' COMPARATIVE STUDIES OF POLLUTION-INDUCED MICROSOMAL NADPH-DEPENDENT CYTOCHROME P-450 MONOOXYGENASE ENZYME COMPLEX OF TILAPIA SPECIES

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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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DEDICATION

TO MY FIANCEE, MISS VICTORIA KUUSANGYELE AND MY DAUGHTER, EVELYN FOR THE MORAL AND FINANCIAL SUPPORT THEY AFFORDED ME DURING THIS WORK

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TABLE OF CONTENTS

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

The baseline levels of hepatic microsomal proteins of cytochrome P-450 monooxygenase enzyme complex of two economically important tilapia species, Oreochromis niloticus and Seratherodon. galilaeus, were measured and compared with the levels of the enzyme in O. niloticus injected with ß-naphthoflavone (ß-NF) a classical inducer of cytochrome P-4501A isozyme, and O. niloticus exposed to effluents from Akosombo Textiles Limited (ATL-E). This was done in order to find out if this enzyme complex could be used as a biomarker to determine the ATL effluent effects on the fish species in the Volta Lake into which the effluent is discharged.

The relationship between exposure to the pollutants and the health status of the fishes was assessed by measuring two biological indices - condition factor (CF) and liver somatic index (LSI). Total protein concentration was determined by the Folin-Lowry method. The activities of NADPH cytochrome P-450 reductase, a component of the monooxygenase enzyme complex, were measured using reduction of exogenous cytochrome c. Cytochrome P-450 enzyme activity was measured using ethoxyresorufin-O-deethylase (EROD) assay which indicates specifically the induction of cytochrome P-4501A isozyme.

The results indicate no relationship between the exposure to the pollutants, ATL-E and ß-NF, and the CF (one of the biological indices). On the other hand, there was a relationship between exposure to ATL-E and the health status of the fish expressed

as liver somatic index (LSI). The results further indicate that the total microsomal protein concentration and the total cytochrome P-450 reductase activity of S. galilaeus were 2-fold and 1.5-fold higher than the values in Q. niloticus. The total microsomal protein concentration of the ATL-E controls, that is fishes at Konkontekope, showed a significant increase relative to the aquarial ß-NF controls. In spite of the higher protein concentration of the ATL-E control fishes over the aquarial controls, there was no difference in their total reductase activity. Both types of control fishes as well as the S. galilaeus did not show any response to the EROD assay indicating the absence of cytochrome P-4501A isozyme in the microsomal proteins induced.

There was a significant increase in the total microsomal protein concentration of test fishes over their controls. Microsomes from fishes exposed to the ATL effluent showed increases in the total NADPH cytochrome P-450 reductase activity compared to their controls, unlike the microsomes from fishes injected with ß-NF. However, microsomes prepared from both ß-NF-injected and ATL-E exposed fishes responded to the EROD assay indicating the presence of cytochrome P-4501A isozyme induction by these pollutants. The induction of total NADPH cytochrome P-450 reductase by the factory effluent suggests that the xenoorganics in the factory effluent could belong to the PB-type or the third type of inducers. That the cytochrome P-4501A protein was induced by the factory effluent, as evidenced by the positive response to EROD assay,indicates that the effluent contains the 3-methylcholanthrene type of inducers.

This study has indicated that the effluent from the Akosombo Textiles Limited contains a mixture of inducers - the 3-MC type, the PB-type and the third type. The three types of inducers may act synergistically to promote the induction exhibited. The molecular weight(s) of the monooxygenase isozymes induced by both ßNF and ATL-E were resolved electrophoretically on 10% SDS-PAG. The BNF and ATL-E exposed fishes were found to induce protein isozymes with similar electrophoretic mobilities with an approximate molecular weight of 53,700 daltons.

LIST OF ABBREVIATIONS

A_{550}	Absorbance change at 550 nm
ATL-E	Akosombo Textiles Limited Effluent
B(α)P	Benzo(α)pyrene
BKME	Bleached Kraft Mill Effluent
BNF	ß-naphthoflavone
BSA	Bovine serum albumin
CF	Condition factor
CoA	Coenzyme A
Concbis	concentrated bisacrylamide solution
C_p	Actual concentration of microsomal protein
Cyt.P-450	Cytochrome P-450
d.f.	Degrees of freedom
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
EROD	Ethoxyresorufin-O-deethylase
EVOS	*EXXON Valdez* Oil Spill
FAD	Flavin adenine dinucleotide
FAO	Food and Agricultural Organization
FMN	Flavin mononucleotide

g	gram
HCl	Hydrochloric acid
i.p.	Intraperitoneal
IAB	Institute of Aquatic Biology
kDa	Kilodalton
Kg	Kilogram
LSI	Liver somatic index
min	minute
ml	millilitre
mmol	millimole
mRNA	Messenger Ribonucleic acid
MT	metallothionein
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium hydroxide
NRDA	Natural Resources Damage Assessment
PAH	Polyaromatic hydrocarbons
PB	Phenobarbital
PCB	Polycyclic/polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-p-dioxin
PCDF	Polychlorinated dibenzo furan
Ph	Benzene
pmol	Picomole
R_f	Relative mobility

rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SO_4^{2-}	Sulphate ion
TEMED	Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultra violet
v (Vol)	Volume
%	percentage
#	Specific activity
ϵ	Extinction coefficient of reduced cytochrome c at 550nm
μl	microlitre
3-MC	3-methylcholanthrene
7-ER	7-ethoxyresorufin

PLATES

LIST OF APPENDICES

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Increasing pollution of the aquatic environment is becoming a threat to the health of organisms inhabiting such ecosystem (Murchelano, 1990) as well as to man, a predator or consumer of such organisms (Dawe, 1990). In heavily polluted areas, fish as well as crabs and shrimps populations are known to have shown high incidence of lesions and neoplasms (Murchelano and Wolke, 1985; Malins et al, 1988) of such vital organs as livers, kidneys and the respiratory tracts (Dawe, 1990).

Increasing incidence of tumours, chromosomal and mitotic aberrations and other genetic as well as physiological disorders in fishes were 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 incidence have also been shown by Porter and Coon (1991) to result from elevated levels of toxic pollutants such as polyaromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCB's), organochlorines, dyes, dibenzofurans and dioxins.

It is known that at lower levels of pollution, when gross pathologies are not evident, biochemical and physiological disturbances may be innately present in fishes and other aquatic fauna and their users. Measurement of such disturbances can provide a more sensitive and reliable indications of stress from pollution than the gross

pathological changes, and have been recommended in monitoring strategies in marine pollution (Stebbing, 1989).

The pressing need to assess the impact of environmental toxicants on the complex ecosystem, particularly the aquatic ecosystem, met with problems that called for the evolution of various strategies designed to facilitate ecosystem health assessment. Initially, the detection and quantitation of pollutants were limited to physicochemical (limnological) parameters only in water, sediments and biota.

The demand for sensitive and specific biological assays in the detection, quantitation and assessment of pollutant levels led to the application of biochemical tools to preserve fish populations
(Förlin et al, 1985; Goksöyr et al, 1986; Stegeman et al, 1986; Goksöyr and Solberg, 1987). The strategies evolved were derived from the concept that the effect of a toxicant on the entire ecosystem originates with a biochemical reaction in an individual member of the ecosystem (Hodson, 1986) and that the primary effect of a toxicant occurs at the subcellular levels.

The effects of exposure to environmental pollutants can be classified and studied at three different levels of the biological organization viz; biochemical effects at the molecular and cellular level, physiological and pathological effects at the tissue, organ and organism level and the ecological effects at the population, community and ecosystem level.

The direct effect of a xenobiotic occurs at the 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 the ecological level. The consequences of this upward transmission are often observed as damage in nature and serve as the basis for risk assessment and regulatory action (Molven and Goksöyr, 1992).

Pollution effects on the ecosystem can therefore be understood and predicted if the toxicant impact is identified at the subcellular levels e.g. at the level of mitochondria or endoplasmic reticulum. Before this, the toxicant impact was evaluated at the population and community level when lesions and a drop in production and/or growth were visible. The toxicant impact was also assessed at the individual level as toxicant accumulation or concentration in muscle and blood (Molven and Goksöyr, 1992). Sensitive and specific biochemical methods can therefore serve as early warning indicators for the survival of organisms and corrective or preventive measures can be taken to obviate any eventual human health hazards and possible extinction of aquatic organisms.

Response to biochemical methods in toxicant monitoring is primarily related to the potential of toxicant assessment. Several biochemical assay systems showed great promise, some of which included assays based on carbohydrate, protein and fatty acid metabolism and blood chemistry (EIFAC, 1975; ICES, 1978; Stegeman, 1981; Hamilton and Mehrle, 1986; Hodson, 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 (Stegeman, 1981) and the other, the 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 metabolise endogenous substrates such as arachidonic acid, fatty acids and steroids.

The metallothioneins (MTs) are cytosolic, low molecular weight metal binding proteins that appear to be present in virtually all vertebrates and invertebrates and are capable of binding metal ions in groups Ib and IIb of the Periodic Table, and Zinc, Copper, Cadmium and Mercury are usually associated with the protein (Dunn et al, 1987). The MTs can be induced by metals and elevated levels of MTs have been correlated with inducing metals in tissue (Durnam and Palmiter, 1981).

Cytochrome P-450 monooxygenase enzyme complex, which metabolises organic pollutants, was the method focused on in this project since early work done on rainbow trout (Oncorhyuchus mykiss), Atlantic cod (Gadus morhua), plaice (Pleuronectes platessa), perch (Perca fluviatilis) [Goksöyr, 1985], scup (Stenotomus chrysops) (Klotz et al, 1983) and a Tilapia species - Oreochromis mossambicus (Ueng et al; 1992) have shown the presence of this enzyme system as a result of induction by pollutants. This indicates that fish, like man, used the NADPH-dependent cytochrome P-450 monooxygenase enzyme system of the liver to get rid of xenobiotics. The monooxygenase enzyme complex, often found in the endoplasmic reticulum, is also referred to as the microsomal mixed-function oxygenase, drug

metabolizing enzyme or the carbon monoxide-binding pigment.

The liver microsomal mixed-function oxygenase enzyme system is specifically induced by xenobiotics and as such, its level reflects the amount of pollutant taken in by the fish (Anderson et al, 1989). The system has been used to establish baseline pollution levels in the aquatic/marine environments such as Hvale Archipelago in Norway (Goksöyr et al, 1991a), where PAH, PCB, DDT/DDE, dibenzofuran, dibenzo-p-dioxin levels in the fish liver and water or sediment were estimated both chemically and biochemically. The use of such methods
to monitor pollution and the response of fishes of economic importance such as plaice, scup, rainbow trout, perch and cod (Goksöyr, 1985) to pollutants has enabled Goksöyr et al (1991a) to devise an immunological assay system e.g. ELISA. The system has been used to obtain results which have influenced government policies on aquatic pollution monitoring, ecotoxicology and fish testing.

Krahn et al, (1986a; 1986b; 1992); Bayer and Goksöyr, 1993 and Collier et al, (1993)) worked on the assessment of crude oil or petroleum hydrocarbon exposure and the resulting biological effects in subtidal fishes of economic importance in the Gulf of Alaska. Examples of fishes studied following the *Exxon Valdez* oil spill (EVOS) included Dolly Vandenchar (Salvelinus malma), yellowfin sole (Limanda aspera), rock sole (Lepidopsetta belineata), flathead sole (Hipoglosoides lassodon) and pollock (Theragra chalcogramma) . All the results, collected under the aegis of the Natural Resources Damage Assessment (NRDA) were used to influence international

oil shipment policies as well as the marine pollution management policies of the North American countries and Alaska (Krahn et al, 1986b).

For a very long time, Africa, and for that matter Ghana, was thought to be free from pollution. However, in recent times, the rapid population growth accompanied by intensive urbanization and bad waste management policies have resulted in the accumulation of heavy and trace metals in both fresh and marine water bodies and hence in fishes of economic importance (Biney, 1991; Biney and Berko, 1991; Biney, 1992). It is now known that industrial activities contribute heavily to the pollution of both marine and fresh water bodies in Ghana.

Mining, textile dyes and agrochemicals are mostly known to be pollutants of inland fisheries. Discharge from such mines, manufacturing industries such as plastics, paint, textiles and cement industries as well as agricultural pesticides have been shown to induce cytochrome P-450 (WHO/OCP, 1985; Goksöyr et al, 1991).

Animal protein contains all the nutrients in terms of essential amino acids and vitamins B_{12}, and it is known to be devoid of harmful materials as gossypol which is characteristic of some vegetable protein. Also, animal proteins are generally palatable and savory, making it preferable to vegetable proteins (Altschul, 1962). Altschul further noted that animal foodstuffs contained, by far, higher protein concentrations than vegetables and increasing the supply of animal foodstuffs is thus one means of attacking the dietary protein deficiency in Africa. Animal husbandry

practices in Ghana are primitive and meat demand far exceeds the supply and hence the high cost of meat particularly in the southern part of this country.

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.

Inland fish production figures reported in the FAO (1989) year book of fisheries statistics for West Africa showed very high percentages. The figures may be underestimated since freshwater fish harvest by subsistent fish farmers, whose production form a sizeable fraction of the harvest, is often not included in fisheries Statistics Bulletins.

It is estimated that Tilapia species accounted for over sixty percent of catches landed in countries like Ghana. This fish is also known to be widely distributed throughout Ghana and therefore a fish of economic importance. Its preserved form is popularly called "koobi". Of the many Tilapia species available in Ghana, Oreochromis niloticus is the most widely distributed inland and therefore a good species to use for a nation-wide study.

Ghana, like other West African countries, has considerable tilapia resources. Irvine (1947) recorded the presence of five tilapias that are now considered commercially important in Ghanaian fisheries and aquaculture. These are Oreochromis niloticus.

Sarotherodon galilaeus, Sarotherodon melanotheron, Tilapia busumana and Tilapia zillii. These are distributed in the two main river basins in Ghana i.e. Volta and the Southern -Western rivers.

Four of the five species listed by Irvine, 1947 are in the Volta basin although S. melanotheron is rare. T. busumana is excluded from the Volta basin but it is found together with S. galilaeus, S. multifaciatus, S. melanotheron and T. zillii in the Southern and Western river basin. The other commercially important species, Tilapia discolour and S. galilaeus multifaciatus occur in Ghana's only natural lake - Lake Bosomtwi.

The formation of the Volta Lake in 1965 provided a large fresh water body in which tilapias including O. niloticus proliferated. Large populations of tilapias developed in the Afram arm and areas such as Kpando. The completion of the Kpong dam in 1981 on lower Volta below Akosombo created a new reservoir for O. niloticus proliferation.

Tilapia, especially O. niloticus has now become abundant in Northern Ghana due to migration from stocks in Burkina Faso, Cote d'Ivoire via The Black, Red and White Volta and from Togo via the Oti river. O. niloticus was selected for this study due to the very widely distributed nature and the position it occupies in the food chain, serving as a mediator between the lower level and man.

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 Ghanaian. Tilapia in its preserved form, "Koobi", has become the major trading commodity for some women in Ghana. It is on record that O. niloticus was exported from this country alive to aquaculture centres in Japan in 1962, to Thailand in 1965 and to the Philippines in 1972. It was also exported to Burkina Faso, Mali and Niger as 'Koobi' as such, acting as foreign exchange earner for Ghana.

Of such commercial importance are tilapias and for that matter O. niloticus that any research, as this one, that seeks to establish the fish response to common pollutants at possible hatchery and breeding as well as feral fishing sites will be of assistance to policy makers regarding health, agriculture and environmental issues.

The overall aim of this proposed research is to establish the presence of a biomarker - NADPH-dependent cytochrome P-450 monooxygenase complex in two economically important Tilapia species i.e., O. niloticus and S. galilaeus; to measure the levels of this biomarker due to induction by a classical pollutant (BNF) and the effluent issuing from Akosombo Textiles Limited (ATL-E), and to use this induction as a sensitive tool in monitoring pollution in our freshwater ecosystems with the view to averting reduction in fish stocks.

The study carried out on Q. niloticus was in two phases. Phase one dealt with β-naphthoflavone (a classical pollutant) induction in aquarial studies at the Institute of Aquatic Biology's aquaculture station at Akosombo while the phase two involved field studies in the Volta Lake at the point where Akosombo Textiles Limited (ATL) effluents enter the Lake. These sites were selected close to one another so that the two phases could be carried out simultaneously. Studies in the Lake were conducted because active fishing takes place here and the species selected is also readily available. The work conducted at the factory effluent-entry point was to find out the inductive effect of the dyes and other xenoorganics issuing from the factory on the mixed-function oxygenase system. To realize these objectives, the goals set were to:

1. take samples of the selected fish species from different freshwater bodies and prepare microsomes from their livers after exposure to ß-NF and ATL-E,
2. measure total microsomal protein concentration of each fish in each group,
3. measure the NADPH-cytochrome P-450 (c) reductase activities of each sample group.
4. determine the response/activity of cytochrome P-450
5. determine the P-450 isozyme induced by ß-NF and ATL effluent.

1.2 LITERATURE REVIEW

1.2.1 Cytochrome P-450 Monooxygenase Enzyme System

1.2.1.1 Overview

The Cytochrome P-450 monooxygenase enzyme system is one of the recent assay systems being employed in monitoring pollutants such as heavy metals and organic compounds due to its sensitivity (Heath and Moore, 1985). The cytochrome in this enzyme is also known as the carbon monoxide-binding pigment, mixed-function oxygenase or the drug-metabolising enzyme (Mason, 1957a) and it represents a unique family of hemoprotein that has a role in the metabolism of a variety of xenobiotics including classical pollutants such as PAH, PCB, the organochlorines (DDT), the hexachlorobenzens, polychlorinated dibenzofurans (PCDF) and dibenzo-p-dioxins (PCDD), crude oil and other petroleum products (Kupfer, 1980; Axelrod, 1983; Waterman et al, 1986). In the case of toxic drugs/chemical or pollutants, the enzyme system chelates or binds to the drug, its substrate, and in the biotransformation reactions that ensue detoxify the pollutant and save the health of the organism. This enzyme system is also involved in the metabolism of endogenous compounds such as fatty acids, prostaglandins as well as steroids (Boobis et al, 1985).

In order to effect the metabolic biotransformation and detoxification, various reactions are catalyzed by this enzyme system. Some of these reactions include epoxidation, hydroxylation, deamination and deethylation (Gillette, 1966) enabling it to operate on a wide range of substrates in reactions of the so-called phase I metabolism.

In all vertebrates, hepatic microsomal monooxygenase system converts lipophilic xenobiotics, drugs and endogenous compounds to water-soluble products through a two-phase process. The first phase involves a change in the molecular structure of the substrate by the introduction of polar groups into the xenobiotic molecules while the phase II involves conjugation of the products of phase I with polar endogenous compounds (Smith, 1968). The less harmful conjugation products are then voided. Substrates of phase I reaction are called type-I substrates and are devoid of groups such as -OH, -COOH, -SH and $-NH_2$ which are groups involved in phase-II reaction. Those substrates used in the phase II reactions are called type-II substrates. The purpose of the phase II reaction is to detoxify and eliminate metabolites, since some of the phase I process may lead to increased toxicity as manifested in benzo(α)pyrene (Varanasi et al, 1987 and Sikka et al, 1990). The insecticides Schradan and Guthan are also relatively non-toxic until metabolism by liver microsomes transforms them into active cholinesterase inhibitors. The substrates or endogenous compounds provided by the body for conjugation are derived from materials involved in carbohydrate, protein and fatty acids metabolism and include glucuronic acid, glycine, cysteine, methionine and SO_4^{2-}.

1.3.1.2 Distribution and Location

The survival of most organisms is linked, in part, to their capacity to induce cytochrome P-450 enzyme system in contaminated environments. It has been reported that cytochrome P-450 enzyme is found in most animal species especially in the hepatic tissues. It was further reported that mixed-function oxygenase abounds in the endoplasmic reticulum and in some cases, the mitochondria (Pedersen, 1978).

In higher animals, cytochrome P-450 monooxygenase system occurs in the endoplasmic reticulum of cells of the gastrointestinal tract lungs, kidneys 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 (Fahl et al, 1978). In addition to 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. The P-450 monooxygenase has also been identified in yeast (Kawaguchi et al, 1973), bacteria where it showed specific catalytic activity and hence, was designated P-450_{cam}. Ambike et al, (1970) demonstrated the presence of P-450 in a fungal strain while Ferris et al 1976) showed the existence of P-450 enzyme system in various strains of fungi.

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 40,000 rpm (Mazel, 1971) or by calcium aggregation (Schenkman and Cinti, 1972) or by acid precipitation (Mazel, 1971).

The method used in this project to isolate microsomes as source of enzyme was

differential ultracentrifugation (Goksöyr et al, 1991). Although the method requires the use of expensive ultracentrifuges and long hours of centrifugation, it has an added advantage of sedimenting both smooth endoplasmic reticulum and the rough ones devoid of haemoglobin and hence good for spectral and enzymatic studies. It is known that acid precipitation inactivates ATPase as well as recording lower or the complete absence of some enzyme activity (Goksöyr et al, 1991b; Jensen et al, 1991).

1.3.1.3 Composition

Strobel et al (1970) successfully solubilized and resolved hepatic microsomes into three fractions which when reconstituted, catalysed the oxidative biotransformation of fatty acids and prostaglandins, steroids, drugs and other xenobiotics. Recent works have established the essential components of the monooxygenase system as involving cytochrome P-450, NADPH-dependent cytochrome P-450 reductase and a phospholipid (Yasukochi and Masters, 1976). Further characterising information on mammalian P-450 monooxygenase by resolution on SDS-PAGE (Gibson and Schenckman, 1978) showed it to contain the components above.

Cytochrome P-450 is an oligomeric complex containing between two to ten protein sub-units with molecular weight range of 45-65 kDa. This component also contains iron protoporphyrin IX in its prosthetic group. The NADPH-dependent cytochrome P-450 reductase was found to contain FAD and FMN prosthetic groups in a ratio 1:1. It is a hexamer with individual protein components each having a molecular weight of approximately 79 kDa. The last component, 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.

The name cytochrome P-450 was established by Omura and Sato (1964a) 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). Sato et al, (1965) noted that the dithionite-reduced CO-difference spectrum was very unusual for a cytochrome but provided no clue as to the nature of the cytochrome.

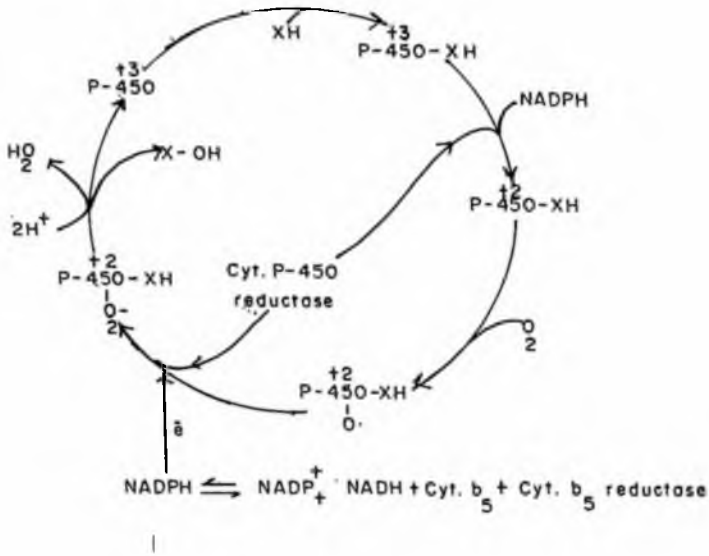
Imai and Sato (1966) also showed that an absorption maximum of 430nm would generally be observed when ethylisocyanide is used as the hemoprotein chelating agent rather than carbon monoxide. The denaturation of this cytochrome to give a form with a diagnostic absorption maximum at 420nm is also another important spectral characteristic of cytochrome P-450.

Studies with hepatic microsomes have shown clearly the existence of at least two electron transport chains localised in the membrane of the endoplasmic reticulum. The first and most important in drug and other xenobiotics metabolism, is composed of at least two proteins viz; a cytochrome P-450 and NADPH-dependent cytochrome P-450 (c) reductase (Hashimoto et al, 1962; Omura and Sato, 1964b).

Estabrook et al, (1976) further showed microsomes to contain a single reductase

molecule linked to between twenty to thirty molecules of cytochrome P-450. In addition, "accessory enzymes" such as epoxide hydrase and uridine diphosphate-glucuronyl transferase (Pullman and Monroy, 1963) are present in the endoplasmic reticulum with functions closely knit to the cytochrome P-450 monooxygenase system i.e. they effect the synthetic or conjugative phase II reactions.

The second type of microsomal electron transport chain consists of at least three protein components; cytochrome b_5, NADH cytochrome b_5 reductase (a flavoprotein) and fatty acyl CoA desaturase. This system uses electrons from NADH in the desaturation of fatty acids (Oshino and Omura, 1973). It has also been reported that this second electron transport system is involved in the reduction of azo and aminoazo dyes (Mueller and Miller, 1949). Here too, evidence by Kozlov and Miker'saar, (1974) shows a single reductase molecule shuttling electrons for thirty molecules of cytochrome b_5. Information now abounds showing the interaction between the cytochrome P-450 and cytochrome b_5 systems to the effect that the cytochrome b_5 system supplies the second electron for the oxidative cycles of cytochrome P-450 in metabolizing xenobiotics as illustrated in Fig. 1a below.



XH = Xenobiotic X-OH = hydroxylated xenobiotic

Fig. 1a. Synergistic oxidation of xenobiotics by cytochromes P-450-linked monooxygenase complex and b_5.

Through selective inhibition of cytochrome P-450 reductase and the determination of this treatment on the enzymatic reduction of cytochrome P-450, Estabrook et al (1976) and Matsubara et al, (1976) found that most of the components of the microsomal electron transport chain sit together in a semipermanent complex, but exchange slowly with free cytochrome and reductase molecules in the membrane.

This accounts for the biphasic (fast and slow) nature of NADPH-dependent reduction of cytochrome P-450 monooxygenases in microsomes and suggests that the fast phase

represents the reduction of cytochrome molecules sitting together with a reductase molecule in a complex; while the slow phase represents the reduction of "free" cytochrome molecules (Estabrook et al, 1963; Matsubara et al, 1976;) through exchanges.

