Muresk Institute
Department of Environmental Biology

Assessment of the health of the Swan-Canning River System using Biochemical Markers of Exposure in Fish

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

Signature: …Diane Webb……

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Abstract

Most environmental studies concerning the environmental health of the Swan-Canning River system have focussed on nutrient inputs from both rural and urban catchments that are the cause of algal blooms. On occasions these algal blooms have resulted in fish deaths attributed to oxygen starvation. Relatively few studies have examined whether non-nutrient contamination is affecting the health of the riverine environment. Those studies that have, have concentrated on measuring the levels of heavy metals, organochlorines, organophosphates, and hydrocarbons in the sediments and water of the river system, and in the flesh of the biota. However, chemical analysis often fails to detect chemicals of concern due to high laboratory detection limits. In addition, analysis of the body burden of contaminants within biota does not necessarily convey if exposure is inducing adverse effects at the individual or ecosystem levels.

The use of biochemical markers as a tool for the assessment of the health of the Swan-Canning River system was examined under a collaborative research project with the Waters and Rivers Commission, established in response to the recognition of the paucity of information from chemical analyses. The present study focussed on the estuarine portion of the Swan-Canning River system, using the black bream (*Acanthopagrus butcheri*), an estuarine dependent fish species, as a biomonitoring tool.

Prior to the commencement of this study it had been determined that the black bream was a suitable fish species for use as a biomonitoring tool when using mixed function oxygenase (MFO) activity induction under laboratory conditions. Biopsies taken from feral black bream collected from eight sites during the period 2000 to 2002 from the estuary confirmed that the use of MFO induction in this fish species as a biomarker of exposure to organic contaminants is a reliable biomarker.

Fish gender was a confounding factor in the interpretation of MFO induction when using the enzyme ethoxyresorufin-*O*-deethylase (EROD) as EROD activity was suppressed in both pre- and post-spawning female black bream. No such

suppression was identified when using the MFO enzyme ethoxycoumarin-*O*-deethylase (ECOD). However, due to differences in the pattern and intensity of the induction of EROD and ECOD activities it was concluded that ECOD activity was not a substitute for EROD activity to detect certain chemical as ECOD activity represents a different cytochrome P450 pattern to EROD activity.

No spatial, seasonal or interannual differences in the level of the enzyme sorbitol dehydrogenase (SDH) in the blood of the black bream were measured indicating that the interpretation of MFO activity induction was not compromised by hepatocellular damage.

This study has shown that the black bream in the Swan-Canning Estuary are exposed to, and are metabolising polycyclic aromatic hydrocarbons (PAHs), notwithstanding that the chemical analysis of the contaminant load of these substances in the estuarine waters is consistently below laboratory detection limits. In addition, biomarker responses such as ECOD activity indicate that various other organic pollutants are present and are being metabolised by the black bream. The measurement of biliary metabolites clearly show that, under winter conditions, the comprehensive drainage system of the Swan Coastal Plain contributes PAHs from pyrogenic sources such as burnt fuels into the estuary although the onset and intensity of rainfall events notably impacts on the volume of stormwater inflow. During the summer months, when freshwater flow is minimal, petrogenic sources of PAHs are dominant.

Metabolic enzyme analysis points to the black bream being challenged in their aerobic capacities during summer, and that gill tissue was the most suitable tissue to evaluate the aerobic and anaerobic capacity of this fish species. Furthermore, there was a significant negative correlation between stress protein (hsp70) expression and DNA integrity in field-collected fish suggesting that the black bream within the estuary are highly stressed.

No gradient of response in biomarker levels was identified in the Swan-Canning Estuary under either winter or summer conditions indicating there are multiple sources of inputs of potential pollutants along the length of the estuary. Stormwater

and road runoff are the primary source of pollutant input into the estuary in the winter months, while summer biomarker levels, particularly PAH, appear to reflect the high usage of the estuary for recreational purposes and runoff from poorly irrigated parks and gardens. Significant rainfall events at any time of the year have the potential to adversely impact the biota of the estuary, particularly when these events result in a flush of water from the drains following long dry periods.

The study shows that the black bream is a suitable fish species to use under field conditions to detect the presence of bioavailable non-nutrient contamination within the Swan-Canning Estuary. A suite of biomarkers in black bream have been tested seasonally and annually but only a small number of biomarkers have proven suitable for routine monitoring of the health of the Swan-Canning Estuary.

This treatise concludes with several recommendations for further investigations into biomarkers of fish health for the purpose of increasing our understanding on the sources and type of contamination entering the estuary, and potential effects on the aquatic biota of the Swan-Canning River system. These recommendations include, but are not limited to: (1) the need to determine baseline levels for the different biomarkers investigated in this study, (2) the examination of the Moore River or the Warren River estuaries as potential reference sites for biomarker studies in the Swan-Canning Estuary, (3) the advantage of identifying a second estuarine-dependent indigenous fish as a biomonitoring tool, (4) the requirement for a targeted study aimed at clarifying the relationship between major drain discharges, biomarker levels and impacts on river biota, and (5) a study of estuarine waters utilising SPMDs be undertaken in tandem with biomarker analysis of field captured fish would be beneficial.

Thesis Organization

This thesis is divided into eight chapters and four appendices. The General Introduction (Chapter 1) introduces aquatic toxicology and biomarker research and includes a literature review of a number of biomonitoring tools with particular emphasis on biochemical markers of fish health. This chapter also presents the general aim of this thesis. Chapter 2 provides background information on the Swan-Canning Estuary, collection site descriptions and expands on the general aim by itemising the specific objectives addressed by the research.

Chapters 3 to 6 are research papers published in peer-reviewed journals and manuscripts in press. Consequently an unavoidable degree of duplication exists throughout the thesis. Chapter 3 describes the results of analyses of biomarkers of exposure undertaken on bioassays formerly collected in the field in the year 2000 when the estuary was under winter conditions. Chapter 4 compares biomarker responses in fish under summer conditions with those measured under winter conditions. Chapter 5 investigates whether any interannual variability existed in fish biomarker responses in either the winter or the summer seasons. Chapters 6 examine biomarkers of effect drawing conclusions to their suitability as biomarkers for assessment of the health of aquatic systems.

Chapter 7 investigates the biomarker of susceptibility, stress protein expression, and a biomarker of effect, DNA integrity. An attempt was made to identify if any relationship can be derived between these two biomarkers to assist in the interpretation of stress protein as a biomarker for use in a suite of biomarkers to assist in an understanding of the health of biota in the Swan-Canning Estuary.

Chapter 8 is a general discussion, which brings together the outcomes of each of the previous research chapters, comments on the relative value of each biomarker for aquatic health monitoring of the Swan-Canning Estuary and identifies areas needing further investigation. Table and figure numbers and some reference formatting from each paper or manuscript has been altered to ensure consistency within the thesis

Appendix A presents conference poster and platform presentations and refereed journal publications, Appendix B shows the data from the chemical analysis of estuarine sediments and from the flesh of the fish, and Appendix C presents photographs of features of the sampling sites in the estuary. Finally, Appendix D gives a full description of the standardized methods adopted for biomarker analysis in this research.

Chapter 1

General Introduction

1.1. History of Aquatic Toxicology

1.1.1. Definition of Ecotoxicology

Aquatic toxicology is a branch of the science of ecotoxicology that is "multidisciplinary in scope and interdisciplinary in practice" (Rand *et al*., 1995). It has evolved as a field of study, borrowing freely from other basic sciences (Fig. 1.1). Aquatic toxicology requires a working knowledge of aquatic ecology, one or more biological disciplines of physiology, biochemistry, histology and behaviour, and environmental chemistry, to enable an understanding of the effects toxic agents have on aquatic organisms (Rand *et al.*, 1995).

The term 'ecotoxicology' was derived from the words *ecology* and *toxicology* and was first introduced by Truhaut in 1969 (Walker *et al.*, 1996). In 1975, Truhaut further defined ecotoxicology to be "the branch of toxicology that studies the toxic effects of natural or artificial substances on living organisms (e.g., fish, birds, plants), whether animal or vegetable, terrestrial or aquatic, that constitute the biosphere" (cited in Rand *et al*., 1995). Others have defined ecotoxicology in simpler terms as "the study of the harmful effects of chemicals upon ecosystems" (Walker *et al.*, 1996).

The study of ecotoxicology focuses primarily on the toxic effects of chemicals and radiation on levels of biological organization from the individual to communities (Wright & Welbourn, 2002). Ecotoxicology has its roots in classical toxicology. Classical toxicology is focused on humans with any studies involving other species were purely as human surrogates (Wright & Welbourn, 2002), while ecotoxicology involves the study of the distribution of substances in the environment together with their fate and the effects they induce. The science of ecotoxicology provides important information to legislative and regulatory bodies regarding the likely adverse effects of new and existing chemicals on individuals and on ecosystems (van der Oost *et al.*, 2003).

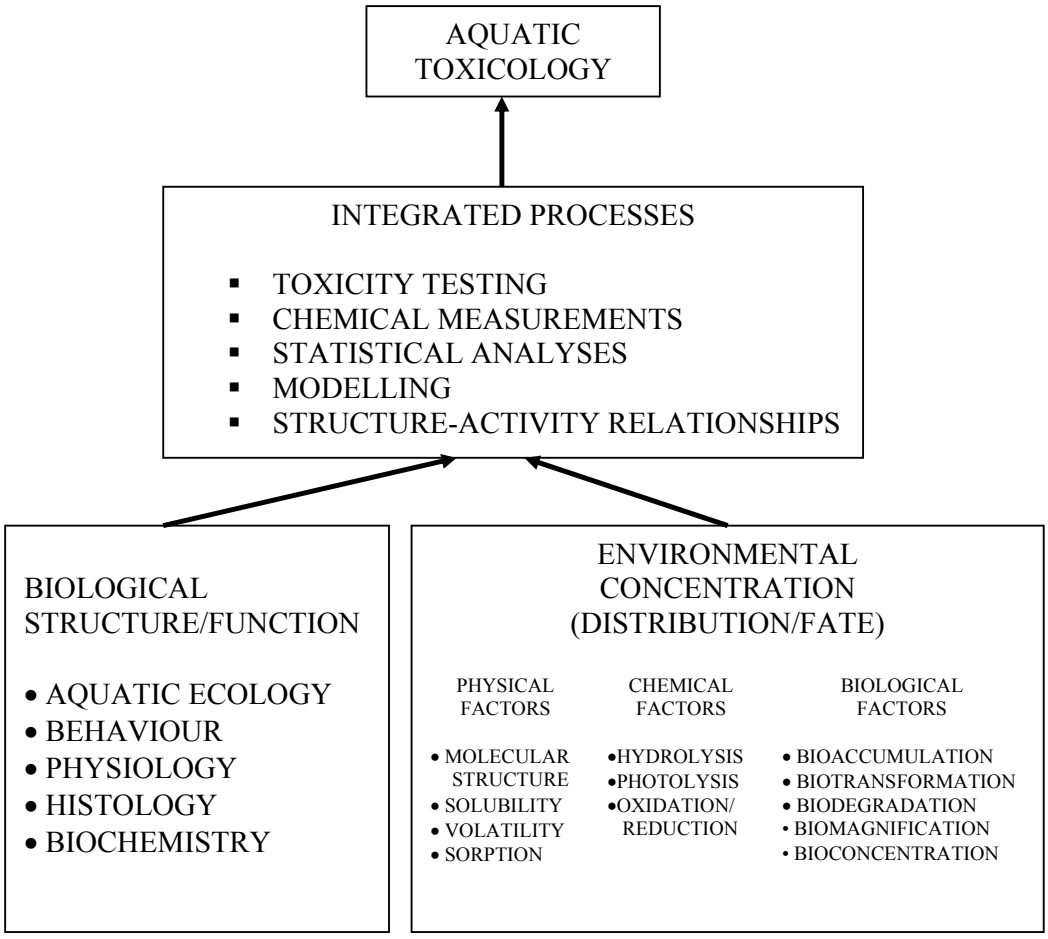

Figure 1.1. *Aquatic Toxicology – a multidisciplinary science (adapted from Rand et al., 1995).*

1.1.2. Development of Aquatic Toxicology

Environmental concern is not an entirely new phenomenon. As early as 500 BC, legislators in Athens passed laws requiring refuse disposal in a designated location outside the city walls, and, the ancient Romans had laws prohibiting the disposal of trash in the River Tiber (Zakrzewski, 2002). Much later in 1775 a London physician, Percival Pott, linked unusually high rates of scrotal cancer in chimney sweeps with exposure to soot (Zakrzewski, 2002), while in 1815 M.J.B Orfila (1787 – 1853) published the first ever book devoted to the harmful effects of chemicals on organisms (Rand *et al*., 1995). The systematic study of the toxic effects in laboratory animals began in the 1920's in response to concerns about the unwanted side effects of food additives, drugs and pesticides (Rand *et al*., 1995). Much of this early work was purely concerned with the detection and determination of chemicals in samples of animals and

plants, with little regard to the effects of these chemicals on the individual animal or on ecosystems (Walker *et al*., 1996).

During and immediately following World War II, chemical industries began to develop rapidly with chemical fertilizers, insecticides, and herbicides coming into widespread use in all nations including developing countries. Grain production more than doubled, between 1950 and 1973 as the world economy expanded by an average of 5% per year. This resulted in rising incomes for all countries, and was paralleled by generally improved health throughout the world e.g. incidence of malaria in India and China declined between 1976 and 1983 due to mosquito control with pesticides (Zakrzewski, 2002). An increasing number of organic trace pollutants, such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzofurans (PCDFs) and dibenzo-*p*-dioxins (PCDDs) were produced, resulting in the environment becoming burdened with foreign organic chemicals (van der Oost *et al.*, 2003). Many of these contaminants ultimately entered the aquatic environment, either by way of direct discharge, hydrologic processes or by atmospheric deposition (van der Oost *et al*., 2003).

The publication of the classic book *Silent Spring* (Carson, 1962) raised the public's awareness of the dangers of contaminating the environment with the indiscriminate use of synthetic chemicals (Rand *et al*., 1995; Wright & Welbourn, 2002; Zakrzewski, 2002). People began to become concerned about the sublethal effects of low-level chemical exposure with the gaining of knowledge of the distribution of OCPs [such as dichlorodiphynyltrichloroethane (DDT)] and their effects on fish and wildlife and the discovery of the widespread occurrence of PCBs within the environment (Rand *et al.*, 1995). Early basic aquatic toxicity tests developed using fish were based on responses of individual fish to acute exposure to single compounds under laboratory conditions. Such fish tests attempted to extrapolate the environmental impacts of compounds under field conditions (Rand *et al.*, 1995).

Acute toxicity testing became the accepted valid parameter for government regulations and guidelines for water pollution control in the 1970s in Canada, the United Kingdom and the United States of America (Rand *et al.*, 1995). During the 1980s, it was recognised that the science of aquatic toxicology needed to move away from single-

species bioassays to ecosystem endpoints with the realisation that the natural environment was subject to mixtures of compounds that were often in very low concentrations. Since this time there have been significant advances in the study of organism responses using biomarkers in fish adapted from human health techniques and a growing acceptance of aquatic toxicology procedures in regulatory bodies worldwide (Rand *et al.*, 1995).

1.2. Environmental Pollution

1.2.1. Definition of a Pollutant

Walker *et al*. (1996) defines a 'pollutant' as a chemical that exceeds normal background levels and has the potential to cause harm. Harm is taken to include biochemical or physiological changes that adversely affect an individual organism's ability to breed, grow or survive (Walker *et al*., 1996).

Rand *et al*. (1995) uses the term 'toxicant' to describe a chemical that can produce an adverse effect in a biological system by damaging its structure or function or inducing death. Toxicants refer to foreign substances (xenobiotics) that may be introduced deliberately or accidentally into an aquatic ecosystem impairing water quality and making it unfavourable for aquatic life (Heath, 1995; Rand *et al.*, 1995). A xenobiotic is a compound that plays no part in the normal biochemistry of an organism (Walker *et al*., 1996). Both pollutants and toxicants have the potential to cause harm however, according to Rand *et al.* (1995), the term pollutant has a broader meaning than the term toxicant, in that a pollutant may also induce abiotic changes such as pH, temperature and salinity in receiving waters.

For the purpose of this thesis, the term pollutant is used to denote all chemicals that have the potential to adversely affect the aquatic biota's ability to survive in their environment, and includes those chemicals that may be classified as toxicants. The term contaminant is used to cover all compounds that exceed background levels, whether they are xenobiotics, naturally occurring or introduced, that do not necessarily harm an organisms ability to survive. A contaminant becomes a pollutant when its presence in the environment alters either biotic or abiotic (or both) conditions thereby impacting on the ability of the aquatic biota to survive within their environment.

1.2.2. Background levels of chemicals in the environment

Both pollutants and contaminants are chemicals that exist at levels above those that are normally present in the environment (i.e., above background levels). Many potentially toxic chemicals, such as heavy metals, sulphur dioxide, nitrogen oxides, methyl mercury and polycyclic aromatic hydrocarbons (PAHs), occur naturally, and were present within the environment before the advent of man. This 'background' level of these chemicals naturally varied from place to place and from time to time (Walker *et al*., 1996). The existence of background levels of chemicals makes it difficult to judge their normal ranges whereas, man-made organic chemicals such as pesticides, did not exist before man and therefore any detectable level in the environment is considered abnormal.

1.2.3. Origin of Pollution

Pollution of aquatic ecosystems can come from both point and non-point sources. A point source is a site where the source of potentially toxic materials into the ecosystem is easy to recognise. Point sources are often deliberate discharges and include sewage outfalls into a water body or a factory pipeline carrying liquid wastes for dispersal in the ocean (Connell *et al.*, 1999a). Non-point sources of pollution can enter a water body via the groundwater, the air, or surface runoff. They are diverse sources and not easily defined in relation to a particular pipeline, outlet or other source. Non-point sources of potential pollutants enter rivers and coastal waters from a variety of different inputs where the actual source of contaminants cannot be pinpointed with certainty. For example, pollutants may enter a river from storm water run-off via road and other drains from a city. The water entering the river has come from all areas of the city and includes contaminants from roads (e.g. PAHs), parks and gardens (e.g. fertilizers and pesticides) and industrial and commercial areas (e.g. various industrial chemicals such as dioxins; Connell *et al.*, 1999a).

1.2.4. Types of Water Pollution

Pollutants can be classified according to their mode of action and target sites (e.g. liver, kidney), their physiological effect (e.g. cancer, mutation, immune responses), use (e.g. pesticide), physical state (e.g. liquid), toxicity potential (e.g. extreme, high, low) or impact on aquatic resources (e.g. tainting; Rand *et al.*, 1995). In addition, pollutants can be grouped according to their chemistry as inorganic ions, organic material,

organometallic compounds, radioactive isotopes and gaseous pollutants (Rand *et al.*, 1995; Walker *et al.*, 1996).

1.2.5. Pollution with the Potential to affect Fish Physiology

1.2.5.1. Organic Materials and Nutrients

Putrefying organic materials result when domestic and industrial wastes are untreated or inadequately treated prior to their release into the environment (e.g. sewage spills or leachate from septic tanks). These wastes are broken down by microbes, which consume oxygen during the decomposition process. This microbial oxygen requirement is termed the biological oxygen demand (BOD). Unless there is considerable mixing of the water, an increase in BOD due to the greater organic load in the water can cause a condition of abnormally low dissolved oxygen (DO; hypoxia; Heath, 1995; Zakrzewski, 2002).

Discharges of nitrogen and/or phosphorous compounds in domestic sewage, agricultural run-off, non-biodegradable detergents and run-off from urban parks and gardens, although not considered toxins in themselves, can have secondary toxic effects on aquatic biota at low concentrations due to eutrophication (Heath, 1995; Connell *et al.*, 1999a). Eutrophication results in large daily changes in dissolved oxygen in waters due to algal photosynthesis during the day and respiration at night. Phytoplankton respiration can cause very low dissolved oxygen immediately before daybreak (Heath, 1995). Additionally, under eutrophic conditions large algal blooms occur that tend to collapse when the limiting nutrient is exhausted (Swan River Trust, 1998) contributing to an increase in BOD.

Hypoxia can have significant physiological effects on fish and other biota in a waterway. There is some evidence to suggest that certain chemicals become more toxic at lower levels of dissolved oxygen (Heath, 1995). For example, Vig and Nemcsok (1989; cited in Heath, 1995) found paraquat combined with hypoxia caused an additive effect in superoxide dismutase induction in gill tissues in carp (*Cyprinus carpio)* due to the accumulation of free radicals in cells. In addition, sublethal exposure to copper for a week resulted in amplified stress responses of bluegill (*Lepomis macrochirus*) to rapid hypoxia exposure [Heath (1991) cited in Heath, 1995].

1.2.5.2. Toxic Chemicals

The Australian Inventory of Chemical Substances (AICS) has in excess of 40 000 industrial chemicals registered for use in Australia (NICNAS, 2004). While, many of the most notorious chemicals, such as DDT, have been taken 'off the market' and the manufacture and use of some chemicals has been tightly regulated, the "body burden" of chemicals in wildlife and humans remains a concern (Brown, 2003). A study by O'Shea and Tanabe comparing the number of chemicals detected in marine mammals in the 1960s to that in the 1990s noted that only five organochlorine compounds and mercury were found in marine mammals in the 1960s. Although the detection capabilities of the 1960s was limited, O'Shea and Tanabe identified 265 organic pollutant and 50 inorganic chemicals in the same species in 1990 (Tanabe, 2002). The major classes of toxic chemicals of concern for aquatic biota are metals, chlorine, cyanides, ammonia, detergents, acids, pesticides, polychlorinated biphenyls, petroleum hydrocarbons and other miscellaneous chemicals (Heath, 1995).

- Metals are natural substances that have been present since the formation of the earth. In most cases metals become pollutants where human activity exposes or releases them from rocks (through mining and smelting) and relocates them into settings where they can cause environmental damage (Heath, 1995; Walker *et al.*, 1996). Metals here are taken to include the heavy metals (e.g. copper, zinc), alkali earth metals (e.g. calcium, magnesium), alkali metals (e.g. sodium, potassium), lanthanides, actinides (e.g. uranium) and metalloids (e.g. silicon, arsenic, selenium).

  The term 'essential elements' refers to the many metals that are required for normal physiological function in animals if the animal is to grow and reproduce normally (Walker *et al.*, 1996). In addition to seven major mineral elements (calcium, phosphorous, potassium, magnesium, sodium, chlorine, and sulphur) animals require a further thirteen other 'trace metals' (including copper, zinc, iron, manganese, cobalt, selenium, iodine; Heath, 1995; Walker *et al.*, 1996). The term 'trace' indicates that these elements are only required in minute quantities (e.g. copper is essential for the normal function of cytochrome oxidase and zinc is an essential component of at least 150 enzymes) and the concentration required varies considerably between species (Heath, 1995;

Walker *et al.*, 1996). Trace elements become toxic when their concentration in the cells becomes sufficiently high to alter physiological function (Heath, 1995; Rand *et al.*, 1995). Many other metals are non-essential (e.g. cadmium, mercury) and, in addition to being toxic above certain levels, may affect organisms by inducing deficiencies in essential elements through competition for active sites in biologically important molecules (Walker *et al.*, 1996).

Metals enter waterways from a wide variety of industrial effluents, domestic pipes and tanks, tyre debris, air conditioning cooling tower bleed-off and old mine sites (Heath, 1995; Rand *et al.*, 1995; Swan River Trust, 1999). A problem in working with toxicity of metals in water is that they tend to complex with organic and inorganic chemicals which may reduce their bioavailability to resident organisms (Heath, 1995). Consequently, a simple analysis for total metals in natural waters may actually overestimate their bioavailability.

- Inorganic, non-metallic toxic chemicals include chlorine, cyanides, nitrogen, phosphorous, boron, ammonia, nitrites, nitrates, and sulphides. Their chemical and toxicological properties may vary with aquatic species and conditions (Rand *et al.*, 1995). Concern for chlorine on the health of aquatic organisms (such as fish) is not for the chloride ion, but with the chemicals that are formed when chlorine gas is introduced into water for antifouling in industrial cooling systems or for disinfection of sewage effluents. The free gas quickly forms HOCl or OCl^- in water which, in the presence of ammonia, is converted into the combined chlorine, monochloramine (NH_2Cl) which, although less toxic than free chlorine, is stable and remains in the water for a long time (Heath, 1995).

  The cyanide radical occurs in many industrial processes, including the manufacturing of synthetic fabrics and plastics, electroplaters, oil refineries, power plants and solid waste combustion (Eisler, 1991; Heath, 1995). Cyanides are readily absorbed across gill membranes in fish and is a potent, rapid acting asphyxiant inducing tissue anoxia and cytotoxic hypoxia (Eisler, 1991).

  Ammonia occurs in effluents and is the result of natural decomposition of organic matter. The non-ionised form of ammonia is toxic to fish but the degree

of its toxicity depends upon the pH and temperature of the water. Increasing pH or temperature increases ammonia toxicity to fish as more ammonia is present in the non-ionised form (Heath, 1995). As the pH and temperature in natural waters can change rapidly during the day, it is often difficult to predict the toxicity of ammonia. In addition, the gill surface of fish has a higher carbon dioxide (CO_2) concentration than the surrounding water and forms carbonic acid when the CO_2 is catalysed by carbonic anhydrase in the mucus, reducing the pH at the gill surface which in turn reduces ammonia toxicity (Heath, 1995).

Although nitrates and phosphates are not particularly toxic, they can cause environmental problems in natural water when used to excess. Fertilisers containing nitrates (NO_2^-) and phosphates (PO_4^{-2}) are used extensively in agriculture and in urban parks and gardens. Both NO_2^- and PO_4^{-2} enter water bodies from surface runoff and additionally, nitrates are released during decomposition of dead plant material which can pass down through the soil, enter the groundwater and eventually find its way into adjacent water courses. An excess of both available NO_2^- and PO_4^{-2} leads to eutrophication of water and fuels algal blooms that have the potential to cause fish death by oxygen starvation (Walker *et al.*, 1996).

Synthetic detergents and washing powders, until the last two decades, have been largely phosphate based and some continue to contain relatively large concentrations of PO_4^{-2} (Wright and Welbourn, 2002). Since 1965, there has been a shift from alkylbenzene sulphonates (ABS) in detergents to the more biodegradable linear alkylate sulphonates (LAS). However, LAS is four times more toxic to fish than ABS but potential toxicity is reduced by rapid biodegradation (Heath, 1995).

- Pesticides are a diverse group of chemicals ranging from simple inorganic substances to complex organic molecules (Rand *et al.*, 1995). Organic pesticides can be derived from plants (e.g., pyrethrins), synthetic derivatives of natural compounds (e.g. fenvalerate) or completely manufactured synthetic substances (e.g., dieldrin; Radhaiah and Rao, 1990; Rand *et al.*, 1995; Walker *et al.*, 1996). All pesticides (insecticides, herbicides, wood preservatives, fungicides, and anti-

fouling agents) are toxic to some form of life and enter natural waters by various means: accidentally during manufacture, during application (e.g. aerial spray drift), water runoff from agricultural or urban land after application, or deliberate introduction (e.g., to control water weeds or vectors of human disease; Rand *et al.*, 1995).

Effective pesticides are designed to be selective with their effects being extremely toxic to target organisms and relatively harmless to others. However, very few pesticides can be said to be completely specific to their targets, therefore other related and non-related species may be affected (Rand *et al.*, 1995). The acute toxicity of pesticides to fish and other aquatic life tends to be greater for organochlorine compounds and pyrethroids than organophosphates. Comparatively, herbicides have relatively low toxicities to fish but secondary effects occur when used for aquatic weed control (Heath, 1995). When used for weed control the decomposing dead plant matter may deplete the oxygen content of the water resulting in fish deaths.

- Organics - the synthesis of organic compounds by man, and their release into the natural environment, assumed significance during the twentieth century, particularly post World War II (Rand *et al.*, 1995; Walker, 2001). Chemicals of concern include the polychlorinated biphenyls, 2,3,7,8-tetrachlordibenzo-*p*-dioxin (TCDD) and related dioxin and furan congeners, polycyclic aromatic hydrocarbons (PAHs) and various organic solvents. The fate, bioavailability and toxicity characteristics of organic chemicals, both within and between various groups, shows very high degrees of variability (Rand *et al.*, 1995).

  Polychlorinated Biphenyls (PCBs) and Polybrominated Biphenyls (PBBs) – both PCBs and PBBs are industrial chemicals that do not occur naturally in the environment. They are lipophilic, stable both chemically and biochemically, and can undergo strong bioconcentration and bioaccumulation to reach relatively high concentrations in the tissues of top predators (Walker, 2001). As a consequence of their environmental effects these chemicals are now banned, or severely restricted, in many countries (Walker *et al.*, 1996).

PCBs are a group (209 congeners) of manufactured organic chemicals. Those PCBs with five or more chlorines have been widely used in a range of manufacturing processes (Rand *et al.*, 1995; Walker *et al.*, 1996). They were principally used as dielectrics in transformers, in heat transfer and hydraulic systems, the formulation of lubricating and cutting oils, plasticisers in paints, and as ink solvents in carbonless paper (Walker, 2001). Exposure to PCBs can alter biochemical activities and cells at the micro and ultrastructure levels, and may adversely affect the reproduction of fish and other organisms (Niimi, 1990). PCBs have been implicated in the decline in the population of several species of fish-eating birds and marine mammals in and near the North Sea (Walker, 2001).

PBBs, formed by the bromination of biphenyl, were introduced in the early 1970s as fire retardants however their production was discontinued in 1974 after the discovery of severe toxic effects on farm animals (Walker, 2001). High concentrations of PBBs have been found in the peregrine falcon, the herring gull, sperm whales, Canadian ringed seals, harbour seals, mussels, and several kinds of fish in Norwegian waters (Brown, 2003).

Perfluorchemicals (PFCs) - are chains of fully fluorinated carbon atoms of varying lengths, yielding chemicals that are extremely resistant to heat, chemical stress, and repel both water and oil (Brown, 2003). Because of these properties PFCs have been widely used since the 1950s as surfactants and emulsifiers and in commercial products including stain or water protectors for carpet, textiles, auto interiors and leather; food packaging; cartons; shampoos; dental cleaners; photographic films; and lubricants. Recently the degradation products of PFCs, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), have emerged as an important class of persistent global pollutants having been found in three species of dolphins as well as whales, bluefin tuna, swordfish, Atlantic salmon, cormorants, seals, sea eagles, bears, turtles, and albatross, with PFOS being the predominant compound (Brown, 2003). Laboratory studies suggests PFOS can cross the blood-brain barrier, interfere with various hormones and is carcinogenic (Austin *et al.*, 2003).

Polycyclic aromatic hydrocarbons (PAHs) - are naturally occurring chemicals in coal and oil deposits. Additionally, PAHs are the products of the incomplete combustion of organic compounds, and are released into the environment both by the activities of man and by certain natural events, e.g. forest fires and volcanic activity (Walker, 2001). They are hydrophobic and lipophilic molecules that interact strongly with sedimentary organic carbon, are sparingly soluble in water and have a relatively low volatility (Burgess *et al.*, 2003). Aromatic hydrocarbons are made up of carbon and hydrogen in one or more aromatic rings, which have a stable alternate double-bond configuration. The PAH group of aromatic hydrocarbons, such as naphthalene, anthracene, and pyrene, have two or more fused rings (Wright and Welbourn, 2002). The structures of some PAHs of environmental interest are given in Fig. 1.2.

Important anthropogenic sources of PAHs include the combustion of coal, crude oil, and natural gas for both industrial and domestic purposes, and the use of these substances in industrial processes such as smelting, the operation of internal combustion engines and the combustion of refuse (Rand *et al.*, 1995; Walker, 2001). Inputs of PAHs into natural waters are most concentrated in estuaries and coastal environments near urban centres. These inputs are from two main sources: (a) the movement of water containing dissolved and particulate constituents from watersheds from both point and non-point sources, and (b) atmospheric deposition both in precipitation and dry deposition (soot) from airsheds (Latimer and Zheng, 2003).

