (Appendix F). The liquid extraction involved the sample to be separated by equal volumes of n-hexane. The funnel was shaken vigorously and allowed to settle. The PCB is dissolved in the hexane observed on the top layer. This layer is eluted and sodium sulphate is added as the drying agent. The drying agent removes the water before separating the PCB from the n-hexane. The resulting solution is distilled to concentrate the sample. The concentrated samples were then analysed by GC-ECD.

5.2.4. Thin Layer Chromatography (TLC)

The samples were spotted on a silica gel HF^{254} TLC plate, about one centimetre from the base. The following solvents were added to a TLC chamber; 5mL of chloroform, 3mL of n-hexane and 1mL of methanol (Vo-Dinh *et al*., 1994). The TLC plate was placed in the TLC chamber and covered with a lid. When the sample rises to the one centimetre mark before the end of the TLC plate, the plate is removed from the TLC chamber. The plate is dried in an oven for 10min, and when removed the plate is placed in a dessicator to cool. The TLC plate is then sprayed with rhodamine and left to air-dry. The rhodamine colours the sample spots pink and the migration is observed (Wilson and Walker, 2000).

5.2.5. Gas chromatography Analysis

A Gas Chromatography-Electron Capture Detector was used to analyse degradation of PCBs (EPA, 1998a). The samples were analysed at Forestry and Forest Products research centre (FFP) at Council of Scientific Industrial Research (CSIR),University of Kwazulu-Natal (UKZN) in South Africa (S.A.). The GC-ECD used was a Clarus 500 Perkin Elmer, autosystem. The column used was an OV17 consisting of a 50% Phenyl Methyl composition with the diameters of 30m x 0.25mm I.D. (0.25µm thickness). A ^{63}Ni electron capture detector was used.

The conditions were optimised as follows: high purity Nitrogen was used as the carrier gas at a flow rate of 1ml/min. The ECD detector make-up gas was ultra-high purity nitrogen at a flow rate of 25mL/min. The column temperature was initially held at 260°C held for 1min and ramped to 290°C, with a 10°C/min increase and held for 8min. The total run time was 12min and resolved the chromatographic peaks from Aroclor 1254 and Aroclor 1260. The sample volume of 1µL was taken up by the automated auto-sampler and injected into a splitless injector. The injector temperature was maintained at 300°C and detector temperature was maintained at 300°C. The samples were processed by Totalchrom Workstation PAX software. The solvent n-hexane, samples, standards Aroclor 1254 and Aroclor 1260 and congeners were analysed by the GC-ECD.

5.3. Results

5.3.1. TLC Analysis

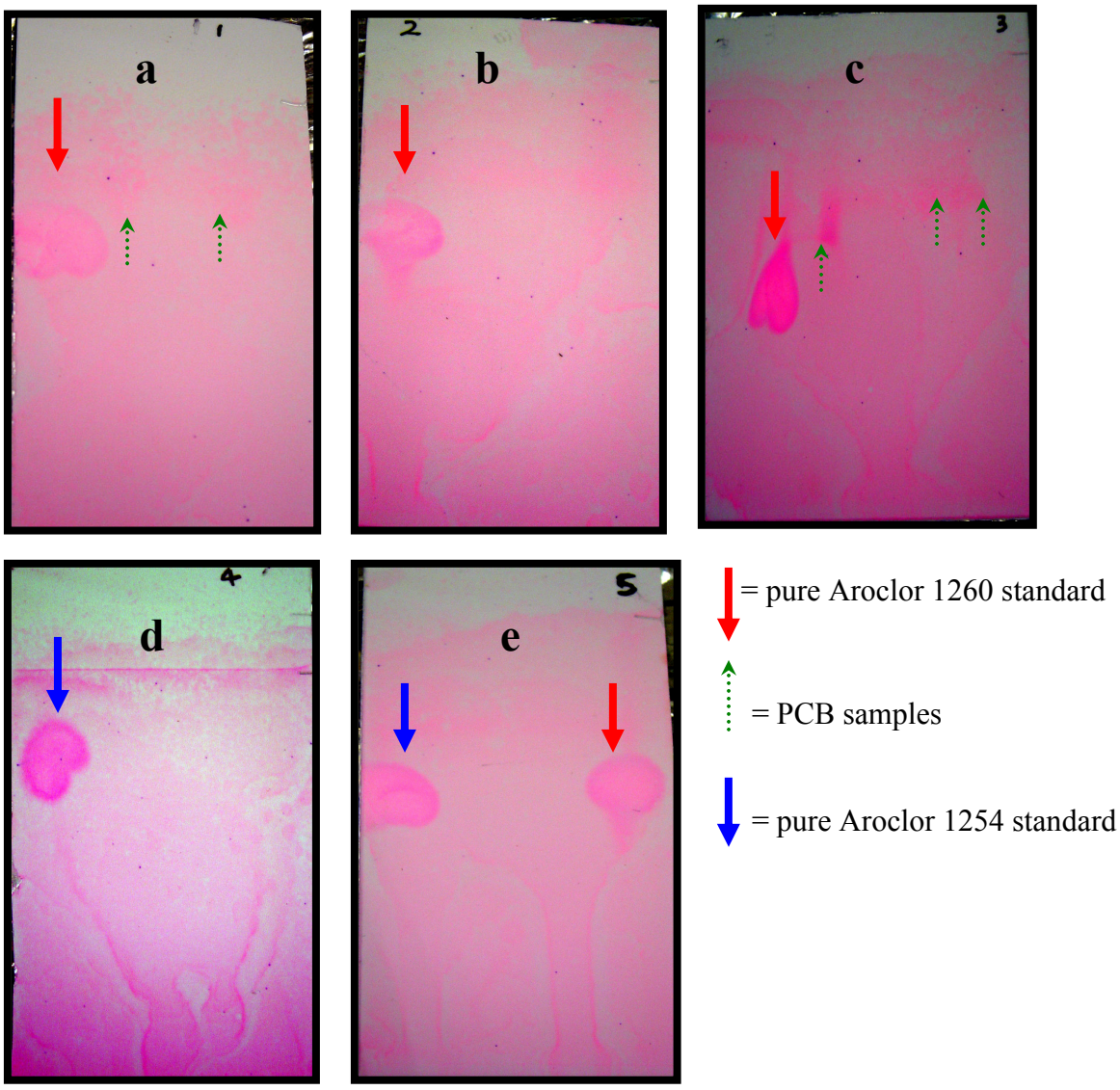

Figure 5.1: TLC plates
(f) Analysis of Aroclor 1260 and two PCB samples that were unclear to detect.
(g) Analysis of Aroclor 1260 and two samples that could not be detected.
(h) Analysis of Aroclor 1260 and three samples that were "tailing", inefficient detection.
(i) Analysis of Aroclor 1254 and three PCB samples that were not detected.
(j) Analysis of Aroclor 1254 and Aroclor 1260 and two PCB sample that were not detected.

The detection of PCBs on TLC plates Fig. 5.1. a – e, were not effective or suitable for the detection of the PCBs in the samples. This was due to the low concentration used in spiking the PCB samples which was less, than 50ppm. A concentration of 50ppm and greater is the required amount to detect samples on a TLC plate. The 5 plates as seen in Fig. 5.1. were only able to detect the standards Aroclor 1254 and Aroclor 1260 as the concentrations used were 50mg/L. There was no detection of the PCB samples in TLC plates 5.1. (b), 5.1. (d) and 5.1. (e). While the detection of the PCB samples in TLC plates 5.1. (a) and 5.1. (c), was inefficient and tailing was observed in TLC plate 5.1.(c) for the Aroclor 1260 standard and the three PCB samples. The tailing effect was due to the change in solvent concentrations during the optimisation of PCB detection and the indication of a low concentration of PCBs in the sample.

5.3.2. GC-ECD Analysis: Quantification of the degradation potential of isolates on Aroclor 1254

Acinetobacter sp. and *Pseudomonas* sp. transformed Aroclor 1254, however the mixed culture degraded the Aroclor 1254 effectively as seen in Table 5.1. The mixed culture was able to completely degrade all the IUPAC specific congeners. *Pseudomonas* sp. was able to degrade congener 2.3'.4'.5 Tetrachlorobiphenyl and 2.2'.4.5.5' Pentachlorobiphenyl, but was unable to transform congener 2.2'.3.5.5'.6 Hexachlorobiphenyl and 2.2'.3.4.4'5.5' Heptachlorobiphenyl. *Acinetobacter* sp. was effective in degrading congener 2.3'.4'.5 Tetrachlorobiphenyl, however congener 2.2'.4.5.5' Pentachlorobiphenyl was transformed by 55 % but the remaining specific congeners could not be metabolised.

Table 5.1: Aroclor 1254 degradation compared to the specific congeners

Peak no.	Congeners	% PCB Degradation		
		***Acinetobacter* sp.**	***Pseudomonas* sp.**	**Mixed culture**
1	2.3'.4'.5 Tetrachlorobiphenyl	100%	100%	100%
2	2.2'.4.5.5' Pentachlorobiphenyl	55%	100%	100%
3	2.2'.3.5.5'.6 Hexachlorobiphenyl	5%	5%	100%
4	2.2'.3.4.4'5.5' Heptachlorobiphenyl	2%	2%	100%