This model was validated by Yang and Strickhart, (1975) in protein reconstitution experiments. Subsequently, work by Stier and Suckman, (1973) to ascertain the relationship between the microsomal monooxygenase proteins and the phospholipid in the membrane bilayer using water-soluble and water-insoluble substances suggested that cytochrome P-450 is enclosed in a phospholipid halo. This halo was found to be more rigid than the bulk of the microsomal lipid. The above work also showed that about 20% of the microsomal phospholipid is located in this halo and thus affects the diffusion of lipid-soluble substrates to, and of lipid-soluble products away from the cytochrome P-450 molecules.

1.3.1.4 **Reactions Catalysed**

The first description of metabolism of xenobiotic compounds by hepatic microsomes was given by Mueller and Miller, (1953) in which they showed liver homogenates catalysing both the reductive splitting of azo linkages and oxidative N-demethylation of aminoazo dyes. Brodie et al, (1955) showed that a similar enzyme system localised in hepatic microsomes was responsible for the metabolic biotransformation and

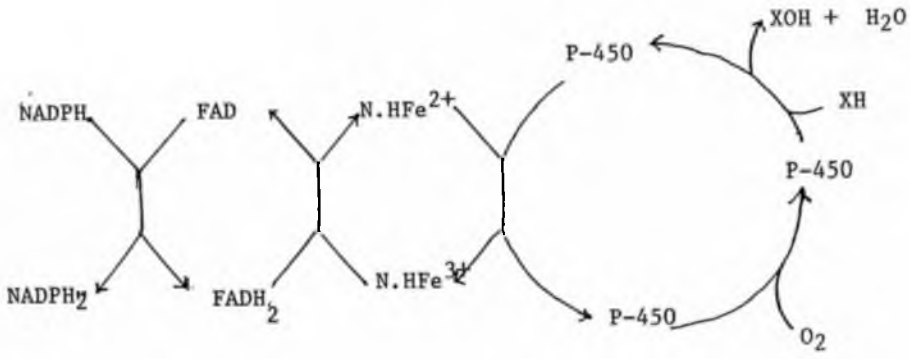
subsequent disposition of drugs and other xenobiotics. The involvement of NADPH

and molecular oxygen in these reactions was reported to be characteristic of mixed-function oxygenase by Mason, (1957a; 1965) and Hayaishi, (1964).

The results of Hayaishi, (1964) with uricase, glucose oxidase, steroid hydroxylases, aerobic cyclization of squalenes to lanosterol and the work on tyrosinase, xanthine oxidase and steroid-11β hydroxylase 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, (1967).

Whereas the second group had both oxygen atoms of molecular oxygen transferred to the substrate enabling it to be described as a dioxygenase or more appropriately as cyclooxygenase or oxygen transferase, the last group, called electron transferring oxygenases or lipoxygenases, reduces molecular oxygen to hydrogen peroxide or water (Mason, 1957b) and oxidizes substrates by one, two or four equivalents.

Microsomal xenobiotic or drug-metabolizing cytochrome P-450 enzyme system is thought of as involving the mixed-function oxygenase mechanism and figure 1b below shows the monooxygenase electron transport chains.



XH = xenobiotic X-OH = hydroxylated xenobiotic

Fig. 1b: Electron transport system involving xenobiotic-metabolizing cytochrome P-450 monooxygenase.

Two key proteins are involved. These are NADPH cytochrome P-450 (c) reductase (a flavoprotein) and cytochrome P-450. This electron transport chain is found in the endoplasmic reticulum of liver and other organs and functions to catalyze the transfer of electrons from NADPH to cytochrome P-450, its natural acceptor. Williams and Kamin, (1962) obtained a molecular weight of 68kDa for the flavoprotein while Masters et al, (1965) showed it to contain equimolar amounts of FMN and FAD. Cytochrome P-450 (c) reductase was also shown to catalyze the electron transfer to a number of artificial electron acceptors including cytochrome c, ferricyanide and dichlorophenolindophene.

A wide variety of oxidative reactions are known to be catalysed by the microsomal mixed-function oxygenase. These reactions include deamination, 0-, N-, and S-dealkylation, hydroxylation of alkyl and aryl hydrocarbons, epoxidation, N- and S-oxidations 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 components of the mixed-function oxygenase together catalyze are as follows.

(a) **Aromatic hydroxylation**

$$Ph \xrightarrow{[OH]} Ph\text{-}OH$$

(b) **Aliphatic hydroxylation**

$$R\text{-}CH_3 \xrightarrow{[OH]} R\text{-}CH_2\text{-}OH$$

c) **N-dealkylation**

$$R\text{-}NH\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}NH\text{-}CH_2\text{-}OH] \longrightarrow RNH_2 + CH_2O$$

(d) **0-dealkylation**

$$R\text{-}O\text{-}CH_3 \xrightarrow{[OH]} [R\text{-}0\text{-}CH_2\text{-}OH] \longrightarrow ROH + CH_2O$$

(e) **Deamination**

$$R\text{-}CH(NH_2)\text{-}CH_3 \xrightarrow{[OH]} R\text{-}C(OH)(NH_2)\text{-}CH_3] \longrightarrow RC0\text{-}CH_3 + NH_3$$

(f) S-oxidation

$$R\text{-}S\text{-}R' \xrightarrow{[OH]} [R\text{-}S(OH)\text{-}R^1] \longrightarrow R\text{-}SO\text{-}R^1 + H^+$$

(g) N-oxidation

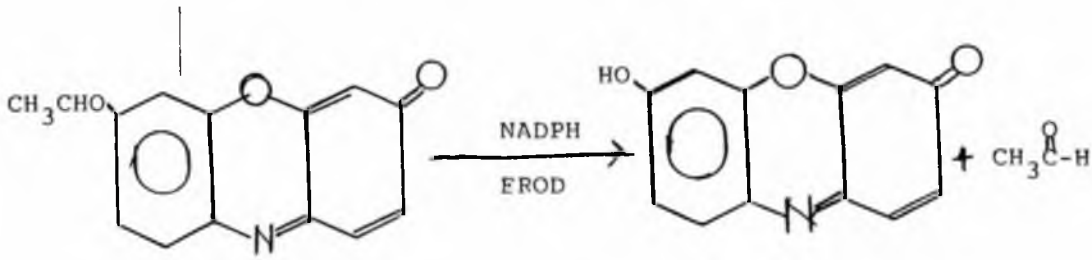
$$Me_3N \xrightarrow{[OH]} [Me_3N\text{-}OH]^+ \longrightarrow Me_3NO + H^+$$

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 PAH induction. Owing to the low specific activity of the NADPH-dependent cytochrome P-450 monooxygenase, assays of high sensitivity e.g. EROD is required to monitor the PAH induction.

Before the EROD assay was developed, a large number of assay systems were used to account for the broad substrate specificity and ready reduction of the mixed-function oxygenase. Those requiring solvent extraction and subsequent quantitation of metabolites involved a number of manipulations resulting in incomplete metabolite recovery as well as extended assay procedures and time.

Several spectrophotometric and spectrofluorimetric assays that reliably follow product analysis continuously have been used of late to obviate the aforementioned problems. Examples of such assays include ethoxycoumarin-O-deethylase, p-nitrophenetole-O-deethylase, p-nitroanisole-O-deethylase and EROD (Netter, 1966).

EROD is one of the few routinely used spectrofluorimetric probes for the catalytic activity of different cytochrome P-450 isozymes (Burke and Mayer, 1974). Of the substrates listed above, only ethoxyresorufin-O-deethylase activity is specific for cytochrome P-450 isozyme commonly induced by 3-methylcholanthrene and ß-NF and other structural analogues. The reaction proceeds as shown in figure 1c below;



7-ER Resorufin

7-ER = 7-ethoxyresorufin

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

The proposed details on the mechanism of action of the xenobiotic metabolising cytochrome P-450 monooxygenase via a hydroxylation reaction is shown in figure 1d. The scheme reflects the native ferric P-450 chelating the xenoorganic with a subsequent reduction to the ferrous state. This sets the stage for molecular oxygen to bind resulting in the second reduction where the oxygen-oxygen bond of molecular oxygen is split, one atom getting lost as water and the other incorporated as activated oxygen into the substrate to form the alcohol group, the discharge of which regenerates the ferric state of the enzyme.

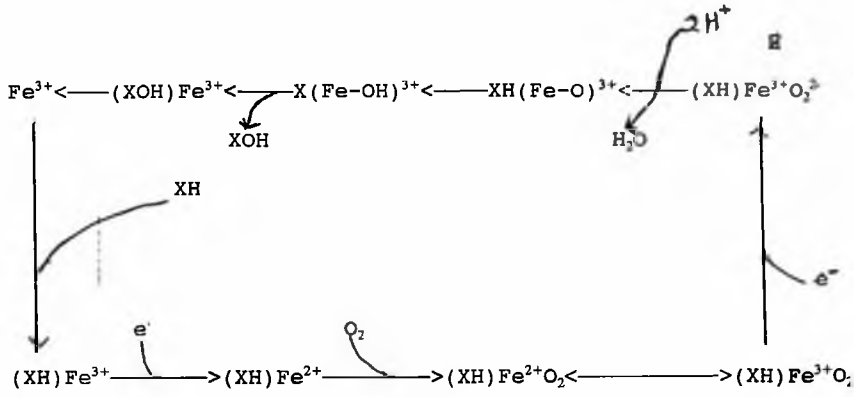


Fig. 1d. Proposed mechanism of action of cytochrome P-450 in the hydroxylation of substrates in the EROD assay.

1.3.1.5 **Isoforms of cytochrome P-450**

Evidence is now amply available implicating mixed-function oxygenase in drug and other xenobiotic metabolism (Hayaishi, 1964; Mason, 1965; Matsubara et al, 1974). 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 i.e. variations in one monooxygenase is due to variations in the substrates (Williams and Buhler, 1983; Goksöyr, 1985; Klotz et al, 1986). Some of

the forms were recently purified and their physical and catalytic properties characterised. Two or three forms of monooxygenases, representing two or three distinct species of carbon monoxide-binding hemoproteins, were speculated each with a unique substrate specificity (Klotz et al, 1986).

It is a popular view that the specificities are imposed upon the monooxygenase system by the existence of multiple forms of cytochrome P-450. Using reconstituted cytochrome P-450 monooxygenase systems, enzymes prepared from controls and phenobarbital (PB) or 3-MC treated rats were shown to exhibited different substrate specificities and that such specificities were confirmed to be residing in the cytochrome component rather than the phospholipid or reductase fractions. Studies on visible spectrophotometry and electron paramagnetic resonance (epr) on 3-MC treated rat microsomes showed induction of cytochrome P-448 form different from cytochrome P-450 of controls (Kumaki et al, 1978).

Treatment of insects with naphthalene or PB induced a hemoprotein spectrally and catalytically different from control cytochrome P-450 (Capdevila et al, 1974) and was labelled cytochrome P-450, (later identified as cytochrome P-448). Capdevila et al, (1974) further showed in PB-induced housefly microsomes the presence of cytochrome P-450 and cytochrome P-420 (later shown to be denatured cytochrome P-450) different from the P-450 of controls.

Polyacrylamide gel electrophoresis (PAGE), in the presence of the anionic detergent sodium dodecyl sulfate (SDS), has been a useful tool for the resolution of protein subunits, the determination of molecular weights and for ascertaining the purity of proteins and this has also been done for cytochrome P-450 monooxygenase system (Moore et al, 1974; Welton and Aust, 1974).

The molecular weight of a given protein can be determined by comparing its electrophoretic mobility in a given gel percentage with known molecular weight marker proteins in the same gel composition. A linear relationship is obtained if the logarithm of the molecular weights of standard protein markers are plotted against their respective mobilities (R_f). The resolution, molecular weight determination and identification of membrane-bound cytochrome P-450 have been pioneered by Moore et al, (1974) and Welton and Aust, (1974). This they did by variously modifying the protocols of Laemmli, (1970).

The SDS-PAGE enabled the above researchers to resolve and partially purify cytochrome P-450 from mammalian liver. Four main bands were resolved with molecular weights ranging from 47-52 kDa. PB induction increased the intensities of the bands with molecular weights 47kDa and 49kDa while 3-MC induction intensified the bands with molecular weights 47, 49 and 52 kDa. These observations were interpreted as different forms of cytochrome P-450 but are now seen as an association of isozymes/ subunits of different proportions. This interpretation looks at cytochrome P-450 as a protein complex formed by several subunits.

The concept gained support due to the increase in molecular weight of cytochrome P-450 complex isolated from mouse and rat microsomes (Matsubara et al, 1974) and the exhibition of oligomerism of one cytochrome P-450 complex isolated from bovine adrenocortex mitochondria. Of the sixteen protein subunits, each had an average molecular weight of 53 kDa (Capdevila et al, 1973). The differential association of subunits reflects an intrinsic value rather than the differences in spectral and catalytic properties and the importance of this intrinsic difference is seen in the capacity of individual subunits to respond to induction by different xenobiotic compound.

The differential induction of cytochrome P-450 subunits has been exploited by various researchers employing different inducing agents on marine and freshwater fishes. This enabled them to resolve, purify and determine the molecular weights of the subunits that respond to the administered inducer. As a result, Klotz et al (1986) and Goksöyr (1985) resolved into five subunits scub microsomal fractions induced with PAH-type inducers, while Stegeman and Kloepper-Sams (1987); Miranda et al, (1989) using cod and rainbow trout, resolved and characterized four subunits in both fishes and five subunits in rainbow trout controls.

Nebert et al, (1987), with a nomenclature based on gene sequence information, described cytochrome P-450 as a superfamily of isozymes. When thereafter the nomenclature was expanded by Nebert et al, (1989) to include chromosomal localization,it was evident that different gene families and subfamilies still respond to different inducing agents differently. Four main families of xenobiotic-induced

cytochrome P-450 in vertebrates were identified: P-450I induced by PAH, PCB, isosafrole and ß-NF, P-450II induced by PB, ethanol and acetone while P-450III and P-450IV are induced by PCN and clofibrate respectively. These gene families surely represent different subunits of cytochrome P-450.

1.4 INDUCTION

1.4.1 Overview

Fouts et al, (1961) were the first to notice that drug-metabolizing enzyme activities/levels were lower in regenerating liver than in normal liver. Conney, (1967) coined the term 'induction' for the monooxygenase system to mean an increased rate of product formation or substrate disappearance. Induction has all along been thought of to occur via one of three possibilities. This may be by either de novo synthesis of novel proteins, the activation of pre-existing but dormant proteins or by the decreased rate of degradation of protein. Induction can be manifested and/or determined in quantitative or qualitative terms.

Proof exists with some purified enzymes to which antibodies have been prepared and immunoprecipitable radioactivity was equated with de novo synthesis of novel protein for such inducible enzymes as α-aminolevulinate dehydratase, tyrosine aminotransferase and glutamine synthetase (Alescio and Moscona, 1969). Induction has now been understood to mean increased or decreased protein levels relative to normal and this has been studied in a wide range of animals including invertebrates and microorganisms.

1.4.2 Induction of cytochrome P-450

The stimulation of liver growth in mammals by exposure to foreign organic compounds has been demonstrated and subsequently, work by (Haugen and Coon, 1975) showed that the molecular basis of induction of mammalian cytochrome P-450 was correlated in mice treated with different PAH levels and further proven to be a de novo process rather than a decreased degradation rate or increased activation of pre-existing protein moieties.

Treatment of rats with PB produced increased levels of drug-metabolizing enzyme activity and the concentration of the hemoprotein, (Remmer and Merker, 1965; Ernster and Orrenius, 1965). However, Sladek and Mannering, (1966) also showed that rats treated with 3-MC produced increased hemoprotein but not its drug-metabolising activities.

The presence of cytochrome P-450 in non-mammalian species has also been proven. These include houseflies (Lee, 1981) yeast (Gallo et al, 1973), bacteria (Cardini and Jurtshuhk, 1968), fungi (Ambike et al, 1970; Ferris et al, 1976) and fish (Lu et al, 1969). These revelations relevantly addressed a number of questions including inducibility of various forms of cytochrome P-450, the evolutionary relationship between these enzyme systems and the response of organisms to pollutants in their environments.

1.4.3 Inducers

The differential effects of PB and its analogues and 3-MC and its analogues enabled researchers to categorise inducers into PB-type and PAH-type inducers. Inducers of hepatic cytochrome P-450 consist of compounds possessing a broad spectrum of structural types, uses and pharmacological activities (Davies 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 classical inducer used for the aquarial studies in this project was ß-NF, a PAH-type inducer similar to 3-MC. It is a hydrocarbon analogue with the structure below:

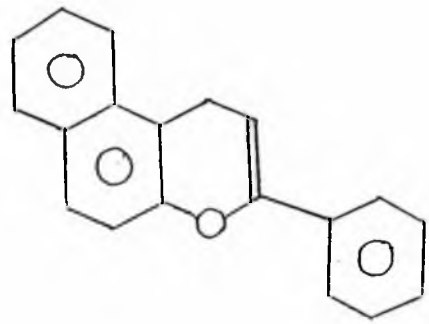


Fig. 1e. The structure of ß-naphthoflavone (ß-NF), a classical P- 450 inducing agent. An example belonging to the PAHs.

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 substrate for, or the capacity to bind to cytochrome P-450 monooxygenase (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. Such chemicals include nicotine and other PAHs in cigarette smoke (Arcos et al, 1961; Welch et al , 1969), DDT, dieldrin, aldrin and andrin (Hart and Fouts, 1965), urea, herbicides (Kinoshita et al, 1966), dyes used in textile industry (Radomski, 1961; Levin and Conney, 1967) and caffeine in food stuffs (Mitoma et al, 1968).

As the number of inducers increased, the classification of inducers was reviewed to three groups focusing on their reaction as inducers. The first is the PB-type which include many drugs and they increase cytochrome P-450 level and cytochrome P-450 (c) reductase activity. 3-MC type which constitute the second group include other polycyclic hydrocarbons and these increase cytochrome P-450 level butnot the NADPH cytochrome P-450 (c) reductase activity. Group three includes the spironolactone and other steroids which are shown to increase NADPH cytochrome P-450 (c) reductase activity and may effect an increase in the cytochrome P-450 levels (Parke, 1975).

Available literature indicates that no disease or sickness inducing cytochrome P-450 is known. However, Diabetes mellitus has been proven as an exception (Morgan et al, 1993). The form of cytochrome P-450 induced by diabetes was classified as cytochrome P-450DM with an electrophoretically determined molecular weight of 51kDa when the microsomes used were from diabetic rats induced with streptozotocin. Cytochrome P-450DM was also found to have a CO-reduced absorption maximum at 452nm, active in aniline hydroxylase activity, has an enhanced O-dealkylation activity towards 7-ethoxycoumarin in the presence of cytochrome b_5 and no response to 7-ER.

Morgan et al, (1993) also showed P-450IV family proteins to be among those increased by diabetes and postulated that diabetes hyperketonemia was responsible for this P-450 family protein induction and effects. Diabetes was further shown to cause a 10-fold elevation of hepatic cytochrome P-450IVA1 mRNA prior to increase in the P-450DM and P-450IV proteins.

1.5. **Aquatic Pollution: Induction in Fish**

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.

ß-NF has been used by Stegeman, (1981); Gooch and Matsumura, (1983); Melancon et al, (1981) and Goksöyr et al, (1991a) to study induction in fishes and their response particularly to EROD assay is known to be specific for cytochrome P-450IA isozyme. 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 (Goksöyr, 1985) 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 elevation in enzyme activities (Stegeman and James, in press). Rainbow trout also showed induction with isosafrol (Vodicnik et al, 1981) while a variable result has been obtained with novel inducer pregnolone-16α-carbonitrile (Förlin, 1980; Vodicnik and Lech, 1983).

In contrast, evidence of PB-type induction in fish is ambiguous. Although some workers reported induction in fish with prototype substrate, following treatment with PB-type inducers eg DDT and non-coplanar PCB, the majority of the studies indicated a general lack of response to PB-induction (Buhler and Rasmusson, 1968). Work done by Payne and Penrose, (1975) first showed increased hepatic catalytic cytochrome P-4501A1 activity (aryl hydrocarbon hydroxylase activity) in brown trout (Salmo trutta). P-4501A1 dependent monooxygenase activities were subsequently shown to be present in fish from waters polluted with oil hydrocarbons, industrial and municipal effluents

containing aromatic and/or chlorinated hydrocarbons (Payne et al, 1984; Lindström-Seppa and Oikari, 1990).

Many fish species are however responsive to induction of cytochrome P-450 enhancing aryl hydroxylase and EROD activities by polychlorinated hydrocarbons and ß-NF (Stegeman, 1981) and measurements of the induction of these enzymes have been suggested as practical biological monitors for aquatic petroleum pollution (Collier et al, 1993).

Although considerable variation exists between aquatic species with regard to basal mixed-function oxygenase enzyme activities and cytochrome P-450 content (Bend and James, 1978), few studies have comprehensively addressed differences in xenobiotic biotransformation patterns among fish species. A review of biotransformation of benzo(α)pyrene [B(α)P] in fish by Stegeman, (1981), showed that fish species can differ substantially in the extent of metabolism at various positions on the molecule. As a generalization, microsomes from fish produce more ring diols (Stegeman and Woodin, 1980) than those from such species as the little skate which forms more phenolic metabolites. Thus, the inducibility by PAH-type inducers of fish cytochrome P-450 monooxygenase system makes it a valuable tool in monitoring environmental pollution either by catalytic assays (Förlin et al, 1985) or by immunochemical techniques (Stegeman et al, 1986; Goksöyr and Solberg, 1987).

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Fish

Oreochromis niloticus for both aquarial and field studies for this research was obtained from the Institute of Aquatic Biology (IAB) aquaculture centre at Akosombo. The fishes were hatched, bred and harvested at this aquaculture station where the aquarial studies were carried out using concrete tanks.

Preliminary studies on the project was carried out with S. galilaeus from a dam site near the Accra-Tema motorway and with O. niloticus from Weija dam (water works). These fishes were caught by gill nets from the wild by fishermen and acclimated for two months in the Zoology Department aquarium, University of Ghana. S. galilaeus was not available in the Weija dam and so was O niloticus from the dam near the motorway.

2.1.2. Chemicals and Reagents.

The following chemicals were obtained from Sigma Chemical Company, St Louis MO, USA: Agarose, ß-naphthoflavone, NADPH, cytochrome c, resorufin, 7-ethoxyresorufin. The already constituted molecular weight marker proteins used for SDS-PAGE were also obtained from Sigma i.e bovine serum albumin, ovalbumin, pepsin, trypsinogen, B-lactoglobulin and lysozyme.

Chemicals obtained from FLUKA were: glycerol, KCl, $CuSO_4.5H_2O$, NaOH, acetic acid, isopropylalcohol, n-butanol, methanol, dipotassium hydrogen phosphate, disodium hydrogen phosphate dodecahydrate, sodium dihydrogen phosphate, sodium potassium tartrate, TEMED, sodium acetate, bovine serum albumin, ammonium persulfate, bromophenol blue, sodium dodecylsulfate (SDS), acrylamide, N,N-methylenebisacrylamide, dithiothreitol (DTT) and coumassie blue. ß-mercaptoethanol was obtained from British Drug House (BDH) Chemicals Limited, Poole, England. Folin-Ciocalteau phenol reagent and tris(hydroxymethyl) aminomethane were obtained from Hopkin and Williams, England.

2.2. METHODS

2.2.1 Preliminary Studies

The main aim of the preliminary studies was to ascertain the constitutive and baseline levels of microsomal cytochrome P-450 related proteins. These dams were regarded as clean and hence, enzyme levels and activities of fishes from them could be compared with those from fishes treated with ß-NF and ATL-effluent.

Initially, feral fishes from the dams near the motorway and Weija were caught using gill nets. The fishes were then killed with a single blow to the head, their livers excised and put on ice and transported to the laboratory for further processing to obtain microsomes (see below, 2.2.4).

When the enzyme activities and protein levels from these microsomal protein preparations showed negligible values, the O. niloticus and S. galileaus were brought in from their respective dams and acclimated at IAB, Accra for two weeks and then transferred to concrete tanks at the Zoology Department aquarium, University of Ghana for two months. They were then killed by a single blow to the head and the livers excised and then processed to obtain microsomes. The average weight of O. niloticus and S. galileaus used in this experiment were 50g and 45g respectively.

2.2.2 **Pretreatment of fish**

Two sets of experiments were conducted in this study. In the first set, ß-naphthaflavone (ß-NF) was injected into fish in an aquarium. The second set of experiment involved a field study at the Akosombo Textile Limited effluent entry point into the Volta Lake at Konkontekope.

For the ß-NF treatment, the O. niloticus used were hatched, bred and kept for five months before the start of the experiments at the Institute of Aquatic Biology (IAB) aquaculture station at Akosombo. The fishes were starved a day prior to the injection and remained starved for the duration of the experiment i.e a maximum of six days.

A standard ß-NF emulsion (10 mg/ml) was prepared in soyabean oil (purchased from the local market) by sonicating in an ultrasound bath (Fretch Laborette 74) for two hours. This emulsion was prepared a day prior to its administration.

The ß-NF dosage of 80 mg/kg was administered by a single intraperitoneal injection (i.p.) close to the anal fin. The volume of ß-NF emulsion administered per kilogram weight of fish was determined from the formula:

$$\mu l \text{ emulsion} = \frac{\text{g fish X 80 mg/kg}}{\text{10 mg/ml}}$$

Hence, the weight of each individual fish was taken and the corresponding volume of ß-NF used. In all, twelve fishes were pretreated with ß-NF. Twelve fishes serving as controls were also injected intraperitoneally with a single dose of soyabean oil.

For the field Studies, fish from the same stock as those used for the aquarial studies were used. Two sets of eight fishes per set were taken from the acclimated stock. One set served as experimental fish while the other set served as controls. Both sets were put into separate cages.

