

**BIODEGRADATION OF POLYAROMATIC HYDROCARBONS BY
BACTERIA ISOLATED FROM THE INTESTINAL GUT LINING OF
COW (*Bos taurus*)**

By

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CERTIFICATION

We certify that this research work titled Biodegradation of Polyaromatic hydrocarbons by Bacteria isolated from the intestinal gut lining of cow (*Bos tarus*) was carried out by Voke Owarieta URHIBO in the department of Microbiology of the University of Benin, Benin-City, Nigeria.

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DEDICATION

This work is dedicated to my parents Chief and Chief (Mrs.) G. U. Owarieta

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I sincerely appreciate God Almighty for making this work possible, for his provision, protection and sustenance. I return all the glory to him. To my mother of Perpetual help Mary, I have always sought your intercession and you have always harkened to my call.

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TABLE OF CONTENTS

	Page
Title	ii
Certification	iii
Approval	iv
Anti-Plagiarism certification	v
Dedication	vi
Acknowledgements	vii
Table of contents	ix
List of Tables	xiv
List of Figures	xvi
List of Plates	xvii
List of Abbreviations	xviii
CHAPTER ONE INTRODUCTION	1
1.1 Statement of the problem	4
1.2 Aim and Objectives	4
CHAPTER TWO LITERATURE REVIEW	6
2.1 Source of polycyclic aromatic hydrocarbons (PAHs)	7
2.2 Occurrence of PAHs	9
2.2.1 PAHs in air	9
2.2.2 PAHs in soil	10
2.2.3 PAH in water	12
2.2.4 PAHs in sediments	13

2.2.5	Occurrence of PAH in foods	14
2.2.6	PAHs in sewage sludge	16
		Page
2.3	Route of Exposure to PAHs	17
2.4	Effects of PAHs	18
2.4.1	Effect on Humans	19
2.4.2	Effects on Animals	22
2.4.3	Effects on Plants	23
2.5	Petrogenic Pollution of PAHs	25
2.6	Persistence of PAHs	25
2.7	Removal of PAHs from the environment	26
2.7.1	Biological degradation of PAHs	27
2.8	Metabolism of PAHs	29
2.10.	Cow and the intestinal gut biota	31
	CHAPTER THREE MATERIALS AND METHODS	36
3.1	Sample collection	36
3.2	Physicochemical characterization of the faecal gulp	36
3.2.1	Total Solids, Moisture Content, Ash Content and Volatile Solids	36
3.3	Isolation and purification of microorganisms	37
3.3.2	Morphological and Biochemical characterization of Microorganisms	38
3.3.3	Microscopy using light microscope (Gram staining)	38
3.3.4	Biochemical Tests for identification of the isolates	39
3.4	Screening of isolates for the PAH degrading potential	42

3.5	API Biochemical test using API20E test kit	42	
3.6	Molecular Identification of selected isolates with best degradation capability	43	
			Page
3.6.1	Genomic DNA Isolation by ZR fungal/Bacterial DNA Miniprep Tm kit	43	3.6.2
	Qualitative DNA assessment (UV-Vis Spectroscopy)	43	
3.6.3	Gel Electrophoresis	44	
3.6.4	Polymerase Chain Reaction (PCR) amplification	44	
3.7	Innoculum development	46	
3.8	Degradation of Benzo[a] Pyrene	47	
3.9	Degradation of Pyrene and Flouranthene	48	
3.10	Determination of Metabolites of Pyrene and Flouranthene during biodegradation by the selected isolates	48	
3.11	Parameters evaluated during biodegradation of BaP, Pyrene and Flouranthene	48	
3.11.1	Viable count monitored during degradation	48	
3.11.2	Physicochemical monitoring during PAH degradation study	48	
3.12	Optimization of the growth of the selected isolates singly and in consortium using one	50	
3.12.1	Effect of incubation period on the degradation of PAHs	50	
3.12.2	Effect of pH	51	
3.12.2	pH and degradation of PAHs by selected isolates singly and in		

combination	51
3.12.3 Effect of Temperature on growth patterns of the isolates	51
3.12.3b Changes in temperature and degradation of PAHs by selected isolates singly and in consortium	52
3.12.4. Effect of varied concentration of PAHs on bacteria isolates and consortium	52
	Page
3.12.5. Effect of Nitrogen/ Phosphorous/Potassium (NPK 15:15:15) sources on growth of the isolates and degradation of PAHs	53
3.13 Detection of PAH Degrading Genes by PCR in the selected bacterial isolates.	53
CHAPTER FOUR RESULTS	55
4.1 Physicochemical characterization of the intestinal chyme	55
4.2 Isolation, Morphological and Biochemical characterization of microorganisms from non PAH enriched media	55
4.3 Testing the potential of the isolated bacteria to degrade PAHs using 2,6-Dichlorophenol Indophenols (DCPIP) Assay	56
4.4 Identification using API 20 E	56
4.5 Molecular identification of selected isolates	65
4.6 Degradation of Benzo[a]Pyrene by bacteria isolates and in consortium	66
4.7 Degradation of Pyrene	79
4.8 Degradation of Flouranthene	87
4.9 Determination of metabolites during degradation of Pyrene and flouranthene at various time intervals	88

4.10	Optimization of growth and degradation of PAHS using one variable at a time	97
4.10.1	Growth profile analysis	102
4.10.2	pH optimization during biodegradation	102
4.10.3	Consequence of varied concentration on the degradation of B(a) by bacteria isolates and consortium	102
4.10.4	Outcome of nutrient (NPK 15:15:15) on percentage degradation of B (a)p, pyrene	102
4.11	Metagenomic evaluation of genes responsible for degradation in <i>E. coli</i> and <i>K. pneumoniae</i>	102
	CHAPTER FIVE DISCUSSION AND CONCLUSION	114
5.1	Discussion	114
5.2	Conclusion	133
5.3	Contribution to Knowledge	134
5.4	Recommendation	134
	References	136
	Appendix	162

LIST OF TABLES

Table	Title	Page
4.1	Physicochemical Characterization of the intestinal chyme	57
4.2	Morphological, Biochemical and sugar fermentation assessment of isolates from direct plating on Nutrient Agar	58
4.3	Morphological, Biochemical and sugar fermentation assessment of isolates on MSM enriched with PAH (Pyrene)	59
4.4	Qualitative screening of isolates from direct plate method for their capability in degrading PAH using 2, 6-Dichlorophenol redox indicator	60
4.5	Qualitative screening of isolates from enrichment method for their capability in degrading PAHs using 2, 6-Dichlorophenol indophenol redox indicator.	61
4.6	Quantitative screening of bacteria from direct plating method by 2, 6-Dichlorophenol indophenol redox indicator using UV-VIS @600nm	62
4.7	Quantitative screening of bacteria from enrichment method by 2, 6-Dichlorophenol indophenol redox indicator using UV-VIS @600nm	63
4.8	Biochemical identification of best selected PAH degrading bacteria using API 20E kit	64
4.9	Qualitative DNA assessment (UV-Vis Spectroscopy) $\pm$ SE	68
4.10	Residual concentration of B(a)P in (mg/L) during degradation By bacterial isolates and consortium	73
4.11	Concentration of Pyrene in (mg/L) during degradation by bacterial isolates and consortium	81
4.12	Concentration of Flouranthene(mg/L) during degradation by bacterial isolate over time	89
4.13	Metabolites produced from degradation of Pyrene by the selected bacterial isolates	95

4.14	Metabolites produced from degradation of Flouranthene by the selected bacterial isolates.	96
Table	Title	Page
4.15	Microbial Growth Pattern at different Temperatures	98
4.16	Effect of varied temperature on the biodegradation of B(a)P by bacterial isolates and consortium	99
4.17	Effect of varied temperature on the biodegradation of Pyrene by bacterial isolates and consortium	100
4.18	Effect of varied temperature on the degradation of Flouranthene by bacterial isolates and consortium	101
4.19	Microbial Growth Pattern at different pH	103
4.20	Effect of varied pH on the degradation of B(a)P by bacterial isolates and consortium	104
4.21	Consequence of varied pH on the degradation of Pyrene by bacterial isolates and consortium	105
4.22	Outcome of varied pH on the degradation of Flouranthene by bacterial isolates and consortium	106
4.23	Effect of varied concentration on the degradation of B(a) by bacterial isolates and consortium	107
4.24	Effect of varied concentration on the degradation of Pyrene by bacterial isolates and consortium	108
4.25	Effect of varied concentration on the biodegradation of Flouranthene by bacterial isolates and consortium	109
4.26	Effect of nutrient (NPK 15:15:15) on percentage degradation of B(a)P, pyrene and Flouranthene and its influence on the growth of isolates	110

LIST OF FIGURES

Figure	Title	Page
4.1	Degradation Percentage of B[a]P by <i>E. coli</i> , <i>K. pneumoniae</i> and consortium	74
4.2	Growth of bacterial isolates during degradation of B[a]P	75
4.3	Changes in Chemical Oxygen Demand (COD) during degradation of Benzo(a)pyrene by bacterial isolates	76
4.4	Changes in Biological Oxygen Demand (BOD) during degradation of Benzo(a)pyrene by bacterial isolates	77
4.5	Changes in pH during degradation of Benzo(a)pyrene by bacterial isolates	78
4.6	Degradation percentage of Pyrene by the selected bacterial and consortium	82
4.7	Growth of bacterial isolates during degradation of Pyrene	83
4.8	Changes in Chemical Oxygen Demand (COD) during degradation of Pyrene by bacterial isolates	84
4.9	Changes in Biological Oxygen Demand (BOD) during degradation of Pyrene by bacterial isolates	85
4.10	Changes in pH values during degradation of Pyrene by bacterial isolates	86
4.11	Degradation Percentage of Flouranthene by the selected bacterial isolates and consortium	90
4.12	Growth of bacterial isolates singly and in consortium during degradation of Flouranthene	91
4.13	Changes in Chemical Oxygen Demand (COD) during degradation of Flouranthene by bacterial isolates	92
4.14	Changes in Biological Oxygen Demand (BOD) during degradation of Flouranthene by bacterial isolates	93
4.15	Changes in pH during degradation of Flouranthene by bacterial isolates	94

LIST OF PLATES

Plate	Title	Page
4.1	Gel Electrophoresis pictogram of the selected bacterial isolates	69
4.2	PCR analysis of all isolates obtained in the study using universal bacterial primers (27F/1492R).	70
4.3	Phylogenetic tree of <i>Morganella morganii</i>	71
4.4	Phylogenetic tree of isolate <i>Raoultella ormithinolytica</i>	72
4.5	PCR analysis using gene specific bacterial primers n-alkanes monooxygenase (<i>alkB</i>)	111
4.6	PCR analysis of isolates using gene specific bacterial primers Naphthalene Dioxygenase (<i>NahAC</i>).	112
4.7	PCR analysis of isolates using gene specific bacterial primers Catechol dioxygenase (230)	113

LIST OF ABBREVIATIONS

Abbreviation	Meaning
ADH	Arginine
AH	Aliphatic hydrocarbons
AhR	Aryl hydrocarbon receptor
AMY	Amydalin
ANOVA	Analysis of variance
API	Analytical profile index
ARA	Arabinose
B(a)P	Benz(a)pyrene
BaP-MEQ	Mutagenic equivalent concentration
BaP-TEQ	Carcinogenic equivalent concentration
BCF	Bio concentration factor
BLAST	Basic local alignment search tool
BOD	Biological oxygen demand
CIT	Citrate
COD	Chemical oxygen demand
DCC	DNA clean and concentrator
DCPIP	Dichlorophenol indophenol
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
GC	Gas chromatograph
GEL	Charcoal gelatin
GLU	Glucose
H ₂ O	Water
HCM	Hydrocarbon mixtures
HPLC	High performance liquid chromatograph
i.e	That is
INO	Inositol

LDC	Lysine
LOD	Limit of detection
LOQ	Limit of quantification
Abbreviation	Meaning
MAN	Mannitol
MC	Moisture content
MEF	Mutagenic equivalency factor
MEF	Mutagenic equivalency factor
MEL	Melibiose
MR	Methyl red
MS	Mass sopectrometer
MSD	Mass selective detector
MSM	Mineral salt medium
NCBI	National centre of Biotechnology information
OD	Optical density
ODC	Ornithine
OH	Hydroxyl group
ONPG	Beta galactosidase
OX	Oxidase
PAH	Polyaromatic hydrocarbons
PCR	Polymerase chain reaction
pH	Potential hydrogen
POCs	Persistent organic carbon
POPs	Persistent organic pollutants
RHA	Rhamnose
ROS	Reactive oxygen species
SAC	Sucrose
SE	Standard error
SIM	Selective ion monitoring
SOR	Sorbitol

TDA	Tryptophan
TEA	Terminal electron acceptor
TEF	Toxic equivalency factor
TSS	Total suspended solids
URE	Urea
Abbreviation	Meaning
UV-VIS	Ultraviolet visible
VOC	Volatile organic compound
VOC	Volatile organic compounds
VP	Voges proskauer
VS	Volatile solids
WAF	Water accommodated fractions

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants that can be found on many surfaces including grasses that are often consumed by ruminants. It was therefore hypothesized that bacteria in the intestine of cow may have the potential to degrade PAHs. The aim of the study was to isolate PAH(s) degrading organisms from the large intestine of cows.

Bacteria were isolated from the intestinal chyme of the large intestine of a healthy cow collected at the point of slaughter by serial dilution/direct plating and enrichment methods. Physicochemical parameters including; pH, temperature, moisture content, total solids, volatile solids and total suspended solids were analysed. The isolates were identified by standard microbiological test protocol (Gram reaction and biochemical tests) and screened for PAH degradation potential using the 2,6-dichlorophenol indophenol (DCPIP) redox dye, quantified by a UV-VIS spectrophotometer. The identity of the two isolates with the highest PAH degradation capacity after preliminary degradation tests, was confirmed following API20e tests analysis and 16S rRNA molecular analysis. The two isolates were used to inoculate carbon free Bushnell Haas medium containing the PAHs in single and combined cultures for the degradation tests. Samples were withdrawn at intervals of three days and analyzed for bacterial growth, pH, temperature, BOD, COD and changes in the concentration of the PAHs for 16 days. The optimum temperature, pH, concentration and nutrient supplementation for efficient degradation was analyzed following standard protocol and genes responsible for degradation identified.

The two test isolates selected after screening and identification were *Escherichia coli* and *Klebsiella pneumoniae*. HPLC/GCMS analyses showed that the concentration of Benzo[a]Pyrene declined by 84.8%, 91.04% and 96.44% by *E. coli*, *K. pneumoniae* and a combination of both respectively after 16 days. The reduction in pyrene concentration was 89.36%, 90.98% and 97.76% after exposure to *E. coli*, *K. pneumoniae* and a combination of both respectively while the decline of fluoranthene concentration stood at 86.4%, 90.3% and 92.37% after similar exposure to the test bacteria. ANOVA confirmed significant differences in the extent of the degradation of the PAHs by the test bacteria and their combined cultures ($P < 0.05$). The growth of the isolates combined peaked at 1.98 log cfu/ml between days 10 and

13 during degradation of BaP. With respect to pyrene, it peaked at day thirteen with a log cfu value of 2.86 while in medium containing fluoranthene day 13 with a log cfu value of 3.23. The pH of medium adjusted to 7.0 declined in the three mediums with the least pH value for BaP, Pyrene and Fluoranthene being 6.5, 6.6 and 6.7 respectively during degradation of the PAHs. Phthalate was the major degradation product in the course of degradation of the PAHs. The optimum temperature and pH conditions for the degradation of the PAHs was 35°C and pH 7, respectively while PAHs ≥ 1000 mg inhibited the growth of the test bacteria. Application of fertilizer (NPK) enhanced growth of the test bacteria and degradation of the PAHs. The genes associated with the degradation of PAHs in *E. coli* and *K. pneumoniae* were found to be alkane monooxygenase (*alkB*), Naphthalene dioxygenase (*NahAC*) and Catechol dioxygenase (*C230*). It can be concluded that the intestine of *Bos taurus* harbor strains of bacteria that are capable of a high degree of degradation of PAHs; and that the consortium of the bacterial strains can be potentially useful for bioremediation of PAH-polluted environment.

CHAPTER ONE

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of chemical compounds containing carbon and hydrogen, conjoined by two or more fused aromatic rings in linear, angular and cluster arrangements without hetero atom or cohere substituent (Masih and Lal, 2014; Hussein and Mona, 2016; Kim *et al.*, 2016). PAHs have high melting and boiling points, low vapor pressure, highly lipophilic with very low aqueous solubility (Masih *et al.*, 2012). They are soluble in many organic solvents with slight variations amongst solvent members.

Polycyclic aromatic hydrocarbon ensue from two main sources: natural (biogenic and geochemical) and anthropogenic activities of man (Takashi *et al.*, 2020) and are extensively spread in soils, sediments, groundwater and the atmosphere (Ranjan *et al.*, 2017). Point and non-point sources of PAHs include petroleum and its allied product spills, manufacturing processes such as coal liquefaction and gasification during coke production, smoldering involving fossil fuels, wood preservatives such as creosote and the spawning of compost from coal gasification plants (Andrés *et al.*, 2017), dumpsites of automobile mechanics and gas factories (Rafin *et al.*, 2009). Natural sources of PAHs include volcanic breakout and forest fires (Selina and Ian, 2005). A number of PAHs from geochemical origin are initiated during pyrolysis: This process involves the subjection of sediments to extreme temperatures during diagenesis (Selina and Ian, 2005). PAHs easily form alliance with heavy metals and other toxicants and becomes recalcitrant in the environment (Ranjan *et al.*, 2017).

PAHs have been tagged hazardous chemicals by various pollution control bodies (Lawal, 2017). Major health effects have been ascribed to acute carcinogenic, mutagenic and teratogenicity (Janošek *et al.*, 2007; Batdelger *et al.*, 2019; Masato and Nobuo, 2020).

Challenges has also been reported on immune system breakdown and reproduction dysfunction resulting in secondary infertility in both females and males (Wang *et al.*, 2016). The routes of entry to humans include ingestion, inhalation, and dermal contact in both occupational and non-occupational settings (Lyu *et al.*, 2014). The rapid absorption of PAHs by humans and other living organisms results in risk occasioned by bioaccumulation in the food chain and food web. The toxicity of PAHs is largely dependent on the number of benzene rings (Igwe and Ukaogo, 2015). Flouranthene, Pyrene, Chrysene and Benz (a) pyrene are considered to be the biomarkers of PAHs due to their presence in the three environmental matrices (soil, water and air) (Adeniji, *et. al.*, 2019). and their implication in certain cancer situation in humans. (Masato and Nobuo, 2020). They are the most ubiquitous and abundant compounds from both petrogenic and pyrolytic sources and their ratios are usually used to pinpoint the origin of PAHs in the environment (Oliveira *et al.*, 2021).

Most crude oil and allied product factories including many factories that exude PAHs have deployed a number of strategies to reduce to the barest minimum the effect of PAHs on both humans and the environment involving both biotic and abiotic processes, such as volatilization, adsorption, photolysis, chemical oxidation and microbial degradation. Among them, microbial degradation involving microorganisms from diverse sources have been used to remodel PAHs to less hazardous or nonhazardous compound. These activities are geared to remove or reduce toxicants in an environmentally affable and cost effective form. They in addition decrease the carcinogenicity, mutagenicity and teratogenicity of PAHs in the environmental matrices restoring the integrity of the environment. Thus the search for suitable microorganisms which will be able to break open the complex bonds of PAHs in good time is required to curb the menace of this toxicant to both human life and the environment. An array

of microorganisms including bacteria, actinomycetes, fungi and algae have been identified and characterized for their capability to utilize PAHs (Zeng *et al.*, 2016).

Ruminant animals including *Bos tarus* exist in the polluted environmental biota and feed on grasses exposed to PAHs. Microorganisms resident in these herbivores has devised means of survival for various toxins as they enter their ecological niche altering its composition (Gupta *et al.*, 2012). Many ruminants are able to ingest and/or digest and metabolize toxic compounds contained in their diet because of the complexity of their stomach. (Oikonomou *et al.*, 2015).

The large intestine of *Bos tarus* is packed with microscopic organisms and enzymes possessing the special ability to digest cellulose and other chemical substances (Natsuga *et al.*, 2014). The ruminant's stomach comprises an ecosystem populated with a variety of organisms that include bacteria, (*Ruminococcus* sp., *Klebsiella* sp, *Raoutella species*, *Prevotella* sp., *Butyrivibrio* sp., *Clostridium* sp., etc), protozoa (*Diplodinium* sp., *Isotricha* sp., *Oscillospira* sp., etc), fungi (*Neocallimastix* sp., *Sphaeromonas* sp., etc) and bacteriophages.

The environmental problems of polyaromatic hydrocarbons (PAHs) are numerous and specific in connection with time and space. Interest in the microbial degradation of pollutants has soared in recent years as man strives to find sustainable ways to clean up its contaminated environments in an ecofriendly manner. There is an increased awareness of challenges posed by PAHs and sometimes its metabolites.

Microorganisms have been isolated from animal wastes and other composted materials and shown to have great potential in the degradation of PAHs. However, there is paucity of information on the use of microorganisms isolated from the unique ruminant intestinal gut environment of cows for the degradation of PAHs. It is therefore possible that bacterial

isolates from the large intestine having survived the rigors of metabolism from the mouth of the cow through the stomach to the large intestine may be capable of degrading or metabolizing PAHs.

1.2 Statement of the problem

Environmental challenges have occurred since the earliest civilization on planet earth. The quality and quantities of pollutants including PAHs in the environment was small and localized. It was generally thought and felt that the world was so vast that it could not be permanently affected by these pollutants (Kim *et al.*, 2013). However, with increased industrialization, widespread pollution of the earth has become a reality leaving behind persistent organic pollutants (POPs). These compounds are chemically stable and resist indigenous microbial attack posing serious danger to the environment and humans (Bhandari, 2009; Hadibarata and Teh, 2014).). Thus it is imperative to stir up systems that would be able to clear the menace of PAHs from the environment in an ecofriendly manner within the shortest time possible.

1.2 Aim and Objectives

The aim of the study was to isolate bacteria capable of degrading polyaromatic hydrocarbons from the large intestine of cow (*Bos taurus*)

The objectives of the study were to:

1. enumerate, isolate and identify bacteria from the large intestine of cow;
2. screen the bacterial isolates for their ability to degrade PAHs and identify the best degraders;
3. ascertain the extent of degradation of the PAHs by selected isolates and the intermediate products;

4. develop a consortium of isolates capable of enhancing the degradation of PAHs;
5. determine the optimum temperature, pH, PAH concentration and incubation periods for degradation of the PAHs by the selected isolates; and
6. identify the genes associated with degradation of the PAHs.

CHAPTER TWO

LITERATURE REVIEW

Polycyclic aromatic hydrocarbons (PAHs) also known as polynuclear aromatic hydrocarbons are potent pollutants that consist of two or more benzene fused aromatic rings (Chen *et al.*, 2014) without hetero atoms or cohere substituents. They are categorized as heavy or light depending on the number of aromatic ring(s) present: compounds containing less than four rings are tagged as light PAHs while those containing four rings and more are heavy PAHs (Igwe *et al.*, 2015). Weighty PAHs are usually more anchored and toxic than the light PAHs (Kuppusamy *et al.*, 2016; Li *et al.*, 2016). PAHs because of their lipophilic nature mix more easily with oil than water and can withstand very high temperatures (Masih *et al.*, 2012). They are soluble in organic solvents and manifest various functions such as light sensitivity, heat resistance, conductivity and emit ability (Akyuz *et al.*, 2010). PAHs have very distinctive UV absorbance spectra which is useful in the identification of PAHs (Parker *et al.*, 2012).

The major source of PAHs is the incomplete combustion of organic material such as coal, crude oil and its allied products and wood (Lawal *et al.*, 2018). PAHs have diagnostic markers for ease of identification as they help determine PAHs pollution emanating from petroleum products, petroleum kindling and bagasse or coal smoldering (Agbozu *et al.*, 2017). PAHs are usually not synthesized chemically for industrial purposes. Nonetheless, some commercial uses of PAHs exist including: some raw materials for drugs, farm products, products for photography, polymers, and many other manufacturing companies for the production of pigments, dyes, resins, asphalt and some roofing compounds (Akyuz *et al.*, 2010). Polycyclic aromatic hydrocarbons available commercially are mostly colorless without aroma or possessing a faintly pleasant aroma (Buha, 2011).

Polyaromatic hydrocarbons have sixteen variants pinned as priority pollutants, eight of which have been classified as possible cancer agents to humans (Alonso-Alvarez *et al.*, 2007; IARC, 2009; Hussein and Moana, 2016). Acenaphthene $C_{12}H_{10}$, Acenaphthylene $C_{12}H_8$, Fluorene $C_{13}H_{10}$, Naphthalene $C_{10}H_8$, Anthracene $C_{14}H_{10}$, Fluoranthene $C_{16}H_{12}$, Phenanthrene $C_{14}H_{10}$, Benzo(a)anthracene $C_{18}H_{12}$, Benzo(b)fluoranthene $C_{20}H_{12}$, Benzo(k)fluoranthene $C_{20}H_{12}$, Chrysene $C_{18}H_{12}$, Pyrene $C_{16}H_{10}$, Benzo(g,h,i)perylene $C_{22}H_{12}$, Benzo(a)pyrene $C_{20}H_{12}$, Dibenzo(a,h)anthracene $C_{22}H_{14}$ and Indeno(1.2.3-cd)pyrene $C_{22}H_{12}$.

2.1 Source of polycyclic aromatic hydrocarbons (PAHs)

The source(s) of Polyaromatic hydrocarbon to the environment are multifarious and generally categorized under natural and anthropogenic. The source identification content of PAH is chiefly tied to practical and analytical environmental samples and chemical dactylogram involving many protocols aiding differentiation amongst groups of PAH sources in both industrial and residential settings (Zang *et al.*, 2009; Wang *et al.*, 2011; Hussein and Mona, 2016). The natural and anthropogenic sources are categorized into three groups based on their origin: Pyrogenic (burning of organic matter), petrogenic (fossil fuels), and biogenic (transformation of natural precursors in the environment by rapid chemical/biological diagenic process (Guo *et al.*, 2010; Lu *et al.*, 2011)

Pyrogenic PAHs ensue from naturally occurring materials exposed to extreme temperature condition when oxygen is infinitesimal or completely absent in a process referred to as pyrolysis. This process may be termed anthropogenic occurring intentionally for example the carbonization fractional process of coal into coke and coal tar, or the catalytic cracking of petroleum into lighter hydrocarbons or unintentional for example during the incomplete combustion of vehicular fuels, or naturally during the incomplete smoldering of wood in

terrestrial and other forest fires. (Lawal *et al.*, 2018). The pyrogenic processes occur at a temperature ranging between (350°C to more than 1200°C). PAHs are abundant in greater concentrations in urban areas and industrialized places. Petrogenic PAH(s) on the other hand are formed at lower temperatures as low as (100–150°C) with crude oil.

PAHs formed from crude oil are termed petrogenic including the exploration, exploitation, maturation, cracking and similar processes including transportation, and storage of crude oil and its allied products. Reported incidents of petrogenic PAHs include oil spills in the aquatic environment, storage facility malfunction (tank leaks), emissions of gases and toxic chemicals associated with transportation (Adetona *et al.*, 2015; Bruns *et al.*, 2015; Pozo *et al.*, 2015; Fernando *et al.*, 2016; Orecchio *et al.*, 2016), incomplete combustion of premium motor spirit together with other refined petroleum products (Martins *et al.*, 2011; Sun *et al.*, 2016) and technical processes involving incomplete combustion (Wang *et al.*, 2013; Eriksson *et al.*, 2014; Ren *et al.*, 2014). Biogenically formed PAHs are usually synthesized by living things including plants and bacteria or formed during the degradation of fossil (Turkovskaya *et al.*, 2019), as well as molten magma eruption and ruins (Inomata *et al.*, 2012). Incomplete combustion natural or anthropogenic derived, is the major contributor of PAHs to the environment. Thus, in categorizing the origin of PAHs to the environment there is no clear distinction as most of the petrogenic sourced also have some pyrolytic origin although many advances are being made research is still on with regards to the identification of the source origin of many polyaromatic hydrocarbons (Nordin *et al.*, 2015).

2.2 Occurrence of PAHs

Polyaromatic hydrocarbons are pervasive nature as a result several of them exist in the various environmental matrices (Huanling *et al.*, 2019). They are emancipated in gaseous outflow, in aqueous and solid effluvium (Sun *et al.*, 2016) affecting the biota of the environment accumulating in both the food chain and food web. The umpteen pedigree of PAHs and the to and fro movement phenomenon of these environmental pollutants aid in the spread of these toxicants, across a wide plane integrating into air, water, soil and sediment (Fernando *et al.*, 2016; Wirasnita and Hadibarata, 2016).

2.2.1 PAHs in air

PAHs are launched to the atmosphere as byproducts of semi-complete combustion contaminating both indoor and outdoor air (Oliveira *et al.*, 2019). In the atmosphere, PAHs are abound in gaseous form and are absorbed in particles (Chen *et al.*, 2007). It occurs in polluted atmospheric environments in (ngm^{-3}) concentration range as a consequence of the ambient conditions (temperature and relative humidity), the type (source and characteristics) of the aerosol, and the nature of the PAH involved (Ravindra *et al.*, 2008). They are detected in air by air samplers monitored over a period of time whether assertive or unassertive (Apostolopoulou *et al.*, 2014; Jariyasopit *et al.*, 2015; Loppi *et al.*, 2015; Terzaghi *et al.*, 2015; Dominguez-Morueco *et al.*, 2016; Parnis *et al.*, 2016; Ewa-Błaszczuk *et al.*, 2019).

Polyaromatic hydrocarbons conjoined by two to three rings (light weight PAHs) reside in vapor phase and in particulate matter while four and more ring(s) members are found majorly in the particulate phase (Yuan *et al.*, 2015). Airborne emissions of PAHs emanates from hot temperature energy outputs (coal, gas, wood, oil, charcoals), manufacturing processes (coke production) (Deng *et al.*, 2014; Kozielska and Koniecznyński, 2015; Xu *et al.*,

2016) and refuse incineration (Chiang, *et al.*, 2012; Li *et al.*, 2014; Liu *et al.*, 2015; Wei *et al.*, 2015; Bayraktar *et al.*, 2016; Bi *et al.*, 2016).

Mans' exposure to atmospheric PAHs can result from emissions from cooking, smoking (White *et al.*, 2016), incense beads and leaves, insect controlling coils (Verma *et al.*, 2015) and PAHs laced materials. Other indoor contamination may result from the use of indoor cooking equipment and/or house warming devices (Shen *et al.*, 2014; Han *et al.*, 2015; Hassanvand *et al.*, 2015; Verma *et al.*, 2016). In urbanized settings sand particles are carriers of polycyclic aromatic compounds and pose as a source of contamination of the environment. Street dust especially in areas that are heavily industrialized is a marker status for PAHs pollution in city areas (Egide *et al.*, 2019). Vehicular emission in traffic situation is a contributory factor to PAHs pollution in the air in cities. The hours of school runs and standard work time is peak for air contamination by toxicants including.....g PAHs (Zhai *et al.*, 2016). Agbozu and co-workers have also reported that gas flaring releases many pollutants into the air including soot and other toxic materials laced with polycyclic aromatics (Agbozu *et al.*, 2016). Many environmental agencies have set acceptable limits for air quality control especially with regards to gas flaring and atmospheric pollution.

2.2.2 PAHs in soil

Soil is a reservoir for many complex materials because it is a recipient of many substances. It is often seen as nature's dustbin (Huanling *et al.*, 2019). Unfortunately, PAHs and many other chemical substances are defiant and persevere in the soil for duration reported as very long (Jones *et al.*, 2011) creating problems for man.

In the United Kingdom, the soil is reported as the primary reservoir for PAHs (Beriro *et al.*, 2016). Wastes which is a constant because of mans' activities are in quote

reduced/recycled partially and finally accumulated in nature's dustbin changing the normal flora and indeed fauna of the soil (Tobiszewski and Namieśnik, 2012). Different naturally occurring compounds present in pest/weed control treatment, biphenyl's, detergents, paints etc. are laced with small quantities of PAHs and are absorbed by the soil (Johnsen *et al.*, 2005). Polycyclic aromatic hydrocarbons (PAHs) are also found in high concentrations at automobile shops where they dispose their spent oil (Akanimo *et al.*, 2019). Sewage is sometimes used to revitalize soils in many parts of the world today however; Paraiba (2011) reported that the addition of sewage to soils increased the amount of polycyclic aromatic hydrocarbons in it leading to the possibility of the pollutants existence in many living systems as the pollutants have no boundary.

Humans' quest for better livelihood has caused an increase in soil contamination throughout the industrialized world especially in oil producing countries with poor regulations (Chengcheng *et al.*, 2019). PAHs in soil has the capacity to change forms subject to availability of friendly environmental conditions including vaporization, abiotic degradation (photolysis and oxidation), microbial degradation, or phyto-oxidation. PAHs in soil travel through the A and B horizons reaching the underground water and settling in the aquifer (Beriro *et al.*, 2016). A study on the Japan Earthquake caused tsunami not only hampered human life but resulted in contamination of the soil by chemicals including PAHs (Bacosa and Inoue, 2015). This affirms that PAHs results from incomplete combustion of natural processes involving incomplete combustion changing the soil structure. They discovered plenty of PAH degrading bacteria in the ruins. Inegite *et al.* (2012) in their study reported that PAHs linger in the soil because of their persistent nature together with co-metabolism with heavy metals whereas the straight chain aliphatics are easily mineralized in soil with microbial treatment.

2.2.3 PAH in water

Water bodies are also recipients of the menace of pollution especially PAHs adversely affecting the zooplankton, phytoplankton and the general hydrosphere. The contamination may be via point sources or non-point sources (Jin *et al.*, 2014). Irrespective of the source of PAHs contamination the effects distort both the fauna and flora of the water bodies (Adeniji *et al.*, 2019). Far reaching oil spills have deleterious consequences on water-bodies especially where there is offshore activities. Severe surface/sediment pollution of the aquatic ecosystem can occur via the breakage of storage facilities, pipeline leakage, faulty well heads, broken down terminals, and oozing from gasworks sites during coke production, leakage in barges during transportation and many explorative/exploitative processes of oil drilling in the high sea, releasing PAHs as well as other organic contaminants to the natural aquatic environment (Hussein and Mona, 2016).

Recent increase of PAHs in the hydrosphere has been detected and is connected with shoot up in the use of oil vessels and fuel engine boats in water bodies (Mojiri *et al.*, 2019). Olajire *et al.* (2005) in their study writes that in aquatic environments PAHs do not homogenize because of their insolubility property rather they accumulate in sediments. They cling to suspended particles and remain in sediments due also to their hydrophobic properties. PAHs in the hydrosphere have prolonged existence and thus transfers to living organisms occur via bioaccumulation (Lawal *et al.*, 2018).

Huanling *et al.* (2019) suggested that wastewater contains high PAH concentrations from industrial run offs, household sewage and road surface run-off. River bodies are the recipients for most of these waste increasing their toxic level. Itodo *et al.* (2019) investigated the fate of PAHs in the riverine areas of Niger Delta, Nigeria including Egbokodo river, River

Ethiopia and Asaba-Ase rivers and wrote that PAHs of several congeners are present. In their scrutiny, they revealed that crude oil and its allied products together with other pyrogenic sources, contributed to the main input of PAHs in the oil rich Niger Delta region in Nigeria

Many rivers have been investigated with links to the petroleum industries and have shown to have above permissible limits PAHs in any water environment including: River Erjien Taiwan, Ekpan river, Nigeria, Lagos Lagoon, Nigeria, Needle Wave Gulf of Mexico, Urie river Isoko, Nigeria, Pearl River Estuary (Anyakora and Coker, 2006; Benson *et al.*, 2014; Botello *et al.*, 2015; Huang *et al.*, 2015; Wang *et al.*, 2016 and Itodo *et al.*, 2019). Others are Abad River, Iran (Kafilzadeh, 2015), Terai belt of North India (Masih and Lal, 2014), suburban area of Japan (Ozaki *et al.*, 2015) river Chaohu, China (Qin *et al.*, 2014), Sydney Basin, Nova Scotia, Xichuan China (Tai *et al.*, 2012) Columbia River Estuary (Counihan *et al.*, 2014), mountainous river California (Conaway *et al.*, 2013), urban river (Huveaune), France (Kanzari *et al.*, 2014), Karoon River, Khuzestan Province, Iran (Keshavarzi *et al.*, 2015), Prai and Malacca Rivers, Peninsular Malaysia (Keshavarzifard *et al.*, 2014), Neuquen River, Argentine Patagonia (Monza *et al.*, 2013), Ovia River, Nigeria (Obinaju *et al.*, 2015), Calabar River Nigeria (Oyo-Ita *et al.*, 2013), Dalian, Northeast China (Hong *et al.*, 2016), Colombian Cauca River (Sarria-Villa *et al.*, 2016) and Lake Chaohu, China (Qin *et al.*, 2013).

2.2.4 PAHs in sediments

Polycyclic aromatic hydrocarbons are hemmed into sediments in the same way that happens to deposits on the true soil. In the riverine, PAHs sorbs to particles abound and settles on top of water bodies by dry or wet deposition. From where they spread by wind currents and integrate with sediments inherent therein (Andrés *et al.*, 2017).

In highly industrialized areas, PAHs atmospheric deposition is extremely high and thus sediments are cleaved to PAHs from waves and other entry portals to the water. A few of the PAHs will be taken in by settled matter and establish their stay joining in the sediment record. This occurrence and its understanding is used by hydro-biologists to keep track of PAH entry into the hydrosphere. (Tehrani *et.al.*, 2012; Botello *et al.*, 2015:). As soon as PAHs get into sediments they become stationary because their non-polar form inhibits them from dissolution in water hence they stick to sediments (Batdelger *et al.*, 2019).

Furthermore, PAHs though insoluble are not absolute and can bio-accumulate if environmental conditions permits. Thus, PAHs may dissolve and stay embedded been held in conjunction with water by forces of adhesion in regions where bioavailable. In situations where water organic colloids are numerous, the concentrations of PAHs will spiral beyond its holding capacity. This is so because the pollutant will be scarf out into the organic colloids. These in turn will make movement into sediment faster. This phenomenon increases the migration and availability of PAHs in sediments (Li *et al.*, 2014).

2.2.5 Occurrence of PAH in foods

The incident of PAH(s) found in unprocessed food reflects the background contamination of the environment. Unprocessed dairy products and other crop products have been investigated to contain large amounts of PAHs. (Kim *et al.*, 2014; Deepa *et al.*, 2018). Transformation of food (drying, boiling, smoking, grilling, roasting, and frying) at temperatures high can generate PAHs (Lee *et al.*, 2016; Zheng *et al.*, 2016). Oils of plant origin (including olive residue oils) may pick PAHS during processes of excessive heating. In this regard, the burnt products may come into contact with the oil seeds (Chen *et al.*, 2012:

Kim *et al.*, 2013) depending on a number of factors including duration, type of fuel, space between the heat source and fat drained (Deepa *et al.*, 2018).

PAHs in food is are graded by the same physicochemical factors that determines their ability to move and occupy different matrices in addition to their uptake by living things (Keefe, 2012). Weighty PAHs clique with particulate matter so atmospheric fall out is a principal route of contamination for food crops (Kim *et al.*, 2013). As a result, green plants, herbivores, and poultry winch may ingest or absorb matter from soil are exposed to contaminants including PAHs sorbed to particles. Leaves and fruits can concentrate PAHs mainly through surface adsorption and they serve as food for both man and animals. The receiving end of most food crop is the surface (peel, outer leaves) although careful washing may remove up to 50% of pollutant overtime may also create problems for consumers. Particle bound PAHs are easily washed off the surface whereas those in the waxy layer are more difficult to remove (Counihan *et al.*, 2014).

Ingestion one of the principal route of human to PAHs is via consumption of contaminated sea anemones that make up part of the human diet (Chen *et al.*, 2012). PAHs in sediments and water are taken in by aquatic animals such as lobsters, muscles, and clams which are either consumed by secondary humans or by primary predators which also form part of the human diet for example squid and fish (Kim *et al.*, 2013). Water is life and consumption of contaminated water may result in certain percentage of PAH transfer. The permissible limit of PAHs in water is usually below 1 ng/L in drinking water. The level of PAHs in water can increase if asphalt or coal tar coating of storage tanks and water distribution pipes are used probably to aid pigging (Adeniji *et al.*, 2019).

2.2.6 PAHs in sewage sludge

Generation of waste and sewage sludge from both industrial and household facilities is inevitable because egestion must occur in living things and waste materials of metabolism and non-metabolic products must find their way out of living systems. With the campaign of waste conversion to wealth, waste is harnessed for useful purposes and sometimes the waste may serve as source of pollution to the lithosphere. Waste so generated is used to irrigate farmland and improve soil properties. However, sewage sludge and waste water may contain various organic pollutants including PAHs (Zielińska *et al.*, 2015). The probability of possible toxicants in sewage sometimes negates the advantage of it been a natural fertilizer. PAHs are like microorganisms ubiquitous with an array of its members found in sewage sludge depending on the source origin of effluents (Ozaki *et al.*, 2015; Ranjan *et al.*, 2017).

In agriculture the use of sewage is controlled by regulatory body under the Sewage Sludge directive, Directive 86/278/EEC, (EC, 1986) terms of reference adopted with the view to encouraging its application in agriculture and to regulate its use, to prevent harmful effects on soil, vegetation, animals and humans. During incineration many cancer causing PAHs are released and they spread in soil treated by sewage mixtures (Deng *et al.*, 2014). Biochar is a common product from the incomplete combustion of sewage. Zielińska and Oleszczuk (2015) evaluated the consequence of biochars in the content of freely dissolved (C_{free}) PAHs in sewage. In his findings excessive heat and particulate size of biochar had a negative result on PAH free concentration reduction. Notable work from Kim Nguu River, Vietnam to explained the toxic levels, spread and accumulation pattern of household sewage from an urbanized area that was directly emptied into the water body indicated shoot up of PAHs on the release of sewage (Kuppusamy *et al.*, 2016). The sludge samples contained PAHs

concentrations far above international limits set for sludge and sediment, such as probable effect levels (PEL), highlighting the biological risk in the used area and in the landfill sites where dredged sludge are dumped.

Aggressive investigation of sewage sludge is a bane in research as the focus is preservation of human life and managing the levels of PAHs from unmanaged sources. Korea sewage sludge incinerators, sewage treatment plant situated in urban river, East China and e-waste recycling sites China. The common feature in all their report was the presence of high levels of PAHs (Busetti *et al.*, 2006; Chen *et al.*, 2014; Ozaki *et al.*, 2015; Krapf *et al.*, 2015; Li *et al.*, 2016)

2.3 Route of Exposure to PAHs

Exposure of humans to PAHs occurs on a regular basis because of the nature of the pollutant. Routes of exposure include ingestion, inhalation, and dermal contact for humans and animals. Some exposures may cut across affecting living things from several routes (Wang *et al.*, 2012) in industrial workplace and domestically from the home. Public health risk may occur to workers at oil facilities, welding and fabrication industries, automobile mechanics, hawkers, street sellers, commuters, workers in coal and gold mining amongst others (Ravindra *et al.*, 2008)

Tobacco and cigarette fumes are heavy on PAHs including B(a)P together with a substantial number of evaluated human carcinogens (Lee *et al.*, 2016). Cigarettes pollutes air posing toxins not just to the smokers but also those inhaling smoke from the environment. Contaminated food products can be ingested in by humans and other living organism and the skin serves as a portal of entry for airborne PAH pollutants because of the pores that allows sorbing of airborne materials. Plants also take in the pollutant via various channels. Many

crops are able to synthesize PAHs taking them via photosynthesis and transpiration into their organs (Loppi *et al.*, 2015).

2.4 Effects of PAHs

Many toxicants released into the biosphere pose serious deleterious effects to human health, animals and plants due to their noxious and tenacity in the lithosphere. Polycyclic aromatic hydrocarbons (PAHs) from biotic and/or man's activities are characterized by their teratogenic, mutagenic and carcinogenic effect on man (Chen *et al.*, 2012). Several epidemiological studies have revealed a relationship between some inauspicious health problems on humans with linkage to plants and animal. Although some physicochemical and biological properties enhance these effects, polycyclic aromatic hydrocarbons (PAHs) are at the forefront of many toxins and heavy metals because of their spread in air (Ravindra *et al.*, 2008; Zhang and Tao, 2009 and Mauro *et al.*, 2012).

