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# CYTOCHROME P-450 MONOOXYGENASE COMPLEX AND GLUTATHIONE-S-TRANSFERASE IN *SAROTHERODON MELANOTHERON* AS BIOMARKERS OF POLLUTION.

A THESIS

BY

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

SEPTEMBER, 1998.

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## DECLARATION

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.

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STUDENT

DATE 21st Oct 1999 SIGNATURE M. Ewurama Addy

SUPERVISOR

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# DEDICATION

TO MY WIFE, MARY ARHIN AND MY SON, CHRISTOPHER RENNER JNR., FOR THEIR UNCEASING LOVE AND CARE.

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**ACKNOWLEDGEMENT.**

With genuine appreciation, I wish to express my hearth-felt gratitude to all those who have contributed in diverse ways to the successful completion of this thesis.

First of all, I gratefully acknowledge the Noguchi Memorial Institute for Medical Research (NMIMR), Legon for providing such excellent facilities without which the laboratory phase of this project could not have been undertaken. To this end, I am thankful to Dr. G. Armah, Head of Electron Microscopy Unit and Mr. Michael Fokuo Ofori, Research Assistant of the Immunology Unit.

I am greatly indebted to my supervisor, Prof. Marian Ewurama Addy whose guidance, unselfish, constructive criticisms and invaluable suggestions enabled me to plan and execute this work. I am particularly appreciative of the promptness with which Prof. Addy attended to all the problems and needs that frequently cropped up during the laboratory work of her students.

Mention must be made of Mr. Lambert Yirbaar Faabeluon, of the Environmental Protection Agency (EPA), for his unmeasurable services and moral support which he rendered for this research work.

This work could not have been done without the appreciated help

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of Mr. Ankrah and his team from the Volta Basin Research Project (VBRP) office. They provided the fish from the Volta lake.

I am also grateful to the National Agricultural Research Project (NARP) for providing the financial support for this study.

Last, but not the least, I wish to thank all my brothers and sisters, colleagues and friends for the concern and contribution to the successful completion of this thesis, especially Papa Ankor for buying me a desktop computer.

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## Abstract

Samples of *Sarotherodon melanotheron* obtained from water bodies of different pollution histories, that is, a site on the Volta lake near Ada, the Fosu lagoon at Cape Coast and aquaria containing the herbicide, 'Roundup', were used to investigate induction of hepatic cytochrome P-450 monooxygenase enzyme system (CYP) and glutathione-s-transferase (GST) in response to pollutants.

Total microsomal protein concentration was determined by the Folin-Lowry method. The activity of NADPH cytochrome P-450 reductase, a component of monooxygenase enzyme system, was measured, using reduction of exogenous cytochrome C. Another component, cytochrome P-450, was measured using CO-difference spectrum. The overall monooxygenase activity was determined using 7-ethoxyresorufin-O-deethylase (EROD) and 7-pentoxyresorufin-O-depentylase (PROD) assays, which indicated specifically, the induction of cytochrome P-4501A and P-4502B isoenzymes respectively. Glutathione-s-transferase activity was measured using 1-chloro-2,4-dinitrobenzene as the substrate.

The results for all the biochemical parameters measured, indicated that the levels of those of the fish from Fosu lagoon were excessively higher than the values recorded for fish from Volta lake and the aquaria, indicating a relatively higher pollution level in the Fosu lagoon. For the CYP the levels measured for both fish from

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the Fosu lagoon and the aquaria were almost similar, indicating higher induction of CYP by 'Roundup'. For the EROD and PROD determinations for the monooxygenase system, activities were detected in livers of fish from Fosu lagoon and the Volta lake. No such activities were detected in fish exposed to 'Roundup', even though CYP induction was high and these monooxygenase enzyme activities were found prior to herbicide exposure. The activity of EROD was 3.5 fold higher than that of PROD, indicating less PB-types of inducers than that of 3-MC type in the Fosu lagoon.

This study has indicated that the Fosu lagoon is polluted with 3-MC types and PB-types of inducers and that the 'Roundup'-induced CYP is not only different from the CYP isozymes responsible for the EROD and PROD assays, it actually inactivates them. GST induction indicated in this study would support the effects of the induced CYP in biotransformation of the xenobiotics which enter the fish in polluted waters.

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## LIST OF ABBREVIATIONS.

| | |
|---|---|
| AhR | Aromatic hydrocarbon receptor |
| arnt | Ah-recptor nuclear translocator |
| ATL-E | Akosombo Textile Limited Effluent |
| BKME | Bleached Kraft Mill Effluent |
| BNF | β-naphthoflavone |
| cDNA | Complementary deoxyribonucleic acid |
| CDNB | 1-Chloro-2,4-dinitrobenzene |
| CYP | Cytochrome P-450 monooxygenase enzyme |
| DDE | Dichlorodiphenyldichloroethane |
| DDT | Dichlorodiphenyltrichloroethane |
| DMSO | Dimethylsulfoxide |
| DTT | Dithiotreitol |
| EAP | Economic adjustment program |
| E.C. | Enzyme Commission |
| EDTA | Ethylenediaminetetraacetic acid |
| EIFAC | European inland Fishries advisory commission |
| ELISA | Enzyme-link immunosorbent assay |
| EROD | Ethoxyresorufin-O-deethylase |
| FAC | Fluorescent aromatic compound |
| FAO | Food and Agriculture Organization |
| GSH | Glutathione (reduced) |
| GST | Glutathione-S-transferase |

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| | |
|---|---|
| hsp90 | Heat shock protein 90 |
| ICES | International commission for exploration of the sea. |
| $LD_{50}$ | Lethal dose of toxicant that will kill 50% of a given group of organisms. |
| MFO | Mixed function oxidase |
| MT | Metallothionein |
| NARP | National Agricultural Research Project |
| OC | Organochlorines |
| PAH | Polyaromatic hydrocarbon |
| PB | Phenobarbital |
| PCB | Polychlorinated biphenyl |
| PCDD | Polychlorinated dibenzo-p-dioxin |
| Ph | Benzene |
| Tris | Tris(hydroxymethyl)aminomethane |
| XRE | Xenobiotic Response Element |
| $\varepsilon$ | Extinction coefficient |
| 3-MC | 3-Methylcholanthrene |
| 7-ER | 7-Ethoxyrosorufin |
| 7-PR | 7-Pentoxyresorufin |

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# CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

## 1.1 General Introduction.

One of the basic premises for sustainable development is the recognition that the environment and development are not exclusive of one another, but are complementary and inter-dependent and, in the long run, mutually reinforcing.

Environment should not suffer because of man's industrial development or economic growth. However, as industrial and human waste increase with the growth of new factories and populations, the aquatic environment is increasingly used as humanity's dustbin. Toxic metals and chemical pollutants enter the surface waters through waste discharge, agricultural runoff, leachate from toxic dumps, accidents and illegal dumping. Increasing pollution of the aquatic environment is becoming a threat to the health of organisms inhabiting such ecosystems as well as to man, a predator or consumer of such organisms (Dawe, 1990).

Developing countries rely mainly on their natural resources for their economic growth. As such the need for economic growth comprises a rational exploitation of the resource base. Industrialization in these countries has been associated with the discharge of all kinds of waste (the types and quantities of which are not fully known) into the aquatic environment (which provides the bulk of the protein requirements) without any form of treatment. In most of these countries, the pollution problems have assumed serious dimensions and resulting in genetic as well as physiological disorders in fishes

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have been shown by Malins and Collier, (1981) and Heath and Moore (1985) to reflect heavy pollution with xenobiotics such as oil and oil products, pesticides and heavy metals by man's activities. Some of the problems of pollution have also been shown by Porter and Conn (1991) to result from elevated levels of toxic pollutants such as polyaromatic hydrocarbons(PAH's), polychlorinated biphenyls (PCB's), organochlorines (OC's), dyes, dibenzofurans and dioxins.

If proper industrial environmental controls and the management of hazardous chemicals are not put in place, environmental pollution will be quite pronounced in the near future. There is, therefore, the need to obtain information on the types and quantities of xenobiotics that are discharged into the aquatic environment, to identify indicator organism that can be used to study and to develop a fast, cheap, reliable and widely applicable tool in order to obtain objective information on the impacts of human pressures on aquatic systems.

In monitoring for environmental contamination, biological indicators (biomarkers) have been developed into an informative tool for detecting exposure and effects of chemical pollutants (McCarthy and Shugart, 1990). Biomarkers have been developed as "biological responses that can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals" (Peakall, 1994). Thus, biomarkers are responses of living organisms that may simply signify exposure to contaminants, may predict future harm, or may themselves be of harmful effect. In other words, a biomarker is a biochemical, physiological, morphological or behavioral response that may be studied in a particular species to assess the status of that species, or in a sentinel species to assess habitat status.

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A study of these biomarkers could enable government legislate mandates regarding release, disposal, treatment and recovery of hazardous wastes into water bodies to avoid damage to the environment. This will also help, for example, to maintain their capacity for sustenance of harvestable aquatic food resources (such as fish) to ensure a safe product for human consumption.

The effect of exposure to environmental pollutants can be classified and studied at three different levels of the biological organisation viz; biochemical effects at the molecular and cellular levels, physiological and pathological effects at tissue, organ, organism level and ecological effects at the population, community and ecosystem level. The direct effect of the xenobiotic occurs at biochemical level and if the defence and repair mechanisms of the cell are unable to handle the challenge, the impact will be transmitted upward to the physiological or pathological and eventually to ecological level.

Concerns about the many chemicals present in the environment range from possible harmful effects on flora and fauna to possible harm to humans consuming such organisms. Complete analyses of these contaminants, even if possible, would only establish the presence of these chemicals, without revealing how available or active they were within the organism (Nott and Nicolaidon, 1990).

Enzymes, proteins and co-factors involved in the binding, biotransformation, and excretion of xenobiotics in aquatic environment as well as intermediate/metabolites produced by the biotransformation process have been proposed as specific biochemical indicators of

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xenobiotic exposure (Payne, 1976; Payne *et al*, 1987)

Several biochemical assay systems showed great promise, some of which were based on carbohydrate, proteins and fatty acid metabolism and blood chemistry (EIFAC, 1975; ICES, 1986; Olsson, 1987). However, two most promising and advanced biochemical methods which have been explored are dependent on elevated levels (induction) of protein biomarkers; one sensitive to metal ions -metallothioneins and the other, cytochrome P-450 monooxygenase system (EIFAC, 1975; Olafson *et al*, 1979; Stegeman, 1981; Olsson, 1987). The same monooxygenase system in tissues other than the liver is used to metabolize endogenous substrates such as arachidonic acid, fatty acids and steroids.

The liver microsomal mixed-function oxygenase complex and glutathione-s-transferase, one of the phase II enzymes, are specially induced by xenobiotics and as such, their levels reflect the amount of pollutant taken by fish (Anderson *et al*, 1989, Vigano; *et al*, 1993). These have been used to establish baseline and pollution levels in fish in the aquatic/marine environments such as Hvale Archipelago in Norway(Goksoyr *et al*, 1991), where PAH, PCB, DDT/DDE, dibenzofuran dibenzo-p-dioxin levels in fish and water or sediment were estimated both chemically and biochemically.

In Ghana, as in any other developing country, there is increasing evidence that excessive demands are being made on limited resources and the carrying capacity of the fragile ecosystem. Improper siting of industrial installations in relation to other activities and residential housing poses considerable problems. The quantity and diversity of industrial waste have increased over the years (Ghana EPA, 1994).

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The major producers of industrial pollutants in the country are textile industries and industries which deal with food processing, petroleum refining and handling and mineral exploitation. For a long time, gold mining has been the most important mining industry in Ghana, one of the major producing countries in the world. However, the nature of gold deposits in Ghana and the process of mining have been contributing toward the pollution of the environment. Analysis of inland waters showed the presence of heavy metals: Hg, Cu, Zn, Cd, Fe, Cr, Mn, and As in the sediment of these waters (Akoto-Bamford *et al*, 1990). Most of the municipal and industrial effluents are pumped directly into coastal waters without any form of pre-treatment and raw sewage is channeled into shallow waters, bays and lagoons. There is hardly any waste recycling or proper management practices in the country.

The yield from fisheries and aquaculture is an important and easily available source of protein. In Ghana, fish consumption exceeds that of meat, and the fishing industry is important in the country's economy (Hinrichsen,1990). According to FAO, (1989), fish proteins are comparable to beef and thus serve as the closest alternative. In Ghana fish contributes about sixty percent of animal protein and inland (freshwater) fisheries provide about forty percent of fish protein in Ghana.

It is estimated that tilapia species account for over sixty percent of catches landed in countries like Ghana. This fish is also known to be widely distributed throughout the country, and therefore a fish of economic importance. Its preserved form is popularly called "Koobi".

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Tilapia serves as a protein source in many Ghanaian homes. It is the main species involved in both commercial and small scale fish farming and hence provides jobs as well as game for some Ghanaians. Tilapia, in its preserved form, "Koobi", has become the major trading commodity for some women in Ghana. It is therefore important to preserve the industry by averting pollution of the aquatic environment. Shown in Fig. 1a is the Fosu lagoon and its immediate environment.

The overall aim of this research is to demonstrate that(the presence of the biomarker of pollution) NADPH-dependent cytochrome P-450 monooxygenase enzyme complex could be used as a biomarker of pollution in the tilapia, *Sarotherodon melanotheron*, an economically important species, and to find if glutathione-s-transferase, a phase II enzyme, is inducible and can be used as a biomarker in this species.

The specific objectives are to:

i. measure the cytochrome P450 monooxygenase complex and glutathione-s-transferase (CYP and GST) levels in fish from pollution-free environment respectively.

ii. measure the CYP and GST levels in fish exposed to a herbicide, 'Roundup' under laboratory conditions.

iii. measure the CYP and GST level in the fish found in a heavily polluted body of water, the Fosu lagoon.

iv. compare the levels of CYP and GST in fish from these three different environments.

