PROTEIN PROFILE AND MITOCHONDRIAL DNA ANALYSIS OF *CLARIAS GARIEPINUS* SURVIVING IN THE KORLE LAGOON.

A THESIS SUBMITTED

BY

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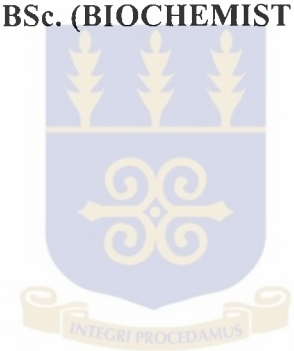

TO THE DEPARTMENT OF BIOCHEMISTRY, FACULTY OF SCIENCE, UNIVERSITY OF GHANA, LEGON, GHANA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF PHILOSOPHY (M.PHIL) DEGREE IN BIOCHEMISTRY

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DECLARATION

I declare that the experimental work for this thesis was carried out by me in the Department of Biochemistry and Noguchi Memorial Institute for Medical Research both of the University of Ghana, Legon, under the supervision of Professor Marian Ewurama Addy and Dr. Alex Kwadwo Nyarko.

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DEDICATION

This thesis is dedicated to Rev. and Mrs. R.A. Addison, National Head of the Church of Pentecost, Nigeria.

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ABSTRACT

Studies have established the presence of some species including fish, in highly polluted water bodies. In order to develop a better understanding of their ability to survive in such environments, this work was undertaken to ascertain the extent of microsomal cytochrome P450 induction, the modification of other proteins and the impact of the pollutants on the genetic material of the surviving organisms. In these studies, hepatic microsomal and cytosolic fractions were prepared from *Clarias gariepinus*, a fish species surviving in the highly polluted Korle lagoon. The same fractions were also prepared from fish samples obtained from the Weija Lake and fishponds at the Ministry of Food and Agriculture aquaculture demonstration centre (MOFA). The microsomal and cytosolic protein concentrations as well as activities of pollution-induced enzymes were assessed using ethoxyresorufin-O-deethylase (EROD), pentoxyresorufin-O-deethylase (PROD) and ρ-nitrophenol (PNP) hydroxylase assays. The values were compared among fish samples from the various water bodies before and after acclimatization to laboratory conditions.

Cytosolic and microsomal protein profiles were obtained by separating the protein using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Mitochondrial DNA was extracted and purified by RNase digestion and the G+C base composition was determined.

In samples both before and after acclimatization to laboratory conditions, cytosolic and microsomal protein concentrations were significantly higher for fish from the Korle lagoon compared to fish from MOFA and Weija Lake. The levels of EROD activities before and after acclimatization to laboratory conditions were significantly higher in fish obtained from the Korle lagoon compared to those obtained from Weija Lake and MOFA. The levels of PNP hydroxylase activities before acclimatization to laboratory conditions were significantly higher in fish obtained from the Korle lagoon compared to those obtained from Weija Lake but not those from MOFA.

After depuration, the PNP hydroxylase activities were significantly higher for fish from the Korle lagoon compared to samples from both the Weija Lake and MOFA. EROD indicating CYP induction by polycyclic aromatic hydrocarbons (PAH), was approximately ten times higher in the samples from the Korle lagoon compared to the samples from the two other water bodies, even after acclimatization to laboratory conditions. PROD activity, an indication of

CYP induction by phenobarbital and its related compounds was significantly lower in the Korle lagoon samples before and after acclimatization. These results indicate the induction of CYP 1A and CYP 2E by polycyclic aromatic hydrocarbons (PAH) and alcohols respectively in fish obtained from the Korle lagoon while induction of CYP 2B isozyme was depressed.

The pattern of cytosolic and microsomal protein profile and the corresponding scan for samples from the various water bodies were similar but the intensity of bands and heights of peaks for fish obtained from the Korle lagoon were much higher even after depuration. Comparison of G+C base composition of mitochondrial DNA for *C. gariepinus* from the water bodies did not show any statistically significant differences.

The study has shown that the induction of higher amounts of particular isozymes of cytochrome P450 such as the 1A and 2E isozymes to an extent that the levels are maintained even in the absence of the inducer might be the property enabling this organism to survive in the highly polluted Korle lagoon.

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ABBREVIATION

AhR	Aromatic hydrocarbon receptor
APS	Ammonium persulphate
BNF	β-naphthoflavone
CYP-450	Cytochrome P-450
DDE	Dichlorodiphenyldichloroethane
DDT	Dichlorodiphenyltrichoroethane
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EMS	Ethylmethanesulfonates
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
EROD	Ethoxyresorufin-O -deethylase
GST	Glutathione-S-transferase
3-MC	3-methylcholanthrene
MOFA	Ministry of Food and Agriculture aquaculture demonstration centre
mtDNA	Mitochondrial deoxyribonucleic acid
PAGE	Polyacrylamide gel electrophoresis
PAHs	Polycyclic aromatic hydrocarbons

PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-p-dioxin
PNP	Paranitrophenol
PROD	Pentoxyresorufin-O-deethylase
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

GENERAL INTRODUCTION

The increase in population and its related human activities such as agriculture, urbanisation and industrialisation have resulted in the production of large volumes of waste (pollutants), which are discharged directly into the atmosphere, streams, rivers, lakes and lagoons without any form of pre-treatment. Water is the basic element for life but the presence of excessive pollutants in the water bodies has caused most fresh water bodies to become detrimental to the health of living organisms. Thus the aquatic environment acts as sinks or reservoir for the deposition of numerous chemicals of natural and anthropogenic origin. Most toxic metal and chemical pollutants enter surface and underground water through direct waste discharge and run off, as well as percolation.

The impact of these contaminants on aquatic organisms and the ecosystem results in changes in community, composition, species abundance and population structure of fish (Munkittrick and Dixon, 1989). Research has shown that there is reproductive impairment, tumour formation and other pathological anomalies as well as behavioural or physiological changes associated with exposure of individual organisms to toxic chemicals (Leatherland and Sunstegard, 1984). High incidence of tumours, chromosomal and mitotic aberrations, genetic and physiological disorders in fishes have been reported in perch (*Perca fluviatilis*) in areas within Swedish Baltic coastal waters contaminated with polycyclic aromatic hydrocarbons (PAHs) (Ericson *et al.*, 1995). Aquatic life in the Great Lakes has also been greatly affected by large amount of toxic chemicals

present in industrial waste discharged directly into the lake without any form of pre-treatment. Genetic impact studies conducted revealed great variation between the organisms found in the Great Lakes and their counterparts found in relatively pristine environment (Murdoch and Hebert, 1994). The ultimate effect of pollution in aquatic ecosystem is the loss of biodiversity and some water bodies are sometimes referred to as "dead water".

Fortunately, some organism, including bacteria, protozoa ciliates, algae, molluscs and some species of fish are found in such polluted ecosystem (Velz, 1970). Some of the surviving fish found in highly polluted water bodies include perch along the Swedish Baltic coast, brown bullhead in the contaminated areas in the Great Lakes (Murdoch and Hebert, 1994), and catfish in the Korle Lagoon (personal communications with inhabitants around the lagoon). Such organisms may have acquired resistance to the pollutants either by the synthesis of new gene products as a result of toxic-induced mutation in the gene structure, adaptation or survival conferred to them by the presence of plasmids which may synthesize proteins necessary for the breakdown of the pollutants.

Ghana and Africa as a whole were thought to be free from industrial pollution. The recent increase in population growth accompanied by intensive formal education, urbanisation, industrialisation and bad waste management policies, as well as improper siting of industrial installation, have resulted in pollution of both fresh and marine water bodies (Biney, 1991). Current assessment of environmental pollution in Ghana has revealed that large volumes of industrial liquid effluent from the brewery, pharmaceutical, textile, food processing, chemical, metal, paper and wood processing industries are not subjected to any form of pre-treatment before being discharged into the environment. This has caused an extensive pollution of most streams and lagoons in the cities, severely destroying the ecosystem.

The Odaw river and Korle lagoon in Accra, Chemu lagoon in Tema, and Aboabo, Sisai and Subin rivers in Kumasi are the most affected water bodies in Ghana (Biney and Amuzu, 1995). About 22 million cubic metres of industrial waste are discharged into some water bodies in Ghana resulting in 40,000 kg biochemical oxygen demand (BOD) daily as opposed to a maximum permissible levels of 50kg BOD daily. The major offenders are the food and beverage industries, which discharge about 5 million cubic metres of waste. The chemical industries follow with 500,000 cubic metres of waste, while the metal industries produce 2,500 cubic metres. These industries thus, destroy life in some lagoons in Ghana (Biney, 1991). Industrial liquid wastes, therefore, need some pre-treatment before being discharged into the aquatic ecosystem.

Pollution destroys many aquatic lives, however other organisms survive in such polluted environment. Characterization of surviving organisms in such environs has shown that biotransfomation of pollutant in the water bodies is important for survival of the organism. Among microbes such as *Alcaligens*, *Pseudomonas, Acinetobactor* and *Flavobacterium,* which are normally found in water bodies that received effluent from US Navy ships, only *Alcaligens* was identified to play a role in the complete denitrification of waste water discharge from US Navy ship boiler tubes which contain high levels of sodium nitrite (Arquiaga *et al.*, 1993). High chitin and melanin found in the cell walls of fungi have been shown to have high metal biosorptive ability that can be used to combat metal pollution from industrial source (Gadd and White, 1993). Filamentous fungi biotransform telluride and selenium into volatile substances (Gharieb *et al.*, 1999) and specific fungi are known to biodegrade PAH, plant polymers, insecticide, herbicide and radionuclei pollutants (Extended Summaries, 1998).

Characterisation of organisms surviving in highly polluted environment have been useful in pollution studies because such studies have led to the utilisation of microbes (bacteria and fungi) and some plant species in biodegradation, bioremediation and biotransformation of industrial and domestic waste in some developed countries. Hydrocarbon utilising microbes were used in the biodegradation and bioremediation of petroleum pollutants in USA during the Alaska oil spills (Atlas, 1995). Mixed microbes entrapped in cellulose triacetate have been used in the treatment of organic waste effluent from food industries (Hsu and Lin, 1996).

Mesophilic and thermophilic microbes have been used to digest and to treat waste effluent from coffee processing industries (Dinsdale *et al.*, 1996). Sasa *et al.*, (1995) reported the use of microorganisms in the biological treatment of wastewater. Water hyacinth, inoculated with *Bacillus maratorium,* was used to biodegrade and biotransform industrial waste containing phenol and petroleum products into utilisable resources. For example, toxic hazardous waste can be used by the water hyacinth for its anabolic processes while the plant can be harvested and used as foliage to feed pigs (Shijun and Jingsong, 1989). Therefore organisms found in Ghanaian polluted water bodies could be exploited in the treatment of industrial waste instead of importing (micro) organisms or chemicals from developed countries for the purpose. In order to develop a better understanding of the ecology of organisms that can biodegrade and biotransform pollutants in our water bodies it is important to assess and characterise them biochemically.

The main objective of this research is to characterize an organism living in a highly polluted water body by comparing its pollution-specific enzyme activities, protein and DNA profiles with those of the same organism living in a relatively pristine water body.

In Ghana, most of the highly polluted water bodies, especially Korle and Chemu lagoons in Accra and Tema respectively, have lost almost all edible fish species (Biney, 1991). Investigation conducted among the indigenous people living around the Korle lagoon suggests that besides some crab species (Molluscs), catfish (*Clarias sp*) are still surviving in the lagoon. Therefore it was worthwhile to subject this species of catfish to biochemical and genetic analysis to assess the impact of pollution and to determine biochemical characteristics that enable this particular fish to survive in the polluted environment.

The specific objectives are to:

i) Identify an organism surviving in a heavily polluted water body;

ii) Assess the extent of protein induction due to pollution in this organism;

iii) Determine the protein profile of the organism from its natural habitat;

iv) Determine G+C base composition of the genetic material of the organism;

v) Compare the extent of protein induction, protein profile and the G+C base content of the same species of organism from aquatic environment, with different pollution histories.

LITERATURE REVIEW

Pollution of Water Bodies

The impact of pollution on aquatic life worldwide has led to the loss of valuable and edible fish species, but there are some, which have survived in such polluted water bodies. Examples include perch found in creosote contaminated river Angermanälven (Sweden) (Ericson *et al.*, 1999) and PAH contaminated Swedish sea (Ericson *et al.*, 1998), Northern pike (*Esox Vucius*) found in PAH contaminated Baltic sea (Ericson *et al.*, 1998) and the brown bullhead in the Great lakes contaminated with PAH and other industrial waste effluent water (Murdoch and Hebert, 1994).

The Korle lagoon in Ghana covers a total area of 25 km^2 (See site plan in fig. 1). It is located to the South West of the central business district of Accra and stretches about 2.8 km inland and drains a total catchment area of about 400 km^2. Majority of the drains in Accra enters the lagoon. The Odaw River is the main tributary of the lagoon. Other channels, flanking the Eastern and Western sides of the lagoon, also drain into it. Most of the manufacturing industries in Accra are sited in its catchment and tributaries (Biney, 1991). Therefore, all waste effluents from these manufacturing industries are carried into the lagoon.

Effluents from industries sited in the catchment area are discharged into the lagoon and this has caused severe and extensive pollution of the lagoon. Effluents and other wastes from artisanal workshop, which are inappropriately sited around the catchment area, are also discharged without any form of pre-treatment into the lagoon. Thus, the general sanitation around the entire lagoon is very poor.

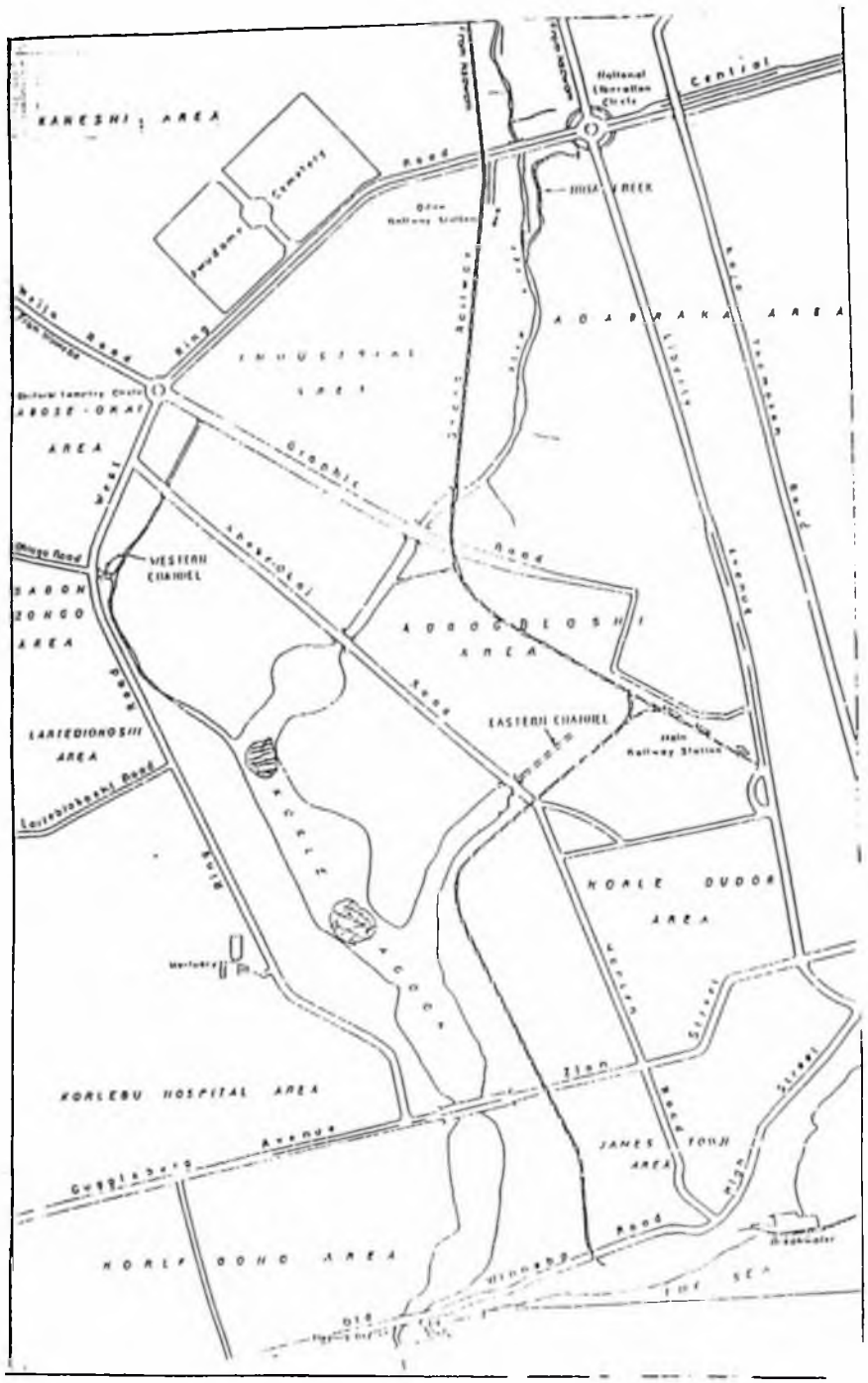

Fig 1 : Korle Lagoon and associated channels. ⊗ *Site of sample collection*

The major pollutants originate from industries, which deal with food, and beverage processing (breweries being the largest contributor), textile, metal and metalloplastic, chemical, paper and pulp. Other sources of major pollutants are domestic, commercial, schools and hospitals, specifically the Korle Bu Teaching Hospital. Solid waste such as paper, food leftovers, scrap metals, old batteries, broken glasses, plastic, textile products, wood cutting, cans and constructional waste are deposited in the lagoon, and this has resulted in the silting of the lagoon (Biney and Asmah, 1994).

The lagoon, until recently, supported both fin and shellfish life. When it was clean, it supported *Caranx hippos* (Horse mackerel), *Tilapia melanotheron*, *Penaeas notalis* (Shrimps), and *Meriophthalmus papilia* (mud skippers) as some of its aquatic organisms (Biney and Amuzu, 1995). It is on record that species caught by fishermen using dragnets in the vicinity of the outlet of the lagoon to the sea included sardinella, anchovies, mugil, barracuda, sea bream crab and octopus (Biney and Asmah, 1994). Thus, the lagoon served as economic livelihood for the indigenous settlers who also used the fish to supplement their protein intake. The present grossly polluted state of the lagoon has almost completely wiped off the fish species, thus depriving the people of their livelihood. The only obvious life around the lagoon is the avian fauna, mainly wild duck; herons and egrets, which are mostly palearctic migrants. The main vegetation includes tufts of grasses (*Paspalluum sp* and *Sporobolus sp*) clumps of *Euphorbia sp*, *Agara sp* and mangrove with *Avicennia germinans* being the dominant species (Biney *et al.,* 1994).