PAHs reside in air as a complicated consortium of congeners having unique molecular weight (Mauro *et al.*, 2012). Each congener has effective toxic level, and are assessed to disseminate each contributor in a given congener especially with regards to its effects on man, plants, animals and the environment. In standard risk measurements, the carcinogenic vibrancy of any congener is calculated based on the (BaP) carcinogenic equivalent concentration (BaP-TEQ), evaluated by calculating the concentration of a congener and its Toxic Equivalency Factor (TEF) (Parales *et al.*, 2000; Boström *et al.*, 2002; USEPA, 2005). In this regard TEF-adjusted concentrations and the vibrancy is estimated as the addition of BaP and TEQ for each congener in a mixture, surmise that the carcinogenic outcome of various congeners is extra (Vu *et al.*, 2011).

Furthermore, with the substitution of TEF with MEF (Mutagenic Equivalency Factors), the mutagenicity of a compound is ascertained related to BaP (BaP-MEQ) thus B(a)P is used as the biomarker to test carcinogenicity/mutagenicity in PAHs related assays.

2.4.1 Effect on Humans

In the human cell, PAHs can affect the DNA and cause major changes in the human body (mutagenic effect). Co-joining of (PAHs) and DNA is a principal role in mutagenic effect (Wang *et al.*, 2016). PAHs associated with particles are broken down to reactive molecules that cleaves with DNA to form bulky DNA adducts. (Batdelger *et al.*, 2019). The reactive molecules pick oxygen by cytochrome P450s to form electrophilic derivatives (*e.g.*, diolepoxides and radical cations) that combines with DNA (Yuan *et al.*, 2015) which finds its way into the inner chambers of a cell via fat split-up, bringing about gradual changes and subsequent mutation in living organelles (Chen *et al.*, 2012).

PAHs align to the purine bases in DNA, forming bulky firm and depurinating adducts (Deng *et al.*, 2014). It has been reported that many types of poly-aromatic compounds unsubstituted PAHs and heterocyclic aromatics have the potential to alter contact inhibition in the liver epithelial cells (Kim *et al.*, 2013). Mutagenic studies with regards to PAHs have indicated that only naphthalene, fluorene and anthracene are not mutagenic among the sixteen priority pollutants (Gupta *et al.*, 2012).

Base on the TEF(s) for PAHs exposure to PAHs is ascribed with inherent cancer risk among the human population especially with increased industrialization (Knafla *et al.*, 2011). Specifically, PAHs have been linked with dermal, lung, liver, pancreas, pharynx, intestinal, mouth, colon plus blood cancers (Yuan *et al.*, 2015). This has broadened the knowledge on the possibility of PAHs to cause trans-activation involving the aryl hydrocarbon receptor

(AhR) leading to liberalization of the cell and invariably massive increase in the epithelial cells, resulting in tumor promotion and in multiplier effect (oncogene) (Ma-White *et al.*, 2016). The origination of DNA adducts may stimulate cellular defense activities which may result in malfunctioned growth (Chramostová *et al.*, 2004).

In the living cells, PAH cleaves to DNA forming units, replicating and stimulating base nucleotide excision repair (Khan and Chakravarti, 2005) inciting the malfunction genes to multiply. A non-genotoxic mode of activity is that which triggers cancers is the perturbation of tissue equilibrium balance as a result of breakage of cell-to-cell connection (Trosko and Upham, 2005). Cigarettes/smoke has been noted as a potential carrier of carcinogenic PAHs and may remotely result in lung cancer to the smokers and others in the same environment. Periodic smokers stand a risk of lung cancer reported in Taiwan, where only a few women are smokers. It has also been documented that husband/wife smoking posed lung cancer risk in the ratio of two to one times in comparison to nonsmoking female population, a lot higher than a 20% excess risk observed in other Western countries (IARC, 2010).

Benzo [a]pyrene and benz[a]anthracene cause a lot of changes in living organisms by shattering the antioxidant defense system in living organisms causing oxidative discomfort. Under normal conditions, the production of ROS (reactive oxygen species) is orchestrated by different cellular processes. These processes consist of antioxidant materials and scavenger proteins, which are apt ROS removal system in the body of living things. When reactive oxygen species (ROS) production surpass the ability of the antioxidants, it will result in oxidative stress and significant system damage (Batdelger *et al.*, 2019). Human beings could via dermal openings face risk of carcinogenic substance benzo[a] pyrene (B[a]P) by contact in polluted environments (Knafla *et al.*, 2011). Epidermal tumors serve as visible tumors in

comparison with systemic tumors after dermal exposure. Although both are relatively dangerous. PAHs skin cancer risk potential should be given serious consideration as a matter of urgent public health issue.

The teratogenic effects of PAHs stems from the facts that high exposure of mothers can affect the fetus in their womb. PAH adducts has been found in the blood of neonates, whose mothers were exposed to PAHs in polluted environments both in China and Poland as observed in test results of mothers and child (Perera *et al.*, 2014). Thus, implicating PAHs agents present in the environment can break the placental barrier and initiate changes in the fetus. A coexisting relationship has been observed between the PAHs in the atmosphere and adduct level ranging from medium to high vascular and neurodegenerative diseases in infants (Chiang *et al.*, 2019). Other health effects accruable to direct contacts with the pollutants may result in deleterious health challenges which may include neurologic jumble (migraine, gagging, wooziness, and lethargy) from volatile organic compound (VOC) vulnerability and respiratory challenges, gastro intestinal tract problems (Adeniji *et al.*, 2019).

Occupational health effects have also been reported from workers in the oil industries exposed to various degrees of the pollutant. Crude oil spills cleaning activities entails certain risk of PAHs to worker involved in the exercise. In a case study of petroleum spill cleanup at Nakhodka Japan (Batdelger *et al.*, 2019), majority of the workforce that partook in the remediating procedure suffered from severe disorders ranging from lumbago, migraines, scarlet eyes and pharyngitis. Details from the Erika spill in 1999 also showed workers with numerous health issues similar to observed in Nakhodka spill. (Monza *et al.*, 2013). Evaluation of health status from Galicia spill cleanup 2002 reported mainly scarlet eyes,

neuralgia, pharyngitis, trauma retching, wooziness, and dyspnea (Oikonomou *et al.*, 2014) all linked to possible polyaromatics present in the spilled products.

2.4.2 Effects on Animals

Animals generally responds to pollutants like humans. Effects have been established between exposure of animals to PAHs and fertility complications, cardiac toxicity, pith toxicity, body defense suppression and organ compromise (Wang *et al.*, 2012). Chemicals may have a cynical effect on the lithosphere and hydrosphere distorting the assortment, myriad and task of the indigenous microbial populations resulting from oil spills and consequent deposit of PAHs (Sandri *et al.*, 2014).

PAHs are abound needed in particles in the hydrosphere and finally released into sediments (Nordin *et al.*, 2015) making them recalcitrant in the aquatic environment and harmful to aquatic life. Generally, because of the importance of fishes in water and consumable resource to humans, they are a sample of choice in different test protocols for toxins especially in forecasting the bio-concentration factor(BCF) (Nordin *et al.*, 2014) of chemical toxicants in the environment. Fish embryos and larvae have been studied in conjunction with PAHs concentration and they have proven to be susceptible to PAHs (Zeng *et al.*, 2019). Glitch resulting from exposure to PAHs include lacrimal and yolk sac hydrops, jaw dropping, conjectural skeletal defects described as spinal disorders such as saddle back or curved spine, and crainocerebral disorders bradycardia and tachycardia has been reported (Nordin *et al.*, 2015).

Anatomic pathology revealed changes in community structures and death of many aquatic species lasting beyond clean up destroying the aqua hydrosphere (Olajire *et al.*, 2003). The percentage of PAHs in the unique organs of four consumable freshwater fish species

including crucian cavin, shellfower fish, turf carp and niggles collected from a Lake in China and analysed by GC–MS disclosed the quantity and consequence of PAHs. The reported percentage was beyond the permissible limit for any aquatic animal (Wanga *et al.*, 2005). Bio surfactants together with other aerosols helped increase the availability PAHs and subsequent consumption by fishes exposed to crude oil as availability of the detoxification oxygenases CYP1A increased by a substantial portion far above water-accommodated fractions (WAF) by itself. (Li *et al.*, 2011)

Laboratory rats have also been used extensively to study the carcinogenic effect of polyaromatics. Polycyclic aromatic hydrocarbons resulted in externalization of ethoxyresorufin in O-deethylase, CYP1A a protein and CYP1A1 mRNA levels in rat liver. Showing variation in the response to individual members of the congener (Wang *et al.*, 2005). The buildup of DNA adducts is in order of enormity higher on the epidermis of mice exposed to B[a]P compared to the dermis and other parts, indicating the possible risk of skin tumors to animals following dermal exposure (Herbst *et al.*, 2013).

2.4.3 Effects on Plants

The repository for many compounds including PAHs is the soil (Huanling, *et al.*, 2019). The lithosphere heavily polluted by PAHs from various sources generates a wave of panic as man is endangered by hazards affecting the primary producer which is in turn important for nutrient cycling. Various chemicals in pest killers/inhibitors (bacteria, fungi, weevils etc.), washing agents, color mixtures and freeze balls contain quantities of PAHs and plants are the recipient stalling plant growth and leading to poor yield of crops (Johnsen *et al.*, 2005). PAHs found in lofty soil concentrations at effluent sites disperse and pollute arable farm soils inhibiting plant growth (Herbst *et al.*, 2019).

Plants are very delicate and succumb easily to foreign bodies including PAHs (Kummerová *et al.*, 2006). PAHs display a tenacity for bioaccumulation having a negative effect on green plants (Chan *et al.*, 2006) hindering photosynthesis. Atmospheric deposits on leaves surpass absorption from soil by roots as a portal of PAH accumulation (Mizwar and Trihadiningrum 2015). Pine needles a common plant was utilized in assessing normal air concentrations of POCs, such as PAHs and dichlorobenzene dioxins in comparison to pure PAHs dissolved in acetone and maintained in H₂O (Beriro *et al.*, 2016). The observed result displayed PAHs on pine needles to have squat photolysis rates. The implication is that the waxes of the pine needles can sustain PAH photolysis (Morzik *et al.*, 2003). PAHs cleaved to the topmost part of pine needles may prompt the production of ROS in the photosynthetic set up, in ways similar to the behavior of the weed killer paraquat (Oguntimehin *et al.*, 2007).

The consequence of hydrocarbon mixture (HCM) containing Maya crude oil and some known PAHs on sprout, development and survival of different grasses was evaluated in comparison to normal growth situation under controlled experiment. Sprout was unaffected for both concentrations but the height of the stems and roots decreased when HCM increased and the development of the studied samples was inhibited by the HCM in comparison to control (Reynoso-Cuevas *et al.*, 2008). Diatoms suffered the same fate when exposed to certain PAHs in HCM from surface sediments sampled in a highly PAH contaminated area of the Mediterranean Sea Italy), due to fierce industrial and anchor activities and the changes on the Diatoms was noxious (Carvalho *et al.*, 2011). PAHs not easily eliminated from soil via leaching and volatilization because of their stable nature. Consequently, they exhibit affinity for cooperation with organic carbon material in the lithosphere forcing themselves on plants

(Juhasz and Naidu, 2000; Jonsson *et al.*, 2007). Contaminating crops and invariably entering the food chain (Brassington *et al.*, 2007).

2.5 Petrogenic Pollution of PAHs

Polycyclic aromatic hydrocarbons found in crude oil and its allied products make its way into the lithosphere and hydrosphere from the exploration, exploitation, transportation, storage and even usage of refined petroleum products (Callén *et al.*, 2011; Nganje *et al.*, 2015; Davies and Abolude, 2016). Depending on the composition of crude and the hydrocarbon bonds that are inherent pollution occurs at various degrees involving many PAHs. Nigeria's sweet crude with low sulphur content has a concoction of total petroleum hydrocarbons (TPH), which comprises forty percent saturated/unsaturated hydrocarbons and twenty-one percent PAHs and thus most of spill leaves the environment exposed to PAHs. (Zabbey *et al.*, 2017). Oil spills accidental or deliberate can have dire effect on hemostasis in aquatic ecosystems (Itodo *et al.*, 2019). This feat has been reported as true for marine environments with situation of the Exxon Valdez, Amoco Cadiz and Prestige oil spills and in Nigeria oil spill at Ogoni land Rivers state. In each case, the flora and fauna of both micro and macro species experienced fatalities. Living organisms in the atmosphere and surrounding lithosphere were not spared (Peterson *et al.*, 2013). The fact that authoconous organisms in any given polluted environment can alter the pollutant from crude and it allied product to other compounds that may be more toxic is a real threat from PAHs emanating from petrogenic source.

2.6 Persistence of PAHs

The tenacity of PAHs in the environment is contingent on parameters pertaining to the principal contaminant: structure, molecular weight, concentration, dispersion and its bioavailability (Bortey-Sam *et al.*, 2014). Physico-chemical conditions also play a role

including but not limited to type of soil, aggregates, textural class, acidity or alkalinity, temperate conditions, aerobic or anaerobic situations, nutritional stimulants, moisture for indigenous microbial proliferation together with other prevailing environmental conditions determines life span of PAHs in the environment (Kim *et al.*, 2013). Generally, bigger ringed PAH molecules have stronger hydrophobicity and toxicity thus will be rooted in the environment (Debajyoti *et al.*, 2016). Furthermore, 'age' or duration of PAHs plays a role in the biodegradability of PAHs in soil because of co-metabolism (Moscoso *et al.*, 2015).

The alliance of PAHs with heavy metals is a major reason why they prolong their lodging time in the environment. Straight chain and indeed simple hydrocarbons and BTEX compounds are readily biodegradable by the autochthonous microbial community comparable to the complex structured PAHs. The struggle for survival by the indigenous species stimulates competition for available oxygen leading to its depletion generating anaerobicity and search for alternative energy source. Naturally the presence of O₂ means life processes are on-going and microbial activities are on top. However, many reactions involving biodegradation under anaerobic conditions have been successfully reported though a slower process. (Debajyoti *et al.*, 2016). The complexity of heavy metals cannot be overlooked in the lithosphere as they could be responsible for inhibiting microbial proliferation and invariably the PAHs utilization.

Furthermore, in the controlled degradation of hydrocarbon the straight chain aliphatics are easier to break and logically the complex structure of PAHs will generally slow down attack as the site where microorganisms can cleave and release enzymes to metabolize the pollutant is more complex and goes through a longer route to eliminate from the environment completely.

2.7 Removal of PAHs from the environment

The providence of PAHs and other toxicants in the ecosystem is analogous with abiotic and biotic processes characterized into physical, chemical and biological methods including vaporization, photonics, synthetic oxidation, sorption, leaching, combustion, photolysis, landfill and ultrasonic decomposition, phytoremediation and microbial transformation (Ouvrard *et al.*, 2014; Debajyoti *et al.*, 2016; He and Chi, 2016; Mehetre *et al.*, 2019). Among the treatment meted on the environment thus far, biological treatments involving bioremediation stratagem is the cheapest and a safer approach: restoring the integrity of the environment. Hence the quest for biological options suitable for degradation of PAHs is high to ensure the integrity of the environment is preserved.

In the microbial population, some can employ PAHs as sole carbon source, stabilize generate energy and by so doing quality of the PAHs would be compromised till carbon dioxide and water emanates or changed to other non-harmful or less harmful substances (Jiménez *et al.*, 2006; Perera *et al.*, 2011; Abdel-Shafy *et al.*, 2015; Mehetre *et al.*, 2019). PAHs responds to treatment metted on it in different manner especially biological treatment. Resulting from the fact that each PAH has a distinctive form and peculiar corporal, synthetic and biotic properties.

2.7.1 Biological degradation of PAHs

Biodegradation of PAHs can be considered in part as the normal processes of nutrient cycling, and also the excommunication of faux elements from the environment. The use of microbes for remediation of PAH polluted places is an incentive technology for recovery of contaminated sites as the natural environment is not damaged further with supposed corrective treatment measures in the degradation stratagem of PAHs. It involves both Phyto and Microbial degradation of toxicants (Abdel-Shafy *et al.*, 2015).

Phyto remediation makes use of plants in combination with some rhizosphere organisms to effect cleanup of PAHs in an ecofriendly manner (Parrish *et al.*, 2006; Tam and Wong, 2007; Corfield *et al.*, 2008). Microbial degradation studies focus on the use of microorganism that utilizes the carbon in the pollutant generally resulting in mineralization (Archana *et al.*, 2008). Microbial degradation employs bioaugmentation (addition of microbes) aerobic and anaerobic organisms and biostimulation (addition of nutrients) in various simulated processes (Peng *et al.*, 2008 and Haritash, 2009).

Microbes are agents that could clean up contaminated places if the contaminant has no barrier and is assessable for utilization by the organism (Debajyoti *et al.*, 2016). PAHs can only be assessed when they are dissolved or are in vaporized and will reabsorb at different rates over time. The major hitch in the availability of PAHs to microorganisms is their insolubility in water and invariable bioavailability. The availability of PAHs is largely dependent on their atomic size (Wang *et al.*, 2009). Angular shaped carbon ring has thermal behavior that is relatively constant, but the gaping places (referred to as bay regions) formed between the tilt benzene rings are subject to attack by enzymes. Making these angular PAHs susceptible to microbial attack than the straight or haphazard structures. PAH degradation can readily succumb to competitive inhibition.

PAH conversion or breakdown can be inhibited if the organism involved in biodegrading them find an alternative route meaning alternative energy source for metabolism other than the carbon source from the PAHs. When binding to alternate substrates occurs the active sites which the enzymes used by bacteria to cleave the needed PAHs as carbon source becomes nonspecific. These enzymes cannot remain in limbo but cleave to the available suitable alternative. If the alternative is readily available then the enzyme will degrade those

chemicals as its carbon source and the PAHs will remain in the environment (Wang *et al.*, 2009). Hence in PAHs degradation studies it is imperative to eliminate alternative carbon sources and other alternative sources in which microorganisms can metabolize ATP for proliferation.

2.8 Metabolism of PAHs

Biological organisms may be able to metabolize the complex structures of poly aromatic hydrocarbons under aerobic or anaerobic conditions. Microorganisms (bacteria, fungi, algae, actinomycetes, protozoa, etc) will need to destroy the benzene ring through variable and constant synthetic changes (Díaz *et al.*, 2013). In analysis involving O₂ and no O₂ trail of PAHs a Terminal Electron Acceptor (TEA) is required. The TEA determines the energy equilibrium and the break down reaction used by microbes (Philipp and Schink, 2012). The breakdown procedures of aromatic hydrocarbon biodegradation are divided into higher pathways and bottom pathways. The first route goes from the actual aromatic structure to the common intermediates, and the lower path goes from the closed cleavage of intermediates through to atoms for biofuels (Cafaro *et al.*, 2004; Carmona *et al.*, 2009)

In the presence of oxygen, oxidation is sped up by monooxygenases (hydroxylases) or by dioxygenases (Huijbers *et al.* 2014). These enzymes cleavage to appropriate positions of the available O₂ bond in which it attaches one O₂ molecule into the structure. The other is broken down to water. Enzymes are important substances produced by living organisms that can sped up reactions having unique nucleotide bases, selective in reactions including distinctive class of electron donor. The first group of enzymes can catalyse a reaction incorporating O₂ into either monoaromatic and polyaromatic hydrocarbons. They act by successively incorporating OH groups to the Benzene structure, using atomic O₂ as substrate,

creating diols and invariably catechols. The dioxygenases act on and speeds up inferential dihydroxylation of the structure taking the form of cis-dihydrodiol then guided by specific cis-dihydrodiol converts to catechols as metabolites. The enzymes act as intermix needing composite het structure held by weak bonds together with proteins (Parales and Resnick, 2004). The first reactions start by cleaving to available O₂ and ends with distinct metabolites, which can be catechols or non-catecholic compounds. Provided an alcoholic group is formed (Tao *et al.*, 2005; Ma *et al.*, 2013). When the reaction proceeds an aldehyde, a ketone and an eventual carboxylic acid may come forth (Fetzner, 2012).

In principle, the initial catalysis of the complex structure involves the de-aromatization of central intermediates, which goes through an ortho, meta or para bifurcation by dioxygenases (Harwood and Parales 2004). Ortho-cleavage, referred to as β -ketoadipate pathway lies sandwiched by two OH groups and catalyzed by intra diol-type dioxygenases using iron dioxide as cofactor (Guzik *et al.*, 2013). Meta-cleavage stands on the C-C bond almost opposite a OH groups and instigates the formation of the α -keto-lactone intermediate, sped up by extradiol-type dioxygenases, using Fe(III) as cofactor (Suenaga *et al.*, 2014). When Ortho cleavage is involved the catechol is catalyzed by catechol 1,2-dioxygenase to cis, cis-muconic acid. The caatechol meta-cleavage is sped up by 2,3-dioxygenase to 2-hydroxy-muconic semialdehyde. If the enzyme continues to metabolize the compound, then mineralization would be reached in which the compound would have been broken down to simple carbon dioxide that could be utilized for nutrient cycling.

2.10. Cow and the intestinal gut biota

Cow (*Bos tarus*) belong to a group of primary consumers known as ruminants. These are a unique group of animals that contribute to the general wellbeing of man as they serve as source of meat, milk, fiber, wool and draft (McCann *et al.*, 2014; Zhu, 2016). Ruminants are cloven hooves animals that chew their cud (Peng *et al.*, 2015; Zhu, 2016). Their diets including supplementation is high on fiber and roughages. They hardly respond positive to diets that are heavy on grains or fat. They possess a unique dental set up that enables them to cut and chew large quantity of leafy feeds. Their dentition is for grinding, and tongues holds together or gather grass and then nip it off between their teeth. They produce a lot of saliva that aids digestion and helps maintain the rumen pH at 6.5-7.2 for microbial activity in the rumen and intestinal gut. They are unique because they have a four compartments stomach the: rumen, reticulum, omasum, and abomasum (Parmar *et al.*, 2015). The rumen is the biggest chamber and it contains heavy microbial population (Ross *et al.*, 2012; Han *et al.*, 2015; Puniya *et al.*, 2015; Sachsenroder, 2016). These microbes interact synergistically with *Bos tarus*, aiding in the digestion of large quantities of foliage. Bacteria and protozoa have a sustainable niche dominating the intestinal gut environment and carry out suitable functions in the conversion of foliage for *Bos tarus* (Yuzhu *et al.*, 2020). These microbes through many processes convert the foliage and produce relevant fat and energy. The fatty acids produced are absorbed simply via the rumen wall generating appropriate energy needed by the *Bos tarus*. It is imperative to note that energy is generated together with proteins including essential amino acids from foliage the cow ingests. Due to the fact that microorganisms in this unique semi anoxic environment make use of available nitrogen generating proteins, cows can metabolize urea and other sources of non-proteinases nitrogen that would destroy non-ruminants. Microorganisms also help produce some essential vitamins needed by the beef cow.

The second compartment reticulum has a honey comb like lining. It is site where the cud is re-crushed that is where rumination takes place. It acts as a police trapping foreign materials ingested by *Bos tarus*. It is a norm to see rocks, nails, and pieces of wire and metal in the reticulum of cows. When cows ruminate, returning eaten food back to their mouth, they are said to be regurgitating a bolus making them super unique herbivores. To enable bacterial and other microbial population digest foliage quickly with maximum production, the foliage must be reduced in size, hence regurgitation occurs several times after feeding. *Bos tarus* eructate or belch several times a day releasing CO₂ and methane. For *Bos tarus* to “lose their cud” stems disaster for the herbivore and a clear indication that it is sick, and the rumen chamber is malfunctioning. This unique conversion chamber is home to many microorganisms and can metabolize an array of feeds, they are susceptible to drastic changes in their diet.

The omasum is “the book” or many piles a title generated as a result of its distinct leaf-like fold form. It is the purifier of *Bos tarus*, sieving unrequired materials and channeling them back to the reticulum creating enablement for fine particles and fluid to go through. Its functions are not distinctly defined however it is the site that aids H₂O reabsorption and recycling and cushion for the saliva (Kasparovska *et al.*, 2016).

The abomasum is the “true stomach.” It can be likened to the human stomach in that active conversions involving acids, enzymes, together with a cascade of other compounds to jump start protein synthesis takes place here. *Bos tarus* may stand at risk of ill health if foliage is unavailable or develop a heath challenge acidosis arising from a displaced abomasum or “twisted stomach.” The displaced abomasum may become torsional inhibiting the flow of nutrients. Treatment is via surgery. However, a displaced abomasum is not a common occurrence in dairy cattle but in beef cattle.

Digestive processes culminate in the small intestine and large intestine like every other complex organism including humans and other mammals. Digesta from the rumen locates the lower digestive tract including some microorganisms and undigested foliage, plus protein and sugars produced by the microorganisms themselves. The intestines are designed in such a way that nutrients which cannot be digested in other parts may see transformation else be channeled out via egestion. Enzymes with the sole responsibility to complex materials glide into the small intestine from the pancreas, and the gall bladder produces bile for metabolizing fats. The small intestine is packed full with enzymes to stimulate digestion and absorption of digested nutrients. Exception exists for product like fatty acids but other nutrients reside freely.

Water is chiefly absorbed in the large intestine and it is the last port of metabolism in the intestinal lining comprising the colon, cecum and rectum. Unutilized feed products, H₂O, metabolic wastes, nutrients etc. are channeled out of the large intestine as fecal material. The final utilization, reabsorption and filtering of wanted and unwanted materials is carried out in the large intestine. Waste products not of metabolic origin are forced down via the rectum to the anus. The consistency of the feces in *Bos taurus* is an indicator of the state of the herbivore and is dependent on water, fiber, and protein content of the feed (Jane-Parish, 2015).

The microbial population of the semi anoxic gut intestinal environment has important defensive, metabolic, and revivifying functions. Aboriginal microorganisms serve a central line aiding digestion and strictly opposing any form of colonization by allochthonous microbes fending off any potential invasion by unwanted bugs. The intestinal gut of the ruminants' animals is lodged by rumen microorganisms termed rumen microbiota (Sachsenroder, 2016). The intestinal gut of ruminant is dominated by bacteria which play major part in the microbial digestion of cellulolytic foilage material (Indugu *et al.*, 2016;

Kasparovska *et al.*, 2016). Bacteria population is more than 95% in the total rumen microbiome of the animal (Sandri *et al.*, 2014; Looor *et al.*, 2016).

The intestinal gut environment has the most diverse ecosystem found in nature comprising microorganisms of various taxonomic groups which are active in degrading different feed component (Kasparovska *et al.*, 2016). Its chambered pouch provides good temperature, pH and anaerobic oxygen during digestion of indigested cellulosic food. Hence after mastication and microbial enzymatic action the digested feeds are channeled to the abomasum or returns back to the rumen for improved digestion (Palmar *et al.*, 2015). Microbiome in the gut lining of ruminants responds rapidly to the variation in feeds, animal genetics, functional metabolism, geographic and environmental conditions (Wu *et al.*, 2012).

Rumen bacteria belong to fibrolytic, amylolytic, lipolytic, ureolytic, tannolytic and proteolytic types based on their enzymatic activities (Samantha, 2013) these bacteria species digest various indigestible plant materials rich in energy and protein (Zhu, 2016). Traditionally, culture dependent method is used in the isolation, identification and characterization of intestinal gut samples (Ahmad *et al.*, 2011). This technique though reliable is limiting in that organisms have specific nutrient requirement and a general purpose medium would identify only a particular group of organism (Nakamura *et al.*, 2008).

The large intestine also known as the large bowel is the niche where microorganisms carry out the final metabolic activity of breaking down complex restructures reabsorbs nutrients and forward out materials not needed from the intestinal tract as faeces (Yuzhu *et al.*, 2020). It contains a semi fluid material known as the intestinal chyme composed of some feacal materials, water and nutrients including unmetabolized food materials. It is the last part of the gastrointestinal tract and the digestive system in cows It is made up of four parts the

cecum, colon, rectum and the anal canal. It is loaded with microorganisms that have survived the rigors of metabolism via the mouth, through the oesophagus, the complex stomach and small intestines.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection

Intestinal chyme was aseptically withdrawn from the sigmoid colon of a male cow immediately after slaughter at the Effurun abattoir (latitude and 5.544230 longitude 5.760269, Delta state using alcohol sterilized containers. The containers were moved to the laboratory in cryo-bags filled with ice for further use.

3.2 Physicochemical characterization of the fecal gulp

The pH and temperature of the intestinal chyme were determined at the point of collection of sample using hand held pH meter and in the laboratory using a Hanna Instrument's multi-parameter (HI9829). Each measurement was done three times and the average determined. All standard procedures were followed according to the manufacturer's instructions.

3.2.1 Total Solids, Moisture Content, Ash Content and Volatile Solids

Other physicochemical parameters including total solids (TS), moisture content, volatile solids (VS) and ash content were also determined: For total solids (TS) and moisture content, 50 ml of each wet sample was weighed for their mass (M_{WS}) and dried at a temperature of 105°C for twelve hours and the mass of the dried sample (M_{DS}) weighed. The volatile solids (VS) and ash content were also ascertained by burning the dried samples to ashes at a temperature of 550°C for four hours and the mass of the ash weighed (MA). They were then calculated using equations (1), (2), (3) and (4) as used by (Van Reeuwijk, 2002; Asam *et al.*, 2011; and Cioabla *et al.*, 2012).

$$(\%) = M_{DS} / M_W \times 100 \dots\dots\dots (1)$$

$$(\%) = (M_{WS} - M_{DS}) / M_{WS} \times 100 \dots\dots\dots (2)$$

$$(\%) = M_A / M_{DS} \times 100 \dots\dots\dots (3)$$

$$(\%) = (M_{DS} - M_A) / M_{DS} \times 100 \dots\dots\dots (4)$$

Where:

M_{WS} = the mass of the wet sample

M_{DS} = the mass of the dry sample

M_A is mass of ash of the sample

3.3 Isolation and purification of microorganisms

Bacteria isolation from the intestinal chyme was carried out by serial dilution method, enrichment method and direct plate methods (Avishai and Charles, 2014). **For serial dilution method**, 1g of the sample was inoculated into test tube (1) containing 9ml of distilled water (stock) and serially diluted to test tube (6). 0.1ml from the serially diluted mixture was plated on nutrient agar and tryptic soy agar plates. All cultures were incubated for 24h at 37°C. The bacterial colonies, which surfaced on the plates, were carefully collected and purified by sub-culturing unto fresh agar plates using the streak plate technique. Unique colonies were selected based on the colony morphology: the appearance, preferment, opacity, coloration and texture of each isolate observed.

For enrichment method, a modified shake flask method of Siddique *et al.*, (2003) as well as Fulekar *et al.*, 2017 was adopted for the PAH spiked enrichment and bacterial isolation technique. A loopful of the stirred intestinal substance was aseptically inoculated into 100ml sterilized MSM broth with composition (0.04 $\text{CaCl}_2 \cdot \text{H}_2\text{O}$; 0.1 KH_2PO_4 ; 0.8 NaCl; 1.0 NH_4Cl ; 0.2 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.1 KCl. Micronutrients used were (mg/L) 0.1 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; 0.425

MnCl₂·4H₂O; 0.05 ZnCl₂; 0.015 CuSO₄·5H₂O; 0.01 NiCl₂·6H₂O; 0.01 Na₂MoO₄·2H₂O; 0.01 Na₂SeO₄·2H₂O. and pH adjusted to 7) spiked with 100 mg/l of solid pyrene dissolved in acetone, prepared separately in a conical flask. The mixture was incubated in a rotatory shaker for 7 days at 120 rpm, 37°C. About 5ml aliquots was aseptically collected and inoculated unto a fresh 100ml sterile MSM broth, supplemented with same amount (100 mg/l) of pyrene and incubated under same condition as reported. After four successive meliorations, the mixture was inoculated on the MSM agar plates containing thin layers of pyrene to obtain the enriched consortium of possible PAHs degrading microorganisms identified by observing a clearing zone around the inoculated region. The isolates obtained were then purified via repeat streaking on tryptic soy/nutrient agar plates by traditional spread plate techniques. Plates were incubated at 37°C for 48 hours after which pure isolates were selected for further identification. All isolates were stored in nutrient agar slant (Zhao *et al.*, 2009).

3.3.2 Morphological and Biochemical characterization of Microorganisms

The bacterial isolates obtained from nutrient and tryptic soy agar from above were first identified based on standard morphological, microscopic (Gram reaction) and biochemical tests in line with methods outlined in Bergey's manual of Determinative Bacteriology and grouped (Zhou *et al.*, 2008).

3.3.4 Microscopy using light microscope (Gram staining)

A clean slide was passed over flame to make it grease free and kept on a rack. A wire loop was sterilized by flaming (red hot) over a burning Bunsen burner and allowed to cool. A drop of sterile deionized water using a sterile pipette was placed on the slide. A small material of the 48-hour culture was added using sterile wire loop and mixed with the water to make a smear. The smear was heat fixed by passing over flame 2-3 times. The slide was covered with

crystal violet for sixty seconds, gently washed with running H₂O and allowed to drain. Slide was covered with Gram's iodine for 60seconds, rinsed with running water and allowed to drain. The smear was de-colored with ninety-five percent alcohol for thirty seconds and washed off with water. The smear was counter stained with saffranin for 30seconds and washed off with water. The slide was air dried and viewed under the Lietz light microscope using X10 and X40.

3.3.4 Biochemical Tests for identification of the isolates

During the biochemical assessment test, duplicate colonies with identical/similar morphological and macro biological features were noted and only one of each was selected for further studies.

Oxidase test: Oxidase test was carried out using the commercially available oxidase reagent (N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride) on a white tile. A loopful of the isolate was placed on the tile and 0.1ml of the oxidase reagent was placed on the isolate. The oxidase test was utilized for the identification of bacteria that metabolizes cytochrome c oxidase. This is bacterial enzyme implicated in the electron transport chain.

Catalase Test: To carryout oxidase test a 1ml of hydrogen peroxide (H₂O₂) solution was dropped on a pristine flat rectangular glass. Sterilized loop was used to pick a small piece of the test isolate and added. Catalase positive result was identified by the production of gas froth while no froth depicted a negative result (Reiner, 2010). If a catalase enzyme is present in a mixture, it sets out to counteract the bactericidal outcome of hydrogen peroxide (Wheelis, 2008). Catalase accelerates the conversion of hydrogen peroxide (H₂O₂) into H₂O and O₂ in a reaction: (2H₂O₂ + Catalase→2H₂O + O₂). This feedback is visible when bubbles form instantly (Clark and Cowan, 1952; MacFaddin, 2000; Harley, 2005).

Motility: Aseptically prepared Triple sugar ion agar (TSI agar) was placed in Bijou bottles (slant) and aseptically stabbed with the isolates using a platinum straight wire and nurtured at thirty-seven degrees for twenty-four hours. A conclusive result was indicated by a hazy growth spread throughout the medium making bottle slightly opaque. Negative result was recorded for growth confined to the stab.

Indole Test: The indole test is a differential test used in the determination of the possibility of microbial isolates to break indole from tryptophan present in buffered peptone H₂O. This identification parameter was to aid the clarification of Gram Negative Bacilli especially in members of the Enterobacteriaceae family. Experimental containers containing peptone H₂O was inoculated with a twenty-four hours' culture. The inoculated medium was nurtured at 37°C for 48 hours. Kovac reagent was added into 1ml of each of the culture medium. The determinant factor was shown by a red or purple color occurring rapidly at the top of the analyzing tube.

Citrate Utilization Test: Simon's citrate agar was used in carrying out the citrate test. Medium solidified in slant form were inoculated with 24 hours' culture of the isolates by carefully stabbing the preparation in the bijou bottles employing aseptic straight inoculating wire. The inoculated bottles were then incubated at 37°C for about 24 hours. Change in color from green to blue after 24 hours of incubation indicated positive result. The Citrate analysis was used to identify isolates which makes use of citrate (ionized form of citric acid) as one of its initiator products for metabolism. As citrate is used up by a bacterial isolate, the pH of the agar changes to alkaline the green color turns blue (alkaline). Blue color is citrate utilized while green color indicates citrate negative (Harley, 2005).

Sugar Fermentation: Triple sugar agar was prepared following instruction as provided by the manufactures with a plop of phenol red as the pH indicator. A 5mls of the preparation was dispensed into test tube and autoclaved at 118-121°C for 15 minutes and cooled in a forty-five-degree position to create deep butts. Using sterile straight inoculating loop, a single colony from a 24-hour culture was picked and stabbed to the butt of the tube. The surface was also streaked and incubated at 37°C for 24hrs.

The different sugars employed were Glucose, lactose and Sucrose. Other sugars evaluated to aid identification were Maltose, Fructose, Xylose, Galactose, L-Arabinose, Mannose, Dulcitol, Inositol, Salicin, Sorbitol and Rhamnose. The sugar fermentation was observed by the presence of shifted spaces (trapped gas) and a change in color (from red to yellow) triggered by the pH indicator, phenol red. The generation of hydrogen sulfide was observed by the blackish precipitate in the bottom of the analyzing container (Islam *et al.*, 2014)

Coagulase Test: The rock tile method was employed. In this method, a loop full of the isolate was dropped on the sterile tile was homogenized with human plasma and rocked for 1 minute. Clump forming agglutinins on slide was used as indicator for positive coagulase reaction (katz, 2010)

Methyl red Test: A 5ml of a 48hr culture in glucose phosphate medium was used on a slide. Five drops of 0.5% methyl red dye were added swirled and observed immediately for result. The positive cultures were bright red and negative culture was yellow.

VP Test: From the methyl red test after 24hours incubation pipette (sterile) 1ml of the broth culture into a sterile container under 5% alpha naphthol and 0.2ml of 40%KOH. Shake

the test tube carefully and allow to remain stagnant for 15minutes. A positive test is red indicating the presence of diacetyl and if no colour change test is negative.

3.4 Screening of isolates for the PAH degrading potential

To test the potential of isolates to degrade PAHs, isolates were subjected to 2,6-Dichlorophenol indophenol testing adopting the method of (Roy *et al.*, 2002; Koma *et al.*, 2003; Korale *et al.*, 2015). The assay mixture containing 2.5ml of carbon free Bushnell Haas medium, 150 µl of FeCl₃.6H₂O suspension and 150 µl of 2, 6-DCPIP was mixed with 300 µl of bacteria standardized at (O.D. 600 nm to 0.1) and 25 µl of pyrene (1000 mg/L in acetone). The reaction mixture was incubated at 32°C under shaking conditions (120 rpm) for 3 days. Pyrene degradation ability of the isolates was observed by recording a discoloration of the medium from blue to colorless. A negative control was also set up containing only the mineral salt medium and the dye without microorganisms and pyrene. The positive control contained mineral salt medium, the dye and pyrene. The set up was left for 96hours. Colour change was monitored (Purple to colourless). The growth of each isolate was also measured using a spectrophotometer at OD_{600nm} (Youssef *et al.*, 2010). Thereafter two (2) isolates with the best degradation capability were selected for further studies.

3.5 API Biochemical test using API20E test kit

The API ® 20E biochemical kit (Biomérieux, France) commercially purchased is a rapid test procedure containing wells for different biochemical tests, The test kit include wells for testing Beta galactosidase (ONPG), Arginine (ADH), Lysine (LDC), Ornithine (ODC), Citrate (CIT), Na thiosulfate (H₂S), Urea (URE), Tryptophan (TDA), Na pyruvate (VP), Charcoal gelatin (GEL), Glucose (GLU), Mannitol (MAN), Inositol (INO), Sorbitol (SOR), Rhamnose (RHA), Sucrose (SAC), Melibiose (MEL), Amygdalin (AMY), Arabinose (ARA)

and Oxidase (OX). Test procedure came with manufacturer's guide and was strictly followed. Results were noted using the API20e test kit guide and identified using the API web™. (Baur and Capone, 1985; Juhasz and Naidu, 2000; Cheeseborough, 2005; Zhou *et al.*, 2008)

3.6 Molecular Identification of selected isolates with best degradation capability

3.6.1 Genomic DNA Isolation by ZR fungal/Bacterial DNA Miniprep™ kit

The two selected pure isolates from the screening test were streaked onto sterile nutrient agar individually and incubated at 35°C for 24 hours in an incubator. Approximately 200mg of streaked samples were scraped into a batter bead™ lysis tube containing 750 µl of the ZR dissolution suspension, vortexed and centrifuged for one min at 10,000rpm. A volume of 400µl of the resultant supernatant was carried in a Zymo spin IV spin sieve, arranged in an assembling bottle and spinned for 1 min at 7,000 rpm. After which 1,200 µl of the DNA stuck up buffer was added to the residue in the assembly tube from the above. An 800µl portion of the mixture in the collection tube was transferred into a Zymo spin IIC column in a collection tube and centrifuged at 10,000 rpm for one min. The supernatant was thrown off and the procedure repeated using 800µl of the suspension remaining in the assembling tube. ZR DNA pre-wash (200µl) and DNA wash (500 µl) buffers were used to pre wash and wash the DNA in the IIC column followed by centrifugation at 10,000 rpm for one min in each case. The extracted DNA was removed by pipetting 50µl of DNA elution solution directly on the IIC column matrix and centrifuged at 10,000 rpm for 1 min.

3.6.2 Qualitative DNA assessment (UV-Vis Spectroscopy)

To evaluate the overall quality of extracted DNA, Purified DNA samples was analysed using a UV-Vis Spectrophotometer. The qualitative ratio was calculated using OD values recorded at 260 nm and 280 nm by the following formula;

$$\text{Concentration of DNA } (\mu\text{l/ml}) = \frac{\text{OD @ 260 (nm)} \times \text{dilution factor} \times 50}{1000}$$

A good DNA preparation generally exhibits an absorbance reading given by; A260/A280 = 1.80

3.6.3 Gel Electrophoresis

Genomic DNA was visualized by gel electrophoresis using one percent agarose. The procedure involved the dissolution of the agarose in 100ml distilled H₂O and heated. A measured quantity of Ethidium bromide was inoculated into the already cooled gel and transferred into the electrophoresis tank with the combs set. The suspension was dipped in Tris- borate-EDTA (TBE) buffer. A five μl of extracted DNA together with same quantity of dye-bromophenol blue was inoculated into the respective wells. A standard DNA ladder (1000 bp) was loaded into the appropriate well. Analysis was done at 120 volts for 40 minutes. Images were then captured using the gel documentation system (Alphamager™).

3.6.4 Polymerase Chain Reaction (PCR) amplification

Three sets of commercially procured forward and reverse bacterial universal primers (Inqaba Biotech South Africa) was used for PCR amplification they were: 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACT-3'), 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGATGTGTACAAGGC-3'), Bact-338 (5'-ACTCCTACGGGAGGCAGC-3') and Bact-1525 (5'-AAGGAGGTGATCCAGCC-3') representing the forward and reverse primers.

PCR procedure

Materials required for the PCR procedure were pieced together in an iced pack bucket and allowed to defrost before the experimental setup. All reagents were kept cold throughout the experiment. The needed materials and instruments like PCR tubes, PCR tube racks with

chilled cryoblocks, ethanol resistant markers and micropipettes needed were arranged on the workbench. The PCR Program (cycling conditions) was inputted into the PCR machine prior to commencement of the reaction. The PCR reaction was done by preparing a master mix (PCR cocktail) totalling 25µl for each isolate, containing 2.5µl of 10X buffer supplemented with 15mM MgCl₂, 100mM Tris-HCl, 500mM KCl, 2.5µl of each oligonucleotide primer (forward and reverse), 2µl of 0.04mM each dNTPs, 2µl of the extracted DNA, 0.25µl of 0.25 units/25µlTaq DNA polymerase and 7.25µl of Nuclease free water. The reaction was run in a Thermocycler by heating at 95°C for 5 min, and then amplification was performed with 35 cycles at 94°C for 2 min, an annealing step at 42°C for 1 min as well as an elongation step at 72°C for 2 minutes. Amplification ended with an elongation phase at 72°C lasting 7 minutes and a final hold at 4°C. At the end of the programme, containers were taken out of the equipment and refrigerated at 4°C.

The PCR products (amplicons) were observed by transferring portions of each reaction into wells of a 1.2% agarose gel which was prepared and stained with 10mg/ml of ethidium bromide. Fifty millilitres (50 ml) of agarose gel containing 0.6g of agarose powder and five micro litre (5 µl) Ethidium bromide was prepared and cast in a casting tray after which it was transferred into an electrophoretic tank submerged with TBE buffer. The PCR Products were loaded into the wells of the agarose gel with 6X loading dye from Thermo Scientific. Two microlitres (2 µl) of the step ladder (O'gene ruler by Inqaba biotech) of molecular size range 1kb was also loaded in the first wells, ran on a Fotodyne 3000 power pack at 140V for 30 min and then visualized using Alpha Innotech Gel documentation system model 201103 (Taiwan).