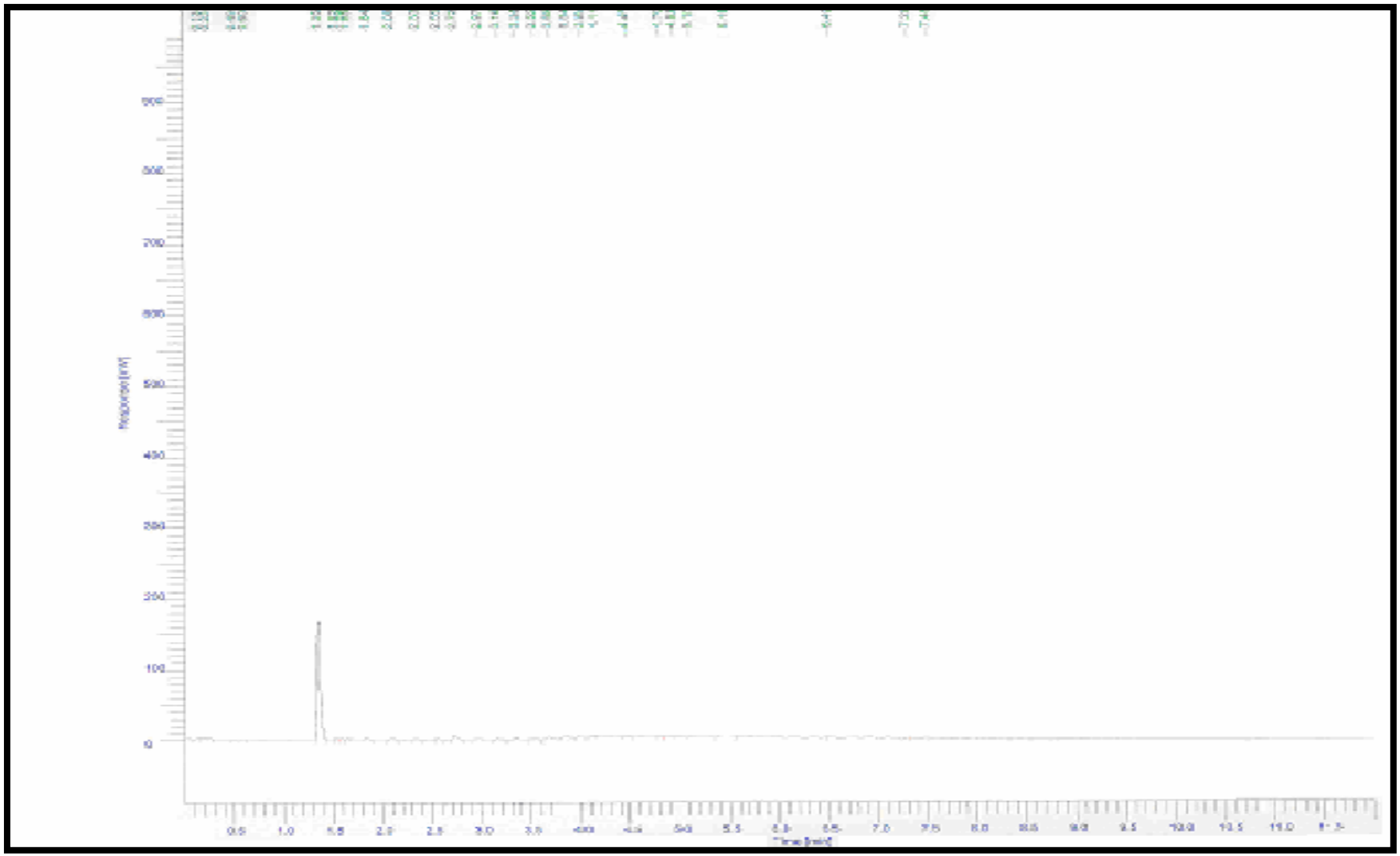

Figure 5.2: n-hexane solvent for the analysis of Aroclor 1254

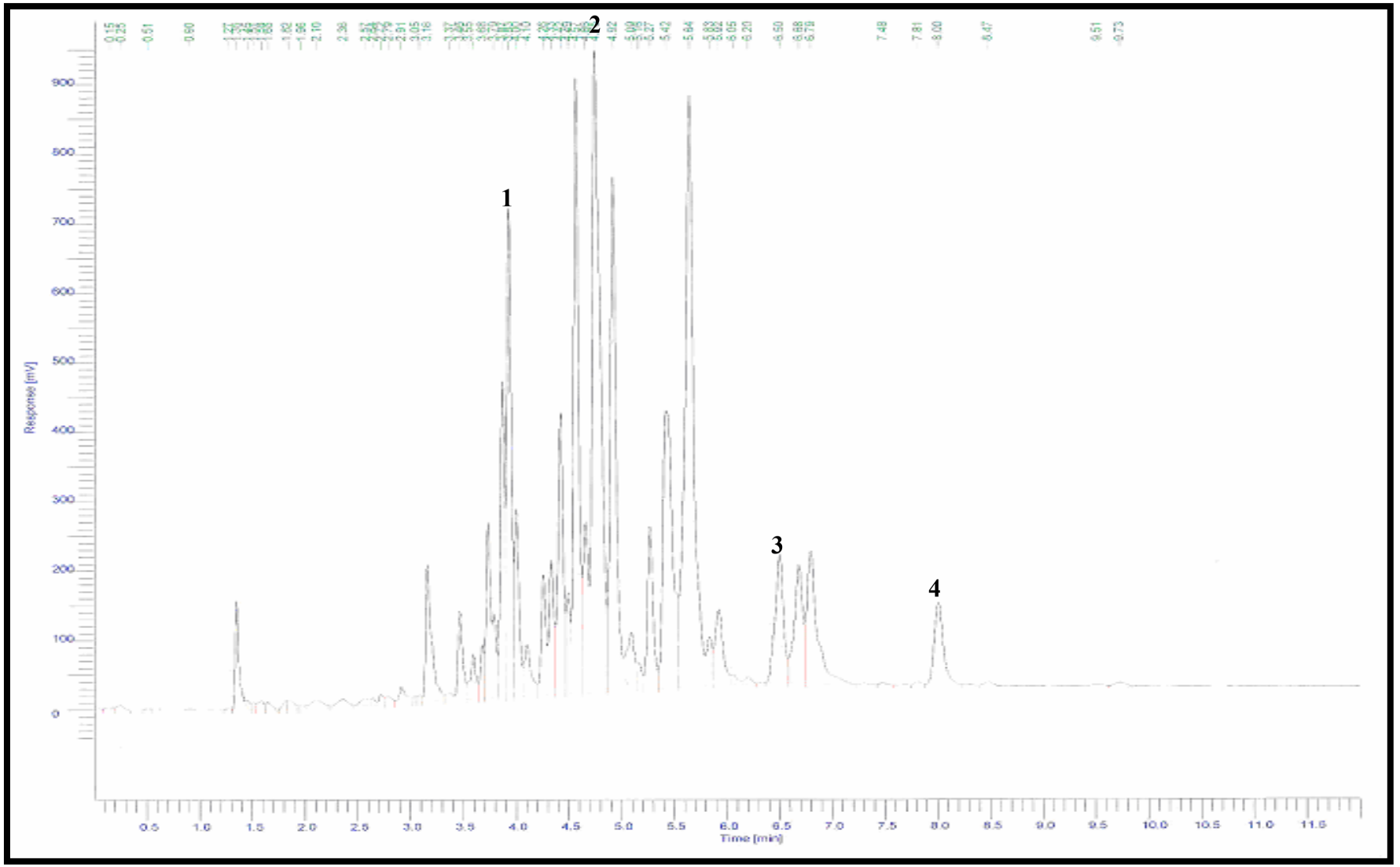

Figure 5.3: Pure standard of Aroclor 1254

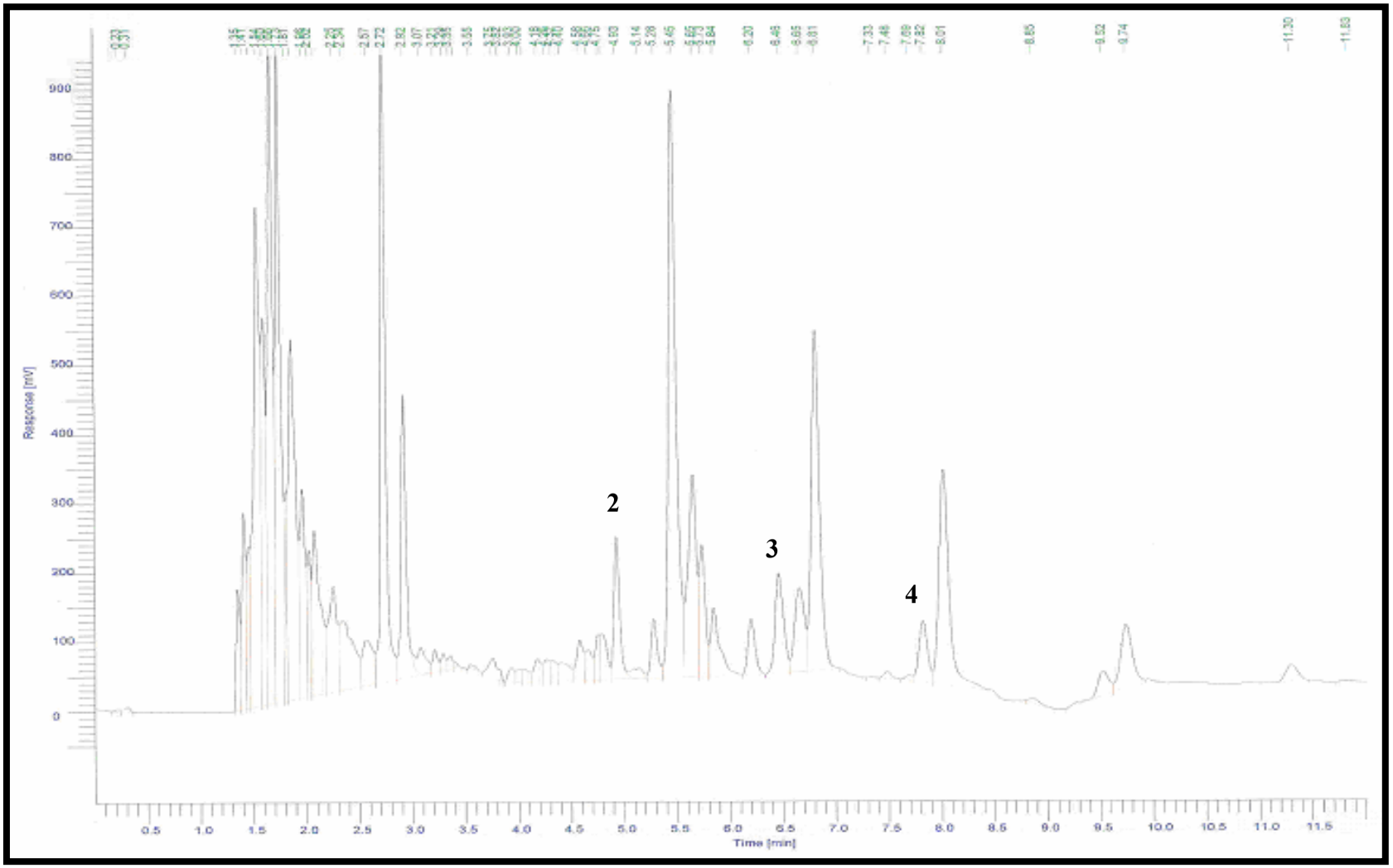

Figure 5.4: PCB sample Aroclor 1254 with organism 4 - *Acinetobacter* sp.

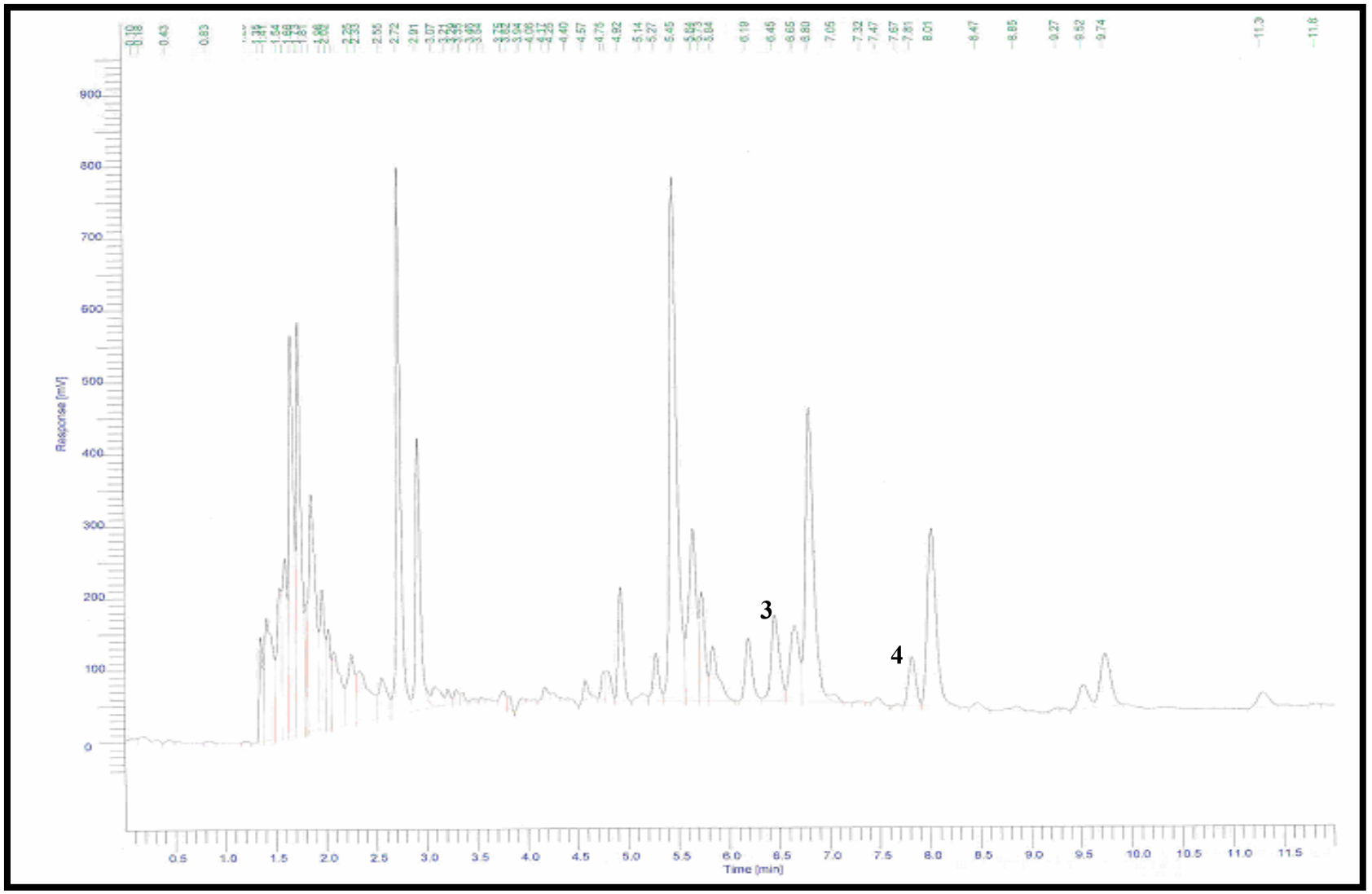

Figure 5.5: PCB sample Aroclor 1254 with organism 5 - *Pseudomonas* sp.

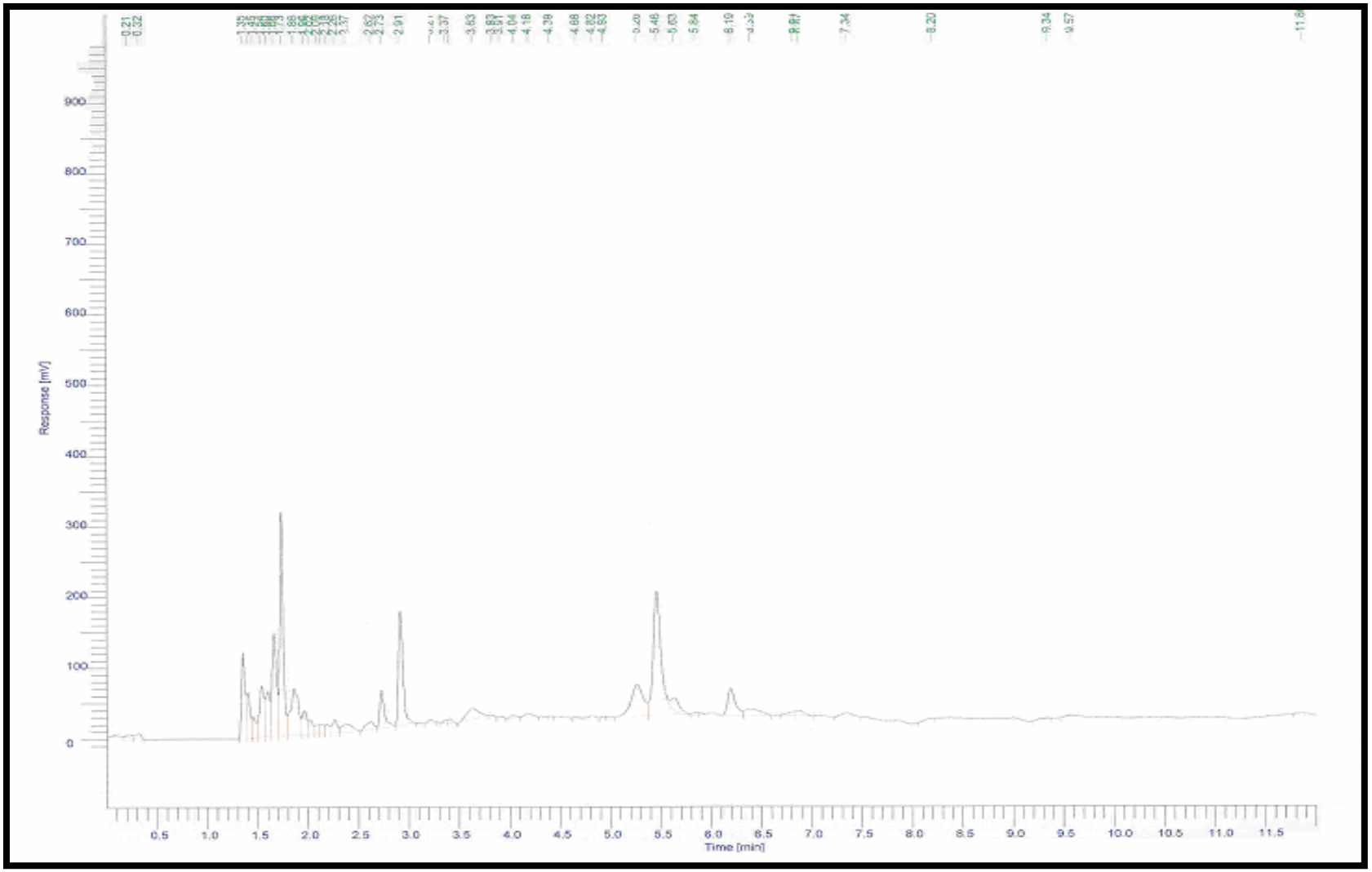

Figure 5.6: PCB sample Aroclor 1254 with the mixture *Acinetobacter* sp. and *Pseudomonas* sp.