Although PAHs are generally chemically unreactive as they have no functional groups they can be oxidised in both the natural environment and biochemically in vitro. It is the transformed reactive products of PAHs that determines their toxicity, in particular, the incorporation of oxygen into the PAH ring has a polarising effect (Walker, 2001). Photodecomposition occurs in air and sunlight to yield toxic oxidative products such as quinone and endoperoxides.

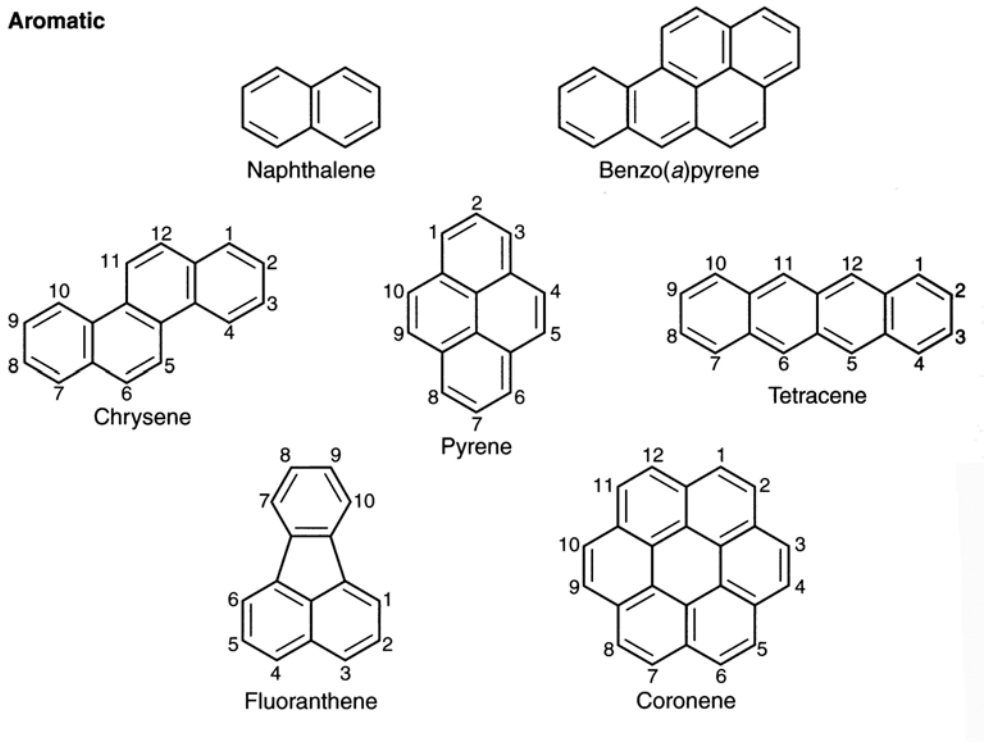

Figure 1.2. *Structures of some polycyclic aromatic hydrocarbons (adapted from Walker, 2001).*

The primary repository of the larger PAHs, four-, five- and six-ring species, in water bodies is the sediment (Latimer and Zheng, 2003). This has consequences in sedimentation areas such as estuaries with the risk of high exposure of benthic organisms to PAH (den Besten *et al.*, 2003). PAHs can be bioconcentrated and/or bioaccumulated by certain aquatic invertebrates low in the food chain that either lack or have a poorly developed capacity for effective biotransformation. Fish and other vertebrates generally transform and eliminate PAHs from the body, therefore bioaccumulation of PAHs in the tissues tends to decline with rising trophic levels (Walker, 2001; den Besten *et al.*, 2003). However, fish, birds, and aquatic mammals that feed on invertebrates may be in the position where their food contains substantial PAH levels. Although they are capable of rapid metabolism of dietary PAH, oxidative metabolism has the potential to produce damaging free radicals with mutagenic and carcinogenic properties (Walker, 2001; Payne *et al.*, 2003).

PAHs can be classified as being pyrogenic, petrogenic, diagenetic, or biogenic according to their source. Pyrogenic PAHs are formed due to the incomplete combustion of organic matter at high temperatures for a short duration, forming a strong interaction with soot carbon, which is of significance for PAH portioning and bioavailability. Petrogenic PAHs are formed at relatively low temperatures over geological time scales (i.e., fossil fuels and petroleum). They are primarily alkylated molecules reflecting the ancient plant material from which the compounds were formed. Diagenetic PAHs come from plant terpenes leading to the compounds retene, perylene, phenanthrene, and chrysene. These PAHs are found in background levels in recent sediments and are dominant in older sediments that predate human industrial activity. Biogenic PAHs, formed by bacteria, fungi, plants, and animals, are in sediments at very low levels (Neff, 1979).

Pyrogenic and petrogenic PAHs are the main concern in environmental studies. Pyrogenic PAHs associated with soot carbon appear to be more persistent being protected from environmental degradation, show little photochemical oxidation, and are resistant to microbial degradation. Petrogenic PAHs are more prone to biogeochemical alteration. Low molecular weight petrogenic PAHs are readily degraded by microbes while the higher molecular weight PAHs are removed from solution by sedimentation. Petrogenic PAHs are generally more bioavailable to organisms as they are more water soluble (Neff, 1979; Burgess *et al.*, 2003).

All the potential pollutants described above can affect living systems at different levels of biological organisation. Their effects depend on their chemical properties as well as on the environment in which they occur, and the organism which absorbs the compound. The next section overviews a number of tools that have been developed to investigate the effects of toxicants on biological systems.

1.3. Biomonitoring Tools

1.3.1. Physiological Indices

Physiological indices are ratios between organ weight and body weight that can be useful in understanding any differences in the health of animals of the same species (Pointet and Milliet, 2000). These parameters are responsive to chronic exposure to contaminants, however they are not very sensitive and may be affected by non-pollutant factors (e.g. season, disease, food availability, life cycle stages; van der Oost *et al.*, 2003). This can make interpretation difficult, requiring a thorough knowledge of the biology of the bioindicator species, and may confuse the interpretation of biochemical markers. Nonetheless, physiological indices may serve as complimentary screening tools to indicate exposure and effects or to provide information on energy reserves (Connell *et al.*, 1999a; van der Oost *et al.*, 2003).

1.3.1.1. Condition Factor

The Condition Factor (CF) is a measure of the fattiness of the fish. The CF is based on the ratio between body weight and length: 100 x [body weight (g)/(length (cm))^3] and allows comparisons to be made between populations living under different conditions (Heath, 1995). This physiological indicator may be affected if food is limited or if food consumption of the fish is impaired due to other stress factors (van der Oost *et al.*, 2003). The CF is relatively insensitive to short-term environmental stress but may be useful in monitoring the nutritional and health status of fish populations over a long experimental period (Hoque *et al.*, 1998).

1.3.1.2. Liver Somatic Index

The Liver Somatic Index (LSI), also known as the hepatosomatic index, corresponds to the ratio between liver weight and total body weight. One of the functions of the liver is the biotransformation and elimination of xenobiotics. Increases in liver size are commonly seen in fish that have been exposed for long periods to organic contaminants. This increase in size is due to either hyperplasia (increased cell number), hypertrophy (increased cell size) or both (Slooff *et al.*, 1983; Heath, 1995). The concurrent increase in LSI can indicate an increased capacity of the liver to metabolise xenobiotics in the presence of pollution.

1.3.1.3. Gonadosomatic Index

The gonadosomatic index (GSI) relates to the ratio between gonad weight and body weight. Under normal, uncontaminated, conditions the index is relatively constant between animals of the same sex, and reflects the maturity of the reproductive organs. A low GSI may indicate that a fish has not yet reached maturity, or is not in a mature reproductive state at the time of capture. However, if the fish are caught immediately prior to spawning, a low GSI may indicate impaired reproductive capacity with the long-term survival of the population in danger (Pointet and Milliet, 2000).

1.3.2. Biomarkers

1.3.2.1. Definition of Biomarkers

Biomarkers are 'biological responses to environmental chemicals which give a measure of exposure and sometimes, also, of toxic effect' (Walker *et al.*, 1996). They are measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the presence and concentration of pollutants, or of the host response (van der Oost *et al.*, 2003).

1.3.2.2. Classification of Biomarkers

Biochemical markers can be classified into 'biomarkers of exposure', 'biomarkers of effect' and 'biomarkers of susceptibility'. Biomarkers of exposure indicate that exposure of an organism to chemicals has occurred, but do not indicate whether any adverse effect has resulted from the exposure. Biomarkers of effect are those that demonstrate that the exposure of the organism to a chemical has caused an adverse effect on that organism (Walker *et al.*, 1996). Biomarkers of susceptibility indicate the inherent or acquired ability of an organism to respond to the challenge of exposure to specific pollutants. They include genetic factors that may increase or decrease an animals ability to survive or cope with a toxic event (Schlenk, 1999: van der Oost *et al.*, 2003).

1.3.2.3. Biochemical Markers

Biomarkers are sensitive indicators for the early detection of adverse effects of chemical contaminants in aquatic organisms before effects are seen at the individual or population level (Holdway *et al.*, 1995; den Besten, 1998; Connell *et al.*, 1999a; Connell *et al.*, 1999b; Gagnon *et al.*, 1999; Schlenk, 1999; Dorward-King *et al.*, 2001).

Evidence of significant effects of toxicant exposure at the population or community levels, and alteration of ecosystem function, may not be apparent for years or decades. Because of the complexities of multicellular organisms, whole animal or tissue residue analyses can not provide an accurate measurement of bioavailability or dose of a compound (Schlenk, 1999). Chemical residues can only indicate exposure to, and uptake of compounds, but not their effects on an animal. Biomarkers in fish, analogous to those applied in human clinical tests, can be used to assess environmental health. Biochemical markers measured in fish integrate the impacts of all toxicants, nutrients, and environmental stressors.

According to Niimi (1990), biochemical and histological evidence can identify possible mechanisms of impairment, which is more beneficial in examining the broader issues of the effects of chemicals on the fish. Toxicants may induce biological responses at different levels of biological organization. At the physiological level, changes may be observed in the animal's ability to hunt, feed, its scope for growth, the ability to avoid predation, or its ability to reproduce. At a biochemical level, toxicants may induce or suppress enzyme levels, alter essential biochemical pathways, and impair normal metabolism by competing with metabolites for active binding sites. At the molecular level, toxicants may bind to DNA, modifying its structure, or induce or suppress the expression of certain genes resulting in altered molecular functions (Connell *et al.*, 1999a).

Biochemical markers can be used to signal, to control, or to predict the presence or effects of pollutants (Gagnon *et al.*, 1999). They can be complemented by chemical analysis in the sediments (Anderson *et al.*, 1996), and in flesh of the fish (Schlenk, 1999), to identify point sources and contamination types.

Organisms have evolved complex enzyme systems to detoxify and eliminate toxins from their bodies. Many organic compounds in the aquatic environment are highly lipid soluble, as a consequence, aquatic organisms bioaccumulate these chemicals in their flesh (Holdway *et al.*, 1995). To convert lipophilic toxicants into more water-soluble forms for elimination, the lipophilic molecule is metabolised into a polar, hydrophilic compound. In vertebrates this is achieved through two sequential phases termed Phase I and Phase II transformations (Walker *et al.*, 1996; Connell *et al.*, 1999a).

- Mixed function oxygenase enzymes are responsible for Phase I transformation, and are primarily found in the liver of fish (Holdway *et al.*, 1995). They are part of a coupled electron transport system composed of two enzyme systems including a cytochrome and a flavoprotein (NADPH – cytochrome reductase; Goksoyr and Forlin, 1992). These enzymes come from a superfamily of structurally and functionally related hemoproteins (Hodson *et al.*, 1991). Specific forms of MFO enzymes, such as cytochrome P-450, are induced by exposure to a variety of lipophilic compounds such as organochlorines, polychlorinated dibenzodioxins, polychlorinated dibenzofurans, PAHs, and PCBs (Connell *et al.*, 1999a). An elevation of MFO enzymes is a reliable biomarker indicating that a fish has been exposed to these classes of contaminants (Hodson *et al.*, 1991).

  Induction of the MFO system begins by binding an organic compound to an enzyme of the MFO family (Lee, 1988). NADPH reduces flavoprotein, which in turn reduces cytochrome P-450. The cytochrome P-450 enzyme then catalyses the foreign compound by the addition of an oxygen atom to the substrate. This increases its polarity, making it more water (blood) soluble, paving the way for further chemical reactions in Phase II transformation (Payne *et al.*, 1987).

  Although, biotransformation by the MFO system attempt to detoxify xenobiotics to protect an organism, it does not always result in detoxification. The detoxification process may induce the formation of free radicals, which under certain conditions can even further damage cells or organs systems in an organism (Lehtinen, 1990). MFO transformation may result in metabolites with greater toxicity than the parent compound, which can exert harmful effects. For example, the initial attack by the MFO detoxification system on benzo(*a*)pyrene (B(*a*)P), while not in itself a carcinogen, can result in various epoxides that tend to be unstable and can quickly reform to become phenols and damaging free radicals and adducts (Au *et al.*, 1999; Walker, 2001; Payne *et al.*, 2003). Fig. 1.3 shows the formation of 7,8 oxide and 4,5 oxide, two important oxidations of B(*a*)P.

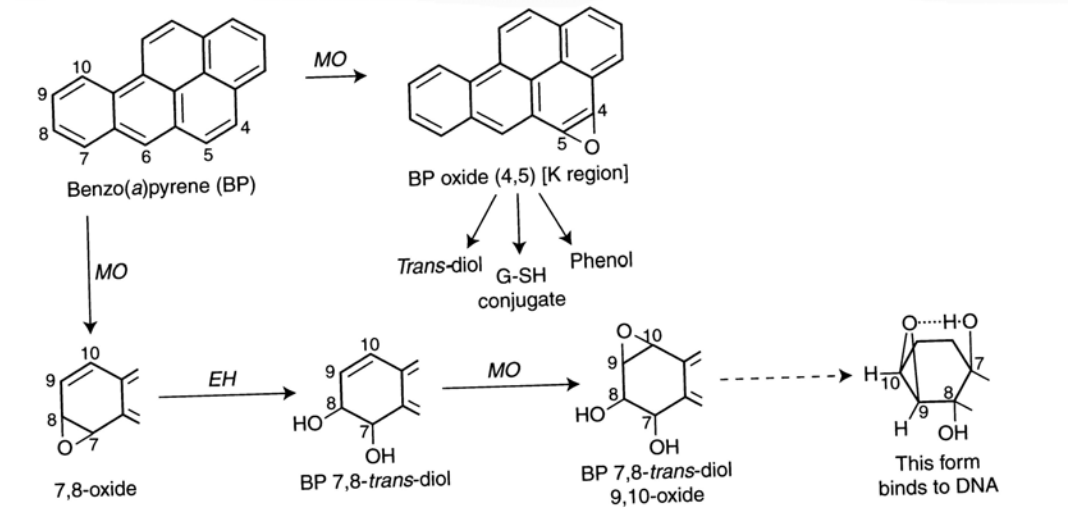

Figure 1.3. *Metabolism of benzo(a)pyrene (source: Walker, 2001).*

The induction of specific isoenzymes of cytochrome 450 caused by exposure to organic compounds, is a very sensitive process, by which a chemical stimulates the rate of gene transcription resulting in increased levels of messenger RNA. The Cytochrome P-450 protein is then synthesised and modified to give the catalytically active enzyme (Goksoyr and Forlin, 1992). Two common MFO isoenzyme activities used for biomonitoring chemical exposure of fish are ethoxyresorufin-*O*-deethylase (EROD) and ethoxycoumarin-*O*-deethylase (ECOD; Holdway *et al.*, 1995; Flammarion and Garric, 1999). MFO induction can be a reliable indicator of contamination of the aquatic environment by compounds known to induce cytochrome P-450, such as many PAHs, PCBs, and organochlorine pesticides (Funari *et al.*, 1987; Collier and Varanasi, 1991; Collier *et al.*, 1993; Smith and Gagnon, 2000).

However, there are important limitations to the use of MFOs in field monitoring. Reproductive status, stress, water temperature, and species differences affect MFO activity in fish. MFO activity is normally reduced in female fish at the onset of sexual maturation as the presence of estradiol can directly inhibit EROD activity (Goksoyr and Forlin, 1992). Suppression of the P-450 enzyme expression can be due to competition between constitutive and inducible enzymes for limited cellular heme pools, leading to accumulation and ultimately

degradation of constitutive P-450 proteins (Waxman, 1988). These factors can influence the activity of the MFO enzymes directly or by affecting the induction process itself (Hodson *et al.*, 1991; Munkittrick *et al.*, 1994).

Both pollutants and reproductive hormones are metabolised by the MFO enzyme system (Stegeman *et al.*, 1982; Lee, 1988; Waxman, 1988). Steroids and some organic pollutants show structural similarities and common metabolic pathways. Therefore, exposure to organic contaminants can suppress the rate of hormone production and/or enhance the clearance rate. Interference with reproductive hormone metabolism can impair the reproductive success of a fish (Lee, 1988).

The induction response to certain pollutants is different between species of fish and even between closely related fish (Funari *et al.*, 1987; Goksoyr and Forlin, 1992). A study by Munkittrick *et al.* (1994), reports that two species of fish belonging to the same genus caught in a polluted area of Lake Superior had different MFO activities. One species, the white sucker (*Catostomus commersonii*), had almost twice the MFO activity when compared to the other species, the longnose sucker (*Catostomus catostomus)*, being collected from the same water body receiving pulp mill effluents. Therefore, the choice of fish species can influence the interpretation of biomarker results (Funari *et al.*, 1987).

- Phase I metabolites are conjugated by Phase II transformation enzymes, such as uridine diphosphate glucuronosyltransferase (UGT) and glutathione-*S*-transferase (GST), into a range of water-soluble metabolites with increased polarity, and water solubility (Ronisz *et al.*, 1999). This facilitates their excretion via urine, bile and through the gills (Payne *et al.*, 1987). Phase II transformation occurs in the cytosol and chemical reactions include deamination, acyclic hydroxylation, aromatic hydroxylation, and dealkylation (Connell *et al.*, 1999a). UGT in fish is involved in processing a wide range of structurally diverse xenobiotics as well as endogenous substrates such as bilirubin and steroids (Ronisz *et al.*, 1999). The substrates for GSTs include epoxides of PAHs metabolised by CYP enzymes, with enzymatic activity measured as EROD activity induction (Ronisz *et al.*, 1999).

Bile is the major excretion route in fish for bile pigments, metals and metabolised xenobiotics such as PAHs (Heath, 1995; Rand *et al.*, 1995). A study by Hellou and Payne (1987), found that several conjugated metabolites of PAHs were found to be accumulated in the bile of trout (*Salmo gairdneri*) exposed to fuel oil. In addition, Krahn *et al.*(1986) found a strong positive correlation between the relative concentration of aromatic compounds in the bile of English sole (*Parophyrys vetulus*) and the presence of liver lesions and other diseases. The presence of metabolites of xenobiotics in the bile indicates that the compounds are bioavailable, and that absorption, metabolism, and elimination of contaminants has taken place (Gagnon and Holdway, 1999). Although adverse effects at the organ level may derive from biologically active metabolites, the measurement of bile metabolites represents a measure of exposure to petroleum compounds rather than a measure of adverse effects.

Many PAHs and their metabolites display strong and characteristic fluorescent properties (Aas *et al.*, 1998). This feature can be used for direct fixed wavelength fluorescent detection of PAH metabolites in bile without the necessity for extraction and clean up prior to analysis. Bile metabolites are therefore measured by fixed wavelength fluorescence, with metabolites classified into naphthalene-, pyrene- or B(*a*)P-types of metabolites (Lin *et al.*, 1996). The simplicity of the method makes it possible to measure PAH exposure on a large number of samples at a low cost (Aas *et al.*, 1998).

- Sorbitol dehydrogenase (SDH) is an enzyme localised primarily in the liver and normally very little, if any, is present in the blood stream (Ozretic and Kranovic-Ozretic, 1993). SDH catalyses the reversible interconversion of fructose and the polyhydric alcohol sorbitol, an oxidation-reduction reaction involving the coenzyme nicotinamide adenine dinucleotide (NADH) (Levine *et al.*, 1978; Dixon *et al.*, 1987). The appearance of SDH in the bloodstream is indicative of hepatocellular injury as serum SDH (s-SDH) is not elevated in any other organ disease but is specific to liver damage (Dixon *et al.*, 1987; Ozretic and Kranovic-Ozretic, 1993). This biomarker is therefore a good marker of liver damage induced by exposure to hepatotoxic contaminants.

Dixon *et al.* (1987) concludes that s-SDH activity is unaffected by fasting, sex or fish weight, and clearly reflects hepatocellular damage induced by exposure of fish to toxic chemicals in a dose-dependent fashion. The rate of conversion of fructose to sorbitol by SDH can be read by a spectrophotometer. The decrease in absorbance is measured as the cofactor NADH is converted to NAD.

Holdway *et al.*(1994) reported that high s-SDH levels were associated with lower hepatic microsomal ECOD and EROD activity levels. Damaged fish livers are less capable of MFO induction than non-injured livers. Therefore, hepatocellular damage represents a confounding factor in the interpretation of MFO induction. Consequently, SDH determination in the serum should be used in conjunction with MFO induction to explain any discrepancies in MFO levels due to cellular damage (Dixon *et al.*, 1987; Gagnon and Holdway, 1999).

- Exposure to contaminants can cause alterations to the metabolic capacity of a tissue. Metabolic capacity, measured by metabolic enzyme activities, occurs in separate but related aerobic and anaerobic processes that can be altered by exposure to specific pollutants or mixtures of pollutants (Priede, 1977; Cordiner and Egginton, 1997).

  Metabolic enzymes are necessary for the generation of energy required for the maintenance of homeostasis in fish living in ever-changing environments. Aerobic capacity can be estimated by the activity of cytochrome C oxidase (CCO), the terminal enzyme of the electron transport system located in the inner membrane of the mitochondria. CCO activity is closely related to the actual oxygen consumption rates for different tissues (Simon and Robin, 1971). Anaerobic capacity is estimated by the activity of lactate dehydrogenase (LDH), the terminal enzyme of glycolysis located in the cellular cytoplasm (Priede, 1985).

  Increasing CCO levels in an organ is a response to the enhanced demand for the generation of ATP. Because of its important role in the processing of cellular energy, disturbances to CCO levels have the potential to profoundly affect aquatic life (Goolish and Adelman, 1987). It has been demonstrated that the

CCO activity in the liver and muscle of a freshwater fish exposed to the pyrethroid, permethrin, was significantly reduced after 24 hours (Singh and Srivastava, 1999). Similarly, the gills of Atlantic salmon exposed to PAH showed reduced CCO activity relative to controls (Gagnon and Holdway, 1999). In addition, pyrethroid pesticides (Szegletes *et al.*, 1995), dimethoate based insecticides (Borah and Yadav, 1996), petroleum hydrocarbons (Cohen *et al.*, 2001), halogenated benzene and phenols (Ogata *et al.*, 1983), and organochlorines (Mishra and Shukla, 1997) have all been shown to have significant impacts on oxygen consumption in fish.

When the availability of oxygen for aerobic metabolism is depleted, anaerobic metabolism becomes a buffer permitting the upper limits of normal metabolism to be exceeded (Priede, 1985). The activity of LDH is a good indicator of the anaerobic capacity of tissues and is inducible by oxygen stress (Wu and Lam, 1997). Studies have demonstrated pollutant-induced changes to LDH activity expression in many fish species. For example, *Clarias batrachus* had depressed LDH activity after exposure to carbofuran (Singh and Sharma, 1998) and to permethrin (Singh and Srivastava, 1999). Additionally, salmon exposed to crude oil had inhibited LDH activity (Gagnon and Holdway, 1999). However, LDH activities increase when muscle, liver or heart muscle is injured from either disease or a toxic compound (Grizzle *et al.*, 1992; Singh and Sharma, 1998).

- Stress (heat shock) proteins (hsps) are involved in the protection and repair of cells following cellular damage due to exposure to a wide variety of stressors, including ultraviolet light, elevated temperatures, salinity, anoxia/hypoxia, pathogens, heavy metals, and other contaminants such as arsenic, cyanide, pesticides, and PAHs. These proteins are highly conserved across a diverse range of phyla from bacteria to humans. The term *stress protein* does not imply that these proteins are only present in organisms subjected to stressful environmental conditions. Some members of the hsp-90, hsp-70 and chaperonins families (all known as molecular chaperones) are synthesised under normal conditions, where they are involved in the maintenance of proper protein folding and the assembly of other cellular proteins (Sanders, 1993).

Under adverse conditions molecular chaperones, such as hsp70, are thought to counter proteotoxic effects by restoring damaged proteins to their native form and vector severely damaged proteins to lysosomes for breakdown (Sanders, 1993; Iwama *et al.*, 1999). The universality, conservation in structure, and consistency with which hsps are induced by a broad spectrum of stressors make them good candidates for the biomonitoring of the environment (Iwama *et al.*, 1998; Feder and Hofmann, 1999; Wright and Welbourn, 2002). Table 1.1 summarises information on the use of hsps as biomarkers.

The accumulation of hsp70 correlates with acquired tolerance to environmental stressors where continuous exposure to mild stressors enable an organism to survive a more stressful event that would otherwise be lethal (Iwama *et al.*, 1999; Bierkens, 2000). However, while small to moderate increases in hsp70 expression may enhance an organisms ability to survive stressful conditions, high concentrations of hsp70 levels may be adverse and directly interfere with cellular ongoing processes, or otherwise alter function to the detriment of the cell (Feder and Hofmann, 1999).

Studies have shown that the expression of hsp70 in rainbow trout is not affected by handling stress, suggesting that the capture, handling and transport of fish for environmental monitoring purposes does not interfere with the use of stress proteins as biomarkers (Vijayan *et al.*, 1997; Washburn *et al.*, 2002). However, the use of stress protein response as a biomarker of effect in fish for on-site ecosystem biomonitoring does need to take into account disease-related expression, the effects of multiple stressors, seasonal variations and the acquisition of tolerance to low background concentrations of chemicals by a species (Feder and Hofmann, 1999; Bierkens, 2000).

- Many pollutants (or their metabolites) may exert toxicity related to oxidative stress. Elevated rates of lesions and neoplasia in fish inhabiting polluted environments can be related to increased oxidative stress associated with pollutant exposure. Reduction products of oxygen [superoxide anion radical (O_2^{-}•), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH•)] are extremely potent oxidants

Table 1.1. The use of stress proteins as biomarkers (adapted from Wright and Welbourn, 2002).

Hsp	Response	Function	Potential as a biomarker
hsp-90	Abundant in normal cells, synthesis increases three- to fivefold upon exposure to stress	May redirect cellular metabolism	Poor when used alone – due to natural abundance and limited response to stressors
hsp-70	Marked increase in synthesis upon exposure to stress	May stabilise or solubilize and remove denatured proteins	Excellent – one of the most highly conserved proteins.
hsp-60/ Chaperonin-60	Increased rate of synthesis in stressed cells	Facilitates translocation and assembly of oligomeric proteins	Good – highly conserved, easily detected response
hsp-20-30	Highly specific to species, found in nuclei of stressed cells	Unknown	Unknown at present - poorly understood function
Ubiquitin	Increased synthesis in response to heat and contaminants	May complement function of hsp-70	Good – easily detected response

capable of reacting with critical cellular macromolecules with the potential to lead to enzyme inactivation, lipid peroxidation, DNA damage, and ultimately cell death (van der Oost *et al.*, 2003). The same metabolic processes that are responsible for the efficient elimination of biodegradable substances (such as PAHs) also have the potential to activate environmental carcinogens to DNA-reactive forms (Dunn, 1991). In environments with high PAH levels, greater MFO induction could contribute to higher steady-state levels of activated carcinogens resulting in a greater formation of DNA adducts. Additionally, high MFO enzyme activity might enhance oxidative DNA damage (Stegeman and Hahn, 1994).

Research into DNA adducts in both human and animal models has focused on the mechanisms of how chemical and physical agents cause genetic disorders and the evaluation of agents for their potential to cause genetic damage, however a more general approach involves the detection of DNA strand breaks (Theodorakis *et al.*, 1994; Everaarts, 1995; van der Oost *et al.*, 2003). DNA strand breakage is a common cellular occurrence caused by heat, UV light and genotoxic chemicals however they are usually repaired rapidly by natural cellular mechanisms. Newsted and Giesy (1987; cited in Steinert *et al.*, 1998) have shown that a number of PAHs are acutely toxic at concentrations below their aqueous solubilities when exposed to solar radiation at levels commonly found in the environment. A study using mussels in San Diego Bay found high levels of DNA damage was induced in mussels exposed to PAHs with low intensity ultraviolet light (Steinert *et al.*, 1998). Chemically induced strand breaks are produced either directly by the toxic chemical (or its metabolite) or by the processing of structural damage (i.e. excision repair enzymes; Shugart, 1996; Lee and Steinert, 2003).

DNA strand breaks are potentially pre-mutagenic lesions (Kammann *et al.*, 2001) and may provide a meaningful indication to the degree of oxidative damage of the DNA (Imlay *et al.*, 1988). They have been induced in fish by *in vivo* and *in vitro* exposure to many chemicals such as BaP, PCBs, metals, and other inorganic and organic chemicals (Shugart, 1988; Shugart, 1995; 1996; Mitchelmore and Chipman, 1998). The increase in the level of strand breaks above background levels can be estimated using the alkaline unwinding assay (Shugart, 1988). The alkaline unwinding assay for this type of damage is easy to perform and cost effective and the assay provides a measure of DNA strand breaks arising from several contaminant-mediated processes (Shugart, 1996).

1.4. Bioindicators and Biomarkers

To date only a few studies have been performed using biomarkers of exposure, and/or effects, to contaminants in fish native to Western Australia with only two species tested, the pink snapper (*Pagrus auratus*; Tugiyono and Gagnon, 2001; 2002) and the banded toadfish (*Torquigenen pleurogramma*; Holme, 2000; Jones, 2001). These investigations

have focused on coastal marine species with only the toadfish studied under field conditions. A pilot laboratory study has investigated the suitability of the native fish, black bream (*Acanthopagrus butcheri*), yellow-tail trumpeter (*Amniataba caudavittata*) and the sea mullet *(Mugil cephalus*), as bioindicators of the environmental health of the estuarine portion of the Swan-Canning River system situated in the south west of Australia (Webb and Gagnon, 2002). The black bream stood out as the most promising fish species to use as a biomonitoring tool in the estuary as it was the most biochemically responsive, of the species tested, to exposure to organic contaminants under laboratory conditions.

This thesis aims to answer the following questions: (1) is the black bream a suitable fish species to use under field conditions to detect the presence of bioavailable contamination using biomarkers, (2) will biomarkers of fish health detect non-nutrient contamination within the Swan-Canning Estuary and, (3) can a suite of biomarkers in black bream be used in a routine monitoring programme to assess the health of the Swan-Canning Estuary? A suite of biomarkers is measured as no single biomarker response is sufficiently unambiguous to evaluate exposure to, and/or affect of pollutant exposure. Additionally the response of one biomarker can be used to improve the interpretation of other biomarkers to gain a better understanding of an ecosystem's health (Schlenk, 1999).