Analysis was run at a hundred and twenty volts for forty minutes. Images were then captured using the gel documentation system (Alphamager™). Test analysis was repeated for

each PCR run optimized. Purification was done by Zymo DNA purifier and concentrator (DCC) kit (Zymo Research) and sequenced with a 3500 ABI genetic analyser at Inqaba Biotech Ltd, South Africa using same forward and reverse primers employed during PCR amplification. Taxonomic identification was achieved by comparing the obtained nucleotide sequences for similarity with reference strains by a BLAST search within the NCBI gene bank (Kumar *et al.*, 2016).

3.7 Innoculum development

A loopful of each selected colony was inoculated unto 5 ml of sterile 0.1% NaCl solution, centrifuged at 4,000 rpm. Pellets were obtained and washed twice in sterile distilled water then absorbance was determined using a UVs-Vis spectrometer at 600nm to ensure the concentration was 0.1 before inoculation (Obuekwe and Al-Zarban, 1998; Al-Wasify *et al.*, 2014).

3.8 Degradation of Benzo[a] Pyrene

Shake flask method (Fulekar *et al.*, 2017) was adopted for degradation and the protocol for the degradation of PAHs were set as follows: sterile bijoux bottles were appropriately labeled and 100µl of B[a]P in acetone was pipetted in and placed in an orbital shaker for 4 hours at 100 rpm for a final concentration of 250 mg/L using the molarity formula for calculation ($\text{molarity} = \frac{\text{mass}}{\text{molar mass}}$) under a biosafety cabinet until the acetone evaporated completely. A 2ml of sterile Bushnell Haas media composition in (Appendix 1) was added to each bijoux bottle containing 250 mg/ml of B[a] P. Each bottle was inoculated with selected pure washed cultures and wrapped in foil (aluminum) paper before incubation in the dark in a rotatory shaker at 100 rev per minute for 16 days. Samples were withdrawn at 3 days' interval: 1, 4, 7, 10, 13, and 16 for analysis.

A separate set of bijou bottles containing 100µl B[a] P and 2ml sterile Bushnell Haas medium, placed under the same conditions served as a control. Unless otherwise stated, this set up was done in triplicate. Residual B[a]P was extracted from each culture vessel using 2ml Dichloromethane. The entire content of each bijou bottle was sonicated for 15 minutes using an ultra sonicator TM. The organic layer was retrieved and evaporated in a suitable evaporator. The resulting residue was then dissolved in 2ml of acetonitrile. B[a]P was analyzed using a Shimadzu 9880 HPLC system (National Institute for Pharmaceutical Research and Development, Abuja, Nigeria). A 20µl of analyte was injected into an immobilized point capillary column measuring (15cm×4.6mm ID) and quantified by UV detector at 254nm. The glide rate of mobile phase (water and acetonitrile) maintained at 1.5 ml/min. Chromatographic peaks in were matched by comparism of the retention time and spectra of control (Wang, *et al* 2016).

3.9 Degradation of Pyrene and Flouranthene

The procedure stated earlier in section 3.8 was followed except that the analyzer of the PAHs (Pyrene and Flouranthene) was GC-MS (Shimadzu7820A- 5975C inert mass spectrometer, Agilent Technologies®). Its operating mass selective detector (MSD) and selective ion monitoring (SIM) together with scan mode were adjusted appropriately for the most sensitive detection of the PAHs wrapped in 5% Phenyl Methyl Siloxane (30m length x 0.32mm diameter x 0.25µm film thickness) (Shimadzu Technologies). The utilized gas was Helium maintained at of 1.48 mL/min at a titular pressure of 1.49 psi with velocity of 44.22 cm/sec. Extracts were fed in split less mode at a temperature of three hundred degrees Celsius. Purge flow to spilt vent was fifteen milligrams per minute for less than one minute with a total flow of 16.67 mL/min; gas saver mode was logged off. Initially oven temperature was 40°C (1

min) and lifted at 12°C/min to 300°C (10 min). Running time was elongated with a 3 min solvent delay. Mass spectrometer was monitored in electron-impact ionization mode at 70eV with ion source temperature of 230°C, quadruple range of 150°C with transfer line at 300°C (Wang and Stout, 2016).

3.10 Determination of Metabolites of Pyrene and Flouranthene during biodegradation by the selected isolates

Metabolites were determined by comparing the additional peaks other than pyrene and flouranthene shown on the chromatogram with known literature and comparing with the library of the GC-MS detailed in Appendix 6.

3.11 Parameters evaluated during biodegradation of BaP, Pyrene and Flouranthene

3.11.1 Viable count monitored during degradation

At three days' interval 1ml of each PAH sample (B(a)P, Pyrene and flouranthene) treated with the selected isolates singly and in combination was suspended in 9mls of 0.85% (w/v) saline and was vortexed vigorously for 1 min. Appropriate dilutions were prepared (10^{-2} to 10^{-6}) and plated in triplicate on nutrient agar. Isolates were nurtured at 37°C for twenty-four hours after which the figure of colonies were counted. Control plates were not inoculated and growth was assessed by logcfu/ml (Babita *et al.*, 2013).

3.11.2 Physicochemical monitoring during PAH degradation study

Dissolved oxygen (DO) was measured during the course of degradation in sections 3.6 and 3.7 using a Dissolved Oxygen meter. Following calibration of the DO meter according to the manufacturers' instruction, the display reading of the DO meter was programmed and zeronized from the control button. The inquest was placed in suspension set to zero for ten minutes to sufficiently polarize the probe. The inquest was removed from the suspension and

immersed in distilled water(100mls). The inquest was positioned about 1 cm above the water and adjusted to display 100 using the slope control. The inquest was then immersed in the PAH sample treated with bacteria singly and in consortium after rocking rigorously. The DO content was then measured in mg/l (Boyd *et.al.*, 2005).

Biological Oxygen demand (BOD) was determined by emptying the entire content of the PAH sample treated with the selected isolates into a 100ml beaker and made up with distilled water. The samples were thoroughly aerated and DO1 was determined. The PAH mixture was then poured in a screw – topped incubation bottle, corked and nurtured in the dark for five days at 20°C. Dissolved oxygen (DO5) was measured from the nurtured sample. BOD is the variance between the two determined DO levels (DO1 and the DO5). (Johnsen *et al.*, 2005).

Chemical Oxygen demand (COD) was measured by centrifuging 20mls of PAHs at 4000rpm for 15minutes and discarding the supernatant. Two milligram of the sediment was then modified with 0.05g of mercury sulphate, 0.5ml of 1.0g potassium dichromate and 2.5ml of concentrated sulfuric acid (containing 5.5mg silver sulfate per gram) waned for two hours at 150°C after cooling. COD was ascertained by absorbance reading on a Hach model DR-2000 spectrophotometer (Hach Company, USA) (Maricy *et al.*, 2011).

pH was determined using a pre-calibrated Hana HI9829 pH/Temperature meter. The glass terminal of the pH meter was immersed in the assayed samples and pH values of each sample bottle was recorded.

3.12 Optimization of the growth of the selected isolates singly and in consortium using one variable at a time Approach (Ova T)

The factors that were varied after the initial assay and evaluated for optimal growth and (PAHs) degradation include; pH, temperature, concentration and nutrient stimulation (Nitrogen, Phosphorus and Potassium. NPK15:15:15). The levels of each factor studied were selected as described below.

3.12.1 Effect of incubation period on the degradation of PAHs

The growth of the selected isolates and percentage degraded PAHs (B(a)P, pyrene and flouranthene) were ascertained by following the protocol: 0.1ml of the PAH(s) was dissolved in 5mls of acetone for a final concentration of 250mg/ml and aseptically inoculated into test bottles and allowed to evaporate in a shaker under a biosafety cabinet. Thereafter, 2mls of Bushnell Hass mineral salt medium with same composition as reported in section 3.3 above was aseptically added and the pH adjusted to 7 by standardized 1M of either HCl or NaCl. The selected isolates were washed in normal saline and adjusted to (OD 0.1 at 600). The combination was done by adding equal volume of the selected washed isolates in a sterile container and inoculating into the PAH/MSM broth. The samples were wrapped and placed in an orbital shaker and adjusted to medium/moderate speed. The effects of the incubation period were evaluated at three days' intervals of (1,4,7,10,13 and 16) Growth was monitored in the form of OD at 600nm while for residual PAHs, the entire content of the experimental flask was subjected to liquid-liquid extraction using ethyl acetate (1:1v/v) after acidification to pH2 with 1M HCl. Ethyl acetate was extracted with same volume of NaOH. The organic phase (neutral fraction) was dried over anhydrous sodium sulphate (Na_2SO_4) and solvent removed in vacuo. The remnant was dissolved in 10ml methanol then used for qualitative

analysis of residual B(a)P, pyrene and flouranthene at 335nm. The percentage degradation of the PAHs (B(a)P, pyrene and flouranthene) was calculated by using the formula

$$\% \text{ degradation} = \frac{A-B}{A} \times 100$$

A Where A=initial concentration, B=Final concentration.

3.12.2 Effect of pH

The tolerance level of the microbes at different pH evaluated by inoculating the selected isolates in nutrient broth, adjusted according with buffer solutions for different pH were prepared: sodium phosphate for (pH 5) with composition: 20.214g of Na₂HPO₄·7H₂O, 3.394g of NaH₂PO₄·H₂O and 800mls of distilled water, potassium phosphate (pH 7) with composition 12.211g K₂HPO₄ and 6.66g of KH₂PO₄ and 800mls distilled water, tris-HCl (pH 9), and glycine–NaOH (pH 11). Growth of isolates was measured using optical density at 600nm using UV-VIS spectrophotometer (AAT Bioquest, 2020).

3.12.2 pH and degradation of PAHs by selected isolates singly and in combination

The effect of pH on concentration of PAHs ((B(a)P, pyrene and flouranthene) and growth of isolates in the various pH mixture was evaluated using the protocol in 3.12.1. and adjusted to pH 5, 7 and 9 with buffer solutions in 3.9.2 above. The sampling bottle was inoculated with the selected isolates singly and in consortium and incubated at 37°C for 96hours. After the sixteenth day of incubation contents from all test tubes were subjected to liquid-liquid extraction and evaluated using a UV-VIS spectrophotometer as before and growth were determined at O.D. 600nm.

3.12.3a Effect of Temperature on growth patterns of the isolates

To determine favourable temperature required for microbial growth, the selected isolates were evaluated in nutrient broth at varied temperatures (20°C, 35°C and 50°C) to test

their viability without exposure to any pollutant. At different time interval (24, 48, 72 and 96) hours, a 2ml portion was poured into a sterile cuvette and placed in the spectrophotometer. Readings were taken at 600nm to ascertain the growth of the isolates.

3.12.3b Changes in temperature and degradation of PAHs by selected isolates singly and in consortium

The effect of temperature and rate of degradation was ascertained by following the protocol in 3.9.1 and nurtured at varied temperatures (20°C, 35°C and 50°C). After 16 days of incubation contents from all test bottles were put through liquid-liquid extraction as before. The residual concentrations/percentage degradation of Benz(a)pyrene, pyrene and flouranthene was determined at 335nm and growth by taking O.D. at 600 nm.

3.12.4. Effect of varied concentration of PAHs on bacteria isolates and consortium

Different concentration of B(a)P, pyrene and flouranthene were prepared using their molarity as before. Each of the PAHs degradation by selected isolates singly and in consortium were studied at 500 mg/L, 750 mg/L, 1000 mg/L and 1250 mg/L. Bottles containing Bushnell Haas mineral salt medium were spiked with different concentrations of B(a)P, Pyrene and Flouranthene respectively. Flasks were then inoculated with 0.1ml inoculums of the selected isolates and consortium from exponential growth phase having 0.1 O.D. at 600 nm. Flasks were incubated in an orbital shaker: 120 rpm at 32°C for a period of sixteen days. Growth was monitored after the 16th day of incubation in the form of O.D. at 600 nm and the entire contents from all bottles were subjected to liquid-liquid extraction by using ethyl acetate as stated earlier. The residual concentration of pyrene was determined by UVVis-spectrophotometer at 335 nm and % degradation was calculated as in 3.12.1

3.12.5. Effect of Nitrogen/ Phosphorous/Potassium (NPK 15:15:15) sources on growth of the isolates and degradation of PAHs

To determine microbial response (individually and in consortium) to additional nutrients and concentration reduction of PAHs, protocol as in 3.12.1 was repeated with amendment of 5% sterile N: P: K15:15:15. The bottles were wrapped as before and incubated in an orbital shaker and the residual PAHs obtained via a UV-VIS by measuring the OD at 335nm and growth by taking O.D. at 600 nm over a 96hour period (Karale *et al.*, 2016)

3.13 Detection of PAH Degrading Genes by PCR in the selected bacterial isolates.

The presence of the genes in the selected bacterial isolates responsible for PAHs degradation was identified based on PCR matches. Three primers were selected for the amplification including: specific bacterial primers: Naphthalene Dioxygenase (*NahAC*), Catechol dioxygenase (*C23O*), n-alkanes monooxygenase (*alkB*). All PCR products were separated in 1.5% agarose gel, stained with ethidium bromide, viewed under UV light then film indexed.

For the screening/detection for the presence of the specific genes, a set of forward and reverse primers were selected including: dioxygenase(*nahAc*) [*nahAc* F-5'-TGGCGATGAAGAACTTTTCC-3' and *nahAc* R-5'-AACGTACGCTGAACCGAGTC-3'] with an expected length of 487bp (Laurie and Jones, 2000), catechol -2-3-dioxygenase(*C23O*)[*C23OF*-5'-AAGAGGCATGGGGGCGCACCGGTTCTGA-3' and *C23OR*-5'-TCACCAGCAAACACCTCGTTGCGGTTGCC-3'] with an expected length of 900bp (Kloss *et al.*, 2006) and n-alkanes monooxygenase(*alkB*) [*alkBF*-5'-AACTACMTGARCAYTACGG-3' and *alkBR*-5'-TGAMGATGTGGTYRCTGTTCC-3'] with an expected length of 100bp (Hesham *et al.*, 2014) were employed. PCR procedure and materials preparation was repeated as before but PCR conditions for gene detection followed

an initial debasing for 5 minutes at 95°C, thirty-five cycles, 40 seconds at 94°C and 55°C respectively, 60 seconds at 72°C, and final elongation for 7 minutes at 72°C, specific for alkane and naphthalene monooxygenase genes (Laurie and Jones, 2000; Guo *et al.*, 2010).

For detection of Catechol 230 dioxygenase, the cycling conditions were as follows: initial debasing for 5 minutes at 95°C 35 cycles with 20 seconds at 94°C, 30 seconds at 63°C, and 45 seconds at 72°C, and final elongation for 5 minutes at 72°C (Hesham *et al.*, 2014). Procedure of detection of amplicons was also repeated as before and ran at 120 volts for 40 minutes. Images were then captured using the gel documentation system (Alphamager™). This series of events was repeated per PCR run optimized. The end product was cleaned using Zymo DNA clean and concentrator (DCC) kit sequenced with a 3500 ABI genetic analyser at Inqaba Biotech Ltd, South Africa using the same forward and reverse primers employed during the first PCR amplification.

CHAPTER FOUR

RESULTS

4.1 Physicochemical characterization of the intestinal chyme

The Physicochemical characteristics of the intestinal chyme from the large intestine are presented in Table 4.1 below. The temperature of the intestinal chyme at the point of collection was within the mesophilic range (20 °C -45 °C) with a mean average of 29.5°C while the pH was 7.15. The moisture content, total solids and volatile solids were substantial in comparison to standard parameters of a healthy cow.

4.2 Isolation, Morphological and Biochemical characterization of microorganisms from non PAH enriched media

After Isolation by the direct culture and enrichment technique(s), several distinct pure cultures each were obtained and identified by their morphological and biochemical test. However, some responded similarly displaying overwhelming similarity based on which 17 and 16 isolates were selected and reported in Tables 4.2 and 4.3 respectively. A total of twenty-four (24) of the thirty-three isolates identified belonged to the Enterobacteriaceae family including *Salmonella*, *Esherichia coli*, *Klebsiella*, *Shigella*, *Rautella*, *Citrobacter* and *Enterobacter*. Other species that surfaced belonged to several variants of the Staphylococcae family and few species belonged to the Microccoccae and Enterococcae families. Most of the organisms were facultative anaerobes with closely related biochemical features. The isolates were predominantly cocci shaped with some displaying diplo-cocci features only a few were rod shaped. All the identified isolates possessed the enzyme catalase as shown in test result.

4.3 Testing the potential of the isolated bacteria to degrade PAHs using 2,6-Dichlorophenol Indophenol (DCPIP) Assay

Tables' 4.4 and 4.5 presents the qualitative assessment of PAHs degradation potential by the bacteria isolates using 2, 6-Dichlorophenol indophenol (DCPIP) redox indicator. The general observation of the indicator is that oxidized forms are coloured (blue) and reduced forms are colourless. PAHs degrading ability of *E. coli* from direct plating method caused the fastest discolouration of the dye as determined by visual observation. For the test bacteria isolates from the enrichment method 70% were discoloured within twenty (24) hours as determined by visual observation. This necessitated the quantitative analysis using the UV-VIS spectrophotometer @ 600nm that measured the degree of clarity and the sample closest to 0.1 was selected as clearest used for further analysis.

Table 4.6 and 4.7 shows the quantitative assessment of the potential of the bacterial isolates to degrade PAHs. *E. coli* stood as the best degrader from the direct plate method while *Raoultella ormithinolytica* was the isolate of choice from the enrichment methods.

4.4 Identification using API 20 E

Table 4.8 showed biochemical test results from API20E for the two selected bacteria isolates *Raoultella ormithinolytica* and *E. coli* from the enrichment and direct plating methods respectively. After incubation and using the APIWEB software, results showed maximal similarity of *Raoultella ormithinolytica* from morphological/biochemical test to be API20E/APIWEB *Raoultella ormithinolytica* 99.7% but *E. coli* showed only a 47% similarity to *Morganella morganii*.

Table 4.1: Physicochemical Characterization of the intestinal chyme

Parameter	Values
Temperature (°C)	29.5 ± 0.50
Ph	7.15 ± 0.02
Moisture Content (%)	96.9 ± 0.33
Total Solids (mg/ml)	13.25 ± 0.41
Volatile solids (mg/ml)	81.26 ± 1.79

NB: All values are expressed as Mean $\pm$ SE after three replicates

Table 4.2: Morphological, Biochemical and sugar fermentation assessment of isolates from direct plating on Nutrient Agar

Morphology	Microscopy	Biochemical Test								Sugar Fermentation						Most likely organism
Shape	Grams Reaction	Urease	Oxidase	Catalase	Coagulase	Indole	VP	MR	Citrate	Glucose	Lactose	Sucrose	H ₂ S	Acid	Gas	
Cocci	+	-	-	+	-	-	+	+	-	+	+	+	-	+	-	<i>Staphylococcus sp</i>
Rod	-	-	-	+	-	+	-	+	-	+	+	+	-	+	+	<i>Morganella morganii</i>
Diplo-cocci	+	-	-	+	-	+	-	-	-	-	-	+	-	+	+	<i>Arthrobacter sp</i>
Cocci	+	-	-	+	-	+			+	-	-	+	-	+	+	<i>Micrococcus hablobils</i>
Rod	-	-	-	+	-	-	-	+	-	+	-	-	+	+	+	<i>Salmonella pullorum</i>
Rod	+	+	+	+	-	+			+	+	+	+	+	+	-	<i>Pasteurella pneumotropica</i>
Diplo-cocci	-	+	-	+	-	+	+	-	+	+	+	+	-	+	+	<i>Raoutella sp</i>
Rod	+	+	-	+	+	+	-	-	-	+	+	+	-	-	+	<i>Staplyloccocus aureus</i>
Cocci	+	-	-	+	-	-	-	+	+	+	+	+	-	+	+	<i>Staphylococcus heamolyticus</i>
Cocci	+	+	-	+	-	+	+	+	-	+	+	+	+	+	+	<i>Citrobacter freundii</i>
Diplo-cocci	-	+	-	+	-	+	-	+	-	+	-	-	-	+	+	<i>Escherichia coli</i>
Diplo-cocci	+	-	-	+	-	-			-	+	+	+	+	+	+	<i>Micrococcus roseus</i>
Cocci	+	-	-	+	-	+	+	=	-	+	+	+	-	+	+	<i>Staphylococcus capitis</i>
Cocci	-	-	-	-	-	-	+	-	+	+	+	+	-	+	+	<i>Enterobacter aerogenes</i>
Rod	-	+	-	+	-	+			-	+	+	+	-	+	-	<i>Leclercia adecarboxylata</i>
Rod	-	+	-	+	-	-	+	-	+	+	-	+	-	+	+/-	<i>Serretia plymuthicas</i>
Rod	+	+	-	+	-	-	+	-	+	+	+	+	-	+	+	<i>Psuedomonas cepacia</i>

Key: - = negative, + = positive.

Table 4.3: Morphological, Biochemical and sugar fermentation assessment of isolates on MSM enriched with PAH (Pyrene)

Morphology	Microscopy	Biochemical								Sugar Fermentation						
Shape	Grams reaction	Urease	Oxidase	Catalase	Coagulase	Indole	VP	MR	Citrate	Glucose	Lactose	Sucrose	H ₂ S	Acid	Gas	Probable Organism
Rod	-	-	-	+	-	-	+	-	-	+	-	-	+	+	+	<i>Salmonella pullorum</i>
Cocci	+	+	-	+	-	+	+	+	-	+	+	+	+	+	+	<i>Citrobacter freundii</i>
Diplo-cocci	+	+	-	+	-	+	-	+	-	+	+	+	+	+	+	<i>Staphylococcus warneri</i>
Rod	-	+	-	+	-	+	-	+	-	+	+	+	-	+	-	<i>Leclercia adecarboxylata</i>
Cocci		+	-/+	+	-	-	+	-	+	+	+	+	-	+	+	<i>Micrococcus varians</i>
Rod	-	-	+	+		-	+	-	+	-	-	-	-	-	-	<i>Pseudomonas alcaligenes</i>
Diplo-cocci	-	+	-	-	-	+	-	+	+	+	-	+	-	+	+	<i>Klebsiella oxytoca</i>
Diplo-cocci	-	+	-	+	-	-	+	-	+	+	-	+	-	+	+	<i>Raoultella ormithinolytica</i>
Diplo-cocci	-	+	-	+	-	+	+	-	+	+	+	+	-	+	+	<i>Raoutella sp</i>
Rod	-	-	-	+	-	+	+	-	+	+	+	+	-	+	+	<i>Khuyvera ascorbata</i>
Cocci	-	+	+	-	-	-	+	-	-	+	+	+	+	+	-	<i>Actinobacillus minor</i>
Cocci	-	-	-	-	-	-	+	-	+	+	+	+	-	+	+	<i>Enterobacter aerogenes</i>
Rod	-	+	-	-	-	-	-	+		+	-	+	-	+	-	<i>Proteus penneri</i>
Diplo-cocci	-	+	-	+	-	+	-	+	-	+	-	-	-	+	+	<i>Morganella morganii</i>
Diplo-cocci	+	-	-	+	-	-	+	-	-	+	+	+	+	+	+	<i>Micrococcus roseus</i>
Rod	+	+	-	+	-	-	+	-	+	+	+	+	-	+	+	<i>Cellobiosococcus sp</i>

Key: - = negative, + = positive.

Table 4.4: Qualitative screening of isolates from direct plate method for their capability in degrading PAH using 2, 6-Dichlorophenol indophenol redox indicator.

Isolate	Colour change after:(h)				
	0	24	48	72	96
CTRL (+ve)	-	-	-	-	-
CTRL (-ve)	-	-	-	-	-
<i>Staphylococcus sp</i>	-	-	-	-	-
<i>Escherichia coli</i>	+	+	+	+	+
<i>Arthrobacter sp</i>	-	-	-	-	+
<i>Micrococcus hablobils</i>	-	-	-	-	-
<i>Salmonella pullorum</i>	-	-	-	-	+
<i>Pasteurella pneumotropica</i>	-	+	+	+	+
<i>Raoutella sp</i>	+	+	+	+	+
<i>Staphylococcus aureus</i>	-	-	-	+	+
<i>Staphylococcus heamolyticus</i>	-	-	-	-	+
<i>Citrobacter freundii</i>	-	-	-	+	+
<i>Morganella morganii</i>	+	+	+	+	+
<i>Micrococcus roseus</i>	-	-	-	-	-
<i>Staphylococcus capitis</i>	-	-	-	+	+
<i>Enterobacter aerogenes</i>	+	+	+	+	+
<i>Leclercia adecarboxylata</i>	+	+	+	+	+
<i>Serratia plymuthicas</i>	-	-	-	+	+
<i>Pseudomonas cepacia</i>	+	+	+	+	+

Key: - = no change, + = discolouration, CTRL +ve = MSM medium + Pyrene+ DCPIP, CTRL-ve= = MSM medium + Pyrene

Table 4.5: Qualitative screening of isolates from enrichment method for their capability in degrading PAHs using 2, 6-Dichlorophenol indophenol redox indicator.

Isolate	Colour change after (h)				
	0	24	48	72	96
CTRL (+ve)	-	-	-	-	-
CTRL (-ve)	-	-	-	-	-
<i>Salmonella pullorum</i>		-	-	+	+
<i>Citrobacter freundii</i>		+	+	+	+
<i>Staphylococcus warneri</i>		-	-	+	+
<i>Leclercia adecarboxylata</i>		-	-	+	+
<i>Micrococcus varians</i>		+	+	+	+
<i>Pseudomonas alcaligenes</i>		+	+	+	+
<i>Klebsiella oxytoca</i>		+	+	+	+
<i>Raoultella ormithinolytica</i>		+	+	+	+
<i>Raoultella sp</i>		+	+	+	+
<i>Kluyvera ascorbate</i>		+	+	+	+
<i>Actinobacillus minor</i>		+	+	+	+
<i>Enterobacter aerogenes</i>		+	+	+	+
<i>Proteus penneri</i>		+	+	+	+
<i>Morganella morganii</i>		+	+	+	+
<i>Micrococcus roseus</i>		+	+	+	+
<i>Cellobiosococcus sp</i>		+	+	+	+

Key: - = No change, + = Discolouration, CTRL +ve = MSM medium + Pyrene+ DCPIP, CTRL-ve=MSM medium + Pyrene

Table 4.6.: Quantitative screening of bacterial from direct plating method by 2, 6-Dichlorophenol indophenol redox indicator using UV-VIS @600nm

Isolate	Incubation Period(h)				
	0	24	48	72	96
CTRL (+ve)	1.66±0.02 ^a	1.63±0.04 ^a	1.60±0.06 ^b	1.60±0.06 ^b	1.59±0.00 ^b
CTRL (-ve)	0.29±0.05 ^b	0.29±0.03 ^b	0.29±0.03 ^b	0.29±0.04 ^b	0.29±0.01 ^b
<i>Staphylococcus sp</i>		1.56±0.01 ^a	1.52±0.02 ^b	1.52±0.03 ^b	1.49±0.07 ^c
<i>Morganella morganii</i>		1.19±0.04 ^a	1.03±0.06 ^b	0.99±0.03 ^c	0.39±0.00 ^d
<i>Arthrobacter sp</i>		1.51±0.06 ^a	1.51±0.02 ^a	1.38±0.03 ^b	1.09±0.07 ^c
<i>Micrococcus hablobils</i>		1.54±0.03 ^a	1.52±0.06 ^a	1.50±0.02 ^b	1.51±0.01 ^b
<i>Salmonella pullorum</i>		1.58±0.05 ^a	1.56±0.03 ^a	1.38±0.02 ^b	1.06±0.03 ^c
<i>Pasteurella pneumotropica</i>		1.57±0.04 ^a	1.25±0.06 ^b	1.06±0.02 ^c	0.92±0.01 ^c
<i>Raoutella sp</i>		1.53±0.02 ^a	1.14±0.03 ^b	0.99±0.01 ^c	0.24±0.01 ^d
<i>Staphylococcus aureus</i>		1.56±0.01 ^a	1.18±0.02 ^b	0.96±0.03 ^c	0.96±0.07 ^c
<i>Staphylococcus heamolyticus</i>		1.59±0.02 ^a	1.49±0.03 ^b	1.11±0.09 ^c	0.99±0.01 ^d
<i>Citrobacter freundii</i>		1.24±0.03 ^a	1.14±0.03 ^b	1.14±0.01 ^b	1.11±0.01 ^b
<i>Escherichia coli</i>		0.87±0.04 ^a	0.56±0.03 ^b	0.49±0.08 ^c	0.11±0.02 ^d
<i>Micrococcus roseus</i>		1.58±0.01 ^a	1.56±0.02 ^a	1.56±0.05 ^a	1.55±0.04 ^a
<i>Staphylococcus capitis</i>		1.47±0.05 ^a	1.45±0.06 ^a	1.36±0.02 ^b	1.29±0.01 ^b
<i>Enterobacter aerogenes</i>		1.31±0.03 ^a	1.00±0.09 ^b	0.98±0.07 ^b	0.99±0.09 ^b
<i>Leclercia adecarboxylata</i>		1.37±0.07 ^a	1.36±0.00 ^a	1.13±0.01 ^b	1.09±0.00 ^b
<i>Serratia plymuthicas</i>		1.45±0.05 ^a	1.36±0.03 ^a	1.19±0.08 ^b	0.97±0.02 ^b
<i>Pseudomonas cepacia</i>		1.12±0.06 ^a	1.07±0.04 ^a	0.99±0.01 ^b	0.87±0.02 ^b

^{abcd}Values across the table with similar superscripts are not significantly different at 5% based on ANOVA

Table 4.7: Quantitative screening of bacterial from enrichment method by 2, 6-Dichlorophenol indophenol redox indicator using UV-VIS @600nm

Isolates	Incubation Period (h)				
	0	24	48	72	96
CTRL (+ve)	1.66±0.02 ^a	1.63±0.04 ^a	1.60±0.06 ^b	1.60±0.06 ^b	1.59±0.00 ^b
CTRL (-ve)	0.29±0.05 ^b	0.29±0.03 ^b	0.29±0.03 ^b	0.29±0.04 ^b	0.29±0.01 ^b
<i>Salmonella pullorum</i>		1.45±0.01 ^a	1.16±0.03 ^b	0.99±0.08 ^c	0.82±0.02 ^c
<i>Staphylococcus hyicus</i>		1.19±0.02 ^a	1.17±0.06 ^a	0.91±0.04 ^b	0.87±0.07 ^b
<i>Staphylococcus warneri</i>		1.25±0.07 ^a	1.15±0.03 ^a	0.99±0.02 ^b	0.73±0.01 ^b
<i>Leclercia adecarboxylata</i>		1.31±0.05 ^a	1.16±0.03 ^b	0.99±0.08 ^c	0.77±0.02 ^d
<i>Micrococcus varians</i>		1.25±0.04 ^a	1.17±0.03 ^b	0.93±0.02 ^c	0.74±0.09 ^d
<i>Pseudomonas alcaligenes</i>		0.97±0.03 ^a	0.65±0.03 ^b	0.23±0.02 ^c	0.23±0.01 ^c
<i>Klebsiella oxytoca</i>		0.99±0.00 ^a	0.87±0.06 ^a	0.56±0.04 ^b	0.21±0.07 ^c
<i>Raoultella ormithinolytica</i>		0.65±0.02 ^a	0.49±0.01 ^a	0.30±0.01 ^b	0.10±0.01 ^c
<i>Raoutella sp</i>		0.88±0.02 ^a	0.65±0.03 ^b	0.23±0.02 ^c	0.17±0.01 ^d
<i>Kluyvera ascorbate</i>		0.99±0.03 ^a	0.87±0.06 ^b	0.71±0.04 ^c	0.77±0.07 ^c
<i>Actinobacillus minor</i>		1.14±0.04 ^a	1.09±0.03 ^a	1.05±0.06 ^b	0.99±0.09 ^b
<i>Enterobacter aerogenes</i>		1.24±0.08 ^a	1.21±0.01 ^a	0.98±0.07 ^b	0.99±0.09 ^b
<i>Buttiauxella agrestis</i>		1.01±0.01 ^a	1.00±0.09 ^b	0.98±0.07 ^b	0.99±0.09 ^b
<i>Proteus penneri</i>		1.03±0.02 ^a	1.01±0.00 ^a	0.99±0.04 ^a	0.96±0.08 ^b
<i>Kluyvera cryocrescens</i>		0.99±0.01 ^a	0.87±0.06 ^b	0.71±0.04 ^c	0.77±0.07 ^c
<i>Cellobiosococcus sp</i>		0.90±0.07 ^a	0.84±0.09 ^b	0.78±0.07 ^b	0.76±0.09 ^c

^{abcd}Values across the table with similar superscripts are not significantly different at 5% based on ANOVA

Table 4.8: Biochemical identification of best selected PAH degrading bacterial using API

20E kit		
Biochemical Tests (Probable)	<i>Escherichia Coli</i>	<i>Raoultella ormithinolytica</i>
Beta galactosidase	+	+
Arginine	-	-
Lysine	+	+
Ornithine	+	+
Citrate	-	+
Na thiosulfate	-	-
Urea	-	+
Tryptophan	-	±
Tryptophan	-	+
Na pyruvate	-	+
Charcoal gelatin	+	-
Glucose	+	+
Mannitol	-	+
Inositol	-	+
Sorbitol	-	+
Rhamnose	+	+
Sucrose	-	+
Melibiose	-	+
Amygdalin	-	+
Arabinose	-	+
Oxidase	+	-
API IDENTIFICATION	<i>Morganella morganii</i> *	<i>Raoultella ormithinolytica</i>

4.5 Molecular identification of selected isolates

Table 4.9 reports the quality of the DNA extracted based on its absorbance reading on a UV- vis spectrophotometer were within the acceptable computational levels to standard 1.8. The spectrophotometer readings were recorded at 260/280 nm and represents values from mean $\pm$ standard error of two sample run for both EC-*Escherichia coli* and RO- *Raoultella ormithinolytica*

The 16SrDNA-based visualization, qualitative and quantitative analysis of extracted genomic DNA as well as amplified fragments via PCR using the BLAST search tool enabled more precise identification of the analysed bacteria isolates *E. coli* and *Raoultella ormithinolytica* via morphological and biochemical tests. Standard 1kb DNA reference ladders were used to qualify genomic DNA whilst 100bp reference ladders were used for all amplification experiments. Plate 4.1 depicts the result of DNA extraction of the two (2) selected isolates presented in Gel Electrophoresis Micrograph of Genomic DNA. Clear bands of genomic DNA were observed from the figure. Molecular identification analysis adopting the isolation and amplification of DNA stratagem revealed a 1,500 base pair average size from the PCR stage. Nucleotide arrangements of the bacteria isolates after sequencing is presented in (Appendix 3). Binomial identification of the bacteria by the Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) were found to share high sequence identities to certain bacteria isolates that are deposited in the Gene Bank. The results of the samples with a strong biochemical signature to *Morganella morganii* exhibited a 98.97% sequence similarity with *Escherichia coli* strain GSH8M-2 and *Raoultella ormithinolytica* 99.30% similarity to *Klebsiella pneumoniae* strain PSB1. The two isolates have been deposited in the Gene Bank

(Plates 4.3 and 4.4). Result of molecular characterization confirmed the bacteria isolates belong to the genus Enterobacteriaceae.

4.6 Degradation of Benzo[a]Pyrene by bacterial isolates and in consortium

Results and calibration of Benzo[a]Pyrene to obtain the line of best fit is reported in (Appendix 4). Following the sampling procedure, analysis via HPLC targeting specifically benzo[a]pyrene using selected pure cultures of *E. coli* and *K. pneumoniae* individually as well as in consortium yielded results shown in table 4.10. Within the first 24h (day1) {225, 217 and 128} mg/L was the residual concentration calculated from the peak area of B[a]P per sample vessel in comparison to initial 250mg/L concentration assayed. There was a steady decline in the residual B[a]P from day (4) four till day (16) with residual concentration 37.80, 22.41 and 8.90 for *E. coli*, *K. pneumoniae* and the consortium respectively.

Diagrammatic representation of the various degrees of degradation is given in Figure 4.1 Data obtained from the Figure revealed a 71%, 86% and 94% degradation of B(a)P at day (16) sixteen for isolate designated *E. coli*, *K. pneumoniae*, and consortium respectively. Chemical oxygen demand COD Figure 4.2 showed a decrease from day 1 to 16 showing a directly proportional relationship with concentration of B(a)P from the initial concentration 7527mg/L to (3301, 3202 and 3122) mg/L for *E. coli*, *K. pneumoniae* and consortium respectively at day sixteen.

Biological oxygen demand (BOD) monitored during the assay run also showed a progressive increase from the initial BOD of the test sample ranging from 211mg/L to 299mg/L. There was progressive increase from day 1 as the microbial population increased showing a directly proportional relationship between the BOD and (CFU) colony forming units of the microbial population Figures 4.3 and 4.4. pH was also evaluated and results presented in Figure 4.5. The graph showed a pH of slight acidity

after dropping from the neutral pH of 7 within the first 24hours and remained in the same level in the assay containing *E. coli* and *K. pneumonia* and in the combination of both bacteria isolates till day sixteen.

Table 4.9. Qualitative DNA assessment (UV-Vis Spectroscopy) $\pm$ SE

Isolate	Absorbance (260nm)	Absorbance (280nm)	Ratio (260/280)
<i>E.coli</i>	0.369 ± 0.103	0.200 ± 0.054	1.845
<i>Raoultella ormithinolytica</i>	0.180 ± 0.009	0.095 ± 0.008	1.841

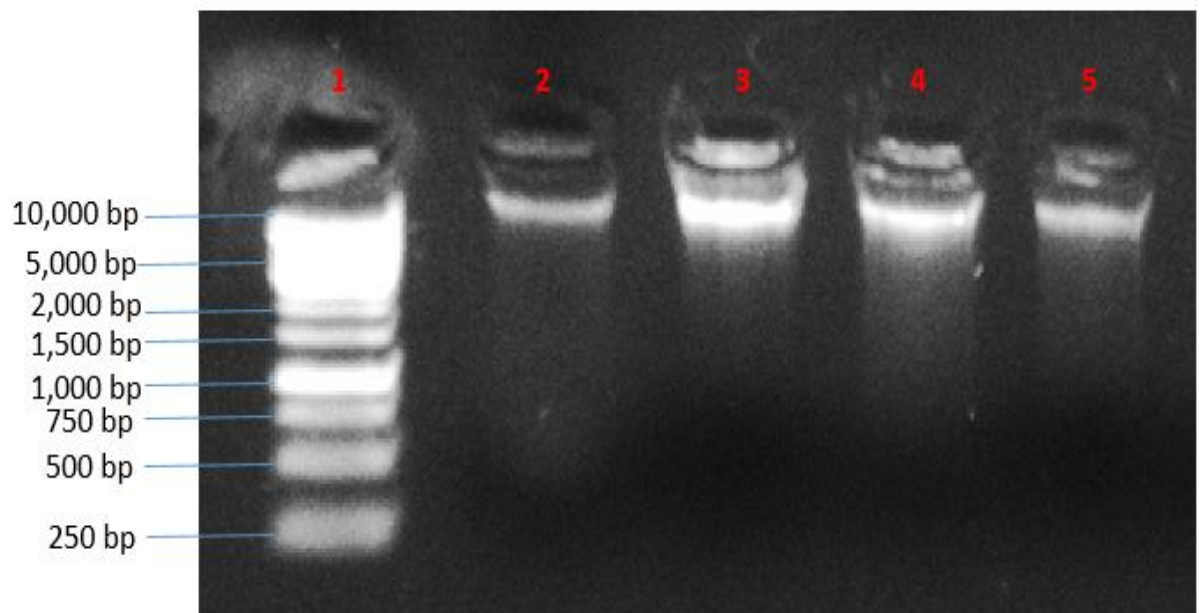


Plate 4.1: Gel Electrophoresis Pictograph of the selected bacterial isolates

Lane 1=GeneRuler™ 1kb DNA ladder (Thermoscientific). Lane 2=Isolate EC1. Lane 3=isolate EC2. Lane 4=isolate RO1. Lane 5=isolate RO2.

(EC1 and EC2 = *Escherichia coli* run in duplicate. RO1 and RO2 = *Raoultellaormithinolyticarun* in duplicate).

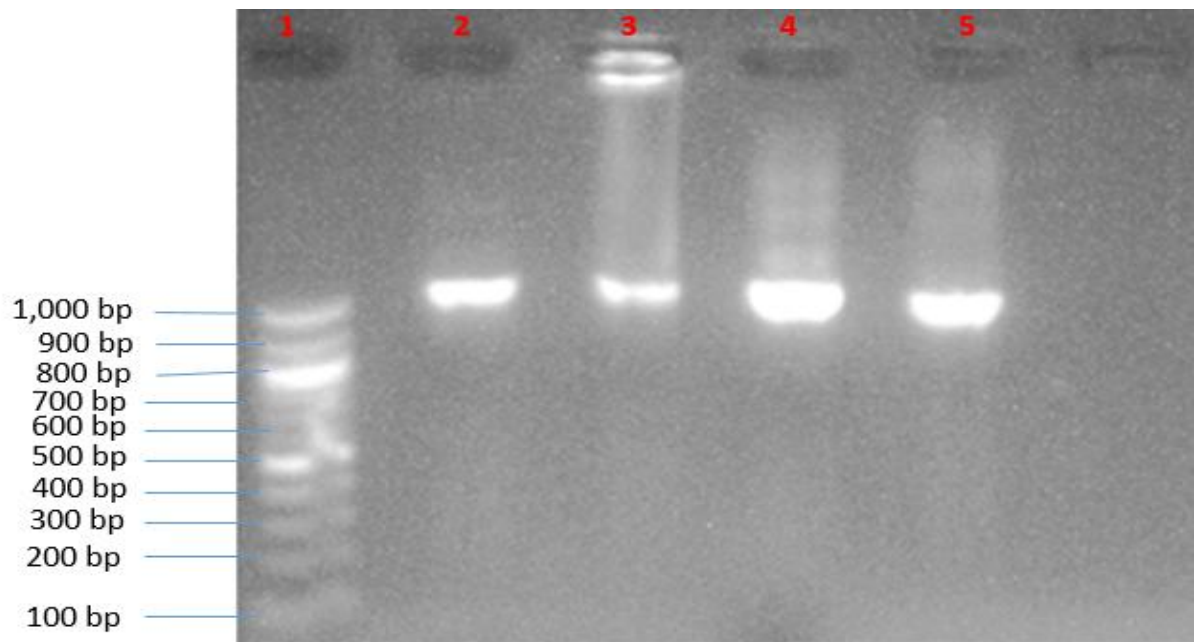


Plate 4.2 PCR analysis of the selected isolates using universal bacterial primers (27F/1492R).

Lane 1= 100bp DNA ladder (Genbiotech). Lane 2=Isolate EC1. Lane 3=isolate EC2. Well 4=isolate RO1. Well 5=isolate RO2.

