School of Chemical and Biological Sciences
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Metabolic Enzymes and Mixed-Function Oxygenase (MFO) system in pink snapper *(Pagrus auratus)*: Biochemical and Histological Relationships

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Declaration

The author declares that:

I. Except where due acknowledgment has been made, the thesis comprises original work by the author;

II. The work has not been submitted previously, in whole or in part, to qualify for any other academic award; and

III. The content of the thesis is the result of work which has been carried out since the official commencement date of the research program

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May god hopefully bless us.....amen.

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Abstract

The environmental health of aquatic ecosystems depends amongst others, on the chemical pollution coming from activities in the catchment's area. In the Swan River Estuary, Western Australia, the chemical pollutants of concern released into the river are petroleum hydrocarbons and sodium pentachlorophenate (NaPCP). Decreased water quality causes a loss of biotic diversity especially amongst fish populations. The health of aquatic ecosystems can be monitored by fish health, especially fish located at higher levels in the food chain. Pink snapper (*Pagrus auratus*), an endemic Western Australian fish species, was tested for its potential as a bioindicator of aquatic environmental health.

This thesis presents data on the responsiveness of pink snapper to the contaminants of concern, using biomarkers such as serum sorbitol dehydrogenase (SDH), mixed function oxygenase (MFO), metabolic enzymes such as citrate synthase (CS), cytochrome C oxidase (CCO) and lactate dehydrogenase (LDH) and the histological alteration such as hepatic cell lesions (hyperplasia and hypertrophy), and glycogen and lipid droplets. The metabolic enzymes CCO and LDH as well as the hepatic MFO induction and histopathology were proven to be the most suitable biomarkers for use for routine monitoring of the Swan River Estuary using pink snapper as a bioindicator. However, CS activity and hepatic cell lesions (hyperplasia and hypertrophy) did not respond to exposure to contamination and are therefore not suited as biomarkers of effects in pink snapper.

The first phase of the study aimed at investigating the responsiveness of juvenile pink snapper to an MFO inducer. Polychlorinated biphenyl isomer # 126 was selected as a model MFO inducer for this study. In the initial experiment, MFO activity was measured as a biomarker of exposure, and serum SDH activity was assessed as a biomarker of liver damage. MFO and SDH activities were of special interest as these biochemical tools have not previously been validated for any Western Australia fish species. Juvenile pink snapper were injected intraperitoneally (i.p.) with 0, 10, 100, 500, 1000 μg PCB-126 per kilogram. Fish were sacrificed 10 days postinjection, and liver and blood were collected for MFO and SDH analysis, respectively. Doses of 10 and 100 μg PCB-126 per kilogram caused the highest MFO induction, while doses of 0 and 1000 μg PCB-126 per kilogram did not result in higher MFO activity relative to carrier-injected (peanut oil) control fish. SDH activities were not significantly different among treatments indicating that hepatocellular damage was not responsible for the reduced MFO activity at the highest dose.

Metabolic enzymes in pink snapper exposed by NaPCP were studied in the second phase of the experiment. The aim of this second experiment was to test the responsiveness of pink snapper to contaminants known to cause metabolic perturbations in vertebrates. Juvenile pink snapper were intraperitoneally (i.p.) injected with 0, 5, 10, 20 mg per kilogram. Oxidative enzymes were assessed by measuring CS and CCO activities and glycolytic enzyme was assessed by measuring LDH activity in liver and white muscle tissues. CS activity remained unchanged in both the white muscle and in the liver. CCO activity was significantly enhanced in liver in all treated fish relative to control fish, but not in the white muscle. LDH

activity was also higher in liver in all treated fish as compared to control fish, while in white muscle, LDH activity significantly increased at the highest dose injected.

The use of a suite of biochemical markers is useful in determining the effects of xenobiotic exposure of aquatic organisms, because it provides a holistic approach with biomarkers at different levels of biological organization. For the third and final phase of the study the suite of biomarkers selected were MFO, metabolic enzyme (CS, CCO and LDH) activities, and histological alternations in combination with physiological indices. The aim of this last experiment was to investigate if a modified liver metabolic activity would alter the MFO induction potential. To test if altered liver metabolism would influence liver detoxication capacities, juvenile pink snapper were i.p. injected with peanut oil (control), or pentachlorobiphenyl # 126 (PCB 126), with sodium pentachlorophenate (NaPCP), or combination of PCB 126+NaPCP. Relative to controls, ethoxyresorufin-*O*-deethylase (EROD) activity was induced in the PCB 126 and PCB 126+NaPCP fish, but not in the NaPCP group. In the liver, CCO activity was enhanced by the treatments while CS activity remained unchanged and LDH activity was increased in the NaPCP treatment only. In the white muscle, only the PCB 126+ NaPCP treatment enhanced CCO activity, with all other enzymatic activities remaining unchanged. Low serum sorbitol dehydrogenase (sSDH) activity and histopathology of the liver indicated no significant alteration of cellular structure, albeit the lipid droplet size was increased in the PCB 126 and in the PCB 126+NaPCP treatments. It is concluded that the hepatic metabolic changes correspond to histopathological observations, but an altered metabolic capacity does not influence the metabolism of xenobiotics by liver enzymes, as measured by EROD activity.

These experiments answered the need to identify a suitable fish species for routine monitoring of the aquatic environment in Western Australia. It also identified the most suitable biochemical markers of exposure and effects, and the suitability of the pink snapper as a bioindicator. Finally, the experiments investigated interactions between biomarkers and provided new knowledge useful to scientists using MFO and/or metabolic enzymes in field or laboratory toxicology.

Thesis Organization

The thesis is divided into five chapters and two appendices. Chapter 1 is a general introduction. The general introduction provides information on biomarker classification, validation, advantages and limitation. This chapter then proceeds with a literature review regarding the most common biomarkers of exposure of native fish to environmental pollution. Chapters 2 to 4 are research papers. These two former are duplications of journal publications. The research described in Chapter 2 and 3 represent initial investigations loading to the main experiment described in Chapter 4. Hence, a certain amount of repetition between the respective introductions in each chapter was unavoidable. Figure and table legends, and the format of reference lists have been altered in all chapters in order to preserve consistency and continuity throughout the thesis. Chapter 5 is a general discussion that provides an overview of the research findings. Finally, a general conclusion reviews the main finding of the 3.5-year research project.

Appendix 1 presents a conference poster and journal publication, and Appendix 2 presents a photograph of histological sections observed by light microscope.

Chapter 1

General Introduction

1. 1. History of Ecotoxicological Science

1.1. 1. Definition of Ecotoxicology

Ecotoxicology was derived from the words *ecology* and *toxicology.* This term was introduced by Truhaut in 1969 who defined it as "the study of the harmful effects of chemicals upon ecosystems". Ecotoxicology deals with movements of pollutants in air, water, soil and sediments, and through food chains, with chemical transformation and biotransformation. However, pure toxicology regards the uptake, distribution, metabolism and excretion of xenobiotics in living organisms (Walker *et al.,* 1996).

Ecotoxicology focuses on the effects of toxic substances not only at the organism and population level but also at the ecosystem level (Stine and Brown, 1996; Jørgensen, 1997). In fact, ecotoxicology is a multidisciplinary science regarding the adverse effects of toxic agents on living systems such as insects, molluscs, amphibians, fish, and birds. Ecotoxicological science involves the fields of chemistry, ecology and toxicology, and is categorised as a new discipline (Richardson,1993). Typical test organisms may include algae, Daphnia, shrimp, honeybees, quail, trout, and fathead minnows (Shugart, 1996; Stine and Brown, 1996).

The concept of ecotoxicology involves the distribution of substances in the environment together with their fate. It is focussed on the effects on populations rather than on individuals (Richardson, 1993, Solbe *et al.,* 1998). This science

provides important information for legislative and regulatory processes regarding the assessment of the likely impact of new and existing chemicals on the environment (Solbe *et al.*, 1998).

1.1.2. Development of Ecotoxicology

During the 1950's and early 1960's, technology and science evolved very rapidly in all nations, including developing countries. The unexpected negative effects of products, and by products of this technological and scientific boom on the environment have become increasingly evident and of concern. At the same time as industrialisation was booming, the effects of diffuse pollution from agriculture were becoming evident. Prior to the 1970's it had been believed that agriculture was a more environment friendly activity when compared to industry. However, with the adverse impacts of the indiscriminate use of pesticides and fertilisers becoming obvious, this image has radically changed (Jørgensen, 1997).

In the second half of the 1960's people began to be concerned with problems such as the reduction of birds of prey populations, especially eagles, because of the biomagnification of dichlorodiphenyltrichloroethane (DDT) and other pesticides via the food chain, unexpected residues of polychlorinated biphenyls (PCBs) in seals, and the effects of air pollutants on human health. Therefore ecotoxicological research started in the 1960's in an effort to elucidate the effects of the presence of toxic substances (pollutants) in the environment. The global pollution problem has become a serious issue since the beginning of the 1980's; of special concern are atmospheric

pollution problems, the green house effect and the reduction of the protective ozone layer (Bickham *et al.*, 2000).

The goal of ecotoxicology is to determine processes of toxicity of all chemicals of interest. There are approximately 100,000 compounds released into the environment in quantities that could threaten the environment. Practices reducing the impacts of agricultural pollutants involved the use of buffer zones between the natural ecosystem and agriculture, development of a new generation of pesticides, a wider use of the biological methods for the control of weeds and herbivorous insects, and the development of strains which do not require the use of pesticides and fertilizers. Ecological engineering, cleaner technology and global concern have been considered in environmental management methods (Jørgensen, 1997; Bickham *et al.,* 2000).

The development of ecotoxicology has shifted from the measurement of acute, lethal effects of chemicals to the assessment of sublethal and chronic effects (Anonymous, 2001). Acute toxicity is toxicity which arises soon after exposure and unless death occurs, recovery is complete (Aldridge, 1988), or the adverse effects occurring within a short time of (oral) administration of a single dose of a substance or multiple doses given within 24 hours (Chan and Hayes, 1989). While chronic toxicity requires prolong or repeated administration of the substance before the toxicity becomes apparent (Aldridge, 1988). The exposure period in chronic studies may vary, depending on the objective of study, the species selected for the study, and the route of administration employed. A generalization which is often made is that chronic

studies do not exceed 10 % of the animal's lifespan (Mosberg and Hayes, 1989; Stevens and Gallo, 1989).

In the past three decades, the scientific community and regulatory agencies have become more concerned about the long-term impacts of environmental stressors on human and environmental health. This shift has occurred in order to understand the long-term toxic effects of various classes of chemicals on individuals and at population level, under natural conditions (Anonymous, 2001). Short term testing cannot be expected to provide detailed information about the action of a chemical in an ecological system or the overall biosphere. Acute testing can, however, provide individual data about the behaviour of selected classes of chemicals, in order to grade of these chemicals into a priority list for extended studies (Korte *et al.,* 1978).

1.2. Environmental Pollutants

1.2.1. Definition of Pollutant

Sometimes the term "pollutant" can differ from the term "contaminant". The term pollutant indicates the chemical that is causing actual environmental harm, whereas the term contaminant indicates the chemical that may not be harmful (Walker *et al.*, 1996). A substance can be defined as a pollutant if its introduction into the environment produces an unwanted or degrading effect (Hughes, 1993). Pollution of the environment is due to the release of substances into any media (water, air, and land) from any process, and capable of causing harm to any living organisms, including man (Hughes, 1993). In the context of ecotoxicology, the term "pollutant" is therefore more appropriate.