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Chapter 2

The Study Area – Swan-Canning Estuary

2.1. Physical Characteristics

2.1.1. Origin of the River

The Swan-Canning Estuary in the southwestern Australia is the focal point around which the city of Perth, with a population approaching 1.5 million people, has developed. The estuary receives water from both the Avon and Swan Coastal Catchments with an approximate surface area of 119 000 km^2 and 20 000 km^2 respectively (Thurlow *et al.*, 1986). The Swan River is an extension of the Avon River (a historical anomaly reflecting binomial naming habits of the early settlers), which is fed waters from the Darling Plateau, 370 metres above sea level, flowing 300 km through the Darling Range down to the coastal plain. The Avon River becomes the Swan River at the confluence of Wooroloo Brook, about 60 km northeast of Perth. The descent to the coastal plain occurs in a gorge falling 127 metres in 80 km, which rejuvenates the river flow before reaching the coastal plain (Riggert, 1978; Thurlow *et al.*, 1986). The estuarine region incorporates a narrow entrance channel (the lower estuary), two large basins (middle estuary) and the saline reaches of the Swan River approximately 60 km from the ocean (upper estuary). The Canning River, a major tributary of the Swan River, is estuarine for about 6 km to the Kent Street Weir (middle Canning; Hodgkin, 1987; Kanandjembo *et al.*, 2001; Fig. 2.1). The Swan-Canning Estuary is a drowned river valley system (Collins, 1987) It has a scoured channel, which after expanding into broad waters in the middle estuary, discharges to sea at Fremantle through a long narrow inlet channel (Thurlow *et al.*, 1986).

2.1.2. Climate

Perth is situated 31^o 57’ latitude (S) and 115^o 52’ longitude (E). The region has a Mediterranean type climate characterised by wet winters and hot dry summers. Summer temperatures average 31.3^{o}C maximum and 18.0^{o}C minimum in February, while winter temperatures range from 8^{o}C minimum to 18.3^{o}C maximum during the coldest months of July and August. The average annual rainfall for the Perth

metropolitan region is 786mm with the majority of rain falling between May and September (Riggert, 1978; Thurlow *et al.*, 1986; BOM, 2004).

The onset of winter rains is associated with the appearance offshore of cyclonic depressions, which move in a south-easterly direction. Cyclical changes in low and high pressure systems affect the weather, tidal levels and wind patterns (Spencer, 1956; Thurlow *et al.*, 1986; Swan River Trust, 1999b). Winds in the Perth region vary from east to south-east in summer with a reliable sea breeze by mid afternoon. The wind pattern over winter is variable with east and north-easterlies changing to stronger north-west winds bringing rain (Riggert, 1978).

2.1.3. Geology

The Swan Coastal Plain around the estuary is over 100 000 years old with surface sediments comprising mainly sand with a poor nutrient-binding capacity. Contemporary estuarine conditions were established about 6 000 years ago by a rise in sea level and estuarine sediments vary from silty sand to black, sulphurous estuarine silt. Sublittoral sandflats fringe much of the estuary shoreline (Collins, 1987; Thomson *et al.*, 2001).

Because of the porous, sandy soil a large amount of freshwater from winter rains accumulates underground every year. In many low-lying areas, the groundwater is less than 5 metres deep from the surface and when the underground storages fill, groundwater flows into the river courses. Consequently, unconfined groundwater is susceptible to contamination from landuse (Appleyard, 1993), however the volume of groundwater entering the estuary is substantially less than winter surface water runoff [Appleyard (1992) cited in Thomson *et al.*, 2001].

2.1.4. Hydrology

The volume of surface water runoff into the system is the principal factor determining the hydrological status of the estuary (Thurlow *et al.*, 1986). As a consequence, the hydrological condition of the estuary displays high seasonality (Fig. 2.2; Spencer, 1956; Riggert, 1978; Stephens and Imberger, 1996; Twomey and John, 2001).

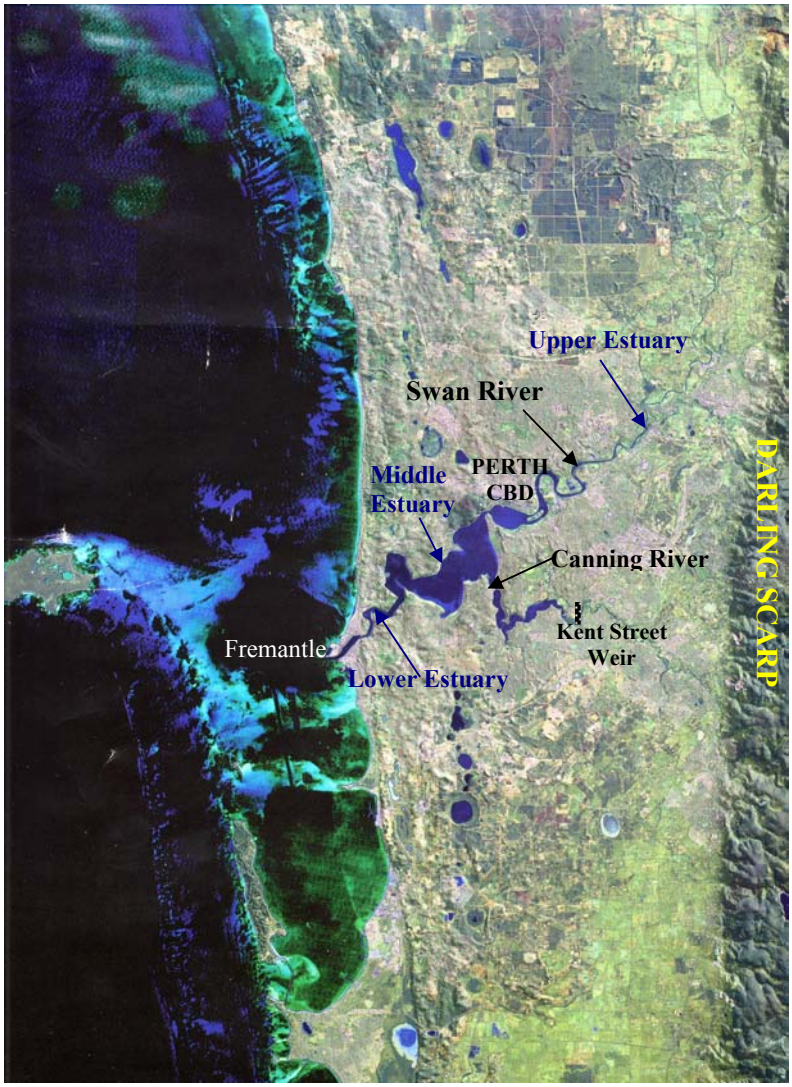

Figure 2.1. *Aerial view of the Swan-Canning estuarine system (source: Dept. of Land Administration, Perth, Western Australia. Landsat TM. SPOT Pan and DEM merged image 25/1/1996).*

Tidal portions of the Swan-Canning system oscillate between being a fresh-to-brackish estuary in winter and a salty ocean-like estuary in summer. Peak runoff occurs following several weeks to two months of rainfall whereby the saline estuarine water is usually flushed from the upper estuary. The freshwater discharge moving into the lower estuary forms a buoyant plume of low salinity or brackish surface water over denser saline waters (Thurlow *et al.*, 1986; Stephens and Imberger, 1996; Thomson *et al.*, 2001).

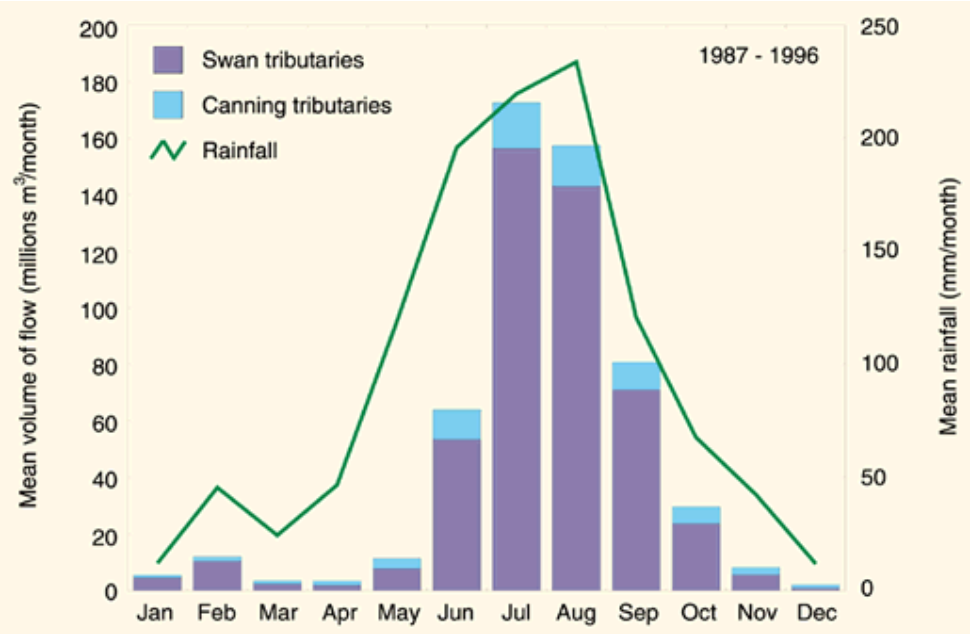

Figure 2.2. *Mean volume of flow and rainfall for the Swan-Canning system (1987-96) (source: Swan River Trust, 1999b).*

In spring, as rainfall (and consequently runoff) decreases, intrusion of salt water into the estuary by the tides displaces freshwater and gradually moves upstream. This mid spring to early summer period is referred to as the 'salt wedge' (Spencer, 1956; Twomey and John, 2001). Although sporadic summer rainfall events result in freshwater inflows during summer; generally there is negligible river flow during the summer to early autumn months (Spencer, 1956; Thurlow *et al.*, 1986; Hodgkin, 1987).

2.1.5. Tides

Prior to the dredging of a limestone sill (barrier) at the mouth of the estuary in Fremantle to permit entry of ships in 1897, estuarine water was mostly fresh to brackish (Riggert, 1978; Spencer, 1987). Tidal influences were probably less than 20 percent of the marine tides, however, since the removal of the sill approximately 80 – 85 percent of the ocean tide now enters the estuary when river flows start to decrease in spring (Riggert, 1978; Stephens and Imberger, 1996).

The estuarine system experiences a tidal range of 0.6 – 0.9 metres as a progressive wave that propagates into the estuary. The tide attenuates at approximately 1 percent per kilometre so by the time the tidal wave reaches Barrack Street it is in the order of 0.5 metre (Hodgkin, 1987; Thomson *et al.*, 2001). The upper limit of the tidal action on the Swan River is in the upper estuary and Kent Street Weir on the Canning River (Thurlow *et al.*, 1986; Hodgkin, 1987).

The passage of atmospheric pressure systems can result in larger variations of up to one metre in tidal range. Falling barometric pressure and characteristic gales associated with winter low pressure systems result in each flood phase being greater than the preceding ebb, so that over a period of a few days there is a marked increase in mean water levels, due to the entry and retention of marine water. Rising barometric pressure results in the ebb phase of each tidal cycle becoming dominant, so that water level falls steadily as water moves out of the estuary (Spencer, 1956). Large but subtle storm surges from cyclonic activity in the north west of Australia can also add or exacerbate tidal levels during summer and autumn.

2.2. Physico-chemical Characteristics

2.2.1. Salinity

Similar to all south western Australian estuaries, the Swan-Canning Estuary exhibits a salt wedge effect resulting from intense seasonal changes in salinity (Hodgkin, 1987). In winter, the seasonal freshwater discharge from tributaries effectively isolates and traps the more dense saline water at the bottom of the water column and either, pushes it downstream, or the estuary becomes highly stratified. Salinities in the order of < 4 ppt are typical in the upper estuary during relatively wet winters while the high winter flow may result in the low salinity layer extending downstream to the middle estuary. Intruding marine water (average 36 ppt) are driven by tides and barometric pressure which keeps the lower estuary well mixed although stratification can occur when river discharge is high (Thomson *et al.*, 2001; Twomey and John, 2001).

As freshwater inflows decline during spring, tidal movement results in intrusion of saline waters that gradually moves into the estuary under the less dense freshwater. Wind mixing and tidal exchange breaks down stratification and salinity increases throughout the estuary. By the end of summer, the estuary is classified as slightly stratified. Salinities can range from mid 20 ppt in the upper estuary some 40 km from the mouth, to > 36 ppt in the lower estuary. In particularly dry years the saline intrusion has been noted up to 60 km upstream from the estuary mouth during autumn (Hodgkin, 1987; Stephens and Imberger, 1996; Twomey and John, 2001). Evaporation from surface waters, over the summer months, can result in the regions of the estuary becoming hypersaline (Thurlow *et al.*, 1986).

2.2.2. Temperature

Thurlow *et al*. (1986) reported average water temperatures in the Swan-Canning Estuary, from November 1979 to November 1985 ranging from 13.8^{o}C in winter to 25.6^{o}C in summer. Thomson *et al*. (2001) reported similar median temperatures between 1994 and 1997, with surface waters ranging from approximately 13^{o}C in winter to summer highs of 24^{o}C (lower estuary), 26.5^{o}C (middle estuary), and, 28 ^{o}C (upper estuary). Bottom water median temperatures ranged from 13 - 16 ^{o}C to 24 - 27^{o}C for all regions. Average temperatures showed the greatest annual variation in the upper estuary while the lower estuary had the least variation.

2.2.3. pH

Median pH values in the lower Swan-Canning Estuary range from 7.7 to 8.2 at the surface and 7.8 to 8.0 at the bottom. Middle estuary pH values at the surface are comparable to the lower estuary (7.6 to 8.1); however bottom median pH values (7.3 to 7.7) are lower. Similarly, the median pH values in the upper estuary are generally similar at the surface, but bottom values are lower again ranging from 7.1 to 7.6. During the months of August and September, when freshwater flow is at its maximum, there is little variation between surface and bottom pH values for the middle and upper estuary (Thomson *et al.*, 2001). Phytoplankton blooms can influence pH levels, becoming slightly higher in surface waters during blooms, while when blooms are decomposing surface waters become slightly acidic. Phytoplankton-induced pH changes can have an effect on the behaviour of metals,

causing metal precipitation and the association of metals with biomass or detritus (Admiraal *et al.*, 1995).

2.2.4. Dissolved Oxygen

In winter, the presence of a deep surface layer of fresh water can cause complete exhaustion of oxygen in the bottom waters of the Swan-Canning Estuary (Spencer, 1956; Thomson *et al.*, 2001). Areas of extremely low oxygen levels (<20%) correspond to the patches of water below areas of severe vertical stratification. In spring and early summer, the saline intrusion transport low oxygen water in the bottom waters creating chronic hypoxic conditions, although by March, under slightly stratified conditions, the entire estuary is often generally well oxygenated (Thomson *et al.*, 2001). Twomey & John (2001) reported a negative linear correlation between salinity and dissolved oxygen during both 1980-81 (r = -0.56) and 1994-95 (r = -0.86).

2.3. Pollution sources in the Swan-Canning Estuary

By the early 1990s, the Swan-Canning estuarine basin was showing signs of stress with algal blooms and toxic blue-green bloom events occurring on a frequent basis, and occasional fish deaths. In 1994, the Government of Western Australia initiated a study to investigate the symptoms of deteriorating health of the Swan and Canning Rivers and the estuary, and develop a clean up management plan. In 1999 a government-endorsed clean up plan was released called the Swan-Canning Cleanup Program (SCCP; Swan River Trust, 1999b). The SCCP has primarily focussed on nutrient inputs into the river system from rural and semi-rural land in the upper coastal catchment, as well as from the lower urban metropolitan catchments, that fuel the highly visible algal blooms. In recent years, there have been significant fish kills attributed to algal blooms, such as a large bloom of *Karlodinium micrum* over the months of April to June 2003. This dinoflagellate, which clogs the gills of fish and is believed to release a toxin that ruptures gill cells, resulted in an estimated 300 000 dead fish (Swan River Trust, 2003a). Substantially less attention has been directed at investigating potentially toxic contaminants, such as heavy metals, pesticides, and other organic compounds, and virtually no effort has been directed at considering

whether inputs of non-nutrient contaminants are influencing the estuarine biota's health.

The Swan-Canning Estuary lacks extensive areas of highly elevated contamination that characterise the polluted waterways of Europe, Taiwan and northeastern and southwestern USA. This is due to a lack of heavy industry inputs and intense shipping activity. Apart from nutrient inputs from the catchment, the main threat facing the Swan-Canning Estuary is contaminant inputs from urban stormwater runoff, contaminated groundwater, sewerage spills, boating facilities (mainly recreational), and a high level of leisure boat traffic (Swan River Trust, 1999c).

2.3.1. Urban Stormwater Runoff

2.3.1.1. Surface Run Off

Much of the Swan Coastal Plain comprises poorly drained, deep leaching Bassendean Sands. These soils have a hardpan at depth that generally restricts recharge of surface waters to deeper groundwater aquifers and the resulting waterlogging limits their capacity to retain nutrients in the soil profile. To prevent urban and agricultural areas from flooding in winter due to the near surface hardpan, there is comprehensive system of man made drains and modified streams (Table 2.1).

The coastal catchment of the Swan and Canning Rivers collects drainage from agricultural, trade and industrial areas, commercial premises and, city and urban roads, and gardens (Fig. 2.3). Although there is no heavy industry sited along the shores of the Swan-Canning Estuary, there are light industries including chemical blending works, automotive industries, printing works and food processing. Waste from these licensed industrial and commercial premises is discharged into reticulated sewerage, however, occasionally illegal waste disposal and accidental spills enter the drainage systems discharging to the Swan-Canning Estuary (Swan River Trust, 1999b).

Table 2.1. Examples of major drains of the Swan Coastal Plain (adapted from Thurlow *et al.*, 1986).

Drain Name	Area of Drainage
Spring Street	Lake Monger, Freeway runoff, market gardens, residential, sewered area, central business district.
Claisebrook	Industrial, intensive residential, restaurant areas, sewered.
Peninsula Rd	Residential, sewered, central business district.
King William St	Industrial, residential, some sewered areas.
Kitchener Rd	Light industrial, residential, some sewered areas, disused fertiliser factory.
Brooke Street	Residential, sewered.
Bennett Brook	Mussel Pool, some vineyards, wetlands, rural, some residential, partially sewered.
Ellen Brook	Light agriculture & animal husbandry, Airforce treated sewage.
Jane Brook	Semi-rural, unsewered, Redhill Tip.
Helena River	Semi-rural, sprawling residential, partially sewered, stock-holding areas, old abattoir.
Airport north	Industrial, marshalling yards, skirts airport to the north, partially sewered.
Airport middle	Direct from airport, light stock area.
Airport south	Industrial, marshalling yards, chlorinated sewage from airport, skirts airport to south.
Belmont Creek	Residential, industrial, fast foods, passes rubbish tip, sewered.
Belmont	Industry, residential, mostly sewered.
PCC – Causeway	Industrial, residential, sewered, stormwater.
PCC – McCallum	Residential, groundwater and stormwater flow, sewered.
Manning	Residential, sewered, rubbish tip.
Collier Pine	Residential, sewered.
Wilson	Residential, sewered.
Mills Street Main Drain	Residential, intensive industry, partially sewered.
Wharf Street	Residential, industrial, partially sewered.
Cockram	Residential, industrial, partially sewered.
Lacey Street	Residential, unsewered.
Yule Brook	Residential, hobby farms, light industry, partially sewered.
Bickley Brook	Residential, semi-rural, industrial, partially sewered.
Helm Street	Residential, rural, mostly unsewered, stormwater.
Ellis Brook	Light residential, rural, unsewered.
Southern River	Residential - sewered, rural, Forrestdale lake, Westfield Treatment Plant (closed).
Thornlie Golf	Residential, partially sewered.
Bannister Creek	Light residential, industrial, partially sewered.
Riley Road	Light residential, sewered.
Marjorie Road	Residential, unsewered.
Modillion Ave	Residential, unsewered.
Sixth Ave	Residential, unsewered.
Bull Creek	Residential, small wetland, sewered, large ground water flows.
Brentwood	Residential, sewered, groundwater from Freeway (acidic).
Bayswater Main Drain	High density residential, commercial, light to medium industry, partially sewered.

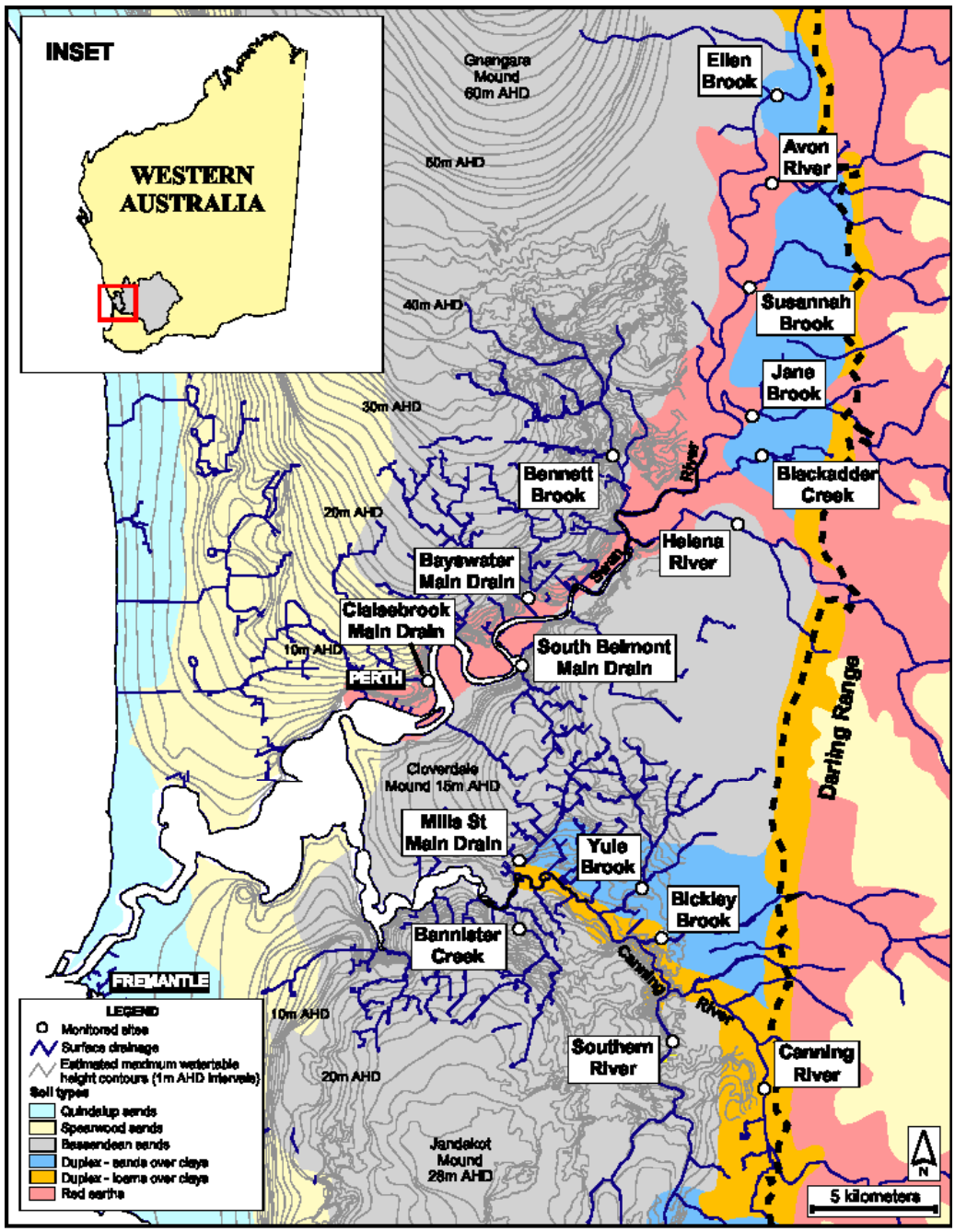

Figure 2.3. *Map of the Swan-Canning estuarine system with monitored catchment sites, soil types and groundwater contours (from Swan River Trust, 2001b)*

The estuary is bounded by an extensive network of sealed road surfaces that deflects water runoff to drains. This runoff carries tyre debris, the wear of asphalt road surfaces, and motor vehicle exhaust fallout. In times of heavy rainfall, the drainage system has the potential to contaminate water, sediment, and biota with heavy metals such as lead, cadmium and copper (Lund, 1992; Gerritse *et al.*, 1998; Swan River Trust, 1999b), polycyclic aromatic hydrocarbons (PAHs), phosphorous, and organochlorines (Gerritse *et al.*, 1995; Stephens and Imberger, 1996; Gerritse *et al.*, 1998; Swan River Trust, 1999b).

The Mills Street Main Drain (MSMD) has been identified as a priority catchment under the SCCP due to its high contribution of nutrients to the estuary. A recent study of the MSMD found elevated concentrations of copper, chromium, lead and zinc in drainage water, particularly during high flows. In addition, high levels of long-chain hydrocarbons have been found in the sediments and dichlorodiphynyltrichloroethane (DDT) and dieldrin were detected during peak storm flows in a compensation basin within the MSMD catchment (Swan River Trust, 2003b). Moreover, a study of heavy metal concentrations in the Bayswater Main Drain (BMD) system found elevated levels of several heavy metals (such as zinc, 7430 mg/L) within a compensation basin in an industrial area of the BMD catchment (DoE, 2003).

2.3.1.2. Groundwater

Leachate from waste disposals sites has the potential to pollute groundwater eventually entering the estuary. This particularly applies to older landfills and rubbish tips (> 10 to 15 years old) that were once located on sandy and riverine soils adjacent to the estuary or next to main drain lines (Thurlow *et al.*, 1986). These sites, which have been closed, still have the potential to leach pollutants into the groundwater (Swan River Trust, 1999b). Leachate production is dependent on the type and stage of decomposition of waste, and the general degradation process. Therefore, leachate may still be entering the estuary via the groundwater many years after the closure of the waste disposal site (Thurlow *et al.*, 1986; Atkins and Klemm, 1987).

In 2001, a fire at Bellevue destroyed a waste control site with a large stockpile of drummed waste chemicals. The large volumes of water applied by fire fighting operations mixed with chemicals, flowed into the stormwater system and discharged on the Helena River floodplain. Sampling of groundwater undertaken several months following the fire found all on-site shallow monitoring bores contained heavy metals, petroleum hydrocarbons, phenols and other semi-volatile organic compounds above expected background levels and in excess of freshwater guidelines (DEP, 2002). This groundwater will eventually enter the estuarine system.

In the autumn of 2003, an assessment of groundwater under a former fertiliser plant found that the water was acidic and had elevated levels of heavy metals. The plume of contaminated groundwater is slowly moving towards the Swan River (Swan River Trust, 2003a). Another study investigated a plume of groundwater containing dissolved phase gasoline that discharges to a estuarine beach on the foreshore of the Canning River. This groundwater contains BTEX (benzene, toluene, ethylbenzene and xylene isomers) compounds and naphthalene originating from an historical and unintentional light non-aqueous phase liquid discharge from an underground storage tank located approximately 80 m from the river's edge (Westbrook *et al.*, 2005).

2.3.2. Sewage Spills

Up to the mid 1930s, treated sewage was discharged directly into the estuary. This practice ceased in 1936 (Atkins and Klemm, 1987) and all sewerage is now diverted through a system of treatment plants, gravity fed pipes and pressure mains to the ocean. Problems with direct sewage discharge now only arise from illegal dumping into stormwater drains and sewage pump station failures (Atkins and Klemm, 1987), or damaged sewer mains.

The Swan River Trust regularly reports on the occurrence of sewage spills from pump station overflows or infrastructure failures. Most incidents are small (30 kL to 600 kL), and although causing temporary closure of sections of the estuary to recreational contact, they are generally considered to have short-term consequences (Swan River Trust, 1997a; 2002; 2003a). However, in October 2004, two and a half million litres of raw sewage poured into the Swan River from a damaged main sewer

pipe (Hewitt, 2004), raising concerns of major oxygen depletion in the water and algal blooms (Swan River Trust, 2004).

2.3.3. Boating Facilities

The Swan-Canning Estuary is used for a wide variety of purposes including fishing, prawning, conservation, tourism, and recreational activities such as boating and other water sports. Boating facilities (including jetties, moorings, and marinas) contribute contaminants to the estuarine environment by the way of fuel spills, bilge water discharges and antifouling paints (Swan River Trust, 1997a; 1999a; 2001a). For example, 1500 litres of sump oil leaked from a yacht club storage tank in 1997 (Swan River Trust, 1999a), 600 litres of diesel was spilt in 2002 at Barrack Street, and additionally, several further incidents of oily bilge discharges and improper refuelling practices have been reported at Barrack Street (Swan River Trust, 2002; 2003a).

2.3.4. Water Craft Traffic

Recreational water craft use on the Swan-Canning Estuary is high, particularly over the summer months, and includes wind surfing, water skiing, jet skiing, canoeing, rowing, yachting, power boating, etc (Spencer, 1987). Apart from erosion, turbulence and noise effects of these craft, marine engine emissions are likely to be of concern. According to the U.S. Environmental Protection Agency, conventional small boat and personal watercraft engines are carburetted two-stroke. With these engines, up to 30% of fuel passes through the combustion chamber unburned or partially burned, thereby being released directly into the water and air as pollution. Petrol discharged to the water elevates concentrations of benzene, naphthalene and other contaminants into the estuary (US Environmental Protection Agency, 2002). In addition, a relatively smaller number of commercial cruise boats, hire craft and ferries operate within the estuarine system throughout the year (Spencer, 1987) potentially releasing contaminants through their vessels.

2.3.5. AtmosphericFfallout

In 1982, the insecticide, toxaphene, was discovered in fish in a lake on Isle Royale in Lake Superior. Isle Royale is a national park with no industry, agriculture, or human

settlements. It was concluded that the chemical pollution of this park can only have occurred via the airborne transport of this chemical (Zakrzewski, 2002).

Globally, the largest emissions of PAHs are into the atmosphere from the incomplete combustion of organic compounds (Walker, 2001). Motor vehicles are a major source of lead and other metals, PAHs and toxic gases discharged to the atmosphere (Connell *et al.*, 1999a). An investigation by Gerritse *et al.* (1995) concludes that PAHs in Ellen Brook, a major tributary of the upper Swan River, are associated with small soot particles that are deposited mainly as dry fallout from combustion processes. On the other hand, according to DAL (1997; cited in Swan River Trust, 1999c) atmospheric fallout is likely not to be significant source of heavy metals in the Swan-Canning Estuary.

2.4. Limitations of Chemical Analysis of Contaminants in the Swan-Canning Estuary

Monitoring of non-nutrient contaminants in the Swan-Canning Estuary has been limited to chemical analysis of sediments, water and biota (Gerritse *et al.*, 1998; Swan River Trust, 1999c; DoE, 2003; Swan River Trust, 2003b). Prior to 1999, there was some data for heavy metal levels within the Swan-Canning system. However, this data was inconsistent for both time and place, and the collection and analysis techniques varied considerably between studies. In addition, the laboratory detection limits for some contaminants were too high to enable comparison with national and international guidelines for water and sediment quality. In 1999, an attempt was made to collate heavy metal and persistent organic data, dating back to 1980, into SCCP Report No. 15 (Swan River Trust, 1999c). This report concluded that apart from some highly localised areas adjacent to drain outfalls and boating facilities; the data were generally below minimum national guidelines for each parameter. However, what the report clearly emphasises was the paucity of data relating to the detection and distribution of potential non-nutrient pollutants in the Swan-Canning estuarine system.

Fish flesh analysis has been primarily concerned purely with the potential risk for human consumption of flesh from these organisms, with little analysis of the

interactions between living organisms and the toxic chemicals in their environment. Accumulation of metals by fish is dependent upon the speciation of metal and the fish organ. For example, inorganic mercury is not taken up by fish but methyl mercury accumulates in the liver and biomagnifies with age and up the food chain. Copper accumulates in the liver and gill, cadmium in the liver, gut, gill and kidney and, lead is found in scales, bone, and other organs with its distribution dependant on whether fish is freshwater, marine or estuarine. The distribution of both zinc and selenium depends on whether exposure was waterborne or via food (Heath, 1995). In addition, a study by Eisler and Gardner (1973; cited in Heath, 1995) found dead fish accumulated more copper and zinc than live fish with regard to waterborne exposure. Dead fish could not excrete the metals and either the skin is extremely permeable to the metals in the dead fish or metals are adsorbed to the mucus that live fish can slough off.