(EC1 and EC2 = *Escherichia coli* run in duplicate. RO1 and RO2 = *Raoultella ormithinolytica* run in duplicate)

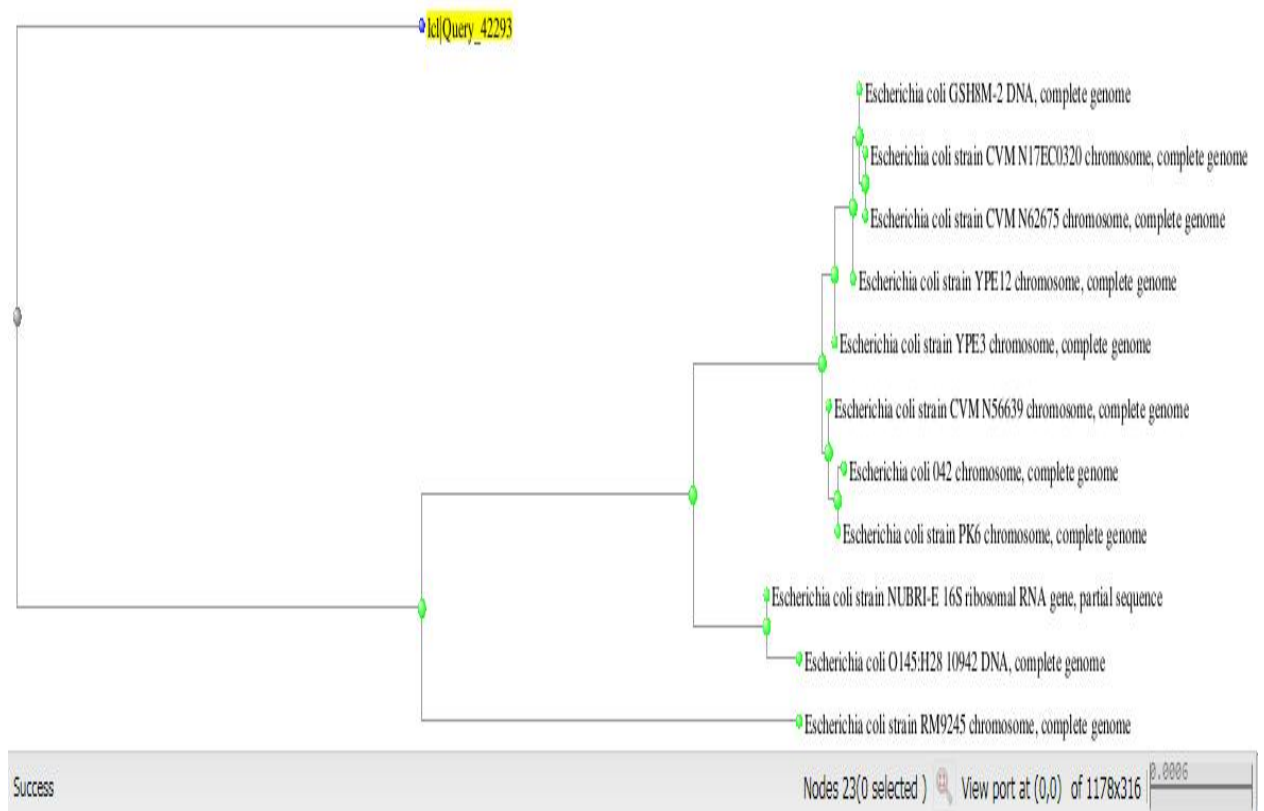


Plate 4.3. Phylogenetic tree of *Morganella morganii* Closest relative with 98.97% sequence similarity is *Escherichia coli* strain GSH8M-2

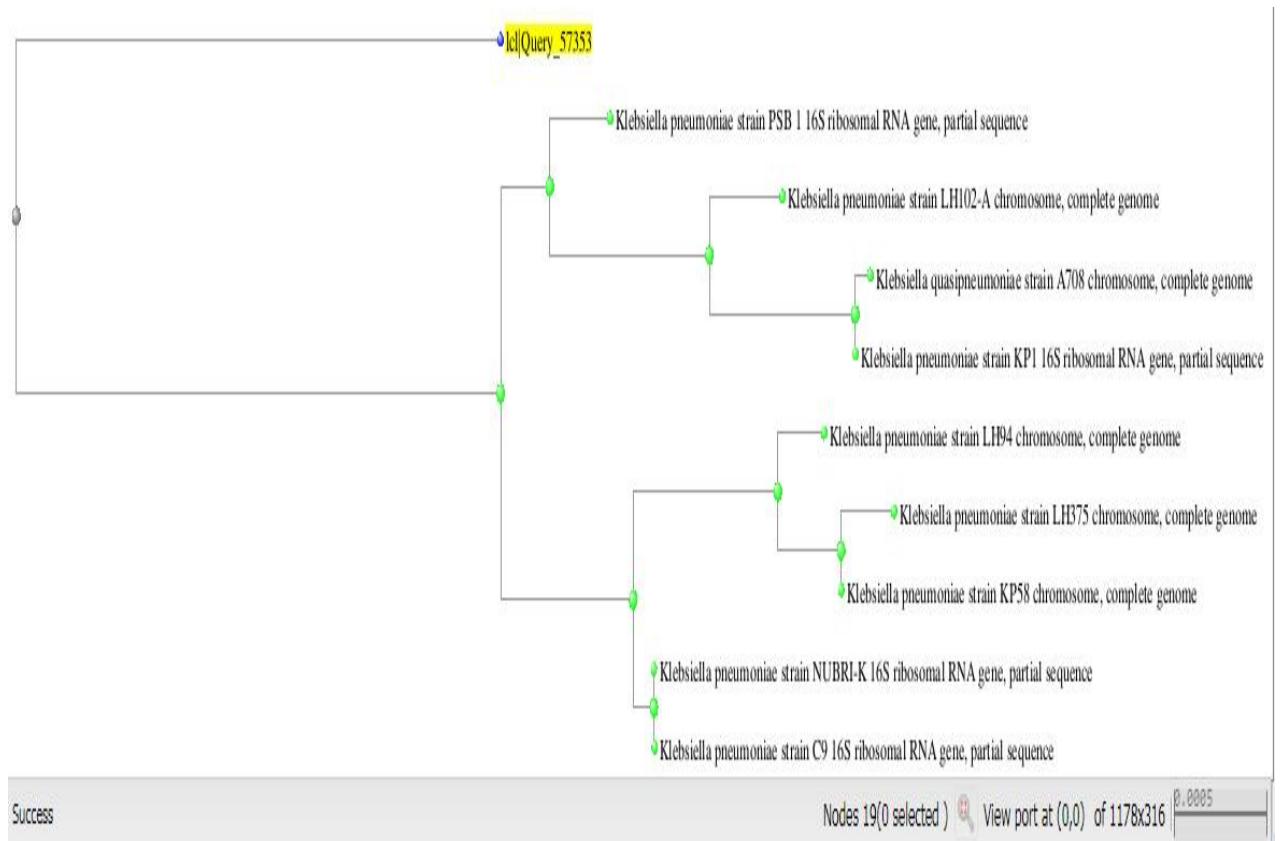


Plate 4.4 Phylogenetic tree of isolate *Raoultella ormithinolytica* Closest relative with 99.30% sequence similarity is *Klebsiella pneumoniae* strain PSB1

Table 4.10: Concentration of B(a)P in (mg/L) during degradation by bacterial isolates and

consortium

Time (d)	Control	<i>E.coli</i>	<i>K. pneumoniae</i>	<i>E. coli</i> + <i>K. pneumoniae</i>	P value
1	249.41±0.63	234.52 ± 0.72	214.43 ± 0.26	219.25 ± 0.99	0
4	230.55 ± 0.21	191.82 ± 0.55	123.53 ± 0.48	114.83 ± 0.11	0
7	229.74 ± 0.49	97.48 ± 0.24	88.53 ± 0.21	62.31 ± 0.01	0
10	222.71 ± 1.08	59.46 ± 0.36	47.6 ± 0.30	44.36 ± 0.17	0
13	222.13 ± 0.48	54.94 ± 0.03	36.47 ± 0.07	27.1± 0.10	0
16	221.25 ± 0.99	37.80 ± 0.43	22.43 ±1.56	8.90 ±0.58	0

*Initial concentration =250; P<0.05

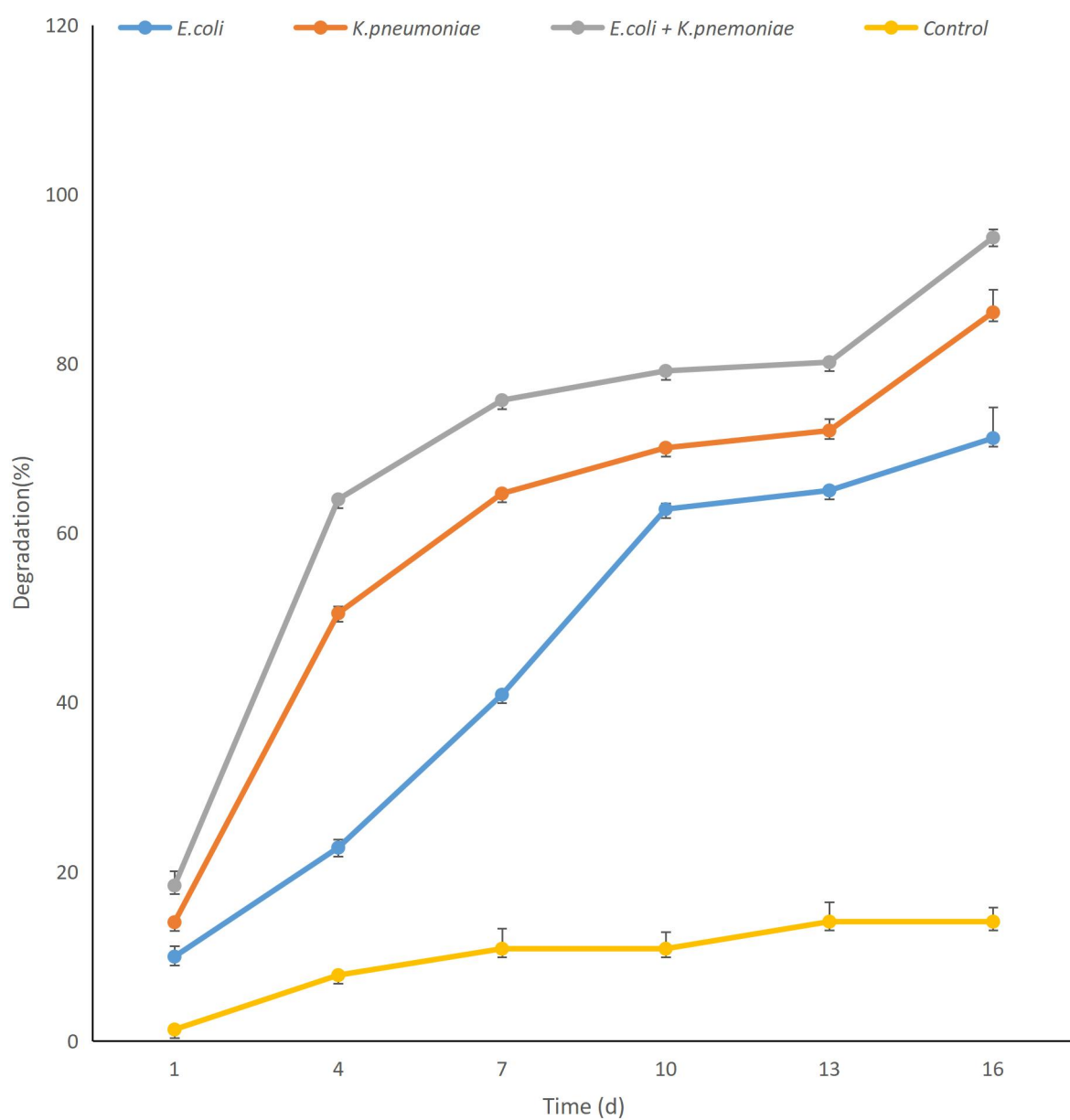


Figure 4.1: Degradation Percentage of B[a]P by *E. coli*, *K. pneumoniae* and consortium

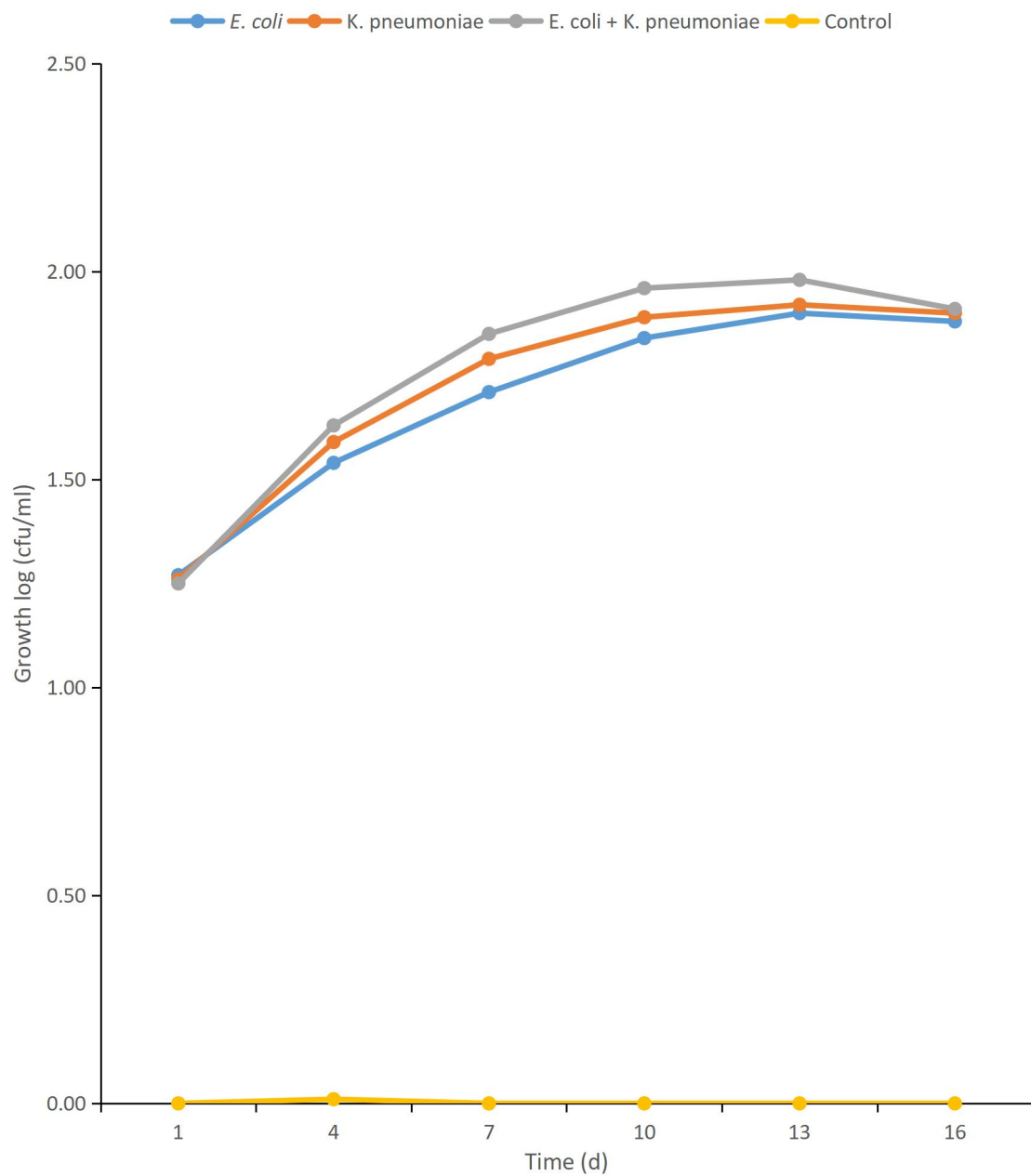


Figure 4.2: Growth of bacterial isolates during degradation of B[a]P

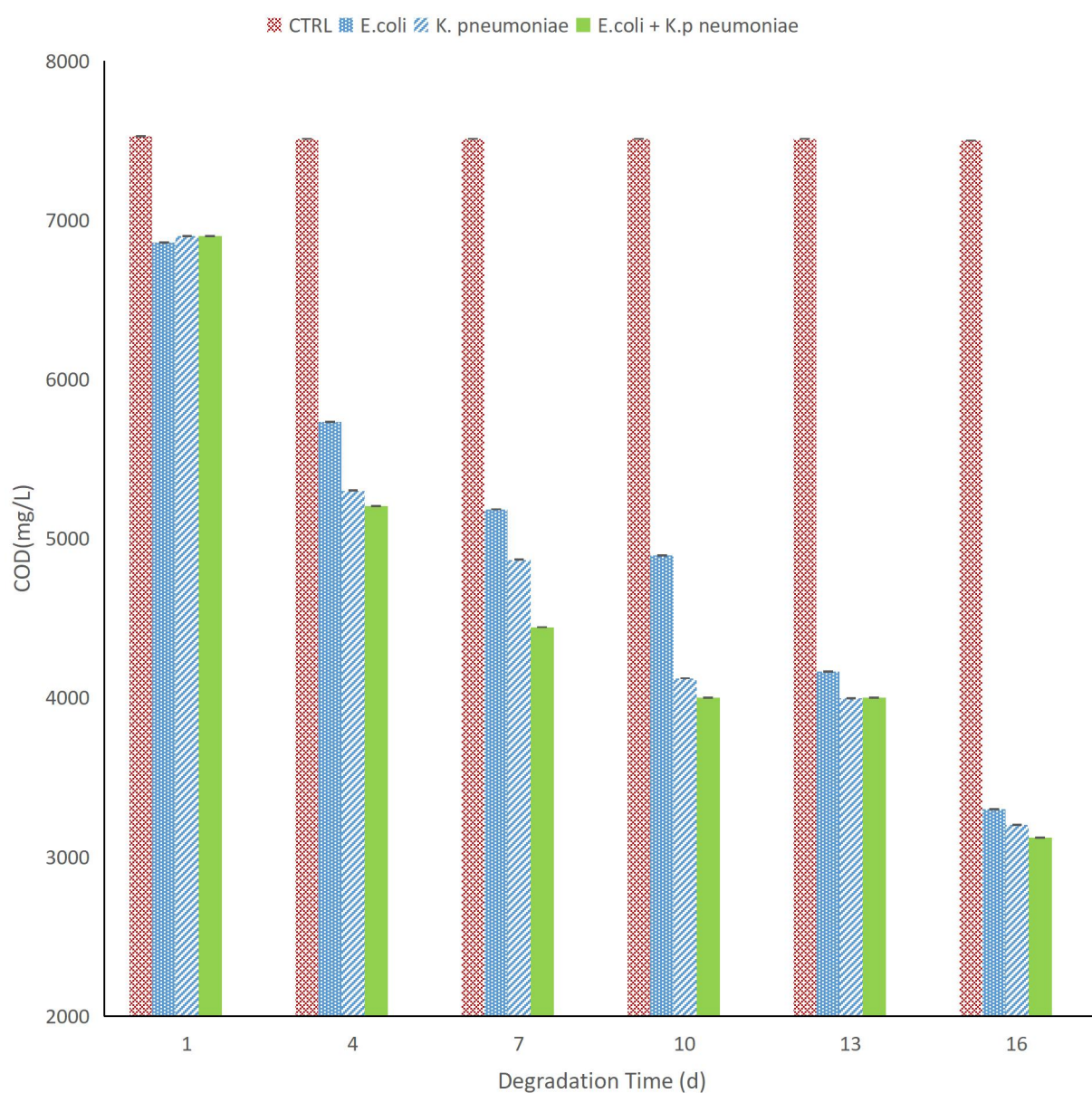


Figure 4.3: Changes in Chemical Oxygen Demand (COD) during degradation of Benzo(a)pyrene by bacterial isolates

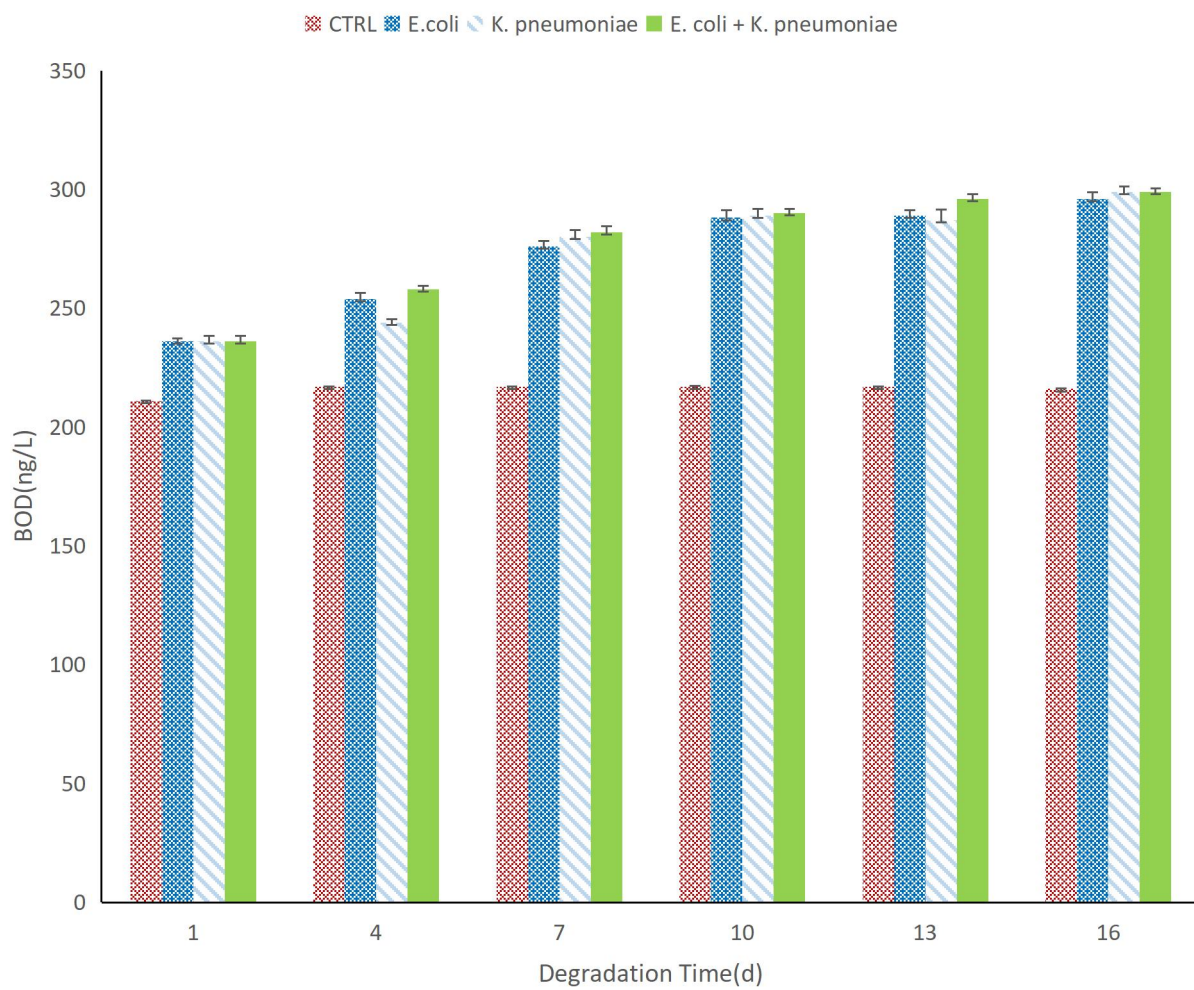


Figure 4.4: Changes in Biological Oxygen Demand (BOD) during degradation of Benzo(a)pyrene by bacterial isolates

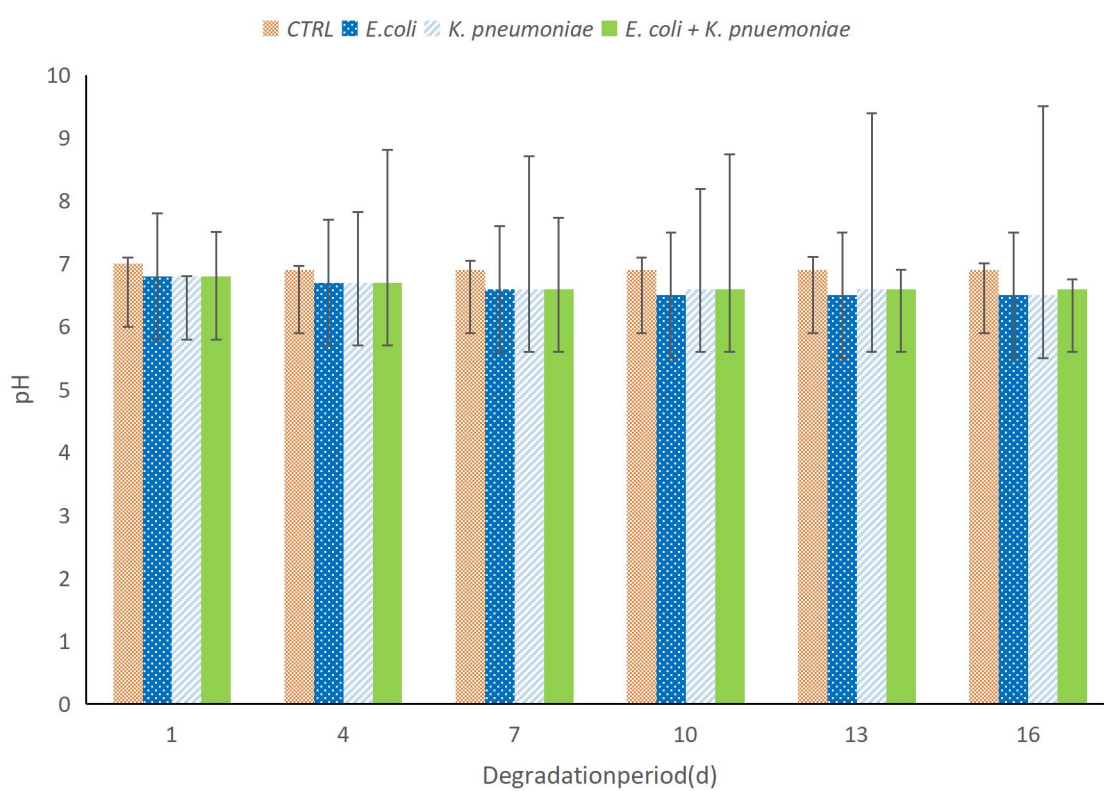


Figure 4.5: Changes in pH during degradation of Benzo(a)pyrene by bacterial isolates

4.7 Degradation of Pyrene

Pyrene stock solution of varied concentration to determine the line of best fit was analysed and result presented in Appendix 4. Following the sampling procedure and report as in 4.6 with the analyser GC-MS, targeting specifically Pyrene. Bacterial isolates and consortium displayed sufficient decline in the residual concentration of Pyrene from 250mg/L from day one (1) to day sixteen (16) for all the samples and the best degraded been the consortium with a residual concentration of as low as 5.72 mg/L Table 4.11. The F. factor from ANOVA pos hoc (turkey) showed significantly recorded differences between each day per assay run with the highest variance also recorded in consortium (Appendix 5). The P factor was less than 0.05 for all the sets compared meaning no significant difference was observed for the various treatment.

Diagrammatic representation of the various degrees of degradation is given in Figure 4.6. Within the first 24h (day 1) 26% of the pollutant has been degraded by the consortium however only 4% of Pyrene was degraded by *E. coli* at day one (1) calculated viz and viz control. Data obtained from the figure revealed an 89.36%, 90.98% and 97.76% for *E. coli*, *K. pneumoniae* and consortium respectively after the 16 days of incubation. Minimal loss of concentration was observed in the control samples of less than 10% during the 16days incubation period. Within the first four days of incubation, the growth of organisms was minimal (lag phase of growth, between the 7th and 13th day the growth of isolates was at the exponential stage and started to decline slightly on the 16th day except for the assay inoculated with *E. coli* that recorded continuous increase even at the 16th day. Chemical oxygen demand (COD) Figure 4.8 showed a decrease from the initial concentration and control concentration for both isolates individually and consortium with a range of 6122mg/L (highest) to 2000mg/L (lowest). Biological oxygen demand (BOD) monitored during the assay run also

showed progressive increase from the initial BOD (day 1) to the sixteenth day 183 mg/L (lowest) to 301 mg/L (highest). The pattern of BOD performance correlated with microbial growth showing a directly proportional relationship between the BOD and (CFU) colony forming units of the microbial population. Figure 4.7 and Figure 4.9. Figure 4.10 showed a graph of pH which slightly decreased from the neutral pH of 7 to acidic pH within day 1 and day 4 thereafter maintaining the pH between 6.7-6.6 for the remaining part of the test analysis till the sixteenth day.

Table 4.11: Concentration of Pyrene in (mg/L) during degradation by bacterial isolates and

consortium

Time (d)	Control	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>E.coli</i> + <i>K. pneumoniae</i>	P Values
1	240.05 ± 1.29	239.05 ± 0.03	219.78 ± 1.58	187.83 ± 2.21	0
4	239.92 ± 1.01	194.33 ± 0.54	97.14 ± 1.26	69.14 ± 0.07	0
7	236.82 ± 2.22	134.73 ± 0.64	69.39 ± 0.40	44.95 ± 0.36	0
10	231.74 ± 0.49	83.34 ± 0.88	34.62 ± 0.81	35.88 ± 0.58	0
13	222.87 ± 0.57	30.69 ± 0.68	29.31 ± 0.27	18.34 ± 0.21	0.01
16	222.19 ± 0.40	25.92 ± 0.50	8.65 ± 0.73	5.72 ± 0.46	0.01

*Initial concentration=250mg/ml; P<0.05

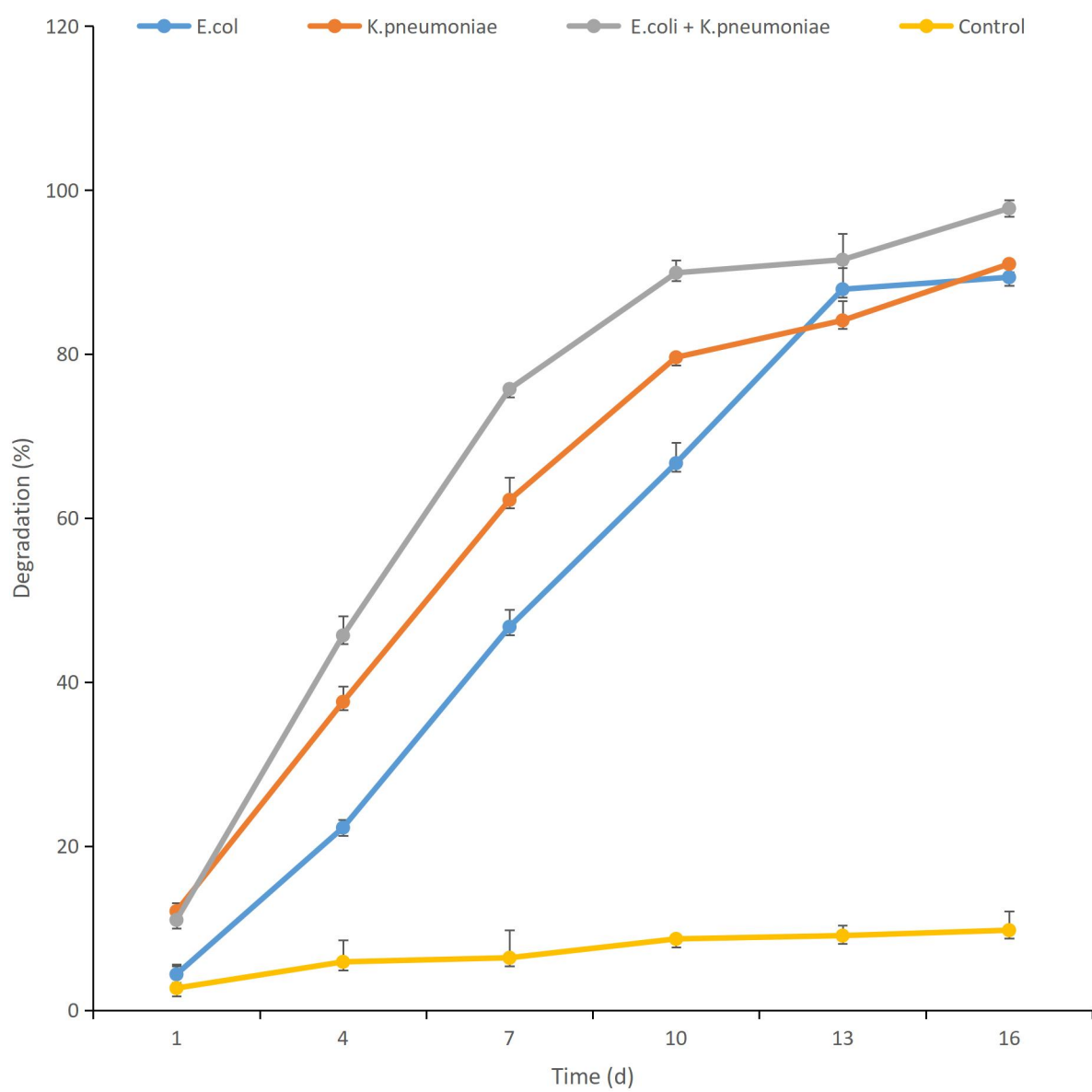


Figure 4.6: Degradation percentage of Pyrene by the selected bacterial and consortium

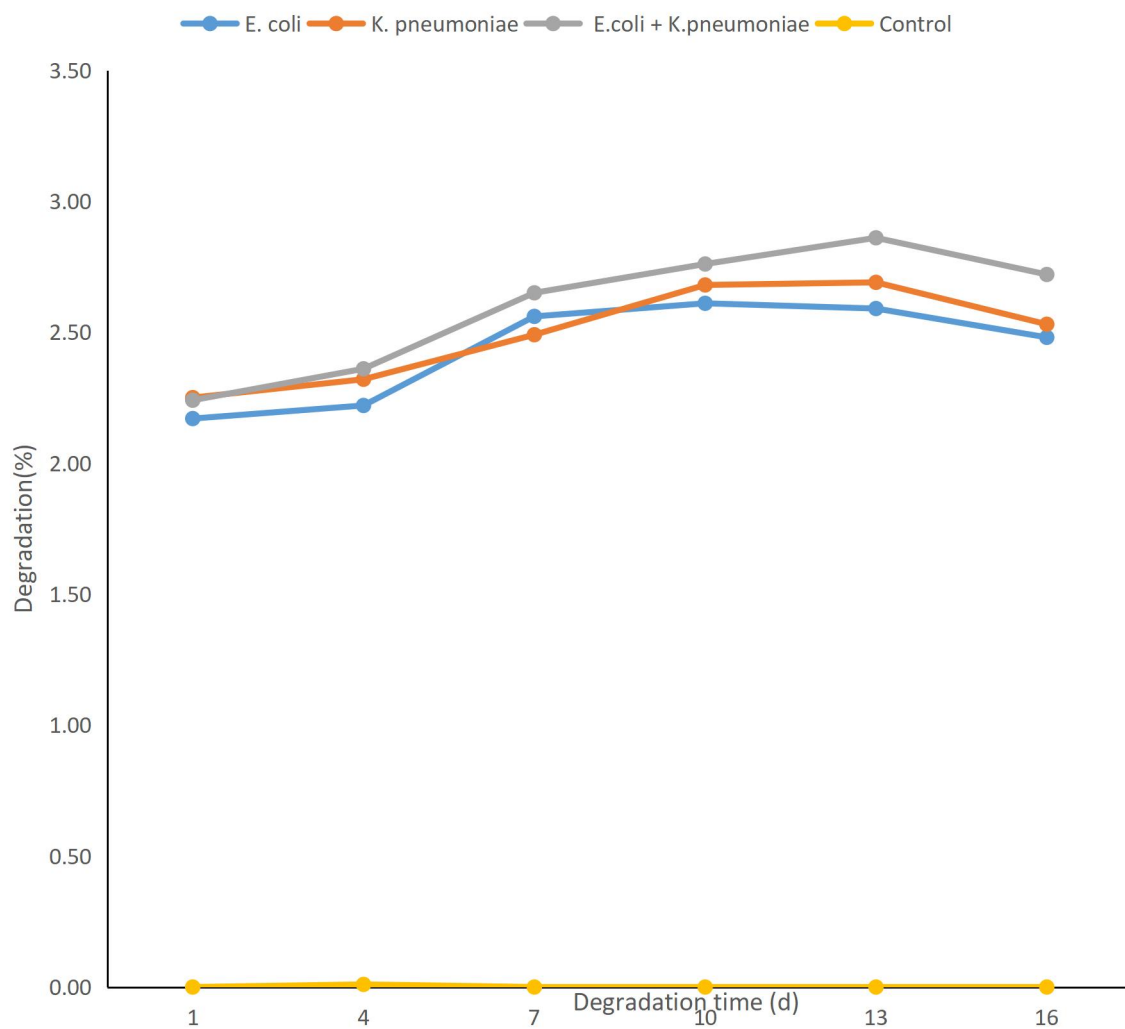


Figure 4.7: Growth of bacterial isolates during degradation of Pyrene

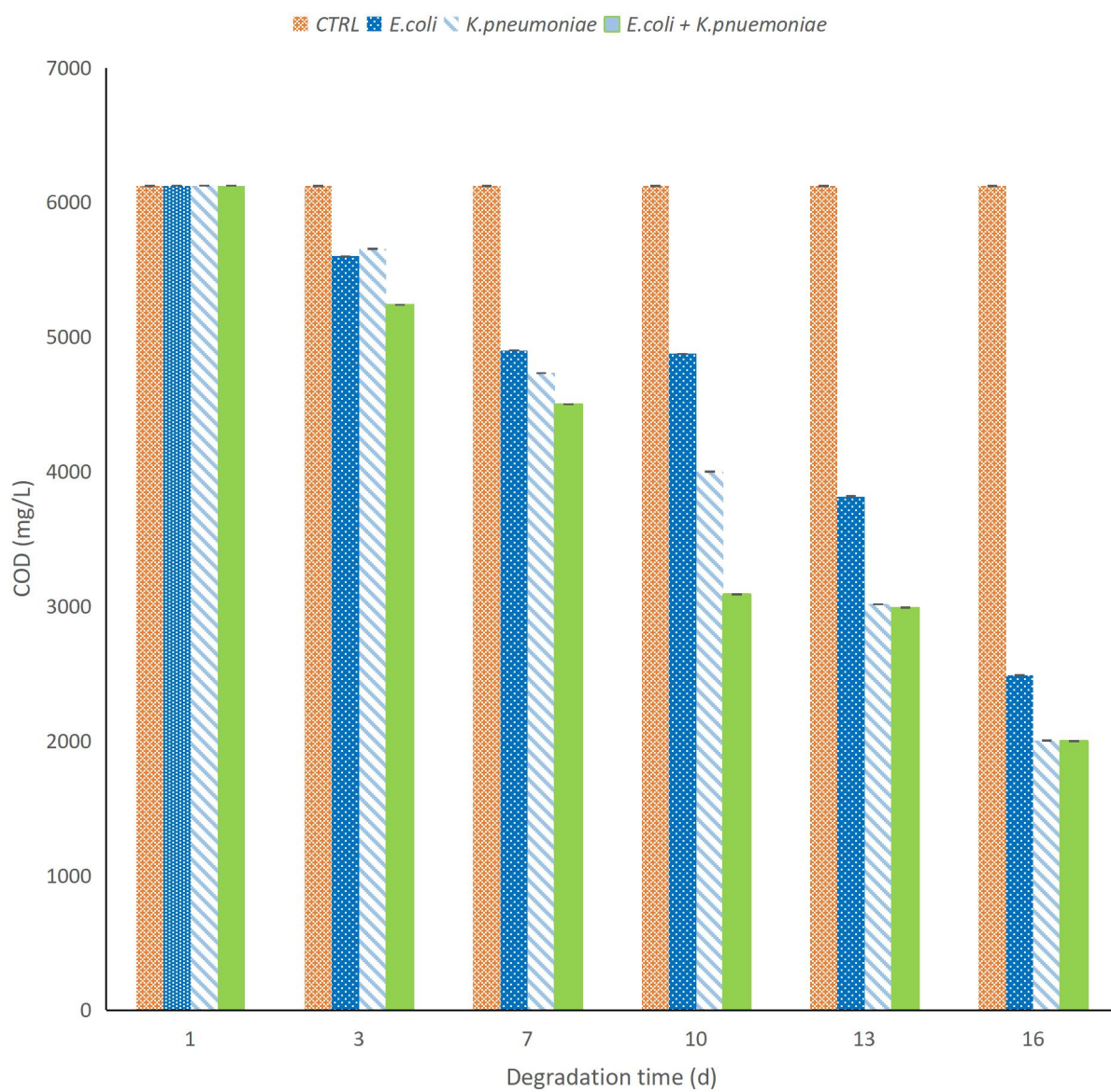


Figure 4.8: Changes in Chemical Oxygen Demand (COD) during degradation of Pyrene by bacterial isolates

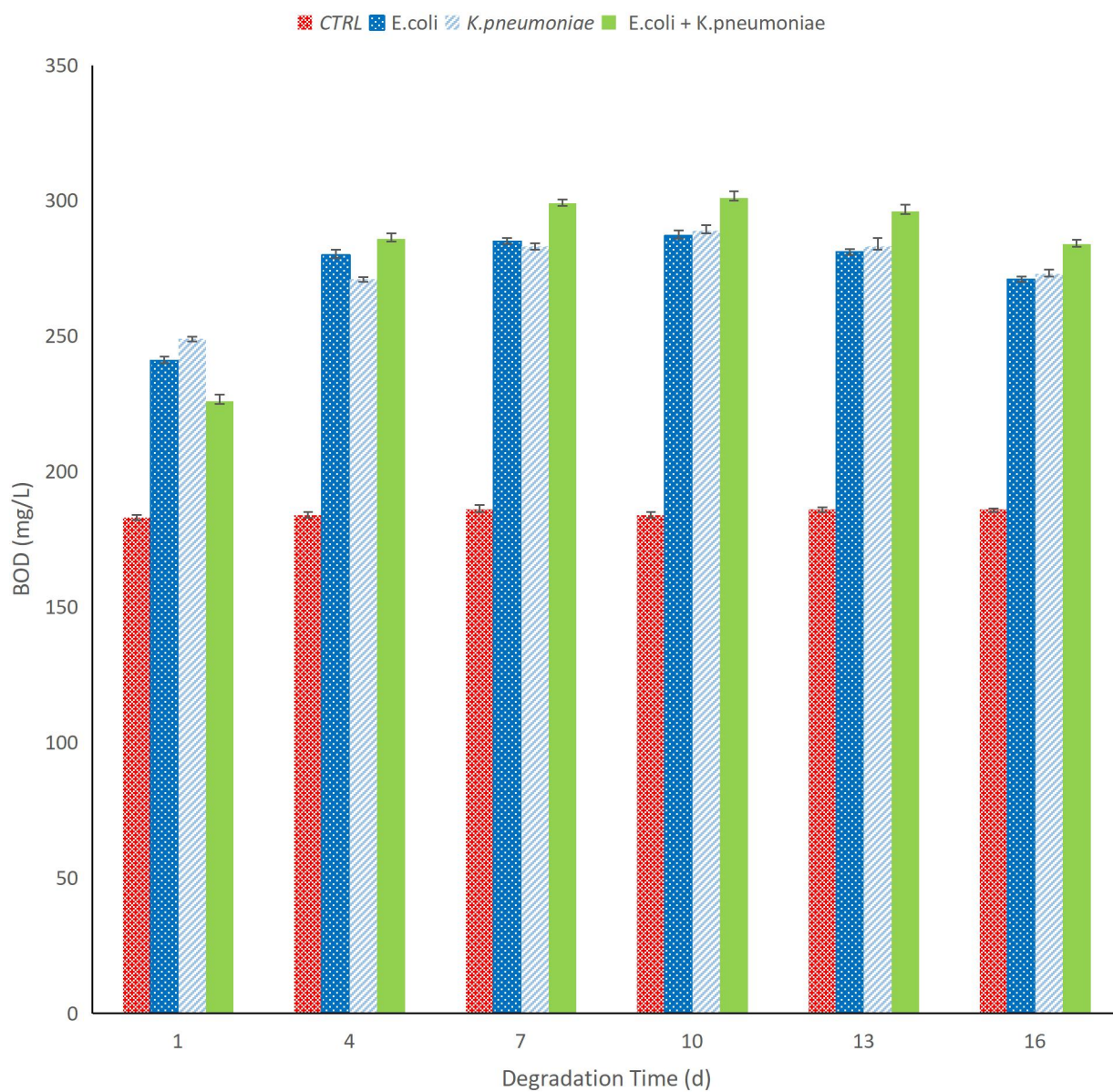


Figure 4.9: Changes in Biological Oxygen Demand (BOD) during degradation of Pyrene by bacterial isolates

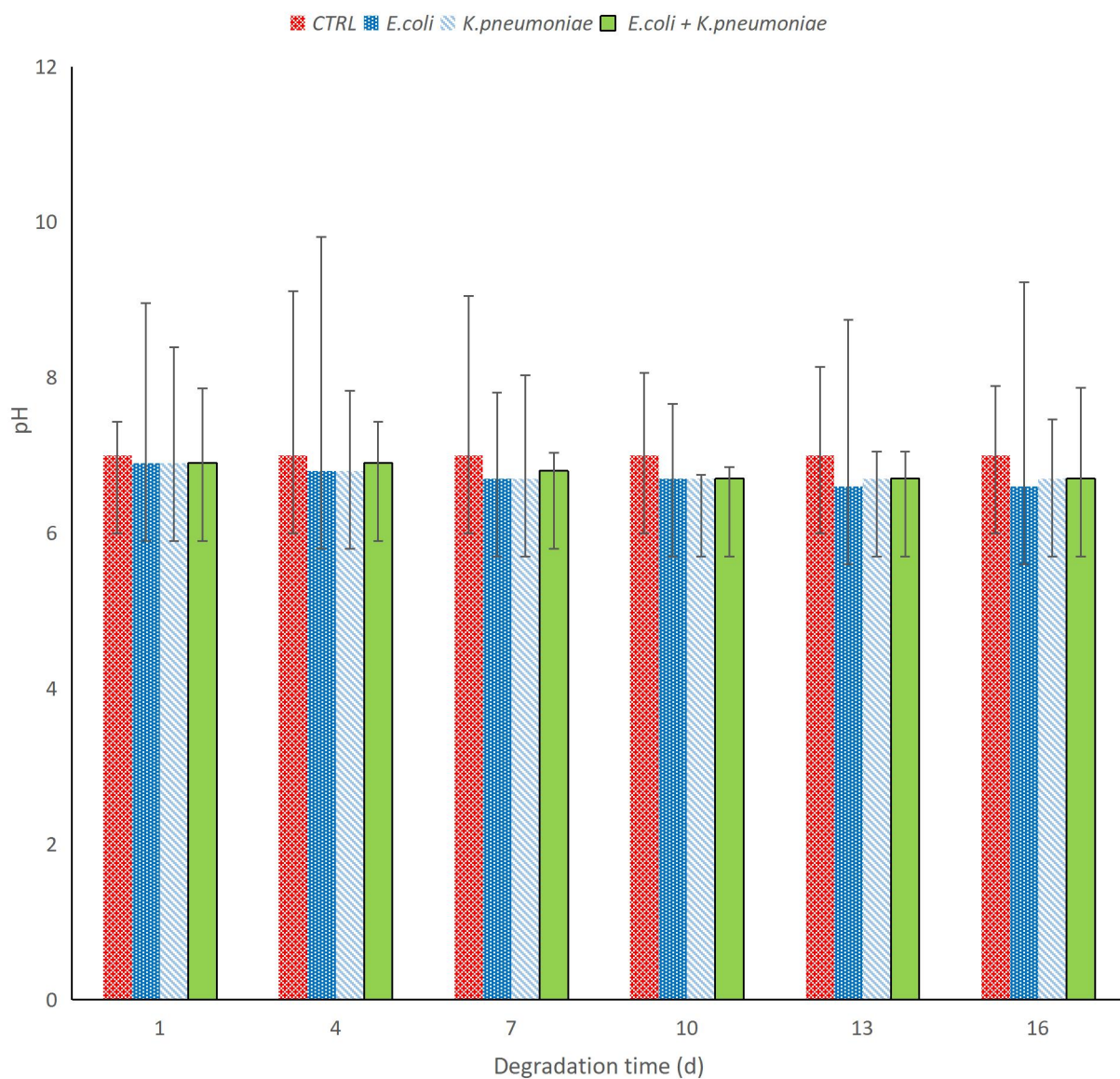


Figure 4.10: Changes in pH values during degradation of Pyrene by bacterial isolates

4.8 Degradation of Flouranthene

Following the sampling procedure as in 4.7 above analysis was done by GC-MS, targeting specifically Flouranthene using individual selected bacteria as well as in consortium. The results showed that there was microbial induced activity in the assay as concentration dropped from the initial 250mg/ml within the first 24 hours that is day 1 substantially Table 4.12 There was a progressive decrease in the concentration of Flouranthene as the days went by. The P value from ANOVA comparing means of each day between groups was not significantly different showing that each treatment was effective but at different degree.