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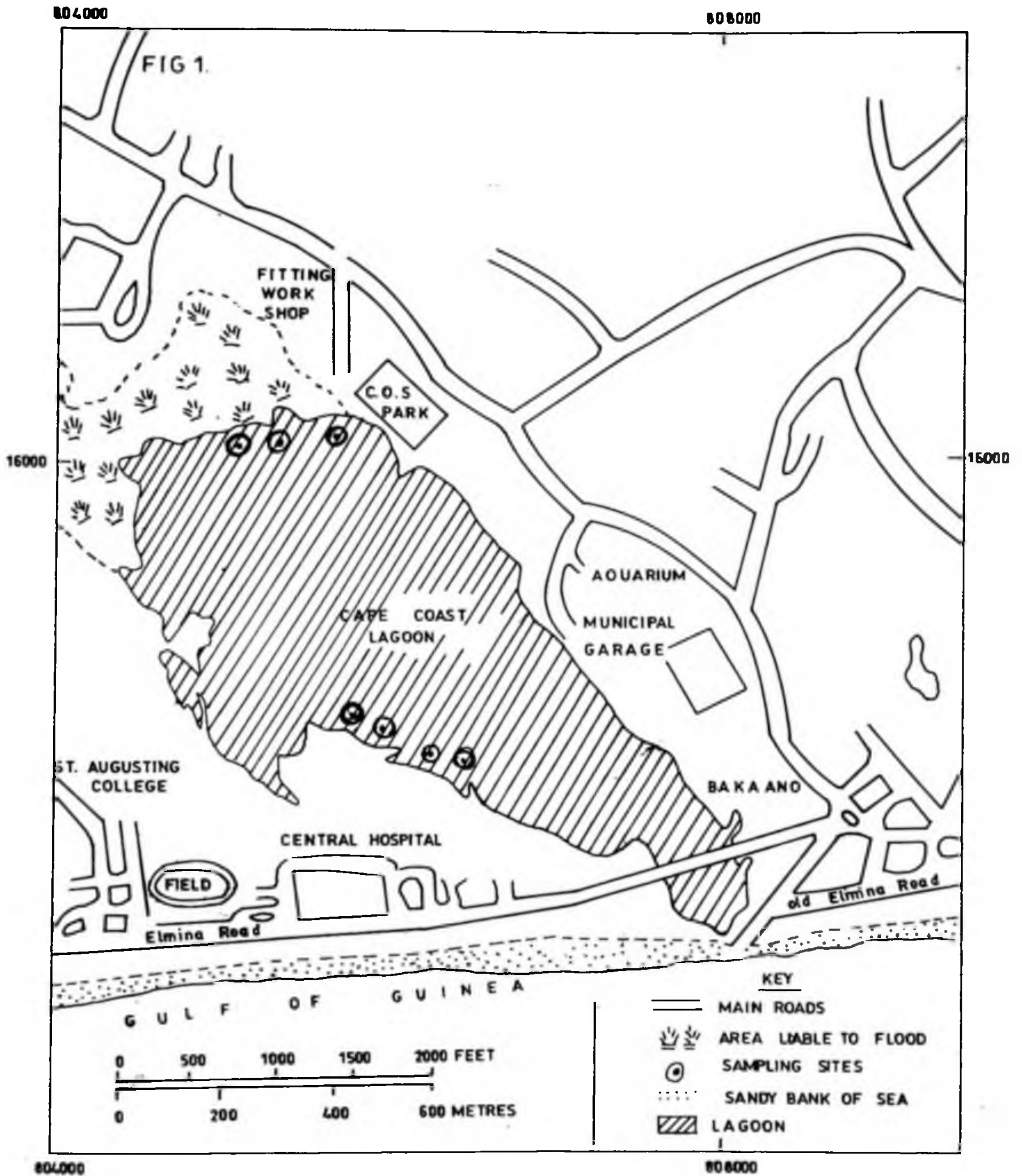


Fig. 1a. The Fosu lagoon and its immediate environment.

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## 1.2 LITERATURE REVIEW

### 1.2.1 Cytochrome P-450 Monooxygenase Enzyme System

The cytochrome P-450 (CYP) enzyme system or complex (E.C. 1.14.14.1, unspecified monooxygenase) comprises a large and expanding superfamily of structurally and functionally related heme proteins that biotransform both endogenous and exogenous compounds. The endogenous compounds include fatty acids, steroids, prostagladins and bile salts and the exogenous ones include polycyclic aromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCBs), dioxins, pesticides, petroleum products, drugs and food additives (Ortiz de Montellano and Correia, (1995; Nelson *et al*, (1996).

The cytochrome P-450 monooxygenase enzyme activity has been incorporated into one of the recent assay systems being employed in monitoring pollutants such as heavy metals and organic compounds due to the sensitivity of the enzyme (Heath and Moore, 1985). The reactions catalysed by these proteins are of a type generally referred to as monooxygenase (oxidative phase I) reactions, formerly mixed-function oxidase, and are considered important in detoxification pathways. The key step in the monooxygenation reaction is the insertion of one atom of molecular oxygen into the substrate. In order to effect the metabolic biotransformation and detoxification, various reactions, catalysed by this enzyme system, follow the initial oxygenation step. Some of these reactions include epoxidation, hydroxylation, deamination and deethylation (Gillette, 1966) enabling the enzyme system to operate on a wide range of substrates in reactions of the so-called Phase I metabolism. The

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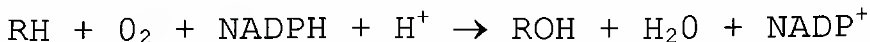
overall monooxygenase reaction catalysed by CYPs is:



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

In all vertebrates, hepatic microsomal monooxygenase systems convert lipophilic xenobiotics, drugs and endogenous compounds, represented by R-OH in the above equation to water soluble products (RH). This is the Phase I metabolism which involves a change in the molecular structure of the substrate, as a result of the introduction of polar groups into the xenobiotic molecule. The Phase II reactions involve conjugation of the products of Phase I reaction with polar endogenous compound (Smith, 1968). The less harmful conjugation products are then voided. The purpose of the Phase II reaction is to eliminate metabolites of xenobiotics.

Induction of cytochrome P-450 monooxygenase enzyme complex, which metabolizes organic pollutants, was the method focused on in this project since early work done on rainbow trout (*Oncorchyuchus mykiss*), Atlantic cod (*Gadus morhua*), plaice (*Pleuronectes platessa*), perch (*Perca fluviatilis*) (Goksoyr, 1985a), scup (*Stenotomus chrysops*) (Klotz *et al*, 1983) and tilapia species - *Oreochromis mossambicus* (Ueng *et al*, 1992), *O. niloticus* and *Sarotherodon galilaeus* (Faabuloun, 1995) has shown the induction of these enzyme systems in fish as a result of pollution.

The monooxygenase enzyme complex, often found in the endoplasmic reticulum, is also referred to as the microsomal mixed function oxygenase (MFO). Another protein, glutathione-s-transferase (GST),

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which metabolises the products of MFO's biotransformation of xenobiotics will also be studied in this project. Work on this protein which is cytosolic has also been done on rainbow trout (*Oncorrhynchus mykiss*) (Vigano *et al*, 1993). It uses endogenous tripeptide, glutathione, to conjugate the products of cytochrome P-450 monooxygenase enzyme system in the liver and eliminate them through the bile.

**Tissue and Cellular Location**

The liver is the major site of CYPIA (a specific CYP isozyme) activity both in mammals and fish, consistent with the role the liver plays in biotransformation of xenobiotics. However, CYPIA induction has been observed in other tissues including those proximal to the environment such as gill (Miller *et al*, 1989), intestine (Van Veld, 1990), and olfactory epithelium (Stegeman *et al*, 1991).

In higher animals, the cytochrome P-450 monooxygenase system is also found in endoplasmic reticulum of cells of the gastrointestinal tract, lungs, kidney and the spleen (Fang and Strobel, 1978). The enzyme is also known to occur in the mitochondria of the adrenal cortex (Watanuki *et al*, 1978) as well as in the nuclear membrane of hepatocytes. In addition to the mammals, cytochrome P-450 monooxygenase has been
shown to be present in birds, frogs ,snakes and fishes.

Lee, (1981) worked on invertebrates and showed them to involve cytochrome P-450 monooxygenase system in the metabolism of xenobiotic and endogenous chemicals. Ambike *et al*, (1970) demonstrated the

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presence of cytochrome P-450 in a fungal strain, while Ferris *et al* (1976) showed the existence of P-450 enzyme system in various strains of fungi.

Histoimmunochemical analyses have identified tissues and cell types expressing CYPIA activity. The evidence of CYPIA associated staining has been reported in gill pillar cells, gill epithelial cells, hepatocytes, enthothelial cells of heart and vasculature in general, gut mucosal epithelium, renal tubules, interrenal cells, nasal epithelium and pseudobranch. (Goksoyr *et al*, 1987; Lorenzana *et al*, 1988; Smolowitz *et al*, 1991;1992 Stegeman *et al*, 1991; Lindstrom-Seppa *et al*, 1994)

When the smooth endoplasmic reticulum cells are physically disrupted by the process of homogenization, there is an active "pinching off" of membranes giving closed vesicles called microsomes. Microsomes are proteinaceous lipoidal spheres that are then isolated via a series of steps with the ultimate recovery through high speed centrifugation at 105,000 x g or by calcium aggregation or by acid precipitation (Mazel, 1971); (Schenkman and Cinti, 1972)..

The method used in this project to isolate microsomes as source of enzymes was differential ultracentrifugation. Although the method requires the use of expensive ultracentrifuges and long hours of centrifugation, it has an added advantage of sedimenting both smooth and rough endoplasmic reticulum. The rough ones are devoid of haemoglobin and hence good for spectral and enzymic studies.

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**Composition**

Strobel *et al*, (1970) successfully solubilized and resolved hepatic microsomes into three fractions, which when reconstituted, catalysed the oxidative biotransformation of fatty acids, prostagladins, steroids, drugs and other xenobiotics. The essential components of the monooxygenase system are cytochrome P-450, NADPH-dependent cytochrome P-450 reductase and a phospholipid (Yasukochi and Masters, 1976). Resolution of mammalian P-450 monooxygenase on SDS-PAGE showed it to contain the components named above (Gibson and Schenkman, 1978).

Cytochrome P-450 is an oligomeric complex containing between two and ten protein subunits, with molecular weight range of 45-65kDa. This component also contains iron protoporphyrin IX in its prosthetic group. The name cytochrome P-450 was established by Omura and Sato (1964) and was subsequently found to be a membrane-bound hemoprotein with an absorption maximum at 450nm when reduced by NADPH or dithionite and complexed with carbon monoxide (Imai and Sato, 1966).

The phospholipid was found mainly to consist of phosphatidylcholine with traces of phosphatidylinositol and phosphatidylethanolamine. The phosphatidylcholine alone accounts for 30-40% of the total phospholipid.

**Reactions Catalysed**

The first description of metabolism of xenobotic compounds by hepatic microsomes was given by Mueller and Miller, (1953) in which they showed that liver homogenates catalysed both the reductive splitting of azo linkages and oxidative N- demethylation of aminoazo dyes.

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Brodie *et al*, (1955) showed that a similar enzyme system localized in hepatic microsomes was responsible for the metabolic biotransformation and subsequent disposition of drugs and other xenobiotics. The involvement of NADPH and molecular oxygen in these reactions was reported to be characteristic of mixed function oxygenase by Mason, (1957a) and Hayaisha, (1964).

The results of Hayaisha's work with uricase, glucose oxidase, steroid hydroxylases, aerobic cyclization of squalenes to lanosterol and the work on tyrosinase, xanthine oxidase and steroid by Thomas *et al*, (1976) enabled oxygenases to be classified into three main groups. In group one, one atom of molecular oxygen is catalytically reduced to water and the other transferred to the substrate and Hayaishi, (1964) gave the name monooxygenases to the group. Further evidence supporting the oxygen utilization as stated above was given by Posner *et al*, (1976).

The second group had both oxygen atoms of molecular oxygen transferred to the substrate, enabling it to be described as a dioxygenase. The last group, called electron transferring oxygenase or lipoxygenase, reduces molecular oxygen to hydrogen peroxide or water (Mason, 1957b) and oxidizes substrate by one, two or four equivalents of electrons. Microsomal xenobiotic or drug-metabolizing cytochrome P-450 enzyme is thought of as involving the monooxygenase or mixed-function oxygenase mechanism and fig. 1b shows the system of electron transport involved.

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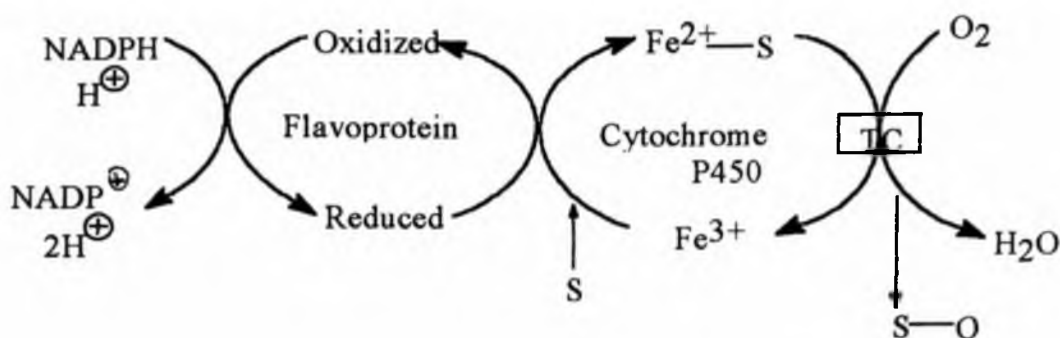


Fig.1b Electron transport system in xenobiotic-metabolizing cytochrome P-450 monooxygenase. S : Xenobiotic, TC : Ternary complex. S-O :Oxygenated xenobiotic

This electron transport chain functions to catalyze the transfer of electron from NADPH to cytochrome P-450, its natural acceptor.
A wide variety of oxidative reactions are known to be catalyzed by the microsomal mixed-function oxygenase. These reactions include deamination, O-, N- and S-dealkylation, hydroxylation of alkyl and aryl hydrocarbons, epoxidation, N- and S-oxidation and dehalogenation. Azo and nitro reductase activities are also reported by Gillette, (1966). Mixed function oxygenase reactions are, unfortunately, always considered simply as hydroxylation reactions and this designation more than obscures the versatility of the enzymes. The various reactions that the enzyme catalyses are illustrated below:

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a) Aromatic hydroxylation.

b) Aliphatic hydroxylation

c) N-dealkylation

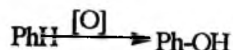
d) O-dealkylation

e) Deamination

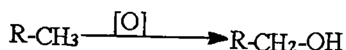
f) S-oxidation

One of the assays used in this project, i.e, 7-ethoxyresorufin-O-deethylation (EROD), reflects the O-dealkylation reaction. This reaction is very specific for the CYP induced by polyaromatic hydrocarbon (PAH). A large number of assay systems were used to determine CYP induction, before the EROD assay was developed for

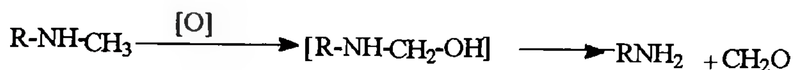
a) Aromatic hydroxylation.



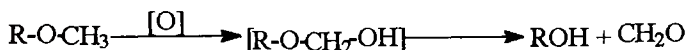

b) Aliphatic hydroxylation




c) N-dealkylation

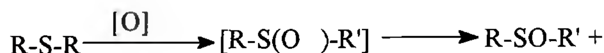



d) O-dealkylation




e) Deamination

f) S-oxidation

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CYP1A. The assay systems requiring solvent extraction and subsequent quantitation of metabolites involved a number of manipulations resulting in incomplete metabolite recovery as well as extended time periods for the assay procedures.

Several spectrophotometric and spectrofluorimetric assays that reliably follow product analysis continuously have been used of late to obviate the aforementioned problems. 7-Ethoxyresorufin-0-deethylase is one of the few routinely used spectrofluorometric probes. The enzyme activity is specific for cytochrome P-450 isozyme commonly induced by 3-methylcholanthrene, β-naphthoflavone and other structural analogues. The reaction proceeds as shown in Figure 1c.

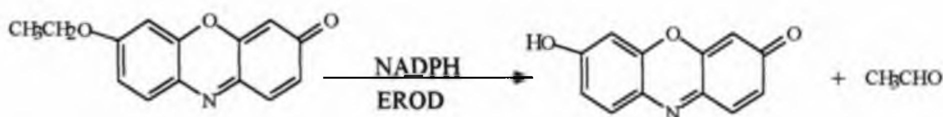


Fig. 1c Hydroxylation reaction involved in the EROD assay.

