

Molecular Characterisation of Bacteria and their Degradation Role on Polychlorinated Biphenyls (PCBs) and Polycyclic Aromatic Hydrocarbons (PAHs) in Wastewater from Gaborone (Botswana) and Mafikeng (South Africa)

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PREFACE

This study was influenced by the global call by the UNEP Chemicals, in cooperation with the Secretariat of the Basel, Rotterdam and Stockholm Conventions (BRS) and in consultation with the PCB Elimination Network (PEN) advisory committee that was held at the Stockholm Convention on Persistent Organic Pollutants in 2001 aimed at eliminating Persistent Organic Pollutants (POPs) from the environment as well striving towards sustainable, yet efficient, means of treating wastewater through bioremediation. This study advocates for complete cleanup of wastewater in an effort to conserve water as a natural resource and minimise environmental pollution. The present thesis contains findings on the topic that was investigated. Peer-reviewed articles from these findings are also presented in the Appendix.

Three (3) papers and a poster were presented at the following national and international conferences:

1. The role of bacteria in the breakdown of carcinogenic substances (PCBs) in wastewater for safe recycling purposes. Presented at the International Conference on Water, Informatics, Sustainability, and Environment, August 2014, Canadian Museum of Civilization, Gatineau-Ottawa, Canada; The abstract is published in Science Direct.
2. The role of bacteria in the breakdown of carcinogenic substances (PAHs) in wastewater for safe recycling purposes. Environmental Education of Southern Africa (EEASA) 32nd Annual Conference and Workshop. September 2014, University of Namibia, Windhoek, Namibia.
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4. Characterization of microorganisms and their effect on the breakdown of recalcitrant compounds from wastewater. Environmental Education of Southern Africa (EEASA) 31st Annual Conference and Workshop. September 2013, Cross Roads Hotel, Lilongwe, Malawi.

AUTHOR PUBLICATIONS

Articles published in peer reviewed journals

1. S. Mathews and N.P. Sithebe, The Role of Bacteria in the Breakdown of Carcinogenic Substances (PCBs) in Wastewater for Safe Recycling Purposes – A Review. International Journal of Environment and Sustainability, Vol. 3 (3), pp 20 -27, 2014
2. Spar Mathews, Biodegradation of Polychlorinated Biphenyls (PCBs), Aroclor 1260, in Wastewater by Isolate MD2 (*Pseudomonas aeruginosa*) from Wastewater from Notwane Sewage Treatment Plant in Gaborone, Botswana. Journal of Bioremediation and Biodegradation, Vol. 5, pp 266 – 272, 2015
3. Spar Mathews, Collins N. Ateba and Nomathamsanqa P. Sithebe. Characterization of bacteria with biodegradation qualities for recalcitrant compounds isolated from wastewater from Gaborone (Botswana) and Mafikeng (South Africa). Merit Research Journal of Microbiology and Biological Sciences. Vol. 3 (2), pp xxx –xxx, 2015.
4. Spar Mathews, Kwenga Sichilongo. Applying QuEChERS method in screening for Polychlorinated Biphenyls (PCBs) from raw and wastewater from Gaborone (Botswana) and Mafikeng (South Africa), International Journal of Environmental Research, Vol. 10 (1), pp 13 – 20, 2016.

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DECLARATION

I, Spar Mathews, declare that the dissertation entitled “**Degradation of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) by bacteria from wastewater in Gaborone (Botswana) and Mafikeng (South Africa)**”, hereby submitted for the degree of Doctor of Philosophy in Biology (Molecular Microbiology), has not previously been submitted by me for a degree at this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

Signed.....this the.....day of.....2015

DEDICATION

This thesis is dedicated to Mr Kekgaoditse Suping, my sons Thabo and Chedu Mathews. Lots of love to you all.

LIST OF ACRONYMS

ATSDR	: Agency for Toxic Substances and Disease Registry
BLAST	: Basic Local Alignment Search Tool
BTEX	: benzene, toluene, ethylbenzene and a mixture of xylenes
cDNA	: complementary deoxyribonucleic acid
DEA	: Department of Environmental Affairs
DGGE	: Denaturing Gradient Gel Electrophoresis
DNA	: Deoxyribonucleic Acid
DWA	: Department of Water Affairs
DWAF	: Department of Water Affairs and Forestry
ECA	: Economic Commission for Africa
EHS	: Environmental, Health and Safety
EPA	: Environment Protection Agency
FAO	: Food and Agriculture Organisation of the United Nations
GC-MS	: Gas Chromatograph Mass Spectrometry
GEF	: Global Environment Facility
HPLC	: High Performance Liquid Chromatography
HWTS	: Household Water Treatment and Safe Storage
IDRC	: International Development Research Centre
IWRM	: Integrated Water Resources Management
IWRM-WE	: Integrated Water Resources Management and Water Efficacy
NCBI	: National Centre for Biotechnology Information
NIST	: National Institute of Standards and Technology
NRW	: Natural Resources and Water

PAH	: Polycyclic aromatic hydrocarbon
PCB	: Polychlorinated biphenyl
PCR	: polymerase chain reaction
PEC	: Probable Effect Concentration
POPs	: Persistent organic pollutants
QuEChERS	: Quick, Easy, Cheap, Effective, Rugged and Safe
SDRA	: Sustainable Development Report on Africa
SRM	: Standard Reference Materials
UNDESA	: United Nations Department of Economic and Social Affairs
UNDP	: United Nations Development Programme
UNECA	: United Nations Economic Commission for Africa
UNEP	: United Nations Environment Programme
UNICEF	: United Nations Children's Fund
UNSGAB	: United Nations Secretary-General's Advisory Board
USGS	: United States Geological Survey
WHO	: World Health Organization
WUC	: Water Utilities Cooperation

DEFINITION OF CONCEPTS

Abiotic: The non living component of the environment or ecosystem.

Aroclor: A commercially prepared polychlorinated biphenyl (PCB) mixture.

Bioaccumulation: A process that results in a molecule being taken up into living cells; it remains in the cells without enzymatic biodegradation and thus results in biosorptive mechanisms, bioprecipitation and intracellular accumulation.

Bio-augmentation: The introduction of bacteria to an area that has been contaminated for purposes of speeding up the removal of the contaminant.

Biodegradation: The metabolic ability of microorganisms to transform or mineralize organic contaminants into less harmful, non-hazardous substances, which are integrated into natural biochemical cycles.

Biomagnification: The increase in concentration of substances with increase in trophic levels in an ecosystem.

Bioremediation: The process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state or to levels below concentration limits established by regulatory authorities.

Biotic: The living component of the environment or ecosystem.

Biotransformation: A step in the biochemical pathway which leads to the conversion of a molecule into a less toxic product.

Carcinogenic: Any substance that tends to stimulate cancer production or uncontrolled proliferation of cells in a living organism (especially animals).

Carbon Catabolite Repression: A regulatory mechanism in bacteria whereby the expression of genes required for the synthesis and activities of proteins necessary for the transport and metabolism of secondary carbon sources is suppressed.

Complementary Deoxyribonucleic Acid (cDNA): DNA formed by reverse transcriptase acting on an RNA as the template.

Congener: Any single, uniquely defined chemical compound in the PCB category with a specific number of chlorine molecules and their positions.

DNA fingerprinting: The process in which genomic DNA base sequence profiles of an organism is obtained.

Ecology: A branch of biology that deals with the relations of organisms to each other and to their natural environment or surroundings.

Ecological niche: The place or function of a given organism within its ecosystem.

Ecosystem: A community of living organisms and their interaction with the non living component of their environment.

Hydrocarbon: These are organic chemical compounds composed of the elements carbon and hydrogen (alkanes, alkenes, alkynes and aromatic).

Pollution: The introduction of contaminants in the environments that cause adverse effects to the biotic and abiotic component of the environment.

Polymerase Chain Reaction (PCR): A technique for the rapid production of millions of copies of a particular stretch of DNA or RNA sequence.

Primary Treatment: The first stage in the sewage or wastewater treatment process where removal of contaminants is through screening and settling processes, resulting in about 40 – 50% removal of contaminants.

Probable Effect Concentration (PEC): This represents the concentration of a contaminant in bed sediment and thus is expected to adversely affect organisms found/living at the bottom.

Recalcitrant Compounds: These are compounds which are non-biodegradable and range from natural organic material such as hair, melanin, lignin, to hydrocarbons and complex polymers such as styrene, pesticides and cyanides.

Ribosomal Ribonucleic Acid (rRNA): These are several types or species of RNA that are incorporated into a ribosome.

Secondary Treatment: The second stage of wastewater treatment process whereby suspended solids are reduced through biological processes and results to removal of about 85 – 95% contaminants.

Standard Reference Materials (SRMs): These are materials that have been well characterised for specific chemical properties such as concentration (denoted as mass fraction) for specific chemical types.

Surfactant: These are surface active agents that reduce surface tension in liquids.

Tertiary Treatment: The third stage in wastewater treatment process which involves filtration and disinfection, resulting in the removal of up to 99.99% of pathogens and suspended solids.

Waste Management: All institutional, financial, technical, legislative, participatory, and managerial aspects related to the handling of wastewater.

Wastewater: Water carrying waste from households, businesses and industries and involves a mixture of water and dissolved or suspended solids.

Xenobiotics: A synthetic chemical substance that is foreign to an ecological system or environment

SUMMARY

This thesis is a study on biodegradation of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in wastewater by bacteria isolated from wastewater as an alternative means of treating wastewater for safe recycling as a possible option to reduce water shortages. The research focused on selected wastewater treatment plants and raw water dams in Gaborone, Botswana and Mafikeng, South Africa. The thesis argues, amongst other things, that water is a scarce resource and this indicates the need to have measures in place to effectively and efficiently treat wastewater for safe recycling to curb the problem of water shortage. Wastewater contains recalcitrant compounds which are not easily removed from the environment through simple conventional processes. Some of these compounds like polycyclic aromatic compounds (PAHs) and polychlorinated biphenyls (PCBs) have carcinogenic and teratogenic effects. These compounds are also considered persistent organic pollutants (POPs) leading to a global agreements such as the Stockholm Convention of 2001 that called for nations to stop the production of these compounds as well as a decree for all nations to come up with a national plan on how these compounds will be eliminated.

The objectives of this study was to isolate and characterise aerobic bacteria that possess biodegradation characteristics for inorganic compounds from wastewater samples obtained from Notwane Sewage Treatment Plant and Setumo/Modimola Dam and surface water samples from Gaborone dam and Disaneng Dam,. A further objective was to analyse the water for the presence of PAHs and PCBs as well as perform degradation tests on PAHs and PCBs using selected isolates.

In the present study, a total of 60 raw water samples and 50 wastewater samples were collected and analysed for bacterial diversity and presence of PCBs and PAHs. The samples and controls were taken from Gaborone and Mafikeng. The identities of 29 bacterial isolates were identified using preliminary (Gram staining, Analytical Profile Index 20E test) and confirmatory (16S rRNA and 16S rRNA gene sequence

analysis using BLAST search). Bacteria species belonging to the genus *Aeromonas*, *Bacillus*, *Pseudomonas*, *Exiguobacterium*, *Kurthia* and *Vibrio* were detected. From these it is evident that wastewater has a highly diverse group of bacteria, some of which might be having potential biodegradation properties for recalcitrant compounds. Isolate MD2 which was identified as *Pseudomonas aeruginosa* and this was detected in wastewater samples obtained from both Gaborone and Mafikeng. The *Pseudomonas aeruginosa* (MD2 isolates) together with *Serratia liquefacians* and *Aeromonas hydrophila* were used to assess their potentials to degrade PAHs and PCBs at concentrations of 1.0 µg/mL. An aliquot of 1.0 µL of the bacterial suspension with an optical density of 1.0 at 600 nm was used as an inoculum of the assay. Isolates were analysed for their ability to degrade PCB (Aroclor 1260) by measuring a shift in the wavemax using Cary 300 UV-visible spectrophotometer for a period of 96 hours. The presence /absence of the compounds was checked using High Performance Liquid Chromatography (HPLC) UFLC Shimadzu using florescence detector pump RF-20A and system gold column C18 (CTO-20A) after 96 hours. PCBs were extracted from wastewater samples from both Gaborone and Mafikeng using the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction kit and analysis was performed using the Gas Chromatography Mass Spectrometer (GC-MS). The bacteria were able to degrade these compounds under different pH values of 5.0, 7.0, 8.0 and 9.0 and temperatures of 20°C, 27°C, 30°C and 35°C. These results showed that degradation occurred at the most at 35°C and the least at 20°C for all the PAH and PCB samples that were used in the study. At 27 and 30°C the activity for the bacteria similar. The bacteria strain MD2 was able to completely degrade Aroclor 1260 that was incorporated into the wastewater samples within 96 hours. This was supported from the fact that there was a shift in the wavelength from 224 nm to 270 nm which indicated that Aroclor 1260 was degraded and therefore forming a chlorobenzoate derivative. From this finding it can be concluded that the wastewater samples did not possess PCB (Aroclor 1260) after treatment with bacteria and can be safely recycled.

The physico-chemical properties indicated that the pH (7.78 – 7.88), temperature (24.4 °C – 26.1 °C) and turbidity (0.01 NTU) values for control samples were within acceptable limits as per Botswana, South Africa

and WHO drinking water standards. On the contrary, turbidity values were rather higher than the set standards for raw water and wastewater, with water samples from Modimola/Setumo dam having recorded the highest range of 25.0 – 200 NTU. The pH values of samples obtained from Modimola/Setumo dam were higher at 9.01 to 9.78.

Wastewater effluent in Notwane Sewage Treatment Plant, Gaborone Dam (both in Gaborone) and Disaneng Dam (South Africa) have polychlorinated biphenyls (PCBs) below detectable levels by the Agilent Gas Chromatography Mass Spectrometer (GC-MS). Only traces of PCBs were detected from wastewater from Modimola dam in Mafikeng. This may be due to the different industries in Mafikeng producing various chemicals compared to Gaborone. The water in Modimola dam therefore requires thorough treatment before it can be returned for domestic consumption as PCBs are toxic compounds that have been found to trigger cancer in humans and also affect the reproduction system resulting in babies that have low intelligence quotient. These results indicate that the isolates obtained and screened in the study may be very useful in the biodegradation of recalcitrant PAHs and PCBs that are usually present in wastewater.

In conclusion, the objectives of the study were fully achieved. Bacterial strains *Serratia liquefacians* and *Aeromonas hydrophila*, together with *Pseudomonas aeruginosa* and isolate ID MD2 were capable of degrading recalcitrant compounds under different environmental conditions. The result from this study also showed that wastewater from Setumo/Modimola dam (Mafikeng) is more polluted than the wastewater from Notwane Sewage Treatment Plant (Gaborone) with regard to the target compounds as well as taking into consideration the turbidity and pH values obtained from the samples. The wastewater from Setumo/Modimola was also found to contain traces of polychlorinated biphenyls. The results from this study therefore suggest that the wastewater from Setumo/Modimola need thorough treatment to render it safe for recycling for purposes of introducing it into treatment plant for potable water production. The target

recalcitrant compounds were not detected in wastewater samples Notwane sewage treatment Plant and raw water from Disaneng and Gaborone dams.

Although it may be concluded that the wastewater from Notwane Sewage Treatment Plant can be diverted to Gaborone dam to enhance the water level and thus be treated for portable water use, more studies still need to be carried out to check for the compounds through studying the vegetation, fish, and beef from cattle using those places as water holes, before a final recommendation can be forwarded to the Water Utilities Cooperation (WUC).

This was the first study on use of bacterial isolates to breakdown PAHs and PCBs to be carried-out on wastewater in these two areas (Gaborone and Mafikeng). Moreover, the study was designed to target recalcitrant compounds, specifically the polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). There is need for extensive research to screen for and identify bacteria strains among the isolates from wastewater treatment plants that can efficiently degrade recalcitrant compounds.. In addition, it is also important to thoroughly screen the wastewater to determine the type of compounds or metabolites that are produced during biodegradation of PAHs and PCBs as well as their effect on other microorganisms that are found in the water bodies. It is therefore suggested that there is the need to conduct further studies designed to detect specific genes that are actively involved in the production of enzymes that catalyse the biodegradation of PAHs and PCBs respectively.

Keywords

Polychlorinated biphenyls (PCBs); polycyclic aromatic hydrocarbons (PAHs); recycling; wastewater; water scarcity; persistent organic pollutants (POPs); biodegradation; bacteria; *Pseudomonas aeruginosa* MD2

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CHAPTER 1

INTRODUCTION AND SIGNIFICANCE OF THE STUDY

1.0 Introduction and Significance of Study

1.1 Background

Water is one of the most essential natural resources that sustain human daily activities and yet it is so scarce. The scarcity of this natural resource is currently a great global concern and therefore the need for it to be conserved. Generally, water scarcity has been reported to be increasing in Africa when compared to other continents and the rapidly growing population in the region (WHO and UNICEF, 2014; UNECA, 2005) worsens the problem. In under-developed and developing countries, wastewater is normally not recycled but if it is done, it is only partially treated and the recycled water is usually used for agricultural purposes such as watering lawns, and not for human consumption (Onda *et al.*, 2012). According to Onda *et al.*, (2012), 40 million m³ of wastewater are recycled in the world everyday of which 70% is channelled into the Agricultural sector. This has been identified as a way of trying to reduce the pressure that the agricultural sector, especially crop production is facing and therefore addressing global cry to fight famine, which is generally directed at lack of adequate water resources (WHO and FAO, 2010). The remaining 30% is used in industrial activities such as boilers and cooling towers (Onda *et al.*, 2012), Figure 1.1).

Wastewater Reuse in the world

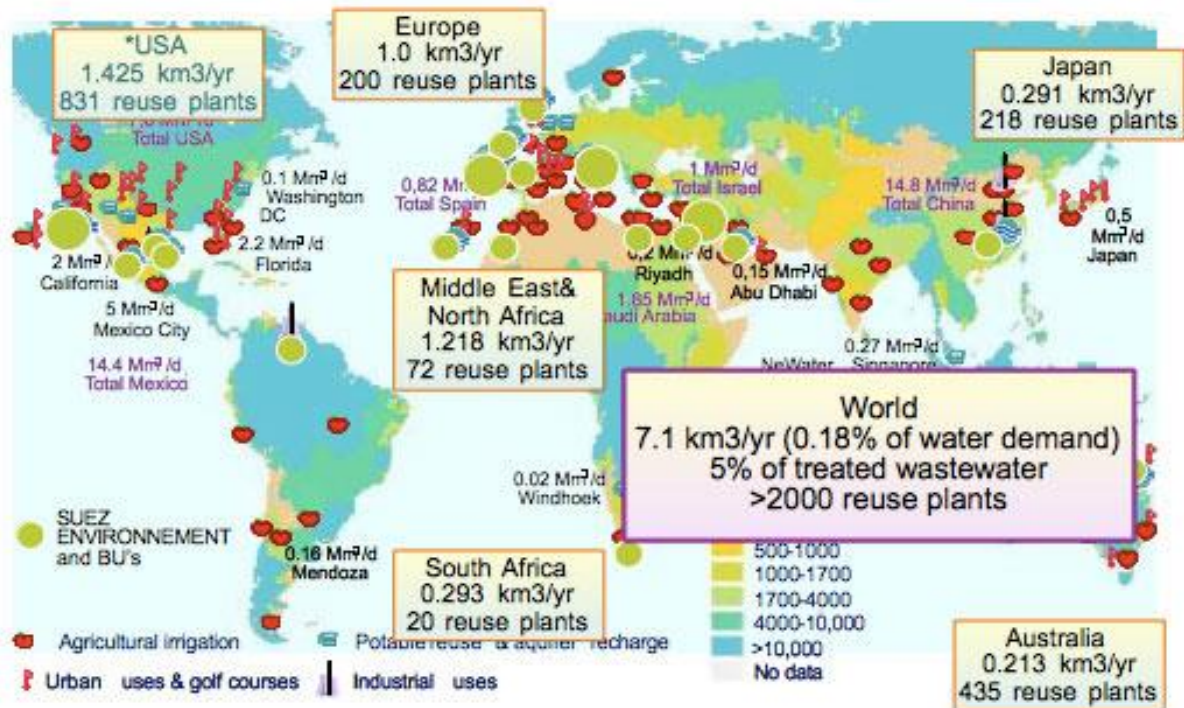


Figure 1.1: Some of the global uses of wastewater (Onda *et al.*, 2012)

Wastewater has not yet been frequently considered to be used to produce drinking water except in Windhoek and Singapore (Onda *et al.*, 2012). However, this study is aimed at carrying out a research whose findings will support the suggestion that grey water as well as sewage water may be treated to an extent that the water is safe for household use and this will curb the problem of shortages in water supply in developing countries such as Botswana. In Botswana, the water from sewage ponds is partially recycled as it can only be used for watering plants in recreation parks and in construction projects, although not always while in South Africa an effort is being made to recycle wastewater but little has been reported on the state of the wastewater in relation to POPs.

The adoption of Millennium Declaration signed at the United Nations General Assembly by World Health Organization (WHO) in September 2000, and its eight (8) goals that were set by WHO and

now known as the Millennium Development Goals (MDGs), led to joint efforts to quickly establish a comprehensive global framework to support the attainment of these goals (WHO and UNEP, 2006; WHO, 2000). The MDGs were to be achieved by 2015 (UNDESA, 2013; WHO and UNEP, 2006) but since most countries had not achieved all the goals by 2015, the United Nations post 2015 Agenda came up with Sustainable Development Goals (SDSN, 2015). Of the eight MDGs, none directly addressed wastewater. When WHO assessed the MDGs, wastewater was found to be infused in most of those goals (MDG goals 1, 4, 7 and 8). The guidelines were built around the Health and Implementation components (WHO and UNEP, 2006) but with SDGs the same issue was directly included as SDG 6, Clean water and Sanitation, so as to address issues surrounding wastewater and sanitation, water scarcity and conservation (SDSN, 2015). In addition, goal number 7 which is “To ensure Environmental Sustainability” was mostly inclined to the use of wastewater to aid in curbing the problem of water shortage and decreased food production (WHO and UNEP, 2006; WHO, 2000). The emphasis though, was that the use of wastewater to alleviate problems associated with water stress and scarcity should not have negative health nor cost implications. Therefore the methods employed in rendering the wastewater in a condition that is safe to be used in the agricultural sector must be effective as well as cost effective (WHO and UNICEF. 2014; WHO and UNICEF. 2013; WHO and UNEP, 2006). This therefore puts a lot of pressure on the developing and middle-income countries as they were the most affected (WHO and UNEP, 2006).

The WHO projections for water supply worldwide, though shows that by 2025 half of the world population will be leaving under water stress conditions (UNDESA, 2013; WHO and UNICEF, 2013). Increase in human population and urbanisation has made the demand for potable water to increase. This have also created new challenges in the wastewater treatment sector (WHO and UNICEF, 2013) and therefore amplifies the need for the development of appropriate technologies for efficient and cost-effective use of water resources and consider ecological sanitation alternatives. The

increase in wastewater production poses health risks due to poor sanitation networks and inadequately functioning wastewater treatment plants, especially in developing and middle income economy countries such as Botswana (WHO and UNICEF, 2014; DWAb, 2013) and South Africa (Ashbolt *et al.*, 2001). In these countries, most wastewater treatment facilities have small holding capacities that get overwhelmed by the growing populations due to urbanisation (WHO and UNICEF, 2013; WHO and UNICEF, 2012). The wastewater eventually spills off these wastewater-holding dams into the environment thus polluting the soil (WHO and UNICEF, 2012).

In 2003, at the Johannesburg World Summit on Sustainable Development, Integrated Water Resources Management (IWRM) was included in the International Policy Framework (IPF) and all countries were tasked with developing their own National IWRM by 2005, especially those countries with serious water scarcity problems. It is envisaged that this will provide countries the opportunity to develop and implement policies regarding the use of wastewater (WHO and UNEP, 2006). Botswana developed its IWRM, known as Botswana Integrated Water Resources Management and Water Efficacy Plan (IWRM-WE) within two years, 2010 – 2012, with the assistance from the United Nations Development Programme (UNDP) and the Global Environment Facility (GEF) (DWA_b, 2013) and South Africa had its IWRM developed in 2000 (DWAF, 2014).

Over the past few years, global warming has led to less rainfall and shorter rainy seasons, and this result in very little available water in the water sources such as boreholes, rivers and dams (WUC, 2011). Figure 1.2 shows the map of Botswana with an overview of rivers and streams that are available.

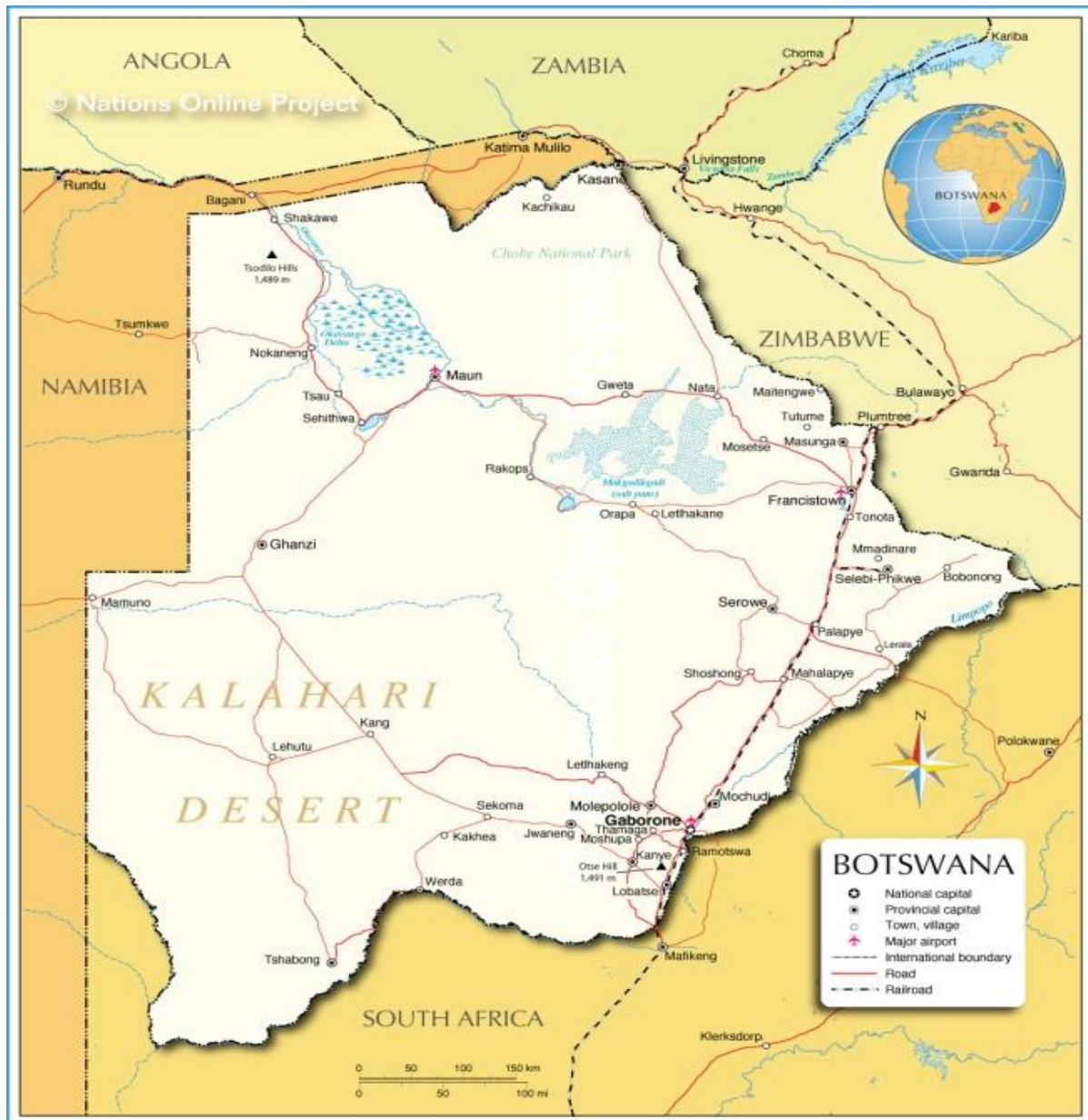


Figure 1.2: Map of Botswana showing the position of Gaborone and few rivers and streams running through the country (WUC, 2013).

In addition, the level of water in the main dams is usually very low and this scarcity of water as a natural resource has greatly been observed in most parts of Botswana including Gaborone (WUC, 2011), Although the situation is greatly improved in south eastern region of Botswana (where Gaborone is situated) where huge amounts of wastewater is available for use in activities such as construction and watering of recreational parks (DWA_b, 2013).

In South Africa residents who live in most rural communities are also faced with the problem of acute shortage of water (Ateba and Maribeng, 2011; DWAF, 1994), Figure 1.3.

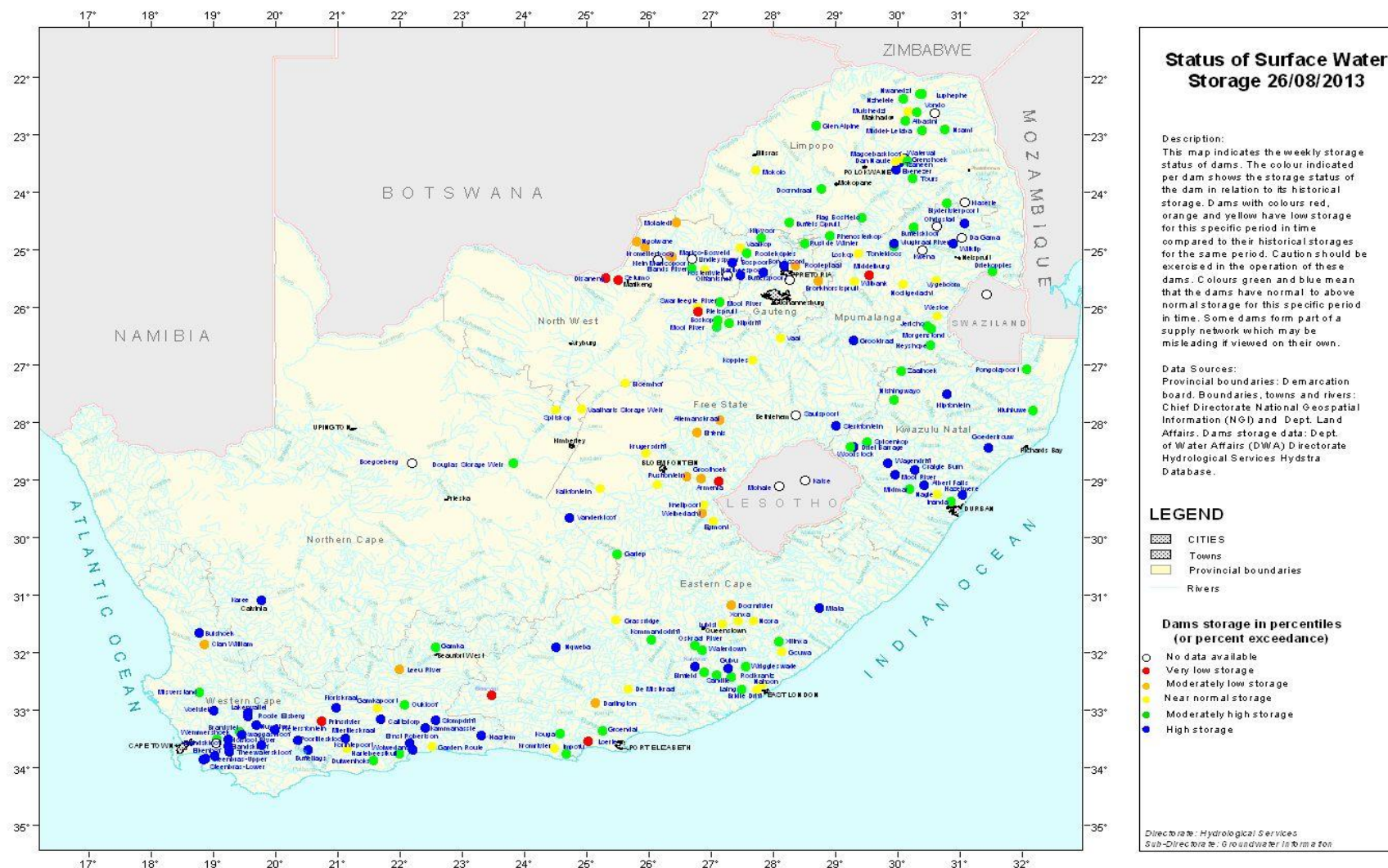


Figure 1.3 A map showing water dams in South Africa (DWAf, August 2013).

Given the water shortage problems mentioned, there is need for recycling of water to help conserve the little that is available. Toole *et al.*, (2007) indicated that recycling of water has to be done safely taking into consideration the health issues involved. UNEP (2004) suggested 10 key areas that municipalities should consider when addressing issues on wastewater and one of these key issues was to “Select appropriate technology for efficient and cost-effective use of water resources and consider ecological sanitation alternatives”.

With the aforementioned as guidelines, sustainable development can only be achieved through the promotion of environmental management and constant search for new technologies to treat vast quantities of waste which includes treatment of wastewater for purposes of recycling (Khadhraoui and Belaid, 2012; Cloete, 2010; Sen and Ashbolt, 2010). The scarcity of water as a natural resource resulting from the aggravating effects of global warming, has resulted to great global concerns to recycle and conserve water, especially in sub-Saharan Africa where the problem of water scarcity has affected most countries (Ateba and Maribeng, 2011). These aggravating changes in climatic conditions has great effects on the natural fresh and raw water resources leading to severe water shortages, especially in rural areas (Onda *et al.*, 2012; WHO and UNICEF, 2012; Ateba and Maribeng, 2011) such as Mafikeng area. In Mafikeng there are two dams, that is, Disaneng and Setumo which are among the dams with the lowest water storage capacities in South Africa as stated in the 2013 report by Department of Water Affairs (DWA_a, 2013).

Although wastewater is fully recycled in other parts of the world (especially the developed countries), this is not the case in most developing countries such as Botswana, although the wastewater effluent has increased from 14.8Mm³ in 1990 to 29.2 Mm³ in 2003 (DWA_b, 2013). Recycling of wastewater (from sewage treatment plants) can greatly relieve the aggravating effects of serious water shortage that is experienced in Botswana and some parts of South Africa (Basson, 2012). This calls for

appropriate and safe measures to be in place to ensure that the recycled water is safe for household use or it is fully potable.

In addition, there is need to ensure the application of Standard Reference Materials (SRMs) (Poster *et al.*, 2004). The use of SRMs will ensure that the quality of water and wastewater with reference to the presence of ubiquitous compounds such as PCBs (Poster *et al.*, 2004) is within acceptable limits. Examples of SRMs, which have been made and are currently used for PCBs, are Aroclors (Poster *et al.*, 2004). In Botswana, sewage water is only partially recycled. This practice of partially recycling sewage water has left a lot of people in Botswana with no option but to use clean/ portable drinking water for all activities including construction and watering gardens. Only 20% of the effluent is currently being used (DWA_b, 2013) for landscaping activities.

If sewage water was fully treated and not rendered unsafe (Mladenov *et al.*, 2005) it would be used for most of these activities and thus saving water and curbing the problem of water shortage. The incorporation of biological means has not been highly infused and thus need to be explored and incorporated in wastewater treatment as it is found to be cost effective and effective in ensuring safe product after the treatment of sewage wastewater (Dhall *et al.*, 2012).

The human activities in developing countries in trying to improve living and survival conditions have also resulted in emerging pollutants and Persistent Organic Pollutants (POPs) are constantly being found in wastewater (Onda *et al.*, 2012) making it even difficult for the low and middle income countries, especially in Sub-Sahara Africa to manage wastewater. The composition of effluent that originate from various industries such as pharmaceutical and mining industries, the agricultural sector, household waste, chemical industries, various manufacturing industries including the oil manufacturers (Heider *et al.*, 2008; Watkinson *et al.*, 2007), increases the potential health threats of such contaminated water systems to humans and animals. The contaminating substances may

remain in the environment (that is soil and water) for long periods of time without being broken down. Compounds that remain for a long time in the environment without being broken down or change in their chemical composition and/or structure are referred to as recalcitrant compounds (Han *et al.*, 2000; Rothmel and Chakrabarty, 1990). Recalcitrant compounds are usually contained in wastewater and are passed on to soil if the water is emptied into the environment or used to water gardens (Watkinson *et al.*, 2007; Han *et al.*, 2000). These resultant compounds are usually taken up by plants or consumed directly in the contaminated water and therefore may pose great health risk to humans and other organisms which may result in an imbalance in the ecosystem (Toole *et al.*, 2007).

Chemicals are used extensively and intensively in the technological society (Cycon *et al.*, 2013; Guillen *et al.*, 2012). Depending on their properties, modes and extent of use, large amounts of these chemicals reach the environment, and have unpredictable environmental and health effects in the long term (Guillen *et al.*, 2012; Toole *et al.*, 2007). In addition, heavy metal and chemical pollution of soil and wastewater is a significant environmental problem (Raja *et al.*, 2009). The recalcitrant compounds, especially the hydrocarbons classified as polycyclic aromatic hydrocarbons (PAHs) and polymers such as polychlorinated biphenyls (PCBs) are highly toxic and can act as mutagens and carcinogens, within living organisms (Suryanto and Suwanto, 2003; Mallick *et al.*, 2007; Hennessee *et al.*, 2009). Certain allergic reactions may also occur in individuals who consume or use recycled water due to the added preservatives (Watkinson *et al.*, 2007).

Internationally, the generally accepted set levels for PAHs and PCBs in soil are 16 mg/kg and 0.89mg/kg respectively (WHO and UNEP, 2006). However, in Botswana, the IRWM focused more on salinity, and general pollution (DWA_b, 2013) than POPs on water quality assessment guidelines although the Stockholm convention urged countries to consider elimination of POPs in wastewater (EPA, 2009; UNEP, 1972; UNEP, 2008). UNEP (2009) reported though that in Africa the key

challenges in relation to management of POPs was mainly due to lack of knowledge and information on hazards, risks and safer alternatives; lack of legislation or reinforcement measures; weaknesses in the technical infrastructure; and a shortage of qualified human resources. These factors are known to account for the extremely minimal available data on PCBs (Ministry of Environment Wildlife and Tourism for Botswana, 2008).

The Department of Environmental Affairs (DEA) of South Africa amended the National Environmental Management Act of 1998 (Act No 107, 1998) to include the phasing out of the general use of polychlorinated biphenyls (PCBs) containing materials and PCBs contaminated materials by 2023 and 2026 respectively (DEA, 2013). According to a report by Ministry of Environment Wildlife and Tourism for Botswana (2008), large quantities of PCB containing equipment (40 000 tonnes) was still in use by 2006 in the country and thus the number of contaminated sources could just have been that high too. These setbacks for African countries were mitigated by the development of country specific IWRM (WHO and UNEP, 2006).

The incorporation of a biological technique known as bioremediation, as a tool to improve the quality of recycled water, has not yet been investigated extensively, especially in Botswana and in some rural parts in South Africa, thus the need for this study. This method of treating wastewater for consumption has been found to be highly cost effective (Faber 2002; Sehnem *et al.*, 2010; Abo-Amer, 2011) and may be of great value to developing countries such as Botswana and South Africa. In addition, the method is not only cost effective but also results in complete clean-up or removal of PAHs and PCBs from the environment (Al-Thani *et al.*, 2009; Nnamchi *et al.*, 2006). Some bacterial strains present in sewage dams, such as *Pseudomonas*, *Acinetobacter*, *Alcaligenes* and *Proteus* species are known to break down various chemical substances, including heavy metals and recalcitrant compounds, during their normal metabolic activities (Raja *et al.*, 2009; Heider *et al.*, 2008). A better understanding of these microbes and the associated metabolic pathways, might lead to the development of improved and safer

water recycling methods (Mallick *et al.*, 2007). Wastewater treatment is also very essential as it addresses the world wide problem of increasing water pollution (Moura *et al.*, 2007).

1.2 Problem Statement

Although studies on bacteria breaking down recalcitrant compounds especially PAHs and other hydrocarbons have been conducted, mostly with isolates from soil (Okere and Semple, 2012; Pacwa-Plociniczak *et al.*, 2011; Phillips *et al.*, 2006), and despite the fact that the degradation of oil in marine environment and freshwater have been studied extensively, no study to the best of our knowledge has been conducted in Botswana and rural areas of South Africa involving wastewater. A study carried out in 2013 on water samples obtained from rivers in the Vaal Triangle area, South Africa, showed that PAHs were present and was at concentrations 0.2 mg/l (Moja *et al.*, 2013) which falls within the acceptable standard reference values. Most studies on the occurrence of PAHS have been conducted extensively in Western countries, Asia and United States of America and there is very limited information on studies on microbial degradation of PAHs and PCBs in Africa, especially Southern Africa (Moja *et al.*, 2013). It is envisaged that the findings from this study may provide information to the Department of Water Affairs and Forestry, South Africa (DWAF) and Water Utilities Corporation (WUC), which may also facilitate the development of strategies to ensure that sewage water is effectively and safely recycled through the process of bioremediation.

The sewage water from Notwane sewage ponds is released to Notwane River instead of recycling it to help address the adverse problem of water shortage in Gaborone and neighbouring villages. This can then be safely practiced in all other towns and big villages in Botswana where vast amounts of water flows through the sewage lines and get wasted to the environment. Very little has been done in Botswana in regard to bacteria capable of degrading recalcitrant compounds PAHs and PCBs aerobically in wastewater though. This probes for studies to be carried out so that the molecular and

biochemical framework on bacteria capable of degrading PAHs and PCBs in wastewater in Gaborone and Mafikeng is established.

1.3 Aim of the Study

The main aim of this study was to isolate and characterize bacteria that are capable of breaking down recalcitrant compounds from sewage or wastewater and raw water in Mafikeng, South Africa and Gaborone, Botswana. A further objective was to determine the efficiency of selected isolated strains to breakdown recalcitrant compounds polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in wastewater thereby improving its quality for safe domestic use.

1.4 Objectives

The objectives of the study were to:

- isolate, identify and characterise bacterial isolates with biodegradation qualities for recalcitrant compounds (PAHs and PCBs) from untreated raw water (from dams) and wastewater (from sewage ponds)
- analyse mixed bacterial population aerobic biodegradation on selected commercially acquired PAHs mixture and PCB aroclors.
- analyse water for polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs).
- determine bacterial enzyme activity on the biodegradation of PAHs and PCBs.

1.5 Significance of the study

The study was designed to provide cost effective and efficient methods for cleaning wastewater with the intention of safe recycling such that the effluent can be safely added to potable water treatment and catchment systems. This option may help to curb the problem of acute water shortages in Gaborone and Mafikeng as well as reduce the occurrence of carcinogenic compounds such as PAHs and PCBs

that are often transmitted through the consumption of contaminated wastewater that flows out into fresh and raw water rivers and dams. This application may also reduce opportunities that lead to the introduction of cancer stimulating compounds into soils thus preventing both soil pollution and human suffering especially in rural communities of developing countries.

CHAPTER 2

LITERATURE REVIEW

2.0 Literature Review

2.1 Introduction

Bacteria have a very simple structure and this provides them with the ability to adapt to changes that occur in the environment and even develop adaptation characteristics to suit any changes that may be brought about by either pollution or climatic alterations (Azhari *et al.*, 2010; Blokesch, 2012; Martins and Peixoto, 2012). In addition, bacteria and other microorganisms are to be able to breakdown recalcitrant and xenobiotic compounds, either through mixed population activity or through mutation of existing microbiota (Azhari *et al.*, 2010; Vrchotova *et al.*, 2013).

The application or use of microbes has been greatly employed in the sewage treatment process, especially at the primary stages of wastewater treatment in which they facilitate the decomposition of sludge (Waksman *et al.*, 1928). This is motivated from the fact that large populations of microorganisms, especially bacteria, has been found to be abundant at the primary stages of wastewater treatment procedures (Waksman *et al.*, 1928). In addition, bacteria are able to convert most chemical substances present in the affluent (Waksman *et al.*, 1928), but because the wastewater is passed through various treatment stages at the treatment plants, this does not give ample time for recalcitrant compounds which may have carcinogenic and teratogenic effects on humans such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), to be broken down (Dhall *et al.*, 2012). The implication is that PAHs and PCBs may be passed to the effluent (Dhall *et al.*, 2012). Recalcitrant compounds are generally not readily and easily biodegradable, and therefore have the potential to persist in the environment without being readily broken down (Paul *et al.*, 2005). PAHs and PCBs are among the most abundant ubiquitous persistent organic pollutants (POPs) in the environment (WHO and UNICEF, 2013; Wongsu *et al.*, 2004). These compounds accumulate in the

food chain and have adverse effects on animals and human health; and are also toxic to other living organisms thus affecting the functioning of the whole ecosystem (Azhari *et al.*, 2010).

Sustainable development requires the promotion of environmental management procedures and the constant search for new technologies to treat vast quantities of waste which may include the treatment of wastewater for purposes of recycling (Khadhraoui and Belaid, 2012; Cloete, 2010; Sen and Ashbolt, 2010). The implementation of these strategies will greatly reduce the biomagnification of PAHs and PCBs and reduce effects of bioaccumulation in the ecosystem. The use of bacteria in the biodegradation of PAHs and PCBs has been found to be effective, non-invasive and cost effective (Guzik *et al.*, 2011; Kumari *et al.*, 2013a, b) and therefore amplifies the importance of this study.

2.2 Bacterial Flora in raw and wastewater

Studies on microbial diversity in sewage treatments have been extensively carried out, especially for pathogenic related studies (Mulamattathil *et al.*, 2014), but there is very little information on the metabolic activities of these isolates in the environment (Okere and Semple, 2012; Phillips *et al.*, 2006). Most of these studies have been carried out at the primary and secondary stages of wastewater treatment in sewage treatment leading to lack of information on microbial diversity in the effluent. The studies though, show that wastewater, unlike raw water, contain a vast population of microorganisms (Han *et al.*, 2013). Bacteria have always been found to play an important role in decomposition of organic material in the ecosystem (Waksman *et al.*, 1928), and it is this process that has been used to study the various metabolic pathways of different bacteria (Noel, 1998). The studies on bacterial pathways were conducted also to find their importance in biodegradation (Anderson and Dawes, 1990; Leahy and Colwell, 1990). Bacteria have the ability to adapt to changes that occur in the environment and thus are able to develop adaptation characteristics to suit any changes that may be brought about by either pollution or climatic alterations (Blokesch, 2012; Martins and Peixoto, 2012). The application or use of microbes has been greatly employed in the sewage treatment plants, especially at the primary

stages of wastewater treatment for purposes of decomposition of sludge (UNEP *et al.*, 2004). This is because of the large population of microorganisms, especially bacteria, has been found to be abundant at the primary stages of wastewater treatment (UNEP *et al.*, 2004). This allows the bacteria to act on most chemical substances present in the affluent (UNEP *et al.*, 2004) but because the wastewater is passed through various treatment stages at the treatment plants, this does not give ample time for recalcitrant compounds to be broken down thus being passed to the surround environment polluting soil and water bodies (Dhall *et al.*, 2012).

The microorganisms with small aerodynamic diameter in water easily get released into the atmosphere and become aerosols and a large proportion of the bacteria in aerosols are affiliated with Proteobacteria and Bacteroidetes (Han *et al.*, 2013), numerous of which emerged from water which is an indication that the bacterial community in bioaerosols is related to the source (Han *et al.*, 2013). Enterobacteriaceae is also another major group of bacteria found in raw and wastewater (Grimont and Grimont, 2006). Although the genus *Pseudomonas* is predominant in sewage wastewater (Lim *et al.*, 2005; Bahig *et al.*, 2008), other genera such as *Chromobacterium*, *Flavobacterium*, *Hyphomicrobium*, *Achromobacter*, *Agrobacterium*, *Alkalegenes*, *Bacillus*, *Serratia* are also found to inhabit in raw water and wastewater (Grimont and Grimont, 2006; Lim *et al.*, 2005) due to their different roles in water such as biodegradation and denitrification processes (Lim *et al.*, 2005).

Treated wastewater and untreated sewage contain bacteria which can be advantageous to the recycling process (La Rosa *et al.*, 2010; Faber, 2002). The capacity of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) degradation by microorganisms (biodegradation) depends on the diversity and characteristics of naturally occurring populations (Dhall *et al.*, 2012; Jayashree *et al.*, 2012; Kapadia and Yagnik, 2013) and their response to environmental conditions rather than on the introduction of new taxa or selective modification of existing ones (Chung and King, 2001; Azhari *et al.*, 2010, Martins and Peixoto, 2012).

2.2.1 Effect of Bacteria on Chemical Substances

Dhall *et al.* (2012) stated that the use of various methods in treatment of sewage have proved not to be effective in rendering the treated water safe for introduction into streams or in the environment. This comes as a result of the water having high loads of substances and microorganisms, some of which can pose a health threat to humans (Dhall *et al.*, 2012). Conventional physical and chemical methods used to decontaminate wastewater from pharmaceutical and textile industries are time and energy consuming (Khadhraoui and Belaid, 2012). The treatment also does not guarantee that the contaminant will be sufficiently removed to allow the water to be recycled (Khadhraoui and Belaid, 2012). After the primary and secondary treatment of wastewater, the effluent is allowed to flow out into the environment and may pose health risks to animals and humans that gain access to this treated wastewater (Bujang *et al.*, 2013). Some of the effluent is intentionally released into nearby streams/rivers as well as dams (Mladenov, *et al.*, 2005). These results in these compounds (recalcitrant), being added to the soil in the surrounding environment and water bodies (Mladenov *et al.*, 2005; Bujang *et al.*, 2013).

This therefore calls for the use of better and improved means in the treatment of wastewater (Dhall *et al.* 2012). The application of the bioremediation process remains a better alternative in treatment of wastewater for safe recycling purposes (Jayashree *et al.*, 2012; Vrchotova *et al.*, 2013; Al-Wasify and Hamed, 2014). Not all microorganisms in wastewater have negative health implications due to their activities or ecological niche (Waksman *et al.*, 1928). The various activities or ecological niche of the various microorganisms in the ecosystem has been summarised as shown in fig 2.1 (Diaz, 2004). The figure presents some of the activities that occur in the environment in relation to bacterial activity and remediation (Lim *et al.*, 2005; Diaz, 2004).

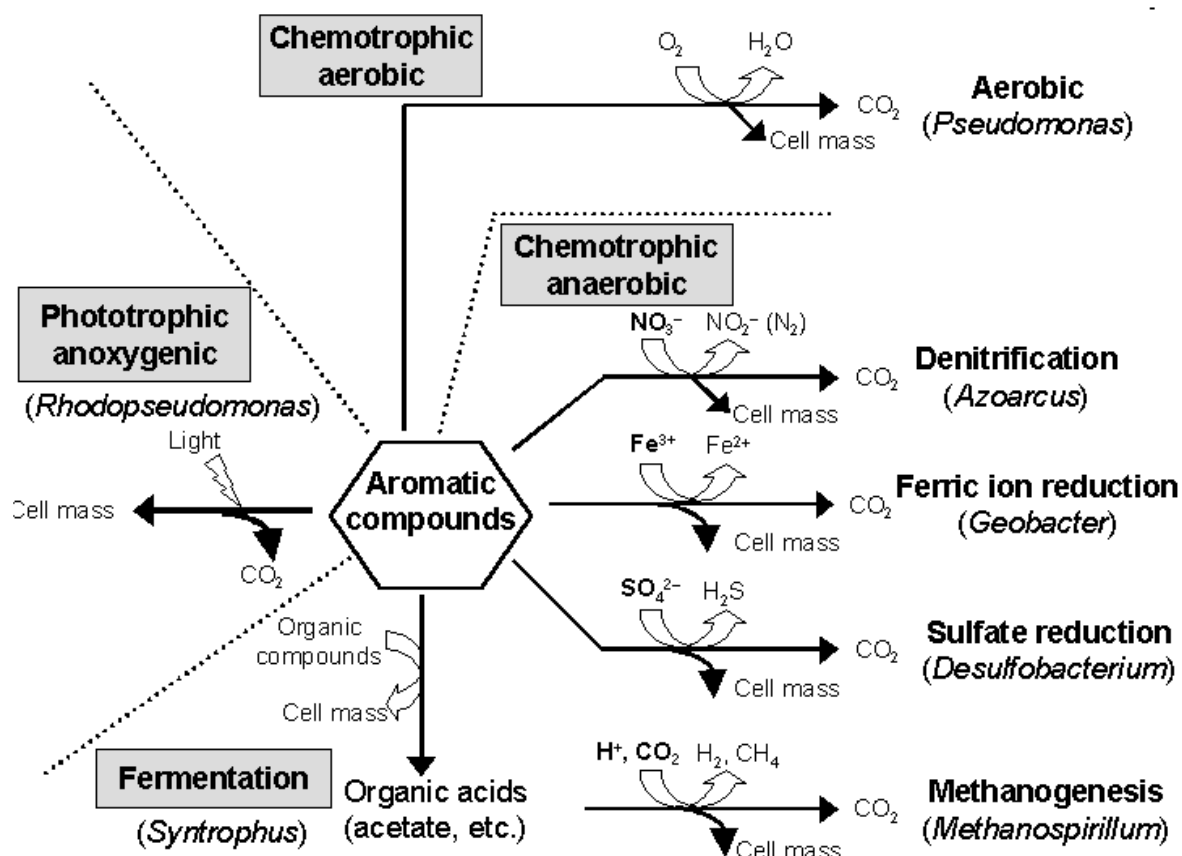


Figure 2.1: Microbial utilization of aromatic compounds (Diaz, 2004)

The use of bacteria results in complete cleanup of wastewater rendering it pollutant free and as such, it is the preferred means of wastewater treatment (Sehnem *et al.*, 2010). The use of microorganisms in wastewater treatment in which waste is biologically broken down, that is, bioremediation (Sehnem *et al.*, 2010) is described as the process of biodegradation (Schinner, 2001; Dhall *et al.*, 2012; Roy *et al.*, 2013). Biodegradation is the metabolic ability of microorganisms to transform or mineralize organic contaminants into less harmful, non-hazardous substances, which are then integrated into natural biochemical cycles (Schinner, 2001; Dhall *et al.*, 2012). Specific bacteria having biodegradative potential for various chemical substances in wastewater as well as untreated (raw) water from water sources, may be used to treat this water (Dhall *et al.*, 2012) for purposes of safely recycling it. Although microorganisms have the ability to naturally breakdown chemical substances, some require several

organisms to work on them at different stages of its molecular status based on their adaptation (Martinkova *et al.*, 2009; Azhari *et al.*, 2010).

According to Sehnem *et al.*, (2010), certain bacteria can be used to eliminate or decrease the amount of the compounds in industrial wastewater. The products of biodegradation may be taken back to the environment through the process of decomposition (Roy *et al.*, 2013). This process causes less or no harm to the environment (Roy *et al.*, 2013). For this process to be more effective, two or more species need to work together to quicken the degradation rate, especially when large compounds (such as PAHs and PCBs) are involved (Warshawsky *et al.*, 2007; Jaward *et al.*, 2012; Dhall *et al.*, 2012). Bahig *et al.*, (2008), stated that organisms such as *Pseudomonas*-like bacteria were isolated from industrially polluted stream and were found to be very detrimental in the treatment of wastewater from sewage effluent. These organisms as well as *Pseudomonas* species were found to have the ability to degrade certain chemicals found in pesticides, which were found to be recalcitrant compounds (Bahig *et al.*, 2008; Heider *et al.*, 2008).

Although *Pseudomonas* species have been the most frequently isolated genus, other genera such as *Escherichia*, *Shigella*, *Citrobacter* and *Enterobacter*, from agricultural soil irrigated with wastewater from sewage treatment plants were also isolated in significant numbers (Bahig *et al.*, 2008) which makes the microbial or bacterial consortia in wastewater to be large. Although microbial communities in the sludge have been studied, the phylogenetic composition of these microbes with relation to biodegradation of recalcitrant compounds is lacking (Wang *et al.*, 2014). For biodegradation by microorganisms to be completely effective and successful, a number of metabolic pathways are usually involved (Heider *et al.*, 2008; Martinkova *et al.*, 2009). This makes it possible for some recalcitrant compounds to be broken down through several steps. For example, the degradation of alkanes and aromatic hydrocarbons involves activation by adding a hydroxyl group by an oxygenase, which is then oxidised to carboxyl group (Mallick *et al.*, 2007). The degradation of phenolic compounds involves several

enzymes, which invariably transform these toxic compounds into protocatechaute and catechol (Azhari *et al.*, 2010). The pathways usually involve the production of intermediates, which are then further broken down until the citrate cycle is reached (Martinkova *et al.*, 2009).

According to Rothmel and Chakrabarty (1990), a number of organisms are naturally able to metabolise synthetic compounds and have genes responsible for dissimilation of these compounds (Suryanto and Suwanto, 2003). *Pseudomonas* species are highly effective in the biodegradation of some recalcitrant compounds may also be modified to enhance their efficiency (Rothmel and Chakrabarty, 1990). Hennessee *et al.*, (2009) stated that bacteria belonging to the genus *Mycobacterium* have been found to have the ability to degrade PAHs in their environment. With the advent of employing microbes in degradation of substances, there is hope that PAHs and PCBs will be highly minimised in the ecosystem (Darvish *et al.*, 2011). The genus *Rhodococcus* has a number of promising species suitable for the biodegradation of recalcitrant compounds too (Martinkova *et al.*, (2009). The xenobiotic aromatic compounds metabolised by these groups of organisms range from aliphatic and aromatic hydrocarbons, oxygenates, halogenated compounds including polychlorinated biphenyls, nitroaromatics, heterocyclic compounds, nitriles and various herbicides (Martinkova *et al.*, 2009).

Although naturally most hydrocarbons will be degraded by microorganisms including bacteria normally found in the environment such as sewage ponds and soils, these have been found to occur slowly as many organisms have to be involved and various metabolic pathways have to be engaged due to recalcitrant characteristic of these compounds (Heider *et al.*, 2008; Martinkova *et al.*, 2009) and thus there has to be enhancement of such organisms for effectiveness of the process (Faber, 2002) for large scale activity (Phillips *et al.*, 2008). This, according to Phillips *et al.*, (2008) allows the organisms to be more effective by optimizing the active gene or enzyme responsible for that particular degradation potential. Biodegradation of hydrocarbons by naturally occurring bacterial populations is important as these compounds are broken down to less toxic or harmless substances (Panda *et al.*, 2013). High

concentrations of PAHs may have toxicity effects on the biodegrading bacteria though thus the need for a mixed bacterial consortium for efficiency (Arbabi *et al.*, 2009; Dhall *et al.*, 2012).

Polychlorinated Biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) are chemical substances that have the tendency of persisting in the environment and get absorbed by the soils, posing a great danger to the ecological balance in the ecosystem (Bahig *et al.*, 2008). Although the presence of heavy metals and other chemical substances such as PAHs and PCBs are hazardous to humans, most microorganisms have detoxification abilities and genetic determinants that make them resistant to or able to breakdown these chemicals (Bahig *et al.*, 2008). According to Al-Jumaily and Al-wahab (2012), bacteria can adapt and grow in the presence of hazardous compounds or any waste stream. These characteristics and the underlying molecular mechanisms thereof, are of great importance in the development of enhanced wastewater recycling methods (Faber, 2002; Dhall *et al.*, 2012; Al-Jumaily and Al-wahab, 2012). These microorganisms can furthermore, also be used to determine the availability of a given chemical compound in the wastewater (Arias *et al.*, 2005).