Furthermore, not all toxic chemicals are biologically available. Some pollutants accumulate in living organisms, some attach strongly to sediments particles effectively becoming unavailable to living organisms, while others are available but are rapidly transformed or broken down within living organisms (Heath, 1995; Connell *et al.*, 1999b). Still other chemicals may be broken down abiotically or by microorganisms (i.e. bacteria and fungi) altering the risk that certain pollutants pose. Once transformed, certain pollutants may become benign while others become more toxic than in their untransformed state (e.g., mercury; Connell *et al.*, 1999a; Walker, 2001).

Solar radiation has the potential to increase the toxicity of some pollutants. Dieldrin residues exposed to sunlight can be converted to the highly toxic and stable photodieldren, photo-oxidation of PAHs form highly toxic oxidative products, and the organophosphorous insecticide malathion is converted to highly toxic iso-malathion under the influence of sunlight and/or high temperatures (Walker, 2001).

PAHs are rapidly metabolised by fish and do not bioconcentrate in tissues to any substantive extent. Biotransformation by mixed function oxygenase (MFO) enzymes may alter the toxicity of a compound, which may be either beneficial (i.e. readily eliminated) or harmful (damaging free radicals and adducts). Therefore, PAHs have

the potential to cause adverse effects with no chemical signature (Payne *et al.*, 2003;van der Oost *et al.*, 2003). Prolonged induction of MFO enzymes at relatively high levels can be considered a risk factor for fish health. Induction of enzyme systems is an energy-utilising process. Any energy loss of a non-essential nature could theoretically adversely affect other biochemical and physiological processes (Payne *et al.*, 2003).

To date, all chemical analyses in the Swan-Canning Estuary have been conducted on classic, untransformed contaminants. Consequently, they provide very limited information on the potential effects/toxicity of these contaminants to the biota.

A further limitation of chemical analysis regards the fact that each potential pollutant is measured individually and fails to take into account interactions between them. Arsenic is a compound that accumulates in riverine sediments, and can be taken up by plants. Some plants are sensitive to arsenic while others do not appear to be affected. Studies using the marsh plants *Phragmites australis* and *Urtica dioica* from the Rhine estuary found that the presence of phosphates in the soil has a stimulating effect on the uptake by these plants of arsenic (Otte *et al.*, 1990). This has ecological implications in aquatic ecosystems where both arsenic and phosphates are present [e.g., Swan-Canning Estuary (DoE, 2003)] for the distribution of macrophytes, the biota that feed on them, and the general food web. In addition, a study of the uptake of copper and lead by a fish, the neon tetra (*Paracheirodon innesi*), found synergistic effects between these two metals where lead accumulation in the fish was enhanced by the presence of copper (Tao *et al.*, 1999).

2.5. Ecological Risk Assessment of the Swan-Canning Estuary using Biomarkers of Fish Health

Although the main emphasis of the SCCP is on nutrient inputs from the Avon, upper Swan, and the Canning River catchments, the lack of knowledge regarding the impact of non-nutrient contaminants on the biota in the estuary was recognised (e.g. Recommendation 9.3 in the SCCP Action Plan; Swan River Trust, 1999b). Consequently, with funding provided by the Australian Research Council (ARC) Strategic Partnerships with Industry Research and Training (SPIRT) Scheme, a

laboratory study was conducted in 2000 to identify a fish species as a biomonitor of the Swan-Canning Estuary using biomarkers of fish health. The black bream (*Acanthopagrus butcheri*), a hardy, long-lived, estuarine dependent fish, was chosen as the most suitable candidate from three fish species investigated (Webb and Gagnon, 2002).

The overall objective of this thesis is to investigate the biochemical marker responses of field captured black bream to chronic exposure to non-nutrient contaminants within the Swan-Canning Estuary, a unique ecosystem that undergoes highly variable seasonal physico-chemical conditions. From the field biomarker responses, it will be determined whether (1) biomarkers are stable or reliable enough to monitor the health of the estuarine system, and (2) if the black bream is a suitable species to use under field conditions for a regular monitoring programme on the health of the Swan-Canning Estuary.

The overall objective will be met by addressing the following specific objectives:

1. To optimise and adapt biochemical markers of exposure in black bream to test if this fish is responsive to contamination under field conditions in late winter by:
    i) evaluating MFO enzyme activity in field caught black bream as measured by ethoxyresorufin-*O*-deethylase (EROD) activity;
    ii) evaluating biliary metabolites to ascertain whether the black bream are exposed to potential organic pollutants such as PAHs in the estuary;
    iii) assessing the presence/absence in the blood of the black bream of serum sorbitol dehydrogenase (s-SDH) enzymatic activity, to ensure interpretation of MFO activity in the liver of black bream is not confounded by heptocellular damage; and
    iv) determining if a gradient in biochemical responses exists between the upper and lower reaches of the estuary when freshwater flow is at its strongest.
2. Investigate whether biomarker responses in the black bream differed between late winter and late summer in response to seasonal changes in the physico-chemical conditions within the estuary by:

i) comparing the EROD activity, s-SDH activity in the blood, and the level of PAH biliary metabolites in the black bream measured in late summer with these biomarker responses measured during late winter;
ii) optimising and evaluating the activity of the MFO enzyme, ethoxycoumarin-*O*-deethylase (ECOD) in black bream, under both winter and summer conditions;
iii) comparing ECOD activity to EROD activity in the black bream to evaluate ECOD activity as a complement to, or an alternative to, EROD as a biomarker of exposure to urban contaminants; and
iv) determining if a gradient in biochemical responses exists between the upper and lower reaches of the estuary under summer conditions and comparing any gradient of response with winter conditions.

3. Determine if the patterns of biomarker responses in the black bream within the Swan-Canning Estuary were sufficiently strong to overcome natural annual variability relating to local climatic conditions (i.e., rainfall patterns) and river flow.
4. Investigate the biomarker of effects, stress protein (Hsp70) expression and DNA integrity in the black bream captured in the Swan-Canning Estuary by:
   i) optimising assay conditions for the determination of Hsp70 expression in black bream from the Swan-Canning Estuary;
   ii) determining baseline levels of Hsp70 under laboratory conditions,
   iii) measuring Hsp70 levels in feral black bream from the Swan-Canning Estuary in two different seasons, and evaluating seasonal and spatial variability of Hsp70 expression in black bream;
   iv) optimizing the alkaline unwinding assay to determine DNA strand breakage in the black bream; and
   v) analysing DNA strand breakage and determining whether DNA strand breakage and Hsp70 expression in black bream vary in a similar fashion.
5. Investigate aerobic and anaerobic metabolism in feral black bream using the metabolic enzymes cytochrome C oxidase (CCO) and lactate dehydrogenase (LDH) by:
   i) measuring CCO and LDH activities in liver, white muscle and gill tissues;

ii) comparing enzyme activity levels in the tissues to identify spatial, seasonal or annual variations; and

iii) evaluating the usefulness of metabolic enzyme activity in black bream as a biomarker of effect for the environmental monitoring of contaminants within the Swan-Canning Estuary.

6. Relate biochemical responses measured in the black bream to the chemical content analysed in the sediment and fish flesh to identify possible links between biomarker response and contamination.
7. Select the most appropriate suite of biochemical markers, to incorporate into future, integrated biomonitoring programmes for the assessment of the health of the Swan-Canning estuarine system.

2.6. Sampling for black bream (*Acanthopagrus butcheri*) in the Swan-Canning Estuary

Field sampling within the estuary was first undertaken during August and September 2000, and then again in April/May 2001. In 2000, black bream were collected from six sites in the Swan River and one site in the Canning River (n = 106), while in 2001 five sites in the Swan and two sites in the Canning River (n = 137) were sampled. At the same time these fish were collected, the Swan River Trust undertook sediment chemical analysis from these sites. Additionally, chemical analysis of the flesh from a sub-sample of the fish from each site was undertaken. The results of both these chemical analyses are tabulated in Appendix B of this thesis.

Further fish sampling for biomarker analysis was carried out in 2002, with fish collected from four sites in the Swan River and one site in the Canning River in both the April/May (n = 85) and August/September (n = 97) periods. Sampling periods were chosen to correspond with seasonal trends in river flow. In the April/May period (i.e. the end of the summer season) marine conditions prevail in the estuary and river flow is minimal. In August/September, winter conditions are dominant, when salinity ranges from freshwater in the upper estuary to brackish in the lower estuary, and freshwater flow predominates.

2.7. Study Sites

Black bream were captured from the Swan River, between 6 km and 40 km from the estuary mouth, and in the Canning River, up to 10 km upstream from its confluence with the Swan River (Fig. 2.4). As all areas in the Swan-Canning Estuary have been impacted by human activity (Swan River Trust, 1999b) none of the sampling sites can be considered a reference site. Photographs showing the main features of study sites are presented in Appendix C (Plates 1 to 8).

2.7.1. Helena – sampled 2000, 2001 and 2002 (Plate 1).

The Helena site is about 40 km upstream from the Swan River Estuary mouth, near the mouth of the Helena River and downstream of Bennett Brook. The area is surrounded by developed land with ≤ 15% natural vegetation. The northern banks of the river are highly susceptible to flooding due to the lack of fringing vegetation (Swan River Trust, 1997b). Drainage from roads, bridges, residential properties, hobby farms, light industry and parks enter this site.

2.7.2. Ascot – sampled 2000, 2001 and 2002 (Plate 2).

Approximately 6 km downstream from the Helena site, the Ascot site is bounded by a racecourse and major residential development. This site receives drainage from the International and the Domestic Airports to the south, and to the north, a major drain (Bayswater Main Drain) with a catchment area of 26.2 km^2, brings stormwater and road drainage from high-density residential, commercial and light to medium industrial areas (DoE, 2003). An important arterial road crosses the river at this point.

2.7.3. Belmont – sampled 2000 and 2001 (Plate 3).

Belmont, a further 6 km downstream from the Ascot site, has the Belmont Park Racetrack on it southern banks which was the site of a major pesticide spill (azinphos-ethyl and fenamiphos) in 1997 (Swan River Trust, 1998a). The embankment at Belmont Park Racecourse is almost vertical due to earthworks. In places the landfill is eroding exposing rubble and rubbish and there is very little riparian vegetation (Swan River Trust, 1997b). A recently constructed major arterial road crosses the river near the site. The site receives drainage from high-density

residential and commercial properties, and from a former power station, brickworks and claypits.

2.7.4. Barrack Street - sampled 2000, 2001 and 2002 (Plate 4).

This site is about 20 km from the mouth near the northern bank of a section of the estuary known as Perth Water, a wide sheltered body of water. The Perth Central Business District (CBD) is on the northern shore of Perth Water and significant residential and commercial development lie along the southern banks. Perth Water has undergone many changes since European settlement with river reclamations, channel deepening and riverbank modifications. There is no riparian vegetation and the channel is regularly dredged for navigational purposes. The banks are bound by major roads and a freeway interchange carrying heavy vehicular traffic. The site has jetties from which river ferries operate, moor and conduct maintenance. Barrack Street receives stormwater drainage from the CBD including air conditioning discharges, road run off from its immediate surrounds and the Spring Street Drain, which collects road and stormwater drainage from high-density urban and commercial development up to 8 km north of the estuary. The Swan River Trust annually reports spillages occurring near the Barrack Street Jetty (e.g. 600 litres diesel in January 2002 and 30 kilolitres sewage in February 2002; Swan River Trust, 2002).

2.7.5. Crawley – sampled 2000 (Plate 5).

A further 3 km downstream from Barrack Street is Crawley, near the northern bank of Matilda Bay in a wide basin known as Melville Water. The riparian zone has been greatly altered due to reclamation and the construction of walls (Swan River Trust, 1997b). The estuarine substrate is of a sandy medium in the shallows and fine grey mud in the deeper waters. The northern bank rises steeply forming a limestone escarpment to Mount Eliza. The Royal Perth Yacht Club and University Boat Club are situated in Matilda Bay and the site receives drainage from a busy arterial road and Kings Park and Botanic Gardens (172 hectares of natural bushland set aside as public open space).

Figure 2.4. *Field study sites in the Swan-Canning Estuary (adapted from Swan River Trust, 1998b).*

2.7.6. Freshwater Bay - sampled 2000, 2001 and 2002 (Plate 6).

Natural riparian vegetation in Freshwater Bay has largely been removed or degraded by urban activities (Swan River Trust, 1997b). Freshwater Bay is approximately 6 km from the estuarine mouth, and has two large yacht clubs with slipping facilities servicing both sail and motorised watercraft. The banks are bounded by long established residential development with roads, parks and gardens, which drain directly into the estuary. This site is subjected to tidal flushing with marine waters in winter when strong frontal systems, with low barometric pressure, cross the coast before winter freshwater discharge stops movement further upstream. On passage of the frontal system, rising barometric pressure forces water out again in a seaward direction flushing the lower estuary on a regular basis (Spencer, 1956). This tidal flushing may influence residence times of potential pollutants within this site.

2.7.7. Salter Point – sampled 2000 and 2001 (Plate 7).

Salter Point is situated in the Canning River about 6 kilometres upstream from its confluence with the Swan River. Some ongoing restoration work to replace fringing

vegetation is being undertaken. The land has been infilled and open drains have replaced the original tributaries. The water front has a thin band of riparian vegetation however, in some parts this riparian zone is so narrow adjacent land use directly impacts on the river (Swan River Trust, 1997b). Stormwater drainage directly enters the estuary via drainage pipes from medium-density residential development along with supporting roads, parks and gardens.

2.7.8. Riverton – sampled 2001 and 2002 (Plate 8).

Riverton, 3.5 kilometres upstream from Salter Point in the Canning River, has banks covered in riparian vegetation, which in winter are swampy and waterlogged (Swan River Trust, 1997b). Within the channel are three large islands. River sediments consist of deep, sulphurous black mud often mixed with large bivalve shells. Slightly downstream from the collection area are two bridges carrying road traffic across the river. The site receives drainage directly from surrounding urban roads and gardens as well as the Mills Street Drain, a priority catchment under the SCCP (Swan River Trust, 2003b), and the Wilson Main Drain that brings runoff from surrounding medium to high-density residential and intensive industrial areas.

The above sites include typical habitats of the Swan-Canning Estuary. Although no true reference site can be found in the estuary, these sites receive a variety of contaminants from industry, residential areas and agriculture.

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Chapter 3

Biomarkers of exposure in fish inhabiting the Swan-Canning Estuary, Western Australia – a preliminary study

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Chapter 4

Interseasonal variability in biomarkers of exposure in fish inhabiting a southwestern Australian estuary

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Chapter 5

Interannual variability in fish biomarkers in a contaminated temperate urban estuary

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Chapter 6

Metabolic enzyme activities in black bream (*Acanthopagrus butcheri*) from the Swan-Canning Estuary, Western Australia

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Chapter 7

Stress protein expression and DNA strand breakage in the black bream

7.1. Introduction

Stress (heat shock) proteins (hsps) are involved in the protection and repair of cells following cellular damage due to exposure to a wide variety of stressors, including ultraviolet light, elevated temperatures, salinity, anoxia, pathogens, plankton blooms, heavy metals, and other contaminants such as arsenic, cyanide, pesticides, and polycyclic aromatic hydrocarbons (PAHs.) These proteins are highly conserved across a diverse range of phyla from bacteria to humans. The term *stress protein* does not imply that these proteins are only present in cells under stressful environmental conditions. Some members of the hsp90, hsp70, and chaperonins families (all known as molecular chaperones) are synthesised under normal conditions, where they are involved in the maintenance of proper protein folding and assembly of other cellular proteins (Sanders, 1993; Iwama *et al.*, 1999; Basu *et al.*, 2002).

Under adverse conditions molecular chaperones, such as hsp70, are thought to counter proteotoxic effects by restoring damaged proteins to their native form and vector severely damaged proteins to lysosomes for breakdown (Sanders, 1993; Di Giulio *et al.*, 1995; Iwama *et al.*, 1999; Basu *et al.*, 2002). The common signal for hsp induction appears to be the presence of abnormal proteins (Parsell and Lindquist, 1993). The universality, conservation in structure, and consistency with which hsps are induced by a broad spectrum of stressors make them good candidates for biomonitoring of the environment (Iwama *et al.*, 1998).

The accumulation of hsp70 correlates with acquired tolerance to environmental stressors where continuous exposure to mild stressors enable an organism to survive a more stressful event that would otherwise be lethal (Iwama *et al.*, 1999; Bierkens, 2000). However, while small to moderate increases in hsp70 expression may

enhance an organisms ability to survive stressful conditions, high concentrations of hsp70 levels may be adverse and directly interfere with ongoing cellular processes, or otherwise alter function to the detriment of the cell (Feder and Hofmann, 1999).

Studies have shown that the expression of hsp70 in rainbow trout is not affected by handling stress, suggesting that the capture, handling and transport of fish for environmental monitoring purposes does not interfere with the use of stress proteins as biomarkers (Vijayan *et al.*, 1997; Washburn *et al.*, 2002). However, the use of stress protein response as a biomarker of effect in fish for on-site ecosystem biomonitoring does need to take into account disease-related expression, the effects of multiple stressors, seasonal variations, and the acquisition of tolerance to low background concentrations of chemicals by a species (Feder and Hofmann, 1999; Bierkens, 2000).

Hsp70 expression in biomonitoring may be of value when combined with markers of genotoxicity such as DNA strand breakage. Schröder et al. (2000) found a highly significant correlation between hsp70 levels and DNA damage in field caught feral dab (*Limanda limanda*).

DNA is present in cells as a functionally stable, double-stranded polymer without breakages, or abnormal structural modifications. It is complexed with proteins in a chromosomal structure and has high integrity (Shugart, 1995). DNA damage, such as strand breakage, can occur as a result of wear and tear of normal cellular activity, by environmental agents such as heat, UV light, or by genotoxic chemicals; however, they are usually repaired rapidly (Shugart, 1988; Theodorakis *et al.*, 1994; Liepelt *et al.*, 1995; Shugart, 1995; Sugg *et al.*, 1995). Newsted and Giesy (1987) (cited in Steinert *et al.*, 1998) have shown that a number of PAHs are acutely toxic at concentrations below their aqueous solubilities when exposed to solar radiation at levels commonly found in the environment. A study using mussels in San Diego Bay found high levels of DNA damage was induced in mussels exposed to PAHs with low intensity ultraviolet light (Steinert *et al.*, 1998). Chemically induced strand breaks are produced either directly by the toxic chemical (or its metabolite) or by the processing of structural damage (i.e. excision repair enzymes; Shugart, 1996; Lee and Steinert, 2003).

DNA strand breaks are potentially pre-mutagenic lesions (Kammann *et al.*, 2001) and may provide a meaningful indication to the degree of oxidative damage of the DNA (Imlay *et al.*, 1988). They have been induced in fish by *in vivo* and *in vitro* exposure to many chemicals such as benzo(*a*) pyrene (BaP), polychlorinated biphenyl (PCB), metals, menadione, and nitrofurantoin (Everaarts, 1995; Sugg *et al.*, 1995; Shugart, 1996; Mitchelmore and Chipman, 1998). The measurement of excessive DNA strand breakage as an environmental biomonitor to measure genotoxicant insult was adopted by the U.S Department of Energy in 1987 for two reasons. Firstly, the alkaline unwinding assay for this type of damage is easy to perform and cost effective and secondly, the assay provides a measure of DNA strand breaks arising from several contaminant-mediated processes (Shugart, 1996). DNA lesions potentially reflect cellular damage with hsps representing an acute response (Schroder *et al.*, 2000).

This study investigates the expression of hsp70 protein and DNA strand breakage in the black bream (*Acanthopagrus butcheri* Munro) a hardy, long-lived estuarine dependent resident in the Swan-Canning Estuary in southwest Western Australia. The Swan River and its major tributary, the Canning River, run through the city of Perth, Western Australia's capital city. Approximately 1.5 million people live in the Perth metropolitan area, with most activity close to the estuarine portion of the river system. The Swan-Canning Estuary has a free connection with the ocean at Fremantle (Fig. 7.1) and experiences a seasonal freshwater discharge from land drainage. The physico-chemical conditions within the estuary are extremely seasonal (Twomey and John, 2001). During the wet winter months freshwater discharge dominates any tidal influence on the movement of marine waters throughout the estuary, while during the hot dry summer freshwater flow ceases and marine conditions prevail. Estuarine conditions can extend inland up to 60 km from the mouth of the Swan River (Stephens and Imberger, 1996).

The Swan-Canning Estuary lacks the extensive areas of highly elevated contaminants that characterise the polluted waterways of Europe, Taiwan, and northeastern and southwestern USA due to a lack of heavy industry inputs and intense shipping activity. The main problem facing the Swan-Canning Estuary is contaminant input

from urban stormwater runoff, sewerage spills, boating facilities (mainly recreational), and a high level of boat traffic (Swan River Trust, 1999b). Direct discharge, surface runoff, and groundwater contribute:

- Pesticides and fertilisers from vineyards, market gardens, parks and recreational areas and residential properties.
- Heavy metal and hydrocarbons (fuel and oil) inputs from residential, commercial, and light industrial areas via spills, illegal waste disposal, and washdowns.
- Runoff from an extensive road network within the estuarine catchment of heavy metals and hydrocarbons from tyre debris, exhaust fallout, and vehicle spillage.
- Wastewater from air-cooling towers containing biocides, anti-corrosives, and anti-scaling chemicals, particularly from the Perth Central Business District.

Examination of the Swan River Trust annual reports since 1997 show that sewage spills from pump station overflows or infrastructure failures occur regularly. Most incidents are small (30 kL to 600 kL), and although causing temporary closure of sections of the estuary to recreational contact, are generally considered to have short-term consequences (Swan River Trust, 1997; 2002; 2003a). However, in October 2004, two and a half million litres of raw sewage poured into the Swan River from a damaged main sewer pipe (Hewitt, 2004), raising concerns for major oxygen depletion of the water and algal blooms (Swan River Trust, 2004). Slipping operations and boating facilities contribute hydrocarbons from fuel spills and wastes from the servicing, maintaining and retrofitting of boats (Swan River Trust, 1999b).

The effect of these contaminant inputs on the health of biota within the estuary is poorly understood. Consequently, this study aims to determine baseline levels of hsp70 under laboratory conditions, measure hsp70 levels in feral black bream from the Swan-Canning Estuary in two different seasons, and evaluate seasonal and spatial variability of hsp70 expression in black bream. Analysis of DNA strand breakage was conducted in parallel to determine whether DNA strand breakage and hsp70 expression in black bream vary in a similar fashion. Finally, the usefulness of hsp70

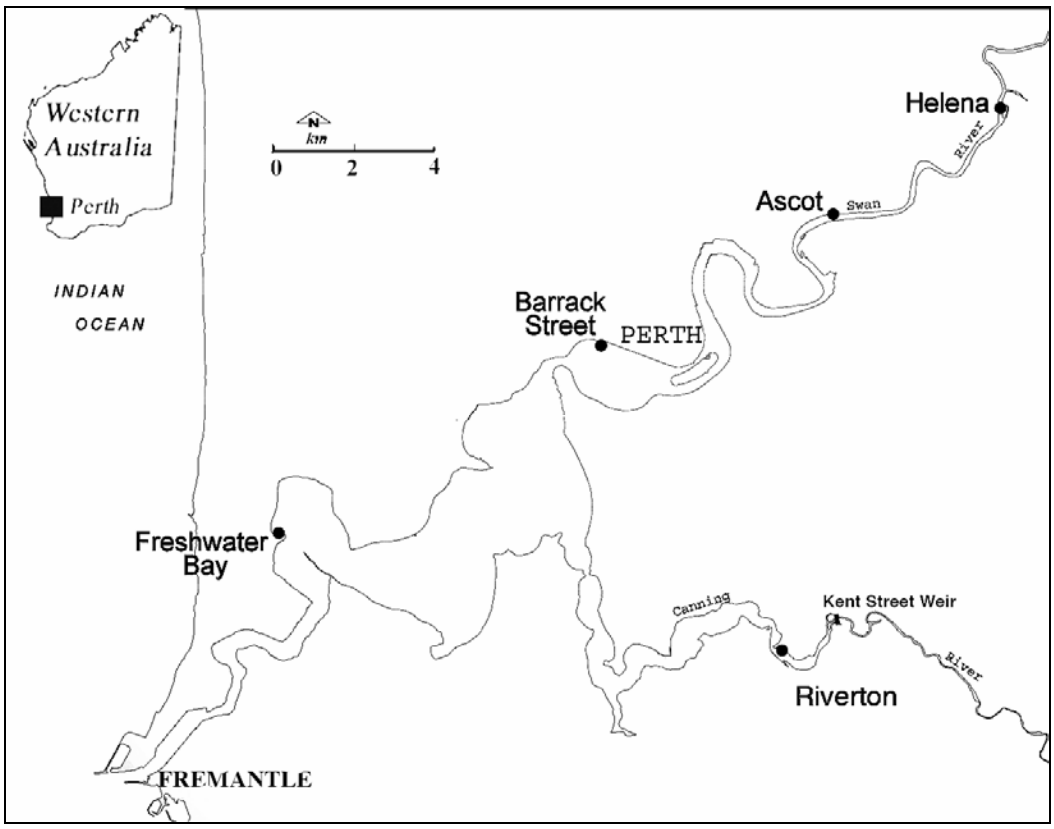

Figure 7.1. *Field collection sites within the Swan-Canning Estuary (adapted from Swan River Trust, 1999a).*

expression in black bream as a biomarker of effect in environmental monitoring of contaminants in the Swan-Canning Estuary is evaluated.

7.2. Methods

7.2.1. Sampling Stations

Feral black bream were collected during late summer (April – May) 2002 and again in the late winter months (August and September) 2002, from four sites in the Swan River ($N = 32$) and one site in the Canning River ($N = 8$; Fig. 7.1).

The following sites were sampled:

Helena: (Appendix C; Plate 1) This site is surrounded by developed land with < 15% natural vegetation. Drainage from roads, bridges, residential properties, hobby farms, light industry, and parks enter this site.

Ascot: (Appendix C; Plate 2) This station has a racecourse adjoining the southern bank, and receives drainage from the International and the Domestic Airports to the south, and a major drain (Bayswater Main Drain) with a catchment area of 26.2 km^2, brings stormwater and road drainage from high-density residential, commercial, and light to medium industrial areas to the north (DoE, 2003). An important arterial road crosses the river at this point.

Barrack Street: (Appendix C; Plate 4) This site lies near the northern bank of a section of the estuary known as Perth Water, has the Perth Central Business District (CBD) on its northern shore and significant residential and commercial development along the southern banks. Perth Water has undergone many changes since European settlement with river reclamations, channel deepening, and riverbank modifications. It is regularly dredged for navigational purposes. The banks are bound by major roads and a freeway interchange carrying heavy vehicular traffic. The site has jetties from which river ferries operate, moor, and conduct maintenance. Barrack Street receives stormwater drainage from the CBD including air conditioning discharges, road run off from its immediate surrounds, and the Spring Street Drain, which collects road and stormwater drainage from high-density urban and commercial development up to 8 km north of the estuary. The Swan River Trust annually reports spillages occurring near the Barrack Street Jetty (e.g. 600 litres diesel in January 2002 and 30 kilolitres sewage in February 2002; Swan River Trust, 2002).

Freshwater Bay: (Appendix C; Plate 6) This site is approximately 6 km from the estuarine mouth, and has two large yacht clubs with slipping facilities servicing both sail and motorised watercraft. The natural vegetation in Freshwater Bay has largely been removed or degraded by urban activity. The banks are bounded by long established residential development with roads, parks, and gardens, which drain directly into the estuary. This site is subjected to tidal flushing with marine in winter when strong frontal systems, with low barometric pressure, cross the coast before winter river flow stops the movement of marine water further upstream. On passage of the frontal system, rising barometric pressure forces water out again in a seaward direction flushing the lower estuary on a regular basis, even during the wet season (Spencer, 1956). This tidal flushing may influence residence times of potential pollutants within this site.

Riverton: (Appendix C; Plate 8) This site is situated in the Canning River, about 9.5 kilometres upstream from its confluence with the Swan River. The sediments at this

site consist of thick, sulphurous, black mud. Slightly downstream from the collection area are two bridges carrying road traffic across the river. The site receives drainage directly from surrounding urban roads and gardens as well as the Mills Street Drain. The Mills Street Drain catchment brings runoff from surrounding medium to high-density residential and intensive industrial areas.

7.2.2. Animals

Indigenous black bream were captured by a commercial fisherman using a 120 m, 100 mm monofilament haul net and sacrificed within 2 hour of capture. Each fish was killed by the method of *Iki Jimi* (spike through the brain), the gills and liver taken and immediately immersed in liquid nitrogen then stored at –80 ^{o}C until analysis.

Juvenile hatchery bred black bream (N = 10) were purchased from the Fremantle Maritime Centre. Five fish were placed into each of two 100 L aquariums and acclimated in seawater (35 ppt) at 17^{o}C (± 0.03) for 10 days. The aquariums were provided with a sponge filter and aeration via an airstone. Ammonia levels were monitored daily to ensure water quality was maintained. At the end of the acclimation period 5 black bream were transferred to a 100 L aquarium with seawater heated to 27^{o}C (positive controls), kept under observation for signs of excessive stress, and then returned to their original aquarium (17^{o}C) at the end of 1 hour. The second group of 5 fish were captured, then immediately returned to their aquarium (17^{o}C; negative controls); then, one hour later these fish were again netted to ensure both groups of fish experienced the same number of capture events. Twenty-four hours following heat shock treatment all fish were sacrificed, gills removed from all black bream and livers were collected from the 5 negative control fish. All samples were immediately immersed in liquid nitrogen then stored at –80 ^{o}C until analysis.

7.2.3. Stress Protein Assay

Stress protein response was measured using the methods of Martin *et al.* (1996) optimised for black bream. Gill tissue was weighed, added to chilled homogenisation buffer [10mM Tris buffer, 0.1M phenylmethylsulfonylfluoride (PMSF) stock] at a 1:4 w/v ratio, and homogenised until completely broken down

(approx. 30 secs) using a Heidolph DIAX 900 homogeniser. The homogenate was centrifuged (Jouan CR3i centrifuge) at 12 000 rpm for 98 mins at 4^{o}C. The supernatant was collected, two 20 μL aliquots set aside for protein determination, and remainder stored at -80^{o}C in aliquots of 100 μL until analysis. Protein content of the supernatant was determined according to Lowry *et al.* (1951).

Supernatant containing ≅ 40 μg of protein was mixed with sample buffer (1 part of 2-mercaptoethanol to 19 parts Laemmli Sample Buffer; Bio-Rad Laboratories Pty Ltd, Regents Park, NSW, Australia) at a ratio of 1:2 supernatant/buffer then placed in a water bath at 95^{o}C for 4 min. Samples were loaded in duplicate into 12% Tris-Glycine iGels (Life Therapeutics, French Forest, NSW, Australia) wells with heat shock standardised control samples loaded into the two outermost wells. The gels were run at 225 V, 120mA (60 mA per gel) for 40 min in a Mini-PROTEAN 3 electrophoresis Cell (Bio-Rad) with power supplied by a Powerpac 300 (Bio-Rad).