Pollution of aquatic ecosystems from both point and non-point sources vary in magnitude, frequency, duration and type, depending on meteorological and hydrological conditions, terrestrial and aquatic system processes and anthropogenic activities. The toxicity of some contaminants is generated by the potency of the substance, the duration of contact with the receptor, and, the concentration at the receptor site (Burton, 1999).

1.2.2. Classification of Environmental Pollutants

The pollutants can be divided into inorganic ions, organic material, organometallic compounds, radioactive isotopes, and gaseous pollution. Some common organic pollutants are hydrocarbons, polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated biphenyls (PBBs), organochlorine insecticides, organophosphorus insecticides, carbamate insecticides, pyrethroid insecticides, phenoxy herbicides, anticoagulant rodenticides, detergents, and chlorophenols (Walker *et al.,* 1996).

Besides its classification based purely on its chemical nature, a pollutant can be divided according to its matrices, being either atmospheric, aquatic or terrestrial (Hughes, 1993). In addition, aquatic pollutants can be further divided into aqueous, particulate or accommodated forms. Alternatively, a pollutant can be classified according its source, namely natural pollutant or man-made substance (xenobiotic). Natural pollutants comprise a wide range of chemicals involving plant products, animal toxins and natural hydrocarbons, whereas the production of man-made substances increases daily in variety and quantity (Livingstone, 1998).

1.2.2.1. PCB Production

Of concern are the industrial chemical compounds of the polyhalogenated aromatic family including polychlorinated biphenyls (PCBs), polychlorinated naphthalenes (PCNs), polychlorinated phenols, polychlorinated terphenyls (PCTs), polybrominated biphenyls (PBBs), and chlorinated phenols, anilines, and benzenes (Walker *et al.*, 1996). In the past, PCBs were widely used as heat transfer fluids, organic dilution agents, plasticizers, lubricant inks, fire retardant, paint additives, sealing liquids, immersion oils, adhesives, deducting agents, laminating agents, waxes, dielectric fluids for capacitors and transformers, and for making carbonless copy paper (Chakrabarty, 1985; Walker *et al.*, 1996).

The commercial application used PCBs because of their physical properties which included stability, resistance to both acidic and basic hydroxides, action against corrosive chemicals, un-reactive viscous liquids, low volatility and low vapour pressure. The main sources of PCBs pollution are manufacturing wastes and the careless disposal or dumping of used liquids (World Health Organisation, 1976; Chakrabarty, 1985; Walker *et al.*, 1996).

As a group of aromatic organic chemicals, PCB encompass 209 congeners sharing a common basic two ring structure. PCBs are made by direct chlorination of biphenyl, a process which replaces hydrogen atoms with chlorine. Congeners of PCBs differ by the number and placement of chlorine atoms on the biphenyl rings (Figure 1). The manufacture of PCBs started in 1929 and continued until quite recently in some

countries. Commercial trade names include Aroclor, Clophen, Phenolor and Kane-Aroclor (Kamrin and Ringer, 1996).

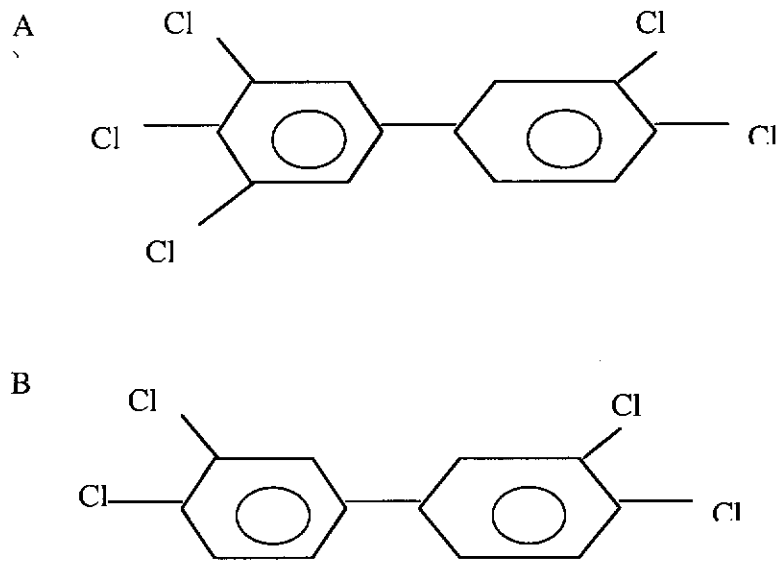

Figure1.1 Structure of PCBs congeners: A. 3, 3',4,4',5-Pentachlorobiphenyl (PCB126) B. 3,3'4,4' tetrachlorobiphenyl (PCB77) (Safe, 1990).

Polychlorinated biphenyls are lipophilic, stable compounds that can accumulate in fluids and tissues of organisms. The degree of lipophilicity and stability increases with the increasing number of chlorine atoms; position of the chlorine atom on the biphenyl molecule also plays a role in stability and lipophilicity. Because of these properties, these compounds will accumulate in the food chain, including fish, wildlife and human adipose tissue, milk, and serum. This has resulted in global environmental problems (Niimi & Oliver, 1989; Safe, 1990; Kimbrough, 1995; Tysklind *et al.,* 1998).

As previously mentioned, the PCB family comprises 209 isomers, but only 36 of these are environmentally relevant (Kimbrough, 1995). The study of the toxicology of these isomers in fish is limited to about 20 monochloro to hexachlorobiphenyls, with most studies restricted to a few biphenyls (Niimi & Oliver, 1989).

The properties of volatility, water solubility and bioaccumulation are of particular importance to introduction into, and transport of PCBs within the aquatic environment. The biodegradation and photodegradation are important factor of PCBs removal. PCBs having highly chlorinated biphenyls, i.e., those containing five or more atoms per biphenyl molecule are commonly found in the environment (Chakrabarty, 1985).

Photolysis may be an important factor in dechlorination (replacement of chlorine by hydrogen) of PCBs in the environment. But this process does not remove PCBs from the environment: it converts highly chlorinated PCBs into a less chlorinated ones (Bunce *et al.*, 1978). The degradation rate of PCBs is extremely slow and the degradation time in the ecosphere is between 20-40 years (Ballschmiter *et al.*, 1978).

The residue of PCBs have been continuously and widely released into the environment in many countries, and are now found to be a world-wide pollutant. Commercial production of PCBs that would result in environmental contamination was stopped in the USA and Sweden during 1970 to 1971, and from 1972 to 1973 in other European countries and Japan (Niimi, 1996). PCBs have been banned (or

strongly restricted) in France since 1990 (Roche *et al.*, 2000). Despite these limitations, significant quantities of PCBs are still released into the environment. The oceans are the final repository in the global cycling of PCBs. The oceanic PCB accumulation which occurred in the last 40 years is mostly located within the upper 10 to 1000 m of the global hydrosphere (Chakrabarty, 1985).

PCBs accumulate in aquatic organisms and can be detected in them when it could not be detected in water. PCB concentrations measured in aquatic organisms can vary by a factor of 10^5, depending on species and sampling sites. Fish found at uncontaminated sites showed the low μg/kg range, while fish collected at contaminated sites show low mg/kg range. Therefore, the waterborne exposure to PCB is not an important pathway for most aquatic organisms because of the low concentrations of dissolved PCBs relative to those in food. However PCBs can cause adverse effects at low mg/kg tissue concentrations. PCBs tissue concentration of > 25 mg/kg in macroinvertebrates and > 50 to 100 mg/kg in fish may cause an adverse effect on growth and reproduction (Niimi, 1996). Exposure to PCBs can alter biochemical activities at the subcellular levels, and may adversely affect reproduction of fish and other organism (Niimi, 1996)

The effects of PCBs at concentrations found in aquatic organisms are difficult to assess directly, mainly because there are no specific clinical symptoms correlated with PCB-induced toxicity in aquatic organisms. In addition, other natural and

anthropogenic organic and inorganic chemicals are invariably present in the organism and its environment (Niimi, 1996). Many PCBs and dioxin are able to bind to a cellular receptor (the aryl hydrocarbon (Ah) receptor), thereby triggering biotransformation enzymes (Molven and Goksøyr, 1993). The Ah receptor is the ligand-activated transcription factor that controls expression of cytochrome P450 1 A genes in response to halogenated aromatic hydrocarbon in fish and mammals (Hahn *et al*, 1998).

Compared to mammals, fish generally have relatively low metabolic capabilities to neutralize or catabolize xenobiotics such as PCBs (Hinz and Matsumura, 1997). The trophic level of fish appears to also be an important factor in the potential accumulation of PCBs. The bivalves, crustaceans and bottom-feeder fish caught from the North West Atlantic had mean PCB residues less than 0.1 μg/g, while carnivorous pelagic fish species had mean PCB residues of more than 0.1μg/g (Slims *et al.,* 1978). The blue fin tuna had mean PCB residues of 3.9 μg/g and the residue level increased with the increasing specimen size (Slims *et al.,* 1978).

Relatively few is known on the fate and effects of sodium pentachlorophenate in the environment. Organic compounds such as sodium pentachlorophenate possess the ability to partition into lipid membranes of mitochondria, and consequently are able to translocate protons across the mitochondrial membrane (Shannon *et al.*, 1991). NaPCP. It is believed that NaPCP has the potential to induce metabolic imbalances in aquatic organisms; however, no studies have tested this hypothesis.

1.3. Biomarkers

1.3.1. Definition of Biomarker

Biomarkers are defined as biological responses to environmental chemicals that give a measure of exposure and sometimes, also, of toxic effect (Walker *et al.*, 1996). In an environmental context, biomarkers are biological tools used as sensitive indicators, demonstrating that toxicants have entered the organisms, been distributed within the tissues, and are eliciting a toxicological effect (McCarthy and Shugart, 1990). Biomarkers are state of the art tools used to estimate the impact of chronic exposure to specific chemicals in the environment (Jørgensen, 1997).

Biomarkers are physiological alterations or manifestations of stress in organisms. A biomarker is a biological reaction used to monitor exogenous exposure, effects of exposure, and early symptoms at the organ or organism level (Schulte, 1995). Biomarkers commonly represent biological responses of individual organism to foreign chemicals or xenobiotics. The biological responses may include, amongst others, 1) enzyme alterations, 2) immune dysfunction, 3) reproductive disturbances, 4) DNA changes, 5) behavioural changes, 6) histopathological lesions and 7) skeletal abnormalities (Ahokas, 1993). Biomarkers have a great potential for use in environmental monitoring of both marine and freshwater ecosystems, and biomarkers have been validated to be included in monitoring programs (den Besten, 1998).

1.3.2. Classification of Biomarkers

Biomarkers have been classified into 2 groups namely biomarkers of exposure and biomarkers of effect. Biomarkers of exposure are a demonstration of chemical

exposure of organism, but do not give information of any biologically important adverse effects that this exposure may have caused. Biomarkers of effects, or more correctly toxic effects, demonstrate that an adverse effect on the organism has occurred due to exposure to pollutants (Molven and Goksøyr, 1993; Lowry 1995; Walker *et al.*, 1996).