Microorganisms have the ability, through the production and action of their enzymes, to breakdown different substances as part of their metabolic activities (Leahy and Colwell, 1990; Jayashree *et al.*, 2012). Although most of the pathways followed by these microorganisms are not well known (Rothmel *et al.*, 1990; Faber, 2002), the main requirements for their metabolic activities are energy and a carbon source (Al-Jumaily and Al-wahab, 2012). The bacteria use most of the chemical compounds in their environment as their sources of carbon (Faber, 2002). During biodegradation, the compounds release energy, which the organisms use for their metabolic activities and further breakdown of other compounds that require more than one metabolic pathway to be completely broken down such as PAHs and PCBs (Dhall *et al.*, 2012). These substances that the microorganisms have to degrade include simple hydrocarbons, PCBs and PAHs (Mallick *et al.*, 2007). Faber (2002) stated that microorganisms could degrade aliphatics and aromatics to produce lighter hydrocarbons, including

methane and the most prevalent bacteria has been found to be the phylum *Proteobacteriaceae* and *Methanobacteriaceae*. Other groups of organisms that have been found to use PAHs and other hydrocarbons were *Staphylococcus* species (Mallick *et al.*, 2007), *Beijerinckia* sp., *Mycobacterium* sp., *Pseudomonas* sp., *Alkalegenes denitrificans* (Nnamchi *et al.*, 2006; Chung and King, 2001; Kanaly and Harayama, 1999); *Micrococcus* species (Leahy and Colwell, 1990). Srujana and Khan, (2012) stated that members of the genera *Beijerinckii*, *Mycobacterium*, *Pseudomonas*, *Alkaligenes*, *Bacillus*, *Corynebacterium*, *Rhodococcus*, *Streptomyces*, *Burkholderia* are some of the bacteria that have been found for a long time, to have the ability to breakdown low molecular weight and high molecular weight PAHs. The production of different enzymes by different bacteria present in an ecosystem plays a major role in the breakdown of these compounds (Azhari *et al.*, 2010).

2.2.2 Role/Effect of Bacterial Enzymes in the biodegradation of PAHs and PCBs

The use of enzymes in the water treatment technology is from as early as 1970s but mainly in the treatment of sludge (Whitely and Lee, 2006). Enzymes help in the biotransformation of compounds making them accessible for biodegradation (Whitely and Lee, 2006). Enzyme assays are analytical tools to visualize enzyme activities (Reymond *et al.*, 2008). Enzyme assays are used in the discovery or optimization of industrial enzymes. Majority of enzyme assays are developed to test isolated enzymes or enzyme containing samples such as culture suspensions (Reymond *et al.*, 2008). Most practical enzyme assays are based on synthetic substrates that release a coloured or fluorescent product upon reaction or induce a directly detectable change in solution such as precipitation (Reymond *et al.*, 2008). Enzyme reactions may also be assayed using indicators, which respond indirectly to product formation or substrate consumption (Roy *et al.*, 2013).

The use of microbial enzymes for biodegradation of PAHs promotes excellent results in the clean-up of different environments which include water (Peixoto *et al.*, 2011; Silva *et al.*, 2012). Enzyme systems are essential in initiating the process of degradation by bacteria as they introduce oxygen into the

substrate (Das and Chandran, 2011). Enzymes help in the biotransformation of compounds making them accessible for biodegradation (Whitely and Lee, 2006; Panda *et al.*, 2013). This can be illustrated as shown in Figure 2.2. The compound was oxidized by laccase to form a biodegradable compound, BaP-1,6-quinone, followed by ring cleavage (Hadibarata, 2012)

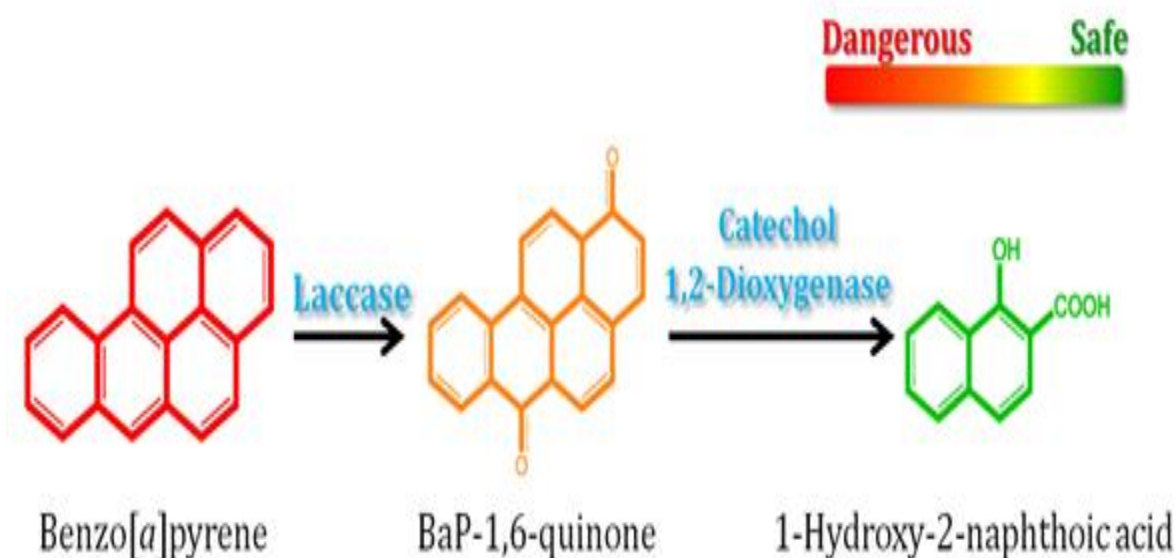


Figure 2.2: A simplified diagram to show biotransformation of Benzo[a]pyrene, a PAH, into a safe biodegradable compound (Hadibarata, 2012)

In degradation of PCBs, chlorobenzoic acids (CBAs) are formed which are biodegradable by most bacteria (Vrchotova *et al.*, 2013). Biphenyl degradation is initiated by a multicomponent non-heme iron oxygenase in some bacteria (Seeger *et al.*, 2010) while the initial step in catechol degradation is operated by catechol 1,2 dioxygenase which catalyses ring fission (Azhari *et al.*, 2010). The result of enzyme based PCB degradation by bacteria may also result in a mixture of CBAs and chlorine atoms depending on the number of chlorination of the degraded PCB congener (Vrchotova *et al.*, 2013). The de-chlorination step is the first while the production of CBAs marks the final stage in the biodegradation of PCB, either through aerobic means or anaerobic (Vrchotova *et al.*, 2013; Seeger *et al.*, 2010). Strains capable of aerobic degradation are the gram negative *Rhodococcus* and *Bacillus* and the gram

positive *Pseudomonas*, *Burkholderia* and *Achromobacter* (Vrchotova *et al.*, 2013; Guzik *et al.*, 2011; Seeger *et al.*, 2010).

According to Camara *et al.*, (2007), in a bacterial mixed consortium culture, most bacteria are able to degrade PCB congeners through various pathways that are identical for most of them. Generally, bacteria will attack a compound and release extracellular enzymes, which initiate catabolic reactions resulting in production of substances that are then taken into the glycolytic pathway for the bacteria to release energy for its own metabolism (Guzik *et al.*, 2013; Fetzner, 2012). Pyruvate acts as a precursor for many anabolic activities, apart from being the precursor for the tri-carboxylic acid (TCA) cycle when it is converted to acetyl Coenzyme A (Diez, 2010).

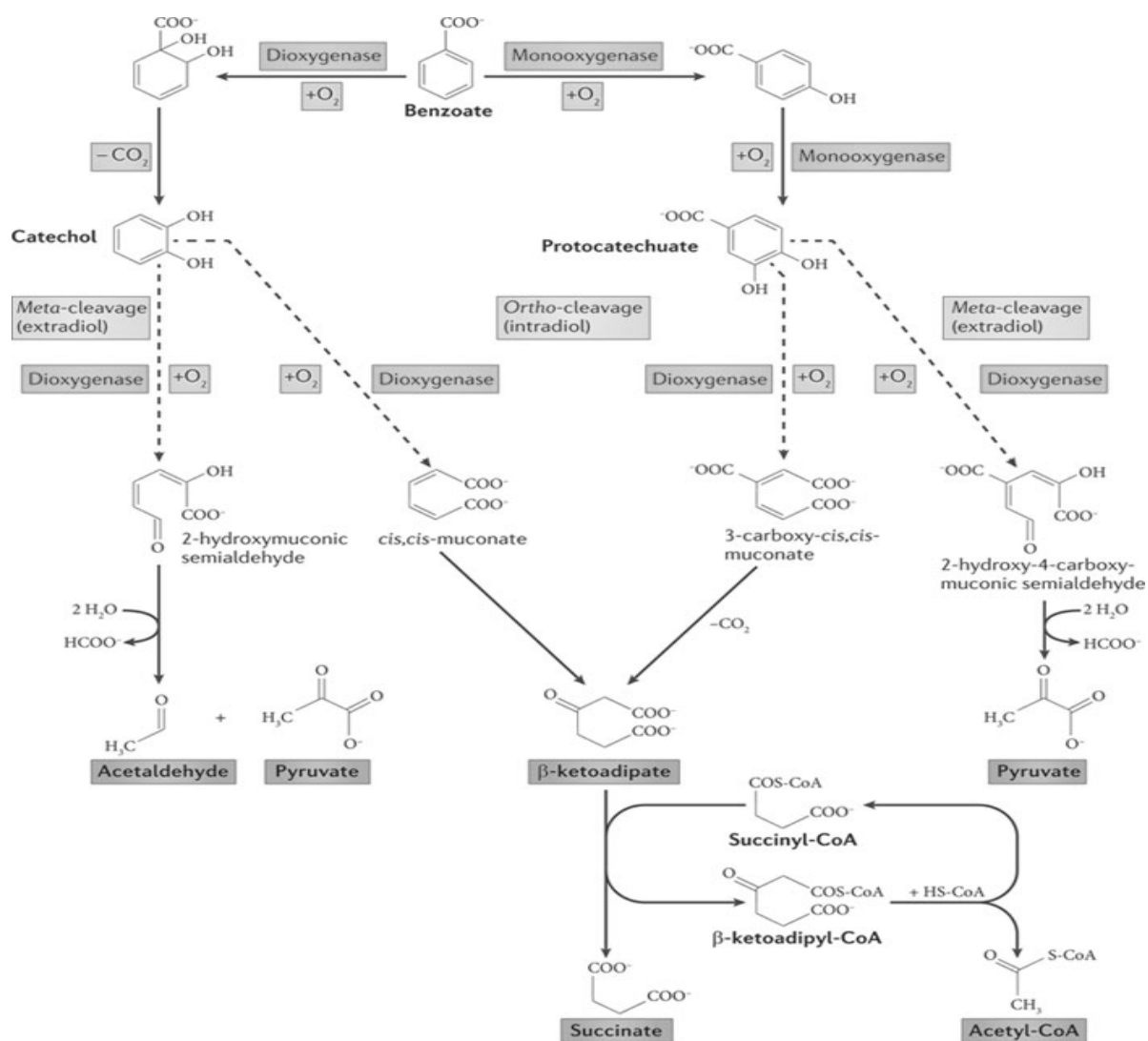


Figure 2.3: Some of pathways that are followed by bacteria during degradation of organic compounds (Fuchs *et al.*, 2011)

CBA's are degraded to catechol resulting in CO₂ and chlorine being released (Vrchotova *et al.*, 2013; Whitely and Lee, 2006). Catechols can be degraded via ortho- or meta- cleavage pathway (Figure 2.3) which eventually results in compounds that are used in the TCA cycle (Vrchotova *et al.*, 2013; Seeger *et al.*, 2010). The ortho- cleavage degradation is catalysed by catechol 1,2 dioxygenase which lead to the cleavage of the bonds between the first and second position of the benzene ring as shown in figure 2.4 (Vrchotova *et al.*, 2013). The result is a *cis,cis*-muconic acid with chlorine atoms in different position as shown in Figure 2. 5 (Vrchotova *et al.*, 2013). Catechol dioxygenase has Fe³⁺ as a possible prosthetic group and belongs to the enzymes that are capable to perform intradiol cleavage (Silva *et al.*,

2012; Peixoto *et al.*, 2011). The other enzyme, catechol 2,3 dioxygenase has four identical subunits of 32KDa and contains a catalytic iron ion (Fe^{2+}) per subunit (Silva *et al.*, 2012).

In the degradation pathways, *meta*- cleavage is very important in the degradation of PCBs as the susceptibility of the enzyme to inactivation by *ortho*- substituted PCBs interferes with the degradation of other PCB congeners (Vrchotova *et al.*, 2013; Fetzner, 2012). This is usually overcome through phyto-degradation or phytoremediation, which is a very slow process, and much is documented on the processes involved (Vrchotova *et al.*, 2013). Although this is feasible, not all these pathways will result in complete breakdown of a large mixture of different congeners of PCBs and in that way enzymes of novel specificities such as biphenyl dioxygenase (BphA) will be essential for a complete clean-up, Figure 2.4 (Vrchotova *et al.*, 2013; Camara *et al.*, 2007).

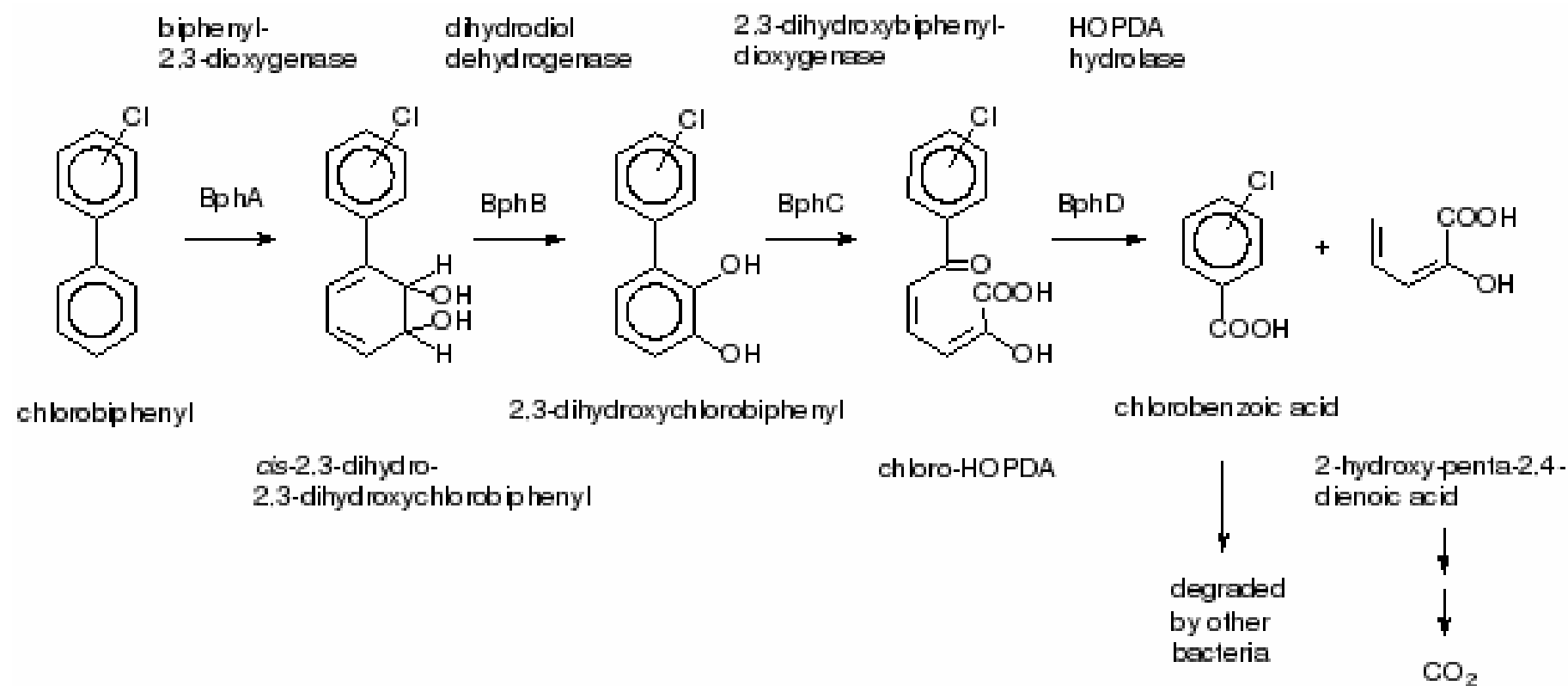


Figure 2.4: Pathway of aerobic degradation of PCBs by diphenyl-oxidizing bacteria (Vrchotova *et al.*, 2013)

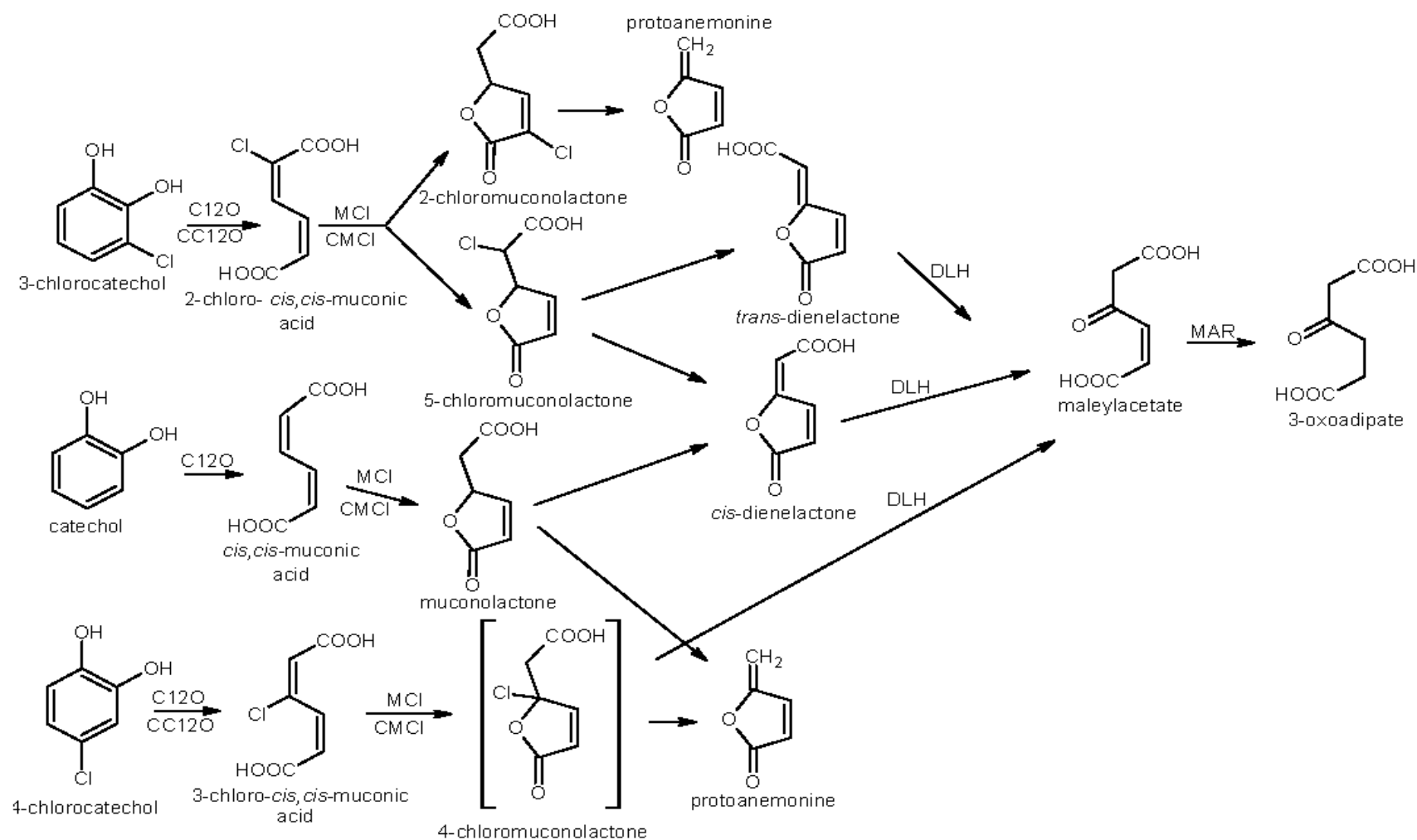


Figure 2.5: Pathway of general aerobic degradation of PCBs (Vrchotova *et al.*, 2013; Koval *et al.*, 2006).

The aromatic compounds aerobic degradation occurs by the formation of few central intermediates such as catechols, protocatechuates, gentisates and hydroxybenzoquinols (Guzik *et al.*, 2011; Koval *et al.*, 2006). This occurs by the introduction of hydroxyl groups in the ortho- or para- position leading to ring cleavage by dioxygenases (Guzik *et al.*, 2011). Enzymes allow peripheral recognition and convert the PAH molecules into biodegradable by enzyme in fission, allowing entry of these molecules to the common routes of energy generation and carbon microbial cells (Silva *et al.*, 2012; Diaz, 2004). Figure 2.6 below shows a simplified diagram for the route taken during biodegradation of PAHs (Diaz, 2004).

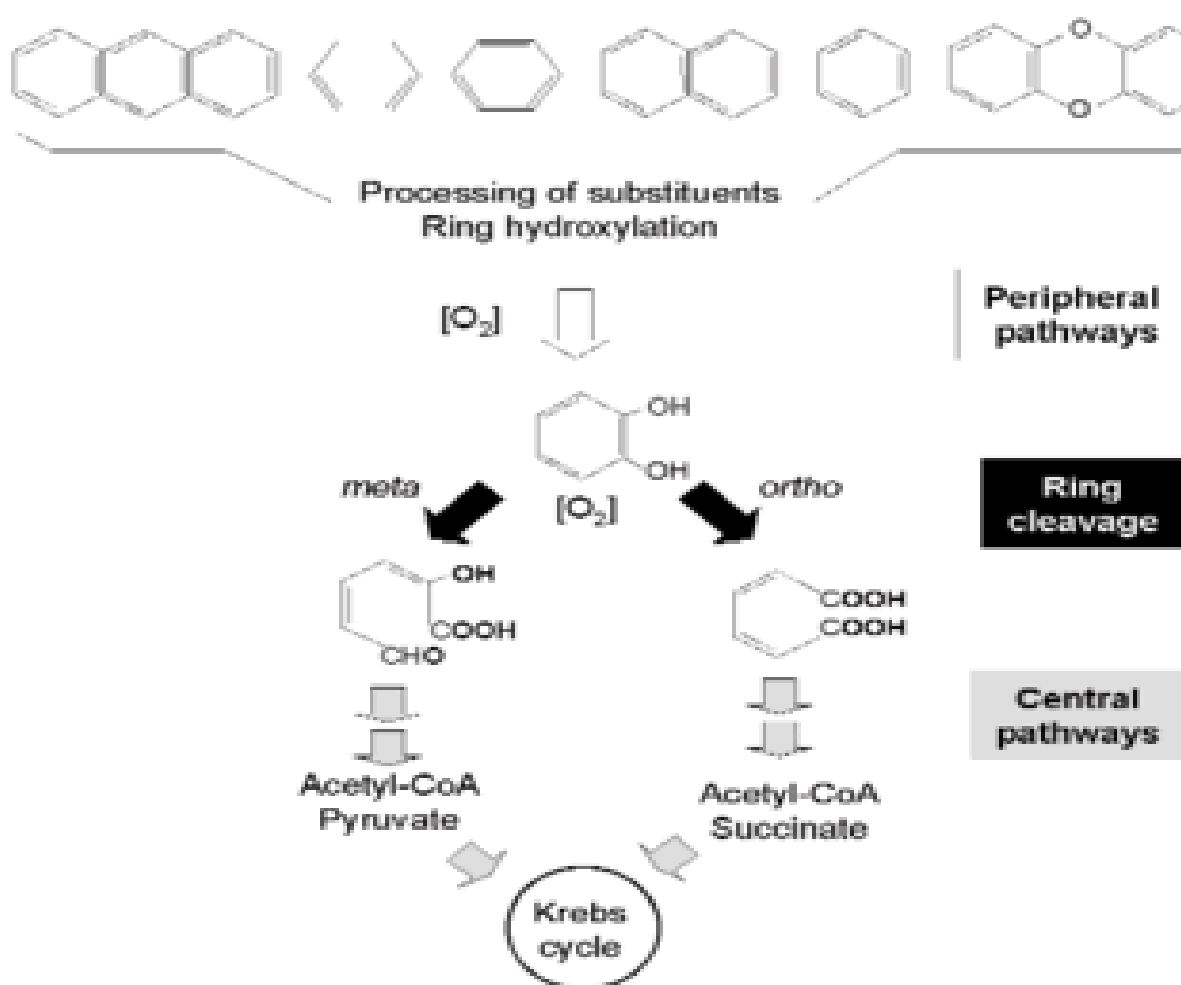


Figure 2.6: A simplified catabolic flow for the aerobic degradation of aromatics (Diaz, 2004)

Some enzymes such as Catechol 1,2 dioxygenase, catechol 2,3-dioxygenase and biphenyl dioxygenase play a critical role in the breakdown of recalcitrant compounds, Figure 2.6 (; Fuchs *et al.*,

2011Camara *et al.*, 2007). The activation of an aromatic substrate in aerobic microorganisms is mainly achieved by hydroxylation reactions and ring-cleaving dioxygenases (Fetzner, 2012). The stage in which ring-cleavage occurs is very essential in the degradation of aromatic compounds by bacteria (Fetzner, 2012) thus the degradation pathway.

The resultant products from these pathways taken by PCBs/PAHs biodegradation bacteria become reactants for the tri-carboxylic acid (Krebs) cycle (TCA) and the glycolytic pathways, Figure 2.18, which are normal metabolic pathways for all microorganisms resulting in the complete biodegradation of the compounds thus complete clean up (Vrchotova *et al.*, 2013; Diez, 2010; Diaz, 2004). Although bioremediation is an effective treatment process through the enzymatic activities of bacteria, slow growth of bacteria or difficulty in controlling and maintaining optimal conditions for their growth is a challenge to biodegradation process (Guzik *et al.*, 2013). The important properties of the degradation enzymes are based on their ability to resist inhibitors from pollutants such as metal ions, alcohol, phenols, hydrocarbons and chelators (Guzik, *et al.*, 2013). Catechol 1,2 dioxygenase is found to be greatly affected by Cu^{2+} , Zn^{2+} , Mn^{2+} , Mg^{2+} , Ca^{2+} , Ag^{2+} and Fe^{2+} (Guzik *et al.*, 2013). The degree of effect varies with catechol 1,2 dioxygenase from different organisms (Guzik *et al.*, 2013).

Although enzymes are the key substances for the metabolic pathways, the substrates should be bioavailable for the organisms to take it up (Panda *et al.*, 2013; Pacwa-Plociniczak *et al.*, 2011). According to Pieper (2005), PCBs are the most superhydrophobic compounds and thus are not bioavailable which stimulate bacteria to secrete surface active substances such as surfactants (Das and Chandran, 2011; Pieper, 2005).

2.2.3 Surfactants

Surfactants are either synthetic (man-made) or produced by microorganisms. Some microorganisms produce biosurfactants (Das and Chandran, 2011; Salihu *et al.*, 2009). These are surface-active

metabolites or compounds derived from living things (Gumaa *et al.*, 2010). Surfactants produced by bacteria are essential in bioremediation (Das and Chandran, 2011; Salihu *et al.*, 2009). They play an important role in the metabolism of these organisms (Das and Chandran, 2011). The synthetic surfactants and biosurfactants are amphiphilic compounds (Gumaa *et al.*, 2010). The surfactants contain the hydrophobic and hydrophilic moieties (Gumaa *et al.*, 2010). The hydrophilic moiety can be a carbohydrate, amino acid, phosphate group or some other compound (Gumaa *et al.*, 2010). The hydrophobic moiety is usually a long fatty acid chain (Gumaa *et al.*, 2010).

2.2.3.1 Synthetic Surfactants

Gross *et al.*, 2007 pointed out that detergents and soaps are the main sources of boron and surfactants found in wastewater. These are usually part of household waste that reaches wastewater (Gross *et al.*, 2007). Synthetic surfactants lower the surface tension between two liquids or between a liquid and a solid (Gumaa *et al.*, 2010, Salihu *et al.*, 2009). Although some may be partially biodegradable, most of these compounds are non-biodegradable (Salihu *et al.*, 2009) hence recalcitrant compounds found in wastewater (Gumaa *et al.*, 2010; Gross *et al.*, 2007). The synthetic surfactants accumulate in the environment, especially waste disposal sites and wastewater (Gross *et al.*, 2007) as a result of their non-biodegradable nature (Makkar and Rockne, 2003). Although synthetic surfactants may be used by bacteria in their metabolism due to their amphiphilic characteristic, their efficiency is very low (Gross *et al.*, 2007; Makkar and Rockne, 2003). In some studies, synthetic surfactants have been found to inhibit the activity or metabolism of bacteria thus interfering with biodegradation process of PAHs (Makkar and Rockne, 2003). These compounds are classified according to their dissociation patterns (Gross *et al.*, 2007).

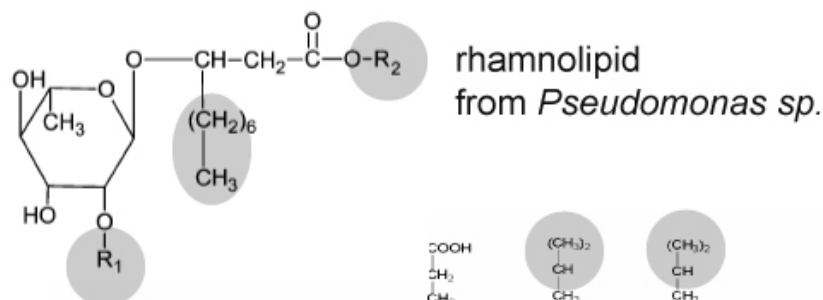
Synthetic surfactants have been found to pose some health threats to humans, (Salihu *et al.*, 2009; Schroder, 2003) which comes as a result of their recalcitrant nature and mostly found in soil and waste water. This is due to the fact that these compounds can be released into rivers or other water sources

from sewage water (Schroder, 2003). According to Schroder (2003), fluorinated surfactants, which are discharged into the environment, have the potential for bioaccumulation. He further stated that reports regarding their presence in wastewater are also rare and the presence of fluorinated compounds in the environment, seem to be a speculation (Schroder, 2003). This has led to biosurfactants being preferred in biodegradation of most recalcitrant compounds (Gumaa *et al.*, 2010; Salihi *et al.*, 2009) over synthetic surfactants.

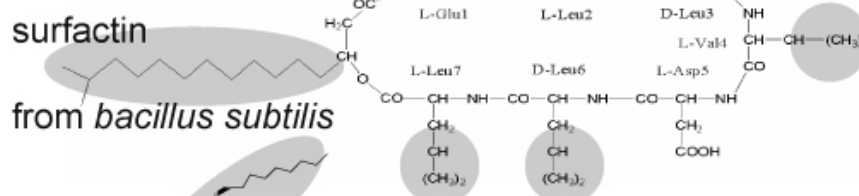
2.2.3.2 Biosurfactants

Biosurfactants can be divided into glycolipids, phospholipids, lipoproteins or lipopeptides and polymeric, based on their overall structures (Fakruddin, 2012; Gumaa *et al.*, 2010). Bacteria produces biosurfactants that have a wide range of structures and some examples and their sources are rhamnolipids, trehalose lipids, sophorolipids which are grouped as Glycolipids biosurfactants; surfactin, iturin and fengycin which are grouped as Lipopeptides and polymeric surfactants (Fakruddin, 2012; Pacwa-Plociniczak *et al.*, 2011; Ron and Rosenberg, 2002) and structural presentation in Figure 2.7.

Glycolipids
rhamnolipids,
sophorolipids,
trehalolipids



Lipopeptides
surfactin,
iturin,
fengycin



Polymeric surfactants



Figure 2.7: Structure and source of some biosurfactants

Biosurfactants may also be categorized by their molecular weight, physio-chemical properties and mode of action and microbial origin (Pacwa-Plociniczak *et al.*, 2011). The production of these substances by microorganisms is favoured by mesophilic conditions for most organisms (Gumaa *et al.*, 2010). Biosurfactants make hydrocarbons, including the recalcitrant polycyclic aromatic hydrocarbons, more soluble in water thus making it possible for them to be broken down (Gumaa *et al.*, 2010; Salihi *et al.*, 2009; Chang *et al.*, 2008; Calvo *et al.*, 2008; Pieper, 2005). Biosurfactants are environmental friendly unlike synthetic surfactants that have recalcitrant properties (Salihi *et al.*, 2009; Abboud *et al.*, 2007). The biosurfactants are non-toxic to microorganisms (Makkar and Rockne, 2003). They are less expensive (Fakruddin, 2012) as the organisms that produce them may do so under conditions of very cheap medium such as waste vegetable oils (Kapadia and Yagnik, 2013; Makkar and Rockne, 2003). The fact that these chemical substances have characteristics of biocompatibility, specificity and digestibility it makes them to have a great advantage if used in biodegradation over synthetic

surfactants (Kapadia and Yagnik, 2013). Some biosurfactants and their properties have been summarised in the Table 2.1.

Table 2.1: Biosurfactants and their use in bioremediation of hydrocarbon contaminated sites (Pacwa-Plociniczak *et al.*, 2011)

Biosurfactant	Microorganism	Application in Environmental Biotechnology
Glycolipids	<i>Pseudomonas aeruginosa</i> , <i>Pseudomonas</i> sp.	Enhancement of degradation and dispersion of different classes of hydrocarbons
	<i>Mycobacterium tuberculosis</i> , <i>Rhodococcus erythropolis</i> , <i>Arthrobacter</i> sp., <i>Nocardia</i> sp., <i>Corynebacterium</i> sp.	Enhancement of the bioavailability of hydrocarbons
Lipopeptides	<i>Bacillus subtilis</i>	Enhancement of the biodegradation of hydrocarbons and chlorinated pesticides
Polymeric biosurfactants	<i>Acinetobacter calcoaceticus</i> RAG-1	Stabilization of the hydrocarbon in water emulsions

Pseudomonas species, *Bacillus subtilis*, *Serratia marcescens* and *Micrococcus luteus* are some of the microorganisms that have those properties of producing biosurfactants and as a result, they can breakdown PAHs and PCBs (Gumaa *et al.*, 2010; Salihu *et al.*, 2009; Bahig *et al.*, 2008). Yin *et al.*, (2009) suggested that the solubility of some PAHs to water can be improved through the addition of biosurfactants owing to their amphipathic structure (Salihu *et al.*, 2009). According to Vasileva-Tonkova and Gesheba (2003), most bacteria that have biodegrading abilities produce biosurfactants and these have been found to help disperse hydrocarbons, increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability thereby stimulating the rate of bioremediation. Some biosurfactants producing strains, *Bacillus subtilis* and *Pseudomonas aeruginosa* were once used

in bioremediation of petroleum oil hydrocarbons contaminated soil (Pacwa-Plociniczak *et al.*, 2011). According to Haritash and Kaushik (2009), *Pseudomonas aeruginosa* can completely breakdown phenanthrene. Some strains of organism such as *Acinetobacter* have the ability to degrade pollutants such as biphenyls from wastewater (Gumaa *et al.*, 2010; Abboud *et al.*, 2007) as well as in the solubilisation of PAHs, phenanthrene and fluoranthene (Pacwa-Plociniczak *et al.*, 2011). Vasileva-Tonkova and Gesheba (2003) suggested that due to the uniqueness of the local conditions in some areas, indigenous microbes have to be introduced in the biodegradation process to increase efficiency on biodegradation of hydrocarbons.

Enterobacter cloacae secrete an emulsifier, which increase the hydrophobicity of the bacterial cell surface and also neutralise the surface charge of cells (Pacwa-Plociniczak *et al.*, 2011). This, as a result increases, the ability of the bacteria to degrade PAHs and PCBs (Fakruddin, 2012; Pacwa-Plociniczak *et al.*, 2011). The diagram below shows chemical structures of some biosurfactants (Fakruddin, 2012).

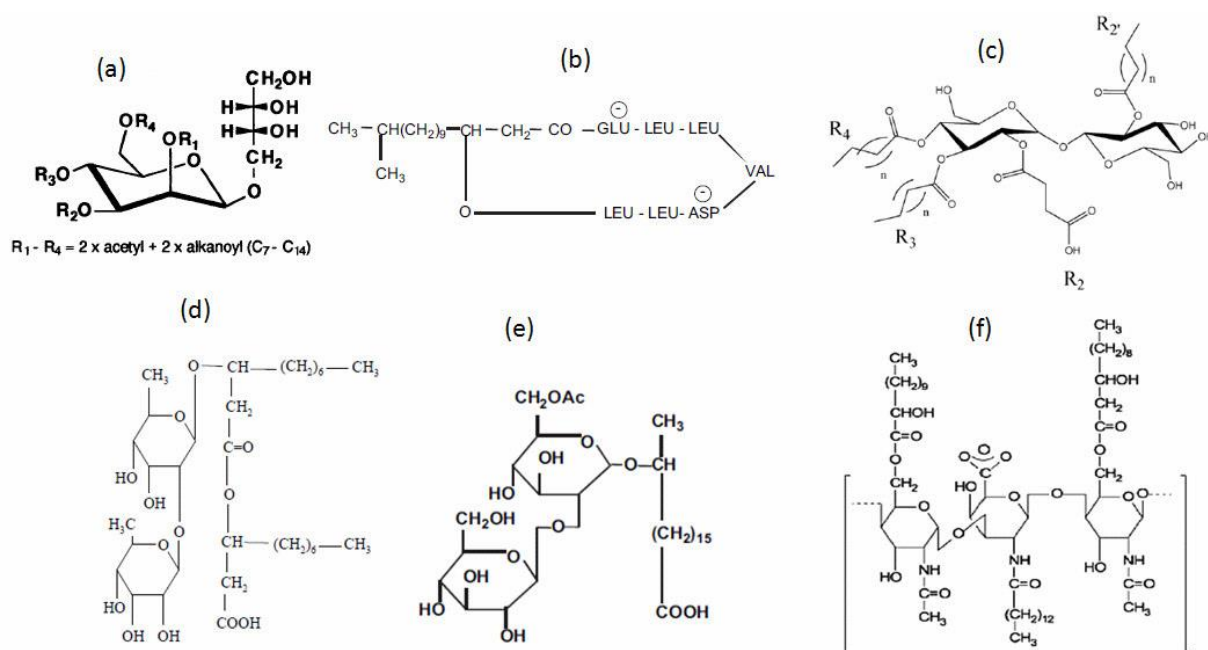


Figure 2.8: Chemical structures of some common biosurfactants (a) Mannosylerythritol lipid (b) Surfactin (c) trehalose lipid (d) Sophorolipid (e) Rhamnolipid (f) Emulsan (Fakruddin, 2012)

Bacteria that are capable of producing biosurfactants are of great importance in the modern world as such properties make them a promising choice for applications in enhancing hydrocarbon bioremediation (Salihi *et al.*, 2009). Biosurfactants have been found to be of paramount importance in the biodegradation of PAHs (Makkar and Rockne, 2003). They tend to facilitate microbial life in the environment dominated by hydrophilic-hydrophobic interface (Kapadia and Yagnik, 2013; Makkar and Rockne, 2003). The ability of most bacteria to release biosurfactants enables the biodegradation of PAHs and PCBs as these surface-active agents emulsify these compounds into solution making them bio-available for removal from the environment (Panda *et al.*, 2013).

Biosurfactants are also effective at the extremes of temperature, pH and salinity (Kapadia and Yagnik, 2013; Fakruddin, 2012), a property that is essential in biodegradation of PAHs and PCBs as they are hydrophobic organic compounds (Pacwa-Plociniczak *et al.*, 2011). This property makes these recalcitrant compounds to be removed through the physic-chemical means or treatments, limited bioavailability to microorganisms and limited availability to oxidative and reductive chemicals when applied in treatments (Pacwa-Plociniczak *et al.*, 2011).

Another property of surfactants that has been found to be important in biodegradation is their ability to permit microorganisms to grow on water immiscible substrates by reducing surface tension at the phase boundary (Fakruddin, 2012). This makes the substrate more readily available for uptake and metabolism (Kapadia and Yagnik, 2013; Fakruddin, 2012; Pacwa-Plociniczak *et al.*, 2011). This characteristic of bioavailability and increase in cell surface interaction is important in biodegradation (Kapadia and Yagnik, 2013; Pacwa-Plociniczak *et al.*, 2011) as it enhances hydrocarbon mobilization, solubilisation or emulsification. This can be summarised as shown in Figure 2.9.

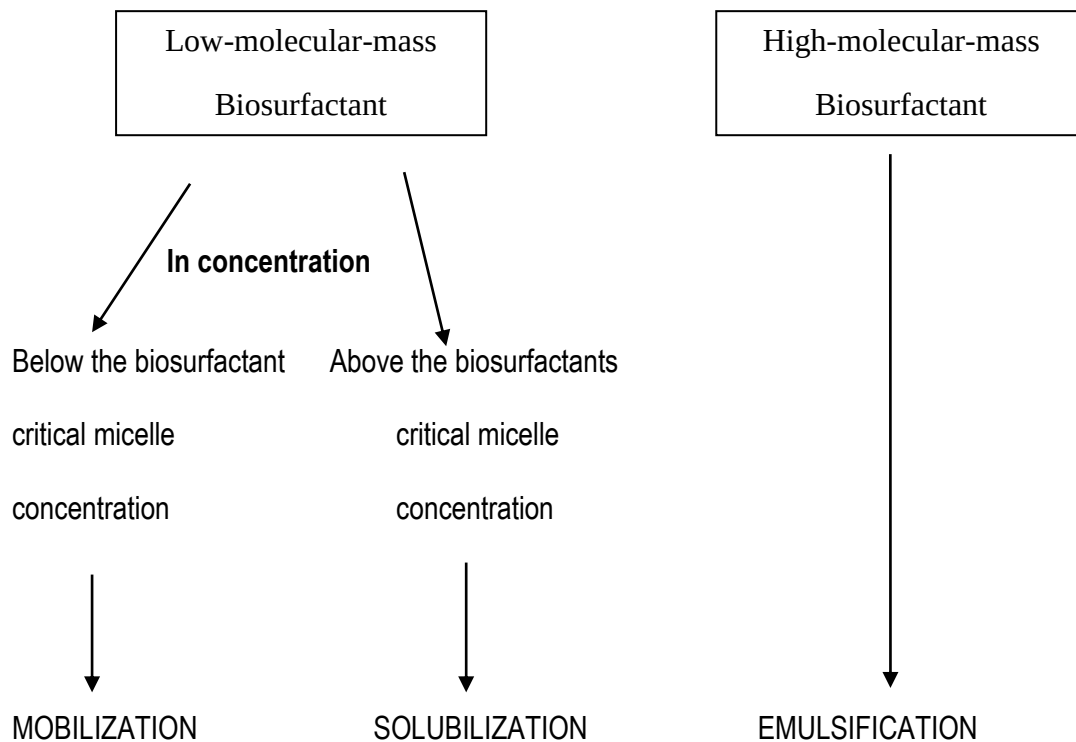


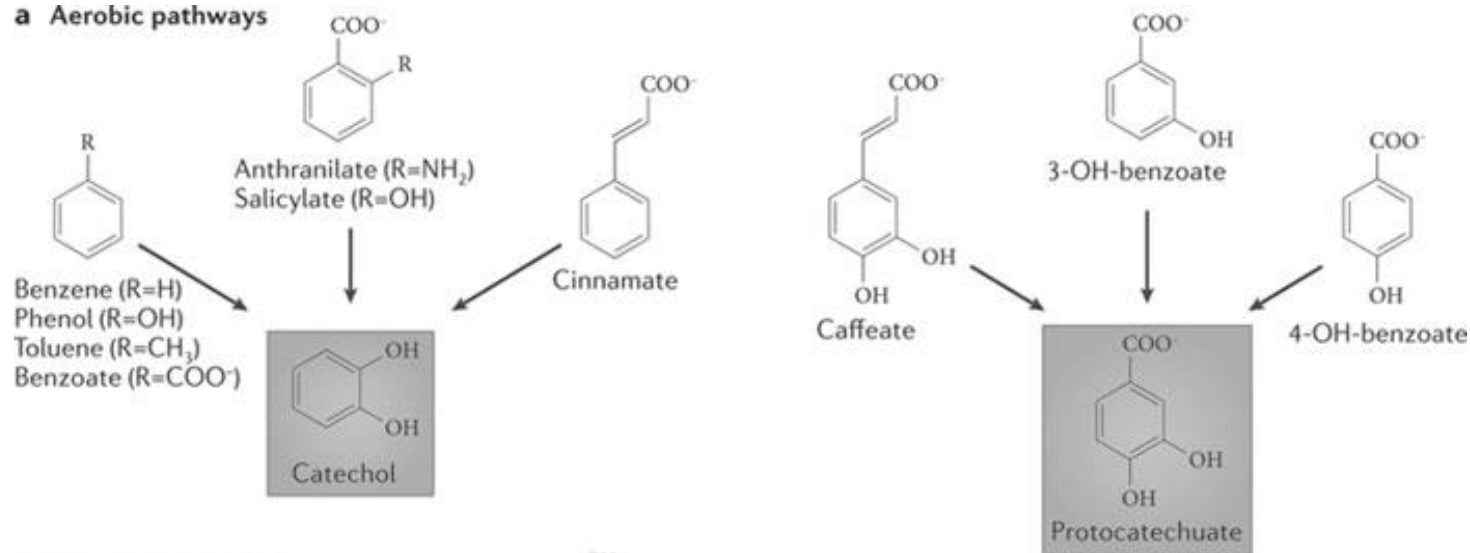
Figure 2.9: Mechanism of hydrocarbon removal by biosurfactants depending on their molecular mass and concentration (Pacwa-Plociniczak *et al.*, 2011).

Biosurfactants production by organisms is affected by the same factors that have been found to affect efficiency of biodegradation of recalcitrant compounds by bacteria (Fakruddin, 2012; Martins *et al.*, 2012). These factors are carbon and nitrogen source, environmental factors (temperature, oxygen, moisture content and pH), aeration and agitation and salt concentration (Fakruddin, 2012). The maximum degradation of hydrocarbons in soil, fresh water and marine water was found to range from 30 - 40°C, 20 - 30°C and 15 - 20°C, respectively (Das and Chandran, 2011). These according to Das and Chandran (2011), has to be coupled with adequate supply of carbon sources or nutrients and pH ranging between 6 and 9.

2.2.4 Factors Affecting biodegradation of high molecular weight hydrocarbons and polymers by Bacteria in wastewater

Bacteria, unlike other organisms, have the ability to interact better with man-made and naturally occurring compounds which result in such compounds being changed structurally and eventually get degraded (Anyasi and Atagana, 2011). This is in a way a better clean-up strategy that can be used in the clean-up of wastewater, as it is environment friendly (Anyasi and Atagana, 2011). Microorganisms degrade chemical and organic compounds in various ways, which may be either aerobic or anaerobic as summarised by Figure 2.10 (Arun *et al.*, 2011; Fuchs *et al.*, 2011, Diaz, 2004).

a Aerobic pathways



b Anaerobic pathways

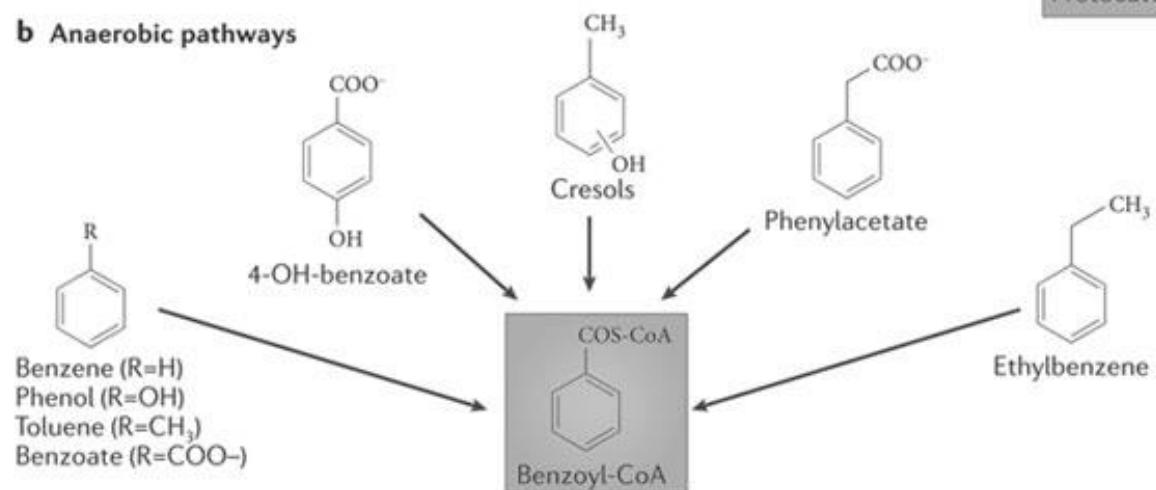


Figure 2.10: Anaerobic and aerobic pathways involved by bacterial during biodegradation (Fuchs *et al.*, 2011)

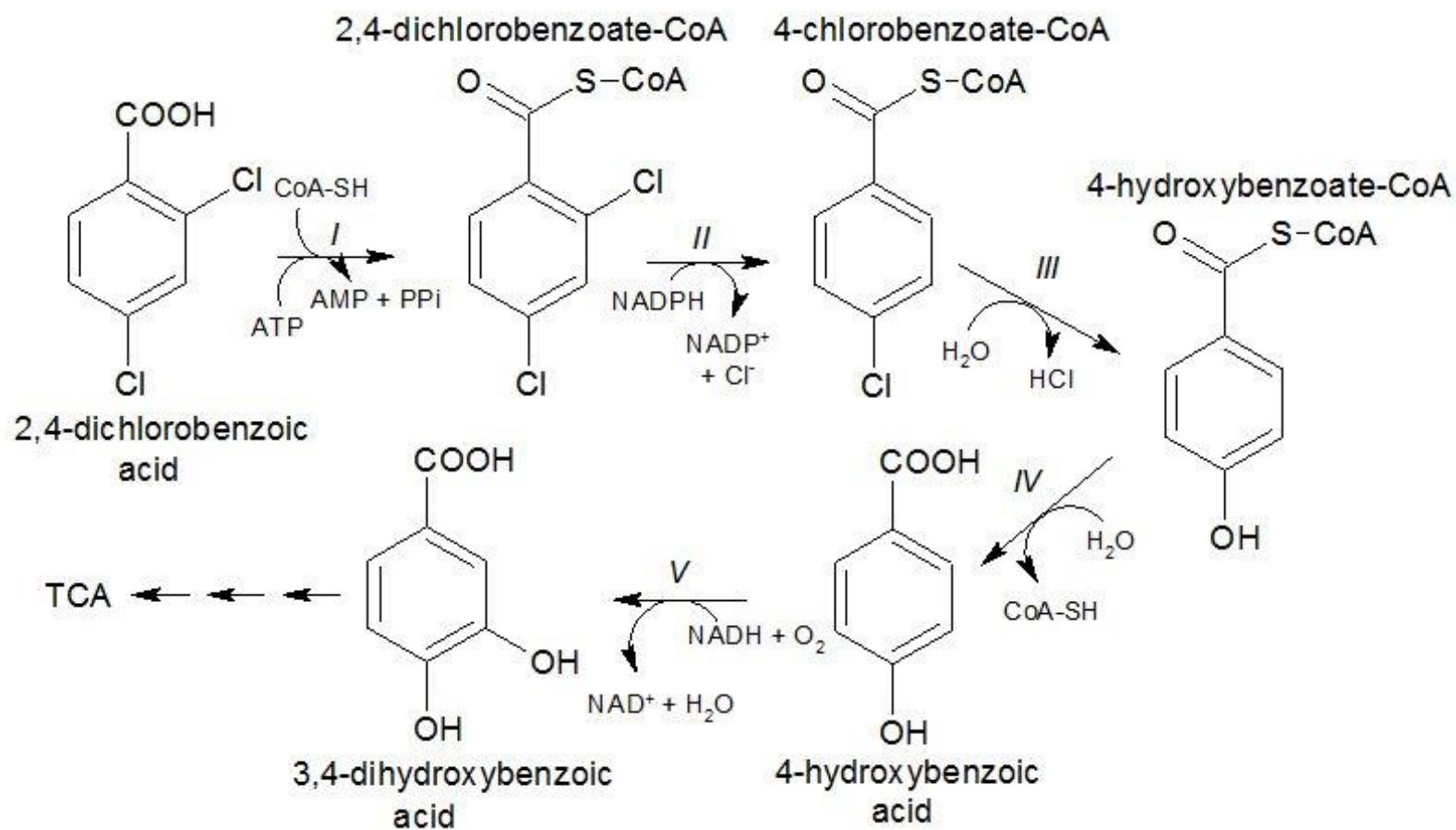


Figure 2.11: A simplified biodegradation route of hydrocarbons (PAHs and PCBs) by bacteria (Vrchotova *et al.*, 2013)

Although the diagram in Figure 2.11 above illustrates breakdown of biodegradable compounds, these are the intermediate compounds which some bacteria degrade the PAHs and PCBs into (Vrchotova *et al.*, 2013). All the intermediate products show that the purpose of the bacteria acting on those recalcitrant compounds is for purposes of obtaining energy (through respiration) thus the product being channelled to the TCA cycle (Vrchotova *et al.*, 2013; Fuchs *et al.*, 2011). Biodegradation of hydrocarbons by microorganisms is affected by several factors (Leahy and Colwell, 1990). These factors include temperature, oxygen, salinity, pressure, pH, nutrition and water activity (Cycon *et al.*, 2013; Martins *et al.*, 2012; Vasileva-Tonkova and Gesheba 2003; Eriksson *et al.*, 2002).

2.2.4.1 Salinity

Salt concentration of a particular medium affects biosurfactant production by bacteria thus generally affecting its cellular activities (Fakruddin, 2012). Alkane biodegradation is lower than 40% in medium with 0g/L NaCl, about 50% at 20g/L and up to 65% at 40g/L (Martins *et al.*, 2012). An increase in salinity results in a decrease in the rate of biodegradation of alkanes to up to 30% but nevertheless, halophytes bacteria are capable of effectively breaking down hydrocarbons (aliphatic and aromatic) even at 30g/L with optimal growth at 45°C (Martins *et al.*, 2012). Increase in salinity in general, may be of advantage to PAHs biodegradation but up to a certain limit which when exceeded it becomes toxic to the bacteria involved in biodegradation (Koul and Furlani, 2013; Vincent, 2006).

2.2.4.2 Temperature

Temperature plays an important role in biodegradation of hydrocarbons by directly affecting the chemistry of the pollutants as well as affecting the physiology and diversity of the microbial flora (Das and Chandran, 2011). Temperature affects bioavailability and solubility of hydrocarbons (Koul and Furlani, 2013). At low temperatures, the onset of biodegradation is delayed due to change in molecular weight and decrease in solubility of hydrocarbons (Koul and Furlani, 2013; Das and Chandran, 2011; Leahy and Colwell, 1990). Various organisms are affected by different ranges of temperature on

biodegradation of compounds (Zhang *et al.*, 2012; Jayashree *et al.*, 2012) with optimum temperature having been observed to be between 30 to 40°C and above 40°C bacterial enzymes are denatured (Koul and Furlani, 2013). According to Das and Chandran (2011), the highest degradation of hydrocarbons in some fresh water environments was found to be between 20 - 30°C. The thermophiles such as *Bacillus thermoleovorans* will perform best at temperatures 60°C and above (Koul and Furlani, 2013).

2.2.4.3 pH

Different pH values can indirectly affect the degradation rates of substrates by changing the existing form of a compound (Zhang *et al.*, 2012). Degradation of some recalcitrant compounds involves hydrolysis and it occurs at its best at low pH in water and soil (Cycon *et al.*, 2009). Some organisms such as *Serratia marcescens* have degradation rate increasing at pH values 6.0 to 7.0 (Zhang *et al.*, 2012). According to Koul and Furlani, (2013), most PAH degrading bacteria perform best when pH is neutral. This is the pH that has been found to promote mineralisation process thus enhancing the overall biodegradation process (Koul and Furlani, 2013). Under extreme pH microbes cannot act on recalcitrant compounds as the enzymatic activity of bacteria will be hampered (Cycon *et al.*, 2009).

2.2.4.4 Nutrients

Bacteria require a carbon sources for their normal glycolytic pathway thus energy release to run all the cellular metabolic activities (Diez, 2010). Apart from a carbon source, bacteria require mineral nutrients such as nitrogen (N), phosphate (P) and potassium (K) for cellular metabolism and growth (Koul and Furlani, 2013). Nitrogen, phosphorus and iron (in some cases) are very important in the process of biodegradation of hydrocarbons (Das and Chandran, 2011). Nitrogen is used by bacteria to make their proteins and for enzyme syntheses (Fakruddin, 2012). The type of carbon, nitrogen and iron sources in some instances may be limiting factors in the degradation ability of bacteria (Das and Chandran, 2011). The carbon and nitrogen sources have to be good for efficiency of bacteria in their metabolic activities

to increase while excessive nutrient concentration inhibits the biodegradation activity (Koul and Furlani, 2013; Fakruddin, 2012).

2.2.4.5 Oxygen

Aerobic biodegradation is the most efficient catabolic pathway for bioremediation of PAHs (Vincent, 2006). The efficient and rapid degradation of PAH, generally, is enhanced by availability of oxygen (Koul and Furlani, 2013; Chung and Holoman, 2008), that is, aerobic aromatic metabolism (Arun *et al.*, 2011). Bacteria use molecular oxygen as co-substrate, which stimulates the oxidation of the aromatic ring (Arun *et al.*, 2011; Mrozik, *et al.*, 2003). In anaerobic conditions the process of biodegradation of PAHs tends to be slow or not occurring at all (Koul and Furlani, 2013; Foght, 2008). Anaerobic biodegradation also results in electron acceptors such as nitrate, Ferric iron and sulphate being reduced thus releasing excess phosphorus and ferrous iron both of which are toxic to the environment, as well as the release of greenhouse gases such as ammonia (CH₄) and Nitrogen dioxide (NO₂) (Koul and Furlani, 2013).

2.3 Recalcitrant Compounds

Man-made chemicals present in nature at high concentrations polluting the environment are known as xenobiotic compounds (Fetzner, 2000). These compounds are not commonly produced by nature (Faber, 2002). Some microbes have been seen to be capable of breaking down xenobiotics to some extent although most of the xenobiotic compounds are non-degradable in nature (Faber, 2002; Fetzner, 2000). Such compounds are known to be recalcitrant in nature (Faber, 2002). The presence of chemicals in the environment calls for quantification of such to come up with risk analysis posed by those chemicals (Urbaniak, 2013; Guillen *et al.*, 2012). Several methods may be used to determine quantitatively, these substances from wastewater, which are mainly from sewage treatment plants (Ying *et al.*, 2009). According to Guillen *et al.*, (2012), substances such as pharmaceuticals, perfluorinated

acids and perfluorosulfonates, PAHs, PCBs, pesticides, and surfactants are mostly found in wastewater some of which have recalcitrance characteristics.

Recalcitrant compounds are those substances that may not be biodegradable directly but requires a series of steps to be broken down by microorganisms (Faber, 2002). The recalcitrant property of xenobiotic compound is directly linked to its complexity such that the higher the complexity the stronger the recalcitrant property (Fetzner, 2000). The properties of xenobiotic compounds attributing to its recalcitrant properties include not being recognised by microbes as substrate, absence of permease, which is needed for transportation of a molecule into a microbial cell, the large size of the molecules, highly stable structure, insolubility to water and toxicity nature to some microbes (Arbabi *et al.*, 2009).