5.3.3. GC-ECD Analysis: Quantification of the degradation potential of isolates on Aroclor 1260

The pure isolates were able to transform Aroclor 1260 more effectively compared to the mixed culture. *Acinetobacter* sp. and *Pseudomonas* sp. clearly transformed Aroclor 1260 effectively as seen in Table 5.2. The mixed culture did not degrade as efficiently as in Aroclor 1254, however sufficient transformation was established by the removal of most of the Aroclor 1260.

Table 5.2: Aroclor 1260 degradation compared to the specific congeners

Peak no.	Congeners	% PCB degradation		
		Acinetobacter sp.	*Pseudomonas* sp.	Mixed culture
1	2.3'.4'.5 Tetrachlorobiphenyl	100%	100%	100%
2	2.2'.4.5.5' Pentachlorobiphenyl	100%	100%	100%
3	2.2'.3.5.5'.6 Hexachlorobiphenyl	40%	67%	45%
4	2.2'.3.4.4'5.5' Heptachlorobiphenyl	100%	100%	100%
5	2.2'.3.4.4'.5.6.6' Octachlorobiphenyl	100%	100%	100%
6	2.2'.3.3'.4.4'.5.5'.6 Nonachlorobiphenyl	100%	100%	100%

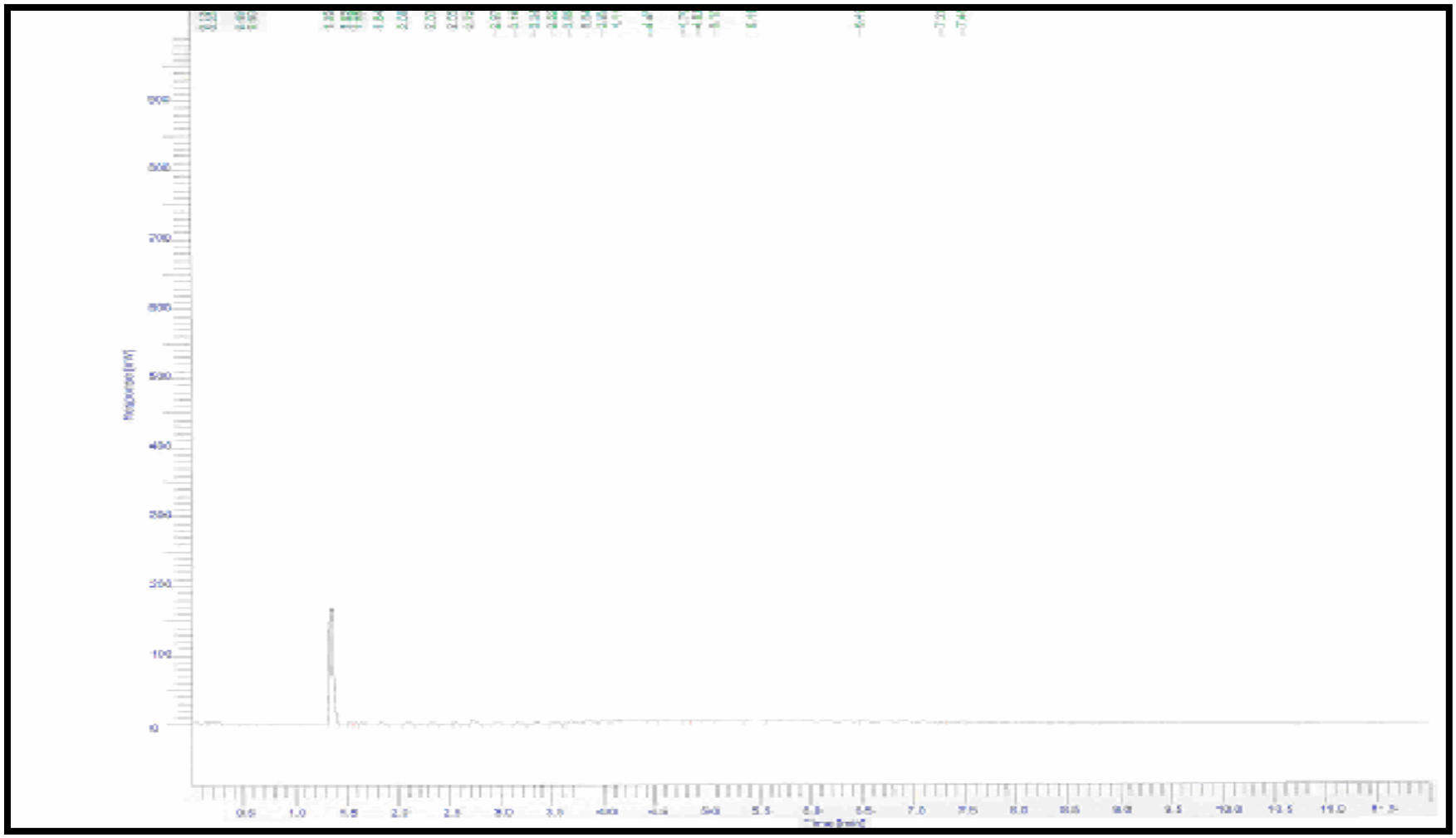

Figure 5.7: n-hexane solvent for the analysis of Aroclor 1260

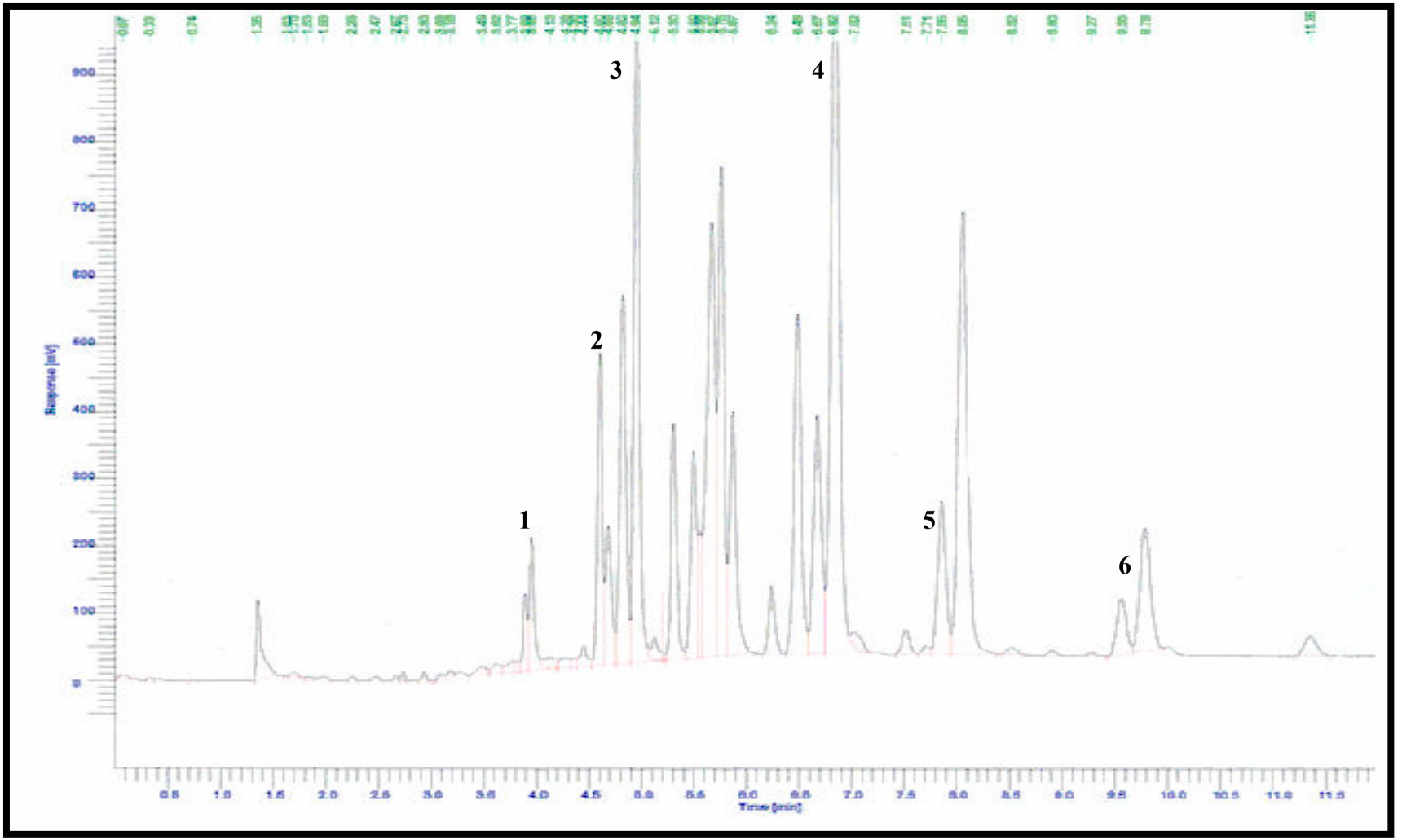

Figure 5.8: Pure standard Aroclor 1260

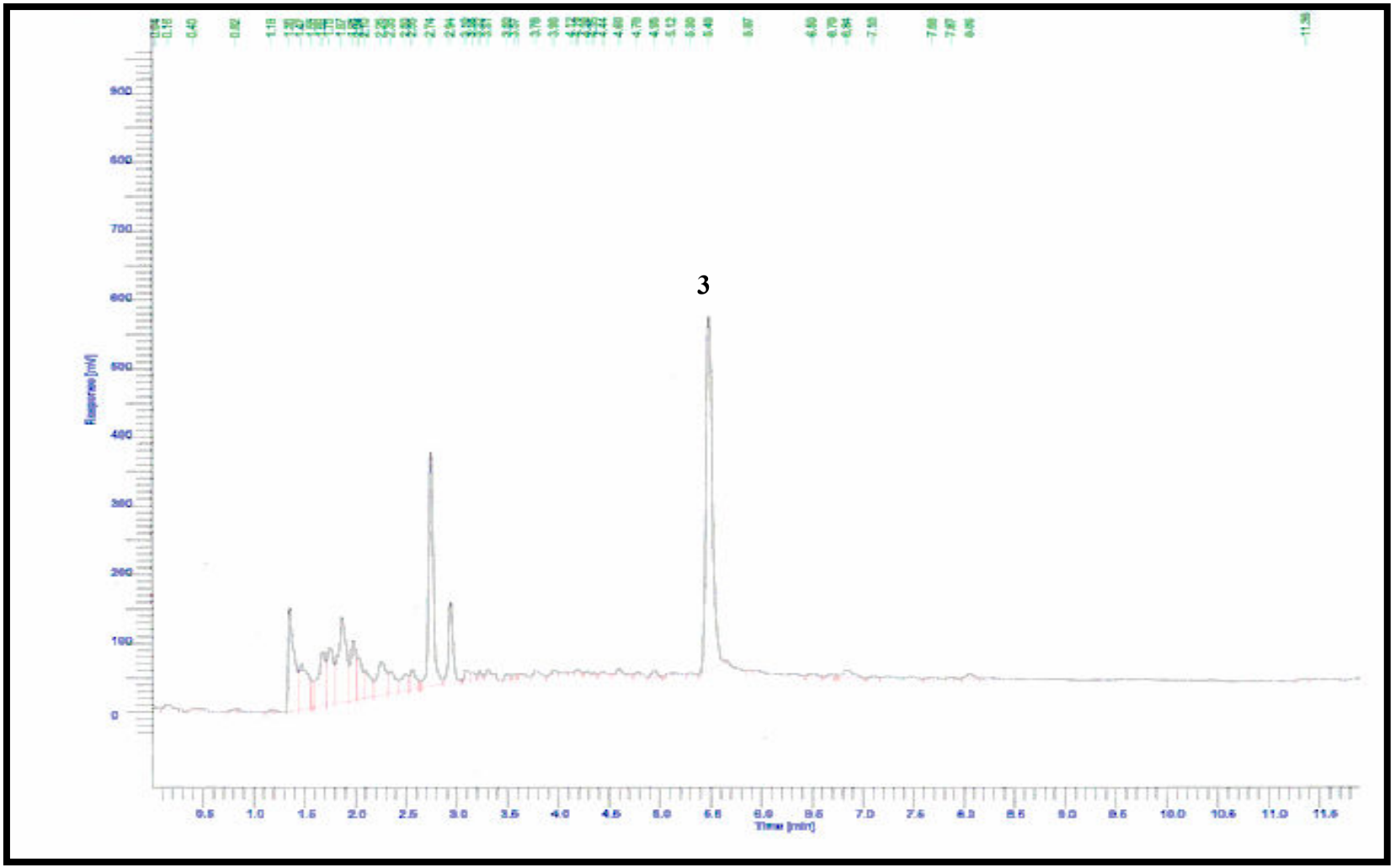

Figure 5.9: PCB sample with Aroclor 1260 organism 4 - *Acinetobacter* sp.