**Isoforms of Cytochrome P-450**

It is becoming increasingly evident that several forms of cytochrome P-450 exist and account for the different monooxygenase activities in liver microsomes due to substrate specificities.

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There has been an explosive growth in number of investigators using catalytic assay, immunoassay or nucleic acid hybridization techniques to detect induction of specific P-450 in fish liver as a measure of contamination by inducing compounds. With this has come a need for a common understanding of terms used to describe P-450 induction and for referring to P-450 forms in different species (Stegeman, 1992).

The classification system (Nebert *et al*, 1991) based on similarities in all known primary (amino acid) sequences of P-450 proteins, whether determined directly or inferred from nucleotide (cDNA or gene) sequences, is being adopted increasingly in the literature to refer to the hydrocarbon-induced P450 in fish. The ctyochrome P-450 genes known are organised into gene families, many with multiple sub-families, and often with more than one member in each subfamily. Currently, recommended nomenclature includes designations CYP, denoting cytochrome P450, arabic number denoting family, letter for subfamily and arabic number for the specific protein. Thus, CYP1A1 refers to the gene for cytochrome P-450 in family 1, subfamily A and the first member of the subfamily 1. A gene transcript could be referred to as CYP1A1 mRNA, or as P-450 1A1 or simply as 1A1. Thus P450 1A1 (CYP1A1) and P-450 1A2 (CYP1A2) refer to two proteins in aryl hydrocarbon-inducible P450 family 1 in mammals (Stegeman, 1992).

It is a popular view that the specificities are imposed upon the monooxygenase system by existence of multiple forms of cytochrome P450. Using reconstituted cytochrome P-450 monooxygenase systems, enzymes prepared from controls and phenobarbital (PB) or 3-MC treated rats were shown to exhibit different substrate specificities and such

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ɔecificities were confirmed to be residing in the cytochrome ɔmponent rather than the phospholipid or reductase component. :udies on visible spectrophotometry and electron paramagnetic ɜsonance (epr) spectroscopy on 3-MC treated rat microsomes showed ıduction of cytochrome P-448 which was different from cytochrome P-50 of controls (Kumaki *et al*, 1978).

**ıduction**

ɔuts *et al*, (1961) were the first to notice that drug-metabolising nzyme activities/levels were lower in regenerating liver than in ɔrmal liver. Conney, (1967) coined the term "induction" for the ɔnooxygenase system to mean an increased rate of product formation r substrate disappearance. Induction has all along been thought of ɔ occur via one of three possibilities: *de novo* synthesis of novel roteins, the activation of pre-existing but dormant proteins or by he decreased rate of degradation of protein. Induction can be anifested and/or determined in quantitative or qualitative terms.

he induction of cytochrome P-450 enzymes in fish liver was first uggested as an indicator of environmental contamination in the early 970s by Payne, (1976). A number of studies between 1975 and 1980 evealed that mixed function oxidase (MFO) activity in fish liver ould be experimentally induced by polynuclear aromatic hydrocarbons, y halogenated aromatic hydrocarbons, and by mixtures of such ompounds (collectively AH), and that the same MFO activities (i.e xthoxyresorufin-O-deethylase (EROD) or aryl hydrocarbon hydroxylase AHH) were elevated in fish from contaminated environment (Bend,

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1978).

The amount of some types of CYP can be induced in response to an organism's exposure to many types of chemical, including many substrates of the enzyme. A typical example is CYPIA, the polyaromatic hydrocarbon (PAH)-inducible subfamily. The induction occurs via an intracellular receptor (Ah-receptor, AhR) located in the cytosol (Poland *et al* 1976; Poland and Knutson, 1982; Nebert and Eisen, 1984; Denison *et al*, 1987; Safe, 1988; Landers, 1991; Swanson and Brafield; 1993; Okey *et al*, 1994). The receptor is predominantly found in the liver but also occurs in extrahepatic tissues ( Okey and Harper, 1994; Stegeman and Hahn, 1994).

Briefly, CYPIA induction is mammals in initiated by binding of the inducer, e.g. tetrachlorodibenzo-p-dioxin, TCDD, or other planar aromatic hydrocarbons, to the AhR (Fig.1d). The association of the receptor with the ligand releases heat shock proteins (hsp90). A cytosolic Ah-receptor nuclear translocator (arnt), translocates the receptor-ligand complex to the nucleus (Hoffman *et al*, 1991). A subsequent interaction between the nuclear form of the AhR complex and xenobiotic receptor elements (XREs) initiates the transcription of the CYPIA genes. CYPIA in RNA is then translated into an apoprotein, followed by binding of heme. The protein is then inserted into the membrane of the endoplasmic reticulum where it performs its catalytic function. In fish, the CYPIA proteins are induced by similar compounds and via the Ah-receptor as in mammals, suggesting a similar mechanism (Heilmann *et al*; 1988; Lorenzen and Okey, 1990; Hahn *et al*; 1994, Safe and Krishnan, 1995).

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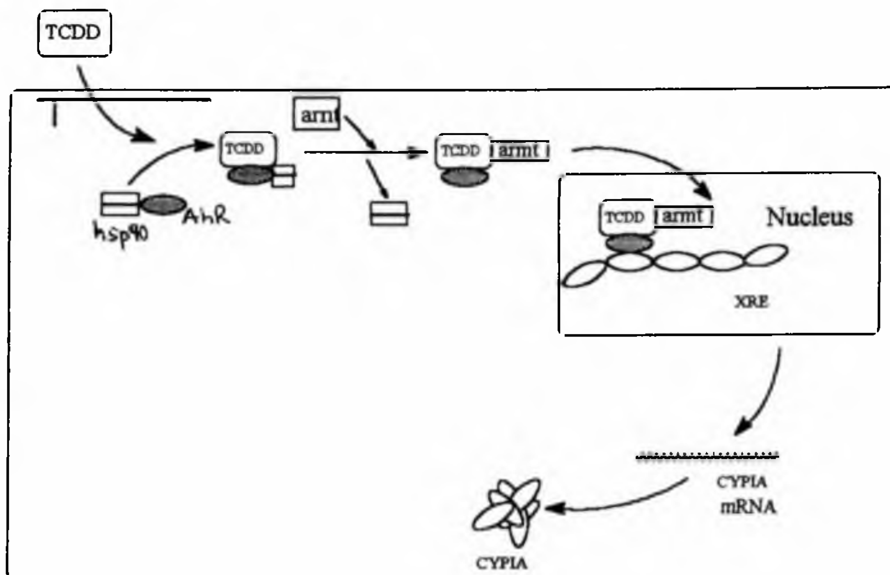


Fig 1d. Molecular mechanism of induction of CYPIA gene expression by TCDD and related compounds

**Inducers**

Inducers of hepatic cytochrome P-450 consist of compounds possessing a broad spectrum of structural types, uses and pharmacological activities (Davis *et al*, 1978). The use and/or pharmacological effects of some of these inducing agents range from carcinogens, antioxidants, sedatives, hypnotics, heat exchange fluids, insulators, lubricants and insecticides. The only common feature shared by all inducers appears to be the high lipid-solubility permitting ready localization in the endoplasmic reticulum and the ability to serve as a substance for, or the capacity to bind to cytochrome P-450

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monooxygenase complex (Parke, 1975). More than two hundred drugs, insecticides, carcinogens and other chemicals are known to stimulate the activity of drug-metabolising enzymes in liver microsomes (Mannering, 1968). Central nervous system stimulants, anticonvulsants, anti-inflammatory drugs, muscle relaxants, analgesics, antihistaminics, alkaloids and steroid hormones are also known to induce cytochrome P-450 monooxygenase activity in animals and can alter the duration and intensity of other drug action.

Induction of cytochrome P-450 in aquatic organisms has been investigated by a number of researchers because the sea and other fresh water bodies serve as recipients of all kinds of anthropogenic waste and also because many of the species, fish in particular, are important human food sources.

β-Naphthoflavone has been used by Stegeman, (1981), Gooch and Matsumura, (1983), Melancon *et al*, (1981) and Goksoyr *et al* (1991), to study induction in fishes and their response particularly to EROD assay. It is known to specifically induce cytochrome P-450IA isoenzyme. Chambers and Yarbrough, (1976) and Bend and James, (1978) established that fishes possessed the ability to perform a wide variety of biotransformation reactions while Stegeman and Kloepper-Sams, (1987) showed cytochrome P-450 monooxygenase to be the central enzyme in the biotransformation of drugs and other chemicals by fish. Induction has been described in detail in a few fishes: perch, rainbow trout, atlantic cod, plaice (Goksoyr, 1985b) and scup (Klotz *et al*, 1983). In fish, evidence for qualitative and quantitative induction of mixed-function oxygenase came largely from experiments with PAH-type inducers which showed approximately a 50-fold

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elevation in enzyme activities (Stegeman and James, in press).

'Roundup' is a, broad-spectrum herbicide. The active ingredient is N-(phosphonomethyl)glycine. It is a water soluble salt. It inhibits an enzyme that is essential to the formation of specific essential amino acids in plants. When sprayed on leaves of plants, this chemical is absorbed and translocated throughout the plant. It has an $LD_{50}$ value of 5.6mg/kg using rats as test organisms. The structure is shown in Fig.1e.

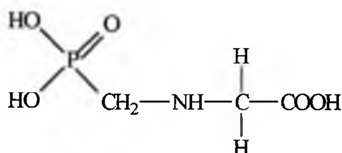


Fig: 1e N-(phosphonomethyl) glycine

**Factors affecting CYP response in fish**

Several biotic (e.g. reproductive and developmental stages, health condition, nutritional status) as well as abiotic factors (e.g. temperature and season) are known to affect background variation in CYP activities and protein levels in natural fish populations (Andersson and Forlin, 1992; Stegeman and Hahn, 1994; Bucheli and Fent, 1995). The most dominant of the abiotic factors that exert influence on CYP induction is temperature. Studies have indicated that cold acclimation and the assay temperature can influence CYP activity (Dewaide and Henderson, 1970). Induction of aryl hydrocarbon hydroxylase (AHH) activity is also found to be suppressed in cold-acclimated animals, at least for several days after treatment, which is accompanied by cold-suppression of CYPIA induction but not in RNA content (Kloepper-Sam and Stegeman, 1992).

Sex of the organisms also affects CYP responses in fish. Many

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studies reported significant increases in CYPIA content and activity in the liver of males compared with females during the reproductive season (Elskus *et al* 1989; Stegeman *et al*, 1989; 1992; Jimenez and Stegeman, 1990; Larsen *et al*; 1992; Stegeman and Hahn, 1994). Evidence exists that in fish, estradiol regulates monooxygenase activity, suppressing CYPIA induction (Elskus *et al*, 1989).. Elskus *et al*, (1992) showed that the endogenous regulation of CYPIA can "override" exogenous induction by even high concentrations of CYPIA inducers. Thus, it is probable that reproductive hormones regulate seasonal changes in CYPIA activity. Studies have also shown that CYPIA activity decreases shortly before or during the spawning season (Jimenez and Stegeman, 1990). Since no such variation has been observed in juveniles, the use of juveniles or male fish may be recommended as a precautionary rule.

Diet and nutrition are known to influence the CYP system in fish. Some diets contain both inducers and inhibitors. Fatty acid composition (Ankley *et al*, 1989; George and Henderson, 1992), and dietary iron (George, 1994; Goksoyr *et al*; 1994) were shown to influence EROD activity. Starvation was observed to significantly reduce EROD activity (Jimenez and Stegeman, 1990), but pollutant can still induce the activity in moderately starved fish (Jimenez and Stegeman, 1990). In field situation, diet and nutrition may significantly influence observations of site differences, as fish in different areas may feed on different pray organisms with different levels of dietary constituents.

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### 1.2.3 Cytochrome P-450 reductase enzyme

Knowledge of NADPH-cytochrome P-450 reductase in man and experimental animals has been associated with the electron transport system of the enzyme complex. It is one of the components of the cytochrome P-450 monooxygenase enzyme complex located in the endoplasmic reticulum. The NADPH-dependent ctyochrome P-450 reductase is a flavoprotein and was found to contain FAD and FMN prosthetic groups in the ratio 1:1. It is a hexamer with individual protein components each having a molecular weight of approximately 79kDa.

Cytochrome P-450 reductase has been shown to catalyze the electron transfer to a number of artificial electron acceptors including cytochrome C, ferricyanide and dichlorophenolindophene (Masters *et al*, 1965). In some organisms, there are iron-sulphur proteins which receive the electrons from the reductase before donating them to cytochrome P-450.

### 1.2.4 Phase II Conjugating Enzyme : Glutathione-S-Transferase.

The most important phase II enzymes in vertebrates are the UDP-glucuronosyltransferases (UDPGT, E.C. 2.4.1.17) and glutathione S-transferases (GSTs, E.C 2.5.1.18). As in the CYPs, both belong to multigene families of enzymes whose isoforms exhibit different but often overlapping substrate specificities for both endogenous and exogenous compounds (Gregus *et al*, 1983; Clarke *et al* 1988; Clarke, 1990; George, 1994). The enzymes play important roles in the

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homeostasis as well as detoxification and clearance of many xenobiotic compounds. In these investigations, the Phase II conjugating enzyme studied was glutathione-s-transferase, the cytosolic enzyme.

Glutathione-s-transferases are enzymes that catalyze the nucleophilic addition of reduced glutathione (GSH) to the electrophilic centre of a wide variety of potentially toxic compounds. This is the first step in the formation of mercapturic acids. Glutathione-s-transferases occur ubiquitously, having been identified in prokaryotes, yeast, higher plants, molluscs, crustaceans, insects, fish, amphibians and mammals (Jakoby, 1978; Mannervik, 1985; Hayes *et al*, 1990).

Cytosolic GSTs are divided into 4 classes: Alpha, Mu, Pi and Beta, each consists of one or more isoenzymes (Beckett and Hayes, 1993). Isoenzymes of GST have distinct substrate specificities, and nuclear material may determine the detoxifying capability of the tissue, (Boyer and Kenney, 1985; Mannervik, 1985). The expression of certain isoenzymes is limited to particular tissues and their measurement in plasma should reflect damage to specific organs (Beckett and Hayes,1993). Glutathione-s-transferases are also involved in metabolizing anti-cancer drugs and therefore high glutathione and GST level in tumour tissue may be a barrier to an effective treatment with some chemotherapeutics (Tsuchida and *Sato*, 1992; Hayes *et al*, (1990).

**Multiple activities of GST.**

Whereas the function of GST is considered primarily to be one of

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detoxifying foreign compounds, these enzymes possess activities other than catalyzing the formation of glutathione-xenobiotic conjugates. For example, GST exhibits peroxidase activity toward organic hydroperoxides and serves to combat oxidative stress. Besides their catalytic properties, the GST can bind numerous non substrate ligands. By binding to lipophilic compounds themselves, GSTs also reduce the likelihood of these compounds binding to other cellular macromolecules such as deoxyribonucleic acids (DNA) (Schelin *et al* ,1983, Van Veld *et al*, 1987; Coles and Ketterer, 1990)

**Enzymology of GST**

The GSTs were originally studied because of their involvement in the mercapturic acid biosynthesis pathway. The conjugation between reduced glutathione (GSH) and xenobiotics represents the first of four steps leading to the formation of mercapturic acid. The existence of an enzyme in rat liver cytosol responsible for catalyzing the conjugation of GSH with foreign compounds was reported first by Coombes and Stakehim,(1961). In these original reports of GST, the conjugation of GSH with 1,2-dichloro-4-nitrobenzene, as well as with 1,2-bromosulfophthalein, was described. However, by the end of the 1960s at least 50 GST substrates had been identified (Boyland and Chasseaud, 1969).