Proteins were transferred from the iGels to 0.2 μm supported nitrocellulose membranes in a Mini Trans-Blot Electrode Module (Bio-Rad) at 100 V, 250 mA for 1 hour. Following Western Transfer the blots were blocked in 5% skim milk powder dissolved in Tween-Phosphate Buffered Saline on a shaker for 1 hour. The blots were probed overnight at 4^{o}C with Monoclonal (Mouse) Anti-Heat Shock Protein 70 Antibody (IgG; Bio-Scientific, Gymea, NSW, Australia), diluted 1:5 000 in Tris Buffered Saline (TBS), then the secondary antibody (Goat Anti-Mouse IgG Peroxidase conjugated; Progen Bioscience, Archerfield, QLD, Australia) was applied, diluted to 1:30 000 in Tween-TBS (TTBS) and allowed to incubate for 2 hours. Between each step, the blots were washed three times with TTBS then finally washed in TBS to remove the Tween.

A working solution of chemiluminescent substrate was prepared by mixing equal volumes of Luminol/Enhancer Solution and Stable Peroxide Solution (Super Signal® West Pico Chemiluminescent Substrate Kit; Progen Bioscience). In a dark room, each blot was wetted with the working solution at the rate of 0.125 mL cm^{-1} of blot surface and incubated for 5 minutes. Following incubation the blot was inserted between plastic sheets, air bubbles removed, placed against auto radiographic film

(CL-XPosure™ X-Ray Film; Progen Bioscience), and exposed for 90 seconds. The film was developed using a 245S X-Ray Processor at a working temperature of 31^{o}C.

Films were scanned using Adobe Photoshop 7.0, saved as TIF files, and the pixel density of the images analysed using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/). The bands on different blots were calibrated to known standards to enable comparison between blots. Hsp70 levels in the black bream are expressed as pixel density per μg of total protein (pixels μg pr^{-1}).

7.2.4. DNA Alkaline Unwinding Assay

DNA strand breaks were determined using the methods of Shugart (1996), which were optimised for black bream. All samples, solutions and equipment were maintained at 4 ^{o}C throughout sample preparation.

Approximately 100 mg of liver was homogenised in 2 mL of DNAzol® (Astral Scientific, Caringbah, NSW, Australia) using a glass/Teflon handheld homogeniser (≅ 10 strokes). The homogenate was centrifuged at 14 000 rpm for 12 mins (Jouan CR3i centrifuge). The supernatant was removed, 100% ethanol added, and mixed to precipitate the DNA. The supernatant/ethanol solution was centrifuged at 11 000 rpm for 5 mins. Excess ethanol was removed leaving the isolated DNA to which 75% ethanol was added and the DNA cleaned by passing in and out of a Pasteur pipette then centrifuged at 4 000 rpm for 2 mins. The cleaning stage was repeated once and the excess ethanol again removed. The DNA pellet was solubilized by adding G-50 Buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM $MgCl_2$, 0.5 mM EDTA) and passing the DNA in and out of a pipette. This final step was performed at room temperature.

The solubilized DNA was added to each of three chilled test tubes (in duplicate). To one of the duplicates, 100 μL of 25 mM NaCL, 5 μL 0.2% SDS (Sodium dodecyl sulfate) in 2 mM EDTA, 3 mL 0.2M Potassium Phosphate buffer pH 6.9, and 3 μL Hoechst Dye 33258 (Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia) were added and incubated for 15 mins at 4 ^{o}C. The fluorescence of the double stranded

DNA (DS) present in the sample was read on a Perkin-Elmer LS-5 Luminescence Spectrometer at excitation/emission wavelengths of 350ex/453em nm (slit 10-ex/10-em).

The remaining duplicate samples had 50 μL of 0.05 M NaOH added. One of these duplicates was incubated in a water bath at 35 ^{o}C for five mins to partially unwind the DNA (DSS) and the other duplicate was incubated at 85 ^{o}C for 30 mins to obtain single stranded DNA (SS). Following incubation, 50 μL of 0.05 M HCl was added to neutralise the solution, followed by 5 μL 0.2% SDS in 2 mM EDTA. The DNA was sheared by forcefully passing the sample 5 times through a 23-gauge needle. To the sheared DNA, 3 mL 0.2 M Potassium Phosphate buffer pH 6.9 and 3 μL Hoechst Dye 33258 were added and the fluorescence read (350 ex/453 em) following incubation at 4 ^{o}C for 15 mins.

Hoechst dye 33258 binds with double-stranded DNA in solution. When DNA is in the single-stranded form, the intensity of fluorescence of the bound dye is reduced to approximately one half of that observed for double-stranded DNA. This development constitutes the basis for determining the amount of double- and single-stranded DNA present in a sample of DNA during the alkaline unwinding assay (Shugart, 1996). The ratio of double-stranded DNA in the sample (F value) was calculated using the equation, F = (DSS - SS)/ (DS - SS); where DSS is the measured fluorescence of partially unwound DNA (incubated at 35^{o}C for 5 mins); SS is the measured fluorescence of single-stranded DNA (incubated at 85^{o}C for 30 mins); and, DS is the measured fluorescence of the double-stranded DNA. In summary, the F value is a measure of DNA integrity with a high value corresponding to high DNA integrity and vice versa.

7.2.5. Statistical analysis

For each biomarker, the data were tested for normality and homoscedasticity. Hsp70 data required log transformation to achieve normality. Data were compared using the one-way analysis of variance (ANOVA), and where significant differences between sites were found ($p \leq 0.05$), Tukey's W test was performed to identify differences between the means. Pearson product-moment correlation analysis was

performed to investigate if a relationship exists between hsp70 expression and the F value. Data are presented as mean ± standard error (SEM) and analysis was done using the SPSS statistical package (Version 10 for Macintosh, 2001; SPSS GmbH, München, Germany).

7.3. Results

Water parameters in the field sampling are shown in Table 7.1. Physical examination of the fish revealed the following anomalies:-
Helena – one black bream in winter samples had a fishhook through the heart ventricle, Barrack Street – 25% had gill fluke infestations in winter 2002, *Freshwater Bay* – 25% in summer and 60% in winter 2002 had gill flukes, *Riverton* – 38% had gill flukes in summer 2002 and 40% had red spot disease in winter.

7.3.1. Stress Proteins

Hsp70 expression in laboratory heat shocked black bream was significantly higher than the untreated control fish ($p = 0.01$). However, the hsp70 levels in laboratory positive control fish (heat shocked) were much lower than hsp70 expression in feral black bream from all field samples ($p = 0.01$; Figure 7.2). No significant differences were recorded in hsp70 expression between field sites in summer ($p = 0.91$) or winter ($p = 0.83$). In addition, no significant seasonal differences were found between summer and winter at any site within the estuary ($p > 0.05$).

7.3.2 DNA damage

Significantly, fewer strand breakages were measured in the laboratory control black bream compared to the field collected fish ($p = 0.02$). No significant inter-site differences were found in DNA strand breaks in either summer ($p = 0.16$) or winter ($p = 0.15$). No seasonal differences were found at any of the sites for DNA strand breaks in feral black bream ($p > 0.05$; Fig. 7.3).

Table 7.1. Water parameters at sites at collection times.

Site	Summer 2002			Winter 2002		
	Temp (^{o}C)	Salinity (ppt)	pH	Temp (^{o}C)	Salinity (ppt)	pH
Helena	19.5	18.9	7.9	15.7	2.8	7.9
Ascot	23.1	24.3	8.2	18.2	3.9	7.8
Barrack Street	19.5	34.0	7.6	18.0	9.1	8.1
Freshwater Bay	21.2	31.5	7.7	17.0	21.6	8.3
Riverton	17.0	7.6	7.6	18.9	1.4	7.9

Note: Measurements taken in water column at approximately 2-meter depth.

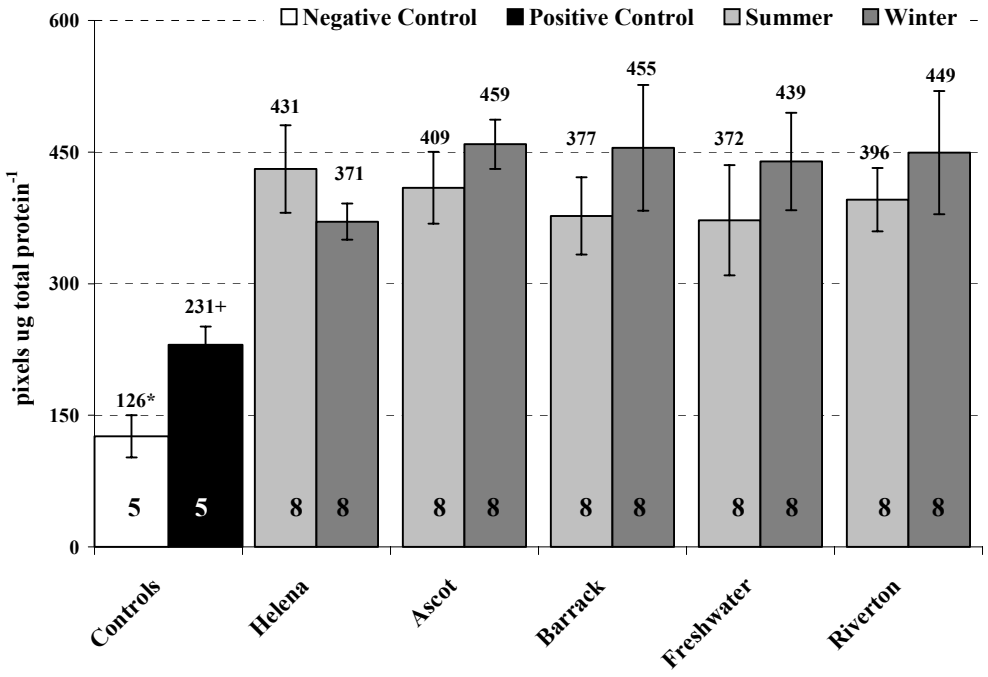

Figure 7.2. *Hsp70 expression (mean ± SEM) in pixels μg protein^{-1} in black bream (Acanthopagrus butcheri). Laboratory control fish are significantly different from all field-collected animals. Numbers within the bars represent the number of fish.*

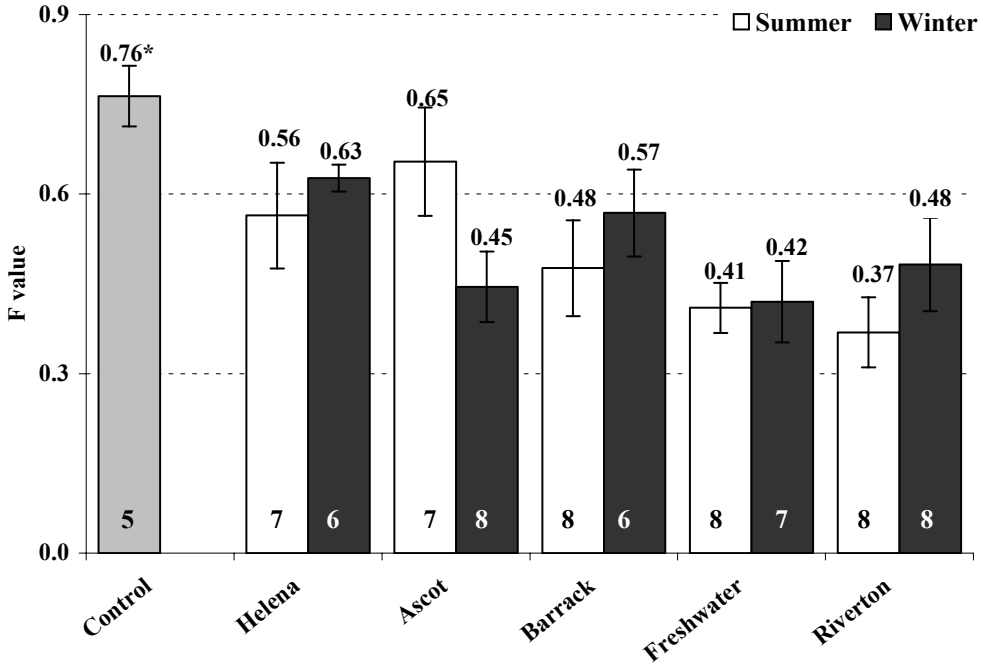

Figure 7.3. *DNA integrity (mean ± SEM) expressed as the F value in black bream (Acanthopagrus butcheri). A higher F value corresponds to higher DNA integrity. Laboratory control fish are significantly different from all field-collected animals. Numbers within the bars represent the number of fish.*

7.3.3 Correlation analysis

A significant negative correlation was identified between hsp70 expression and F values in field captured feral black bream in summer (r = -0.66, $p < 0.001$) and in winter (r = - 0.77, $p < 0.001$; Fig. 7.4).

7.4. Discussion

There are no areas of the Swan-Canning Estuary which have not been impacted by anthropogenic activities (Swan River Trust, 1999a) therefore no valid reference sites were available. Consequently, an attempt was made to determine baseline levels for both biomarkers using hatchery-bred juvenile fish under laboratory conditions.

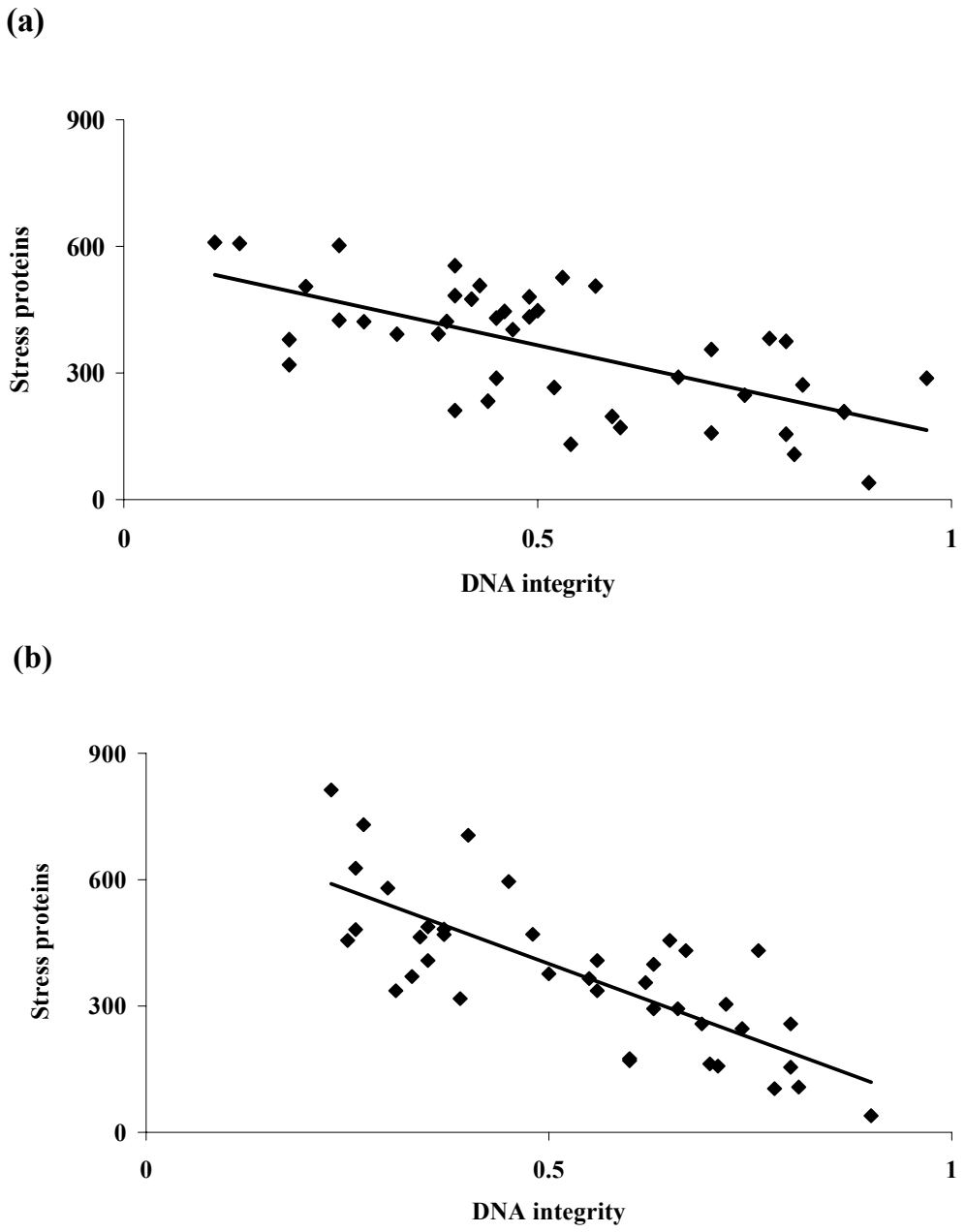

Figure 7.4. *Relationship between hsp70 level (pixels μg protein^{-1}) in gills and DNA integrity (F value) in the liver of black bream (Acanthopagrus butcheri) collected from five sites in the Swan-Canning Estuary in 2002. (a) correlation between hsp70 and DNA in late summer (April-May 2002). (b) correlation between hsp70 and DNA in late winter (August-September 2002).*

Using laboratory bred juvenile black bream as controls for biomarker responses in field caught adult black bream is problematic. Observed stress responses in fish are an expression of both genetic and environmental factors such as season, rearing history and nutritional state (Iwama *et al.*, 1999). The laboratory raised black bream used in this study were from parental stock that originated from the Swan-Canning Estuary, therefore from stock with a similar genetic make up. However, the laboratory fish were bred and reared in solid walled vats (seawater 35 ppt) and fed at regular intervals on a formulated pelleted food while the feral black bream were captured in a highly seasonal estuarine environment. Differences in food type and availability (Williams *et al.*, 1996), the accumulation of hsp70 (Bierkens, 2000) and enhanced DNA repair mechanisms (Bombail *et al.*, 2001) due to long term chronic exposure (acquired tolerance) of black bream to low level contaminants within the estuary need to be accounted for.

In addition, the laboratory raised black bream originated from a relatively stable environment prior to transportation to smaller glass tanks in the experimental laboratory. These fish underwent substantially greater handling than the field caught black bream. It has been shown that the stress hormone adrenaline at physiological (10^{-7} M) and pharmacological (10^{-5} M) levels caused an increase in hsp70 concentration in primary cultures of rainbow trout hepatocytes (Mazur, 1996 cited in Iwama *et al.*, 1998). However, the relationship between stress hormones and hsp70 induction may be modulated by *in vivo* factors as Mazur is also reported as finding that the handling of rainbow trout significantly reduced the induction of both hsp30 and hsp70 (Mazur, 1996 cited in Iwama *et al.*, 1998). However, neither Vijayan *et al.* (1997) nor Washburn *et al.* (2002) found any affect on hsp70 by the physical handling of rainbow trout prior to experimentation. Both the negative and positive control laboratory reared black bream were subjected to similar treatment. As there was a significant increase in hsp70 expression on the heat shock control fish compared to the negative control fish it is concluded that handling stress did not significantly influence hsp70 expression in the black bream.

A study of four fish species, found significant seasonal differences in hsp70 expression indicating that caution was needed when interpreting 'normal' levels of heat shock proteins (Fader *et al.*, 1994). The Swan-Canning Estuary experiences

highly seasonal conditions varying from hyper-saline conditions in late summer to freshwater conditions in late winter (Spencer, 1956; Twomey and John, 2001). Water parameters within the estuary were within expected ranges during 2002 with the exception of Riverton in May where salinity was much lower. This was the result of a severe thunderstorm event two days prior to sampling, which resulted in heavy localised runoff at Riverton (BOM, 2003) with surface salinity of only 7.6 ppt. Notwithstanding the high seasonality of the Swan-Canning Estuary, no seasonal differences were detected in hsp70 expression or DNA strand breaks in black bream under field conditions. The black bream is a moderately mobile fish (K. Littleton, professional fisher, pers. comm.) within the estuary. This could account for the similarities in hsp70 in the fish and preclude it as a biomarker for inter site differences in the black bream; however, hsp70 could still have a value as a biomarker in the event of a major event affecting local sections of the estuary.

With the exception of hsp70 expression in summer, no downstream/upstream gradient existed in either biomarker indicating that there are multiple sources of contaminant input into the estuary. Hsp70 expression in the black bream in the summer sampling tends to decline moving downstream from Helena to Freshwater Bay in the Swan River. However, this gradient of response was statistically insignificant.

DNA integrity in black bream was lower, although not significantly, at Riverton and Freshwater Bay in both seasons and at the Ascot station in winter when compared to the remaining field sites. Riverton is near a major drain (Mills Street Drain) bringing runoff from nearby urban and industrial areas. A recent study of water and sediments within this drainage system identified elevated concentrations of arsenic (0.02 mg L^{-1}) copper (0.06 mg L^{-1}), chromium (0.13 mg L^{-1}), lead (0.12 mg L^{-1}) and zinc (1.2 mg L^{-1}) in drainage water, particularly during high flows (Swan River Trust, 2003b). The organochlorine pesticides dichlorodiphynyltrichloroethane (DDT) (39 ng L^{-1}) and dieldrin (4 ng L^{-1}) were detected during peak storm flows in the one of the drain's compensation basins. Additionally, toluene (0.6 – 2.7 µg L^{-1}), trimethylbenzene (0.8 – 12.0 µg L^{-1}) and xylene (2.2 µg L^{-1}) were detected in parts of the drainage system and a "few" sampling sites had elevated long chain petroleum hydrocarbons (C10 – C28) at 65 – 630 µg L^{-1} (Swan River Trust, 2003b). Sediment

sampling conducted at Barrack Street in September 2000 detected high levels of lead (656 ppm), zinc (773 ppm), chlordane (0.053 ppm), dieldrin (0.062 ppm) and ∑DDT (0.23 ppm; DoE, unpublished data).

About 40% of the fish from the Riverton site had 'red spot' disease (i.e., epizootic ulcerating syndrome), a bacterial infection that appears during late winter. According to a local commercial fisherman, up to 90% of black bream in the Canning River, and 10% in the Swan River, have this condition during late winter in some years (K. Littleton pers. comm.). The occurrence of this condition is evidence that the fish in the estuary are stressed, although the condition does not appear to be fatal with many fish captured showing scars from past infection. The fish from Freshwater Bay had a high incidence of parasitic copepod (gill fluke) infestations in the gills, particularly in winter 2002. However, the lack of site differences or trends in the biomarker results suggest that neither of these conditions were responsible for the hsp70 levels recorded or the lower F values in the field caught fish to any significant extent.

Correlations between chemical concentrations and biomarker responses would provide evidence of effect even without a reference location however, chemical analysis for organophosphates, PAHs and BTEX within the estuary have consistently failed to find these compounds above their detection limits (<0.01 ppm, <1.0 ppm and <0.5 to <2.5 respectively; DoE, unpublished data). In contrast, analysis of the biomarker of exposure, biliary metabolite levels, has shown that the black bream in the Swan-Canning Estuary are exposed to, and metabolizing, the PAHs - naphthalene, pyrene, and benzo(*a*)pyrene (Webb and Gagnon, 2002). Biomarker levels measured indicated that there are multiple sources of inputs of PAHs along the length of the estuary, and that the proximity of major roads and drains into the Swan-Canning Estuary is the most significant factor influencing these biomarker responses in the black bream following winter rains.

There is a significant negative correlation between the level of hsp70 in the gills and the F values representing DNA integrity of the liver. This can indicate that the elevated level of hsp70 in the feral black bream is due to factors other than acquired tolerance and that the fish are stressed due to contaminant exposure. However,

observed stress responses in fish can have many causes such as adverse water conditions (extreme conditions of pH, dissolved oxygen, temperature, etc), contaminant exposure (metals, chlorines, phenols, PCBs, hydrocarbons, and pesticides) or plankton blooms especially under hypoxic conditions (Iwama *et al.*, 1999). The alkaline unwinding assay does not differentiate between single and double DNA strand breaks with exposure to chemical pollutants causing single strand breaks, while ionising radiation causes both double and single strand breaks (Shugart, 1996). The data obtained from the field in this study suggest that further investigation is required to determine if the elevated stress in the black bream is the result of natural stressors, anthropogenic contamination or both, by way controlled laboratory experiments into the expression of hsp70 and DNA stand breaks in black bream relating to defined stressors. The use of the comet assay under alkaline conditions to evaluate single-strand DNA breaks may be of value to differentiate between environmental and chemically induced DNA damage (Singh *et al.*, 1988; Shugart, 1996). However, a study using butterfish (*Pholis gunnellus*) in Scotland concluded that the comet assay is not a suitable genotoxicity biomarker compared to the measurement of nuclear anomalies in the cells in field studies (Bombail *et al.*, 2001).

The interpretation of the biomarker levels recorded in this study has a number of limitations. Firstly, hsp70 and DNA integrity were measured in different tissues, which was due to two factors; (1) insufficient liver tissue from each fish to enable hsp70 measurement to be conducted following the analysis of mixed function oxygenase enzyme activity, metabolic enzyme and DNA strand break analyses, and (2) the loss of all sample material following the failure of the -80^{o}C freezer in which the samples were stored. Secondly, the use of hatchery-raised juvenile fish as a control introduces an age variable to the project design as well as important differences in life histories of these fish and the adult field captured fish. Finally, only five fish were used for each of the controls and they had been depurated in the laboratory for only one week, which may not have been long enough to establish baseline levels for hsp70 and DNA integrity.

This study has shown that hsp70 levels in field collected black bream appear to be elevated, that DNA integrity is compromised and there is a correlation between

hsp70 levels in the gills and DNA strand breaks in the liver, when compared to hatchery-raised, juvenile black bream. As mentioned above the use of hsp70 as a biomarker of effect is problematic. However, if used in conjunction with a suite of biomarkers such as bile metabolites, mixed-function oxygenase activity, metabolic enzymes, DNA damage and other hsp isoform expression, hsp70 may have a value as a tier 1 biomarker.

7.5. References

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Chapter 8

General Discussion and Conclusions

8.1. Black bream (*Acanthopagrus butcheri*) as a bioindicator species

The black bream was chosen as a bioindicator species into the health of the Swan-Canning River system because it is a native to Western Australia. The black bream is a large, extremely hardy, long-lived, relatively abundant fish confined to the estuarine region of the river system. Its biology is relatively well known (Loneragan *et al.*, 1987; Chaplin *et al.*, 1998; Sarre and Potter, 1999; Sarre *et al.*, 2000) and it has been shown to be responsive to contaminants (Webb and Gagnon, 2002). Black bream forms the basis of a large amateur rod and line fishery in the estuary (Loneragan *et al.*, 1987) and has been successfully hatchery bred so juveniles and sub adults are available for both aquaculture and research purposes. The fish is a multiple spawner typically reproducing between September and December each year (Sarre and Potter, 1999). In the Swan-Canning Estuary, its diet consists mainly of benthic filter organisms such as molluscs, crustaceans and polychaetes along with the red algae *Gracilaria verrucosa* (Sarre *et al.*, 2000).

Feral black bream, in the southwest of Western Australia, is an estuarine dependant fish, which is only found in coastal waters near estuarine mouths when freshwater river flow is strong enough to flush a small number of fish out to the ocean (Chapter 3; Potter and Hyndes, 1999). These fish return to their natal estuary as soon as the strength of fresh water outflow declines sufficiently enough to enable them to swim upstream. Assemblages of black bream in the different estuaries of Western Australia have been shown to be genetically distinct (Chaplin *et al.*, 1998) providing evidence that the fish does not readily move between estuaries on the west coast contrary to the situation in south-eastern Australia (Farrington *et al.*, 2000).

Black bream have a somewhat widespread distribution throughout the estuarine system with this study finding them in relative abundance at Freshwater Bay (approximately 6 km from the estuary mouth), at Helena (40 km upstream in the Swan River) as well as in the estuarine portion of the Canning River at all sampling

times. Although black bream are reputed to be a highly mobile fish capable of travelling up to 50 km a day, when a cohort of fish move into an area of the estuary they tend to remain in that area from a few days to several weeks (K. Littleton, commercial fisher, pers. comm.). Other studies have stated that the majority of large fish (>250 mm total length) were caught in salinities below 20 ppt, and the fish migrated into the upper estuary in spring to spawn (Sarre and Potter, 1999; Partridge and Jenkins, 2002). However, in the present study black bream > 300 mm total length were captured in the lower estuary under both summer and winter conditions in salinities ranging from 2 ppt to 38 ppt. Anecdotal claims state that any black bream flushed into the middle and lower estuaries when freshwater outflow is at it strongest tend to stay in the lower estuary and the population of black bream at Freshwater Bay is stable and spawns in that area (K. Littleton, commercial fisher, pers. comm.). We have observed that the black bream captured in the upper estuary had darker skin pigmentation than those captured in the lower estuary and in the Canning River, while those in the middle estuary were a mixture of light and dark coloured fish. It is hypothesised that this colour variation is due to the access of black bream in the upper estuary to tannin rich waters of tributary streams and drains. Once the fish move into the lower estuary, they appear to gradually lose their darker colouration over time.

8.2. Biomarker responses in black bream compared to chemical analysis of estuarine sediments and flesh

In September 2000 and again during May 2001, the Waters and Rivers Commission (now the Department of Environment) arranged sediment and fish flesh chemical analysis to be undertaken from each of the sampling stations. A fillet of white muscle (approximately 70 grams including skin and scales) was removed from each fish at the same time biopsies were taken for biomarker analysis. Each fillet was taken from an area below the dorsal fin down to the lateral line, and extending from behind the left hand operculum to the beginning of the tail shaft in line with the rear of the dorsal fin. The fillet was wrapped in methanol washed aluminium foil and stored at – 80^{o}C until sent to the analytical laboratory. Sediment sampling was undertaken by the Swan River Trust staff. Chemical analysis of the 2000 sediment and flesh samples was performed by the Australian Government Analytical

Laboratories (AGAL), while the Chemistry Centre of WA analysed the 2001 samples. The results of these analyses are tabulated in Appendix B.

The tables in Appendix B clearly show that, apart from metals, some organochlorine pesticides and total petroleum hydrocarbons ≥ C15, the majority of compounds were below the detection limits for sediments. Only metals were detected in the fish flesh. For the May 2001 samples, hydrocarbons were not analysed by The Chemistry Centre in either sediments or flesh, due to cost and the inability to lower laboratory detection limits.

The use of fish flesh in chemical analysis has more to do with human health issues associated with consumption of the flesh than to do with fish health, and its use to measure pollution levels in a water body is problematic. White muscle is not necessarily the final repository for a pollutant taken up by a fish. Firstly, fish are very efficient in the metabolism and elimination of polycyclic aromatic hydrocarbon (PAHs) compounds (den Besten *et al.*, 2003) and PAHs do not bioaccumulate in any tissues, although metabolism can lead to the formation of reactive intermediaries (Chapter 2; Schlenk, 2001). Secondly, a chemical, once absorbed via gills or gut, is usually bound to a protein and then transported by blood either to a storage point (e.g. fat), or to the liver for transformation and/or storage. If transformed by the liver, it may be stored there, excreted in the bile, or passed back to the blood for excretion by the kidney or the gills, or stored in body fat (Heath, 1995). Thirdly, insecticides, such as dieldrin, carbaryl and parathion, bind to both albumin and lipoproteins in the plasma. Hydroxylated metabolites of PAHs bind primarily to albumin while unmetabolised PAH associate with plasma proteins. Albumin bound compounds tend to be absorbed in the endothelial cells in organs (such as the liver) while lipoproteins diffuse into all tissues and exhibit less specificity (Heath, 1995). Finally, the accumulation of metals in organs/tissues varies in distribution according to metal and species (Chapter 2; Heath, 1995). The concentration of a chemical found in different organs after environmental exposure can vary considerably. The total body burden of a foreign chemical will be a weighted average of all tissues, which may differ from each other in concentration of the chemical by many orders of magnitude (Heath, 1995).