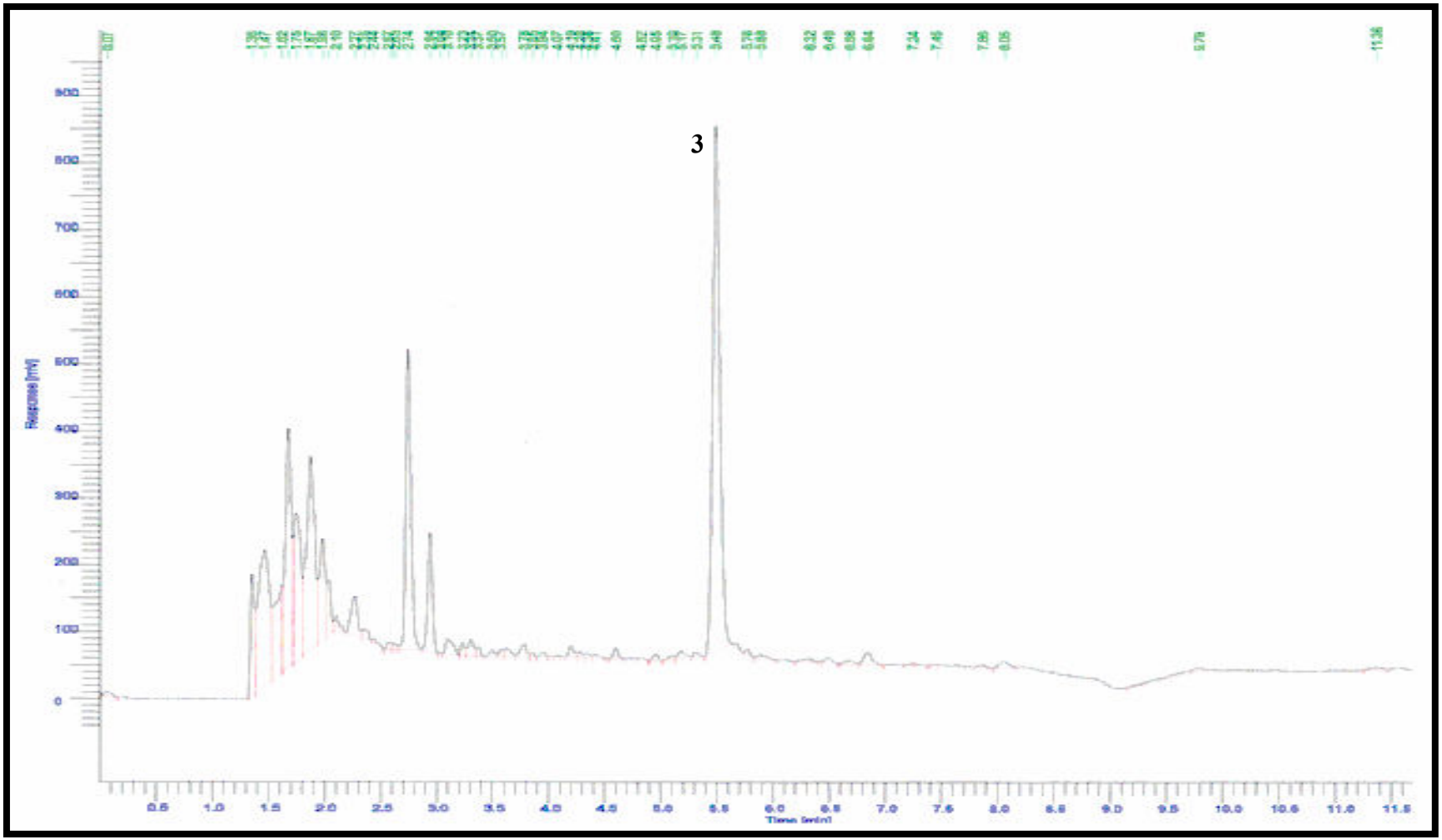

Figure 5.10: PCB sample with Aroclor 1260 organism 5 - *Pseudomonas* sp.

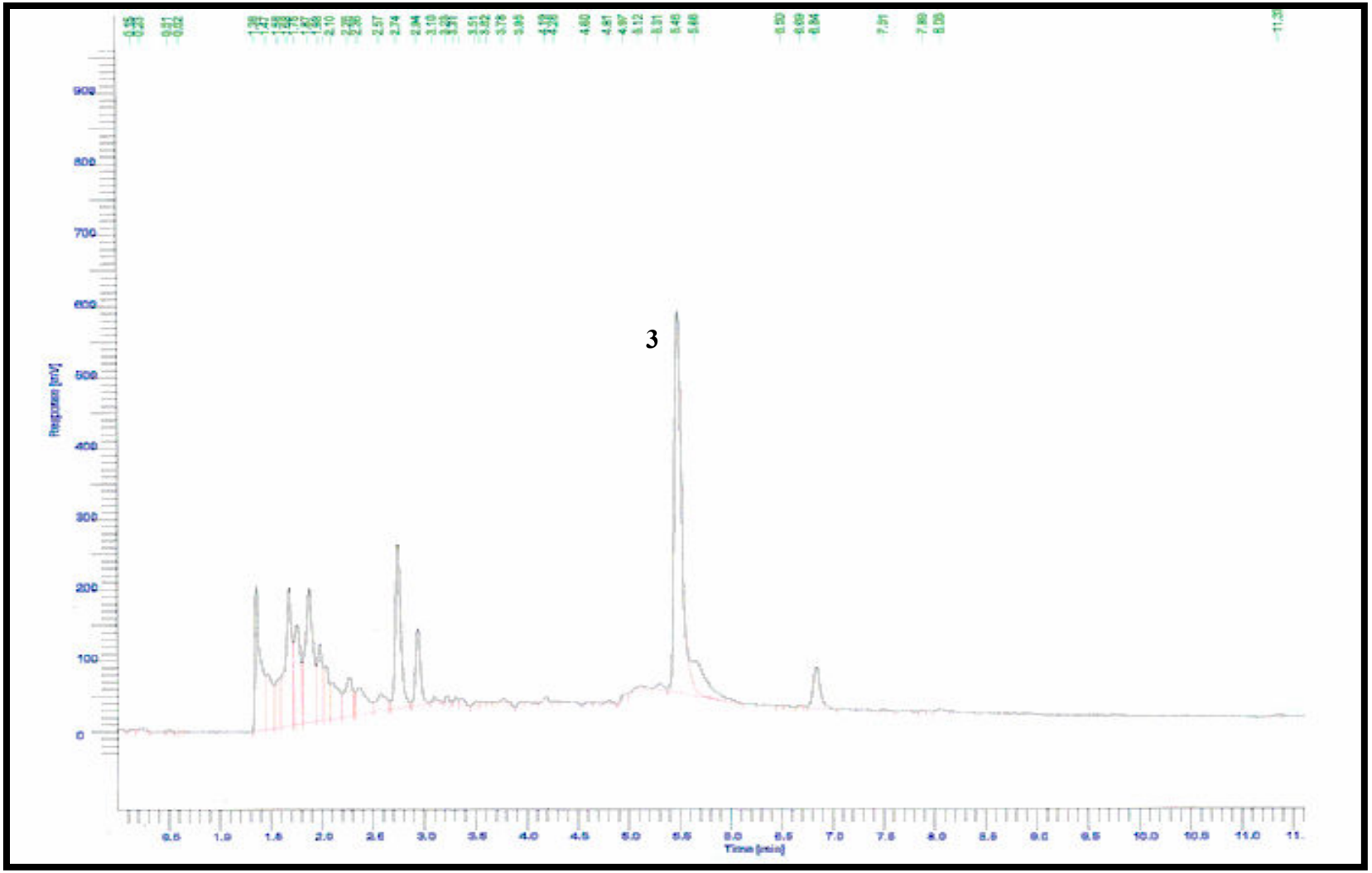

Figure 5.11: PCB sample Aroclor 1260 with the mixture *Acinetobacter* sp. and *Pseudomonas* sp.

5.3.4. Congener Analysis

Table 5.3: Congener peak numbers and retention times

Congener	*IUPAC no.	Peak #	RTime (sec)
2.3'.4'.5 Tetrachlorobiphenyl	**70**	1	2.7
		2	3.7
		3	4.7
2.2'.4.5.5' Pentachlorobiphenyl	**101**	1	4.0
		2	5.0
		3	8.6
2.2'.3.5.5'.6 Hexachlorobiphenyl	**151**	1	3.7
		2	4.6
2.2'.3.4.4'5.5' Heptachlorobiphenyl	**180**	1	6.7
2.2'.3.4.4'.5.6.6' Octachlorobiphenyl	**204**	1	2.7
		2	6.7
2.2'.3.3'.4.4'.5.5'.6 Nonachlorobiphenyl	**206**	1	2.7
		2	11.3

* IUPAC = International Union of Pure and Applied Chemistry

Peak # = peak number

RTime = Retention Time

The comparison of congeners as seen in Fig. 5.12. – 5.17. to the *Acinetobacter* sp., *Pseudomonas* sp. and the mixed culture's ability to metabolise PCBs as seen in figures 5.2. – 5.11. were investigated. The observed congener chromatographs compared to samples indicated that *Acinetobacter* sp. and *Pseudomonas* sp. cannot transform higher congeners in Aroclor 1254 i.e. 2.2'.3.4.4'5.5' Heptachlorobiphenyl with retention time of 6.7sec as shown in Table 5.3. The congener 2.2'.3.4.4'5.5' Heptachlorobiphenyl is also common for the congener 2.2'.3.4.4'.5.6.6' Octachlorobiphenyl. The congener 2.2'.3.4.4'5.5' Heptachlorobiphenyl was still present for Aroclor 1254 for both the *Acinetobacter* sp. and *Pseudomonas* sp., however the mixed culture was able to

completely transform Aroclor 1254 including congener 2.2'.3.4.4'5.5' Heptachlorobiphenyl.

Congener analysis of Aroclor 1254 indicated that *Acinetobacter* sp. was transformed to a pentachlorobiphenyl due to the higher concentration of the remaining congener peak in Fig. 5.4. The remaining congener 2.2'.4.5.5' Pentachlorobiphenyl had a higher concentration than the Hexachlorobiphenyl and Heptachlorobiphenyl peaks. The *Pseudomonas* sp. was transformed to a Hexachlorobiphenyl due to the higher concentration of the remaining peak in Fig. 5.5. The congener 2.2'.3.5.5'.6 Hexachlorobiphenyl had a higher concentration than the Heptachlorobiphenyl congener. The *Acinetobacter* sp., *Pseudomonas* sp. and the mixed cultures was able to transform all the congeners of Aroclor 1260 except the congener 2.2'.3.5.5'.6 Hexachlorobiphenyl. This could be due to the toxic effect on the organisms. However it was observed that the *Pseudomonas* sp. was able to degrade the 2.2'.3.5.5'.6 Hexachlorobiphenyl congener by 67%.