Attempts to classify the enzymes by chemical structure of the electrophilic substrates resulted in the use of such terms as aryl transferase, alkene transferase and epoxide transferase. The inadequacy of nomenclatures based on substrate specificity became apparent when homogenous prepartions of individual separate

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isoenzymes were analyzed and shown to exhibit overlapping activities toward many of the substrates. (Coombes and Stakehim, 1961).

The susceptibility of different fish species to chemical carcinogenesis may be modulated by the activity of GST (Varanasi *et al*, 1987). The model substrate of choice in assessing the activity in piscine species is 1-chloro-2,4-dintrobenzene (CDNB) (Jansen and Arias, 1977; James, 1987; Donnarumma *et al* 1988).

Induction of phase II enzymes is less pronounced, unlike CYPIA levels, when animals are exposed to xenobiotic compounds which induce appreciable quantities of CYP1A1 (Andersson *et al*, 1985, George, 1994). Attempts to determine chemically induced levels of phase II enzymes in fish collected from the field have yielded conflicting results. Some studies indicated induced levels while others observed no elevated levels (James and Bend, 1980; Collier and Varanasi, 1984; Andersson *et al*, 1985; Lindstrom-Seppa and Oikari, 1989; Van Veld *et al*, 1991; Gadagbui *et al*, 1996).

### 1.2.5 **Fish as indicator organism.**

Fish are an indispensable component of the ecosystem. The importance of fish in ecotoxicology is a logical consequence of ecological and economic factors. Fish are capable of inhabiting all zones of the aquatic habitat. Although there may be many other species in the aquatic environment, which are of equal or greater sensitivity to changes in water quality, fish, by their size alone and by the attention which is paid to them by commercial and recreational

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fishermen, constitute excellent indicator organisms that can be used to assess the health of the aquatic environment. Most fish are universal in their feeding habit as they do not normally depend on restricted food types. They may feed at any level in the environment; at the surface, in the mid-depth or at the bottom; in this way, they are exposed to chemical contaminants contained in the water, food sources, or sediments. A continuous exposure is obtained by the passage of water through the gills and by contact with the water that always surrounds them. The tropical region is endowed with an extremely diverse fish fauna, many of the fishes possessing extraordinary specializations for feeding and reproduction.

Ghana, like other West Africa countries, has considerable Tilapia resources. Irvine (1947) recorded the presence of five species of Tilapia that are now considered commercially important in Ghanaian fisheries and aquaculture. These are *Oreochromis niloticus, Sarotherodon galilaeus, Sarotherodon melanotheron, Tilapia busmana* and *Tilapia zilli.* These are distributed in the two main river basins in Ghana, that is, the Volta lake and Southern-Western rivers.

Characteristic of population of *S. melanotheron* is the presence of intense black patches on the lower jaw, throat, branchiostegal membrane, lower part of operculum, pre- and interoperculum that have given the species the name of 'black-chinned tilapia' among aquarists. Tilapia for this study was selected as an experimental animal based on the following:

Tilapia are distributed widely in the tropics where they are widespread in inland waters, rivers and lagoons. They have a short generation time of 3-6 months and exhibit successive breeding; their fast

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growth, herbivorous or omnivorous feeding habits, high food conversion efficiency, ease of spawning, ease of handling, resistance to disease, tolerance of wide temperature and salinity ranges and good acceptance make the fish highly popular in aquaculture in Africa, Asia and other countries.

Fish for studies of the heavily polluted area was obtained from the Fosu Lagoon at Cape Coast (Fig. 1a), approximately 150km from Accra, Ghana. At maximum water level, the lagoon has a surface area of $0.61km^2$, and a mean depth of 1.5 metres. The lagoon is closed to the sea by a sand bar which is occasionally breached by rain floods or human activity. Thus, relatively, this lagoon has a poor water exchange; it is fed principally by rain water and domestic effluents, although sometimes sea water spills into it over the sand bars.

Large quantity of pollutants are discharged into the Fosu lagoon, which is the main source of freshwater fish for the Cape Coast municipality and its environs. Active fishing occurs in the lagoon. A bad odour emanates from the environs around the lagoon. Most of the larger drains in the township empty their contents into this lagoon. The biggest government hospital in the Central Region, Cape Coast Central Hospital, as well as St. Augustine's College Laboratory, are situated on the banks of the lagoon and their effluents drain into the lagoon. The waste consists of discharge from surgical, mortuary, out patients departments (OPD), maternity, isolated wards and dispensary. In addition, oil spillage from the way-side mechanics, situated at the other side of the bank are washed into the lagoon. It is believed that high occurrence of epidemiological diseases in the municipalities is due to the 'diseased' fish fed on by the populace.

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# CHAPTER TWO: MATERIALS AND METHODS.

## 2.1 MATERIALS.

### 2.1.1 Fish

*Sarotherodon melanotheron* samples for this research work were obtained from the Fosu lagoon at Cape Coast, in the Central Region and from the lower basin of the Volta lake near Ada, in the Greater Accra Region. The fish were caught from the wild by fishermen, using gill nets. Some of the fish were acclimated for 21 days in aquaria under laboratory conditions.

*S. melanotheron* samples from a remote part of the Volta lake at Ada were used as fish from pollution-free environment, providing baseline values for the parameters measured in this project. The fish samples were provided by personnel from the Volta Basin Research Project (VBRP). Some of the samples, which were kept in aquaria in the laboratory, were fed with specially prepared fish meal (Wardley Premium Pellets) from USA. The fish were fed daily, *ad libitum.* The weight of *S. melanotheron* ranged from 28.3 to 36.6g.

### 2.1.2 Chemicals and Reagents.

The following chemicals were obtained from Sigma Chemical Company, St. Louis, MO, USA: Nicotinamide adenine dinucleotide phosphate (reduced), cytochrome C, resorufin, 7-ethoxyresorufin, 7-pentoxyresorufin, glutathione and bovine serum albumin.

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Chemicals obtained from Fluka were glycerol, potassium chloride, copper sulphate pentahydrate, sodium hydroxide, ethanol, dipotassium hydrogen phosphate dodecahydrate, sodium dihydrogen phosphate, sodium potassium tartate, sodium carbonate, sodium dithionite and dithiotreitol (DTT).

Folin-Ciocalteau phenol reagent, ethylenediaminetetraacetic acid (EDTA) and tris (hydroxymethyl) aminomethane were obtained from Hopkins and Williams, England. Carbon monoxide was obtained from Praxair, Belgium. 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Aldrich Chemical Company (Germany). Dimethylsulphoxide (DMSO) was obtained from Wako Pure Chemical Industries, Japan.

## 2.2 METHODS

### 2.2.1 Pretreatment of Fish.

Fish stock from the Volta lake, a site near Ada, were kept in aquaria for 21 days in order to rid them of xenobiotics, if any. Levels of proteins/enzymes recorded for these fish samples were regarded as baseline values.

For the herbicide treatment, fish stock from the Volta lake acclimated in aquaria to the laboratory conditions for 21 days, were introduced into other aquaria containing two concentrations (1.0ppm and 5.0ppm) of 'Roundup' and kept for 14 days. The volume of chlorine-free water used in each aquarium was 100L. For the control, fish samples were kept in

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the aquaria without any herbicides for 14 days.

### 2.2.2 **Sampling of Fish.**

Fish from the Fosu lagoon were caught with gill nets and transported to a laboratory near by (Holy Child school laboratory, Cape Coast) where they were killed with a blow to the head and livers excised (without gall bladder). The livers were transported, in liquid nitrogen, to the laboratory at the University. Live fish from a remote area of the Volta lake near Ada, were supplied by the personnel from the VBRP.

Fish livers from each "exposure group" were pooled and used for the preparation of microsomes. Each set consisted of 8 fish livers.

### 2.2.3 **Preparation of microsomes**

Fish livers excised freshly or those stored in liquid nitrogen and thawed on ice, were blotted with tissue paper and weighed. The livers were then minced and homogenised in 1:4 volume of ice-cold homogenization buffer (see appendix) with a Glas-Col homogenizer.

The homogenates were spun in a Hitachi 20PR-52D centrifuge at 10,000 x g for 10 minutes at 4°C. The supernatant fractions were removed and centrifuged at 16,800 x g for 10 minutes (Fig 2a). The supernatant fractions were centrifuged again at 105,000 x g (40,000 rpm) for 1 hour

in Hitachi 80P-7 Ultracentrifuge at 4°C. The pellets were washed in a homogenizing phosphate buffer of pH 7.6 to remove any haemoglobin not removed and to clean microsomes of cytochrome $b_5$ contamination which often interferes with cytochrome P-450 spectral studies. The supernatant fraction obtained after 105,000 x g centrifugation, was used as the source of the GST enzymes. The washed microsomal pellets were rehomogenised in 2.0ml/g liver weight of storage buffer pH 7.6. (see appendix) and distributed into labeled cryotubes and stored in liquid nitrogen or at -80°C. The supernatant fraction (source of GST) was aliquoted into cryotubes and stored similarly.

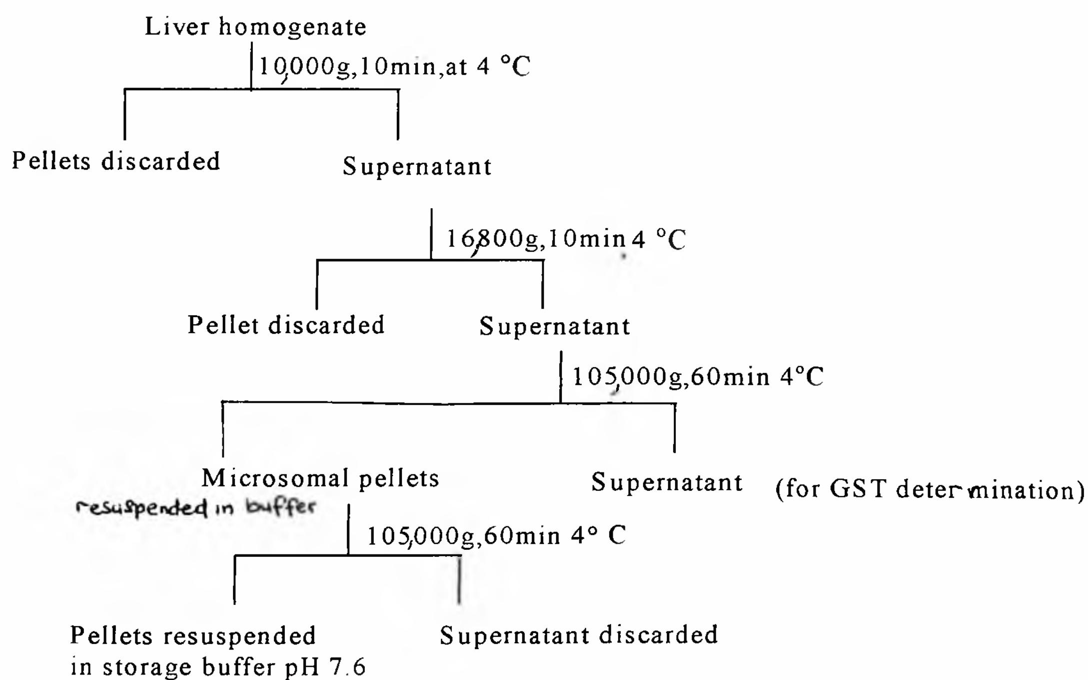


Fig. 2a. Flow Chart of the process involved in the preparation of microsomes.

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### 2.2.4 **Protein Determination**

The method of Lowry *et al.* (1951) in which absorbances of coloured complexes, emanating from a reaction between alkaline copper-phenol reagent and tyrosine and/or tryptophan, are measured at 750nm, was used for the determination of total protein in this work.

Prior to the determination, the microsomes and supernatant fraction were diluted 1:100 and 1:25 respectively, with 0.5M NaOH. An aliquot of 1000µl of the diluent were taken in duplicate. To 1000µl of each sample, 5ml of alkaline copper-phenol reagent were added, the mixture mixed thoroughly by vortexing, and allowed to stand for 10 minutes. A 0.5ml of Folin reagent was added to each sample in alkaline reagent in a test tube, and mixed immediately and completely. The mixture was allowed to stand for 30 minutes. The absorbances were then read at 750nm on a double beam spectrophotometer, Schimadzu UV-190, after zeroing with a blank containing all the reagents except the microsomes or supernatant fraction. The protein concentration of the microsomes and that of the supernatant fraction were directly interpolated from a standard curve constructed with bovine serum albumin (BSA).

To prepare the standard curve, 1.0mg of BSA was dissolved in 10ml of 0.5M NaOH. This was regarded as stock solution (100µg/ml) and the following volumes, 0, 0.2, 0.4, 0.6, 0.8 and 1.0ml of this stock BSA solution (equivalent to 0, 20, 40, 60, 80 and 100µg respectively) were taken and made up to a final volume of 1ml with 0.5M NaOH. Alkaline

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copper-phenol reagent and Folin reagent were added as described above for the microsomal and supernatant fractions, and the absorbances at 750 nm read. These absorbances were then used to construct the standard curve of absorbances against BSA concentrations (µg/ml). The alkaline copper reagent was prepared freshly by mixing 2% w/v $Na_2CO_3$ in 0.1M NaOH, 1% w/v $CuSO_45H_2O$ in water in a ratio of 100:1:1 by volume respectively.

### 2.2.5 **Determination of NADPH-cytochrome P-450 reductase activity.**

NADPH-cytochrome P-450 reductase enzyme activity was measured using exogenous cytochrome C (oxidized, ferric form) as an artificial electron acceptor. The principle involved here is that the reduced form of cytochrome C has a characteristic band at 550 nm which is absent in the oxidized form.

The method used was that by Blecker *et al.* (1973). In this method, 250µl of a solution of cytochrome C (5mg/ml made by dissolving 50mg of cytochrome C in 10ml of water) was mixed with 2.15ml of 0.1M tris-HCl buffer pH 7.6 and the mixture put in a sample cuvette. A 100µl of microsomal preparation that has been diluted to a protein concentration of 4mg/ml with storage buffer, was added. A reference blank solution was also prepared which contained all the above reagents, including the microsomal fraction, and put in a reference cuvette. A 25µl of storage buffer was added to the reference cuvette to a total volume of 2.525ml. Both cuvettes were placed in a Schimadzu UV-190 double beam

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spectrophotometer and the reaction initiated by addition of 25µl 2% w/v NADPH solution to the sample cuvette. This was thoroughly and completely mixed and the absorbance was monitored at 550nm for 3 minutes.