Diagrammatic representation of the various degrees of degradation in relation to the various concentration and initial concentration showed the effectiveness of the organisms in biodegradation of Floranthene with an 83.1%, 93.7% and a 97.7% loss of flouranthene for *E. coli*, *K. pneumoniae* and consortium respectively after 16days Figure 4.11. The viability test of isolates in the assay showed a good tolerance of the PAH. There was accumulation to new environment showed between day (1) one and (4) four representing the lag phase of growth Figure 4.12. There was progressive increase in growth from day (7) through day 16 for the individual isolates except for the combo that experienced a slight decline in the microbial population at day 16

Results in Figure 4.13 showed a sharp decline in the chemical oxygen demand from the initial between day zero and four thereafter for all test set up the decrease was gradual with a range of 6961 mg/L (higest) and 1889 mg/L (lowest). Figure 4.14 showed result of the biological oxygen demand which increased progressively as the days went by till the sixteenth with an initial value of 168 mg/L to 298 mg/L for *K. pneumoniae* and consortium.

The pH of the assay dropped from the neutral pH to slightly acidic pH. From day 10 pH dropped to 6.6 and remained the same till the 16th day of the experiment figure 4.15

4.9 Determination of metabolites during degradation of Pyrene and flouranthene at various time intervals

Metabolites are observed as additional peak other than pyrene and flouranthene shown in (Appendix 6). Observed metabolites, incubation period, chemical formula and chemical groups are presented in (Table 4.13 and 4.14). It was observed that phthalate was distinctive through the degradation period. And additional peaks revealed a diol group, an aldehyde and a ketone group for both Pyrene and Flouranthene

Table 4.12: Concentration of Flouranthene (mg/L) during degradation by bacterial isolates over time

Time (d)	Control	<i>E.coli</i>	<i>K.pnuemoniae</i>	<i>E. coli</i> + <i>K. pnuemoniae</i>	P Values
1	248.39 ± 0.75	192.03 ± 0.50	186.13 ± 2.01	182.24 ± 1.75	0.01
4	240.11± 0.27	145.63 ± 0.26	156.91 ± 2.05	126.52 ± 0.27	0.26
7	240.03 ± 0.50	119.52 ± 0.24	100.64 ± 0.25	85.33 ± 0.23	0
10	232.13 ± 1.19	96.33± 0.56	63.7± 0.19	36.63 ± 0.24	0
13	231.63± 0.95	79.67 ± 0.23	58.01 ± 0.05	11.30 ± 0.22	0
16	230.04 ± 1.11	42.33 ± 0.97	15.81 ± 0.09	6.75 ± 0.14	0

*Initial concentration=250mg/ml; P<0.05

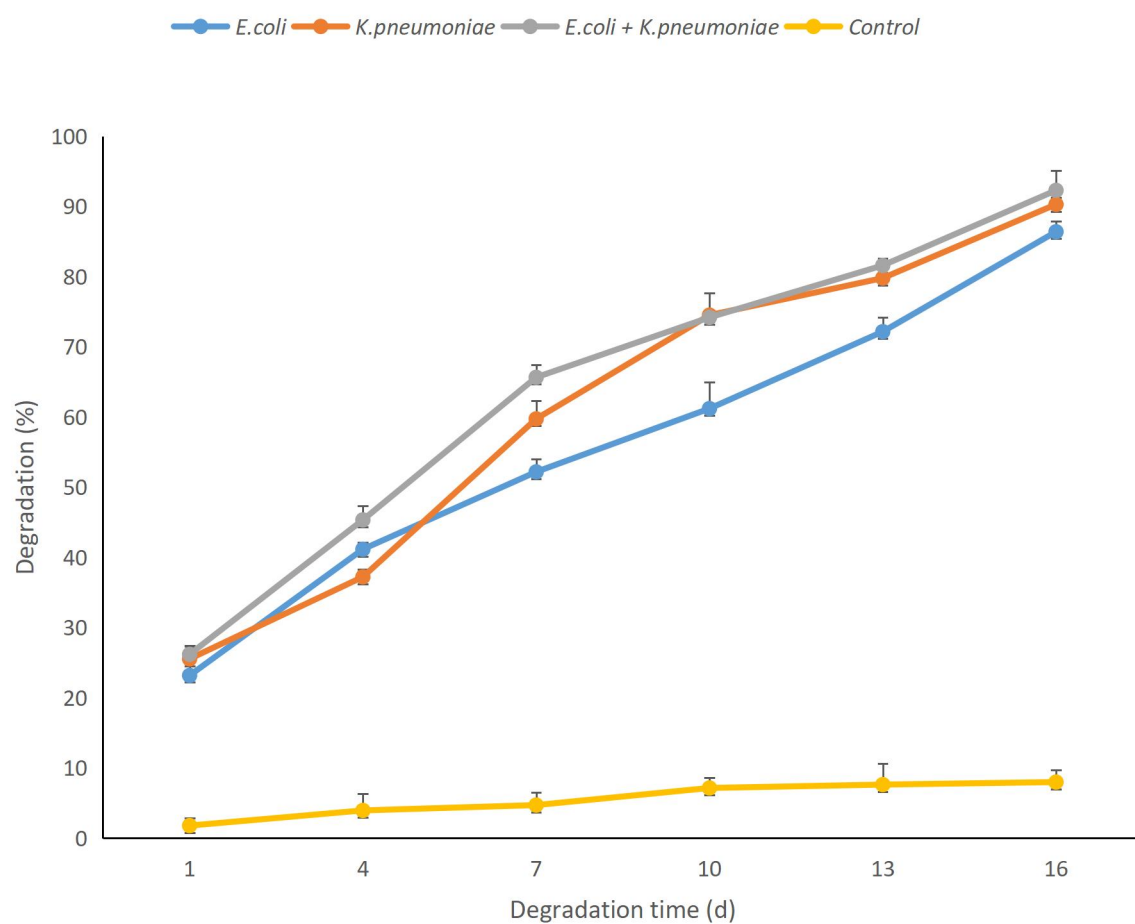


Figure 4.11: Degradation Percentage of Fluoranthene by the selected bacterial isolates and consortium

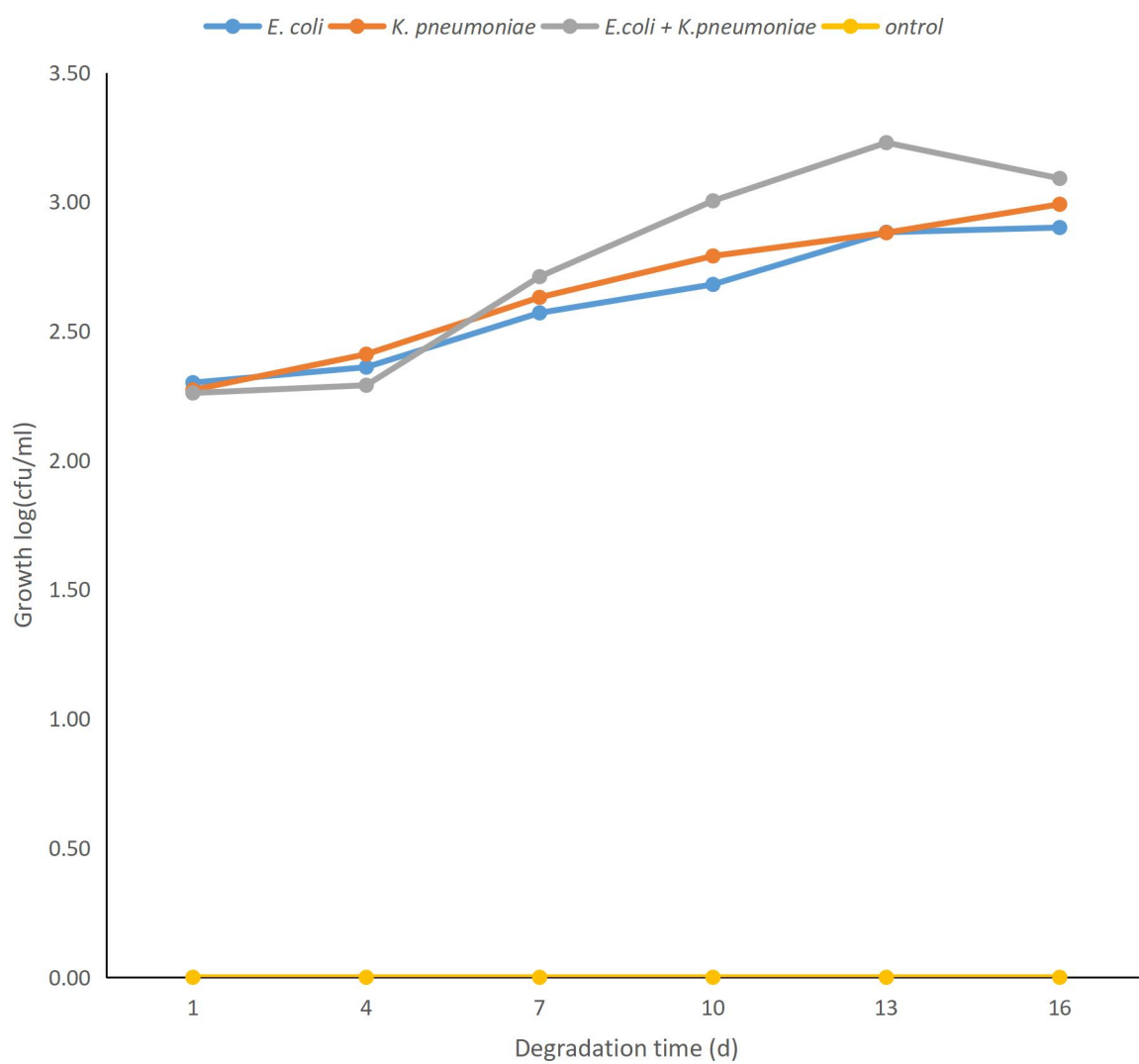


Figure 4.12: Growth of bacterial isolates singly and in consortium during degradation of Flouranthene

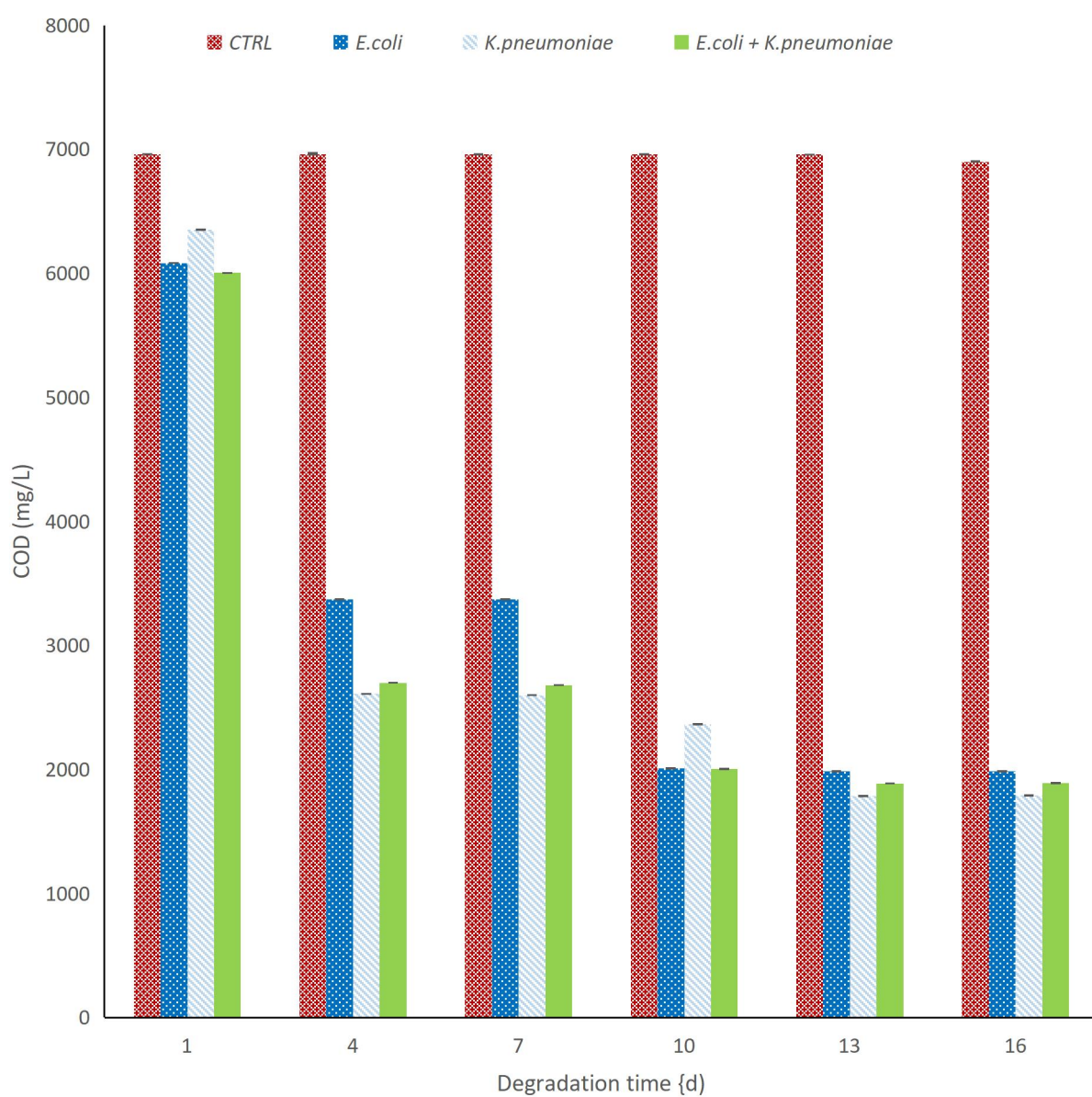


Figure 4.13: Changes in Chemical Oxygen Demand (COD) during degradation of Flouranthene by bacterial isolates

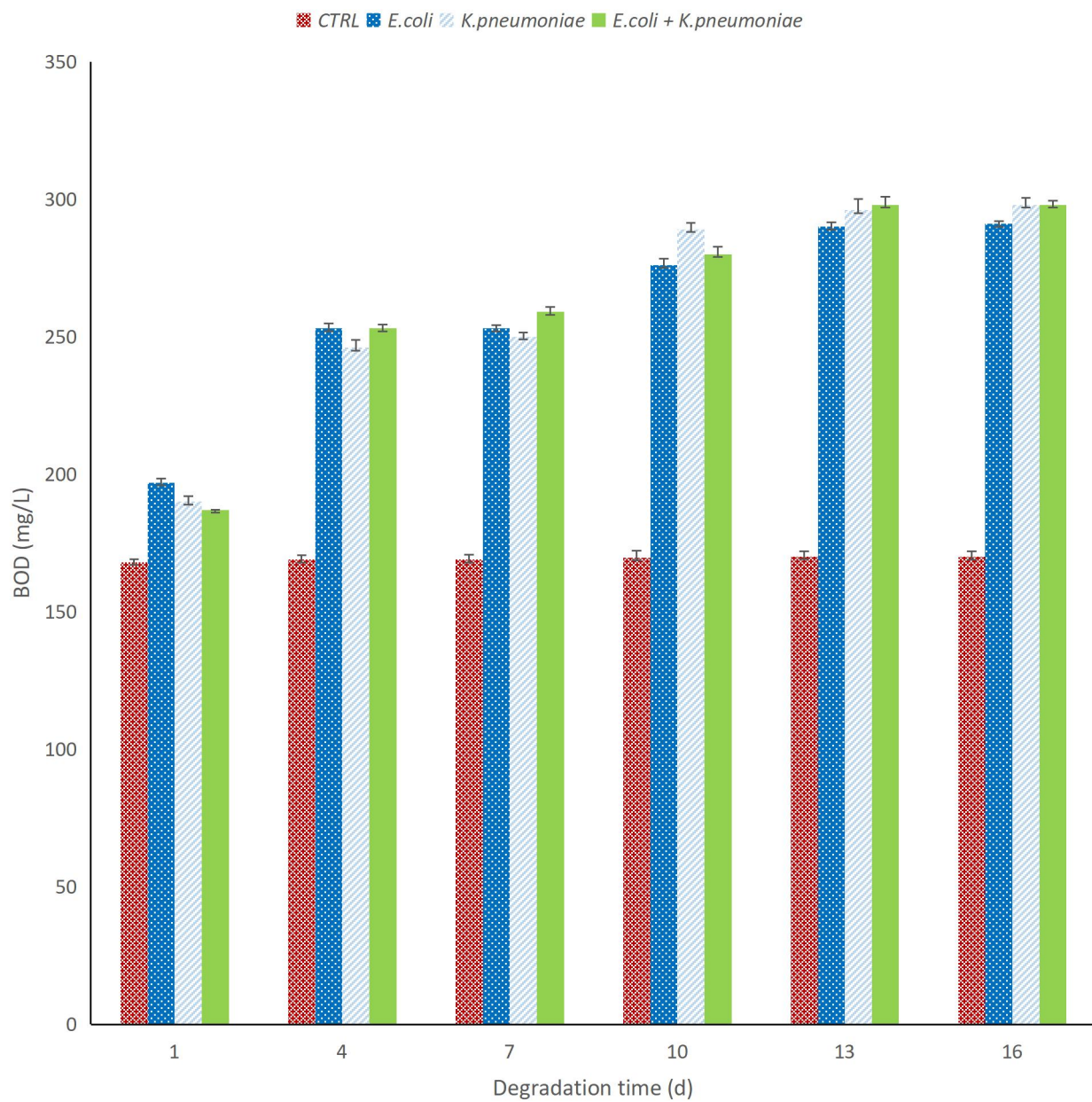


Figure 4.14: Changes in Biological Oxygen Demand (BOD) during degradation Flouranthene by bacterial isolates

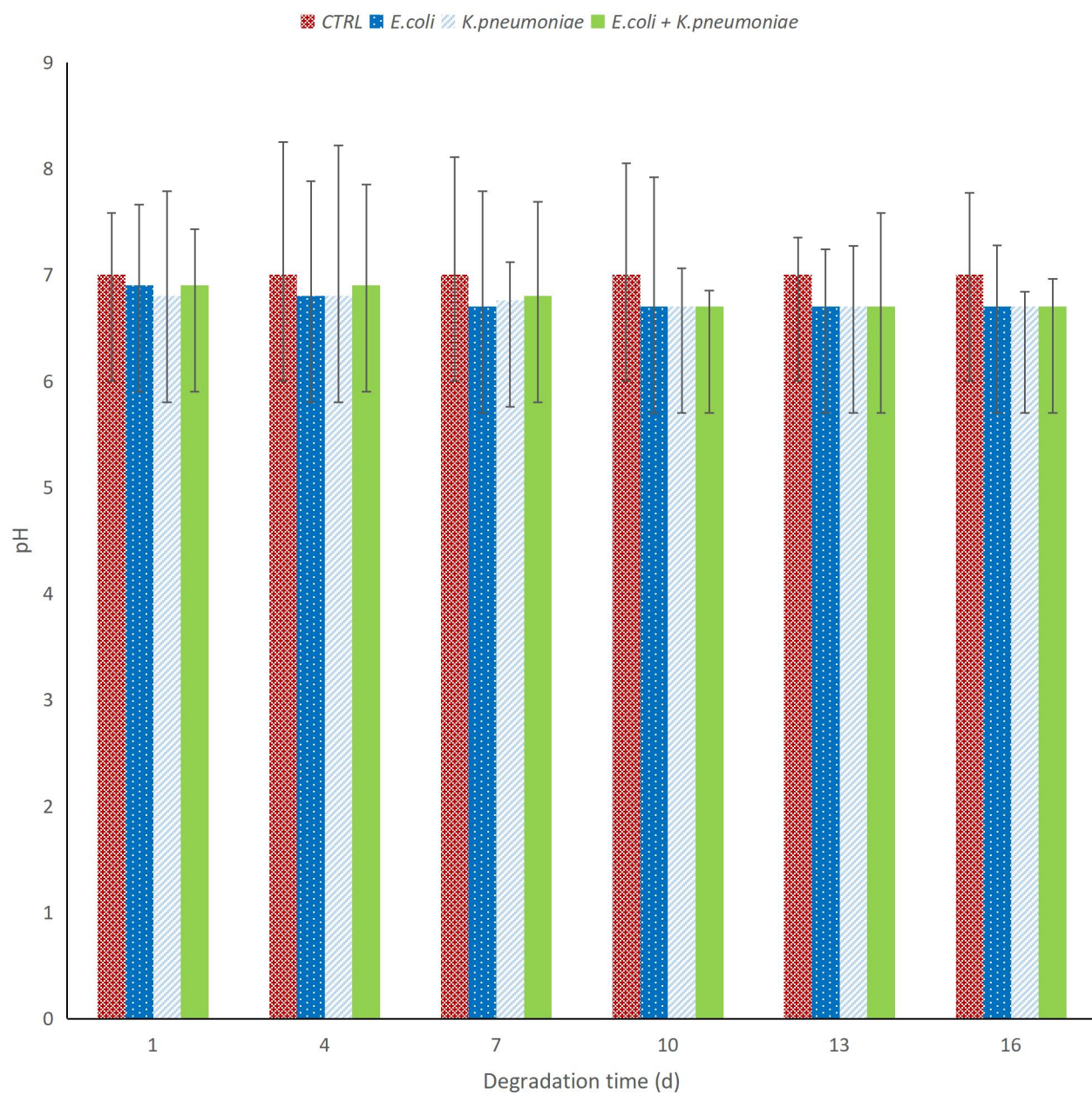


Figure 4.15: Changes in pH during degradation of Fluoranthene by bacterial isolates

Table 4.13: Metabolites produced from degradation of Pyrene by the selected bacterial isolates

Time (d)	Compound	Chemical formula	Group
1	Pyrene	C ₁₆ H ₁₀	
4	Phenaleno[1,9-bc]thiophene	C ₁₄ H ₈ S	
	4,5-dihydroxydihdropyrene	C ₁₆ H ₁₂ O ₂	Diol
7	2H-1- Benzopyran-2-one	C ₁₅ H ₁₈ O ₄	Ketone
	4 –hydroxyperinaphthenone	C ₈ H ₈ O ₂	Ketone
	1,2- Benzenedicarboxylic acid	C ₁₀ H ₁₂ O ₆	
10	Phthalic acid	C ₁₆ H ₁₀	
	Cyclobutylhoptylester		
	1,2-Benzenedicarboxylic acid	C ₁₆ H ₂₂ O ₄	
	1-2 Benzenedicarboxylic acid	C ₂₆ H ₄₂ O ₄	
13	Dibutyl phthalate	C ₁₆ H ₂₂ O ₄	
	Isophthalic acid	C ₁₈ H ₁₆ FNO ₆	
	Phthalic acid	C ₂₅ H ₃₈ O ₄	
	1, pyrenecarboxyldehyde	C ₁₇ H ₁₀ O	Aldehyde
16	Isophthalic acid	C ₁₈ H ₁₆ FNO ₆	
	Phenathrene-4-caboxylic acid	C ₁₅ H ₁₀ O ₂	Carboxylic acid

Table 4.14: Metabolites produced from degradation of Fluoranthene by the selected bacterial Isolates

Time (d)	Compound	Formula	Group
1	Fluoranthene	C ₁₆ H ₁₀	
4	7,8 dihydroxyfluoranthene	C ₁₅ H ₁₀ O ₄	Diol
	Phthalic acid	C ₂₅ H ₃₈ O ₄	
7	Isophthalic acid	C ₁₈ H ₁₆ BRFO ₄	
	2-bromo-4-fluorophenyl butyl ester	C ₁₈ H ₁₆ BRFO ₄	Ketone
10	Phthalic acid	C ₂₅ H ₃₆ O ₄	
	Isoflurane	C ₃ H ₂ CLF ₅ O	
	1,2 dihydroacenaphthylenyl	C ₃ H ₂ CLN ₃ O ₂	Ketone
	Tetrazolo[1,5-a]pyrimidine	C ₆ H ₇ O ₅	
	Anthracene	C ₁₆ H ₁₂	
13	2 hydroxyl,1-2,dihydroacenaphthylenyl	C ₆ H ₁₂ O ₂	Aldehyde
	2,2-dimethyl-propanesulfinyl sulfone	C ₁₀ H ₃₂ S ₃ O ₂	Aldehyde
	1,2,3-Trimethyldiaziridine	C ₄ H ₁₀ N ₂	
	2,4,6-Tris(1,1-dimethylethyl)-4-methylcyclohexa-2,5-dien-1-one	C ₁₉ H ₃₂ O	Aldehyde
16	9-fluoronone-1carboxylic acid	C ₁₄ H ₈ O ₃	
	Terephthalic acid	C ₂₅ H ₃₆ O ₄	Carboxylic acid
	Phthalic acid	C ₂₅ H ₃₈ O ₄	
	n-Decanoic acid	C ₁₀ H ₂₀ O ₂	

4.10 Optimization of growth and degradation of PAHS using one variable at a time

4.10.1 Growth Profile Analysis

Table 4.15 shows the thermos tolerant levels for the isolates *E. coli* and *K. pneumoniae* in nutrient medium without exposure to PAHs. The results show that at optimum temperature of 35°C there was rapid growth of *E. coli* and *K. pneumoniae* from 24h to the 96th h indicated by the steady increase in the absorbance values. The turbidity observed at 20°C was also heavier qualitatively as compared to that at 50°C confirmed by the quantitative reading via the UV-VIS spectrophotometer in nm.

Thermotolerance level of the selected isolates and consortium was also evaluated with the PAHs following the initial assay protocol as before with temperatures adjusted accordingly to observe optimization. The Tables 4.16, 4.17 and 4.18 showed percentage concentration loss of the PAHs as well as the response in terms of population of the isolates *E. coli* and *K. pneumoniae* used singly and in combination. for B(a)P, Pyrene and Fluranthene respectively.

From the tables there was an inversely proportional relationship between the concentration and population increase/decrease of the bacteria isolates. The growth of *E. coli* and *K. pneumoniae* in the mesophilic temperature 35°C showed better concentration losses in comparison to the thermophilic and psychrophilic temperatures as observed in tables 4.16, 4.17 and 4.18. The response of the bacteria to thermophilic temperature was better than the psychrophilic temperature for B(a)P and Fluranthene but for Pyrene the response of both bacteria strain was similar for both the thermophilic and mesophilic temperatures.

Table 4.15. Microbial Growth Pattern of bacterial isolates at different Temperatures

Time (h)	Isolate	Temperature		
		20°C	35°C	50°C
24	<i>E. coli</i>	0.445	0.891	0.941
	<i>K. pneumoniae</i>	0.341	0.518	0.438
48	<i>E. coli</i>	0.691	0.946	0.985
	<i>K. pneumoniae</i>	0.484	1.309	0.609
72	<i>E. coli</i>	1.471	1.599	1.195
	<i>K. pneumoniae</i>	1.169	1.978	1.041
96	<i>E. coli</i>	1.259	1.672	0.733
	<i>K. pneumoniae</i>	0.849	1.979	0.445

Table 4.16: Effect of varied temperature on the biodegradation of B(a)P by bacterial isolates and consortium

	Temp (°C)	Degradation (%)	Growth (nm)
<i>E. coli</i>	20	43.8	0.099
	35	66.9	0.257
	50	37.3	0.05
<i>K. pneumoniae</i>	20	46.4	0.178
	35	70.1	0.358
	50	21.5	0.124
<i>E. coli</i> + <i>K. pneumoniae</i>	20	49.2	0.199
	35	76.5	0.387
	50	36.4	0.145

Table 4.17: Effect of varied temperature on the biodegradation of Pyrene by bacterial isolates and consortium

	Temp (°C)	Degradation (%)	Growth (nm)
<i>E. coli</i>	20	51.3	0.121
	35	73.4	0.317
	50	41.8	0.101
<i>K. pneumoniae</i>	20	49.1	0.124
	35	72.9	0.318
	50	40.4	0.12
<i>E.coli</i> + <i>K. pneumoniae</i>	20	53.2	0.144
	35	75.8	0.487
	50	42.5	0.121

Table 4.18: Effect of varied temperature on the degradation of Flouranthene by bacterial

isolates and consortium			
	Temp (°C)	Degradation (%)	Growth (nm)
<i>E. coli</i>	20	50.1	0.196
	35	71.5	0.257
	50	42.2	0.121
<i>K. pneumoniae</i>	20	46.9	0.124
	35	70.5	0.308
	50	40.7	0.121
<i>E. coli</i> + <i>K. pneumoniae</i>	20	51.9	0.211
	35	73.3	0.387
	50	42.6	0.202

4.10.2 pH optimization during biodegradation

Table 4.19 shows the results of the response of the bacteria isolates to varied pH evaluated in a nutrient medium without a contaminant. The best three chosen for their high spectrophotometric reading were pH 5, 7 and 9. This was also observed visually as each day the turbidity got heavier. On exposure to the PAHs the optimum pH suitable for degradation by the bacterial isolates was the neutral pH 7 for B(a)P, pyrene and flouranthene respectively Tables 4.20, 4.21 and 4.22 respectively

4.10.3: Consequence of varied concentration on the degradation of B(a) by bacteria

isolates and consortium

Variation of the concentration of the various PAHJs showed a tolerable threshold of 1000 and below as growth of the bacterial isolated were inhibited at concentration above 1000 for the selected bacteria species Table 4.23, 4.24, 4.25 for B(a)P, pyrene and flouranthene respectively.

4.10.4: Outcome of nutrient (NPK 15:15:15) on percentage degradation of B(a)P, Pyrene and Flouranthene and its influence on the growth of isolates

For all the studied treatment protocol addition of more nutrients in the form of agriculturally used NPK 15:15:15 sped up the degradation process for B(a)P, pyrene and flouranthene respectively.

4.11 Metagenomic evaluation of genes responsible for degradation in *E. coli* and *K. pneumoniae*

The metabolic genes used to trace the specific involvement of the bacteria (*E. coli* and *K. pneumoniae*) degradation of PAHs were (*alkB*, (*nahAc*)and(*C23O*). The presence of the genes was detected in the clear PCR amplification bands shown in plates 4.5, 4.6 and 4.7 respectively.

Table 4.19: Microbial growth pattern of bacterial isolates at different pH

Time (h)	Isolate	pH				
		3	5	7	9	11
24	<i>E. coli</i>	0.049	0.918	1.341	0.934	0.048
	<i>K. pneumoniae</i>	0.159	0.443	0.676	0.768	0.173
48	<i>E. coli</i>	0.093	1.342	1.211	1.149	0.338
	<i>K. pneumoniae</i>	0.226	1.383	1.151	1.151	0.21
72	<i>E. coli</i>	0.086	1.066	1.298	1.049	0.236
	<i>K. pneumoniae</i>	0.675	0.959	1.341	0.702	0.345
96	<i>E. coli</i>	0.034	0.972	1.173	0.938	0.375
	<i>K. pneumoniae</i>	0.471	0.755	1.126	0.311	0.109

Table 4.20: Effect of varied pH on the degradation of B(a)P by bacterial isolates and consortium

	pH	Degradation (%)	Growth (nm)
<i>E. coli</i>	5	50.1	0.304
	7	61.5	0.457
	9	51.2	0.31
<i>K. pneumoniae</i>	5	29	0.204
	7	63.3	0.458
	9	43	0.301
<i>E. coli</i> + <i>K. pneumoniae</i>	5	50.7	0.204
	7	64.6	0.358
	9	48.9	0.224

Table 4.21: Consequence of varied pH on the degradation of Pyrene by bacterial isolates and consortium

	pH	Degradation (%)	Growth (nm)
<i>E. coli</i>	5	47.5	0.205
	7	73.6	0.457
	9	41.9	0.321
<i>K. pneumoniae</i>	5	40.1	0.224
	7	73.4	0.558
	9	30.8	0.298
<i>E. coli</i> + <i>K. pneumoniae</i>	5	54.7	0.445
	7	76.4	0.587
	9	49.3	0.212

Table 4.22: Outcome of varied pH on the degradation of Flouranthene by bacterial isolates and consortium

	pH	Degradation (%)	Growth (nm)
<i>E. coli</i>	5	49.7	0.204
	7	70.6	0.457
	9	49.9	0.221
<i>K. pneumoniae</i>	5	32.4	0.124
	7	71.5	0.458
	9	56.5	0.278
<i>E. coli</i> + <i>K. pneumoniae</i>	5	51.5	0.145
	7	70.3	0.498
	9	59.7	0.312

Table 4.23: Effect of varied concentration on the degradation of B(a) by bacterial isolates and consortium

	Conc (mg/ml)	Degradation (%)	Growth (nm)
<i>E. coli</i>	500	56.8	0.31
	750	50.6	0.29
	1000	45.9	0.27
	1000	20.7	0.16
<i>K. pneumoniae</i>	500	50.1	0.33
	750	51.2	0.31
	1000	39.9	0.29
	1250	16.6	0.11
<i>E.coli</i> + <i>K. pneumoniae</i>	500	53.2	0.38
	750	53.1	0.36
	1000	42.2	0.29
	1250	21.6	0.25

Table 4.24: Effect of varied concentration on the degradation of Pyrene by bacterial isolates and consortium

	Conc (mg/ml)	Degradation (%)	Growth (nm)
<i>E. coli</i>	500	60.1	0.41
	750	55.6	0.4
	1000	39.2	0.31
	1250	35.5	0.24
<i>K. pneumoniae</i>	500	62.9	0.45
	750	60.3	0.41
	1000	41.1	0.39
	1250	36.7	0.26
<i>E. coli</i> + <i>K. pneumoniae</i>	500	63.4	0.86
	750	61.5	0.87
	1000	41.1	0.66
	1250	36.9	0.51

Table 4.25: Effect of varied concentration on the biodegradation of Flouranthene
by bacterial isolates and consortium

	Conc (mg/ml)	Degradation (%)	Growth (nm)
<i>E. coli</i>	500	59.5	0.49
	750	54.9	0.44
	1000	33.7	0.24
	1250	20.1	0.15
<i>K. pneumoniae</i>	500	58.9	0.42
	750	54	0.39
	1000	39	0.26
	1250	21.6	0.24
<i>E. coli</i> + <i>K. pneumoniae</i>	500	60.7	0.52
	750	62.5	0.51
	1000	44.3	0.39
	1250	23.9	0.28

Table 4.26: Effect of nutrient (NPK 15:15:15) on percentage degradation of B(a)P,
Pyrene and Flouranthene and its influence on the growth of isolates

	Isolates	Degradation (%)	Growth (nm)
B(a)P	CTRL	11	0.218
	<i>E. coli</i>	72.5	0.411
	<i>K. pneumoniae</i>	74.8	0.355
	<i>E. coli</i> + <i>K. pneumoniae</i>	79.4	0.588
Pyrene	CTRL	6	0.111
	<i>E. coli</i>	79.4	0.361
	<i>K. pneumoniae</i>	81.5	0.314
	<i>E. coli</i> + <i>K. pneumoniae</i>	93.6	0.371
Flouranthene	CTRL	7	0.118
	<i>E. coli</i>	89.4	0.411
	<i>K. pneumoniae</i>	80.5	0.425
	<i>E. coli</i> + <i>K. pneumoniae</i>	95.4	0.493

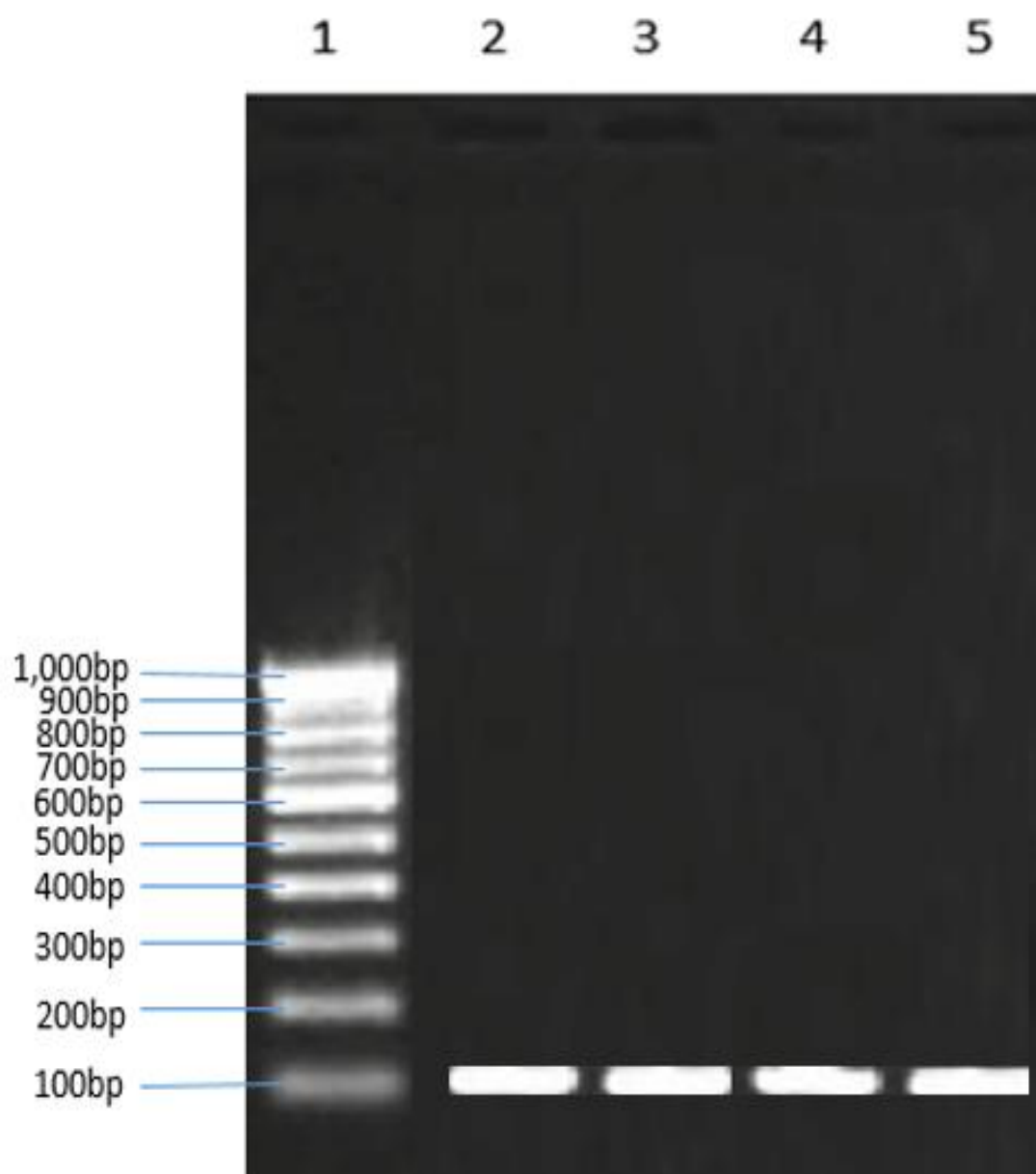


Plate 4.5. PCR analysis of all isolates obtained in the study using gene specific bacterial primers n-alkanes monooxygenase (*alkB*).
 lane 1= 100bp DNA ladder (Genbiotech). Lane 2= Ec1. lane 3= Ec2. Lane 4= Kb1. Lane 5= Kb2.
 Key: Ec1 and Ec2 = *Escherichia coli* run in duplicate. Kp1 and Kp2 = *Klebsiella pneumoniae* run in duplicate.