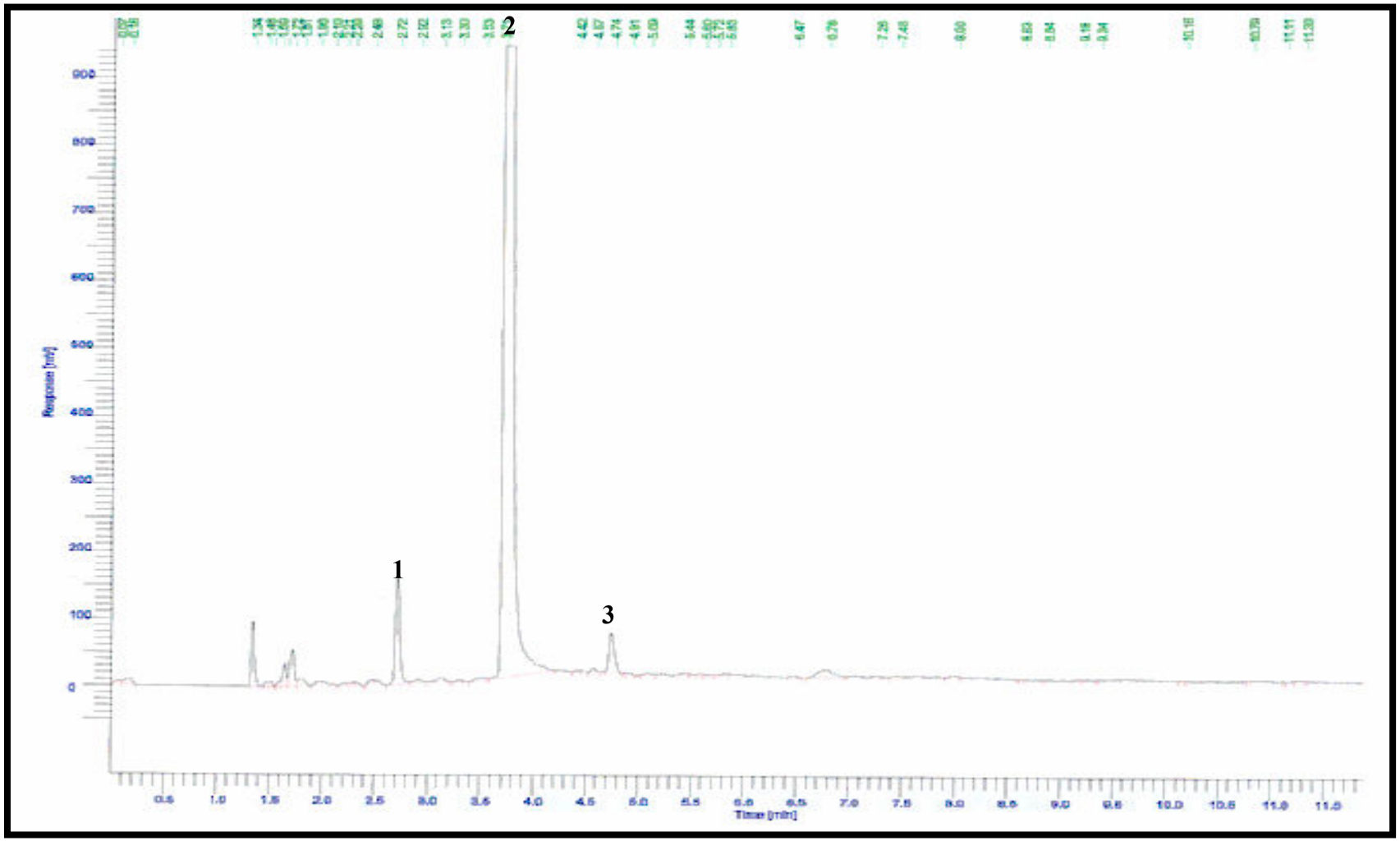

Figure 5.12: Congener 2.3'.4'.5 Tetrachlorobiphenyl pure standard

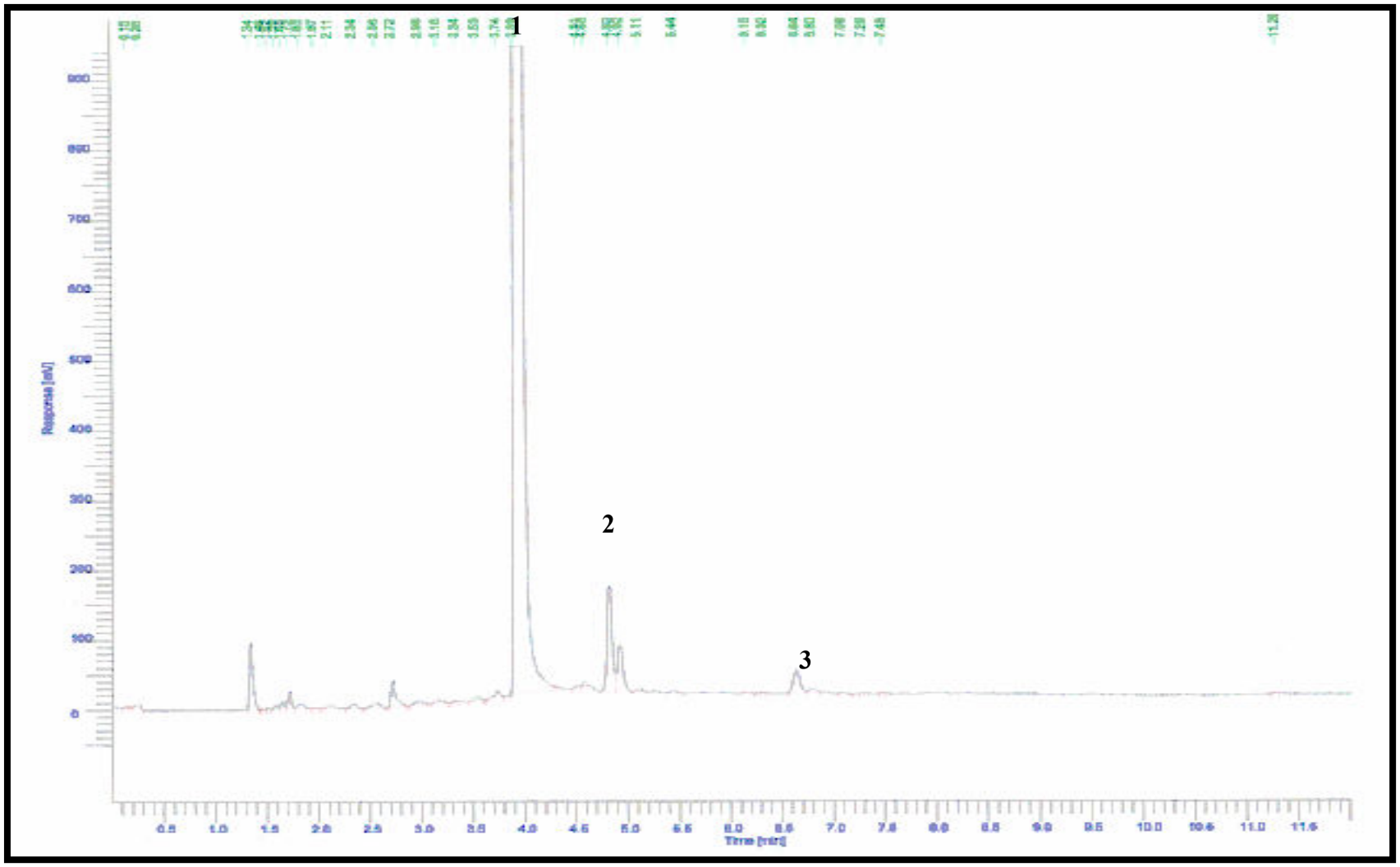

Figure 5.13: Congener 2.2'.4.5.5' Pentachlorobiphenyl pure standard

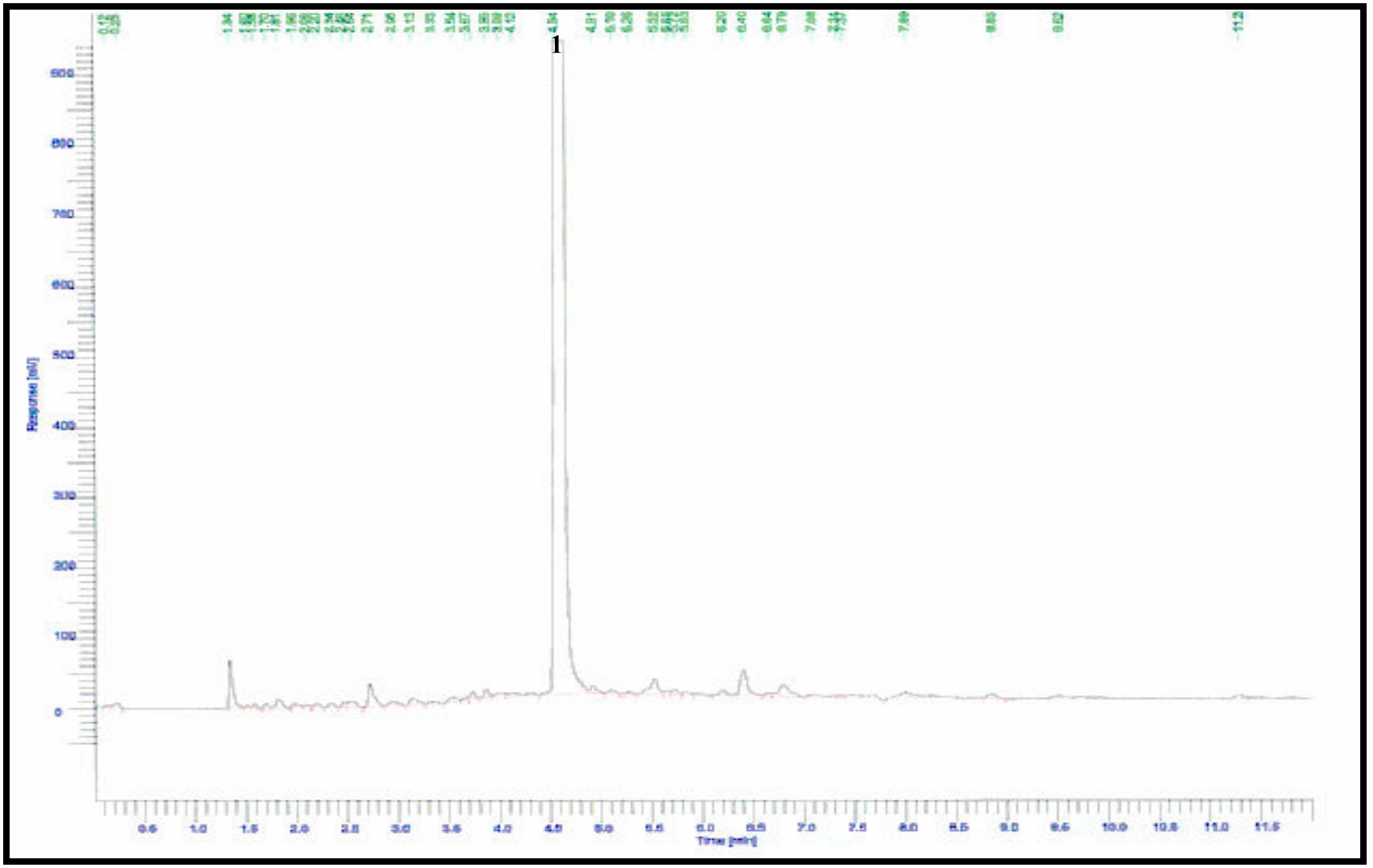

Figure 5.14: Congener 2.2'.3.5.5'.6 Hexachlorobiphenyl pure standard

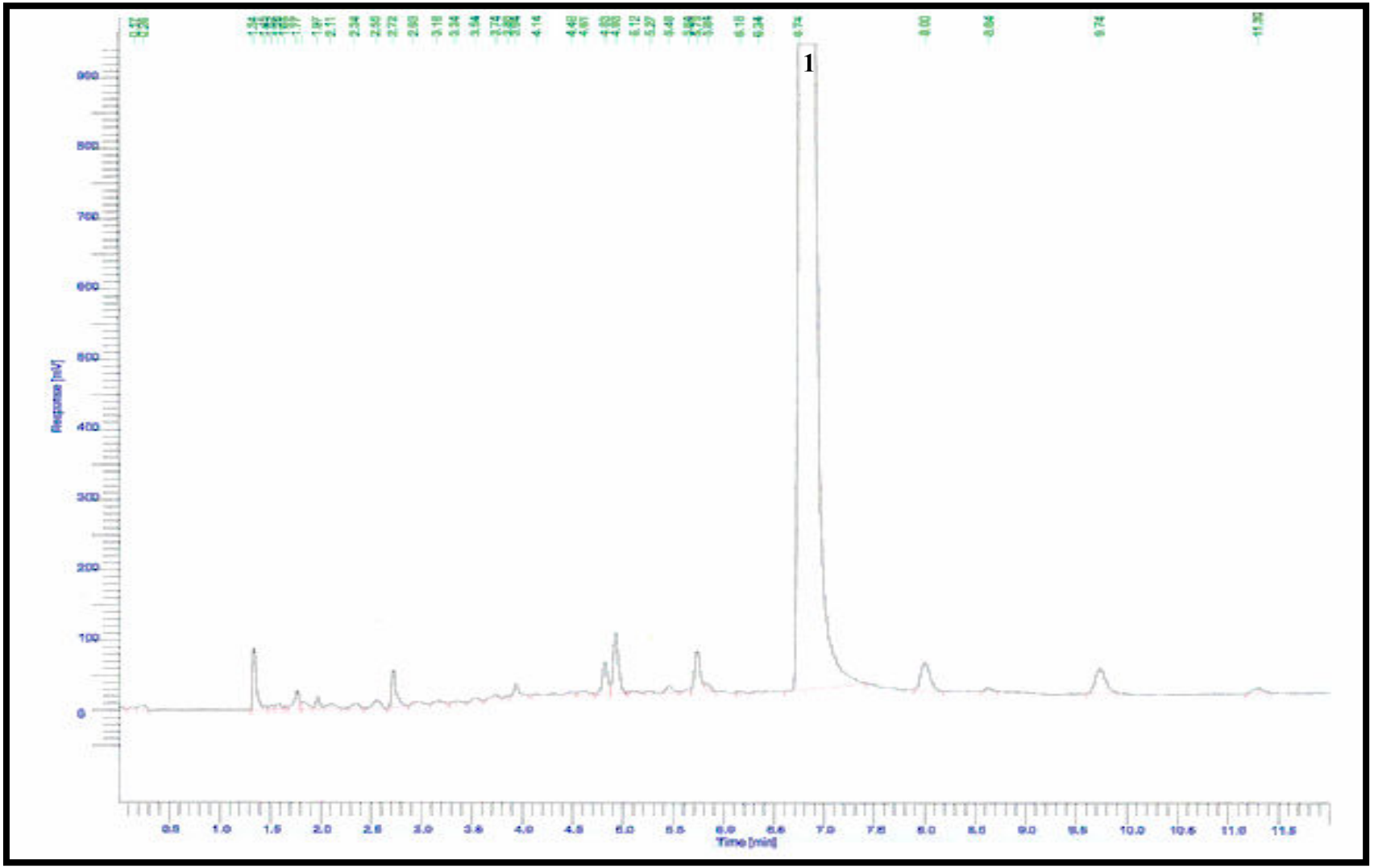

Figure 5.15: Congener 2.2'.3.4.4'5.5' Heptachlorobiphenyl pure standard

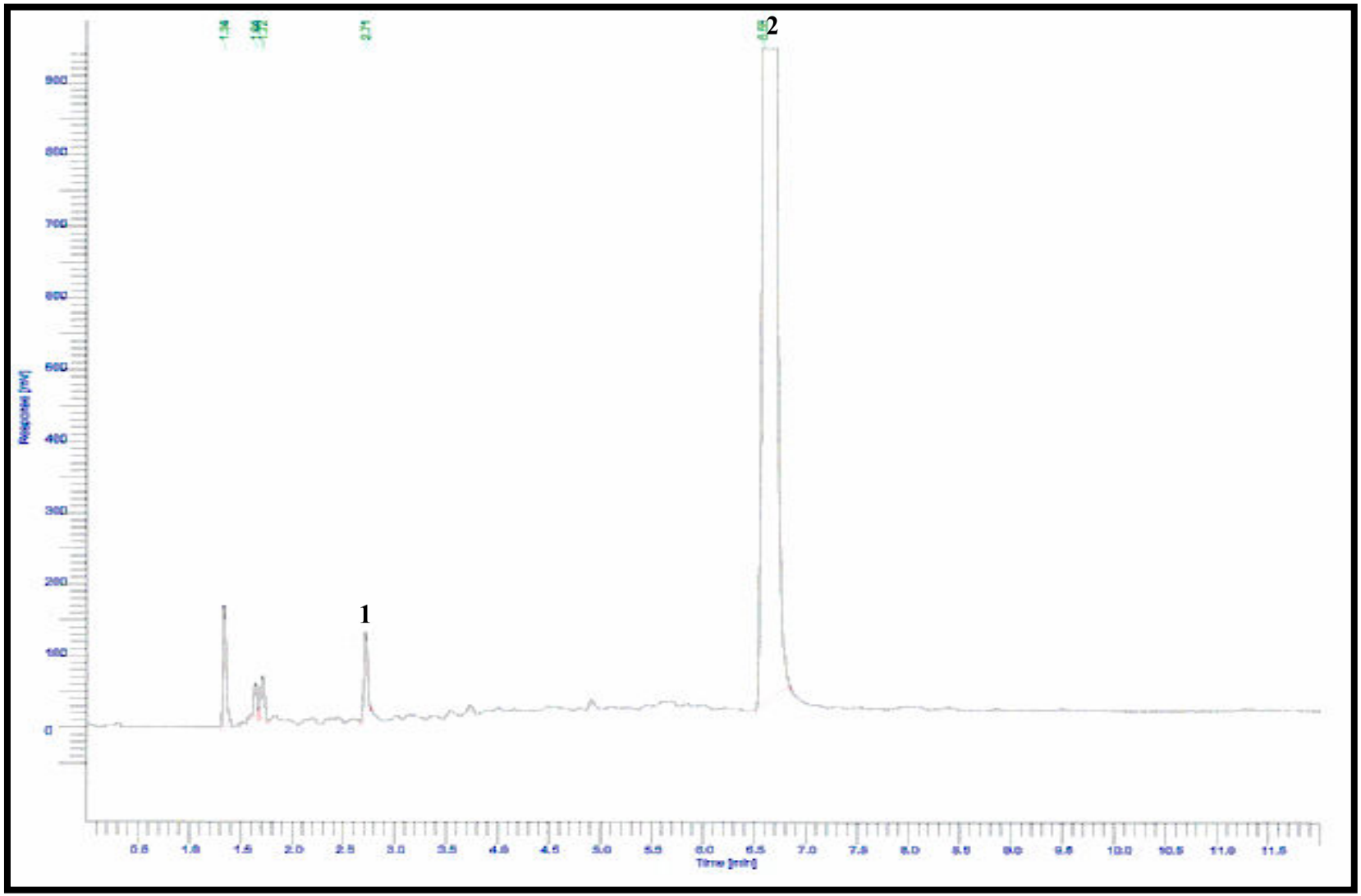

Figure 5.16: Congener 2.2'.3.4.4'.5.6.6' Octachlorobiphenyl pure standard

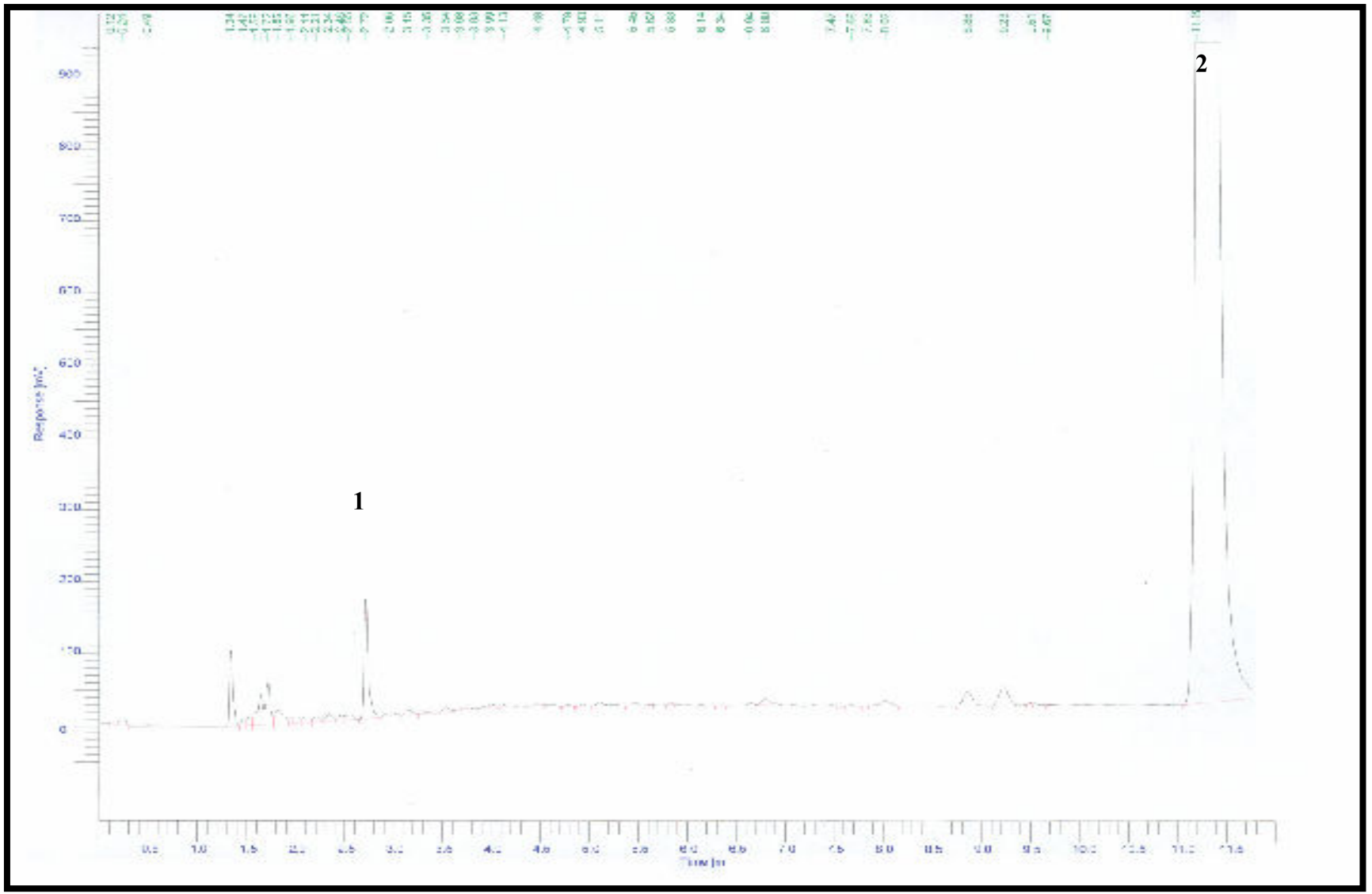

Figure 5.17: Congener 2.2'.3.3'.4.4'.5.5'.6 Nonachlorobiphenyl pure standard

5.4. Discussion

The GC-ECD analysis showed that the isolates were able to transform both Aroclor 1254 and Aroclor 1260 to PCB metabolites. Commercial PCB mixtures containing predominantly monochlorobiphenyls and dichlorobiphenyls readily undergo primary biodegradation by activated sludge microorganisms (Tucker *et al*., 1975). The use of TLC plates was not suitable for the detection of PCBs as seen in Fig. 5.1. a-e, due to the use of PCB concentrations of less than 50ppm in the samples. Detection of PCBs on a TLC plate requires a concentration of 50ppm and greater. The use of TLC to detect degradation was not efficient which could be attributed to the low concentration of the PCB metabolites that were transformed during the process that could not be detected (Vo-Dinh *et al*., 1994). The GC-ECD was then utilised as it is able to detect very low concentrations and is sensitive to PCB compounds.

The degradation potential was analysed by comparative GC analysis of selected PCB congeners (EPA, 1998b); 2.3'.4'.5 Tetrachlorobiphenyl (IUPAC 70), 2.2'.4.5.5' Pentachlorobiphenyl (IUPAC 101), 2.2'.3.5.5'.6 Hexachlorobiphenyl (IUPAC 151), 2.2'.3.4.4'5.5' Heptachlorobiphenyl (IUPAC 180), 2.2'.3.4.4'.5.6.6' Octachlorobiphenyl (IUPAC 204) and 