A study conducted in Ghana (IMDC, 1993) described the Odaw river and the Korle lagoon as the most polluted surface water bodies in Accra. Effluent from the breweries and food processing industries as a whole have high content of organic matter with high BOD that depletes dissolved

oxygen and cause euthrophication of the lagoon and hence contributes to the loss of aquatic life (IMDC, 1993). These water bodies have high BOD level, low dissolve oxygen, elevated levels of phosphates, nitrates, and ammonia compared to non-polluted water bodies. Anoxic conditions prevailing in these water bodies generate methane and hydrogen sulphide gases which give rise to the characteristic smell and odour in the environs of the lagoon (IMDC, 1994). Compared to the natural seawater background, the polluted lagoon also has high levels of heavy metal including Hg, As, Cd, Pb, Zn, Ni, Sn, Se, and Cr. Hg, Pb, and Sn contamination are believed to be responsible for the loss of aquatic fauna (Biney *et al.*, 1994).

Typical biochemical, physiological and morphological symptoms in most fish found in contaminated water are fin erosion, reduced gonad weight, liver enlargement, induction of liver ethoxyresorufin-O-deethylase (EROD) activity, changes in carbohydrate metabolism and disturbed ion balance. Other abnormalities include stimulated red blood cell production and altered white blood cells, indicating weakened immune system (Andersson *et al.*, 1988). Most of these disturbances were detected in perch and other fish studied in Finland, Canada and USA that were exposed to bleached pulp mill effluents (Lindström-Seppa and Oikari, 1989; Munkittrick *et al.*, 1991; Adams *et al.*, 1996). Research has also shown that although the effect of toxins on behaviour, physiology and the population structure may be mitigated within few generations after the reduction of the pollutants, the genetic consequence of exposure to the pollutants usually persists. These include the introduction of contaminant-induced mutation and culling of genetic variation due to natural selection or population collapse (Eisenstedt *et al.*, 1982).

The biological organisation of organisms in aquatic ecosystems affected by the effects of exposure to toxic environmental pollutants can be studied and classified at three different levels.

It can be studied through biochemical effects at the molecular and cellular levels, physiological and pathological effect at the tissue, organ and organism level and ecological effect on the population, community and the entire ecosystem level. Studies on the effects of xenobiotics have shown that their biochemical effect can be transmitted to the physiological and pathological level, which eventually appears at the ecological level.

Biomarkers of Pollution

Biomarkers are indicators of biological responses, which result from the interaction of xenobiotics (toxic pollutants) and the physiological systems of an organism exposed to such pollutants. The biological responses are related to the type of toxic pollutant that an organism is exposed to (Peakall, 1994). These biological indicators or biochemical markers have been developed and used as information tools to assess the extent and effect of chemicals and the susceptibility of aquatic organisms to such pollutants. Biomarkers may be used to elucidate the cause-effect and dose-effect relationships in health risk assessment for environmental pollution monitoring purpose (McCarthy and Shugart, 1990). They are specific and sensitive biological responses, which confirm the presence of pollutants in the environment, and serve as early warning indicators in surviving organisms. These indicators may predict future harm and therefore, enable corrective or preventive measures to be taken to avert destruction of biodiversity. Biomarkers also enable governments to legislate on industrial waste disposal, treatment and recovery of hazardous waste to avoid possible extinction of aquatic organisms and further damage to the environment.

Several biochemical assay systems are based on carbohydrate, protein, fatty acid metabolism and blood chemistry (EIFAC, 1975; ICES, 1978; Stegeman, 1981). The most prominent and

advanced biochemical methods depend on the induction of protein biomarkers such as metallothioneins (Olsson, 1978) and the cytochrome P-450 (CYP-450) monooxygenase system (EIFAC 1975; Stegeman, 1981).

Cytochrome P-450 - Dependent Monooxygenase

Many exogenous chemical compounds and pollutants induce CYP-450 systems. These compounds (xenobiotics) include polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane (DDT) and dibenzofuran (Andersson *et al.*, 1985). Xenobiotics are classified as carcinogens, mutagens and clastogens (Houk, 1992). Field investigations have revealed that most of the biochemical and physiological effects observed in fish exposed to toxic effluents disappear or show a weak response. For example, gonad size and hematocrite value revert to normal after sometime. However, certain other parameters such as delay in sexual maturity and EROD activity indicate an incomplete recovery from toxic substances, which may be biotransformed into genotoxic substances (Larsson *et al.*, 1999).

CYP 450 detoxifies xenobiotics in eukaryotes. Detoxification of xenobiotics in eukaryotes occurs in the liver in two phases. Phase I reactions involve the conversion of certain hydrophobic compounds to a hydrophilic form through reactions including epoxidation, hydroxylation, deamination and deethylation (Gillette, 1966). Phase II reactions involve the conjugation of the product(s) of phase I with endogenous polar compounds (Smith, 1968) which facilitates their subsequent removal. The enzyme involved in phase I reaction, the cytochrome P-450 monooxygenase is found in the endoplasmic reticulum (ER).

This enzyme system is a member of the haemoprotein superfamily that catalyses the biotransformation of both endogenous and xenobiotic compounds. Steriods, prostaglandins, bile salts and fatty acids are some examples of endogenous compounds, which are biotransformed by the monooxygenase enzyme complex. Some xenobiotics metabolized by the enzyme include PAH, PCB, DDT, polychlorinated dibenzo-p-dioxin (PCDD), pesticides, dioxins, petroleum products, hexachlorobenzenes, drugs and food additives (Nelson *et al.*, 1996).

The monooxygenases are a family of enzymes that are membrane bound (integral protein). They are bound to ER and upon disruption of the cell, the ER fragments into small vesicles called microsomes, which can be separated from other organelles through a series of differential centrifugation. The enzyme is found in the ER of the liver, kidney, lungs, spleen and gastrointestinal tract in vertebrates. It can also be found in the nuclear membrane of hepatocytes and mitochondria of the adrenal cortex (Watanuki *et al.*, 1978). Ambike *et al.* (1970) established the presence of the enzyme in the fungus *Claviceps purpurea* and their studies showed a direct correlation between alkaloid production and enzyme level. Their studies identified the involvement of the monooxygenase in the hydroxylation of anisole in yeast and bacteria.

Studies conducted on the CYP 450 have established that there are several forms of the protein and each isoform is substrate specific (Klotz *et al.*, 1986). The characterisation of the various isoforms was based on the amino acid sequence, which were either determined directly or from the gene sequence (Nebert *et al.*, 1991). CYP 450 gene has multiple sub-families with each sub-family also having more than one member. There are four main families of CYP 450 in vertebrates. These include P450 I which can be induced by PAH, PCB, isosafrole and ß-naphthaflavone (B-NF); P450 II which is induced by phenobarbital, ethanol (alcohols) and acetone; P 450 III is induced by steroids and macrolide antibodies; and P-450 IV is induced by

clofibrate. These gene-families represent different subunits of CYP 450 (Nelson *et al.*, 1996). Lately the nomenclature has been expanded to include chromosomal localisations that respond to different inducing agents differently.

The essential components of the drug metabolizing enzyme system are cytochrome P450, NADPH-dependent cytochrome P450 reductase and phospholipids (Gibson and Schenckman 1978). Phosphatidylcholine and traces of phosphatidylinositol and phosphatidylethanolamine are the main phospholipids found in the membrane bound cytochrome P450 monooxygenase enzyme complex. The NADPH dependent cytochrome P450 reductase is a hexamer that consists of FAD and FMN prosthetic groups. The cytochrome P-450 is an oligomeric complex, which contains iron protoporphyrin IX as its prosthetic group. This complex has maximum absorption at wavelength of 450nm when reduced by NADPH or dithionite and complexed with carbon monoxide (Yasukochi and Masters, 1976).

The key step in the oxygenation reaction is the insertion of one atom of molecular oxygen into the substrate to produce an unstable intermediate, which breaks down to yield the final polar product. The overall monooxygenase reaction can be summarised by the equation:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$$

In the above reaction, a lipophilic xenobiotic RH is converted to a hydrophilic product ROH. This product ROH can then be conjugated by phase II enzymes such as glutathione-S-transferase (GST) for subsequent elimination. In the metabolism of xenobiotics, NADPH cytochrome P-450 reductase, which is a flavoprotein, catalyses the transfer of electrons from NADPH to the cytochrome P-450. The monooxygenase functions as an electron transport chain, and is shown in figure 2

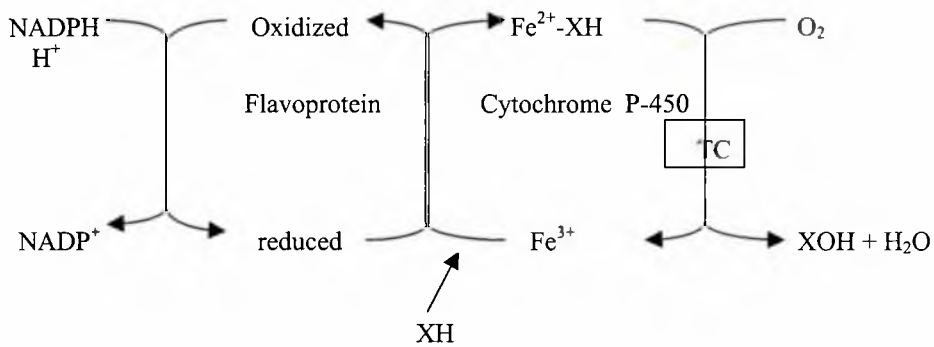

Figure 2: *Electron transport system in xenobiotic-metabolizing cytochrome P-450 monooxygenase. XH=Xenobiotic, TC = Ternary complex, XOH= Oxygenated xenobiotic.*

The CYP 450 system catalyzes a variety of reactions in the metabolism of xenobiotics. These include:

Aromatic hydroxylation

$Ph—H \xrightarrow{[O]} Ph—OH$

Aliphatic hydroxylation

$R—CH_3 \xrightarrow{[O]} R—CH_2—OH$

N—dealkylation

$R—NHCH_3 \xrightarrow{[O]} [R—NHCH_2—OH] \longrightarrow RNH_2 + HCHO$

O—dealkylation

$R—OCH_3 \xrightarrow{[O]} [R—OCH_2OH] \longrightarrow R—OH + HCHO$

In this research, one of the assays used is EROD which reflects O-dealkylation reaction and is specific for CYP 450 IA induced by PAH. The EROD assay is shown figure in 3:

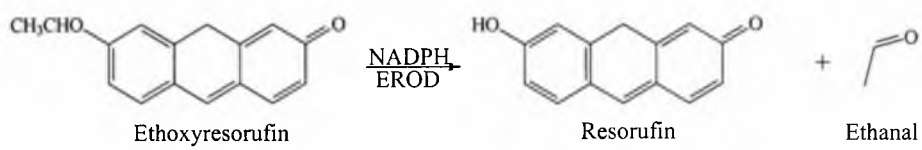

Fig 3. *Hydroxylation reaction involved in EROD assay*

EROD activity is measured by means of spectrofluorometer, which is specific and very sensitive. The assay evaluates the conversion of ethoxyresorufin into a fluorescent metabolite, resorufin. Resorufin fluoresces at specific excitation and emission wavelength that are different from those of the substrate (Burke *et al.,* 1985).

Pentoxyresorufin O-deethylase (PROD) assay was also used to assess induction of CYP 45O 2B usually induced by phenobarbital and its related compounds, thus indicating whether some of the pollutants in the water bodies belonged to this class. In addition, ρ-nitrophenol hydroxylation (PNP) assay was used as a marker for aromatic hydroxylation reaction, specific for CYP450 2E which is induced by ethanol. This induction can be enhanced by higher alcohols which are associated with increase in hepatic drug metabolism, acetaminophen hepatoxicity and cancer (Lieber, 1988).

Genotoxicity

The DNA constitution of the genome of a particular species of organism remains very much the same and only few changes occur due to recombination that occur in the nucleus. In eukaryotes the changes that occur in the base sequence are at a very slow rate as opposed to prokayotes. Physical and chemical agents facilitate the changes by causing mutation of the genetic material. The damaged DNA sequence may be corrected in some areas by an in-built repair mechanism although most of the DNA damage cannot be directly corrected (Darnell *et al.*, 1990).

The effect of genotoxic substances on higher organisms may not become obvious until a long time after the exposure. Chemical modification of DNA is generally accepted to be a critical initiating step in chemical carcinogenesis and cytotoxicity. Following the initial exposure, future generations can be affected through reduced embryonic viability and genetic disorders. There are several reports of liver lesions and other histopathological abnormalities in the benthic fish species found in industrially contaminated areas (Vethaak *et al.*, 1992; Myers *et al.*, 1994). Furthermore, exposure to mutagens increases germline mutation rates, which may decrease fitness of affected species (Würgler and Kramers, 1992).

Xenobiotics (pollutants) present in industrial effluent discharged into water bodies without any form of pre-treatment are usually carcinogenic and have a detrimental effect on most aquatic organisms. Due to their lipophilic nature, they accumulate in tissues such as gonad, liver and kidney. In the gonad, they induce heritable mutations in the germ line cells, and this is the main cause of variations in organisms in contaminated water.

There are a number of factors other than pollution that may affect the genetic diversity of organisms. These include radiations and geographical location. Indeed some major historical and

geographical events have been implicated as factors influencing the evolutionary processes leading to the extant patterns of interspecific genetic diversification and systematic relationships (Brundin, 1965; Bot *et al.*, 1989). However, industrial and domestic pollutants discharged into the environment have been a major historical and current factor influencing the evolutionary processes leading to intra and interspecific genetic diversification.

PAHs are the main component of industrial waste and are easily accumulated in aquatic organisms, especially fish, due to their lipophilic nature. Highly reactive intermediates are produced during the biotransformation of PAH in the liver microsomes, which bind to the biological macromolecules such as proteins and nucleic acids (Millar and Millar, 1947). Several studies have demonstrated an elevated level of DNA adduct in fish species found in areas contaminated with PAHs (Dunn *et al.*, 1987; Ericson *et al.*, 1998). Laboratory exposure of fish to PAH have also shown hepatic DNA adducts (French *et al.*, 1996).

The genetic patterns among the Atlantic cod (*Gadus morhua*) from different geographical regions have been studied and used to classify the genetic population structure and the gene flow in that fish species (Pogson *et al.*, 1995). van der Meer *et al.*, (1992) have reviewed the molecular mechanism of genetic adaptation to xenobiotic compounds by microbes in their natural habitat. The mitochondrial DNA (mtDNA) composition was used to characterise three *Chlamydomonas species* isolated from soil in relation to metal resistance (Spanier *et al.*, 1992).

DNA adducts serve as a biomarker for exposure to PAH, and adduct levels in livers of fish correlate with the degrees of PAH contamination (Stein *et al.*, 1992) Also, DNA adducts being cellular reaction products are the integrated outcome of several pharmacokinetic processes, such

as uptake, distribution, metabolism, excretion, DNA repair and cellular turnover (van der Oost *et al.*, 1994).

There are certain chemicals, which interact with DNA to induce mutations. These chemicals fall into two classes. The first group of compounds, which include dimethylsulfate, nitrogen mustard and methylnitrosourea, acts directly on DNA without any metabolic activation. The second group of compounds are chemically inert (carcinogens), which require metabolic activation. The indirect acting carcinogens include benzo(a)pyrene, dibenz(a,h)anthracene, 2-naphthylamine, dimethyl nitroamine, vinyl chloride, aflatoxin B (*Aspergillus flavus*) and 2-acetylaminofluorene. These chemicals are metabolised by microsomal CYP 450 that is involved in detoxification of noxious chemicals into highly reactive electrophiles. Figure 4 shows the metabolic activation of benzo(a)pyrene, an inert PAH that is metabolized into a reactive carcinogenic intermediate. This reactive intermediate or electrophile reacts with negatively charged centres in DNA, RNA and protein that lead to the modification of both free and circular DNA, for subsequent phenotypic expression. Cells or organisms exposed to chemical carcinogens often induce permanently altered state in circular DNA (Darnell *et al.*, 1990).

While some mutations have detrimental effect on the organism, others show no observable effect, because they either occur in parts of the DNA that do not encode vital information, or have no effect on the coded information. Some mutations are beneficial because they enable the organism to adapt to the environment, an example is haemoglobin AS.

Mitochondrial monooxygenase in rat liver has been shown to be capable of activating carcinogen, which then modifies mtDNA. For example, aflatoxin B1, a hepatic carcinogen is metabolized by rat liver monooxygenase into an electrophilic reactive form, which preferentially

covalently modifies mtDNA (Niranjan *et al.*, 1982). Similarly, studies using tissue culture cells, indicate that bioactivated benzo(a)pyrene also causes modification of both mtDNA and nuclear DNA but affects mtDNA more (Allen and Coombs, 1980).

Carcinogenic alkylating agents modify mtDNA by a factor of about five times greater than the nuclear DNA from the same cell (Wunderlich *et al.*, 1970). Furthermore, evidence suggests that mtDNA is the genetic target of lipophilic contaminants (Allen and Coombs, 1980) that form DNA adducts, thus enhancing the probability of mutation (Eisenstadt *et. al.*, 1982). Maternal transmission of mtDNA ensures that variation can be generated only by mutation unlike the nuclear genome in which variation can be introduced through recombination (Murdoch and Hebert, 1994). Mutations accumulate in mtDNA at a more rapid rate than in the nuclear DNA (Brown *et al.*, 1979), suggesting that the mtDNA replication enzyme complex lacks the editing function. These factors alone could contribute greatly to a high mutation rate in addition to the fact that mtDNA has a higher turnover rate than nuclear DNA in tissues (Clayton *et al.*, 1974; Niranjan *et al.*, 1982).

At the population level, natural levels of mtDNA diversity may be drastically reduced by either strong selection pressures or population collapse associated with environmental degradation. Degradation may be due to contaminant input, reduced water and/or habitat alteration. When the selective pressure is reduced (or the effective population rebounds) the genetic diversity will be restored through the process of immigration of variant genotype and mutation. When the fish species have low immigration and therefore low rate of gene flow, such geographically separated population will remain genetically distinct. The renewal of genetic diversity through immigration or mutation may, therefore, be a slow process requiring many generations. At the individual level female fish exposed to genotoxic compounds during oogenesis may incorporate

mutated mtDNA molecules into their eggs, and hence produce offspring that are heteroplasmic for mtDNA. Such variations will reflect in the population after sometime due to random assortment of mtDNA molecules at reproduction, which ensure the transition from heteroplasmy to fixation within individuals of newly arisen mutation (Murdoch and Hebert, 1994).