In order to calculate the specific enzyme activity, the actual concentration of microsomal protein (Cp) in the reaction mixture was determined as follows:

$$Cp = \frac{\text{volume of microsomes taken} \times 4.0\text{mg/ml}}{\text{volume of reaction mixture}}$$

$$= 0.158\text{mg/ml}$$

The specific activity (#) of the reductase enzyme was calculated from the formula:

$$(\#) = \frac{A_{550}}{\varepsilon Cp}$$

Where, $\varepsilon$ is the extinction coefficient for reduced cytochrome C, at 550nm and has a value of $19.6mM^{-1}cm^{-1}$ and $A_{550}$ is the absorbance change for the linear portion of the curve over three (3) minutes. Enzyme activities were expressed as pmol cytochrome C reduced per minute per mg protein.

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### 2.2.6 Determination of cytochrome P-450 content.

The method used to determine cytochrome P450 content was that by Mastubara *et al (1976)*. In this method, 2ml each of microsomal preparation containing 2mg/ml protein were placed in both the test and reference cuvettes. A baseline scan between 400 and 500nm was recorded on Schimadzu UV-190 spectrophotometer. A few grains of solid sodium dithionite were added to both cuvettes and carbon monoxide was bubbled only through the sample cuvette, for one minute. The spectrum was re-scanned from 400-500nm. The cytochrome P-450 content, C, was calculated as follows:

$$C = \frac{\Delta A}{\varepsilon \times L}$$

where $\Delta A$ = Absorbance difference between 450 and 490nm and $\varepsilon$= extinction coefficient for the wavelength couple 450 - 490nm, with a value = 91 $mM^{-1}cm^{-1}$ and L is the pathlength of the cuvette taken by light and has a value of 1.

Therefore, $C = 10.989 \times \Delta A$ $\mu mol.ml^{-1}$

The specific cytochrome P-450 content in the liver sample (#), was calculated taking into consideration the dilution factor used and the protein concentration of the tissue as fellows:

$$(\#) = \frac{10.989 \times \Delta A \times f}{B} \; \mu mol mg^{-1} \text{ protein}$$

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where, f, is the dilution factor and B the protein concentration of the microsomes.

### 2.2.7 Determination of glutathione-s-transferase activity

Glutathione-s-transferase activity was determined with the model substrate, 1-chloro-2,4-dinitrobenzene. The procedure used was that by Habig *et al.* (1974), and as described by Gadagbui *et al.* (1996). To each of two 3ml spectrophotometric cuvettes, 0.1ml of 30mM glutathione, 0.1ml 1-chloro-2,4-dinitrobenzene and 2.2ml of 0.1M sodium phosphate buffer, pH 7.4, were added. These mixtures were incubated at 34°C for 3 minutes. The cuvettes were then placed in the reference and test compartment of the Schimadzu UV-190 double beam spectrophotometer. The reaction was initiated by adding 0.6ml of diluted cytosolic fraction of a protein concentration of 10mg/ml to the test cuvette and 0.6ml of the homogenizing buffer to the reference cuvette. Increase in absorbance with time was recorded at 340nm, over a five minute period. The total reation mixture was 3.0ml.

The specific activity (#) of GST was calculated from the formula:

$$\# = \frac{A_{340}}{\varepsilon \times Wp \times L}$$

Where, $\varepsilon$ is the extinction coefficient for glutathione adduct at 340nm and has a value of $9.6mM^{-1}cm^{-1}$, and $A_{340}$ is the absorbance change for the linear portion of the curve per minute, and Wp is the actual

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cytosolic protein concentration in the reaction mixture, calculated as follows:

$$Wp = \frac{0.6\ (ml) \quad x \quad 10\ (mgml^{-1}}{(3.0\ (ml)}$$

$$= \quad 2\ mg/ml$$

The enzyme activity was expressed in nmol product formed per min per mg protein.

## 2.2.8 ..Monooxygenase Assays

The 7-ethoxyresorufin-O-ethylase (EROD) and 7-pentoxyresorufin-O-depentylase(PROD) assays as measures of overall cytochrome P-450 monooxygenase activity, were determined spectrofluorometrically, essentially as described by Burke and Mayer, (1974). A 0.41mM stock solution of the substrates, 7-ethoxy-, and 7-pentoxyresorufin, and 0.85mM of the product, resorufin were made in dimethylsulfoxide (DMSO). The buffer for the assay was 0.1M $NaH_2PO_4.2H_20$ in 0.1M NaOH. The optimum pH of this buffer determined for *O. niloticus*, which is 6.8, (Gadagbui *et al* 1996) was used.

The absorption spectra of both substrates and product were recorded using double-beam spectrophotometer, Schimadzu UV-190. For this, 10 µl of each of the stock solutions were separately stirred into a cuvette with the $NaH_2PO_4$ buffer to a final volume of 2.0ml. The spectra were recorded to obtain the wavelength of maximum absorption (lambda max), the absorbance of which is required for the determination of true

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extinction coefficient at that wavelength, a value used later in the determination of the actual concentration of the substrates in the specrofluorometric assays.

The $\varepsilon$max, that is, the extinction coefficient at the determined lambda max, was estimated from Beer-Lambert Law as follows:

$$\varepsilon_{max} = \frac{\text{Absorbance}}{[\text{substrate conc.}(\mu\text{Mml}^{-1})]}$$

The emission spectra of resorufin, 7-pentoxyresorufin(7-PR) and 7-ethoxyresorufin (7-ER) were obtained using the SFM-25 spectrofluorimeter with VERS 8603 recorder attached. The total volume of solution used was 2.0 ml made up of 1990µl of buffer, pH 6.8, and 10µl of 0.41mM 7-ER, 7-PR or 0.85mM resorufin. The excitation and emission wavelengths were set at 535nm and 650nm respectively on the lambda-drive.

For the EROD assay, a resorufin working solution was made by diluting one part of stock solution in two parts of DMSO to give a working solution of concentration 17.9 $\mu molL^{-1}$ . The microsomal preparations were diluted with the $NaH_2PO_4$ buffer to obtain a protein concentration of 0.2 mg/ml. A volume of 1960 µl of this buffer, 10µl of 7-ER solution and 20 µl of 0.2 mg/ml microsomal preparations were mixed in a cuvette. The cuvette was placed in the fluorometer and the emission fluorescence recorded for 30 seconds at the excitation and mission wavelengths of 535nm and 585nm, respectively.

The deethylation/depentylation reaction was then initiated by the addition of 10µl of 10mM NADPH to the cuvette followed by thorough mixing. The velocity of the reaction was measured by recording the

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change in fluorescence on the time-drive plot over a period of time, approximately two minutes.

During the time period that the recording of the change in fluorescence was still being made, 10µl of resorufin working solution was added as an internal standard to a final concentration of 17.9 pmol/L. The reaction solution was mixed again and the sudden increase in fluorescence (spike) was recorded for another minute. The reactions of EROD assay were all carried out at room temperature in subdued light.

The EROD activity was calculated using the linear change in fluorescence with time as a result of NADPH addition until the addition of the internal standard. The specific enzyme activity ($pmol min^{-1} mg^{-1}$ protein) was estimated by the following formula:

$$\text{pmol resorufin formed} = \frac{S \times C \times D}{t \times R \times \text{mg protein}}$$

D = dilution factor for the sample = 2000/20

C = concentration of resorufin

S = sample fluorescence (blank), mm recorder response

R = resorufin fluorescence (spike), mm recorder response

t = time over which velocity was measured

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## 2.3 Statistical analysis

The statistical analyses were performed using the Sigmastat analytical system. Statistical significance in the differences between values obtained for samples from the Volta lake after acclimatiom and other 'exposure sites' (Fosu lagoon and 'Roundup'-exposed fish in aquaria) were evaluated by standard student's *t* test. Also, values for fish from the same site, before and after acclimation were evaluated for statistically significant differences. The level of significance was set at $p < 0.05$ in all cases.

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# CHAPTER THREE :RESULTS.

The biochemical parameters studied are grouped into two, those found in microsomes and one that is cytosolic.

The biochemical data analysed included total protein concentration and the protein components of cytochrome P-450 monooxygenase complex, i.e, NADPH-cytochrome C (P-450) reductase and cytochrome P-450. Also measured were the 7-ethoxyresorufin-O-deethylase (EROD) and 7-pentoxyresorufin-O-depentylase (PROD) activities of the proteins to indicate the type of cytochrome P450 present.

The other biochemical parameter studied is the glutathione-s-transferase activity.

With the aquaria experiment, 70% mortality was recorded in 5 days when the fish were exposed to 5ppm of the 'Roundup' in the aquaria. No mortality was recorded when 1.0ppm of the Roundup was applied.

## 3.1 Total microsomal protein concentration.

For the determination of the total microsomal protein concentration, a bovine serum albumin calibration curve was plotted using $A_{750}$ versus bovine serum albumin (BSA)

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concentration, as shown in Fig. 3a. From this curve microsomal protein concentrations were read off using their $A_{750}$ values. The vaues obtained were corrected.

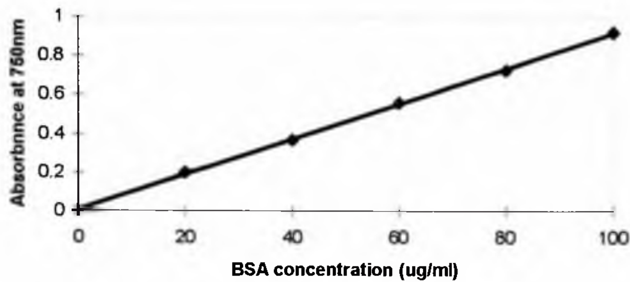
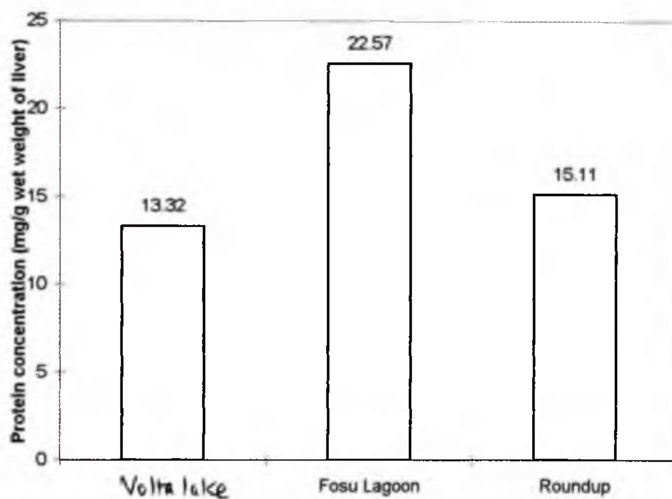



**Fig. 3a: BSA calibration curve used for protein determination**




-g. 3b. Mean total hepatic microsomal protein concentration, expressed as mg/g wet weight of liver from fish with different pollution histories.

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The yields of total microsomal protein, in mg/g wet liver weight for *S. melanotheron* obtained from water bodies of different pollution histories, are shown in Fig. 3b.

The results indicated that *S. melanotheron* obtained from the Fosu lagoon had approximately 69% more hepatic protein than fish from the Volta lake. *S. melanotheron* exposed to 'Roundup', a herbicide, had approximately 13% more protein than that of the fish from the Volta lake (Fig. 3b).

The mean total liver microsomal protein concentration for *S. melanotheron* from the Volta lake and that from the Fosu lagoon were 13.11 and 16.46 mg/g wet weight respectively, after both samples of fish were acclimated to the laboratory conditions for twenty-one (21) days (Fig. 3c).

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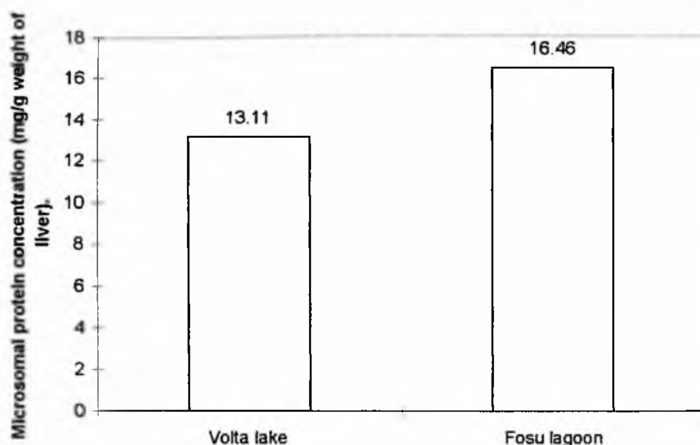


**Fig. 3c. Mean total microsomal protein concentration, mg/g wet weight of fish liver acclimated to laboratory conditions.**

The results show that the values for the fish from the Volta lake and Fosu lagoon were reduced by approximately 2% and 27% respectively, when they were transferred from the wild and acclimated to the laboratory conditions for 21 days in chlorine-free water. Because of the very small change in protein concentration after acclimation, fish from the Volta lake were taken to represent fish from pollution-free environment and used in other experiment in which fish were exposed to 'Roundup'.

The results of statistical analysis, using the student's *t* test, is shown in Table 3.1. There was a significant difference in the concentration of total microsomal protein for the fish obtained from the Fosu lagoon and those from the Volta lake, as well as the fish from the Fosu lagoon and those exposed to 'Roundup' but no significant difference between the values for fish from the lake and those exposed to 'Roundup'.

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Statistical analysis indicated that there was no significant difference in the hepatic microsomal protein obtained for *S. melanotheron* from the Fosu lagoon and the fish from the Volta lake, when acclimated to the laboratory conditions for 21 days. Analysis showed significant difference in the hepatic microsomal protein concentration for the fish from the Fosu lagoon, before and after acclimation to the laboratory conditions, whereas no significant difference existed between the values obtained for the fish from the Volta lake before and after acclimation (Table 3.1).

### 3.2 NADPH-cytochrome C (P450) reductase activity.

The assay for this enzyme showed the highest activity in the microsomes prepared from *S. melanotheron* obtained from the Fosu lagoon. The next higher activity was found in the fish exposed to the herbicide, 'Roundup'. The least activity was found in the microsomes prepared from livers of fish from the Volta lake. These results are shown in Fig. 3d.

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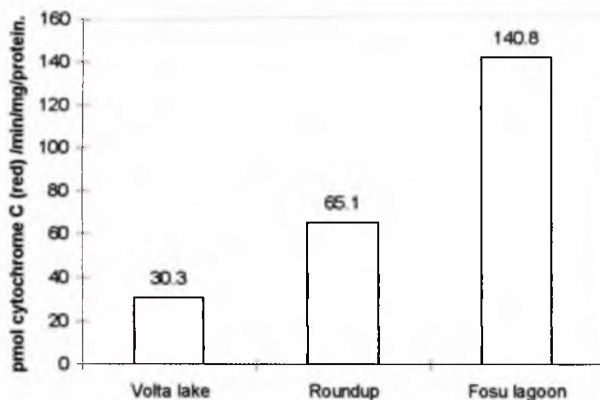


**Fig. 3d. Mean specific NADPH-Cytochrome C (P-450) reductase activity for *S. melanotheron* from different 'exposure sites'.**

Reductase enzyme activity of the microsomes prepared from livers of *S. melanotheron* from Fosu lagoon was higher by approximately 365% compared to the activity observed for the microsomes obtained from *S. melanotheron* from the Volta lake. The activity observed in microsomes obtained from fish exposed to 'Roundup', was approximately 115% higher than that of the unexposed fish from Volta lake. Comparing the enzyme activities in microsomes from fish in the Fosu Lagoon and those exposed to 'Roundup', showed an increase of approximately 116% for the fish from the Fosu lagoon.