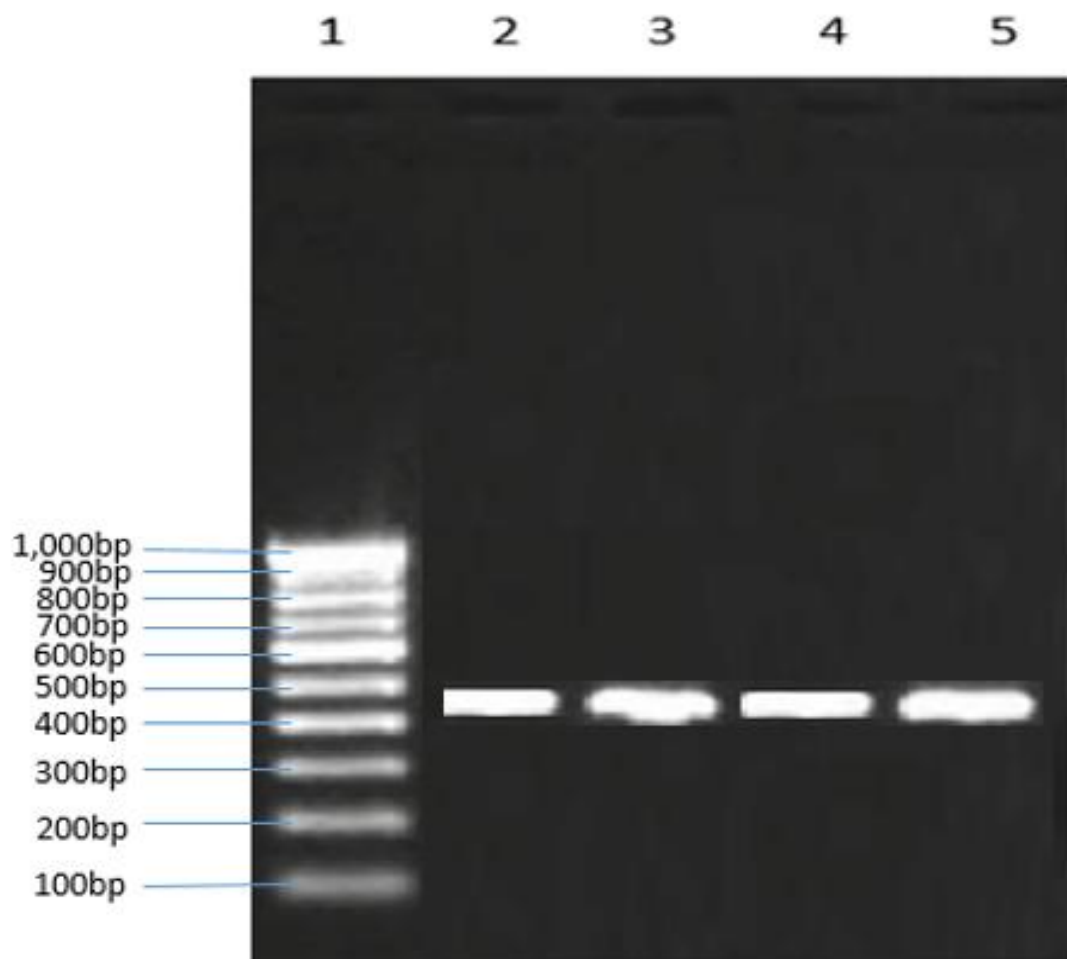


Figure 4.6. PCR analysis of all isolates obtained in the study using gene specific bacterial primers Naphthalene Dioxygenase (*NahAC*).
 Well 1= 100bp DNA ladder (Genbiotech). Lane 2= Ec1. Lane 3= Ec2. Lane 4= Kb1. Lane 5= Kb2.
 Key: Ec1 and Ec2 = *Escherichia coli* run in duplicate. Kp1 and Kp2 = *Klebsiella pneumoniae* run in duplicate.

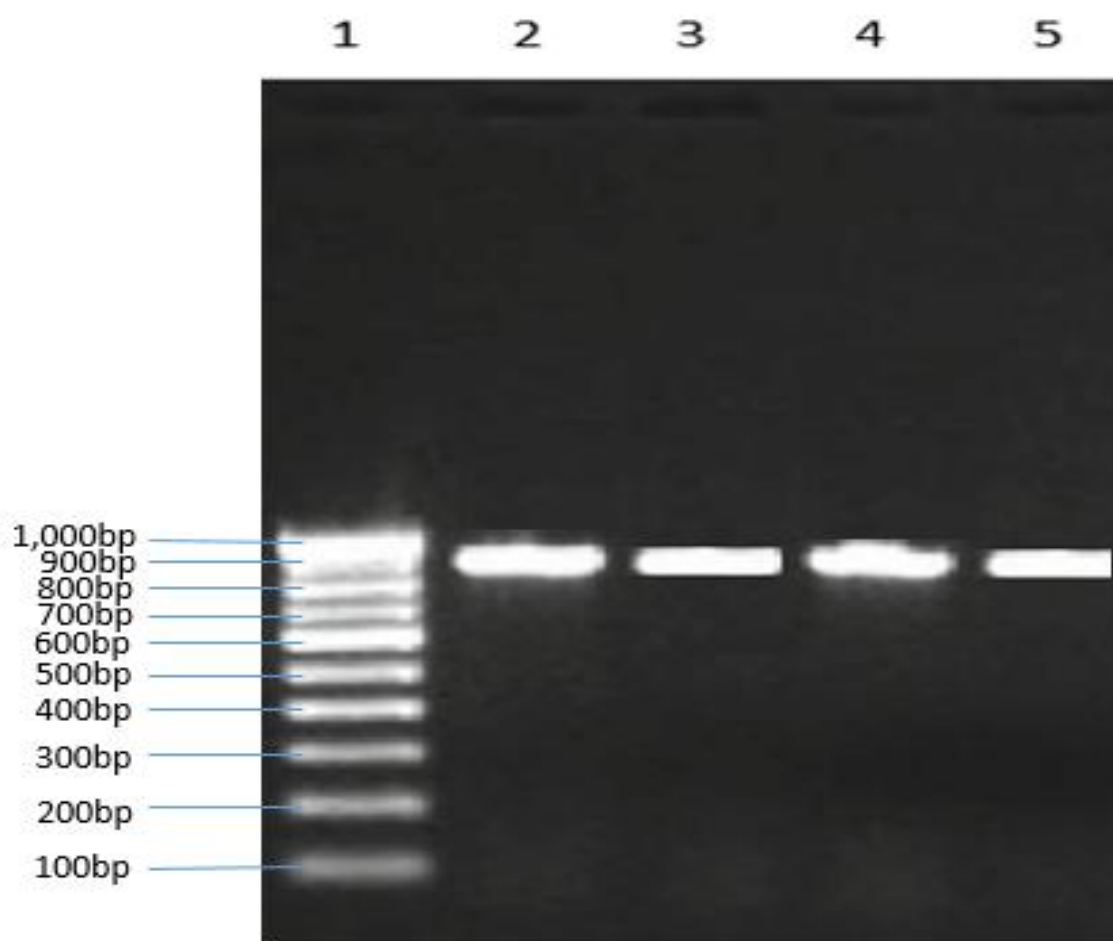


Figure 4.7. PCR analysis of all isolates obtained in the study using gene specific bacterial primers
Catechol dioxygenase (*C23O*).

Lane 1= 100bp DNA ladder (Genbiotech). Lane 2= Ec. lane 3= Ec2. Lane 4= Kb1. Lane 5=Kb2.

Key: Ec1 and Ec2 = *Escherichia coli* run in duplicate. Kp1 and Kp2 = *Klebsiella pneumoniae* run in duplicate.

CHAPTER FIVE

DISCUSSION

5.1 Discussion

Microorganisms are ubiquitous with the inherent ability to flourish within different ecological niches where they are exposed and are continuously bombarded by an array of organic as well as inorganic compounds. Such exposure, coupled with a cascade of intrinsic metabolic biomolecules, boosts their ability to assimilate and metabolize diverse range of substances towards ensuring growth and survival (Deepa *et al.*, 2018).

The microbiota obtained from the intestinal chyme of *Bos taurus* in this study was diverse due to the rich array of materials that they consume, as well as the highly nutritional and moist environment of the large intestine which supports the propagation of several hundred species of microorganisms (Kim *et al.*, 2011; Creevey *et al.*, 2014). Experimental procedures have been published on the use of cow dung and other composted materials for remediation of PAHs including works of Neethu *et al.*, (2019). However, little is known about the PAHs degrading microorganisms from the unique semi anoxic intestinal gut environment of *Bos taurus* which undergoes several metabolic processes including breaking down of cellulolytic materials and other conversion of chemical products serving as pollutants including PAHs (Khafipour *et al.*, 2009; Chow *et al.*, 2010). Undeniably, leaves tainted by pollutants including PAHs serve as a discrete measurement for environmental stress (Loppi *et al.*, 2015) and forms the major food products for *Bos tarus*.

The microorganisms obtained in this study were members of the autochthonous microbial community which act via symbiosis to digest a wide range of vegetation for the nourishment of the vertebrate herbivores. The studied test result (Table 4.1) implied

that the environmental niche of the cow where the intestinal chyme used in this study was collected possessed a near neutral pH 7.15. This is in line with the pH range of the intestinal gut lining of cows (6.6-7.8) Jane-Parish (2017) who explained that as materials leave the stomach they pick free radicals together with moisture and become less acidic.

The large intestine had high moisture content of between 96-98%; an ideal growth environment for the mass propagation of diverse microorganisms. This is in line with findings of (Kamra, 2005) that 90-98.5% of the fluid in both the small and large intestine of cows are moisture laced. The pH and moisture content from the ecological niche of *Bos taurus* is thought to be maintained throughout the lifespan of the host animal with slight increment in either the acidity or alkalinity contemplated due to cloistered activities of organic acids via the digestive process, coupled with splintering gaseous exchange events to restore carbon dioxide and methane balance. The average moisture content from the rumen of the studied animals aligned with data obtained from other research findings by a consortium of scientists globally (Zeng *et al.*, 2019). The total solids (TS) obtained from the rumen fluid was moisture laced, which upon drying was expected to display a moderate to high percentage level owing to the fact that the large intestine represents the go-between the anus and small intestine whereby bioconversion activities take place and the end point of metabolic activities before unused and undigested food product is egested out.

Subsequent on the actions of a gamut of intestinal enzymes on feedstock, an arsenal of biomolecules gets released such that they are soluble in gastric acids and other intestinal juices as well as saliva, thus making it easier to detect solid materials culminated in the term total solids (TS) (Lopp, *et al.*, 2015) and the amount present in this study was substantial for a healthy beef cow.

Successfully isolating bacteria and purifying them is crucial for research experimental work therefore in choosing isolation methods, care has to be taken to be able to obtain viable organisms thus two methods were employed in this study one direct plating method chosen to obtain the possible bacteria that could be isolated culturally outside the ecological niche of the organisms in a general purpose rich nutrient medium and the other method was to expose the inherent microbes to a minute quantity of the pollutant to create recognition and adaptation when a larger portion of the pollutant is encountered. In this study targeting possible aerobes or facultative anerobes the direct plate method displayed a lot of similar isolates screened out for their similarities (morphological and biochemical tests). The results displayed a variety of species both hydrocarbon and non-hydrocarbon degraders alike. It is conceivable that exposure to any pollutant would trigger the rise of more dominant resistant microorganisms within the same ecological niche as animals have a strong defence mechanism against any foreign body. To test/validate this theory the enrichment technique was used to expose the isolates to the pollutants and test their level of survival in a small quantity of the pollutant (PAHs). Data generated from the enrichment technique Table 4.3 though not as much as that of the direct plating method showed colonies which were more distinct thus easier to obtain pure cultures after several repeats. This feat corresponds to the research findings of Chang *et al.* (2019) that though the enrichment method is more time consuming and laborious the eventual yield is better than the direct plating methods.

From the biochemical assessment of the pure cultures obtained in this study a good number of the identified bacteria belong to the Enterobacteriaceae family probably due to the fecal proponents of the environment from which the isolates were drawn. Within the last decade, species of Enterobacteriaceae have been found to be prominent

heterotropic petroleum degraders including PAHs, particularly the asphaltene fractions of crude oil (Jahromi., 2014; Patel., 2014; Subramanian & Menon, 2015; Mohamed., 2015). The results from this study also align with published data obtained in other reports like that of Sari and co-workers who isolated and positively identified *Enterobacter* species from rumen of cattle (Sari *et al.*, 2017; Lauková *et al.*, 2018). Many species in this genus (Enterobacteriaceae) have adapted to exposure to a combination of extrinsic (temperature, relative humidity), implicit (microbe-microbe interaction, influence of peculiar feedstock) and intrinsic (acidity, moisture content) factors. Another visible family dominating the intestinal chyme sample from the large intestine was the Staphylococcaceae family (Tables 4.2 and 4.3). Numerous species of *Staphylococcus* have been isolated from cows both dairy and beef species (Keefe, 2012; Kateete *et al.*, 2013; De Visscher *et al.*, 2014; Boss *et al.*, 2016; Capra *et al.*, 2017; Srednik *et al.*, 2017). Roberts and colleagues found a wide distribution of *Staphylococcus* species including *Staphylococcus haemolyticus* isolated from the ruminal compartment of seven sampled cows, *Staphylococcus cohnii* was also implicated to be present (Roberts *et al.*, 2018). Other families that surfaced was the Micrococcaeae and Streptococcaeae which have been implicated to be found in the intestinal lining of *Bos taurus* (Kay, 1964).

It was observed that some isolates from direct plating method shared similar biochemical identity with isolates from the spiked mineral salt medium. However, their performance in the DCPIP experiment showed that recognition from enrichment had a part in responses of isolates to pollutants. Isolates from the enrichment technique exposed to the pollutant showed survival and adaptability. The results from this research also agree with the hypothesis that enrichment brings recognition and adaptation (Hilyard *et al.*, 2008). Results also tally with the findings of Thauer and co-

workers as well as Angelidaki and colleagues who reported that amongst the methanogenic microbiota culture from cattle rumen, substrate metabolism by its microorganisms is achieved mainly via acetogenesis, acidogenesis, hydrolysis, methanogenesis, as well as other catalytic events including but not limited to tanninolytic, proteolytic and ureolytic events (Krause., 2003; Thauer., 2008; Russell, 2009; Angelidaki *et al.*, 2011; Wright and Klieve, 2011).

It was imperative to test the thirty-four pure cultures identified biochemically for their ability to degrade PAHs and prune the number of isolates for further analysis. The redox dye 2, 6-Dichlorophenol Indophenol was used and is considered to be the most reliable, cheaper and less tasking of all other screening methods for hydrocarbons (Desai and Vyas, 2006). In this study, DCPIP was employed in a manner similar to that of Mariano and co-workers who examined methods for screening potential hydrocarbon degrading microorganisms for which alternative petroleum oils were utilized as the only carbon source for screening (Mariano *et al.*, 2008). This approach has also been described as both a quantitative and qualitative indicator method whereby upon metabolism of the hydrocarbon in the broth mixture, a colour change from blue to colourless; triggered by oxidation or transfer of electrons to one of several electron acceptors including but not limited to carbon dioxide (Grishchenkov *et al.*, 2000; Boopathy, 2002; Massias *et al.*, 2003; Bidoia *et al.*, 2010).

Data obtained qualitatively showed four of the identified species from direct plating been decolourized within the 96h period and eleven out of the sixteen isolates from the enrichment method showing positive DCPIP within the first twenty-four hours necessitating the quantitative assessment of each sample via UV-VIS spectrophotometer. Data generated revealed that the best hydrocarbon degrading microorganisms using the DCPIP were probable isolates from biochemical's designated

Escherichia coli and *Raoultella ormithinolytica*. These organisms have long been known for their petroleum degradation abilities, particularly when assayed using the DCPIP protocol (Roy *et al.*, 2002; Joshi and Pandey, 2011; Patil *et al.*, 2013; Adegbola *et al.*, 2014; Selvakumar *et al.*, 2014). The suspected *Escherichia coli* was amongst several genera of bacteria isolated from various environments by other researchers who found it capable of metabolizing polycyclic aromatic hydrocarbons; naphthalene utilized as carbon solely (Nnamchi *et al.*, 2006; Pizzul *et al.*, 2007). In their studies, they found that *Escherichia coli* exhibited a high response to utilization of crude at 600nm via a UV-VIS spectrophotometer (Nnamchi *et al.*, 2006; Pizzul *et al.*, 2007). Similar finding was more recently reported by Subramanian and Menon, as well as Wanjohi and co-workers in Kenya Subramanian and Menon, 2015; Wanjohi *et al.*, 2015).

Raoultella genus of bacteria is commonly isolated from oil polluted sites and have demonstrated excellent petroleum degrading capabilities when activated (Nwaguma *et al.*, 2016). Interpretation of the Absorbance reading of data obtained from the DCPIP assay suggests that isolates obtained using mineral salt medium spiked with pyrene were better metabolizers of hydrocarbons than their non-PAH-spiked variants. From the result of DCPIP UV-VIS analysis the best two hydrocarbon degraders were selected and subjected to a more robust identification method via the API 20E kit.

It is noteworthy that the probable conclusion drawn from the biochemical test enabled the choice of a suitable API test kit which was a rapid test kit less cumbersome with fewer material and tools needed in comparison to conventional biochemical tests but with limitation of dependence on the Gram reaction and oxidase test for completion of test procedure Table 4.8. Results of the API test for the two samples selected for further analysis were confirmed as *Morganella morganii* and *Raoultella ormithinolytica*.

The *Morganella morganii* was lowly discriminated because the percentage similarity was the highest but less than 70% of others in the APIWEB and result sheet giving a “low discrimination” score card raising an iorta of doubt necessitating further confirmatory tests. *Raoutella ormitholytica* on the other hand had a 99.9% similarity in matching result to the APIWEB *Raoutella ormitholytica*. Comparing the biochemical testing and API one major observation was the fact that both *Escherichia coli* and *Raoutella ormitholytica* were lactose negative in their biochemical tests while the API test showed them to be late lactose fermenters. This was also validated using MacConkey agar. Thus reliance on a test protocol for identification can be misleading. The organisms had the ability to utilize the enzyme permease and penetrate the bacterial cell wall indicating further that the organisms are late lactose fermenters in line with the findings by Sargar *et al.* (2019).

Molecular identification uses the DNA of isolates and presents an undisputed option for identification of microorganisms. In the application of gel electrophoresis towards DNA extraction, an expected band size of $\geq 10,000\text{bp}$ sought after as genomic deoxynucleic acids (gDNA) are large molecules and are prone to remain within the well whilst loading the gels, leading to the appearance of a smear above the bands. The appearance of a smear below or above the DNA band is evidence of an RNA or protein contamination in the sample. A higher concentration of agarose gel was required for the separation of PCR amplicons as these products are much smaller than gDNA and are normally in the range of 100bp-1500bp. The strong bands that appeared suggested that the annealing temperature of the primers was selected properly as such a phenomenon known as primer-dimer did not take place. Furthermore, no smear was observed below the bands, thereby indicating that the concentration of template DNA used for this reaction was sufficient and within the 20-30ng range for PCR reactions.

The PCR amplicons obtained appeared to be approximately 1,000bp in size in comparison with the 100bp reference DNA ladder. PCR analysis of the two (2) isolates (*Escherichia coli* and *Raoutella orminolytica*) based on the DCPIP assay revealed amplicons of about 1kb in size (Plate 4.1). Amplification was also attempted with other universal bacteria PCR primer sets; 63F and 1387R, BACT338/BACT1525, however, the combination of 27F/1492R gave the best amplified results of the 3 universal bacteria primers. Following the purification, concentration and sequencing of the amplified PCR products, raw nucleotide sequences exported from the sequenced-chromatograms using the BioEdit software followed by (BLAST) software database determined sequence similarities with already known organisms within the NCBI archive. The results showed that isolate *E. coli*, suspected to be *Morganella morganii* based on API20E analysis, shared a 98.97% sequence homology with *Escherichia coli* strain GSH8M-2 (plate 4.3.). The results showed that *Raoutella orminolytica* suspected to be *Raoutella orminolytica* based on API20E analysis, shared a 99.30% sequence homology with *Klebsiella pneumoniae* strain PSB1 (Plate 4.4). Studies have revealed that the *Raoutella* family of microorganisms consists predominantly of Gram-negative, oxidase negative, non-motile, capsulated, aerobic and facultative anaerobic rods, which originated from the *Klebsiella* family only to be redefined and separated based on advanced recombinant DNA techniques (Drancourt *et al.*, 2001). Both *Raoutella* and *Klebsiella* belong to the Enterobacteriaceae super family with the major difference being the presence of the *rpoβ* gene in the former (Ponce-Alonso *et al.*, 2016).

It is imperative to note that the biochemical test though effective has the challenge of members of a particular genus having same response to the chemicals making it difficult to identify the species inherent. *Raoutella* has within the new millennium been found to be easily mis-identified as sub-types of *Klebsiella*, namely

Klebsiella ornithinolytica and *Klebsiella planticola* (Drancourt *et al.*, 2001). The mis-identification of both *Raoutella* and *Klebsiella* stems from their association with histamine formation for which *Klebsiella pneumoniae* and *Klebsiella oxytoca* are still being misconstrued to be *Raoutella planticola*. This mis-match further extends to unique species of the *Klebsiella* family such that *Klebsiella trevisanii* that has been re-classified as *Raoutella planticola* (Bollet *et al.*, 2002). The advent of recombinant DNA technology has however prompted the proper identification of both genera based on 16S rRNA and *rpoB* gene sequences (Drancourt *et al.*, 2001). *Klebsiella* and *Raoutella* species represent a large group of commonly isolated bacteria found in 100% of rumen samples in dairy farms across the United States (Zadoks *et al.*, 2011). These researches put to rest the disparity observed between the identification of the isolate identified as *Raoutella ornithinolytica* by API technique and *Klebsiella pneumoniae* identified by the molecular technique.

Escherichia coli(S13) and *Klebsiella Pnuemoniae*(PSB8) showed themselves strong in the degrading (B[a]P), Pyrene plus Flouranthene. These pure compounds after preparation and standardization were quantified using a Gas Chromatography- Mass spectrophotometer to establish their peaks and retention time together with recovery. The GC-MS detected pyrene and flouranthene as at the appropriate retention time peaks were observed. However, the GC-MS could not detect the (B[a]P) compound after the initial quantification (LOD) and (LOQ) analysis run with the GC-MS. The HPLC was able to detect the (B[a]P) peak and thus utilized as the analyzer to detect the residual concentration during biodegradation in relation to time.

Researches have shown that the response of microorganisms to pollutants in a medium is varied and is dependent on the type and concentration of the pollutants (Gauri *et al.*, 2019). Established record have it that there is threshold of acceptance for

very toxic compounds, invariably affecting their degradation potential. If concentration in a given environment is beneath acceptable threshold, it will not be available for uptake by microorganisms and thus not utilized, while if above range of concentration (i.e, too high), the xenobiotics will be toxic to the organisms and may inadvertently inhibit growth (Doick *et al.*, 2005; Nwaguma *et al.*, 2016) thereby stripping them of their degradative abilities as observed in the monitoring of behaviour of the bacteria in response to the varied concentration of PAHs Tables 4.23, 4.24 and 4.25 for BaP, Pyrene and Flouranthene respectively.

Data obtained from HPLC analysis of (B[a]P) showed that isolates from the large intestine had the capacity to reduce (B[a]P) although at different rates. *Klebsiella pneumoniae* exhibited a higher degradation efficiency (14.1%) than *Escherichia coli* strain (9.9%) and a combination of both isolates of the initial 250 µg/ml of B[a]P after 24 hours on incubation (Table 4.10 and Figure 4.1). While the initial kick off after 24hrs saw *Klebsiella pneumoniae* performing better than *E. coli* and a combination of both isolates the pick-off of the isolates as degradation went on showed the synergy in which *E. coli* and *K. pneumoniae* have working together to attain a 94.8% degradation better than the 71.2% for *E. coli* and 74.8% after sixteen days. This corresponds with the findings of Nwaguma *et al.* (2016) that *E. coli* and *K. pneumoniae* from human faeces could degrade petroleum hydrocarbon. The expectation was that the control sample will remain at the initial concentration since it was aseptically prepared without microorganisms however there was slight decrease in its concentration. This may have been due to volatilization as prolonged volatilization has been implicated in reduced PAH concentrations during pilot studies reported by Kong *et al.* (2015). Whilst utilization of B[a]P for energy generation was verified in individual and mixed cultures, observed pattern showed variation in regards to their specific growth rates (Figure 4.2.)

Bacteria growth on B[a]P was relatively slow for *E. coli* the combination of *E. coli* and *K. pneumoniae* was better though still not fantastic. Probably due to the acclimatization rates (substrate metabolism) of *E. coli* to the B[a]P substrate and limited transport or cytotoxicity (Sowada *et al.*, 2014). Studies have shown that degradation of B[a]P under aerobic conditioning is usually via a stepwise oxygenation process (Cébron *et al.*, 2008). In that same report, it was deduced that bacterial utilization of weighty PAHs like B[a]P, flouranthene and pyrene is usually achieved using mixed microbial consortiums. As observed in this study, the lower rates of PAH biodegradation by individual bacteria opposes the energy consumption required for the complete bioconversion/bioremediation (Andersson and Hughes, 2010; Haiser and Turnbaugh, 2013)

It has been established that, the concentration of the hydrocarbon pollutants (in a controlled environment) affects the survival of microorganisms as the survival of the organism depends on the utilization of carbon from the substrate for energy. This research work agrees with the above observations. Accordingly, there was progressive growth for isolates singly and in combination. Although B(a)P was degraded but it was not completely mineralized hence there was no decrease in population as concentration decreased up until the sixteenth day.

Pyrene and flouranthene showed similar response of the isolates to the PAHs even with a different analyser. Results revealed that the concentration of pyrene/flouranthene degraded had a relationship with the response of the bacterial isolates singly and in consortium as the control recorded an infinitesimal to a near no growth within the period of the assay run probably due to the lack of a carbon in assay. This data supports reports by Kim and colleagues (2007) who found that *Escherichia coli* like other PAH degrading bacteria possesses certain resistant genes, which upon

escalated expression in response to environmental stimuli, enable them to survive preventing against oxidative, membrane and protein damage (Kim *et al.*, 2007). The results revealed that both organisms worked synergistically in degrading Pyrene however significant values from ANOVA in which P was less than 0.05 ($P < 0.05$) gave the inference that the individual isolates were also super degraders of Pyrene.

With flourantheneas stated earlier, degradation pattern was similar although the percentage degraded was not as much as pyrene. This is due most likely to the distinctly different structural forms of pyrene and flouranthene. Fluoranthene has a pentagon and three hexagons while pyrene has four closely fused hexagon (Díaz *et al.*, 2014). Therefore, giving the same time the enzymes coding for the genes responsible for degradation will cleave to the hexagon and recycle before ortho cleaving with the pentagon.

Bacteria viability testing, revealed that growth of the *K. pneumoniae* was higher than *E. coli* for both pyrene and flouranthene but highest in a consortium. This phenomenon was observed in all viability assessment against pyrene and flouranthene. Probably due to the fact that *K. pneumoniae* had prior exposure to the pollutant so acclimatized quicker than its *E. coli* partner or maybe attributed to the unique enzyme cascade, crucial to both the electron transfer and bioconversion of hydrocarbon principle of which are the mono- and dioxygenase genes (*AlkB*, *C23O* including *PAHRHDα* as reported by many researchers (C'ebon *et al.*, 2008; Guo *et al.*, 2010). The structural system spectrum of one and multiple oxygenase enzyme play a major part in the initiation of PAHs degradation aerobically (Vaidya *et al.*, 2018) thus throughout the period of the assay the dissolved oxygen level was analyzed and maintained by ensuring agitation using an orbital shaker.

Chemical oxygen demand (COD) was observed to have a direct proportional relationship with the concentration of the PAHs. The COD decreased with corresponding decrease in concentration of PAHs in MSM. Cogent decrease in COD concentrations was observed at each sampling day till the sixteenth day. BOD on the other hand, showed an increase as concentration decreased during the assay run for the experimented PAHs showing an inversely proportional relationship with growth of the organism. This finding tallies with the research of Fulekar *et al.* (2017) who reported similar findings with lower molecular weight PAHs.

Degradation of PAHs by microorganisms depends on some physicochemical parameters, which includes: incubation period, pH, temperature, concentration and stimulants such as NPK 15:15:15. In nature the response of organisms to pollutants follow the normal growth curve pattern in which on exposure experience the period of acclimatization (lag phase) and with time may pick up to exponential phase, stationary and then decline phase. The response of the pollutant in respect to growth and degradation is dependent on some variables including microbial proliferation rate, percentage loss of pollutant including intermediaries culminating in determination of PAHs reduced in a medium. The obtained result for the PAHs analyzed revealed the metabolism of the hydrocarbon been dependent on the microbial number which in turn correlates to the time of exposure.

The response of the bacterial isolates to different pH and temperature to ascertain their survival rate and viability without the PAHs was impressive. During the initial assay pH was monitored and there was slight variation as degradation progressed due to the release of possible metabolites and the bioavailability of the PAHs. However, with the optimization study it was observed that PAH(s) degradation by the bacterial isolates and consortium was pH dependent. It was observed that testing the viability of

the isolates in nutrient broth there was steady increase in growth of *K. pneumoniae* with *E. coli* performing better than *K. pneumoniae*. The *E. coli* is known for its sporadic growth and one of the most studied bacteria (Dudhagara *et al.*, 2016).

Microbial growth analysis at different temperatures revealed that under psychrophilic conditions, the *E. coli* strain exhibited a better absorbance range daily over the 96h incubation period than *K. pneumoniae*. Both organisms grew best under mid-mesophilic conditions (35°C). It is expected that as the temperature increases, enzymatic functions accelerate, causing a rapid increase in cell size. Beyond the temperature threshold of different microorganisms' metabolic machinery, enzymatic functions deteriorate and denatures, coupled with a decline in cellular growth rate. This is depicted in the absorbance readings taken for *E. coli* at thermophilic conditions in comparison to *K. pneumoniae* (Tables 4.16, 4.17 and 4.18) for B[a]P, Pyrene and Flouranthene. Increased temperatures boosted the degree of B[a]P degradation in *E. coli*. At the temperature optima of 35°C, *E. coli* exhibited a 52.6% rate of B[a]P degradation and the corresponding growth absorbance also increased. The *K. pneumoniae* shared the same feat with the consortium showing better degradation for all the PAHs analysed. The concentration for each PAH tested was greatly reduced by a combination of both isolates at the mid mesophilic temperature of 35°C. At higher temperature the synergy of the isolates to be able to hold stay was strong in comparison to lower temperatures with two-fold decline in concentration and lower growth yield of the test organisms. Publications on degradation of PAHs have unraveled the aerobic enzymes requirements for PAHs metabolism having optimum reaction temperatures in the mesophilic range (Patel *et al.*, 2012; Vaidya *et al.*, 2017). Similar trend was observed when measurements were taken whilst substituting B[a]P with Pyrene and Flouranthene.

This research also confirmed that acidic and alkaline environments altered the general growth and biodegradation efficiency of individual bacterial strains. The degradation and growth rate were considerably enhanced in the consortium degradation experiment under neutral conditions thus indicating that molecular and enzymatic functions of the bacterial isolates operates best at neutral pH 7. This phenomenon was also observed when bacterial isolates were grown in media kept at neutral pH. This corroborates with findings of a Revathy *et al.* (2015) that the pH of the medium during biodegradation is a function of the percentage degraded and growth profile of microorganisms. Result also agrees with other research findings showing that a pH close to neutral is mostly required by PAHs degrading bacteria Zhou *et al.* (2016).

Significantly, stimulation of these bacteria with nutrients from various sources including more sugars, nitrogenous substances enhances the rate of degradation. Commercially obtained fertilizer with appropriate composition was able to speed up degradation by the bacterial isolates and consortium thereby prompting the use of extrinsic factors to boost polycyclic aromatic hydrocarbon consumption. Electron rich reduced compounds is a necessity for microbes to catabolically provided free electrons during PAH degradation. The stimulant NPK 15:15:15 provides such ions and alternative energy levels for the metabolism of the pollutants by the microorganisms singly and in combination. The observed results in Table 4.26 showed the percentage degradation of PAHs and rapid growth rate of *E. coli* and *K. pneumoniae* when NPK was added to medium.

Suspensions of the specific identity, let alone presence or absence thereof of critical genes coding for enzymes responsible for metabolism of the PAHs used in this study were analyzed via recombinant DNA techniques. The presence or absence thereof of selected hydrocarbon degrading enzymes gene, was assayed via polymerase chain

reaction (PCR). From the amplification observed all isolates obtained possessed the dioxygenase (*nahAc*) as amplification using the gene specific primers was visible as the PCR amplicons between (300-500) bp in length. This corresponds with literature that has shown that the fragment size of this gene is approximately 487bp (Laurie and Jones, 2000). The n-alkane monooxygenase gene as the name implies has been implicated as the major activator of the metabolic pathway geared towards the degradation of alkanes, which gave rise to the notion of the Alk enzyme system (Guo *et al.*, 2010). This gene codes for the Alk enzyme which major function is the transmembrane activation of aliphatic hydrocarbon hydroxylation aerobically, utilizing a complex set of enzymes and co-factors namely; Transmembrane alkane monooxygenase (*alkB*), Rubredoxin (*alkF*), and Rubredoxin reductase (*alkG*).

Together, these component parts have proven efficient and effective in environmental (bioremediation) and industrial applications including but not limited to biocatalysis, drugs, pravastatin and other synthetic compounds (Kloos *et al.*, 2006). The presence of this gene by way of PCR amplification (Plate 4.6) suggests that these microorganisms could be applied towards the biotransformation of alkanes in petroleum polluted soils. The use of the *alkB* gene specific primers for the characterization of several microorganisms revealed that as a biomarker, there is a large diversity of this gene widely distributed in nature and although the nucleotide sequence encoding this gene from specie to specie varies, it is still the gold standard for the detection of potential alkane-degrading bacteria Wang *et al.* (2014). All the tested bacterial isolates possessed the n-alkanes monooxygenase (*alkB*) gene. The 100bp band size of the *alkB* gene detected during assessment aligns with the band size amply reported by Powell and colleagues as well as by Hesham and co-workers (Kloos *et al.*, 2006; Powell *et al.*, 2006; Hesham *et al.*, 2014).

Amplification of catabolic *alkB* gene served as an important indicator that these isolates could be adapted towards the remediation of short-chain n-alkanes which the cattle would invariably come into contact with and would otherwise be toxic. Of particular interest is the presence of the naphthalene dioxygenase (*nahAc*) gene because the enzyme coded by this gene is so versatile that it participates in numerous PAH cracking, not only naphthalene. It is inferred to be one of the primary genes responsible for the degradation of B[a]P, fluoranthene and pyrene in this study. Similar PAH degradation patterns as observed in this study and attributed to this specific gene is displayed by many bacteria (Guo *et al.*, 2010; Lu *et al.*, 2011). Catechol dioxygenase genes (*C230*) was also amplified and from the observation of the possible metabolites where a Cis-Cis diol group was observed it is possible that the dioxygenase group was involved in the cleavage resulting in this alcoholic group during degradation.

The breaking open of an aromatic ring whilst picking up a hydroxyl group, prior to the degradation and subsequent conversion to a ketone then an aldehyde and finally a carboxylic group, all culminate into the tricarboxylic acid cycle (TCA) all form part of the gene-enzyme specific functions of *nahAc* and its associated enzyme system. Pyrene biodegradation by the isolated strains together with their consortium appeared to follow the o-phthalate metabolic pathway based on the intermediates seen in the library and comparison to existing literature; 4,5-dihydroxydihdropyrene, 4 – hydroxyperinaphthenone, phthalic acid, isophthalic acids, 1, pyrenecarboxyldehyde, Phenanthrene-4-carboxylic acid and Terephthalic acid other than the pyrene peaks were some of the detected compounds by the GC-MS library conforming with the degradation pattern of polyaromatic hydrocarbons.

Similarly Fluoranthene biodegradation by the isolated strain of *E. coli* and *K. pneumoniae* sailed on same route the o-phthalate metabolic pathway also based on the

intermediates seen; 7,8-dihydroxyfluoranthene, phthalic acid, isophthalic acids, isoflurane, 9-fluoronone-1-carboxylic acid and Terephthalic acid other than the fluoranthene including Diketone alcohol, 2-pentanone which further degradation by ring-cleaving dioxygenases produced intermediates captured by the TCA cycle via the *o*-phthalate degradation pathway. These peaks were comparatively selected from the GC-MS library in consonance with the degradation pathway.

Overall, the two selected bacterial isolates utilized in this study via the DCPIP assay, coupled with all the isolates obtained via the culture dependent isolation on nutrient and tryptic soy agar showing to have strong potentials to degrade PAHs, appear mostly similar to microorganisms isolated from the gut and milk of cows by other researchers (Quigley *et al.*, 2013). Biodegradation of PAHs has been well established among members of the *Klebsiella* genus, particularly *Klebsiella oxytoca* naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, pyrene, and fluoranthene has been reported (Zhang *et al.*, 2018). Both studied bacteria in this research work validated other published work by several scientists and the knowledge base of PAHs biodegraders from the unique semi anoxic intestinal gut environment of *Bos tarus*.

This data obtained pertaining to *Escherichia coli* supported reports by Kim and colleagues (2007) who found that *Escherichia coli* like other PAH degradable bacteria possesses certain resistant genes especially laccases coded by a *CueO* gene that is more predominant and widely distributed in this organism than most. The 5-ringed structure of B[a]P appear to favour the growth and metabolism of *E. coli* better than that of *K. pneumoniae*, which upon escalated expression in response to environmental stimuli, enable them to degrade certain toxic chemicals in order to prevent oxidative, membrane and protein damage, usually brought about upon prolonged exposures to PAHs simple and complex structures alike (Kim *et al.*, 2007).

Therefore, a combination of *E. coli* and *K. pneumoniae* is efficient in the degradation of the PAHs used in this study and should be considered in the remediation stratagem of contaminated environments by poly aromatic hydrocarbons. The competence developed by microorganism from enriched consortium from the rich intestinal gut of rumen showed great capability to use and reduce PAHs generating survival energy through phthalic acid reduction pathway. It also laid bare the fact that the physicochemical parameters/prevailing environmental conditions have profound contributory roles in the removal or conversion of polyaromatics. The degradation of the PAHs was positively affected by addition of nutrient further confirming that biostimulation and bioaugmentation will yield better results in biodegradation of pollutants involving PAHs.

The bacterial isolates displayed varied degrees of catalytic activity with enzymes capable of breaking down hydrogen peroxide into water and oxygen for all isolated organisms whereas only a few possessed the rare *Cytochrome oxidase* that permits microbial participation in electron transport with capability of splitting indole from tryptophan thereby confirming the presence of *tryptophanase*.

5.2 Conclusion

The findings discussed in 5.1 above showed that *E. coli* and *K. pneumoniae* had good synergy in the degradation of 4-5 ring PAHs. These enterobacteria were isolated from the intestinal gut lining of *Bos taurus* suggesting that the microbiota from the large intestine possess a strong molecular gene cluster based on the presence of selected hydrocarbon degrading genes in the selected isolates that have been implicated for PAH degradation in organisms from varied sources. Implying that that the combination of *E. coli* and *K. pneumoniae* from the intestinal gut lining of *Bos taurus* was efficient in the degradation of PAHs through the phthalic acid degradation pathway.

5.3 Contribution to Knowledge

- The study demonstrated that bacterial strains from the large intestine of cows were capable of degrading PAHs.
- Intestinal strains of *E. coli* and *K. pneumoniae* were established as strong degraders of PAHs however, the consortium of both isolates had the highest degradation.
- The *o-phthalate* pathway is crucial in the use and reduction of PAHs.

5.4 Recommendation

- Laboratory analysis usually gives buoyant outcomes as environmental conditions are controlled, making it difficult to predict the performance of bioremediation strategies in the field where there is less control of environmental parameters and increased heterogeneity. An upgrade to field scale will be necessary to corroborate with the conclusions drawn from this study.
- A robust metagenomic profiling will give a clearer understanding of the arsenal of microorganism in the intestinal gut lining as the culturable bacteria from the intestinal gut lining of cows did not give a true picture of the vast array of organisms present in the gut.
- Screening for an array of genes that possess PAH reduction potentials will save time, mitigate losses and improve the search for hydrocarbon degraders.
- It is recommended that the following set of gene specific primers be employed for further gene detection studies in order to acquire the *alkB* gene at a higher level of specificity if present; *RHOSE*, *RHOAS*, *alkB1*, *alkB2*, *alk-H*.
- Biostimulants from organic origin will also help to speed up the rate of degradation of PAHs in the natural environment.

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APPENDIX 1

Composition of Nutrients

Nutrient agar

Meat extract	-----1g/l
Yeast extract	----- 2g/l
Peptone	-----5g/l
Sodium chloride	----- 5g/l
Agar	----- 15g/l
pH	----- 7.4 ±0.2

Tryptic soy agar

Casein peptone (pancreatic)	----- 17g/l
Soya peptone (papain digest)	----- 3g/l
Sodium Chloride (NaCl)	----- 5g/l
Di potassium hydrogen sulphate	-----2.5g/l
Glucose	----- 2.5g/l
pH	-----7.3 ± 0.2

Mineral Salt medium

Disodium phosphate (Na ₂ HPO ₄)	----- 1g/l
Monopotassium phosphate (KH ₂ SO ₄)	----- 1g/l
Ammonium sulphate(NH ₄) ₂ SO ₄	----- 1g/l
Magnesium Sulphate(MgSO ₄ .7H ₂ SO ₄)	----- 0.1g/l
Iron Chloride(FeCl)	-----0.05g/l
Calcium Chloride(CaCl)	----- 0.01g/l
Trace elements include	
Magnesium oxide(MgO)	-----10.75g/l

Calcium carbonate(CaCO_2)	-----2.0g/l
Iron Sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	-----4.5g/l
Zinc Sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)	-----1.44g/l
Maganese (II) Sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$)	-----1.12g/l
Copper (II) Sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)	----- 0.25g/l
Cobalt(II) Sulphate ($\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$)	-----0.28g/l
Dihydrogen Borate (H_2BO_3)	-----0.06g/l
Hydrochloric acid (HCl)	-----51.3mg/ml
Distilled H_2O	----- 1000mls

Adjust with 0.1M NaOH to pH 6.5 and add 500mg/l Griseofulvin for bacteria

MacConkey Agar (Lactose fermenters)

Peptone	----- 17g
Proteose peptone (meat and casein)	----- 3g
Lactose monohydrate	----- 10g
Bile Salts	----- 1.5g
Sodium Chloride	----- 5g
Neutral red	----- 0.03g
Crystal violet	-----0.001g
Agar agar	----- 13.5g
Distilled water	-----1000mls
pH	-----7.1+ 0.1 @25oC

Triple Sugar Iron agar

Beed extract	-----3.0g
Yeast extract	-----3.0g
Peptone	----- 20g

Glucose-----	71.0g
Lactose -----	10.0g
Sucrose -----	10.0g
Ferrous Sulphate-----	0.2g
NaCl -----	5.0g
Sodium Thiosulfate-----	0.3g
Phenol red -----	0.02g
Agar -----	13.0g
Distilled water -----	1000mls

APPENDIX 2

API20E



Plate A

Plate B

Appendix 2A - Initial picture of inoculated selected isolates

Appendix 2B - Outcome of selected isolate after 24 hours

apiweb™ - Identification x

localhost:8080/apiweb/servlet/Identify

BIOMÉRIEUX

apiweb™

API 20 E V5.0 Printout Export New test Modify

REFERENCE DATE 2/27/19

COMMENT

API

API 10S
API 20 A
API 20 C AUX
API 20 E
API 20 NE
API 20 STREP
API 50 CHB
API 50 CHE
API 50 CHL
API CAMPY
API CANDIDA
API CORYNE
API LISTERIA
API NH
API STAPH
RAPID 20 E

ID32

EXCELLENT IDENTIFICATION

Strip	API 20 E V5.0		
Profile	5 3 5 5 7 7 3		
Note			

Significant taxa	% ID	T	Tests against
Raoultella ornithinolytica	99.9	1.0	

Next taxon	% ID	T	Tests against
Klebsiella oxytoca	0.1	0.5	ODC 0%

Appendix 2C - Positive Raoultella Ornithinolytica result film dexed from API WEB.