Some biomarkers are not yet clearly classified, and weather they are biomarker of exposure or effect is open to debate. While an induction of P450 enzymes may be measured, a direct link to any adverse biological response at higher levels of significance (eg reproductive, behavioural, morphological) has yet to be established. A general classification of biomarkers is shown in Table 1.

Table 1.1 Biomarkers of potential value in monitoring for exposure and effects in the environment.

Biomarkers of exposure	Bile analysis
	DNA adducts
	P450 induction
Biomarkers of effect	
Biochemical indices	
	Phase II induction
	Acetylcholinesterase inhibition
	Oncogene activation
	Heat-shock protein induction
	RNA/DNA ratio
Cellular pathology	Lipid peroxidation
	Oxygen radicals
	Lysosome stability
	Early liver lesions
	Antioxidant concentrations
	Energetic substrates
Reproduction indices	Hormonal alterations
	Vitellogenin/eggshell protein secretion
	Gonad development
	Sperm motility
	Spawning success
	Hatching success
	Time to hatch
	Viability
	Developmental defects
Immunological markers	
Condition indices	

Source: Molven and Goksøyr (1993).

The broad development, application, and validation of biomarkers based environmental monitoring will require coordination and integration of teams of researchers whose expertise encompasses a range of biomarker responses. Anatomical and cytological abnormalities are classic endpoints that have long been used as indicators of deleterious exposure to pollutants in the environment. Biochemical and immunological responses such as the induction of the cytochrome P-450 mixed function oxidase (MFO) system, reproductive competence, genotoxicity and stress protein have been useful as biomarkers (McCarthy and Shugart 1990).

1.3.2.1. Biochemical Markers

Exposing animals to xenobiotics causes alterations at the cellular level, and involves modifications of biochemical pathways. The measurable variations in biological systems are called biochemical markers, commonly referred to as biomarkers (Landis & Yu, 1995).

In order to measure the exposure and physiological effects of a chemical agent on an organism, physiological and biochemical markers are used (Lowry, 1995). The concept of a biomarker is that a toxic effect will become apparent at the subcellular level before the effects appear at higher levels of biological organization (Stein *et al* 1998; Walker 1998). The cellular targets for toxicant interaction and observed responses are: a) cellular membranes: disruption of permeability, b) enzymes: loss of enzymatic activity, 3) protein biosynthesis: dysfunction and d) DNA: structural damage (Shugart,1996). Therefore biochemical markers can be used to detect the exposure to environmental contaminants and quantify specific toxicological responses in exposed organisms (Black, 1997). Because liver is the detoxification

organ in vertebrates, liver detoxification enzymes are one of the most commonly used enzymatic biomarker in wildlife.

1.3.2.1.1. Mixed Function Oxygenase (MFO) enzymes

Each animal has a suite of biotransformation enzymes, usually present in highest concentrations in the liver (vertebrates) or tissues associated with the processing of food (invertebrates). The major function of these enzymes is to convert hydrophobic lipid-soluble organic compounds to water-soluble metabolites. Biotransformation affects the disposition, residence time, and toxicity (detoxication or activation) of xenobiotics in an organism (Timbrell, 1989; Livingstone, 1998).

Xenobiotic metabolism, also known as biotransformation process, transforms the lipophilic chemicals into more water-soluble compounds, representing an important sequence of reaction for detoxification and excretion of xenobiotics (Landis & Yu, 1995). This metabolism can be simply divided into two phases. The first phase is called the oxidative step, catalysed by the cytochrome P-450 (CYP) monooxygenase system located in the smooth endoplasmic reticulum of the cell. This phase alters, or modifies the original molecule by adding on a functional group (-OH, -COOH, -NO_2 etc) to the parent compounds. Phase II is called the conjugation reaction. This step involves large endogenous compounds conjugated to the oxygenated metabolite with the aid of the different families of transferase enzymes, thereby transforming a lipophilic compound into a polar and water-soluble end product. Finally, the water soluble compound is excreted from the organism within the bile or urine or over the gills (Timbrell,1989; Safe, 1990; Goksøyr and Forlin, 1992; Livingstone 1998). The

activity of hepatic MFO enzymes and excretion of metabolites are important processes preventing accumulation of xenobiotic compounds in organisms (Holdway *et al.*, 1994).

Cytochromes P-450 are monooxygenases which catalyse oxygenation of various organic substrates using NADPH and molecular oxygen as co-substrates. The cytochrome P-450, also called polysubstrate multifunction oxygenases (PSMOs) are a family of endogenous enzymes that increase the water solubility of aromatic and lipophilic compounds such as steroids (Hodson *et al.*, 1991). The cytochrome P-450 system encompass a large superfamily of heme proteins involved in the oxidative metabolism of lipophilic exogenous and endogenous compounds, such as drugs, aromatic hydrocarbons, pesticides, fatty acids, prostaglandin and steroids (Goksøyr, 1995).

The P-450 enzymes also acts as a peroxidase that is utilising organic hydroperoxides and hydrogen peroxide as co-substrates in hydroxylation reactions, and as an electron carrier that is reducing certain compounds. Water formation is characteristic of cytochrome P-450 functioning as a monooxygenase. That is why the cytochrome P-450 is also termed a mixed function oxygenase (MFO) (Archakov and Zhukov, 1989). Enzymes of the P-450 family absorb light at 450 nm, which give them their name "P-450".

The cytochrome P-450 is divided into 36 subfamilies based on the similarities of sequence (Shugart, 1996). One of these superfamilies is CYP1A effectively induced

by planar aromatic and chlorinated hydrocarbons (Gosksøyr,1995). The induction of cytochrome P 4501A (CYP1A) in fish can be used a reliable indicator of aquatic contamination by some of anthropogenic compounds (Bogovski *et al.*, 1998). The cytochrome P450 system of fish is specifically induced by planar organochlorines and PAHs (Molven and Goksøyr, 1993). Other inducers include 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene (BaP), planar poly-chlorinated biphenyls (PCBs), and other compounds with similar structures (Goksøyr and Forlin, 1992).

In eukaryotes most cytochrome P-450 isoenzymes are found in membranes, mainly in the endoplasmic reticulum and mitochondria (Goeptar *et al.*, 1995). The liver, which contains the highest concentration of this enzyme, is the major site for metabolism of xenobiotics and steroids (Timbrell, 1989; Honkakoski and Negishi, 1997).

Generally, the methods for measuring the activity of the cytochrome P-4501A1 in fish are by catalytic assay, which is performed using specific substrates. In the wild, the substrates to be metabolised by the P-4501A1 enzymes are the aquatic contaminants previously described. In the laboratory however, one of the most common substrates for assessing P-4501A1 activity is ethoxyresorufin. The transformation of ethoxyresorufin by the P-4501A1 enzyme into the fluorescent product resorufin allows the level of enzyme activity to be measured by fluorimetry (Hodson *et al.*, 1991; Stegeman and Lech, 1991). EROD (7-ethoxyresorufin-*O*-deethylase) activity is assayed for measuring the catalytic activity of cytochrome P-450 system. EROD activity is such a reliable indicator of aquatic pollutant that it has been recently proposed as a biological parameter for the international monitoring program in the North Sea (Köhler and Pluta, 1995; Palace *et al.*, 1996).

MFO activity is useful for governmental regulatory and monitoring purposes, because MFO enzyme activities are very well correlated to organic pollutants, and indicate a sublethal effects or adverse effects detectable by routine monitoring programs (Hansen, 1993).

The hepatic mixed function oxygenase (MFO) activity, as indicated by 7-ethoxyresorufin-*O*-deethylase (EROD) activity, is a sensitive indicator of the ability of the fish to detoxify pollutants such as coplanar polychlorinated biphenyls (PCBs), planar chlorinated dibenzodioxins (CDDs), chlorinated dibenzofurans (CDF) (Hansen, 1993), drugs, agrochemicals and industrial contaminants (Safe, 1990).

1.3.2.1.2. PCB126 as an MFO inducer

PCB126 (3,3',4,4',5 -Pentachlorobiphenyl) is the most toxic coplanar PCB congener, is a potent MFO inducer, and is of great environmental concern (Safe, 1990). PCB126 more intensively induces cytochrome P4501A induction than PCB 77 (Huuskonen *et al.,* 1996). In mammalian and fish toxicology, several experiments revealed the induction potential of PCB126 on MFO enzymes (Safe, 1990). In general, fish exposed to PCB126 have highly induced MFO activity, indicating aquatic contamination (Palace *et al.,* 1996). MFO activities in fish livers have also proven to be a good indicator of pollution in Port Phillip Bay (Smith and Gagnon, 2000). Multiple positive correlations between PCB126 concentrations in liver of fish with mixed function oxygenase enzyme activities were obtained (Palace *et al.,* 1996).

Because of its reliability in inducing MFO enzymes in fish and vertebrates, PCB126 has been widely used as a model contaminant in laboratory toxicology. In this regard, PCB126 acts as a surrogate for several classes of MFO-inducing pollutants found in aquatic environments, namely petroleum hydrocarbons, dioxins and furans and organochlorine pesticides.

1.3.2.1.3. Sorbitol dehydrogenase

Another hepatic enzyme of importance is sorbitol dehydrogenase (SDH). Sorbitol dehydrogenase is an enzyme found primarily in the liver. It is involved in the interconversion of fructose and sorbitol. Under normal conditions SDH concentration is negligible in the bloodstream, but its presence in the blood indicates that hepatocellular injury has occurred (Dixon *et al.*,1987; Ozretic and Ozretic,1993). Fish liver with cellular injuries due to xenobiotic exposure are less capable of MFO induction than are healthy livers (Holdway *et al.*, 1998). Therefore, in monitoring programs of aquatic environmental health, measurement of serum SDH activity can be used in conjunction with MFO activity to explain discrepancies in measured MFO levels due to cellular liver damage (Holdway *et al.*,1994).

1.3.2.1.4. Metabolic Enzymes

Biomarkers of effects are used to assess the biologically significant adverse effects following chemical exposure. Alteration of the metabolic capacity of a tissue is a common reaction following exposure to contaminants. Metabolic capacity, as measured by metabolic enzyme activities, occurs in separate, but related processes

(aerobic and anaerobic) and can be altered by exposure to specific contaminants or contaminant mixtures (Cordiner and Egginton 1977; Priede 1997).

Measurement of metabolic enzyme activities at cellular and subcellular levels could support and explain observed alterations in organism activity level, growth and reproduction consequent to modified energy metabolism. The consequences of perturbed metabolism in biological organization can involve an impaired locomotor activity and reduced survival probabilities in wild fish (Cordiner and Egginton, 1997; Priede 1977). Sustained chemical stress also elevates basal metabolic energy demand, causing a reduction in growth rate with possible consequences on reproductive outputs (Giesy *et al.,* 1988).

Metabolic enzymes, are necessary for generating the energy required for adaptation of fish to their changing environment. Common metabolic enzymes used to measure metabolic capacity are citrate synthase (CS), a key enzyme of the citric acid cycle, cytochrome C oxidase (CCO), a representative of the oxidative capacity of an organ and lactate dehydrogenase (LDH), which expresses the glycolytic capacity (Giesy *et al.*, 1988; Stryer 1988; Philip *et al.*, 1995).