2.2'.3.3'.4.4'.5.5'.6 Nonachlorobiphenyl (IUPAC 206) (Fig. 5.12. - 5.17.). The *Acinetobacter* sp. (SWA 4) did not show the most efficient degradation as seen in Table 5.1. and Fig. 5.4. The *Acinetobacter* sp. was not able to transform the hexachlorobiphenyl and heptachlorobiphenyl but the isolate was able to transform congener 2.2'.4.5.5' Pentachlorobiphenyl by 55%. *Pseudomonas* sp. (SWA 5) showed the best degradation and transformation to lower chlorinated biphenyls in Aroclor 1254 as seen in Table 5.1. and Fig. 5.5. It was able to degrade the different numbers of chlorines. Most of the mono, bi- and tri-chlorobiphenyls were transformed. Based on the comparative congener analysis the mixed culture was an efficient degrader however it was not able to fully degrade the hexachlorobiphenyl and heptachlorobiphenyl congeners compared to the *Acinetobacter* sp. It was observed in Table 5.1. and Fig. 5.6 that the mixed culture was effective in completely transforming Aroclor 1254 with the removal of all 4 congeners shown in Fig. 5.6. The *Acinetobacter* sp. and *Pseudomonas* sp. were able

to degrade the PCBs effectively. The *Pseudomonas* sp. are well known as aerobic decomposers and biodegraders of PCBs. *Acinetobacter* sp. and *Pseudomonas* sp. are renowned for their abilities to degrade compounds which are highly toxic to other organisms, including other environmental pollutants (Prescott *et al*., 1996). These species have a long history of aromatic ring cleavage, from benzoate to phenylacetate. This allows the manipulation of the organism's traits to degrade PCBs (Geng *et al*., 2006). Congener analysis of Aroclor 1254 indicated that *Acinetobacter* sp. was transformed to a pentachlorobiphenyl due to the higher concentration of the remaining congener peak in Fig. 5.4. The *Pseudomonas* sp. was transformed to a Hexachlorobiphenyl in Aroclor 1254 due to the higher concentration of the remaining peak in Fig. 5.5.