Studies on rapid evolution of animal mtDNA revealed that the degree of divergence of higher animals were at the site of recognition and cleavage of restriction endonuclease (Brown *et al.*, 1979). Bogenhagen and Clayton (1974) have inferred that organisms with gene mutation in mitochondria survive because the mitochondrion is polyploid and each cell contains at least one copy of the mitochondrial genome. Hence, a mutation inactivating a gene in one genome might have little or no effect on the fitness of the organism.

DNA adducts in both nuclei and mitochondria have been studied and used to classify organisms in relation to geographical and geological patterns as well as the effect of pollution. Taylor and Dodson (1995) studied the molecular diversity of Holarctic fish in relation to biogeographic events. Ericson *et al.*, (1999) used DNA adducts as a biomarker to study perch (*Perca fluviatilis*) in relation to creosote contamination. The effect of PAH on the DNA and histopathological abnormalities as well as organosomatic indices in perch and northern pike (*Esox lucius*) have been studied and classified (Ericson *et al.*, 1998). Unlike the perch and Northern pike the mtDNA of catfish has not been investigated. Such studies could help in determining how pollutants in water bodies in Ghana affect DNA of organisms that are found in these water bodies.

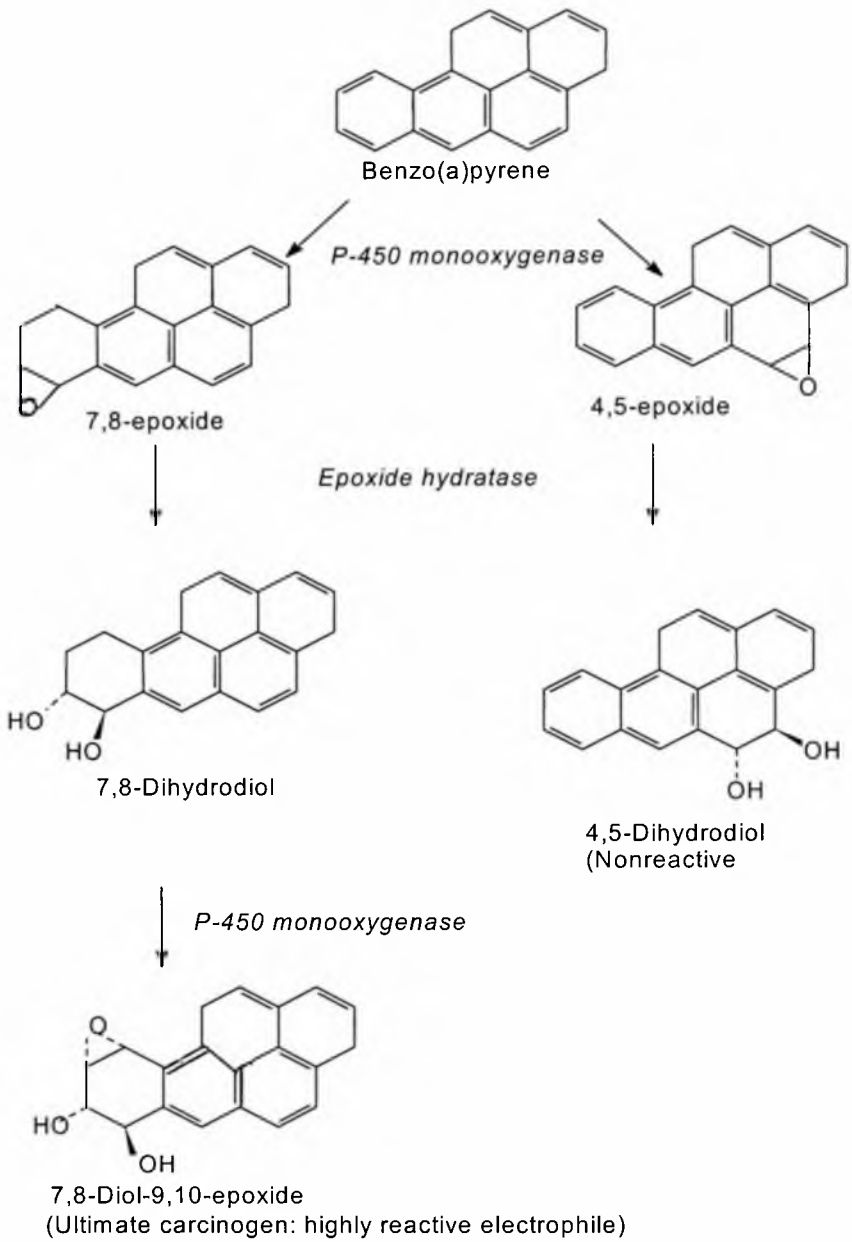

Fig 4: The metabolic activation of benzo(a)pyrene, a polycyclic aromatic hydrocarbon that is a powerful carcinogen.

DNA techniques used in the classification of organisms include G+C base composition. This has been used to classify both mitochondrial and nuclear DNA. Pot *et al.*, (1989) used this technique among others to study and classify the intra- and inter-generic relationships of the genus *Oceanospirillum* into five species. De Ley (1970) has also used this method to examine the association between melting point, bouyant density and chemical base composition of DNA in bacteria. Stanley *et al.*, (1992) characterised *Campylobacter helveticus sp* isolated from domestic animals faeces based on their DNA base composition and total protein profile. In their study, they showed that the isolates had the same base composition while their protein profile, rRNA gene profile and genomic DNA homology were different in each isolate. Thus, since organisms of the same genus and species are expected to have the same genomic DNA chemical composition any differences may be attributed to mutation that have occurred over a period of time.

Protein profile

Proteins are gene products and any influence of xenobiotic (environmental pollutant) on the genetic material is expected to be reflected in the gene product. Environmental pollution can result in the production of non-functional proteins, isozymes or proteins, which may have different molecular weight, compared to those of natural gene products.

Protein resolution is performed on polyacrylamide-gel electrophoresis (PAGE). This technique separates charged molecules such as nucleic acid and proteins on the basis of their movement though a polymatrix gel under the influence of an electric field. The technique employs a discontinuous buffer system in the presence of denaturing anionic detergent, sodium dodecyl sulphate (SDS). The SDS-PAGE technique separates proteins by a more conservative parameter of molecular weight. PAGE of whole cellular protein has been used to characterise organisms.

SDS-PAGE of whole cell proteins have been shown to be relatively simple, easy, reproducible and reliable procedure for identification. The outer membrane protein profile of *Serratia marcescens* has been studied and used to characterise the organism into 3 major groups (Larsen and Biedermann, 1993). This protein profile technique was used by Bouchara *et al.*, (1993) to study inducible proteinase synthesis in *Aspergillus fumigatus* in the presence of other protein.

The studies reviewed on the effect of pollution on the induction of CYP, DNA and protein profile provide the rationale for the present research reported in this thesis.

CHAPTER TWO

MATERIALS AND METHODS

Fish Sample

Catfish (between 500g and 600g) used for this research were obtained from the Korle Lagoon between the old Fadama street and the Korle Bu bridge in Accra, the Weija the Lake and Ministry of Food and Agriculture aquaculture demonstration centre (MOFA) at Ashiaman, all in the Greater Accra Region. The fish from each locality were divided into two and one part kept for 21 days in aquaria under laboratory conditions.

The samples from the Weija Lake and MOFA were used as fish from relatively pristine (pollution free) aquatic environment. These provide baseline values for comparison in this project. The fish kept in the aquarium were fed with feed prepared by MOFA Fishery Department. Every catfish used in this project was sent to the Department of Zoology for identification.

Chemicals and Regents

Ethylenediaminetetracetic acid (EDTA), bovine serum albumin (BSA), resorufin, 7-ethoxyresorufin, 7-pentoxyresorufin, ρ-nitrophenol, nicotinamide adenine dinucleotide phosphate (NADPH), Tris(hydroxymethyl) aminomethane (Tris), acrylamide, sodium acetate N, N^1-methylenebisacrylamide, sodium dodecyl sulphate (SDS), dimethylsuphoxide (DMSO), Proteinase K, RNase and SDS molecular weight markers were obtained from Sigma Chemical Company, (St. Louis, MO, USA). Sodium dihydrogen phosphate, potassium chloride, Folin-Ciocalteau phenol reagent and magnesium chloride were obtained from Hopkins and Williams, England. Chemicals obtained from Fluka were dithiothreitol (DTT), sodium hydroxide, sodium potassium tartrate, ammonium persulphate, N,N,N^1,N^1-tetramethylethylenediamine (TEMED),

Coomassie brillant blue R250, phenol, trisodium citrate and sodium chloride. Copper sulphate pentahydrate, sodium carbonate, glycine, methamol, glacial acetic acid, α-amylase, ethanol, chloroform and isoamylalcohol were obtained from BDH Limited, England. All chemicals were of analytical grade.

METHODS

Pre-treatment of fish samples

Catfish were caught with gill nets from their natural environment and immediately put in cages of water and transported to the Research Laboratory of the Department of Biochemistry. The catfish in each set were divided into two. Half from each set were kept in an aquarium while the other half was stunned by a blow to the head and the livers were excised and the gallbladder carefully removed. The livers were stored at -80°C in a homogenising buffer (Appendix 1), until they were used.

Those kept in the aquaria for depuration were killed and the liver removed after 21 days. A total of 30 fish, comprising 14 from the Korle lagoon and 8 each from the Ministry of Food and Agriculture aquaculture demonstration centre (MOFA) and Weija Lake respectively, were used for the studies.

Preparation of microsomes and other subcellular fractions.

The frozen liver tissues were thawed on ice, weighed, diced with a pair of scissors and homogenized in a volume of ice-cold homogenization buffer with a Glas-Col homogenizer. The

homogenates were diluted with the homogenization buffer to give 4ml-homogenization buffer per gram of liver tissue.

The homogenates were centrifuged at 10,000g for 10 minutes at 4°C in a Hitachi 20 PR-52D centrifuge with RPR20-2-1128 rotor. The pellets were discarded. The supernatant fractions were further centrifuged at 16,800g for 10 minutes at 4°C. The pellets, which contained mitochondria, were stored at -20°C for DNA extraction. The supernatant fractions were centrifuged at 105,000g for 60 minutes using Hitachi 80P-7 preparative ultracentrifuge with RP65T 453 rotors. The supernatant fractions were aliquot into labelled eppendorf tubes and stored at -20°C for further analysis. The pellets were washed to remove pigments from the microsomes and centrifuged at 105,000g. The washed pellets were suspended in storage buffer pH 7.6 (see appendix 1) in a ratio of 1g liver weight to 2ml buffer (2ml/1g liver weight). The re-suspended microsomal pellets were distributed into labelled 1.5ml eppendorf tubes and stored at - 80°C.

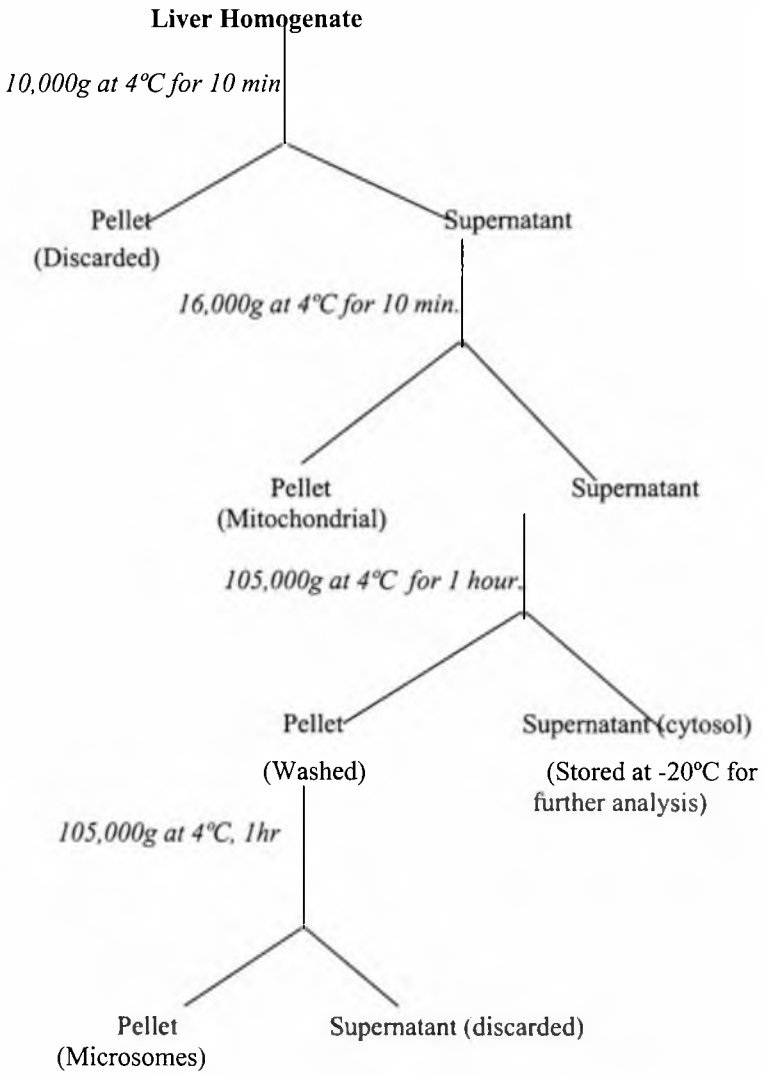

*Fig 5: Flow chart for the preparation of microsomes and other subcellular fra*ctions.

Protein Determination

Microsomal and cytosolic protein concentrations were determined by the method of Lowry *et al.*, (1951). The method depends on coloured complexes emanating from a reaction between alkaline copper-phenol reagent and tyrosine and/or tryptophan, and absorbance measured at 750nm. Prior to the determination, a standard curve of bovine serum albumin (BSA) was prepared. In this, 1mg of BSA was dissolved in 10ml of 0.5M NaOH, which constituted the stock of 100ug/ml-protein solution. Various concentrations between 0 and 50ug/ml (0, 10, 20, 30, 40, 50) were prepared. To 1ml of each diluent, 5ml of alkaline copper phenol reagent (see appendix) were added, mixed thoroughly by vortexing, and allowed to stand for 10 minutes. To each mixture, 0.5ml of Folin reagent was added, mixed immediately and allowed to stand for 30 minutes. The absorbances were read at 750nm in a double beam spectrophotometer, (Schimadzu UV-190). The blank contained all reagents except the BSA. A standard curve of absorbance against BSA concentrations (ug/ml) was plotted and used to determine the actual microsomal and cytosolic protein concentrations of the fish samples.

The microsomal and cytosolic fractions were diluted 1:20, and 1:25 respectively with 0.5M NaOH. The alkaline copper phenol reagent and Folin reagent were added as described above for BSA standard curve. The absorbance was measured and the protein concentration read from the BSA standard curve (shown in appendix 2).

Monooxygenase Assays

7-ethoxyresorufin-O-deethylase (EROD) assay:

The 7-ethoxyresorufin-O-deethylase (EROD) assay was determined spectrofluorometrically as described by Burke *et al.,* (1985). The method essentially measures the overall cytochrome-P-450 monooxygenase activity due to the induction of CYP 1A protein by PAH.

The excitation and emission spectra of resorufin and 7-ethoxyresorufin (7-ER) were obtained using the SFM-25 spectrofluorimeter, a total volume of 2ml solution, made up of 1990µl 0.1M NaH_2PO_4 buffer pH7.6 (see appendix 1) and 10 µl of 0.41mM 7-ER or 0.85mM resorufin. The excitation and emission wavelengths were set at 510nm and 585nm respectively.

Each reaction was performed in a 2ml-reaction mixture, which was made up of 1930µl of 0.1M NaH_2PO_4 assay buffer pH 7.6, 50µl of microsomal preparation with a protein concentration of 0.2mg/ml and 10 µl 0.41nM 7-ER. The reaction mixture was incubated for 5 minutes at 37°C and the fluorescence measured at 510 and 585 excitation and emission wavelength respectively for 30 seconds. A 10µl aliquot of 50mM NADPH was stirred into the mixture to start the deethylation reaction. The progressive increase in fluorescence (as 7-ER was converted to resorufin) was recorded for 3 minutes. An internal resorufin standard of known concentration was added and the sudden increase in fluorescence recorded for another minute. The reactions were performed under subdued light at room temperature.

7-Pentoxyresorufin-O-deethylase (PROD) assay.

PROD activity was determined from the rate of formation of resorufin from 7-pentoxyresorufin (PR). The assay was carried out as described above for EROD except that 10µl of 0.41mM 7-PR was used instead of 7-ER.

Both the EROD and PROD activities were calculated using the linear change in fluorescence with time as a result of the addition of NADPH. The specific enzyme activity was estimated by the formula:

Specific enzyme activity (nmol resorufin formed)

$$= \frac{F \times C \times D}{t \times S \times P} \quad (\text{nmol min}^{-1}\ \text{mg}^{-1}\ \text{microsomal protein})$$

C = Concentration of internal resorufin standard

D = Dilution factor for sample (2000/50=40)

F = Fluorescence of sample reaction

P = Concentration of microsomal protein (mg)

S = Spike or fluorescence of internal resorufin standard

t = time over which fluorescence was measured.

ρ-Nitrophenol (PNP) assay

The PNP hydroxylation activity was determined as described by Reinke and Moyer (1985). Briefly, the assay involves the formation of 4-nitrocatechol from ρ-nitrophenol by ρ-nitrophenol hydroxylase. The 4-nitrocatechol is measured spectrophotometrically (Shimadzu CL-720) under alkaline condition.. Each reaction assay was performed in 1ml reaction mixture (made up of 500µl of 0.1M Tris buffer pH 7.4, 50µl of 10mM NADPH, 300µl of 0.0167M $MgCl_2$ and 100µl

microsomal preparation, to give a final concentration of 0.05M Tris buffer, 5mM $MgCl_2$, 0.4g/ml NADPH and 0.3mg microsomal protein). The reaction was initiated by the addition of 50 µl of 2.0×10^{-6} PNP to each reaction mixture. Incubation was carried out for 10 minutes in a shaking water bath at 37°C. The reaction was terminated by the addition of 0.5ml of 0.6N $HClO_4$ to the incubation mixture. The mixture was centrifuged at 610g using Hitachi SCT 5BA centrifuge to remove precipitated proteins. An aliquot of 1ml of the supernatant fraction was mixed with 100µl of 10M NaOH. The absorbance of the mixture was read at 546nm. The amount of 4-nitrocatechol formed was calculated using the molar extinction coefficient of $10.28 mM^{-1}\ cm^{-1}$. The specific enzyme activity was estimated using the formula:

4-nitrocatechol formed (µmol/min/mg microsomal protein) =

$$\frac{A \times D}{E \times t \times P}$$

A = Absorbance of 4-nitrocatechol formed

D = Dilution factor for sample

E = Extinction coefficient ($10.28 mM^{-1}\ cm^{-1}$)

t = time over which reaction was performed

P = Concentration of microsomal protein used

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel Preparation

The SDS-PAGE was performed using slight modification of the procedure of Laemmli (1970). The gel cassettes were assembled using clean glass plates 12cm by 14cm as instructed by the manufacturer. A spacer of 1.5mm was used to obtain a gel of 1.5mm thickness.