The results from biochemical marker analysis clearly show that the black bream are exposed to chemical pollutants (such as PAHs) in the estuary despite the chemical analysis data being below detection limits for most organic compounds (Chapter 3 to 5 incl.). As shown in both Chapters 6 and 7, this exposure has the potential to have a detrimental effect on the fish, which in turn may affect their ability to survive environmentally stressful periods (e.g. algal blooms and hypoxic conditions). Chemical analysis for compounds and their metabolites and/or derivatives in cells, tissues, body fluids and/or excreta (bioaccumulation markers) used in conjunction with analysis of biomarkers of exposure needs to reflect the distribution of the chemical and its metabolites throughout the organism (van der Oost *et al.*, 2003). That is to say, fish chemical analysis needs to be performed on the whole fish, or simultaneously on several organs, and not just the muscle tissue. It is only then that this measure, along with sediment chemical analysis, can be used to complement biomarker analysis to gain a comprehensive picture of pollutant distribution and the potential impact of those pollutants on the biota within the estuary.

No trend could be identified between the chemical analysis of both sediment and fish flesh and any of the biomarker levels measured in the black bream from the Swan Canning Estuary.

8.3. Selection of a suite of biomarkers for aquatic health monitoring of the Swan-Canning Estuary

The present research has shown that the use of biliary metabolites and MFO activities (Chapters 3, 4 & 5), and the alteration in metabolic enzyme activities (Chapter 6) are all appropriate biomarkers to use with black bream for the assessment of the aquatic health of the Swan-Canning Estuary.

Chapter 3, 4 and 5 clearly show that PAH biliary metabolite detection in black bream is an extremely sensitive biomarker for the detection of PAHs within the Swan-Canning Estuary. The results confirm that the fish are exposed to PAHs and that metabolism of these compounds is taking place. The use of ratios between naphthalene-type and benzo(*a)*pyrene (B(*a*)P)-type biliary metabolites identifies the primary sources of PAH input into the estuary (Neff, 1990; Aas *et al.*, 2000b). In

both summer samplings naphthalene-type compounds of petrogenic sources from unburnt fuels (outboard motors, fuel spills or leakage from industry) dominated the estuary (Chapters 4 & 5). Winter PAH sources were more variable. In winter 2000, the naphthalene-type:B(*a*)P-type ratio indicated the dominance of PAHs originating from burnt fuels (eg motor vehicles) from winter run-off into the estuary (Chapter 3). In contrast, winter 2002 ratios were very close to the summer ratios that indicated the PAHs stem from petrogenic sources (Chapter 5). The possible explanation for the difference in winter PAH ratios in the black bream in 2000 compared to 2002 is the below average rainfall experienced in the Perth metropolitan along with lower stormwater runoff and river flow in the winter of 2002 (Chapter 5).

The risk to the biota from PAH differs according to groups. Petrogenic sources dominated by naphthalene are generally considered to be more volatile, have shorter residence times in the aqueous environment, and lower toxicity to aquatic biota when compared to PAHs such as B(*a*)P (Anderson *et al.*, 1974), however, as they are more water soluble they are more bioavailable to organisms (Neff, 1979; Burgess *et al.*, 2003). Naphthalene has been shown to cause phagocyte-induced peroxidative damage in the tissues of the fish *Anguilla anguilla* (Ahmad *et al.*, 2003) and chlorinated naphthalenes competitively inhibited hepatic CYP1A enzymes in rainbow trout fry (*Oncorhynchus mykiss*; Pesonen *et al.*, 2000). Combustion derived PAHs [e.g., B(*a*)P] have been linked in many studies to mutagenesis and carcinogenesis (Aas *et al.*, 2000a) and used engine oil has been found to contain higher levels of heavy metals, chlorinated solvents, PCBs additives and antioxidants that in 'fresh oil' (Upshall *et al.*, 1993).

Knowledge of the sources from which PAH are entering the estuarine system is a valuable tool for ecosystem managers in their endeavours to identify and remediate potential pollutant inputs. Remediation to control the input of PAHs from pyrogenic sources will require the redesign and restoration of stormwater drains discharging into the estuary, a costly exercise, particularly in long established suburbs. The control of petrogenic sources of PAHs will require reduction in inputs of fuel from spillages through education and increased penalties and a move away from the use of two-stroke carburetted engine to the more fuel efficient four-stroke engine on personal water-craft.

Differences in ethoxyresorufin-O-deethylase (EROD) activity in the male compared to the female black bream made interpretation of this biomarker difficult. EROD activities in the female black bream were much lower than in the male fish and at some sites female black bream greatly outnumbered the males, a factor that further complicated analysis of this biomarker. However, the heavy rainfall event at Riverton in May 2002, resulted in a greatly induced EROD activity in both male and, to a lesser extent, female black bream (Chapter 5) showing that under certain circumstances EROD activity has a high value as a biomarker of exposure to organic contaminants. Sexual differences in the ethoxycoumarin-O-deethylase (ECOD) activity in the black bream were not identified (Chapter 4 and 5). The differences in the pattern and intensity of the measured ECOD activity in the black bream compared to EROD activity indicates that ECOD activity may represent a different cytochrome P450 pattern to EROD activity (Machala *et al.*, 1997;Stegeman *et al.*, 1997). Therefore, ECOD activity is not a substitute for EROD activity as a biomarker to detect PAH exposure in black bream but may have a value as a biomarker for exposure for a mixture of contaminants other than PAHs, such as non-planar PCBs, chlorinated pesticides (e.g. DDT and chlordanes) and phenobarbital (Machala *et al.*, 1997; Troisi and Mason, 1997).

The presence of the enzyme serum sorbitol dehydrogenase (s-SDH), an indicator of liver damage, in the blood can be used in conjunction with MFO enzyme activity. In this context SDH has a value to explain any discrepancies in measured MFO activity levels due to hepatocellular damage (Holdway *et al.*, 1998). In the present study, no spatial or seasonal difference were detected in s-SDH levels in the black bream in either the winter or summer sampling periods indicating that comparisons of the measured EROD and ECOD activities were not prejudiced by liver damage.

Metabolic enzyme analysis clearly highlighted that the black bream were challenged in their aerobic capacities at the Barrack Street site (Chapter 6) during summer. Comparison of the aerobic and anaerobic capacities of liver, muscle and gill tissue found the latter to be the most suitable tissue to evaluate the aerobic and anaerobic capacity of fish over the summer months.

Furthermore, both the induction of the stress protein (hsp70) and the quantification of DNA strand breakage (Chapter 6) have considerable potential as biomarkers of susceptibility in the Swan-Canning Estuary black bream due to contaminant exposure. Results from this study show that the hsp70 in the field collected black bream are significantly higher than heat shocked laboratory-raised fish from the same genetic stock. Furthermore, the black bream within the estuary displayed low DNA integrity compared to the laboratory fish. There was a significant negative correlation between hsp70 expression and DNA integrity in field-collected fish suggesting that the black bream within the estuary are highly stressed.

8.4. Limitations to the interpretation of the results from this study

8.4.1. Sexual bias in the black bream sampled

Webb & Gagnon (2002) failed to identify any sex-related differences in EROD activity in feral black bream injected with PCB after acclimation to laboratory conditions. These fish were captured from the estuary in May 2000 and fed daily throughout the laboratory acclimation and experimental period with live shrimps (*Acetes* sp. and *Palaemonetes australis*) also collected from the estuary. However, the fish captured in the estuary in May 2001 and May 2002 did demonstrate sex-related differences in EROD activity (Chapters 4 & 5). Extrapolation of laboratory-based toxicity studies, using one chemical under controlled conditions, to field conditions where the feral fish are exposed to a mixture of contaminants as well as to seasonal variability in water conditions, and food types has its drawbacks.

To overcome the sex-related differences in the measured EROD activity in the black bream, sample sizes need to be large enough to adequately represent both male and female fish. As mentioned in the previous section female black bream greatly outnumbered male fish at some of the sites. For example, at Ascot in winter 2000, only two male fish were present in a sample catch of 19 black bream in 2000, and females outnumbered male black bream in summer 2001 by 5 to 1 (Chapter 4). At Freshwater Bay, the female black bream outnumbered males by a ratio of 3 to 1 in three of the four sampling times (Chapters 3, 4 & 5).

No sexual bias was reported by Sarre and Potter (1999) and Sarre *et al.* (2000) in black bream collected from the Swan-Canning Estuary after collecting at least 60 fish from each of 3 sites, although their study concentrated on black bream in the upper Swan estuary only. Sarre and Potter (1999) reported that there is no evidence that black bream change sex from male to female during the course of their life despite hermaphrodism being a feature in the life cycle of many Sparidae fish.

All fish captured for this current study were within the size range 268 to 364 mm and 400 to 800 grams for each of the sampling periods. There did not appear to be a sex/ size bias. Both the Helena and Riverton sites had sex ratios closer to 50:50 compared to the other sites. It is assumed that the bias toward female fish at some sites was random and related to small sample sizes.

The collection of a larger number of fish to ensure an adequate number of males are present would possibly overcome this unbalanced sex ratio. However, although black bream are relatively abundant throughout the estuarine system, they are not necessarily easy to capture. The fish captured for analysis under this study were caught by a professional fisherman with many years experience. The black bream tend to spend the daylight hours in the deeper waters of the estuary and move into the shallows at night to feed. This movement into the feeding grounds is dependant upon many factors, such as moon phases/brightness, barometric pressure, prevailing winds and the changing of tides within the estuary. Notwithstanding the experience of the fisherman, there were nights when the fish did not move into the shallows when expected and during 2002, extremely low water conditions were experienced throughout the estuary making the fish difficult to locate and capture. Therefore, the collection of a large enough sample number to ensure adequate numbers of male black bream has been, on some occasions, problematic.

The use of laboratory bred fish, placed in cages at each of the sites, as an alternative to capturing estuarine fish was considered but rejected due to, (1) the inability to ensure the security of the fish in the cages as the black bream are a highly prized fish among many recreational anglers, (2) the potential for vandalism of the cages at all sites, and (3) the long term placement of cages in areas that will come into conflict with the use of the estuary for navigation and water sports.

8.4.2. Delays in collection of fish in each sampling period

As reported in Chapter 5, in 2002 the black bream had limited access to the mussel beds, their preferred feeding grounds due to the low water conditions, and remained in the deeper channels. Low water conditions in the Swan-Canning Estuary are associated with the predominance of high barometric pressures and offshore winds with successive ebb phases dominant (Spencer, 1956), usually in spring. This phenomenon was dominant throughout 2002 which when combined with low average rainfall in that year (Chapter 5) resulted in the extremely low water levels in the estuary throughout the year. Additionally, in the 2000 and 2001 samplings, adverse weather conditions (heavy rains and/or strong southerly winds) delayed sampling at most sites. The result of all these factors was a delay in the collection of fish at the various sites that extended sampling over five to six weeks whereas a one to two week period would have been preferred to strengthen the validity of site comparisons, especially when biomarkers are influenced by the sex of the animal.

8.4.3. High variability in biomarker responses

Biomarker responses in the black bream displayed a high inter-fish variability. It has been reported that this variability is due in part to the rates at which individual fish take up and metabolise aromatic compounds (Krahn *et al.*, 1986). It has also been suggested that the variability in EROD activity is due to the non-linear dose-response of this biomarker (Vignier *et al.*, 1994). This high inter-individual variability represents an important constraint in field studies and can mask significant differences between sites. However, by increasing the number of individual fish collected in the field at each site, this constraint can, to an extent, be partially overcome (Hodson *et al.*, 1993).

As well as the high inter-individual variability in biomarker responses, there was a high spatial inter-seasonal and inter annual variability within the black bream from the Swan-Canning Estuary (Chapter 4 & 5). There were no consistent upstream or downstream trends suggesting multiple sources of contaminant input into the estuary. The patterns of responses by the black bream indicate that the proximity of major roads and drains into the Swan-Canning Estuary is the most significant factor influencing biomarker responses in winter. The onset and finish of winter rains, and

the number of significant rainfall events determines the level of runoff into the estuary. As the strength of freshwater outflow will influence the level of input of potential pollutants into the estuary from the catchment drainage, and their residence time within the estuary (Gerritse *et al.*, 1998; Twomey and John, 2001), yearly rainfall episodes will have a strong influence on the pattern and strength of biomarker responses in the estuarine biota. Therefore, assumptions from one season/year cannot be used to predict biomarker levels in the black bream in subsequent seasons/years.

The elevated biomarker levels measured in the black bream at the Riverton site immediately following the heavy rainfall event late summer 2002 (Chapter 5) clearly highlighted the impact of stormwater drainage on the biota within the estuarine system. The Mills Street Main Drain catchment has been identified as a catchment of concern under the Swan-Canning Cleanup Program (SCCP) (Swan River Trust, 2003). Analysis of sediments taken from the collection site in summer 2001 show that PAH, organochlorine and organophosphate levels are all below their respective detection limits (Appendix B) whereas DDT and dieldrin has been detected in water within the drain itself (Swan River Trust, 2003). The pulse of runoff water from the Mills Street Main Drain into the Swan-Canning Estuary resulted in the elevated EROD and ECOD activity levels recorded at Riverton two days following the rainfall event.

8.4.4. Mobility of the black bream

The mobility of the black bream within the estuarine system may hinder the value of the black bream as a biomarker species. MFO enzyme activities and bile metabolite levels represent recent exposure (past 4 – 7 days) to contaminants. This was clearly demonstrated in Chapter 5 where mixed function oxygenase (MFO) activities were clearly elevated in the black bream at Riverton, immediately following a rainfall event in May 2002, which resulted in significant runoff of freshwater from local drainage, in comparison to the other sites. This is a potential link between elevated black bream MFO enzyme activity and inducing substances brought in by stormwater runoff from residential and industrial areas. The fish captured in the middle estuary at Barrack Street displayed elevated biliary metabolites at the end of the winter months (Chapter 3), particularly B(*a*)P-type. This again shows the

potential for the contribution of stormwater and road drainage to contaminant levels within the estuary.

8.4.5. Weaknesses in the interpreting stress protein and DNA damage results

The use of laboratory raised black bream, bred and maintained under controlled conditions, as the negative control (reference) for the determination of hsp70 expression and DNA integrity in a field study has limitations. The problem with this approach has been fully explained in Chapter 7 (p. 187) and includes environmental, life experience and dietary differences. The results do not inform if the higher levels for hsp70 expression in the field-captured fish is due to acquired tolerance to a highly stressful environment or whether the fish were stressed to the point of adverse effects. The laboratory fish were depurated for only one week prior to heat shock and tissue collection, which was due to lack of space for long term depuration, and raises the question as whether one week was long enough.

Due to the paucity of data for chemical concentrations (in sediment, water and fish) (Chapter 2 & Appendix B), comparisons of hsp70 expression and DNA integrity in the black bream to contaminant distribution within the estuary could not be conducted. It was concluded that the high mobility of the black bream could explain the inter site similarities in Hsp70 and rule it out as a biomarker for inter-site differences. Stress in fish can have many causes such as adverse water conditions, contaminant exposure or plankton blooms especially under hypoxic conditions (Iwama *et al.*, 1999).

The alkaline unwinding assay does not differentiate between DNA strand breaks caused by exposure to chemical pollutants and those due to environmental factors (Shugart, 1996). Shugart (1996) recommends the use of the comet assay to distinguish between environmental and contaminant induced DNA strand breaks. However, it has been suggested that strand breakage is not a suitable genotoxicity biomarker for field studies (Bombail *et al.*, 2001) and that the measurement of nuclear anomalies, such as micronuclei formation, in fish is a more relevant and accurate measure of DNA damage for this purpose.

8.5. Conclusions

This study has shown that the black bream in the Swan-Canning Estuary are exposed to, and are metabolizing PAHs notwithstanding that the chemical analysis of the contaminant load of these substances in the estuarine waters is consistently below laboratory detection limits. In addition, biomarker responses such as EROD and ECOD activity indicate that a variety other non-nutrient type pollutants enter the estuary via surface road and stormwater runoff and by way of the groundwater. The Swan-Canning Estuary, like many estuaries around the world, is subjected to inputs of a mixture of both organic and inorganic contaminants from both point and non-point sources. These pollutants have the potential to affect the aerobic and anaerobic metabolism of the black bream and are impacting on this fish's ability to survive additional seasonal stresses, such as algal blooms and periods of hypoxia.

Induction of the MFO enzyme system reflects recent exposure to potential pollutants such as PAHs, PCBs, PBBs and dioxins (Payne *et al.*, 1987; Arcand-Hoy and Metcalfe, 1999), while the measurement of biliary metabolites in fish is a rapid and inexpensive measure that clearly reflects short-term PAH exposure (Aas *et al.*, 2000b). The results from this preliminary study clearly indicate that the measurement of both MFO enzyme activity and biliary metabolites in black bream are invaluable and reliable tools for environmental risk assessment of the health of the Swan-Canning Estuary. Nonetheless, caution needs to be taken in the interpretation of EROD activity induction due to influence of the gender of black bream on this biomarker.

The measurement of the metabolic enzyme activities, cytochrome *C* oxidase, and lactate dehydrogenase have shown that the black bream at the Barrack Street site are challenged during the summer months. The comparison of gill, liver and white muscle tissues has concluded that the gill is the most suitable tissue to use to measure aerobic and anaerobic capacities in the black bream.

This study has shown that hsp70 levels in the field collected black bream are elevated, that DNA integrity is compromised and there is a statistically significant correlation between hsp70 expression and DNA strand breaks. However, the

measurement of these biomarkers in feral black bream during this study did not distinguish between environmental effects (e.g. UV light, temperature, salinity, dissolved oxygen) and pollutant effects. Further investigations are required to validate these biomarkers of susceptibility and effect; heat shock protein response and DNA stand breaks, under field conditions.

No gradient of response in biomarker levels was identified in the Swan-Canning Estuary under either winter or summer conditions indicating there are multiple sources of inputs of potential pollutants along the length of the estuary. Stormwater and road runoff are the primary source of pollutant input into the estuary in the winter months, while summer biomarker levels, particularly PAH, appear to reflect the high usage of the estuary for recreational purposes and runoff from poorly irrigated parks and gardens. Significant rainfall events at any time of the year have the potential to adversely impact the biota of the estuary, particularly when these events result in a flush of water from the drains following long dry periods.

8.6. Recommendations for further investigation

- Further investigations are required to determine baseline levels of all biomarkers in the healthy black bream and to determine the extent of natural variability.
- A recent study has found sex-related differences in the cellular stress response using hsp70 in juvenile chinook salmon (*Oncorhynchus tshawytscha)* (Afonso *et al.*, 2003). Sex-related differences were not considered in Chapter 7; however, in light of the results of Afonso *et al.* (2003), this aspect requires further examination in the black bream.
- The assessment of DNA strand breakage using either the alkaline unwinding assay or the comet assay may not necessarily be the best genotoxicity biomarker for a field study (Bombail *et al.*, 2001) into the health of black bream in the Swan-Canning Estuary. A comparison of the comet assay with the micronucleus assay is recommended to ensure the most suitable biomarker of genotoxic effects on feral black bream is selected.
- This study lacks a reference site/river. It is recommended that an investigation be conducted into relatively ‘clean’ non-urban waterways.

Black bream are found in the Moore River to the north of the Swan-Canning Estuary as well the Warren River to the south. However, both these river systems run through agricultural lands and are likely to be impacted by agricultural pesticide runoff, and the Warren River is likely to be influenced by runoff from the timber industry.

- An alternative to the use of a reference involves the capture of black bream from the Swan-Canning Estuary and the long term depuration of these fish for a period of two to three months in 'clean' water. This will enable baseline biomarker levels to be established and overcome the problem of comparing fish with genetic variations from other southwestern Australian estuaries.
- To date only the black bream has been investigated as a bioindicator species for the biomonitoring of the health Swan-Canning Estuary. It is recommended that the biomarker levels in a second estuarine-dependent indigenous species be investigated.
- Conclusions have been drawn in this study that the proximity of major roads and drains are the major source of non-nutrient pollution into the Swan-Canning Estuary. A targeted study to clarify the relationship between major drain discharges, biomarker levels and impacts on river biota is required.
- The water in the stormwater drains is fresh water while the estuarine waters are brackish to marine. The drains tend to be quite shallow, therefore the estuarine fish may move short distances into the drains to feed when conditions are suitable, but do not reside there on a long term basis. The linking of the health effects of drain discharges and river health will lead to a greater understanding of and better management of drains. Therefore, a resident freshwater species resident within the drains needs to be identified and a suite of biomarkers measured on this species.
- Metallothionein, an enzyme found in the liver, kidney, and other organs of a fish, is induced in the presence of metals and is believed to play an antioxidative role to protect cells from radicals. As metals are known to be present in the sediment of the Swan-Canning Estuary (Appendix B) it is recommended that this enzyme be analysed in the black bream.
- Chemical analysis of water, sediments and fish flesh consistently fails to identify PAHs within the Swan-Canning Estuary (Swan River Trust, 1999). Where other organic and inorganic contaminants are identified, they may not

necessarily be bioavailable to the estuarine biota. Bioavailable contaminants, including PAHs, can be measured using a device known as a passive sampler or semi-permeable membrane device (SPMD). Contaminant levels collected in the SPMD can be directly related to biomarker responses in fish. It is recommended that a study of estuarine waters utilising SPMDs be undertaken in tandem with biomarker analysis of field captured fish.

8.7. References

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Appendix A
Publications

The following publications have resulted from this PhD project.

A1. Conference Proceedings

A1.1. Posters

*Webb, D. & M.M. Gagnon. 2002. MFO activity in fish under differing salinity, Swan River estuary, Western Australia. Abstract No. 32. p 196. SETAC/ASE Asia-Pacific 2003, Christchurch, NZ. 28 September - 1 October 2003.

Webb, D. & *M.M. Gagnon. 2004. HSP70 and DNA integrity in feral black bream (*Acanthopagrus butcheri*) in the Swan-Canning estuary, Western Australia. Abstract No.PM215. p 264. Society of Environmental Toxicology and Chemistry 25th Annual Meeting, Portland, Oregon USA. 14 – 18 November 2004.

A1.2. Platform Presentations

*Webb, D. & M.M. Gagnon. 2002. Seasonal variability of biliary PAH in fish, Swan River estuary, Western Australia. Abstract No. 562. p 122. Society of Environmental Toxicology and Chemistry 23rd Annual Meeting, Salt Lake City, USA. 16 – 20 November 2002.

*Webb, D., & M.M. Gagnon. 2003. Expression of hsp70 in fish inhabiting the Swan River estuary, Western Australia. Abstract No. 31. p 190a. SETAC/ASE Asia-Pacific 2003, Christchurch, NZ. 28 September - 1 October 2003.

*Webb, D., T.H. Rose & M.M. Gagnon. 2004. Inter-annual variability in fish biomarkers, Swan Canning estuary, Western Australia. Abstract No. W2E p 140. Interact 2004. Australasian Society for Ecotoxicology, Gold Coast, Queensland, Australia, July 2004.

A2. Journal Publications

Webb, D. & M.M. Gagnon. 2002. Biomarkers of exposure in fish inhabiting the Swan-Canning Estuary, Western Australia - a preliminary study. Journal of Aquatic Ecosystem Stress and Recovery. 9(4): 259-269.

Webb, D., M.M. Gagnon & T. Rose. 2005. Interseasonal variability in biomarkers of exposure in fish inhabiting a southwestern Australian estuary. Environmental Toxicology. 20(5): 522-532.

Webb, D., M.M. Gagnon & T.H. Rose. 2005. Interannual variability in fish biomarkers in a contaminated temperate urban estuary. Ecotoxicology and Environmental Safety. 62(1): 53-65.

Webb, D., M.M. Gagnon & T. Rose. 2005. Metabolic enzyme activities in black bream (*Acanthopagrus butcheri)* from the Swan-Canning Estuary, Western Australia. Comparative Physiology and Biochemistry, Part C. 141:356-365.

Appendix B

Results of the chemical analysis of sediments and fish flesh.

Table B1: Sediment chemical analysis in the Swan-Canning estuary September 2000 as analysed by AGAL, Perth, Western Australia.

	Methods	Units	Limit of Reporting	Sites						
				Helena	Ascot	Belmont	Barrack Street	Crawley	Freshwater Bay	Salter Point
Trace Elements										
Arsenic	**NT2_49**	**mg/kg**		**2.1**	**4.4**	**7.6**	**14**	**1.1**	**0.8**	**1.7**
Cadmium	**NT2_49**	**mg/kg**		**<0.05**	**<0.05**	**0.7**	**1.3**	**<0.05**	**<0.05**	**<0.05**
Copper	**NT2_49**	**mg/kg**		**20.7**	**55**	**85.3**	**170**	**5.4**	**8.9**	**6.1**
Lead	**NT2_49**	**mg/kg**		**25.3**	**60.3**	**136.7**	**656.7**	**9.2**	**7.7**	**15.7**
Mercury	**NT2_49**	**mg/kg**		**<0.02**	**<0.02**	**<0.02**	**0.8**	**<0.02**	**<0.02**	**<0.02**
Phosphorous	**NT2_49**	**mg/kg**		**370**	**700**	**590**	**416.7**	**45.7**	**61.7**	**57**
Selenium	**NT2_49**	**mg/kg**		**0.9**	**1.2**	**1.4**	**1.4**	**<0.05**	**<0.05**	**<0.05**
Sulphur	**NT249_253**	**mg/kg**		**10870**	**8047**	**14133**	**18400**	**883**	**883**	**1923**
Zinc	**NT2_49**	**mg/kg**		**57.7**	**156.7**	**413.3**	**773.3**	**25**	**17.7**	**33.7**
Organochlorine (OC) Pesticides										
HCB	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
HCH(BHC) Total alpha, beta, delta	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Lindane (gamma-BHC)	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Heptachlor	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Heptachlor epoxide	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Chlordane	NR_19	mg/kg	<0.01	<0.01	0.011	<0.01	0.053	<0.01	0.012	<0.01
Alpha Endosulphate	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Beta Endosulphate	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Endosulphan sulphate	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Aldrin	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Dieldrin	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	0.062	<0.01	<0.01	<0.01
Endrin	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
p,p-DDE	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	0.037	<0.01	<0.01	<0.01
p,p-DDD	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	0.054	<0.01	<0.01	<0.01
p,p-DDT	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	0.138	<0.01	<0.01	<0.01
Metoxychlor	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Total OC,s	**NR_19**	**mg/kg**	**<0.2**	**<0.2**	**<0.2**	**<0.2**	**0.45**	**<0.2**	**<0.2**	**<0.2**
Organophosphate (OP) Pesticides										
Mevinphos	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Diazinon	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Chlorpyrifos-methyl	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fenchlofos	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Parathion-methly	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Chlorpyrrifos	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Malathion	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fenitrothion	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
ParathionChlorfenvinphos	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Bromophos-ethyl	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Tetrachlorvinphos	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Ethion	NR_19	mg/kg	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Total OP's	**NR_19**	**mg/kg**	**<1.0**	**<1.0**	**<1.0**	**<1.0**	**<1.0**	**<1.0**	**<1.0**	**<1.0**
Total Organic Carbon	**NW_SL4**	**mg/kg**		**28610**	**35753**	**31947**	**80607**	**2157**	**1867**	**4280**
Poly Aromatic Hydrocarbons										
Naphthalene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Acenaphthylene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Acenaphthalene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Fluorene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Phenanthrene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Anthracene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Fluoranthene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Pyrene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Benz(a)anthracene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Chrysene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Benzo(b+k)fluoranthene	WL206	mg/kg	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
Benzo(a)pyrene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Indeno(1,2,3,c,d)pyrene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Bibenz(a,h)anthraceene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Benzo(h,I)perylene	WL206	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Total PAH's	**WL206**	**mg/kg**	**<16**	**<16**	**<16**	**<16**	**<16**	**<16**	**<16**	**<16**
BTEX										
Benzene	WL230	mg/kg	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
Toluene	WL230	mg/kg	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
Ethylbenzene	WL230	mg/kg	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50	<0.50
Xylene	WL230	mg/kg	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
Total BTEX	**WL230**	**mg/kg**	**<2.5**	**<2.5**	**<2.5**	**<2.5**	**<2.5**	**<2.5**	**<2.5**	**<2.5**
Benzene	WL244	mg/kg	<1.0	<1.0	<1.0				<1.0	<1.0
Toluene	WL244	mg/kg	<1.0	<1.0	<1.0				<1.0	<1.0
Ethylbenzene	WL230	mg/kg	<1.0	<1.0	<1.0				<1.0	<1.0
Xylene	WL244	mg/kg	<2.0	<2.0	<2.0				<2.0	<2.0
Total BTEX	**WL244**	**mg/kg**	**<5.0**	**<5.0**	**<5.0**				**<5.0**	**<5.0**
Total Petroleum Hydrocarbons										
TPH C6-C9	WL230	mg/kg	<25	<25	<25	<25	<25	<25	<25	<25
TPH C10 - C14	WL230	mg/kg	<50	<50	<50	<50	<50	<50	<50	<50
TPH C15 - C28	WL230	mg/kg	<100	230	270	<100	720	<100	<100	<100
TPH C29 - C36	WL230	mg/kg	<100	155	170	<100	900	<100	<100	<100
Total TPH	**WL230**	**mg/kg**	**<275**	**390**	**435**	**<275**	**1600**	**<275**	**<275**	**<275**
TPH C6-C9	WL244	mg/kg	<25	<25					<25	<25
TPH C10 - C14	WL203	mg/kg	<25	<25					<25	<25
TPH C15 - C28	WL203	mg/kg	<100	<100	390				260	<100
TPH C29 - C36	WL203	mg/kg	<100	540	313.3				540	<100
Total TPH	**WL203**	**mg/kg**	**<250**	**540**	**443.3**				**810**	**<250**
Inorganics										
Total Nitrogen	**WL132WL119**	**mg/kg**	**<50**	**2017**	**3243**	**2847**	**3080**	**253**	**323**	**540**

Table B2: Sediment chemical analysis in the Swan-Canning estuary May 2001 as analysed by The Chemistry Centre, Perth, Western Australia.