The recalcitrant xenobiotic compounds can be divided into different groups depending on their chemical composition (Fetzner, 2000). These groups, according to (Mallick *et al.*, 2007; Suryanto and Suwanto, 2003; Faber, 2002) are halocarbons, polychlorinated biphenyls, synthetic polymers, alkylbenzyl sulphonates, oil mixtures and hydrocarbons (Mallick *et al.*, 2007).

According to Schafer and Kegley (2002), halocarbons consist of a halogen group in their structure. The substances/compounds are mainly used in solvents, pesticides, propellants (EPA, 2013). They are highly volatile and escape into nature leading to destruction of ozone layer of atmosphere and some of these compounds are present in insecticides, pesticides (Mallick *et al.*, 2007; Suryanto and Suwanto, 2003). Polychlorinated biphenyls (PCBs) consist of a halogen group and benzene ring (EPA, 2013). They are mainly used in plasticisers, insulator coolants in transformers (EPA, 2013). They are chemically and biologically inert adding on to its recalcitrant nature (Vrchotova *et al.*, 2013; Schafer and Kegley, 2002). Synthetic polymers are mainly used to form plastics such as polyester, polyvinyl chloride etc. They are insoluble in water and of high molecular weight explaining the recalcitrant property. Alkylbenzyl Sulphonates consist of a sulphonate group, which resists break down by microbes. They

are mostly found in detergents. Oil mixtures and Hydrocarbons are a result of used oil spills and covering a huge area making the break down by action of microbes becomes non effective (Al-Thani *et al.*, 2009; Ophardt, 2003). They become recalcitrant as they are insoluble in water and some components of certain oils are toxic in higher concentrations.

Hydrocarbons can be either saturated (carrying a carbon bound to four or more atoms through single bonds) or unsaturated containing either double or triple bonds (Ophardt, 2003). Due to this, hydrocarbons are grouped into alkanes, alkynes, alkenes and aromatic compounds (Ophardt, 2003). Alkanes are hydrocarbons with carbon-to-carbon interaction of a single bond, C–C. Alkenes are hydrocarbons with C=C bonds and alkynes are hydrocarbons with C≡C bonds. Hydrocarbons classified as recalcitrant are mostly the aromatics, especially PAHs (Al-Thani *et al.*, 2009; Mallick *et al.*, 2007; Suryanto and Suwanto, 2003). Aromatics are divided or classified into three major categories, which are PAHs, heterocyclics and substituted aromatics (Seo *et al.*, 2009). Some compounds of interest, which are associated with wastewater, are monoaromatics. According to Farhadian *et al.*, (2007), monoaromatics that include benzene, toluene, ethylbenzene and a mixture of xylenes (BTEX) reach sewage through industrial waste. These compounds have serious health threats as most of them are found to have carcinogenic effects (Martinkova *et al.*, 2009; Hennessee *et al.*, 2009; Farhadian *et al.*, 2007), with PAHs comprising the largest class of chemical carcinogens (Abou-Arab, 2010). Although these compounds are soluble compared to other hydrocarbons, they are highly mobile as they get adsorbed by soil (Farhadian *et al.*, 2007) which requires removal of these compounds from the wastewater.

2.3.1 Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) have a common singular feature that is based on two or more fused benzene rings (Al-Thani *et al.*, 2009; Seo *et al.*, 2009; Nnamchi *et al.*, 2006). The rings are in linear, angular or cluster arrangements (Seo *et al.*, 2009). These are mainly formed during incomplete

combustion or high temperature pyrolysis of coal, oil, gas, wood, fossil fuels, garbage or other substances such as tobacco and char boiled meat (Abou-Arab *et al.*, 2010). Some of the 16 PAHs are presented in Figure 2.12.

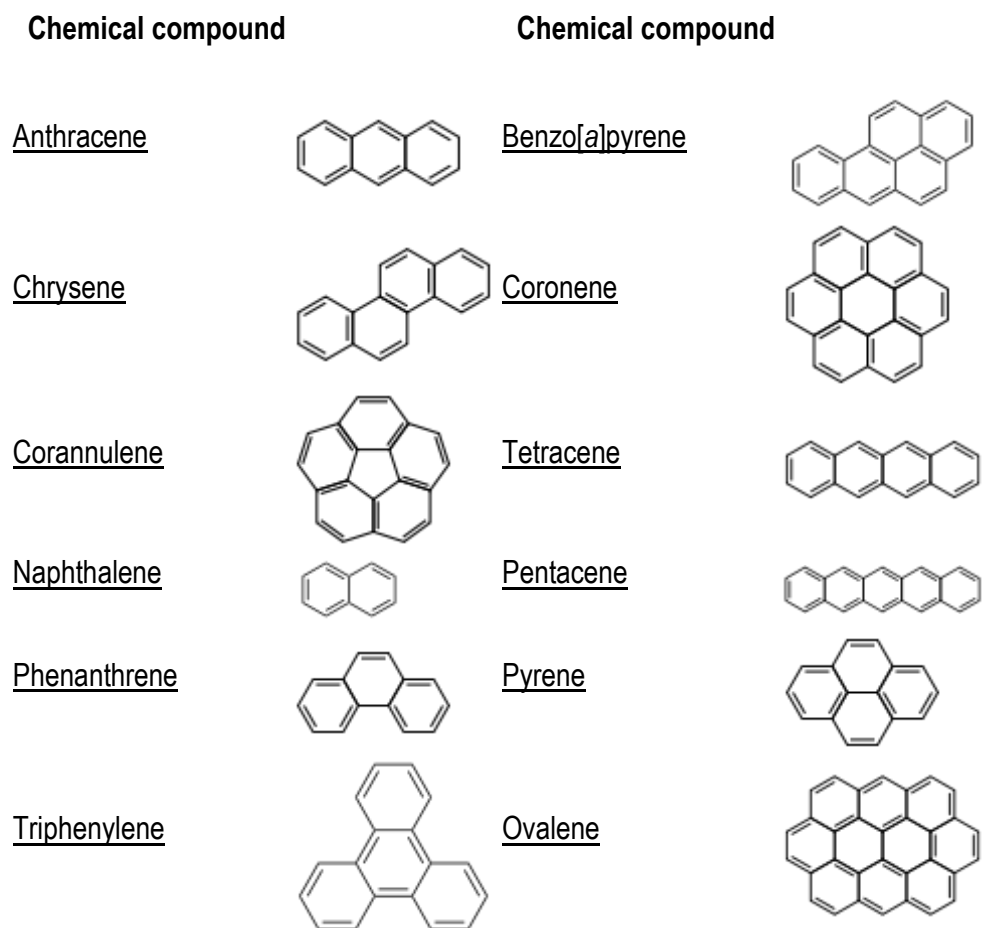


Figure 2.12: Some of PAHs (EPA, 2013)

The physical and chemical properties of PAHs vary with the number of rings and hence their molecular weight (Seo *et al.*, 2009). Chemical reactivity, aqueous solubility and volatility of PAHs decrease with increasing molecular weight (Al-Thani *et al.*, 2009; Seo *et al.*, 2009). This has resulted in these compounds differing in their fate in the environment and their effects on the biological system of bacteria (Seo *et al.*, 2009). Their biochemical persistence in the environment arises from dense clouds of π -electrons on both sides of the ring structure making them resistant to biodegradation (Nnamchi *et*

al., 2006), the recalcitrant nature. This is due to their low aqueous solubility (highly lipophilic) and thus low bioavailability (Blokesch, 2012; Nnamchi *et al.*, 2006).

PAHs with high molecular weight have been a great concern as they have been found to be highly associated with recalcitrance (Perrin, 2012; Seo *et al.*, 2009). Although they have been of greater concern, some low molecular weight PAHs such as naphthalene, anthracene and phenanthrene have also been found to have hazardous health effects, although mild (Nnamchi *et al.*, 2006). These properties are closely linked to the non-biodegradable nature of PAHs (Pawar, 2012; Seo *et al.*, 2009) in a single phase. Most PAHs contain a bay-region, K-region and an L-region, diagram in Figure 2.13.

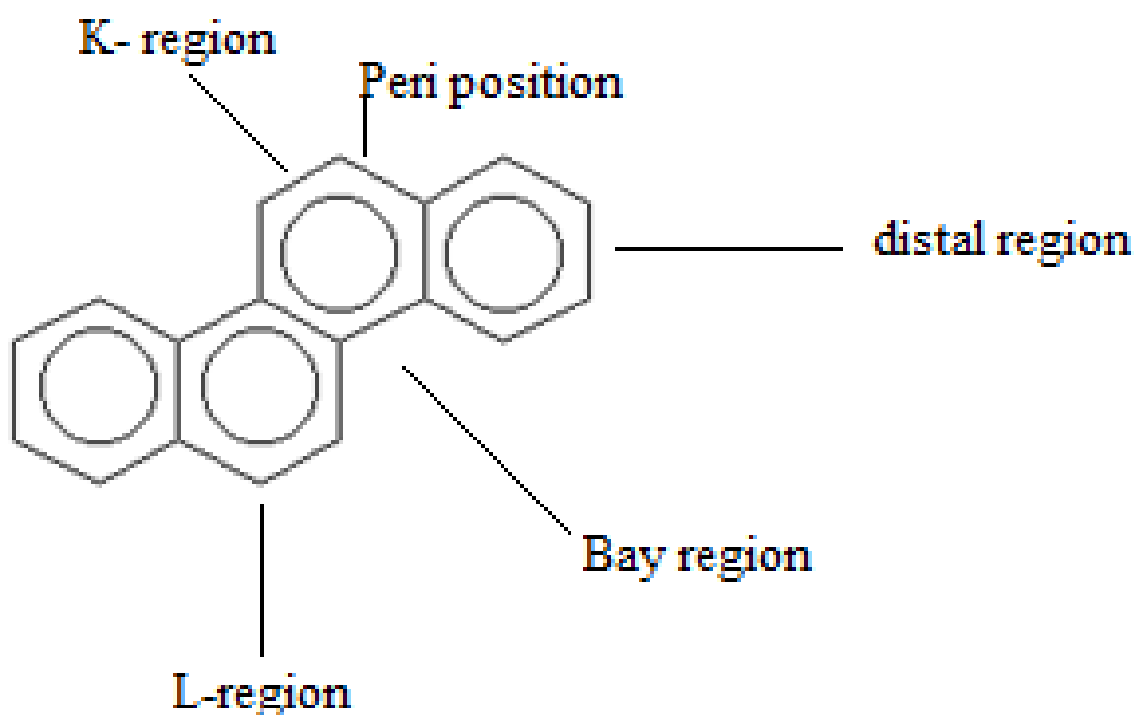


Figure 2.13: Different regions of biological activity of PAHs (Pawar, 2012)

The bay-region is an internal inner corner such as can be seen in the structure of phenanthrene (Pawar, 2012; Seo *et al.*, 2009). The K-region is an external closed corner while the L-region represents the pair of opposed anthracenic point atoms (Pawar, 2012; Mrozik *et al.*, 2003). The bay and K-regions have been found to be chemically reactive and have developed metabolically and biologically (Pawar,

2012). Environment containing PAHs are considered hazardous, as studies using animals have shown their specific carcinogenic, mutagenic and teratogenic effects (Nnamchi *et al.*, 2006).

2.3.1.1 Sources of PAHs

There are three major sources of PAHs identified as petrogenic, pyrogenic and biogenic (Seo *et al.*, 2009). According to Seo *et al.*, (2009); the petrogenic are petroleum and petroleum derived products. They enter the environment as releases into air from volcanoes, forest fires, residential wood burning, cigarette smoke, asphalt roads, coal tar, agricultural burning, municipality industrial waste incineration, hazardous waste sites and exhausts from automobiles and trucks (Abou-Arab *et al.*, 2010). These are marked by the abundance of alkyl – substituted PAHs; the pyrogenic are produced from combustion processes and are predominantly unsubstituted PAHs while the biogenic aromatic compounds include aromatic amino acids, lignin and their derivatives of biotransformation origin.

Martinkova *et al.*, (2009) stated that Xenobiotic aromatic compounds are released into the environment from various industrial processes and thus form hazardous environmental contamination. They further stated that the major sources of aromatic pollutants are chemical and pharmaceutical industries.

2.3.1.2 Effects of PAHs on Human Health

The hazards posed by xenobiotics are huge (Faber, 2002; Fetzner, 2000). These compounds are highly toxic in nature and affect the survival of lower as well as higher eukaryotes (Al-Thani *et al.*, 2009). It also poses health hazards to humans such as skin problems, reproductive system and even known to trigger proliferation of cells (cancer) (Al-Thani *et al.*, 2009). These compounds are persistent and remain in the environment for many years leading to bioaccumulation or biomagnification (Pacwa-Plociniczak *et al.*, 2011; Al-Thani *et al.*, 2009). They also find a way into the food chains and the concentrations of such compounds was found to be high even in organisms that do not come in contact with xenobiotics directly (Faber, 2002; Al-Thani *et al.*, 2009). The most common PAHs associated with

causing human cancer include naphthalene, acenaphthene, acenaphthylene, fluorene, anthracene, phenanthrene, fluoranthene, chrysene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(a)pyrene, benzo(k)fluoranthene and chrysene (Al-Thani *et al.*, 2009; Hennessee *et al.*, 2009; Farhadian *et al.*, 2008). The effects of PAHs have been associated with cancer of the pulmonary, gastrointestinal, renal, dermatological and hematopoietic systems (Srujana and Khan, 2012; Al-Thani *et al.*, 2009). To aid in minimising these problems/health effects, biodegradation of polycyclic aromatic hydrocarbons (PAHs) is a possible way to clean up polluted soils and water system (Srujana and Khan, 2012; Eriksson *et al.*, 2002). This is due to the fact that environments contaminated with PAHs are considered hazardous (Al-Thani *et al.*, 2009; Hennessee *et al.*, 2009; Nnamchi *et al.*, 2006). PAHs have also found to lead to reproductive problems, which in most cases results in birth defects (EPA, 2008; Al-Thani *et al.*, 2009).

Other forms of recalcitrant compounds such as carbazole, 2, 4-dichlorophenol, and DL-camphor have also been reported to have the same effects (Nnamchi *et al.*, 2006) on humans. Tebuconazole has been reported to result in alteration of metabolism in humans which lead to death (Srujana and Khan, 2012; Sehnem *et al.*, 2010). Some of the stated PAHs such as benzo[a]pyrene (Figure 2.14) has been associated with lung and skin cancer; and reproductive effects, while benzo[a]anthracene, benzo[b]fluoranthene, benzo [k]fluoranthene, chrysene, dibenzo[ab]anthracene and indeno[1.2.3-cd]pyrene have been associated with low birth weight, premature birth, heart malformations and low IQ in humans (Srujana and Khan, 2012; Sehnem *et al.* 2010) and have similar effects as the other recalcitrant groups carbazole, 2,4-dichlorophenol and DL-camphor (Nnamchi *et al.*, 2006). Naphthalene has been associated with breakdown of red blood cells leading to anaemia (Sehnem *et al.*, 2010). The structure of some of the toxic PAHs is presented in Figure 2.14.

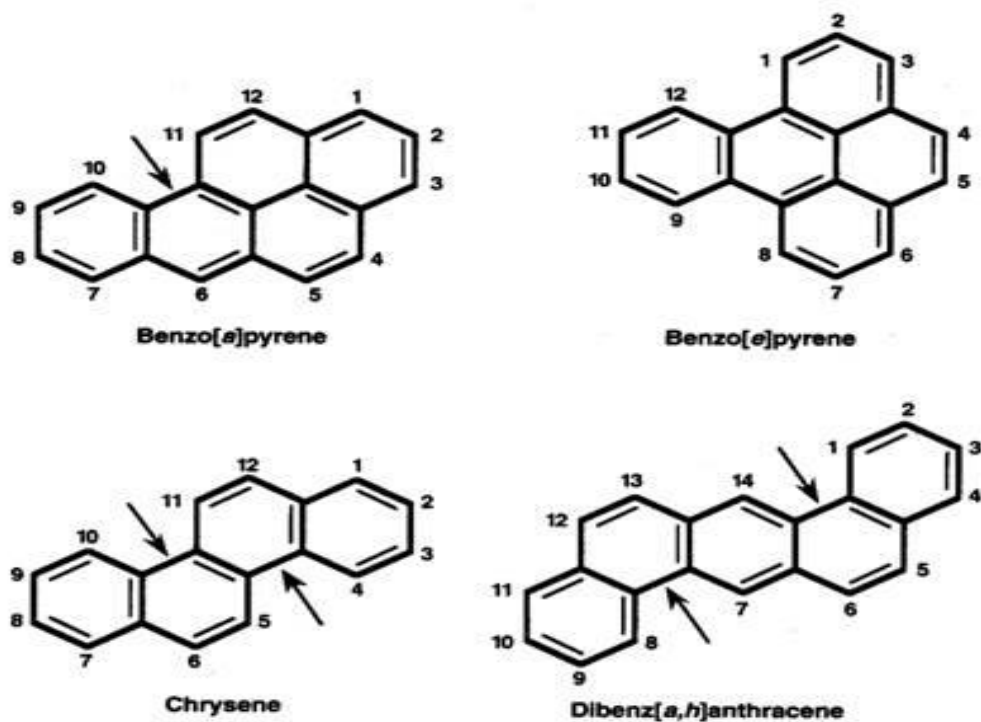


Figure 2.14: Some PAHs associated with health risks (Ravindra *et al.*, 2007).

2.3.2 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (PCBs), consists of two benzene rings with a carbon to carbon bond between carbon 1 on one ring and carbon 1 on the second ring, Figure 2.15 (EPA, 2013). The general chemical formula for PCBs is $C_{12}H_{10-n}Cl_n$, where n is any number between 1 and 10 (WHO, 2000). PCBs have varying number of chlorines in their structure (EPA, 2013). The diagram below is a simplified structure of a PCB molecule (Barbalace, 2003).

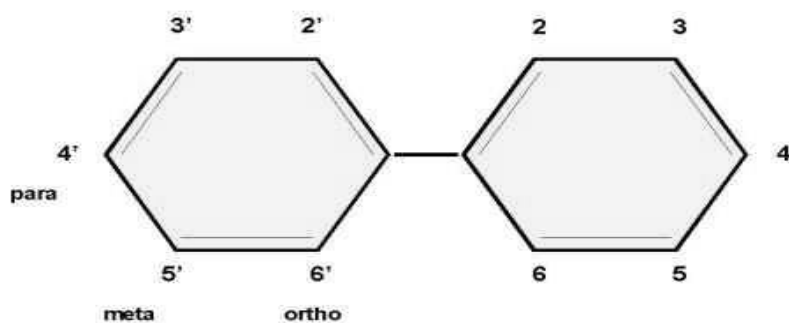


Figure 2.15: Structure of PCBs (Barbalace, 2003).

All PCBs are lipophilic and the lipophilicity increases with the number of chlorines attached to the molecule thus their low solubility to water (WHO, 2000). Toxicity of PCBs is dependent upon the number of chlorines present on the biphenyl structure and their position, Figure 2.16 (Barbalace, 2003). The more chlorine molecules contained a compound renders that compound less biodegradable (EPA, 2013). These properties result in bioaccumulation of these compounds (EPA, 2013; Barbalace, 2003; Schafer and Kegley, 2002).

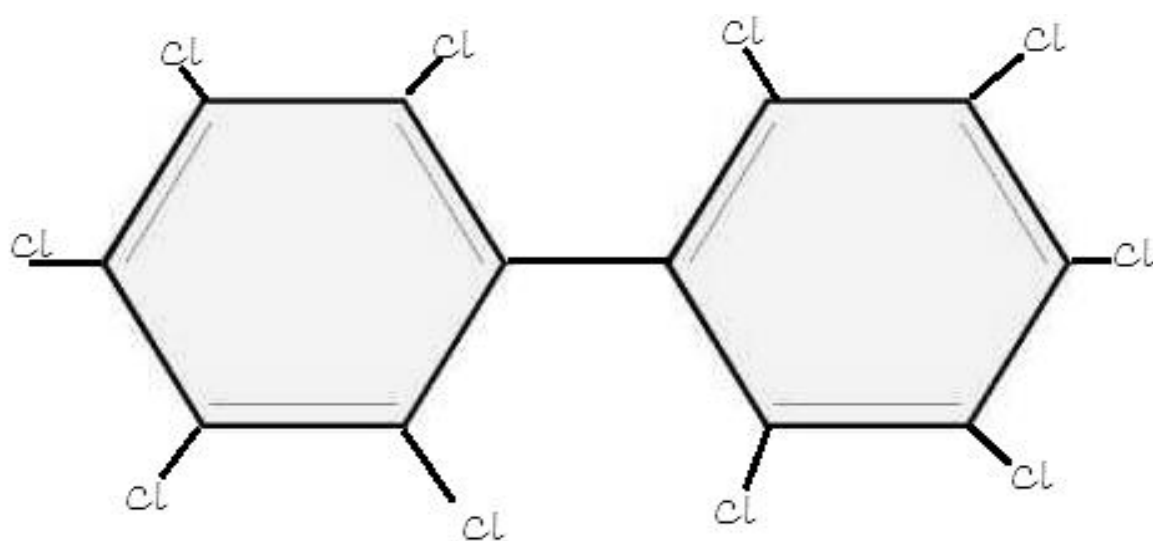


Figure 2.16: PCB diagram with chlorine molecules attached to it

In terms of structural relationship to toxicity, PCBs fall into 2 distinct categories, referred to as coplanar or non-*ortho*-substituted “arene” substitution patterns and non-coplanar or *ortho*-substituted congeners (Jensen, 2006). The coplanar group members have a fairly rigid structure, with the biphenyl rings in the same plane. This gives the molecule a structure similar to polychlorinated di-benzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans, and allows it to act in the same way as these molecules as an agonist of the aryl hydrocarbon receptor (AhR) in organisms (Arsalan *et al.*, 2007). These types of PCBs are considered as contributors to overall dioxin toxicity (Arsalan *et al.*, 2007).

Non-coplanar PCBs, with chlorine atoms at the *ortho* positions, have not been found to activate the AhR, and are not considered part of the dioxin group; however, studies have indicated some neurotoxic and immunotoxic effects and thus increased concern by regulatory bodies (Rudel *et al.*, 2008; Wethington and Hornbuckle, 2005).

PCBs are very stable compounds and do not decompose readily (Rudel *et al.*, 2008). This is due to their chemical inability to oxidize and reduce in the natural environment. They have low solubility in water as well as low electrical conductivity (WHO, 2000), and as a result they are highly resistant to thermal degradation thus the characteristic that made them to be used as dielectric isolators in electric equipment (WHO, 2000). Furthermore, PCBs have a long half-life (8 to 15 years) and are insoluble in water, which contributes to their stability, bioaccumulation and recalcitrant property (Hernandez-Sanchez, *et al.*, 2013; Rudel *et al.*, 2008; WHO, 2000). Their destruction by chemical, thermal, and biochemical processes is extremely difficult, and presents the risk of generating extremely toxic dibenzodioxins and dibenzofurans through partial oxidation (EPA, 2013; Arsalan *et al.*, 2007). Figure 2.17 shows generalised structure of PCB congeners with varying chlorine molecules (EPA, 2013).

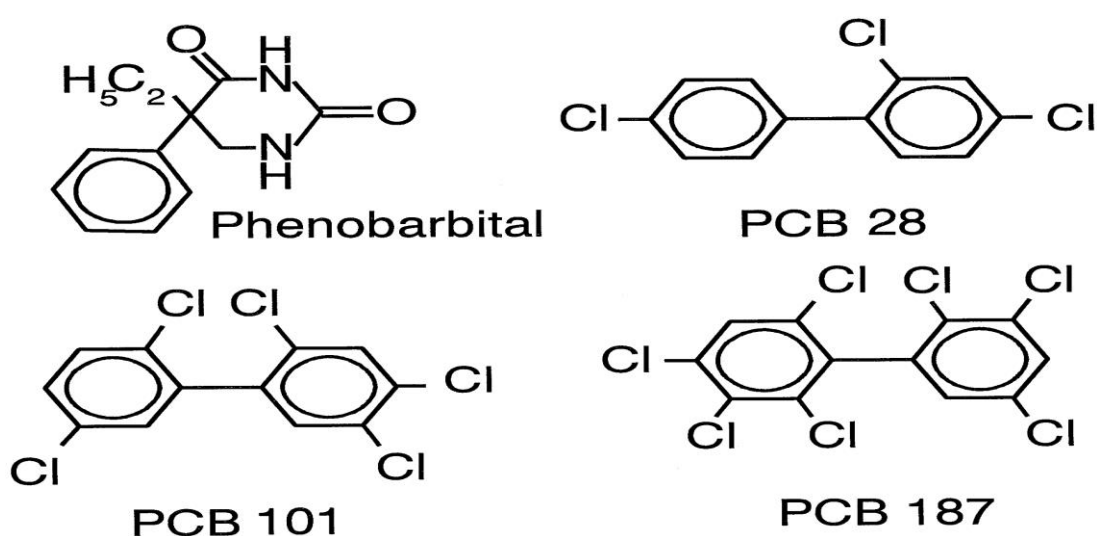


Figure 2.17: Generalized structure of PCB congeners (EPA, 2013)

The more chlorine molecules contained in a compound renders that compound less biodegradable (EPA, 2013). PCBs are mostly hydrophobic and some are less hydrophilic (Muir and Lohmann, 2013; Nwinyi, 2011). These properties result in bioaccumulation of these compounds as they do not dissolve in water and thus render them difficult to be biodegraded (EPA, 2013; Muir and Lohmann, 2013; Barbalace, 2003; Schafer and Kegley, 2002).

PCBs have a variety of uses, which include making electrical transformers, heat exchange fluids in machinery, additives to paints, plastics, rubbers and flame-retardants, lubricants or adhesives, inks and dyes, and in insulating materials (EPA, 2013). The following physio-chemical properties of PCBs have greatly contributed to their use in the industry: low aqueous solubility, non-flammability, resistance to oxidation, resistance to hydrolysis and low electrical conductivity and most of these properties make the compounds recalcitrant (EPA, 2013; Man *et al.*, 2011).

PCBs are introduced into the environment by being released during their manufacture and use in industry and/or disposal of products containing PCBs (Man *et al.*, 2011; Lynch *et al.*, 2012). They may also be introduced through their application of contaminated sewage sludge to soils (Man *et al.*, 2011). Biphenyls (PCBs) can enter the body either by inhalation of air containing polychlorinated biphenyls, ingestion of contaminated food or water, or by dermal contact with polychlorinated biphenyls or products containing polychlorinated biphenyl (Nabavi *et al.*, 2013; Zhao *et al.*, 2009).

2.3.2.1 Sources of Polychlorinated Biphenyls (PCBs)

Ying *et al.*, (2009) noted that the presence of pharmaceutically active compounds in wastewater, are a major concern. Although the production of PCBs has been prohibited since 1970s and the regulation of these compounds by all nations and augmented during the Stockholm Convention, 2001 (Muir and Lohmann, 2013; van Leeuwen *et al.*, 2013; ATSDR, 2001), a lot of these compounds are still present in the environment as a result of bioaccumulation and biomagnification in organisms (Nabavi *et al.*, 2013;

Lunch *et al.*, 2012; Petric *et al.*, 2007). More of these ubiquitous substances were still being introduced into the environment through various human activities (Teran *et al.*, 2012).

According to Ying *et al.*, (2009), although most research done regarding the removal of these substances were carried out, it was mainly on soil and activated sludge and very little done on the wastewater. The wastewater is allowed to flow out into the environment and may pose health risks to animals and humans (WHO and UNICEF, 2014). This also results in these compounds being added to the soils in the surrounding environment. These soils become polluted with PCBs, which poses a risk not only to plants that grows in those soils but also to human using the soils for farm produce (McDougall, 2004). Since these compounds are not easily broken down, they accumulate in the environment (bioaccumulation) and can be passed from one trophic level to the other through feeding relations (Nabavi *et al.*, 2013; McDougall, 2004) thus the need to have these compounds completely eliminated from the environment.

2.3.2.2 Effects of Polychlorinated Biphenyls (PCBs) on Human Health

PCB mixtures have been associated with cancer incidents in animals from long time back (Nabavi *et al.*, 2013; Lynch *et al.*, 2012; Pavuk *et al.*, 2004; Ross, 2004). PCBs were found to induce liver tumours, thyroid adenomas, intestinal metaplasia and adenocarcinomas in rats and mice (Nabavi *et al.*, 2013; Lynch *et al.*, 2012; Pavuk *et al.*, 2004). Exposure to some environmental chemicals such as DDT and PCBs have been associated with a drop in sperm count, breast cancer, testicular cancer, hypospadias which are all associated with endocrine disruption caused by these chemicals (Rogan and Ragan, 2007). This comes as a result of some PCB congeners being able to occupy thyroid receptors thus interrupting its action (Rogan and Ragan, 2007; Ross, 2004). PCBs have also been greatly found to suppress the immune system (EPA, 2013). This has major implications on human health, as the immune system is vital in ensuring the person remains healthy. When the immune system is

suppressed the affected person is prone to many infections as well as increased chances of developing PCB-induced cancer (EPA, 2013).

PCBs accumulate in the fats of organisms and are passed on from one organism to the other in food chains (Man *et al.*, 2011; Zhao *et al.*, 2009) thus causing bioaccumulation. The PCBs get entry into the human body and animals through the skin, lungs and gastrointestinal tract (Anyasi and Atagana, 2011). This get distributed to various parts of the body via blood and get to accumulate in different tissues (Anyasi and Atagana, 2011; Man *et al.*, 2011). The effects of PCBs on humans depend on age, sex and part of the body affected by the chemicals (Anyasi and Atagana, 2011). The liver, as the major organ for removal of toxins in the body, is usually highly affected by PCBs (Anyasi and Atagana, 2011; Pavuk *et al.*, 2004). Humans become exposed to PCBs through consumption of contaminated fish, meat and dairy products (Lynch *et al.*, 2012) and also through grains grown in PCB contaminated soils (Lynch *et al.*, 2012; Anyasi and Atagana, 2011). PCBs have been isolated from human milk and serum (Man *et al.*, 2011; Linderholm *et al.*, 2010), and has been found to have effects on breastfed children leading to low IQ, and endocrine related ailments (Lynch *et al.*, 2012; Man *et al.*, 2011; Linderholm *et al.*, 2010). Some studies have shown an increase in cancer mortality in workers exposed to PCBs (Anyasi and Atagana, 2011).

2.4 Biochemical Mechanism of Bacteria on PAHs and PCBs

The presence of chemicals in the environment calls for quantification of such to come up with risk analysis posed by those chemicals (Guillen *et al.*, 2012). According to Guillen *et al.*, (2012), substances such as pharmaceuticals, perfluorinated acids and perfluorosulfonates, PAHs, PCBs, pesticides, and surfactants are mostly found in wastewater. Ying *et al.*, (2009) also noted that the presence of pharmaceutically active compounds in wastewater, are a major concern. Several methods may be used to determine quantitatively, these substances from wastewater, which is mainly from sewage treatment plants (Ying *et al.*, 2009). According to Ying *et al.*, (2009), although most research done regarding the

removal of these substances were carried out, it was mainly on activated sludge and nothing done on the wastewater.

2.4.1 Biodegradation

The maintenance of a bacterial cell depends on highly coordinated biochemical reactions (Mckee and Mckee, 2013). With a carbon source being highly essential in driving these reactions, bacteria devise means of surviving even in environments where the readily available sources of carbon such as carbohydrates are in lack thus the utilisation of even the xenobiotic compounds (PAHs and PCBs) leading to biodegradation in the process (Simarroa *et al.*, 2013; Seeger *et al.*, 2010). The biochemical reactions that occurs leading to biodegradation ensure that intermediates products like pyruvate, which are key compounds in glycolysis enroute TCA cycle, are available for the release of energy needed by the bacteria for normal metabolism (Mckee and Mckee, 2013). The processes also result in compounds such as NADPH, which are reducing agents in many cellular reactions (Mckee and Mckee, 2013). Use of mixed population of microbes is usually recommended as it has been seen to yield faster results as different microbes attack different parts of a compound through different mechanisms resulting in effective break down of the compound (Martinkova *et al.*, 2009; Farhadian *et al.*, 2007). This activity also creates a condition of co-metabolism (Martinkova *et al.*, 2009; Pieper, 2005).

When the xenobiotic aromatic compounds, monoaromatic or polyaromatic hydrocarbons are biodegraded, they are converted to a catechol structure and the cleavage of the catechol structure are the key steps in aerobic BTEX degradation (Martinkova *et al.*, 2009; Farhadian *et al.*, 2007). The figures below show a summary of some pathways that are involved during the biodegradation of PAHs (Guzik *et al.*, 2013; Martinkova *et al.*, 2009). The compounds are broken down into biodegradable compounds such as catechol for final removal from the environment (Guzik *et al.*, 2013).

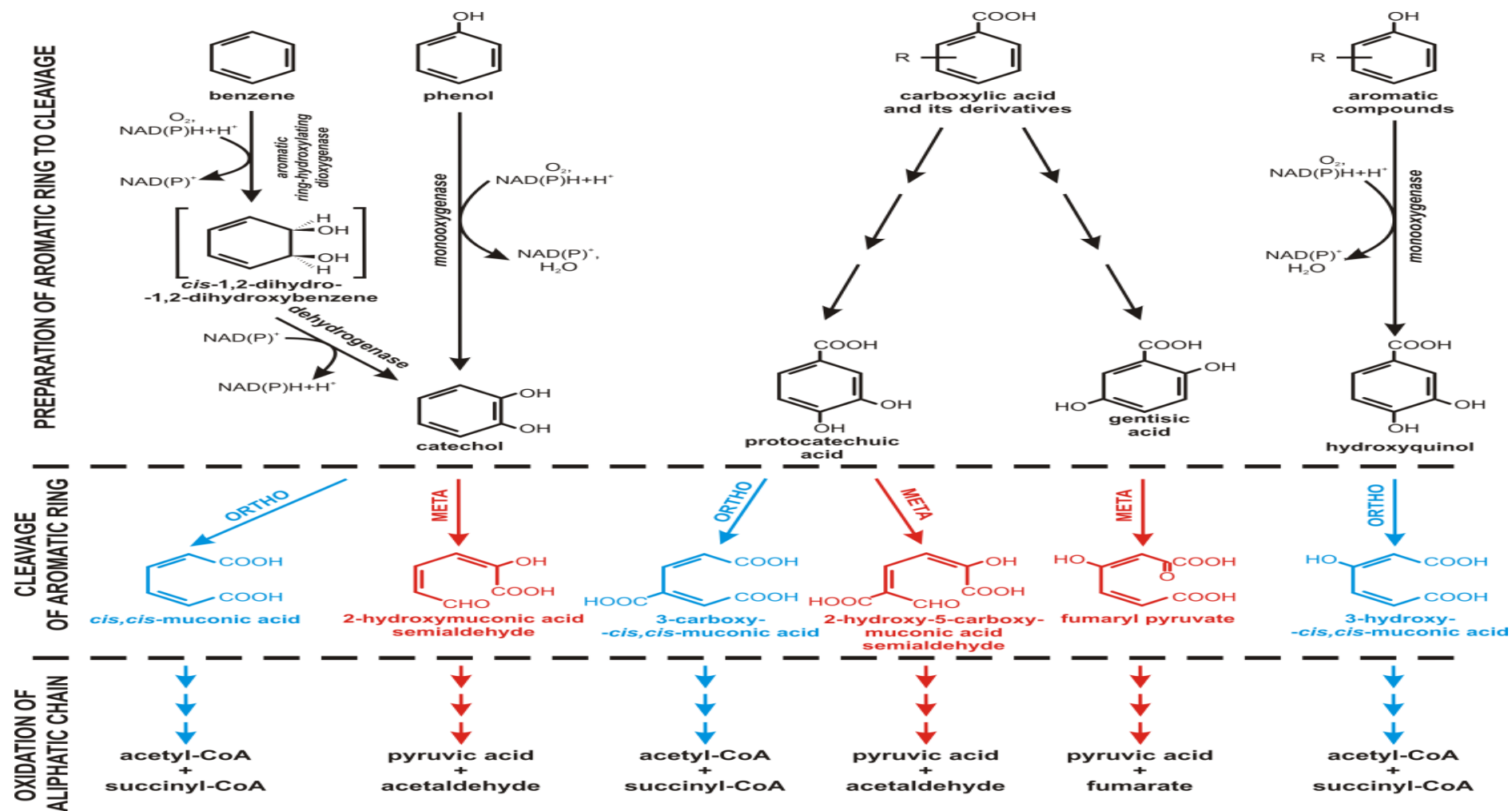


Figure 2.18: Pathways of aromatic compounds degradation (Guzik *et al.*, 2013).

Figure 2.19 shows a pathway for a specific organism *Rhodococcus* species on PAH degradation (Martinkova *et al.*, 2009).

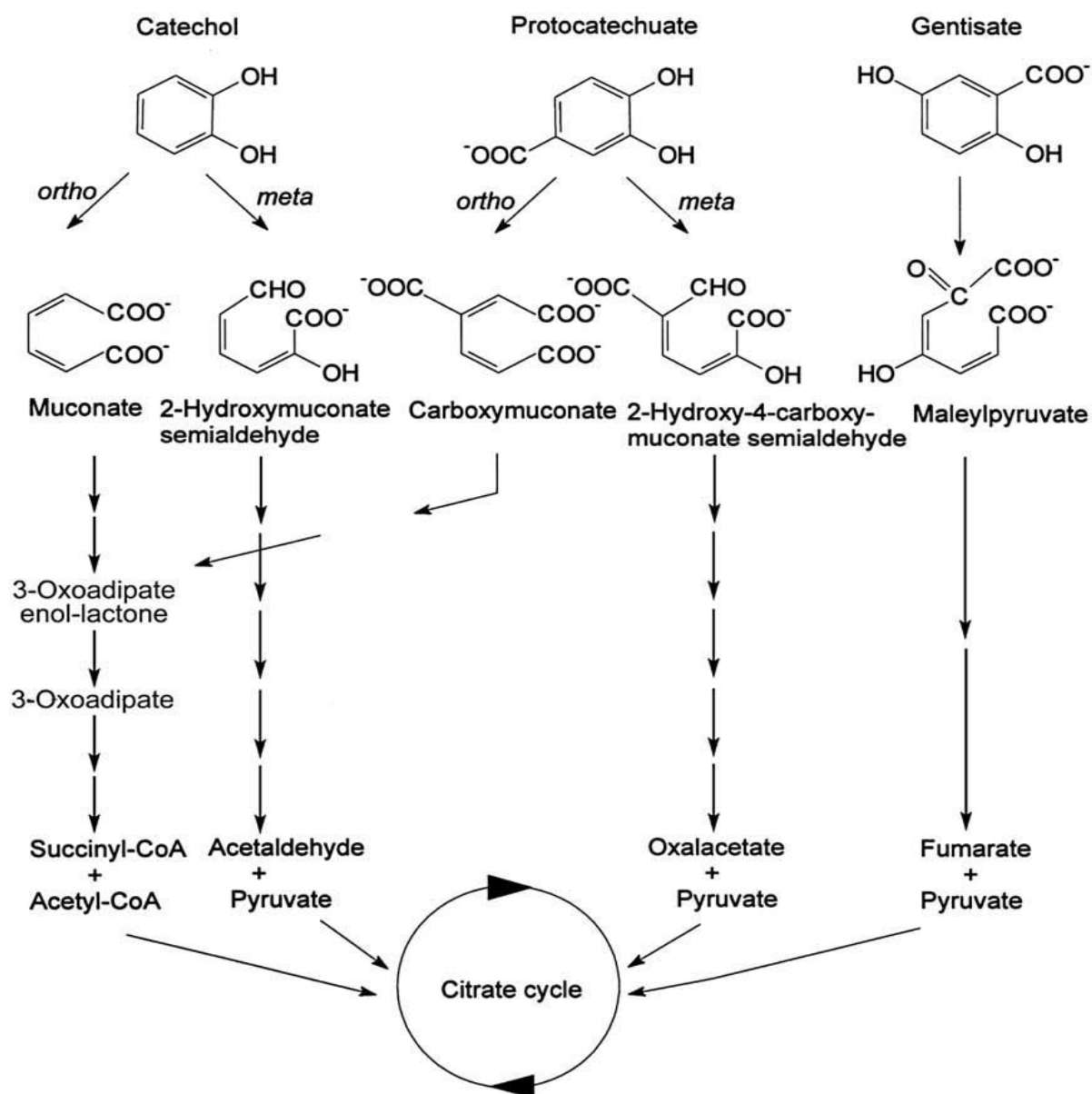


Figure 2.19: Central pathways of catabolism of aromatic compounds in rhodococci (Martinkova *et al.*, 2009).

According to Martinkova *et al.*, (2009), only a few groups of microorganisms, particularly the gram-negative bacteria of *Pseudomonas*, *Sphingomonas*, *Acinetobacter*, *Ralstonia* and *Burkholderia* and the

gram-positive genus *Rhodococcus*, can biodegrade PAHs effectively. Although natural aromatic compounds are naturally easily biodegraded by microorganisms, the same does not happen with the xenobiotic aromatic compounds, that is, PAHs and PCBs (Martinkova *et al.*, 2009) as some of these compounds are toxic to most microorganisms (Arbabi *et al.*, 2009). Those that are capable to survive the toxicity therefore are able to use those compounds as their sources of energy through various means such as CCR (Blokesch, 2012). Those bacterial species degrade the PCBs into easily biodegradable compounds such as benzoate (Seeger and Pieper, 2010). Figure 2.20 shows PCB biodegradation pathway (Seeger and Pieper, 2010) with benzoate being a product that is eventually broken down into acetylaldehyde and pyruvate, which are used in the TCA cycle (Pieper, 2005).

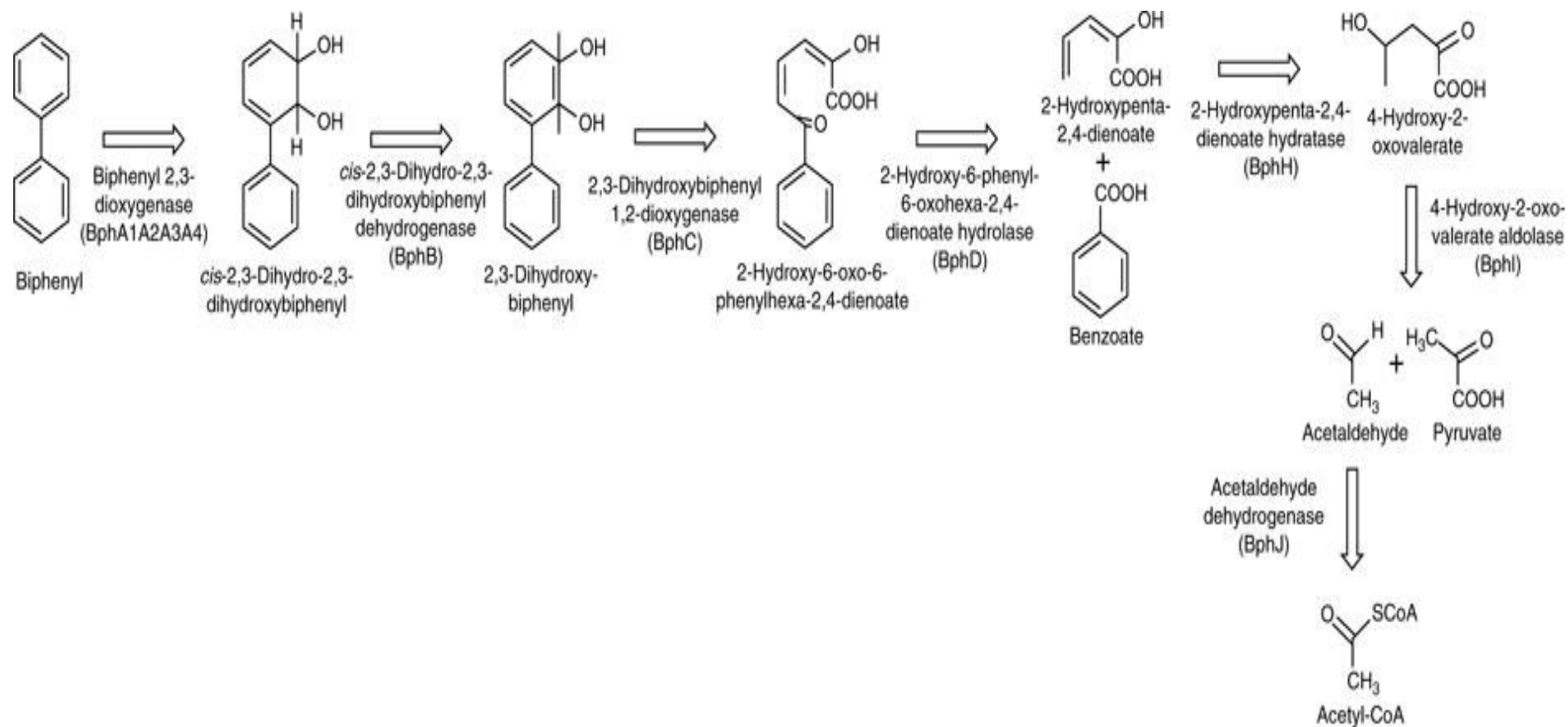


Figure 2.20: A biphenyl being biodegraded by aerobic bacteria (Seeger *et al.*, 2010)

Certain microbes on continuous exposure to xenobiotics develop the ability to degrade the same as a result of adaptations (Heider *et al.*, 2008). These adaptations result in modification of gene of microbes so that the active site of enzymes is modified to show increased affinity to xenobiotics (Heider *et al.*, 2008). Certain adaptations result in developing new enzymatic pathway for xenobiotic degradation (Heider *et al.*, 2008). The diagram in Figure 02.21 is a generalised presentation on the various pathways that can occur in the environment as bacteria become adapted to act on various compounds to render them biodegradable (Hernandez-Sanchez, *et al.*, 2013). The illustration shows a pathway for action of bacteria on biphenyls (Hernandez-Sanchez, *et al.*, 2013).

Engineered Hybrid Pathway for Chlorobiphenyl Mineralization

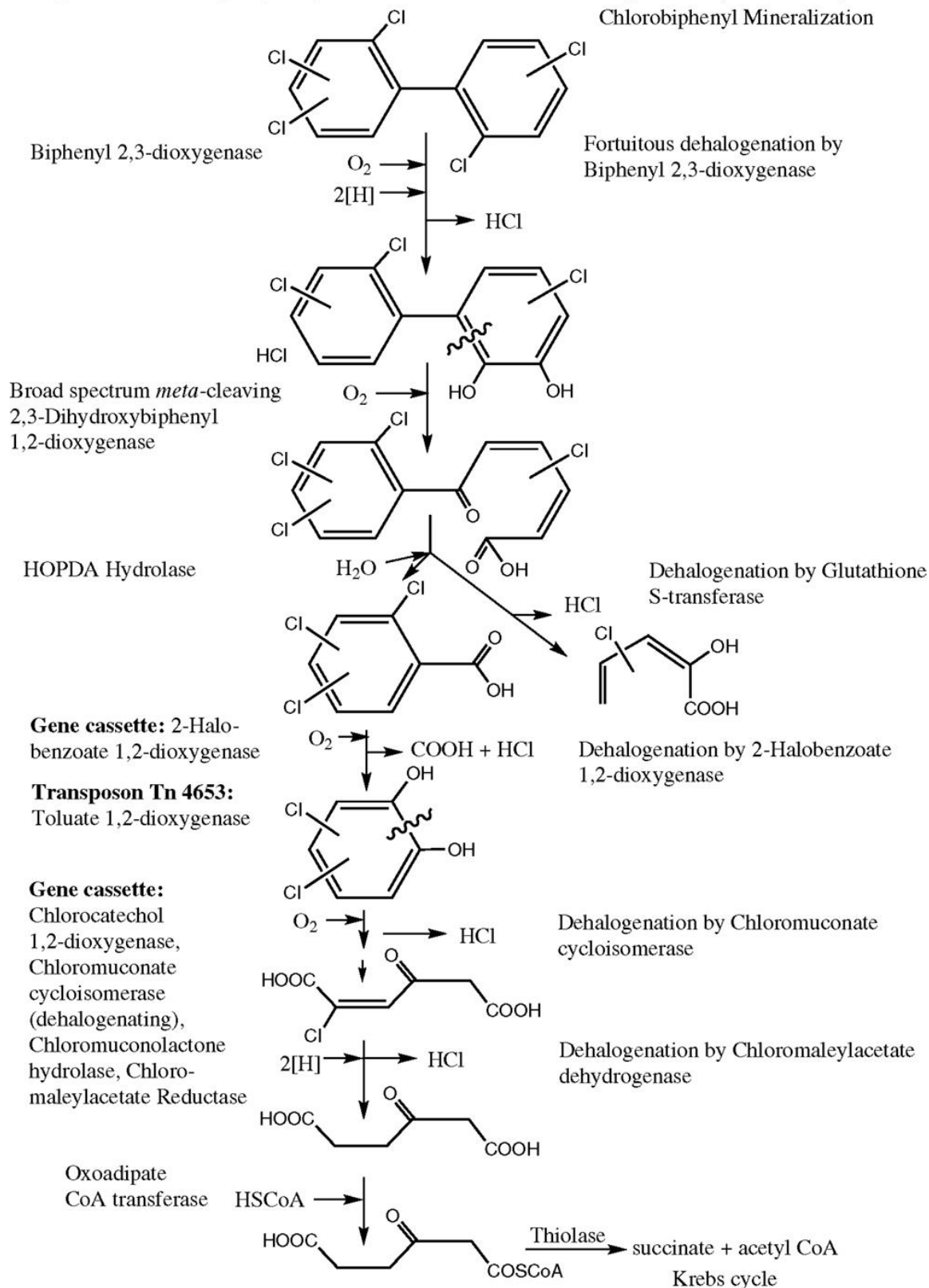


Figure 2.21: Proposed catabolic pathways for the mineralization of halogenated biphenyls by bacterial strains (Hernandez-Sanchez, *et al.*, 2013).

Microbial diversity has been reported crucial for soil functioning and health (Smalla *et al.*, 2007) and as a result it is essential to understand variability of microbial community structure and functions.

2.4.2 Mechanisms involved in biodegradation of xenobiotics

Xenobiotic compound owing to its recalcitrant nature is hard to break down and degrade (Heider *et al.*, 2008). The complexity of its chemical composition adds to this recalcitrant property (Pacwa-Plociniczak, 2011). For breaking down such compounds, the enzymes act on certain groups present in the compound (Heider, 2008). For example, in the halocarbons the halogen group is targeted, with enzymes such as oxygenases playing a major role (Pacwa-Plociniczak, 2011). The bonds like ester-, amide-, or ether bonds present in the compounds are first attacked leading to breaking down of compounds (Cycon *et al.*, 2013; Abor-Amer, 2011). The biodegradation of PCBs results in a compound hydroxyquinol as a key intermediate (Guzik *et al.*, 2013). This is the compound that stimulates the secretion of extra-cellular hydroxyquinol metabolized enzymes by bacteria which they will for the degradation of various aromatic xenobiotics (Guzik *et al.*, 2013).

The site and mode of attack depends on the action of enzyme, its concentration and the favourable conditions (Abor-Amer, 2011). Often it is seen that the xenobiotics do not act as a source of energy to microbes and as a result they are not degraded (Abor-Amer, 2011). The presence of a suitable substrate induces its breakdown (Cycon *et al.*, 2013). These substrates are known as co-metabolites and the process of degradation are known as co-metabolism (Cycon *et al.*, 2013). Gratuitous metabolism is another process in which the xenobiotics serve as substrates and are acted upon to release energy (Pacwa-Plociniczak *et al.*, 2011).

This cannot be achieved through the use of *Moringa oleifera* and related species in treating wastewater to remove PAHs and PCBs. The removal of these compounds have not been fully studied with the use of the plant protein, although the protein has been found to be slightly efficient with the reduction of

faecal coliforms and other bacteria as (Kawo and Daneji, 2011; Dalen *et al.*, 2009). The use of *Moringa oleifera* seed powder in water treatment has been found to target mainly microorganisms thus reduction in turbidity (Mumuni *et al.*, 2013). Although this mode of water treatment has been used, especially in rural areas of the developing countries, the synthetic polymers, aluminium sulphate, ferric chloride and poly aluminium chlorides used together with this powder have been reported to be unsafe (Lea, 2014; Mumuni *et al.*, 2013; Darnel *et al.*, 2009). The action of *Moringa oleifera* seed powder has been reported to be based on the ability of the protein contained in the seeds to be able to form coagulants, which reduce water turbidity, by acting on coliforms (Mangale *et al.*, 2012). The bacteria found to be mainly involved in biodegradation of POPs and PCBs have been found mostly not to be coliforms (Blokesch, 2012; Anyasi and Atagana, 2011). The remaining bacteria after treating water with *Moringa* seed powder, that is, the $10^1 - 10^5$ of bacteria left (Mangale *et al.*, 2012) may be those that are capable of biodegradation of xenobiotic compounds by using them as their sole carbon sources (although no report to that effect has been found).

CHAPTER 3

MATERIALS AND METHODS

3.0 Materials and Methods

This is an exploratory laboratory study that involved screening of wastewater samples and untreated raw water (from dams) samples for the presence of bacteria species capable of carrying out the process of biodegradation on selected POPs. The water samples were also screen for recalcitrant compounds.

3.1 Sampling Sites

Water samples were obtained from Gaborone dam and effluent from Notwane Sewage Treatment Plant in Gaborone, Botswana. Notwane sewage treatment plant was selected since is the largest wastewater treatment plant in Gaborone, the capital city of Botswana. This wastewater treatment plant receives wastewater from the residential areas, industrial and agricultural sectors in Gaborone. This increases the chances of having a variety of chemical substances in wastewater. Water samples were also obtained from Disaneng dam and Setumo/Modimola Wastewater Treatment Plant effluent, which is in Mmabatho, South Africa. Disaneng dam was chosen as it has raw water unlike Modimola/Setumo dam, which has wastewater. Figure 3.1 is a point map to show the sampling sites.

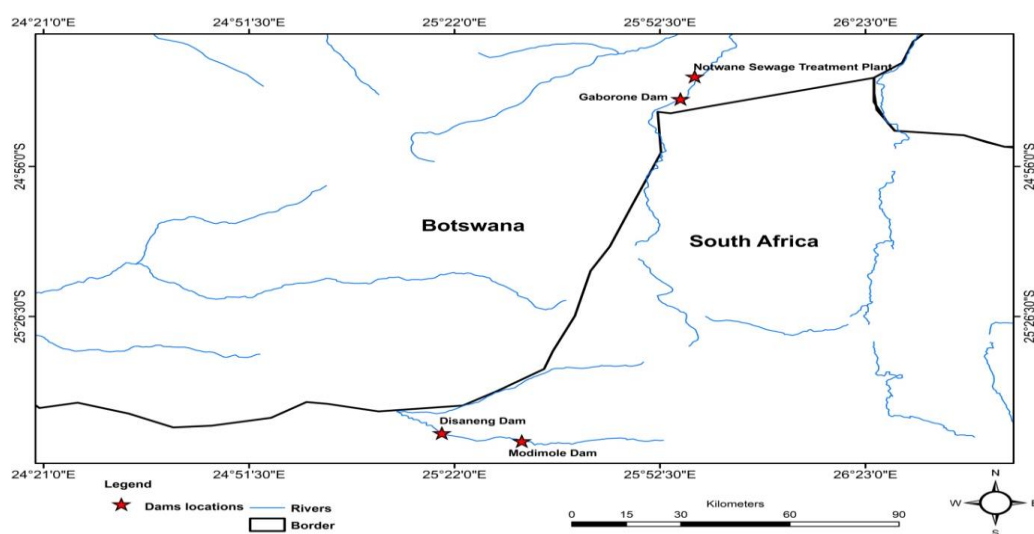


Figure 3.1: A sketch map to show the sampling sites.

The purpose of sampling in Gaborone and Mafikeng/Disaneng areas was to broaden the area of analysis based on the differences in geographical position, the different activities that are found around the different sampling areas. For example, the Mmabatho region has pharmaceutical industries and mines around whereas Gaborone region has none of these industries.

3.2 Water Collection

Raw water samples were collected from Gaborone dam (site A, 30 samples) and Disaneng dam (Site B, 30 samples), and wastewater samples were collected from Notwane Sewage Treatment Plant effluent dams (Site C, 25 samples) and Setumo/Modimola dam/wastewater treatment effluent (Site D, 25 samples) between the months of April 2013 and March 2014. Random sampling was used throughout the study. Tap water samples obtained from Gaborone and Mafikeng were used as negative controls. Samples were collected during the different seasons of the year. Water samples were collected using sterile 250 ml and 500 ml Duran Schout bottles and properly labelled and transported on ice to Department of Biological Sciences, North-West University - Mafikeng Campus for analysis. Samples were analysed within 24 hours from the time of collection. Samples for chemical analysis were also collected simultaneously.

3.3 Analysis of water samples

3.3.1 Determination of physical parameters of the water samples

Physical parameters that included pH, temperature and turbidity of samples were determined immediately at sampling sites during collection. The turbidity was measured using Orbeco Hellige TB 200™ Potable Turbidimeter from LASEC, South Africa. The pH and temperature (°C) were measured using Crison PH 25 – Spain supplied by LASEC, South Africa.

3.3.2 Microbiological isolation of bacteria

The water samples were first checked for the presence of suspended solids. All solids were filtered where necessary to remove any solid material as described by Ateba and Maribeng, 2011; Moura *et al.*, 2007; Zaugg *et al.*, 2007. An aliquot of 1 ml of each water sample was mixed with sterile molten Plate Count Agar (PCA) (Sigma Aldrich, supplied by Lehlabile Scientific, South Africa) in 90 mm petridishes for initial isolation of heterotrophic aerobic bacteria (Andrews, 1992; Swanson *et al.*, 1992). The inoculated plates were allowed to solidify and were incubated aerobically at 37°C for 24 hours (Andrews, 1992; Swanson *et al.*, 1992). Colonies with different morphologies were picked using a sterile loop and sub-cultured in Tryptone Soy Agar (TSA) (Sigma Aldrich, supplied by Lehlabile Scientific, South Africa) to obtain pure cultures (Andrews, 1992). Plates were incubated aerobically at 37°C for 24 hours. Pure bacteria cultures were sub-cultured in TSA slants and incubated at 37°C for 24 hours. The slants were kept at room temperature until further analysis.

3.4 Bacteria Identification tests

3.4.1 Gram staining

Morphology of bacteria cells were determined using standard gram staining technique (Andrews, 1992).

3.4.2 Analytical Profile Index (API) 20E

A total of 25 isolates (both gram negative and gram positive) were randomly picked and analysed using API 20E strips (BioMérieux, Marcy-L'Etoile / France). This is designed to identify members of the Enterobacteriaceae. The test was performed according to the manufacturer's instructions (BioMérieux, Marcy-L'Etoile / France) with modifications where necessary. Of the 25 isolates, 5 were selected from sites B, C and D. The remaining 20 isolates were selected from site A. The indices were generated for the different isolates and these were used to determine their identities using the API Web™ identification software.