A separation gel solution (24ml) of final concentration of total acrylamide content of 10% with 2.67% N, N-methylenebisacrylamide cross linker content was prepared as follows: A volume of 8ml acrylamide monomer solution, 5ml separating gel buffer (Tris-HCl pH 8.8) and 0.2ml of 10% SDS solution were added to 6.67ml deionized H_2O and mixed. The gel was polymerized chemically by the addition of 0.1ml 10% w/v of ammonium per sulphate (APS) and 10µl of N,N,N^1,N^1-tetramethylethylencdiamine (TEMED). The final concentration of other components in the gel solution were 0.375M Tris-HCl, pH 8.8, 0.1% w/v SDS, 0.05% APS and 0.05% TEMED. (See appendix 1 for stock solution preparation). By means of a dropping pipette, the separation gel solution was dispensed into the gel cassette gently, avoiding air bubbles. It was overlaid with deionized H_2O and allowed to polymerize under fluorescent light. The deionized H_2O was drained by inverting the gel cassette after polymerization.

The separating gel was overlaid with a stacking gel of total acrylamide content of 5% with crosslinker content of 2.67%, prepared by adding 830µl of acrylamide monomer solution, 1250µl of stacking gel buffer and 50µl of 10% SDS to 2850µl deionized H_2O and mixing. The concentration of other components were 0.125M Tris-HCl pH 6.8, 0.1% SDS, 0.05% APS and 0.1% TEMED. The gel was polymerized chemically by the addition of 25µl of APS and 5µl

TEMED, the stacking gel solution being immediately dispensed gently onto the separation gel and the PTFE comb (14 wells) inserted, care being taken to avoid any air bubbles below or on the side of the teeth of the comb. The gel was allowed to polymerize under fluorescent light. After polymerization, the comb was gently removed (to avoid disturbance of the stacking gel) and the wells washed several times with deionized H_2O and filled with tank buffer (see appendix 1). The sandwich plate with the gel was placed in the electrophoretic tank and filled with electrode running (tank) buffer pH 8.3.

Sample preparation and application

A sample buffer comprising 0.0625M Tris HCl pH 6.8, 2% SDS, 10% glycerol and 5% dithiotreitol (DTT) with 0.001% bromophenol blue was prepared (see appendix 1). The samples (cytosolic and microsomal fractions) from the Korle Lagoon were diluted with sample buffer in the ratio 1:4 while those from Weija Lake and MOFA were diluted in the ration of 1:2 to give approximate protein concentration of 20ug/ml. The proteins were completely denatured by immersing the samples (in eppendorf tubes) in boiling water for 3 minutes. By means of a micro syringe and needle, 20µl of samples mixed with the sample buffer were loaded onto the wells. SDS molecular weight markers ranging between 14000 dalton and 66000 dalton were also loaded.

The gels were subjected to electrophoresis using an initial current of 20mA. The current was increased to 50mA after the tracking dye had moved from the stacking gel to about a centimeter into the separation gel. The run was terminated when the tracking dye reached the bottom of the gel. The gels were carefully removed and simultaneously fixed and stained overnight in a staining solution containing 0.10% Coomassie brillant blue (appendix I). The gels were

destained in the same staining solution but without the Coomassie brillant blue. The process of destaining was repeated several times on a shaker until the background gel became clear. The gels were stored in 20% glycerol and later photographed. The gels were scanned on a Cosmo densitometer.

DNA extraction and analysis

Mitochondrial DNA was extracted by the method of Ericson *et al.,* (1998). The mitochondrial pellets (about 100mg) were suspended in 700µl of 50mM Tris and 20mM EDTA buffer pH 8.0, with 0.5% SDS. They were incubated with 35µl of 20mg/ml proteinase K (final concentration 1mg/ml) for 3 hours at 37°C. Proteins were removed by sequential extraction with phenol, phenol-chloroform-isoamylalcohol (25:24:1) and chloroform-isoamylalcohol (24:1). The nucleic acids were precipitated by adding 0.1 volume of 5M NaCl and 1 volume of -20°C absolute ethanol. The mixture was kept on ice for 30 minutes and centrifuged. The supernatant fractions were discarded and the nucleic acid pellets suspended in 700µl of 50mM Tris-HCl and 20mM EDTA at pH 8.0. The suspended pellets were incubated with 5µl of 20mg/ml RNase and 20µg of ∝-amylase (for hydrolysis of liver glycogen) for 30 minutes at 37°C. Protein extraction with the organic solvents was repeated. The DNA was precipitated by the addition of 0.1 volume 5M NaCl and 1 volume -20°C ethanol and kept on ice for 30 minutes. The DNA solutions were centrifuged at 500g and pellets dissolved in 10mM Tris and 1mM EDTA buffer pH 7.4. The purity of the DNA was assessed by measuring UV absorbance at 260nm and 280nm using double beam spectrophotometer (Schimadzu UV-190). The ratio of UV absorbance at 260m and 280nm (A_{260}/A_{280}) was used to assess the purity of the DNA. The samples were kept at -20°C for further analysis.

G+C base content

The G+C base content of the DNA was determined using the ultraviolet absorbance- temperature profile method developed by Mandel and Marmur (1968). The DNA sample was diluted with standard saline citrate (see appendix 1) to obtain a final DNA concentration of 20ug/ml. The initial absorbance A_{25} was measured at 25°C at 260nm wavelength using a double beam spectrophotometer (Schimadzu UV-190). Based on a preliminary rough determination of melting temperature of the DNA, the temperature of the chamber was raised to 60°C by means of a temperature regulated water bath. The absorbance was measured at 60°C and the temperature gradually increased by intervals of 2°C. The absorbance was measured with increasing temperature until there was no significant change in absorbance with temperature increase.

The absorbance was corrected for concentration dilution caused by solvent expansion using tables provided by Mandel and Marmur (1968). The relative absorbance was determined using A_t/A_{25}; where:

A_t = Absorbance at temperature t
A_{25} = Absorbance at 25°C (room temperature).

The melting temperature T_m (50^{th} percentile) was obtained from a graph of relative absorbance against temperature. The G+C base content was calculated using the linear relationship: G+C = (Tm - 69.3) 2.44.

Statistical Analysis

The Student's t-test was used to test for the level of significance between means obtained for catfish from the different waterbodies. Also, the differences between the means obtained before and after acclimatization to laboratory conditions for 21 days for sample from the same water body was evaluated. P values less than 0.05 were considered significant. In the calculation of the t-values, two-sample assuming unequal variances were used.

CHAPTER THREE

RESULTS

The catfish obtained from the Korle lagoon as the only surviving fish in this highly polluted water body was identified as *Clarias gariepinus* at the Department of Zoology, University of Ghana. The biochemical parameters measured as markers of pollution included total cytosolic and microsomal protein concentrations and the activities of 7-ethoxyresorufin-O-deethylase (EROD), 7-pentoxyresorufin-O-deethylase (PROD) and paranitrophenol hydroxylase (PNP). These microsomal protein activities indicated the type of CYP-450 induced. The establishment of a protein profile involved the separation of cytosolic and microsomal proteins using both denatured and native gels.

The DNA analysis involved the determination of G+C base content of the mitochondrial (mt) DNA to determine whether the effect of pollution in the Korle lagoon had changed the base composition of the mtDNA.

Total Protein

Microsomal protein concentration

The results of total microsomal protein concentration for *C. gariepinus* obtained from the various water bodies of different pollution histories before and after acclimatization under laboratory conditions for 21 days are presented in fig. 6. The results show that total microsomal protein concentration in the fish from the Korle lagoon is higher than the level found in fish obtained from the Weija Lake and the aquaculture demonstration centre of the Ministry of Food and

Agriculture (MOFA). The fish from MOFA had the least concentration of microsomal protein. The result showed that the protein concentration of fish obtained from the Korle lagoon was approximately 90% and 100% more than that of fish from the Weija Lake and MOFA respectively, before acclimatization to the laboratory conditions. The results further showed a decrease in microsomal protein concentration after acclimatization to laboratory conditions for 21 days. The decrease in protein concentration for fish from the Korle lagoon was 32%, that for fish from the Weija Lake was 58% and that from MOFA was 44%. The protein concentration after acclimatization for fish from the Korle lagoon was 300% and 260% higher than those from Weija Lake and MOFA respectively; that for fish from MOFA was 16% higher than that for fish from Weija Lake.

The statistical analysis, using the Student's t-test as shown in table 1, indicates a significant difference in microsomal protein concentration between fish from the Korle lagoon and those from the Weija Lake and MOFA, before and after acclimatization. In contrast, no statistically significant differences were observed between protein concentrations of fish from Weija Lake and MOFA before and after acclimatization to laboratory conditions. Table 1 further shows a significant difference in microsomal protein concentration before and after acclimatization to laboratory condition for fish from the various water bodies.

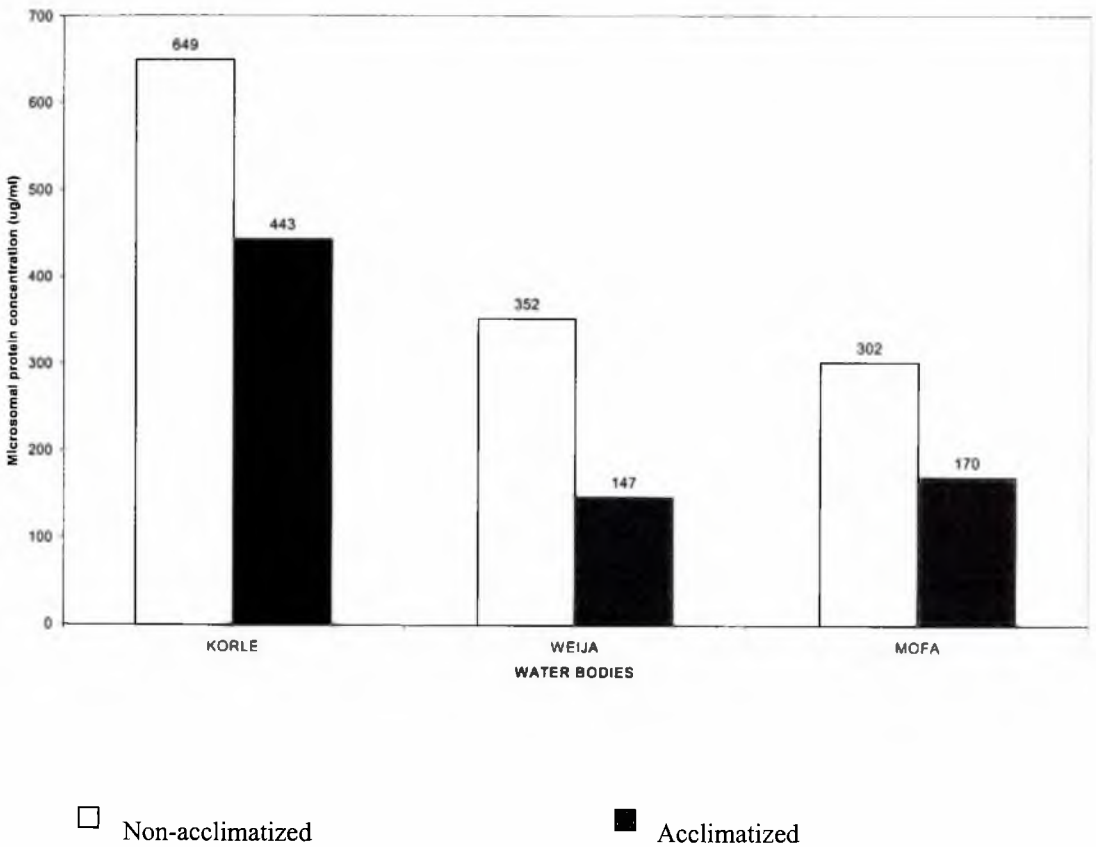

Fig 6 : Mean total hepatic microsomal protein concentration for C. gariepinus before and after acclimatization

Table 1: Student's t-test of pairs of microsomal protein concentration for *C. gariepinus* from different water bodies.

Source of C. gariepinus	t-statistics	t-tabulated	P
Non acclimatized			
Korle lagoon and Weija Lake	3.728	2.145	<0.05
Korle lagoon and MOFA	4.842	2.228	<0.05
Weija Lake and MOFA	1.398	2.571	n.s
Acclimatized			
Korle lagoon and Weija Lake	6.738	2.571	<0.05
Korle lagoon and MOFA	5.067	2.262	<0.05
Weija Lake and MOFA	-0.689	2.447	n.s
Non acclimatized and acclimatized			
Korle lagoon	2.460	2.131	<0.05
Weija Lake	5.673	2.571	<0.05
MOFA	4.000	2.571	<0.05

P value <0.05 for significant level

n.s no significant differences

P - significance level.

Cytosolic Protein Concentration

The results of total cytosolic protein concentration for *C. gariepinus* are shown in fig. 7. indicating a high cytosolic protein concentration in the fish obtained from the Korle lagoon before and after acclimatization to laboratory conditions for 21 days. The protein concentration of fish from the Korle lagoon was approximately 300% higher than those obtained from the Weija Lake and MOFA, before acclimatization to laboratory conditions. The results further showed a small decrease in protein concentration for fish from Weija and MOFA after acclimatization to laboratory whereas the decrease after acclimatization was 20% for fish from the Korle lagoon. The protein concentration of fish from the Korle lagoon was more than those from Weija and MOFA by more than 200% after acclimatization.

The summary of the statistical analysis is shown in table 2. The table shows a significant difference in cytosolic protein concentration between fish from the Korle lagoon and those from Weija Lake and MOFA before and after acclimatization. Comparing fish from the wild and those acclimatized to laboratory conditions, no significant differences were observed for the cytosolic protein concentrations for fish from the various water bodies.

Total Microsomal CYP-450 Monooxygenase Activity

Ethoxyresorufin-O-deethylase (EROD) activity.

The use of the EROD assay was to indicate the presence of CYP 1A induction by polycyclic aromatic hydrocarbon (PAH) compounds.The results for EROD assay before and after acclimatization are shown in fig. 8. The results indicate that EROD activity in rom the Korle lagoon before and after acclimatization was approximately1000% or 10

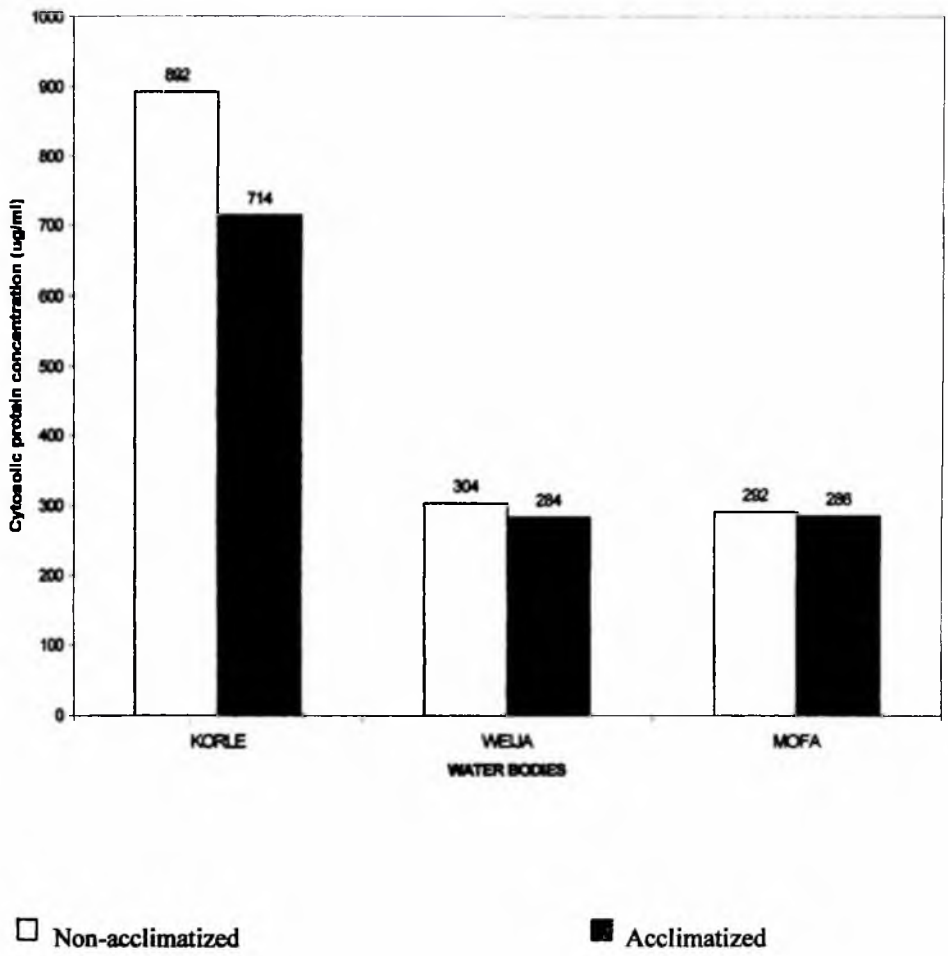

Fig 7: Mean total cytosolic protein concentration for C. gariepinus before and after acclimartization to laboratory conditions

Table 2: Student's t-test of pairs of data on cytosolic protein concentration for *C. gariepinus* from different water bodies.