	Methods	Units	Limit of Reporting	Sites						
				Helena	Ascot	Belmont	Barrack Street	Freshwater Bay	Salter Point	Riverton
Trace Elements										
Arsenic	**iAS2STVG**	**mg/kg dry**		**1.8**	**11.4**	**2.7**	**5.4**	**0.9**	**1.7**	**1.5**
Cadmium	**iELE2STIM**	**mg/kg dry**	**<0.03**	**0.17**	**0.79**	**0.22**	**0.71**	**<0.03**	**0.15**	**0.09**
Copper	**iMET2STIP**	**mg/kg dry**		**13.9**	**95.3**	**41.6**	**105.8**	**10.2**	**11.8**	**6.9**
Lead	**iPB1STFA**	**mg/kg dry**		**0.04**	**0.29**	**0.06**	**0.17**	**0.06**	**0.04**	**0.02**
Mercury	**iHG2STVG**	**mg/kg dry**		**12.1**	**8.2**	**82.3**	**265**	**8.8**	**11.8**	**17.0**
Phosphorous	**eP1STCO**	**mg/kg dry**		**209.3**	**591.7**	**144.7**	**250.0**	**132.0**	**169.3**	**93.3**
Selenium	**iSE2STVG**	**mg/kg dry**		**0.48**	**0.62**	**0.22**	**0.25**	**0.10**	**0.38**	**0.17**
Sulphur	**iMET2SIP**	**mg/kg dry**		**15017**	**18333**	**6367**	**13780**	**933**	**11983**	**3627**
Zinc	**iMET2STIP**	**mg/kg dry**		**61.2**	**513.3**	**201.3**	**459.0**	**23.3**	**54.0**	**49.8**
Organochlorine (OC) Pesticides										
HCB	iOC1SOILGC	mg/kg dry	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Lindane	iOC1SOILGC	mg/kg dry	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Heptachlor	iOC1SOILGC	mg/kg dry	<0.01	<0.01	<0.01	<0.01	0.15	<0.01	<0.01	<0.01
Chlordane (total)	iOC1SOILGC	mg/kg dry	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Endosulphan (total)	iOC1SOILGC	mg/kg dry	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Aldrin	iOC1SOILGC	mg/kg dry	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Dieldrin	iOC1SOILGC	mg/kg dry	<0.01	<0.01	<0.01	<0.01	0.03	<0.01	<0.01	<0.01
Endrin	iOC1SOILGC	mg/kg dry	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
DDT & met.	iOC1SOILGC	mg/kg dry	<0.01	<0.01	<0.01	<0.01	<0.01	0.14	<0.01	<0.01
Metoxychlor	iOC1SOILGC	mg/kg dry	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Total OC,s		**mg/kg dry**	**<0.1**	**<0.1**	**<0.1**	**<0.1**	**0.21**	**0.24**	**<0.1**	**<0.1**
Organophosphate (OP) Pesticides										
Diazinon	iOC1SOILGC	mg/kg dry	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Chlorpyrifos	iOC1SOILGC	mg/kg dry	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Malathion	iOC1SOILGC	mg/kg dry	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Fenitrothion	iOC1SOILGC	mg/kg dry	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Total OP's	**iOC1SOILGC**	**mg/kg dry**	**<1.0**	**<1.0**	**<1.0**	**<1.0**	**<1.0**	**<1.0**	**<1.0**	**<1.0**
Total Organic Carbon	**eTOCSOIL**	**% dry**		**5.3**	**3**	**0.8**	**8.1**	**0.3**	**0.4**	**1.8**
Poly Aromatic Hydrocarbons										
Naphthalene			<1.0	NA	NA	NA	NA	NA	NA	NA
Acenaphthylene			<1.0	NA	NA	NA	NA	NA	NA	NA
Acenaphthalene			<1.0	NA	NA	NA	NA	NA	NA	NA
Fluorene			<1.0	NA	NA	NA	NA	NA	NA	NA
Phenanthrene			<1.0	NA	NA	NA	NA	NA	NA	NA
Anthracene			<1.0	NA	NA	NA	NA	NA	NA	NA
Fluoranthene			<1.0	NA	NA	NA	NA	NA	NA	NA
Pyrene			<1.0	NA	NA	NA	NA	NA	NA	NA
Benz(a)anthracene			<1.0	NA	NA	NA	NA	NA	NA	NA
Chrysene			<1.0	NA	NA	NA	NA	NA	NA	NA
Benzo(b+k)fluoranthene			<2.0	NA	NA	NA	NA	NA	NA	NA
Benzo(a)pyrene			<1.0	NA	NA	NA	NA	NA	NA	NA
Indeno(1,2,3,c,d)pyrene			<1.0	NA	NA	NA	NA	NA	NA	NA
Bibenz(a,h)anthraceene			<1.0	NA	NA	NA	NA	NA	NA	NA
Benzo(h,I)perylene			<1.0	NA	NA	NA	NA	NA	NA	NA
Total PAH's			**<16**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**
BTEX										
Benzene	eBTEXSoil	mg/kg dry	<0.50	<0.5	<0.50	<0.5	<0.50	<0.5	<0.50	<0.5
Toluene	eBTEXSoil	mg/kg dry	<0.50	<0.5	<0.50	<0.5	<0.50	<0.5	<0.50	<0.5
Ethylbenzene	eBTEXSoil	mg/kg dry	<0.50	<0.5	<0.50	<0.5	<0.50	<0.5	<0.50	<0.5
Xylene	eBTEXSoil	mg/kg dry	<0.50	<1.0	<0.50	<1.0	<0.50	<1.0	<0.50	<1.0
Total BTEX		**mg/kg dry**	**<5.0**	**<5.0**	**<5.0**	**<5.0**	**<5.0**	**<5.0**	**<5.0**	**<5.0**
Total Petroleum Hydrocarbons										
TPH C6-C9			<25	NA	NA	NA	NA	NA	NA	NA
TPH C10 - C14			<50	NA	NA	NA	NA	NA	NA	NA
TPH C15 - C28			<100	NA	NA	NA	NA	NA	NA	NA
TPH C29 - C36			<100	NA	NA	NA	NA	NA	NA	NA
Total TPH			**<275**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**
Inorganics										
Total Nitrogen	**eN1STCO**	**% dry**		**0.3**	**0.17**	**0.05**	**0.3**	**0.04**	**0.04**	**0.1**
Total Phosphorous	**eP1STCO**	**mg/kg dry**		**251.70**	**591.7**	**96.5**	**323.3**	**136**	**67.7**	**116.2**

NA - Not Analysed

Table B3: Fish flesh chemical analysis in the Swan-Canning estuary September 2000 as analysed by AGAL.

	Methods	Units	Sites						
			Helena	Ascot	Belmont	Barrack Street	Crawley	Freshwate Bay	Salter Poin
Date of Capture			21/8/00	23/8/00	30/8/00	15/8/00	15/8/00	21/9/00	14/9/00
Trace Elements									
Arsenic	**NT2_49**	**mg/kg**	**0.42**	**0.39**	**0.56**	**1.2**	**1.47**	**2.38**	**1.21**
Cadmium	**NT2_49**	**mg/kg**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
Copper	**NT2_49**	**mg/kg**	**0.16**	**0.19**	**0.18**	**0.16**	**0.19**	**0.18**	**0.27**
Lead	**NT2_49**	**mg/kg**	**0.01**	**<0.01**	**0.09**	**0.02**	**0.01**	**<0.01**	**0.01**
Mercury	**NT2_49**	**mg/kg**	**0.07**	**0.13**	**0.05**	**0.05**	**0.03**	**0.04**	**0.04**
Selenium	**NT2_49**	**mg/kg**	**0.49**	**0.59**	**0.51**	**0.5**	**0.54**	**0.6**	**0.54**
Zinc	**NT2_49**	**mg/kg**	**3.92**	**4.42**	**3.68**	**3.94**	**4.12**	**4.86**	**4.56**
Organochlorine (OC) Pesticides									
HCB	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Lindane (gamma-BHC)	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Heptachlor	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Aldrin	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
BHC (other than g-BHC)	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Heptachor epoxide	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Chlordane (trans and cis)	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
DDE	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Dieldrin	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Endrin	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
DDD	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
DDT	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Metoxychlor	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Total Endosulphan	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Organophosphate (OP) Pesticides									
Demeton-S-Methyl	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Diazinon	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Dimethoate	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Pirimiphos-Methyl	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Chlorpyrifos	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Parathion	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Malathion (maldison)	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Fenithion	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ethion	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Azinphos-Methyl	NR_19	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Others									
Surrogate OC Rec.	NR_19	%	79.4	84.4	90.6	69.8	74.6	89.6	87.8
Surrogate OP Rec.	NR_19	%	77.8	80.4	79	70.6	77.8	76.6	80.2
BTEX									
Benzene	NGCMS_11	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Toluene	NGCMS_11	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Ethylbenzene	NGCMS_11	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Xylene	NGCMS_11	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Total Petroleum Hydrocarbons									
TPH C6-C9	NGCMS_11	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
TPH C10 - C14	NGCMS_11	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
TPH C15 - C28	NGCMS_11	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
TPH C29 - C36	NGCMS_11	mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Surrogate									
Surrogate 1 Rec.		%	101.2	104.2	103	113.2	109.4	104.2	105
Surrogate 2 Rec.		%	97.6	105.8	110	104.4	99.2	100.8	100.6

Table B4: Fish flesh chemical analysis in the Swan-Canning estuary May 2001 as analysed by The Chemistry Centre.

		Sites						
	Units	Helena	Ascot	Belmont	Barrack Street	Freshwater Bay	Salter Point	Riverton
Date Collected		7/5/01	9/4/01	3/4/01	1/4/01	29/4/01	10/4/01	30/4/01
Trace Elements								
Arsenic	**mg/kg**	**0.04**	**<0.05**	**<0.05**	**0.07**	**0.02**	**<0.05**	**<0.05**
Cadmium	**mg/kg**	**<0.06**	**<0.06**	**<0.06**	**<0.06**	**<0.06**	**<0.06**	**<0.06**
Copper	**mg/kg**	**0.6**	**0.4**	**0.4**	**0.5**	**0.1**	**0.4**	**0.3**
Lead	**mg/kg**	**0.08**	**<0.06**	**<0.06**	**<0.06**	**<0.06**	**<0.06**	**<0.06**
Mercury	**mg/kg**	**0.11**	**0.08**	**0.07**	**0.08**	**0.1**	**0.06**	**0.04**
Selenium	**mg/kg**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.05**
Zinc	**mg/kg**	**13.5**	**13.5**	**13.2**	**23**	**9.2**	**9.8**	**13.3**
Organochlorine (OC) Pesticides								
HCB	mg/kg	NA	NA	NA	NA	NA	NA	NA
Lindane (gamma-BHC)	mg/kg	NA	NA	NA	NA	NA	NA	NA
Heptachlor	mg/kg	NA	NA	NA	NA	NA	NA	NA
Aldrin	mg/kg	NA	NA	NA	NA	NA	NA	NA
BHC (other than g-BHC)	mg/kg	NA	NA	NA	NA	NA	NA	NA
Heptachor epoxide	mg/kg	NA	NA	NA	NA	NA	NA	NA
Chlordane (trans and cis)	mg/kg	NA	NA	NA	NA	NA	NA	NA
DDE	mg/kg	NA	NA	NA	NA	NA	NA	NA
Dieldrin	mg/kg	NA	NA	NA	NA	NA	NA	NA
Endrin	mg/kg	NA	NA	NA	NA	NA	NA	NA
DDD	mg/kg	NA	NA	NA	NA	NA	NA	NA
DDT	mg/kg	NA	NA	NA	NA	NA	NA	NA
Metoxychlor	mg/kg	NA	NA	NA	NA	NA	NA	NA
Total Endosulphan	mg/kg	NA	NA	NA	NA	NA	NA	NA
Organophosphate (OP) Pesticides								
Demeton-S-Methyl	mg/kg	NA	NA	NA	NA	NA	NA	NA
Diazinon	mg/kg	NA	NA	NA	NA	NA	NA	NA
Dimethoate	mg/kg	NA	NA	NA	NA	NA	NA	NA
Pirimiphos-Methyl	mg/kg	NA	NA	NA	NA	NA	NA	NA
Chlorpyrifos	mg/kg	NA	NA	NA	NA	NA	NA	NA
Parathion	mg/kg	NA	NA	NA	NA	NA	NA	NA
Malathion (maldison)	mg/kg	NA	NA	NA	NA	NA	NA	NA
Fenithion	mg/kg	NA	NA	NA	NA	NA	NA	NA
Ethion	mg/kg	NA	NA	NA	NA	NA	NA	NA
Azinphos-Methyl	mg/kg	NA	NA	NA	NA	NA	NA	NA
Others								
Surrogate OC Rec.	%	NA	NA	NA	NA	NA	NA	NA
Surrogate OP Rec.	%	NA	NA	NA	NA	NA	NA	NA
BTEX								
Benzene	mg/kg	NA	NA	NA	NA	NA	NA	NA
Toluene	mg/kg	NA	NA	NA	NA	NA	NA	NA
Ethylbenzene	mg/kg	NA	NA	NA	NA	NA	NA	NA
Xylene	mg/kg	NA	NA	NA	NA	NA	NA	NA
Total Petroleum Hydrocarbons								
TPH C6-C9	mg/kg	NA	NA	NA	NA	NA	NA	NA
TPH C10 - C14	mg/kg	NA	NA	NA	NA	NA	NA	NA
TPH C15 - C28	mg/kg	NA	NA	NA	NA	NA	NA	NA
TPH C29 - C36	mg/kg	NA	NA	NA	NA	NA	NA	NA
Surrogate								
Surrogate 1 Rec.	%	NA	NA	NA	NA	NA	NA	NA
Surrogate 2 Rec.	%	NA	NA	NA	NA	NA	NA	NA

NA - Not Analysed

Appendix C
Field Collection Sites

C1. Helena

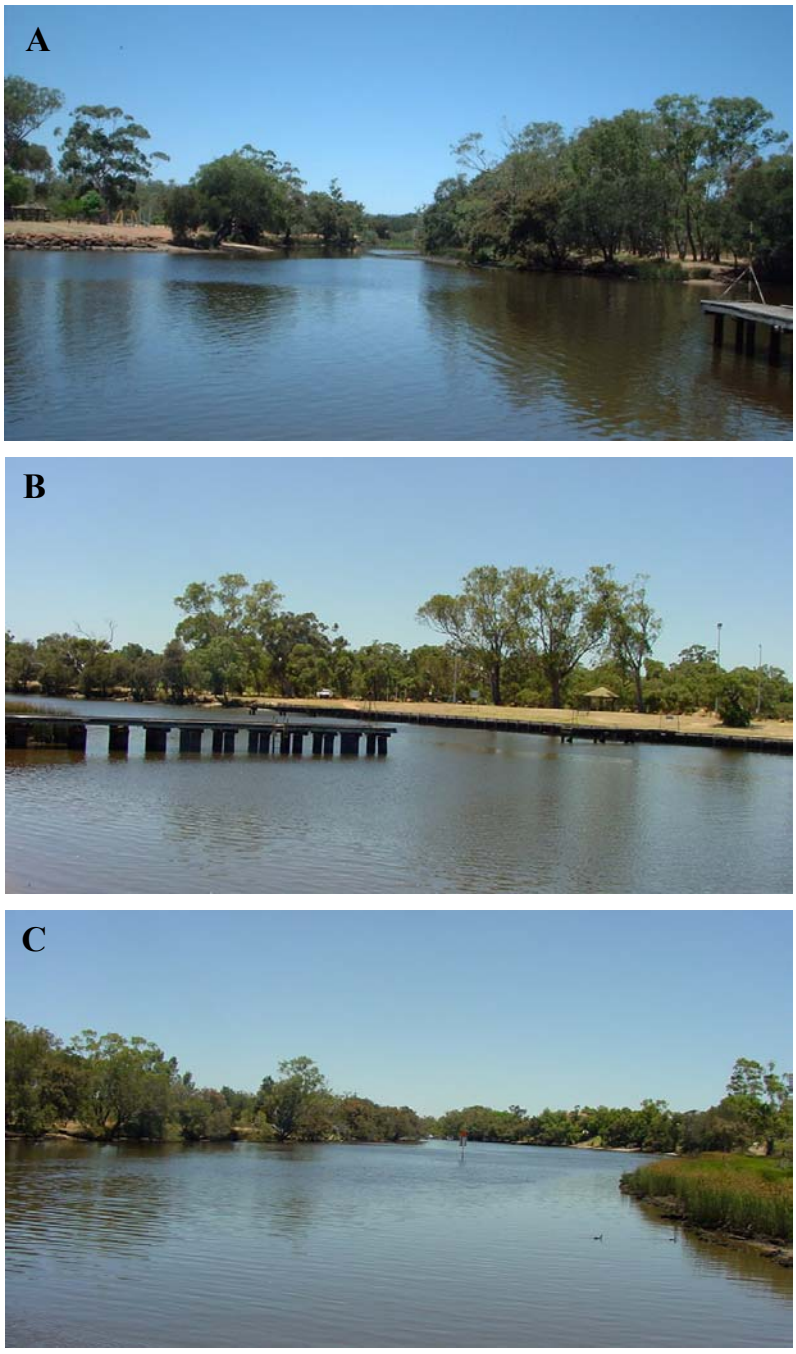


Plate 1: *Helena collection site: (A) facing the confluence of the Helena River with the Swan River, (B) Kings Meadow Reserve on eastern bank of Swan River. (C) Swan River downstream from mouth of the Helena River.*

C2. Ascot

Plate 2: *Ascot collection site, (A) facing the Ascot Racecourse from northern bank of Swan River upstream collection site, (B) entry of Bayswater Main Drain into the Swan River upstream from collection site,(C) Garret Road Bridge, collection site summer at low tide.*

C3. Belmont

Plate 3: *Belmont collection site, (A) stormwater and road runoff directly into Swan River, (B) facing Belmont Park Racecourse from northern bank of Swan River, (C) Windan Bridge downstream of collection site, which carries considerable road traffic along the Graham Farmer Freeway.*

C4. Barrack Street

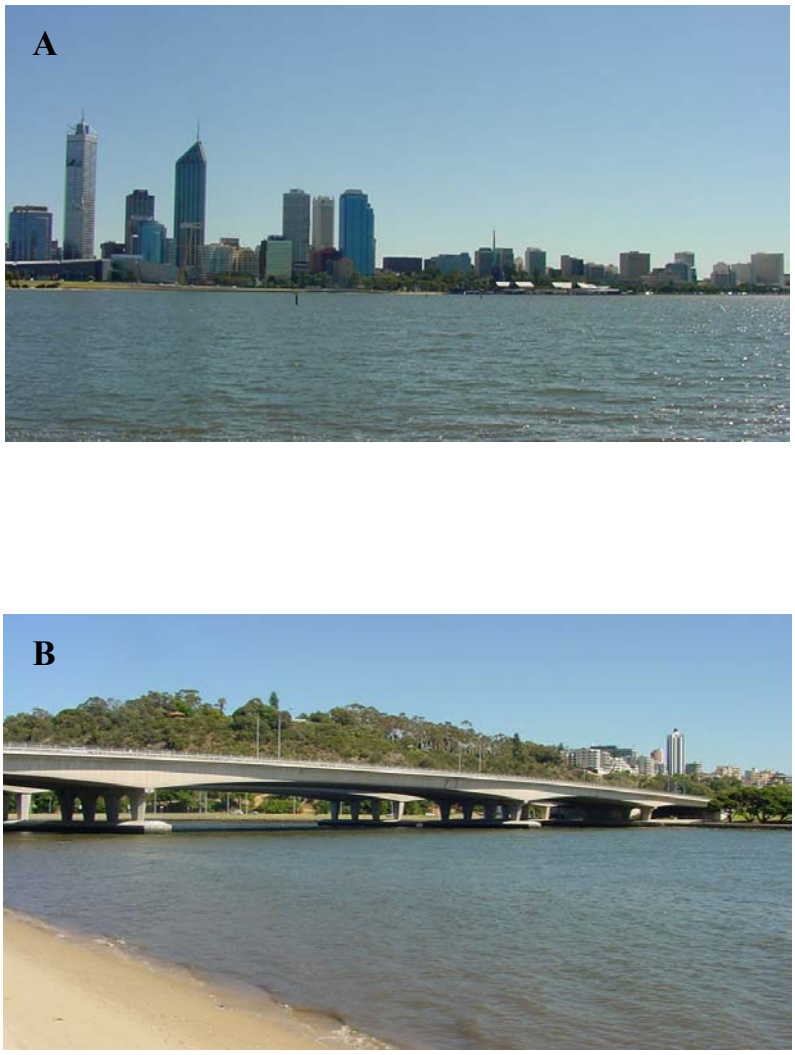


Plate 4: *Barrack Street collection site, (A) Perth CBD and Barrack Street site taken from the southern banks of the Swan River across Perth Water, (C) Narrows Bridge, carries heavy road traffic cross estuary.*

C5. Crawley

Plate 5: *Crawley collection site, (A) looking downstream towards the Royal Perth Yacht Club, (B) University Boat Shed, (C) drain brings runoff directly to Swan River at Crawley from nearby roads and residential properties.*

C6. Freshwater Bay

Plate 6: *Freshwater Bay collection site, (A) boat pens at Freshwater Bay Yacht Club, (B) example of drain outlet at Freshwater Bay that brings run off directly to the estuary from roads and surrounding areas, (C) Freshwater Bay showing surrounding residential development and typical foreshore recreational summer activity.*

C7. Salter Point

Plate 7: *Salter Point collection site, (A) facing southern banks showing residential development, (B) facing upstream (east), (C) drainage outlet at Salter Point bringing run off directly to Canning River from bordering residential areas.*

C8. Riverton

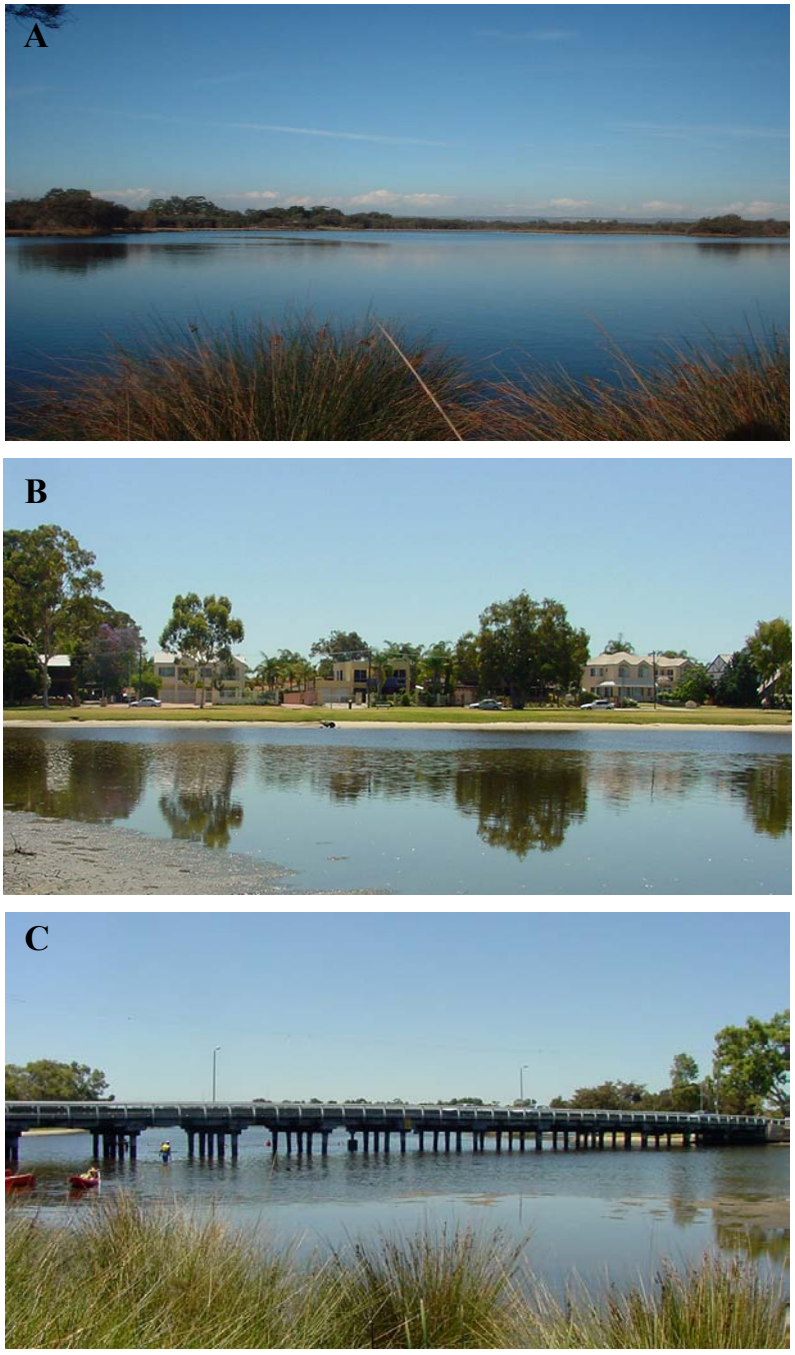


Plate 8: *Riverton collection site, (A) viewed from the southern bank, (B) Residential development on the southern bank of the Canning River, (C) Riverton Bridge downstream from collection site.*

Appendix D

Standardised methods for measuring biomarkers in black bream: Swan-Canning Estuary, Western Australia

D1. Mixed function oxygenase (MFO) assays

The quantification of the activity of the MFO enzyme 7-ethoxycoumarin-*O*-deethylase (ECOD) was adapted for black bream (*Acanthopagrus butcheri*) from the methods of Holdway *et al.*, 1998). The method for the measurement of the MFO enzyme ethoxyresorufin *O*-deethylase (EROD) was modified from Hodson *et al.*, 1991). All chemicals used in the MFO assays were purchased from Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia.

D1.1. Preparation of the S9 post-mitochondrial suspension (PMS)

Materials

0.2M HEPES	4.77 g
0.15M KCl	11.18 g

Dissolve in 1 litre of distilled (DI) water. Adjust pH to 7.5 with NaOH.

Procedure

Thaw liver samples on ice then take approximately 1 g of liver and add 4 mL of ice cold 0.2M HEPES buffer pH 7.5 and place into vials and homogenise. Centrifuge the resultant homogenate at 9800 rpm for 20 minutes at 4^{o}C. The PMS was collected using a Pasteur pipette, taking care to avoid the pellet and floating lipid layer.

D1.2. Batch assay for ECOD (7-ethoxycoumarin O-deethylase)

Materials

<u>7-ethoxycoumarin substrate</u>

Dissolve 15.2 mg ethoxycoumarin in 500 µL DMSO. Add this to 39.5 mL of 0.1 M Tris buffer pH 7.4 preheated to 50 ^{o}C. Cool and store at 4^{o}C.

<u>Cofactors</u>

10 mM MgCl2 - Dissolve 0.5083 g in 250 mL DI H_2O

200 mM KCl - Dissolve 3.7275 g in 250 mL DI H_2O

1.25 mM NADPH - 300 µg (0.0003g) NADPH dissolved in 3 mL DI H_2O (100 µg/mL). NAPDP has a short reactive life so prepare shortly before start of reaction.

Reaction Buffer

0.1M Tris Buffer pH 7.4 - Dissolve 12.11 g Trizma base in 1000 mL DI H_2O. Adjust pH to 7.4 with HCl.

Kill Reagents

5% $ZnSO_4$ - Dissolve 1.4377 g $ZnSO_4.7H_2O$ in 100 mL DI H_2O.

Saturated $Ba(OH)_2$ - Dissolve excess $Ba(OH)_2$ in 100 mL DI H_2O.

Alkalising Reagent

0.5 M Glycine-NaOH buffer pH 10.4:-

A) Dissolve 18.77 g glycine in 500 mL DI H_2O

B) Dissolve 10 g NaOH in 500 mL DI H_2O

Mix 250 mL of A + 193 mL of B.

D.1.2.1. ECOD standard curve

Stock Solution

A) 10.1 mg Umbelliferone + 12.5 mL ethanol

B) 1 mL of A + 49 mL DI H_2O

C) 1 mL of B + 9 ml DI H_2O

= 10 mL of 10 µM 7-hydroxycoumarin (16 µg umbelliferone)

= 1.6 µg/mL

= 1600 ng/mL

Standard Curve

D) 375 µL of C + 3.625 mL DI H_2O = 4 mL 150 ng/mL

E) 250 µL of C + 3.75 mL DI H_2O = 4 mL 100 ng/mL

F) 250 µL of C + 7.75 mL DI H_2O = 8 mL 50 ng/mL

G) 2000 µL of F + 2.00 mL DI H_2O = 4 mL 25 ng/mL

H) 1000 µL of F + 4.00 mL DI H_2O = 5 mL 10 ng/mL

I) 500 µL of F + 4.50 mL DI H_2O = 5 mL 5 ng/mL

ECOD concentrations

Mol weight of 7–hydroxycoumarin 162.1 g

Concentration (ng in 100 µL)	pmol
0	0
1.0	0.617
5.0	3.084
10.0	6.169
25.0	15.422
50.0	30.844
100	61.687
150	92.535
200	123.375
400	246.75

D1.2.1.2. ECOD Procedures

	Samples (x2) µL	Blank µL	Standard µL
0.1 M Tris buffer pH 7.4	1350	1350	1250
10 mM $MgCl_2$	25	25	25
200 mM KCl	25	25	25
0.125 mM NADPH	25	25	25
microsomes	100	100	100

Incubate for 2 minutes in water bath @ 30 ^oC

Add

2 mM ethoxycoumarin substrate	100	-	-

Incubate for 10 minutes in water bath @ 30 ^oC

Remove and place on ice

Kill reaction with

1. 5% $ZnSO_4$	500	500	500
2. Saturated $Ba(OH)_2$	500	500	500

Add

2mM ethoxycoumarin	-	100	100
Known concentration of 7-hydroxycoumarin standard	-	-	100

Vortex to mix, then centrifuge @ 3000 rpm for 10 mins @ 10 ^oC.

Remove 1 mL of supernatant from each tube and add 500 µL of 0.5 M glycine-NaOH buffer pH 10.5.

Read on Fluorimeter @ ex/em 380/452 nm slit 10/10.

Calculation

1) Protein concentration in mg/mL x dilution (usually 20)
2) Average ECOD (pmol)/protein = ECOD pmol H mg pr^{-1}

3) ECOD pmol mg pr^{-1}/10 minutes = ECOD pmol H mg pr^{-1} min^{-1}.

D1.3 Batch assay for EROD (ethoxyresorufin O-deethylase)

Materials

7-ethoxyresorufin substrate

Dissolve 5 mg 7-ethoxyresorufin in 166 mL DMSO to give a final concentration of 0.03 µg per mL.

Cofactors

1.28M $MgSO_4$ - Dissolve 15.4 g in 100 mL DI H_2O

40 mg mL^{-1} bovine serum albumin (BSA) - Dissolve 4 g in 100 mL DI H_2O

0.036 mM NADPH - 90 µg (>0.0001g) NADPH dissolved in 3 mL DI H_2O (approx 30 µg/mL). NAPDH has a short reactive life so prepare shortly before start of reaction.

Reaction Buffer

1M HEPES Buffer pH 7.8 - Dissolve 23.83 g HEPES in 1000 mL DI H_2O. Adjust pH to 7.8 with NaOH.

Kill Reagent

HPLC grade methanol

D1.3.1. EROD standard curve

Stock Solution

10 mg resorufin in 100 mL DMSO = 100 µg mL^{-1}

Standard Curve

A) 1 mL of stock solution + 3 mL DMSO = 4 mL 25 µg/mL
B) 500 µL of stock solution + 4.5 mL DMSO = 5 mL 10 µg/mL
C) 250 µL of stock solution + 4.75 mL DMSO = 5 mL 5 µg/mL
D) 100 µL of stock solution + 9.9 mL DMSO = 10 mL 1 µg/mL
E) 2000 µL of D + 2.0 mL DMSO = 4 mL 0.5 µg/mL
F) 1000 µL of D + 3.0 mL DMSO = 4 mL 0.25 µg/mL
G) 400 µL of D + 3.6 mL DMSO = 4 mL 0.1 µg/mL

EROD concentrations

Mol weight of resorufin 235.2 g

Concentration (µg mL^{-1})	pmol
0	0
0.1	4.251
0.25	10.6275
0.5	21.255
1.0	42.51
5.0	212.55
10.0	425.1
25.0	1062.575

D1.3.1.2. EROD Procedures

	Samples (x2) µL	Blank µL	Standard µL
1 M HEPES pH 7.8	1250	1250	1250
1.28 M $MgSO_4$	10	10	10
BSA (40 mg mL^{-1})	50	50	50
0.036 mM NADPH	30	30	30
PMS	50	50	50
2 mM ethoxyresorufin substrate	20	-	-

Incubate for 2 minutes at room temperature

Kill reaction with

HPLC methanol	2500	2500	2500

Add

Known concentration of resorufin standard	-	-	10

Vortex to mix then centrifuge @ 3000 rpm for 10 mins @ 10 ^{o}C.

Read on Fluorimeter @ 535/585 nm slit 10/10.

Note - adding ethoxyresorufin

Isolate 12 samples (tubes) then follow routine with a timer.

At time 0 add 20 µL ethoxyresorufin substrate to first tube - vortex. Repeat this at time 10 seconds with second tube, 20 with third, 30, 40, 50, 0, 10, 20, 30, 40, 50 (12th tube). Go back to the first tube and add 2.5 mL methanol at time 0 and mix. Repeat procedure with remaining tubes at 10, 20, 30, 40, 50, 0, 10, ...50 seconds.