The aerobic metabolic capacity of a tissue can be determined by the activity of citrate synthase (CS). Because white muscle represents the majority of a fish's biomass, measurement of CS activity in fish white muscle reflects the whole oxygen demand of the fish (Yang and Somero, 1993). Citrate synthase, the first enzyme of the Krebs (citric acid) cycle, is located within the mitochondrial matrix. The function of this

enzyme activity is to catalyse the conversion of acetyl-CoA and oxaloacetate into citryl CoA, which is then hydrolysed to citrate and CoA (Stryer, 1988; Dickson *et al.*, 1993). Its presence is related with the density of cell mitochondria (Pelletier *et al.*, 1995).

Atlantic salmon (*Salmo salar*) exposed to the water accommodated fraction of light crude oil and chemically dispersed crude oil showed an inhibition of CS activity in the gills (Gagnon and Holdway, 1999). Similarly, variations of CS activity have been obtained in gills of contaminated crucian carp (*Carassius carassius*) (Lind, 1992).

Cytochrome C oxidase (CCO), the terminal enzyme of the electron transport system (oxidative phosphorylation), is found in the inner mitochrondrial membrane (Goolish and Adelman, 1987; Dickson *et al.*, 1993). In eukaryotic cells oxidative phosphorylation provides 95% of the total ATP requirement (Khan *et al.*, 1986; Stryer, 1998). The increasing CCO levels in an organ is a response to enhanced demand for the generation of aerobic ATP (Goolish and Adelman, 1987). As a consequence, CCO activity relates to the aerobic capacity of tissues (Bostrom and Johansson, 1972). Its density depends on the total membrane surface of mitochondria or mitochrondrial shape (Pelletier *et al.*, 1995). Because of its important role in the processing of cellular energy, perturbations in CCO levels have the potential to profoundly affect aquatic organisms (Stryer, 1988).

Experiments using CCO activity as a biomarker have found that CCO activity in liver and muscle of freshwater fish *Channa striatus* treated with 40 % of the LC_{50} of

pyrethroid permethrin for 24 hours was reduced to about 75% of the control (Singh and Srivastava, 1999). Similarly, gills of Atlantic salmon exposed to petroleum compounds showed a reduced CCO activity relative to the control (Gagnon and Holdway, 1999). Similar results have been obtained in rat liver where CCO activity was inhibited by a high concentration of PCP (Weinbach, 1954).

When the availability of oxygen for aerobic metabolic enzyme is depleted, anaerobic metabolism becomes a buffer permitting the upper limit of normal metabolism to be exceeded (Priede, 1985). The activity of lactate dehydrogenase (LDH) is a good indicator of the anaerobic capacity of a tissue and is a terminal enzyme of the glycolyctic process, which is important in biological systems (Childress and Somero, 1979; Dickson *et al.*, 1993). LDH enzyme activity is inducible by oxygen stress (Wu and Lam, 1997). LDH, located in cellular cytoplasm, catalyses pyruvate into lactate (Verma *et al.*, 1982).

1.3.2.1.5. PCP as an Inducers of Metabolic Perturbation

Polychlorinated phenols (PCP) is commonly used for domestic, agriculture and industrial purposes because of its potent biocide properties. Its application includes wood and textile protection (Muir and Eduljee 1999), in pulp mills as a bleaching agent (Gifford *et al.*, 1996), agricultural pesticides (Schecter *et al.*, 1996), molluscide (Tanaka and Tsuji, 1997) and fungicide (Alcock and Jones, 1997). The presence of phenol compounds, especially the chlorinated forms in the aquatic environment, is of great concern as it has the potential to affect all forms of aquatic life, even at low concentrations (Davi and Gnudi,1999).

Chlorophenols exhibit weak acidic properties in water. This means chlorophenols dissociate in alkaline the water but remain un-dissociated in water with low pH. Therefore, the concentration of chlorophenols in acidic water is usually higher than in non-acidic water. PCP concentration in pike (*Esox lucius*) caught from alkaline lakes was significantly lower than in those caught from acidified lakes with similar contamination levels (Larsson *et al.,* 1993). PCP is the strongest acid of the chlorophenols family; chlorophenol acidic properties normally decrease with decreasing chlorine substituents. Because of its water solubility, PCP is more available for absorption via the gills of aquatic organisms (Larsson *et al.,* 1993). Toxicity of chlorophenols also increases with the number of chlorine atoms, however for chlorophenols having the same number of chlorine atoms, the toxicity decreases in the order of non-, mono-, and di-ortho-chlorophenols (Kishino and Kobayashi 1996a).

Trace concentrations of PCP compounds have a potential to cause adverse effects in aquatic organisms (Bostrom and Johansson, 1972; Muir and Eduljee, 1999). Polychlorinated phenols causes an inhibition of oxygen consumption in fish at µg/liter concentrations (Brodeur *et al.,* 2001). Polychlorinated phenols is also known to uncouple oxidative phosphorylation (Schüürmann *et al.,* 1997) and act as an energy transfer inhibitor in various respiration stages (Ogata *et al.,* 1983). When bioaccumulated, PCP is stored in hepatic lipid reserves and strongly bound to mitochondrial proteins (Bostrom and Johansson, 1972). Polychlorinated phenols

elevates maintenance energy demands causing a reduction of growth rate, which can be used as a sub-lethal indicator of fish stress (Webb and Brett, 1973).

For most aquatic invertebrates tested (annelids, molluscs and crustaceans) as well as for fish, the acute toxicity of PCP compounds is below 1 mg/L (WHO, 1987). PCP clearly caused reduced growth rate and inhibited swimming performance of sockeye salmon (*Oncorhynchus nerka*) (Webb and Brett, 1973). Pelagic and benthic piscivorous fish appear at greatest risk amongst the fish, followed by pelagic and benthic omnivorous fish (Bartell *et al.*, 1999). The fish chronically exposed to phenols showed a reduction in feeding rate, growth rate, delayed maturity and lower fecundity relative to control, while fish acutely exposed to phenol showed a respiratory distress, and excess mucous secretion from the skin and gill (Saha *et al.*, 1999).

Experimental results measuring metabolism showed that NaPCP stimulated anaerobic activity in gill, brain and liver and caused various impairments of gill function of the fish *Notopterus notopterus* (Verma *et al.*, 1982). In addition, anaerobic activity was shown to increase significantly after 24 hours exposure to 50 ppm of 2,4-diamin (herbicide) in the serum of fish *Cyprinus carpio* (Oruc and Uner, 1999).

However, other experiments showed that anaerobic metabolism was significantly inhibited in the gill of salmon exposed to crude oil (Gagnon and Holdway, 1999). Whereas Bostrom and Johansson (1972) showed that anaerobic metabolism was reduced after 4 days of PCP treatment in eel liver. Interpretation of data need to consider that activities of certain anaerobic enzymes such as lactate dehydrogenase

(LDH) increase when muscle, liver or heart is injured whether from disease or exposure to a toxic compound (Singh and Sharma, 1998; Grizzle and Lovshin, 1996).

1.3.2.2. Histological Alterations

The impact of xenobiotics on an organism is reflected through alteration in its physiology, cellular structure, and biochemical balances (Najle *et al.,* 2000). The hepatocytes are very adaptable and may rapidly be stimulated to increase activity following exposure to a xenobiotic stressor (Brown *et al.,* 1998). Liver lesion may be neoplastic, preoplastic, non-neoplastic proliferative or unique degenerative/necrotic lesion. These lesion types have been positively correlated with contaminant exposure and may be promising as biomarkers predictive of pathological effects (Myers *et al.,* 1998). Especially early liver lesions may be a good indicator of environmental contaminants (Molven and Goksoyr, 1993).

Besides causing cell structure alteration, a xenobiotic may also cause alterations to glycogen and lipid storage. Glycogen is a branched polymer of glucose and increases as well as decreases in glycogenolysis. this can occur due to toxicant-induced stress, which results in either depletion or accumulation of glycogen. In most cases the stress condition causes depletion of both glycogen and lipid storage (Giesy *et al.,* 1988).

Ecotoxicological studies demonstrated drastic glycogen depletion in barbel (*Barbus barbus*) treated with food containing 2.5 μg/gr of Aroclor 1260 (PCB) for 30 days (Hugla and Thome, 1998), and an elevation in lipid droplets storage in tilapia

(*Oreochromis mossambicus*) injected with 50 µg/Kg of PCB125 for 5 days exposure (Quabius *et al.,* 1998). Similarly, the liver of flounder (*Platichthys flesus*) caged at a contaminated site showed an increase in lipid/glycogen vacuoles compared to fish caged at a reference area (Husøy *et al.,* 1996). These alterations indicate an up-regulation of cellular metabolism with consequences on energy use and storage (Quabius *et al.,* 1998).

1.3.2.3. Physiological Indices

Physiological indices such as condition factor (CF), liver somatic index (LSI) and gonad somatic index (GSI) are important indicators of exposure to chronic concentrations of xenobiotics (Huuskonen and Lindstrom-Seppa, 1995). These indices have been known to reflect environmental stresses involving contaminants (Molven and Goksøyr, 1993).

Condition Factor

Fulton's formula, [Weight/(length)^3] X 100, is used to determine the nutritional state (condition factor) of fish. It is a useful evaluation of the fish's fattiness (Lucky, 1977) as well as being an indicator of the health or fitness of the fish (Estudillo *et al.*, 2000).

The condition factor is relatively insensitive for short-term environmental stress, but it may be useful in monitoring the nutritional and health status of fish populations for a long experimental period (Hoque *et al.*, 1998). Condition factor is independent of size, age, sex, maturity and area of fish (Lloret and Rätz, 2000).

Liver Somatic Index (LSI)

Conditions of stress caused by exposure to chemicals commonly cause the enlargement of the liver, which can be measured in animals as a higher liver somatic index. A higher somatic index is usually the result of cell hyperplasia (Brown *et al.*, 1998) or enlarged nuclei (anisokaryosis) (Walter *et al.*, 2000). Previous experiments in which the LSI was measured showed that an increase in liver size, relative to body weight (LSI) was obtained in waterborne clofibric acid (CLO) exposed channel catfish (*Ictalurus punctatus*) (Perkins and Schlenk, 1998), as well as in the crussian carp *Carrassius carrassius* exposed to pulp mill effluent (Kukkonen *et al.*, 1999). A range of chemicals can induce liver enlargement; for example, increased LSI was observed in Atlantic salmon (*Salmo salar*) exposed to diets containing 17β-estradiol, nonylphenol or di-2-ethylhexyl phthalate (Norrgren *et al.*, 1999). Similarily, the LSI was slightly increased in perch (*Perca fluviatilisi)* collected in environments with multiple contaminants (Huuskonen and Lindstrom-Seppa, 1995).

Multiple factors such as reproductive stage, exposure to contaminants or nutritional status have the potential to influence the liver somatic index. These confounding factors have to be considered when interpreting the LSI.

1.3.3. Biomarkers at Different Levels of Biological Organization

Biomarkers at Ecosystem Levels

Alterations in the species composition within an ecosystem are the most dramatic impacts that can be observed. Acid rains for example have been noted to cause

dramatic alterations in both aquatic and terrestrial ecosystems. Introduction of nutrients is also known to increase the rate of eutrophication. Global temperature changes have had dramatic effects upon species distributions (Landis and Yu, 1995).