Source of C. gariepinus	t-statistics	t-tabulated	P
Non acclimatized			
Korle lagoon and Weija Lake	6.708	2.365	<0.05
Korle lagoon and MOFA	6.818	2.365	<0.05
Weija Lake and MOFA	0.063	2.262	n.s
Acclimatized			
Korle lagoon and Weija Lake	10.254	2.450	<0.05
Korle lagoon and MOFA	10.415	2.570	<0.05
Weija Lake and MOFA	0.097	2.365	n.s
Non-acclimatized and acclimatized			
Korle lagoon	2.155	2.201	n.s
Weija Lake	0.821	2.262	n.s
MOFA	0.619	2.262	n.s

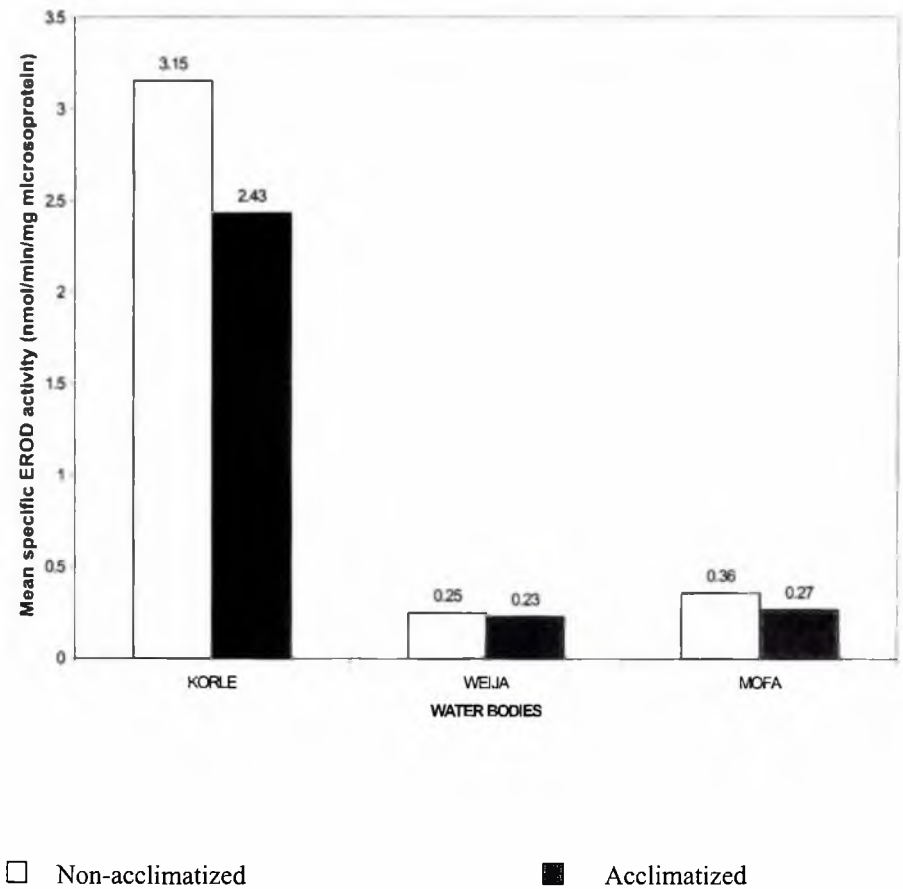

Fig 8: *Mean specific EROD activity for C. gariepinus before and after acclimatization.*

Table 3: Student's t-test of pairs of data on mean specific EROD activity for *C. gariepinus* from different water bodies.

Source of C. geriepinus	t-statistics	t-tabulated	p
Non acclimatized			
Korle lagoon and Weija Lake	15.833	1.998	<0.05
Korle lagoon and MOFA	15.101	1.997	<0.05
Weija Lake and MOFA	-3.388	2.004	<0.05
Acclimatized			
Korle lagoon and Weija Lake	18.500	2.064	<0.05
Korle lagoon and MOFA	17.289	2.064	<0.05
Weija lake and MOFA	-0.968	2.110	n.s
Not acclimatized and acclimatized			
Korle lagoon	3.287	1.988	<0.05
Weija Lake	0.243	2.045	n.s
MOFA	2.547	2.020	<0.05

times higher compared to the EROD activity recorded for fish from Weija Lake and MOFA

The statistical analysis shown in table 3 indicates significant differences in EROD activity for fish from the Korle lagoon, and those from the Weija Lake and MOFA before and after acclimatization to laboratory condition. There was also a significantly higher EROD activity in the MOFA fish samples compared to those from the Weija Lake before acclimatization. However, the difference in EROD activity after acclimation was not statistically significant. The EROD activity was higher in fish from wild than after acclimatization. The 25% and 22% reduction in EROD activity for fish from the Korle lagoon and MOFA respectively were statistically significant. In contrast, the 8% reduction in EROD activity in the fish from the Weija Lake before and after acclimatization was not statistically significant.

Pentoxyresorufin-O-deethylase (PROD) activity

PROD activity is an indication of CYP 2B induction by phenobarbital and its related compounds. The results for mean specific PROD activity before and after acclimatization to laboratory conditions for 21 days are presented in fig. 9.

For the non acclimatized samples, PROD was higher for fish from MOFA with those from the Korle lagoon showing minimum activity. The enzyme activity in fish from MOFA was about 35% higher than those from the Korle lagoon and about 20% higher than those from the Weija Lake. PROD activity observed for fish from the various water bodies were statistically significant for fish from the wild as shown in table 4. The highest PROD activity was not observed in fish obtained from the Korle lagoon which is thought to be the most highly polluted in this study. This observation is in contrast to that

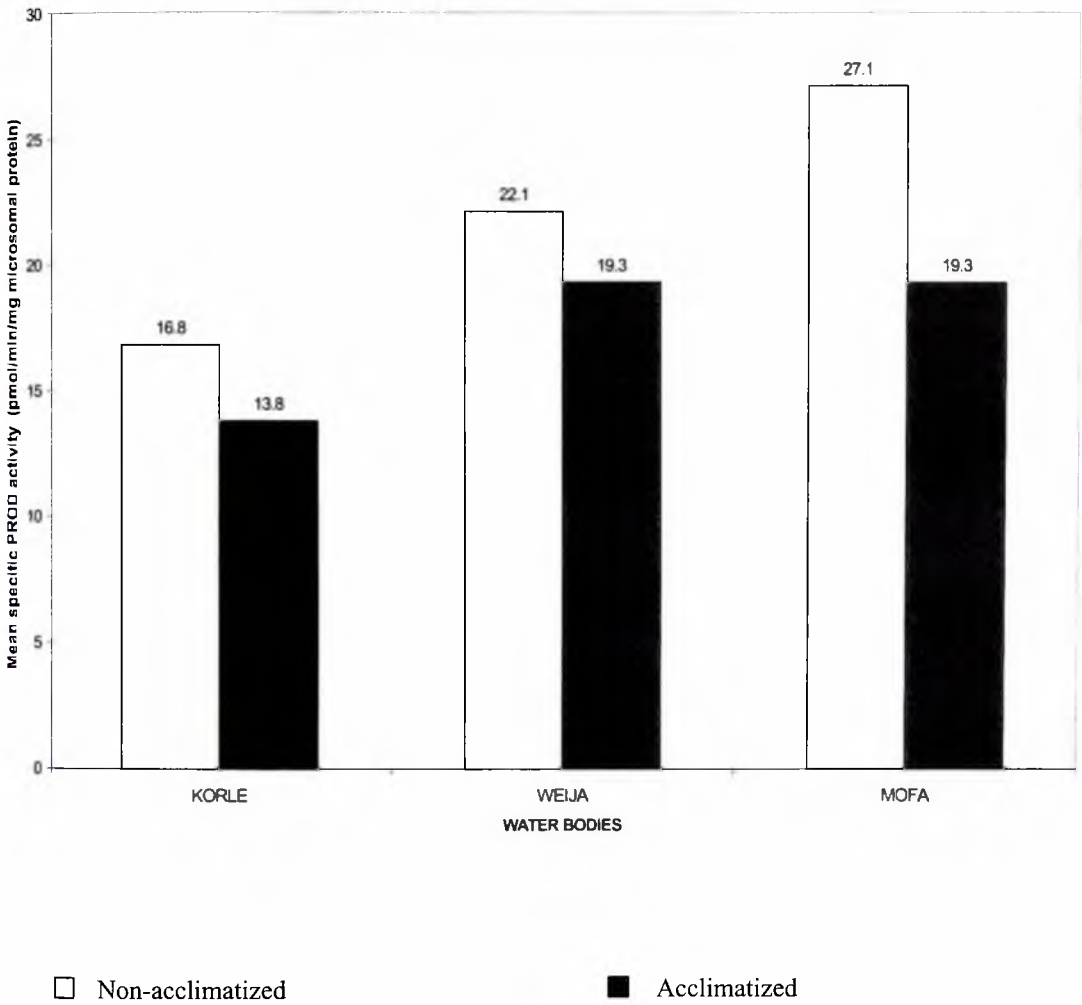

Fig 9: *Mean specific PROD activity for C. gariepinus before and after acclimatization to laboratory conditions.*

Table 4: Student's t-test of pairs of data on mean specific PROD activity for *C. gariepinus* from different water bodies.

Source of *C. gariepinus*	t-statistics	t-tabulated	P
Non acclimatized			
Korle lagoon and Weija Lake	2.949	2.093	<0.05
Korle lagoon and MOFA	4.626	2.086	<0.05
Weija Lake and MOFA	2.242	2.101	<0.05
Acclimatized			
Korle lagoon and Weija Lake	7.078	2.145	<0.05
Korle lagoon and MOFA	7.078	2.145	<0.05
Weija Lake and MOFA	0.032	2.002	n.s
Non acclimatized and acclimatized			
Korle lagoon	2.242	2.060	<0.05
Weija Lake	1.931	2.228	n.s
MOFA	2.452	2.160	<0.05

for the EROD activity. The results showed a 31% lower PROD activity for fish obtained from the Korle lagoon than those from Weija.

The PROD activity was lower after acclimatization to laboratory condition for 21 days. The reduction in activity was 17%, 13% and 29% respectively for fish obtained from the Korle lagoon, Weija Lake and MOFA. PROD activity after acclimatization was comparable in fish from Weija and MOFA. Activity of the enzyme is however lower in fish from the Korle lagoon. While the reduction in PROD activity was statistically significant for fish obtained from the Korle and MOFA after acclimatization, that for fish from Weija Lake was not. Furthermore, the difference in PROD activity between the Korle lagoon samples and those from the Weija Lake and MOFA was statistically significant, but that for samples from Weija Lake and MOFA were not.

Paranitrophenol hydroxylase (PNP) activity

The PNP enzyme system is specific for CYP-2E isozyme induced by ethanol. The induction response is enhanced by higher carbon chain alcohols such propanol, butanol and pentanol.

The results of PNP hydroxylase enzyme activity of microsomes prepared from fish samples from different water bodies are shown in fig. 10. The statistical analysis of the result is shown in table 5. The activity of PNP hydroxylase was high in fish obtained from the Korle lagoon and MOFA compared to fish from the Weija Lake. The activity for the Korle lagoon samples were higher although not statistically different from those from MOFA. PNP activities for Korle lagoon and MOFA samples were more than 100%

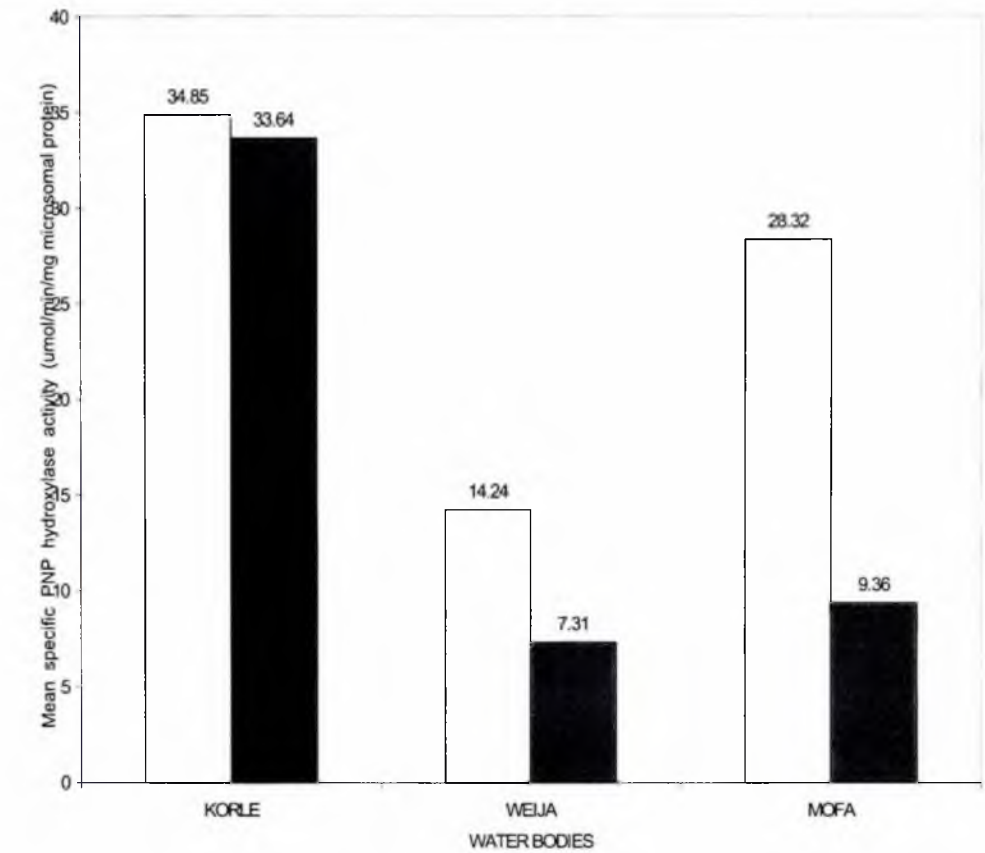

Fig 10: *Mean specific PNP hydroxylase activity for C. gariepinus before and after acclimatization to laboratory conditions*

Table 5: Student's t-test of pairs of data on mean specific PNP activity for *C. gariepinus* from different water bodies.

Source of *C. gariepinus*	t-statistics	t-tabulated	P
Non acclimatized			
Korle lagoon and Weija Lake	3.195	2.068	<0.05
Korle lagoon and MOFA	0.885	2.042	n.s
Weija Lake and MOFA	-3.089	2.120	<0.05
Acclimatized			
Korle lagoon and Weija Lake	8.314	2.365	<0.05
Korle lagoon and MOFA	-7.700	2.262	<0.05
Weija lake and MOFA	-5.467	2.262	<0.05
Non acclimatized and acclimatized			
Korle lagoon	0.175	2.055	n.s
Weija Lake	3.403	2.365	<0.05
MOFA	4.625	2.201	<0.05

higher than the activities obtained for the Weija Lake samples. The differences were statistically significant.For the acclimatized samples, the PNP hydroxylase activity for fish from the Korle lagoon was significantly higher, than those from Weija Lake (at least 450%) and MOFA (at least 36%). The PNP activities for the acclimatized Weija samples were significantly lower than that of MOFA samples by 20%. Comparing fish from the wild and those acclimatized to the laboratory conditions, the results indicated 50% and 67% reduction in activity for fish in Weija Lake and MOFA respectively. These decreases in PNP hydroxylase activity were statistically significant. In contrast, the percentage change in enzyme activity for the fish obtained from the Korle lagoon, was not statistically significant.

Protein Profile

The SDS-PAGE electrophoregram of cytosolic protein under denaturation condition is shown in fig. 11a. All the fish samples from the various water bodies showed similar patterns of protein bands, even though the protein bands in the sample from Weija appear faint. However, there are many more protein bands in the 3 samples from the Korle lagoon. Fig. 11b shows the scan of the electrophoregram presented in fig. 11a. The pattern of peaks corresponds to the protein bands. The electrophoregram indicated that the intensity and number of the protein bands of the Korle lagoon samples were higher than those from Weija Lake and MOFA (fig 11a). The pattern of protein peaks in fig 11b identified proteins, which were very faint and could not be seen in fig. 11a. The cytosolic protein profile for sample of fish from the Korle lagoon shows very high protein peaks that exceeded the set range for the densitometer used. The peaks recorded for the Weija and MOFA samples were not as high as those from the Korle lagoon samples. Furthermore these peaks correspond to proteins, which did not appear in fig. 11a. The peaks for MOFA samples were higher than those from Weija samples but lower than those from the Korle

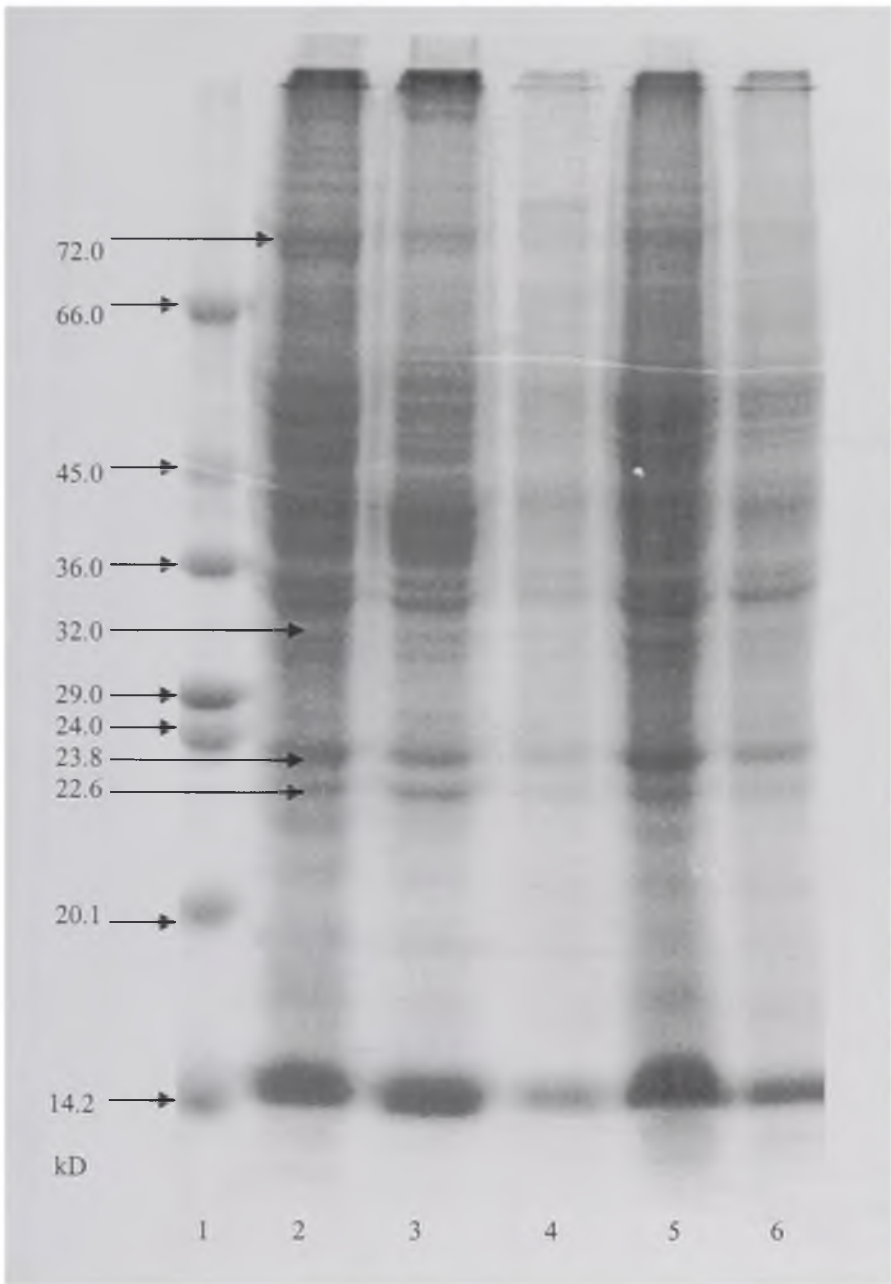

Fig 11a: *SDS-PAGE of cytosolic protein from C. gariepinus obtained from the various water bodies. Cytosolic fraction of 0.2mg/ml protein concentration was applied to each lane. Lane 1, standard molecular weight markers; lane 2,3 and 5,Korle lagoon samples; lane 4, Weija Lake sample and lane 6, MOFA sample.*