3.4.3 DNA Isolation and amplification of bacterial 16S rRNA gene fragments

3.4.3.1 Isolation of DNA from Bacterial strains

Pure isolates from TSA (Sigma Aldrich) were inoculated into 5 ml of nutrient broth and incubated aerobically at 37°C for 24 hours while shaking at 120 rpm. Genomic DNA was extracted from all isolates using Zymo Research Genomic DNA™ –ZR Fungal/Bacterial DNA MiniPrep kit (Catalogue No. D6005-USA supplied by Bio-Rad Laboratories, South Africa) according to the manufacturer's instructions. Briefly, 100 µl from the broth culture was aliquot into Bashingbead™ lyses tube, followed by addition of 750 µl lyses solution. The contents were processed using a bead beater (Disrupter Genie™) at maximum speed for 5 minutes. The tube was centrifuged at 10000 rpm for 1 minute using a bench-top Hermle Z300 high speed microcentrifuge (Kendro, Germany). An aliquot of 400 µl of the supernatant was transferred to Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged at 7000 rpm for 1 minute and 1 200 µl of DNA binding buffer was added to the filtrate. The mixture was transferred to a Zymo-Spin™ IIC column in a collection tube and the contents of the tube were centrifuged at 10000 rpm using a bench-top Hermle Z300 high speed microcentrifuge (Kendro, Germany) for 1 minute. The supernatant was discarded and the pellet was re-suspended in 200 µl of DNA Pre-buffer and centrifuged at 10000 rpm and the resulting supernatant was discarded. An aliquot of 400 µl of g-DNA Wash Buffer was added to the spin column and centrifuged at 10000 rpm for 1 minute. The resulting supernatant was also discarded. Finally, 100 µl of DNA Elution Buffer was added to each column. The columns were incubated for 5 minutes at room temperature and then centrifuged at 13500 rpm for 30 seconds to elute the DNA. DNA samples were stored at -20°C for PCR analysis.

3.4.3.2 Isolation of total Bacterial DNA from water samples

DNA was also directly isolated from water samples using the same procedure.

3.4.3.3 Amplification of bacteria 16S rRNA gene fragments

The 16S rRNA genes from isolates were amplified through PCR from genomic DNA as described by Cai *et al.*, (2003) and Phillips *et al.*, (2006) with modifications. The isolates were selected for API tests were subjected to PCR analysis. 16SrRNA gene target sequences were amplified from all the 19 isolates using the primer sequences U758 (R) CTACCAGGGTATCTAATCC and U341 (F) CCTACGGGAGGCAGCAG (Hilyard *et al.*, 2008; Mallick *et al.*, 2007; Muyzer *et al.*, 1993). The 16S RNA universal primers, U758 (R) CTACCAGGGTATCTAATCC and U341 (F) CCTACGGGAGGCAGCAG (Phillips *et al.*, 2006) were used to amplify about 600 base pair gene fragments in isolates. Amplifications were carried out using a C1000 Touch TM Thermal Cycler (Bio-Rad, Johannesburg, South Africa). PCR amplification was performed in a total volume of 25 µl made up of 12.5 µl master mix (Thermo scientific PMM), 8.5 µl of nuclease free water, 1.5 µl of loading buffer, 0.5 µl oligonucleotide primer set and finally, 2.0 µl of DNA template. All the reagents were obtained from Inqaba Biotec Ltd, Sunnyside- South Africa. Amplification was achieved through the following parameters: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The PCR products were stored at 4°C before separation by electrophoresis.

3.4.3.4 Denaturing Gradient Gel Electrophoresis (DGGE) PCR analysis

The DNA was amplified using total bacterial DNA from ten randomly selected isolates that constituted isolates 20 to 29 on Table 4.4. The primer sets EUB 968F (AACGCGAAGAACCTTAC) and UNV 1392R (ACGGGCGGTGTGTRC) with the EUB 968F GC-clamp CGCCCGGGGCGCGCCCGGGCGGGGCGGGGGCACGGGGGG (Chang *et al.*, 2006) coupled with BA 338F (ACTCCTACGGGAGGCAGCAG) and UN 518R (ATTACCGCGGCTGCTGG) with BA 338F GC-clamp CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGG (Manzano *et al.*, 2012) were used for amplification. PCR was performed at 95°C for 5 minutes, followed by 30 cycles of

denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. Amplification was performed using a C1000 Touch™ Thermal Cycler (Bio-Rad, Johannesburg, South Africa). The amplicons were resolved using a Bio-Rad DCode™ system Model 475 gradient delivery system supplied by Bio-Rad, South Africa. An 8% (w/v) acrylamide gel with 40% to 60% denaturant gradient was used for DGGE analysis. The Bio-Rad DGGE profiling Gel volumes protocol for 10 -100% denaturant (40% acrylamide) described in DCode™ Universal Mutation Detection System Manual, Bio-Rad, UK, was followed.

3.4.3.5 Agarose gel electrophoresis

The PCR products were separated by electrophoresis on a 1% (w/v) agarose gel. The gel was run for 60 minutes at 80 V using 1X TAE buffer (40 mM Tris, 1 mM EDTA and 40 mM glacial acetic acid, PH 8.0) using a horizontal Pharmacia biotech equipment system (model-BCMSCHOICE; Biocom, UK). Each gel contained a 1kb DNA molecular weight marker (Fermentas, USA). The gel was stained in ethidium bromide (0.1 µg/ml) and amplicons were visualized under UV light at 420 nm. A ChemiDoc Imaging System (Bio-Rad ChemiDoc™ MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software. Isolates were further subjected to PCR sequencing and blasting.

3.4.3.6 Bacterial 16S rRNA sequencing and sequence analysis

The PCR products were sequenced at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. The sequenced products were analysed using the Chromas Lite (version 2) and the BIOEDIT Sequence Alignment Editor Program was used to align sequences. The sequences were then compared using the packages DNA DIST and Bootstrap and distance trees were generated using phylograms. Cleaned sequences were subjected to MEGA 6.06 (6140226) and MAFFT programs for characterisation of the organisms and construction of a phylogenetic tree.

3.5 Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) and Polychlorinated Biphenyls (PCBs)

Strains of *Pseudomonas aeruginosa* ATCC 27853™ (Lot No. 353-147-1), *Aeromonas hydrophila* ATCC 7966™ (Lot No. 870-32-1) and *Serratia liquefaciens* ATCC 27592™ (Lot No. 838-33-6) were commercially obtained from Microbiologics (USA) and supplied by Quantum Biotechnologies, South Africa. In addition, an environmental isolate MD2 obtained from wastewater during the study and positively identified as *Pseudomonas aeruginosa* was also used in the PAH and PCB biodegradation analysis. The isolate was selected as it was found to be present in the two wastewater treatment plants used in this study. These strains were used to determine the effect of bacteria on the biodegradation of commercially obtained PCBs and PAHs. *Pseudomonas aeruginosa* and *Serratia liquefaciens* strains were revived by sub-culturing on TSA and Nutrient Agar plates obtained from Sigma Aldrich supplied by Lehlabile Scientific, South Africa. *Aeromonas hydrophila* was sub-cultured on a 10% (w/v) Sheep Blood Agar supplied by Bio-Rad Laboratories, South Africa. The plates were incubated at 35°C for 24 hours.

The chemical or recalcitrant compounds (PAHs and PCBs) were obtained from SUPELCO Solutions Within™ - USA, through Lehlabile Scientific, South Africa. The PAHs were supplied as SS TCL Polynuclear Aromatic Hydrocarbons Mix (Lot LB99887) in a 1 mL ampoule at a concentration of 2000 µg/ml dissolved in Methylene Chloride (CH₂Cl₂): Benzene (C₆H₆) at 1:1 ratio. As indicated by the supplier (SUPELCO Solutions Within™ – USA), the mixture contained Acenaphthene (99.9% purity), Acenaphthalene (99.2% purity), Anthracene (99.1% purity), Benzo[a]anthracene (99.8% purity), Benzo[a]pyrene (99.6% purity), Benzo[b]fluoranthene (99.5% purity), Benzo[G,H,I]perylene (96.3% purity), Benzo[k]fluoranthene (99.9% purity), Chrysene (99.9% purity), Dibenzo[A,H]anthracene (99.3% purity), Fluoranthene (98.3% purity), Fluorene (99.1% purity), Indeno [1,2,3-CD]pyrene (99.6% purity), Naphthalene (99.9% purity), Phenanthrene (99.0% purity) and Pyrene (99.1% purity). The wavemax

(λ_{max}) was determined using HPLC at a UV wavelength of 254 nm. The PCBs were supplied as Aroclors. Aroclor 1242 (Lot No. LB8851), 1248 (Lot No. LB88969) and 1260 (Lot No. LB92109) were obtained in a 1 mL ampoule at concentration 1000 $\mu\text{g/ml}$ dissolved in isooctane. Stock solutions were prepared using the commercially obtained PAHs and PCBs by mixing with 99 mL sterile normal (0.9%) saline according to Ogbonna *et al.*, (2012). Working solutions, 1.0 $\mu\text{g/ml}$, of the recalcitrant laboratory stocks were used for biodegradation analysis.

3.5.1 Bacteria Inoculum Preparation

Mineral Salt Medium (MSM) was prepared by dissolving 75.2 g Sodium diphosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$), 30 g of Potassium Phosphate (KH_2PO_4), 5 g Sodium Chloride (NaCl) and 5 g Ammonium Chloride (NH_4Cl) in 1000ml distilled water and the pH was adjusted to 7.0 using Sodium hydroxide (NaOH). The liquid medium was sterilised by autoclaving at 121°C for 15 minutes. In addition, 100 mL stock solutions of Magnesium sulphate (MgSO_4) and Calcium chloride (CaCl_2) respectively were prepared. These solutions were also sterilised by autoclaving. The MSM used during the study was in 100 mL volumes that included 99 mL of the salt mixture, 200 μL MgSO_4 and 10 μL CaCl_2 as described by Shafie *et al.*, (2006) with slight modifications to suit the study. CaCl_2 was added just before the experiment as it precipitates.

The final bacteria inoculum for all the experiments was prepared by growing each strain in 50 mL of Mineral Salt Medium (MSM). The flasks were incubated at 30°C on a rotary shaker at 150 rpm overnight (Abor-Amer, 2011). After 24 hours of incubation, cells were harvested by centrifugation at 12 000 rpm for 5 minutes at 4°C.

Pelleted cells were washed twice in 25 mL of sterile 50 mM phosphate buffer (pH 7.2) and the numbers of cells were enumerated using the standard plate count technique. The optical density (OD) of the bacteria inoculum was adjusted to 1.0 at 600 nm using a spectrophotometer (model Spectroquant 300,

Merck, South Africa) as previously described (Abor-Amer, 2011) but with certain modifications. For the standardization experiments in this study, 10^6 CFU/mL of bacterial culture was used to inoculate MSM and wastewater samples. Flasks were covered with aluminium foil to exclude light so as to avoid photodegradation of PAHs and PCBs.

3.5.2 Degradation of PAHs and PCBs

A colony from each 24 hours old culture of the three strains of bacteria, (*Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Serratia liquefaciens*), was used to inoculate 100 mL of MSM supplemented with 100 μ L of either PAH or PCB respectively in a 250 mL conical flask. As a quality control measure 100 mL of MSM supplemented with the similar quantities of PAH and PCB respectively but without any bacteria inoculum was also utilized. The conical flasks were wrapped with aluminium foil to exclude light and incubated at 30°C on a rotary shaker at 150 rpm in the dark to avoid photo degradation of PAHs and PCBs (Shafie *et al.*, 2006; Roy *et al.*, 2013). Two (2) mL of samples were aseptically taken at intervals of 10 hours from both the treatment and the control flasks. The optical densities of these samples at 600 nm were used to evaluate bacteria growth.

3.5.3 Screening for factors that affect the degradation of PAHs and PCBs

3.5.3.1 pH

The inoculum densities of bacterial strains in MSM was amended with 100 μ L of PAH and PCB, respectively. The effect of pH on the degradation of PAHs and PCBs was determined by subjecting the bacterial strains to the same media whose pH values differed and were adjusted to 5.0, 7.0, 8.0 and 9.0. The experiments were maintained at 30°C for 24 hours on a rotary shaker at 150 rpm in the dark to avoid photo degradation of PAHs and PCBs (Shafie *et al.*, 2006; Roy *et al.*, 2013).

3.5.3.2 Temperature

The effect of temperature on the degradation of PAHs and PCBs was determined by incubating media containing both the inoculum of each of the test strains (*Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Serratia liquefacians*) and each of the commercially obtained recalcitrant compounds at 20, 27, 30, and 35°C in the dark. Similarly, a control experiment that consisted of 100 mL of MSM supplemented with same amount of either PAH or PCB respectively, and without bacteria inoculum was also evaluated at each of the treatment temperatures. Aliquots of 2 mL samples were collected from each treatment flasks and the controls at 24 hours' intervals for up to 96 hours. The effect of temperature on the degradation of PAHs and PCB was determined by measuring the optical density at 200 – 800 nm using a UV spectrophotometer (model Cary 300 UV-VIS Series Spectrophotometer, Agilent Technologies, South Africa and Cary Win UV software).

3.5.3.3 Effect of the concentrations of PAHs and PCBs on their biodegradation

An analysis of the effect of different concentrations of the commercially obtained PAHs and PCBs were determined. Aliquots of 10, 40 and 100 µL of each of PAH and PCB were added to 100 mL of MSM in a 250 mL flasks and inoculated with a mixed culture of the test bacterial strains (Shafie *et al.*, 2006), but with certain modifications. The flasks were incubated at 30°C in the dark in a rotary shaker at 150 rpm and 2 mL samples were drawn from each treatment and controls at 24 hours' intervals. These samples were assessed for the level of PAH and PCB degradation by evaluating the optical density at 200 – 800 nm range using a Cary 300 UV-VIS Series Spectrophotometer obtained from Agilent Technologies, South Africa. The data was analysed through Cary Win UV software.

3.5.4 Evaluation of the biodegradation of PAHs and PCBs introduced directly into wastewater using Spectrophotometry analysis

Wastewater samples that were collected from Notwane Sewage Treatment Plant (Site C - wastewater 1) and Modimola/Setumo dam (Site D - wastewater 2) (Section 3.2) were used to evaluate the level of biodegradation of PAH and PCB when introduced directly in to the wastewater samples. The samples were divided into two parts, one part was sterilised by autoclaving at 121°C for 15 minutes and the other half was left unsterilized. The unsterilized and sterilized wastewater was further divided into two parts. The wastewater samples were treated with PAHs and PCBs separately. To each 100 mL of unsterilized wastewater sample that was contained in a 250 mL flask, 100 µL of PAHs and PCBs, respectively, was added. In addition, bacteria inoculum was added to one of the flasks containing the unsterilized wastewater. A 100 mL portion of the sterilised wastewater samples were inoculated with the test organisms (*A. hydrophila*, *S. liquefacians* and *P. aeruginosa*) while the other portions served as negative controls (without bacteria inoculation) (Control 2). The water samples were incubated at 30°C in the dark in a rotary shaker at 150 rpm and 5 mL of the samples were taken at 24 hour intervals. These samples were used to determine the change in PAHs and PCBs levels at wavelengths of 200 – 800 nm using Cary 300 UV-visible spectrophotometer.

3.5.5 Evaluation of the biodegradation of PAHs and PCBs introduced directly into wastewater using HPLC analysis

The High Performance Liquid Chromatography (HPLC) (model --- Gilson 361E2Q012) was also used to assess the biodegradation of PAH and PCB when introduced into wastewater at different concentrations (Roy *et al.*, 2013; Shafie *et al.*, 2006). In achieving this, 1mL of water sample was analysed for residual PAH and PCB. The compounds were extracted by adding 10 mL each of dichloromethane and acetone. The mixture was incubated in a rotary shaker for 24 hours at 30°C and later centrifuged for 10 minutes at 12 000rpm at 4°C using Hermle Z326k high speed micro-centrifuge,

Labortechnik GmbH (LASEC, South Africa). The extra water was pipetted and 4 g of anhydrous sodium sulphate was mixed with the recalcitrant compounds (PAH or PCB) to remove residual water. The extract was concentrated to 1.5 mL using rotary evaporator Stuart RE300DB, LASEC, South Africa and filter sterilized with 0.45 µm PTFE syringe filters. The resulting extracts were used to perform the HPLC analysis using a fluorescence detector pump (RF-20A) and a system gold column C18 (CTO-20A) on a UFLC Shimadzu equipment as described by Shafie *et al.*, (2006). The excitation level was set at 254 nm and the emission level was at 390 nm. The mobile phase used was a mixture of acetonitrile and water (80:20) as described by (Shafie *et al.*, 2006). Data analysis was computed using Real-time Analysis. All chemicals used were of HPLC grade products supplied by Sigma Aldrich through Lehlabile Scientific, South Africa.

3.5.6 Enzyme Activity on biodegradation of PAHs/PCBs

3.5.6.1 Screening of bacteria strains for enzyme activities

Test organisms were grown in MSM supplemented with PAHs and PCBs in separate flasks and incubated at 35°C in the dark for 18 hours. The cells were harvested by centrifugation at 9200 rpm for 10 minutes using a bench-top Hermle Z326k high speed micro-centrifuge, Labortechnik GmbH (LASEC, South Africa) as described by Roy *et al.*, (2013) with modifications. The pellets obtained were washed two times with phosphate buffer (pH 7.0). The cells were re-suspended in the same buffer for 15 minutes. The cells were then lysed using Disruptor Genie, Scientific Industries cell lyses. The extracts were centrifuged at 14500rpm for 40 minutes using bench-top Hermle Z326k high speed micro-centrifuge, Labortechnik GmbH (LASEC, South Africa) at 4°C. The resultant clear supernatant was decanted into sterile 1 ml centrifuge tubes and stored at 4°C for further studies. Confirmatory test for presence of enzymes was performed by a general qualitative protein test since enzymes are protein molecules. This was achieved by addition of 1% Sodium hydroxide solution and 1% Copper(II)Sulphate solution to 0.5 mL of the resultant supernatant (Galewska *et al.*, 2013). The mixture changed from blue

colour to a violet indicating that the Cu^{2+} were reduced to Cu^+ , a positive result for protein since the enzymes are protein molecules (Galewska *et al.*, 2013). The product was used to carry-out the effect of enzymes on the degradation of PAHs and PCBs (Roy *et al.*, 2013).

3.5.6.2 Evaluation of the effect of bacteria enzymes on the degradation of PAHs and PCBs

The activity of enzymes obtained from bacteria on the degradation of PAH and PCB was evaluated by determining the rate at which these compounds were hydrolysed. Spectral changes at 30°C were monitored using a Cary 300 UV-visible spectrophotometer (Agilent Technologies) and the data was analysed using the Cary Win UV software. Aliquots of 10 μL of the test compounds were added to 2 mL of the enzyme extract prepared in section 3.5.5.1. The optical density of the mixture determined using a Cary 50 CONC UV-visible spectrophotometer Varian Single Cell Peltier Accessory that was preset at 30°C. To monitor the effect of the enzyme on the degradation of the recalcitrant compounds, the spectrophotometer was set to record spectral readings every 30 minutes for 2 hours. The same set-up was repeated by incubating a similar mixture at 30°C on a rotary shaker at 150rpm in the dark. Product formation was also checked by taking spectral readings by measuring optical density of the mixture using UV-visible spectrophotometer at 30 minutes' intervals for 2 hours, at a wavelength of 200 - 800nm (Roy *et al.*, 2013).

3.6 Gas Chromatography Mass Spectrometer (GC-MS) analysis

3.6.1 Preparation of Quality Control Standards and water samples for GC-MS Analysis

Quality control was set using the water samples and five randomly selected pesticides that were available from the supplier (Sigma-Aldrich, Germany). These pesticides utilized were Aldrin, 4,4-DDT, 4,4-DDD, 4,4-DDE and Dieldrin. Stock solutions of the pesticides were prepared by dissolving 5 mg in 1 Litre of HPLC grade hexane obtained from Sigma Aldrich, South Africa. Spiked PAH dissolved in saline and PCB standards were also prepared (SUPELCO Solutions Within™ – USA). GC-MS calibration standard solutions were concentrated in HPLC grade toluene (Sigma Aldrich, South Africa). Standard

stock solutions (1 ppm and 5 ppb) were prepared in HPLC grade acetonitrile (Sigma Aldrich, South Africa). These were used to spike water samples in preparation for GC-MS analysis.

As a quality control measure, water samples were divided into two parts of 10 mL each. Each part was further divided into two and these were spiked individually with 1 ppm and 5 ppb of each pesticide (Aldrin, 4,4-DDT, 4,4-DDD, 4,4-DDE and Dieldrin) stock solution. However, the other portions were not spiked with the pesticides and recalcitrant compounds. The main rationale is that upon GC-MS analysis the pesticides and recalcitrant compounds should be detected in the spiked samples when compared to the unspiked water samples. In addition, this procedure was used to evaluate the effectiveness of the QuEChERS method that is designed to extract compounds from water samples (Furlani *et al.*, 2011).

To extract compounds from water samples aliquots of 10 mL of water sample (both spiked and unspiked) were transferred into a 50 mL polypropylene centrifuge tube and 10 mL of HPLC grade acetonitrile was added. The mixture was vortexed (model Vortex Genie 2) for 1 minute. Four grams of anhydrous MgSO_4 and 1g of NaCl were added to the mixture and vortexed for 1 minute. The mixture was transferred into a 15 mL polypropylene centrifuge tube and centrifuged for 5 minutes at 5000 rpm using a Labofuge 200 centrifuge (Heraeus Sepatech Germany) supplied by Phala Scientific (PTY) LTD, Gaborone. The centrifuged product was cleaned by transferring 4 mL aliquot of the upper layer (supernatant) into a polypropylene centrifuge tube containing 200 mg PSA and 600 mg anhydrous MgSO_4 (Furlani *et al.*, 2011). This was vortexed for 30 seconds using a Vortex Genie 2 supplied by Scientific Industries. The product was then centrifuged for 3 minutes at 3500 rpm using the Labofuge 200 centrifuge (Heraeus Sepatech Germany) supplied by Phala Scientific (PTY) LTD, Gaborone. A 4ml aliquot of the supernatant was transferred into a vial and concentrated by blowing with nitrogen gas until it was dry. The product was topped up to 0.5 ml with toluene (Furlani *et al.*, 2011) in a vial and used for GC-MS analysis. The vials were placed in an auto-sampler tray in Gas Chromatography equipment (model 7890A– Agilent Technologies 5975C Mass Spectrometer Inert XL EI/CI MSD) that

possessed a Triple axis detector and samples were subjected to GC-MS analysis. The runs were performed under the following GC-MS conditions: 260°C Inlet –F temperature, 220°C oven temperature, 1.5 column 1 flow, 280°C Aux -2 temperature, 200°C MS source, 150°C MS quad (Brondi *et al.*, 2011; Furlani *et al.*, 2011).

3.6.2 Analysis of PAHS and PCBs from water samples using Gas Chromatography Mass Spectrometer (GC-MS)

The raw water samples were obtained from the surface of the water bodies at depths of 50 and 100 meters but at different locations in each dam, with depth of the dams being about 3 meters for Gaborone dam and about 10 meters for Disaneng dam. Gas Chromatography Mass Spectrometer (GC-MS) was used to detect presence of PAHs and PCBs in water samples (Kawaguchi *et al.*, 2012; Mallick *et al.*, 2007). To achieve this, 250 mL polyethylene (PET) bottles were washed with nitric acid to remove impurities. Composite water samples (250 mL) were prepared by mixing samples from the different depths collected from the four sample sites of a particular dam. The identities of resolved products were determined by comparing results with the profiles of GCMS reference compounds.

CHAPTER 4

RESULTS AND INTERPRETATION

4.0 Results and Interpretation

4.1 Determination of physical parameters in water samples

The mean pH, turbidity and temperature of the water samples were determined and results are presented in Table 4.1. As shown in Table 4.1, samples obtained from Site D (Modimola dam) had the highest turbidity (with mean values of 25.10 – 200.6) and pH (mean values of 9.01 – 9.78) values while those from Site C (Notwane Sewage treatment plant) had the highest temperature (mean values of 10.1 – 26.2) values. In addition, the turbidity values of both control samples (tap water) were 0.01 respectively when compared to the results obtained for the environmental wastewater samples. Generally, the pH, turbidity and temperature values for the control water samples were within the acceptable standard reference values for drinking water.

Table 4.1: Mean values of physical parameters (pH, turbidity and temperature) of water from the four sampling sites. The results ^a to ^d imply that pollutants may be adsorbed to particulate matter or dissolved organic carbon.

Sample source	Mean Turbidity (NTU)	Mean pH	Mean Temperature (°C)
A	10.12 – 10.68 ^a	8.26 – 8.64	9.2 – 24.5
B	13.31 – 25.09 ^b	8.96 – 9.04	8.0 – 26.7
C	12.44 – 20.34 ^c	8.99 – 9.77	10.1 – 26.2
D	25.10 – 200.6 ^d	9.01 – 9.78	9.0 – 24.2
Control – 1	0.01	7.78	24.4
Control – 2	0.01	7.88	26.1
EPA reference value for drinking water	≤1.0	6.5 – 9.5	25.0
EPA reference value for wastewater	≤5.0	5.5 – 10.0	≤ 40.0
WHO reference value for drinking water	≥ 1 - ≤5.0	6.5 – 8.0	25.0
WHO reference value for wastewater	5.0	6.5 – 8.0	30.0
BW and ZA reference value for drinking water	0.5 – 5.0	6.5 – 8.5(BW) 5.5 – 9.5 (ZA)	25.0
BW and ZA reference value for wastewater	25 - 30.0	6.5 – 8.5	30.0

BW=Botswana; ZA = South Africa

4.2 Morphological and biochemical profile (API 20E) analysis for identification of bacteria isolates

The morphological (Gram status) and biochemical profiles (oxidase test, catalase test and API 20E results) of the isolates including their identities are shown in Table 4.2. As shown in Table 4.2, a total of 40 isolates were randomly selected based on differences in colonial characteristics from all the sites and subjected to these identification tests. A total of 17 isolates were positively identified. Bacteria species belonged to the genus *Aeromonas*, *Serratia*, *Enterobacter*, *Ewingella* and *Pantoea* (Table 4.2). However, large proportions (35.2% and 41.2%) of the isolates were *Serratia* and *Aeromonas* species respectively. Generally, the samples that were obtained from Sites A and C (both in Gaborone, Botswana) possessed a larger variety of bacteria species when compared to those from Sites B and D (both in Mafikeng, South Africa). On the contrary, no bacterial isolates were recovered from all the control water samples and this was a good observation from a public health point of view. None of the isolates produced hydrogen sulphide gas and a large proportion (97.5 % to 87.5 %) were unable to utilize both indole and urea. On the contrary, large proportions (82.5%) of these isolates were rod shaped bacteria amongst which 51.5% were Gram negative. However, only 15.5% of the isolates were vibrio and cocci. A large proportion (94%) of the rod shaped bacteria were catalase positive while all the vibrio isolates were catalase negative.

Table 4.2: Preliminary identification test results for isolates obtained in the study

Site	Isolate ID	Gram Stain	Glucose Fermentation	Catalase	Oxidase	Indole	Urea	H ₂ S production	API ID and % identity
A	GD1	- rod	+	-	+	-	-	-	<i>Aeromonas</i> sp., 89.8
	GD2 (S5)	+ rod	+	+	+	-	-	-	-
	GD3	+ rod	+		+	-	-	-	-
	GD4		+		+	-	-	-	-
	GD5	- rod	+		-	-	-	-	<i>Serratia ficaria</i> , 98.0
	GD6 (S18)	+ rod	+	+	+	-	+	-	-
	GD7	- rod	+	-	+	-	-	-	<i>Aeromonas</i> sp., 98.9
	GD8	- rod	+	+	-	-	-	-	<i>Enterobacter sakazakii</i> , 98.4
	GD9(S19)	+ rod	+	+	+	-	-	-	<i>Aeromonas</i> sp., 95.3
	GD10	+ cocci	+	-	-	-	-	-	-
	GD11 (S16)	+ rod	+	+	-	-	-	-	-
	GD12 (S13)	+ rod	+	+	-	-	-	-	-
	GD13	- rod	+	+	-	-	-	-	<i>Enterobacter sakazakii</i> , 98.4
	GD14	- rod	+	+	-	-	-	-	<i>Serratia liquefaciens</i> , 90.1
	GD15	- rod	+	+	-	-	-	-	<i>Serratia ficaria</i> , 98.0
	GD16	- rod	+	+	-	-	-	-	<i>Serratia ficaria</i> , 98.0
	GD17	- rod	+	+	-	-	-	-	-
	GD18 (S15)	+ rod	+	-	+	-	-	-	-
	GD19 (S12)	+ rod	+	+	+	-	-	-	-
B	DS1 (S2)	- rod	+	+	+	+	-	-	-
	DS2	- cocci	+	+	+	-	-	-	-
	DS3	- rod	+	+	+	-	-	-	<i>Aeromonas</i> sp., 83.0
	DS4	- rod	+	+	-	-	-	-	<i>Serratia marcescens</i> , 95.6
C	GS1	- rod	+	+	-	-	-	-	<i>Serratia liquefaciens</i> , 100%
	GS2 (S6)	+ rod	+	+	+	-	-	-	-
	GS3	- rod	+	-	+	-	-	-	<i>Aeromonas hydrophila</i> , 98.0
	GS4 (S17)	- vibrio	+	-	-	-	-	-	-
	GS5	- rod	+		-	-	+	-	-
	GS6 (S7)	- vibrio	+	-	-	-	-	-	<i>Aeromonas hydrophila</i> , 99.0
	GS7 (S14)	+ rod	-	+	-	-	-	-	-
	GS8 (S1)	+ rod	-	+	+	-	+	-	-
	GS9		+		+	-	-	-	-
	GS10 (S9)	+ rod	-	+	-	-	-	-	-
	GS11 (S10)	+ rod	+	+	+	-	-	-	-
	GS12 (S11)	- vibrio	+	-	-	-	-	-	<i>Aeromonas</i> sp., 85.0

D	MD1 (S3)	- cocci	+	-	+	-	+	-	-
	MD2 (S4)	- cocci	+	+	+	-	-	-	-
	MD3	- rod	+	+	-	-	-	-	<i>Ewingella americana</i> , 99.6
	MD4	- rod	+	+	-	-	+	-	<i>Pantoea</i> sp, 95.6

Key: - negative, + positive

4.3 Bacterial 16S rRNA gene PCR amplification

A total of 19 isolates were subjected to 16S rRNA PCR analysis and 500 bp amplicons were obtained. In addition, 16S rRNA gene fragments were also amplified using the total DNA that was directly isolated from water samples. Figure 4.1 indicates a 1% (w/v) agarose gel of the PCR products that were amplified in the study. The sequence results obtained were aligned with Blast search of NCBI databases and results are presented in Tables 4.3 and 4.4 for bacterial 16S rRNA gene fragments amplified from DNA obtained from isolated strains and total DNA from water samples respectively. .

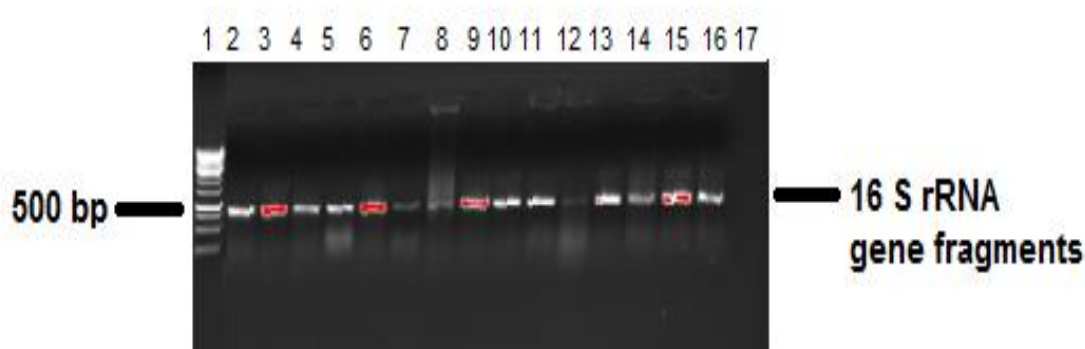


Figure 4.1: A representative picture of a 1% (w/v) agarose gel depicting bacterial 16S rRNA gene fragments amplified from isolates and total DNA from water samples respectively in the study. Lane 1=100 bp molecular weight marker; Lane 2

4.4 Bacterial 16S rRNA sequence analysis

Bacterial 16S rRNA gene fragments were sequenced by Inqaba Biotec (Pty) Ltd, Pretoria, South Africa and sequence data were used to determine the identities of the isolates. The bacterial sequences were aligned using Blast search and compared with sequences in the NCBI database. The data in Table 4.3 depicts the identities of isolates after blasting sequence data, the accession numbers of closely related strains or sequences and the percentage similarities of the amplified sequences to those in Genbank.

All the isolates obtained in the present study showed very close similarities (90% to 99%) to those found in Genbank. A large proportion of the rod shaped bacteria were identified as *Bacillus* species while the vibrio shaped were *Vibrio cholerae*. In addition, isolates were also related to *Exiguobacterium* species, *Pseudomonas* species and *Kurthia gibsonii*.

Table 4.3: Organisms as identified based on bacterial 16S rRNA gene sequence analysis using BLAST search.

Site	Isolate ID	Identification of isolates based on 16S rRNA gene sequence analysis	Accession number of closely related sequence and percentage similarity
A	GD2 (S5)	<i>Bacillus subtilis</i>	FJ864727, 96%
	GD6 (S18)	<i>Bacillus megaterium</i>	JX475126, 90%
	GD9 (S8/S19)	<i>Bacillus subtilis</i>	FJ544346, 98%
	GD19 (S12)	<i>Bacillus aryabhattai</i>	KC550562, 98%
	GD11 (S16)	<i>Bacillus megaterium</i>	GQ274925, 95%
	GD12 (S13)	<i>Exiguobacterium indicum</i>	JF935075, 96%
	GD18 (S15)	<i>Bacillus megaterium</i>	HF570085, 93%
B	DS1 (S2)	<i>Pseudomonas aeruginosa</i>	KJ101516, 99%
C	GS8 (S1)	<i>Bacillus subtilis</i>	EU373328, 94%
	GS4 (S17), GS6 (S7), GS12 (S11)	<i>Vibrio cholerae</i>	KJ413142, 90%, HM590223, 97%, DQ991212, 94% respectively
	GS10 (S9)	<i>Bacillus cereus</i>	KF478221, 90%
	GS7 (S14)	<i>Kurthia gibsonii</i>	KJ396180, 93%
	GS2 (S6)	<i>Exiguobacterium acetylicum</i>	KF963623, 94%
	GS11 (S10)	<i>Bacillus aryabhattai</i>	KJ186075, 95%
D	MD1 (S3)	<i>Pseudomonas</i> sp.	KF708909, 98%
	MD2 (S4)	<i>Pseudomonas aeruginosa</i>	CP006832, 95%

Table 4.4: The identities of bacteria species that were obtained through sequence analysis of 16S rRNA gene fragments that were amplified using total bacterial DNA from water samples

Site	Sample ID	Identification of bacteria based on 16S sequence analysis	Accession number of closely related sequence and percentage similarity
A	GDa20(S21)	<i>Aeromonas hydrophila</i>	KM013811, 80%,
	GDb22(S22)	<i>Aeromonas hydrophila</i>	KF183982, 87%
	GDb25(S27)	<i>Aeromonas hydrophila</i>	KF681381, 94%
	GDa27 (S25)	Uncultured bacterium clone	JF709658, 96%
B	DSa7 (S20)	<i>Enterococcus</i> sp.	JX471098, 98%
	DSa15 (S26)	<i>Bacillus anthracis</i>	CP008854, 100%
C	GSa17 (S23)	<i>Morganella morganii</i>	KM222639, 88%
	GSb20 (S28)	<i>Serratia marcescens</i>	KC435001, 91%
D	MDa10 (S24)	<i>Bacillus thuringiensis</i>	CP005935, 76%
	MDb15 (S29)	Uncultured bacterium clone	DQ807646, 87%

4.5 Genetic relatedness of bacteria isolates

The results obtained from the Tables 4.3 and 4.4 were used to draw phylogenetic trees. Three phylogenetic trees were generated since three different universal primers were used in the 16S rRNA amplification procedure. The phylogenetic trees are presented in Figure 4.2, 4.3 and 4.4 respectively.

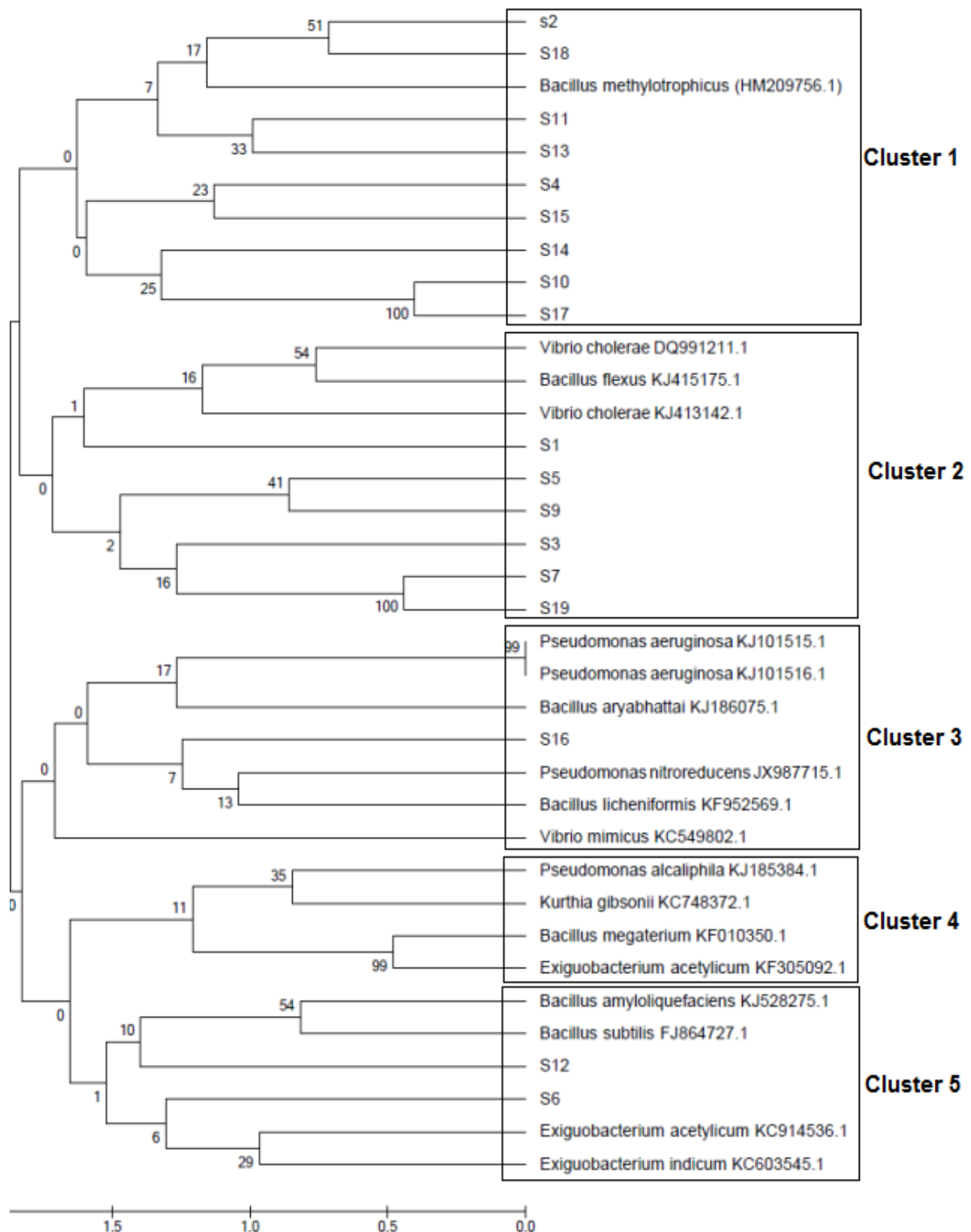


Figure 4.2: Phylogenetic relationship of the isolates to database bacteria

The evolutionary history was inferred using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and this produced an optimal tree with 44.02349177 as the sum of branch length. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test

(1000 replicates) is shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 178 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

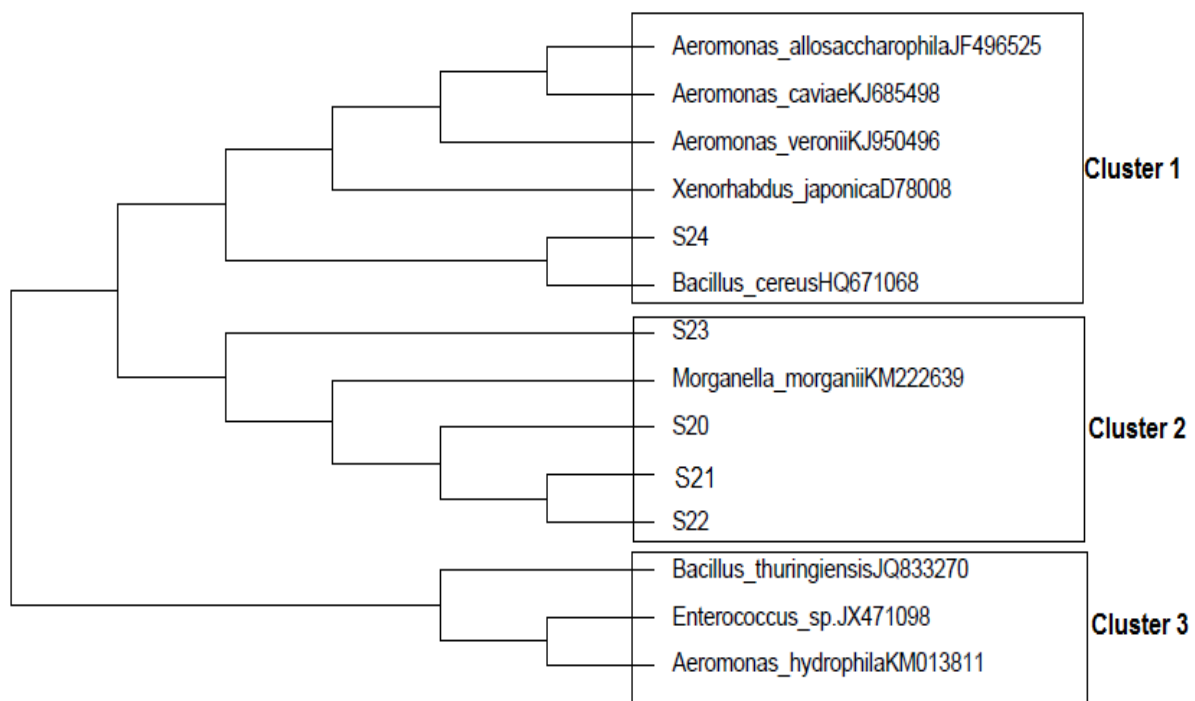


Figure 4.3: Phylogenetic relationship of the bacterial species from total DNA bacterial strains in the database

The evolutionary history was inferred using the UPGMA method resulting in the optimal tree with the sum of branch length = 16.16673765. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis involved 14 nucleotide sequences. Codon positions included were

1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 347 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

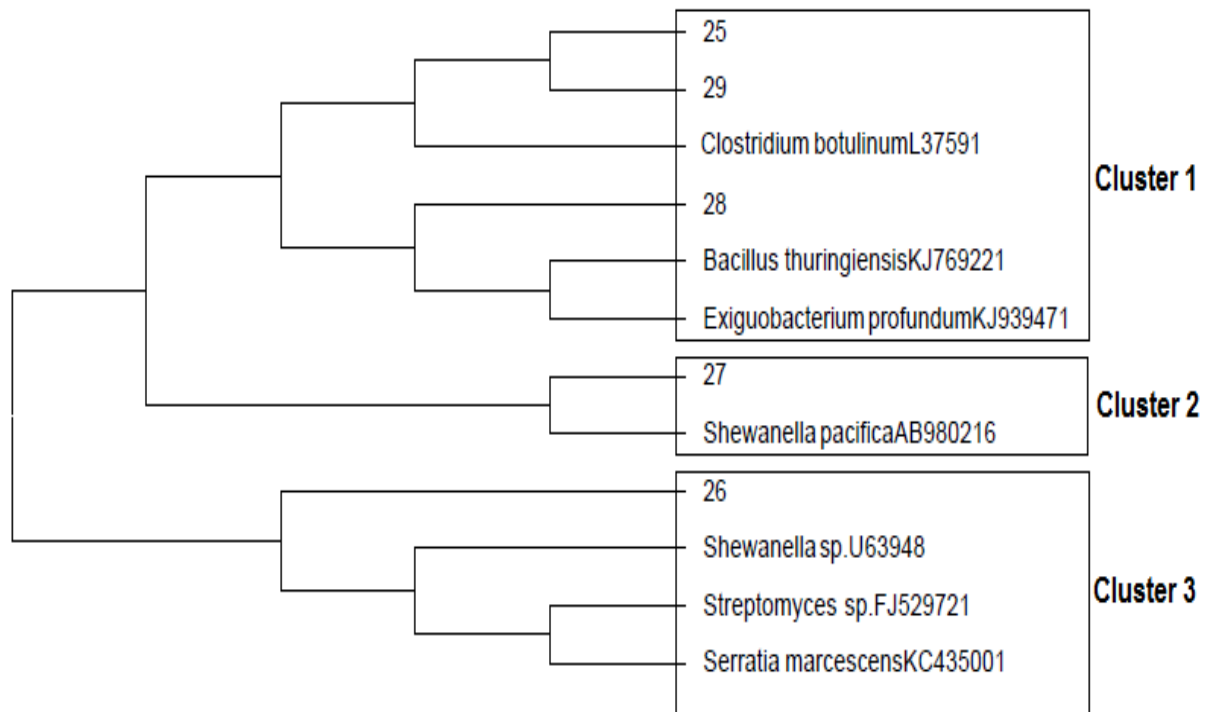


Figure 4.4: Phylogenetic relationship of the bacterial species from total DNA bacterial strains in the database

The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 347 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

4.6 Hydrocarbon Degradation Experiments

4.6.1 Standardization

Following inoculum preparation of the organisms, that is *Aeromonas hydrophila*(Ah), *Pseudomonas aeruginosa* (Pa) and *Serratia liquefaciens* (Sl), each organism was grown in Mineral Salt Medium (MSM) enriched with 100 μ l (1.0 μ g/ml) of each of the chemical compounds under investigation, which are a mixture of PAHs and PCB Aroclors 1242, 1248, 1260. After 24 hours incubation at pH 7 and 35°C, spectral scan was carried out using a UV-VIS spectrophotometer and results were as shown in Figures 4.5 and 4.6. A full scan was performed at a wavelength range of 200 – 800 nm and absorbance from 0.0 to 10.0.

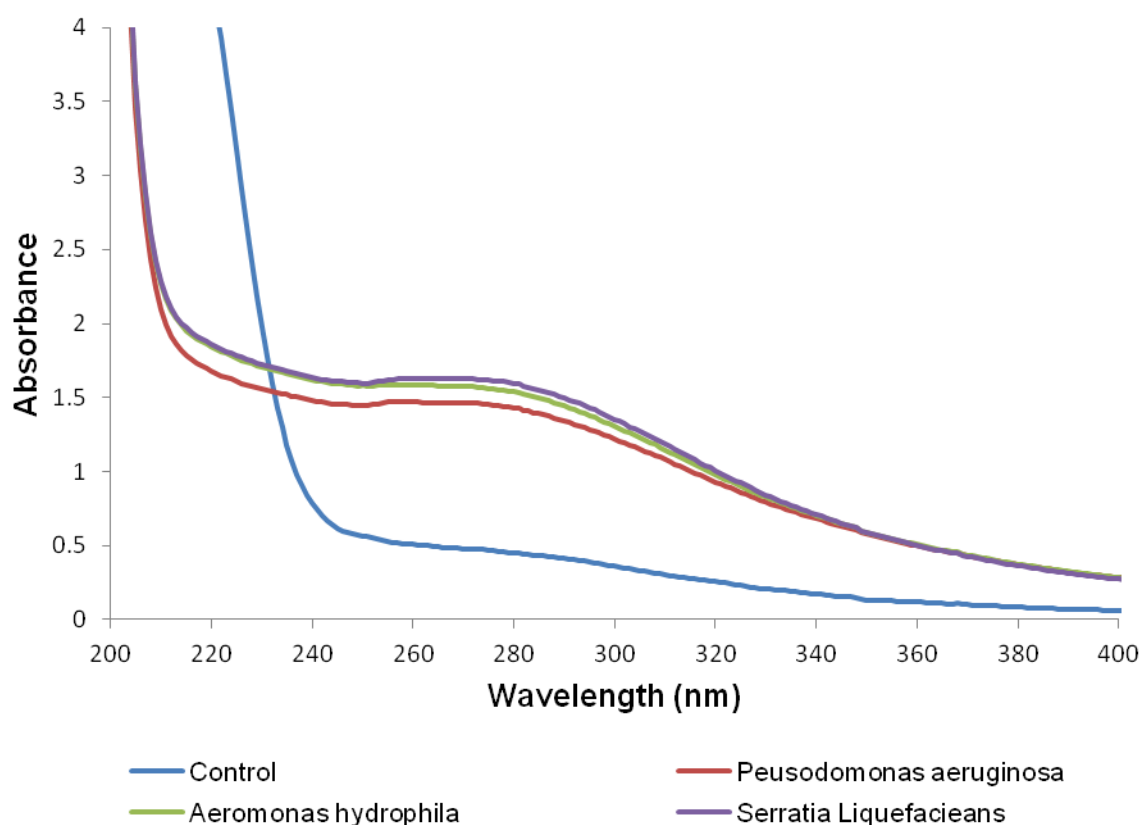


Figure 4.5: Spectral scan for Standardization test for individual organism's action on PAHs.

The test organisms were also each grown in MSM enriched with one of the PCB Aroclors, 1242, 1248 and 1260, used in the study at pH 7 and 35°C. The last two numbers represent the percentage of chlorine present in each PCB Aroclor.

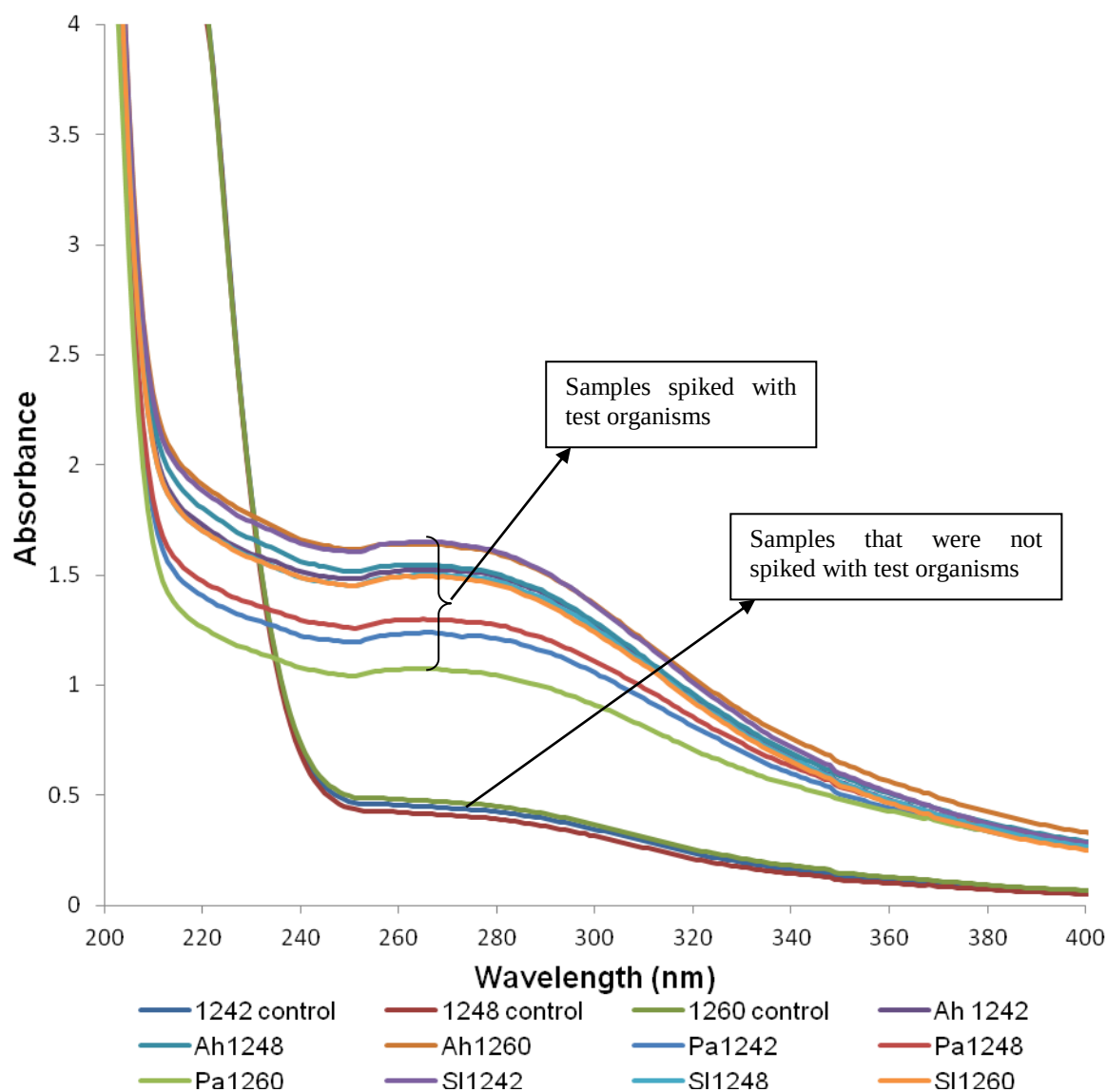


Figure 4.6: Spectral scan for the standardization test to evaluate action on PCBs Aroclors 1242, 1248 and 1260 by the individual organisms.

Confirmation on growth of the organisms in the presence of the compounds was re-confirmed by streaking a loopfull of the samples from each of the experiment and control flasks after 24 hours of incubation on TSA and sheep blood agar plates. The plates were incubated at 35°C and growth of the cultures was determined after 18 hours of incubation. The control samples were not spiked with test organisms. As shown in Figure 4.6 the absorbance which indicates the degree to which the organisms were able to breakdown the polychlorinated compounds was higher for the spiked when compared to the unspiked samples.

4.6.2 Degrading of Polycyclic Aromatic Hydrocarbons (PAHs) and Polychlorinated Biphenyls (PCBs) by mixed consortia of the test organisms

After standardization the three organisms were mixed into one culture and used for hydrocarbon assay. The samples were incubated for up to 96 hours at pH 7 and 35°C. However, after 48 hours no absorbance was obtained indicating that the organisms had used up all the hydrocarbons in the samples. Results are shown in Figure 4.7.

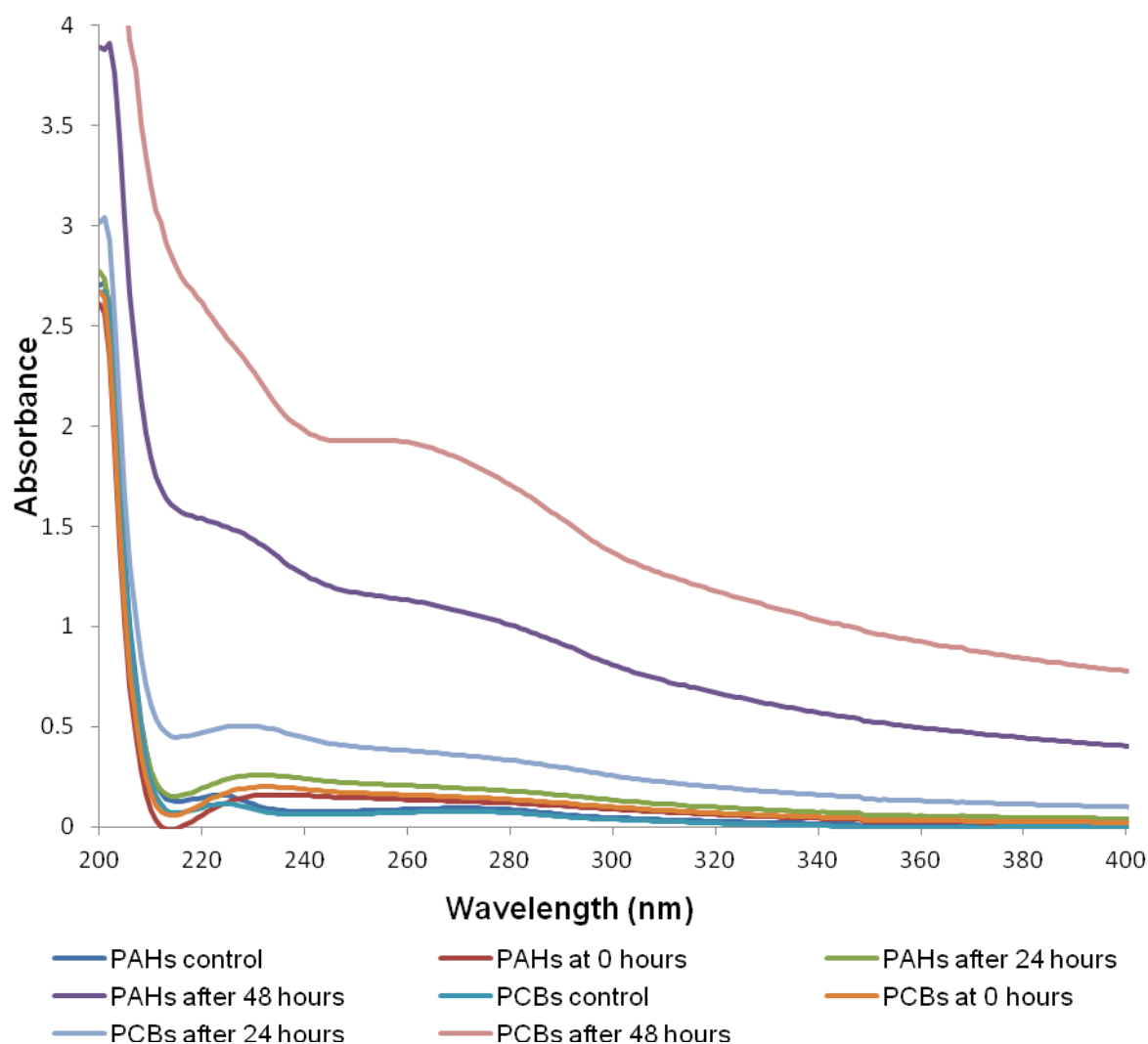


Figure 4.7: UV-vis absorbance indicating the effect of test bacterial consortia on PAHs and PCBs in MSM

4.6.3 Effect of pH and Temperature on PAH/PCB degradation

Mixed bacteria consortia was grown in MSM enriched with PAHs and PCBs and incubated under pH 5, 7, 8 and 9 at 30°C for 96 hours. The same set-up was repeated with different temperature treatments at pH 7.0. Readings were obtained at wavelength 200 – 800 nm and absorbance from 0.0 to 10.0. The results are presented in Figures 4.8a – h and 4.9a - c.