There are two processes that are commonly studied in ecosystems. These are energy flow and material cycling. Pollution can influence either one of those. A toxin may be transported as gases or particulates via the air, dissolved or adsorbed on the surface of particles in water, or leach through soils. A toxicant can also be carried by or concentrate in biological tissues with physical processes (such as filter feeding) or with chemical processes (Newman 1998). Monitoring the key organisms or *sentinel species* can be used as an early warning system for detecting toxicant effects on ecosystem health. This practice is called biomonitoring (Stine and Brown, 1996).

Biomarker at Community Level

The structure of a community may be an indication of environmental stress. For example euthrophication processes emphasize the impact of pollution as species composition and energy flow of aquatic ecosystem is altered (Newman, 1998). The most common index of community structure is the Shannon-Wiener species diversity index. Species diversity should be examined closely as to its worth in determining xenobiotic impacts upon biological community (Landis and Yu, 1995).

Biodiversity, one of the most important characteristic of community, is an important measure of relative abundance of each species in a community. The type of community that develops in a given area depends on factors such as climate, soil, and

other physical conditions. The community structure will change over time as a reflection of environmental changes (Newman 1998). Toxins can affect community structure and its function in several ways. Currently it is difficult to pick up a parameter that describes the health of biological community that can form the basis of predictions (Landis and Yu, 1995; Stine and Brown, 1996).

Biomarkers at Population Level

Population density is affected by competition for resources (food, water, shelter etc) and predation, and density-independent factors such as environmental conditions e.g. weather, toxicants etc. (Stine and Brown, 1996). The indication of population stress includes the number of individuals within the structure of the population. Additionally, as younger life stages are considered to be more sensitive to a variety of pollutants, shifts in age structure to an older population may indicate environmental stress (Landis and Yu, 1995).

Determination of alterations in the genetic structure of populations has become increasingly popular. Alteration of competitive abilities of organisms can be an indication of pollution. Xenobiotics may also affect species diversity if a particularly competitive species is more sensitive to a particular toxicant (Landis and Yu, 1995).

Biomarkers at the Cellular, Organ and Organism Level

Interactions of xenobiotic and biomolecules at the molecular level determines the impact of the pollutant. Some research has been done on the development of a variety of molecular and physiological analyses to be used as indicators and perhaps

eventually as predictors of the effect of toxicants. Biomarkers can be highly specific; for example, an enzyme of the haempathway amonolevulinic acid dehydratase (ALAD) is inhibited specifically by lead; another example is the inhibition of acetylcholinesterase (AchE) which is specific to the organophosphorus and carbamate pesticides. Biomarkers can also be non specific: the induction of monooxygenases and effects on the immune system can be caused by a variety of chemicals (Walker *et al.,* 1996). Numerous biomarkers such as stress proteins, liver, spleen, gonad somatic indices, and DNA adducts and strand breaks are non-contaminant specific (Landis and Yu, 1995).

The presence of certain enzymes in the blood system can be used as indication of lesions or damage of specific organ. For instance, serum sorbitol dehydrogenase (sSDH) is an indication of hepatocellular injury (Ozretic and Ozretic, 1993). Aspartate aminotransferase (AST) is an indication of tissue injury such as muscle, liver, kidney or heart injury (Grizzle and Lovshin, 1996).

The organ-specific damage can often be observed at the organism level. This observation is based on the fact that an animal often exhibits deformation in bone structure, damage to liver and other organs, which can be easily observed. Furthermore, lesions and necrosis in tissues have been the cornerstone of much environmental pathology, and the cytogenetic examination of miotic cells can reveal damages to genetic baggage, and reflect effects of xenobiotics at the individual level (Landis and Yu, 1995).

Biomarkers assess the biological and ecological responses to contaminants present in the environment. These responses can be observed at several levels of biological organization from the molecular level, where pollutants can cause damage at cellular and elicit defensive strategies such as detoxification, to the organism level, involving adverse effect on growth, reproduction, developmental abnormalities or decreased survival. Furthermore, perturbations at the individual level may possibly translate into effects at the population, community, or even at ecosystem levels (Shugart 1996; Walker *et al.*, 1996).

1.3.4. Advantages of Biomarkers

Measurement of biochemical responses to contaminant exposure offers the potential of providing information that cannot be obtained from measurements of chemical concentrations in sediments or in body burdens (McCarthy and Shugart, 1990). The use of biomarkers in monitoring the effects of a pollutant (xenobiotic) at sublethal levels is a rapid, inexpensive and effective way of measuring impacts of water pollution (Lubet *et al.*, 1990; Hugla and Thome 1999; Agradi *et al.,* 2000). These can reveal actual effects of complex mixtures and provide information on the integrated response (Ahokas, 1993; Wu and Lam, 1997). In field monitoring programs, the biochemical indicators of stress are essential to relate any alteration in the measured biomarker to an adverse effect on the organism's growth, reproduction or survival (Giesy *et al.*, 1988).

1.3.5. Limitations of Biomarkers

Assessing either exposure to or effects of environmental contaminants is fraught with uncertainties. Exposure is difficult to assess because of the wide diversity of potential routes of exposure (air, water, soil and food chain), the large differences in biological availability of contaminants associated with the different environmental media and the inter-individual variability in response. The adverse health or ecological effect that has resulted from environmental exposure is even more difficult to describe than the exposure itself. The adverse effects will depend on the magnitude and duration of exposure, the mode of action of the toxicant, length of time required to manifest a diseased state, and susceptibility of the organisms (McCarthy and Shugart, 1990).

Inherent variability among individuals due to season, geographic clines, genotype, and natural perturbations such as turbidity makes it difficult to demonstrate toxicant-induced alteration (Giesy *et al.,* 1988). Additionally, contaminant-specific biochemical markers vary according to species, sex, season, temperature, diet, synergistic or antagonistic compounds and hepatocellular injury (Jimenez *et al.,* 1990; Molven and Goksøyr, 1993; Holdway *et al.,* 1998). However, by choosing the appropriate organism, tissue and enzyme for particular species in a particular ecosystem, these confounding effects can be minimized (Giesy *et al.*, 1988). Obviously, field monitoring programmes have to be site-specific.

1.4 Application of a Suite of Biomarkers in a Biomonitoring Programme of Aquatic Environmental Health in Western Australia

Along Perth's coastal waters and the Swan-Canning River, industries such as fertilizer plants, metal processing plants, petroleum refinery, and gas and chemical plants release liquid effluents into water bodies. However, assessment of the health of this aquatic environment to date has been limited to studies of diversity of benthic invertebrates and distribution of sea grass cover to plankton communities (Department of Environmental Protection, 1996). It is believed that aquatic ecosystem health can be better reflected by the health of native fish populations (Raymond and Shaw, 1997).

To date few studies using biomarkers of exposure, or of effect, to contaminants in native fish have been done in Western Australia. Preliminary studies have commenced into the suitability of native fish such as the black bream (*Acanthopagrus butcheri*), yellowtail trumpeter (*Amniataba caudavittata*) and sea mullet (*Mugil cephalus*) as biological indicators of environmental health for the Swan-Canning River system (Webb, 2000). Pink snapper (*Pagrus auratus*) was a potential bioindicator species of aquatic health monitoring when MFOs and hepatic metabolic enzymes such as CCO and LDH were used as a biomarkers (Tugiyono and Gagnon 2001; Tugiyono and Gagnon, 2002a), and hepatic metabolic change correspond to histopathological alteration (Tugiyono and Gagnon, 2002a). Furthermore, in Eastern Australia, some endemic species have been used in biochemical marker analysis such as spikey globefish (*Atopomycterus nichemerus*) (Holdway *et al.*, 1998), carp (*Cyprinus carpio*) (Ahokas *et al.*, 1994), sand flathead (*Platycephalus bassensis*)

(Holdway *et al.*, 1994, 1995; Brumley *et al.*, 1995), bluethroat wrasse (*Notalabrus tetricus*) and sixpin leatherjacket (*Meushenia frycineti*) (Smith and Gagnon 2000). Despite all these tested candidates, few species have been shown to be suitable. To date, pink snapper appears to be one of the most promising fish species to be used as a biological tool.

The present research will attempt to demonstrate that the native fish pink snapper (*Pagrus auratus*) is a suitable sentinel species to use in monitoring of the aquatic environment in Western Australia. The suite of biomarkers selected in this study include the MFO activity as detoxification capability, sSDH as indication of liver damage, metabolic perturbations by the measurement of CS, CCO and LDH activities, and histological alterations. The histological alteration involved the hyperplasia and hypertrophy of hepatocytes, as well as the accumulation and depletion of glycogen and lipid droplets.

A suite of biomarkers is measured because no single biomarker response is sufficient to unequivocally evaluate exposure, and/or effect. In addition, the response of one biomarker can provide information that improves interpretation of other biomarkers (Jimenez *et al.*, 1990). Multiple biomarkers are measured to minimize misinterpretation and to gain a better understanding of effects induced by exposure to xenobiotics (Jimenez *et al.*, 1990). Biomarkers such as EROD, carbohydrate metabolic enzyme activities and histological alterations are considered complementary to assess exposure of fish to xenobiotics (Perez *et al.*, 2000).

This Study.

Exposing animals to chemical contaminants causes alterations at the cellular level, as well as modifications of biochemical pathways. These measurable variations in biological systems are called biochemical markers, commonly referred to as biomarkers. A biomarker is a biological reaction used to monitor exogenous exposure, effects of exposure, and early symptoms at the organ or organism level (Schulte, 1995).

In order to measure the exposure and physiological effects of a substance on an organism, physiological and biochemical biomarkers are used (Lowry, 1995). The basic concept is that a toxic effect will occur at the subcellular level before it will be apparent at higher levels of biological organisation (Stein *et al.*, 1998, Walker, 1998). Therefore biomarkers can be used to detect the exposure to environmental contaminants and quantify specific toxicological responses in exposed organisms (Black, 1997). Generally, biomarkers can be differentiated into two major groups, namely biomarkers of exposure and biomarker of effects. A biomarker of exposure indicates that a contaminant has been absorbed by the organism, but does not provide information or indicate any possible adverse effects related to the intake of the xenobiotic. A biomarker of effects is used to assess the adverse effects on an exposed organism following exposure to contaminants (Lowry, 1995).

Field studies investigating environmental health more commonly use biomarkers of exposure than biomarkers of effects. Examples of biomarker of exposure are the chemical quantification of tissue content, or the activity of the mixed function

oxygenase (MFO) enzymes measured in the liver of fish, indicating the biovailability of compounds such as petrol hydrocarbons and or planar PCB (Stein *et al.* 1998). These widespread chemical or biochemical markers inform on the bioavailability, uptake and transformation of contaminants, but do not provide information on possible adverse effects caused by the xenobiotics (Gagnon 1998).

Biomarkers of effects relate to the physiological and/or biochemical measurements with adverse health effects caused by chemical insult. For example: high activity enzymes of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in serum denote injured muscle tissue in channel catfish (*Ictalurus punctuates*) (Grizzle and Lovshin 1995), or increased activity enzymes of sorbitol dehydrogenase (SDH) and glutamate dehydrogenase (GLDH) in plasma can indicate hepatic damage in grey mullet (*Mugil auratus* Risso) (Ozretic and Ozretic 1993).