Stones were used to submerge the cages. The cage with the experimental fish was placed at the point where the ATL effluent enter into the Volta Lake while the controls were submerged at a point upstream the factory-effluent entry point and at an area where the inhabitants of Konkontekope fetch their drinking water.

2.2.3 **Sampling of fish.**

For the aquarial studies, samples were taken 2, 3 and 5 days after i.p injections. For the field studies, sampling was done on two days i.e day-2 and day-5 after pretreatment.

Four fishes were taken from both the aquarial and field groups on each sampling day. The fishes taken from each group were then killed with a single blow to the head and the weights (g) and lengths (cm) of each fish was measured. The fish was then carefully dissected and the liver excised free of the gall bladder.

The excised liver was put in ice-cold perfusion solution (0.154M KCl) to remove haemoglobin and then transferred to a cryotube containing ice-cold 30% glycerol sufficient to cover the liver. The tube was put on ice. When all the samples were taken, the tubes were transferred into a tank of liquid nitrogen until further processing. The weights and lengths of the fishes taken were used to calculate the 'Condition factor' an assessment of the health status of each fish from Fulton's formula

$$\text{Condition factor (CF)} = \frac{\text{g wet weight of fish} \times 100}{(\text{cm fish length})^3}$$

The power 3 is the surface area to volume ratio of the fish. The condition factors were calculated for all test and control fishes sampled.

2.2.4 Preparation of Microsomes

The livers stored in liquid nitrogen were removed, thawed on ice, blotted dry with tissue paper and weighed. The liver weights together with the wet weight of each fish were used to calculate the organ health status using a second Fulton's formula:

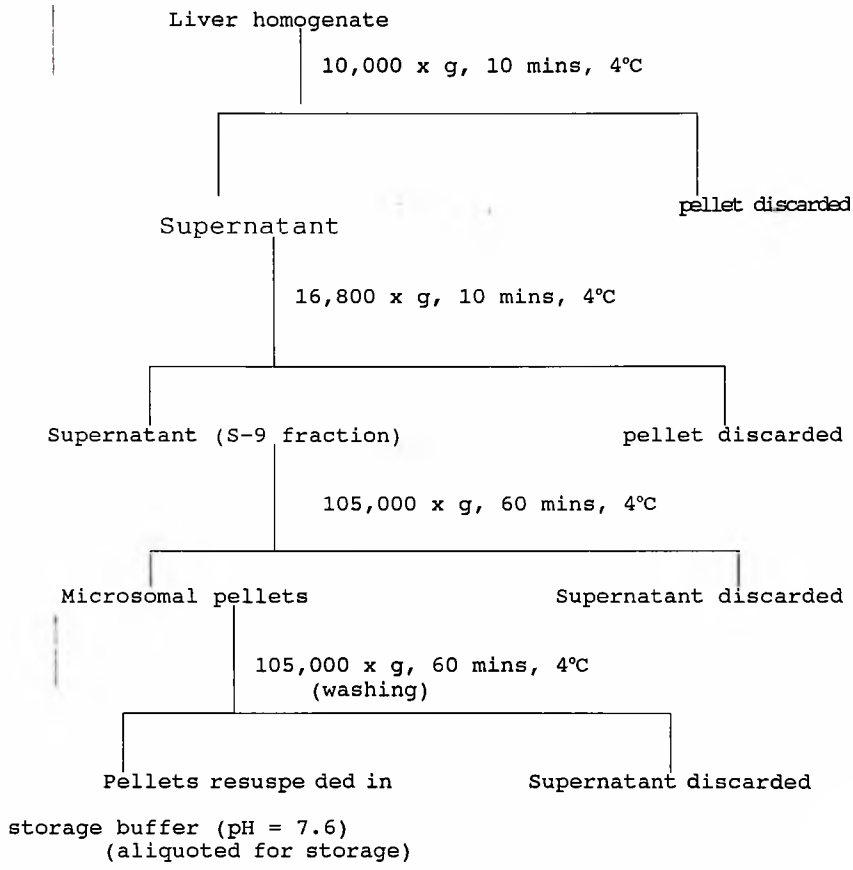
$$\text{Liver somatic index (LSI)} = \frac{\text{g wet weight of liver x 100.}}{\text{g wet weight of fish}}$$

The liver was then homogenised in a 1:4 volumes of ice-cold homogenization buffer with a Polytron homogenizer. The homogenization buffer (a sodium phosphate buffer) of pH 7.6 included 0.154M KCl 10% glycerol and 1mM EDTA.

The homogenates were spun in a Tomy-20 centrifuge at 10,000 x g for 10 mins at 4°C. The supernatants were removed and centrifuged at 16,800 x g for 10 mins. The supernatants obtained, often referred to as the S-9 fractions were again centrifuged at 40,000rpm (105,000 x g) for 1 hour in Hitachi 80P-7 ultracentrifuge at 4°C. The pellets were washed in 0.1M $NaPO_4$ buffer in 0.154M KCl, 1.0mM EDTA, pH 7.6 to remove any haemoglobin not removed at the perfusion stage and to clean microsomes of cytochrome b_5 contamination which often interferes with cytochrome P-450 spectral studies. The washed pellets were then rehomogenised in 2ml g^{-1} liver weight of storage buffer consisting of 0.1M $NaH_2PO_4.H_2O$, 0.154M KCl, 1mM EDTA, 1mM DTT and 20% glycerol at pH 7.6 and distributed into six previously labelled cryotubes and stored in liquid nitrogen or at -71°C.

Throughout the preparation of microsomes, all operations were performed on ice to minimize enzyme degradation.

Fig. 2.0 Flow chart of the processes involved in the preparation of microsomes.



2.2.5 Protein Determination

The method 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 750 nm was used for the determination of total protein in this work.

The microsomal fractions were diluted 1:100 with 0.5M NaOH. An aliquot of 200μl or 400μl of the diluted microsomes was taken in duplicate and made to a final volume of 1000μl with 0.5M NaOH. To each sample, 5ml of "alkaline copper-phenol" reagent were added, the mixture mixed thoroughly by vortexing, and allowed to stand for 10 minutes. 0.5ml of 1N Folin reagent was added 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. The microsomal protein content was directly interpolated from a standard curve constructed with bovine serum albumin (BSA).

To prepare the standard curve, 1.0mg of BSA in 10ml of 0.5M NaOH was made as stock solution (100μg/ml). The following volumes: 0, 0.2, 0.4, 0.6, 0.8, and 1.0ml of the standard stock BSA solution (equivalent to 0, 20, 40, 60, 80 and 100μg/ml respectively) were taken and made up to a final volume of 1.0ml with 0.5M NaOH. Alkaline copper-phenol reagent and Folin reagent addition were made as described above for the microsomal fractions, and the absorbances at 750nm read. The absorbances read from the BSA dilutions were then used to construct the standard curve of absorbances against BSA concentration (μg). The alkaline Copper reagent was prepared freshly by mixing 2%w/v Na_2CO_3 in 0.1M NaOH, 1%w/v $CuSO_4$ (hydrated) in water and 2%w/v sodium potassium tartrate in water in a ratio of 100:1:1 by volume respectively.

2.2.6 Determination of cytochrome P-450 (c) reductase activity

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

The method used is that by Bleecker et al, 1973. In this method, 250μl of a 5 mg/ml solution of cytochrome c (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. 100μl of microsomal solution that has been diluted to a protein concentration of 4mg/ml with resuspension buffer was added. A reference blank solution was also prepared which contained all the above reagents including the microsomal fraction and put in a cuvette. 25μl of resuspension 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 spectrophotometer and the reaction initiated by the addition of 25μl of 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 volume was determined as follows:

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

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

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

$$\# = \frac{A_{550}}{\epsilon Cp}$$

where ϵ is the extinction coefficient for reduced cytochrome c at 550nm and has a value 19.6 mM^{-1} cm^{-1} and A_{550} is the absorbance change for the linear portion of the curve over three (3) minutes. The above equation was used to determine the specific cytochrome c reductase activity for the samples. The results were expressed as pmol cytochome c reduced per minute per mg protein.

The total reductase enzyme activity was then calculated taking into account the volume of microsomal protein in the analytical cuvette and the total volume of microsomal protein prepared from the excised liver and expressed as pmol min^{-1} g^{-1} liver wet weight.

2.2.7 **Ethoxyresorufin-O-Deethylase (EROD) Assay**

The 7-ethoxyresorufin-O-deethylase (EROD) assay as a measure of cytochrome P-450 monooxygenase activity was determined spectrofluorometrically essentially as described by Burke and Mayer, (1974). A 0.41mM stock solution of the substrate 7-ethoxyresorufin and 0.85mM of the product, resorufin were made in dimethylsulfoxide (DMSO). The buffer for the EROD assay was 0.1M $NaH_2PO_4.H_2O$ in 0.1M NaOH. The optimum pH of this buffer determined for O. niloticus, that is 6.8, (Gadagbui, 1993) was used.

First, the absorption spectra of both substrate and product were chartered using Specord-40. For this, 10μl of each of the stock solutions was separately stirred into a cuvette with 0.1M 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 the true extinction coefficient at that wavelength, to be used later in the determination of the actual concentration of the substrate in the EROD assays.

The $\epsilon_{max.}$, that is, the extinction coefficient at the determined lambda max., was estimated from Beer-Lambert law as follows:

$$\epsilon_{max.} = \frac{\text{Absorbance}}{[\text{Substrate conc. } (\mu M\ ml^{-1})]}$$

The emission spectra of resorufin and 7-ethoxyresorufin were obtained using the SFM-25 spectrofluorometer with VERS 8603 recorder attached. The total volume of solution used was 2.0ml made up of 1990μl of EROD buffer pH 6.8 and 10μl of 0.41mM 7-ER 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 $\mu molL^{-1}$ and the microsomal preparations were diluted with EROD buffer to obtain a protein concentration of 0.2mg/ml. 1960μl of EROD buffer, 10μl of 7-ER solution and 20μl of 0.2mg/ml microsomal preparation were mixed in a cuvette. The cuvette was placed in the fluorometer and the emission fluorescence recorded for 30 seconds at the excitation and emission wavelengths of 535nm and 585nm respectively.

The deethylation 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 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 $pmolL^{-1}$. The reaction solution was mixed again and the sudden increase in fluorescence (spike) was recorded. The recording of the change in

fluorescence was continued 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/mg 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 = conc. of resorufin

S = sample fluorescence (blank), mm recorder response

R = resorufin fluorescence (spike), mm recorder response

t = time over which velocity was measured.

The total EROD activity was calculated taking into account the volume of microsomal protein used in the cuvette, the total volume of the microsomal preparation and the weight of liver used in preparing the microsomes. The total EROD activity was expressed as pmol min^{-1} g^{-1} liver weight.

2.2.8 Molecular weight estimation of cytochrome P-450

2.2.8.1 Gel Preparation

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used in the resolution and characterization of microsomal cytochrome P-450. The separation gel composition was 10% while the stacking gel was 5%. The slab gels used for the separation was made by mixing 4.2ml of Concbis (a millipored solution of 40g acrylamide and 1.5g of bisacrylamide), 3.0ml of 10x buffer (0.4M tris-acetate buffer pH 7.4 containing 0.2M sodium acetate and 20mM EDTA), 0.3ml of 10%w/v SDS and 18.0ml of doubly distilled water. The mixture was deaerated for two minutes and 1.5%w/v ammonium persulfate solution was added. Lastly, 50μl of a catalyst, TEMED was stirred into the mixture which was immediately transferred by a pasteur pipette into the slab on the Biorad mould. A drop of n-butanol was put on top to prevent contamination by dust and evaporation from the surface.

The stacking gel was similarly mixed and poured on top of the running gel with the combs fixed to create the wells for sample loading.

2.2.8.2 Sample Preparation

The sample buffer made up of 50mM Tris-HCl, pH 8.0 was used to dilute the microsomal preparation to a protein concentration of 4.0mg/ml. The sample to be loaded onto the gel was then prepared using the protocol below:

20 parts of sample buffer

60 parts of 4.0mg/ml microsomal protein

10 parts of 10%w/v SDS

5 parts of ß-mercaptoethanol

5 parts of bromophenol blue as a tracking dye.

50μg of each sample protein prepared as above was then loaded per well onto the gel alongside marker protein standards already constituted as follows:

Protein	Molecular Weight(Da)
bovine serum albumin	66,000
ovalbumin	45,000
pepsin	34,700
Trypsinogen	24,000
ß-lactoglobulin	18,400
lysozyme	14,300

The concentration of the molecular weight marker protein was diluted to 3.0 mg/ml before 40μg of it was loaded onto the gel.

2.2.8.3 Electrophoresis Conditions

The electrophoresis buffer was made by mixing 100ml of 10X buffer, 10ml of 10%w/v SDS and 890ml of doubly distilled water. This solution was then used to pre-electrophorese the gel for 30 minutes at 36 volts in the cold room before the samples were loaded onto the gel. The pre-electrophoresis exercise was to equilibrate the gels and remove any excess SDS.

After loading the gels, they were run first at 36 volts in the cold room to concentrate the samples onto the running gel. The voltage was then increased to 80 volts and the gel was run until the tracking dye had just run off. Five additional minutes was allowed for all the heme to run off to prevent any possible recombination with the resolved proteins during protein fixation.

2.2.8.4 Protein Fixation and Detection on Gels

In order to facilitate the detection of the resolved proteins on the gel, they must first be fixed. Protein fixation was achieved electrophoretically at 36 volts for 60 minutes using the following solvent combination: isopropylalcohol: acetic acid: water (25:10:65). Alternatively, the gel was soaked in this solution overnight. The fixed gel was then rinsed twice with distilled water.

In the absence of benzidine dihydrochloride and tetramethylbenzidine which are specific for cytochrome P-450, the fixed proteins were stained by the general protein staining technique i.e. coumassie blue for one hour or overnight.

For the detection of the proteins on the gels, two solutions proved quite effective in clearing the gel background. These were methanol: acetic acid: water (50:75:875) and isopropylalcohol: acetic acid: water (10:10:80). However, the latter was the better of the two and changing the destaining solution thrice was sufficient to clear all the gel background. The destained gels were preserved in 10% glycerol until pictures of the gels were taken.

The mobilities of the resolved microsomal protein bands as well as the already constituted molecular marker proteins were all measured as R_f values relative to the gel lengths using R_f = distance moved by protein band/gel length. The R_f values and the corresponding $\log_{10}$ molecular weights of the constituents of the constituted marker proteins were used to construct a standard curve from which the microsomal protein bands molecular weights were estimated.

2.2.9 **Statistical Analysis**

Statistical analyses were performed manually and the differences between groups were analyzed by ANOVA, multiple range, interval factors for mean and the standard Student's t-test with the level of significance set at $p < 0.05$ in all cases. In the calculation of the t-values, significance between two (2) means for paired and unpaired samples were considered. The effects of sampling time were evaluated by ANOVA.

CHAPTER THREE

RESULTS

The parameters investigated are grouped into two i.e biological and biochemical.

3.1 Biological Data

The biological data analyzed included the 'condition factor' (CF) and the 'Liver somatic index' (LSI) of Bagenal and Tesch, (1978).

3.1.1 Condition Factor (CF)

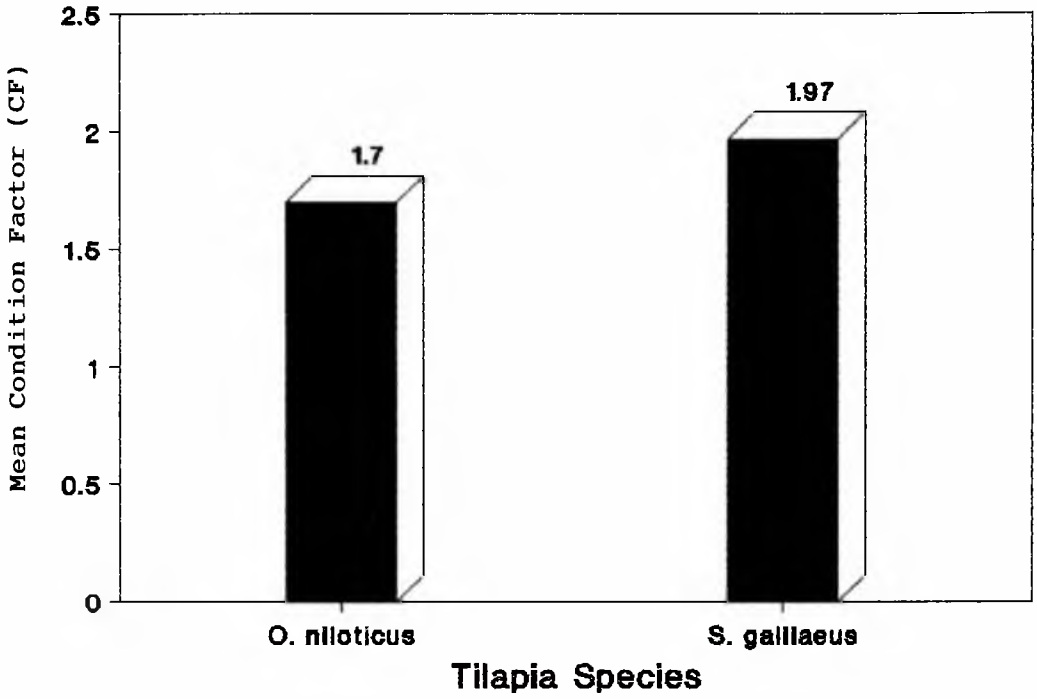
3.1.1.1 Preliminary Result

Feral Q. niloticus from Weija dam and S. galileaus from the dam near the Motorway, both of which were used in the preliminary investigations, had average CF values as 1.70 and 1.97 respectively (Fig. 3a). Since both species were held under the same laboratory conditions viz: temperature, food supply (nutrition) and photoperiodicity and since CF reflects nutritional status of the fish, it could be said that S. galileaus is a better converter of feed than Q. niloticus since both fishes were not starved prior to sample taking.

3.1.1.2 Aquarial Studies

The CF for a non-fasting Q. niloticus from the stock where the experimental fishes were taken at the IAB aquaculture station at Akosombo were calculated and shown to be 1.27 for fishes of average weight and length of 50.0 g and 15.5 cm respectively. This was taken to be the standard value for Q. niloticus.

Fig. 3a: Condition factor (CF) for preliminary studies



The mean CF values for the fishes used in the aquarial studies at the Akosombo station are as represented in Fig. 3b. Both the control (soyabean oil-injected) and the test (ß-NF-injected) fishes have their mean values below the standard. The t-test values at $p < 0.05$ confidencelevel and 6-(d.f.) for the aquarial studies are shown in the table 3.0 below:

Table 3.0; Student's t-table of CF values for aquarial studies.

BNF and control	t-tabulated	t-calculated
Day-2	2.45	0.394
Day-3	2.45	0.726
Day-5	2.45	0.656

As the results indicate, there is no relationship between the CF and the BNF treatment since there is no significant difference between the values for the test and controls. This was confirmed by ANOVA (appendix I)

3.1.1.3 Field Studies

The fishes here were exposed to ATL-E for as long as the aquarial studies were carried out and the fishes used were from the same stock as those used in the aquarial studies. The mean CF value for day-2 and day-5 ATL-E fishes were higher than the standard. However, on both day-2 and day-5 of ATL-E exposure, the CF values were higher than their controls as shown in Fig. 3c.

Fig. 3b: Condition factor (CF) for aquarial studies

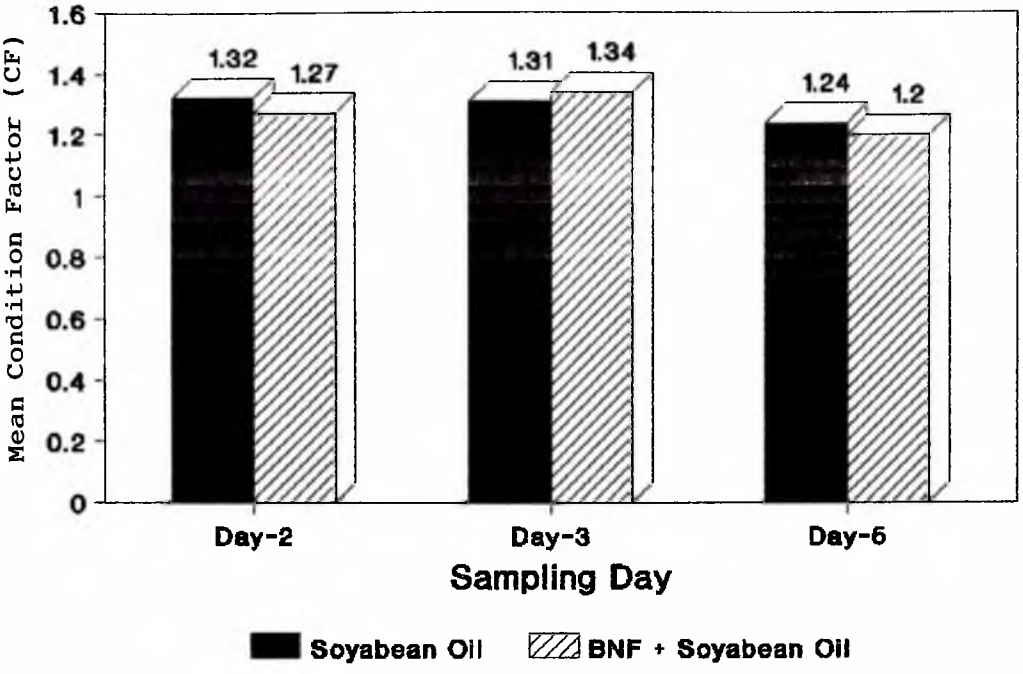
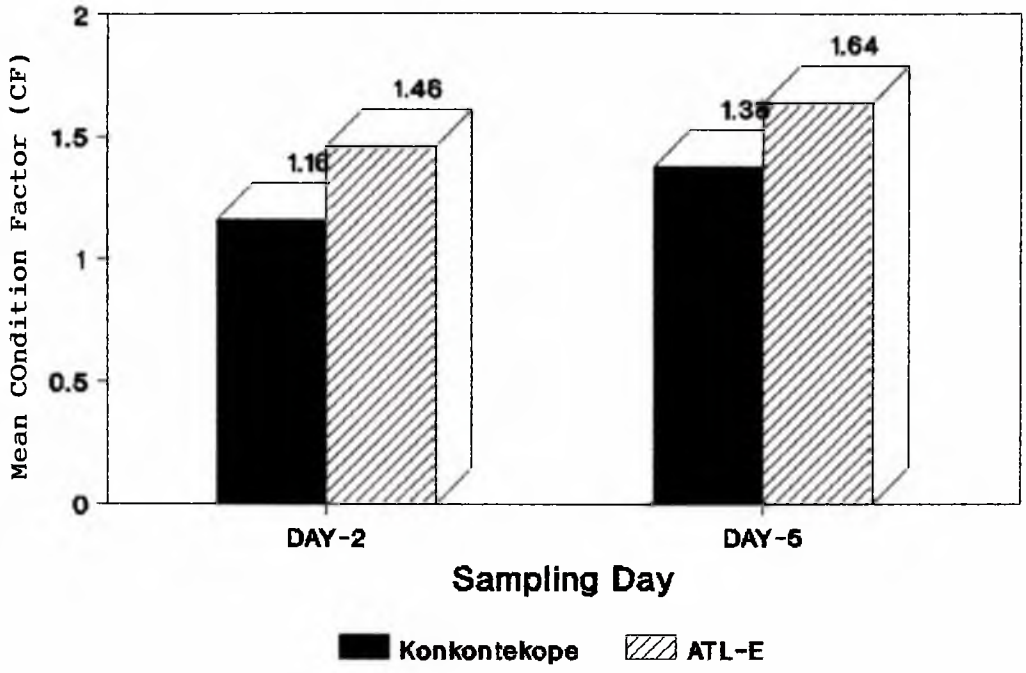


Fig. 3C: Condition factor (CF) for field studies



The Student's t-values in the table below show all the ATL-E exposure resulted in significant differences between the exposed fishes and their controls at Konkontekope, indicating a relationship between exposure to ATL-E and the CF values.

Table 3.1; Student's t-table for CF values for field studies.

ATL-E & control	d.f	t-tabulated	t-calculated
Day-2	6	2.45	2.75
Day-5	4	2.87	4.69

3.1.2 Liver Somatic Index (LSI)

The other biological parameter considered was the LSI which, at elevated levels, is indicative of some induction and may either be due to hyperplasia or hypertrophy. Both processes are known to cause liver cells to become larger than normal when exposed to some pollutants.

3.1.2.1 Preliminary Result

The mean LSI values for feral O. niloticus from Weija dam and S. galileaus from the Motorway dam used in the preliminary studies were 1.03 and 1.41 respectively (fig. 3d).