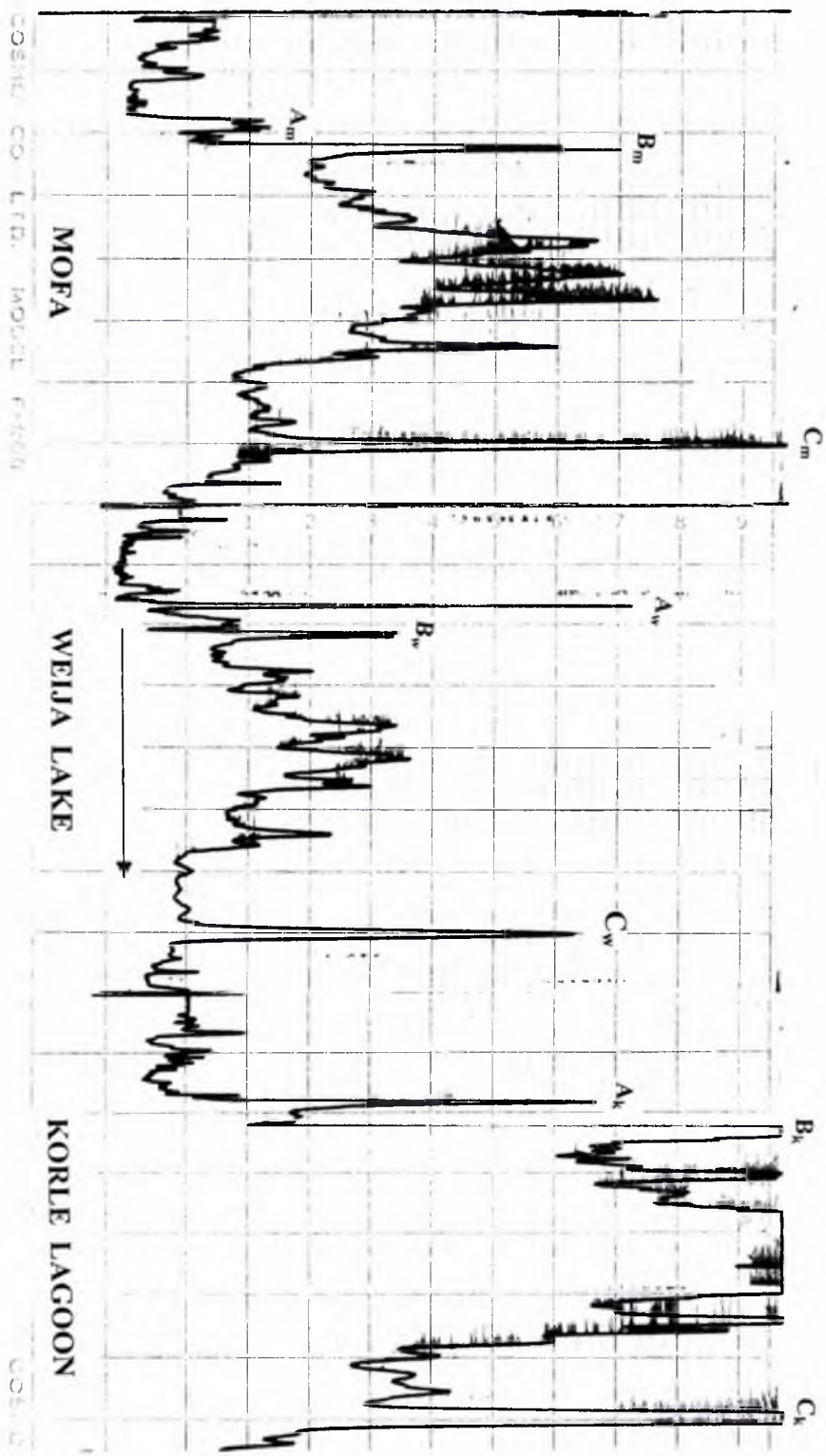

Fig 11b: *Electrophoregram scan of cytosolic protein profile under denaturation conditions for* C. variegatus *from the various water bodies.*
Direction of scan: Higher molecular weight to lower molecular weight.

lagoon samples. The scanning of the electrophoregrams for the three samples showed similar protein profiles but differed in peak heights for fish from the various water bodies. Two distinct peaks B_k and C_k (Korle samples) were considerably higher than the corresponding peaks for the Weija (B_w and C_w) and MOFA (, B_m and C_m) samples. Peak heights of B and C, like most of the other peaks were consistent with the trend of protein concentration for the various water bodies, that is, highest for samples from the Korle lagoon. In contrast the peaks labelled A were not; the heights were approximately the same for the Weija and Korle samples with the samples from MOFA having the least value.

The results in fig 12a display the electrophoregram for the cytosolic protein analysed under non denaturation condition. The native protein profile shows more intense protein for samples from the Korle lagoon compared to samples from the other two water bodies.

The results of the electrophoregram scan for the cytosolic proteins under non-denaturation conditions are shown in fig. 12b. The height and the patterns of peaks correspond to the results shown in fig. 12a. The pattern of cytosolic protein peaks shows two distinct protein peaks D and E. The peaks D_k and E_k are considerably higher than their counterpart D_w, D_m and E_w, E_m. The peak E_k exceeded the set range for the densitometer used while E_m was the lowest peak among the E peaks. In contrast, D_m (MOFA) was higher than D_w (Weija), with the D_k (Korle) being the highest peak.

The results presented in fig. 13a show the electrophoregram of the microsomal proteins under denaturation conditions. The major protein patterns are similar except for the relatively lower molecular weight proteins, which were absent in MOFA and Weija samples. The low molecular weight bands (32kD to 43kD) for the Korle lagoon samples

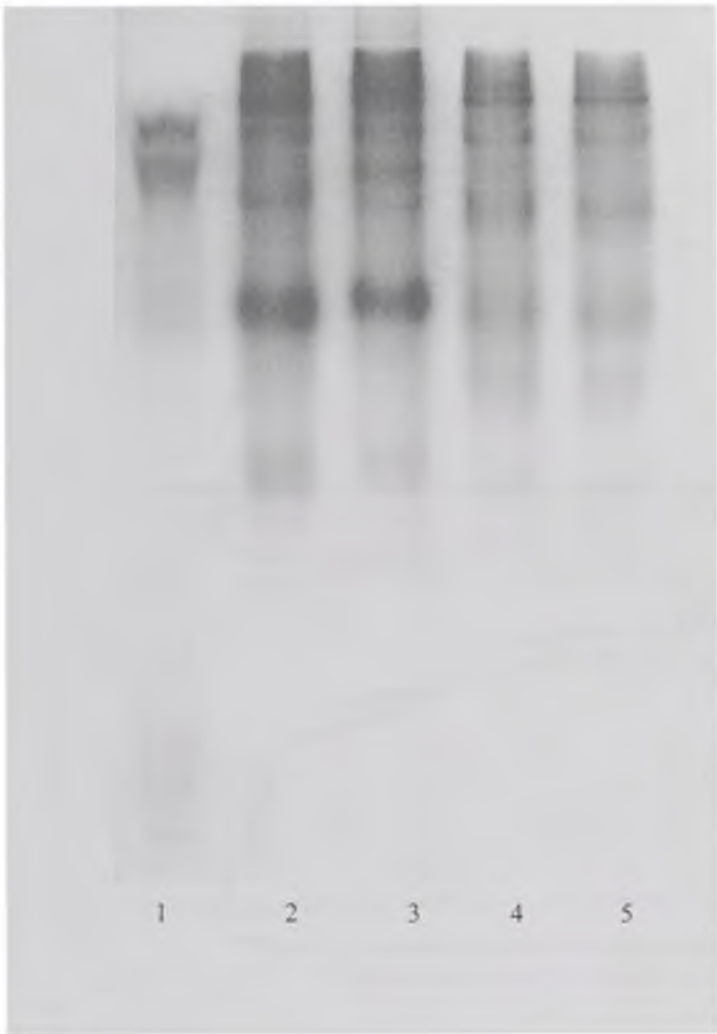

Fig 12a: *Page of cytosolic protein from C. gariepinus obtained from the various water bodies. Cytosolic fraction of 0.2mg/ml protein concentration was applied to each lane. Lane 1; standard molecular weight markers; lane 2 and 3, Korle lagoon samples; lane 4, Weija Lake sample and lane 5, MOFA sample.*

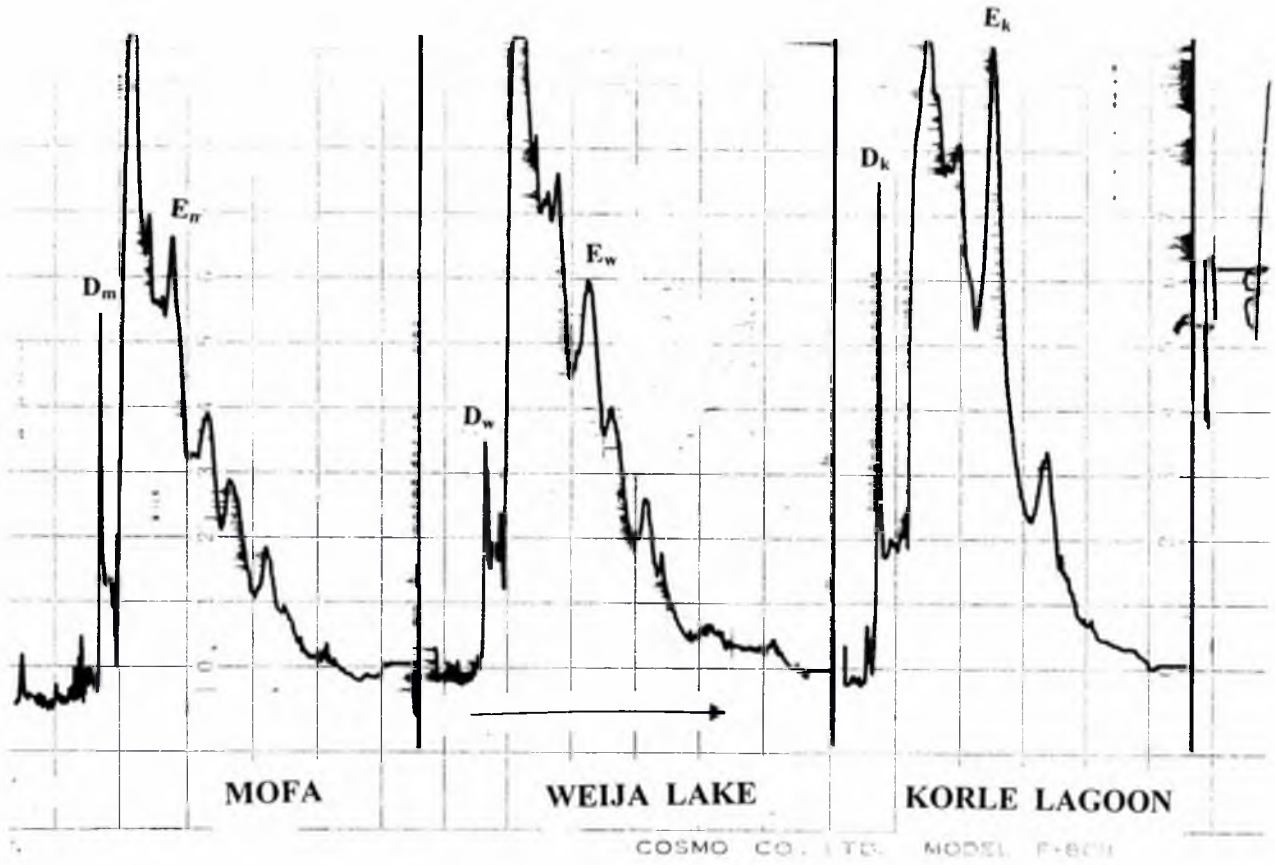

Fig 12b: *Electrophoregram scan of cytosolic protein profile under non-denaturation conditions for C. gariepinus from the various water bodies* ⟶ *Direction of scan: Higher molecular weight to lower molecular weight.*

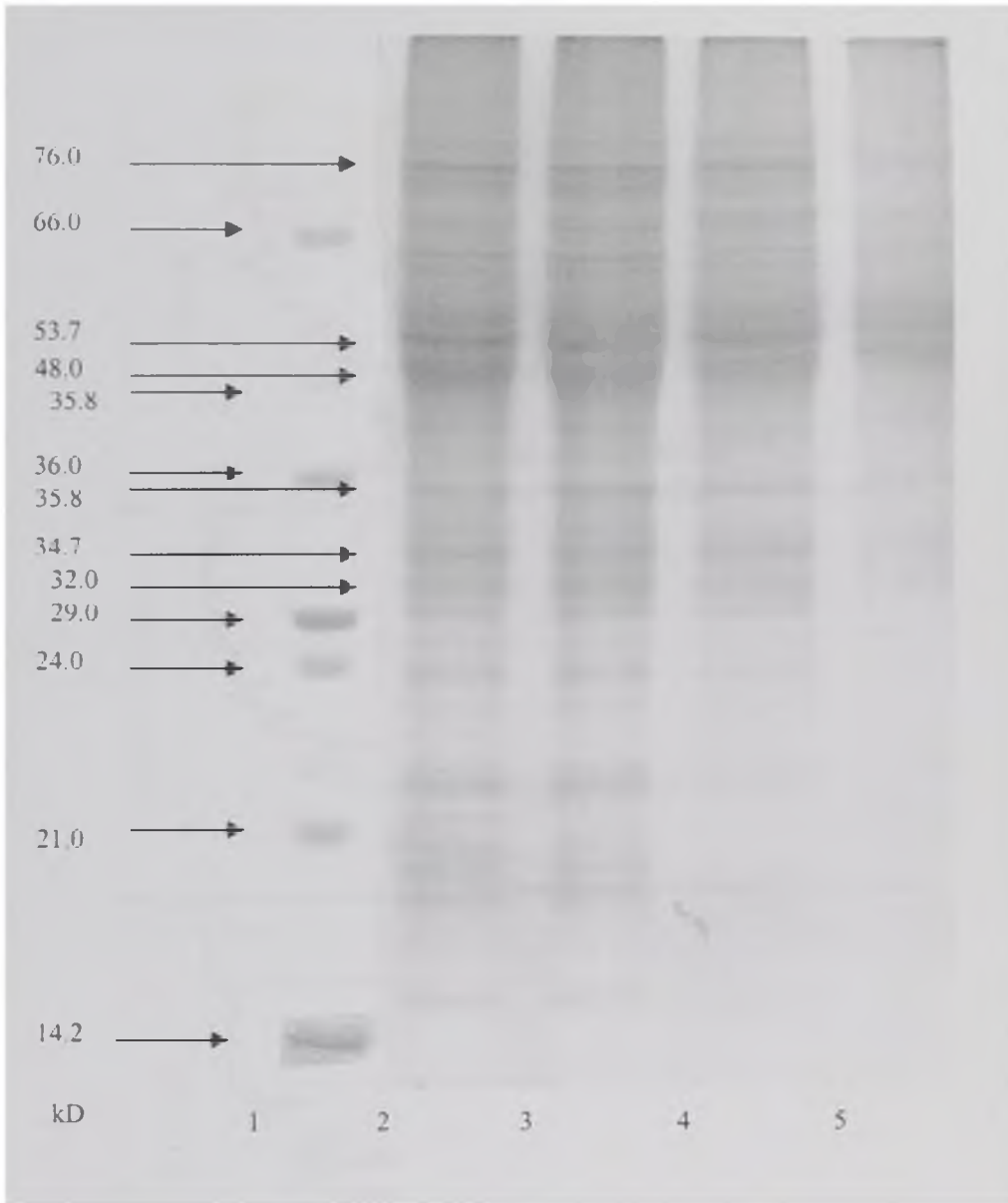

Fig 13a: *SDA-PAGE of microsomal protein from C. gariepinus obtained from the various water bodies. Microsomal fraction of 0.2mg/ml protein concentration was applied to each lane. Lane 1, standard molecular weight markers; lane 2 and 3, Korle lagoon samples; lane 4, Weija Lake sample; and lane 5, MOFA sample.*