Calculation

1. Protein concentration in mg/mL x dilution (usually 20)
2. Average EROD (pmol)/protein = EROD pmol R mg pr^{-1}
3. EROD pmol mg pr^{-1}/2 minutes = EROD pmol R mg pr^{-1} min^{-1}

D2. Biliary Metabolites

Evaluation of biliary metabolites was undertaken using the methods of Krahn *et al.*, 1986) for pyrene-type metabolites and Lin *et al.*, 1996) for naphthalene-type and benzo(*a*) pyrene (B(*a*)P)-type metabolites. All chemicals used in bile metabolite determination were purchased from Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia.

D2.1. Preparation and quantification of standard curves

Materials

HPLC grade methanol

1-naphthol

1-hydroxypyrene (1-OH pyrene)

Procedure

i) 1-naphthol standard curve

a) 10 mg 1-naphthol in 10 mL HPLC grade 50/50 methanol/H_2O = 10 mL 1 mg/mL

b) 0.1 mL of a + 9.9 mL 50/50 methanol/H_2O = 10 mL 10 000 ng/mL

c) 1.0 mL of b + 9.0 mL 50/50 methanol/H_2O = 10 mL 1 000 ng/mL

d) 0.5 mL of b + 9.5 mL 50/50 methanol/H_2O = 10 mL 500 ng/mL

e) 0.1 mL of b + 9.9 mL 50/50 methanol/H_2O = 10 mL 100 ng/mL

f) 1.0 mL of e + 1.0 mL 50/50 methanol/H_2O = 2 mL 50 ng/mL

g) 1.0 mL of e + 9.0 mL 50/50 methanol/H_2O = 10 mL 10 ng/mL

h) 1.0 mL of g + 1.0 mL 50/50 methanol/H_2O = 2 mL 5 ng/mL

Read fluorescence at ex/em 290/335 nm slit 10/10.

ii) 1-OH pyrene standard curve

a) 10 mg 1-OH pyrene in 10 mL HPLC grade methanol = 10 mL 1 mg/mL

b) 0.1 mL of a + 9.9 mL 50/50 methanol/H_2O = 10 mL 10 000 ng/mL

c) 1.0 mL of b + 9.o mL 50/50 methanol/H_2O = 10 mL 1 000 ng/mL

d) 0.5 mL of b + 9.5 mL 50/50 methanol/H_2O = 10 mL 500 ng/mL

e) 0.1 mL of b + 9.9 mL 50/50 methanol/H_2O = 10 mL 100 ng/mL

f) 1.0 mL of e + 1.0 mL 50/50 methanol/H_2O = 2 mL 50 ng/mL

g) 1.0 mL of e + 9.0 mL 50/50 methanol/H_2O = 10 mL 10 ng/mL

h) 2.0 mL of g + 2.0 mL 50/50 methanol/H_2O = 4 mL 5 ng/mL

i) 1.0 mL of h + 1.0 mL 50/50 methanol/H_2O = 2 mL 2.5 ng/mL

For pyrene-type metabolites, read the fluorescence of the standard curve at ex/em 340/380 nm slit 10/10

For B(*a*)p-type metabolites read the fluorescence at ex/em 380/430 nm slit 10/10.

D2.2. Determination of biliary metabolites

Dilution of bile

A. 20 µL of pure bile + 1980 µL 50/50 HPLC grade methanol/H_2O = 2 mL of 1:100 dilution
B. 100 µL of 1:100 + 4900 µL 50/50 methanol/H_2O = 5 mL 1:5 000 dilution
C. 200 µL of 1:100 + 3800 µL 50/50 methanol/H_2O = 4 mL 1:2 000 dilution
D. 400 µL of 1:100 + 3600 µL methanol/H_2O = 4 mL 1: 1 000 dilution
E. 800 µL of 1:100 + 3200 µL methanol/H_2O = 4 mL 1:500 dilution.

Naphthalene-type metabolites - read the fluorescence of 1:2 000 diluted bile against the 1-naphthol standard curve at ex/em 290/335 nm slit 10/10.

Pyrene-type metabolites – read the fluorescence of 1:500 diluted bile against the 1-OH pyrene standard curve at ex/em 340/380 nm slit 10/10.

B (*a*)p-type metabolites – read the fluorescence of 1:500 diluted bile against the 1-OH pyrene standard curve at ex/em 380/430 nm slit 10/10.

Dilutions of bile may need to be adjusted to ensure fluorometric measurements for biliary metabolites fall within the ranges of their respective standard curves.

D3. Determination of protein

The concentration of protein in a sample was determined using the method of Lowry *et al.*, 1951). All chemicals used in protein determination were purchased from Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia.

D3.1. Materials

Reagent A: 100 g Na_2CO_3 (anhydrous) in one litre 0.5M NaOH.

Reagent B: 1 g $CuSO_4.5H_2O$ in 100 mL millipore filtered DI H_20.

Reagent C: 2 g Sodium Potassium Tartrate in 100 mL millipore filtered DI H_20.

Folin & Ciocalteu's Phenol Reagent (2N Folin phenol): purchase premixed.

0.3 mg mL^{-1} BSA

Add 30 mg of bovine serum albumen to 100 mL millipore filtered DI H_20.

Standard curve (in duplicate test tubes)

A. 0 µL 0.3 mg mL^{-1} BSA + 1000 µL DI H_20 = 1 mL of 0.0 mg mL^{-1}
B. 100 µL 0.3 mg mL^{-1} BSA + 900 µL DI H_20 = 1 mL of 0.03 mg mL^{-1}
C. 300 µL 0.3 mg mL^{-1} BSA + 700 µL DI H_20 = 1 mL of 0.09 mg mL^{-1}
D. 500 µL 0.3 mg mL^{-1} BSA + 500 µL DI H_20 = 1 mL of 0.15 mg mL^{-1}
E. 700 µL 0.3 mg mL^{-1} BSA + 300 µL DI H_20 = 1 mL of 0.21 mg mL^{-1}
F. 1000 µL 0.3 mg mL^{-1} BSA + 0 µL DI H_20 = 1 mL of 0.3 mg mL^{-1}

D3.2. Dilution of samples

Dilutions for protein determination in black bream depend on the tissue used and the assay being conducted.

EROD/ECOD – dilute 50 µL of PMS in 950 µL of DI H_20. Add 50 µL of this diluted PMS to 950 µL of DI H_20 to determine protein concentration (20X dilution).

Bile metabolites – Add 50 µL of bile in 950 µL of DI H_20 to determine protein concentration. (1X dilution).

Metabolic enzymes – Gill - Add 20 µL of homogenised gill supernatant in 980 µL of DI H_20 to determine protein concentration. (50X dilution). Liver - Add 50 µL of homogenised liver supernatant in 950 µL of DI H_20 to determine protein concentration. (20X dilution).

Stress protein - Add 20 µL of homogenised gill supernatant in 980 µL of DI H_20 to determine protein concentration. (50X dilution).

Prepare samples in duplicate test tubes.

D3.3. Procedure

Mix reagents A, B & C in the ratio 20:1:1.

Add 1 mL of this solution to each test tube, Vortex and allow tubes to incubate at room temperature for 15 minutes.

While the tubes are incubating, add 5 mL of 2N Folin phenol reagent to 50 mL of DI H_20 in a beaker and mix.

At the end of the 15 minutes, add 3 mL of the Folin solution to each tube and vortex immediately.

Incubate tubes for 45 minutes at room temperature.

Read the absorbance of the standard curve on a spectrophotometer at 540 nm after incubation first, followed by the samples.

D4. Serum sorbitol dehydrogenase (s-SDH) (EC1.1.1.14)

D4.1. Preparation of serum

Centrifuge chilled fresh whole blood at 3000 rpm for 10 minutes. Collect the separated serum using a Pasteur pipette, put the serum in cryovials and immediately place in liquid nitrogen. Transfer to –80^{o}C freezer until analysis.

D4.2. Measurement of s-SDH activity

Materials

Sigma Diagnostics Sorbitol Dehydrogenase assay kit – catalogue # 50-UV.

Alternatively, if this kit is not available make up the reagents as follows:-

<u>0.1M Tris Buffer pH 7.5</u>

Dissolve 1.211 g Trizma base in 100 mL DI H_2O. Adjust pH to 7.5 with HCl.

<u>Fructose solution</u>

Dissolve 7.2 g D-Fructose in 10 mL DI H_2O

<u>1.28 µM ß-Nicotinamide Adenine Dinucleotide, reduced form</u>

Add 0.2 mg ß-NADH to 2 mL of 0.1M Tris Buffer pH7.5. Make this up immediately prior to assay.

All chemicals used in the SDH assay were purchased from Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia.

Procedure

a. Thaw serum on ice.
b. Place 50 µL of serum into a cuvette.
c. Add 450 µL ß-NADH/Tris Buffer solution and mix by gently shaking
d. Incubate for 10 minutes at room temperature to allow for the reaction of keto acids in the serum.
e. Add 100 µL of Fructose solution to start the reaction.
f. Immediately read the rate of decrease in absorbance (ΔA) over one minute at 340 nm in a spectrophotometer.

Calculation

Calculate the SDH activity in the serum using the following equations:-

$$\text{milli-International Units (mU) of SDH activity} = \frac{\Delta A/\text{min} \times 0.6}{0.00622 \times 0.05}$$

Where: 0.6 = Reaction volume (mL)

0.00622 = Micro molar absorptivity of NADH at 340 nm (McComb *et al.*, 1976)

0.05 = Sample volume (mL)

One International Unit (U) of an enzyme is defined as that amount which will convert 1 µM of substrate per minute.

D5. Stress Proteins

The method for the analysis of stress protein response was adapted from the methods of Martin *et al.*, 1996) then optimised for black bream. Unless otherwise indicated all chemical used for stress protein analysis were purchased from Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia.

Materials

3x buffer stock, pH 6.8	
10 mM Tris base	0.305 g
Millipore DI H_2O	250 mL
100 µM PMSF (Phenylmethylsulphonylfluoride) (store at -20°C)	
PMSF	0.174 g
isopropanol	10 mL
Homogenisation buffer	
3x buffer stock pH 6.8	10 mL
100 µM PMSF	30 µL
Millipore DI H_2O	20 mL
Sample Buffer	
2-mercaptoethanol	25 µL
Laemmli Sample Buffer	475 µL

Dilute sample with sample buffer just prior to loading into wells

(2 parts buffer: 1 part sample). Laemmli Sample Buffer (catalogue # 161-0772) was purchased from Bio-Rad Laboratories Pty Ltd, NSW, Australia.

Running Buffer, pH 8.3

10x Tris/Glycine/SDS Buffer (catalogue # BG-141 purchased from Life Therapeutics, French Forest, NSW, Australia)	100 mL
Millipore DI H_2O	900 mL

Store at 4°C.

Transfer Buffer, pH 8.3

10x Tris/Glycine Buffer (# 161-0771 Bio-Rad)	100 mL
20% v/v HPLC grade methanol	200 mL
Millipore DI H_2O	700 mL

Store at 4°C

PBS Stock Solution

2.7 M NaCl	157.79 g/L
29 nM KH_2PO_4	3.95 g/L
162 mM Na_2HPO_4	23.0 g/L
54 mM KCl	4.0 g/L

Prepare 1 Litre stock solution in millipore DI H_2O

PBS-Tween

Tween 20 (# 170-6531 Bio-Rad) to PBS stock solution	1 ml/L

Blocking Solution

Add 5% w/v non-fat dry milk powder to PBS-Tween.

TBS (Tris-Buffer Saline) – 20mM Tris, 500mM NaCl, pH 7.5

20x Tris-Buffered Saline (catalogue # AM07888 purchased from Astral Scientific, Caringbah, NSW, Australia)	50 mL
Millipore DI H_2O	950 mL

OR

10x Tris-Buffered Saline (# 170-6435 BioRad)	100 mL
Millipore DI H_2O	900 mL

TTBS (wash solution)

Add 0.05% (0.5mL/L) Tween 20 to TBS

Primary Antibody

Monoclonal (Mouse) Anti-Heat Shock Protein 70 Antibody (IgG1) (catalogue # MA3-006 purchased from Bio-Scientific, Gymea, NSW Australia) (Dilute to 1:5000

with TBS in 2 mL aliquots) - follow instructions included with product. Store at –20^{o}C.

Secondary Antibody

ImmunoPure Goat Anti-Mouse IgG Horseradish Peroxidase conjugated (catalogue # 31430 purchased from Progen Bioscience, Archerfield, QLD, Australia). Comes in a powdered form. Reconstitute with 2.0 mL millipore DI H_2O to the vial and close tightly with parafilm. Store at 4^{o}C.

Others consumables

Super Signal® West Pico Chemiluminescent Substrate Kit (catalogue # 34080 from Progen Bioscience).

Pre-stained SDS PAGE standard (# C3437 from Sigma-Aldrich).

12% Tris-Glycine iGel (# NG21-012 from Life Therapeutics).

Mini Trans Blot Thick Filter Paper (# 171-3932 Bio-Rad).

Thin Filter Paper

Supported nitrocellulose membrane 0.2um (catalogue # WP2HY00010 purchased from Medos Co Pty Ltd, Mt Waverley, Vic, Australia)

CL-Xposure film 5x7 Clear Blue X-Ray film (# 34090 Progen Bioscience).

Equipment

Mini-PROTEAN 3 Electrophoresis Cell/Mini Trans-Blot Module (# 165-3317 Bio-Rad).

Powerpac 300 (# 165-5051 Bio-Rad).

Fuji G8 X-Ray cassette

242S X-Ray Processor (working temperature of 31°C)

D5.1. Heat shock control samples

Quantification of stress proteins in field collected black bream requires standardisation with stress protein levels measured from black bream that have undergone a known stress event. This is achieved by subjecting 5 black bream to 10^{o}C heat shock. Five fish are acclimated to water at 17^{o}C. At the end of one week, these fish are placed in water heated to 27^{o}C, kept under observation for signs of excessive stress, then returned to their original aquarium (17^{o}C) at the end of 1 hour. After twenty-four hours at 17^{o}C, the fish are killed and gill tissue collected.

Run the electrophoresis of the heat shock control fish with the outer wells of the gel loaded with Pre-stained SDS PAGE standard (a molecular weight marker) to ensure that proteins in the 70-kDa range are correctly identified.

D5.2. Preparation of supernatant

Thaw the gill tissue on ice and cut the gill rays from the gill arches. Place approximately 200 to 500 mg of gill tissue in a homogenisation tube, record the weight and add 1x homogenisation buffer in the ratio of 1:4 w/v. Homogenise the tissue until completely broken down (approx 30 seconds). Spin the homogenate at 12 000 rpm at 4^{o}C for 98 minutes, collect the supernatant and place in aliquots of 100 µL. Put aside 1 aliquot for immediate protein determination and store the remainder at –80^{o}C until analysis.

Conduct the Lowry assay to determine the concentration of protein in the supernatant. Once the protein concentration has been determined, calculate the amount of supernatant required to ensure there is 40 µg of proteins in 19.8 µL of sample/buffer for stress protein determination.

D5.3. Electrophoresis

D5.3.1. Sample preparation

a) Add sufficient H_2O to the supernatant containing 40 µg protein to make up to 16.5 µL in an eppendorf tube.
b) Add 33 µL sample buffer to the tube to bring total volume to 49.5 µL.
c) Place the tube in a water bath at 95^{o}C for 4 minutes.

D5.3.2. Gel preparation

a) Wash the gels in DI H_2O, then rinse the wells with Running buffer.
b) Place the gels in the electrode assembly with short plates facing inwards. Clearly mark the plates A and B.

D5.3.3. Running the gels

a) Lower the electrode assembly into the Mini Tank. Fill the inner chamber with 125 mL, and the Mini Tank with 200 mL, of Running Buffer.
b) Place the Mini Tank in a small water bath.

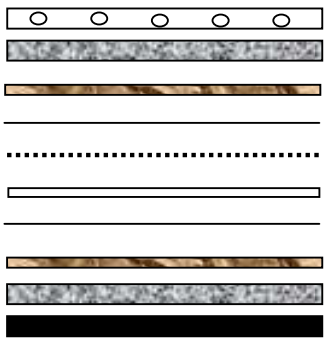
c) Miss the first two wells on each end of the gel, the load 19.8 µL of sample/buffer into each of the inner wells using a pipette with a gel-loading tip.
d) Load 19.8 µL of heat shock control samples into the outer two wells on each end of the gel.
e) Place the lid on the tank aligning the colour coded cathode/anode plugs and fill the water bath with ice, packing it around the tank to ensure the samples do not over heat.
f) Run the gels at 225 V, 120 mA (2 gels) or 60 mA (1 gel) for 40 minutes.

D5.4. Western Transfer

a) While the electrophoresis is running, cut nitrocellulose membranes and thin filter papers to the size of the iGels. Wear gloves and use tweezers when handling the membrane. Cut the top right hand corner of each membrane and mark the flat side with a pencil (A or B).
b) Soak the membranes, thick and thin filter papers and fibre pads in Transfer Buffer for at least 15 minutes to ensure soaked.
c) When the electrophoresis is complete remove the inner chamber, pour off the Running Buffer, and open cams.
d) Open gel cassettes taking care not to tear gel. Trim top of gel, being careful bits of gel do not stick to the gel. Gently agitate the gel cassette in Transfer Buffer until the gel separates from the cassette.
e) Place the Mini Trans-Blot Module cassette with the black side down
f) Place – 1 fibre pad on black side
1 thick paper on fibre pad
1 thin paper on thick paper
gel on the thin paper with the PROBED SIDE UP – remove bubbles
nitrocellulose membrane on the gel – smooth (shiny) side in contact with the gel – remove air bubbles
1 thin paper on the nitrocellulose membrane
1 thick paper on thin paper
1 fibre pad on thick paper – as shown on the following diagram

Close the cassette and secure with white latch.

Note: Make sure gel is black side of cassette – probed side up, and that the smooth side of the nitrocellulose membrane is in contact with the gel, otherwise the transfer of proteins from the gel to the membrane will not occur.



g) Place the cassette in the Mini-Trans Blot assembly with the black side of the cassette facing the black cathode electrode panel.
h) Add a stir bar and the Mini-Trans Blot ice block in the assembly.
i) Fill buffer chamber with Transfer Buffer to the level of the top row of circles of the cassette. Do NOT OVERFILL.
j) Place assembly on stirrer, connect to PowerPac and run at 100 V (250 mA) for 60 minutes.

D5.5. Immunoblotting

D5.5.1. Blocking

a) Place the membrane in a flat bottom container and cover with Blocking solution.
b) Agitate in the Blocking solution, on a shaker, for 60 minutes.

D5.5.2. Primary Antibody

a) Decant the Blocking solution and rinse the membranes in TTBS three times at 5 minutes each on the shaker. After the 3rd wash remove from TTBS and place membrane face (shiny side) up on a piece of Glad Wrap®.
b) Cover membrane with 2 mL of 1^{o} antibody ensuring even coverage. Wrap in the Glad Wrap to ensure membrane does NOT DRY OUT.
c) Probe with the primary antibody for 2 hours.

D5.5.3. 2^{ndary} Antibody

a) At the end of the 2 hours, unwrap membranes from the Glad Wrap and return to containers.
b) Wash the membranes three times in TTBS for 5 minutes each wash. Gently agitate during this time on a shaker.
c) After the third wash, cover the membrane with 2^{ndary} antibody at a 1:30 000 dilution. (5 µL antibody in 150 mL TTBS).
d) Incubate for 2 hours with gentle agitation on a shaker.

D5.6. Chemiluminescence

D5.6.1. Preparation of membranes

a) At the end of 2 hours, wash the membranes in TTBS, three times, 5minutes each wash while gently agitating on a shaker.
b) Wash for a further 10 minutes in TBS to remove the Tween-20. Gently agitate.
c) Decant the solution and replace with sufficient fresh TBS to keep the membrane moist.

D5.6.2. Developing the proteins

For each membrane to be developed, mix 3 mL each of the Stable Peroxide Solution and the Luminol Enhancer Solution from the Super Signal® West Pico Chemiluminescent Substrate Kit in a brown glass bottle. Take membranes and bottles of developing solution to a dark room.

a) Take the membranes from the TBS and place in clean flat bottom container clearly marked to correspond to the marks of the respective membranes.
b) Pour the developing solution over each membrane. Gently shake the boxes to ensure even coverage.
c) Allow sit for 5 minutes using a timer with alarm.
d) Place a plastic sheet, folded in half, into the film cassette to ensure the X-ray film will not touch the developing solution.
e) At the end of 5 minutes remove the membranes from the developing solution and blot the membrane with clean tissues.
f) Wearing gloves and using tweezers, place the membranes within the folded plastic sheet in the film cassette (2 membranes fit in the cassette) and remove air bubbles.

g) Remove X-ray film from box, cut a corner of the film to indicate the top (use a different cut for each film to clearly distinguish between each development time).
h) Place X-ray film in cassette on top of the plastic sheet, ensuring the developing solution does not come in direct contact with the film.
i) Develop film for 60 seconds then place film in X-ray Processor.
j) Insert a 2nd film in the cassette, develop for 90 seconds, then place in X-ray Processor.
k) Each film takes about 2 minutes to develop.

D5.7. Quantification

a) Scan the films using Adobe Photoshop 7.0 using a minimum 300 dpi.
b) Save in Grayscale mode as TIF files.
c) Analyse the pixel density of the images using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/).

Calculation

a) Calculate the ratio for each heat shock control fish (HSC) relative to the one with the highest pixel density on the standard films = Proportion.
b) For each sample film calculate the relationship that the HSC from the sample films has to the HSC from the standard film = Factor 1.
c) Calculate the average pixel density µg protein^{-1} as follows:
    - Divide the average pixels of the duplicates for each sample by Factor 1 = A.
    - Divide A by the proportion value of the HSC use on the sample film = B.
    - Divide B by the total protein in 19.8 µL of sample = standardised number of pixels per µg of total protein.

D6. Metabolic Enzymes

Assay conditions for enzyme activity were optimised for black bream based on the methods of Sidell *et al.*, 1987). All chemicals used in metabolic enzyme

determination were purchased from Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia.

Materials

50 mM Imadiazole Buffer pH 8.0

3.404 g Imadiazole added to 1000 mL millipore DI H_2O.

50mM Imadiazole Buffer pH 7.4

1.702 g Imadiazole added to 500 mL millipore DI H_2O. Adjust the pH to 7.4 with HCl.

70 µM CCO Dosing Solution

0.0087 g Cytochrome C (from horse heart) in 10 mL 50mM Imadiazole Buffer pH 8.0. Reduce all the Cytochrome C by adding 0.015 g of sodium hydrosulphite. The excess sodium hydrosulphite is then removed by gently bubbling with air for 45 to 60 seconds using a small aquarium aerator and airstone. Prepare immediately before use.

0.33% w/v Potassium ferricyanide solution

0.33 g $K_3Fe(CN)_6$ in 100 mL millipore DI H_2O.

Phosphate Buffer pH 7.4

Mix 40.5 mL 0.2 M Na_2HPO with 9.5 mL of 0.2 M NaH_2PO_4.

0.2 M Na_2HPO – Add 14.2 g Na_2HPO to 500 mL millipore DI H_2O.

0.2 M NaH_2PO_4 - Add 13.8 g NaH_2PO_4 to 500 mL millipore DI H_2O.

The two phosphate solutions can be stored in the refrigerator for several weeks. The pH may need to be adjusted slightly after mixing the two solutions.

LDH Dosing Solution

0.16 mM NADH - Add 0.006 g of NADH to 50 mL Phosphate Buffer pH 7.4.

0.8 mM Pyruvic acid - Add 5µL of pyruvic acid to 50 mL 0.16 mM NADH solution. Prepare immediately before use.

Procedures

D6.1. Preparation of homogenate

a) Thaw each tissue sample on ice and weigh out approximately 0.5 g of tissue.
b) Add 4.5ml of 50 mM Imidazole buffer pH 8.0 and homogenise – 1:10 w/v.
c) Centrifuge the homogenate at 5000 rpm for 5 mins at 4^{o}C and collect the supernatant for immediate use using a Pasteur pipette.

d) For lactate dehydrogenase assay take 100 µL of above homogenate and add 900 µL 50 mM Imadiazole Buffer pH 7.4.

D6.2. Batch assay for CCO (cytochrome C oxidase) (Cytox; EC1.9.3.1)

a) Prepare a control by adding 100 µL 0.33% (w/v) $K_3Fe(CN)_6$ to 900µL 70 µM CCO Dosing Solution A.
b) Measure the decrease in absorbance on a spectrophotometer at 550 nm as the cytochrome C is oxidized.
c) In duplicate, take 10 to 20 µL of tissue homogenate (pH 8.0) and add sufficient 70 µM CCO Dosing Solution to take the sample volume to 1000 µL. Measure the decrease in absorbance at 550 nm over 2 minutes.

D6.3. Batch assay for LDH (lactate dehydrogenase) (LDH; EC 1.1.1.27)

a) The amount of tissue homogenate required for LDH analysis in the black bream differed according to the tissue being analysed. Liver required 10 to 100 µL, gill 100 µL and white muscle needed 400 µL.
b) Add sufficient LDH Dosing Solution to the tissue homogenate (pH 7.4) to bring the sample volume to 1000 µL. Prepare samples in duplicate.
c) There is no control required as the enzymatic activity is always nil in the phosphate buffer in the absence of NADH.
d) Measure the decrease in absorbance, as NADH is consumed, on a spectrophotometer at 340 nm over 2 minutes.

Calculation

a) Measure the protein content of the homogenate using the Lowry method.
b) Calculate CCO enzymatic activity using a molar extinction coefficient of 29.5 for cytochrome c reduced.
c) Calculate LDH enzymatic activity using a molar extinction coefficient of 6.22.
d) Calculate the enzymatic activity in international units (U) normalised per milligram of protein. One U is equivalent to the conversion of 1 mol of substrate to product per minute.

Enzymatic Activity (U min^{-1}) =

$$\frac{(\Delta \text{ Ab/2 mins}) \times \mu\text{L final volume} \times \text{tissue homogenate dilution}}{}$$

$$U\ mg\ Pr^{-1}\ min^{-1} = \frac{\text{Extinction coefficient X } \mu L \text{ tissue homogenate}}{U\ min^{-1}} \Big/ mg\ protein$$

D7. Alkaline Unwinding Assay

The method for the determination of DNA strand breaks by the alkaline unwinding assay was adapted from Shugart, 1996), then optimised for black bream. All samples, solutions and equipment were maintained at 4 ^{o}C throughout sample preparation. Unless otherwise indicated, all chemicals used in the alkaline unwinding assay were purchased from Sigma Aldrich Pty Ltd, Castle Hill, NSW, Australia.

Materials

G-50 Buffer

150 mM NaCl	0.8764 g
10 mM Tris pH 7.4	0.1211 g
1 mM $MgCl_2$	0.0203 g
0.5 mM EDTA	0.0186 g

Make up to 100 mL with millipore DI H_2O.

25 mM NaCl - Add 0.073 g NaCl to 50 mL millipore DI H_2O.

0.2M Potassium Phosphate buffer pH 6.9 - Add 27.22 g KH_2PO_4 to 1000 mL millipore DI H_2O. Adjust pH to 6.9.

DNAzol® (catalogue # DN127 purchased from Astral Scientific, Caringbah, NSW, Australia)

0.05 M HCL

0.05 M NaOH

SE - Add 0.2885 g Sodium dodecyl sulfate (SDS) to 50 µL millipore DI H_2O then add 0.3722g EDTA

Hoechst Dye 33258

Procedure

D7.1. Preparation of homogenate

a) Thaw each liver sample on ice and weigh out approximately 100 mg.
b) Add 2 mL of DNAzol® and homogenise using a glass/Teflon handheld homogeniser (≅ 8 - 10 strokes). Let stand for 10 minutes.
c) Centrifuge the homogenate at 14 000 rpm for 12 mins at 4°C.
d) Avoiding the fatty layer, collect the supernatant for immediate use using a Pasteur pipette and place in a clean, chilled centrifuge tube.

D7.2. Isolation of DNA

a) Add 1 mL of 100% ethanol to the supernatant and mix by inversion 6 times, to precipitate the DNA.
b) Centrifuge the supernatant/ethanol solution at 11 000 rpm for 5 mins.
c) Remove the excess ethanol leaving the isolated DNA and add 1 mL of 75% ethanol.
d) Clean the DNA by passing in and out of a Pasteur pipette then centrifuge at 4000 rpm for 2 mins.
e) Repeat steps c and d and finally remove the excess ethanol.
f) Solubilize the DNA pellet by adding 1 mL of G-50 Buffer and passing the DNA in and out of a pipette. Perform this final step at room temperature.
g) Store sample on ice.

D7.3. Time and temperature optimisation for the DNA Alkaline Unwinding Assay using black bream

a) Prepare sufficient homogenate for all assays and isolate the DNA.
b) Add 100 µL of solubilized DNA to test tubes.
c) Prepare double stranded (DS), single stranded (SS) and partially unwound (DSS) samples in duplicate as per the procedure detailed in *D7.4*.
d) Store the DS sample at 4°C for 15 minutes and then read on the fluorimeter at 0, 20, 30, 40, 50, and 60 minutes. Return to the refrigerator between readings.
e) Incubate the DSS samples at 38°C for 20, 30, 40, 50, and 60 minutes.
f) Incubate the SS samples at 85°C for 20, 30, 40, 50, and 60 minutes.

g) Place each DSS and SS sample in the refrigerator for 15 minutes following incubation then, read the fluorescence of each DSS and SS sample.
h) Plot the change in fluorescence over time. The optimum time is when SS starts to level off.
i) Repeat the above over a range of temperatures making small adjustments to times until the optimum time/temperatures are achieved. For black bream, the optimum conditions were DSS - 35^{o}C/5 minutes, and SS – 85^{o}C/30 minutes.

D7.4. Alkaline Unwinding Assay for determination of DNA strand breaks

Once the optimum assay time and temperatures have been determined, the samples can be analysed.

Double Stranded (DS)

a) To two chilled test tubes add:-
   - 100 µL of 25 mM NaCL
   - 5 µL SE
   - 3 mL 0.2M Potassium Phosphate buffer pH 6.9
   - 3 µL Hoechst Dye 33258
   - Vortex
   - 100 µL of solubilized DNA
   - Vortex
b) Incubate for 15 mins at 4 ^{o}C (refrigerator).
c) Read the fluorescence of the DS present in the sample at excitation/emission wavelengths of 350ex/453em nm (slit 10-ex/10-em).

Single Stranded (SS)

a) To two chilled test tubes add:-
   - 100 µL of solubilized DNA
   - 50 µL 0.05 M NaOH
b) Vortex then seal tubes with parafilm
c) Place tubes in water bath heated to 85^{o}C and incubate for 30 minutes
d) Remove from water bath and add:-
   - 50 µL 0.05 M HCl

- 5 µL SE, then pass the solution 5 times through a 23 G needle to shear the DNA
- 3 mL 0.2 M Potassium Phosphate buffer pH 6.9
- 3 µL Hoechst Dye 33258

e) Place in the refrigerator for 15 minutes

f) Read the fluorescence of the SS present in the sample at excitation/emission wavelengths of 350ex/453em nm (slit 10-ex/10-em).

Partially Unwound DNA (DSS)

a) To two chilled test tubes add:-
   - 100 µL of solubilized DNA
   - 50 µL 0.05 M NaOH

b) Vortex then seal tubes with parafilm

c) Place tubes in water bath heated to 35^{o}C and incubate for 5 minutes

d) Remove from water bath and add:-
   - 50 µL 0.05 M HCl
   - 5 µL SE, then pass the solution 5 times through a 23 G needle to shear the DNA
   - 3 mL 0.2 M Potassium Phosphate buffer pH 6.9
   - 3 µL Hoechst Dye 33258

e) Place in the refrigerator for 15 minutes

f) Read the fluorescence of the DSS present in the sample at excitation/emission wavelengths of 350ex/453em nm (slit 10-ex/10-em).

Calculation

The ratio of double-stranded DNA in the sample (F value) was calculated using the equation, F = (DSS - SS)/(DS - SS).

D8. References

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