3.1.2.2 Aquarial Studies

LSI data for the aquarial experiments at Akosombo are as presented in fig. 3e. The day-2 and day-3 ß-NF treated fishes had mean LSI values higher than the standard whereas the day-3 control and day-5 fishes

Fig. 3d: Liver somatic index (LSI) for preliminary studies

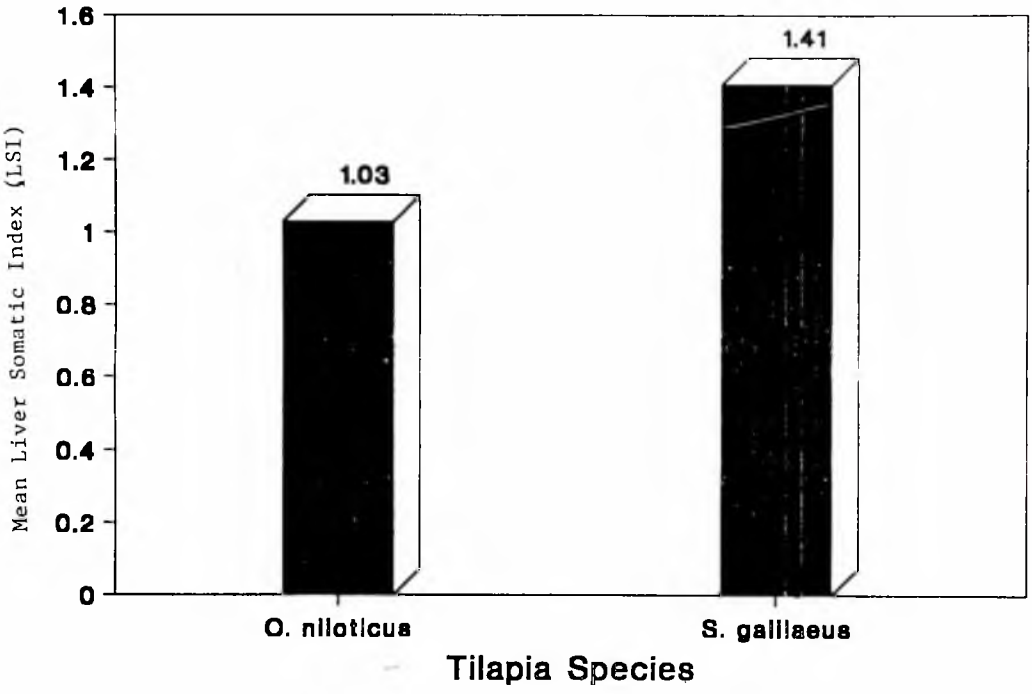
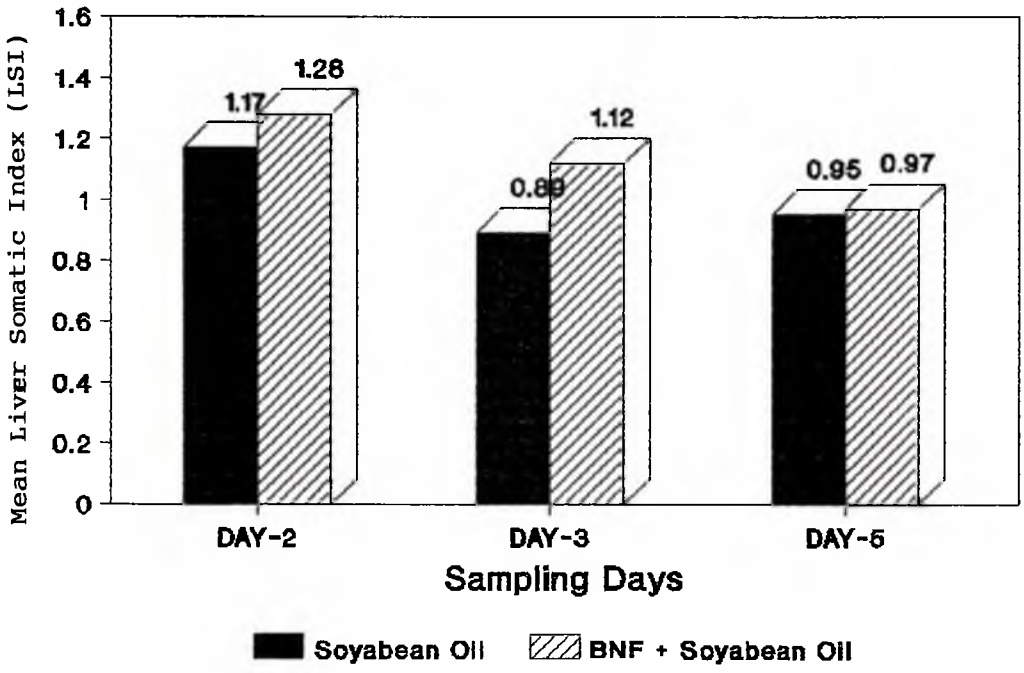


Fig. 3e: Liver somatic index (LSI) of O. niloticus in the aquarial studies.



show mean LSI values below the standard. While there is a general decrease in the mean LSI for the BNF-injected fishes, the same cannot be said of the controls. The aquarial LSI t-values at $p < 0.05$ and 6-(d.f.) presented in Table 3.2, the ANOVA table (appendix IIa) and the mean intervals of factor means (Appendix IIb) all indicate no significant difference with regards to all the ß-NF pretreated fishes and their controls.

Table 3.2: Student's t-table for LSI values for the aquarial studies

ß-NF & Control	t-tabulated	t-calculated
Day-2	2.45	1.43
Day-3	2.45	1.40
Day-5	2.45	0.187

The multiple range analysis of mean LSI by sampling days as shown in appendix IIc indicates no relationship existing between day-2 and day-3 but rather between day-2 and day-5 as well as day-3 and day-5.

3.1.2.3 **Field Studies.**

Fig. 3f represents the data for the ATL-E fishes and their controls set up at Konkontekope. On both day-2 and day-5, the ATL-E exposed fishes had higher LSI values compared to the controls. It further shows that there is a decrease from day-2 to day-5 for both controls and ATL-E fishes.

Fig. 3f: Liver somatic index (LSI) of O. niloticus in the field studies.

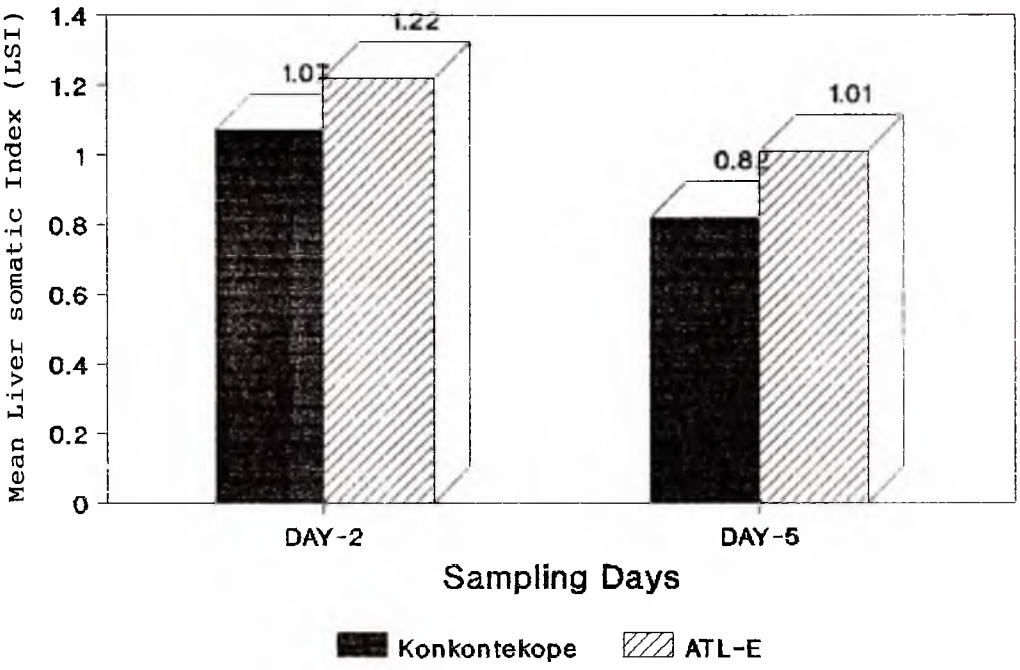
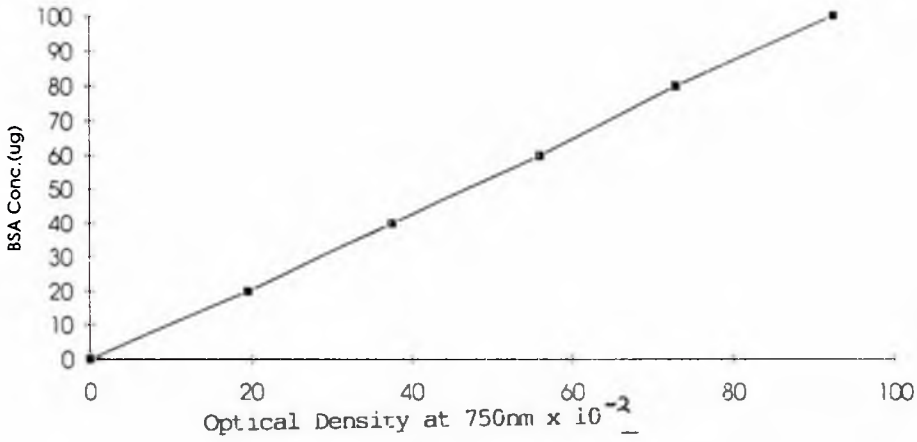


Fig. 4a BSA Calibration Curve used in Protein determination



Below is the Student's t-table (table 3.3) for the field studies at $p < 0.05$, and together with ANOVA (Appendix IId), they reveal a significant difference between the ATL-E fishes and their controls.

Table 3.3: Students t-table for LSI values for field studies

ATL-E & control	d.f	t-tabulated	t-calculated
Day-2	6	2.45	3.13
Day-5	4	2.87	2.93

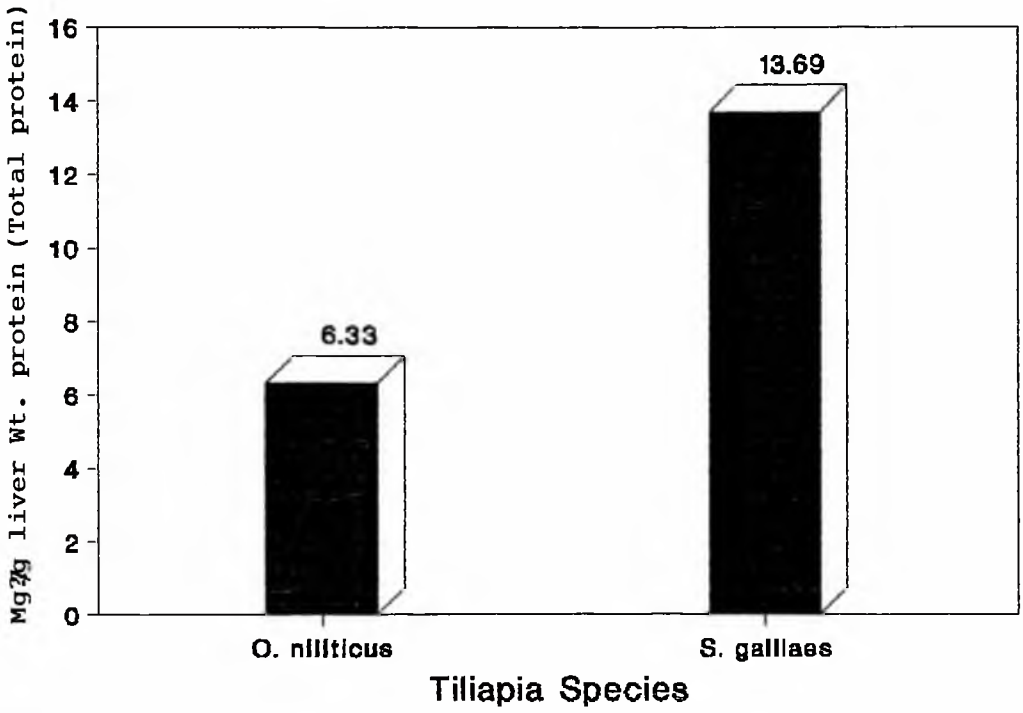
3.2 Biochemical Data

The biochemical parameters investigated included total protein concentration, NADPH-cytochrome (P-450) c reductase (as a component of the monooxygenase activity as well as the integrity of the endoplasmic reticulum) and EROD as a measure of cytochrome P-450 dependent monooxygenase activity. The cytochrome P-450 isoform(s) being induced was investigated using SDS-PAGE.

3.2.1 Total Microsomal Protein Concentration

In the determination of the total microsomal protein concentration, a bovine serum albumin calibration curve plotted as OD_{750} versus bovine serum albumin concentration, as shown in Fig. 4a, was used.

Fig. 4b: Mean total microsomal protein (mg/g liver weight) of fishes in the preliminary studies



3.2.1.1 Preliminary Results

The yield of total microsomal protein in mg/g liver weight for the feral fish showed that S. galileaus, obtained originally from the dam near the Motorway, had approximately 54% more protein than of O. niloticus obtained from the Weija dam (fig. 4b). These mean total protein values for O. niloticus and S. galileaus were taken to be their basal values since both species were acclimated in tap water for two months before being used in the experiments.

3.2.1.2 Aquarial studies

The results of the aquarial studies are as presented in fig. 4c showing evidence that in all three days of sampling, the ß-NF pretreatment increased mean total microsomal protein to levels higher than those of the soyabean oil injected fishes the controls. It further showed that the difference, expressed as a percentage increase in the total microsomal protein concentration appear to be similar for all three (3) days i.e. by a factor ranging between 2.3 and 2.5.

From the ANOVA (Appendix IIIa) and the Student's t-table below, it is evident that there were significant differences in the ß-NF treated fishes over the controls for all the days.

Fig. 4c: Mean total microsomal protein (mg/g liver weight) of aquarial O. niloticus

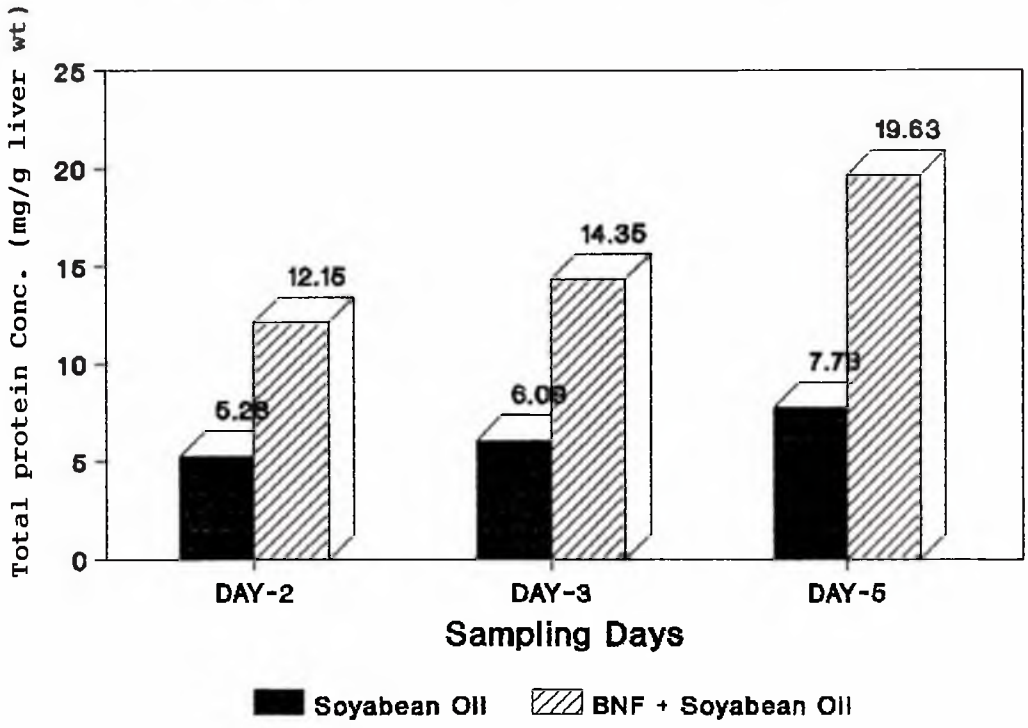


Fig. 4d: Mean total microsomal protein (mg/g liver weight) of fishes in the field studies

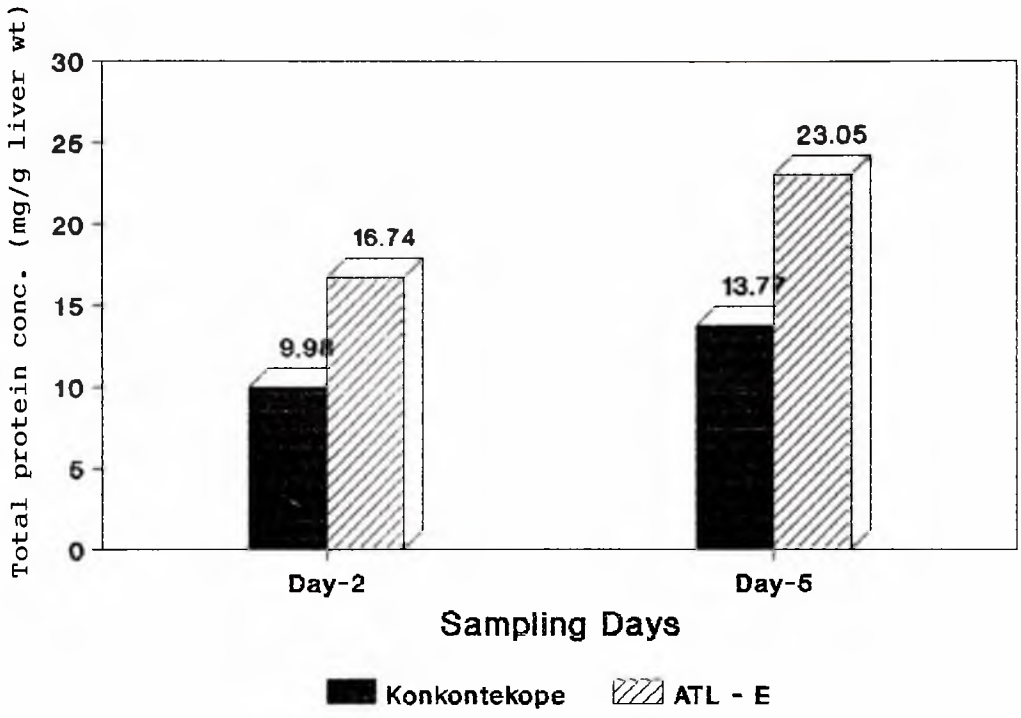


Table 3.4: Student's t-table for total microsomal protein concentration for the aquarial studies at $p < 0.05$ for 6-(d.f.).

ß-NF & control	t-tabulated	t-calculated
Day-2	2.45	4.63
Day-3	2.45	4.28
Day-5	2.45	5.01

Appendix IIIb represents the multiple range analysis for total microsomal protein by sampling days and it indicates that there are differences between the day-2 and day-5, day-3 and day-5 but not between day-2 and day-3 treatments.

3.2.1.3 **Field Studies**

Fig. 4d represents the results of the field studies and shows the ATL-E affected fishes as having higher mean total microsomal protein concentration over their controls by a factor of 1.68. Both test and control fishes for day-5 have higher values than their day-2 counterparts.

The ANOVA table (Appendix IIIa) and the Student's t-table below (Table 3.5), indicate a significant difference between the ATL-E fishes and their controls. Also, the significant differences in the protein levels between day-2 and day-5 test fishes were shown in multiple range analysis (Appendix IIIb).

Fig. 5a. Mean specific NADPH-cytochrome c (P-450) reductase activity of fishes in the preliminary studies.

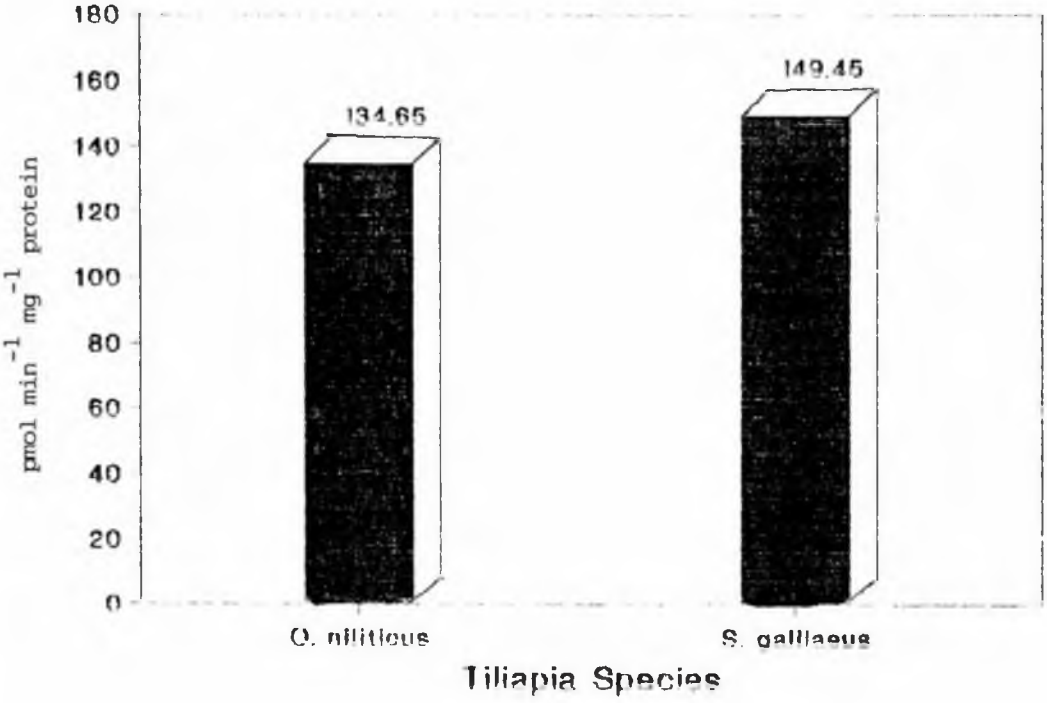


Table 3.5; Student's t-table for the total microsomal protein of field studies at $p < 0.05$.

ATL-E & control	d.f.	t-tabulated	t-calculated
Day-2	6	2.45	3.61
Day-5	4	2.87	3.97

3.3.1 NADPH-dependent cytochrome P-450 reductase activity

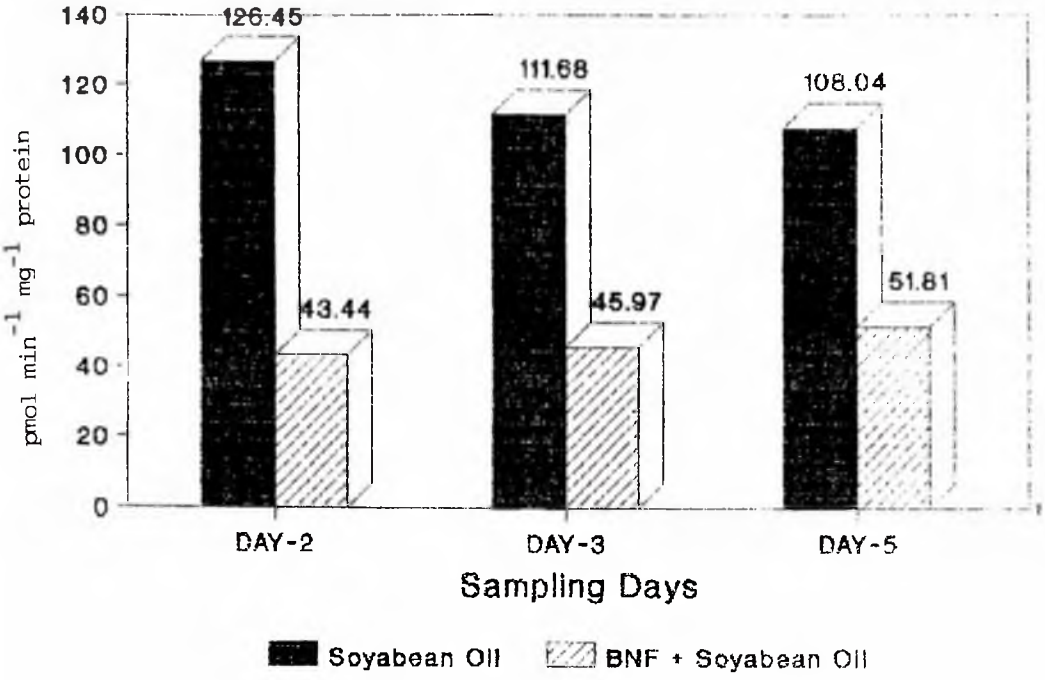
3.3.1.1 Preliminary Results

The electron transport potential of the monooxygenase system of both O. niloticus and S. galileaus was evaluated as NADPH-dependent cytochrome P-450 (c) reductase specific activity. The O. niloticus and S. galileaus used in the preliminary studies recorded values of 134.65 and 149.45 pmol of cytochrome c reduced per milligram of protein per minute respectively (fig. 5a). This represents a difference of 11% activity in the S. galileaus over the O. niloticus.

3.3.1.2 Aquarial Studies

The results of the specific reductase activities reflecting the electron transport potential of the microsomal monooxygenase enzyme system of O. niloticus in the main experiments are as indicated in fig. 5b. The control values are quite comparable to the values of the preliminary studies (fig 5a). The data further shows clearly a depressed cytochrome c reduction per milligram protein. This apparent depression in

Fig. 5b. Mean specific NADPH-cytochrome c (P-450) reductase activity of aquarial O niloticus.



the NADPH-dependent cytochrome P-450 (c) reductase activities in the test fishes compared to the controls could be attributed to the relatively higher protein concentrations registered by the test fishes over their controls. Table 3.6 shows the Student's t-table for specific reductase activity for the aquarial studies at $p < 0.05$ and six degrees of freedom.

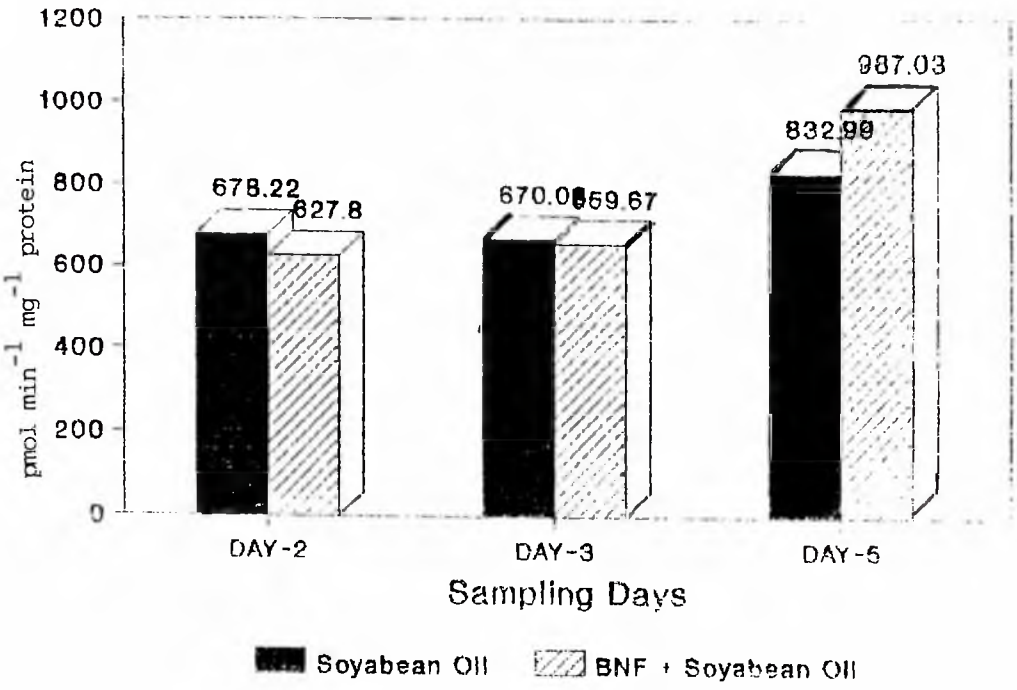
Table 3.6: Student's t-table for the mean specific cytochrome P-450 (c) reductase activity for the aquarial studies at 6-(d.f.).