4.6.3.1 Effect of pH on PAHs and PCBs biodegradation by *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Serratia liquefacians*

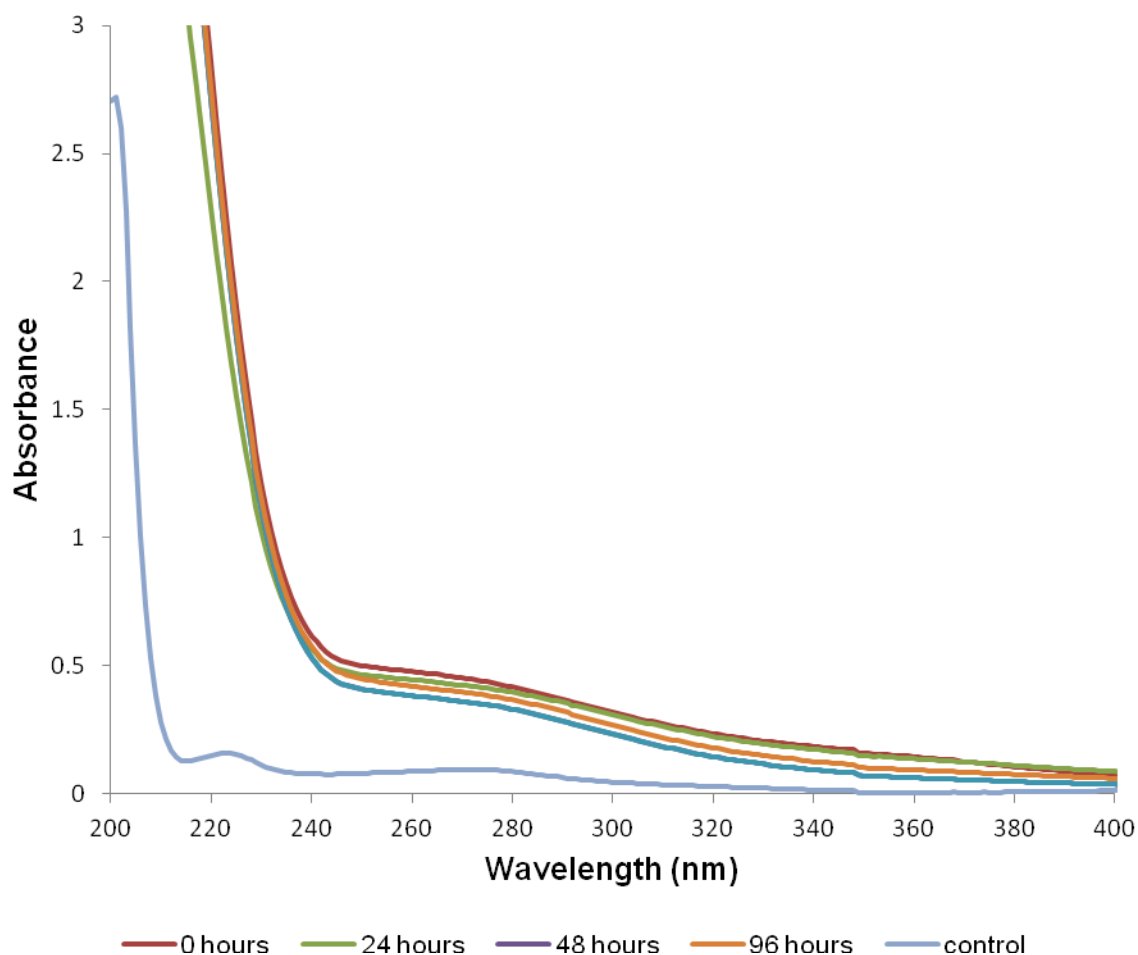


Figure 4.8a: Degradation of PAHs by mixed bacteria consortia in MSM at pH 5

The chromatogram obtained for the experiments when compared to the control indicates a shift in λ_{max} from about 230 nm to about 260.

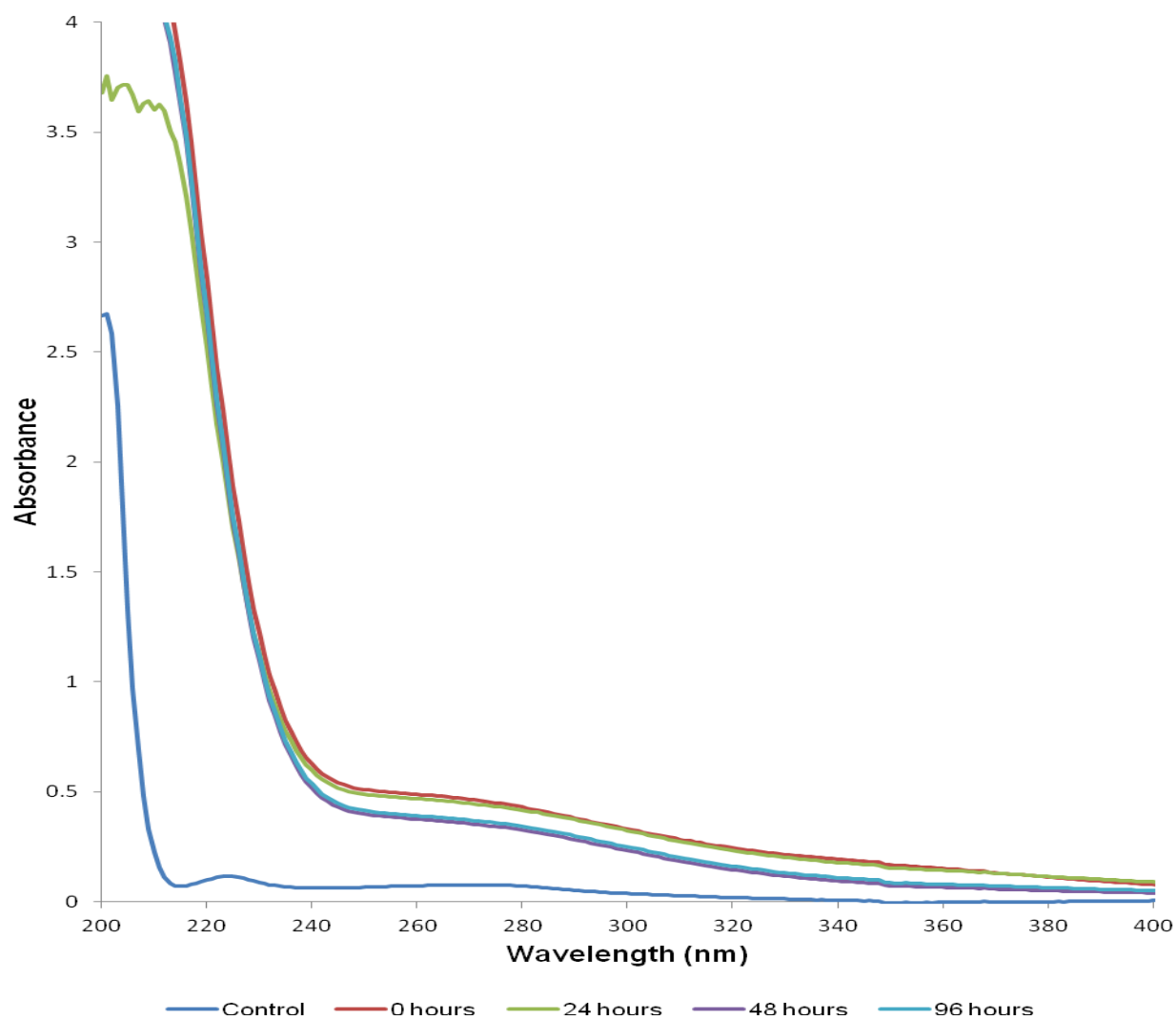


Figure4.8b: Degradation of PCBs by mixed bacteria consortia in MSM at pH 5.

The chromatogram obtained for the experiments when compared to the control indicates a shift in $\lambda_{\max}$ from about 230 nm to about 254 to 264 nm.

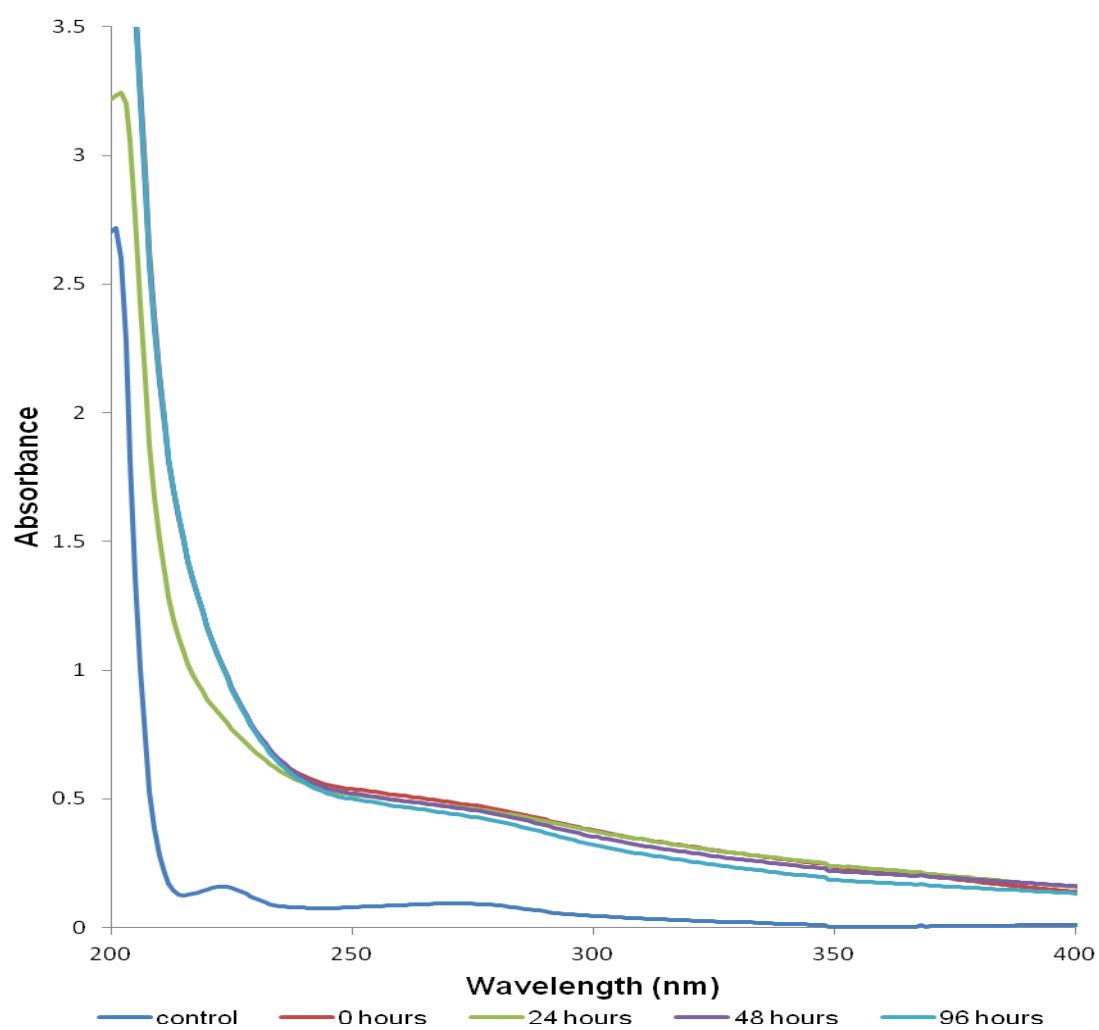


Figure 4.8c: Degradation of PAHs by mixed bacteria consortia in MSM at pH 7

The chromatogram obtained for the experiments when compared to the control indicates a shift in $\lambda_{\max}$ from about 230 nm to about 254 nm.

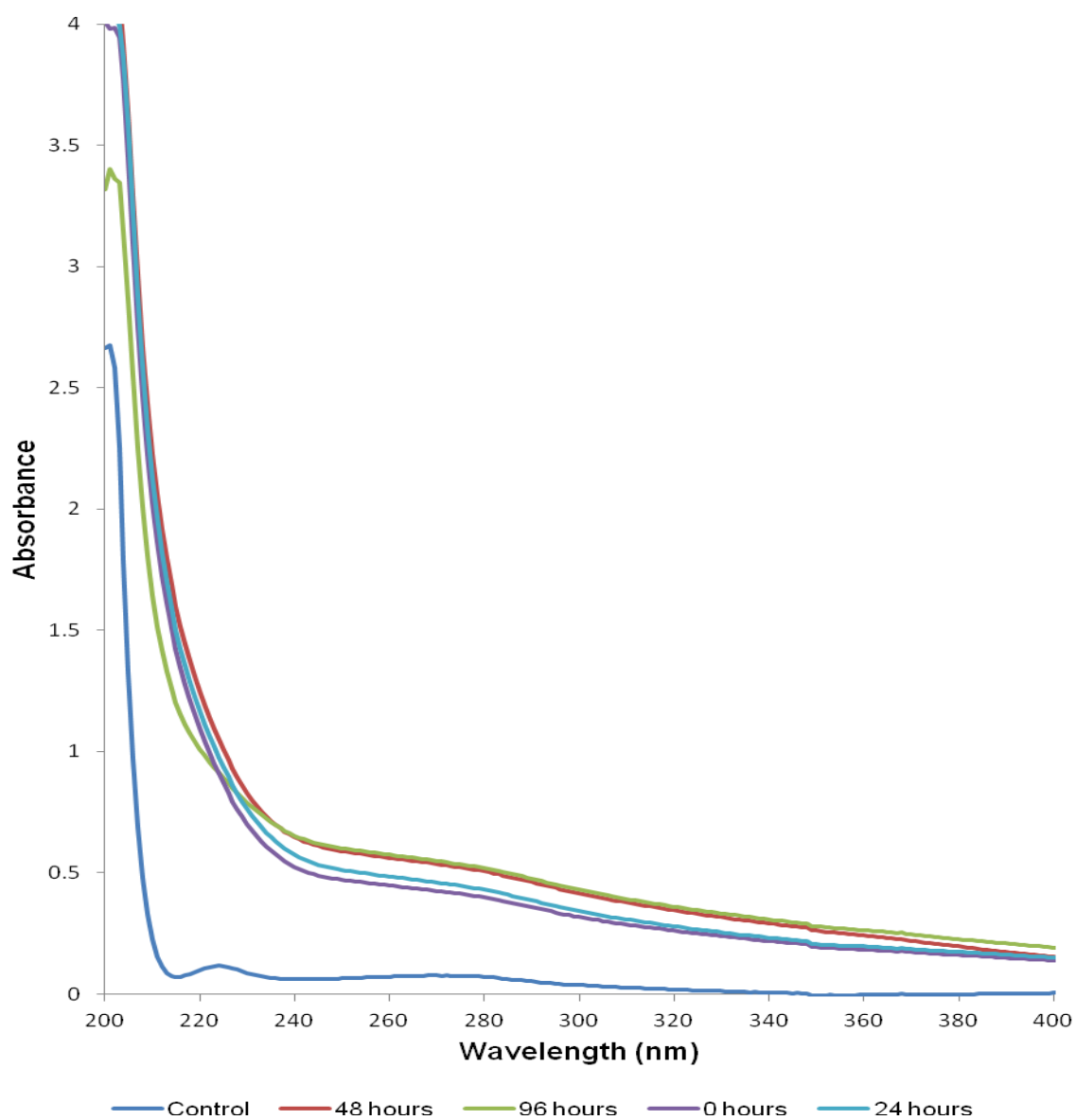


Figure 4.8d: Degradation of PCBs by mixed bacteria consortia in MSM at pH 7.

The chromatogram obtained for the experiments when compared to the control indicates a shift in $\lambda_{\max}$ from about 230 nm to about 264 nm.

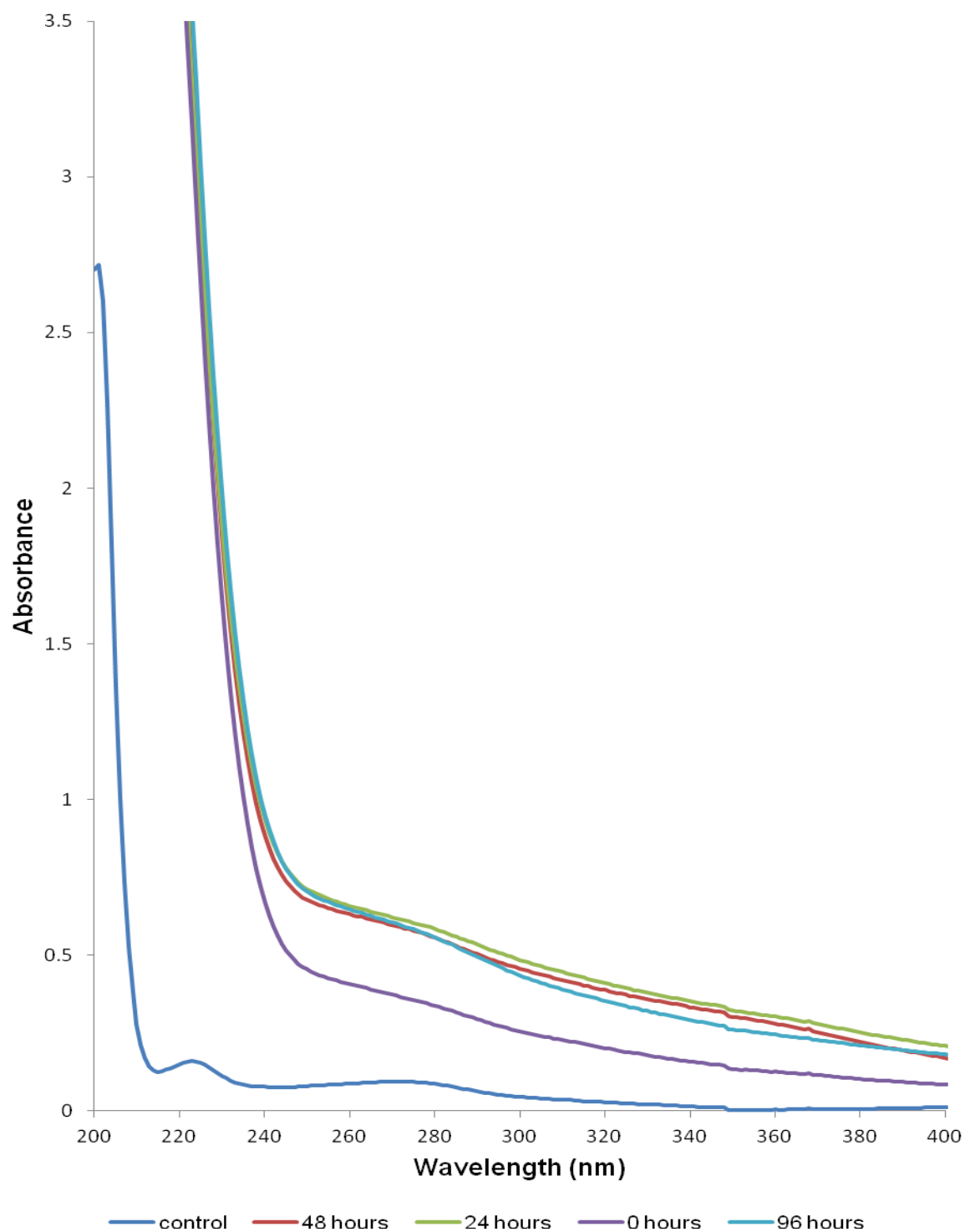


Figure 4.8e: Degradation of PAHs by mixed bacteria consortia in MSM at pH 8.

The chromatogram obtained for the experiments when compared to the control indicates a shift in $\lambda_{\max}$ from about 230 nm to about 254 to 264 nm.

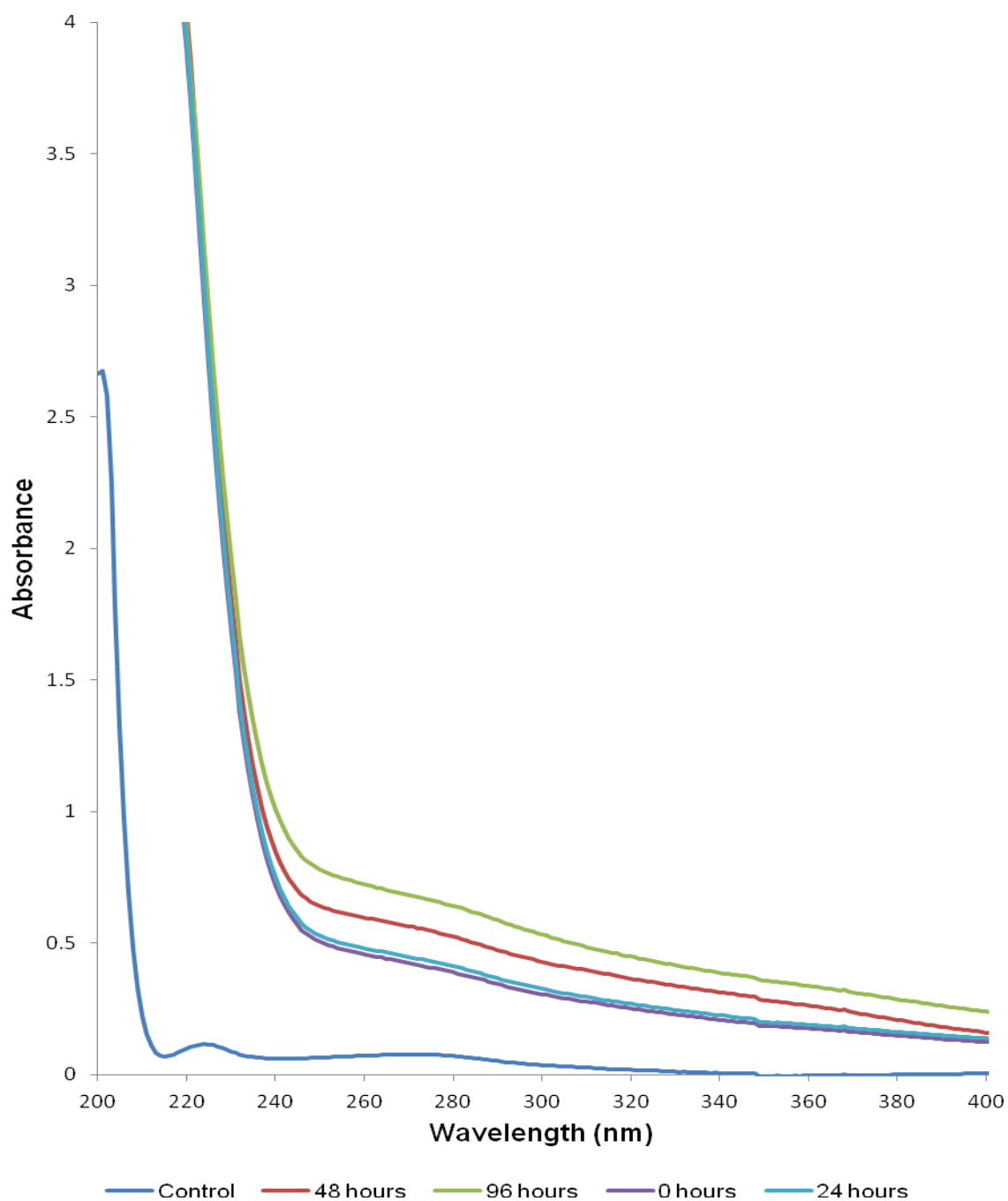


Figure 4.8f: Degradation of PCBs by mixed bacteria consortia in MSM at pH 8.

The chromatogram obtained for the experiments when compared to the control indicates a shift in $\lambda_{\max}$ from about 230 nm to about 264 nm.

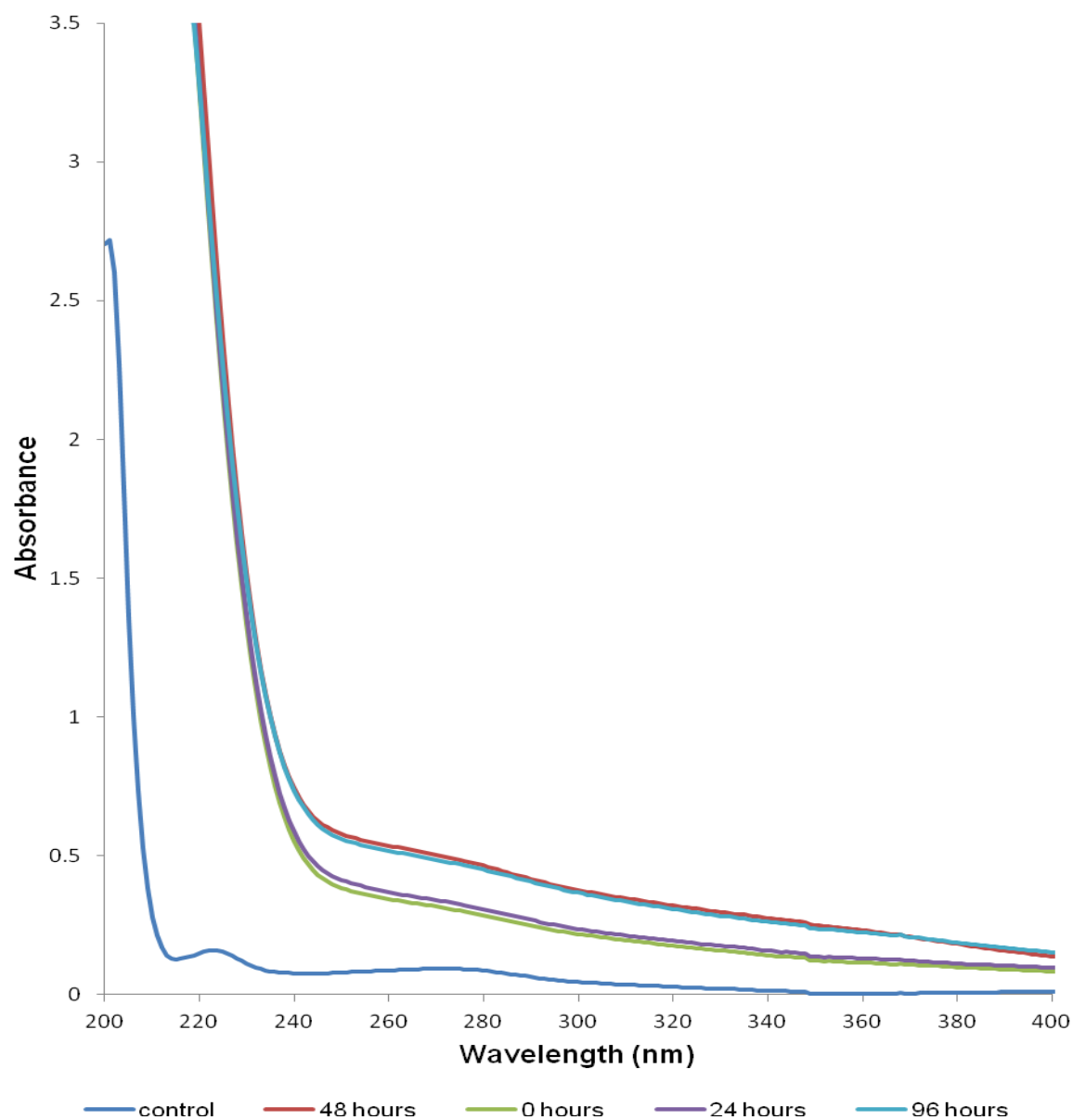


Figure 4.8g: Degradation of PAHs by mixed bacteria consortia in MSM at pH 9.

The chromatogram obtained for the experiments when compared to the control indicates a shift in $\lambda_{\max}$ from about 230 nm to about 254 to 264 nm.

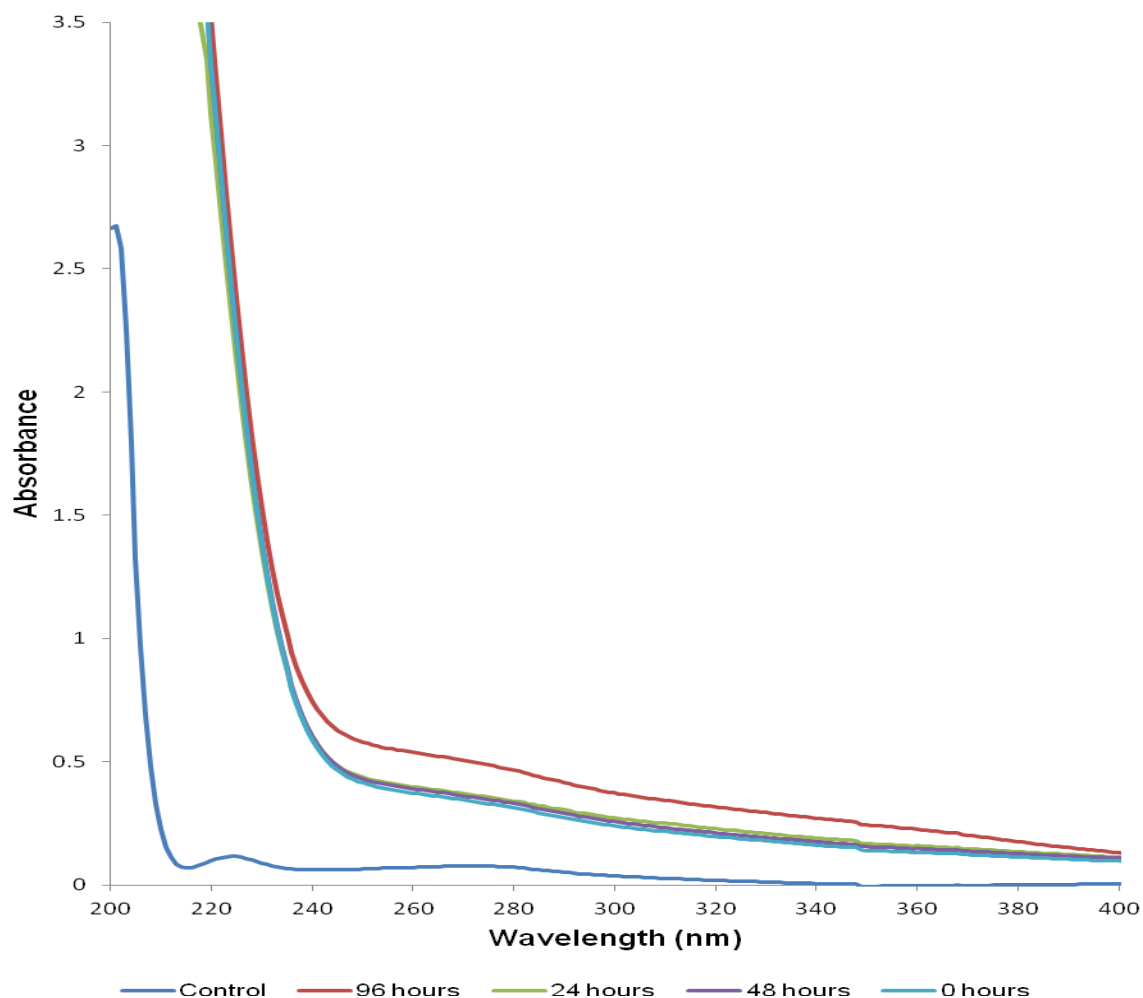


Figure 4.8h: Degradation of PCBs by mixed bacteria consortia in MSM at pH 9.

The chromatogram obtained for the experiments when compared to the control indicates a shift in $\lambda_{\max}$ from about 230 nm to about 264 nm.

The degradation of PAHs and PCBs is an important remediation process and its success depends on the optimal condition for the degrading isolates. Despite the fact that pH also has been found to have an important effect on biodegradation of these polychlorinated compounds results obtained in the current study revealed that there was no relationship between the biodegradation of the compounds with increase in pH.

4.6.3.2 Effect of temperature on PAHs and PCBs biodegradation by *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Serratia liquefacians*.

The readings were obtained at wavelength range of 200 to 800 nm and absorbance read from 0.0 to 10.0. The results obtained were as shown in Figures 4.9a –c.

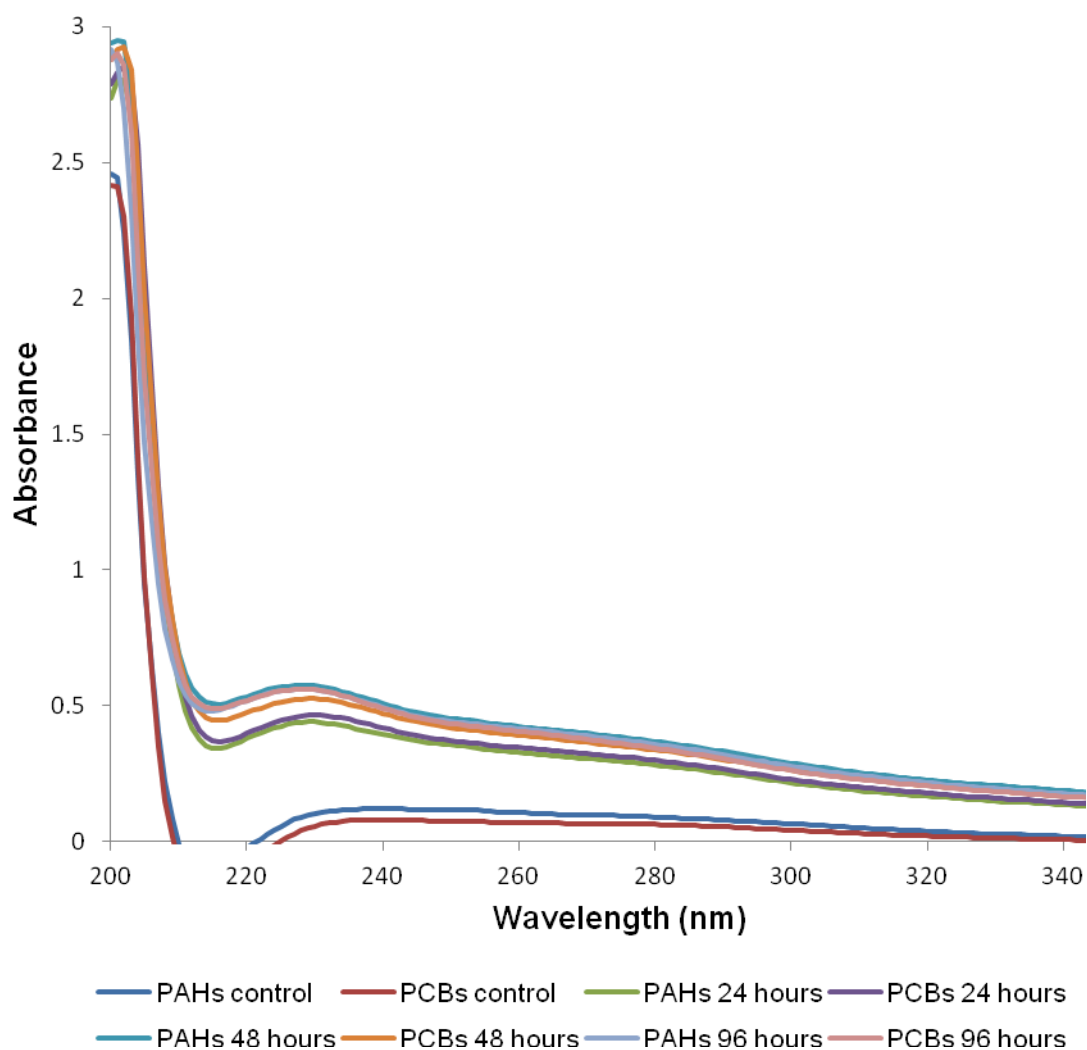


Figure 4.9a: Spectral Changes of PCBs and PAHs in MSM inoculated with the three test organisms (*Pseudomonas*, *Serratia* and *Aeromonas* species) and incubation at 20°C for 96 hours.

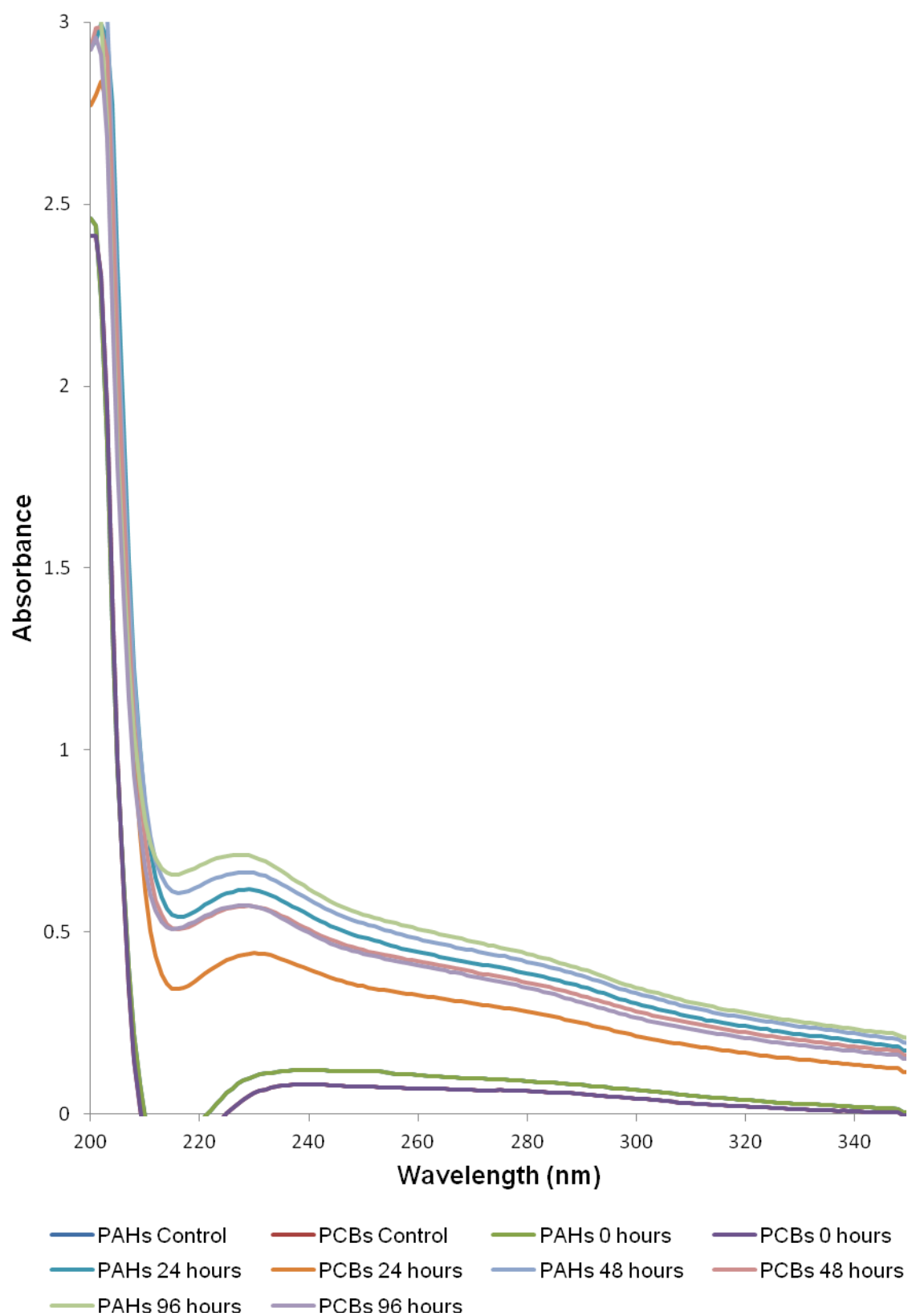


Figure 4.9b: Spectral Changes of PCBs and PAHs in MSM inoculated with the three test organisms (*Pseudomonas*, *Serratia* and *Aeromonas* species) and incubated at 27°C for 96 hours.

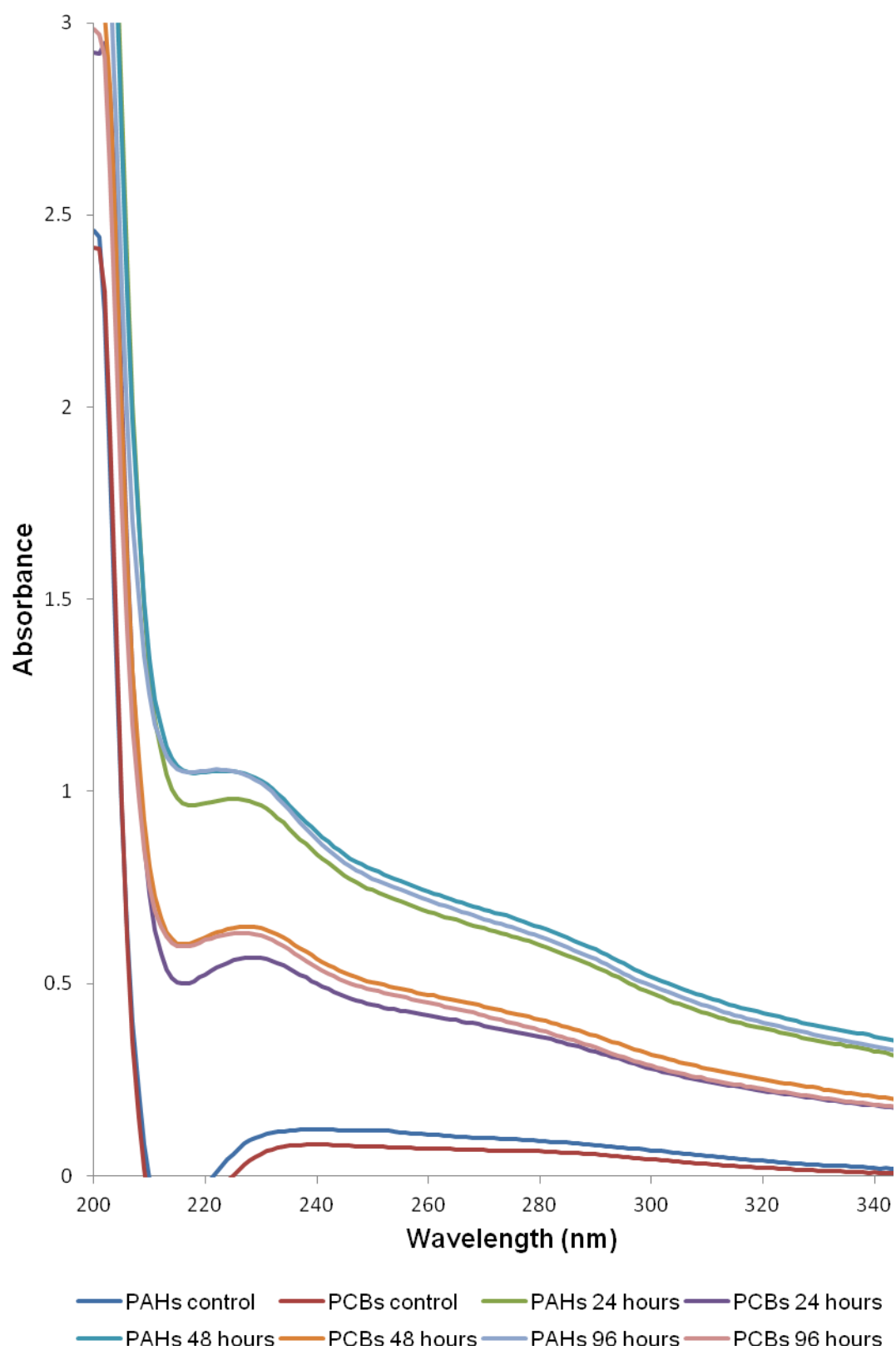


Figure 4.9c: Spectral Changes of PCBs and PAHs in MSM inoculated with the three test organisms (*Pseudomonas*, *Serratia* and *Aeromonas* species) and incubated at 35°C for 96 hours.

4.6.4 PAHs and PCBs biodegradation in wastewater

Samples of wastewater from sampling sites C and D were used in this study to find out the degree of biodegradation of the test compounds in wastewater un-inoculated and inoculated with the test organisms - *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Serratia liquefacians*. Spectral changes from the UV-VIS spectrophotometer were used and results presented in Figures 4.10a and 4.10b.

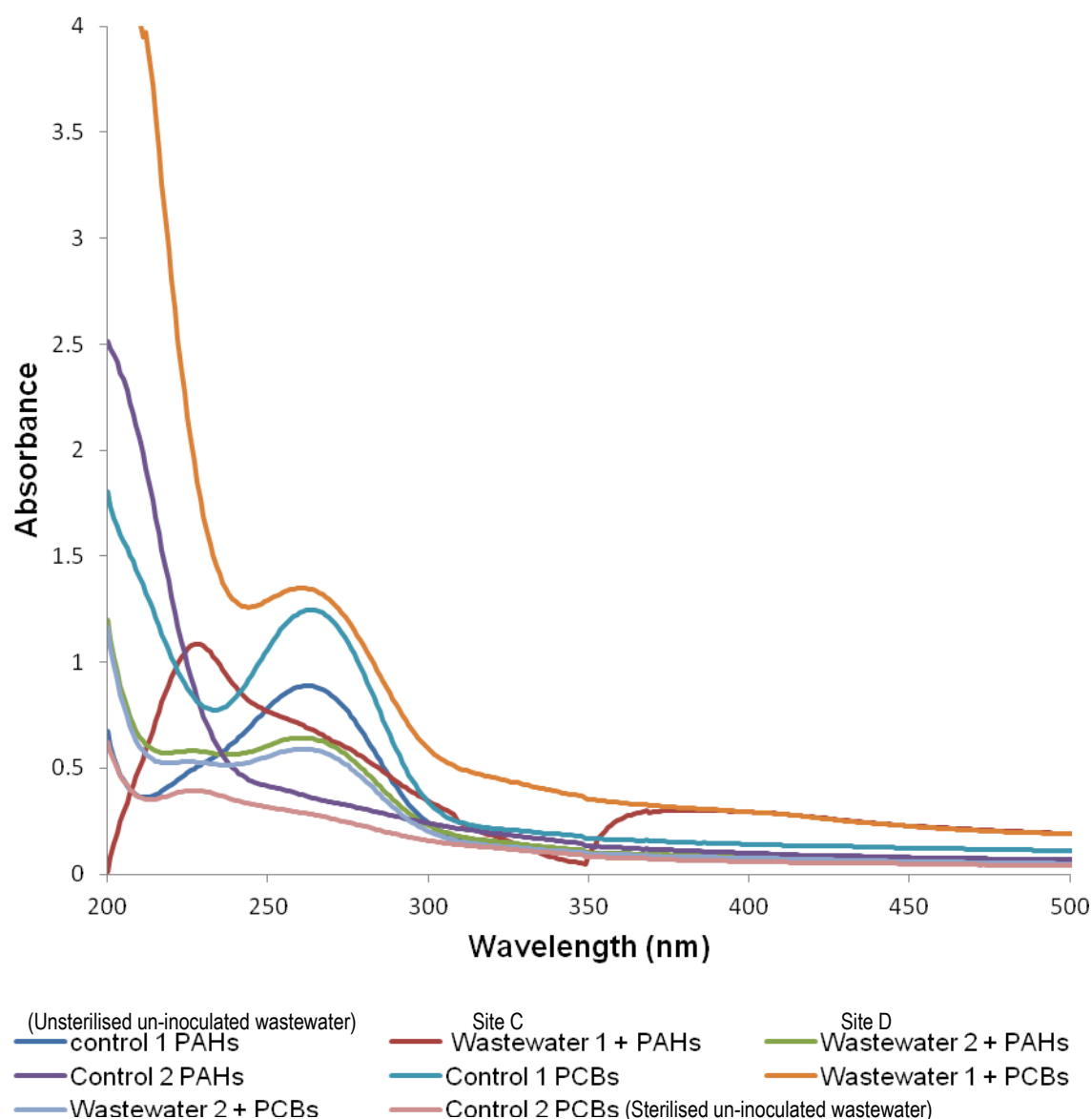


Figure 4.10a: Absorbance obtained after 24 hours of incubation of treated wastewater

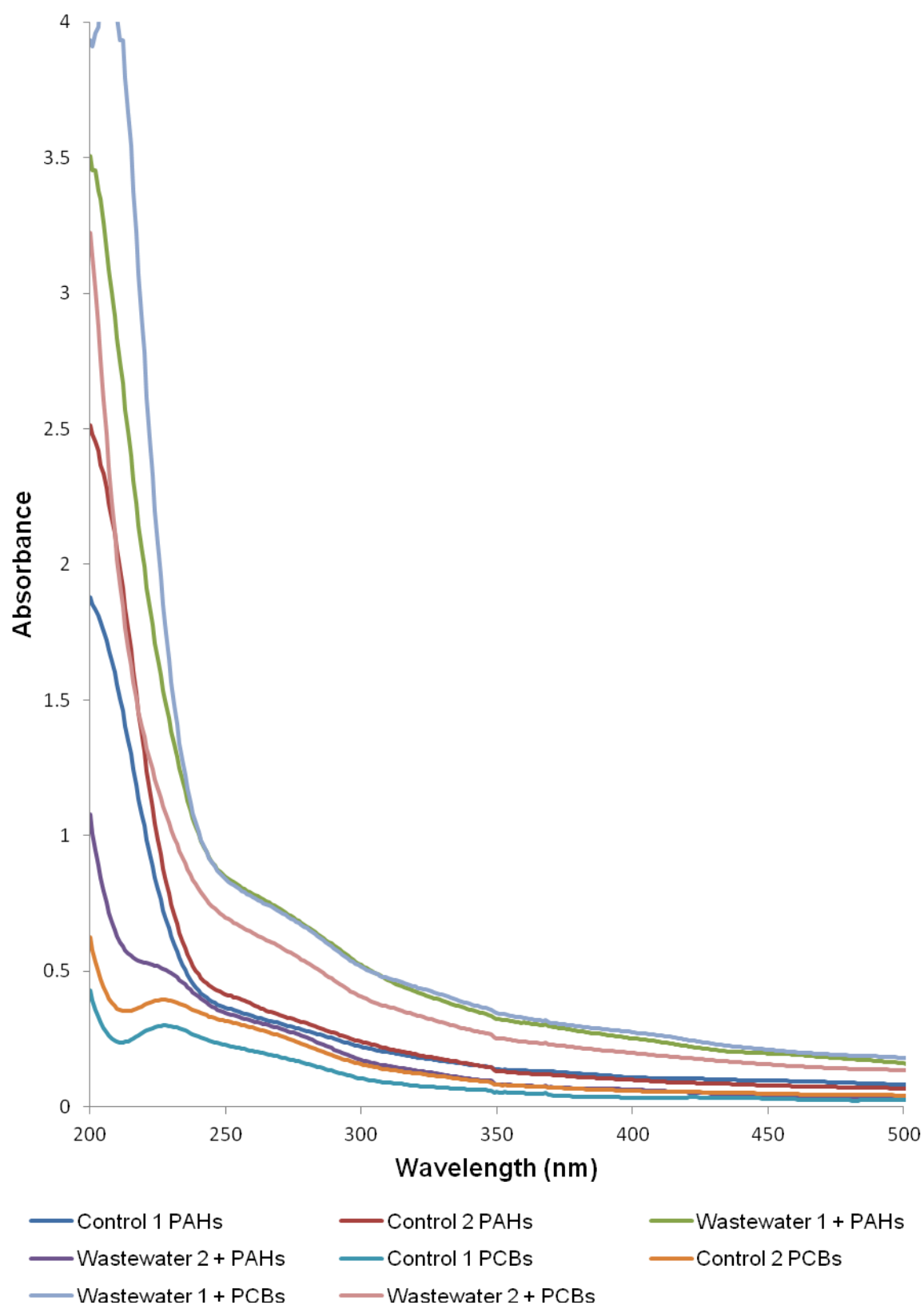


Figure 4.10b: Absorbance obtained after 96 hours of incubation of treated wastewater

4.6.5 Effect of time of incubation on the biodegradation of PAHs and PCBs

For this study a concentration of 1.0 µg/ml that is 100 µl of test compounds added to either wastewater or MSM was used to assess the effect of time on the biodegradation of PAHs and PCBs. . The organisms were grown in MSM at pH 7 and temperature of 35°C. Results are shown in Figures 4.11 and 4.12.

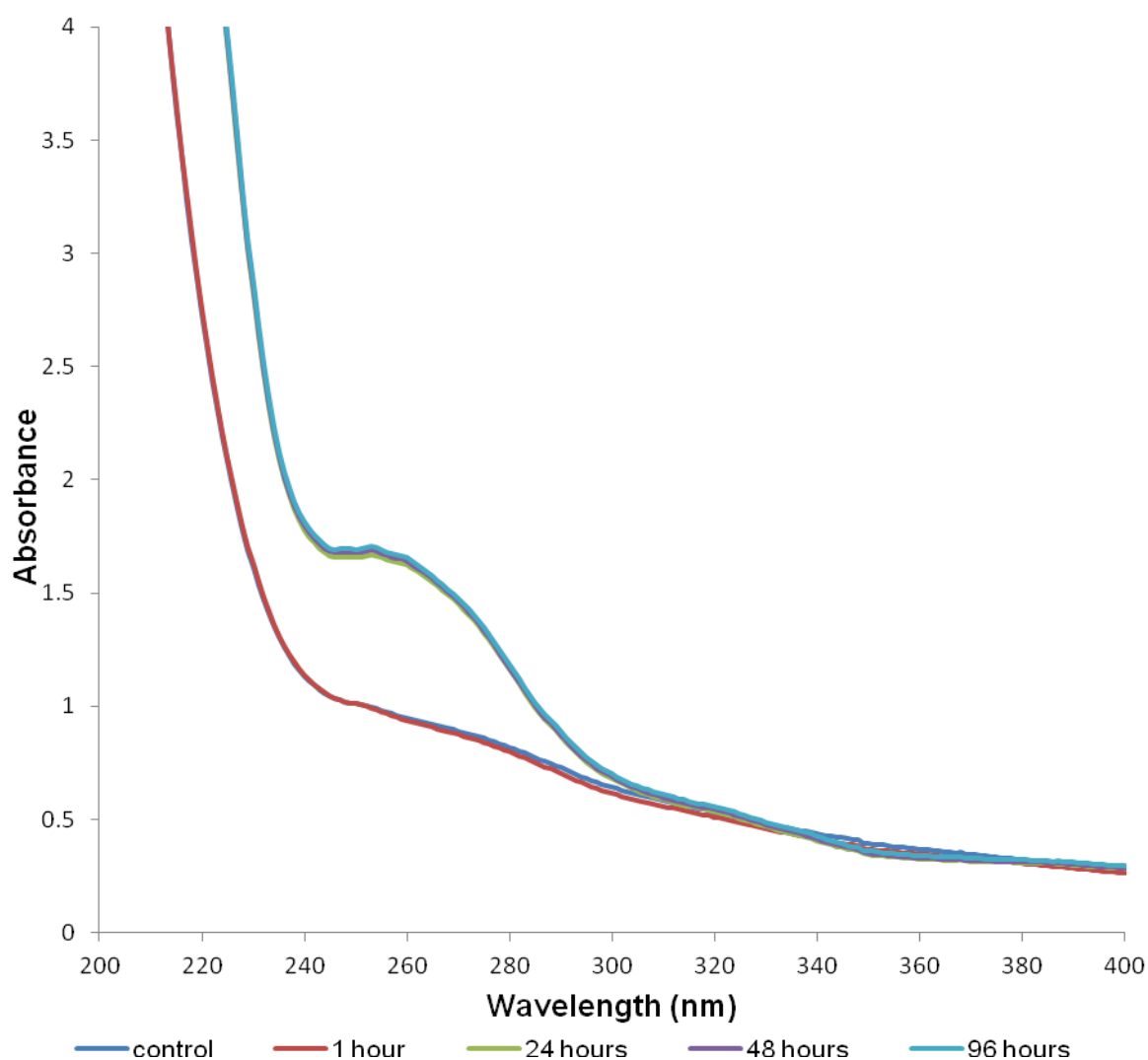


Figure 4.11: Effect of time on PAHs (0.25 µg/ml) degradation by mixed bacteria consortia

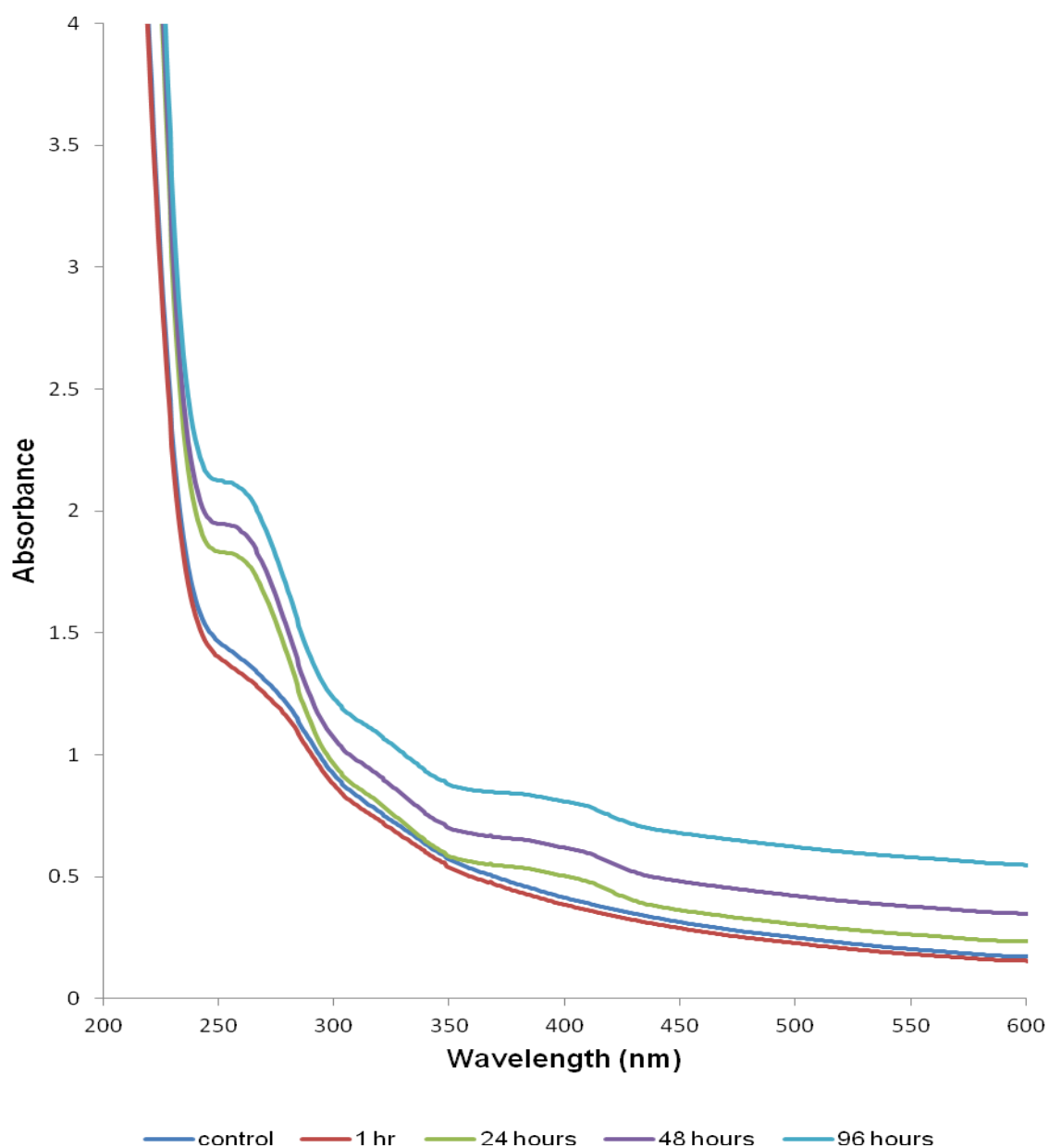


Figure 4.12: Effect of time on degradation PCBs Aroclors mixture (0.25 $\mu\text{g/ml}$) by mixed bacteria consortia

The results in Figure 4.11 and Figure 4.12 show that absorbance increased with time. This was an indication that there was degradation of the compounds by the bacteria.

