The characterisation of Australian freshwater fish immune systems and their response to immunomodulators

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

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**Declaration**
I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and any editorial work, paid or unpaid, carried out by a third party is acknowledged.

_____________________      _________
Andrew Harford      Date

**Parts of this work have been presented at the following scientific forums:**


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<th>Description</th>
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<tbody>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>3MC</td>
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<td>ethoxyresorufin O-deethylase</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
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<td>forward scatter</td>
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<td>γ-aminobutyric acid</td>
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<td>halogenated aromatic hydrocarbons</td>
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<tr>
<td>HEPES</td>
<td>N-(2hydroxyethyl)piperazine-N-2-ethane sulfonic acid</td>
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<tr>
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<td>hen egg white lysozyme</td>
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</tr>
<tr>
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<td>iC3b</td>
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<tr>
<td>IC₅₀</td>
<td>concentration that results in 50% inhibition</td>
</tr>
<tr>
<td>ADI</td>
<td>acceptable daily intake</td>
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<td>AF</td>
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<td>Ah</td>
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<td>alkaline phosphatase</td>
</tr>
<tr>
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<tr>
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<td>acute phase protein</td>
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<tr>
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<td>concanavalin A</td>
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<tr>
<td>CPM</td>
<td>counts per minute</td>
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<tr>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DBT</td>
<td>dibutyltin</td>
</tr>
<tr>
<td>DDT</td>
<td>dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12 dimethylbenzo(a)anthracene</td>
</tr>
<tr>
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<td>dimethylsulfoxide</td>
</tr>
<tr>
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</tr>
<tr>
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<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>NTE</td>
<td>neuropathy target esterase</td>
</tr>
<tr>
<td>NTP</td>
<td>National Toxicology Program (U.S.A.)</td>
</tr>
<tr>
<td>OC</td>
<td>organochlorine</td>
</tr>
<tr>
<td>OMPI</td>
<td>L-2-oxo-3-(2-mercaptoethyl)-5-phenyl-imidazolidine</td>
</tr>
<tr>
<td>OP</td>
<td>organophosphate</td>
</tr>
<tr>
<td>OPIDN</td>
<td>OP-induced delayed neurotoxicity</td>
</tr>
<tr>
<td>PAH</td>
<td>polyaromatic hydrocarbons</td>
</tr>
<tr>
<td>PBB</td>
<td>polbrominated biphenyls</td>
</tr>
<tr>
<td>PCB</td>
<td>polychlorinated biphenyls</td>
</tr>
<tr>
<td>PCDD</td>
<td>polychlorinated dibenzodioxin</td>
</tr>
<tr>
<td>PCDF</td>
<td>polychlorinated dibenzofuran</td>
</tr>
<tr>
<td>PDT</td>
<td>6-phenyl-2,3-dihydroimidazo(2,1-b) thiazole</td>
</tr>
<tr>
<td>PFC</td>
<td>plaque-forming cell</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>propridium iodide</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane Ca(^{2+})-ATPase-type pump</td>
</tr>
<tr>
<td>PME</td>
<td>pulp mill effluents</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>ppb</td>
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</tr>
<tr>
<td>pptr</td>
<td>parts per trillion</td>
</tr>
<tr>
<td>PVC</td>
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<td>PWM</td>
<td>Pokeweed mitogen</td>
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<tr>
<td>RBC</td>
<td>red blood cells</td>
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<td>ROS</td>
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<td>SAA</td>
<td>serum amyloid A</td>
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<tr>
<td>SERCA</td>
<td>sarco-endoplasmic reticular Ca(^{2+})-ATPase-type pump</td>
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<td>SS</td>
<td>side scatter</td>
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<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
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<td>USEPA</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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</table>
Summary

The Murray-Darling basin is the largest river system in Australia with significant economic, social, recreational and cultural value. It supplies water for drinking and agriculture to a large inland area of the eastern and southern states of Australia. It is also the ultimate sink for many environmental contaminants that result from human activities within the catchment. Aquatic organisms live intimately with their environment and may be continuously exposed to these contaminants through the water column or the food chain. Some chemicals are bioaccumulated and biomagnified in tissue to reach high body burdens. Populations of native fish species within the Murray-Darling basin have been in decline since human settlement, yet little is known about the lethal and sublethal effects of environmental pollutants on native freshwater fish and many of the Australian water quality guidelines are based on data from exotic fish species.

Researchers have correlated levels of pollution with immune dysfunction and an increased incidence of disease amongst wildlife populations. Many of the pollutants of the Murray-Darling basin have known immunotoxicity in both mammals and exotic fish species. The immune system is a sensitive target organ because, in order to maintain integrity, it requires constant renewal through the rapid proliferation and differentiation of cells.