ß-NF & Controls	t-tabulated	t-calculated
Day-2	2.45	15.80
Day-3	2.45	13.09
Day-5	2.45	6.26

The above results show that the soyabean oil-treated fishes (controls) have higher specific reductase activity than the ß-NF treated fishes.

The total microsomal NADPH-cytochrome P-450 reductase activity for this group of fishes represented in fig. 5c, shows the total reductase activity in the ß-NF-induced fishes to be the same as their controls for day-2 and day-3. On the contrary, the value for the controls of day-5 was lower than for the test fishes. This difference for the day-5 was found to statistically significant (Student's t-test; t-calculated = 2.97, t-tabulated = 2.45) and the day-2 and day-3 result not significant, in all cases, at the

Fig. 5c. Mean total NADPH-cytochrome c (P-450 reductase activity of aquarial O niloticus.



6-(d.f.) for $p > 0.05$ level. The higher total reductase activity shown in day-5 buttresses an earlier statement that the depression in the specific reductase activity is due to higher amounts of proteins.

3.3.1.3 Field Studies

Results of the field studies presented in fig. 5d shows a higher specific reductase activity for the controls over the ATL-E fishes only for day-2. The day-5 control and test fishes had the same value which was close to the value for the day-2 test fishes. The Student's t-table (Table 3.7) below indicates that there exists a significant difference for the value for day-2.

Table 3.7: Student's t-table for the field studies mean specific cytochrome P-450 (c) reductase activity at $p < 0.05$.

ATL & Controls	d.f	t-tabulated	t-calculated
Day-2	6	2.45	3.48
Day-5	4	2.87	0.329

The results of the mean total microsomal NADPH-dependent cytochrome P-450 (c) reductase activity of the field studies are represented in fig. 5e. There was an increase in total reductase activity for both day-2 and day-5 over their controls confirming higher protein as a key to depressed specific reductase activity. The significant increase for the day-5 relative to the day-2 test fishes by a factor 1.6 was statistically confirmed by the Student's t-test (day-2: t-calculated = 1.01, t-tabulated = 2.45; day-5: t-calculated = 5.21, the t-tabulated = 2.87).

Fig. 5d. Mean specific NADPH-cytochrome c (P-450) reductase activity of fishes in the field studies.

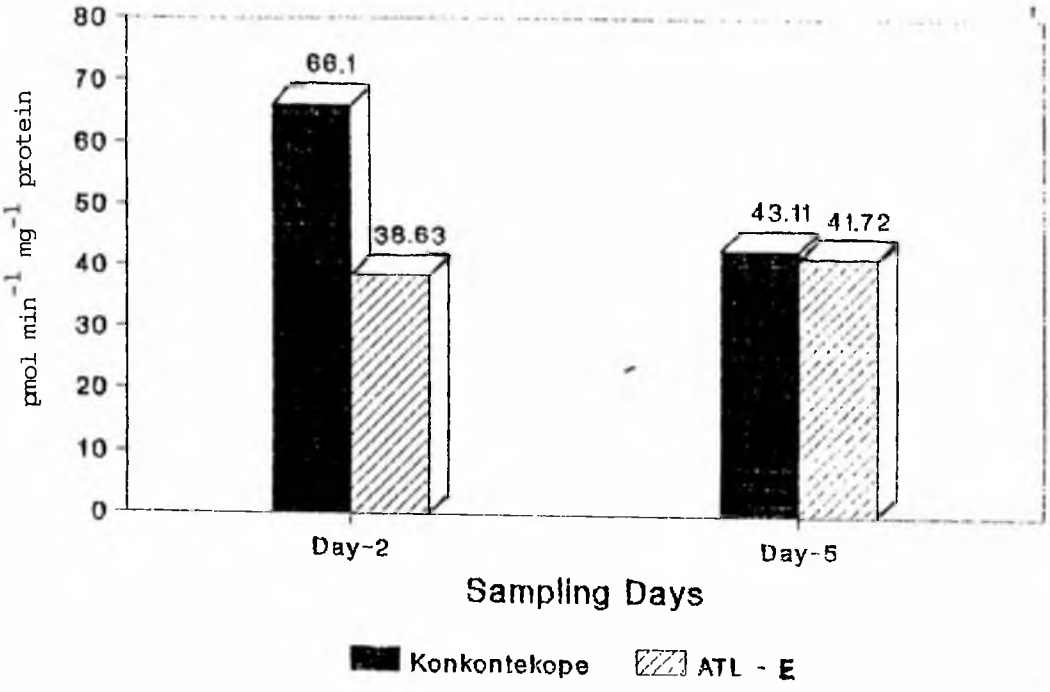
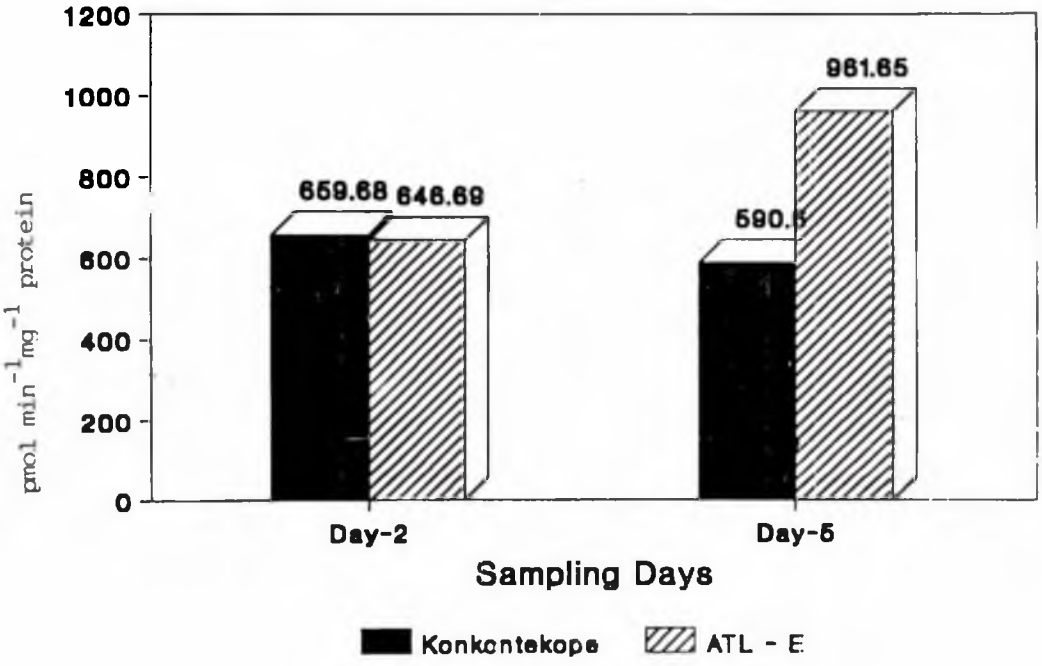


Fig 5e. Mean total NADPH-cytochrome c (P-450) reductase activity. for field O. nilocicus



3.3.2 Ethoxyresorufin-O-deethylase (EROD) activity

3.3.2.1 Purity of Substrate and Internal Standard

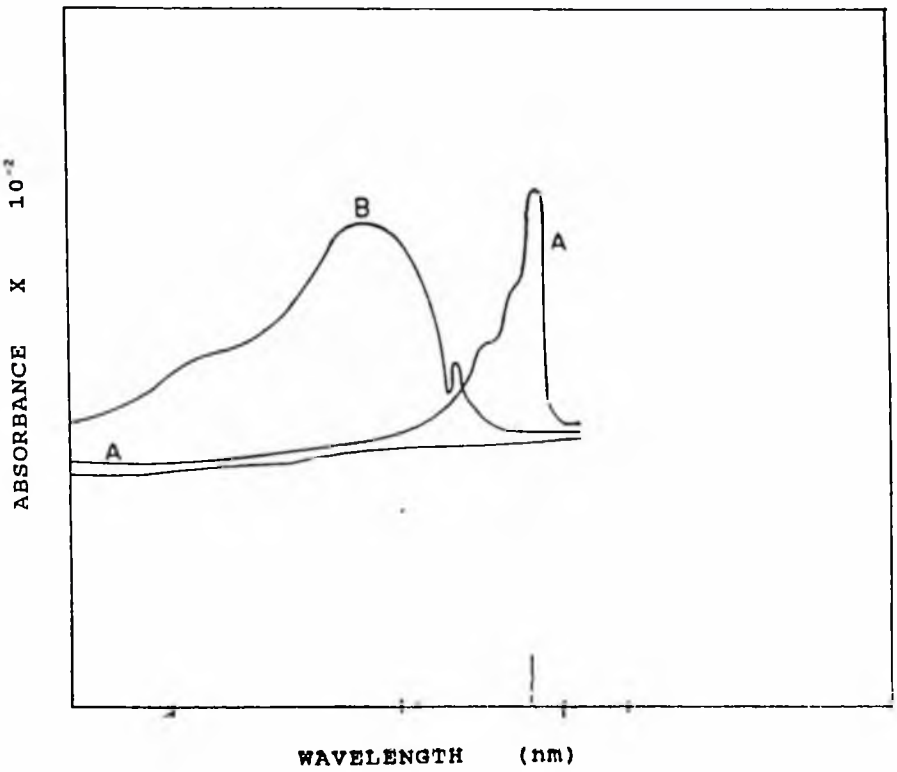
Fig. 6a and fig. 6b are the absorption spectra of both resorufin (which was used as the internal standard) and 7-ethoxyresorufin (substrate) in EROD buffer respectively, chartered on Spectrophotometer Model Specord-M40, to facilitate the determination of the wavelengths of maximum absorbance and the absorbance values for both substrate and product. The charts indicated maximum absorbance of 8.09×10^{-2} $M^{-1}cm^{-1}$ at 464.7nm instead of 2.25×10^{4} $M^{-1}cm^{-1}$ at 482nm reported by Prough et al, (1978) for 7-ER and a maximum absorbance of 9.36×10^{-2} $M^{-1}cm^{-1}$ at 592.4nm rather than 4.0×10^{4} $M^{-1}cm^{-1}$ at 572nm also reported for resorufin. For the values reported by Prough et al , (1978), the substrate 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 (ϵ) at the determined wavelength of maximum absorbance to be calculated from Beer-Lambert's law. The corrected ϵ for the substrate, 7-ER at 464.7nm is 46.0 $mM^{-1}cm^{-1}$ while that of resorufin is 69.37 $mM^{-1}cm^{-1}$. The literature value of ϵ reported for 7-ER at different times and places are 22.32 ± 0.31 $mM^{-1}cm^{-1}$ and 22.5 $mM^{-1}cm^{-1}$ while those reported for resorufin ranged from 20.1 to 73.2 ± 2.7 $mM^{-1}cm^{-1}$ (Prough et al, 1978; Burke and Mayer, 1974; Klotz et al, 1984; ICES-IOC, 1991).

Fig. 6 UV–Visible absorption spectra of resorufin and 7-ethoxyresorufin

(A) Resorufin (1.8 uM) in DMSO

(B) 7-ethoxyresorufin (4.8 uM) in DMSO



λmax 7-ER = 464 nm 0.0809

λmax Reso. = 592 nm 0.0936

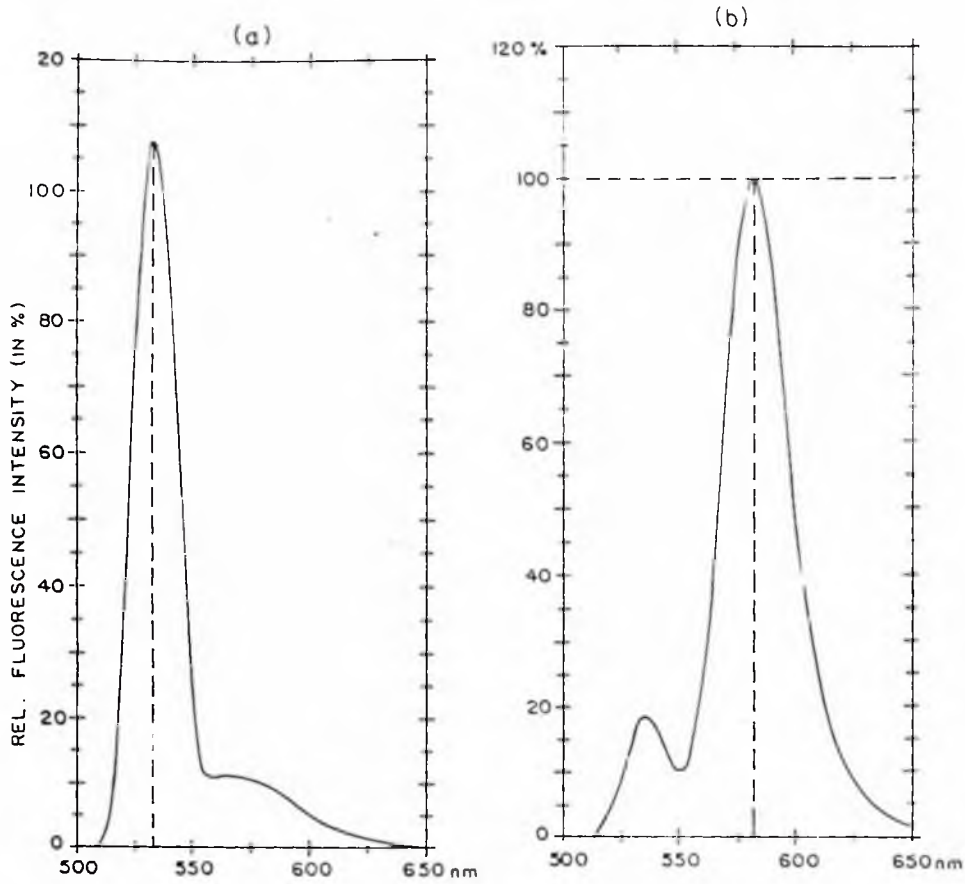


Fig. 7 EMISSION FLUORESCENCE SPECTRA ON THE LAMBDA DRIVE OF

(a) 7 – ethoxyresorufin (substrate)

(b) Resorufin (product)

Figs. 7a and 7b show the emission fluorescence spectra of both the substrate and the product respectively on the lambda drive of the spectrofluorimeter used. This was done for both within the scan range of 150nm (i.e 500nm and 650nm) to determine the wavelength of maximum fluorescence and ascertain their purity as described earlier in 2.2.7.

Both spectra were shown to lie within the visible region of the electromagnetic spectrum and the resorufin showed 100% purity while attaining a maximum fluorescence at 582 nm. On the other hand, the substrate showed more than 100% purity at 534 nm indicating that whatever impurity the substrate might contain also fluoresces at the same wavelength of maximum fluorescence accounting for the excess fluorescence above 100%. These wavelengths of maximum fluorescence conform to literature values as reported by Burke and Mayer, (1974) and standardised at the ICES-IOC EROD Intercalibration workshop at Aberdeen, (1991).

3.3.2.2 **Enzyme Assay**

The cytochrome P-450 monooxygenase activity was evaluated as 7-ethoxyresorufin-O-deethylase (EROD) activity. The enzyme activity is known to be elevated by polyaromatic hydrocarbons such as ß-NF, 3-MC, B(α)P and other structural analogues. EROD assay using microsomes prepared from all the control samples of O. niloticus including the preliminary ones, showed no fluorescence response to the assay system as indicated in Figs. 8a, 8b, 8c respectively and 8d for S. galilaeus.

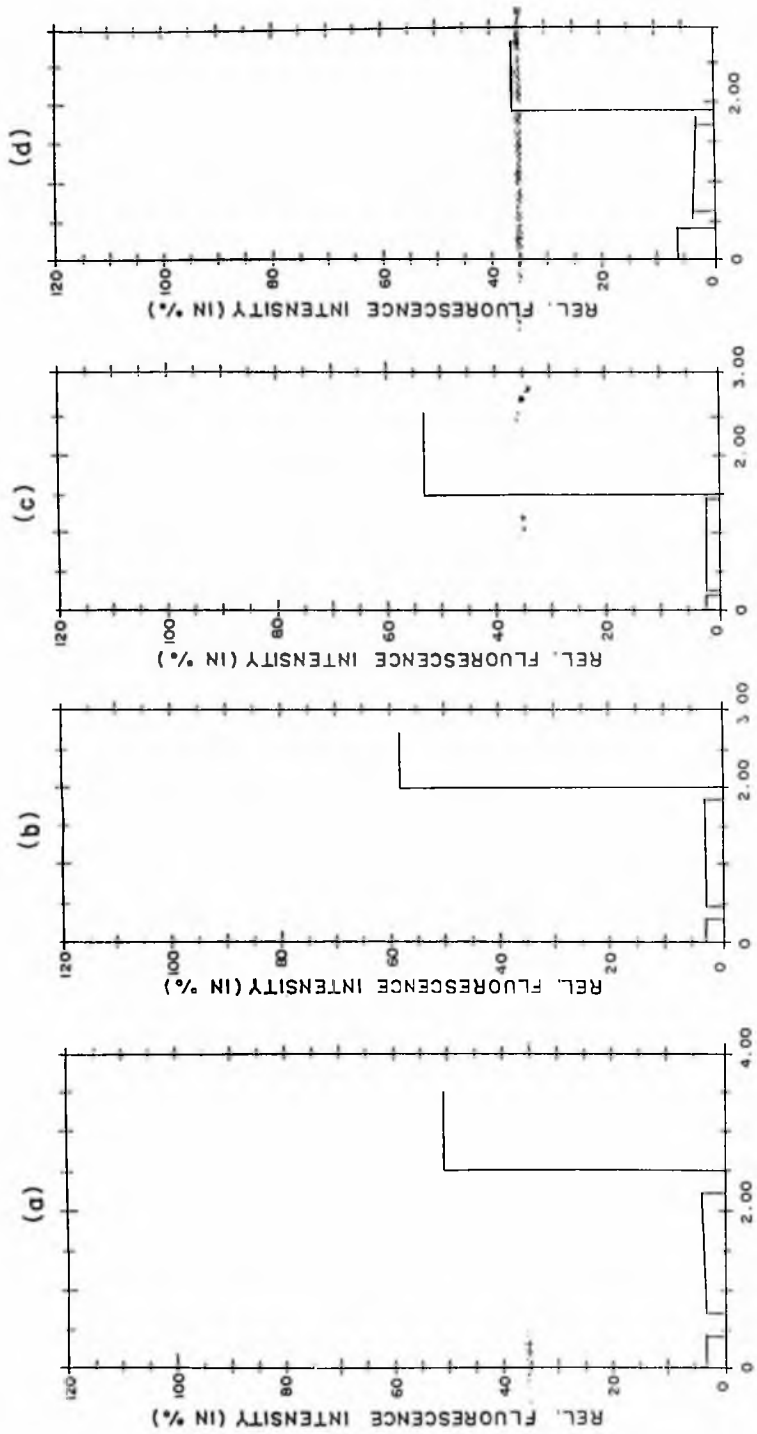


FIG. 8 FLUORESCENCE (EROD) RESPONSES BY MICROSOMES FROM O niloticus

(a) Konkontekope (ATL-E controls)
(b) Soyabean injected (BNF controls)
(c) Weija dam
(d) S galileaus from the dam near the motorway

3.3.2.3 Aquarial Studies

Fishes treated with ß-NF responded to the EROD assay as shown in Fig. 9a. Estimations from such response charts of S, R and D as defined in 2.2.7 enabled the calculations of all the EROD activity to be done. The means of these activities are presented in Fig. 9d. It is reported by Goksöyr and Förlin, (1992) that the bile of fish exhibit some inhibitory effects on EROD assay. Inhibition studies carried out by the addition of 10μl of bile to a progressing EROD assay produced a slight depression in the level of resorufin being produced (shown in fig. 9c).

The results generally indicate an increase in response/activity due to ß-NF over the controls (which showed no enzyme activity). There was a progressive decrease from day-2 through to day-5 for the ß-NF treatment. It is also noted that this decrease is by a factor of 1.4 between day-2 and day-3 and 1.9 between day-3 and day-5. The difference in specific EROD activity between day-2 and day-3 ß-NF treatment at the 5% level, were not statistically significant, while a statistically significant difference existed between the values for day-2 and day-5 as well as day-3 and day-5 as shown in Table 3.8 below.

Table 3.8: Student's t-table for specific EROD activity at the 5% level for unpaired aquarial studies.

ß-NF Treatment	d.f	t-tabulated	t-calculated
Day-2 & Day-3	6	2.45	1.20
Day-2 & Day-5	4	2.87	4.19
Day-3 & Day-5	4	2.87	5.88

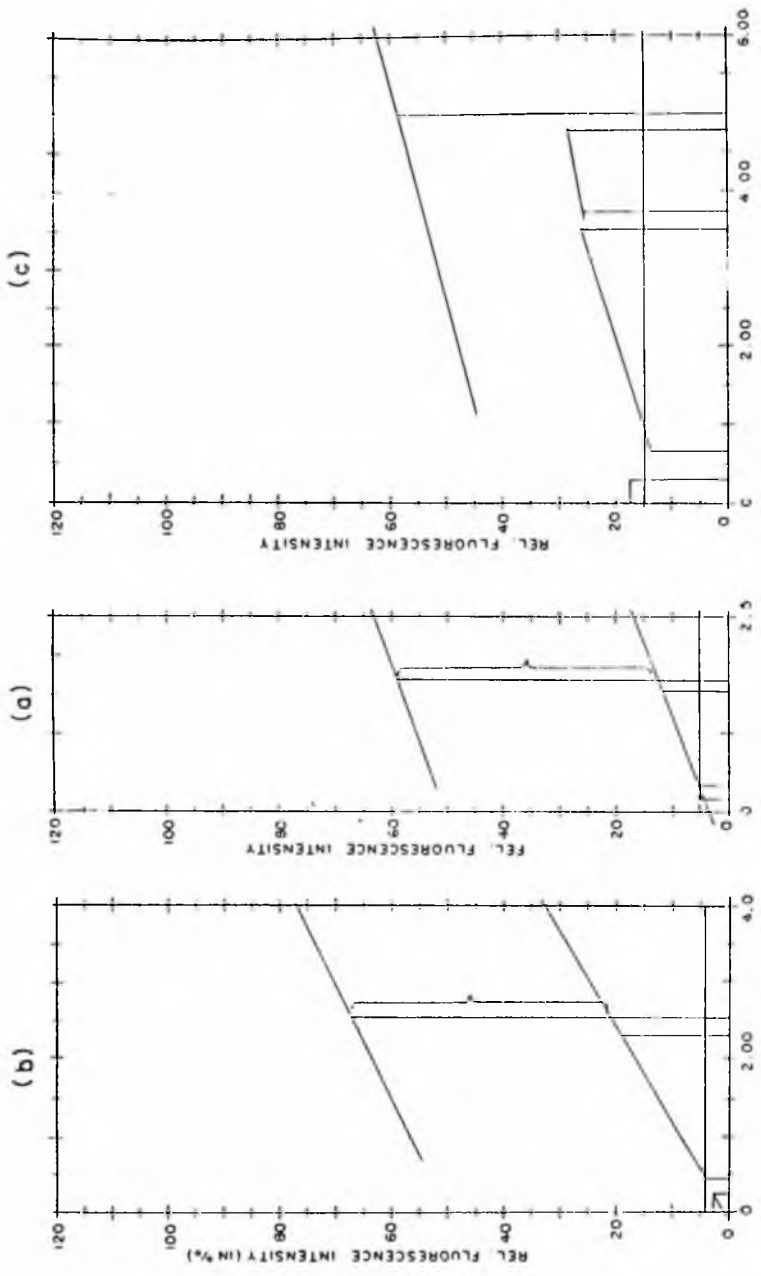
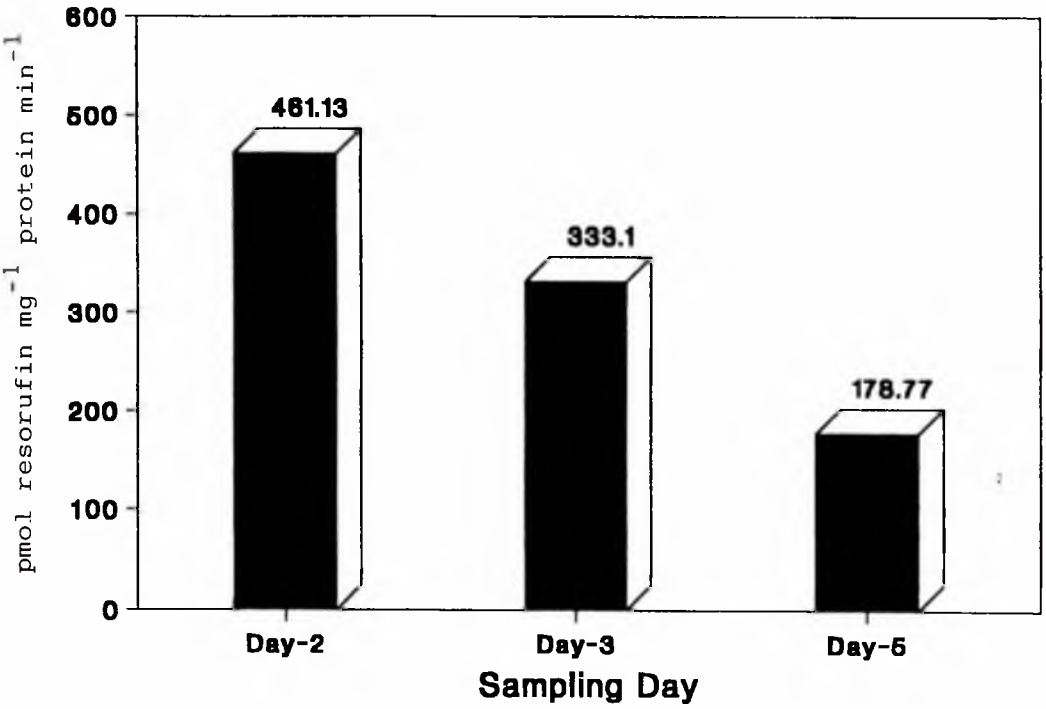


FIG. 9 FLUORESCENCE RESPONSES OF O. niloticus

(b) Treated with BNF
(a) Exposed to ATL-E
(c) ATL-E bile inhibition

Fig. 9d. Mean specific EROD activity for aquarial O niloticus



The total EROD activities were calculated and are as presented in fig. 9e. A maximum was attained on day-2 with the subsequent decrease possibly due to self-destruction of the protein after it has performed its functions. The results further show from the Student's t-test that there were significant differences between day-2 and day-3 (t-calculated = 4.79, t-tabulated = 2.45), day-2 and day-5 (t-calculated = 6.03, t-tabulated = 2.87) and then day-3 and day-5 (t-calculated = 4.84, t-tabulated = 2.87).