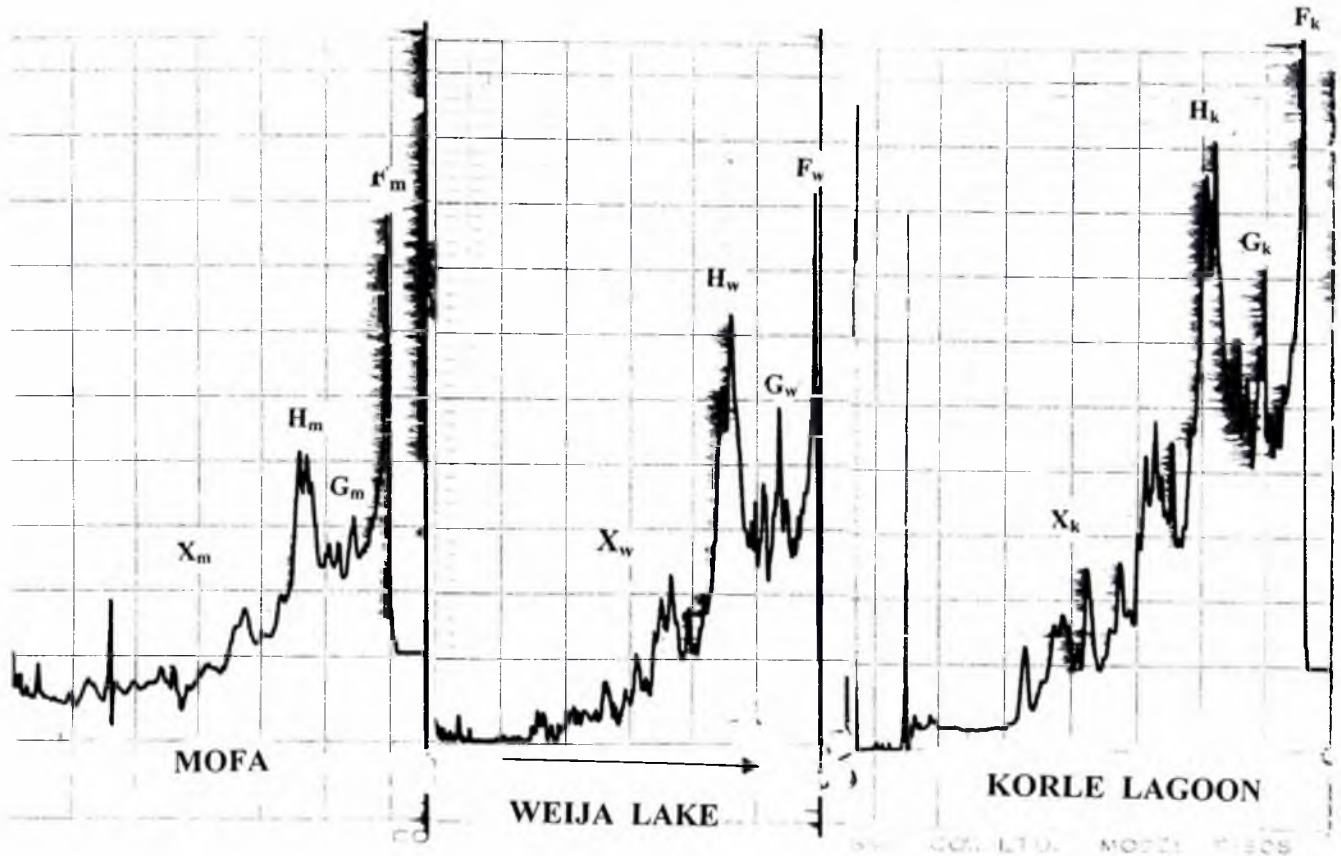

Fig 13b: *Electrophoregram scan of microsomal protein profile under denaturation conditions for C. gariepinus from the various water bodies.* ——→ *Direction of scan: lower molecular weight to higher molecular weight.*

are of higher intensity than those for MOFA and Weija samples. A scan of the electrophoregram is shown in fig 13b. The peaks for the Weija and MOFA samples were lower than those from the Korle lagoon samples. Compared to the Korle lagoon samples, there were virtually no peaks for the lower molecular weight proteins for the MOFA and Weija samples. Three conspicuous protein bands (about 53.5kD, 58.5kD and 78kD molecular weight) were observed in the profile for the Korle lagoon samples. These bands corresponded to peaks H, G and F respectively in fig 13b. The peak H_k for Korle sample was the highest while its corresponding peak H_m for MOFA sample was the lowest. Generally, the protein bands for samples from the Korle lagoon were more intense than those from MOFA and Weija Lake. Figs 13a and 13b show that the samples from the Korle lagoon have higher microsomal protein concentration than those from MOFA and the Weija Lake. MOFA samples had the lowest concentration and number of proteins in the microsomes.

Mitochondrial DNA

The temperature profile for the mtDNA denaturation is shown in fig. 14. All the fish samples obtained from the various water bodies showed similar pattern of increasing relative absorbance with increasing temperature. However, fish obtained from the Korle lagoon showed higher relative absorbance than those from the Weija Lake and MOFA. The mean melting temperature (50^{th} percentile) of the Korle lagoon samples was 93.2°C while fish obtained from Weija Lake and MOFA were 93.0°C and 92.9°C respectively.

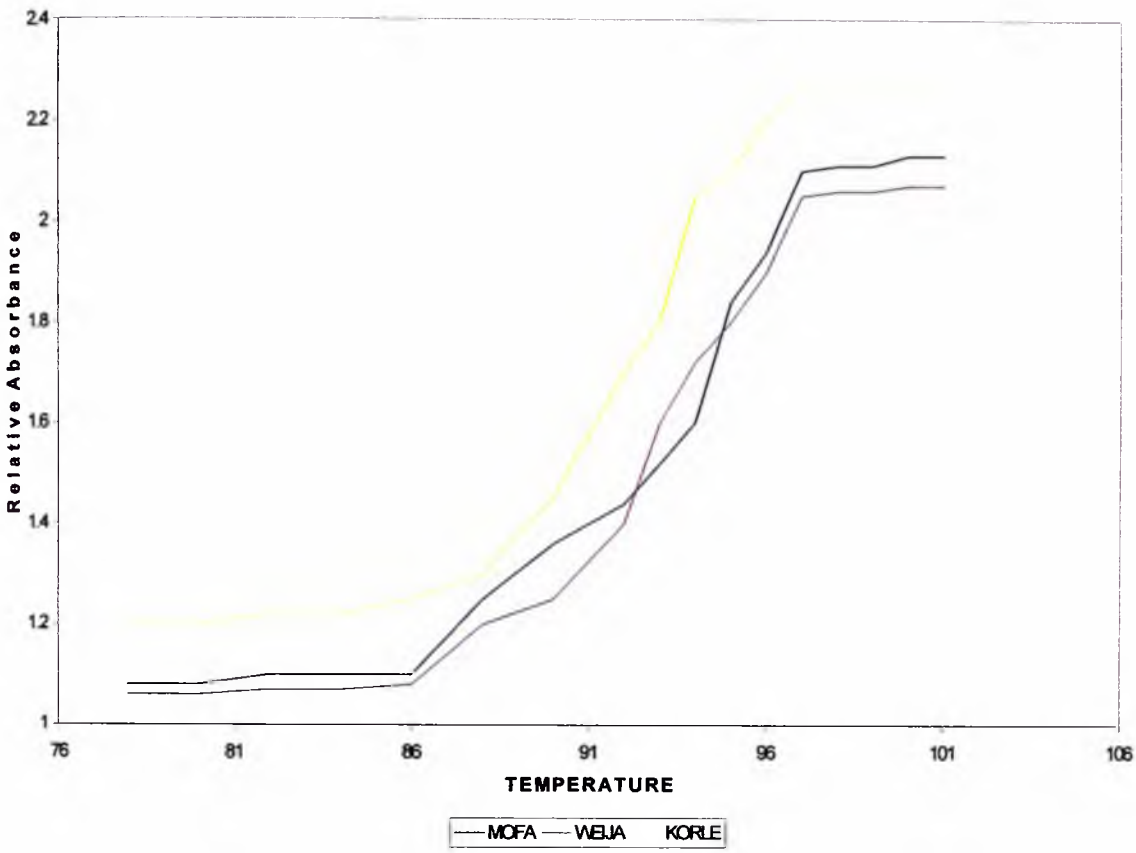

Fig 14: *Thermal denaturation curves of mtDNA for C. gariepinus obtained from the various water bodies.*

G+C base content of the purified mtDNA isolated from the various samples after homogenization was determined using ultraviolet absorption. The results are shown in table 6 and results for t-test of significance is shown in table 7.

Table 6: G+C base content of the mitochondrial DNA for *C. gariepinus* from different water bodies.

Source of *C. gariepinus*	UV Absorbance		Ratio	G+C content (Mean ± SD) mol%
	260nm	280nm	260/280	
Korle lagoon	0.608	0.349	1.74	58.21 ± 2.10
Weija Lake	0.176	0.101	1.75	57.93 ± 1.80
MOFA	0.249	0.138	1.80	57.69 ± 1.85

Table 7: Student's t-test of pairs of data on mean G+C base content.

Source of *C. gariepinus*	t-statistics	t-tabulated	P
Korle lagoon and Weija Lake	0.893	2.101	n.s
Korle lagoon and MOFA	1.506	2.101	n.s
Weija Lake and MOFA	0.668	2.101	n.s

The samples from the Korle lagoon had the highest G+C base content but statistically there was no significant difference between G+C base content of mitochondrial DNA in fish samples from the various sources.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

Pollution affects aquatic life worldwide, leading to the loss of valuable and edible fish species. In Ghana, the impact of pollution on the Korle lagoon and other heavily polluted water bodies has been documented (Biney, 1991). The studies reported in this thesis were designed to examine some of the biochemical and molecular changes that facilitate the survival of an organism in this lagoon. Thus, the first objective of this study was to identify an organism, preferably eukaryote, surviving in the Korle lagoon.

The presence of *Clarias gariepinus* as the only surviving fish species in the highly polluted Korle lagoon is in contrast to the report of the Korle lagoon ecological studies conducted by Biney and Amuzu (1995). According to that report, the grossly polluted state of the Korle lagoon had resulted in the complete loss of all fish species in the main lagoon body where the *C. gariepinus* for this study was found. The identification of *C. gariepinus* in the Korle lagoon suggests that other fish species might have adapted to the effect of toxic xenobiotics and are surviving in the lagoon. Therefore, further ecological studies of the main Korle lagoon need to be conducted in order to identify all fish species that have adapted to the toxic xenobiotics in the Korle lagoon.

Enzyme activities that are specific to inducible proteins were measured in order to ascertain the extent of protein induction due to pollution by xenobiotics discharged into the Korle lagoon. Enzyme activities measured for samples from the Korle lagoon were compared with those

obtained from relatively less polluted water bodies. The same comparison was for activities measured after acclimatization in the laboratory tanks filled with clean water. Cytosolic and microsomal protein concentrations were also measured.

The present study indicates that there were significantly higher cytosolic and microsomal protein concentrations in fish samples from the highly polluted Korle lagoon compared to those from the Weija Lake and MOFA. This is likely to be due to the presence of excessive amounts of pollutants in the Korle lagoon. These findings are consistent with previous investigations of protein induction in *Oreochromis niloticus* in response to xenobiotic pollutant discharged by the Akosombo Textile Limited (ATL) into the Volta Lake (Addy *et al.*, 1995), and also in *Sarotherodon melanotheron* present in the Fosu lagoon (Renner, 1998). These investigations clearly demonstrate that induction of both cytosolic and microsomal hepatic proteins occur in fish that are obtained from polluted water bodies compared to fish found in relatively pristine water bodies.

The mean total microsomal protein concentration for fish sample from the Korle lagoon was almost twice those from Weija Lake and MOFA. The total microsomal protein content of fish samples from the wild gave an indication of more protein induction in fish from the Korle lagoon, which reflects the extent of pollutants discharged into the lagoon. The differences in microsomal protein concentrations between Korle lagoon sample and the other samples after acclimatisation to laboratory condition for 21 days were significant. When fish from Weija lake and MOFA were acclimatised in tap water for 21 days in the laboratory, the decrease in microsomal protein concentrations was over 40%. Samples from the Korle lagoon showed the smallest decrease (32%) in microsomal protein. Inspite of the decrease, the protein concentration for fish from the Korle lagoon remained high after acclimatisation. Lech *et al.*,

(1982) reported that microsomal protein induction due to xenobiotics was reversed when feral fishes were acclimatised in tap water for 7 days. The results obtained in this study indicate that a much longer period of time in tap water is required for total depuration. It is also possible that in the case of fish from Korle lagoon, the pollution induction process cannot be reversed completely. Further studies need to be conducted to ascertain the time required for complete reversal of induction in the absence of an inducer, if this is possible.

There was no significant difference between protein concentration for fish from MOFA and Weija Lake after acclimatization to laboratory conditions. In each case, however, there was a significant decrease after depuration, indicating that those water bodies are also polluted but not to the same extent as the Korle lagoon.

The results of cytosolic protein concentrations of fish from the various water bodies showed trends similar to those of the microsomal protein concentrations, indicating induction of cytosolic proteins in the highly polluted Korle lagoon. Values for the Weija and MOFA samples were the same, comparing pre- and post- acclimatization results; the reduction in cytosolic protein was not statistically significant for both samples. This is in contrast to the results of the microsomal protein concentration. Difference between cytosolic protein concentration whether before or after acclimatization for MOFA and Weija samples were not statistically significant, suggesting this protein concentration to be the constitutive level of cytosolic protein of *C. gariepinus*.

Unlike the Weija Lake and MOFA samples, acclimatization caused a reduction in cytosolic protein concentration recorded for *C. gariepinus* from Korle lagoon. The decrease in protein level after depuration was however, not statistically significant but the actual protein level was

still much higher than those from Weija Lake and MOFA. As with the microsomal proteins, probably a longer period of depuration is required for the proteins to revert to constitutive levels. These results confirm the observation of induction of cytosolic protein as reported by Andersson *et al.*, (1985).

Researchers have put forward hypotheses to account for the elevated levels of proteins which include the presence of xenobiotics in aquatic environments (Andersson and Forlin, 1992) and temperature variation (Snegaroff and Bach, 1990). The presence of excessive xenobiotics in the Korle lagoon may contribute to the rise in total protein levels in *C. gariepinus* samples from the Korle lagoon. Temperature variation between the Korle lagoon and the other water bodies may also contribute to the difference in protein induction due to the continued or excessive microbial activities in the lagoon as compared to other water bodies.

The microsomal and cytosolic protein concentrations were determined primarily for the calculation of specific activities of enzymes and amount required for SDS-PAGE. However, the significant difference between fish samples from the Korle lagoon and those from the Weija and MOFA (relatively less polluted water bodies) suggests that in this type of study, total protein concentration, whether microsomal or cytosolic, could be used as pollution markers instead of the specific proteins, when facilities are limited for such specific assays.

In this study, different assays (i.e. EROD, PROD and PNP) were used to assess or characterise the type of hepatic monooxygenase activity, and hence the cytochrome P450 isozyme induced in *C. gariepinus* when exposed to water bodies with different pollution histories.

The EROD was used as a marker of CYP 1A induction by polycylic aromatic hydrocarbons (PAH). The results obtained for the EROD activity before and after acclimatisation to laboratory

conditions in clean water showed a lower activity for fish from MOFA and Weija Lake compared to fish from the Korle lagoon. The difference in EROD activity after depuration was insignificant for fish from MOFA and Weija Lake, suggesting that proteins responsible i.e., CYP 450 1A monooxygenase complex of *C. gariepinus* were at their constitutive level. Since these proteins are inducible, the results would further suggest that the two water bodies were devoid, or had low levels of PAH compounds that would induce the proteins that were monitored.

The results obtained before and after acclimatization suggests that the Weija Lake was the most pristine water body of the three, since there was no significant change in EROD activity and also the lowest EROD activity was recorded for the fish samples from the Lake. The EROD activity for fish from the Korle lagoon was at least, 10 fold higher than those from the Weija Lake before and after acclimatisation to laboratory conditions. This level of high enzyme activity is a reflection of massive induction of CYP 1A isozyme in the Korle lagoon samples, which also reflects accumulation of large amounts of PAH compounds. The significantly high EROD activity even after depuration suggests that fish from the Korle lagoon have adapted to their polluted habitat by producing an apparently high constitutive CYP 1A protein to get rid of the xenobiotic pollutants, which are always present in their environments. For this reason, this fish is able to survive in the highly polluted water body as opposed to other fish species, which were present before the onset of pollution (Biney, 1991). CYP 1A activity has been shown to be inducible by PAH in all fish studied so far in relation to pollution (Stegeman, 1989). This has been confirmed in studies of polluted water bodies in Ghana (Addy *et al.*, 1995; Renner, 1998). The results obtained in this study for *C. gariepinus* from the Korle lagoon are no exception. Since PAHs are the main components of industrial waste and are easily accumulated in aquatic

organism due to their lipophilic nature, these results with EROD activity confirm the polluted nature of the Korle lagoon.

The PROD enzyme assay is an indication of CYP 2B induction by phenobarbital and its related analogues. The PROD activity pattern was in contrast to the pattern observed for EROD activity; PROD activity was significantly lower for samples of fish obtained from the Korle lagoon than those from Weija Lake and MOFA. The percentage difference in activity between fish from the Korle lagoon and those from Weija Lake and MOFA were not as high as those obtained for the EROD activity. This observation in EROD and PROD activities in *C. gariepinus* is in contrast to findings made when similar studies were conducted in *S. melanotheron* from the Fosu lagoon (Renner, 1998), which showed that both EROD and PROD activity were elevated. Furthermore, where as the percentage increase in EROD activity for fish from that polluted water body was approximately 5 fold, the percentage increase in EROD activity recorded for this study was about 10 fold.

The results indicate that the induction of CYP 2B isozyme is minimal in the Korle lagoon and highest in the MOFA. The relatively low and insignificant percentage decrease in PROD activity recorded after acclimatization of *C. gariepinus* samples from the Weija Lake indicates that the Lake is relatively clean with respect to CYP 2B inducers and suggests that the PROD activity recorded is at constitutive level of this particular monooxygenase activity. This level was reached after depuration with the MOFA samples, again indicating that this was the constitutive levels in *C. gariepinus*. Furthermore the low PROD activity recorded for fish from the Korle lagoon which is in contrast to that of the Fosu lagoon (Renner, 1998) could be due to a high CYP 1A induction by PAH in the highly polluted Korle lagoon which suppressed the induction of CYP 2B. Induction of this CYP was generally low (picomolar quantities) in this

study, and these results are consistent with the general lack of induction of this CYP isozyme in fishes (Williams and Buhler, 1982).

The results for the PROD assay suggest that either there is absence of the inducer in the Korle lagoon or the other xenobiotics present suppress the induction of CYP 2B. The PROD results further suggest that the levels present is the constitutive level since the percentage differences between fish from the various water body were very low as compared to EROD and PNP activities, even though the differences were significant.

Increased PNP hydroxylase activity is an indication of CYP 2E induction by ethanol and its related analogues. This has been demonstrated in cultures of chick embryo and rat hepatocyte treated with ethanol and isopentanol (Louis *et al.*, 1993). The PNP hydroxylase activity prior to acclimatization was high in the Korle lagoon and MOFA samples compared to the fish from the Weija Lake. These results suggest that *C. gariepinus* found in the Korle lagoon and MOFA are exposed to relatively high levels of alcohol related compound which induced PNP hydroxylase activity. Fish from Weija Lake and MOFA environments during the acclimatization period eliminated the pollutant hence the decrease in PNP activity. The activity was low for fish from Weija Lake before and after acclimatization again indicating this water body to be the cleanest of the three. The reasons for the sustained PNP activity for the Korle lagoon samples after depuration is not clear but it is likely that the fish has adapted to high constitutive levels of PNP because of increase pollution levels.

A plausible reason for the significant level of induction of this enzyme for fish from the Korle lagoon may be that of excessive microbial fermentation process in the lagoon and the discharge from breweries and waste from the Korle Bu Teaching Hospital, which are close to the lagoon.