Efforts to increase numbers of native fish in the wild have led to an aquaculture industry that produces fingerlings for the restocking of waterways. In more recent years, this industry has matured and now produces table-size native freshwater fish for local and international markets. Although the industry has researched areas of reproduction, nutrition and stocking, there is little understanding of the immunology or immunotoxicology of Australian freshwater fish. This research project investigated the immunology of three large native fish species (i.e.
Murray cod, golden perch and silver perch), which are the basis of the native freshwater aquaculture industry. Additionally, a small fish species native to the basin (i.e. crimson-spotted rainbowfish) was studied as an alternative to the use of large fish. Of the four species, Murray cod possessed characteristics that made it an excellent candidate for eco-immunotoxicity testing.

In this project three immune functional assays were successfully optimised to assess the immunotoxic responses of Australian freshwater fish to environmental contaminants and immunostimulants used in aquaculture:

- The lysozyme assay measured the bactericidal activity of fish serum, however little change was seen in this parameter when fish were exposed to chemicals.
- The specific response of fish lymphocytes to a number of mitogens was investigated in the mitogenesis (i.e. mitogen-induced lymphoproliferation) assay. PHA-induced mitogenesis was chosen to assess the cell-mediated (i.e. T-cell) immune functions of fish exposed to chemicals, as it provided a consistent day-to-day response. However, some difficulties were experienced in achieving the level of stimulation observed in other species such as rainbow trout.
- Phagocytic capacity of fish granulocytes was measured via flow cytometry. The flow cytometric method was highly sensitive and useful, as it not only assessed the innate immune function of the fish but also the relative number of lymphocytes and granulocytes in head kidney tissue, which gave an indication of toxicity directed towards specific cell subpopulations.

These assays were used to assess the immunomodulatory effect of a variety of important pollutants and immunostimulants in four aforementioned fish species using in vitro and in vivo exposure methodologies. The test compounds included:
• the organotins, tributyltin (TBT) and dibutyltin (DBT),
• the organochlorine (OC) pesticide, endosulfan,
• the organophosphate (OP) pesticide, chlorpyrifos,
• the cyanobacterial toxins, microcystin-LR and cylindrospermopsin, and
• two immunostimulants (i.e. levamisole and β-glucan), which are commonly used in the aquaculture of exotic species to increase fish health.

The organotins displayed immunotoxic activity in both *in vitro* and *in vivo* immune assays, with DBT being more immunotoxic than TBT. Although the pesticide endosulfan was highly toxic to fish, immunotoxicity was only seen at doses nearing lethal concentrations. This indicates that endosulfan is not a specific environmental immunotoxin, although chronic exposure to low levels should be investigated as endosulfan has some propensity to bioaccumulate. Chlorpyrifos was only immunotoxic at doses where signs of neurotoxicity were observed, indicating it was also unlikely to be a specific environmental immunotoxin. Preliminary *in vitro* studies on the effects of the cyanobacterial toxins demonstrated that they have the ability to stimulate granulocyte phagocytosis at low doses, a finding that warrants further investigation. Both immunostimulants showed some immunostimulatory effects *in vitro*. However, the *in vivo* dosage regime used in this study failed to generate immunostimulation of fish immune functions, although levamisole did increase splenic somatic indices and β-glucan marginally increased granulocytes numbers in the head kidney.

This project has addressed the paucity of information available on the immunology of a number of highly-valued Australian freshwater fish. Moreover, it has demonstrated that immune functional assays can be used to assess the immunotoxicity of xenobiotics in native freshwater fish.
Aims of this research

Studies from abroad have demonstrated that many aquatic pollutants are immunotoxic to exotic species of fish, however very little research has been conducted on the effects of these chemicals on freshwater Australian fish. In addition, very little is known about immunology of Australian freshwater fish that are now the focus of a native aquaculture industry. Researchers have used immunostimulants to increase the immune functions of fish to aid in their resistance to infections but it is unknown if these treatments are effective in native species. This project aimed to increase the knowledge of native freshwater fish immune systems and apply immune functional assays to assess the immunotoxicity of environmental pollutants and aquaculture immunostimulants. More specifically the aims were to:

(1) Investigate the immune functions of Australian freshwater fish using assays adapted from exotic fish and mammalian studies.

(2) Optimise and assess immune functional assays to measure the immunotoxic responses of Australian freshwater fish.

(3) Examine the immunotoxicity of a number of key environmental pollutants and immunostimulants to Australian freshwater fish and compare their sensitivity.

(4) Investigate the suitability of different native fish in ecotoxicology testing.
Chapter 1: General Introduction