After three months of storage at -71°C, the microsomes were re-assayed for the EROD activity. The results showed a decay in the enzyme activity of the ß-NF-treated fish microsomes from 461.13 to 380.91 (a 28% decrease), 333.18 to 292.86 (a 12% decrease) and 178.77 to 173.41 (a 3% decrease) {all in pmol per minute per mg protein} for day-2, day-3 and day-5 respectively .

3.3.2.4. **Field Studies**

The response to the EROD assay for the ATL-E fishes and their controls are shown in Figs. 9b and 8a respectively. Whilst the controls exhibited no response at all, the activities calculated for the ATL-E are shown in fig. 9f. These results reflect very high activity for day-2 and day-5 with the day-5 registering the highest value and by a factor of 1.2 over day-2. The results of Student's t-test performed on the paired and unpaired samples at 5% level are shown in table 3.9.

Fig. 9e. Mean total EROD activity for field O niloticus

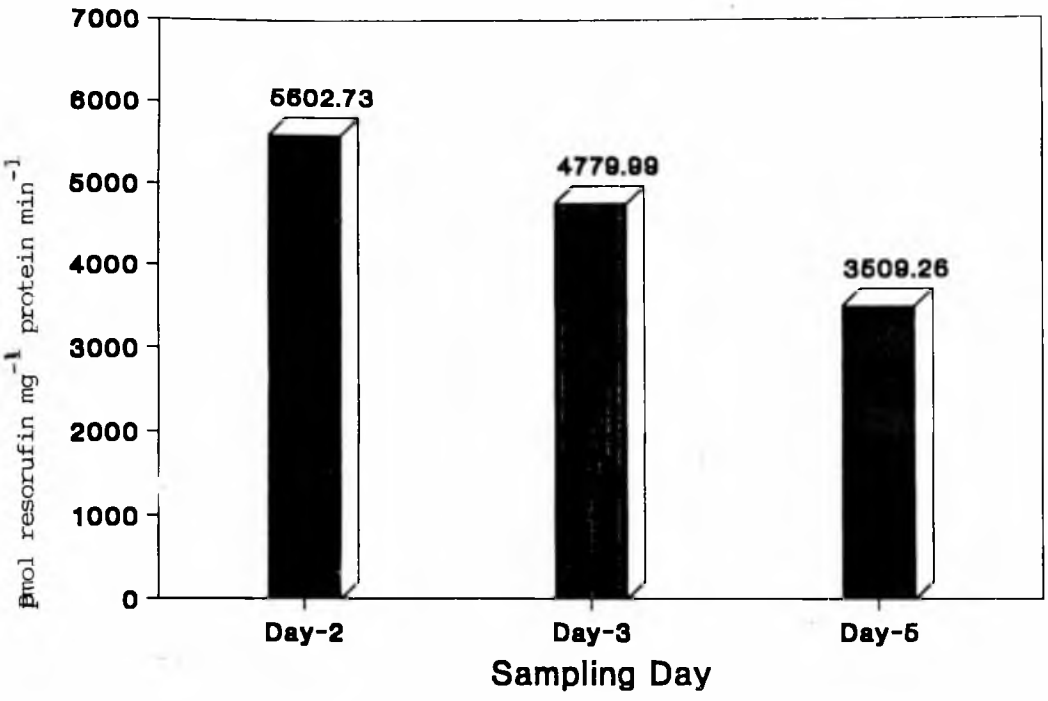


Fig. 9f. Mean specific EROD activity for field O niloticus

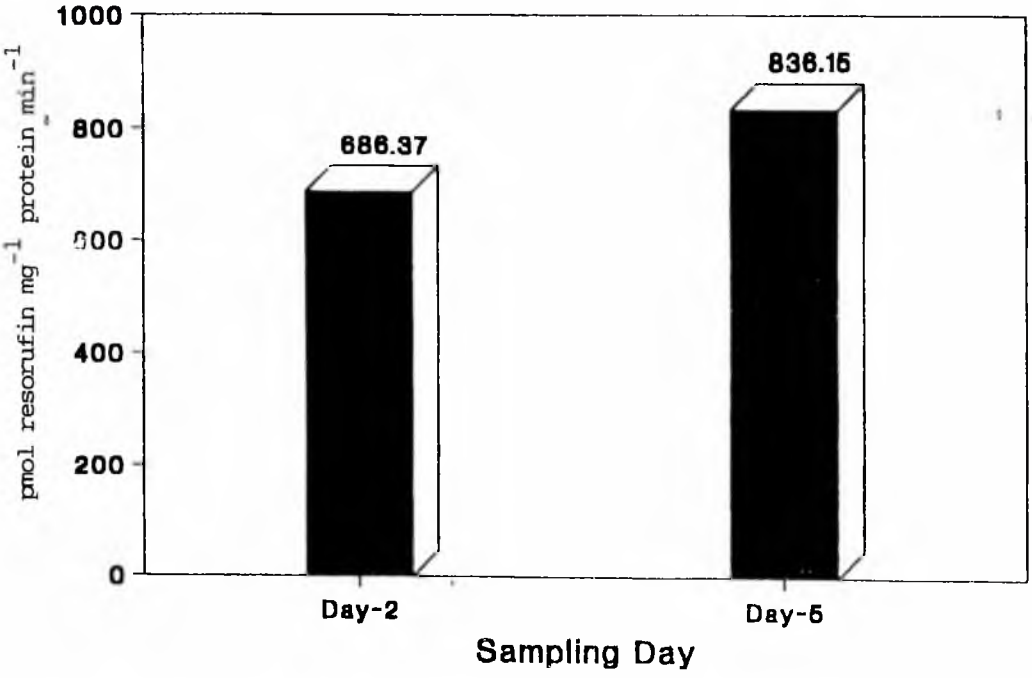


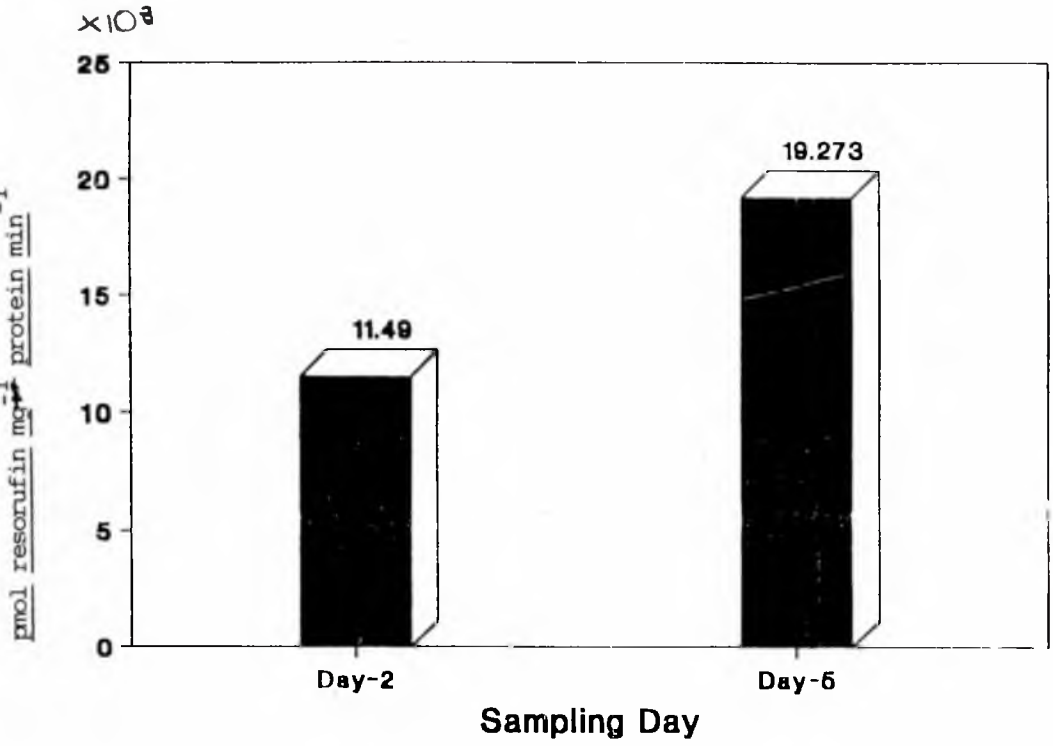
Table 3.9: Student's t-table for specific EROD activity at the 5% level comparing both types of treatments on test samples.

Treatment	d.f	t-tabulated	t-calculated
Day-2 ATL-E & ß-NF	6	2.45	2.62
Day-5 ATL-E & ß-NF	4	2.87	7.65
Day-2 & 5 ATL-E	4	2.87	2.05

From the results, there is no significant difference between the day-2 and day-5 ATL-E EROD activities while there are significant differences between the Day-2 ATL-E and ß-NF-treated as well as the day-5 ATL-E and ß-NF treatments. The difference between the two types of treatments are clearly shown in figs. 9d and 9f where the EROD activities due to the ATL-E (continuous exposure) far exceeds that due to ß-NF (single injection) for the days specified.

The total EROD activities calculated from the specific and the total microsomal protein and presented in fig. 9g shows that there is a significant difference between day-2 (minimum) and Day-5 (maximum). This is in consonance with the suggestion that this increase is due to continous exposure.

Fig. 9g. Mean total EROD activity for field *O niloticus*



3.3.3 SDS-PAGE

Microsomal proteins from all the controls, ß-NF-treated and ATL-E exposed Q. niloticus and S. galileaus livers were subjected to both denaturing and reducing conditions and subsequently resolved on 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the inductive effects of ATL-E and ß-NF on cytochrome P-450 proteins. One major protein band was detected in the 50,000 - 60,000 dalton molecular weight region (Plates A and B) where fish cytochrome P-450 hemoproteins generally migrate (Stegeman and Kloepper-Sams, 1987).

Plate A shows the electrophoretogram of Q. niloticus protein profile for controls and test for both ß-NF and ATL-E samples, applying 40μg protein per well and run parallel with marker protein in lane 10. Fig. 10 represents the calibration curve for electrophoretic mobilities of marker protein bands of Plate A (lane 10) and the average molecular weights of microsomal proteins from the mobilities of the resolved bands (5 out of the 8 bands) are as shown in Table 4.0 below.

Table 4.0: Molecular weights of resolved microsomal proteins from aquarial and field Q. niloticus and their control.

Marker Protein (Daltons)	ATL-E Exposed (Daltons)	ß-NF-injected (Daltons)
	108,800	109,300
66,000	68,900	70,700
45,000	53,700	53,700
34,700	44,700	43,500
24,000	33,500	32,100

Fig. 10. Calibration curve for Molecular weight Determination.

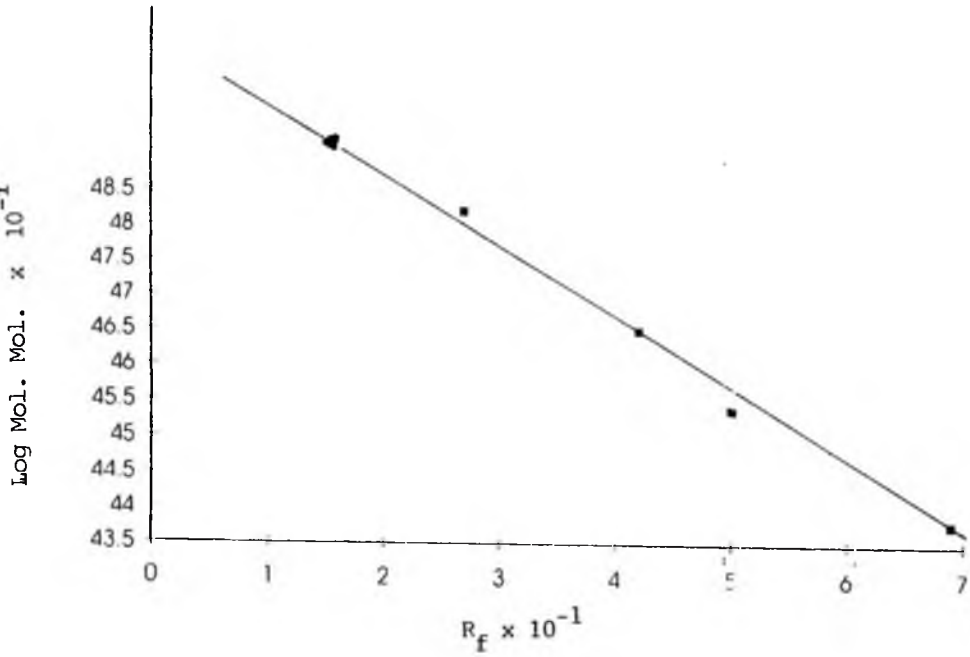


PLATE: A: Microsomal protein profile of controls, BNF-injected & ATL-E exposed O niloticus

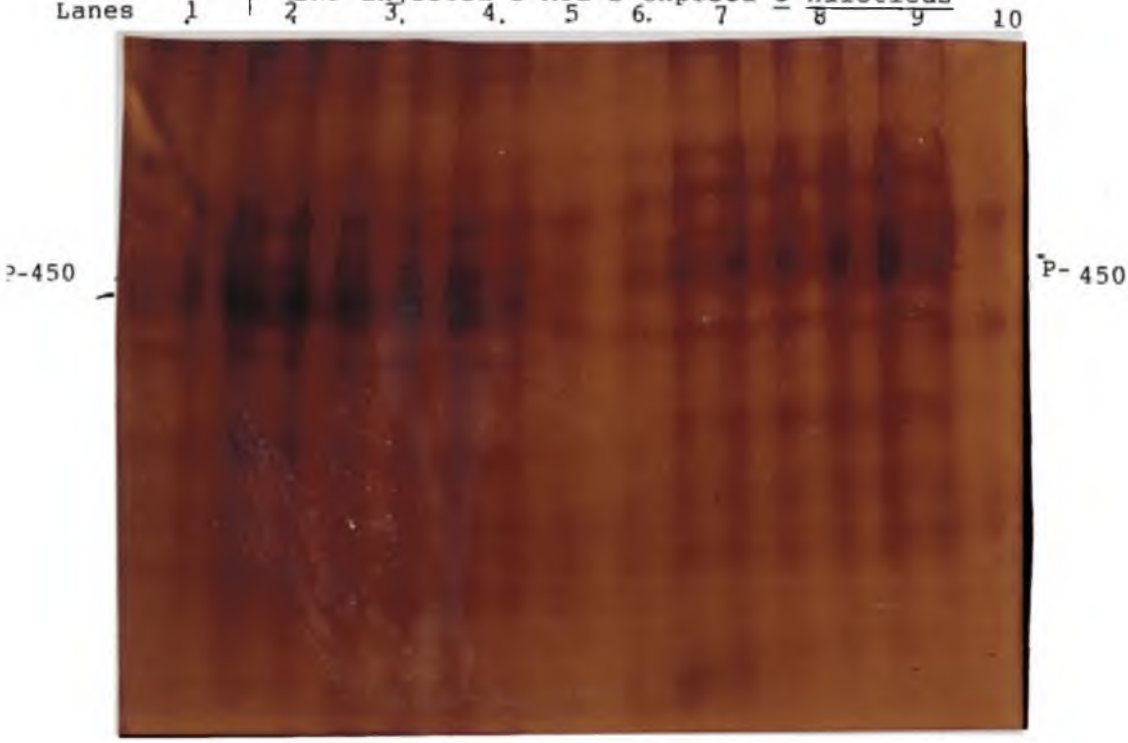
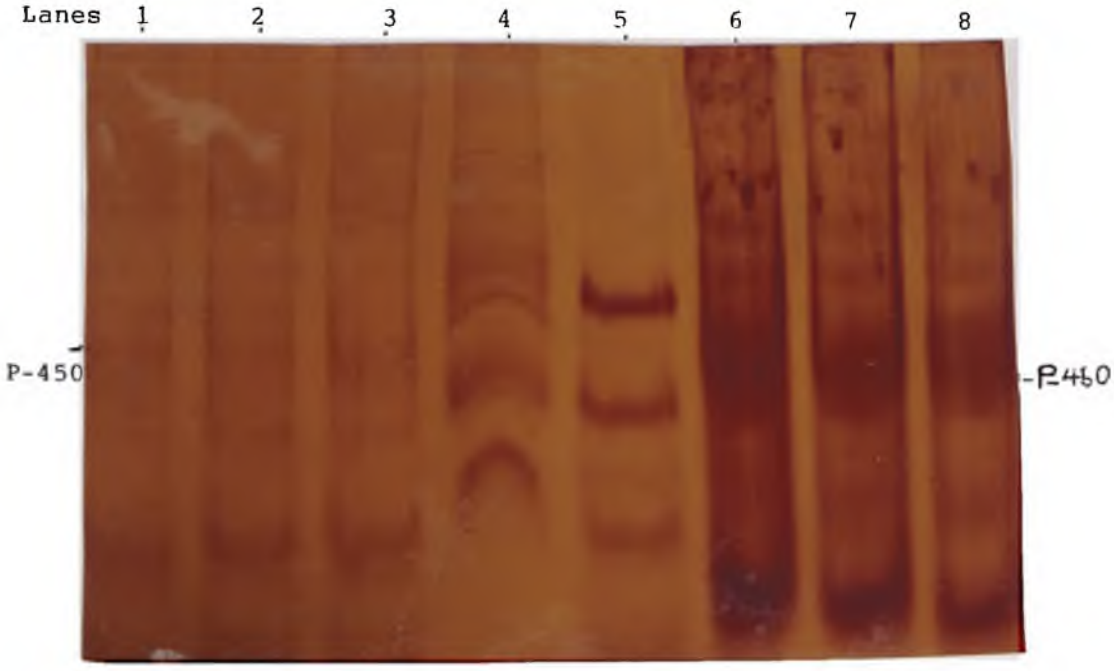


PLATE B: Microsomal protein profile of O niloticus form Weija dam and S galilaeus from the dam near the Tema motorway.



Both controls show a similar protein profile pattern, particularly the suspected P-450 band at 53,700 dalton which may be regarded as constitutive cytochrome P-450 band. However, the cytochrome P-450 band of the ATL-E controls appears more intense than the ß-NF controls. Comparing the protein profiles of the controls of both the aquarial and field studies with those of the ß-NF and ATL-E pretreatment against the sampling days shows an amplification of the 53,700 daltons bands of the ß-NF and ATL-E over their controls. It also shows a more intense 53,700 daltons protein band in the ATL-E pretreated fishes compared to the ß-NF pretreatment.

The day-2 (lane 2 and 3, Plate A) for ATL-E pretreatment registered the most intense cytochrome P-450 band (53,700 daltons) followed by day-5 [lane 4 -(40μg) and lane 1 (25μg)]. Lanes 5 and 6 represent the aquarial control (Soyabean oil injection) and field control (Konkontekope) respectively. From the electrophoretogram, the bands for these two control lanes are the least intense and suggests the baseline (constitutive) cytochrome P-450 in O. niloticus. Lanes 7, 8 and 9 define the days 5, 3 and 2 profiles respectively of the ß-NF samples.

Similarly, Plate B represents the resolved electrophoretic profile of O. niloticus from Weija dam and S. galileaus from the dam near the Motorway on 10% SDS-PAGE. Lane 5, which is the marker protein, shows 4 clear bands out of the expected 6. To the right of lane 5, are protein profiles of S. galileaus (5 bands) while to the left of lane 5 are profiles of O. niloticus (5 bands).

Table 4.1: Molecular weights for protein bands for feral Q. niloticus and S. galilaeus in daltons.

Marker Protein	Q. niloticus	S. galilaeus
	109,600	108600
66,000	70,700	71,600
45,000	53,700	54,300
34,700	44,700	42,500
24,000	32,100	32,400

Both species of tilapia show bands within the cytochrome P-450 region. However, while the Q. niloticus cytochrome P-450 band had molecular weight as 53,700 daltons, that of S. galileaus was at 54,300 daltons (a broad band). The broad nature of the S. galilaeus target band relative to thay of the Q. niloticus could be attributed to poor resolution of the microsomal proteins.

Lane 5 represents the profile for the marker proteins and shows 4 bands out of the expected 6. To the left of the standards, are replicated lanes of the Weija Q. niloticus microsomal protein profiles. On the right side are the profiles for the S. galilaeus from the dam near the Tema motorway. It is clear from Plate B that the pattern of protein bands for both tilapia species appear to be different e.g. 44,700 and 42,500 daltons.

CHAPTER FOUR

4.0 DISCUSSION AND CONCLUSION

The increasing awareness of fish as useful models for carcinogenesis, toxicity testing and monitoring studies in the aquatic environment has resulted in expanding interests in studying the enzymatic basis of xenobiotic metabolism in fishes (Payne et al, 1987; Powers, 1989; Stegeman and Lech, 1991). Tilapia mossambicus, the most widely distributed species in Taipei, Taiwan has been used by Ueng et al (1992) to monitor the effects of industrial effluents discharged into the Damsui river as was also done with perch (Perca fluviatilis) to monitor Bleached Kraft Mill effluents in Sweden (Anderson et al, 1988; Södergren, 1989 and Förlin et al, 1991).

In Ghana, Oreochromis niloticus is becoming a popular model in aquatic pollution monitoring for trace metals (Biney, 1991) and heavy metals (Biney and Beeko 1991; Biney, 1992). The present study, for the first time, has characterized a biochemical mechanism used in the metabolism of xenoorganics in two economically important tilapias O. niloticus and Seratherodon galilaeus and has revealed interesting properties in consonance with results of other fishes studied elsewhere. The study has shown the O. niloticus monooxygenase enzyme system to be significantly responsive to stimulatory effects of pollutants, ß-NF - a classical one and those present in the effluent from a textile factory. This indicates that the fishes in both treatments were exposed to xenobiotics (such as PAH and PCB) and mixtures with proven inducing properties on the cytochrome P-450 monooxygenase system in fishes. The environmentally inducible properties together with the wide distribution and economic

importance of O. niloticus (dwells in freshwater with muddy bottoms) and S. galilaeus (inhabits in freshwater with stony bottoms) suggests these species as potential models for xenobiotic metabolism and environmental pollutionmonitoring studies in our freshwater bodies.

4.1 Preparation and Storage of Microsomes

It is reported by Förlin et al (1994) that erroneous results could be obtained if great care was not taken in the sampling and handling of samples. The activity levels of the cytochrome P-450 monooxygenase system in O. niloticus and S. galilaeus were noted to be influenced by the previous handling of the organ processed for their assays - the liver in this case. Low enzyme activities were recorded in the preliminary studies when the samples were transported frozen over long distances before microsomal preparation. The CO-difference spectra showed a maximum peak at 420nm reminiscent of degraded microsomes just as has been reported for other fishes (Förlin et al, 1994). These results therefore show that the membrane-bound P-450 of O. niloticus and that of S. galilaeus are susceptible to inactivation during sampling time (after death of fish), freezing before the preparation of microsomal fractions, enzyme fractionation (frothing during pellet rehomogenization) and storage of the enzyme fraction before enzyme analysis.

In subsequent work, degradation of the P-450 system was averted by sacrificing, dissecting and excising the livers of the fishes as quickly as possible. In this operation, rupturing of the gall bladder to spill the bile was avoided since Goksöyr and Förlin, (1992) reported the bile content to contain some P-450 inhibitors. The inhibitory effect of the bile content was

demonstrated in this report as has been indicated in Fig. 9c.

All the procedures after the excision of the liver involved the use of iced buffers and storage in liquid nitrogen or at -71°C. Because samples were taken in the field without immediate access to centrifuges, the livers in cryotubes, were layered with ice-cold 30% glycerol to give increased protection during storage. In processing the frozen livers into the postmitochondria supernatant (S-9fraction) and then microsomes, they were thawed on ice to prevent damage to the endoplasmic reticulum. To further enhance the viability of the microsomes in terms of activity, the phosphate buffers used to resuspend the microsomes contained antioxidants such as DTT and EDTA (which is also a chelating agent) in addition to the glycerol.

4.2 Biological Data

Condition factor is an indication of the nutritional status of fish and is reported to be different for different fish species, requiring a standard to be determined for each fish species. Starving fishes are expected to register lower CF values relative to feeding fishes. With respect to this index, all Q. niloticus used in this study, except those exposed to ATL-E for 5 days, registered CF values lower than the mean of non-starving fish from the same stock. There was no difference between the aquarial test and control fishes because both groups were not fed. However, there was a significant difference between the the test (ATL-E) and their controls at Konkontekope as evidenced in Fig. 3c. One can conclude that for Q. niloticus, there exists a relationship between the condition factor and feeding habits for the fishes at the ATL-E site as opposed to the control fishes at Konkontekope.