Results of enzyme activity in the microsomes from the fish acclimated to the laboratory conditions for 21 days, indicated that the activity was still higher for the fish from Fosu lagoon, approximately 164%, of that from the Volta lake.

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Comparing the fish from the wild and those acclimated to the laboratory conditions for 21 days, it was shown that the reductase activity reduced by approximately 55% for fish from the Fosu lagoon and 20% for fish from the Volta lake

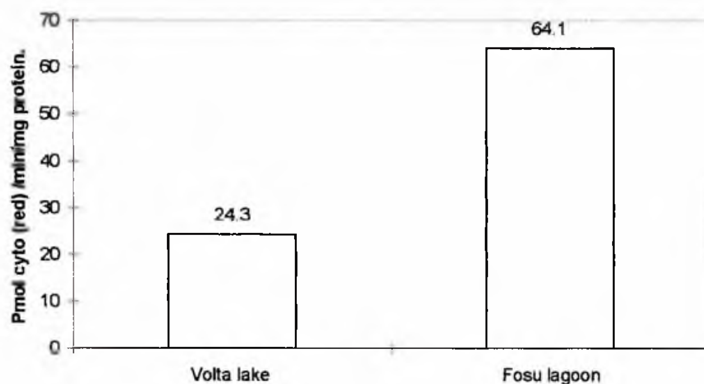


**Fig.3e. Mean specific NADPH-cytochrome C (P- 450) reductase activity for *S. melanotheron* acclimated to the laboratory conditions.**

Statistical analyses revealed that there were significant differences between enzyme activities in the fish from Volta lake, Fosu lagoon and those exposed to 'Roundup' (Table 3.1).

The results of reductase activities of fish from Fosu lagoon and Volta lake acclimated to the laboratory conditions, showed that there was a statistically significant difference between the two groups. Also analysis showed significant differences in reductase activities in fish from both Fosu lagoon and Volta lake, after the period of depuration, compared to the values before depuraion.

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## 3.3 Cytochrome P-450 content.

Determination of the cytochrome P-450 content in microsomes obtained from *S. melanotheron* from different 'exposure sites', indicate that the highest content was obtained for fish from the Fosu lagoon, followed by the fish exposed to 'Roundup'. The least cytochrome P-450 content was found in the microsomes obtained from *S. melanotheron* from the Volta lake. These results are shown in Fig. 3f.

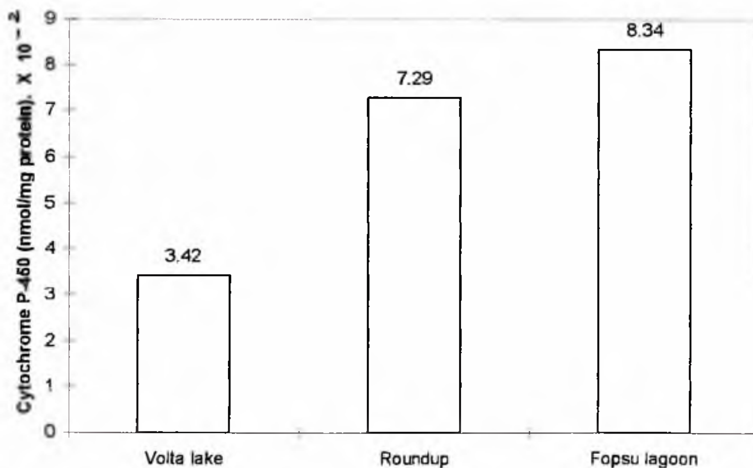


**Fig. 3f. Mean cytochrome P-450 content for *S. melanotheron* from different 'exposure sites'.**

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The Cytochrome P-450 content of the microsomes obtained from *S. melanotheron* from the Fosu lagoon is approximately 144% higher than that obtained from fish from the Volta lake. The content of cytochrome P-450 of the microsomes from the *S. melanotheron* exposed to 'Roundup' showed an increase of approximately 113% over the value for the fish from Volta lake. The cytochrome P-450 content of fish from the Fosu lagoon was approximately 14% higher than that of the fish exposed to 'Roundup'.

The results obtained when the fish were acclimated to the laboratory conditions for 21days, indicated that cytochrome P-450 content of the microsomes from fish from the Fosu lagoon was still higher, approximately 37% higher than that from the Volta lake.

Comparing the fish from the wild and those acclimated to the laboratory conditions, the results indicated that the cytochrome P-450 content reduced by approximately 51% for the fish from the Fosu lagoon and 12% for those from Volta lake.

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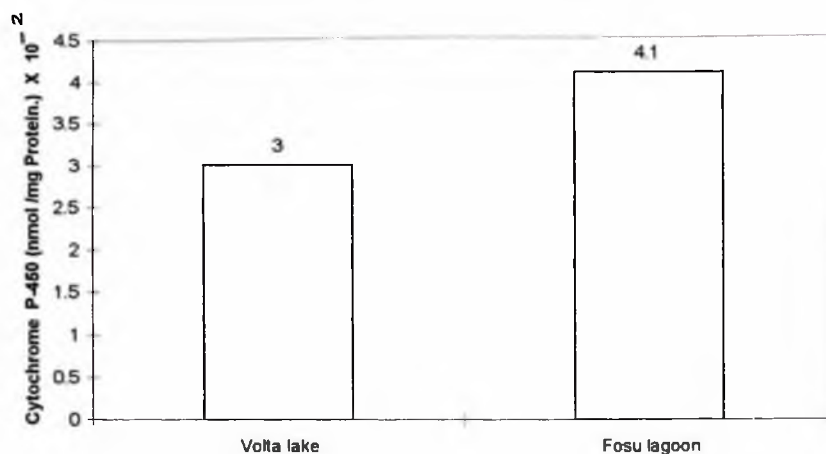


**Fig. 3g. Mean cytochrome P-450 content for *S. melanotheron* acclimated to the laboratory condition.**

Statistical analysis indicated (Table 3.1), a significant difference between the cytochrome P450 values for the fish exposed to 'Roundup' and those not exposed; fish from Fosu lagoon and those from the Volta lake, but not for the values obtained for fish from the Fosu lagoon and those exposed to 'Roundup'. The analysis also showed that there was no statistically significant difference between the CYP values obtained after the fish had been acclimated to the laboratory conditions, whether they were originally from the Volta lake or from the Fosu lagoon. Significant differences were shown in CYP content for fish from the Fosu lagoon, before and after depuration, whereas the differences for the fish from the Volta lake were insignificant

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## 3.4 Monooxygenase enzyme activity

**Purity of substrates and internal standard.**

Figs.3h, 3i and 3j are the absorption spectra of resorufin (which was used as the internal standard) and substrates (7-pentoxy- and 7-ethoxyresorufin) in EROD buffer respectively. The spectra were obtained to facilitate the determination of the wavelengths of maximum absorbance and absorbance values for both substrates and product.

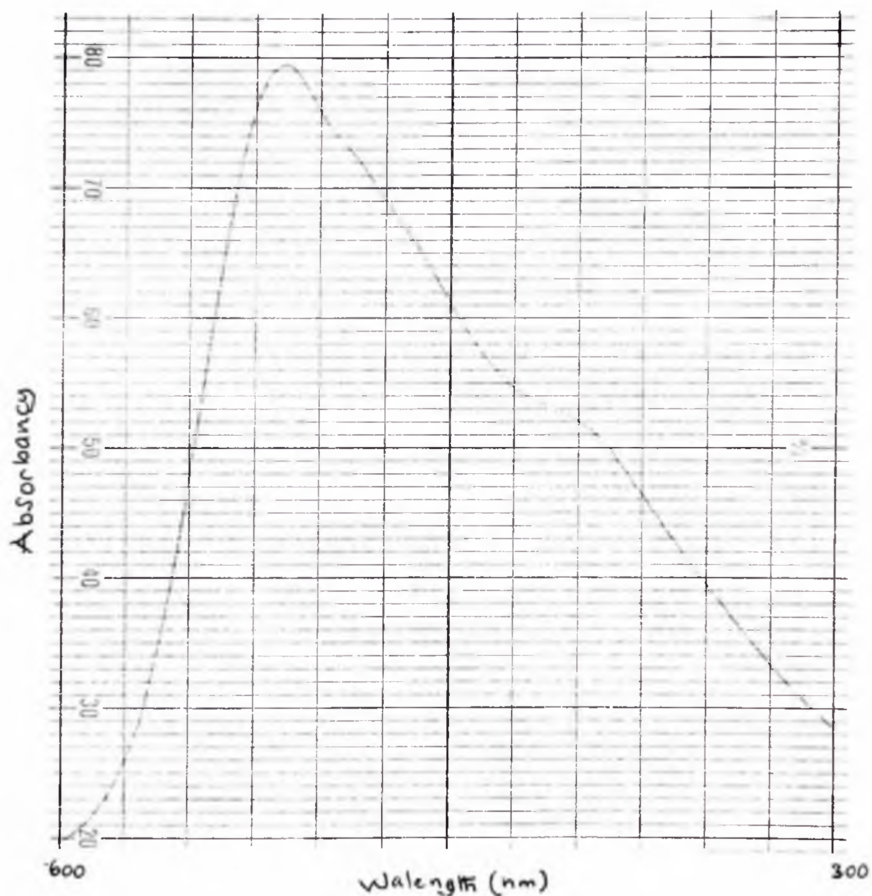


Fig. 3h. UV-Visible absorption spectrum of resorufin.

λ max. for Resorufin = 571nm 0.067

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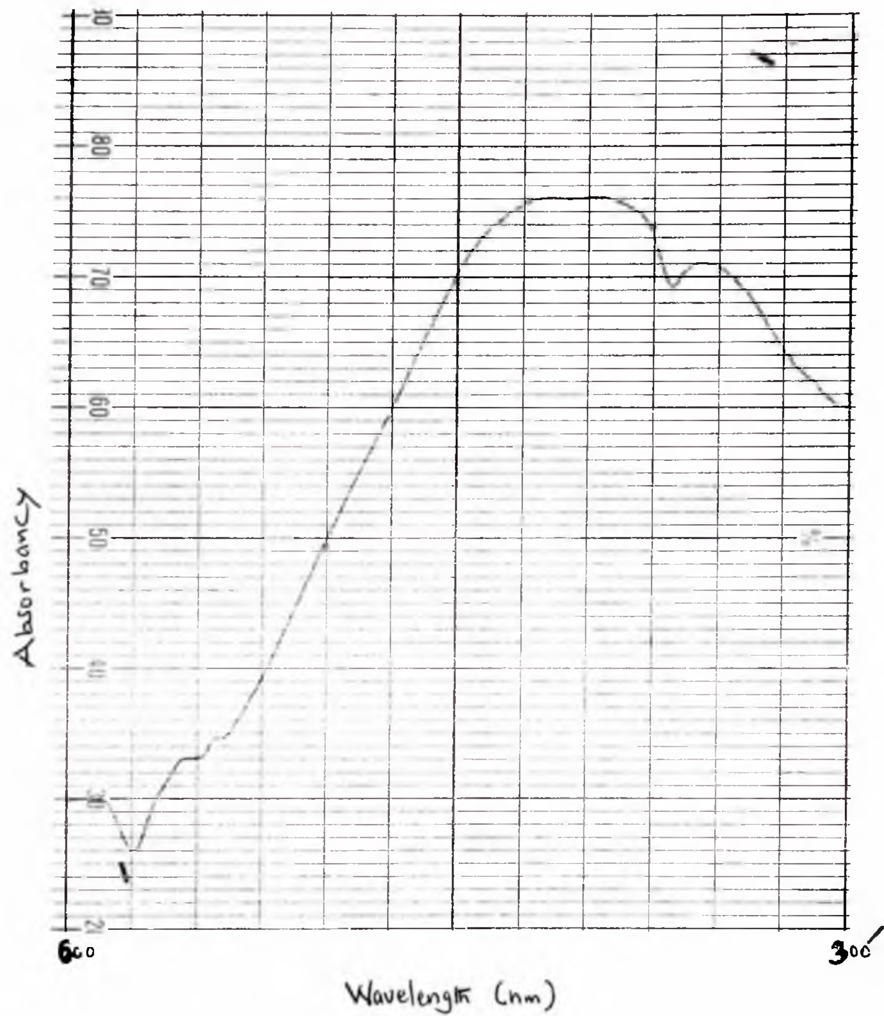


Fig. 3i. UV-Visible absorption spectrum of 7-pentoxyresorufin.

λ max. for 7-PR = 472nm ,0.026

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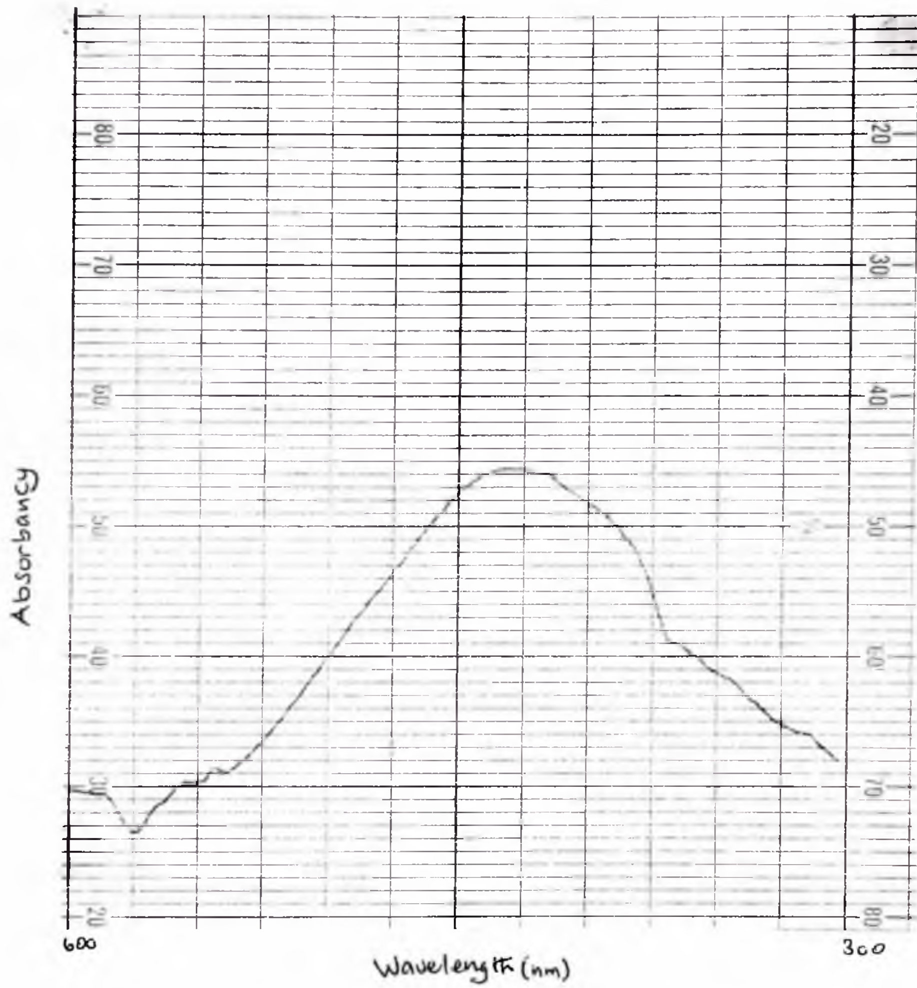


Fig. 3j UV-Visible absorption spectrum of 7-ethoxyresorufin

λ max. for 7-ER = 492nm ,0.026

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The charts indicated maximum absorbance of 2.6 $x10^{-2}$ at 492nm, instead of 2.25 x $10^{4}$ $M^{-1}cm^{-1}$ at 482nm reported by Prough *et al*, (1978) for 7-ER and a maximum absorbance of 6.7 $x10^{-2}$ at 571nm rather than 4.0 x $10^{4}$ at 572nm also reported by Prough *et al*, (1978) for R. Also, the maximum absorbance recorded for 7-PR in DMSO was 2.6 x $10^{-2}$ at 472nm. The values reported by Prough *et al*, for substrates and product were both dissolved in ethanol instead of dimethylsulfoxide (DMSO).