1.1: The Murray-Darling Basin

The Murray-Darling Basin is the largest and most economically important river system in Australia and covers 1.06 million square kilometres or 14% of the nation’s landmass. The principle waterway of the basin is the Murray River, although it includes many major tributaries such as the Darling, Murrumbidgee and Goulburn rivers. The basin stretches through the three eastern Australian states of Queensland, New South Wales (NSW) and Victoria and finishes its path in South Australia (figure 1.1). The annual runoff from the basin is 24,300 GL, however, in comparison to other major river systems, it carries a relatively small and highly variable volume of water. Presently, this runoff is highly regulated with a large amount of the water being used in the irrigation of crops and a small amount also being used as drinking water (MDBMC, 2002b). The rivers of the basin provide drinking water for Adelaide and other towns and yield $A23 billion for Australia’s gross domestic production (MDBMC, 2002b). Within the basin there is 465 million hectares of agricultural land from which $A10 billion is produced annually, which equates to 40% of Australia’s gross agricultural production. This production is the most significant of any defined region in Australia (Smith and Maheshwari, 2002).
Despite the Murray-Darling basin being one of the largest river systems in the world, the number of freshwater fish that complete their life-cycle within the basin is relatively small. The Murray-Darling Basin Commission (MDBC, 2001) has listed 35 native species that spend at least some of their life-cycle within the basin. Table 1.1 shows a list of 27 species that occur in the NSW and Victorian section of the Murray-Darling basin and complete all of their life-cycle within the basin. The species used in this study were the crimson-spotted rainbowfish, silver perch, golden perch and Murray cod.

Figure 1.1: The location of the Murray-Darling basin in Australian (MDBC, 2001).

1.2: Fish species of the Murray-Darling Basin

Despite the Murray-Darling basin being one of the largest river systems in the world, the number of freshwater fish that complete their life-cycle within the basin is relatively small. The Murray-Darling Basin Commission (MDBC, 2001) has listed 35 native species that spend at least some of their life-cycle within the basin. Table 1.1 shows a list of 27 species that occur in the NSW and Victorian section of the Murray-Darling basin and complete all of their life-cycle within the basin. The species used in this study were the crimson-spotted rainbowfish, silver perch, golden perch and Murray cod.
<table>
<thead>
<tr>
<th>Common name</th>
<th>Spawning triggers</th>
<th>Migrations</th>
<th>Intolerance</th>
<th>Distribution, conservation status and state listings[^]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Australian smelt (Retropinna semoni)</strong></td>
<td>15-18°C</td>
<td>No</td>
<td>No</td>
<td>Widespread</td>
</tr>
<tr>
<td><strong>Bony herring (Nematalosa erebi)</strong></td>
<td>No</td>
<td>No</td>
<td>Yes Barriers</td>
<td>Widespread</td>
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<tr>
<td><strong>Barred galaxias (Galaxias fuscus)</strong></td>
<td>Yes</td>
<td>No</td>
<td>Yes Barriers</td>
<td>Threatened species, upland; Vic – Critically endangered, protected under FFG[^]</td>
</tr>
<tr>
<td><strong>Climbing galaxias (broad-finned galaxias)</strong></td>
<td>Yes</td>
<td>No</td>
<td>Yes Barriers</td>
<td>Recently introduced into Murray-Darling Basin</td>
</tr>
<tr>
<td><strong>Common jollytail (common galaxias)</strong></td>
<td>No</td>
<td>Yes</td>
<td>Yes Barriers</td>
<td>Widespread</td>
</tr>
<tr>
<td><strong>Crimson-spotted rainbowfish (Melanotaenia fluviatilis)</strong></td>
<td>20-25°C</td>
<td>No</td>
<td>No</td>
<td>Widespread, Vic- restricted, protected under FFG</td>
</tr>
<tr>
<td><strong>Darling river hardy-head (Craterocephalus amniculus)</strong></td>
<td>-</td>
<td>No</td>
<td>No</td>
<td>Threatened species, restricted distribution</td>
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<tr>
<td><strong>Dwarf flatehead gudgeon (Philypnodon sp)</strong></td>
<td>No</td>
<td>Yes</td>
<td>Yes Barriers</td>
<td>Lower MDB and Murrumbidgee and Murray rivers; Vic – protected under FFG</td>
</tr>
<tr>
<td><strong>Flathead gudgeon (Philypnodon grandiceps)</strong></td>
<td>No</td>
<td>Yes</td>
<td>Yes Barriers</td>
<td>Widespread common</td>
</tr>
<tr>
<td><strong>Murray hardy-head (Craterocephalus fluviatilis)</strong></td>
<td>&gt;23.5°C</td>
<td>No</td>
<td>No</td>
<td>Threatened species, restricted distribution; Vic-endangered, protected under FFG; NSW - endangered species</td>
</tr>
<tr>
<td><strong>Freshwater catfish (Tandanus tandanus)</strong></td>
<td>&gt;24°C</td>
<td>No</td>
<td>No</td>
<td>Declined population numbers but was once widespread; Vic – Vulnerable, protected under FFG; NSW – Protected from commercial catch</td>
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<tr>
<td><strong>Golden perch (Macquaria ambigua)</strong></td>
<td>&gt;23°C</td>
<td>Yes</td>
<td>Yes Barriers</td>
<td>Fragmented and in low abundance; Vic – Endangered, protected under FFG</td>
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<tr>
<td><strong>Hyrtl’s tandan (Neosilurus hyrtlii)</strong></td>
<td>20-34°C</td>
<td>Yes</td>
<td>No</td>
<td>Northern MDB</td>
</tr>
<tr>
<td><strong>Macquarie perch (Macquaria australasia)</strong></td>
<td>14-21°C</td>
<td>Yes</td>
<td>Yes Water quality</td>
<td>Threatened species, restricted distribution; ACT – endangered; Vic – endangered protected under FFG</td>
</tr>
<tr>
<td><strong>Mountain galaxias (Galaxias olidus)</strong></td>
<td>No</td>
<td>No</td>
<td>Upland areas and slopes</td>
<td></td>
</tr>
<tr>
<td><strong>Murray cod (Maccullochella peeli)</strong></td>
<td>16-21°C</td>
<td>No</td>
<td>Yes Barriers</td>
<td>Fragmented and in low abundance; Vic – Endangered, protected under FFG</td>
</tr>
<tr>
<td><strong>Murray hardyhead (Craterocephalus fluviatilis)</strong></td>
<td>No</td>
<td>Yes</td>
<td>Yes Water quality</td>
<td>Threatened species, restricted distribution; Vic – endangered, protected under FFG; NSW – endangered species</td>
</tr>
<tr>
<td><strong>Flathead galaxias (Galaxias rostratus)</strong></td>
<td>9-14°C</td>
<td>No</td>
<td>Widespread common</td>
<td></td>
</tr>
<tr>
<td><strong>Olive perchlet (Ambassis agassizii)</strong></td>
<td>&gt;25°C</td>
<td>Yes</td>
<td>No</td>
<td>Threatened species, declining in lower MDB</td>
</tr>
<tr>
<td><strong>Purple spotted gudgeon (Mogurnda adspersa)</strong></td>
<td>20-34°C</td>
<td>Yes</td>
<td>No</td>
<td>Threatened species once widespread; Vic – critically endangered, protected under FFG</td>
</tr>
<tr>
<td><strong>River blackfish (Gadopsis marmoratus)</strong></td>
<td>&gt;16°C</td>
<td>No</td>
<td>No</td>
<td>Lower MDB and cooler regions</td>
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<tr>
<td><strong>Silver perch (Bidyanus bidyanus)</strong></td>
<td>&gt;23°C</td>
<td>Yes</td>
<td>Yes Water quality and barriers</td>
<td>Threatened species declining; ACT – endangered; Vic – critically endangered protected under FFG</td>
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<tr>
<td><strong>Southern pygmy perch (Nannoperca australis)</strong></td>
<td>No</td>
<td></td>
<td>Lower MDB uncommon; NSW – Vulnerable</td>
<td></td>
</tr>
<tr>
<td><strong>Spangled perch (Leiopotherapon unicolour)</strong></td>
<td>20-26°C</td>
<td>Yes</td>
<td>Yes Temperature</td>
<td>Common, mid to upper MDB</td>
</tr>
<tr>
<td><strong>Trent cod (Maccullochella macquariensis)</strong></td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Threatened species, two known populations; ACT – endangered; Vic-critically endangered listed under FFG; NSW – endangered and protected</td>
</tr>
<tr>
<td><strong>Two spined blackfish (Gadopsis bispinosus)</strong></td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Upland species, lower MDB; ACT – Vulnerable</td>
</tr>
<tr>
<td><strong>Carp gudgeons (Hypseleotris sp.)</strong></td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Widespread</td>
</tr>
</tbody>
</table>