River system and coastal marine ecosystem are under stressing from urban and industrial development, which continuing discharge toxic substances into adjacent aquatic ecosystem. Because of that, the health status of aquatic ecosystems should be assessed at regular intervals to determine trend in pollutant exposure to organisms within those ecosystems. Determine of a suite of biomarker of exposure (MFOs) and of effects (liver metabolic enzyme such as LDH, CCO and CS) and liver histological alteration in fish can provide an early warning system before significant deterioration at population, community or ecosystem level of biological organisation occurs.

The general aim of this study is to demonstrate that the native fish pink snapper (*Pagrus auratus*) is a suitable sentinel species to use in monitoring of the aquatic environmental health in Western Australia. The suite of biomarkers selected in this study include the mixed function oxygenase (MFOs) activity as detoxification capability, serum sorbitol dehydrogenase (SSDH) as indication of liver damage, metabolic enzyme disturbance as measured by citrate synthase (CS), cytochrome C oxidase (CCO) and lactate dehydrogenase (LDH) and histological alteration. The histological alteration involved the hyperplasia and hypertrophy of hepatocytes, as well as the accumulation and depletion of glycogen and lipid droplets.

Specific aims are:

(1) to assess if a native Western Australian fish species, pink snapper (*Pagrus auratus*), can be used as a bioindicator species by evaluating the biochemical responses in the liver of this species;

(2) To evaluate if a common contaminant, sodium pentachlorophenate (Na-PCP), triggers biochemical responses in pink snapper, as measured by enzymes of the metabolism;

(3) To evaluate if biochemical responses of the liver, metabolism of the liver, and liver histology are detectable in a comparable time frame following triggered biological responses.

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Tugiyono and Gagnon, M.M. (2001). Pink snapper (Pagrus auratus) as a bioindicator of aquatic environmental health in Western Australia. *Environmental Toxicology,* 16:449-454

(Co-ordinator, ADT Project (Bibliographic Services), Curtin University of Technology, 11/12/03)

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Tugiyono and Gagnon, M.M. (2002). Metabolic enzymes as biochemical markers of effect following exposure of fish to sodium pentachlorophenate (NaPCP). *Bulletin of Environmental Contamination and Toxicology,* 69:570-575

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Tugiyono and Gagnon, M.M. (2002). Metabolic disturbances in fish exposed to sodium pentachlorophenate (NaPCP) and 3,3',4,4',5-pentachlorobiphenyl (PCB126), individually or combined. *Comparative Biochemistry and Physiology, Part C,* 132:425-435

Chapter 5

General Discussion

5.1. Pink Snapper (*Pagrus auratus*) as a Bioindicator of Exposure to Xenobiotics

Pink snapper has been chosen in these experiments because it is a native Western Australia fish species, and has commercial and recreational value. In addition, it has been successfully farmed so that juveniles are available from reared stock for aquaculture or research purposes. In the wild juvenile pink snapper are found mainly in coastal embayments and estuaries, while adult fish are found in coastal embayments (Chapter 1) (Fisheries Western Australia, 1998). Because of their distribution, pink snapper is potentially exposed to xenobiotics originating from industrial effluents discharged along Perth's coastal water. Industries located along Perth's coastal waters include fertiliser plants, metal processing plants, petroleum refineries, and gas and chemical plants (Department of Environmental Protection, 1996).

Juvenile pink snapper has been shown to be a suitable indicator of environmental health when MFO induction (Chapter 2) and metabolic enzyme alteration (Chapter 3) are used as biomarkers. Alterations of cellular metabolism has also proven a good biomarker of exposure of pink snapper to xenobiotics (Chapter 4). In our experiments, the juvenile stage was used because of stock availability, but also because juvenile stage are usually more responsive to pollutants (Landis and Yu, 1995). In addition to the ease of handling, the use of fish in their juvenile stage

eliminated the confounding factor of sexual maturity in the interpretation of metabolic enzyme activity, MFO activities and physiological indices such as liver somatic index (LSI) and condition factor (Gagnon and Holdway 1996; Dobrowska *et al.*, 2000; Norris *et al.*, 2000).

The hepatic tissue was the most responsive organ for the measurement of MFOs and metabolic enzymes in the present study. In fish as in mammals, the majority of xenobiotic and steroid metabolising enzymes are located primarily, although not exclusively, in the liver (Cravedi *et al.*, 1999). The metabolic profiles of chemicals as measured in plasma, bile and urine are largely a consequence of their hepatic metabolism (Timbrell, 1989; Livingstone, 1998). The exposure of organisms to xenobiotics is reflected by alteration in physiological, cellular, and biochemical markers of the liver tissue (Najle *et al.*, 2000). Liver enlargement of fish exposed to xenobiotics is an indication of induced metabolic disturbances and/or enhanced activity of xenobiotic biotransformation enzymes (Andersson *et al.*, 1987). This study demonstrated that measurable MFO activity can be induced in pink snapper (Chapter 2), that CCO and LDH activities are altered by exposure to contaminants (Chapter 3) and that liver metabolism is perturbed as measured by the store of lipid droplet in hepatocytes (Chapter 4). In liver tissue, CS activity was not affected by exposure to contaminants. Similar conclusions were reached with CCO and CS activities in the white muscle, while LDH activity in this organ was strongly altered by exposure to contaminants (Chapter 3 and 4).

5.2. Metabolic Enzymes as Biomarkers of Effect

Activity or synthesis of specific enzymes can be induced by individual chemicals or mixture of contaminants (Giesy *et al.*, 1988). Often, non-specific response such as the induction of MFO enzymes and effects on the immune system are caused by a variety of xenobiotics (Walker *et al.*, 1996). Because it is often unknown which chemicals are present, it is useful to measure non chemical-specific alterations in exposed animals.

MFO activity involves the oxidative metabolism of lipophilic, exogenous and endogenous compounds such as drugs, aromatic hydrocarbons, pesticides, fatty acids, prostaglandins and steroids (Goksøyr, 1995). One of the super families of cytochrome P-450 is cytochrome P-4501A, which is very specific to xenobiotic exposure to compounds such as with planar organochlorines and polyaromatic hydrocarbons (PAH) (Molven and Goksøyr, 1993), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene (BaP) and planar polychlorinated biphenyls (PCBs) (Goksøyr and Forlin, 1992). The present study showed that MFO activity, as measured by EROD activity, was induced by PCB126 in pink snapper (Chapter 2). Chapter 3 demonstrated, through the measurement of metabolic enzymes, that metabolism was perturbed in pink snapper following exposure of the fish to environmentally relevant concentrations of sodium pentachlorophenate (Na-PCP). Finally, this study showed that biochemical responses were occurring at similar time as histological changes in the liver, and that MFO activity was not induced by NaPCP (Chapter 4).

The perturbations of metabolic enzymes responsible for the generation of energy, also called the cellular respiration process, is an indication that the fish is under stress. The CCO and LDH activities were stimulated in the liver by both NaPCP (Chapter 3) and by PCB126 (Chapter 4) whereas CS activity was not significantly changed. These metabolic enzymes, even though occurring in separate processes, can be altered by exposure to individual or by a mixture of xenobiotics (Cordiner and Egginton, 1977; Priede 1977).

Metabolic imbalances must be regarded as biologically relevant effects, as they may alter the energy balance, especially during periods of enhanced energy demand such as during sexual maturation or under condition of stress and starvation (Andersson *et al.*, 1988). For instance, elevated LDH activity in tissue adversely affects the enzymes involved in tricarboxylic acid cycle (TCA) cycle such as succinate dehydrogenase and malate dehydrogenase (MDH) (Philip *et al.*, 1995). In return, an imbalance of the glycolysis and TCA cycles will affect oxidative phosphorylation, the third stage of respiration, which involves CCO activity (Stryer, 1988; Campbell, 1996). A similar phenomenon was shown by Bhagyalakshmi *et al.* (1984), who demonstrated that elevated LDH and decreased succinate dehydrogenase and MDH activities in the hepatopancreas of the fresh water rice field crab (*Oziotelphusa senex senex*) indicated the development of anaerobic conditions at the tissue level in stressed crabs.

5.3. The Use of a Suite of Biomarkers in Aquatic Environmental Health Monitoring Programs

The use of a suite of biomarkers is important to minimize misinterpretation and provide a better understanding of the effects induced by xenobiotic exposure (Jimenez, *et al.*, 1990). Serum sorbitol dehydrogenase (sSDH), an indicator of liver damage, is used in conjunction with MFO, in order to explain discrepancies in measured MFO levels due to hepatocellular damage (Holdway *et al.*, 1998). In chapter 4 sSDH was used along with histopathology and liver somatic index (LSI) to further ascertain the condition of the liver. The parallel use of several biomarkers is supported by Perez *et al.* (2000) who showed that EROD activity, haematological and histological analyses in fish may be considered as suitable set of biochemical tools to assess fish exposure to environmental pollutants.

Additionally, Chapter 4 describes a suite of biomarkers including MFO, metabolic enzymes and histological analysis as indicators of fish exposure to individual or a mixture of xenobiotics. The purpose of this analysis not only was to determine the specific effect of xenobiotics on selected biomarkers but also to understand the effects of synergistic or antagonistic action of the xenobiotics. Especially, the experiment was designed to investigate if altered liver metabolism would influence the EROD activity. EROD activity was stimulated by injections of PCB126, but combined injections of both PCB126 and NaPCP did not result in a different EROD activity relative to the PCB126 inducer alone. Conversely, CCO activity in

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Extrapolation of laboratory-based toxicity studies is very precarious, mainly due to the fact laboratory studies are performed under strictly controlled environmental conditions while "real-life" exposure of wild fish occurs under ever-changing environmental parameters (Johnsen *et al.*, 1998). In addition, field-collected organisms integrate the exposure to contaminant mixtures, inter- and intra-specific interactions, inter-seasonal variations and various stresses. Field collected organisms also have a wide variation in size and life history, as well as sex-related differences which represent confounding factors during the interpretation stage. However, field studies deliver a real measure of environmental impacts of contamination.

Field studies or in situ-monitoring programmes attempt to understand the environmental problems by analysing a suite of parameters in natural fish populations, which reflect the situation in the field rather than the standardised conditions of laboratory experiments (Walker *et al.*, 1996). For instance, in routine environmental monitoring programs, the induction of monooxygenase system

activity in fish by aquatic pollutants serves as an important tool for the detection of pollution (Leitao *et al.*, 2000). In fact, the induction in fish liver of MFO enzymes activity provides the earliest biological warning signal of exposure to pollutants (Arillo, *et al.*, 1992)

However, prior to using biomarkers of exposure and of effect under field conditions, the biological responses have to be measured and validated in the laboratory (Landis and Yu, 1995). The research presented in Chapters 2, 3, and 4 provides validation of several biomarkers for use in pink snapper. It also provides some prediction of the response of the fish to some common pollutants of concern in the Swan River Estuary where juvenile pink snapper are found. It is known that petroleum hydrocarbons found in the estuary are potent MFO inducers (Webb and Gagnon, 2002); it has also been established that NaPCP is widely found in the river, as a result of agricultural practices along the river system (Swan River Trust, 1998). The laboratory validation of a suite of biomarkers for use with pink snapper allows for implementation of routine monitoring of the Swan River Estuary using the biological responses tested during this research.