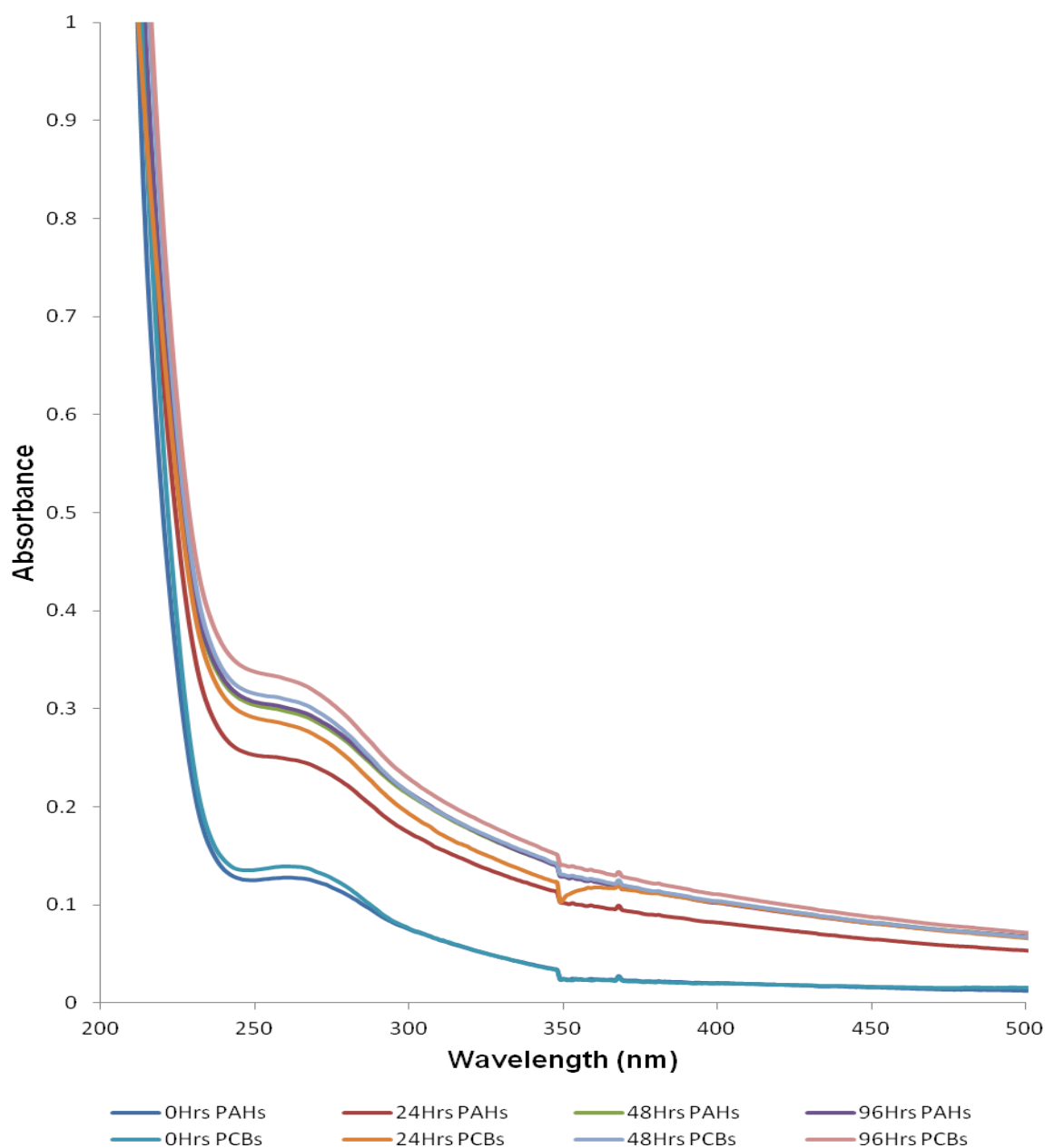


Figure 4.13: Spectral Changes of PCBs and PAHs degradation in MSM inoculated with isolate MD2

The results obtained after isolate MD2 was used in the experiment to analyse its ability to degrade the polychlorinated compounds showed an increase in absorbance with time for both PAHs and PCBs. This was an indication that, like the other commercially obtained strain of *Pseudomonas*, the isolate was also able to degrade the compounds.

4.6.6 Enzyme Activity of PAHs and PCBs

Enzyme assay was carried out using enzymes extracted from the mixed bacteria cells for the test bacteria used in this study. 1.5 ml of enzyme extract was mixed with 100 μ l of test compounds and each assay performed separately for 2 hours. Results taken by measuring absorbance through spectral observation from UV-VIS spectrophotometer are presented in Figures 4.14 and 4.15.

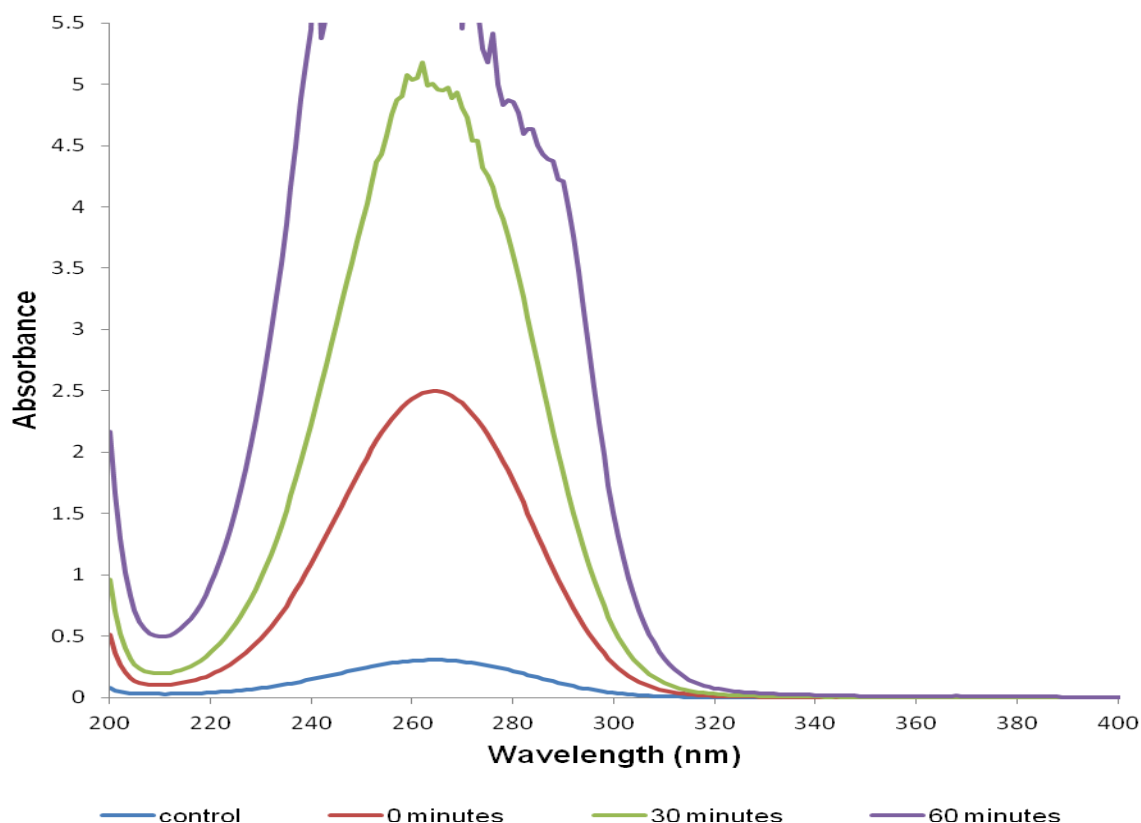


Figure 4.14: Enzyme kinetics on PCBs degradation over a 2-hour period

The results indicate that there was increase in absorbance as time increased. This is a clear indication that there was enzyme activity on the PCBs in this experiment. The bacterial enzymes were able to degrade PCBs at wavelength of about 262 nm. The action of enzymes on PCBs was faster compared to PAHs (Figure 4.15) as the recordable chromatogram was up to 60 minutes while for PAHs it went through to 90 minutes.

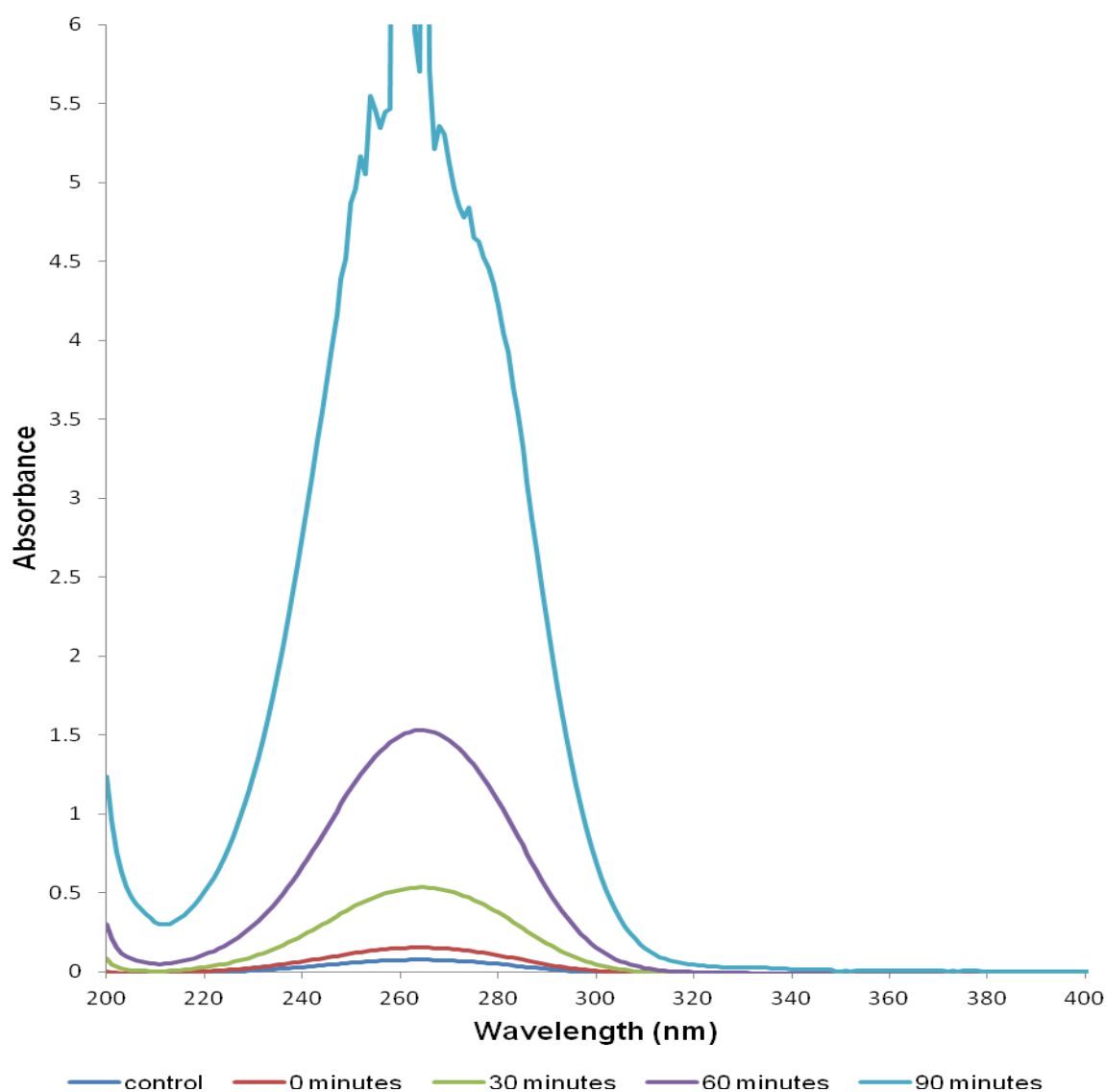


Figure 4.15: Enzyme kinetics on PAHs degradation over 2 hour period

The results indicate that there was increase in absorbance as time increased. This is a clear indication that there was enzyme activity on the PAHs in this experiment. The bacterial enzymes were able to degrade PAHs at wavelength of about 264 nm. After 90 minutes the chromatogram was no smooth an indication that absorbance could not be read clearly by the equipment, all the compounds were completely broken down.

4.6.7 High Performance Liquid Chromatography (HPLC) Results

The results obtained from HPLC showed that there was difference in the chromatogram obtained from the experiments, the controls and the standards. Although concentrations were not determined, the results indicated absence of some of the PAHs and PCBs after incubation, which is an indication that the bacteria degraded the compounds. The chromatogram obtained for the experiments were compared with the chromatogram for the standards using the retention times for the compounds in the standards. These results are presented in figures 4.16 to 4.19.

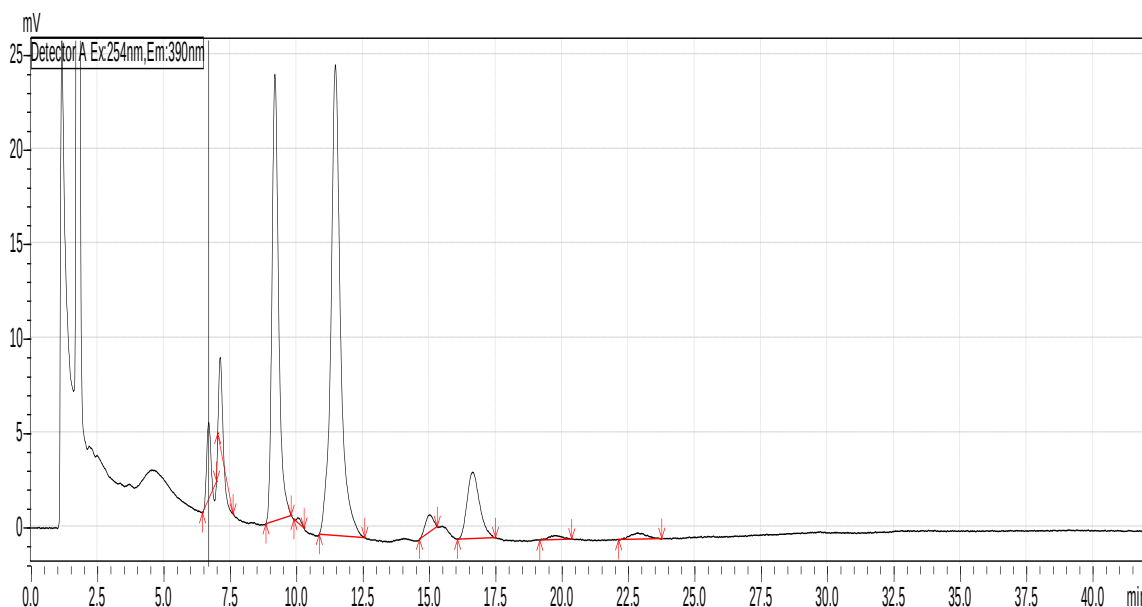


Figure 4.16: HPLC Chromatogram for PAHs standard using a Fluorescence detector method at 10 μ l injection volume

The compounds in the PAH standard were detected at times ranging from the 1st minute to the 25th minute. From the results it shows that the PAH standard was made up of fourteen (14) components out of sixteen (16) indicated by the supplier (Acenaphthene, Anthracene, Benzo[a]anthracene, Benzo[a]pyrene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Chrysene, Dibenzo[A,H]anthracene, Fluoranthene, Fluorene, Indeno [1,2,3-CD]pyrene, Phenanthrene and Pyrene. Acenaphthalene and Benzo[G,H,I,]perylene detection times are closer to the Acenaphthene and Dibenzo[A,H]anthracene respectively, and this might have led to their peaks being masked.

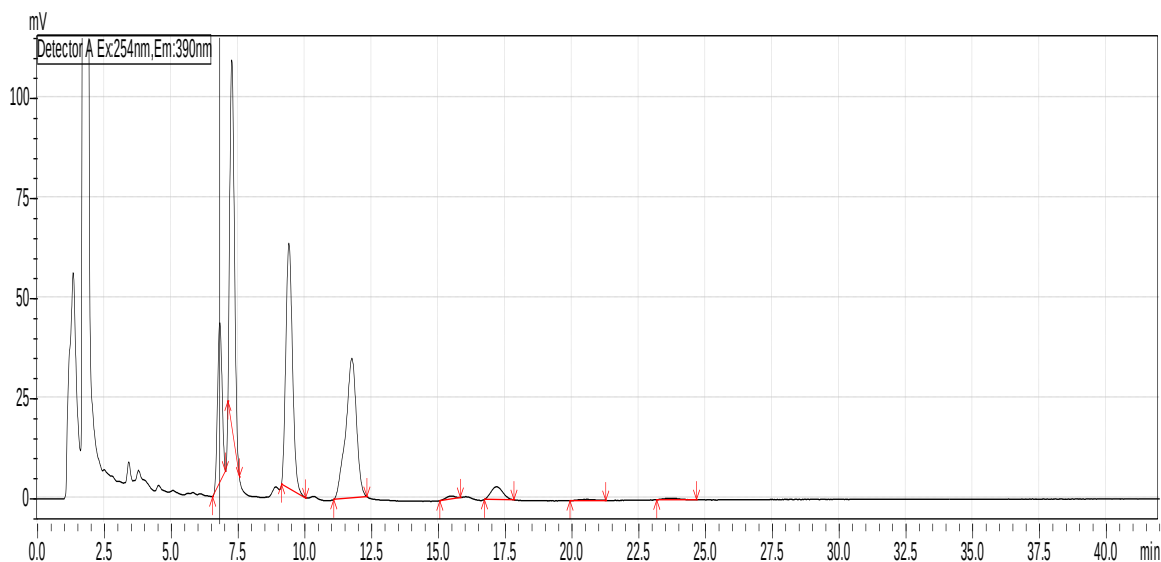


Figure 4.17: HPLC Chromatogram for Control for PAH degradation.

Sterilised Wastewater with PAHs added to it and incubated at the same time and conditions as the experiment. The chromatogram presents all the peaks at same detection time as was shown in Figure 4.16 for the standard. This is an indication that there was no bacteria to breakdown the PAHs proves that the breakdown of PAHs that occurred in the experiment set up (results in Figure 4.18 and Figure 4.19) was due to the test bacteria used in this study.

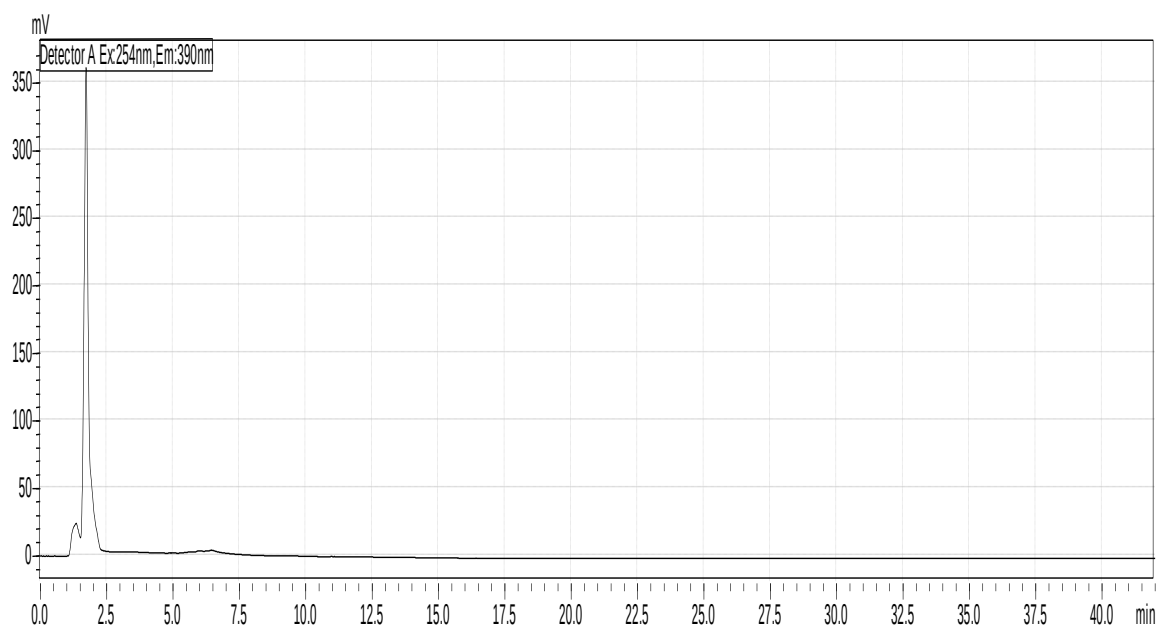


Figure 4.18: HPLC chromatogram for PAH degradation in wastewater experiment after 96 hours of incubation

The water was not sterilised and it was inoculated with the test organisms. There was no peak detected which indicates that all there was no compound that was detectable by the fluorescent light of the HPLC. All compounds were broken down in 5 days.

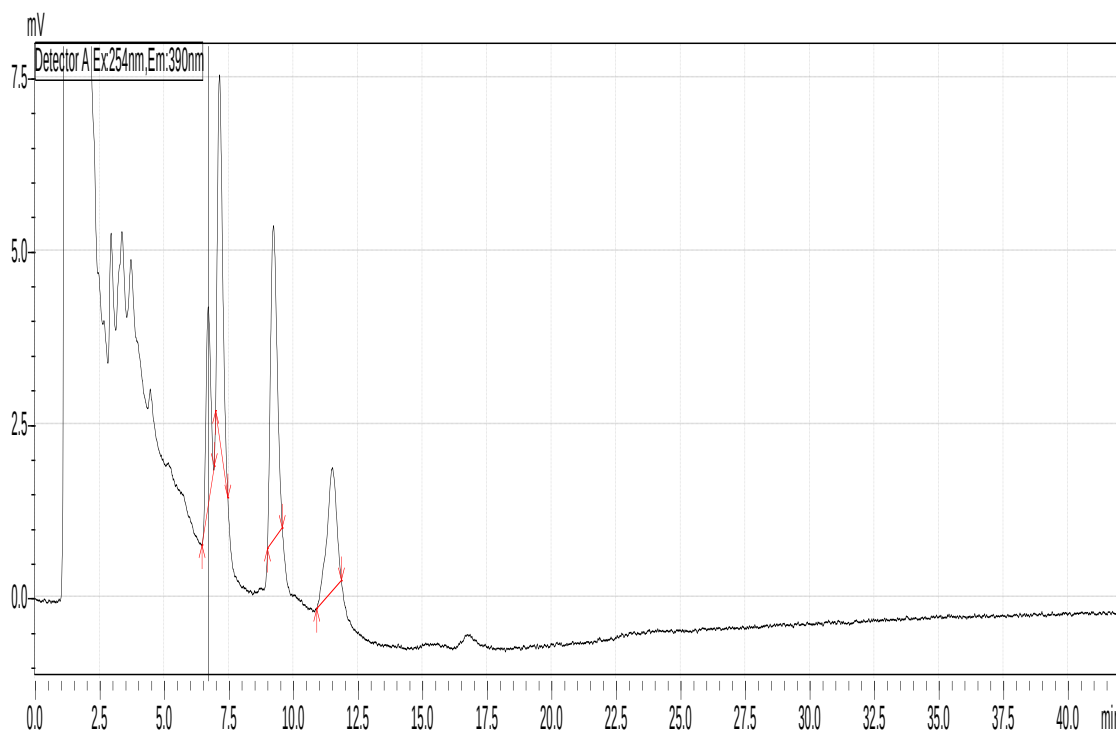


Figure 4.19: HPLC Chromatogram obtained after an experiment to analyse PAH degradation in wastewater.

The wastewater was sterilised and thereafter inoculated with the test organisms and PAHs added. The peaks shown in the chromatogram do not represent any of the compounds in the PAHs mixture. This is an indication that the compounds were broken down and the peaks are for resultant intermediate products after the biodegradation process.

The HPLC chromatogram results for PCBs are shown by Figure 4.20 – 4.23. The detection of the compounds was observed to occur between the 3rd and 15th minute. A total of ten (10) compounds (Figure 4.20 (a) and (b)) were detected by the equipment under the set conditions stated in the protocol.

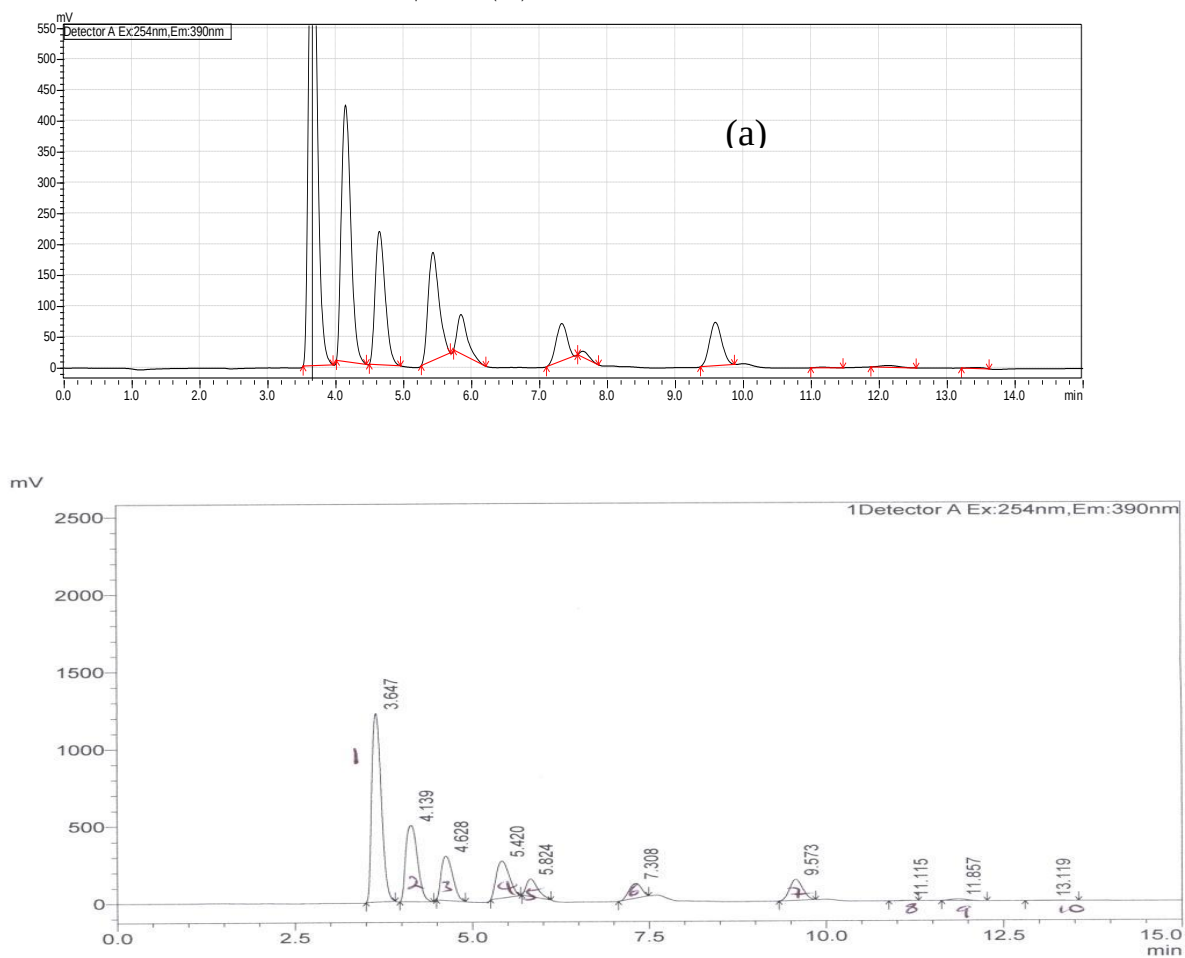


Figure 4.20 (a) and (b): HPLC Chromatogram for PCBs standard using a Fluorescence detector method at 10 µl injection volume

The use of the equipment in this study allowed separation of ten (10) different components of the commercial mixture of PCB standard in less than 20 minutes (Figure 4.20).

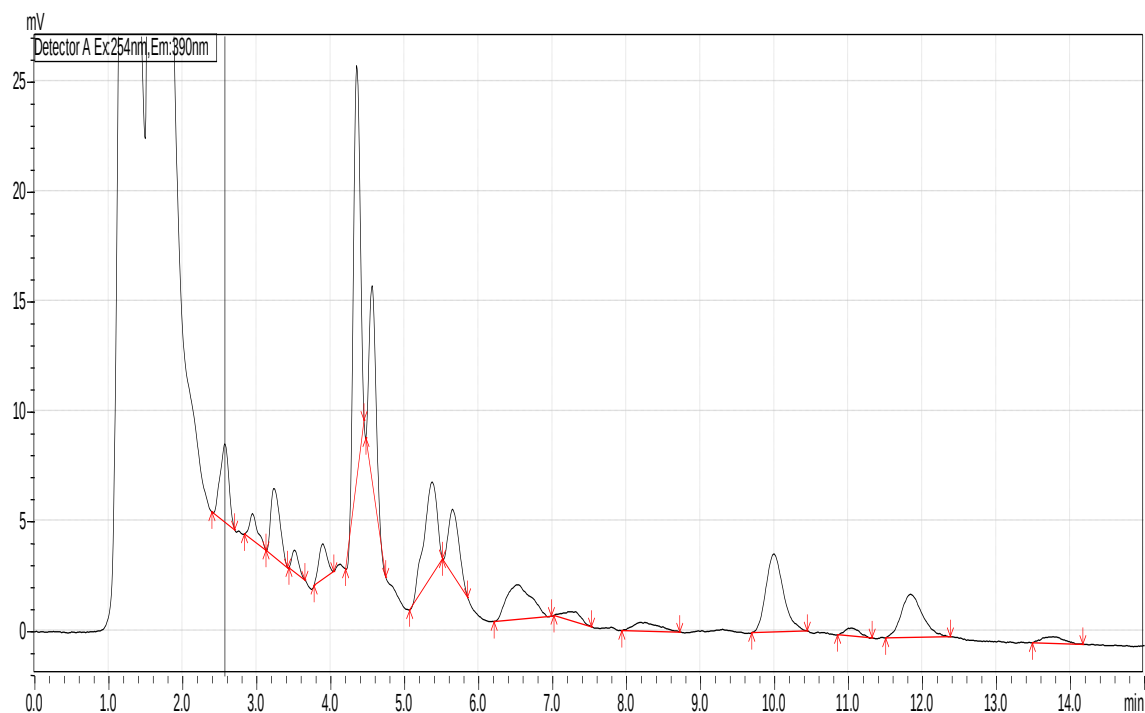


Figure 4.21: HPLC Chromatogram for Control for PCB degradation.

Sterilised Wastewater with PCBs added to it and incubated at the same time and conditions as the experiment. The chromatogram results are same as that for the standard (Figure 4.20 (a)). This is an indication that the PCBs were present and in same status as in the standard.

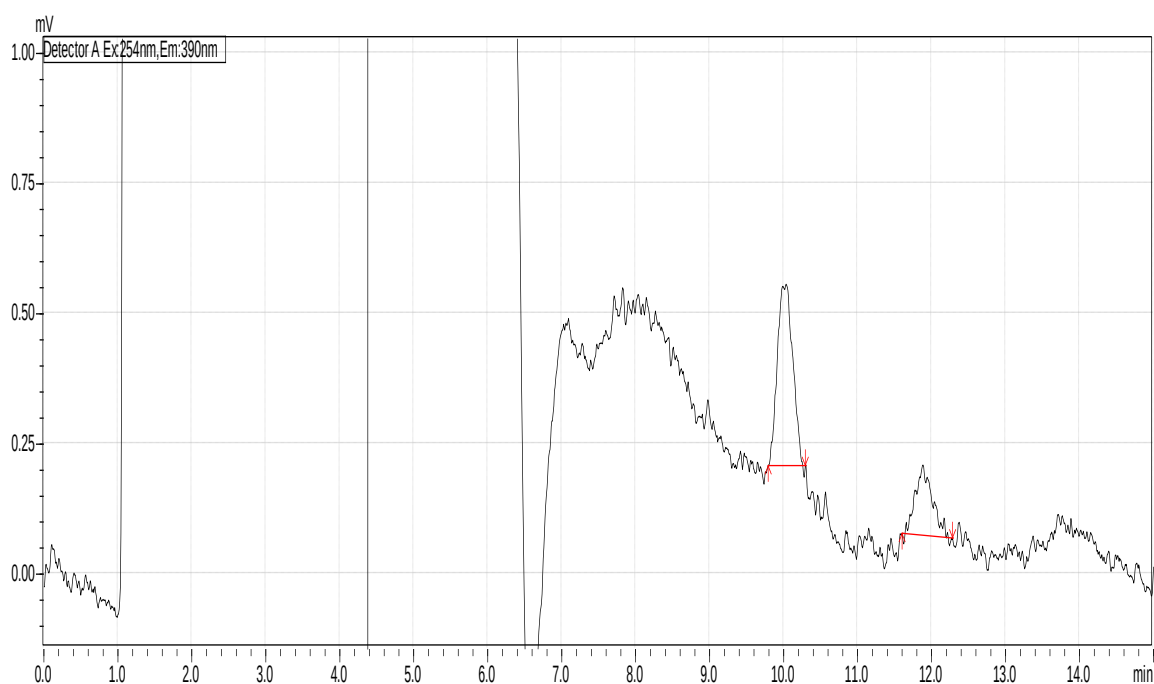


Figure 4.22: HPLC Chromatogram obtained after an experiment to analyse PCB degradation in wastewater.

The wastewater was sterilised and thereafter inoculated with the test organisms and PCBs mixture added. The peaks shown in the chromatogram in Figure 4.22 do not represent any of the compounds in the PCBs mixture which are shown in the chromatogram in Figure 4.20. This is an indication that the bacteria was able to breakdown the PCB compounds and the peaks are for resultant intermediate products after the biodegradation process.

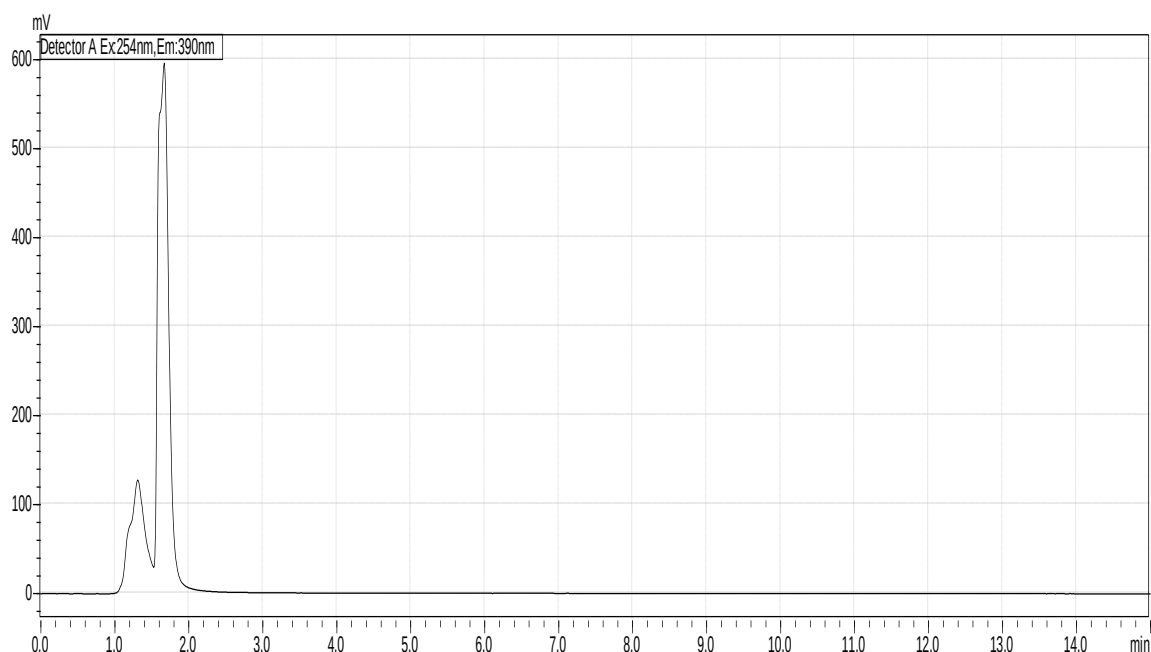


Figure 4.23: HPLC chromatogram for PCB biodegradation experiment after 96 hours of incubation.

The results for the experiment in which water sample used was not sterilised and test organisms (*Pseudomonas*, *Serratia* and *Aeromonas* species) were added to the water sample incubated at pH 7 and 35°C for 96 hours showed that there was no peak detected. This was an indication that all the PCBs were broken down. There was no compound that was detectable by the fluorescent light of the HPLC.

4.6.8 Analysis of Water samples for PAHs and PCBs using GC-MS

The results showed that the PAHs and PCBs were below the detection limit of the instrument used. This was confirmed through the use of standards which indicated that the equipment detection limit for the compounds was 17.1 ppb. This was obtained from the equipment detection limit using the results for the standard. The chromatogram from the standards is shown in Figure 4.24a, b, and c. The summarised results are presented in Table 4.5.

Table 4.5: PAHs and PCBs Screening results from the GC-MS

Sample	Sample Code	Aldrin (mg/kg)	Dieldrin (mg/kg)	4,4 DDD (mg/kg)	4,4 DDE (mg/kg)	4,4 DDT (mg/kg)	PCB (mg/kg)	PAH
1	A1	ND	ND	ND	ND	ND	ND	ND
2	A2	ND	ND	ND	ND	ND	ND	ND
3	A3	ND	ND	ND	ND	ND	ND	ND
4	A4	ND	ND	ND	ND	ND	ND	ND
5	A5	ND	ND	ND	ND	ND	ND	ND
6	B1	ND	ND	ND	ND	ND	ND	ND
7	B2	ND	ND	ND	ND	ND	ND	ND
8	B3	ND	ND	ND	ND	ND	ND	ND
9	B4	ND	ND	ND	ND	ND	ND	ND
10	B5	ND	ND	ND	ND	ND	ND	ND
11	C1	ND	ND	ND	ND	ND	ND	ND
12	C2	ND	ND	ND	ND	ND	ND	ND
13	C3	ND	ND	ND	ND	ND	ND	ND
14	C4	ND	ND	ND	ND	ND	ND	ND
15	D1	ND	ND	ND	ND	ND	ND	ND
16	D2	ND	ND	ND	ND	ND	ND	ND
17	D3	ND	ND	ND	ND	ND	ND	ND
18	D4/Blank	ND	ND	ND	ND	ND	Traces	ND
Control (Gaborone)	1 cont 1	ND	ND	ND	ND	ND	ND	ND
Control (Mafikeng)	2 cont 2	ND	ND	ND	ND	ND	ND	ND

Legend: ND = Not Detected; A = Gaborone Dam; B = Disaneng Dam; C = Notwane Sewage Treatment Plant; D = Setumo/Modimola Dam

Abundance

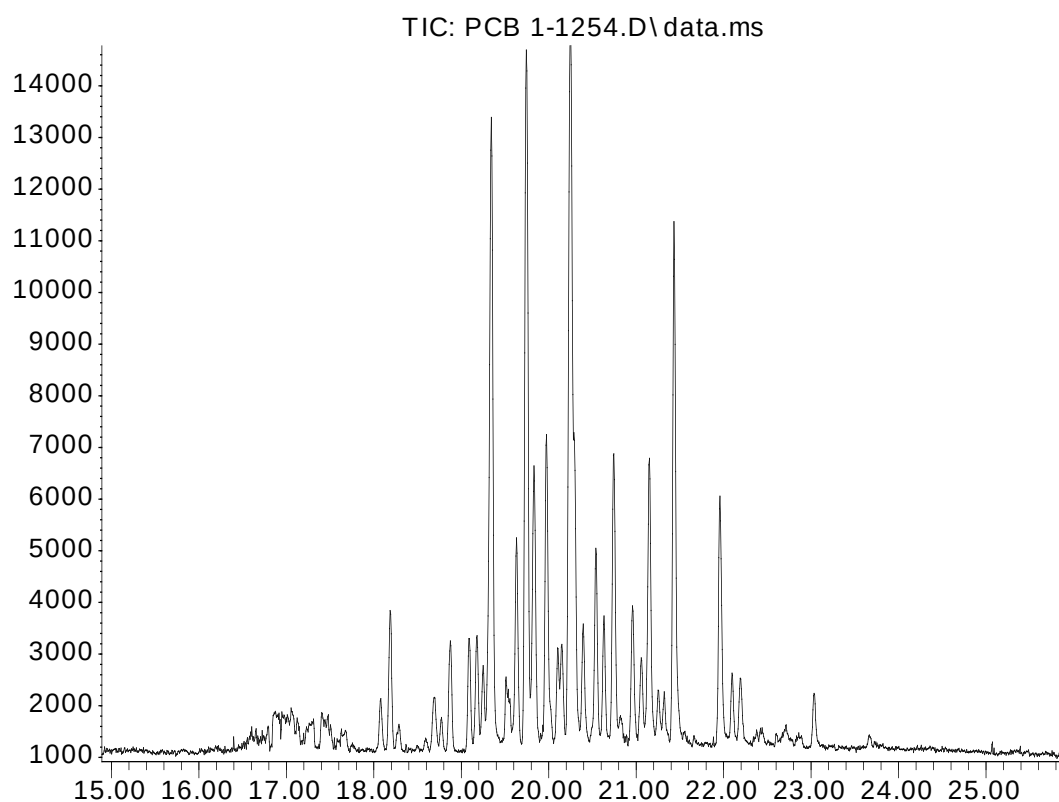


Figure 4.24a: GC-MS chromatogram for PCB standard.

The compounds contained in the standard were found to have retention times ranging from 18.8 to 23.7. The molecular weight ranged between 326 and 430. The GC-MS equipment was able to separate the components of the commercial mixture of PCB standard used.

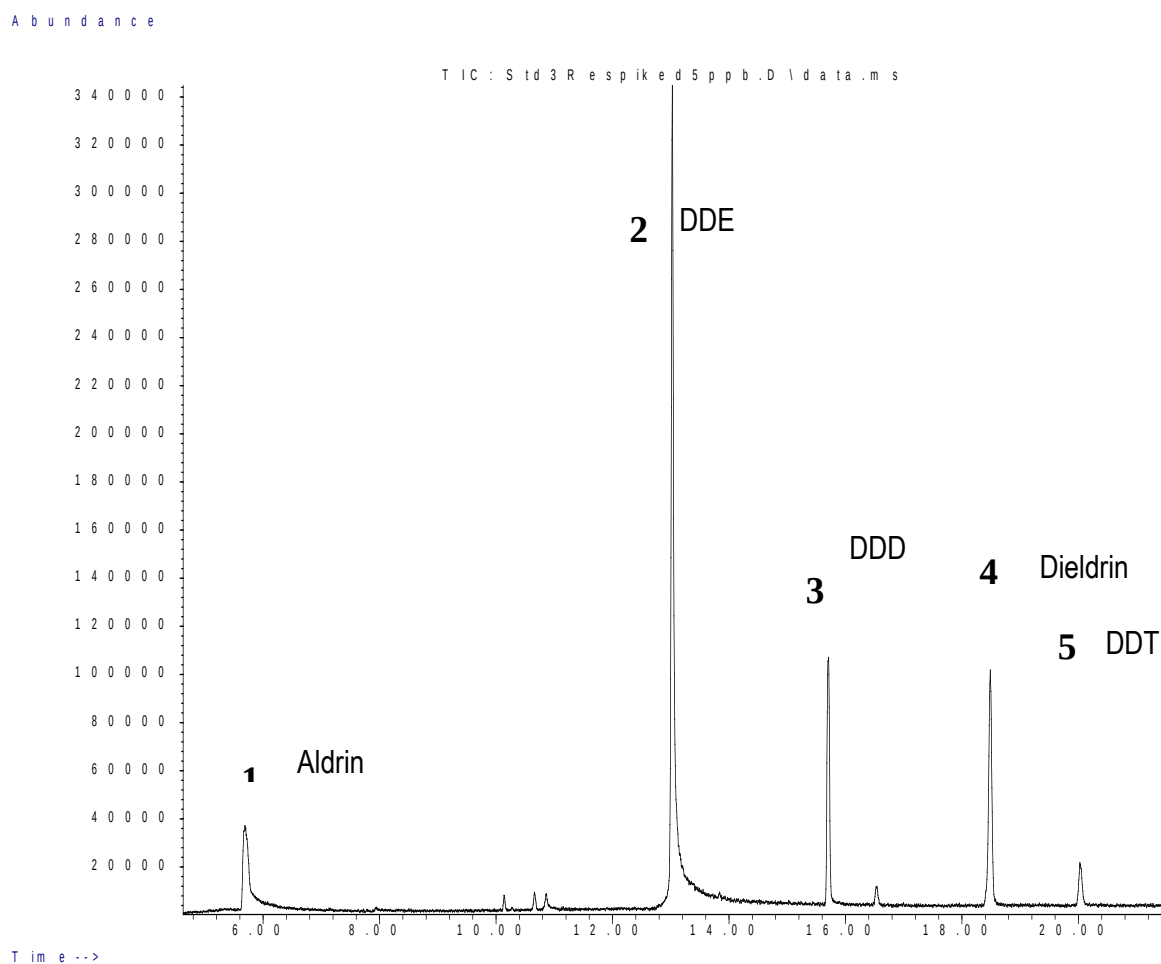


Figure 4.24b: GC-MS chromatogram for standard 2 (pesticides mixture) at 5 µg/L concentration

The pesticides used to formulate this standard were DDD, DDE, Dieldrin, Aldrin and DDT. The equipment separated all the pesticides.

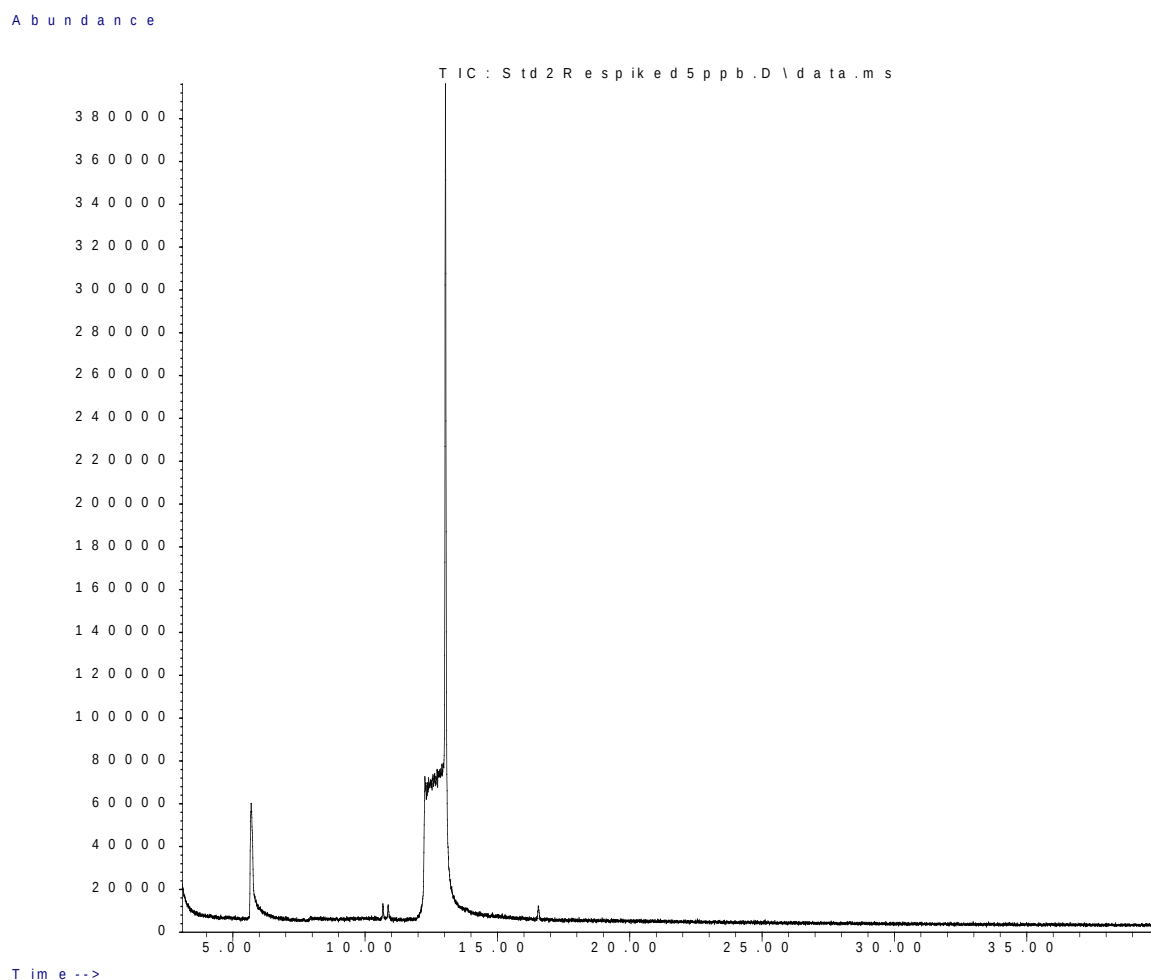
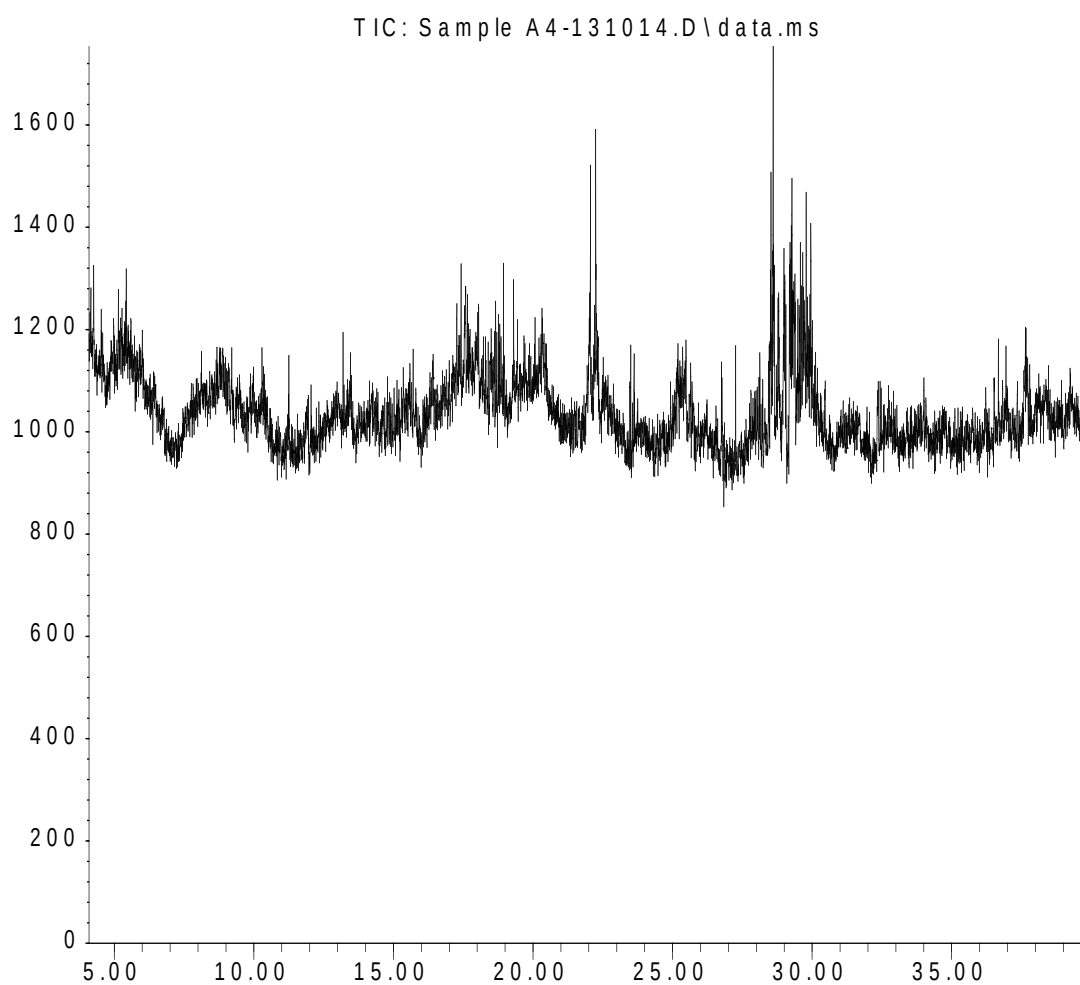


Figure 4.24c: GC-MS chromatogram for spiked PAH standard at 5 µg/L

This concentration was used to show that at concentrations as low as 5 µg/L the compounds, both PAHs and PCBs could not be detected from water by the GC-MS that was used in this study.

The results for PAH and PCBs analysis using Agilent 5975 GC-MS from water obtained from Gaborone dam, Disaneng dam, Notwane sewage treatment plant and Modimola/Setumo dam were as presented in the Figures 4.25a to 4.25d. The chromatogram for showed that no PAHs or PCBs were detected.

Abundance

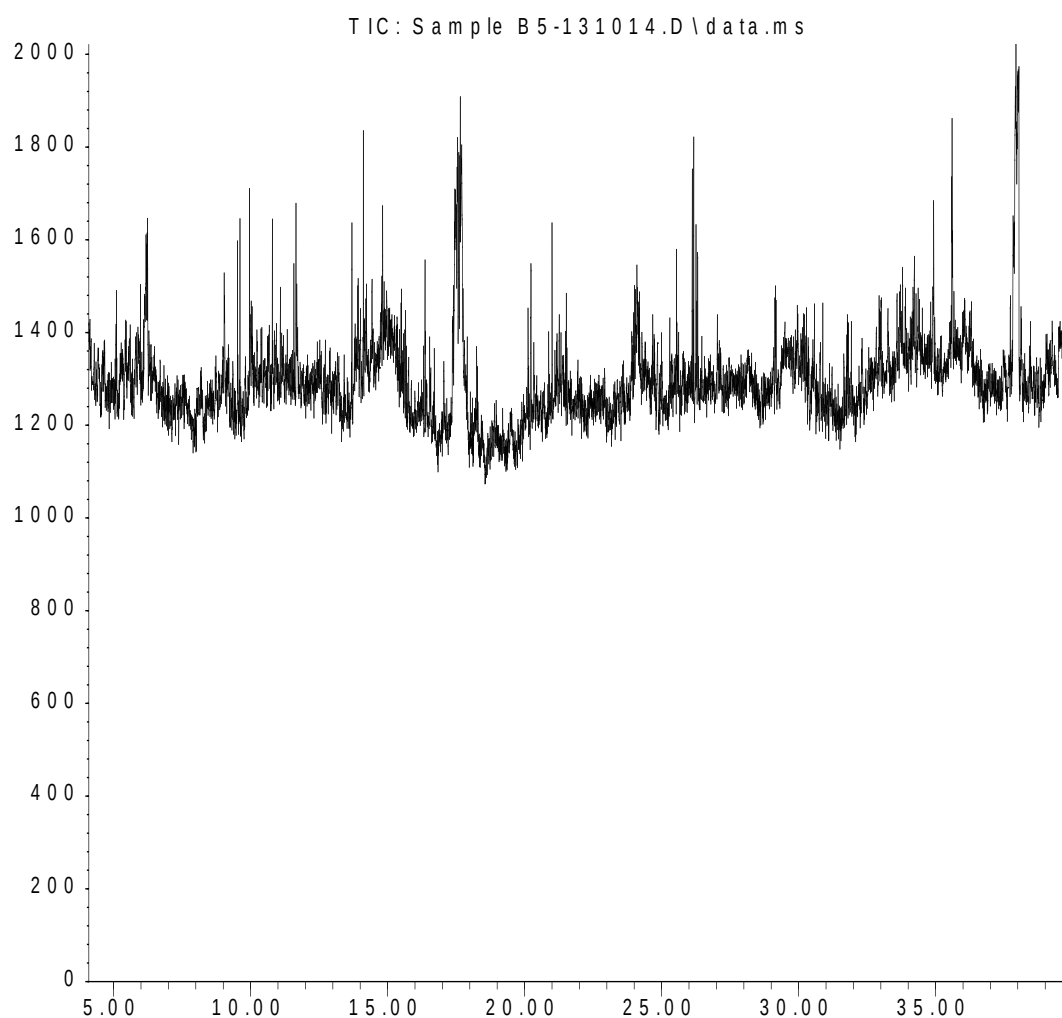


Time-->

Figure 4.25a: GC-MS Chromatogram for water sample from Gaborone Dam

As observed for raw water from Gaborone dam, Figure 4.25a, no compound was detected from the raw water from Disaneng dam.

Abundance



Time -->

Figure 4.25b: GC-MS Chromatogram for water sample from Disaneng dam

The chromatogram below clearly shows that there was no detection of any compound from wastewater from Notwane Sewage Treatment plant in Gaborone.

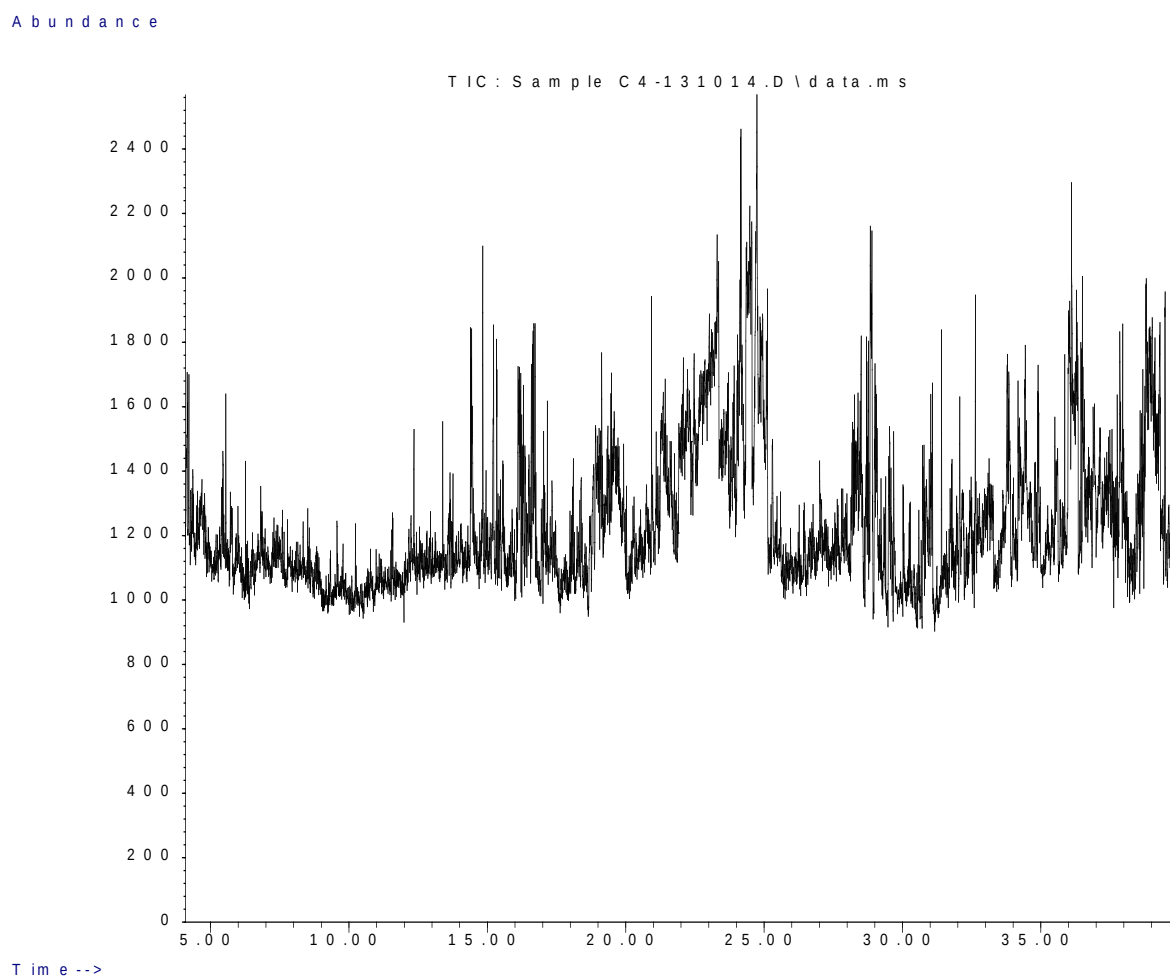


Figure 4.25c: GC-MS Chromatogram for water sample from Notwane Sewage Treatment plant

The GC chromatogram presented in Figure 4.25d indicates that some traces of compounds were detected whose retention times were close to those depicted in the chromatogram in Figure 4.25 a which is for PCB standard. Due to the very low concentrations, the peaks were not well defined thus no specific compound could be picked.