For the acclimated feral fishes in the preliminary studies, the results suggest that S. galilaeus is a better feeder than O. niloticus. This better feeding habit correlates positively with the mean total microsomal protein which were higher in S. galilaeus (Fig. 3a), establishing a possible relationship between the feeding habits, increase in condition factor and total microsomal protein.

Liver somatic index is another Fulton's index that measures the health status of fish organs such as the liver. It differs from one species of fish to another. Hyperplasia, hypertrophy and the development of cancerous conditions or protein synthesis in the livers of fish are usually associated with diseases or the presenceof xenobiotics and are reflected in the rise in LSI. The mean LSI value for unexposed and hence standard O. niloticus was 1.01. The absence of any significant difference between the LSI values for the ß-NF-treated fishes and their controls (Table 3.2) clearly indicates the lack of any relationship between this index and ß-NF injection. However, the significant difference between ATL-E and Konkontekope fishes establishes a relation between LSI and exposure to the effluents from ATL factory. The factory effluent could be carcinogenic.

4.3 **Biochemical Indices**

Studies on microsomal protein concentrations and activities of specific enzymes were carried out in order to examine the usefulness of biochemical responses as health indicators in fishes injected with the classical pollutant (ß-NF) and fishes exposed to effluents from the ATL factory. The responses were to be used to elucidate the extent of lethal/toxic effects on the fish population exposed to ATL-E as was similarly done in Sweden on fish exposed to

Bleached Kraft Mill Effluent (BKME) by Förlin et al (1985), Andersson et al (1988), Södergren (1989) and Lindström-Seppa & Oikari (1990).

4.3.1 Total Microsomal Protein

The mean total microsomal protein concentrations of the acclimated fishes in the preliminary studies showed the S. galilaeus to have higher values than the O. niloticus (fig. 4b). The over 100% increase in S. galilaeus protein concentration with respect to O. niloticus probably reflects species differences. Since acclimation of feral fishes in tap water reverses any induction in seven (7) days (Lech et al, 1979), the longer period of acclimation in these experiments (2 months) permits one to conclude that the reported values are for the basal/constitutive proteins and hence, cannot tell any pollution history about the water bodies from which these fishes were taken.

The significant differences in mean total microsomal protein concentration recorded for both the aquarial fishes and those in the field over their controls confirm the occurrence of induction due to the xenobiotics. In comparing the induced protein levels of the aquarial and field fishes, the higher values recorded for the ATL-E exposed fishes can be attributed to the ATL-E having a higher concentration of xenobiotics. One can, however, also conclude that this effect was due to the continuous exposure relative to the single i.p. injection of ß-NF (80mg/kg body weight) given per fish in the aquarial studies. But the fact that this result may also be a reflection of the more potent nature of the xenobiotics in the ATL-E as an inducer cannot be ruled out.

The total microsomal protein for both treatments was the highest by the last day of sampling (day-5). It may be appropraite to extend the experimental period beyond day-5, irrespective of the fact that about half (50%) of the fish exposed to the ATL-E had died by day-5, in order to find out the period for maximum induction. The high percentage mortality (50%) recorded for the ATL-E exposed fishes may be a confirmation of the breakdown of the cytochrome P-450 system, the first line of defence of the fishes, or the high toxicity of ATL-E.

It should also be noted that the high mean total proteins shown by fishes at Konkontekope, the control site for the field studies, compared to the controls of the aquarial studies, indicates some induction and protein synthesis but the induced protein was devoid of P-4501A activity that characteristically respond to EROD assay. The high protein concentration could be attributed to the presence of some inducing agents that induce P-450 isozymes that do not respond to EROD. In the light of the foregoing, the general conclusions that the absence of any response to EROD are due to the absence of pollutants, could be misleading.

Of the three hypotheses put forward to account for the elevated levels of microsomal protein, the presence of xenobiotics (ß-NF, agrochemicals, domestic sewage and ATL-E) in the fish environment plausibly explained the rise in total protein in both the ATL-E and ß-NF treated fishes (Porter and Coon, 1991; Andersson and Förlin, 1992). Since all the control and test fishes were held at the same temperature, water pH 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 fishes used in this project, the surge in vitellogenesis could not also be the factor involved in the elevated protein levels.

4.3.2 Enzyme Assay

Inducers were classified by Gillette (1971) to reflect the type of reaction a class of chemicals/xenobiotics undergoes. Thus, in addition to elevating the total protein levels, inducers can also specifically affect the P-450 level and NADPH-dependent reductase activity. Based on the above, inducers can be classified into three viz; PB-type inducers which increase both the P-450 level and the NADPH-dependent cytochrome P-450 (c) reductase activity, 3-MC-type inducers that increase the P-450 level but not the reductase activity (this can either decrease or remain constant) and finally, the third group which primarily increases the reductase activity and may or may not affect the P-450 level.

The fact that there was no induction of cytochrome P-450 (c)reductase in ß-NF-treated fishes (test values were not significantly different from control values, fig. 5c) confirms ß-NF as belonging to 3-MC class of inducers which increase the P-450 levels but not the cytochrome P-450 (c) reductase activity. The lower specific reductase activity in the test fishes compared to their controls in the aquarial studies could be a mere reflection of the higher microsomal protein concentration of the former.

That there was a statistically significant increase in the mean total cytochrome P-450 (c) reductase activity for the day-5 test fishes in the field over their controls (fig. 5e) suggests induction of this protein in response to constant exposure of fish to pollutants in the ATL-E which are different from the ß-NF type. The mean specific NADPH-dependent cytochrome c (P-450) reductase activities of the controls were significantly higher than the test fishes in both types of exposures except for ATL-E day-5, where the specific enzyme activity was the same for both test and control fishes, inspite of the significantly higher protein concentration of the test fishes compared to that of the controls. All these taken together indicates that the ATL-E induced protein has reductase activity and so the xenoorganic(s) in the effluent could belong to the PB or the third type of inducers.

The significantly lower mean specific reductase activity of the fishes at Konkontekope on day-5 compared to the value on day-2 seems to indicate that the additional proteins synthesized by day-5 at the control site did not have any reductase activity, suggesting that the pollutants present at Konkontekope belong to neither the PB nor the third type of inducers. Another possible reason for this could be the presence of reductase inhibiting chemicals (such as cyanide and heavy metals) at the site. The fact that the mean specific cytochrome P-450 (c) reductase activities of the exposed fishes were fairly constant also indicates the possibility that the induction of the reductase is a process that is not affected by the time period employed in this assay.

Ethoxyresorufin-O-deethylase assay/response was used to indicate cytochrome P-450 dependent monooxygenase activity since the protein could not be assayed by CO-difference spectra. Since fishes acclimated in tap water usually register a decline in the P-450 enzyme activity, the results obtained implies that the increased level in EROD activity was due to the ß-NF in the case of the aquarial studies and xenoorganic dietarycomponent(s) in the ATL-E exposure. The xenoorganics present in the ATL-E must belong to, or are structural analogues of the class of inducers of polyaromatic and polychlorinated hydrocarbons such as lindane and the dioxins (Boon et al, 1992) that induce the cytochrome P-4501A isozyme of which the P-4501AI gene product is the only known one in the fishes so far studied.

It is clear from figs. 9f and 9g that the EROD and P-4501A activities due to ATL-E exposure were higher than that due to ß-NF induction and this can possibly be explained by the continuous exposure of the fishes to ATL-E in relation to the single i.p dosage of 80mg Kg^{-1} ß-NF injected. One other explanation could be that the factory effluent (pollutants) are more potent and persistent inducers, or there could be a mixture of xenoorganics which act synergistically in inducing the monooxygenase complex in fishes (Gooch et al, 1989, Skaare et al, 1991, van der Weiden et al, 1992, Boon et al, 1992).

One other result worth noting is the fact that while both specific and total EROD activities for the ATL-E exposed fishes increased with time, those of the ß-NF-injected fishes followed a time-course decline. This may be attributed to the continuous exposure of field test fishes to ATL-E as against the single i.p. ß-NF injection in the case of the aquarial studies where enough biomarker was synthesized to overcome the ß-NF by day-2 (highest EROD value).

This enzyme is not a "house keeping enzyme". As such, once induced to perform the destined duties, it subsequently follows a path of self-destruction. The other plausible reason may also be thedifferent xenoorganic components in the two pollutant sources used in the field (ATL-E) and aquarial (ß-NF) work.

Even though the actual organic components of the ATL-E was unknown, and hence no conclusion can be drawn as to which component(s) are responsible for the induction, the results obtained indicate that the ATL-E is made up of a mixture of different types of cytochrome P-450 inducers including PAHs, PCBs and possibly, dioxins and other dyes used in the textiles industry. No responses to the EROD assay were observed for S. galilaeus (fig. 8d) and all the controls including those for the field studies which had higher microsomal protein concentration indicating induction of protein synthesis. This indicates that cytochrome P-4501A was not present among the microsomal proteins induced by the pollutants in the Volta Lake at Konkontekope and and the other water bodies used in the preliminary studies. The increased mean total protein correlates positively with the induced monooxygenase system responding to EROD assay in O. niloticus exposed to the ATL-E and indicates induction of cytochromme P-4501A proteins in addition to the reductase as a result of the exposure.

The increased mean total protein concentration in the aquarial studies does not correlate with the EROD or the reductase assays. This suggests that the induction of other proteins peculiar to tilapia and could possibly explain why tilapias can tolerate a high degree of pollution (Ueng et al, 1992).

Fishes exposed to the ATL-E manifested induction to the xenoorganics present in increased microsomal protein concentration, increase in total reductase activity, as well as increased cytochrome P-450 (P-4501A). That P-4501A protein was induced by the factory effluent as evidenced in the positive response to the EROD assay suggests the factory effluent contains 3-MC-type inducers. The induction of reductase in the test fishes in the field studies indicates the presence of PB-type or the third type (or a mixture of both types) of inducers in the ATL effluent.

The different types of microsomal protein synthesized reflect different pollution histories. The pollution at Konkontekope, the control site for the factory effluent, could be due to the domestic sewage from the Akosombo township and chemical wastes from the hospital which discharge into the Lake upstream Konkontekope and the field experimental sites. It could also be attributed to agrochemicals such as dieldrin sprayed to control Segatoga a banana and plantain virus in plantations upstream the hydropower generation station or may be as a result of agrochemicals including DDT and gamelin-20 washed into the Lake from surrounding cocoa farms. DDT and dieldrin have been proven by Axelrod, (1983) and Waterman et al, (1986) to elevate fish microsomal protein level, cytochrome P-450 (c) reductase activity but the P-450 monooxygenase enzyme complex induced does not respond to EROD assay.

The cytochrome P-450 monooxygenase system is a superfamily of twenty-eight isozymes (Nebert et al, 1991). The most common isozyme in fish is the P-4501A which when induced is known to specifically catalyse EROD activity (Goksöyr et al, 1991b; Husöy et al, 1993).

Lower levels of, or no P-4501A/EROD activity have been reported during spawning in several fishes (Koivusaari et al, 1981; Larsen et al, 1992). This could not have been responsible for the lack of response to EROD assay in the S. galilaeus, all the control O. niloticus as well as the fishes from Weija dam since there was no physiological evidence regarding maturation of the fishes used.

4.3.3 SDS-PAGE

The SDS-PAGE electrophoretograms showed the liver microsomal protein profiles of the ATL-E exposed and the ß-NF-injected O. niloticus and their controls to be similar, having a common protein band of molecular weight 53,700 daltons. This band can apparently be taken as being that of the constitutive cytochrome P-450 protein inthe control fish. That the intensity of the bands due to the ATL-E (lanes 1, 2, 3 and 4) and ß-NF (lanes 7, 8 and 9) were higher than their controls (lanes 6 and 5) of plate A suggests that the induction due to both pollutants is not qualitative (since no new bands showed up in the electrophoretogram) but quantitative. However, for the absence of new bands, the possibility of poor resolution of the different bands cannot be ruled out. The amplified band intensities (53,700 daltons) due to ATL-E and ß-NF probably represents the cytochrome P-450 monooxygenase protein responsible for the positive EROD assay.

From plate A and the total EROD activities (Figs. 9e and 9g), it is evident that the induction and hence EROD activity for both ß-NF and ATL-E treatments peaked by day-2. Subsequently, a process of self destruction of the induced functional proteins commenced. The band intensities due to ATL-E (lanes 2, 3 and 4 in Plate A) were higher than those for

ß-NF (lanes 7, 8 and 9). The higher band intensities registered can be attributed to additional P-450 isozyme induced by ATL-E with electrophoretic mobility falling within the range specified for fish microsomal cytochrome P-450 isozymes (Stegeman and Kloepper-Sams, 1987). Since it has also been shown (Goksöyr et al, 1991b; Husöyr et al, 1993 and Nebert et al, 1989) that for fish, only one kind of isozyme is induced by PAHs such as ß-NF and that this protein responds to EROD assay, one can conclude that the intense bands in the P-450 mobility zone due to ß-NF induction could be the P-450AI gene product.

It is also evident from the protein profile of Plate A that ATL-E induces P-450 isozyme (P-450IA, molecular weight of approximately 53700 daltons) of similar mobility as the isozyme induced by ß-NF, with the induction due to ATL-E being stronger as reflected in the higher intensity of the P-450IA suspected band. The isozyme induced by the ATL effluent may or may not be the same as that induced by the ß-NF but it is clear that the isozyme(s) involved in both treatments belong to the same family.

For the protein profiles of the preliminary studies involving acclimated feral O. niloticus and S. galilaeus. (Plate B) there were distinct differences between the two tilapia species. While they induced almost the same constitutive protein in the P-450 mobility zone which cannot be used as any distinguishing factor, the protein bands outside the P-450 zone show marked molecular weight differences. Thus, microsomal protein profiles on SDS-PAGE may serve as an additional biochemical tool for differentiating between species when the orthodox taxonomic tools (meristic and physiological characteristics), often used, tend to be obscure or confuse the classification.

The presence of one type or a mixtures of planar PAHs, PCBs, dioxins, dibenzo-p-furan pollutants and/or their structural analogues in the ATL-E and their induction of NADPH-cytochrome P-450 monooxygenase enzyme complex in tilapia species have been established in this work by the response of the fish to the catalytic activity of two enzymes, 7-ethoxyresorufin-O-deethylase and NADPH-dependent cytochrome P-450 (c) reductase. The catalytic activity of or response to the EROD assay in the present study has confirmed ß-NF as a classical xenobiotic that can induce the monooxygenase enzyme system in fish (Bend et al, 1979; Lech et al, 1982), and has shown that the ATL effluent contains 3-MC-type inducers or structural analogues. The study also demonstrated that ATL-E has the capacity to induce this biomarker system in O. niloticus. It is further noted that the ATL-E has actually induced NADPH-dependent cytochrome P-450 (c) reductase enzyme suggesting that the ATL effluent may contain PB-type or the other types of inducers. The ATL-E components, thus, have been shown to exhibit properties relating to the three classes of inducers defined by Gillette, (1971) which may act synergistically.

This work has demonstrated the inducibility of tilapia monooxygenase enzyme system by xenobiotics such as ß-NF and those in the effluent from the Akosombo Textile Factory. Therefore the ERODassay, which is specific for cytochrome P-450A1 monooxygenase enzyme complex, can be used as an index for indicating or confirming fish exposure to xenobiotics. The detection of such a biomarker, as has been done in this work with a model fish like O. niloticus, can serve as an early warning signal of environmental pollution and as a tool for assessing pollution impact on fish stock.

Appendix I: Analysis of Variance for Mean Condition Factor (CF).

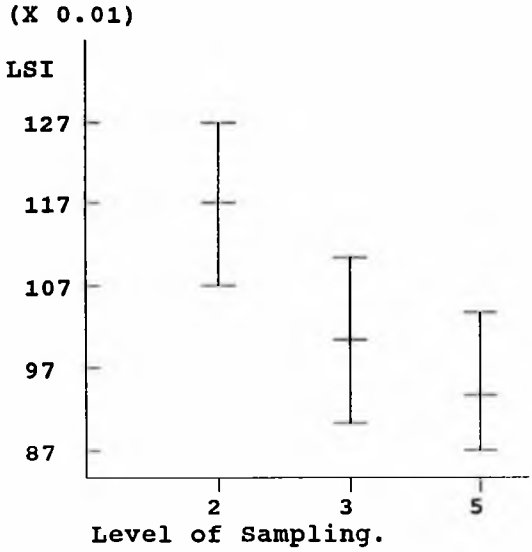
Source of Var.	Sum of squares	d.f.	Mean Square	F-ratio	Slevel
AIN EFFECTS	0.07 4475	6	0.01 8619	1.055	0.4643
Sampling days	0.0138875	3	0.0069437	0.369	0.7087
Level of treat	0.0465125	1	0.0465125	2.471	0.1768
Level of cont.	0.0250000	3	0.0250000	1.328	0.3012
RESIDUAL	0.0941125	5	0.0188225		
TOTAL (C0RR)	0.1735600	9			

Appendix IIa Analysis of Variance for mean Liver Somatic Index (LSI)

Source of Var.	Sum of squares	d.f.	Mean square	F-ratio	S.Level
MAIN EFFECTS	0.0138875	6	0.0069437	0.369	0.679
Sampling days	0.0794475	3	0.0198619	1.055	0.464
Level of treat	0.0079380	1	0.0079380	2.921	0.148
Level of Cont.	0.0490000	3	0.0490000	1.328	0.301
RESIDUAL	0.0082589	7	0.0020109		
Total (CORR)	0.1866236	11			

S. level = Significance level

Appendix IIb: Intervals for Factor means at 95% confidence for mean Liver somatic index against sampling days.



Appendix IIc: Multiple range analysis for mean LSI by sampling day for both BNF-treated Fishes. Method: 95% confidence intervals

Level	Count	average	Homogeneous Groups
3	4	0.9375000	*
2	2	1.0050000	*
5	4	1.1855000	*

Appendix IIIb: Multiple range analysis for total microsomal protein concentration Of BNF-treated fish by Sampling day. Method: 95% confidence intervals.

Level	Count	Average	Homogeneous Groups
3	2	10.220000	*
2	4	11.036750	*
5	4	16.057500	*

Appendix II(d): Analysis of Variance for Mean Liver Somatic Indix (LSI) for field studies.

Source of Var.	Sum of Square	d.f.	Mean Square	F-Ratio	S.Level
MAIN EFFECTS	0.1853340	4	0.463335	17.047	0.0041
Sampling Day	0.1333333	2	0.0666667	24.529	0.0026
Level of Treat	0.0079380	1	0.0079380	2.921	0.0482
Level of Cont.	0.0492804	2	0.0492804	18.132	0.0086
RESIDUAL	0.0135896	5	0.0027179		
Total (CORR)	0.1989236	9			

Appendix III(a): Analysis of Variance for Total Microsomal Protein Concentrations at the 95% Confidence Limit.

Source of Var.	Sum of Square	d.f.	Mean Square	F-Ratio	S.Level
MAIN EFFECTS	296.94615	6	74.23654	42.504	0.0005
Sampling Day	51.72464	3	25.86232	14.807	0.0079
Level of Treat	43.66919	1	43.66919	25.003	0.0041
Level of Cont.	185.14948	3	185.14948	106.007	0.0001
RESIDUAL	8.7328965	5	1.7465793		
Total (CORR)	305.67904	9			

Appendix III(c): Multiple range analysis for total Microsomal Protein Concentration of Test Fishes at the level of Treatment (BNF-Injection and ATL-E Exposure) by Sampling day. Method: 95% Confidence Intervals.

Level	Count	Average	Hommogeneous Groups
1	6	10.880500	*
2	4	15.883500	*

REFERENCES

Anderson J.W., Bay S.A. and Thompson B.E. (1989). Characteristics and effects of contaminated sediments from Southern California. In: Oceans '89. Vol. **2**. Ocean Pollution, Marine Toxicology Society, Ocean Engineering Society of the IEEE , Seattle: 449-451

Andersson T. and Förlin L. (1992). Regulation of cytochrome P-450 enzyme system in fish. Aquat. Toxicol. **24**, 1-20.

Andersson T., Förlin L., Hardig J. and Larsson A. (1988). Physiological disturbances in fish living in coastal waters polluted with bleached pulp mill effluents. Can. J. Fish Aquat. Sci. **45**, 1525-1536.

Arcos, J.S., Conney A.H. and Buu-Hoi N.P. (1961). Induction of microsomal enzyme synthesis by polycyclic aromatic hydrocarbons of different molecular sizes. J. Biol. Chem. **236**, 1291-1296.

Alescio and Mascona, (1969). Immunological evidence for enzyme synthesis in the hormonal induction of glutamine synthetase in embryonic retina in culture. Biochem Biophys. Res. Commun. **43**, 176-182.

Altschul A.M. (1962). Seed Protein and world protein problems ; Economic Botany. Vol. **16**,2.

Ambike S.H., Baxter R.M., Zahid N.D. (1970). Phytochem. **9**, 1953

Axelrod J. (1983). The discovery of the microsomal drug-metabolizing enzymes. In: Drug-metabolism and distribution. Lamble J.W. (Ed).Current Rev. Biomed. **3**, 1-6. Elsevier Biomedical Press. Amsterdam.

Bailey G.S., Hendricks J.D., Nixon J.E. and Pawloski N.E. (1984). The Sensitivity of rainbow trout and other fishes to carcinogens. Drug Metab. Res. **15**, 725-750.

Bayer J. and Goksöyr A. (1993). Cytochrome P-450 observations in Gulf Fish. Mar. Pollut. Bull. **27**, 293-296.

Bagenal T.B. and Tecsh F.W. (1978). Methods for assessment of fish production in freshwater. Age and growth. Blackwell Scientific Publications, Oxford.

Bend J.R. and James M.O. (1978). Xenobiotic metabolism in marine and freshwater species: In: Biochemical and Biophysical Perspectives in Marine Biology. Vol **4**. 125-188. (D.C. Malins and J.A. Sargent Ed). Academic Press. New York.

Bend J.R., Ball L.M., Elmamlouk T.H., James M.O. and Philpot R.M. (1979). Microsomal mixed-function Oxidation of untreated and polycyclic aromatic hydrocarbon treated fish. In: Pesticides and Xenobiotic metabolism in aquatic organisms. (M.A.Q Khan, J.J Lech and J.J.Menn Ed), ACS symposium Series, American Chemical Society. Washington, DC. Page 297.

Biney C. (1991). A baseline study of trace metals in marine organisms from Ghana. In: 'Coastal Zone 1991', ASCE, Longbeach, California. 1155-1167.

Biney C. (1992). Pollution and Environmental degradation relation to inland fisheries and aquaculture. African Fisheries and the Environ. (in press).

Biney C. and Berko C.A. (1991). Trace metal concentrations in fish and sediments from the Wiwi: A small urban river in Kumasi, Ghana. Tropical Ecology. **32** (2), 197-206.

Bleecker W., Capdevila J., Agosin M. (1973). Sequential solubilization of microsomal mixed-function oxidases. J. Biol. Chem.**248**, 8474-8481.

Boobis A.R., Caldwell J., DeMatteis F. and Elcombe C.R. (Ed), (1985). Microsomes and drug oxidation. Page 144. Taylor and Francis, London.

Boon J.P., Everaats J.M., Hillebrand M.T.J., Egaas M.L., Pijnenburg J. and Goksöyr A. (1992). Changes in the levels of hepatic biotransformation enzymes and haemoglobin levels in female plaice (Pleuronectes platessa) after oral administration of technical polychlorinated biphyl mixture (Clophen A40). Sci. Tot. Environ. **114**, 113-133.

Brodie B.B., Axelrod J., Cooper J.R., Gandette L., La Du B.N., Mitoma C. and Underfund S. (1955). Detoxification of drugs and other foreign compounds by liver microsomes. Science. **121**, 603-604.

Buhler D.R. and Rasmusson M.E. (1968). The Oxidation of drugs by fishes. Comp. Biochem. Physiol. **25**, 223-239.

Burke M.D. and Mayer R.T. (1974). Ethoxyresorufin: Direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-MC. Drug Metab. Disp. **2 (6)**, 583-588.

Capdevila J., Saeki Y., Prough R.A. and Estabrook R.W. (1974). Carcinogenesis: Fundamental mechanisms and Environmental effects. In: B.Pullman, P.O.P T'so and H.V. Gilbion ed. Page 113. Reidel, Dordredt. Holland.

Cardini G. and Jurtshuk P. (1968). Cytochrome P-150 involvement in the oxidation of n-octane by cell-free extracts of Corynebacterium sp. strain 7E1C. J. Biol. Chem. **243**, 6070.

Chambers J.E. and Yarbrough D.J. (1976). Xenobiotic biotransformation systems in fish. Comp. Biochem. Physiol. **55c**, 77-84.

Collier T.K., Krahn M.M., Krone C.A., Johnson L.L., Myers M.S., Cha S-L, Varanasi U. (1993). Oil exposure and effects in subtidal fish following the *EXXON Valdez* oil spill. Proceedings of 1993 International oil spill conference on Prevention, Preparedness and Response ; Tampa, Florida.

Conney, A.H. (1967). Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. **19**, 317-366.

Dawe C.J. (1990). Implications of aquatic animals health for human health. Environmental Health Persp. **86**, 245-255.

Davies E., Dumont J.E. and Vassart G. (1978). Improved techniques for the isolation of intact thyroglobulin-synthesizing polysomes. Anal. Biochem. **80**, 289-297.

Dunn M.A., Blalock L.T. and Cousins R.J. (1987). Metallothionein. Proc. Soc. Exp. Biol. Med. **185**, 107-119.

Durnam D.M. and Palmiter R.D. (1981). Transcrptional regulation of the mouse metallothionein-1 gene by heavy metals. J. Biol. Chem. **256**, 5712-5716.

EIFAC. (1975). Report on fish toxicity testing procedures prepared by the European Inland Fisheries Advisory Commission (EIFAC). Working Party on Toxicity testing procedures. EIFAC Technical paper **24**.

Ernster L. and Orrenius K. (1965). Substrate induced synthesis of the hydroxylating enzyme system of liver microsomes. Fed. Proc. **24**, 190-199.

Estabrook R.W., Cooper D.Y. and Rosenthal O. (1963). The light reversible carbon monoxide inhibition of the steroid C21-hydroxylase system of adrenal cortex. J. Biochem. **338**, 741-755.