Blanks indicate insufficient information. The species used in this study are highlighted in bold
[^]FGG – Victorian flora and fauna guarantee act of 1988
[^]Murray-Darling Basin Ministerial Commission, 2000a
[^]Murray-Darling Basin Commission, 2001
[^]MBD= Murray-Darling Basin; Vic = Victoria, NSW= New South Wales, ACT = Australian Capital Territory
1.3: Aquaculture in the Murray-Darling basin

Hume and Barlow (1993) identified only 4 species native to the Murray Darling system that with potential for aquaculture, although other species have been grown for food markets (National Aquaculture Council, 2002). Silver perch, golden perch, Murray cod and the native freshwater catfish, *Tandanus*, all possess characteristics that have made them the focus of native aquaculturalists interested in producing local species for domestic and international markets. The majority of the “outdoor” industry is located in the warmer parts of NSW and Queensland, where growth rates in ponds are more economically viable for these warm water fish. However, new intensive indoor systems are being employed for silver perch and Murray cod aquaculture, improving the potential for southern states to increase production and their market share. Currently, the dominant market sector involves silver perch aquaculture, however aquacultured Murray cod is likely to overtake silver perch sometime in the near future. Technical difficulties in growing cod are being overcome and consumer demand is greater for cod, which is reflected in the cod product being worth twice the value of silver perch per kilogram.

1.3.1: Silver perch aquaculture

Silver perch aquaculture was the first to take off and currently has the largest market sector of the four species. This was due to a number of factors such as; good growth rates from low protein diets, omnivorous feeding habits and a lack of cannibalism (Kibria *et al.*, 1996). In 1996, silver perch aquaculture was described as “booming” due to interest from countries such as China and Taiwan (Kibria *et al.*, 1996). During 1998/1999 the NSW aquaculture industry produced 163 tonne of silver perch worth around $A1.55 million, which was stated by Frances *et al.* (2000), to be well within its potential. In 2001/2002 the value of the market had doubled to $A3 million (Love and Langenkamp, 2003).
1.3.2: Murray cod aquaculture

Murray cod aquaculture is an emerging industry and continues to grow, especially in Victoria, producing both table-sized fish (500g) for human consumption and fingerlings for the stocking of waterways. In 2001, in excess of 70 tonnes of Murray cod was produced for human consumption, which was approximately double the total production from the commercial capture fisheries from the whole of the Murray-Darling basin. In that year, the Murray cod market was estimated to be worth $A2 million and its value continues to grow (Kearney and Kildea, 2001; Love and Langenkamp, 2003).