Abundance

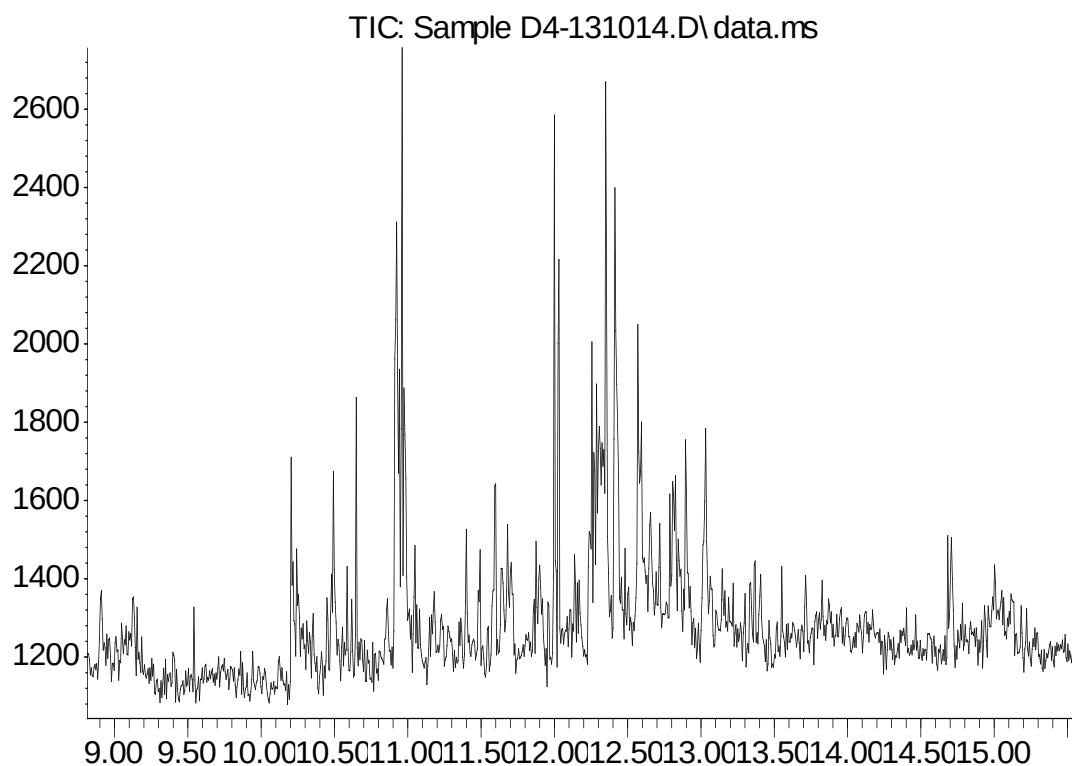


Figure 4.25d: GC-MS Chromatogram for water sample from Modimola dam

The peaks could not be clearly separated which is an indication that although the equipment could detect presence of some compounds, the concentration could not allow for clear retention times.

CHAPTER 5

DISCUSSION

5.0 Discussion

Water is an important resource for life and despite this; most countries in Sub-Saharan Africa strive to meet the MDGs that were drafted to address problems such as water scarcity, lack of proper sanitation as well as the management of wastewater (WHO and UNICEF, 2014; WHO and UNICEF, 2013). However, in most developing countries, including South Africa and Botswana access to safe water and inadequate sanitation continue to be a danger to human health and untreated water constitutes one of the main sources of drinking water supply (WHO and UNICEF, 2014; WHO and UNICEF, 2013; Ateba and Maribeng, 2011). Despite the fact that the MDGs were to be achieved by 2015 (UNDESA, 2013; WHO and UNEP, 2006), none of the eight goals directly addressed wastewater management and recycling regardless of the fact that wastewater was found to be infused in most of the goals (WHO and UNEP, 2006). In addition, the MDG guidelines were aimed at improving public health through the implementation of specific policies as well as monitoring systems to ensure environmental sustainability (WHO and UNEP, 2006).

Achieving and sustaining environmental sustainability is a challenge, especially with the emerging threat of climate change. Reports documented thus far are still biased towards ensuring that the water is free from pathogens and very little is reported on POPs and other chemicals (WHO and UNICEF, 2014). It is therefore suggested that in order to improve access to water and sanitation, countries must concentrate efforts in rural areas and low-income groups, as urban–rural income disparities in access are holding back progress (African Development Bank Group, 2016).

Despite the fact that the limit of detection of the Gas Chromatograph – Mass Spectrophotometer (GC-MS) was 17.1 ppb, none of the POPs (PAHs and PCBs) were detected in all the water samples except

for sample 18 that was collected from Modimola dam in Mafikeng. Traces of PCB were detected in this sample. Despite the fact that the turbidity values for the water samples from Mafikeng (-DWA – ZA, 2013) and Gaborone (-DWA BW, 2013 (BOS 93:2004)) were within the acceptable limits, they were relatively high. In addition, the water sample from Modimola dam from which traces of PCB were detected within the Limit of detection of 17.1 ppb had a turbidity value of up to 200.6 NTU. Given that the major source of turbidity, which refers to how clear water is, has been reported to be affected by the presence of particulate matter such as clays and silts from shoreline erosion, re-suspended bottom sediments and organic detritus from stream and/or wastewater discharges, it was difficult to conclude that the water samples were free of PCBs and PAHs. In addition, particulates also provide attachment sites for heavy metals such as cadmium, mercury and lead, and many toxic organic contaminants such as PCBs, PAHs and some pesticides.

The primary aim of this study was to conduct a biochemical analyse waste and raw water obtained from Mafikeng, South Africa and Gaborone, Botswana with reference to recalcitrant polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) in order to determine their association with physical parameters in water. Physical parameters investigated included the pH, turbidity, and temperature.

Water from Setumo / Modimola dam (D) was the most turbid with a mean turbidity value ranging from 25.10 to 200.6 NTU while samples from Gaborone dam (A) had the least mean turbidity readings (10.12 to 10.68 NTU). Although turbidity does not provide an indication of the microbial quality or the chemical composition of water, it may provide information on the level of pollutants present in the water. Setumo/Modimola dam receives effluent from the Setumo wastewater treatment plant while Gaborone dam and Disaneng dam store raw water. Water from Disaneng dam was more turbid than effluent obtained from Notwane sewage treatment plant in Gaborone. Water samples from all the sites investigated had turbidity values >5.0 NTU which is above the limits set by the Target Water Quality

Range (TWQR) for both Botswana and South Africa (DWA-BW and DWA-ZA, 2013). The turbidity of the control water samples was both 0.01 NTU, which was within the acceptable limits. The low turbidity values for raw water and wastewater samples from Gaborone might have resulted from the fact that very fewer human or animal activities occur at those sites. On the contrary, Setumo / Modimola and Disaneng dams are surrounded by villages with inhabitants who fish in the water and livestock in the area also use the water as drinking holes resulting in a lot of activity. This has previously been identified to be the main factor that increases the turbidity through the addition of pollutants to the water bodies (Mulamattathil *et al.*, 2014).

The mean pH values for all the sites sampled ranged from 7.78 to 9.78 (Table 4.1). The implication is that wastewater; raw water and the tap water assessed during the study were slightly alkaline and were slightly above the limits set by TWQR (DWA-BW and DWA-ZA, 2013). According to Zhang *et al.*, (2012) bacteria growth as well as the efficiency of bacteria to biodegrade recalcitrant compounds may not be influenced by a shift in the pH of water. Despite this the most suitable pH range for biodegradation of PAHs and PCBs by bacteria in general, has been found to be between pH 6.0 and 7.0 (Cycon *et al.*, 2009). In addition, the bacteria isolated from these water samples were able to grow optimally at pH of 7.0. This implies that despite the fact that bacteria are capable of breaking down the PAHs and PCBs in these water bodies and wastewater treatment plants, they are more likely to be limited by the high pH that these water bodies have.

Despite the fact that the shift of the pH values obtained from the generally acceptable limits might be regarded as insignificant it may provide opportunities for easy manipulation of the effluent by introducing bacteria that are able to breakdown of recalcitrant compounds taking into consideration that bacteria as single celled organisms are able to easily adapt to changes in environmental conditions (Blokesch, 2012; Martins and Peixoto, 2012). This results through changing their sources of carbon and therefore enhancing their potential for biodegradation of PAHs and PCBs (Vrchotova *et al.*, 2013).

Water samples were collected early in the mornings around 07:00 hours and at about 14:00 hours to the latest. The temperature readings for water samples ranged from as low as 8°C to 27°C. The temperature of water samples collected in winter was 8.0°C – 19.7°C while those for autumn, summer and spring were 11°C- 23.1°C, 20.1°C – 26.7°C and 20.4° - 26.1°C respectively. According to Das and Chandran (2011), effective degradation of hydrocarbons in some fresh water environments usually occurs at temperatures ranging from 20°C to 30°C. In the current study, temperature of 35°C had a significant effect on the degradation of PAHs and PCBs by *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Serratia liquefacians*. In this study the bigger shift in wave-max ($\lambda_{\max}$) during experiment on biodegradation of PAHs and PCBs using the test organisms was observed at temperatures 27°C and 35°C (Figure 4.10b and c). At 20°C (Figure 4.10a) very little change in wave-max ($\lambda_{\max}$) and absorbance was observed. This implies that although the temperatures would be low during part of the day and other seasons during the year, most of the time temperatures favour the activity of many bacterial populations that are capable of PAH/PCB biodegradation as the temperature increases especially in Mafikeng and Gaborone where environmental temperatures can rise to as high as 37°C (DWA -BW and DWA-ZA, 2013). The findings of this study indicate that bacterial strains *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Serratia liquefacians* were able to degrade the recalcitrant compounds effectively as a mixed bacterial consortium and similar observations have been reported (Dhall *et al.*, 2012; Jayashree *et al.*, 2012).

A further objective of the study was to isolate and characterise bacteria from raw and wastewater samples. Thirty bacteria isolates obtained through pour plate technique on Plate Count Agar were characterised using biochemical and molecular assays. Gram negative rod-shaped isolates were identified using the API 20E assay and results indicated the presence of *Aeromonas hydrophila*, *Enterobacter sakazakii*, *Serratia liquefacians*, *Serratia ficaria*, *Serratia marcescens*, *Ewingella Americana*, *Aeromonas caviae* and *Aeromonas sobria*. A large proportion of the Gram positive rods

were identified as *Bacillus* species. *Bacillus* species have been identified as the most diverse bacteria that can colonise any environment (Maughan and Auwera, 2011) and this may explain why it was isolated from almost all the sites sampled. *Exiguobacterium* species have the tendency of developing resistance to xenobiotic compounds such as PAHs thus able to degrade these compounds (Jeswani and Mukherji, 2012).

Bacterial 16S rRNA gene sequence of isolates revealed that a large proportion of the rod shaped bacteria after were identified as *Bacillus* species had 90% to 100% similarities to *Bacillus* species in the gene bank with accession numbers as follows: S18 similar to *Bacillus megaterium* (Accession No: JX475126); S9 *Bacillus cereus* (Accession No: KF478221); S15 *Bacillus megaterium* (Accession No: HF570085); S1 *Bacillus subtilis* (Accession No: EU373328S26); S10 *Bacillus aryabhattai* (Accession No: KJ186075); S16 *Bacillus megaterium* (Accession No: GQ274925); S5 *Bacillus subtilis* (Accession No: FJ864727); S8/S19 *Bacillus subtilis* (Accession No: FJ544346); S12 *Bacillus aryabhattai* (Accession No: KC550562); S26 *Bacillus anthracis* (Accession No: CP008854) while the vibrio shaped isolates S17, S7, S11 revealed 90% to 97% similarity *Vibrio cholerae* Accession numbers KJ413142, HM590223 and DQ991212 respectively. Isolates S6 and S13 revealed 94% and 96% similarity to *Exiguobacterium acetylicum* (Accession No: KF963623) and *Exiguobacterium indicum* (Accession No: JF935075) respectively. Isolates S2, S3, S4 (MD2) revealed a 95% to 99% similarity to *Pseudomonas aeruginosa* (Accession No: KJ101516) *Pseudomonas* sp. (Accession No: KF708909) and *Pseudomonas aeruginosa* (Accession No: CP006832) respectively. Isolate S14 was revealed to be 93% similar to *Kurthia gibsonii* (Accession No: KJ396180) while isolates 21, 22 and 27 revealed 80% to 94% similarity to *Aeromonas hydrophila* of accession numbers KM013811, KF183982, KF681381 respectively. Other isolates (S20, S23, S25, and S28) were revealed to *Enterococcus* sp. (Accession No: JX471098 (98%)), *Morganella morganii* (Accession No: KM222639 88%), Uncultured bacterium clone (Accession No: JF709658 (96%)) and *Serratia marcescens* (Accession No: KC435001, (91%)).

All these organisms except for *Aeromonas hydrophila* were isolated in all the four sampling sites. However, an isolate of *Aeromonas hydrophila* was detected from raw water obtained from sampling sites A (Gaborone dam) and B (Disaneng dam). This finding is consistent with previous reports that *Aeromonas* species are commonly found in freshwater environments (Janda and Abbott, 2010) but might not be restricted to these habitats (Ogugbue and Sawidis 2011). This might also be an indication that the genus may be used as an indicator organism for environment polluted with chemicals from such industries.

All the organisms isolated in the study have been reported to possess biodegradation potentials for PAHs and PCBs (Koul and Fulekar, 2013; Srujana and Khan, 2012-), except for *Pantoea* species whose role in biodegradation has not been fully established. Despite the fact that *Ewingella americana*, *Vibrio cholera*, *Aeromonas hydrophila*, are currently associated with some major human clinical complications they have also have been reported to play important roles in the breakdown of recalcitrant compounds in wastewater (Koul and Fulekar, 2013; Khleifat, 2006). In addition, *Pseudomonas*, *Haemophilus*, *Rhodococcus*, *Mycobacterium*, *Bacillus*, *Burkholderia* and *Serratia* have been reported to efficiently degrade polycyclic aromatic compounds (PAHs) and polychlorinated biphenyls (PCBs) in the soil (Jayashree, *et al.*, 2012; Haritash and Kaushik, 2009). According to Seo *et al.*, (2009) and Koul and Fulekar (2013) there is a variety of bacteria that are capable of biodegrading these compounds in any contaminated environment but the presence of a mixed population is essential for complete clean-up (Dhall *et al.*, 2012; Arbabi *et al.*, 2009).

Aeromonas, *Serratia* species have been found to play an important role in biodegradation through the production of biosurfactants (Lakshmi, 2013; Gumaa *et al.*, 2010). Production of biosurfactants is considered a very vital characteristic of bacteria in as far as biodegradation of PAHs and PCBs which are mostly recalcitrant (Kapadia and Yagnik, 2013; Lakshmi, 2013). *Enterobacter* species and *Serratia* species are also of vital importance when biodegradation of recalcitrant compounds is considered due

to their ability to produce biosurfactants and use petroleum products and petroleum hydrocarbons as their sources of carbon (Cycon *et al.*, 2013; Abo-Amer, 2011; Darvish *et al.*, 2011; Erdogan *et al.*, 2011) which implies that they are capable of using various sources of carbon for their metabolic activities. This is a good sign that having these organisms in the wastewater sites are of importance despite the environmental limiting factors which may interfere with their biodegradation effectiveness on recalcitrant compounds.

Although a variety of bacteria were isolated from the different sampling sites, the number of types of bacteria was small compared to similar studies that were done in soil as indicated in a report by Seo *et al.*, (2009). This attributes to the type of primers that were used. Although universal primers U341F and U758R are meant for bacteria, they have been found to have been designed to target a 418pb fragment corresponding to positions 341 – 758 in *Escherichia coli* sequence and covered the reserved regions v3 and v4 (Yeung *et al.*, 2010; Hendrickx *et al.*, 2006; Muyzer *et al.*, 1993), and this may have limited the amplification process and eventually omitting some of the bacterial isolates. According to Mao *et al.*, (2012), PCR efficiency and accuracy may be reduced by many factors that include Primer-template mismatches, reactant concentrations, the number of PCR cycles, annealing temperatures, the complexity of the DNA template, among others. No one combination of primers and read length works best in all environments and no universal primer is truly 'universal' (Soergel *et al.*, 2012). Primers such as 338F has been designed for use in pyrosequencing (Mao *et al.*, 2012) and not Sanger sequencing which was used in this study, which might have been the reason towards the small number of amplifications obtained from PCR and thus a small number of bacteria identified in this study especially that it was paired with 518R, which might not have been the best pair for isolates from aquatic environments. A report on the efficiency of primers by Ghyselinck *et al.*, (2013) noted that although primer 518R if used with 518F showed the highest coverage amongst primers, it was not only targeting bacteria but also eukaryotic DNA thus making it less suitable for use while on the other hand 338F used

with 338R showed the least coverage but specific for bacteria. Having 338F and 518R must have led to the less sensitivity for PCR amplification thus decreasing the efficiency of the PCR.

In a study by Nielsen *et al.*, (1999) using the same primers (EUB 968F and UNV 1392R) with isolates from sludge, resulted in a completely different set of bacteria from what was identified in this study (Figure 4.2 to 4.4). This might have been brought about by techniques that were used in this study and the study carried out by Nielsen *et al.*, (1999). This was alluded to by Mao *et al.*, (2012) who observed that several factors affect the efficiency and accuracy of PCR and Soergel *et al.*, (2012) and Hendrickx *et al.*, 2006 that no one combination of primers works best in all environments.

Another objective of the study was to assess the biodegradation of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) by control strains *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Serratia liquefacians* as well as an environmental isolate MD2 (*Pseudomonas aeruginosa*-). The breakdown of recalcitrant compounds was studied using commercially produced mixtures of PAHs and Aroclors of PCBs using bacterial strains *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Serratia liquefacians* as well as isolate MD2. Isolate MD2 was obtained from wastewater Mineral Salt Medium (MSM) was used in the biodegradation analysis of PAHs and PCBs by the bacteria strains. However, a separate experiment in which wastewater was used as the medium without enhancement or addition of any carbon source was included. The use of MSM in studies involving biodegradation of chemical compounds, especially hydrocarbons has been widely documented (Silva *et al.*, 2012). The three test bacteria were found to positively grow in MSM enriched with the test compounds (PAHs and PCBs). This was proved by inoculating MSM enriched with each compound with each bacterial strain separately and incubated at 30°C on a rotary shaker for 24 hours after which absorbance at 600 nm (OD₆₀₀) was checked using a spectrophotometer the reading of which was compared with the OD₆₀₀ was taken before incubation. The absorbance increased which showed that the degree of light penetrating through the liquid culture reduced as a result of bacteria

growth. The results were further confirmed by inoculating TSA agar plates and growth of each strain was still observed. This was to ensure that whatever lack of growth that may occur afterwards with experiments on PAHs and PCBs it will be clear that it is not because of the bacterial strains not being able to grow in enriched MSM but the different treatments that would have been employed to test the ability of the test bacterial consortia to degrade the compounds that are being tested.

The bacterial strains were also found to grow in the MSM when it was enriched with PAHs mixture and PCBs Aroclors, separately. This was clearly shown by the results obtained from the UV-VIS spectrophotometer presented in Figure 4.5 and Figure 4.6. The wavelength maximum ($\lambda_{\max}$) for PAHs for all the three organisms was found to have shifted from 257nm to 260nm on average and from 264nm to 269nm on average for PCBs (Figure 4.5 and Figure 4.6). These results indicated that the compounds were broken down forming new products with different wavelength and thus the change in the absorbance which implies that the bacteria were able to breakdown the PAHs and PCBs and use them as their sole sources of carbon thus biodegradation. The shifts are an indication of the presence of initial ring oxidation metabolites and ring fission metabolites (Seeger *et al.*, 2010). PCBs first get degraded into chlorobenzoates (Seeger *et al.*, 2010; Pieper, 2005) that have been found to have $\lambda_{\max}$ ranging from 210 – 214nm in the B-band and 244 – 270nm in the C-band when dissolved in water (Forbes, 1960), a range that was observed in the results obtained after 24 hours of growing the three test organisms used in this study in PCBs aroclor mixture (Figure 4.7) which proves that the organisms were able to breakdown the PCBs. The product chlorobenzoate is biodegraded into benzoate and eventually pyruvate and acetylaldehyde, which are essential in the tricarboxylic cycle (TCA) (Seeger *et al.*, 2010). During the biodegradation of PAHs muconic acid is one of the products when the ortho-cleavage route is considered (Guzik *et al.*, 2013; Koul and Fulekar, 2013) that has a $\lambda_{\max}$ ranging from 268 to 274nm (Sharma and Vaidyanathan, 1975). The other route during biodegradation involves the meta-cleavage in which 2,hydroxyl-muconic semi-aldehyde is the intermediate product (Koul and Fulekar, 2013). These compounds have $\lambda_{\max}$ different from the PAHs. These shifts in $\lambda_{\max}$ was observed

in all the experiments carried out in this study to check the biodegradation ability of *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Serratia liquefacians* under different conditions as show in Figure 4.8a - h and Figure 4.9a - c. Although in other studies *Serratia marcescens* was mostly used (Gumaa *et al.*, 2010; Seo *et al.*, 2009), this study still showed that *Serratia* species can be used in biodegradation of recalcitrant compounds PAHs and PCBs.

When PCBs aroclor mixture and PAHs mixture was added to wastewater from the sampling sites and the test organisms added, the results showed a significant shift in $\lambda_{\max}$ when compared to the wastewater was sterilised before introducing the test organisms for PCBs than PAHs (Figure 4.10a – b). The test organisms were able to degrade PAHs and PCBs under different conditions and concentrations (Figure 4.11 and Figure 4.12). When the compounds were added to MSM inoculated with isolate MD2, the results indicated that there was degradation of the compounds as shown in Figure 13. The results were similar to what was obtained with reference isolate *Pseudomonas aeruginosa* ATCC 27853 TM. The experiment on enzyme assay proved that these bacteria are capable of biodegrading PCBs and PAHs as the results showed a significant shift in $\lambda_{\max}$ for both types of compounds (Figure 4.14 and Figure 4.15).

A further objective was to screen raw and wastewater samples for the presence of PAHs and PCBs. The water samples were screened for the recalcitrant compounds PAHs and PCBs using GC-MS and HPLC. The results showed that the compounds were not detected by the instruments used. There were traces of PCBs from Modimola dam though, although the quantities could not be determined as the levels were below quantification and thus traces. The results indicate that since these compounds are highly lipophilic (WHO, 2000) they need certain surfaces to which they can adhere and require fats in which they dissolve making it difficult for them to be detectable in water. This explains why most studies have only been able to detect these compounds from fish, plant material and soils. This also explains why these compounds highly result in bioaccumulation (EPA, 2013; Nabavi *et al.*, 2013) and thus very

undesirable in the ecosystem. The implication from the results obtained during this study is that these compounds if they get attacked by bacteria, they are biodegraded as these will absorb the compounds and use them as their carbon sources thus biodegradation as was described by Azhari *et al.*, (2010) and Vrchotova *et al.*, (2013). If these compounds go through the wastewater treatment plant undegraded, they end up into the soils where the effluent is released, or sediments of dams and rivers thus polluting the environment (WHO and UNICEF, 2013). The pollution will result in the increase in concentration as the compounds go up the trophic levels (Azhari *et al.*, 2010) such that when humans eat fish, meat from livestock drinking from those polluted water bodies and eating vegetation in the polluted environment or vegetables containing these compounds, they will experience the effects of the compounds the most as they will receive high concentrations thus the increase in cancer incidences and related ailments which come as a result of these compounds.

CHAPTER 6

COCLUSION AND RECOMMENDATIONS

6.0 Conclusion and Recommendations

6.1 Conclusion

The objectives of the study were fully achieved. Bacterial strains *Serratia liquefacians* and *Aeromonas hydrophila*, together with *Pseudomonas aeruginosa* and isolate ID MD2 were capable of degrading recalcitrant compounds under different environmental conditions. The result from this study also showed that wastewater from Setumo/Modimola dam (Mafikeng) is more polluted than the wastewater from Notwane Sewage Treatment Plant (Gaborone). The wastewater from Setumo/Modimola was also found to contain traces of polychlorinated biphenyls. The results from this study therefore suggest that the wastewater from Setumo/Modimola need thorough treatment to render it safe for recycling for purposes of introducing it into treatment plant for potable water production.

As for the wastewater from Notwane sewage treatment Plant and raw water from Disaneng and Gaborone dams, the recalcitrant compounds that were of target in this study were not detected. Although it may be concluded that the wastewater from Notwane Sewage Treatment Plant can be diverted to Gaborone dam to enhance the water level and thus be treated for portable water use, more studies still need to be carried out to check for the compounds through studying the vegetation, fish, and beef from cattle using those places as water holes, before a final recommendation can be forwarded to the Water Utilities Cooperation (WUC). From these results it is therefore concluded that the use of *Serratia liquefacians* and *Aeromonas hydrophila*, together with *Pseudomonas aeruginosa* (isolate ID MD2, S4) which was isolated from wastewater is the originality of this study. The biodegradation of PAHs and PCBs in wastewater which was also proved by the enzyme action on the same compounds is unique for this study as no such study was carried out in Gaborone or Mafikeng on biodegradation of recalcitrant compounds, especially PAHs and PCBs. These were my own findings as

to the ability of these organisms in the biodegradation of PAHs and PCBs in wastewater from Gaborone and Mafikeng which is my contribution to the knowledge of biodegradation of recalcitrant compounds by bacteria.

6.2 Recommendations

This was the first study on use of bacterial isolates to breakdown PAHs and PCBs to be carried-out on wastewater in these two areas (Gaborone and Mafikeng). Moreover, the study was designed to target recalcitrant compounds, specifically the polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). There is need for extensive research to screen for and identify bacteria strains among the isolates from wastewater treatment plants that can efficiently degrade recalcitrant compounds. In addition, it is also important to thoroughly screen the wastewater to determine the type of compounds or metabolites are produced during biodegradation of PAHs and PCBs as well as their effect on other microorganisms that are found in the water bodies. It is therefore suggested that there is the need to conduct further studies designed to detect specific genes that are actively involved in the production of enzymes that catalyse the biodegradation of PAHs and PCBs respectively.

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Appendix 1

Water Quality Standards

		WUC
VARIABLE		Recommended Maximum Limit
Physical Requirements		(mg/l)
Turbidity	NTU	0.5
Colour	TCU	15
Taste & Odour		non-objectionable
Chemical Requirements		(mg/l)
Chlorine Residual	Cl ₂	0.3-0.6
pH Value		6.5-8.5
Total Dissolved Solids	TDS	450
Total Hardness	(as CaCO ₃)	20
Sulphate	So ₄	200
Calcium	Ca	80
Nitrite	No ₂	3
Potassium	K	0.25
Chloride	Cl	100
Sodium	Na	100
Magnesium	Mg	30
Iron	Fe	0.3
Manganese	Mn	0.5
Ammonium	Nh ₄	1.0
Aluminium	Al	0.1
Copper	Cu	1.0
Zinc	Zn	3.0
Toxic Substances		(mg/l)
Nitrate	No ₃	45
Fluoride	F	0.7
Lead	Pb	0.01
Cadmium	Cd	0.03
Cyanide	CN	0.7
Microbiological Variable		(mg/l)
E.coli/100ml		0
Faecal streptococci/100ml		0
Total Coliforms/100ml		10

The Role of Bacteria in the Breakdown of Carcinogenic Substances (PCBs) in Wastewater for Safe Recycling Purposes – A Review

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Abstract

Water is one of the most essential natural resources for daily human activities, yet it is so scarce. Treated wastewater and untreated sewage contain bacteria that can be advantageous to the recycling process. The composition of effluent originates from various industries, such as pharmaceutical industries, mining industries, agricultural sector, household waste, chemical industries, and various manufacturing industries, including the oil manufacturers. These introduce potential health-threatening compounds to wastewater. These compounds that remain for a long time without being broken down or changed in their chemical composition and/or structure in the environment are referred to as recalcitrant compounds. Polychlorinated Biphenyls (PCBs) are examples of such compounds. These compounds have serious negative health effects on humans (act as mutagens and carcinogens). The capacity of Polychlorinated Biphenyl's (PCBs) degradation by bacteria (biodegradation) depends on the diversity and characteristics of naturally occurring populations and their response to environmental conditions. Conventional physical and chemical methods that are used to decontaminate wastewater contaminated by these substances are time and energy consuming. The use of *Moringa oleifera* seeds, which is currently under trial, is also very costly and not sustainable. This threatens the economic security of most developing countries. Biodegradation is the metabolic ability of microorganisms to transform or mineralize organic contaminants into less harmful, non-hazardous substances, which are then integrated into natural biochemical cycles. This process is not only cost effective, but it is also environmentally friendly. Wastewater that has gone through effective biodegradation can be fully recycled, thus conserving raw water and at the same time ensuring safe available water for all.

Key words: Recycling; wastewater; polychlorinated biphenyls (PCB); water conservation; safer recycled water; biodegradation; sustainability

Introduction

Sustainable development requires the promotion of environmental management and the constant search for new technologies to treat vast quantities of waste, which includes treatment of wastewater for purposes of recycling (Khadhraoui and Belaid, 2012). The scarcity of water as a natural resource, resulting from the aggravating effects of global warming, has resulted in great global concern to

recycle and conserve water, especially in sub-Saharan Africa where the problem of water scarcity has affected most countries (Ateba and Maribeng, 2011). South Africa is faced with freshwater scarcity that is exacerbated by its increasing demand, pollution, unsustainable use, and climate change (DWA, 2012).

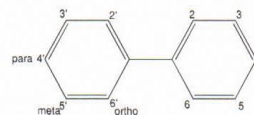
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The presence of chemicals in the environment calls for quantification of such in order to come up with risk analyses posed by those chemicals (Guillen et al., 2012; Urbaniak, 2013). According to Guillen et al., (2012), substances such as pharmaceuticals, perfluorinated acids and perfluorosulfonates, PAHs, PCBs, pesticides, and surfactants are mostly found in wastewater. Ying et al. (2009) also noted that the presence of pharmaceutically active compounds in wastewater are a major concern. Several methods may be used to quantitatively determine these substances from wastewater, which are mainly from sewage treatment plants (Ying et al., 2009). According to Ying et al., (2009), although most research done regarding the removal of these substances was carried out, it was mainly on activated sludge and not done on wastewater.

Some strains of organism *Acinetobacter* have the ability to degrade pollutants such as biphenyls from wastewater (Gumaa et al., 2010). *Enterobacter cloacae* secrete an emulsifier that increases the hydrophobicity of the bacterial cell surface and neutralises the surface charge of cells (Pacwa-Plociniczak et al., 2011). This, as a result, increases the ability of the bacteria to degrade PCBs (Pacwa-Plociniczak et al., 2011; Fakruddin, 2012). Biosurfactants are also effective at the extremes of temperature, pH, and salinity (Kapadia and Yagnik, 2013; Fakruddin, 2012), a property that is essential in biodegradation of PCBs, as they are hydrophobic organic compounds (Pacwa-Plociniczak et al., 2011). This property makes these recalcitrant compounds removable through physico-chemical means or treatments, limited bioavailability to microorganisms, and limited availability to oxidative and reductive chemicals when applied in treatments (Pacwa-Plociniczak et al., 2011).

Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) consist of two benzene rings with a carbon-to-carbon bond between carbon 1 on one ring and carbon 1 on the second ring (EPA, 2013). The PCBs have varying numbers of chlorines in their structure (Barbalace, 2003; Anyasi and Atagana, 2011; EPA, 2013) the diagram below.



Structure of PCBs (Barbalace, 2003)

Toxicity of PCBs is dependent upon the number of chlorines present on the biphenyl structure and their position, that is, the co-planar congeners (Barbalace, 2003; Anyasi and Atagana, 2011). The PCB congeners that have been seen to be highly toxic were those that had chlorine atoms attached to the 3, 4-ortho positions, followed by those with 5-10 chlorine atoms in the para and meta positions (Anyasi and Atagana, 2011).

In terms of structural relationship to toxicity, PCBs fall into two distinct categories, referred to as coplanar, or non-*ortho*-substituted “*arene*” substitution patterns, and noncoplanar, or *ortho*-substituted congeners (Jensen, 2006). The coplanar group members have a fairly rigid structure with the biphenyl rings in the same plane. This gives the molecule a structure similar to polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans and allows it to act in the same way as these molecules as an agonist of the aryl hydrocarbon receptor (AhR) in organisms (Arsalan et al., 2007). These types of PCBs are considered contributors to overall dioxin toxicity (Arsalan et al., 2007).

Noncoplanar PCBs, with chlorine atoms at the *ortho* positions, have not been found to activate the AhR and are not considered part of the dioxin group; however, studies have indicated some neurotoxic and immunotoxic effects, but at levels much higher than normally associated with dioxins, and thus, of much less concern to regulatory bodies (Rudel et al., 2008; Wethington and Hornbuckle, 2005).

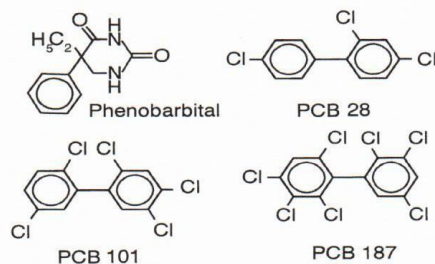
PCBs are very stable compounds and do not decompose readily (Rudel et al., 2008). This is due to their chemical inability to oxidize and reduce in the natural environment. Furthermore, PCBs have a long half life (8 to 15 years) and are insoluble in water, which contributes to their stability and recalcitrant property (Rudel et al., 2008). The biodegradability (and solubility in water) of PCBs is also dependent upon the number of chlorine molecules it has (Anyasi and Atagana, 2011; EPA, 2013). The more chlorine molecules contained in a

compound renders that compound less biodegradable (EPA, 2013). PCBs are mostly hydrophobic and some are less hydrophilic (Nwinyi, 2011; Muir and Lohmann, 2013). These properties result in bioaccumulation of these compounds, as they do not dissolve in water and thus, render them difficult to be biodegraded (Schafer and Kegley, 2002; Barbalace, 2003; EPA, 2013; Muir and Lohmann, 2013).

Elimination of PCBs

Although the Stockholm Convention on Persistent Organic Pollutants (POPs) (of which PCBs are part of) signed in 2001 was aimed at eliminating and/or restricting the production and use of POPs (Lammel et al., 2009), more of these ubiquitous substances were still being introduced into the environment through various human activities (Teran et al., 2012). Water has become a widely used environmental matrix for monitoring POPs (Muir and Lohmann, 2013; van Leeuwen et al., 2013), although more studies on PCBs have been carried out on contaminated soils than water (Leigh et al., 2006).

The destruction of PCBs by chemical, thermal, and biochemical processes is extremely difficult and presents the risk of generating extremely toxic dibenzodioxins and dibenzofurans through partial oxidation (Leigh et al., 2006; Arsalan et al., 2007; US EPA, 2013).



Generalized Structure of PCBs (US EPA, 2013)

Effects of Polychlorinated Biphenyls (PCBs) on Human Health

PCB mixtures have been associated with cancer incidents in animals from a long time back (Pavuk et al., 2004; Ross, 2004; Lynch et al., 2012). PCBs were found to induce liver tumours, thyroid adenomas, intestinal metaplasia, and adenocarcinomas in rats and mice (Pavuk et al., 2004). Exposure to some environmental chemicals such as DDT and PCBs have been associated with a drop in sperm count, breast cancer, testicular cancer, and hypospadias, which are all associated with endocrine disruption caused by these chemicals (Rogan and Ragan, 2007). This comes as a result of some PCB congeners being able to occupy thyroid receptors, thus interrupting its action (Ross, 2004; Rogan and Ragan, 2007).

PCBs accumulate in the fats of organisms and get passed on from one organism to the other in food chains (Zhao et al., 2009; Man et al., 2011), thus causing bioaccumulation. The PCBs get entry into the human body and animals through the skin, lungs, and gastrointestinal tract (Anyasi and Atagana, 2011). This gets distributed to various parts of the body via blood and gets to accumulate in different tissues (Anyasi and Atagana, 2011; Man et al., 2011). The effects of PCBs on humans depend on age, sex, and part of the body affected by the chemicals (Anyasi and Atagana, 2011). The liver, as the major organ for removal of toxins in the body, is usually highly affected by PCBs (Pavuk et al., 2004; Anyasi and Atagana, 2011). Humans become exposed to PCBs through consumption of contaminated fish, meat, and dairy products (Lynch et al., 2012) and also through grains grown in PCB contaminated soils (Anyasi and Atagana, 2011; Lynch et al., 2012). PCBs have been isolated from human milk and serum (Linderholm et al., 2010; Man et al., 2011) and have been found to have effects on breastfed children leading to low IQ and endocrine related ailments (Linderholm et al., 2010; Man et al., 2011; Lynch et al., 2012). Some studies have shown an increase in cancer mortality in workers exposed to PCBs (Anyasi and Atagana, 2011).

Biodegradation

Biodegradation is the metabolic ability of micro-organisms to transform or mineralize organic

contaminants into less harmful, non-hazardous substances, which are integrated into natural biochemical cycles (Leigh et al., 2006; Dhall et al., 2012). Specific bacteria, having bio-degradative potential for various chemical substances in wastewater and raw water, may be used to treat water (Dhall et al., 2012) for purposes of safely recycling. Bacteria, unlike other organisms, have the ability to interact better with man-made and naturally occurring compounds, which results in such compounds being changed structurally and eventually getting degraded (Anyasi and Atagana, 2011). This is, in a way, a better cleanup strategy that can be used in the cleanup of wastewater, as it is environmentally friendly (Anyasi and Atagana, 2011). Use of mixed population of microbes is usually recommended, as it has been seen to yield faster results as the two different microbes attack different parts through different mechanisms resulting in effective breakdown of a compound (Farhadian et al., 2007; Martinkova et al., 2009). This activity also creates a condition of co-metabolism (Martinkova et al., 2009).

Although PCBs are not readily biodegradable, some bacteria species, such as *Vibrio cholera*, *Acinetobacter lwoffii*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Rhodococcus* sp., *Bacillus* sp., and *Burkholderia* sp., have been found to have a role in the biodegradation of these compounds (Petric et al., 2007; Hamzah et al., 2010; Nwinyi, 2011; Anyasi and Atagana, 2011; Blokesch, 2012). This may be achieved through co-metabolism and mineralisation (Anyasi and Atagana, 2011; Blokesch, 2012) and the use of a metabolic pathway similar in all these bacteria, which comprises four steps catalysed by enzymes BphA, BphB, BphC, and BphD (Petric et al., 2007). The pathway, according to Petric et al., 2007, is initiated by insertion of two oxygen atoms at the carbon positions 2, 3 of one aromatic ring. This is then followed by dehydrogenation meta-cleavage and hydrolysis which results in the formation of a 5-carbon compound, thus a biphenyl catabolic pathway (Petric et al., 2007). Due to the hydrophobic moiety of PCBs, the presence of surfactants has been found to be essential in the biodegradation (Das and Chandran, 2011).

Surfactants produced by bacteria are essential in bioremediation (Salihu et al., 2009; Das &

Chandran, 2011). They play an important role in the metabolism of these organisms (Das & Chandran, 2011). The surfactants are amphiphilic compounds (Gumaa et al., 2010). The hydrophilic moiety can be a carbohydrate, amino acid, phosphate group, or some other compound (Gumaa et al., 2010). The fact that these chemical substances have characteristics of biocompatibility, specificity, and digestibility (Kapadia and Yagnik, 2013) makes them advantageous for use in biodegradation (Fakruddin, 2012; Martins et al., 2012).

Biosurfactants

Biosurfactants have a wide range of structures, and some examples and their sources are given in Figure 1.

Another property of surfactants that has been found to be important in biodegradation is their ability to permit microorganisms to grow on water immiscible substrates by reducing surface tension at the phase boundary (Fakruddin, 2012). This makes the substrate more readily available for uptake and metabolism (Fakruddin, 2012; Pacwa-Plociniczak et al., 2011; Kapadia and Yagnik, 2013). This characteristic of bioavailability and increase in cell surface interaction is important in biodegradation (Pacwa-Plociniczak et al., 2011; Kapadia and Yagnik, 2013), as it enhances hydrocarbon mobilization, solubilisation, or emulsification. This can be summarised as shown in Figure 2.

Biosurfactants are also effective at the extremes of temperature, pH, and salinity (Kapadia and Yagnik, 2013; Fakruddin, 2012), a property that is essential in biodegradation of PAHs and PCBs, as they are hydrophobic organic compounds (Pacwa-Plociniczak et al., 2011).

Mechanisms Involved in Biodegradation of Xenobiotic Compounds

Xenobiotic compound, owing to its recalcitrant nature, is hard to break down and degrade (Heider et al., 2008). The complexity of its chemical composition adds to this recalcitrant property (Pacwa-Plociniczak, 2011).

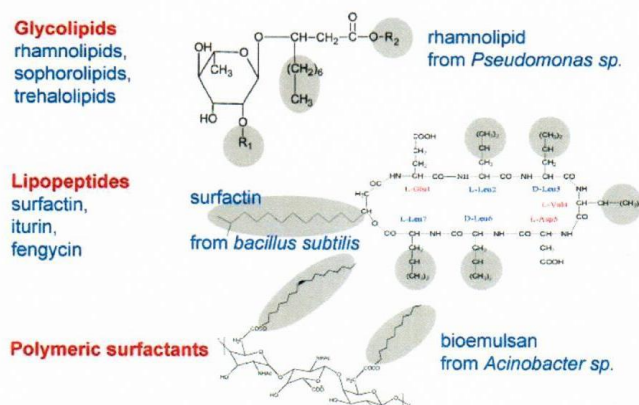


Figure 1: Structure and Source of Some Biosurfactants

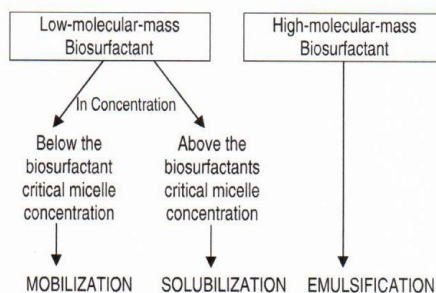


Figure 2: Mechanism of Hydrocarbon Removal by Biosurfactants Depending on their Molecular Mass and Concentration (source: Pacwa-Plociniczak et al., 2011).

For breaking down such compounds, the enzymes act on certain groups present in the compound (Heider, 2008). For example, in the halocarbons, the halogen group is targeted, with enzymes such as oxygenases playing a major role (Pacwa-Plociniczak, 2011).

The bonds like ester-, amide-, or ether bonds present in the compounds are first attacked, leading to breaking down of compounds (Abor-Amer, 2011; Cycon et al., 2013). In some cases, the aliphatic chains, and in aromatic compounds, the aromatic components, may be targeted (Abor-Amer, 2011). The site and mode of attack depends on the action of the enzyme, its concentration, and

the favourable conditions (Abor-Amer, 2011). Often it is seen that the xenobiotics do not act as a source of energy to microbes, and as a result, they are not degraded (Abo-Amer, 2011). The presence of a suitable substrate induces its breakdown (Cycon et al., 2013). These substrates are known as co-metabolites, and the process of degradation is known as co-metabolism (Cycon et al., 2013). Gratuitous metabolism is another process in which the xenobiotics serve as substrates and are acted upon to release energy (Pacwa-Plociniczak et al., 2011).

This cannot be achieved through the use of *Moringa oleifera* and related species in treating wastewater to remove PCBs. The removal of these compounds has not been fully studied with the use of the plant protein, although the protein has been found to be slightly efficient with the reduction of fecal coliforms and other bacteria (Dalen et al., 2009; Kawo and Daneji, 2011). The use of *Moringa oleifera* seed powder in water treatment has been found to target mainly microorganisms, thus reducing turbidity (Mumuni et al., 2013). Although this mode of water treatment has been used, especially in rural areas of the developing countries, the synthetic polymers, aluminium sulphate, ferric chloride and poly aluminium chlorides used together with this powder have been reported to be unsafe (Danel et al., 2009; Mumuni et al., 2013; Lea, 2014). The action of *Moringa oleifera* seed powder has been reported to be based

on the ability of the protein contained in the seeds to be able to form coagulants that reduce water turbidity by acting on coliforms (Mangale et al., 2012). The bacteria found to be mainly involved in biodegradation of POPs and PCBs has been found mostly not to be coliforms (Anyasi and Atagana, 2011; Blokesch, 2012). The remaining bacteria, after treating water with *Moringa seed* powder, that is the $10^1 - 10^5$ of bacteria left (Mangale et al., 2012), may be those that are capable of

biodegradation (although no report to that effect has been found). Tree growth and productivity relies on environmental conditions and thus, production and maintaining the *Moringa* tree species may not be sustainable given the global warming related environmental problems. The indoor growth of plants compared to bacteria (microorganisms in general) is quite costly, although Lea (2014) argues that propagation is affordable.

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Biodegradation of Polychlorinated Biphenyls (PCBs), Aroclor 1260, in Wastewater by Isolate MD2 (*Pseudomonas aeruginosa*) from Wastewater from Notwane Sewage Treatment Plant in Gaborone, Botswana

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Abstract

Notwane Sewage Treatment Plant is situated on the eastern part of Gaborone emptying its effluent into Notwane River which flows along the periphery of Gaborone. The wastewater treatment plant receives wastewater from most major industries, and most of the residential waste water for the city of Gaborone. The wastewater is not recycled despite the great shortage of water in Gaborone and surrounding villages and the drying up of Gaborone dam.

Wastewater samples from the treatment plant was collected and taken to Department of Biological Sciences in Mafikeng for analysis of PCB (Aroclor 1260) degradation by bacteria. Biodegradation of polychlorinated biphenyls using MD2 was conducted using Aroclor 1260 added to wastewater obtained from Notwane Sewage Treatment Plant by measuring shift in the wavemax (λ_{max}) nm using Cary 300 UV-visible spectrophotometer, for a period of 96 hours. The presence/absence of the compounds was checked using High Performance Liquid Chromatography (HPLC) UFLC Shimadzu using florescence detector pump RF-20A and system gold column C18 (CTO-20A) after 96 hours.

The bacterial strain MD2 was found to have completely degraded the Aroclor1260 in wastewater after 96 hours. A shift in maximum wavelength was observed to be from 224 nm to 270 nm, an indication that Aroclor 1260 was degraded forming a chlorobenzoate derivative. This renders the wastewater free from polychlorinated biphenyls thus can be safely recycled.

Keywords: Aroclor 1260; Biodegradation; Polychlorinated biphenyls; Recycle; Wastewater

Introduction

Water scarcity has been reported to be increasing in Africa in general and the problem is worsened by the rapidly growing population in the region [1]. Wastewater treatment is also very essential as it does not only address the global problem of increasing water pollution [2] but may also be a means of alleviating the problem of global water scarcity. According to Onda et al. [3], 40 million m³ of wastewater are recycled in the world everyday of which 70% is channelled into the Agricultural sector. The remaining 30% is used in the industrial activities such as boilers and cooling towers [3]. The human activities in developing countries in trying to improve living and survival conditions have also resulted in emerging pollutants and POPs being found in wastewater [3] making it even difficult for the low and middle income countries, especially in Sub-Sahara Africa to manage wastewater. The wastewater eventually spills off these wastewater holding dams into the environment thus polluting the soils [4].

Chemicals are used extensively and intensively in our technological society [5,6]. Depending on their properties, modes and extent of use, these large amounts of chemicals reach the environment through wastewater, and have unpredictable environmental and health effects in the long term [6]. Heavy metal and chemical pollution of soil and wastewater is a significant environmental problem [7]. Some of the recalcitrant compounds such as polychlorinated biphenyls (PCBs) are highly toxic and can act as mutagens and carcinogens, in the bodies of living organisms [8].

Wastewater has not yet been frequently considered to be used to produce drinking water except in Windhoek and Singapore [3,9]. However, this research suggests that grey water as well as sewage water may be treated to an extent that this water is safe for household use

and this will curb the problem of water supply shortage in developing countries such as Botswana. In Botswana, the water from sewage ponds is partially recycled as it can only be used for watering plants in recreational parks and construction projects, although not always.

The adoption of Millennium Declaration signed at the United Nations General Assembly in September 2000 and its eight (8) set goals, the Millennium Development Goals (MDGs), by WHO led to joint efforts to establish a comprehensive global framework to support the attainment of these goals [10]. The MDGs were to be achieved by 2015 by all [10]. Of the eight MDGs, none directly addressed wastewater but when WHO assessed the MDGs, wastewater was found to be infused in most of those goals with goal number 7 (To ensure Environmental Sustainability) mostly inclined but since the guidelines were built around the health component and implementation component, the issues on wastewater were infused in most of these goals [11]. The emphasis though, was that the use of wastewater to alleviate problems associated with water stress and scarcity should not have negative health nor cost implications and therefore the methods employed in rendering the wastewater in a condition to be used in the agricultural sector must be effective as well as cost effective [11]. This put a lot of

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pressure to the developing and middle income countries as they were the most affected by this [10]. The WHO projections for water supply worldwide though shows that by 2025 half of the world population will be leaving under water stress conditions [12] despite the fact that large volumes of wastewater get lost without being recycled.

The incorporation of biological means or rather the use of microorganisms has not been highly infused and thus need to be explored and incorporated as it is found to be cost effective and effective in ensuring safe product after the treatment of sewage wastewater [13].

UNEP [14] reported though that in Africa the key challenges in relation to management of POPs was mainly due to lack of knowledge and information on hazards, risks and safer alternatives; lack of legislation or reinforcement measures; weaknesses in the technical infrastructure; and a shortage of qualified human resources which led to availability of data on PCBs being extremely minimal although these compounds are among the most abundant ubiquitous persistent organic pollutants (POPs) in the environment [15,16]. These compounds accumulate in the food chain and have adverse effects on animals and human health; and are also toxic to other living organisms thus affecting the functioning of the whole ecosystem [17]. This was confirmed in the report by MEWT [18] for Botswana.

Conventional physical and chemical methods used to decontaminate wastewater from pharmaceutical and textile industries are time and energy consuming [19]. The treatment also does not guarantee that the contaminant will be sufficiently removed to allow the water to be recycled [19]. This results in compounds (recalcitrant), being released to the environment in effluent thus added to the soils in the surrounding environment and water bodies [20,21]. The use of bacteria results in complete cleanup of wastewater rendering it pollutant free and as such it is the preferred means of wastewater treatment [22]. Some bacterial strains present in sewage dams, such as *Pseudomonas*, *Acinetobacter*, *Alcaligenes* and *Proteus*, can break down various chemical substances, including recalcitrant compounds, during their normal metabolic activities [7,23]. Although wastewater is passed through various treatment stages at the wastewater treatment plants, this does not give ample time for recalcitrant compounds some of which have carcinogenic and teratogenic effects on humans such as polychlorinated biphenyls (PCBs), to be broken down thus the possibility of having those compounds passed to the effluent [13].

According to Al-Jumaily [24], bacteria can adapt and grow in the presence of hazardous compounds or any waste stream. These characteristics and the underlying molecular mechanisms thereof, are of great importance in the development of enhanced wastewater recycling methods [13,24]. In degradation of PCBs, chlorobenzoic acids (CBAs) are formed which are biodegradable by most bacteria [25].

The incorporation of biological means (bioremediation), as a tool to improve the quality of recycled water, has not yet been investigated extensively, especially with regard to wastewater in Botswana, thus the need for this study.

Materials and Method

Sample collection

Water released from the wastewater treatment plant (effluent) was obtained from Notwane Sewage Treatment Plant situated in Gaborone, Botswana. Water samples were collected using sterile 250 mL and 500 mL Duran Schout bottles and properly labelled and transported on ice to Department of Biological Sciences, North-West University -

Mafikeng Campus for analysis. Samples were analysed within 24 hours from the time of collection. Samples were analysed within 24 hours after sampling.

Biodegradation of PCBs in wastewater by isolate MD2

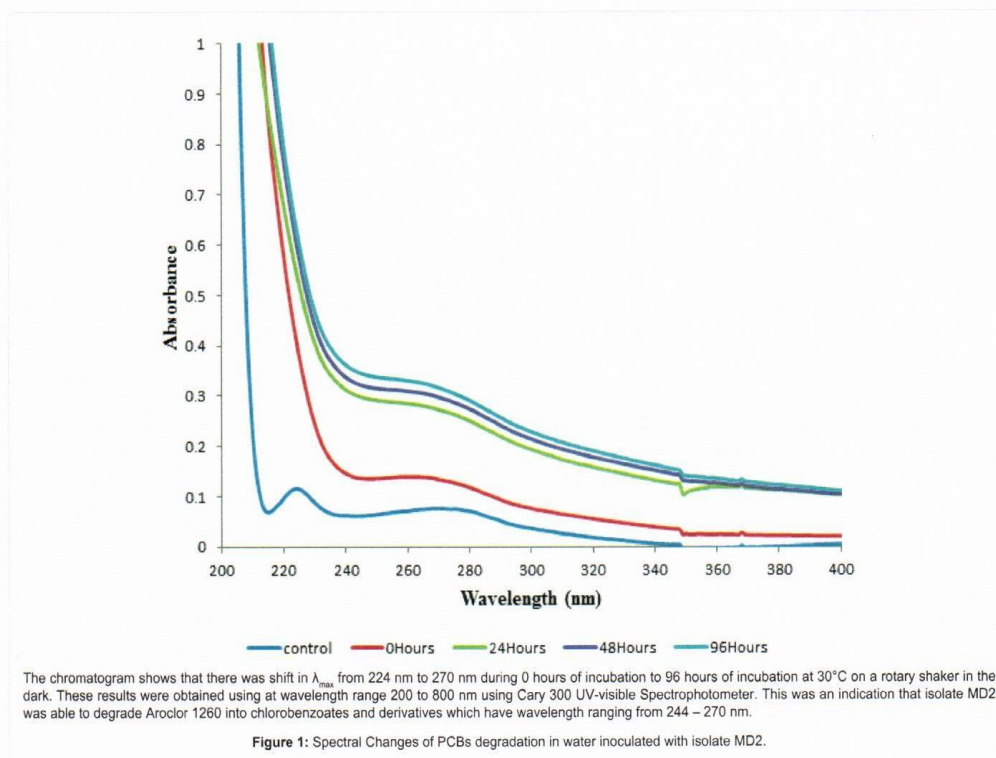
Wastewater samples that were collected from Notwane Sewage Treatment Plant was used to evaluate the level of biodegradation of PCB (Aroclor 1260) when introduced directly in to the wastewater samples. The samples were divided into two parts, one part was sterilised by autoclaving at 121°C for 15 minutes and the other half was left unsterilized. The unsterilized and sterilized wastewater was further divided into two parts. The wastewater samples were treated with PCBs. To each 100 mL of unsterilized wastewater sample that was contained in a 250 mL flask, 100 µL of Aroclor 1260 was added. In addition, bacteria inoculum was added to one of the flasks containing the unsterilized wastewater. A 100 mL portion of the sterilised wastewater samples were inoculated with the test organism (Isolate MD2, *Pseudomonas* species) while the other portions served as negative controls (without bacteria inoculation). The water samples were incubated at 30°C in the dark in a rotary shaker at 150rpm and 2 mL of the samples were taken at 24 hour intervals. These samples were used to determine the change in PCBs level at wavelengths of 200-800 nm using Cary 300 UV-visible spectrophotometer.

Evaluation of the biodegradation of PCB (Aroclor 1260) introduced directly into wastewater using HPLC analysis

The High Performance Liquid Chromatography (HPLC) (model UFLC Shimadzu) was also used to assess the biodegradation of PCB when introduced into wastewater [26]. In achieving this 1mL of water sample was analysed for residual PAH and PCB. The compounds were extracted by adding 10 mL each of dichloromethane and acetone. The mixture was incubated in a rotary shaker for 24 hours at 30°C and later centrifuged for 10 minutes at 12000 rpm at 4°C using Hermle Z326k high speed micro-centrifuge, Labortechnik GmbH (LASEC, South Africa). The extra water was pipette and 4 g of anhydrous sodium sulphate was added to remove residual water. The extract was concentrated to 1.5 mL using rotary evaporator Stuart RE300DB, LASEC, South Africa and filter sterilized with 0.45 µm PTFE syringe filters. The resulting extracts were used to perform the HPLC analysis using a fluorescence detector pump (RF-20A) and a system gold column C18 (CTO-20A) on a UFLC Shimadzu equipment as described by Roy et al. [26]. The excitation level was set at 254 nm and the emission level was at 390 nm. The mobile phase used was a mixture of acetonitrile and water (80:20) as described by Roy et al. [26]. Data analysis was computed using Real-time Analysis. All chemicals used were of HPLC grade products supplied by Sigma Aldrich through Lehlabile Scientific, South Africa.

Results and Discussion

Analysis for biodegradation of polychlorinated biphenyls (PCBs) in wastewater was achieved by introducing the compound (Aroclor 1260) in samples of wastewater obtained from Notwane Sewage Treatment Plant in Gaborone and inoculating the water with bacteria isolate MD2. Following 24 hours incubation the results obtained from the UV-VIS Spectrophotometer showed that there was a shift of maximum wavelength from 224 nm to 258 nm (Figure 1). Furthermore, the absorbance had increased from 0.116 to 0.314. These results indicated that the PCBs were broken down forming new products with different wavelength and thus the change. After 96 hours incubation the maximum wavelength was observed to be at 270 nm



(Figure 1) with absorbance of 0.332. This implies that the bacteria were able to use PCBs as their sole sources of carbon thus biodegradation of these compounds added to wastewater. The increase in absorbance is an indication that there has been bacterial growth which might be an indication that the bacteria isolate MD2, a *Pseudomonas* isolate from Notwane sewage treatment plant wastewater, was capable of breaking down Aroclor 1260 which is a PCB with 60% chlorine atoms during its metabolic activities and use it for growth. The HPLC results confirmed that these results were reliable as the screening for the compound following extraction of the same aliquot product of the experiment did not show the presence of the compound. The degradation of PCBs by *Pseudomonas* strains from Notwane Sewage Treatment Plant has not been reported before and thus these results suggest that strains from Gaborone wastewater have the ability to degrade recalcitrant compounds as reported from other studies in different areas [7,25,27].

The shifts are an indication of the presence of initial ring oxidation metabolites and ring fission metabolites [25,27] PCBs first get degraded into chlorobenzoates [27] that have been found to have λ_{max} ranging from 244-270nm in the C-band when dissolved in water [28], a range that was observed in the results obtained after 24 hours of incubation of treated wastewater with bacterial isolate MD2 used in this study and PCBs aroclor 1260 (Figure 1) which proves that the bacteria was able to breakdown the compound. The product chlorobenzoate is biodegraded into benzoate and eventually pyruvate and acetylaldehyde which are essential in the tricarboxylic cycle (TCA) [25,27].

The results obtained from HPLC were confirmatory for the

degradation of Aroclor 1260 by isolate MD2. The results indicated that there was difference in the chromatogram obtained from the experiments, the controls and the standards. Although concentrations were not determined, the results indicated absence of Aroclor 1260 after 96 hours of incubation which is an indication that the bacteria degraded the compounds. The chromatogram obtained for the experiments were compared with the chromatogram for the PCB standard using the retention times for the compounds in the standard. The results for the chromatogram obtained for the PCB standard was presented in Figure 2.