Estabrook R.W., Woerringloer J.., Masters B.S.S., Johen H., Matsubara T., Ebel R., O'Keefe D. and Petersson J.A. (1976). In: Structural basis of membrane function. Y. Hatefi, L. Djavadi, Ohaniance (ed). Page 429-445. New York.

Fahl W.E., Jefcoate C.R. and Kasper C.B. (1978). J. Biochem. **253**, 3106.

Fang W.F. and Strobel H.W. (1978). The drug and carcinogen metabolism system of rat colon microsomes. Arch. Biochem. Biophys. **186**, 128.

FAO. (1989). Yearbook of Fisheries Statistics. Vol. **68**. 6-12.

Ferris J.P., MacDonald L.H., Patrie M.A. and Martin M.A. (1976). Aryl hydroxylation activity in the fungus Cunnhamella bainieri: Evidence for the presence of cytochrome P-450. Arch. Biochem. Biophys. **175**, 443.

Förlin L. (1980). Effects of Clophen A50, 3-Metylcholanthrene, Pregnenolone-16 -carbonitrile and phenobarbital on the hepatic microsomal cytochrome P-450-dependent monooxygenase in rainbow trout (Salmo gairdneri) of different age and sex. Toxicol. Appl. Pharmacol. **54**, 420-430.

Förlin L., Andersson T., Bengtsson B.E., Hardig T. and Larsson A.(1985). Effects of Pulp Bleached Plant Effluents on hepatic xenobiotic biotransformation enzymes in fish: Laboratory and Field Studies. Mar. environ. Res. **17**, 109-112.

Förlin L., Goksöyr A. and Husoy A.M. (1994). Cytochrome P-450 monooxygenase as indicator of PCB, dioxin-like compounds in fish. In: Biological monitoring of Estuarine and Coastal waters. (K. Kramer, ed), Boca Raton, Florida, CRC Press (in press).

Gadagbui K. M.B. (1993). Optimization of pHs of EROD assay buffers for different fresh water fish species. Personal communication.

Gibson G.G. and Schenckman J.B. (1978). Purification and properties of cytochrome P-450 obtained from liver microsomes of untreated rats by lauric acid affinity chromatography. J. Biol. Chem. **253**, 5957.

Gillette J.R. (1966). Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. Adv. Pharmacol. **4**, 219-261.

Gillette J.R. (1971). Metabolism. **20**, 215.

Goksöyr A. (1985). Purification of hepatic microsomal cytochrome P-450 from ß-NF-treated Atlantic cod (Gadus morhua), a marine teleost fish. Biochim. Biophys. Acta. **840**, 409-417.

Goksöyr A., Andersson T., Buhler D.R., Stegeman J.J., William D.E., Zhang Y. and Förlin L. (1986). An immunological comparison of microsomal ß-NF inducible cytochrome P-450 isozymes in different fish species. In: Proceedings Toxic chemicals and aquatic life symposium; Seattle, Sept. 17-19 (abstract).

Goksöyr A., Andersson T., Buhler D.R., Stegeman J.J., Williams D.E. and Förlin L. (1991b). Immunological cross reactivity of ß-naphthoflavone-inducible cytochrome P-450 (P-4501A) in liver microsomes from different species and rats. Fish Physiol. Biochem. **9**, 1-13.

Goksöyr A., Larsen H.E. and Husoy A.M. (1991a). Application of a cytochrome P-4501A1 ELISA in environmental monitoring and ecotoxicity testing in fish. Comp. Physiol. Biochem. (in press).

Goksöyr A. and Förlin L. (1992). The cytochrome P-450 system in fish; aquatic toxicology and environmental monitoring. Aquatic Toxicol. **22**, 287-312.

Goksöyr A. and Solberg T.S (1987). Cytochrome P-450 in fish larvae: Immunological detection of responses to pollution. Sarsia. **72**, 23-29.

Gooch J.W., Elskus A.A., Kloepper-Sams P.J., Haln M.E. and Stegeman J.J. (1989). Effects of ortho and non-ortho polychlorinated biphenyl congeners in hepatic monooxygenase system in scup. Toxicol. Appl. Pharmacol. **98**. 422-433.

Gooch J.W. and Matsumura F. (1983). Characteristics of the hepatic microsomal monooxygenase system of the dogfish (Carssius auratus) and its induction with ß-NF. Toxicol. Appl. Pharmacol. **68**, 380-391.

Hamilton S.J. and Mehrle P.M. (1986). Metallothioneins in fish; a review of its importance in assessing stress from metal contaminants. Trans. Am. Fish Soc. **115**, 596-605.

Hart L.G. and Fouts J.R. (1965a). Further studies on the stimulation of hepatic microsomal drug metabolizing enzymes by DDT and its analogs. Arch. Exp. Pathol. Pharmacol. **249**, 486-500.

Hashimoto Y., Yamano T. and Mason H.S. (1962). An electron spin resonance study of microsomal electron transport. J. Biol. Chem. **237**, PC3843-PC3845.

Haugen, D.A., Nebert D.W. and Coon M.J. (1975). Induction of multiple forms of mouse liver cytochrome P-450: Evidence for genetically controlled de novo protein synthesis in response to treatment with and PB. J. Biol. Chem. **251**, 1817-1827.

Hayaishi O. (1964). Oxygenases. In: International Congress on Biochemistry. 6th New York Proceedings of the Planery sessions and the program. Vol. **33**, 31-43. Washington DC.

Heath G.W. and Moore M.N. (1985). Responses of Marine organisms to pollutants In: Marine Environ. Res. **17**, 2-4

Hodson P.V. (1986). Water quality criteria and the need for biochemical monitoring of contaminants effect on aquatic ecosystem. In: Water quality management. Fresh Water ecotoxicity in Australia. Hart B.T (ed). Water studies Center. Chrisholm Institute of Technology; Melbourne, Australia. 7-21

Husöy A.M., Willis M.J., Myers M.S., Collier T.K. and Goksöyr A. (1993). Immunological localization of cytochrome P-4501A1 in different organs of Atlantic cod (Gadus morhua). In: The science of total environment, supplement 1993; Elsevier science Publishers. B.V Amsterdam. PP 671-680.

ICES. (1978). Working group on pollution baseline and monitoring studies in the Oslo commission and ICNAF areas. Report of the subgroup on the feasibility of effects monitoring. Cooperative Research report **75**. International commission for the exploration of the Sea (ICES). Charlottenlund. Denmark.

Imai Y. and Sato R. (1966). Substrate interaction with hydroxylase system in liver microsomes. Biochim. Biophys. Res. Commun. **22**. 620-626.

Jensen E.G., Skaare J.U., Egaas E. and Goksöyr A. (1991). Responses of xenobiotic metabolizing enzymes in rainbow trout (Oncorhynchus mykiss) to endosulfan, detected by enzyme activities and immunological methods. Aquatic Toxicol. **21**, 81-92.

Kawaguchi K., Ishidate K. and Tagawa K. (1973). Non-mitochondrial electron transport system in anaerobically grown yeast cells. J. Biochem. **74**, 817.

Kinoshito, F.K., Franley J.P. and Dubois K.P. (1966). Quantitative measurement of induction of hepatic microsomal enzymes by various dietary levels of DDT and toxaphene in rats. Toxicol. Appl. Pharmacol. **9**, 505-513.

Klotz A.V., Stegeman J.J., Woodin B., Snowberger E., Thomas P. and Walsh C. (1986). Cytochrome P-450 isozymes from marine teleost Stenotomus chrysops: Their roles in steroids hydroxylation and the influence of cytochrome b_5. Arch. Biochem. Biophys.**249**, 326-336.

Klotz A.V., Stegeman J.J. and Walsh C. (1983). An aryl hydrocarbon hydroxylating hepatic cytochrome P-450 from a marine fish Stenotomus chrysops. Arch. Biochem. Biophys. **226**, 578-592.

Klotz A.V., Stegeman J.J. and Walsh C. (1984). An alternative 7-ethoxyresorufin-O-deethylase activity assay : A continuous visible spectrophotometric method for the measurement of cytochrome P-450 monooxygenase activity. Anal. Biochem. **140**. 138-145.

Koivusaari U., Harri M. and Hanninen O. (1981). Seasonal variation of hepatic biotransformation in female and male rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. **70**, 149-157.

Kozlov I.A. and Mikel'saar H.N. (1974). On the subunit structure of soluble mitochondrial ATPase. FEBS Letts. **43**, 212-214.

Krahn M.M., Kittle Jr. L.J., and MacLeod Jr. W.D. (1986a). Evidence for exposure of fish to oil spilled into the Columbia river. Mar. Environ. Res. **20**, 291-298

Krahn M.M., Moore K.L. and MacLeod Jr. W.D. (1986b). Standard Analytical proceedings of the NOAA National Analytical Facility, 1986. Metabolites of Aromatic compounds in fish bile. Technical memorandum NMFS/F/NWC-102. Springfield, Vancouver: 25

Krahn M.M., Burrows D.G., Ylitalo G.M., Wigren C.A., Collier T.K., Chan S-L and Varanasi U. (1992). Mass Spectrometric determination of metabolites of aromatic compounds in the bile of fish captured from Prince Williams Sound, Alaska after the "EXXON Valdez oil spill. Environ. Sci. Technol. **v26**, 116-126.

Kumaki K., Sato M., Kon H. and Nebert D.W. (1978). Correlation of Type I, II and reverse type I difference spectra with absolute changes in spin state of hepatic microsomal cytochrome P-450 iron form five mammalian species. J. Biol. Chem. **253**, 1048.

Kupfer D. (1980). Endogenous substrates of monooxygenases. Fatty acids and prostaglandins. Pharmac. Ther. **11**, 469-496.

Laemmli U.K. (1970). Cleavage of structural Proteins during he assembly of the Head of Bacteriophage T4. Nature. **227**, 680-685.

Larsen H.E., Celander M. and Goksöyr A. (1992). The cytochrome P-450 system in Atlantic salmon (Salmo solar). II. Variations in hepatic catalytic activities and isozyme pattern during an annual reproductive cycle. Fish Physiol. Biochem. **10**, 291-301.

Lee R.F. (1981). Mixed-function Oxygenase in marine invertebrates. Marine Biol. Letts. **2**, 87-105.

Levin W. and Conney A.H. (1967). Stimulatory effects of polycyclic hydrocarbons and aromatic azo derivatives on the metabolism of 7,12-dimethylbenzo(α)anthracene. Cancer Res. **27**, 1931-1938.

Lindström-Seppa P. and Oikari A. (1990). Biotransformation activities of feral fishes in waters receiving bleached pulp mill effluents. Environ. Toxicol. Chem. **9**, 1415-1424.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951). Protein measurements with Folin-phenol reagent. J. Biol. Chem. **193**, 265-275.

Lu A.Y.H., Junk P. and Coon M.J. (1969). Resolution of cytochrome P-450 containing w-hydroxylation system of liver microsomes into three components. J. Biol. Chem. **244**, 3714-3721.

Malins D.C. and Collier T.K. (1981). Xenobiotic interactions in aquatic organisms: Effects on biological systems. Aquat. Toxicol. **1**, 257-289

Malins D.C., McCain B.B., Landahl J.T., Myers M.S., Kahn M.M., Brown D.W., Chan S-L and Roubal W.T. (1988). Neoplastic and other diseases in fish in relation to toxic chemicals. An overview. Aquat. Toxicol. **11**, 43-67.

Mannering G.J. (1968). Significance of stimulation and inhibition of drug metabolism. In: Selected Pharmacological Testing Methods. Edited by Alfred Burger. Page 51-119. Marcel Dekker, New York.

Mason H.S. (1965). Oxidases. Ann. Rev. Biochem. **34**. 595-634.

Mason H.S. (1957a). Mechanisms of oxygen activation. In: Advances in Enzymology. Nord F.F (Ed). Interscience. New York. 79-233.

Mason H.S. (1957b). Mechanisms of oxygen metabolism. Science. **125**, 1185-1188.

Masters S.S.B., Karmin H., Gibson Q.H. and Williams C.H.Jr. (1965). Studies on the mechanism of microsomal triphosphopyridine nucleotide cytochrome c reductase. J. Biol. Chem. **240**, 920-931.

Matsubara T., Baron J., Peterson L.L. and Peterson J.A. (1976). NADPH- cytochrome P-450 reductase. Arch. Biochem. Biophys. **172**, 463-469.

Matsubara T., Prough R.A., Burke M.D. and Estabrook R.W. (1974). The preparation of microsomes from fractions of rodent respiratory tract and their characterization. Cancer Res. **34**, 2196-2203.

Mazel P. (1971). Cytochrome P-450 and drug metabolism. In: Fundamentals of drug metabolism and distribution. La Du B.N. et al, (ed). Page 529, Baltimore: Williams and Wilkins, 1971.

Melancon M.J., Elcombe, C.R., Vodicnik M.J. and Lech J.J. (1981). Induction of cytochrome P-450 and mixed-function Oxidase activity by polychlorinated biphenyls and ß-NF in carp (Cyprinus carpio. Comp. Biochem. Physiol. **69c**, 219-226.

Miranda C.L., Wang J.L., Henderson M.C. and Buhler D.R. (1989). Purification and characterization of hepatic steroid hydroxylases from untreated rainbow trout. Arch. Biochem. Biophys. **268**, 22238.

Mitoma C., Sorich T.J. and Neubauer S.E. (1968). The effects of caffeine on drug metabolism. Life Science. **7**, 145-151.

Molven A. and Goksöyr A. (1992). Ecotoxicity monitoring. Edited by Mervyn Richardson. VCH Publishers. Weinheim. New York. Basel. Cambridge. Tokyo.

Mueller G.C. and Miller J.A. (1949). The reductive cleavage of 4-dimethylaminoazobenzene by rat liver microsomes: The intracellular distribution of the enzyme system and its requirements for triphosphopyridine nucleotide. J. Biol. Chem. **180**, 1125-1136.

Mueller G.C. and Miller J.A. (1953). The metabolism of methylated aminoazo dyes. II. Oxidative demethylation by rat liver homogenates. J. Biol. Chem. **202**, 579-587.

Moore R.W., Welton A.F. and Aust S.B. (1974). Detection of hemoproteins in SDS polyacrylamide. In: Microsomal electron transport and cytochrome P-450. Methods in Enzymology. **52**.

Morgan E.T., Donahue B.S., Tenney K.A. and Furgeson N.L. (1993). Pretranslational induction of CYP4A1 in diabetic rats and reversal by vanadate treatment. FASEB J. (abst.). **7**. 664.

Murchelano R.A. (1990). Fish health and Environmental Health. Environmental Health Persp. **86**, 257-259.

Murchelano R.A. and Wolke C.D. (1985). Fish health and Environmental health ter flounder, Pseudopleuronectes americanus. Science. **228**, 587-589.

Nebert D.W., Adesnik M., Coon M.J., Estabrook R.W., Gonzalez F.J., Guengerich P.F., Gonsalus I.C., Johnson E.F., Kemper B., Levin W., Philip J., Sato R. and Waterman M.R. (1987). The P-450 superfamily: Recommended nomenclature. DNA. **6**, 1-11.

Nebert D.W., Adesnik M., Coon M.J., Estabrook R.W., Gonzalez, F.J., Guengerich P.F., Gonsalus I.C., Johnson E.F., Kemper B., Levin W., Philip J., Sato R. and Waterman M.R. (1987). The P-450 superfamily: Update listing of all genes and recommended nomenclature for the chromosomal loci. DNA. **8**, 1-13.

Nebert D.W., Nelson D.R, Coon M.J, Estabrook R.W., Fayereisen R., Fujii-Kuriyana Y., Gonzalez F.J., Guengerich F.P., Gonsalus I.C., Johnson E.F., Loper J.C., Sato R., Waterman M.R. and Waxman D.J. (1991). The cytochrome P-450 superfamily: Update on new sequences, gene mapping and recommended nomenclature. DNA Cell Biol. **10**, 1-14.

Netter K.J. (1966). N. Schmied. Arch. Pharmakol. **225**, 151-162.

Olafson R.W., Sim R.G. and Boto K.G. (1979). Isolation and chemical characterization of the heavy metal binding protein - metallothioneins from marine vertebrates. Comp. Biochem. Physiol. **62**, 407-416.

Olsson P.E. (1987). Metallothioneins in Fish: Aspects of Biochemistry and function. Ph.D. Thesis; Department of Zoophysiology. University of Gothenburg, Gothenburg, Sweden.

Omura T. and Sato R. (1964a). The CO-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. **239**, 2397.

Omura T. and Sato R. (1964b). The CO-binding pigment of liver microsomes II. Solubilization, purification and properties. J. Biol. Chem. **239**, 2370.

Oshino N. and Omura T. (1973). Immunological evidence for the participation of b_5 in microsomal Stearyl-CoA desaturase reaction. Arch. Biochem. Biophys. **157**, 395-404.

Parke D.V. (1975). Cytochrome P-450 and its functions. In: Enz. Induction. Parke D.V. (Ed). Page 207. New York. Plenum Press.

Payne J.F. and Penrose W.R. (1975). Induction of aryl hydrocarbon (Benzo<[α]pyrene) hydroxylase in fish by petroleum. Bull. Environ. Contam. Toxicol. **14**, 112-116.

Payne J.F., Bauld C., Dey A.C., Kiceniuk J.W. and Williams U. (1984). Selectivity of mixed-function oxygenase enzyme induction in flounder (Pseudopleuronectes americanus) collected at the site of the Baie Verte, Newfoundland oil spill. Comp. Biochem. Physiol. **79c**, 15-29.

Payne J.F., Fancey L.L., Rahimtula A.D. and Porter E.L. (1987). Review and perspective on the use of mixed-function oxidase enzymes in biological monitoring. Comp. Biochem. Physiol. **86c**, 233-245.

Porter T.D. and Coon M.J. (1991). Cytochrome P-450 : Multiplicity of isoforms, substrates and catalytic regulatory mechanisms. J. Biol. Chem. **266**, 13469-13472.

Posner H.S., Graves A., King C.T.G. and Wilk A. (1961). Experimental alteration of the metabolism of chlorcyclizine or the incidences of cleft palate in rats. J. Pharmacol. Exp. Ther. **155**, 494-505.

Prough R.A., Burke M.D. and Mayer R.T.(1978). Direct Fluorimetric methods for measuring mixed-function oxygenase activity. Methods. Enzymol. **52**, 37-77.

Powers D.A. (1989). Fish as model systems. Science. **246**, 352-358.

Pullman M.E. and Monroy G.C. (1963). A naturally occurring inhibitor of mitochondrial adenosine triphosphatase. J. Biol. Chem. **238**, 3762-3769.

Remmer H. and Merker H.J. (1965). Effects of drugs on the formation of smooth endoplasmic reticulum and drug-metabolizing enzymes. Ann. N.Y. Acad. Sci. **123**, 79-97.

Radomski J.L. (1961). The absorption, fate and excretion of citrus Red No.2 (2,5-dimethylphenyl-azo-2-naphthol) and external D and C No.14 (1-xylylazo-2-naphthol). J. Pharmacol. Exp. Ther. **134**, 100-109.

Saad M.A.H., Amuzu A.T., Biney C., Calamari D., Imevbore A.M., Naeve H.F. and Ochumba P.B. (1990). Scientific basis for pollution control in African inland waters. Domestic and industrial organic loads. FAO Fisheries Rep. **437**, 6-24.

Sato R., Omura T. and Nishibayashi H. (1965). Co-binding hemoprotein and NADPH-specific flavoprotein in liver microsomes and their roles in microsomal electron transfer. In: Oxidases and related redox systems. C.T. King, H.S. Mason and M. Morrison Ed). New York, Wiley. Page 861.

Schenkman J.B. and Cinti D.L. (1972). Preparation of microsomes with calcium. Life Science. **11**, 247.

Sikka H.C., Rutkowski J.P. and Kandaswami C. (1990). Comparative metabolism of Benzo(α) pyrene by liver microsomes from brown bullhead and carp. Aquatic Toxicol. **16**, 101-112.

Skaare J.U., Gram-Jensen E., Goksöyr A. and Egaas E. (1991). Responses of xenobiotic-metabolizing enzymes in rainbow trout to the mono-ortho substituted polychlorinated PCB congener 2,3,4,4',5-pentachlorophenyl (PCB 118) detected by enzyme activity and immunochemical methods. Arch. Environ. Contam. Toxicol. **20**, 349-352.

Sladek N.F. and Mannering G.J. (1966). Evidence for new P-450 hemoprotein in hepatic microsomes from methylcholanthrene treated rats. Biochem. Biophys. Res. Commun. **24**, 668-674.

Smith J.N. (1968). The comparative metabolism of xenobiotics. In: Advances in comparative Physiology and Biochemistry, edited by O. Lowenstein, **Vol. 3**, Page 173-231. Academic Press. New York.

Snegaroff J. and Bach J. (1990). The effects of temperature on the activity of cytochrome P-450 in rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. **95B**, 515-519.

Södergren A. (1989). Biological effects of bleached mill pulp effluents. National Swedish Environmental Protection Board. Report 3558, Stockholm. Page 139.

Stebbing A.R. (1989). Monitoring the North Sea: Biological Techniques in an integrated Strategy. In: ten Hallers C.C., Bijsma A. (ed). Distress Signals, Proc. 3rd North Sea Seminar 1989 Werkgroep Noordzee, Amsterdam: 61-68.

Stegeman J.J. (1981). Polynuclear aromatic hydrocarbons and their metabolism in marine environment. In: Polycyclic hydrocarbons and Cancer. Gelboin H.V. and Ts'O (Ed). Academic Press. New York. Vol. **3**, 1-60.

Stegeman J.J. and James M.O. Individual variations in patterns of Benzo(α)pyrene metabolism in the marine fish, Scup. Marin. Environ. Res. (in Press).

Stegeman J.J. and Lech J.J.(1991). Cytochrome P-450 monooxygenase system in aquatic species: Carcinogen metabolism and biomarkers for carcinogens and pollutants exposure. Environ. Health Persp. **90**, 101-109.

Stegeman J.J., Kloepper-Sams P.J. and Farrington J.W. (1986). Monooxygenase induction and chlorobiphenyls in the deep sea fish, Coryphaenoides armatus. Science. **231**, 1287-1289.

Stegeman J.J. and Kloepper-Sams P.J. (1987). Cytochrome P-450 isozymes and monooxygenase activity in aquatic animals. Environ. Health Persp. **17**, 87-95.

Stegeman J.J and Wooden B.R. (1980). Patterns of benzo(α)pyrene metabolism in liver of the marine fish Stenotomus versicolor. Fed. Proc. **39**, 1752.

Strobel H.W., Lu A.Y., Heidman D. and Coon M. (1970). Phosphatidylcholine requirement in the enzymatic reduction of hemoprotein P-450 and in fatty acid, hydrocarbon and drug hydroxylation. J. Biol. Chem. **245**, 4851-4854.

Thomas P.E., Lu A.H.Y., Ryan D., West S.B., Kawalek J. and Levin W. (1976). Multiple forms of rat liver cytochrome P-450: Immunological evidence with antibodies against cytochrome P-488. J.Biol. Chem. **251**, 1385-1391.

Ueng T.H., Ueng Y.F. and Park S.S. (1992). Comparative induction of cytochrome P-450-dependent monooxygenase in the liver of tilapia and carp. Aquat. Toxicol. **23**, 49-64.

van der Weiden M.E.J., Celander M., Seinen W., van der Berg M., Goksöyr A. and Förlin L. (1992). The use of immunological determinations of P-4501A1 and EROD activity assay in rainbow trout and carp treated with 2,3,7,8-TCDD. Mar. Environ. Res. **34**, 215-219.

Varanasi U., Stein J.E., Nishimoto M., Reichert W.L. and Collier T.K. (1987). Chemical carcinogenesis in feral fish: Uptake, activation and detoxification of organic xenobiotics. Environ. Health Persp. **71**, 155-170.

Vodicnik M.J. and Lech J.J.(1983). Effects if sex steroids and pregnenolone-16 -carbonitrile on the hepatic microsomal monooxygenase activity of rainbow trout (Salmo gairdneri). J. Steroid Biochem. **18**, 323-328.

Vodicnik M.J., Elcombe C.R. and Lech J.J. (1981). The effects of various types of inducing agents on hepatic microsomal monooxygenase activity in rainbow trout. Toxicol. Pharmacol. **59**, 364-374.

Watanuki M., Tilley B.F. and Hall P.F. (1978). Cytochrome P-450 for 11ß- and 18-hydroxylase activity of bovine adrenocortical mitochondria: One enzyme or two. Biochem. **17**, 1227.

Waterman M.R.E., Malivakal E.J. and Simpson E.R. (1986). Regulation of synthesis and activity of cytochrome P-450 enzymes in physiological pathways. In: cytochrome P-450, Structure, Mechanism and Biochemistry. (Ortiz de Montellano P. R. Ed.) Plenum Press. New York and London. Pages 345-385.

Welch R.M., Harrison Y.E., Conney A.H. and Burns J.J. (1969). An experimental model in dogs for studying interactions of drugs with bishydroxycoumarin. Clinical Pharmacol. Ther. **10** (6), 817-825.

Welton A.F. and Aust S.D. (1974). Multiplicity of cytochrome P-450 hemoproteins in rat liver microsomes. Biochem. Biophys. Res. Commun. **56**, 898.

WHO/OCP (1985). Ten years of Onchocerciasis control in West Africa. OCP/GVA/85. 18. Geneva.

William D. and Buhler D. (1983a). Comparative properties of purified cytochrome P-488 from ß-NF-treated rats and rainbow trout. Comp. Biochem. Physiol. **75c**, 25-32.

William C.H. Jr. and Kamin H. (1962). Microsomal triphosphopyridine nucleotide cytochrome c reductase of liver. J. Biol Chem. **237**, 587-595.

Yang C.S. and Strickhart F.S. (1975). Interactions between solubilized cytochrome P-450 and hepatic microsomes. J. Biol. Chem. **250**, 7968-7972.

Yasukochi Y. and Masters B.S.S. (1976). Some properties of a detergent-solubilized NADPH-cytochrome c (P-450) reductase by biospecific affinity chromatography. J. Biol. Chem. **251**, 5337.