1.3.3: Golden perch aquaculture

Golden perch fingerlings are produced in large numbers for the stocking of dams, lakes and waterways. However, the production of table-sized fish has not fully developed, due to technical difficulties with their feeding and growth rates and a lack of research into the culture of this species. In 2001/02, New South Wales produced 1.1 tonne of golden perch valued at $16 400, although figures from Victoria were not available because of the small number of producers. Recent feeding trials have reported that golden perch can be weaned onto an artificial diet, and this development could lead to the large-scale production of table-size fish (Love and Langenkamp, 2003).

1.4: Fish Immunology

The immune system serves to protect the host from infectious diseases and developing neoplastic cells and is highly conserved across all vertebrate species, with remnants also existing in invertebrates (Roitt et al., 1998). The teleost immune system shares many structural and functional similarities with the mammalian immune system and humoral, cell-mediated and non-specific immune responses have all been described. Fish are able to reject allografts, exhibit hypersensitivity responses, produce specific antibodies following antigenic
stimulation, respond to mitogens and elicit a mixed lymphocyte reaction (van Muiswinkel et al., 1985). Differences between mammals and fish are seen in the types of lymphoid tissues and immunoglobulins produced (Zelikoff, 1994).

Only a small sample of the more than 20,000 known fish species have had their immune systems investigated (Zelikoff, 1994), while a very limited number of studies have investigated the immunology or immunotoxicology of Australian fish. Comparative immunologists started studying fish in the 1960s and began to document the phylogenetic differences between the basic, but highly functional, teleost immune system and the more complex defence mechanisms of mammals. Since the 1970s, most studies have been conducted in fish species with high economic value, such as rainbow trout (Oncorhynchus mykiss), Atlantic salmon (Salmo salar), common carp (Cyprinus carpio), channel catfish (Ictalurus punctatus) and bluefin tuna (Thunnus maccocyii). These studies were motivated by the commercial interests of aquaculturalists to combat and control potential stock losses caused by fish diseases (Scapigliati et al., 1999; van Muiswinkel et al., 1985). Many of these on-going studies have used functional immune assays to assess the effectiveness of vaccines and immunostimulant therapies that aid in the health of fish held during high density and “stressful” conditions (Anderson, 1992; Gudding et al., 1999; Sakai, 1997). In more recent years, fish immunology has begun to include wild and feral species in ecotoxicological studies to assess the immunotoxicity of environmental pollutants. The ultimate aim of these studies is to use fish species to monitor the health of ecosystems (Bols et al., 2001; Zelikoff et al., 2002).

1.4.1: Lymphoid and myeloid tissue

Teleost lymphoid tissues are not as defined in fish as those of higher vertebrates. Primitive teleosts do not possess such specific lymphoid and myeloid tissue but still have areas with an
intermingling of both types of tissue, in which high concentrations of immune cells and lysosomal enzymes can be found. The major lymphoid tissues in teleosts are anterior (or head) kidney, spleen, thymus and mucosa-associated tissue (Fänge, 1982; Scapigliati et al., 2002).

Fish do not possess bone marrow, which is the source of myeloid cells in higher vertebrates (Kennedy-Stoskopf, 1993). Therefore, the key lymphoid organ in teleosts is the head kidney, which has a mixture of lymphoid and myeloid characteristics, while ultra-structural studies have confirmed its similarity to bone marrow. The head kidney also plays other roles in endocrine secretion and haematopoiesis (Fänge, 1982). The spleen in fish is a discrete organ possessing lymphoid cells (a mixture of T- and B-cells), plasma cells and red and white pulp, but it lacks germinal centres (Corbel, 1975; Kennedy-Stoskopf, 1993).

1.4.2: Immunocytes

Immune cells in fish share many similarities with their mammalian counterparts in both structure and function and thus, fish immunocyte subpopulations have been described as B-lymphocytes (Kaattari, 1992; Koumans-Van Diepen et al., 1994b; Miller et al., 1994), T-lymphocytes (Lin et al., 1992; Nakanishi et al., 2002), granulocytes (Ainsworth, 1992; Hine, 1992; Slierendrecht et al., 1995), macrophages (Romano et al., 1998; Sarmento et al., 2004; Weyts et al., 1997) and non-specific cytotoxic cells (Cuesta et al., 2003; Evans et al., 1992; Hogan et al., 1996; Ishimoto et al., 2004; Yoder, 2004).