APPENDIX 3

MOLECULAR SUPPLEMENTARY MATERIALS

E. coli

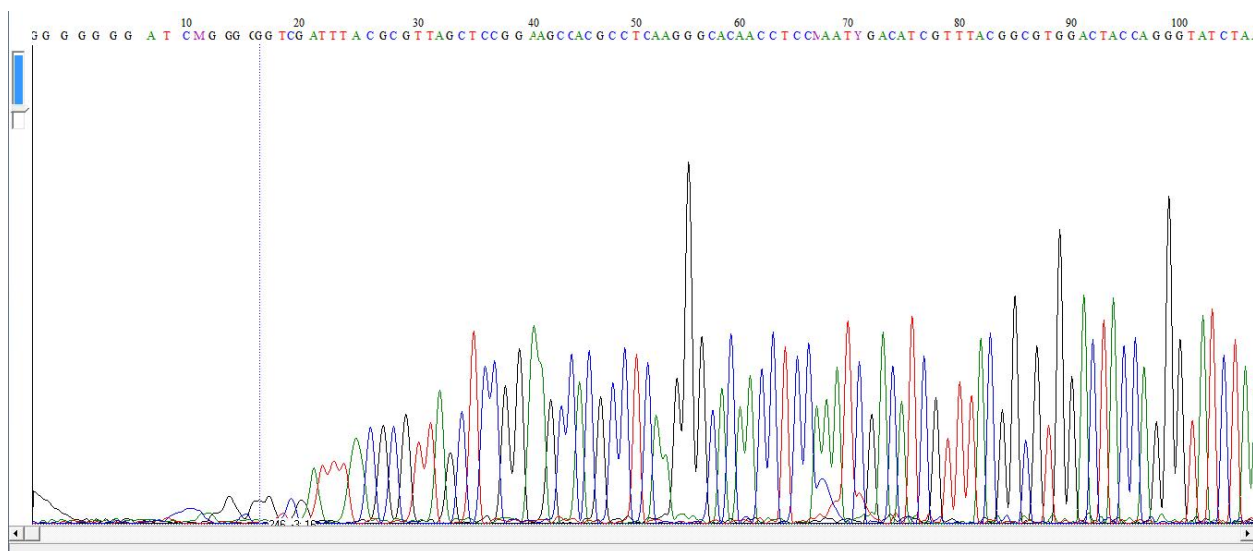
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CCCAGGTTGAGCCCGGGGATTTACATCTGACTTAACAAACCGCCTGCGTG
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CAAAGGTATTAACTTTACTCCCTTCCTCCCCGCTGAAAGTACTTTACAACCC
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GCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTC
CAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCCTWGGTGA
GCCGTTACCCACCWACWAGCTAATCCCATCTGGGCACATCCGATGGCAA
GAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCTA
CCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCA
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ACTCT

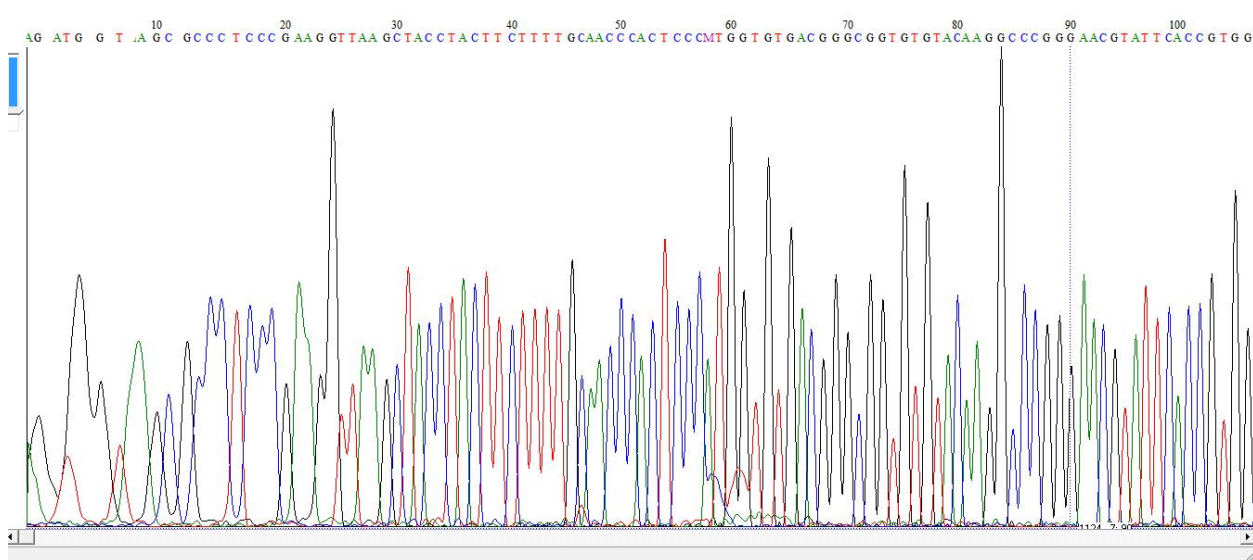
(Reverse Sequence)

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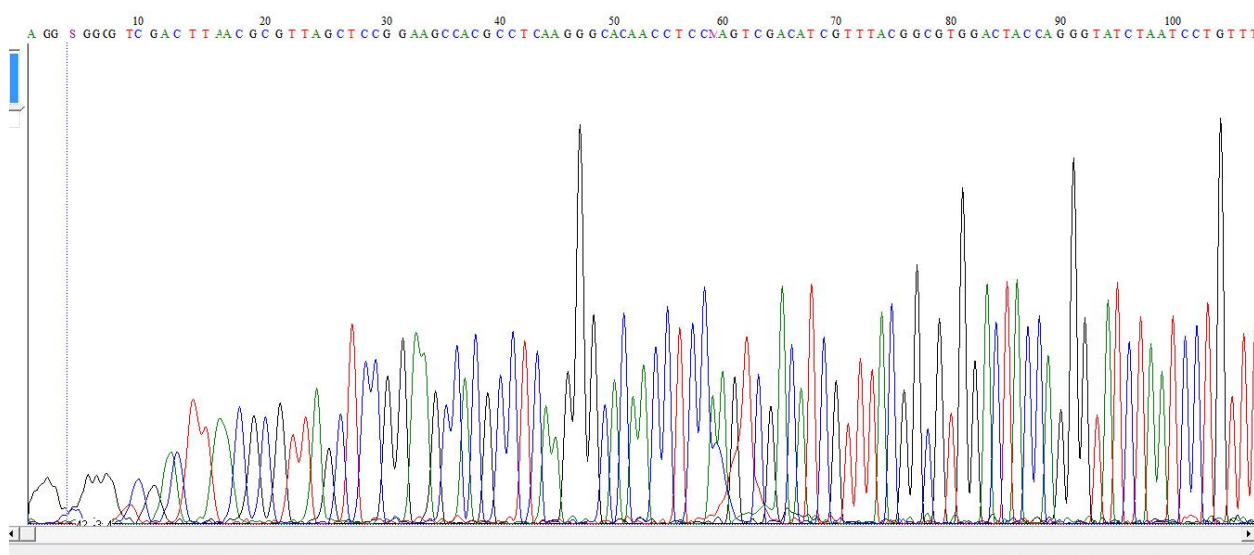
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GCGCTCGTTGCGGGACTTAACCCAACATTTACAAACACGAGCTGACGACAG
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CCTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAG
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TCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGC
ATTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCTTGC
CAGTATCAGATGCAGTTCCCAGTTGAGCCCGGGGATTTACATCTGACTTA
ACAAACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGAC
CCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCG
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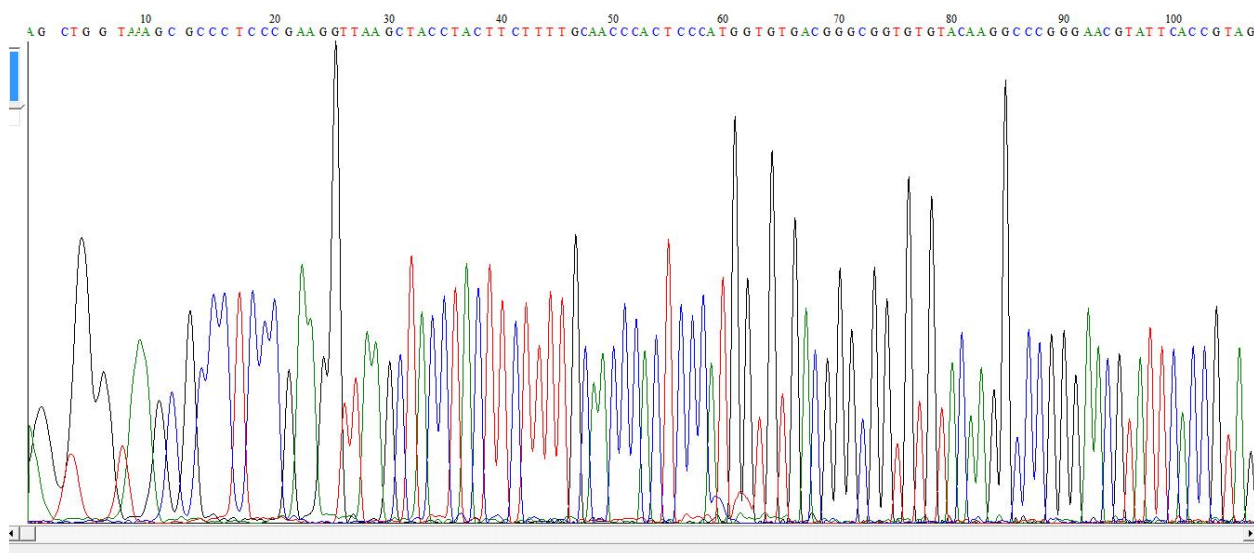
Appendix 3a: Raw FASTA Sequences of Forward and Reverse sequenced data for *E.coli*



Appendix 3b Forward and Reverse Primer Sequencing chromatogram from ABI sequencer for isolate *E. coli*



Appendix 3c: Raw FASTA Sequences of Forward and Reverse sequenced data for *Raoutella orminiticus*



Appendix 3d. Reverse Primer Sequencing chromatogram from ABI sequencer for isolate

Raoutella orminiticus

(Forward Sequence)

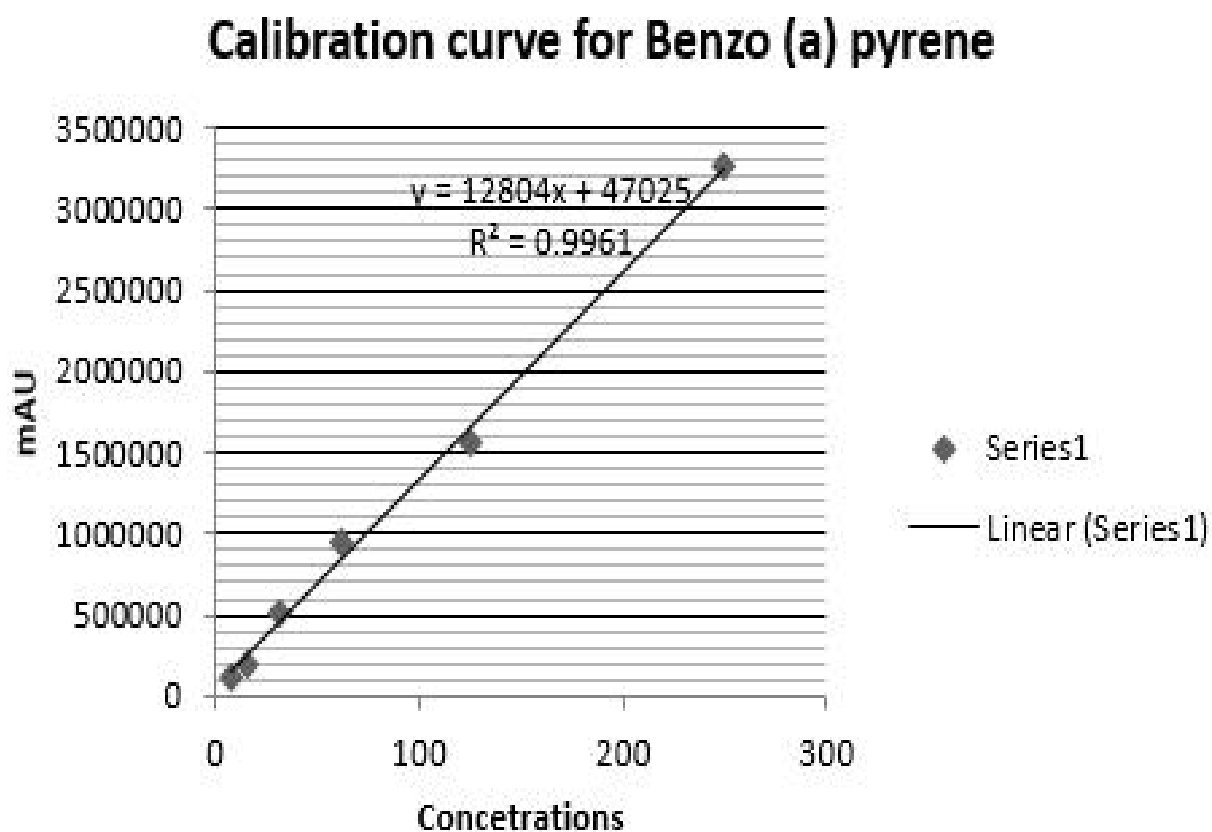
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GCCATTGTGCAATATCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTG
TCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGC
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TGGCATGAGGCCCCGAAGGTCCCCCACTTTGGTCTTGCGACRATTATGCGGTAT
TAGCTACCGTTTCCAGTAGTTATCCCCCTCCATCAGGCAGTTTCCCAGACAT
TACTACCCGTCCGCCGCTCGTCACCCGAGAGCAAGCTCTCTGTGCTACCGC
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MAAMCTCTA

(Reverse Sequence)

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APPENDIX 4



Appendix 4a. Standard calibration curve of Benzo[a]Pyrene

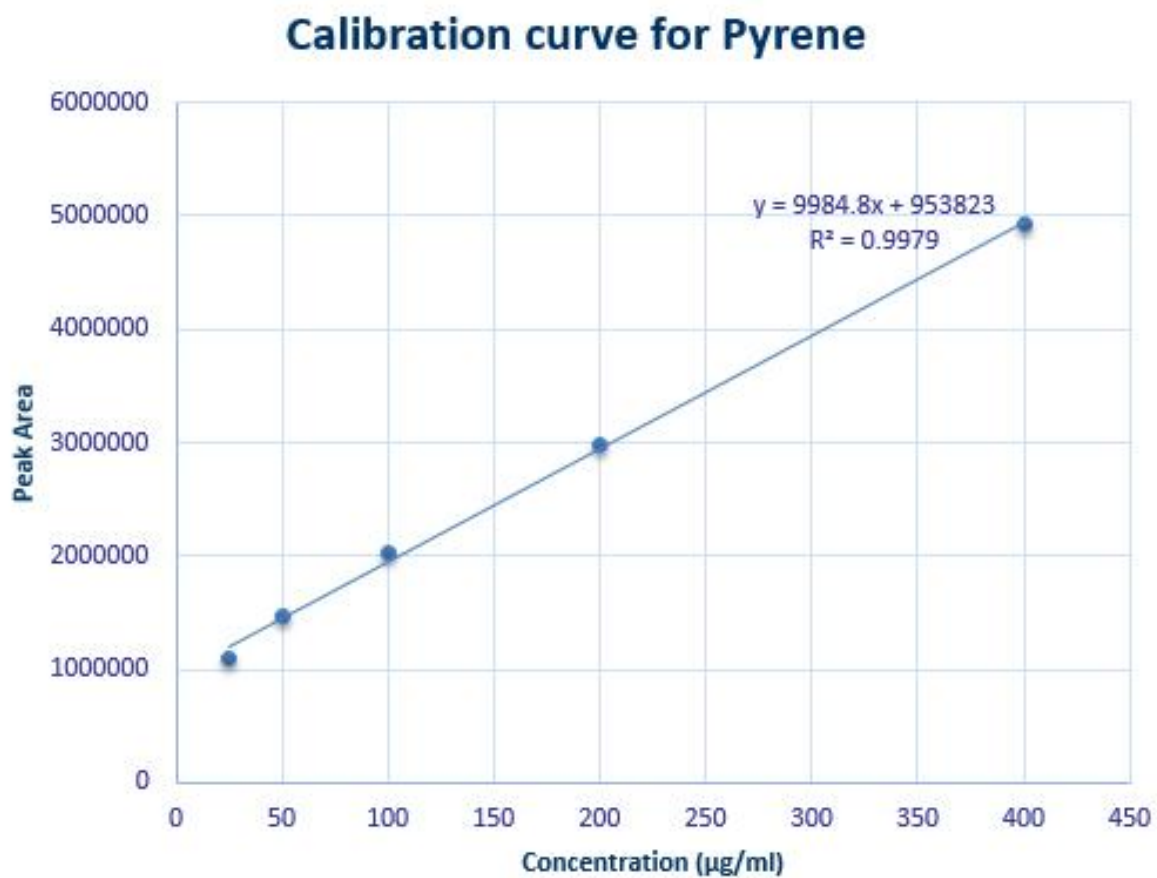
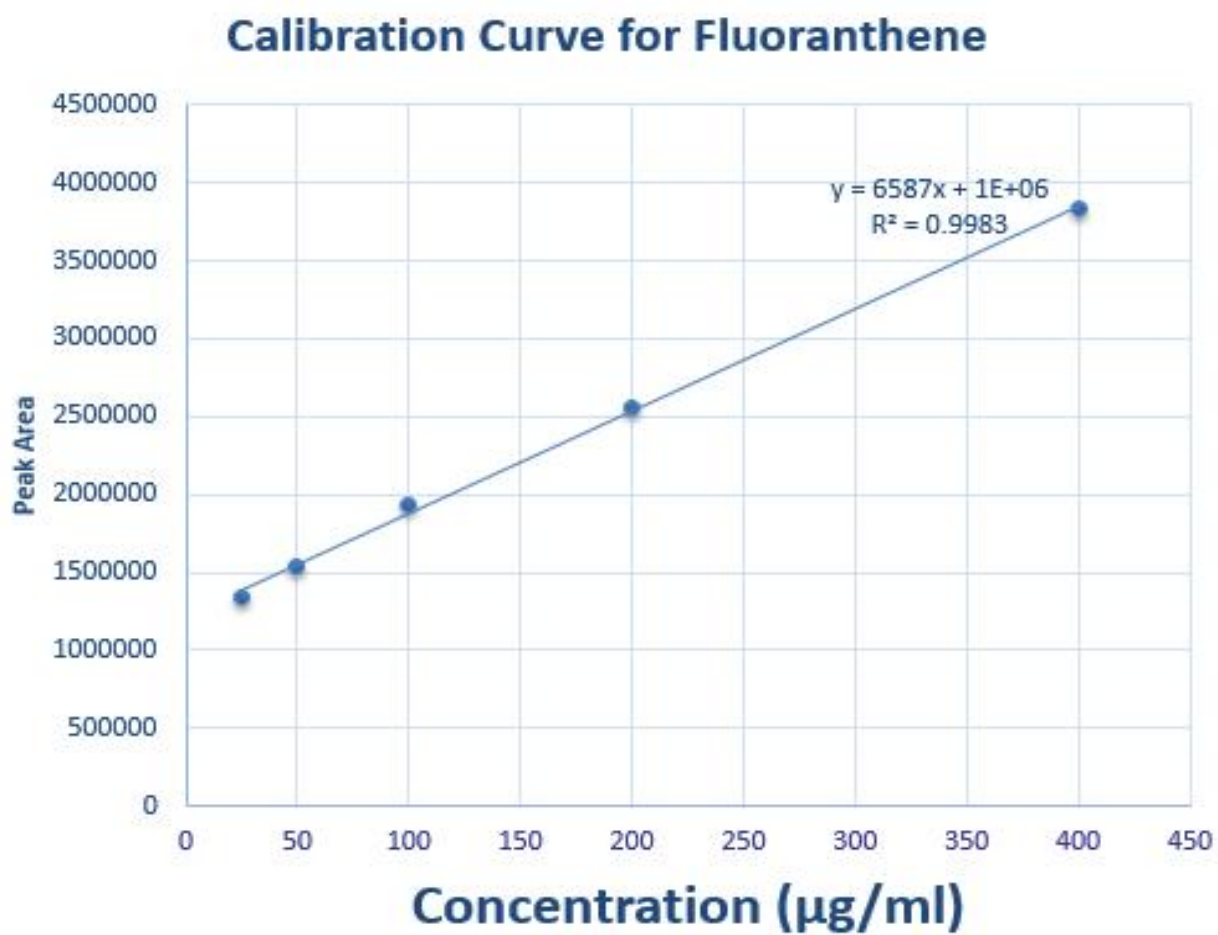
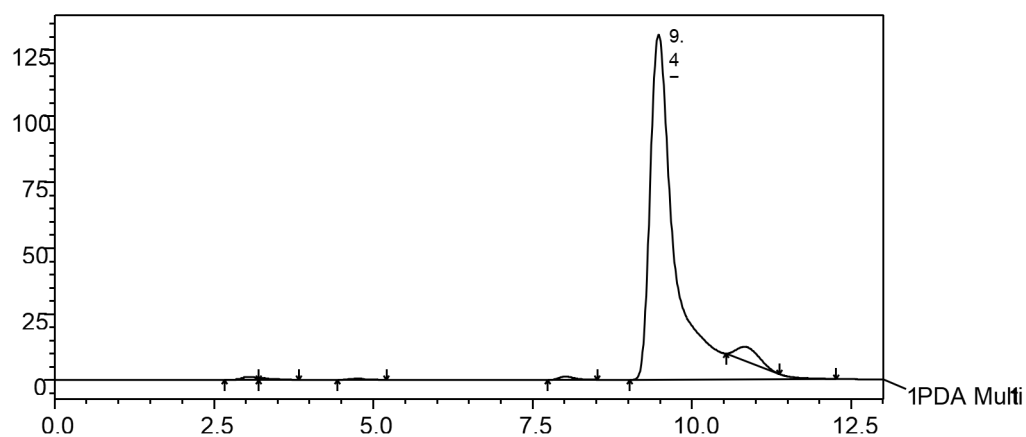


Figure 4b. Standard Calibration curve of Pyrene

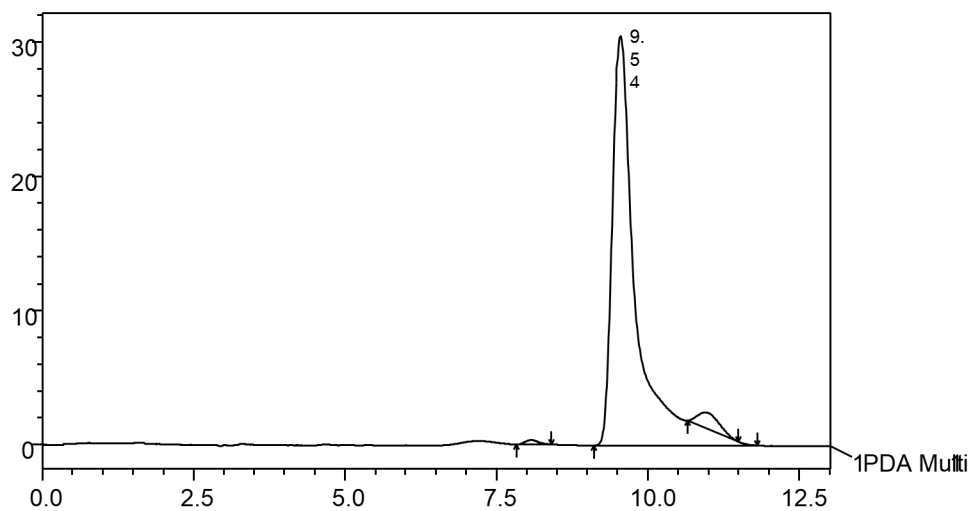


Appendix 4c. Standard calibration curve of Fluoranthene

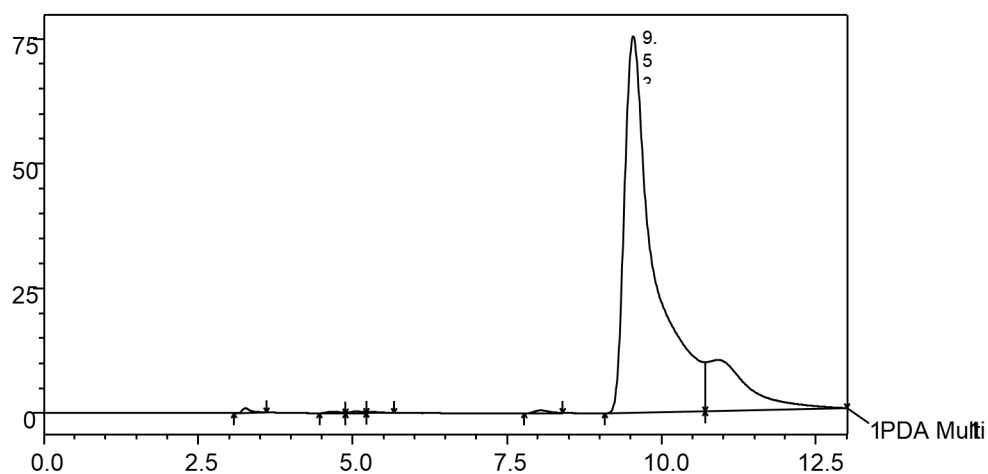
APPENDIX 5



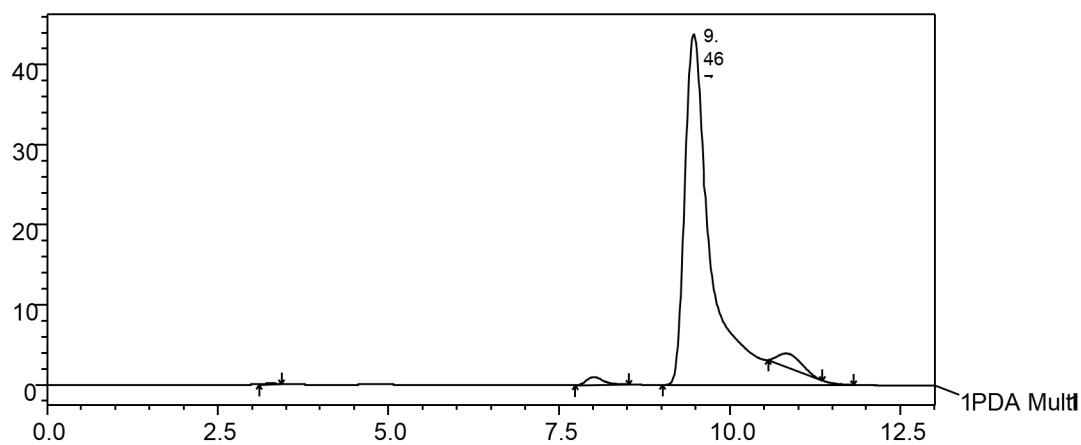
Appendix 5a Chromatograph of HPLC analysis of BaP amended with *E. coli* day 1



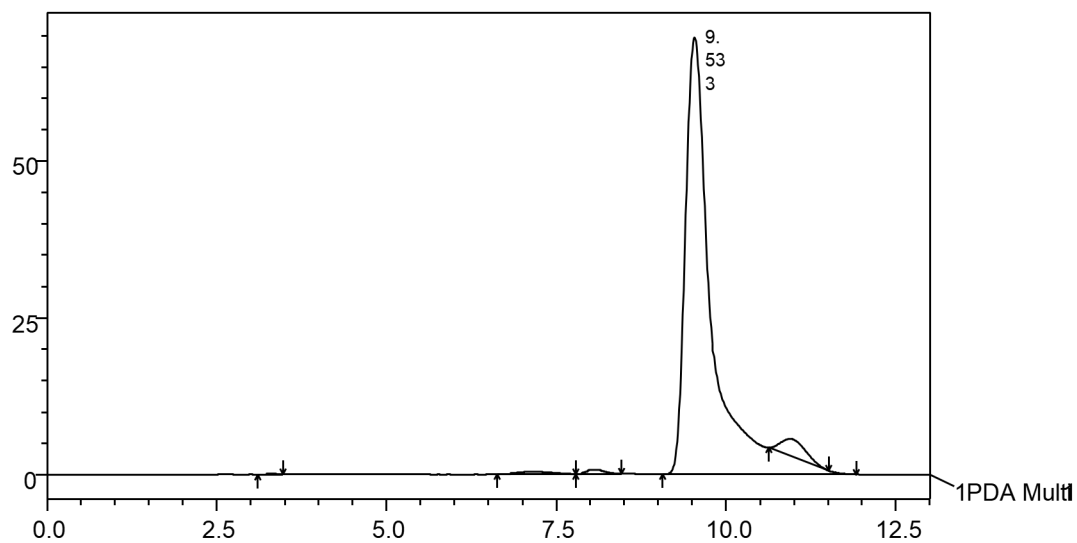
Appendix 5b Chromatograph from HPLC showing degradation of B(a)P amended with *E. coli* at day 16.



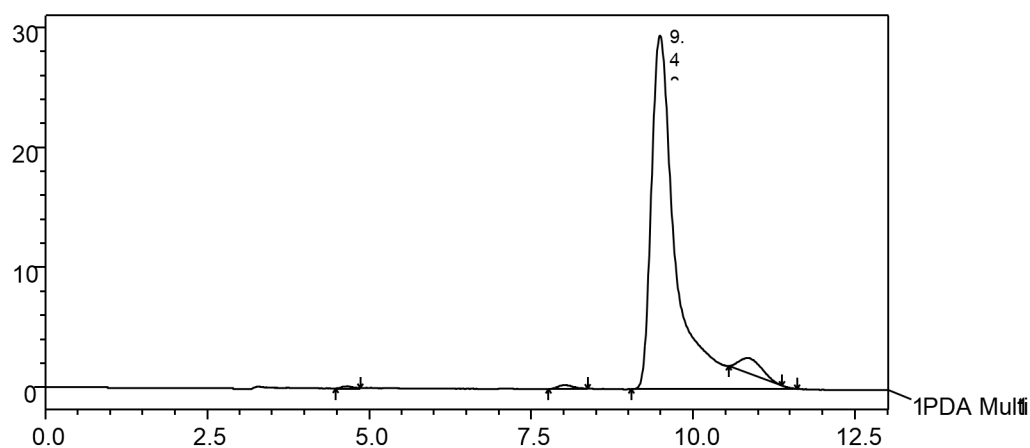
Appendix 5c: Chromatograph from HPLC showing degradation of B(a)P amended with *K. pneumoniae* at day 1.



Appendix 5d: Chromatograph from HPLC showing degradation of B(a)P amended with *K. pneumoniae* at day 16



Appendix 5e: Chromatograph of HPLC analysis of BaP amended with *E. coli* + *K. pneumoniae* day 1

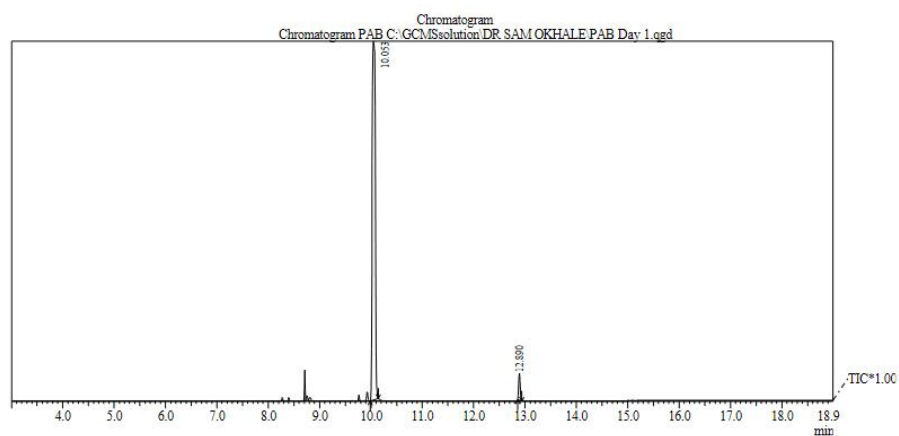


Appendix 5f: Chromatograph of HPLC analysis of BaP amended with *E. coli* + *K. pneumoniae* day 16

APPENDIX 6

Chromatographs and library of GC-MS for pyrene and Fluoranthene

Pyrene Day 1



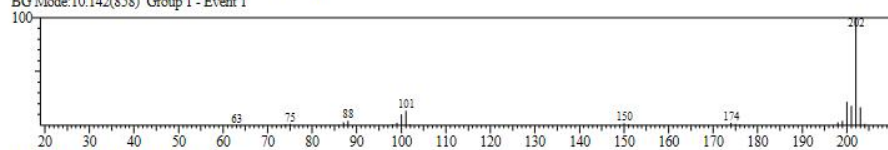
Library

<< Target >>

Line# 1 R.Time:10.058(Scan#:848) MassPeaks:24

RawMode:Single 10.058(848) BasePeak:202.05(370461)

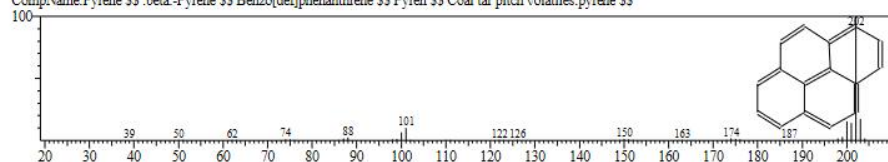
BG Mode:10.142(858) Group 1 - Event 1



Hit# 1 Entry:44184 Library:NIST08.LIB

SI:96 Formula:C₁₆H₁₀ CAS:129-00-0 MolWeight:202 RetIndex:1984

CompName:Pyrene \$\$.beta.-Pyrene \$\$ Benzo[def]phenanthrene \$\$ Pyren \$\$ Coal tar pitch volatiles:pyrene \$\$



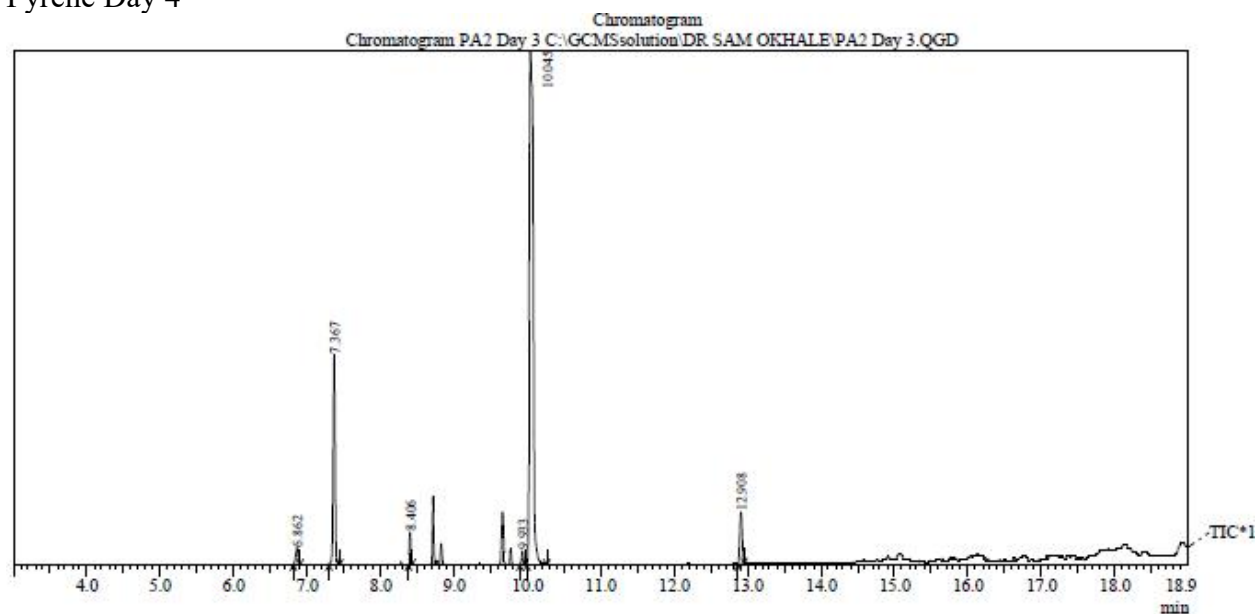
Day 1: Pyrene (Consortium)

Hit#:2 Entry:110629 Library:NIST08.LIB

SI:85 Formula: C₁₆H₁₀ CAS:0-00-0 MolWeight:202 RetIndex:1984

CompName: Pyrene

Pyrene Day 4

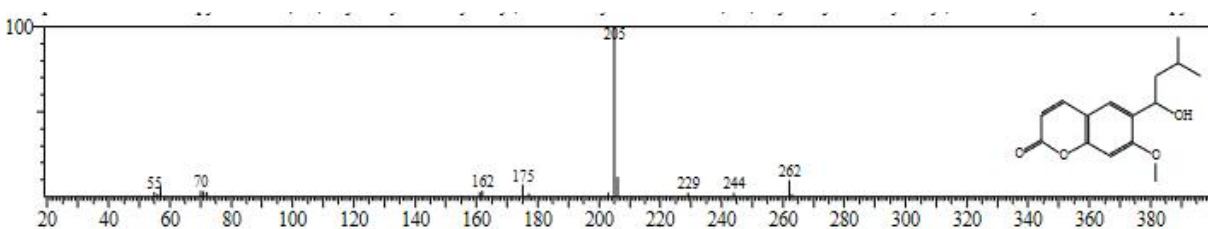
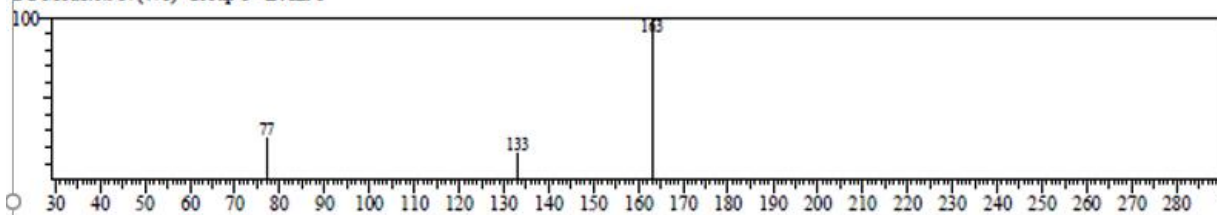


<< Target >>

Line# 1 R.Time: 6.858(Scan# 464) MassPeaks: 3

RawMode: Single 6.858(464) BasePeak: 163.05(15193)

BG Mode: 6.917(471) Group 1 - Event 1



Day 4: Pyrene Consortium

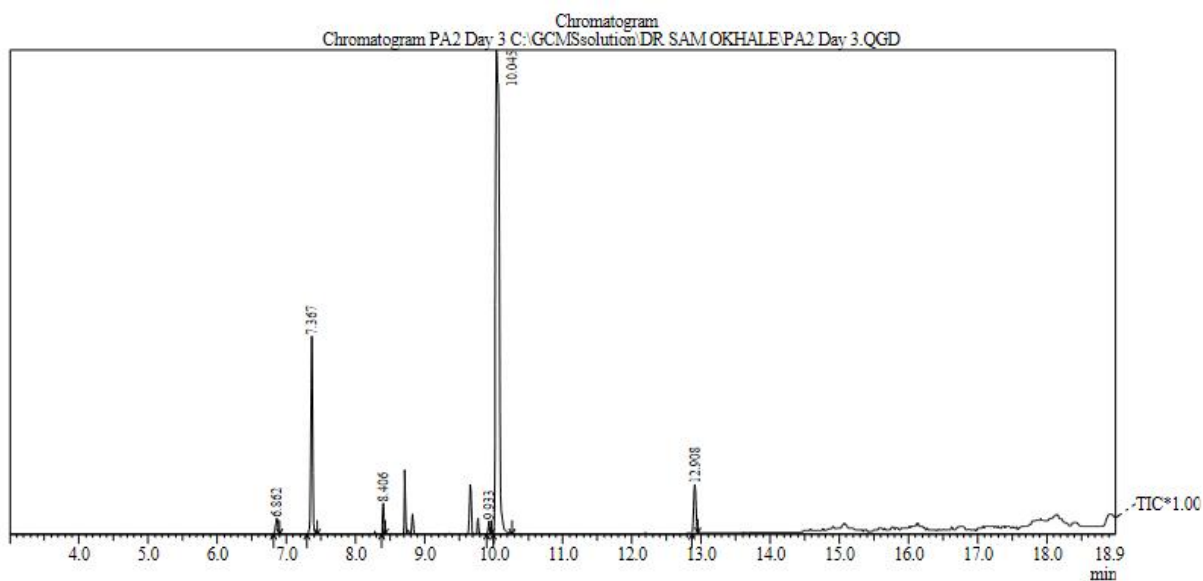
Hit#: 2 Entry: 110629 Library: NIST08.LIB

SI: 85 Formula: C₁₆H₁₂O₂ CAS: 0-00-0 MolWeight: 298 RetIndex: 2384

CompName: 4,5-dihydroxydihdropyrene

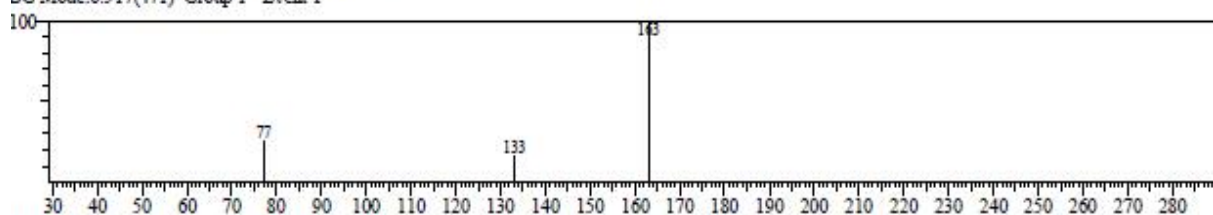
Pyrene Day

7

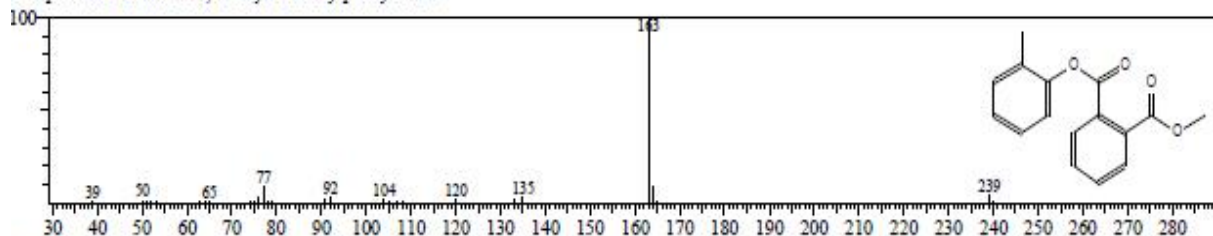


<< Target >>

Line# 1 R.Time: 6.858 (Scan#: 464) MassPeaks: 3
 RawMode: Single 6.858 (464) BasePeak: 163.05 (15193)
 BG Mode: 6.917 (471) Group 1 - Event 1



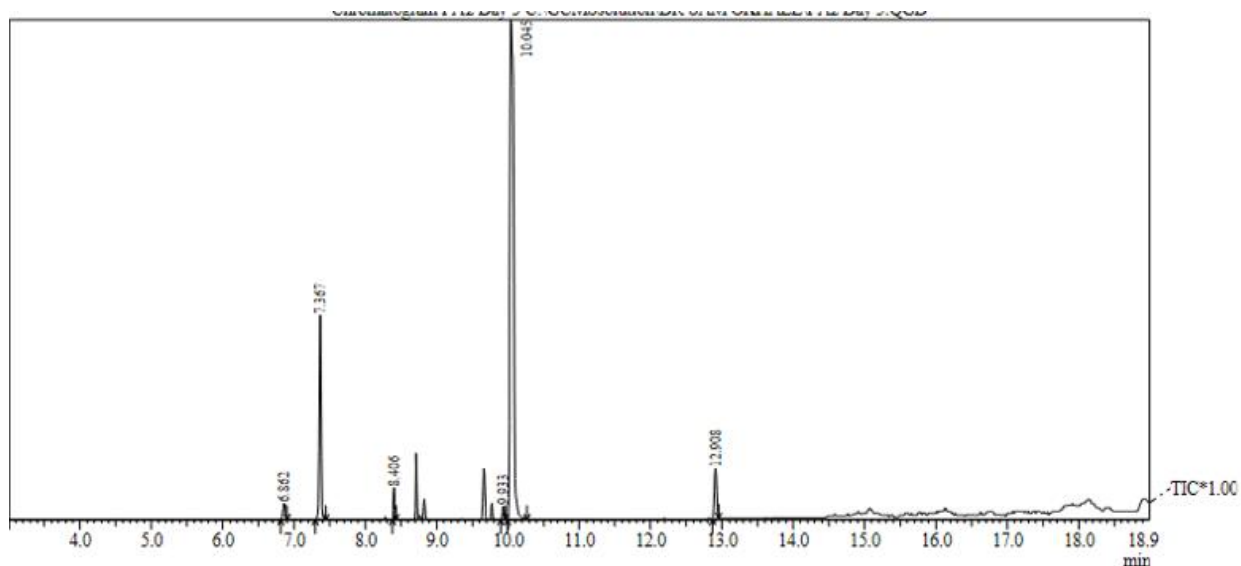
Hit# 4 Entry: 90494 Library: NIST08.LIB
 SI: 84 Formula: C₁₆H₁₄O₄ CAS: 0-00-0 MolWeight: 270 RetIndex: 2127
 CompName: Phthalic acid, methyl 2-methylphenyl ester



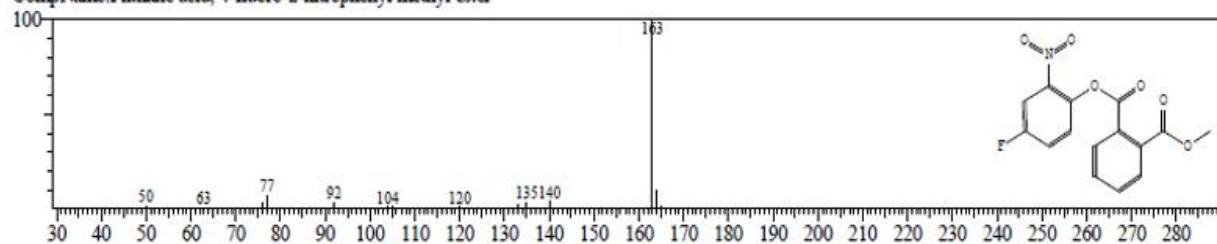
Day 7: Pyrene Consortium Hit# 2 Entry: 110629 Library: NIST08.LIB