Knowing the concentration of each solute in the reaction cuvette enabled the correct extinction coefficient ($\varepsilon$) at the determined wavelength of maximum absorbance to be calculated from Beer-Lambert's law. The corrected $\varepsilon$ for the substrates, 7-ER at 492nm is 12.68 $mM^{-1}cm^{-1}$ and 7-PR at 472nm is 10.0 $mM^{-1}cm^{-1}$, while that of the resorufin is 59.16 $mM^{-1}$ $cm^{-1}$ at 571nm.

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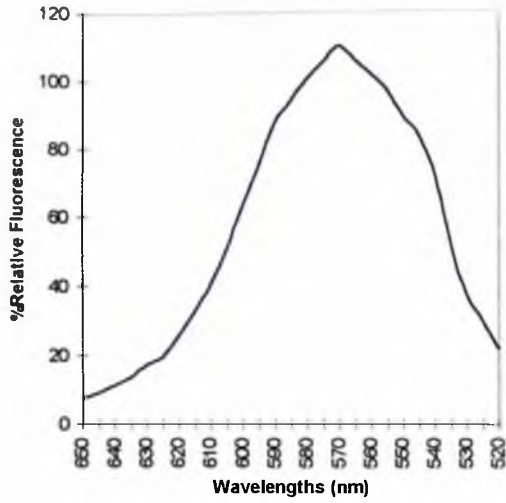


**Fig. 3k: % Relative Emission Fluorescence for 7-ER**

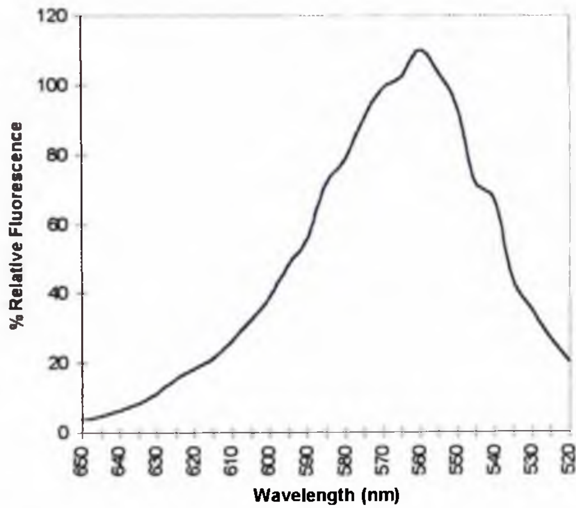


**Fig. 3l. % Relative Emission Fluorescence for 7-PR.**

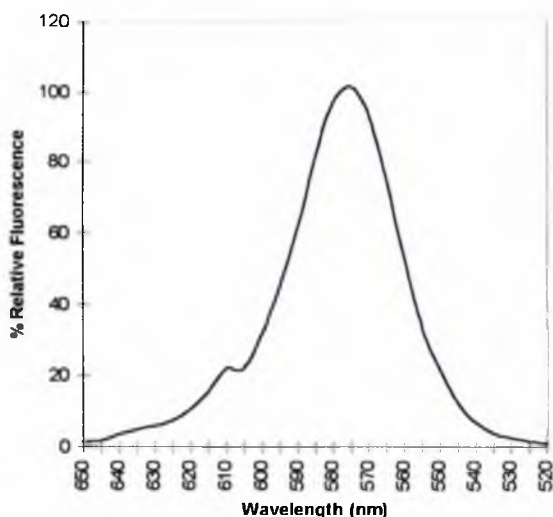


**Fig. 3m: % Relative Emission Fluorescence for Resorufin**

Figs. 3k, 3l and 3m show the emission fluorescence spectra of the substrates (7-ER and7-PR) and product (resorufin) respectively on the lambda drive of the spectrofluorometer used. These were done for both substrates (7-ER and 7-PR) and product, within the scan range of 130nm (i.e, 520 and 650nm) to determine wavelength of maximum fluorescence and ascertain their purity as described previously.

Spectra were shown to lie within the visible region of the electromagnetic spectrum and the resorufin (Fig. 3m) showed 100% purity, while attaining a maximum fluorescence at 581 nm. On the other hand, the subtrates showed more than 100% purity: at 560 nm for 7-PR (Fig. 3l) and at 570 nm for 7-ER (Fig. 3k), indicating that whatever impurities the substrates might contain also

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fluoresce at the same wavelength of maximum fluorescence accounting for excess fluorescence above 100%. These wavelengths of maximum fluorescence conform to literature values as reported by Burke and Mayer, (1974) and standards at the ICES-IOC EROD intercalibration workshop (ICES, 1991).

**7-Ethoxyresorufin-O-deethylase (EROD) assay.**

Enzyme activity for the EROD assay was highest in the microsomes prepared from *S. melanotheron* obtained from the Fosu lagoon. No activity was recorded for fish exposed to 'Roundup' (Fig. 3n).

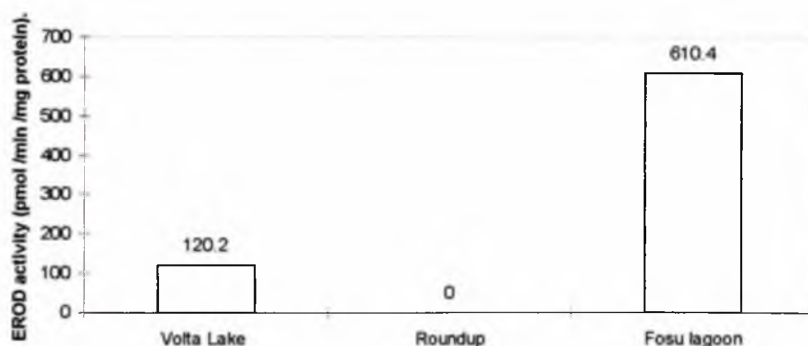


**Fig. 3n. Mean EROD activity for *S. melanotheron* from different 'exposure sites'.**

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Enzyme activity of the microsomes obtained from *S. melanotheron* from Fosu lagoon showed an increase of approximately 408% over that recorded for microsomes obtained from *S. melanotheron* from the Volta lake. After acclimation to the laboratory conditions, results show that EROD activity of microsomes from S. *melanotheron* obtained from Fosu lagoon was still higher, approximately 121% over that for fish from the Volta lake (Fig.3o.).

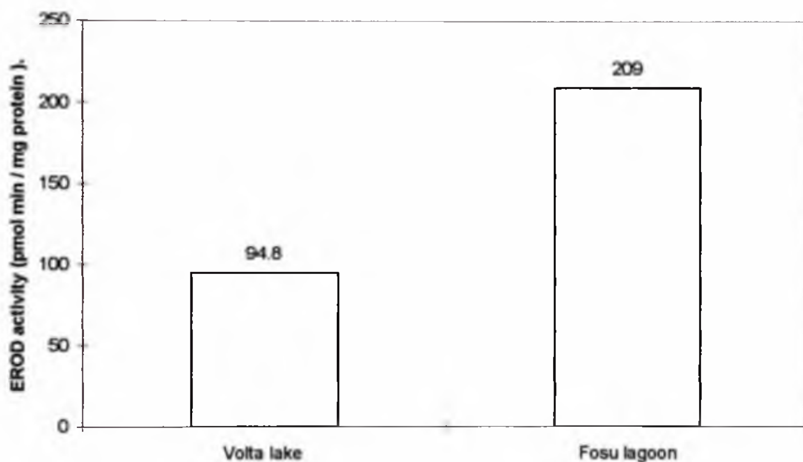


**Fig. 3o. Mean EROD activity for *S. Melanotheron* acclimated to the labortory conditions.**

Comparing the fish from the wild and those acclimated to the laboratory conditions, results indicated that the EROD activities were lowered by approximately 21% and 66% for fishes from Volta lake and Fosu lagoon respectively when acclimated to the laboratory conditions.

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From statistical analysis, it was evident that there was a significant difference in the EROD activities of microsomes from the fish in the Fosu lagoon compared to that from the Volta lake, both before and after acclimation (Table 3.1).

Analysis also showed significant differences in EROD activities in microsomes prepared from fish from Fosu lagoon before and after depuration but no significant difference in the values obtained for fish from the Volta lake.

**7-pentoxyresorufin-O-depentylase assay.**

This assay is specific for the CYP2B isozyme and is induced mainly by phenobarbital and its analogues. The assay indicated higher enzyme activity in the microsomes prepared from fish from the Fosu lagoon compared to fish obtained from the Volta lake at a site near Ada. These results are shown in Fig. 3p.

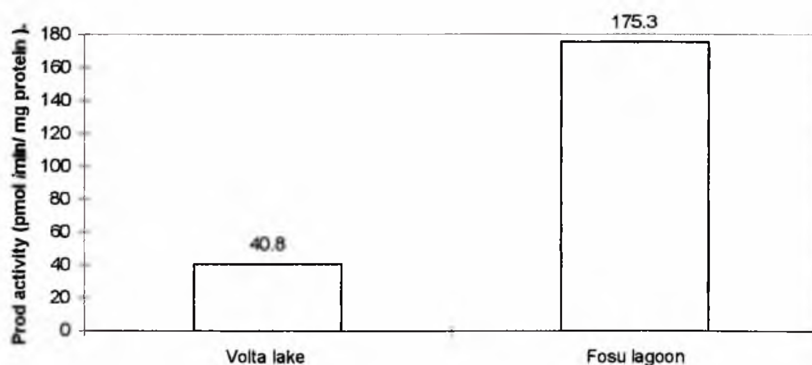


**Fig. 3p. Mean PROD activity for *S. melanotheron* from different 'exposure sites'.**

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The results indicated that PROD activity of the microsomes obtained from *S. melanotheron* from the Fosu lagoon showed an increase of approximately 330% compared to microsomes obtained from fish from the Volta lake. There was no PROD activity in livers from fish exposed to the herbicide, 'Roundup'.

Results from the fish acclimated to the laboratory conditions, showed that the PROD activity of the fish from Fosu lagoon was approximately 262% higher than fish obtained from the Volta lake (Fig. 3q).

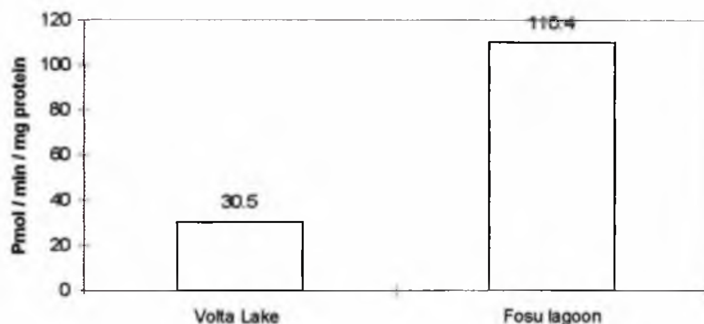


**Fig. 3q. Mean PROD activity for *S. melanotheron* acclimated to laboratory**

Comparing fish from the wild and those acclimated to the laboratory conditions, the results indicated that the PROD

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activities reduced by approximately 37% and 25% for fish from Fosu lagoon and Volta lake respectively.

There was a statisticaly significant difference between the values obtained for the fish from Volta lake and those from Fosu lagoon before and after acclimation to laboratory conditions. The difference in values for fish from the same exposure site before and after was also statistically significant, but not for fish from the Volta lake.

### 3.5 **Glutathione-s-transferase activity.**

The cytosolic enzyme investigated in this study, was glutathione-s-transferase. The activity was highest in *S. melanotheron* obtained from the Fosu lagoon. The activity in the cytosol from *S. melanotheron* exposed to 'Roundup' was almost the same as that observed for the fish from the Volta (Fig. 3r).

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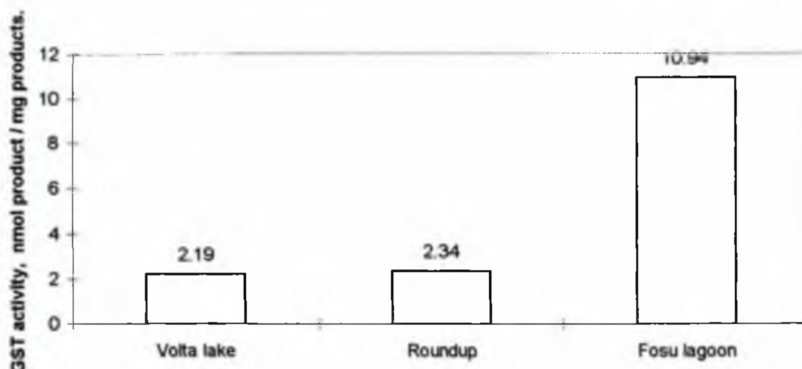


**Fig. 3r. Mean glutathione-s-transferase activity for *S. melanotheron* from different 'exposure sites'.**

The results indicated that S *melanotheron* obtained from the Fosu lagoon have activity approximately 400% higher than those from Volta lake. Those from the *S melanotheron* exposed to the herbicide (Roundup), showed an activity of approximately 15% higher than those from Volta lake. *S. melanotheron* from the Fosu lagoon showed an activity of approximately 368% higher than fish samples exposed to the 'Roundup'.

The GST activity of the fish from Fosu lagoon was approximately 101% higher than those from the Volta lake when the fish were acclimated to laboratory conditions (Fig. 3s).

The GST activity dropped by approximately 61% and 2% for fish Fosu from lagoon and Volta lake respectively, after acclimation to laboratory conditions.

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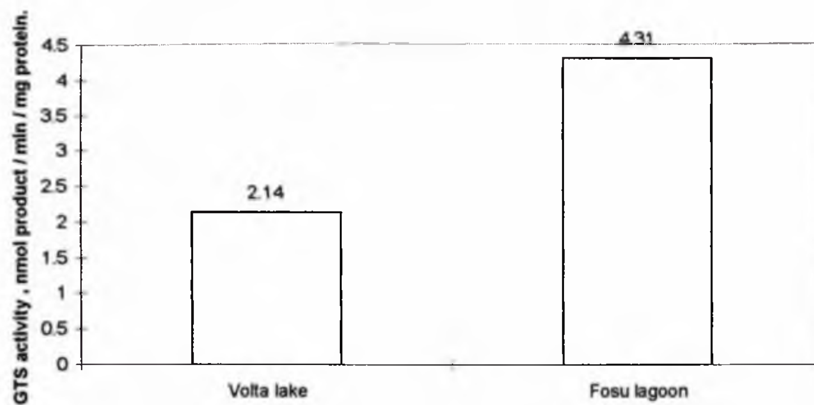


**Fig. 3s. Mean glutathione-s-transferase activity for *S. melanotheron* acclimated to the laboratory conditions.**

The difference between the mean values of GST activity for *S. melanotheron* from Fosu lagoon and Volta lake was found to be statistically significant and so was the difference between the activity of the enzyme for fish from the Fosu lagoon and those exposed to 'Roundup' (Table 3.1).