Teleost B-lymphocytes share many characteristics with their mammalian counterparts including surface immunoglobulins (sIgM), proliferation in the presence of B-cell mitogens (i.e. lipopolysaccharide, LPS) and production of antibodies in response to antigens (Kaattari, 1992). In addition, cellular immunoglobulin-positive plasma cells that have been identified in
the head kidney of fish are able to secrete large amounts of antibody following a secondary exposure, demonstrating that the teleost immune response has a memory component (Koumans-Van Diepen et al., 1994b). T-lymphocytes are the effective cells in cell-mediated immune responses and lack sIgM, respond to T-cell mitogens (i.e. phytohemagglutinin, PHA) and defend against viral infections (Nakanishi et al., 2002). T-cell receptor subunits and major histocompatibility complex products (MHC) for fish have been cloned, sequenced and described, while monoclonal antibodies to detect cells possessing T-cells receptors have also been produced in a number of species (Charlemagne, 1997; Flajnik et al., 1999; Hordvik et al., 2004; Park et al., 2001; Scapigliati et al., 2000; Timmusk et al., 2003).

Granulocytes are large phagocytic cells with cytotoxic granular particles within their cytoplasm and are found in the blood and other strategic areas, such as the peritoneal cavity, the head kidney and intestinal tract (Hine, 1992). In mammals, further distinctions between granulocytes are made based on the staining of the cell, i.e. neutrophils, eosinophils and basophils. In fish however, this nomenclature can be confusing due to differences in staining (Ainsworth, 1992). In the studies described in this thesis, although no specific stains were used, the term granulocyte was used for large cells of the head kidney that emitted a high level of side-scatter (SS) in flow cytometry, which is caused by particles and granules in their cytoplasm. Tissue macrophages and circulating monocytes are phagocytic cells with a pivotal role in the immune system, which includes antigen processing and presentation (Vallejo et al., 1992) and chemical signalling to modulate the function of other cells (Chihara, 1992). It has been demonstrated that the function of fish granulocytes and macrophages can be enhanced by a variety of substances such as adjuvants, LPS, glucans, chitin and synthetic chemicals, such as levamisole (Secombes, 1994).
1.4.3: Immunoglobulins

Compared to mammals that possess five classes of immunoglobulins (i.e. IgA, IgG, IgE, IgD and IgM), most teleosts have just two classes of immunoglobulins (Watts et al., 2001a). The first identified immunoglobulin isotype in fish is now the well-characterized tetrameric IgM-like molecule (600 - 800 kDa), consisting of subunits of two light and heavy chains held together by disulphide bonds (Anderson and Zeeman, 1995; Elcombe et al., 1985; Siwicki et al., 1998; Smith et al., 1993). IgM molecules are broad-range, low specificity immunoglobulins, which exist on the surface of B-cells in both mammals and fish (i.e. sIgM). When sIgM reacts with an antigen, the cell undergoes rapid proliferation and secretes specific antibodies against the antigen that stimulates the cells. The major difference between fish and mammals, is that fish B-cells are unable to undergo isotype switching to higher affinity immunoglobulins (e.g. IgG) and they are not able to produce progressively higher affinity IgM molecules through the selection of high affinity somatic mutants (Du Pasquier, 1993). Nevertheless, fish are able to mount a secondary response that is faster than the primary response. Further differences exist between mammals and fish in the structure of the IgM molecule. In mammals, the IgM structure is pentameric and is linked by a J chain. In some fish, the J chain is absent in the tetrameric structure, which is thought to introduce flexibility in the molecule allowing for the bonding of more epitopes (Coscia et al., 2000; Wilson and Warr, 1992). More recently, a small IgD molecule has been identified in some species of teleosts. However, its function remains to be determined, not only in fish, but also mammals (Hirono et al., 2003; Hordvik et al., 1999; Stenvik et al., 2001; Wilson et al., 1997).

Numerous studies have purified and characterised fish IgM molecules from over ten species of fish, including but not limited to; rainbow trout (Oncorhynchus mykiss) (Elcombe et al., 1985; Thuvander et al., 1990), channel catfish (Ictalurus punctatus) (Ghaffari and Lobb, 1989), Atlantic salmon (Salmo salar) (Hordvik et al., 1999; Pettersen et al., 2000), gilthead
seabream (*Sparus aurata*) (Navarro *et al.*, 1993), snapper (*Pagrus auratus*) (Morrison *et al.*, 2002), Pacific herring (*Clupea pallasi*) (Davis *et al.*, 1999), blue fin tuna (*Thunnus maccoyii*) (Watts *et al.*, 2001b), turbot (*Psetta maxima*) (Estevez *et al.*, 1993), Nile tilapia (*Oreochromis niloticus*) (Mochida *et al.*, 1994) and emerald rockcod (*Trematomus bernacchii*) (Coscia *et al.*, 2000). Furthermore, a number of these studies have raised monoclonal antibodies against IgM molecules for use in immunostaining and identification of B-cells (i.e. surface IgM positive cells) and their functions (Davis *et al.*, 1999; Morrison *et al.*, 2002; Scapigliati *et al.*, 1999; Thuvander *et al.*, 1990).