5.4. Where and When in Western Australia/Australia can the Results of this Study be Useful?

EROD methods are standardised, and have been widely applied by most authors reporting experimental results following exposure of fish to xenobiotics (Stagg and Addison, 1995). In most instances, EROD activity is significantly induced in fish

captured in urban and agricultural catchments (Cavanagh *et al*, 2000, Webb and Gagnon, 2002). Similarly, EROD activity in sand flathead collected in Port Phillip Bay was highest in fish collected in close proximity to an industrial discharge (Holdway *et al*., 1994).

The results of this present 3.5 year study will be useful for routine aquatic health monitoring, especially in areas that are suspected of having high concentrations of petroleum hydrocarbons, organochlorine and organophosphate pesticides and phenol compounds. In Western Australia, such areas may be:

1) The Swan-Canning River and Swan-Canning Estuary which drains a variety of rural, agriculture (horticulture, vineyards, etc), urban, commercial and industrial lands (Swan-Avon Integrated Management Coordinating Group, 1996);
2) Perth Coastline including Cockburn Sound, Owen Anchorage and Warnero Sound which are the recipients of industrial effluents, and are also receiving 95% of Perth's reticulated domestic wastewater (i.e. sewage effluent) (Department of Environmental Protection, 1996); and,
3) Offshore petroleum activities including production platforms off the west and north-west coast (Swan *et al*., 1994).

Chapter 5

General Discussion

5.1. Pink Snapper (*Pagrus auratus*) as a Bioindicator of Exposure to Xenobiotics

Pink snapper has been chosen in these experiments because it is a native Western Australia fish species, and has commercial and recreational value. In addition, it has been successfully farmed so that juveniles are available from reared stock for aquaculture or research purposes. In the wild juvenile pink snapper are found mainly in coastal embayments and estuaries, while adult fish are found in coastal embayments (Chapter 1) (Fisheries Western Australia, 1998). Because of their distribution, pink snapper is potentially exposed to xenobiotics originating from industrial effluents discharged along Perth's coastal water. Industries located along Perth's coastal waters include fertiliser plants, metal processing plants, petroleum refineries, and gas and chemical plants (Department of Environmental Protection, 1996).

Juvenile pink snapper has been shown to be a suitable indicator of environmental health when MFO induction (Chapter 2) and metabolic enzyme alteration (Chapter 3) are used as biomarkers. Alterations of cellular metabolism has also proven a good biomarker of exposure of pink snapper to xenobiotics (Chapter 4). In our experiments, the juvenile stage was used because of stock availability, but also because juvenile stage are usually more responsive to pollutants (Landis and Yu, 1995). In addition to the ease of handling, the use of fish in their juvenile stage

eliminated the confounding factor of sexual maturity in the interpretation of metabolic enzyme activity, MFO activities and physiological indices such as liver somatic index (LSI) and condition factor (Gagnon and Holdway 1996; Dobrowska *et al.*, 2000; Norris *et al.*, 2000).

The hepatic tissue was the most responsive organ for the measurement of MFOs and metabolic enzymes in the present study. In fish as in mammals, the majority of xenobiotic and steroid metabolising enzymes are located primarily, although not exclusively, in the liver (Cravedi *et al.*, 1999). The metabolic profiles of chemicals as measured in plasma, bile and urine are largely a consequence of their hepatic metabolism (Timbrell, 1989; Livingstone, 1998). The exposure of organisms to xenobiotics is reflected by alteration in physiological, cellular, and biochemical markers of the liver tissue (Najle *et al.*, 2000). Liver enlargement of fish exposed to xenobiotics is an indication of induced metabolic disturbances and/or enhanced activity of xenobiotic biotransformation enzymes (Andersson *et al.*, 1987). This study demonstrated that measurable MFO activity can be induced in pink snapper (Chapter 2), that CCO and LDH activities are altered by exposure to contaminants (Chapter 3) and that liver metabolism is perturbed as measured by the store of lipid droplet in hepatocytes (Chapter 4). In liver tissue, CS activity was not affected by exposure to contaminants. Similar conclusions were reached with CCO and CS activities in the white muscle, while LDH activity in this organ was strongly altered by exposure to contaminants (Chapter 3 and 4).

5.2. Metabolic Enzymes as Biomarkers of Effect

Activity or synthesis of specific enzymes can be induced by individual chemicals or mixture of contaminants (Giesy *et al.*, 1988). Often, non-specific response such as the induction of MFO enzymes and effects on the immune system are caused by a variety of xenobiotics (Walker *et al.*, 1996). Because it is often unknown which chemicals are present, it is useful to measure non chemical-specific alterations in exposed animals.

MFO activity involves the oxidative metabolism of lipophilic, exogenous and endogenous compounds such as drugs, aromatic hydrocarbons, pesticides, fatty acids, prostaglandins and steroids (Goksøyr, 1995). One of the super families of cytochrome P-450 is cytochrome P-4501A, which is very specific to xenobiotic exposure to compounds such as with planar organochlorines and polyaromatic hydrocarbons (PAH) (Molven and Goksøyr, 1993), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo(a)pyrene (BaP) and planar polychlorinated biphenyls (PCBs) (Goksøyr and Forlin, 1992). The present study showed that MFO activity, as measured by EROD activity, was induced by PCB126 in pink snapper (Chapter 2). Chapter 3 demonstrated, through the measurement of metabolic enzymes, that metabolism was perturbed in pink snapper following exposure of the fish to environmentally relevant concentrations of sodium pentachlorophenate (Na-PCP). Finally, this study showed that biochemical responses were occurring at similar time as histological changes in the liver, and that MFO activity was not induced by NaPCP (Chapter 4).

The perturbations of metabolic enzymes responsible for the generation of energy, also called the cellular respiration process, is an indication that the fish is under stress. The CCO and LDH activities were stimulated in the liver by both NaPCP (Chapter 3) and by PCB126 (Chapter 4) whereas CS activity was not significantly changed. These metabolic enzymes, even though occurring in separate processes, can be altered by exposure to individual or by a mixture of xenobiotics (Cordiner and Egginton, 1977; Priede 1977).

Metabolic imbalances must be regarded as biologically relevant effects, as they may alter the energy balance, especially during periods of enhanced energy demand such as during sexual maturation or under condition of stress and starvation (Andersson *et al.*, 1988). For instance, elevated LDH activity in tissue adversely affects the enzymes involved in tricarboxylic acid cycle (TCA) cycle such as succinate dehydrogenase and malate dehydrogenase (MDH) (Philip *et al.*, 1995). In return, an imbalance of the glycolysis and TCA cycles will affect oxidative phosphorylation, the third stage of respiration, which involves CCO activity (Stryer, 1988; Campbell, 1996). A similar phenomenon was shown by Bhagyalakshmi *et al.* (1984), who demonstrated that elevated LDH and decreased succinate dehydrogenase and MDH activities in the hepatopancreas of the fresh water rice field crab (*Oziotelphusa senex senex*) indicated the development of anaerobic conditions at the tissue level in stressed crabs.

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Chapter 6

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This study has successfully demonstrated that pink snapper is a good biological tool for environmental monitoring of Western Australia aquatic environments when MFOs and metabolic enzymes such as cytochrome C oxidase (CCO) and lactate dehydrogenase (LDH) activities are measured. The initial experimental have shown that pink snapper is responsive to both compounds, ie PCB126 as MFO inducer, and NaPCP and an inducer of metabolic perturbations..

The final experiment showed that induction of MFO detoxification enzymes occurred independently of metabolic perturbations. This phenomenon was clearly demonstrated when metabolic perturbations in the liver (as measured by increased CCO and LDH activities) did not result in differential hepatic EROD activity in the PCB 126 + NaPCP treatment.

Pink snapper may potentially be used as a bioindicator species in Western Australia when a suite of biomarkers is used. From the work performed during the course of this study, it is concluded that:

1. Pink snapper may potentially be used as a bioindicator species for Western Australian waters;

2. MFO induction can be used as a biomarker of exposure when pink snapper is targeted as a bioindicator species.
3. The activities of the metabolic enzymes CCO and LDH can also be used as biomarkers of effects in the liver of pink snapper exposed to xenobiotics.
4. The liver tissue of pink snapper appeared to be the most reactive and sensitive organ, while white muscle was irresponsive to treatments.
5. MFO induction potential as measured by ethoxyresorufin-*O*-deethylase activity is not affected by metabolic perturbations of liver.
6. During short-term exposure, liver hyperplasia and hypertrophy, as well as glycogen accumulations, were not suitable markers of exposure. However, the accumulation of lipid droplets in hepatocytes appeared to be altered by short-term exposure to xenobiotics.
7. A suite of biomarkers involving MFO activity, metabolic enzymes and histopathology determined in pink snapper has a potential to provide a sensitive early warning approach, indicative of environmental deterioration in the Swan River system.

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Appendix 1

Conference Presentations

Tugiyono and Gagnon, M.M., 2001. Pink snapper (*Pagrus auratus*) as bioindicator of aquatic environmental health in Western Australia. Envirotox 2001, Australian Society for Ecotoxicology, Canberra, Australia. 12-14 February 2001.

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Tugiyono and Gagnon, M.M., 2001. Pink snapper (*Pagrus auratus*) as bioindicator of aquatic environmental health in Western Australia. Environmental Toxicology 16: 494-454.

Tugiyono and Gagnon, M.M., 2002. Metabolic enzymes as biochemical markers of effect following exposure of fish to sodium pentachlorophenate (Na-PCP). Bulletin of Environmental Contamination and Toxicology,69:570-575..