When the polychlorinated biphenyl (PCB) was added to wastewater sample with isolate MD2 not added, the results showed that there was breakdown of PCBs (Figure 3). The resultant products had retention times different from those obtained for the standard used in the study. This was used as an indication that biodegradation did occur resulting in new compound as was also the findings in other studies [26].

In Figure 4 the HPLC results depicted many peaks which proved that with absence of bacteria there was no degradation of Aroclor 1260 in the wastewater during the time of incubation. The results confirmed the role of the bacteria isolate MD2 in the biodegradation process of PCBs.

The wastewater was sterilised and thereafter inoculated with the test organism and Aroclor 1260 was also added. Figure 5 showed that no peaks were detected by the HPLC which implies that there was no detectable compound. This is an indication that the polychlorinated

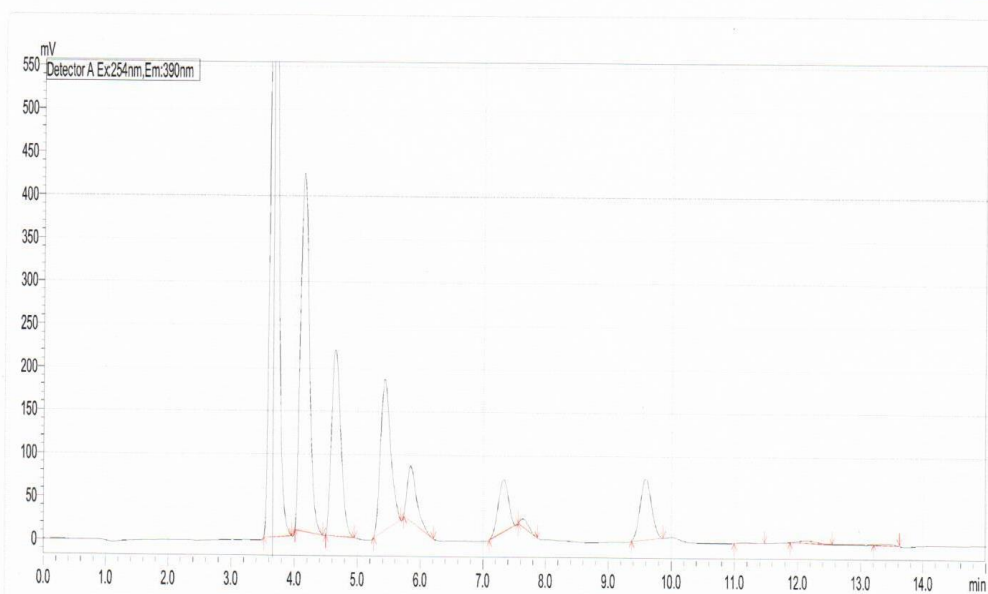


Figure 2: HPLC Chromatogram for PCBs standard using a Fluorescence detector method at 10 ml injection volume.

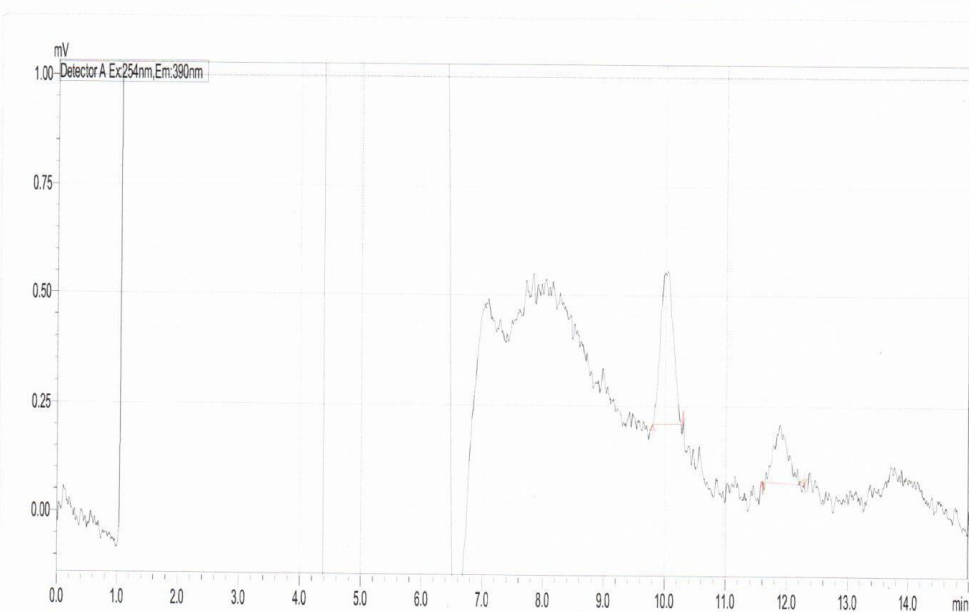
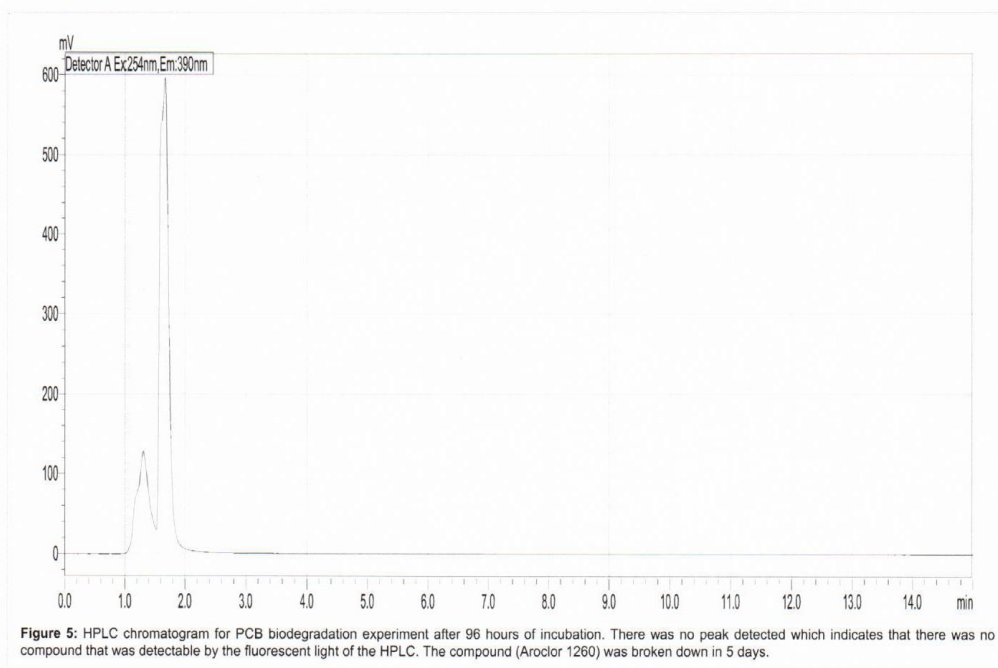
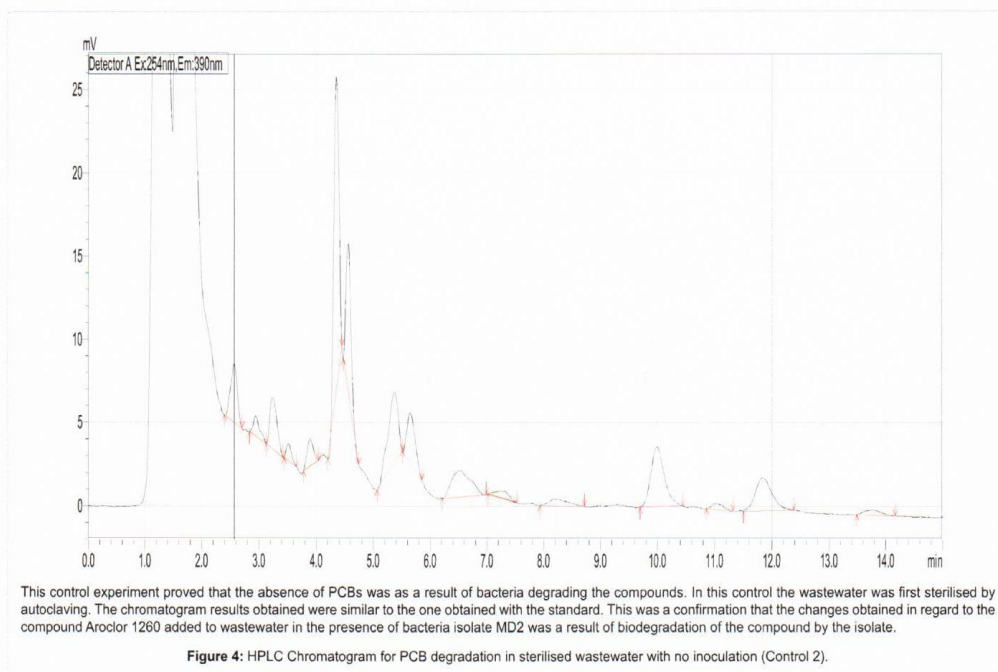


Figure 3: HPLC Chromatogram obtained after an experiment to analyse PCB degradation in unsterilized wastewater (Control 1).



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biphenyls added to the wastewater were completely broken down by isolate MD2 resulting in complete elimination of the compound. The results from the HPLC confirmed that the compound was biodegraded by the bacteria isolate MD2 as presented in Figure 5 which was also proved by Raja et al. [7], Heider and Rabus [23] and Roy et al. [26] in their studies. No PCB compound was detected after 96 hours of exposure to the bacteria in wastewater.

Conclusion

This study proves that the *Pseudomonas* strain, MD2, isolated from wastewater in Notwane Sewage Treatment Plant can be used in biodegradation of recalcitrant polychlorinated biphenyls thus rendering the water free from these carcinogenic compounds. The water can be safely recycled fully or redirected to Gaborone dam thus curbing the problem of water shortage, taking into consideration other factors, such as total coliforms, *Escherichia coli* and other pathogenic organisms and chemicals, that have to be within the expected standards according to international World Health Organization (WHO) standards as well as Botswana Bureau of Standards (BOBS) limits.

Acknowledgements

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Original Research Article

Characterization of bacteria with biodegradation qualities for recalcitrant compounds isolated from wastewater from Gaborone (Botswana) and Mafikeng (South Africa)

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Abstract

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Water is a scarce resource and this indicates the need to have measures in place to effectively and efficiently treat wastewater for safe recycling to curb the problem of water shortage especially in the rural areas of middle income and developing countries such as Botswana and South Africa. Wastewater does contain recalcitrant compounds, some of which are polycyclic aromatic compounds (PAHs) and polychlorinated biphenyls (PCBs) that have carcinogenic effects. A mixed bacterial population is often required for biodegradation of these compounds. A total of 60 raw and 50 wastewater samples were collected from Gaborone, Botswana and Mafikeng, South Africa. Analysis of bacterial 16S rRNA gene sequence using BLAST revealed the presence of *Aeromonas*, *Bacillus*, *Pseudomonas*, and *Vibrio* species. Interestingly these organisms are known to have the potential of degrading different compounds, including those that display recalcitrant characteristics, such as, PCBs and PAHs. *Vibrio* species are highly pathogenic but they have carbon catabolite repression (CCR) that is very crucial in the biodegradation of recalcitrant compounds in aquatic environments. This is of great economic importance in the water purification industry especially for middle income countries since the use of microorganisms in biodegradation is cost effective, reliable and results in complete clean-up of wastewater.

Keywords: Biodegradation, Polymerase chain reaction, recalcitrant compound, recycling, wastewater

INTRODUCTION

Water is an essential natural resource which needs to be conserved. Potable water is still a challenge in most parts of developing countries as well as in some rural settings in developed countries in Sub-Sahara Africa. Despite the fact that there is serious water shortage, a lot of water is used but not taken back into the water reticulation system for reuse. Wastewater is still inadequately treated thus lost to the environment as effluent as it contains hazardous compounds that have recalcitrant

characteristics. These compounds require a mixed bacterial population for complete biodegradation. This study was sought to isolate such bacteria.

The aim of this study, therefore, was to characterise bacteria isolated from raw water and wastewater and to use these organisms in the biodegradation of recalcitrant compounds viz; polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) with an objective to render wastewater potable for human consumption

(recycling purposes).

Wastewater has to be carefully and effectively treated to ensure that it is safe and made potable as it comes from various industries, factories, farms and households. Chemicals are used extensively and intensively in the technological society (Cycon et al., 2013; Guillen et al., 2012) and some of these compounds are recalcitrant with harmful effects to humans and animals (Raja et al., 2009). Recalcitrant compounds such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) have very serious harmful effects ranging from being carcinogenic and immune suppressive to being teratogenic in humans and other animals (Hennessee et al., 2009).

Depending on their properties, modes and extent of use, large amounts of these compounds reach the environment, and have unpredictable environmental and health effects in the long term (Guillen et al., 2012). Wastewater has been found to contain vast populations of microorganisms that have some biodegradation qualities for these recalcitrant compounds (Han et al., 2013; Lim et al., 2005). Enterobacteriaceae is one of the major groups of bacteria found in raw and wastewater (Grimont and Grimont 2006). Although the genus *Pseudomonas* is predominant in sewage wastewater (Bahig et al., 2008), other genera such as *Chromobacterium*, *Flavobacterium*, *Hyphomicrobium*, *Achromobacter*, *Agrobacterium*, *Alkalegenes*, *Bacillus*, *Serratia* are also found to inhabit both raw and wastewater (Grimont and Grimont 2006; Lim et al. 2005) due to their different roles in water such as biodegradation and denitrification processes (Lim et al., 2005). Use of microorganisms in wastewater treatment is both cost effective and efficient resulting in completely cleans up of all pollutants (Abor-Amer 2011; Sehnem et al., 2010; Al-Thani et al., 2009).

MATERIALS AND METHODS

Collection of Water samples

A total of 110 samples were collected from four sites viz: Gaborone dam (A, 30 samples) and Notwane (C, 25 samples) both in Botswana; Disaneng dam (B, 30 samples) and Modimola/Setumo (D, 25 samples) both in South Africa, between the months of April 2013 and March 2014. Normal tap water was used as controls were obtained from Gaborone and Mafikeng. pH and turbidity were measured at sampling sites (in-situ). Water samples were collected in sterile 250ml Duran bottles with screw-cap and immediately placed on ice in a cooler box. The samples were taken to Department of Biological Sciences Molecular Microbiology Research Lab, North-West University for analysis. Samples were analysed within 24 hours after collection.

Analysis of Water Samples

Determination of physical parameters in water samples

The pH, temperature and turbidity of samples were taken at sampling sites during sample collection. The turbidity (Nephelometric Turbidity Units (NTU)) was measured using Orbeco Hellige TB 200™ Potable Turbidimeter from LASEC, South Africa. The pH and temperature (°C) were measured using Crison PH 25 – Spain supplied by LASEC, South Africa at sampling sites.

Microbiological isolation of bacteria

A volume of 1ml of sample water was mixed with sterile molten Plate Count Agar (PCA) (BIOLAB) for initial isolation of bacteria. The inoculated plates were allowed to solidify before they were incubated at 37°C for 24 hours. Different colonies of isolates were picked using a sterile loop and sub-cultured on Tryptone Soy Agar (TSA) (Sigma Aldrich) to obtain pure cultures. Pure isolates were sub-cultured in TSA slants and incubated at 37°C for 24 hours. The slants were kept at 4°C for later analysis. The replica isolates were subjected to various bacteria identification tests.

Bacteria Identification tests

Gram staining

The Gram staining standard technique (Andrews, 1992) was used to determine the morphological characteristics of the isolates.

Analytical Profile Index (API) 20E

A total of 25 isolates (both gram negative and gram positive) were randomly picked and analysed using API 20E strips (BioMérieux, Marcy-L'Etoile / France) for Enterobacteriaceae. This type of API was chosen as the isolation was general (not target specific). The test was performed according to the manufacturer's instructions (BioMérieux, Marcy-L'Etoile / France). Of the 25 isolates, 5 were selected from sites B, C and D. The other isolates were selected from isolates obtained from site A as it had more isolates that looked different from each other from the morphological observation. Results were read with or without the addition of reagents. The indices were generated for the different isolates and these were used to determine their identities using the API Web™ Identification software.

DNA Isolation and Polymerase Chain Reaction (PCR) amplification of bacteria 16S rRNA gene fragments

DNA Isolation

Pure isolates from TSA (Sigma Aldrich) were inoculated into 5ml of nutrient broth and incubated aerobically at 37°C for 24 hours while shaking. Genomic DNA was extracted from all isolates using Zymo Research Genomic DNA™ –ZR Fungal/Bacterial DNA MiniPrep kit (Catalogue No. D6005-USA supplied by BioRad, South Africa) following manufacturer's instructions. Briefly, 100µl from the broth culture was aliquoted into Bashingbead™ lyses tube, then 750µl lyses solution. The contents were processed using a bead beater (Disrupter Genie™) at maximum speed for 5 minutes. This was followed by centrifuging at 10000 rpm for 1 minute using a bench-top Hermle Z300 high speed micro centrifuge (Kendro, Germany). 400µl of the supernatant was transferred to Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged at 7000 rpm for 1 minute. 200µl of DNA binding buffer was added to the filtrate. The mixture was transferred to a Zymo-Spin™ IIC Column in a collection tube. The contents of the tube were centrifuged at 10000 rpm using a bench-top Hermle Z300 high speed micro centrifuge (Kendro, Germany) for 1 minute and the supernatant in collecting tube was discarded. The pellet was re-suspended in 200µl of DNA Pre-buffer to each spin column in a new collection tube, centrifuged as mentioned above and the resulting supernatant was discarded. This was followed by adding 400µl of g-DNA Wash Buffer to the spin column and centrifuged at 10000 rpm for 1 minute and the resulting supernatant was discarded. Finally, 100µl of DNA Elution Buffer was added to each tube, the tubes were incubated for 5 minutes and then centrifuged at 13500 rpm for 30 seconds to elute the DNA into the new eppendorf tube. DNA samples were stored at -20°C for future use.

PCR for the amplification of bacteria 16S rRNA gene fragments

Nineteen (19) of the initial 25 isolates selected for API tests were further subjected to PCR analysis. Bacterial 16S rRNA gene target sequences were amplified from all the 19 isolates using previously tested primer sequences (Hilyard et al., 2008; Muyzer et al., 1993). The 16S rRNA universal primers, U758 (R) CTACCAGGG-TATCTAATCC and U341 (F) CCTACGGGAGGCAG-CAG (Muyzer et al., 1993) were used to amplify about 500 base pair (bp) gene fragments. Amplifications were carried out using a C1000 Touch™ Thermal Cycler (Bio-Rad, South Africa). PCR amplification was performed in a total volume of 25µl made up of 12.5µl master mix (Thermo scientific PMM), 8.5µl of nuclease free water, 1.5µl of loading buffer, 0.5µl oligonucleotide primer set

and finally, 2µl of DNA template. All the reagents were obtained from Inqaba Biotech Ltd, South Africa. Amplification was achieved through the following parameters: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The PCR products were stored at 4°C before separation by electrophoresis.

Agarose gel electrophoresis

The PCR products were separated by electrophoresis on a 1% (w/v) agarose gel. The gel was run for 60 minutes at 80V and 250MA using 1x TAE buffer (40mM Tris, 1mM EDTA and 40mM glacial acetic acid, pH 8.0) using a horizontal Pharmacia biotech equipment system (model-BCMSCHOICE; Biocom, UK). Each gel contained a 1kp DNA molecular weight marker (Fermentas, USA). The gel was stained in ethidium bromide (0.1µg/ml) and amplicons were visualized under U.V light at 420nm wavelength. A ChemiDoc Imaging System (Bio-Rad ChemiDoc™ MP Imaging System, UK) was used to capture the image using Gene Snap (version 6.00.22) software. Isolates were further subjected to DNA sequencing and blasting.

Bacterial 16S rRNA sequencing and sequence analysis

The PCR product was sent to Inqaba Biotechnical Industries (Pty) Ltd, South Africa for sequencing. Sequencing was done using the Sanger technique. The sequenced products were analysed using the Chromas Lite version 2 and BIOEDIT Sequence Alignment Editor Program. The cleaned sequences were subjected to MEGA 6.06 (6140226) and MAFFT programs for characterisation of the organisms and construction of a phylogenetic tree.

RESULTS

Physical Parameters

The mean pH, turbidity and temperature of the samples are presented in Table 1. As shown in the Table, samples obtained from Site D (Modimola dam) had the highest turbidity and pH values while those obtained from Site C (Notwane Sewage treatment plant) had the highest temperature values. In addition, the turbidity values of both control samples (tap water) was 0.01NTU when compared to the samples analysed. Moreover, the pH values and turbidity for the control samples were within the acceptable standard reference for drinking water.

Table 1. Average Presentation of the physical properties of water from four sampled sites

Sample source	Mean Turbidity (NTU)	Mean pH	Mean Temperature (°C)
A (Gaborone Dam)	10.12 – 10.68	8.26 – 8.64	9.2 – 24.5
B (Disaneng Dam)	13.31 – 25.09	8.96 – 9.04	8.0 – 26.7
C (Notwane Sewage Treatment Plant)	12.44 – 20.34	8.99 – 9.77	10.1 – 26.2
D (Modimola Dam)	25.10 – 200.6	9.01 – 9.78	9.0 – 24.2
Control – 1	0.01	7.78	24.4
Control – 2	0.01	7.88	26.1

Samples obtained from Site D (Modimola dam) had the highest turbidity and pH values while those obtained from Site C (Notwane Sewage treatment plant) had the highest temperature values. The pH values and turbidity for the control samples were within the acceptable standard reference values for drinking water.

Table 2. Preliminary identification test results for isolates obtained in the study

Site	Isolate ID	Gram Stain	Glucose Fermentation	Catalase	Oxidase	Indole	Urea	H ₂ S production
A	GD2 (S5)	+ rod	+	+	+	-	-	-
	GD6 (S18)	+ rod	+	+	+	-	+	-
	GD8 (S21)	- rod	+	+	-	-	-	-
	GD9 (S19)	+ rod	+	+	+	-	-	-
	GD11 (S16)	+ rod	+	+	-	-	-	-
	GD12 (S13)	+ rod	+	+	-	-	-	-
	GD14 (S23)	- rod	+	+	-	-	-	-
	GD18 (S15)	+ rod	+	-	+	-	-	-
	GD19 (S12)	+ rod	+	+	+	-	-	-
	DS1 (S2)	- cocci	+	+	+	+	-	-
B	DS2 (S25)	- rod	+	+	+	-	-	-
	DS4 (S20)	- rod	+	+	-	-	-	-
	GS1 (S24)	- rod	+	+	-	-	-	-
C	GS2 (S6)	+ rod	+	+	+	-	-	-
	GS4 (S17)	-vibrio	+	-	-	-	-	-
	GS6 (S7)	-vibrio	+	-	-	-	-	-
	GS7 (S14)	+ rod	-	+	-	-	-	-
	GS8 (S1)	+ rod	-	+	+	-	+	-
	GS10 (S9)	+ rod	-	+	-	-	-	-
	GS11 (S10)	+ rod	+	+	+	-	-	-
	GS12 (S11)	-vibrio	+	-	-	-	-	-
	MD1 (S3)	- cocci	+	-	+	-	+	-
	MD2 (S4)	- cocci	+	+	+	-	-	-

73.9% of the isolates were rod shaped bacteria of which 70.6% were gram positive. 94.1% of these rod shaped bacteria were catalase positive. 13.0% of the isolates were gram negative vibrio and cocci, respectively. Out of the total number of isolates, 95.7% of the isolates were negative for indole utilization, 87.0 % were negative for urea breakdown and none of the isolates gave a positive result for production of hydrogen sulphide gas.

Morphological and Some Analytical Profile Index (API) 20E Results

The samples obtained from Sites A and C (both in Gaborone) were found to be more contaminated than those from sites B and D (both in Mafikeng region). No bacterial isolates were obtained from the control samples. A total of 23 isolates were randomly selected from all the sites and subjected to further analysis using biochemical tests. The selection criterion was mainly based on differences in colonial characteristics. These were

subjected to Gram staining, oxidase tests, catalase and API 20E tests. The results are presented in Table 2.

As shown in Table 2 a large proportion (73.9%) of the isolates were rod shaped bacteria. Amongst these rod shaped cells, 70.6% were Gram positive bacteria. On the contrary, only small proportions (13.0%) of the isolates were Gram negative vibrio and cocci respectively. A large proportion (94.1%) of the rod shaped bacteria were catalase positive while all the vibrio isolates were catalase negative. 95.7% of the isolates were negative for indole utilization, 87.0% were negative for urea

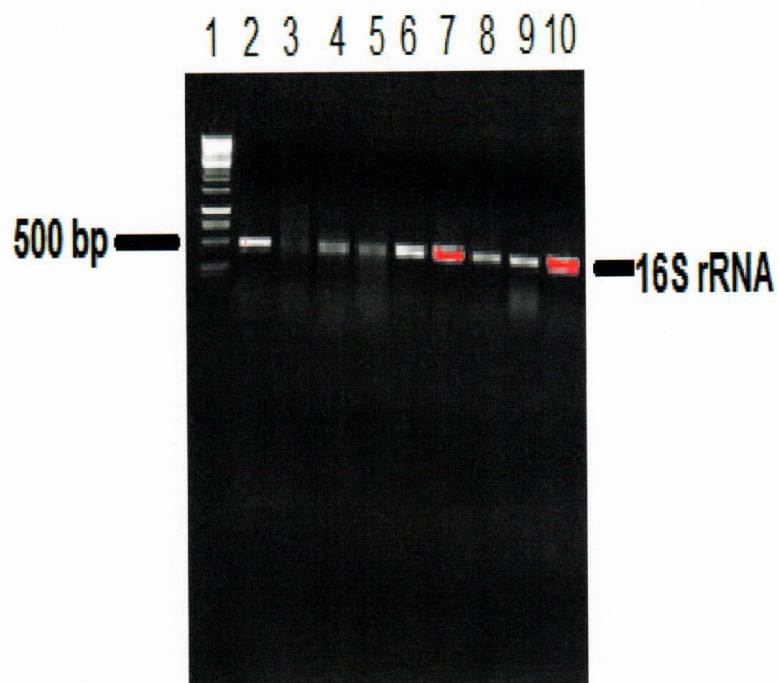


Figure 1. A 1% (w/v) agarose gel depicting bacterial 16S rRNA gene fragments amplified from isolates in the study. Amplification of bacterial 16S rRNA produced a 500 bp gene fragments. 1% (w/v) agarose gel of the PCR products was obtained.

Table 3. Organisms as identified based on bacterial 16S rRNA gene sequence analysis using BLAST search.

Site	Isolate ID	Identification based on 16S sequence analysis	Accession No closely related sequence and percentage similarity
A	GD2 (S5)	<i>Bacillus subtilis</i>	FJ 864727, 96%
	GD6 (S18)	<i>Bacillus megaterium</i>	JX475126, 90%
	GD9 (S19)	<i>Bacillus subtilis</i>	FJ544346, 98%
	GD19 (S12)	<i>Bacillus aryabhattai</i>	KC 550562, 98%
	GD11 (S16)	<i>Bacillus megaterium</i>	GQ 274925, 95%
	GD12 (S13)	<i>Exiguobacterium indicum</i>	JF 935075, 96%
	GD18 (S15)	<i>Bacillus megaterium</i>	HF570085, 93%
B	DS1 (S2)	<i>Pseudomonas aeruginosa</i>	KJ 101516, 99%
C	GS8 (S1)	<i>Bacillus subtilis</i>	EU373328, 94%
	GS4 (S17), GS6 (S7), GS12 (S11)	<i>Vibrio cholerae</i>	KJ413142, 90%, HM 590223, 97%, DQ991212, 94% respectively
	GS10 (S9)	<i>Bacillus cereus</i>	KF 478221, 90%
	GS7 (S14)	<i>Kurthia gibsonii</i>	KJ 396180, 93%
	GS2 (S6)	<i>Exiguobacterium acetylicum</i>	KF 963623, 94%
	GS11 (S10)	<i>Bacillus aryabhattai</i>	KJ186075, 95%
	MD1 (S3)	<i>Pseudomonas species</i>	KF708909, 98%
D	MD2 (S4)	<i>Pseudomonas aeruginosa</i>	CP 006832, 95%

All the isolates obtained in the present study showed very close similarities (90% to 99%) to those found in Genebank. A large proportion of the rod shaped bacteria were identified as *Bacillus* species while the vibrio shaped were *Vibrio cholerae*.

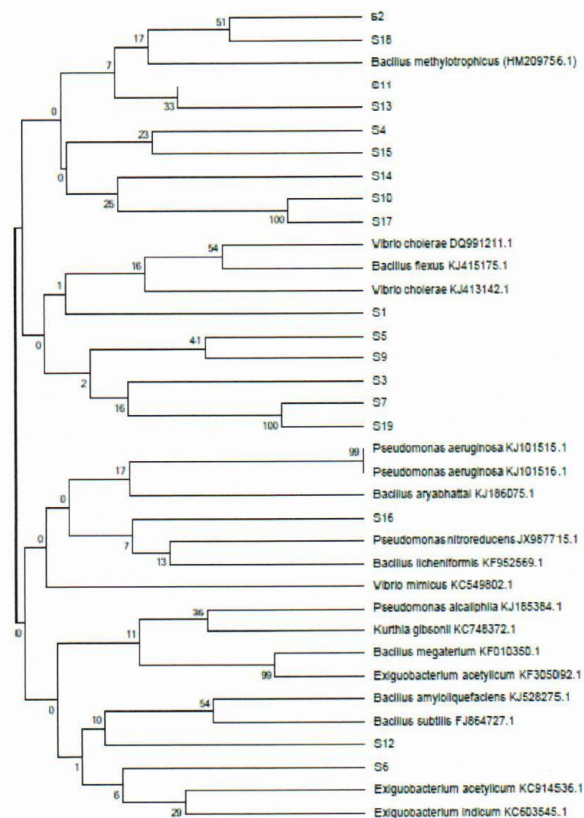


Figure 2. Phylogenetic tree showing relatedness of isolates to other previously identified bacteria.

The evolutionary history was inferred using the UPGMA method resulting in optimal tree with the sum of branch length = 44.02349177. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 178 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

breakdown and none of the isolates showed positive results for production of hydrogen sulphide gas (Table 2).

Bacterial 16S rRNA gene amplification

Amplification of bacterial 16S rRNA produced a 500 bp gene fragments. The results are presented in Figure 1 indicates a 1% (w/v) agarose gel of the PCR products.

Sequencing and sequence analysis

Bacterial 16S rRNA gene fragments were sequenced and analysed to determine the identities of the isolates. The data in Table 3 depicts the identities of isolates, the accession numbers of closely related strains or sequences and the percentage similarities of the amplified sequences test isolates to those that are found in Genbank. All the isolates obtained in the present

study showed very close similarities (90% to 99%) to those found in Genbank. A large proportion of the rod shaped bacteria were identified as *Bacillus* species while the vibrio shaped were *Vibrio cholerae*.

The results in Table 3 were used to draw a phylogenetic tree that is shown in Figure 2. This was performed to determine the genetic relatedness of the isolates to organisms in Gene bank. The evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the Maximum Composite Likelihood. The analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6.

DISCUSSION

The mean pH values for all the water samples was found to be above 7.0 with mean temperatures ranging from as low as 8°C to as high as 27°C (Table 1). The alkaline conditions and moderate temperatures may not be favourable for the bacteria that have been found to be effective in the process of biodegradation of recalcitrant compounds. The most suitable pH range for biodegradation of PAHs and PCBs by bacteria in general, has been found to be between pH 6.0 and 7.0 and temperatures between 20 - 30°C (Zhang et al., 2012; Das and Chandran 2011; Cycon et al., 2009).

Since effective biodegradation of recalcitrant compounds requires a mixed bacterial consortium (Kapadia and Yagnik 2013), the presence of many different types of bacterial isolates (Table 3) that included *Bacillus*, *Pseudomonas*, and *Vibrio* species should positively enhance the biodegradation of PAHs and PCBs in the water. Bacteria belonging to other genera such as *Exiguobacterium* and *Kurthia* were also identified and all these organisms are capable of carrying out biodegradation of PAHs and PCBs (Khleifat 2006). *Vibrio Cholera* although it has been found to be one of the major human clinical pathogen, it has been found to play an important role in the breakdown of phenol (Blokesch 2012; Khleifat 2006). *Vibrio* species are known to be highly pathogenic, but are also very crucial in the biodegradation of recalcitrant compounds in aquatic environments as they possess the carbon catabolite repression (CCR) characteristic (Blokesch 2012). This characteristic is essential in microorganisms as it enables them to utilise the not so readily available carbon sources (Blokesch 2012). *Pseudomonas* species have been found to play an important role in biodegradation of various compounds including POPs, through the production of biosurfactants (Lakshmi 2013) which are essential in the biodegradation of PAHs and PCBs (Kapadia and Yagnik 2013; Lakshmi 2013). Biosurfactants enables bacteria to bind to these

compounds as well as to increase their chances of hydrolysis as they decrease the compounds' hydrophobicity (Pacwa-Plociniczak, Plaza et al., 2011). These chemical substances (biosurfactants) have characteristics of biocompatibility, specificity and digestibility (Kapadia and Yagnik 2013; Pacwa-Plociniczak et al., 2011), it makes them to have a great advantage for bacteria involved in biodegradation.

Enterobacter species and *Serratia* species are also of vital importance when biodegradation of recalcitrant compounds is considered due to their ability to use petroleum products and petroleum hydrocarbons as potential sources of carbon (Cycon et al., 2013; Abor-Amer 2011; Darvish et al., 2011; Erdogan et al., 2011). *Exiguobacterium* species have tendency of developing resistance to xenobiotic compounds such as PAHs making them able to degrade such compounds (Jeswani and Mukherji 2012). In addition, *Bacillus* species was identified as the most diverse bacteria that can colonise any environment (Maughan and Auwera 2011) and this may explain why it was isolated from almost all the sites sampled.

The phylogenetic tree in Figure 2 showed that S12 and S16 were closely related to *Bacillus* species; S6 shared close genetic relationship with *Exiguobacterium acetylicum*. Similar observations had been reported (Vishnivetskaya et al., 2009).

CONCLUSION

There is diversity in the types of bacteria isolated. This has been shown by the phylogenetic tree. This is a positive indication for biodegradation of recalcitrant compounds. The presence of these organisms in the wastewater, despite the alternating changes in environmental conditions which may act as limiting factors, biodegradation of recalcitrant compounds such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls may be possible. This is still to be explored further though. The effects of varying pH and temperature in water on the breaking down PAHs and PCBs should also be studied. The effect of the PCBs and PAHs on the physio-chemical properties of wastewater (especially pH) will also be explored.

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Applying QuEChERS Method in Screening for Polychlorinated Biphenyls (PCBs) from Raw and Wastewater from Gaborone (Botswana) and Mafikeng (South Africa)

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ABSTRACT: Wastewater contains a variety of chemical substances owing to the different sources that contribute to affluent into sewage treatment plants. Raw and wastewater samples were obtained from the surface of water bodies at depths of 50 and 100 metres but at different locations in each sampling site. The water samples were subjected to extraction of polychlorinated biphenyls (PCBs) using QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction kit before analysis using the Gas Chromatography Mass Spectrometer (GC-MS). The control samples had turbidity, temperature and pH values within the acceptable levels as per Botswana, South Africa and WHO drinking water standards. Turbidity values were rather higher than the set standards for raw and wastewater, with water samples from Modimola/Setumo dam having recorded the highest range of 25.0 – 200 Nephelometric Turbidity Units (NTU). The same sampling site also had water samples with pH higher at 9.01 to 9.78. Wastewater effluent in Notwane Sewage Treatment Plant, Gaborone Dam (both in Gaborone) and Disaneng Dam (South Africa) have polychlorinated biphenyls (PCBs) below detectable levels by the Agilent Gas Chromatography Mass Spectrometer (GC-MS). Only traces of PCBs were detected from wastewater from Modimola dam in Mafikeng. This may be due to the different industries in Mafikeng producing various chemicals compared to Gaborone. The water in Modimola dam therefore requires thorough treatment before it can be returned for domestic consumption as PCBs are toxic compounds that have been found to trigger cancer in humans and also affect the reproduction system resulting in low IQ.

Key words: Wastewater, Polychlorinated biphenyls, Recycling, QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), Extraction

INTRODUCTION

Chemicals are used extensively and intensively in the technological society (Cycon *et al.*, 2013; Guillen *et al.*, 2012). Depending on their properties, the modes and extent of use, large amounts of these chemicals reach the environment, and have unpredictable environmental and health effects in the long term (Guillen *et al.*, 2012). Chemical pollution of soil and wastewater is a significant environmental problem (Raja *et al.*, 2009). The recalcitrant compounds, especially hydrocarbons and polymers such as polychlorinated biphenyls (PCBs) are highly toxic and can act as mutagens and carcinogens, within living organisms (Hennessee *et al.*, 2009; Mallick *et al.*, 2007).

However, in Botswana, the Integrated Resources Water Management (IRWM) focuses more on the salinity and general pollution (DWA, 2013) than POPs on water quality assessment guidelines although the

Stockholm convention urges countries to consider the elimination of POPs in wastewater (EPA, 2009; UNEP, 2009). UNEP (2009) reported that in Africa, the key challenges in relation to management of POPs was mainly due to lack of knowledge and information on hazards, risks and safer alternatives; lack of legislation or reinforcement measures; weaknesses in the technical infrastructure; and a shortage of qualified human resources. These factors are known to account for the extremely minimal available data on PCBs (Ministry of Environment, Wildlife and Tourism of Botswana, 2008).

The Department of Environmental Affairs (DEA) of South Africa amended the National Environmental Management Act of 1998 (Act No 107, 1998) to include the facing out of the general use of polychlorinated biphenyls (PCBs) containing materials and PCBs contaminated materials by 2023 and 2026 respectively

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(DEA, 2013). According to a report by the Ministry of Environment, Wildlife and Tourism of Botswana (2008), large quantities of PCB containing equipment (40 000 tonnes) was still in use in the country by 2006 and thus the number of contaminated sources could just have been that high too. These setbacks for African countries were mitigated by the development of country specific IWRM (WHO and UNEP, 2006). This study was therefore aimed at establishing the state of raw and wastewater with regard to POPs, and main focus being the polychlorinated biphenyls (PCBs).

The presence of chemicals in the environment calls for quantification of such so as to come up with risk analysis posed by those chemicals (Urbaniak, 2013; Guillen *et al.*, 2012). Substances such as polychlorinated biphenyls (PCBs) and pesticides are found in wastewater. The general chemical formula for PCBs is $C_{12}H_{10-6}Cl_n$, where n is any number between 1 and 10 (WHO, 2000). PCBs have varying numbers of chlorines in their structure (EPA, 2013). All PCBs are lipophilic and the lipophilicity increases with the number of chlorines attached to the molecule, thus, their low solubility to water (WHO, 2000). PCBs are mostly hydrophobic and some are less hydrophilic (Muir and Lohmann, 2013; Nwinyi, 2011).

PCBs are very stable compounds and do not decompose readily (Rudel *et al.*, 2008). Furthermore, PCBs have a long half life (8 to 15 years) and are insoluble in water (Rudel *et al.*, 2008). The biodegradability of PCBs is also dependent upon the number of chlorine molecules it has (Anyasi and Atagana, 2011; EPA, 2013). The more chlorine molecules contained in a compound renders that compound less biodegradable (EPA, 2013). PCBs are lipophilic and thus are mostly hydrophobic (Muir and Lohmann, 2013). These properties result in bioaccumulation of these compounds as they do not dissolve in water and thus render them difficult to be biodegraded (EPA, 2013; Muir and Lohmann, 2013).

Literature review revealed that a lot of work on removal of these pollutants from activated sludge has been done and little or no work has been done on effluents. According to Ying *et al.*, (2009), although most research conducted on the removal of these substances have been carried out, it is mainly on soil and activated sludge and very little done on wastewater. Wastewater is allowed to flow out into the environment and may pose health risks to animals and humans (WHO and UNICEF, 2014). This also results in these compounds being added to the soils in the surrounding environment. These soils become polluted with PCBs which poses a risk not only to plants that grow in those soils, but also to human being using the soils for farming (McDougall, 2004). Since these

compounds are not easily broken down, they accumulate in the environment (bioaccumulation) and can be passed from one trophic level to the other through feeding relations (Nabavi *et al.*, 2013; Sapozhnikova and Lehotay, 2013; McDougall, 2004) thus the need to have these compounds completely eliminated from the environment.

Several methods have been developed and used to determine quantitatively, these pollutants in wastewater derived mainly from sewage treatment plants (Ying *et al.*, 2009). For these PCBs to be removed from the wastewater, this study aimed at screening for their presence in the water. QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach is being used in determination of pollutants in several types of samples (Vera *et al.*, 2013). This is mainly based on its salting-out extraction with a solvent, followed by a clean-up procedure (Vera *et al.*, 2013). QuEChERS have been mainly used in the determination of pesticides in soils (Vera *et al.*, 2013).

The use of QuEChERS has not been employed in the screening of these compounds in water but in tissue samples of either fish or plant material from polluted water bodies (Sadowska-Rociek *et al.*, 2014; Sapozhnikova and Lehotay, 2013). This study therefore aims at closing this gap.

MATERIALS & METHODS

Water samples were obtained from Gaborone dam and effluent from the Notwane Sewage Treatment Plant in Gaborone, Botswana. The Notwane Sewage Treatment Plant was selected on the basis that it is the major wastewater treatment plant in Gaborone, receiving large volumes of wastewater from different industries, schools, medical facilities and households. Water samples were also obtained from Disaneng dam and the Setumo/Modimola Wastewater Treatment Plant effluent which is in Mmabatho, South Africa. Disaneng dam was chosen because of its raw water unlike Modimola/Setumo dam which has wastewater. These wastewater treatment plants receive wastewater from residential areas, industrial and agricultural sectors. Fig. 1 is a point map to show the sampling sites.

Ten (10) water samples from each site were collected for analysis using Gas Chromatography Mass Spectrometer (GC-MS). Tap water samples obtained from Gaborone and Mafikeng were used as negative controls. Samples were collected during the different seasons of the year. The samples were carried from sampling sites in cooler boxes filled with ice to keep samples at about 4°C. Physical parameters that included pH, temperature and turbidity of samples were determined immediately at the sampling sites during collection. Turbidity was measured using Orbeco Hellige

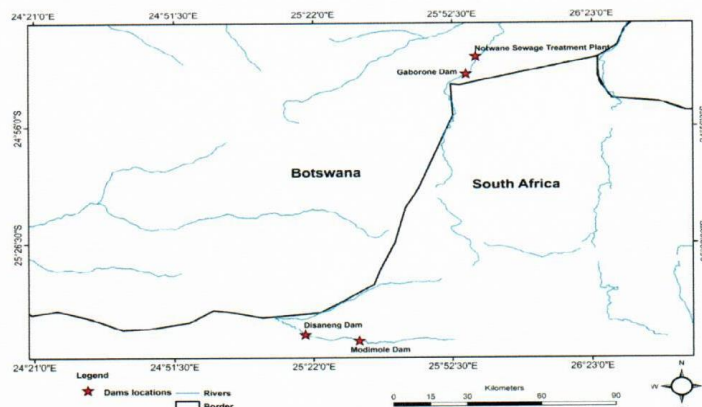


Fig. 1. A sketch map to show the sampling sites

(The sampling sites were in Gaborone, Botswana and Mafikeng area, South Africa so as to analyse the effect of the different industries in these two areas on components of wastewater/sewage effluent, in terms of PCBs composition.)

TB 200™ Potable Turbidimeter from LASEC, South Africa. The pH and temperature (°C) were measured using Crison PH 25 – Spain supplied by LASEC, South Africa. The raw water samples were obtained from the surface of the water bodies at depths of 50 and 100 metres but at different locations in each dam. Gas Chromatography Mass Spectrometer (GC-MS) was used to detect the presence of PCBs in water samples (Kawaguchi *et al.*, 2012; Mallick *et al.*, 2007). To achieve this, 250 mL polyethylene (PET) bottles were washed with nitric acid to remove impurities. Composite water samples (250 mL) were prepared by mixing samples from the different depths collected from the four sample sites of a particular dam. The identities of resolved products were determined by comparing results with the profiles of GCMS reference compounds.

Quality control was set using the water samples and five randomly selected pesticides that were available from the supplier (Sigma-Aldrich, Germany). The pesticides utilised were Aldrin, 4,4-DDT, 4,4-DDD, 4,4-DDE and Dieldrin. Stock solutions of the pesticides were prepared by dissolving 5 mg in 1 Litre of HPLC grade hexane obtained from Sigma Aldrich, South Africa. Spiked PAH dissolved in saline and PCB standards were also prepared (SUPELCO Solutions Within™ - USA). GC-MS calibration standard solutions were concentrated in HPLC grade toluene (Sigma Aldrich, South Africa). Standard stock solutions (1 ppm and 5 ppb) were prepared in HPLC grade acetonitrile (Sigma Aldrich, South Africa). These were used to spike water samples in preparation for GC-MS analysis. QUECHERS kit-AOAC, part number 5982-5755, Agilent Technologies, South Africa was used in this study.

As a quality control measure, water samples were divided into two parts of 10 mL each. Each part was further divided into two and spiked individually with 1 ppm and 5 ppb of each pesticide (Aldrin, 4,4-DDT, 4,4-DDD, 4,4-DDE and Dieldrin) stock solution. However, the other portions were not spiked with the pesticides and recalcitrant compounds. The main rationale is that, upon GC-MS analysis, the pesticides and recalcitrant compounds should be detected in the spiked samples when compared to the unspiked water samples. In addition, this procedure was used to evaluate the effectiveness of the QuEChERS method in the extraction of recalcitrant compounds from water samples (Furlani *et al.*, 2011).

To extract polychlorinated biphenyls from water samples, aliquots of 10 mL of water sample (both spiked and unspiked) were transferred into a 50 mL polypropylene centrifuge tube and 10 mL of HPLC grade acetonitrile added to it. The mixture was vortexed (model Vortex Genie 2) for 1 minute. Four grams of anhydrous $MgSO_4$ and 1g of NaCl were added to the mixture and vortexed for 1 minute. The mixture was transferred into a 15 mL polypropylene centrifuge tube and centrifuged for 5 minutes at 5000 rpm using a Labofuge 200 centrifuge (Heraeus Sepatech Germany) supplied by Phala Scientific (PTY) LTD, Gaborone. The centrifuged product was cleaned by transferring 4 mL aliquot of the upper layer (supernatant) into a polypropylene centrifuge tube containing 200 mg PSA and 600 mg anhydrous $MgSO_4$ (Furlani *et al.*, 2011). This was vortexed for 30 seconds using a Vortex Genie 2 supplied by Scientific Industries. The product was then centrifuged for 3 minutes at 3500 rpm using the

Labofuge 200 centrifuge (Heraeus Sepatech Germany) supplied by Phala Scientific (PTY) LTD, Gaborone. A 4ml aliquot of the supernatant was transferred into a vial and concentrated by blowing with nitrogen gas until it dried up. The product was topped up to 0.5 ml with toluene (Furlani *et al.*, 2011) in a vial and used for GC-MS analysis.

The vials were placed in an auto-sampler tray in Gas Chromatography equipment (model 7890A–Agilent Technologies and 5975C Mass Spectrometer Inert XL EI/CI MSD) with a Triple axis detector and samples were subjected to GC-MS analysis. The runs were performed under the following GC-MS conditions: 260°C Inlet –F temperature, 220°C oven temperature, 1.5 column l flow, 280°C Aux -2 temperature, 200°C MS source and 150°C MS quad (Brondi *et al.*, 2011; Furlani *et al.*, 2011).

RESULTS & DISCUSSION

The mean pH, turbidity and temperature of the water samples were determined and results are presented in Table 1. As shown in Table 1, samples obtained from Modimola dam had the highest turbidity (with mean values of 25.10 – 200.6) and pH (mean values of 9.01 – 9.78) values while those from Notwane Sewage treatment Plant had the highest temperature (mean values of 10.1 – 26.2) values. In addition, the turbidity values of both control samples (tap water) were 0.01 respectively compared to the results obtained for the environmental wastewater samples. Generally, the pH, turbidity and temperature values for the control water

samples were within the acceptable standard reference values for drinking water.

The site which was found to have the most turbid water was Setumo/Modimola dam with a mean turbidity value ranging from 25.10 to 200.6 NTU while Gaborone dam had the least record of mean turbidity readings with a value range of 10.12 to 10.68 NTU. Modimola/Setumo Dam receives effluent from the neighbouring Setumo Wastewater Treatment Plant while Gaborone Dam (like Disaneng Dam) stores raw water. Water from Disaneng Dam was found to be more turbid than effluent obtained from the Notwane Sewage Treatment Plant in Gaborone. The samples from all sites had turbidity of >5.0 NTU above what is set out in the Target Water Quality Range (TWQR) (DWA-BW and DWA-ZA, 2013) while the control water samples were both at 0.01 NTU which is acceptable according to the set level. With both the raw water and wastewater from Gaborone being less turbid, this might be as a result of the fewer activities that occur at these sites while in Modimola/Setumo and Disaneng dams there is a lot of activity carried out there such as fishing which is done with the aid of engine boats. A large number of livestock also use the sites as drinking holes, a factor that was highlighted by Mulamattathil *et al.*, (2014). All these may result in the addition of pollutants to the water bodies resulting in reduced light penetration.

The mean pH values for all the sites from which samples were obtained was found to range between

Table 1. Mean values of physical parameters (pH, turbidity and temperature) of water from the four sampling sites

Sample source	Mean Turbidity (NTU)	Mean pH	Mean Temperature (°C)
Gaborone Dam	10.12 – 10.68	8.26 – 8.64	9.2 – 24.5
Disaneng Dam	13.31 – 25.09	8.96 – 9.04	8.0 – 26.7
Notwane Sewage Treatment Plant	12.44 – 20.34	8.99 – 9.77	10.1 – 26.2
Modimola/Setumo Dam	25.10 – 200.6	9.01 – 9.78	9.0 – 24.2
Control – 1	0.01	7.78	24.4
Control – 2	0.01	7.88	26.1
EPA reference value for drinking water	≤1.0	6.5 – 9.5	25.0
EPA reference value for wastewater	≤5.0	5.5 – 10.0	≤40.0
WHO reference value for drinking water	⇒ 1 - ⇒5.0	6.5 – 8.0	25.0
WHO reference value for wastewater	5.0	6.5 – 8.0	30.0
BW and ZA reference value for drinking water	0.5 – 5.0	6.5 – 8.5 (BW) 5.5 – 9.5 (ZA)	25.0
BW and ZA reference value for wastewater	25 – 30.0	6.5 – 8.5	30.0

BW = Botswana; ZA = South Africa; WHO = World Health Organization; EPA = Environment Protection Agency; Control 1= Gaborone; Control 2= Mafikeng (Water samples from Modimola/Setumo Dam presented high turbidity values and pH, above the set standards for raw and wastewater. The control sample from both Gaborone and Mafikeng were found to have turbidity, pH and temperature within acceptable levels/standards as set by Botswana, South Africa and World Health Organization (WHO))

pH7.78 and pH9.78 (Table 1). This shows that the wastewater, raw water and the tap water from the sampling locations are slightly alkaline with a 0.28 pH value above the Target Water Quality Range (TWQR) which is between pH5.5 and pH9.5 (DWA-BW and DWA-ZA, 2013). Turbidity does not determine the

microbial quality or give a clue on the chemical composition of water but may be an indication of increased pollution although the presence of some chemical pollutants may result in an increased or low pH in water. The results showed that the PCBs were below the detection limit of the instrument used. This

Table 2. Recalcitrant Compounds Screening results from the GC-MS

Sample	Sample Code	Aldrin (mg/kg)	Dieldrin (mg/kg)	4,4 DDD (mg/kg)	4,4 DDE (mg/kg)	4,4 DDT (mg/kg)	PCB (mg/kg)
1	A1	ND	ND	ND	ND	ND	ND
2	A2	ND	ND	ND	ND	ND	ND
3	A3	ND	ND	ND	ND	ND	ND
4	A4	ND	ND	ND	ND	ND	ND
5	A5	ND	ND	ND	ND	ND	ND
6	B1	ND	ND	ND	ND	ND	ND
7	B2	ND	ND	ND	ND	ND	ND
8	B3	ND	ND	ND	ND	ND	ND
9	B4	ND	ND	ND	ND	ND	ND
10	B5	ND	ND	ND	ND	ND	ND
11	C1	ND	ND	ND	ND	ND	ND
12	C2	ND	ND	ND	ND	ND	ND
13	C3	ND	ND	ND	ND	ND	ND
14	C4	ND	ND	ND	ND	ND	ND
15	D1	ND	ND	ND	ND	ND	ND
16	D2	ND	ND	ND	ND	ND	ND
17	D3	ND	ND	ND	ND	ND	ND
18	D4	ND	ND	ND	ND	ND	Traces
Control 1	cont 1	ND	ND	ND	ND	ND	ND
Control 2	cont 2	ND	ND	ND	ND	ND	ND

ND = Not Detected; A = Gaborone Dam; B = Disaneng Dam; C = Notwane Sewage Treatment Plant; D = Setumo/Modimola Dam; Control 1 = Gaborone; Control 2 = Mafikeng (PCBs levels in the water sample from Gaborone Dam. Disaneng Dam, Notwane Sewage Treatment Plant were below detectable level by GC-MS (7890A/5975C model))

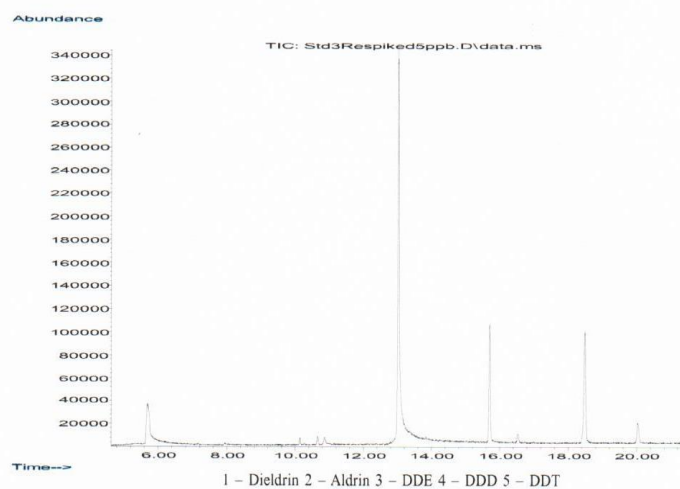


Fig. 2. GC Chromatogram for pesticides used as a standard

(All the pesticides used in the quality control experiment were detected by the GC-MS used in this study. This was used as reference for the analysis of PCBs in water. This therefore shows that the PCBs were below detectable levels in wastewater. This might have been due to their lipophilic characteristic)

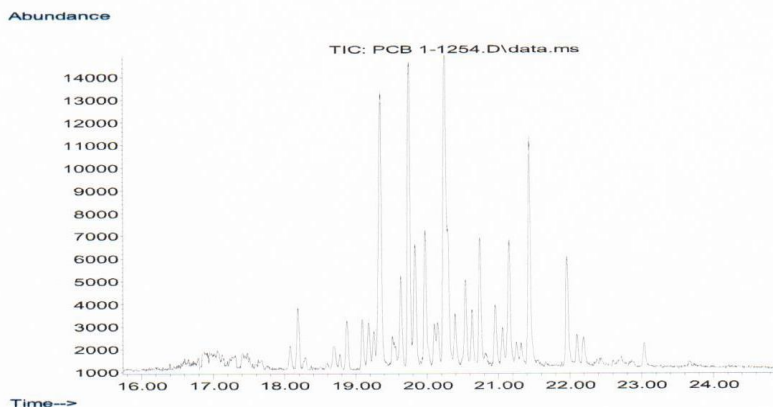


Fig. 3. GC Chromatogram for PCB Aroclor 1254 used as a standard

(The PCB standard used in the quality control experiment was detected by the GC-MS used in this study. This therefore shows that the PCBs were below detectable levels in wastewater)

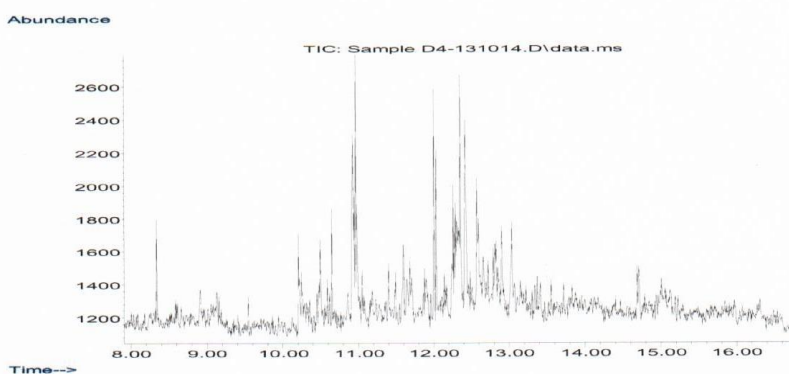


Fig. 4. GC chromatogram for water sample from Modimola/Setumo dam

(Only traces of PCBs were detected from wastewater collected from Modimola/Setumo Dam. Further analysis is required though, on other types of samples, other than water to verify the traces and for identification of those compounds)

was confirmed through the use of standards which indicated that the equipment detection limit for the compounds was 17.1 µg/L. This was obtained from the equipment detection limit using the results for the standard. The results are presented in Table 2. Although the study was targeting PCBs, none of the insecticides that were used for quality control were detected in the water samples. The results are also presented in Table 2.

The screening results were also shown in GC-MS chromatograms. These results are shown in Figs. 2 to 4. The chromatogram from the standards is shown in Figs. 2 and 3. The standard pesticides used in the study as standards are represented by peaks 1 to 5 in Fig. 2.

The results showed that the compounds were not detected. There were traces of PCBs from Modimola/Setumo dam though, although the quantities could not be determined as the levels were below quantification and thus traces, as presented in Fig. 4.

The results indicate that since these compounds are highly lipophilic (WHO, 2000) they required certain surfaces to which they can adhere and require fats in which they dissolve. This explains why most studies have only been able to detect these compounds from fish, plant material and soils. This also explains why these compounds highly result in bioaccumulation (EPA, 2013; Nabavi *et al.*, 2013) and thus very undesirable in the ecosystem. The implication from the

results obtained during this study may be that these compounds if they get attacked by bacteria, they are biodegraded as these will absorb the compounds and use them as they carbon sources thus biodegradation as was described by Azhari *et al.*, (2010) and Vrchotova *et al.*, (2013) or they get taken up into tissues of organisms found in the water bodies (Nabavi *et al.*, 2013). The PCBs that manage to pass through the wastewater treatment plant un-degraded, they will be contained in the effluent which has a low bacterial population and as a result they will end up into the soils where the effluent is released, or sediments of dams and rivers into which effluent reach thus polluting the environment (WHO and UNICEF, 2013).

CONCLUSIONS

The targeted recalcitrant compounds, the polychlorinated biphenyls (PCBs), were not detected in the water samples from the studied sites. Only traces (un-quantifiable levels) of PCBs were detected from wastewater sample from Modimola/Setumo dam. The use of QuEChERS procedure to extract PCBs from water may not to be very effective as these compounds are highly lipophilic and thus higher levels of these compounds would be required for them to be extracted from water. PCBs although they get access into wastewater from the different industries, their lipophilic property make them not be easily isolated directly from the water. There is need to analyse for these compounds in the living organisms and soils where the wastewater is discharged to ensure that the compounds are not re-introduced into the environment through effluent. The physical parameters of drinking water in the two (2) regions where the study was conducted are within the global acceptable standards.

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