SI: 85 Formula: C₈H₈O₂ CAS: 0-00-0 MolWeight: 270 RetIndex: 2262
 CompName: 4-hydroxyperinaphthenone

Pyrene Day 10



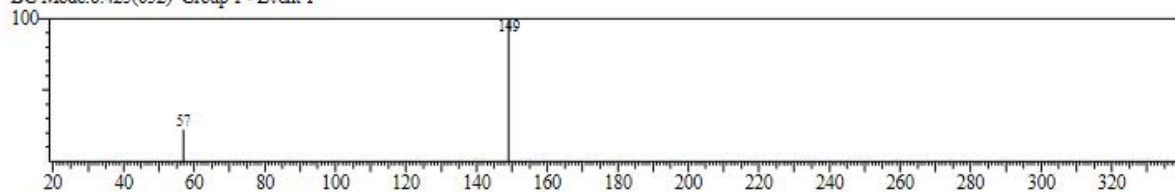
Hit#1 Entry:125162 Library:NIST08.LIB
 SE:85 Formula:C15H10FNO6 CAS:0-00-0 MolWeight:319 RetIndex:2384
 CompName:Phthalic acid, 4-fluoro-2-nitrophenyl methyl ester



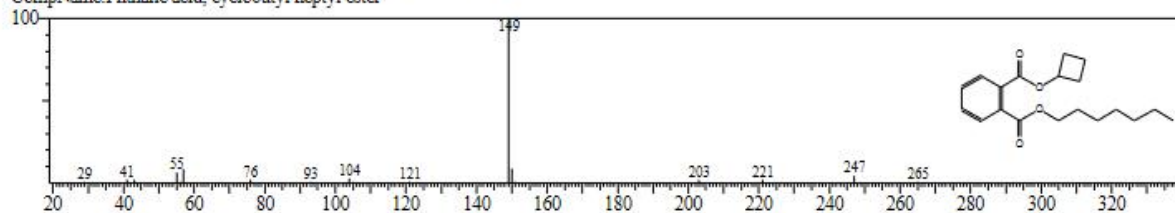
Pyrene Day 13

<< Target >>

Line# 1 R.Time: 8.400(Scan#: 649) MassPeaks: 2
RawMode: Single 8.400(649) BasePeak: 149.00(15346)
BG Mode: 8.425(652) Group 1 - Event 1



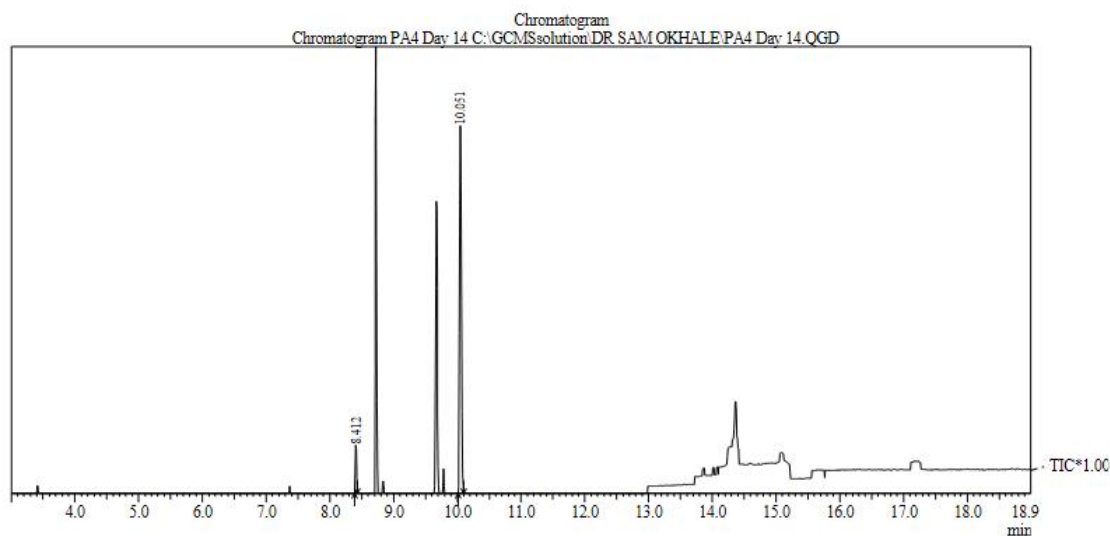
Hit# 4 Entry: 124741 Library: NIST08.LIB
SI: 86 Formula: C₁₉H₂₆O₄ CAS: 0-00-0 MolWeight: 318 RetIndex: 2357
CompName: Phthalic acid, cyclobutyl heptyl ester



Day 13: Pyrene Consortium

Hit# 2 Entry: 110629 Library: NIST08.LIB
SI: 85 Formula: C₁₇H₁₀O CAS: 0-00-0 MolWeight: 318 RetIndex: 2262
CompName: 1, pyrenecarboxyldehyde

Pyrene Day 16



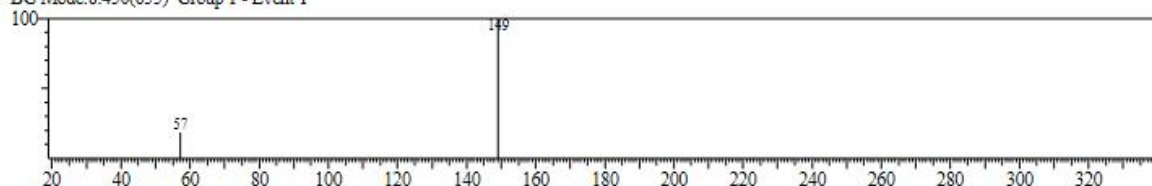
Library

<< Target >>

Line# 1 R.Time: 8.408 (Scan# 650) MassPeaks: 2

RawMode: Single 8.408 (650) BasePeak: 149.00 (11202)

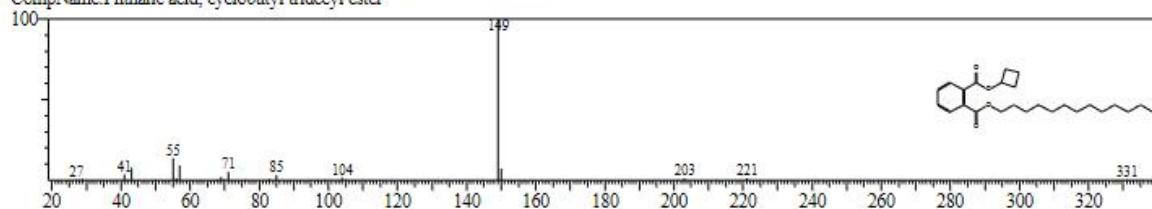
BG Mode: 8.450 (655) Group 1 - Event 1



Hit# 1 Entry: 168608 Library: NIST08.LIB

SI: 88 Formula: C₂₅H₃₈O₄ CAS: 0-00-0 MolWeight: 402 RetIndex: 2954

CompName: Phthalic acid, cyclobutyl tridecyl ester

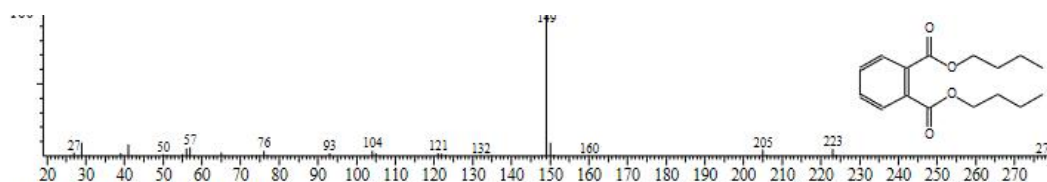


Day 16: Pyrene Consortium

Hit# 2 Entry: 110629 Library: NIST08.LIB

SI: 85 Formula: C₁₅H₁₀O₂ CAS: 0-00-0 MolWeight: 298 RetIndex: 2262

CompName: Phenanthrene-4-carboxylic acid



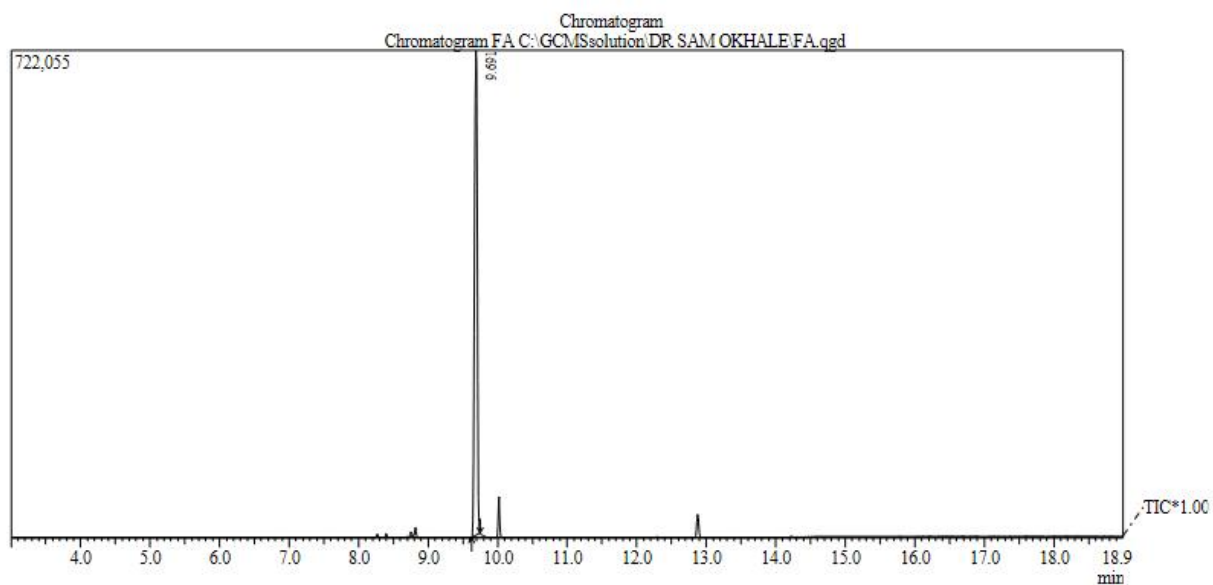
Hit# 3 Entry: 96162 Library: NIST08.LIB

SI: 98 Formula: C₁₆H₂₂O₄ CAS: 17851-53-5 MolWeight: 278 RetIndex: 1973

CompName: 1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester \$ Phthalic acid, butyl isobutyl ester \$ 1-Butyl 2-isobutyl phthalate # \$

Flouranthene day

1



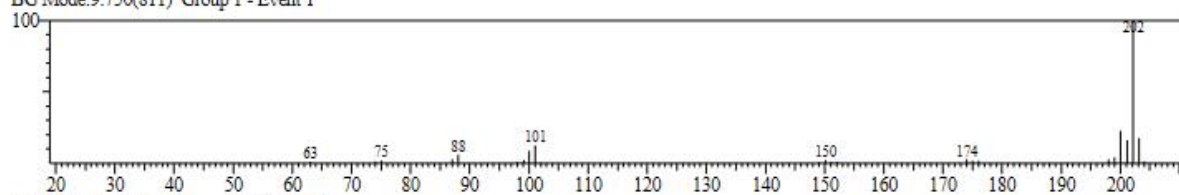
Library

<< Target >>

Line#:1 R.Time:9.692(Scan#:804) MassPeaks:24

RawMode:Single 9.692(804) BasePeak:202.10(333141)

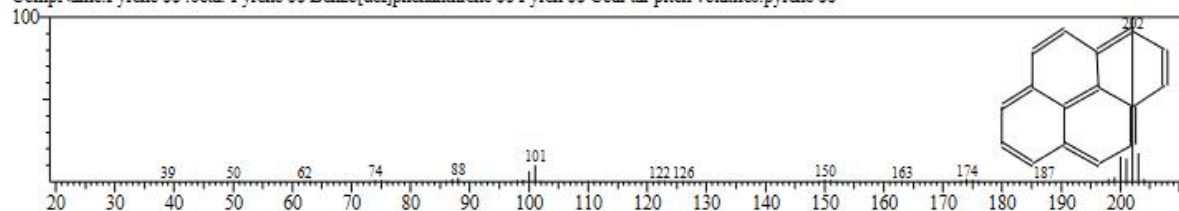
BG Mode:9.750(811) Group 1 - Event 1



Hit#:1 Entry:44184 Library:NIST08.LIB

SI:95 Formula:C16H10 CAS:129-00-0 MolWeight:202 RetIndex:1984

CompName:Pyrene \$\$.beta.-Pyrene \$\$ Benzo[def]phenanthrene \$\$ Pyren \$\$ Coal tar pitch volatiles:pyrene \$\$

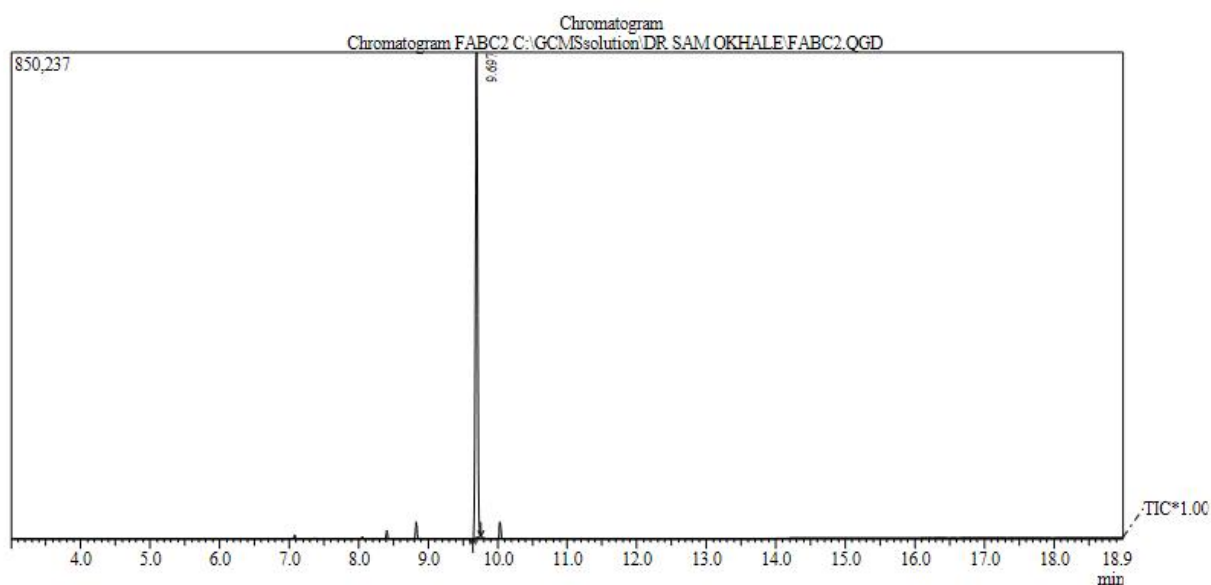


Hit#:2 Entry:16863 Library:NIST08s.LIB

SI:95 Formula:C16H10 CAS:206-44-0 MolWeight:202 RetIndex:1802

CompName:Fluoranthene \$\$ Benzene, 1,2-(1,8-naphthalenedivl)- \$\$ Benzo[k]fluorene \$\$ Idv1 \$\$ 1,2-(1,8-Naphthylene)benzene \$\$ Benzo[k]fluorene, idv1

Flouranthene Day 4

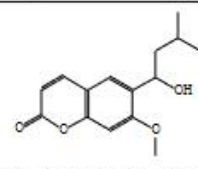
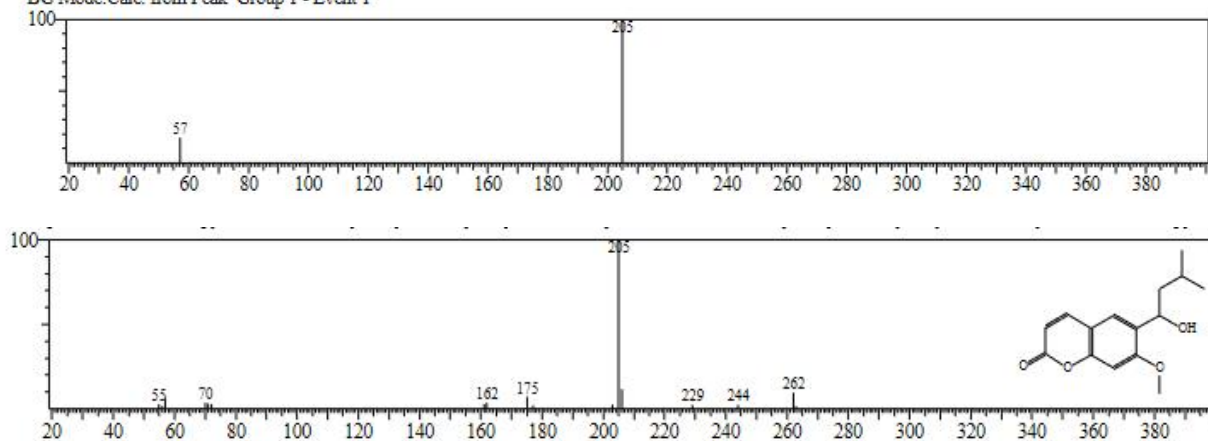


<< Target >>

Line#:1 R.Time:7.083(Scan#:491) MassPeaks:2

RawMode:Averaged 7.075-7.092(490-492) BasePeak:205.05(4675)

BG Mode:Calc. from Peak Group 1 - Event 1



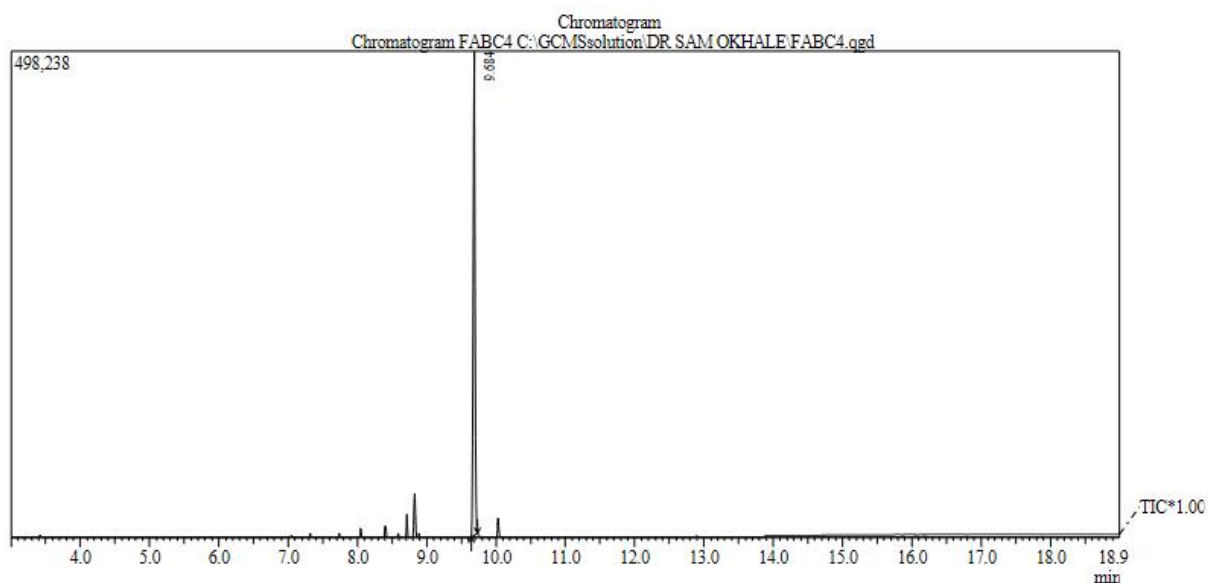
Day 4: Flouranthene

Hit#:2 Entry:110629 Library:NIST08.LIB

SI:85 Formula $C_{15}H_{10}O_4$ CAS:0-00-0 MolWeight:262 RetIndex:2262

CompName 7,8 dihydroxyflouranthene

Flouranthene Day 7



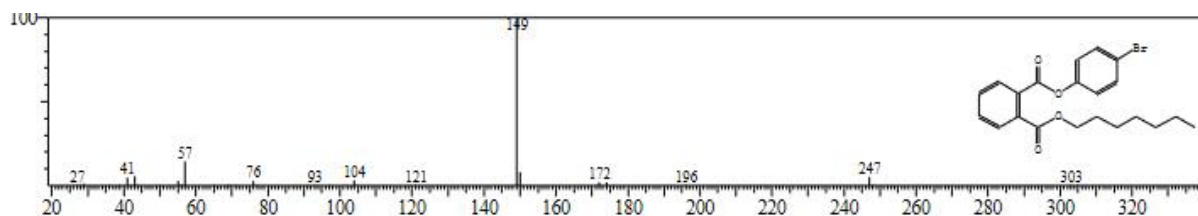
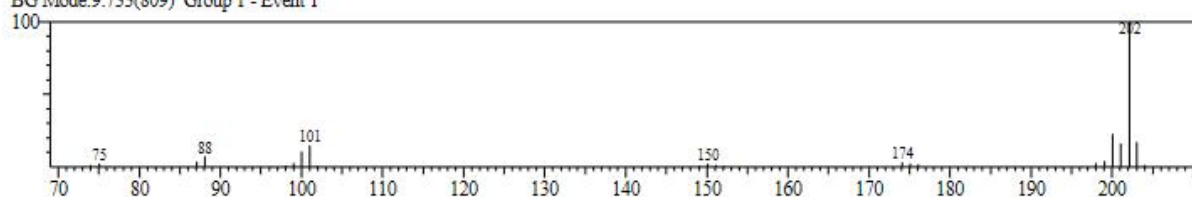
Library

<< Target >>

Line#:1 R.Time:9.683(Scan#:803) MassPeaks:23

RawMode:Single 9.683(803) BasePeak:202.10(220623)

BG Mode:9.733(809) Group 1 - Event 1



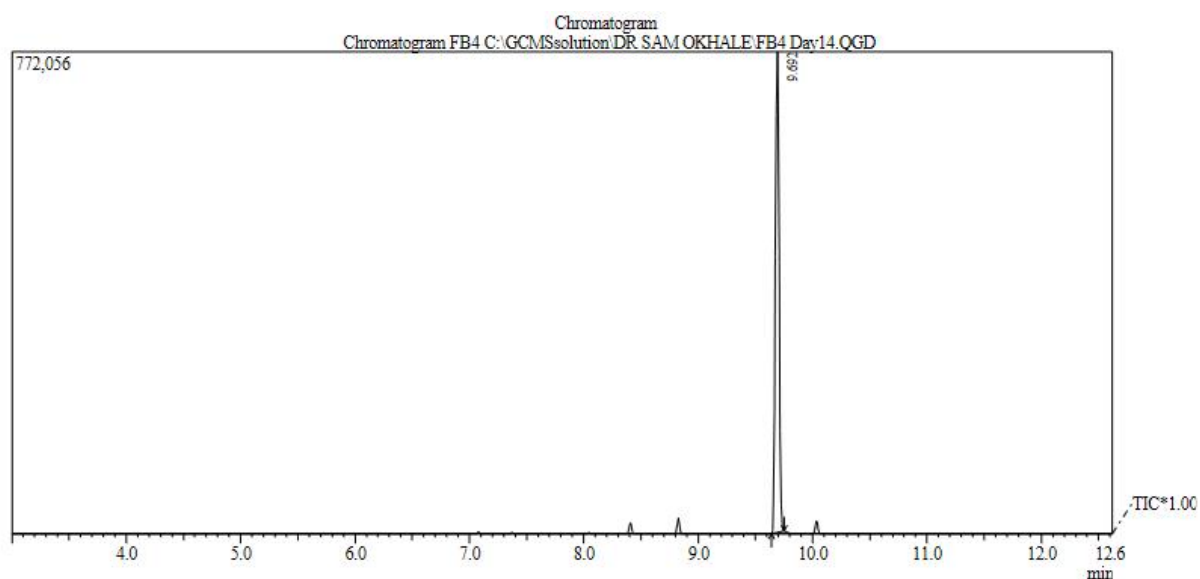
Day 7: Flouranthene

Hit#:2 Entry:110629 Library:NIST08.LIB

SI:85 Formula C₁₈H₁₆BrFO₄CAS:0-00-0 MolWeight:262 RetIndex:2262

CompName 2-bromo-4-fluorophenyl butyl ester

Flouranthene Day 10



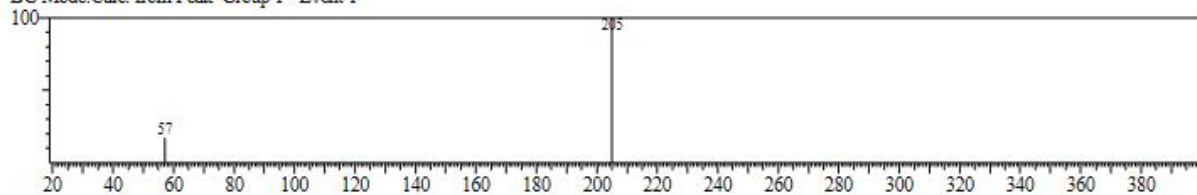
Library

<< Target >>

Line#: 1 R.Time: 7.083(Scan#: 491) MassPeaks: 2

RawMode: Averaged 7.075-7.092(490-492) BasePeak: 205.05(4441)

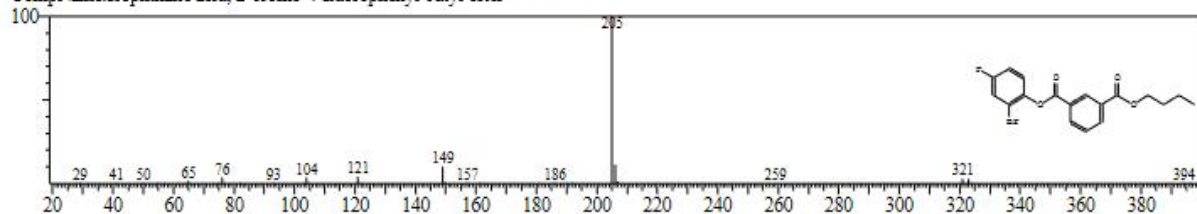
BG Mode: Calc. from Peak Group 1 - Event 1



Hit#: 1 Entry: 165545 Library: NIST08.LIB

SI: 79 Formula: C₁₈H₁₆BrFO₄ CAS: 0-00-0 MolWeight: 394 RetIndex: 2607

CompName: Isophthalic acid, 2-bromo-4-fluorophenyl butyl ester



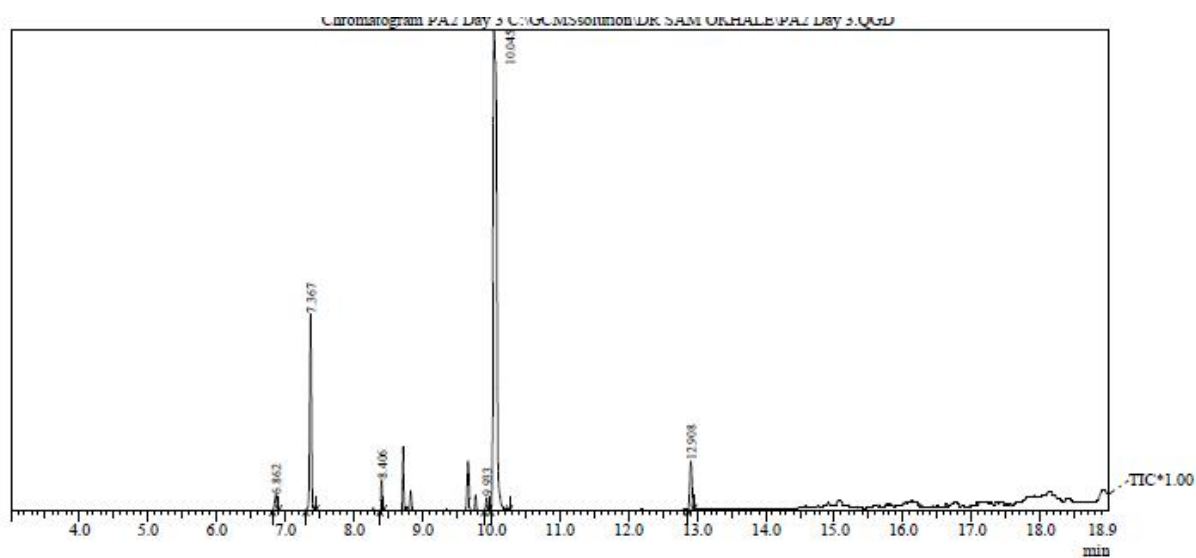
Day 10: Flouranthene

Hit#: 2 Entry: 110629 Library: NIST08.LIB

SI: 85 Formula: C₂₅H₃₆O₄ CAS: 0-00-0 MolWeight: 262 RetIndex: 2262

CompName: Pthalitic acid: Isoflurane

Floranthene Day 13



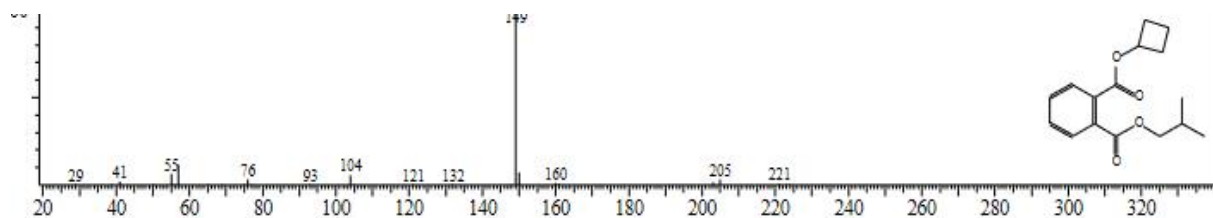
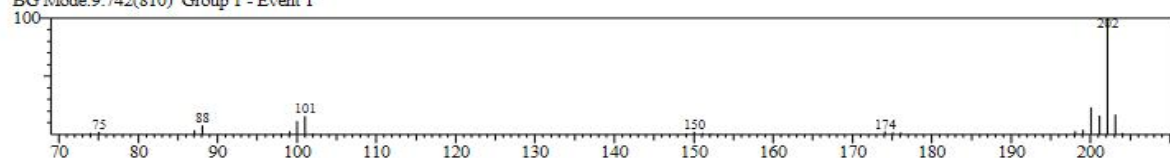
Library

<< Target >>

Line#:1 R.Time:9.692(Scan#:804) MassPeaks:23

RawMode:Single 9.692(804) BasePeak:202.10(313717)

BG Mode:9.742(810) Group 1 - Event 1



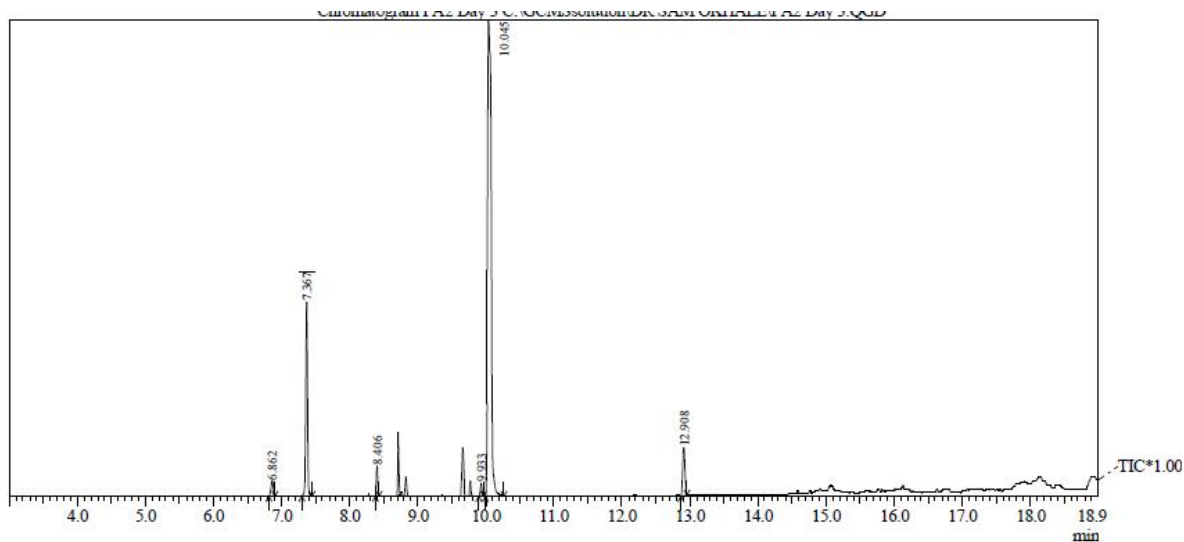
Day 13: Flouranthene

Hit#:2 Entry:110629 Library:NIST08.LIB

SI:85 Formula C₁₉H₃₂O CAS:0-00-0 MolWeight:262 RetIndex:2262

CompName Pthalitic acid: 2,4,6-Tris(1,1-dimethylethyl)-4-methylcyclohexa-2,5-dien-1-one

Flouranthene Day 16



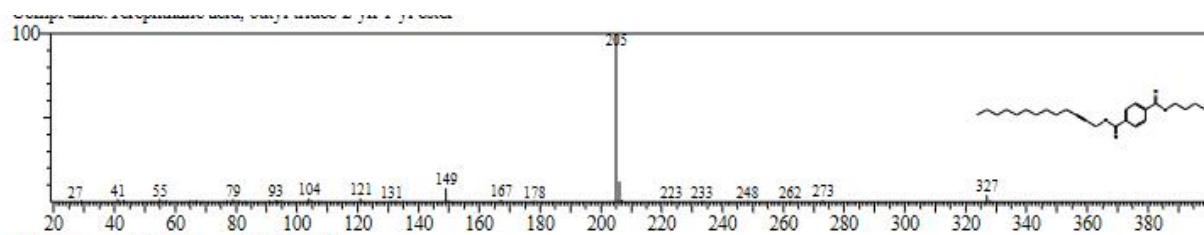
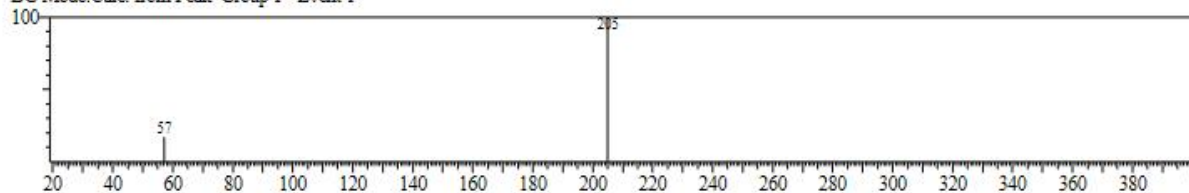
Library

<< Target >>

Line#:1 R.Time:7.083(Scan#:491) MassPeaks:2

RawMode:Averaged 7.075-7.092(490-492) BasePeak:205.05(4441)

BG Mode:Calc. from Peak Group 1 - Event 1



Day 16: Flouranthene

Hit#:2 Entry:110629 Library:NIST08.LIB

SI:85 Formula $C_{14}H_8O_3$ CAS:0-00-0 MolWeight:262 RetIndex:2262

CompName 9-fluoronone-1carboxylic acid

APPENDIX 7

Appendix 7a

ONEWAY

ONEWAY/DATA ANALYSIS OF BaP BY SPSS

DESCRIPTIVES /POSTHOC=DUKEY LSD ALPHA (0.05).

day1 day4 day7 day10 day13 day16 BY VAR00001

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
Days						Lower Bound	Upper Bound		
1	Control	3	249.41	0.24	0.63	249.01	250.19	249.42	249.87
	<i>E. coli</i>	3	230.55	1.25	0.21	221.41	227.63	223.09	225.41
	<i>K. pneumoniae</i>	3	222.74	0.47	0.49	213.27	215.59	213.99	214.92
	<i>E.coli + K. pneumoniae</i>	3	222.71	1.72	0.99	197.98	206.52	201.01	204.21
4	Control	3	230.55	2.65	0.21	227.03	240.17	230.55	235.20
	<i>E. coli</i>	3	191.82	0.96	0.55	189.42	194.21	191.05	192.90
	<i>K. pneumoniae</i>	3	123.53	0.85	0.48	121.43	125.64	123.01	124.51
	<i>E.coli + K. pneumoniae</i>	3	114.83	0.20	0.11	114.34	115.31	114.62	115.01
7	Control	3	229.74	1.33	0.49	220.97	227.57	222.74	225.05
	<i>E. coli</i>	3	97.49	0.42	0.24	96.44	98.53	97.00	97.73
	<i>K. pneumoniae</i>	3	88.54	0.36	0.21	87.64	89.43	88.30	88.95
	<i>E.coli + K. pneumoniae</i>	3	62.32	0.01	0.01	62.29	62.35	62.31	62.33
10	Control	3	222.71	0.18	0.10	221.73	222.61	222.01	222.36
	<i>E. coli</i>	3	59.46	0.67	0.39	57.80	61.14	58.69	59.87
	<i>K. pneumoniae</i>	3	47.60	0.53	0.31	46.29	48.91	46.99	47.91
	<i>E.coli + K. pneumoniae</i>	3	44.60	0.31	0.18	43.60	45.13	44.01	44.55
13	Control	3	222.13	0.21	0.48	218.72	219.77	219.05	219.47
	<i>E. coli</i>	3	54.95	0.06	0.03	54.81	55.08	54.91	55.01
	<i>K. pneumoniae</i>	3	36.47	1.36	0.07	33.09	39.86	35.41	38.01
	<i>E.coli + K. pneumoniae</i>	3	27.10	0.17	0.10	26.67	27.53	26.99	27.30
16	Control	3	221.25	0.09	0.99	213.91	214.33	214.03	214.20
	<i>E. coli</i>	3	37.80	7.68	0.43	18.73	56.88	30.01	45.36
	<i>K. pneumoniae</i>	3	22.43	2.72	1.56	15.69	29.18	19.30	24.02
	<i>E.coli + K. pneumoniae</i>	3	8.90	1.01	0.58	6.39	11.42	8.02	10.01

NB: 0=control;1=*E. coli*;2= *K. pneumoniae*; 3= *E.coli + K. pneumoniae*

APPENDIX 7B:

ANOVA for BaP

tme (Days)	Outcome	F	Sig.
1	btw /within group	1014	0

4	btw /within group	4450	0
7	btw /within group	30300	0
10	btw /within group	10360	0
13	btw /within group	50890	0
16	btw /within group	1651	0

APPENDIX 7C:

ONEWAY

ONEWAY/DATA ANALYSIS OF Pyrene BY SPSS

DESCRIPTIVES /POSTHOC=TUKEY LSD ALPHA (0.05).

day1 day4 day7 day10 day13 day16 BY VAR00001

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	Min		Max
Days						Lower Bound	Upper Bound		
1	0	3	240.24	0.17	0.10	239.83	240.65	240.05	240.36
	1	3	239.05	0.05	0.03	238.93	239.16	239.00	239.09
	2	3	219.78	2.73	1.58	212.99	226.56	217.31	222.71
	3	3	187.83	3.83	2.21	178.31	197.34	183.82	191.45
4	0	3	235.81	1.75	1.01	231.45	240.16	234.61	237.82
	1	3	194.33	0.94	0.54	192.00	196.65	193.26	195.01
	2	3	97.14	2.19	1.26	91.71	102.57	95.75	99.66
	3	3	69.14	34.75	20.07	-17.19	155.47	29.01	89.41
7	0	3	236.63	0.37	0.21	235.72	237.54	236.21	236.85
	1	3	133.73	1.11	0.64	130.96	136.49	133.05	135.01
	2	3	69.39	0.70	0.40	67.65	71.12	68.66	70.05
	3	3	44.95	0.62	0.36	43.42	46.49	44.31	45.54
10	0	3	231.75	0.02	0.01	231.72	231.79	231.74	231.77
	1	3	83.34	1.53	0.88	79.55	87.14	82.01	85.01
	2	3	34.62	1.40	0.81	31.13	38.10	33.05	35.76
	3	3	35.88	1.01	0.58	33.37	38.39	35.01	36.99
13	0	3	222.66	0.23	0.13	222.08	223.23	222.41	222.87
	1	3	30.69	1.17	0.68	27.77	33.61	30.01	32.05
	2	3	29.31	0.47	0.27	28.16	30.47	29.01	29.85
	3	3	18.34	0.36	0.21	17.44	19.24	18.07	18.75
16	0	3	222.24	0.13	0.08	221.91	222.57	222.14	222.39
	1	3	25.92	0.87	0.50	23.76	28.07	25.01	26.74
	2	3	8.65	1.27	0.73	5.50	11.80	7.85	10.11
	3	3	5.72	0.80	0.46	3.73	7.70	5.04	6.60

NB: 0=control;1=*E. coli*;2= *K. pneumoniae*; 3= *E. coli* + *K. pneumoniae*

APPENDIX 7D:

ANOVA for Pyrene

Days	Outcomes	Mean	F	P.sig
		Square		
1	btw /within group	1795.74	324.32	0.01
4	btw /within group	18656.94	61.34	0.01
7	btw /within group	21979.95	39290.00	0
10	btw /within group	25970.85	19500.00	0
13	btw /within group	29063.33	65200.00	0
16	btw /within group	32940.17	43700.00	0

APPENDIX 7E:

ONEWAY

ONEWAY/DATA ANALYSIS OF Fluoranthene BY SPSS

DESCRIPTIVES /POSTHOC=DUKEY LSD ALPHA (0.05).

day1 day4 day7 day10 day13 day16 BY VAR00001

Descriptives

			N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max
							Lower Bound	Upper Bound		
1	0	3		248.27	0.23	0.13	247.69	248.84	248.00	248.41
	1	3		192.03	0.87	0.50	189.87	194.19	191.22	192.95
	2	3		186.13	3.50	2.02	177.44	194.82	183.33	190.05
	3	3		182.24	3.04	1.75	174.69	189.79	180.24	185.74
4	0	3		240.16	0.05	0.03	240.05	240.27	240.12	240.21
	1	3		145.63	0.46	0.26	144.49	146.76	145.12	146.01
	2	3		156.90	35.54	20.52	68.61	245.19	135.75	197.93
	3	3		126.52	0.48	0.27	125.34	127.70	126.01	126.95
7	0	3		240.03	0.03	0.01	239.96	240.09	240.00	240.05
	1	3		119.52	0.43	0.25	118.46	120.58	119.21	120.01
	2	3		100.64	0.44	0.25	99.54	101.73	100.15	101.01
	3	3		85.28	0.41	0.23	84.27	86.29	85.01	85.75
10	0	3		232.18	0.19	0.11	231.69	232.66	232.01	232.39
	1	3		96.98	0.98	0.57	94.55	99.42	95.99	97.95
	2	3		63.70	0.34	0.20	62.86	64.54	63.34	64.01
	3	3		36.63	0.43	0.25	35.57	37.69	36.16	36.99
13	0	3		231.44	0.38	0.22	230.49	232.39	231.00	231.69
	1	3		79.59	0.40	0.23	78.59	80.58	79.34	80.05
	2	3		58.01	0.88	0.51	55.83	60.19	57.29	58.99
	3	3		11.30	0.40	0.23	10.32	12.29	10.99	11.75
16	0	3		230.46	0.22	0.12	229.92	230.99	230.22	230.64
	1	3		42.02	1.69	0.98	37.82	46.23	40.07	43.05
	2	3		15.82	0.16	0.09	15.42	16.22	15.70	16.00
	3	3		6.75	0.25	0.14	6.14	7.36	6.51	7.00

NB: 0=control;1=*E. coli*;2= *K. pneumoniae*; 3= *E. coli* + *K. pneumoniae*

APPENDIX 7F:**ANOVA for Flouranthene**

Time(Days)	Outcome	Sum	Mean	F	Sig.
1	btw/within group	8646.03	2882.01	517.53	0.01
4	btw/within group	22648.65	7549.55	23.90	0.26
7	btw/within group	44745.70	14915.23	109900.00	0
10	btw/within group	67786.00	22595.33	69780.00	0
13	btw/within group	81680.42	27226.81	87950.00	0
16	btw/within group	100225.15	33408.39	44600.00	0