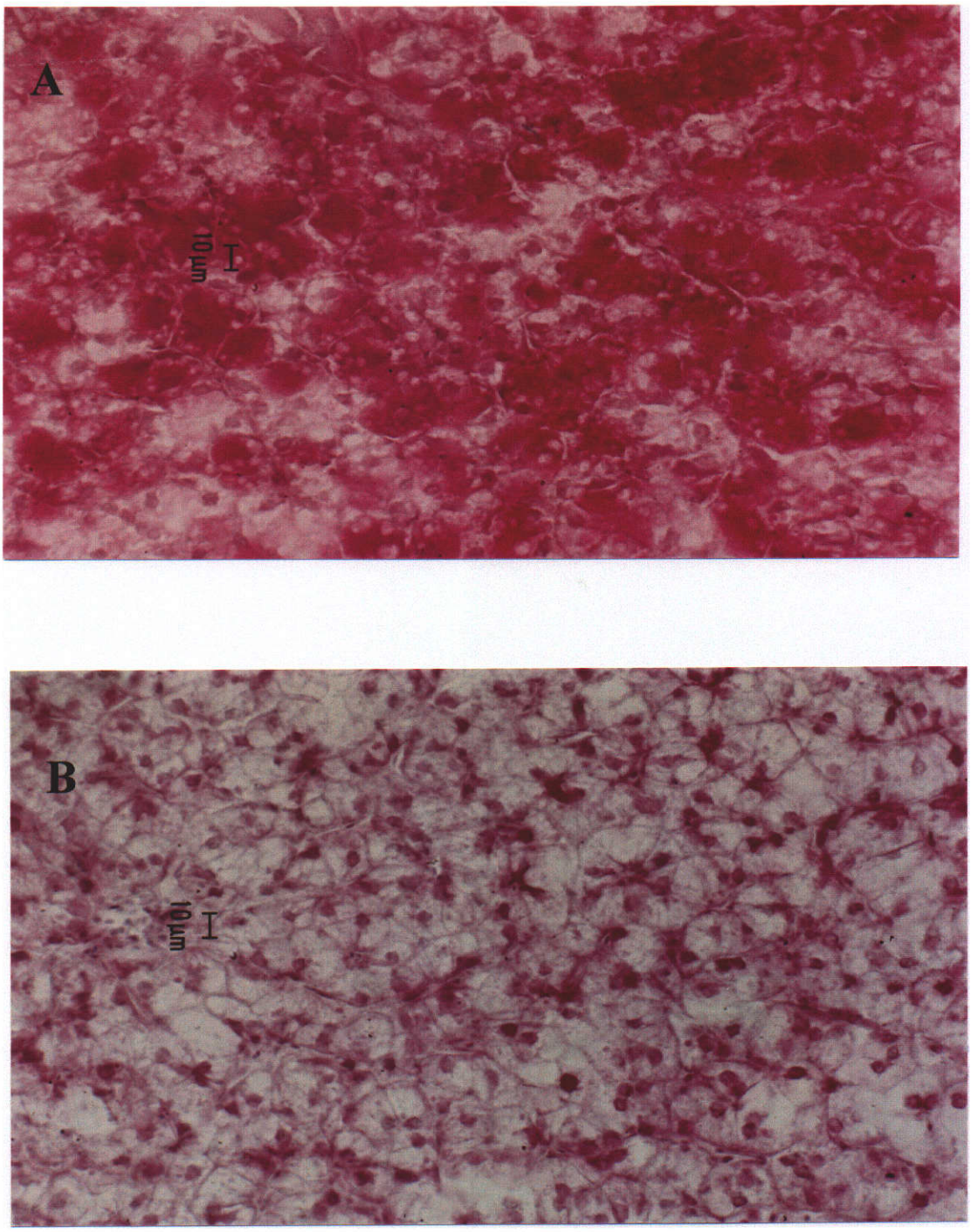
Tugiyono and Gagnon, M.M., 2001. Metabolic disturbance in pink snapper (*Pagrus auratus*) exposed to sodium pentachlophenate (NaPCP), 3,3',4,4',5-pentachlorobiphenyl (PCB126) individually or combined. Comparative Biochemistry and Physiology Part C, 132:425-435.

Appendix 2

Figure A. Glycogen granules in pink snapper hepatocytes exposed to 10 mg/kg NaPCP for 10 days. Cryostat (frozen) section, X 400, (A) P.A.S. positive, (B) P.A.S. negative/diastase reaction (McManus and Mowry, 1964; Hibiya, 1982; Bancroft and Cook, 1994).

Figure B. Lipid droplets in hepatocytes of pink snapper injected with (A) peanut oil (controls), (B) 100 μg/kg PCB126. Cryostat (frozen) section, oil Red O, X 400. Lipids are stained as small red-purple droplets (Mc Manus and Mowry, 1964; Hibiya, 1982; Bancroft and Cook, 1994).

Figure C. Hepatic cells structure of pink snapper injected with (A) peanut oil (controls); (B) 10 mg/kg NaPCP. 10% formalin, HE, X 1000, cell membrane (▶), cytoplasm (→), nucleus (➡) (Mc Manus and Mowry, 1964; Hibiya, 1982; Bancroft and Cook, 1994).



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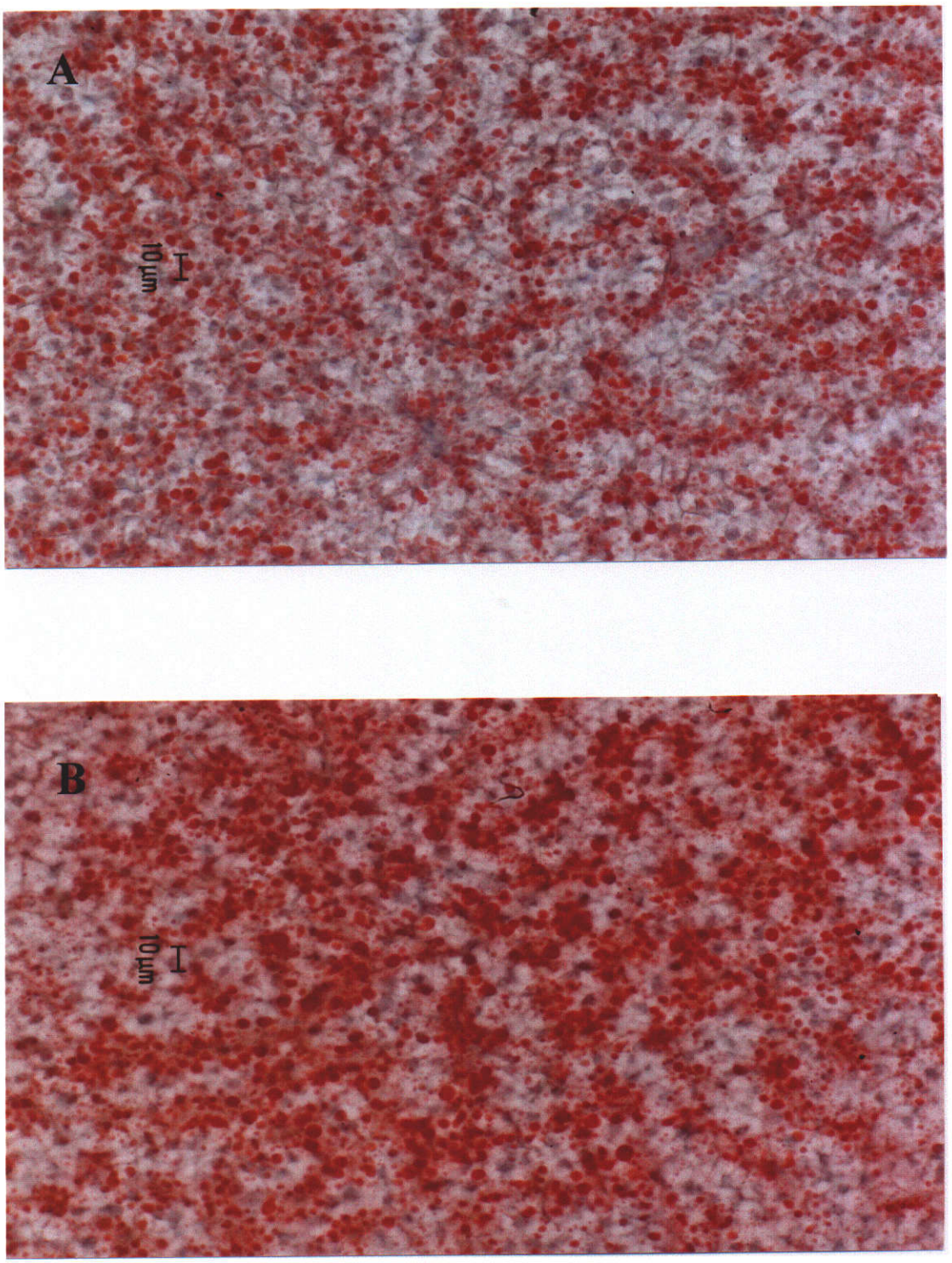

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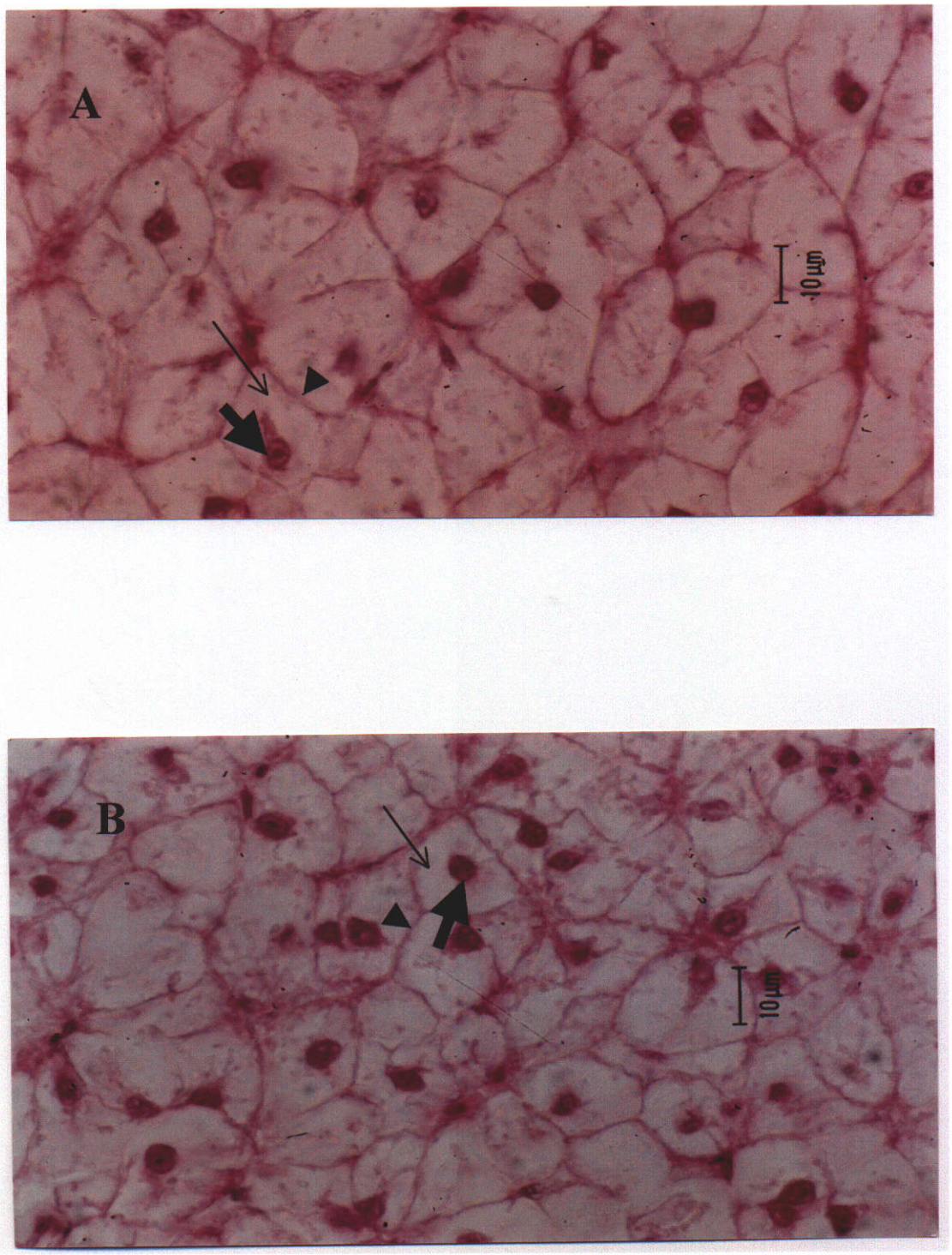

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