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Table 3.1 Student's t table of biochemical parameters for *S. melanotheron* from different sources. The t-test values at $p < 0.05$ confidence level and degrees of freedom = 6 t-tabulated = 2.776.

| Source of fish samples being compared | t-calculated | | | | | |
|---|---|---|---|---|---|---|
| | Total Protein | Reductase | Cytochrome P450 | EROD | PROD | GST |
| VL & FL | 4.231 | 6.041 | 5.049 | 6.732 | 4.329 | 4.430 |
| VL & RU | 1.037 | 4.321 | 4.603 | _ | _ | 0.147 |
| FL & RU | 2.978 | 3.979 | 1.605 | _ | _ | 4.333 |
| VL, before and after depuration | 0.785 | 2.910 | 1.470 | 2.556 | 2.114 | 0.791 |
| FL, before and after depuration | 2.818 | 4.001 | 2.915 | 3.728 | 3.601 | 4.308 |
| VL & FL , after depuration | 0.753 | 3.651 | 1.274 | 3.349 | 3.183 | 3.109 |

VL = Volta lake, FL = Fosu lagoon, RU = 'Roundup'

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# CHAPTER FOUR : DISCUSSION AND CONCLUSION.

The chemical identification of unknown compounds in aqueous effluents can be prohibitively expensive, time-consuming and even of little value if there is no information on biological effects produced by the chemicals on the resident organisms in the aquatic ecosystem.

The increasing awareness of fish as useful models for carcinogenesis, toxicity testing, and environmental monitoring studies in aquatic environment has documented that fish possess cytochrome P-450 dependent monooxygenase and glutathione-s-transferase, and that induction of these enzymes activities can provide a useful early warning marker to assess the exposure to some important classes of pollutants (Payne *et al*, 1987; Stegeman and Klopper-Sams, 1987; Andersson and Forlin,1992; Vigano *et al*, 1994).

This study, carried out using *Sarotherodon melanotheron* ,was in two phases. One phase dealt with induction in aquaria studies in the laboratory, with a commonly used herbicide, 'Roundup', as the pollutant. Phase two involved a field study, in the Fosu lagoon in Cape Coast, in the Central Region of Ghana. This site was chosen because active fishing occurs here and this species is readily available. This field study was to assess the effect of variable xenobiotics that are thrown into the lagoon from different sources, on the protein components of the NADPH-dependent monooxgenase enzyme system and glutathione-s-transferase.

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In this study, we chose the herbicide, 'Roundup', which is commonly used on agricultural farms, and are washed into aquatic ecosystems through surface run-off waters. We therefore assessed its effect on the fish in fresh waters.

The present study, for the first time, has characterised the monooxygenase system and glutathione-s-transferase in the liver of *Sarotherodon melanotheron,* and has shown interesting properties in consonance with results of other fish studied elsewhere.

Specific protein and enzyme activities were measured to ascertain the usefulness of biochemical responses as health indicators in fishes when exposed to pollutants. The effects/responses were to be used to determine the extent of lethal/toxic effects on the fish population when exposed to variable toxicants/pollutant. Such studies have been conducted in Sweden on fish exposed to Bleached Kraft Mill Effluent (BKME) by Lindsrom-Seppa and Oikari (1990). Similar response of fish to Akosombo Textile Limited Effluent (ATL-E) were conducted in Ghana in 1995(Addy *et al,1995* and 1996 by Faabeluon and Gadagbui.

The mean total microsomal protein concentration of the fish from the Fosu lagoon studied showed an approximately 69% more microsomal protein than fish obtained from the Volta Lake (Fig.3b). Fish exposed to the herbicide, 'Roundup' for 14 days had approximately 13% more microsomal protein than fish from the Volta lake.

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But when fish from the Volta lake and Fosu lagoon were acclimated in chlorine-free tap water for 21 days, the mean total microsomal protein concentration reduced by 2% and 27% respectively (Fig. 3c). One may speculate that this fall in values reflects the extent of pollution in these two freshwater bodies. Acclimation of feral fishes in tap water reverses any induction in seven (7) days (Lech *et al*, 1982). Therefore, these results indicate that the Fosu lagoon is highly polluted, since the microsomal protein concentration remains high after 21 days of acclimation to the laboratory conditions. A much longer time period in clean water, longer than 21 days is needed for depuration.

A high percentage mortality (70%) was recorded for the fish exposed to 'Roundup' in 5 days when a concentration of 5ppm was used. This may be an indication of the breakdown of the cytochrome P-450 system, the first line of defence against xenobiotics, in response to the high toxicity of the 'Roundup' applied. When 1ppm of the herbicide was used, there was no mortality but protein induction.

The difference in the total microsomal protein concentration before and after depuration was insignificant for the fish from Volta lake, suggesting this concentration to be the constitutive level of hepatic microsomal protein of *S. melanotheron* in freshwater. The significant difference in mean total microsomal protein concentration recorded for fishes from the Volta lake and Fosu lagoon indicates the occurrence of induction due to xenobiotics discharged into the lagoon from different sources.

in the Fosu lagoon

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Of the three hypotheses put forward to account for elevated levels of microsomal protein, the presence of xenobiotics (agrochemical, domestic, laboratory and hospital waste) in the fish environment, plausibly explained the rise in the microsomal protein in both Fosu lagoon and Roundup treated fish (Porter and Coon, 1991; Andersson and Forlin, 1992). Since all the controls and test fishes were held at the same water temperature and photoperiodicity, the alternative hypothesis of environmental temperature, implied by Snegaroff and Bach (1990) to influence P-450 protein synthesis and activity was discounted. Because of the absence of any evidence relating to differential sexual maturation in the fish used in this project, the surge in vitellogenesis could also not be the factor involved in the elevated protein levels.

Therefore one could infer that total hepatic microsomal protein can be used as a tool to measure pollution levels when the pollution is very high.

Functionally, the induced cytochrome P-450 protein of mixed function oxygenase is to transfer reducing equivalent from endogenous NADPH through a reductase to bring about reduction of xenobiotics introduced into the living system.

In the absence of statistically significant changes in the cytochrome P-450 content of *S. melanotheron* from the Volta lake when acclimated to the laboratory conditions and before, one may regard the value obtained to be the constitutive level of the cytochrome P-450 in the liver. The high value recorded for fish from the Fosu lagoon decreased by a significant margin (51%),

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after depuration, which indicates pollution in the Fosu lagoon. The high level of cytochrome P-450 content was due to the continued exposure to xenobiotics/pollutants. In the absence of pollutants/xenobiotics in the aquaria, the cytochrome P-450 content reduced due to the absence of induction.

Similarly, induction or cytochrome P450 was observed when acclimated fish stock from the Volta lake were exposed to a dose of 'Roundup' for 14 days. There was an increase of 113% over the basal/constitutive level. Evidence from total microsomal protein determination indicated that the amount of protein induced was insignificant, yet the cytochrome P450 content induced was as much as that of the fish from the Fosu lagoon, since there was no statistically significant difference between the two. This result may be a reflection of the more potent nature of the 'Roundup' as a CYP inducer.

From these results, one can conclude that measurement of cytochrome P450 content of *S. melanotheron* can be used to monitor pollution levels in freshwater bodies.

The reductase assay showed a high level of enzyme activity in the fish obtained from the Fosu lagoon (Fig. 3d). Although the level of activity was significantly reduced, the activity remained high after acclimation to the laboratory conditions, compared to the fish obtained from the Volta lake. The decline in the activity after acclimation indicated the absence of the inducer(s). However, the high level indicates a possibly slow breakdown of the induced protein.

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The fish from the Volta Lake showed a statistically significant reduction in the reductase activity when acclimated to the laboratory conditions. This is an indication of some degree of induction of this enzyme in the fish from the lake. However, there was no significant change in the hepatic microsomal protein and cytochrome P450 when the fish were acclimated to the laboratory conditions. Therefore one could speculate that the higher reductase activity before depuration could be ascribed to some changes, eg due to diet changes in the internal system of the fish, and not necessarily due to the presence of pollutants. since the reductase is required to shuttle reducing equivalents from NADPH and NADH to electron acceptors

Significant level of reductase enzyme activity was observed in fish treated with the 'Roundup' over its control. This is an indication that the reductase, as a component of the cytochrome P-450 monooxygenase enzyme system, was induced by the 'Roundup', and might probably aid in its detoxification. Comparing the levels of this enzyme activity in fish from the Fosu lagoon and those exposed to 'Roundup', a statistical significant change was observed, unlike the levels of induced CYP. One can conclude that reductase assay for *S. melanotheron* cannot be used to monitor pollution levels, since the activity was higher when cytochrome P450 was not high.

The EROD (as indicator of CYP1A induction) and PROD (as indicator of CYP2B induction) assays were used to charaterize the type of hepatic monooxygenase activity induced in *S. melanotheron*.

The results obtained were interesting in that no EROD and PROD activities were detected when the acclimated fish were treated with 'Roundup' (Fig. 3n and 3q.) Yet there was evidence to indicate that these two enzyme activities were present in the livers of the acclimated fish which were treated with 'Roundup' in the aquaria, (Fig.3d and 3f). Explanation of the observation, that is, the elimination of the protein responsible for the EROD and PROD activities, could be the structure of 'Roundup', since the CYP protein induced is related to the general structure of the inducer: CYP1A is induced by polycyclic aromatic hydrocarbons and its analogues and CYP2B by phenobarbital and its analogues. The 'Roundup's active ingredient is N-(phosphonomethyl)-glycine, an organophosphate, which is not structurally related to the inducers mentioned above, thereby inducing different CYP protein other than CYP1A and CYP2B. Another plausible explanation could be due to the fact that there was an inhibition of monooxygenase activities by 'Roundup' which had accumulated in the liver from which the microsomes were prepared.

Results of the overall monooxygenase enzyme activities in fish from the Fosu lagoon, showed that CYP1A activity was 3.5 fold higher than CYP2B. This could be ascribed to the fact that there are less PB-type of inducers than 3-MC type in the Fosu lagoon. The difference could also be due to the 3-MC types there being more potent inducers than the PB-types.

In general, samples of *S. melanotheron* collected from Fosu lagoon, compared with those collected from the Volta lake, had significantly higher hepatic microsomal monooxygenase enzyme as measured by total microsomal protein, reductase and cytochrome

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P450 content. In addition EROD and PROD activities were relatively higher. The Volta lake appears to represent a relatively clean body of water probably because it receives less domestic or commercial affluents (discharges) and new water from the upper part of the Volta lake. One may conclude that the higher level of activities of proteins/enzymes associated with pollution, which were recorded for fish from the Fosu lagoon are due to the non-flow nature of the lagoon and continuous discharge of waste into it.

Glutathione-s-transferase activity was statistically significantly higher in fish obtained from Fosu lagoon compared to those from the Volta lake (Table 3.1). The activity was not significantly increased in fish acclimated to the laboratory conditions and exposed to 'Roundup' (Fig.3r). The higher GST enzyme activity of fish from Fosu lagoon could be due to the fact that variable amount of toxicant/xenobiotics enter the fish in the lagoon, thereby causing the induction of this enzyme for their elimination, following transformation of these xenobiotics by the monooxygenases.

It has been observed that GST has a relatively slow induction response and its induction can be enhanced by both polycyclic and polychlorinated hydrocarbons (Zhang *et al*,1990; Andersson *et al*, 1985). A plausible reason for the insignificant level of induction of this enzyme by the herbicide could be due to the fact that the active ingredient in 'Roundup' is an organophosphate and not a polychlorinated hydrocarbon which has been found in previous studies to enhance GST induction. These observations indicate

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that GST could be used as a tool to monitor pollution in freshwater bodies, except for organophosphate based-pollutants.

In conclusion, the data generated in the present study indicate that the pollutants/xenobiotics discharged into the Fosu lagoon, as well as 'Roundup', are capable of inducing some biomarkers, i.e, reductase, CYP and GST, all used in hepatic biotransformation and elimination of xenobiotics. It is not possible to determine which particular constituents are responsible for the biomarker responses in the fish from the Fosu lagoon, but polyaromatic hydrocarbons and polychlorinated biphenyls are likely to be major contributors. The results suggest that either the effluent contains some highly potent inducers of CYP1A and CYP2B activities, or that synergism between two or more compounds or classes of compounds may be occurring with regards to the induction of these activities.

Results from this study have shown that depuration of fish from the Fosu lagoon should be extended beyond 21 days, probably 60 days or more, to enable complete removal of the pollutants. Since depuration is time dependent, much time must be allowed for complete eradication of the pollutant, if any.

The results also show that the water body used by Lee et al (1979) was less polluted relative to the Fosu lagoon, since depuration was completed in 7 days.

These results indicating protein induction in apparent healthy fish by pollutants, demonstrate the utility of the cytochrome P-

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450 monooxygenase enzyme system and glutathione-s-transferase as valuable early-warning biomarkers of pollution, as well as biomarkers to detect exposure of aquatic fish resource to contamination in tropical freshwater bodies.

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# Appendix

## Homogenizing Buffer pH 7.4

This was prepared by dissolving 13.804g of $NaH_2PO_4.H_2O$ and 11.203g of KCl in 500ml distilled water. A 0.372g EDTA was added and to the resulting solution was added 115ml of 87% glycerol. The pH of the final solution was adjusted to 7.4 with few drops of concentrated solution of NaOH and distilled water added to 1L, to obtain buffer containing 0.1M $NaH_2PO_4.H_2O$, 0.154M KCl. 1mM dithiotreitol (DTT), 1mM EDTA and 10% glycerol.

## Storage Buffer pH 7.4 :

Preparation and components are the same as that for homogenizing buffer, except the percentage of the glycerol which was increased to 30%. This was obtained by addition of 345mL of the 87% glycerol.

## Alkaline copper-phenol reagent:

The following solutions were made:

(i) sodium carbonate, 2% w/v in 0.1M NaOH

(ii) Copper sulphate (hydrated) 1% w/v, in water,

(iii) Sodium potassium tartate, 2% w/v in water.

Copper-phenol reagent was prepared freshly by mixing the above sodium carbonate, copper sulphate and sodium potassium tartate

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solutions in the ratios of 100 :1:1 v/v respectively. Water used here was doubly distilled.

**0.10M Tris Buffer pH 7.4 :**

This was prepared by dissolving 6.057g of Tris and 100ml of 100% glycerol in 250ml of distilled water. The pH of the resulting solution was adjusted to 7.4 with few drops of concentrated solution of NaOH and distilled water added to make 500mL buffer of 0.10M tris and 20% glycerol.

**2% w/v NADPH.**

A 10mg of NADPH was dissolved in 0.5mL distilled water. This was prepared fresh.

**EROD/PROD buffer, pH 6.8:**

This was prepared by dissolving 7.8001g of $NaH_2PO_4.2H_2O$ in 350ml of 0.1M NaOH. The pH was adjusted with 0.1 NaOH to pH 7.6.

**10mM NADPH:**

10mM βNADPH corresponds to 8mg dissolved in 1mL distilled water. This solution could be stored at -20°C for 1 week.

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