1.4.4: Cytokines

Cytokines are soluble, non-antibody proteins that are secreted by activated lymphocytes and initiate and regulate inflammatory responses. They are pleiotropic in nature, acting on multiple cell types and possessing a broad range of activities. There is also an element of redundancy in their activity, which indicates an overlapping of activities by different cytokines (Engelsma *et al.*, 2002). In mammals, an inflammatory insult results in a cascade of cytokine secretion, which begins with the release of tumour necrosis factor-α (TNF-α). This stimulates the release of interleukin-1β (IL-1β), which is then followed by the release of interleukin-6 (IL-6). The initiation of inflammation leads to the release of a myriad of other cytokines, which include chemoattractants that signal neutrophils and macrophages to migrate to the site of injury (i.e. chemokines). Ultimately, cytokines also up-regulate the production of cyclooxygenase and inducible nitric oxide synthetase, which are two enzymes with a key role in inflammation (Secombes *et al.*, 2001). Furthermore, they increase the production of acute phase proteins in the liver (Baumann and Gauldie, 1994).

In recent years, a flurry of research activity has lead to a number of fish cytokines and their genes being identified and characterised. The TNF-α gene and its receptor was first cloned in
the Japanese flounder (Hirono et al., 2000) and was later identified in rainbow trout (Laing et al., 2001) and common carp (Saeij et al., 2003). Additionally, functional tests in fish have proven a role for TNF-α in nitric oxide induction and the stimulation of cell proliferation (Saeij et al., 2003). Multiple IL-1 genes have been cloned from both rainbow trout (Zou et al., 1999) and carp (Engelsma et al., 2003; Fujiki et al., 2000), and the cytokine has also been identified in sea bass (Scapigliati et al., 2001), gilthead seabream (Pelegrin et al., 2001) and other fish, including the more primitive cartilaginous sharks (Bird et al., 2002). Its mRNA expression \textit{in vivo} is increased in experimentally-infected fish and modulated by cortisol. \textit{In vitro}, its expression is up-regulated by LPS stimulation and other lymphokines (Engelsma et al., 2003). In addition, researchers have begun to identify IL-1 receptors (both I and II types) in a number of fish (Engelsma et al., 2002; Sangrador-Vegas et al., 2000; Subramaniam et al., 2002). The anti-inflammatory cytokine, transforming growth factor-β3, has been identified in some fish (Hardie et al., 1998; Harms et al., 1997; Laing et al., 1999; Yin and Kwang, 2000) and a number of chemokines have also been identified (Dixon et al., 1997; Huising et al., 2003; Liu et al., 2002; Sangrador-Vegas et al., 2002).

1.4.5: Acute Phase Proteins and Non-cellular non-specific mechanisms

Acute phase proteins (APPs) or acute phase reactants are plasma proteins that are produced by cells in response to an inflammatory signal (i.e. TNF and interleukins). APPs may be antimicrobial, anti-inflammatory or have roles in repair processes, thus initiating a return to normal function of the body after traumatic events (Baumann and Gauldie, 1994). Fish possess a myriad of proteins and glycoproteins of a non-immunoglobulin nature, that have the ability to react with a wide range of environmental antigens. These include microbial growth inhibitory proteins (transferrin, metallothionein and ceruloplasmin), interferons, enzyme inhibitors (α2 macroglobulin and other α−globulins), hydrolase enzymes (lysozyme, chitinase, chitobiase) and non-specific lysins and agglutinins (including toxins with
bacteriolytic activity) (Alexander and Ingram, 1992; Bayne and Gerwick, 2001; Bayne et al., 2001). Complement, ILs and clotting proteins may also be classified as APPs, as they either aid in the initial stages of the defensive response or the clean up and repair of tissue. The large majority of these proteins are produced by hepatocytes and then enter the circulatory system but they may also be produced by monocytes, endothelial cells and fibroblasts (Bayne and Gerwick, 2001).

1.4.6: Fish immunophysiology

Fish immunophysiology is a discipline that investigates the interactions of the immune system with other physiological systems. Fish require good communication between their immune, nervous and endocrine systems to achieve homeostasis in a highly variable and rapidly changing environment. Therefore, the immune system responds not only to antigens and cytokines, but also internal factors such as hormones, neuropeptides, and neurotransmitters (figure 1.2) (Plytycz and Seljelid, 1995). Furthermore, immune organs are innervated by sympathetic and peptidergic nerve fibres, which allows for rapid and direct sympathetic effects on immune cells (Flory, 1989; Weyts et al., 1999). Receptors for neurohormones and neurotransmitters have also been identified on the surface of fish leukocytes (Flory and Bayne, 1991; Jozefowski et al., 1995; Weyts et al., 1998). A large number of cytokines are now known to interact with the hypothalamus–pituitary–adrenal (HPA) axis in mammals, and cells that are an integral part of the nervous system also produce cytokines, which were originally thought to only be produced by cells of the immune system (Weyts et al., 1999). The pro-inflammatory cytokines, IL-1, TNF-α and IL-6, are considered the main effectors in this communication between the immune system and the HPA axis in mammals (Engelsma et al., 2002).
Figure 1.2: Schematic representation of communication pathways between the hypothalamo-pituitary-interrenal axis and the immune system in teleost fish. The speculative central role of the head kidney in immune-endocrine interactions is shown. In the head kidney, cortisol- and catecholamine-producing cells are intermingled with leukocytes, enabling direct, paracrine interactions. Abbreviations: CNS, central nervous system; CRH, corticotropin-releasing hormone; TRH, thyrotropin-releasing hormone; DA, dopamine; ACTH, adrenocorticotropic hormone; $\alpha$-MSH, alpha-melanocyte-stimulating hormone; CA, catecholamine; IS, immune system. Dotted lines and question marks refer to potential communication pathways (from Weyts et al., 1999).