The mixed culture is clearly seen in Table 5.1., Table 5.2., Fig. 5.6. and Fig. 5.11. to predominantly degrade all the chlorinated biphenyls to a substantially lower chlorinated PCB metabolite. This can be attributed to the ability of co-metabolism of the *Acinetobacter* sp. and *Pseudomonas* sp. During the transformation of Aroclor 1254 *Acinetobacter* sp. was only able to transform congener 2.2'.4.5.5' Pentachlorobiphenyl by 55% (Table 5.1. and Fig. 5.4.). Aroclor 1254 contains primarily pentachlorobiphenyls and are resistant to microbial attack by *Acinetobacter* sp. (Furukawa *et al*., 1983). In the mixed culture the influence of the *Pseudomonas* sp. was able to transform congener 2.2'.3.5.5'.6 Hexachlorobiphenyl by 100%. This is also observed in Table 5.2. of Aroclor 1260, that the *Pseudomonas* sp. transformed congener 2.2'.4.5.5' Pentachlorobiphenyl by 67% and *Acinetobacter* sp. transformed congener 2.2'.3.5.5'.6 Hexachlorobiphenyl by 40% but with *Pseudomonas* sp. the transformation was increased by 5%. The most noted result was the 100 % transformation of all the congeners in Aroclor 1254 as seen in Fig. 5.11. by the mixed culture. The inability of the cultures to decrease congeners 2.2'.3.5.5'.6 Hexachlorobiphenyl and 2'.3.4.4'5.5' Heptachlorobiphenyl any further can be attributed to the toxicity of the congeners on the isolates. However the ability of the mixed culture to completely transform Aroclor 1254 can be attributed to the ability of one of the organisms in the mixed culture to transform the higher chlorinated biphenyls thereby completing transformation (Hernandez *et al*., 1995). The increase degradation of trichlorobiphenyl, tetrachlorobiphenyl and pentachlorobiphenyl decreases the rate of

degradation which has been observed for similar results for various Aroclors by *Pseudomonas* sp. (Liu, 1980).

The ability of *Pseudomonas* sp. to transform PCBs effectively classifies this strain as a versatile PCB degrader (Kohler *et al*., 1988). Both the organisms were able to utilise both the higher and lower chlorinated PCBs which goes against literature of aerobic PCB degraders that are unable to degrade higher chlorinated biphenyls (Yadav *et al*., 1995). *Pseudomonas* sp. transformed congener 2.2'.3.5.5'.6 Hexachlorobiphenyl and 2.2'.3.4.4'5.5' Heptachlorobiphenyl in Aroclor 1254 (Fig. 5.5.) at a very low rate which keeps to the *Pseudomonas* sp. ability to predominantly transform low chlorinated biphenyls (Pieper, 2004; Tiedje, 1993). However in Aroclor 1260 it was able to decrease congener 2.2'.3.5.5'.6 Hexachlorobiphenyl up to 67% (Fig. 5.10.) compared to the mixed culture and *Acinetobacter* sp. However both the organisms were unable to transform congener 2.2'.3.5.5'.6 Hexachlorobiphenyl. This could be attributed to certain PCB congeners having toxic properties similar to dioxins (Kim *et al*., 2004). It could also be due to the metabolites formed by the congeners that might affect the relative reactivity of the Aroclors.

Substantial degradation of individual congeners was observed. The complete removal of congener peaks 2.3'.4'.5 Tetrachlorobiphenyl, 2.2'.4.5.5' Pentachlorobiphenyl and 2.2'.3.5.5'.6 Hexachlorobiphenyl by the pure cultures. and mixed culture (Table 5.1. and Table 5.2.) is much lower in Aroclor 1260 than Aroclor1254. The reason can be observed in the difference in the Aroclor mixtures that vary qualitatively in the congener composition. (Yadav *et al*., 1995). This explains the different percentages of PCB degradation.

Only a few strains are able to degrade PCBs with more than 4 chlorines (Bedard *et al*., 1986). Aerobic bacterial strains capable of metabolizing polychlorinated biphenyls (PCBs) in the pure cultures because they are able to use these compounds as a carbon source. Some PCBs display toxic effects and in some cases, metabolites, can inhibit the PCB degradation in bacteria. Thus, a more efficient aerobic degradation and

dechlorination of low-chlorinated biphenyls seems to occur in defined mixed bacterial cultures containing both PCB degrading bacteria (Fabio, 1998). This was observed with the mixed culture compared to the pure cultures efficiency for transformation of Aroclor 1260.

The microorganisms isolated are capable of utilizing PCBs as a carbon and energy source. This robust metabolism gives them a high capacity for adaptation, which explains why these bacteria are found in very diverse environments. They have illicted a growing interest in environmental applications for PCB bioremediation Aroclor 1254 and Aroclor 1260 have over 60 congeners and these pure cultures and the mixed culture were able to utilise the PCB and transform the PCBs to lower chlorinated metabolites. These organisms are novel in that they were isolated from industrial wastewater treatment plants and the species have an affinity to transform Aroclors both in the higher and lower chlorinated congeners in aerobic conditions.

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

The enrichment and isolation of bacteria from industrial wastewater sufficed for screening isolates for potential PCB degraders. The aerobic isolates were predominantly gram negative rods. The media was efficient in purifying and screening potential PCB degraders. The screening process using different concentrations of PCBs was able to establish the isolates ability to utilise the PCBs at higher concentrations. Comparatively only SWA 4 and SWA 5 were able to utilise the PCBs at $10mgmL^{-1}$ and $30mgmL^{-1}$. These bacteria were able to survive and utilise the PCB as a sole carbon source. The remaining isolates may have been able to withstand the toxicity but could not utilise the PCBs effectively and therefore with the increase of concentration the isolates had a minimum growth. The UV/Vis spectrophotometer was able to indicate 10 organisms from the original number of bacteria isolated in this study as potential PCB degraders by the degree of absorbance with increasing degree of concentration of the PCBs. Isolates SWA 4 and SWA 5 were selected for further characterisation and determination of their growth kinetics. The remaining 23 isolates will be used for future PCB studies.

The two isolates growth rates were analysed by the Live/dead stain. The stain luminance's the live bacteria, to appear green under a fluorosine filter on an epifluorescence microscope. Both SWA 4 and SWA 5 had a maximum cell activity at 9hrs. The active cells were used in the PCB batch assay for investigation of potential PCB degradation. The pure bacteria and a combination of SWA 4 and SWA 5 were characterised by a UV/Vis spectrophotometer to investigate the potential PCB degradation ability between a pure culture and a mixed culture. The mixed culture showed efficient transformation of Aroclor 1254 and Aroclor 1260. The pure culture SWA 5 presented good potential for degradation of Aroclor 1260. The two aerobic bacterial strains SWA 4 and SWA 5 were presumptively identified as *Acinetobacter* sp. and *Pseudomonas* sp. respectively using an API kit.

The degradation potential was analysed by comparative GC analysis of selected PCB congeners (EPA, 1998b); 2.3'.4'.5 Tetrachlorobiphenyl (International Union of Pure and Applied Chemistry-IUPAC 70), 2.2'.4.5.5' Pentachlorobiphenyl (IUPAC 101), 2.2'.3.5.5'.6 Hexachlorobiphenyl (IUPAC 151), 2.2'.3.4.4'5.5' Heptachlorobiphenyl (IUPAC 180), 2.2'.3.4.4'.5.6.6' Octachlorobiphenyl (IUPAC 204) and 2.2'.3.3'.4.4'.5.5'.6 Nonachlorobiphenyl (IUPAC 206). The GC-ECD degradative studies revealed that the mixed culture was able to transform all the congeners in Aroclor 1254. The *Acinetobacter* sp. and *Pseudomonas* sp. were unable to transform the higher chlorinated congeners of Aroclor 1254. However the mixed culture was able to transform Aroclor 1254 completely. This can be attributed to the presumptive synergistic action of *Acinetobacter* sp. and *Pseudomonas* sp. Aroclor 1260 was transformed by *Acinetobacter* sp. by 80%, *Pseudomonas* sp. by 95% and the mixed culture by 85%. This was due to the inability of the cultures to transform congener, 2.2'.3.5.5'.6 Hexachlorobiphenyl. The congener was clearly toxic to the cultures and apprehended further transformation of Aroclor 1260.

Comparison of Aroclor 1254 and Aroclor 1260 found that in the mixed culture the *Pseudomonas* sp. was influential in the degradation process. This was seen by the higher transformation of Aroclor 1254 and Aroclor 1260 by *Pseudomonas* sp. than the *Acinetobacter* sp. The ability of the mixed culture to completely transform all the congeners was dependant on the *Pseudomonas* sp. which had the ability of completely transforming congener 2.2'.3.5.5'.6 Hexachlorobiphenyl in Aroclor 1254. This is also observed in Aroclor 1260, the *Pseudomonas* sp. transformed congener 2.2'.4.5.5' Pentachlorobiphenyl by 67% and *Acinetobacter* sp. transformed congener 2.2'.3.5.5'.6 Hexachlorobiphenyl by 40% but with the addition of *Pseudomonas* sp. the transformation is increased by 5%.

The complete transformation of Aroclor 1254 and 95% transformation of Aroclor 1260 by the mixed culture can be attributed to the synergistic relationship of *Acinetobacter* sp. and *Pseudomonas* sp. as versatile chlorinated degraders. It was concluded that the mixed culture of *Acinetobacter* sp. and *Pseudomonas* sp. proved efficient to transform both

Aroclor 1254 and Aroclor 1260. The *Pseudomonas* sp. showed the most efficient transformation of Aroclor 1254 and Aroclor 1260.

6.2. Recommendations

- The use of the pure cultures isolated can be adapted to metabolise a wide variety of pollutants. PCBs contaminate the environment mainly in the form of Aroclors. The growth substrates and parameters investigated will allow the efficient growth of the microorganisms and will thus optimise degradation of PCBs.

- A practical approach is the use of environmental congener analysis for quantification of PCBs. PCBs analysed by congener-specific analysis is necessary to understand the source, fate and transport of the PCB in the environment. The congener analysis is a rapid method of identifying microorganisms that are able to degrade PCBs.

- Further studies will focus on the biochemistry of PCB degradation and will have an impact on the development of effective biodegradative processes for treatment of PCBs in the environment.

- These novel PCB-degraders would prove useful for South Africa, in combating a wide variety of PCB pollutants. The strains have a high tolerance to the toxicity of the Aroclors and thus have a good application for the bioremediation of PCBs.

- A lab scale pilot reactor for PCB biodegradation with a continual fed batch system would allow the manipulation of parameters for the optimisation of efficient PCB degradation by the isolates. Further laboratory studies with pure cultures, mixed cultures and *in-situ* lab scale reactors would obtain a better understanding of the importance of the PCB dehalogenation process in a contaminated environment. Understanding the microbial interaction and the

influences on the environment are essential for possible degradation of aged PCBs in an environment. Quantification of each specific congener for the specific Aroclor 1254 and Aroclor 1260 should be selected based on the seven different chlorobiphenyls i.e. from monochlorobiphenyl to decachlorobiphenyl. This would represent a variety of structural features to confirm PCB transformation. The use of internal standards will also be a good confirmation of degradation.

- It is generally accepted that an ideal PCB-degrader should be a highly tolerant microorganism that is expressed by the degradation enzyme with a wide specificity without the accumulation of toxic intermediates. Therefore the study of cellular activity is essential in predicting the survival ability of the microorganism. The use of enzymes for the identification of PCB metabolites allows the elucidation of the metabolic pathways during the transformation of the Aroclors. The key enzymes will be identified by linking the different catabolic pathways for mineralization.

- Regular sampling of industrial wastewater should be undertaken to compare the load of organics and solid suspensions to the number of potential PCB-degraders. The microbial carbon sources and biochemical characteristics are necessary to optimise the efficiency and effectiveness of the PCB degradation without compromising the environment and animal and human health.

- Determination of the respective organism's species level will be investigated using appropriate molecular techniques.

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APPENDIX A

NUTRIENT AGAR WITH 0.2% GLUCOSE

The nutrient agar used was manufactured by Biolab, 1997 and the additional glucose added was for enrichment purposes.

Composition in grams per litre (g/L):

- Meat Extract …… 1.0g/L
- Peptone ……. 5.0g/L
- Yeast Extract …… 2.0g/L
- Sodium Chloride ……... 8.0g/L
- Agar …….. 15.0g/L

Additional

- Glucose …… 2.0g/L

pH 7.1 ± 0.1

Preparation

The components were added into a 1Lt schott bottle and distilled water was added to make up a Nutrient broth of 1Lt. The solution was autoclaved at 15psi pressure, at 121^{o}C for 15min.

APPENDIX B

SELECTIVE POLYCHLORINATED BIPHENYL MEDIA

The media was modified for this study to isolate potential PCB degraders using Bedard *et al*., 1986 and Sierra *et al*., 2003 media components.

Solution A (make up 1 Litre (Lt) stock solution)

- di-Potassium hydrogen
  Orthophosphate Anhydrous (K_2HPO_4) ……... 56.77g/L
- Potassium dihydrogen (KH_2PO_4) …… 21.94g/L
- Ammonium Chloride (NH_4Cl) …… 12.96g/L

pH: 7.0

Solution B (make up 1 Litre stock solution)

- Magnesium Sulphate ($MgSO_4$) …… 39.0g/L
- Manganous Sulphate
  anhydrous ($MnSO_4$. H_2O) ……..... 5.0g/L
- Ferrous Sulphate
- Heptahydrate ($FeSO_4$. $7H_2O$) …….. 1.0 g/L
- Calcium Chloride ($CaCl_2$. $2H_2O$) ……. 0.5g/L

pH: 7.0

- A few drops of Hydrogen Sulphate was added to Solution B to prevent precipitation of the salts.

Stock Solution of Aroclor 1254 and Aroclor 1260

The Aroclor 1254 and Aroclor 1260 were each purchased as a 50mg (dehydrated form). The dehydrated Aroclors were made-up to 10mL using HPLC grade n-hexane. The concentration of Aroclor 1254 and Aroclor 1260 was therefore 1mL stock solution from 50mg in 10mL of HPLC grade n-hexane.