The high PNP activity for MOFA samples from the wild may be due to the presence of ethanol in aquarium due to the fermentation of left over feed by microbes. In the MOFA samples, unlike the samples from the Korle lagoon, this activity reduced considerably after acclimatization to laboratory conditions. The difference in enzyme activity between samples from the wild and those after acclimatization, with respect to fish from the Korle lagoon was not statistically significant. This is in contrast to the Weija Lake and MOFA samples and suggests that with the Korle samples, the induction of CYP 2E isozyme, just like that of CYP 1A continues even when the inducer is absent. The significantly higher PNP hydroxylase activity even after depuration (at least 3.6 fold higher than MOFA and Weija Lake samples) suggests that with fish from the Korle lagoon, CYP 2E protein is no longer induced but constitutively expressed to get rid off excessive alcohols which may be present in the environment continuously. There is also the possibility that the xenobiotics causing the induction are in the muscles or some other tissue of the fish, and it will take longer than 21 days to get rid of all of them. Unlike EROD, PNP hydroxylase activity in MOFA samples was high, but was reduced with depuration. This would suggest that in the MOFA samples, the enzyme is still inducible and not constitutively expressed as in the Korle lagoon samples.

The results obtained for overall monooxygenase enzyme activities in *C. gariepinus* from the various water bodies showed a heightened induction of CYP 1A and CYP 2E isozymes in Korle samples but not CYP 2B.

The pattern of cytosolic protein shown in fig. 11a and its corresponding scan in fig 11b, show that although the pattern of protein bands and peaks for fish from the various water bodies looked the same, the intensity of bands and the height of peaks from fish from the Korle lagoon were much higher than those from other water bodies. Like the enzyme activities these increase

in protein levels are due to the presence of xenobiotics in the Korle lagoon. Although the cytosolic fractions were diluted to give approximately the same protein concentration per gram of tissue before electrophoresis, yet the protein bands in the Korle lagoon samples were higher in intensity while samples of fish from the Weija Lake had the least intensity.

The protein band and peak intensity patterns are indications of the level of protein induction in the various water bodies with different pollution histories. The protein profiles and the monooxygenase activities confirm the induction of proteins. The EROD activity, which indicates the induction of CYP 1A by PAH, was significantly higher for the Korle samples compared to samples from MOFA and Weija Lake. Glutathione-S-transferase (GST) is a cytosolic protein which has been reported to be induced by xenobiotics specifically PAHs (Andersson *et al.*, 1985). This enzyme conjugates reduced glutathione with a number of xenobiotics. GST is a dimeric protein with molecular weight ranging from 23kD to 27kD. SDS-PAGE analysis of cytosolic protein from malignant human liver identified 3 isozyme protein bands of 24.8kD, 25kD and 26.7kD molecular weight for π, $\propto$, and μ-GST. GST purified from lindane induced *Spodoptera littoralis* larva had molecular weight of 27kD (Lagadic *et al.*, 1993).

The prominent band of approximate molecular weight of 24kD in the SDS-PAGE of the Korle samples may be due to GST. The presence of this band and the high EROD activity observed in fish from the Korle lagoon suggest the induction of GST by pollutants in the Korle lagoon, the bulk of which may be PAH.

The electrophoresis of cytosolic protein under non-denaturation conditions also showed a similar pattern. Under this condition, the secondary, tertiary and quartenary structures of the proteins

are maintained and proteins are separated in their native form. This native form makes separation very difficult because of the bulky nature of the native proteins.

The microsomal protein profiles (fig. 13a and 13b) indicate that samples from the Korle lagoon have high microsomal protein concentrations. CYP 450s are microsomal proteins and the levels normally depend on the presence of exogenous compounds, which cause induction of more CYP 450 proteins and increase the level above their constitutive levels. Studies have shown that the molecular weight of benzo(a)pyrene-induced CYP 450 protein ranges from 51kD to 58.6kD (Fuch *et al.*, 1992; Gilewich *et al.*, 1987; Kirchin and Winston 1992). The SDS PAGE analysis of microsomal fraction indicated the presence of two prominent protein bands of 48kD and 53.7kD molecular weight in the protein profile for the Korle samples. These may be proteins induced by PAH, which are responsible for the high EROD activity recorded for the Korle samples. The intensity of this band was low in samples from both MOFA and Weija Lake. Another prominent band corresponding to a high molecular weight of 76kD is present in all samples. This protein may be associated with electron transfer, since other studies have identified proteins of molecular weight ranging from 76.4kD to 80kD that are associated with electron transfer in PAH induced systems (Koener et al., 1993; McManus *et al.*, 1989). This protein is NADPH dependent cytochrome P450 reductase, which is one of the major components of the monooxygenase enzyme system. Previous work done by Renner (1998), showed significantly high reductase activity in fish obtained from the highly polluted Fosu lagoon. Thus the prominent 76kD protein band in the profile for the Korle sample is likely to be NADPH dependent CYP 450 reductase.

The microsomal protein measurements (fig 6) and their SDS-PAGE (fig 13a and 13b) suggest that MOFA was the most pristine among the water bodies studied. These results are, however,

not consistent with the three pollution-induced monooxygenase activities (EROD, PROD and PNP) measured, indicating that the Weija Lake was the least polluted. This provides evidence for the need to use pollution specific enzyme activities to assess the extent of aquatic pollution when pollution has not reached its climax. The high peaks recorded for microsomal protein profile for fish from the Korle lagoon samples confirm the enzyme assays, that the Korle lagoon is the most polluted among the water bodies studied.

The presence of three distinct bands of low molecular weight of 32kD, 34.7kD and 35.8kD and their corresponding peaks labelled X (fig 13b) may suggest the induction of those proteins by some xenobiotics in the Korle lagoon. These bands were faint and virtually absent in the profile for the Weija Lake and MOFA samples.

The genomic DNA composition of a particular species or organism remains very closely the same and changes occur via recombination or via mutations. The changes that occur in eukaryote are at a slower rate as opposed to changes occurring in prokaryotes. Studies have revealed that carcinogenic alkylating agents modify mtDNA more than nuclear DNA of the same species by 5 fold (Wunderlick *et at.*, 1970). The mtDNA has been identified as the genetic target of lipophilic contaminants (Allen and Coombs, 1980) hence the probability of enhanced mutation in the mtDNA is high. The mtDNA base composition determined in this study for fish from the various water bodies showed a higher G+C base composition for the Korle samples, compared to the other two samples but statistically the differences were not significant. Thus, there might be PAH-induced mutation in the Korle lagoon samples but either they do not affect the G+C bases or the changes are not significant, comparing the size of the mtDNA and the number of bases that have mutated.

Dunn *et al.*, (1987) reported of mutation in DNA by PAH and other xenobiotics. This mutation preferentially occurs in the mitochondria due to the absence of the editing function of the replication enzyme complex. The high EROD activity recorded for fish from the Korle lagoon suggests the presence of large of amounts of PAH, which could cause mutation in the mitochondrial DNA. The insignificant difference in G+C composition recorded in this study suggests that there are no significant PAH induced mutation in the mtDNA which affects these two bases.

The negative relationship between the genetic diversity and contaminated site imply that the mutation rate is not significant in the population of *C. gariepinus* in the Korle lagoon. It is likely restriction digest of the mtDNA, e.g. Restriction Fragment Length Polymorphism, analysis would reveal some differences, and since some of the xenobiotics-induced mutation may occur within the recognition sequence of restriction endonucleases. DNA adduct studies (*Ericson et al.*, 1998) could determine the effect of PAHs on DNA in fish found in contaminated environment and could have provided a better insight. These studies were not carried out for lack of resources.

It appears that the xenobiotics affected the genomic DNA in such a way as to cause inducible gene to be expressed constitutively but do not significantly influence the composition of bases in *C. gariepinus* mtDNA.

The G+C base composition recorded for *C. gariepinus* in this study ranged from 55.84 to 60.42 mol % (mean: 57.94 ± 2.50 mol %). This value was higher than the range of 36.1 to 41.8 mol % G+C base composition recorded for teleostean fishes by Gold and Korel (1988) using the same denaturation method used in this study and developed by Mandel and Marmur (1968).

The differences in mol % recorded in this study and that of Gold and Korel may be due to differences in the organisms used.

In conclusion, the present study has confirmed the claims by the indigenous people living in the vicinity of the Korle lagoon that fish identified as *C. gariepinus* is present in the lagoon in contrast to studies conducted by Biney (1991).

The presence of hepatic proteins with high EROD and PNP activities as well as high intensity of protein bands and peak height for fish samples from Korle lagoon reported here is an indication of the effect of pollution in the lagoon. CYP 1A and CYP 2E proteins appear to be no longer induced but constitutively expressed to get rid of excessive xenobiotics which are always present in the lagoon. In addition to the microsomal CYP 450, the reductase enzyme also appears to be induced, allowing an efficient CYP-dependent monooxygenase activity, which enables this organism to live in the highly polluted water body. Therefore this organism combines its natural ability to dwell in mud with its increased ability to induce the proteins responsible for biotransforming and eliminating excessive amounts of xenobiotics

There is the need to extend these studies to cover GST and NADPH-dependent CYP 450 reductase enzyme activities and also the actual measurement of CYP 450 protein content. Furthermore, there is the need to isolate, purify and study the characteristics of the proteins that assist this fish to survive in its environment in order to exploit their properties in the treatment of industrial waste of compounds similar to that found in the lagoon.

Further molecular studies need to be done to consolidate what has been achieved in this study. These include both mitochondrial and nuclear DNA analysis using restriction endonucleases DNA adduct studies and other genetic tools to ascertain the real impact of pollution on the genetic material of *C. gariepinus* in the lagoon.

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APPENDIX 1

1. **Homogenizing buffer pH 7.4**

This was prepared by dissolving 15.601g $NaH_2PO_4.2H_2O$ and 11.4822g KCl in 500ml distilled H_2O. An amount of 0.372g EDTA, 0.1543g DTT and 100.5ml 99.5% glycerol were added and stirred and pH adjusted 7.4 with few drops of conc. NaOH and volume made up to the 1000ml mark. The resulting buffer contains O.1M NaH_2PO_4, 0.15M KCl, 1mM DTT, 1mM EDTA and 10% glycerol.

2. **Storage buffer**

The same as homogenising buffer except the percentage of glycerol which was increased to 30%.

3. **Separation gel buffer: 1.5M Tris-HCl pH 8.8**

This was prepared by dissolving in 36.3g Tris in 100ml deionized H_2O and 17.5ml 5M HCl added. The pH was adjusted to 8.8 with few drops of Conc NaOH and the volume made up to 200ml mark.

4. **Stacking gel buffer :0.5M Tris-HCl pH 6.8**

This was prepared by dissolving 6.05g Tris in 60ml dissolved H_2O and 9.5ml 5M HCl added. The pH was adjusted to 6.8 and the volume made up to 100ml.

5. Acrylamide monomer solution

An amount of 58.4g acrylamide and 1.6g Bisacrlylamide were dissolved in deionized water and made up to the 200ml mark. The solution was filtered and stored in the dark. The final concentration of the monomer solution was 30% acrylamide and 2.67% bisacrylamide.

6. Electrophoretic tank buffer pH 8.6

This was prepared by dissolving 30g Tris, 144g glycine and 10g SDS in 1 litre deionized water. (For native gels, the tank buffer did not contain SDS).

7. Staining and fixing solution

This was made up of methanol, glacial acetic acid and deionized water in the ratio 40:7:53 and 0.1g coomassie brillant blue R250 (For every 100ml solution).

8. Destaining solution

The same as the staining and fixing solution but lacked the Coomassie brillant blue 250.

9. 50mM Tris - 20mM EDTA pH 8.0

An amount of 1.5138g of Tris and 1.8661g EDTA were dissolved in 200ml doubled distilled water (DDW) and pH adjusted to 8.0. The solution was topped to 250ml mark autoclaved and stored at 4°C.

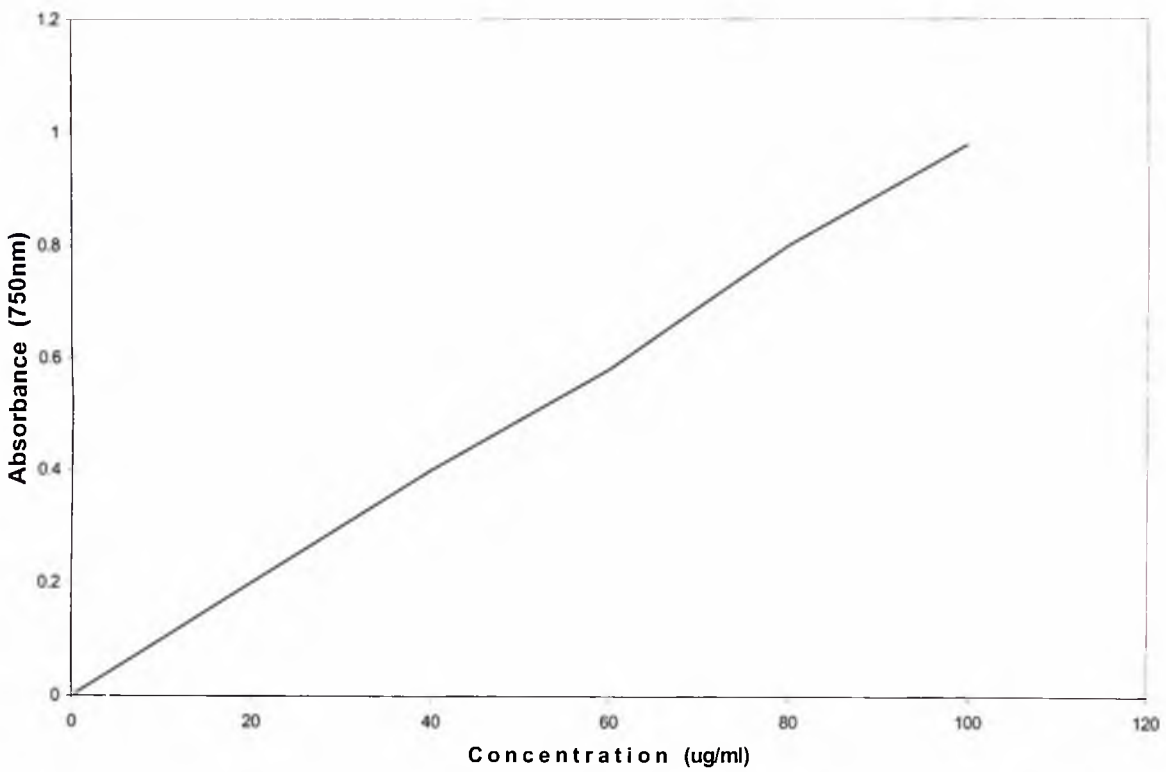
10. 10X gel buffer : TAE pH 8.3

This was prepared by dissolving 24.22g Tris acetate and 3.722g EDTA in 400ml double distilled water (DDW) and the pH adjusted to 8.3. The resulting buffer contain 400mM Tris acetate and 20mM EDTA. The solution was autoclaved and stored at 4°C.

11. Standard saline citrate buffer pH 7.0 (SSC).

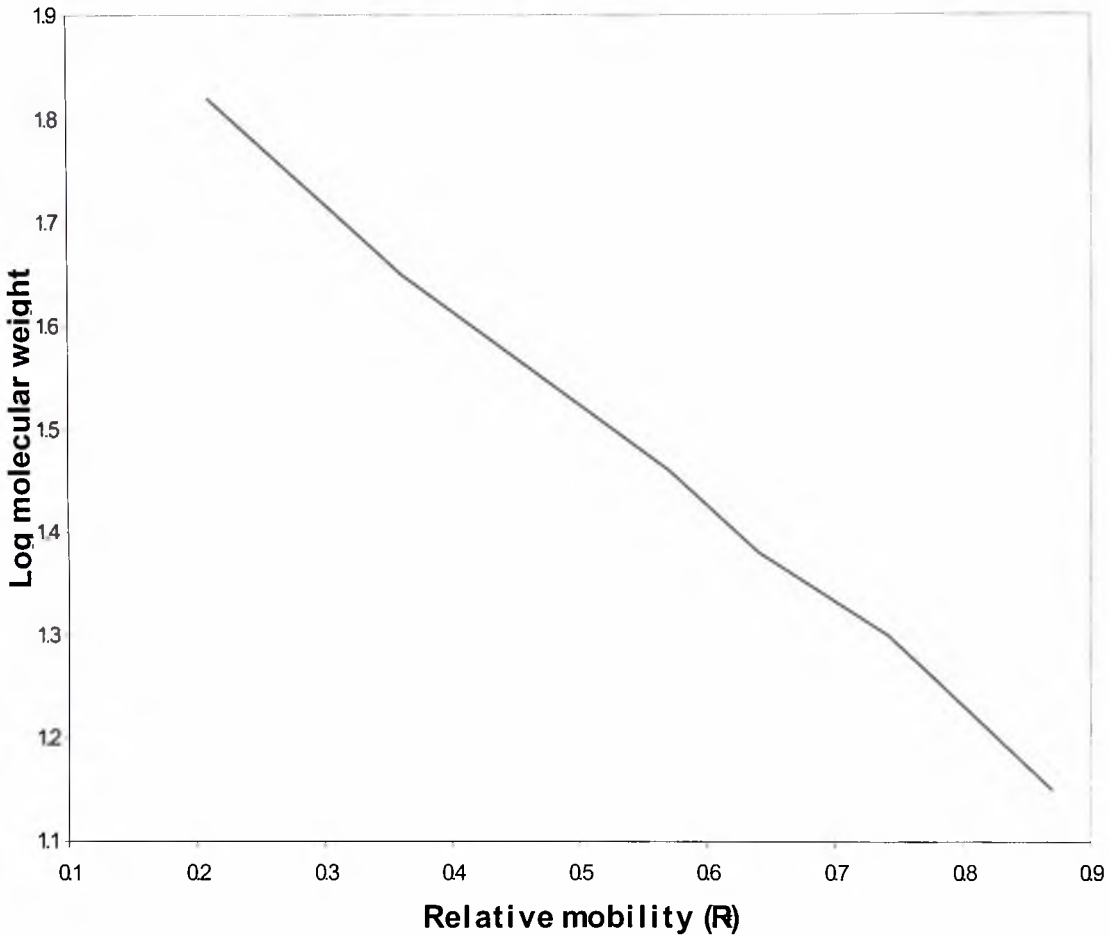
This was prepared by dissolving 0.8775g NaCl and 0.4412g Trisodium Citrate in 90ml DDW. The pH was adjusted to 7.0 and the volume topped up to the 100ml mark. The resulting SSC contained 0.15M NaCl and 0.015M Na_3 Citrate.

APPENDIX 2



Bovine Serum Albumin standard curve for protein determination

APPENDIX 3



A graph of molecular weight standards against R_f for the determination of molecular weight of proteins.

$$R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$