Method: (makes up 1 Litre)

1. **PCB source:** A volume of 1mL of the stock solution of Aroclor 1254 or Aroclor 1260 (50mg in 10mL of HPLC grade n-hexane) was added to a 1 Litre (contaminant free and autoclaved) schott bottle.

2. **Phosphate Buffered Medium Concentrate (PA):** Yeast Extract (0.1g) was added to a 1 Lt Schott bottle. 77.5mL of Solution A was added to this solution and made up to 1Lt with distilled water. The pH was corrected to 7.0 before autoclaving.

3. **Phosphate-Buffered Mineral Salts Medium (PAS):** A volume of 10mL of Solution B was pipetted into a 20mL schott bottle and the pH was corrected to 7.0 before autoclaving.

- Plate Agar Medium: Add 1.5% weight per volume ($^{w}/_{v}$) of agar into Solution A (i.e. Bacteriological Agar 19g/L)

The solutions were autoclaved, i.e. solution PA and PAS at 121^{o}C for 15min. Once cooled the solutions were added to the PCB source schott bottle. The broth or agar was poured into the erlenmeyer flasks or plated out.

APPENDIX C

GRAM STAIN (Modified Hucker Method)

(Jenkins *et al*., 1986)

Solution 1

- Crystal Violet ……. 2.0g/L
- 95% Ethanol …….. 20mL
- Ammonium Oxalate …….. 0.8g/L
- Distilled Water ……... 80mL

Method for Solution 1

Crystal Violet and 95% Ethanol were first mixed. Ammonium Oxalate and distilled water were mixed separately. These two solutions are then combined and stirred well to form Solution 1.

Solution 2

Iodine ……... 1.00g/L

Potassium Iodide ……. 2.00g/L

Distilled water ……. 300mL

Method for Solution 2

The iodine and potassium iodide were added to 300mL distilled water and stirred well.

Solution 3

Safrinin O …….. 0.6g

95% Ethanol …….. 10mL

Distilled Water …….. 100mL

Method for Solution 3

The Safrinin O was added to 10mL of 95% Ethanol. A volume of 10mL the Safrinin O solution was added to 100mL of distilled water.

Staining a microscope slide

A thin bacterial smear was prepared on a slide and allowed to air dry. The slide was stained for 1min with Solution 1 and rinsed with water for a second. The slide was then stained with Solution 2 for 1min and rinsed with water. The slide was finally decolourised with 95% ethanol and stained with Solution 3 for 1min. The stained slide was rinsed with water and air dried. The slide was then observed for the gram reaction using a microscope.

APPENDIX D

LIVE/DEAD STAIN FOR GROWTH CURVE

(Molecular Probes, 2001)

Kit contents for viability kit, L-7012

- STO-9 dye, 3.34mM (component A), 300µL solution in DMSO (di-methyl sulphoxide) solution.
- Propidium iodide, 20mM (component B), 300µL solution in DMSO (di-methyl sulphoxide) solution.

Method

1. Equal volumes of component A and B were thoroughly mixed in an eppendorf tube.
2. A volume of 3µL of the dye was added to each millilitre of bacterial suspension. When used at the recommended dilutions the reagents contribute 0.3% DMSO to the staining solution. Higher DMSO concentrations may adversely effect staining.
3. The sample was then mixed thoroughly and incubated at room temperature in a dark cabinet for 15min.
4. A volume of 5µL of the stained bacterial suspension was added on a slide and a cover slip was placed on top of the slide. The edges of the cover slip on the slide were painted with clear nail polish to prevent leakage or movement during microscopic examination.
5. A fluorescent microscope was used, equipped with specific omega and chroma filters to observe the red and green colours.

APPENDIX E

API KIT

(BioMérieux® S.A., 2002)

Content of the kit

Kit for an API test (ref. 20 100)

- An API 20 E strip
- An incubation box
- A result sheets
- 1 package Insert

Composition of the strip

The composition of the API 20 E strip was given in the Reading as seen in Table Appendix E.2.

Reagents and material required but not provided

Reagents

- API 20 E Reagent kit (Ref. 20 120) or individual reagents: TDA (Ref. 70 402)
  : JAMES (Ref. 70 542)
  : VP 1 + VP 2 (Ref. 70 422)
- Mineral oil (Ref. 70 100)
- API 20E identification software (BioMérieux)

Material

- Pasteur pipette
- General microbiology laboratory equipment

Specimen Preparation

The microorganisms were grown in nutrient broth (Appendix A) for 24hrs at 30^{o}C, according to standard microbiological techniques.

Instructions for use

Preparation of the strip

- An incubation box (tray and lid) was prepared and 5mL of distilled water was distributed into the honey-combed wells of the tray to create a humid atmosphere.
- The strains reference codes were recorded on the elongated flap of the tray.
- The strip was removed from the package and placed in the incubation box.

Preparation of the Inoculum

Using a Pasteur pipette 1mL of the inoculum from the nutrient broth was used to inoculate the microtubes.

Inoculation of the strip

- Using the same Pasteur pipette both tube and capule of the tests CIT , VP and GEL were filled with the bacterial suspension.
- Only the tube (and not the cupule) of the other tests were filled.
- An anaerobic condition was created in the tests ADH, LDC, ODC, H_2S and URE by overlaying the microtubes with mineral oil.
- The incubation box was closed and incubated at 30°C for 24hs.

Reading and interpretation

Reading the strip

- After the 24hr incubation period, the strip is read by referring to the Reading Table as seen in Table Appendix E.2.
- The reactions which required addition of reagents were first performed and recorded:

  - TDA Test: 1 drop of TDA reagent produced a reddish brown colour indicating a positive reaction.

  - IND Test: 1 drop of JAMES reagent produced a pink colour that developed in the whole cupule indicating a positive reaction.

  - VP Test: 1 drop each of VP 1 and VP 2 reagents were added. It was left for 10min. A pink or red colour indicated a positive reaction.

NOTE: The indole production test was performed last since as this reaction releases gaseous products which may interfere with the interpretation of other tests on the strip. The plastic incubation tray was not replaced after the addition of the reagent.

Interpretation

Identification was obtained by the **numerical profile** as seen in Fig. E.1.

- Determination of the numerical profile:

  On the result sheet, the tests were separated into groups of 3 and a value 1, 2 or 4 was indicated for each. The corresponding positive reactions were added together within each group. A 7-digital profile number was obtained for the 20 tests of the API 20E strip.
- Identification:

  The identification was performed by entering the numerical code into the BioMérieux identification software database (V4.0).

Figure Appendix E.1.: Example of 7-digital profile number

READING TABLE

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS/ENZYMES	RESULTS	
				NEGATIVE	POSITIVE
ONPG	2-nitrophenyl-ßD-galactopyranoside	0.223	ß-galactosidase (Ortho NitroPhenyl-ßD-Galactopyranosidase)	colorless	yellow (1)
ADH	L-arginine	1.9	Arginine DiHydrolase	yellow	red / orange (2)
LDC	L-lysine	1.9	Lysine DeCarboxylase	yellow	red / orange (2)
ODC	L-ornithine	1.9	Ornithine DeCarboxylase	yellow	red / orange (2)
CIT	trisodium citrate	0.755	CITrate utilization	pale green / yellow	blue-green / blue (3)
H2S	sodium thiosulfate	0.075	H2S production	colorless / greyish	black deposit / thin line
URE	urea	0.76	UREase	yellow	red / orange (2)
TDA	L-tryptophane	0.38	Tryptophane DeAminase	TDA / immediate	
				yellow	reddish brown
IND	L-tryptophane	0.19	INDole production	JAMES / immediate	
				colorless pale green / yellow	pink
VP	sodium pyruvate	1.9	acetoin production (Voges Proskauer)	VP 1 + VP 2 / 10 min	
				colorless	pink / red (5)
GEL	Gelatin (bovine origin)	0.5	GELatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	1.9	fermentation / oxidation (GLUcose) (4)	blue / blue-green	yellow / greyish yellow
MAN	D-mannitol	1.9	fermentation / oxidation (MANnitol) (4)	blue / blue-green	yellow
INO	inositol	1.9	fermentation / oxidation (INOsitol) (4)	blue / blue-green	yellow
SOR	D-sorbitol	1.9	fermentation / oxidation (SORbitol) (4)	blue / blue-green	yellow
RHA	L-rhamnose	1.9	fermentation / oxidation (RHAmnose) (4)	blue / blue-green	yellow
SAC	D-sucrose	1.9	fermentation / oxidation (SACcharose) (4)	blue / blue-green	yellow
MEL	D-melibiose	1.9	fermentation / oxidation (MELibiose) (4)	blue / blue-green	yellow
AMY	amygdalin	0.57	fermentation / oxidation (AMYgdalin) (4)	blue / blue-green	yellow
ARA	L-arabinose	1.9	fermentation / oxidation (ARAbinose) (4)	blue / blue-green	yellow
OX	(see oxidase test package insert)		cytochrome-OXidase	(see oxidase test package insert)	

(1) A very pale yellow should also be considered positive.
(2) An orange color after 36-48 hours incubation must be considered negative.
(3) Reading made in the cupule (aerobic).
(4) Fermentation begins in the lower portion of the tubes, oxidation begins in the cupule.
(5) A slightly pink color after 10 minutes should be considered negative.
• The quantities indicated may be adjusted depending on the titer of the raw materials used.
• Certain cupules contain products of animal origin, notably peptones.

Table Appendix E.1.: Reading Table

APPENDIX F

LIQUID-LIQUID EXTRACTION & SIMPLE DISTILLATION FOR CONCENTRATING

(Standard Methods, 1985)

Equipment

50mL Separating Funnels
n-hexane
Schott Bottles
Erlenmeyer Flasks
Sodium Sulphate
Distillation apparatus

Method

The PCB sample and n-hexane was added to a 50mL separating funnel. A volume of 25mL of the sample and 25mL of n-hexane was added to the separating funnel. On addition of the sample and n-hexane the flask was shaken vigorously for a minute and placed into a residing holder to settle. The process was continued as described above after each separation. The top layer contained the PCBs dissolved in the n-hexane was collected in a schott bottle. The bottom layer was discarded. Two spoons of sodium sulphate was added to the extracted sample to absorb water.

The distillation apparatus was setup and the sample was heated to 70^{o}C. The recycled n-hexane from the receiving arm of the apparatus was collected in an erlenmeyer flask. The concentrated PCB sample in the round bottom flask was pipetted into a 2mL amber bottle for GC-ECD analysis.

RAW DATA

Cell Count Data of Live/Dead Growth Curve Analysis

Table 1: Organism SWA 4 Live/Dead Count

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Avg.**
1hr	6	7	9	11	12	11	14	7	7	6	13	12	6	12	12	**10**
2hr	16	13	18	18	16	18	16	18	17	13	15	13	12	19	14	**16**
3hr	21	17	22	21	22	19	17	19	22	21	14	22	22	21	22	**20**
4hr	25	23	25	27	25	28	32	25	23	24	27	25	25	23	25	**25**
5hr	54	53	53	54	52	52	53	52	55	51	53	51	55	52	48	**52**
6hr	75	85	73	77	76	84	87	84	78	73	75	76	86	84	85	**80**
7hr	126	125	129	121	128	129	123	127	128	129	124	123	125	122	127	**126**
8hr	135	134	153	152	153	152	153	152	152	153	154	154	155	153	153	**151**
9hr	165	172	172	172	169	170	173	173	175	166	173	172	176	178	176	**172**
10hr	167	167	167	168	168	168	163	165	164	163	162	163	162	162	162	**165**
11hr	165	168	161	161	162	162	162	162	162	162	161	162	162	162	162	**162**
12hr	154	153	155	156	152	153	152	152	152	152	154	155	156	152	157	**154**
13hr	156	153	156	156	156	157	157	157	153	152	151	156	156	156	156	**155**
14hr	154	153	154	154	154	153	152	153	152	151	151	151	151	151	151	**152**
15hr	144	145	143	144	144	145	145	144	145	144	141	142	143	144	144	**144**

Avg. = The average of the 15 counts

Table 2: Organism SWA 5 Live/Dead Count

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Avg.**
1hr	6	5	8	6	4	23	10	5	9	6	8	5	9	12	11	**8**
2hr	25	23	22	24	23	34	26	23	31	22	35	22	25	22	23	**25**
3hr	46	41	44	40	47	44	45	44	42	48	48	44	45	48	44	**44**
4hr	53	53	66	66	67	64	66	63	53	67	63	66	66	66	66	**63**
5hr	67	69	60	69	69	69	71	69	68	69	59	70	69	70	68	**69**
6hr	67	74	77	76	77	76	77	76	77	76	77	78	76	77	78	**76**
7hr	85	97	86	87	87	87	93	93	90	85	82	91	98	80	98	**95**
8hr	110	111	112	104	112	111	112	113	114	114	115	112	112	112	112	**104**
9hr	127	125	125	124	127	123	124	123	124	124	125	127	127	127	127	**125**
10hr	123	122	123	124	122	121	120	122	121	124	123	121	121	121	121	**124**
11hr	121	121	122	121	121	122	122	122	122	121	122	122	121	121	122	**122**
12hr	123	123	124	124	124	124	124	124	123	124	124	124	124	124	124	**124**
13hr	122	122	123	123	123	123	123	123	124	124	124	123	123	122	123	**123**
14hr	112	111	112	112	113	112	112	122	113	122	112	113	113	113	112	**114**
15hr	113	114	112	113	112	112	114	115	112	112	112	114	122	112	113	**113**

Avg. = The average of the 15 counts