In fish, the head kidney serves both systems in the one organ, as it is involved in haematopoiesis, antibody production, and cortisol and catecholamine production. Thus, the term hypothalamus–pituitary-interrenal (HPI) axis is used in fish instead of HPA axis. The term “stress axis” has also been used in fish due to the clear interaction of stress stimuli on the functions of cells within the HPI axis (see section 1.4.7.) (Weyts et al., 1999).
1.4.7: Sensitivity to stress

The definition of stress has received much debate, but for this thesis it is broadly defined as a condition in which the homeostasis of an animal is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly called “stressors” (Wendelaar Bonga, 1997). The process involves a coordinated set of behavioural and physiological responses, thought to be compensatory and/or adaptive, enabling the animal to overcome the threat. However, in some situations the stress response may lose its adaptive value, which can result in the inhibition of growth, reproductive failure and immunosuppression (Wendelaar Bonga, 1997; Weyts et al., 1999). Identified stressors in fish are numerous and include sudden and extreme changes in the physical environment (e.g. temperature, salinity and turbidity), animal interactions (e.g. predation, parasitism, competition for space, food and sexual partners), human interference (including aquaculture practices e.g. netting, handling, crowding, transport and anaesthesia) and water pollution (e.g. low pH, high ammonia, heavy metals and organic pollutants) (Barton and Iwama, 1991; Bly et al., 1997; Gerwick et al., 1999; Wendelaar Bonga, 1997; Weyts et al., 1999). Of interest to this study are reports that chemical exposure above a certain threshold may result in an integrated stress response, which can cause immunosuppression or immunoactivation (Wendelaar Bonga, 1997).

The HPI-axis is the key feature in the stress response of fish as it conveys cognitive stress stimuli from the brain to the periphery, through a cascade of biochemical messengers that results in the secretion of glucocorticosteroids as the main end-products (e.g. cortisol in fish) (Bly et al., 1997; Weyts et al., 1999). Cortisol has been implicated in mediating the inhibitory effects of stressors on the immune response. In vivo experiments with fish have shown that a high plasma cortisol concentration is associated with a decrease in resistance to pathogens, reduced lymphocyte numbers and antibody production, an increase in circulating neutrophils that have an impaired ability to migrate, and a decrease in other non-specific
immune functions (Barton and Iwama, 1991; Bly et al., 1997; Wendelaar Bonga, 1997). *In vitro* studies have found that cortisol can directly suppress mitogenesis and induce apoptosis in lymphocytes, as well as inhibit phagocytic activity (Barton and Iwama, 1991; Wendelaar Bonga, 1997).

### 1.4.8: Sensitivity to temperature

Fish are ectothermic organisms that are unable to regulate their body temperature and are therefore susceptible to the influence of environmental temperatures. Although temperature can be considered a stressor, it is also considered separately as the effects observed differ from other stressors and there is a direct metabolic consequence that has specific effects on an individual (Bly et al., 1997). It is generally agreed that higher temperatures (i.e. about 5-10°C above ambient temperature) enhance specific immune responses, whereas lower temperatures suppress the specific immune response of fish (Bly et al., 1997; Miller and Clem, 1984). Conversely, conflicting studies have been reported on the effects of temperature on non-specific immunocyte functions, with some studies proposing that suppression of specific immunity is offset by an enhanced non-specific immunity (Le Morvan et al., 1996; Le Morvan-Rocher et al., 1995).

Specific lymphocyte functions may acclimatise to lower environmental temperatures by homeoviscous adaptation, although it appears that B-cells are more efficient at this process and are therefore less susceptible than T-cells. Adaptation to low temperatures involves the conversion of saturated fatty acids to more viscous unsaturated fatty acids, to maintain membrane fluidity and trans-membrane signalling. Additionally, suppression of T-cells appears to occur in the early stages of activation and has been shown to effect naive immature T-cells rather than memory cells (Bly and Clem, 1992).
Non-specific immune functions, such as natural cell-mediated cytotoxic (NCC) activity, phagocytic activities and the alternative complement pathways, have been reported to increase in cyprinid fish acclimatised to low temperatures during both *in vivo* and *in vitro* experiments (Collazos *et al.*, 1994a; Collazos *et al.*, 1994b; Le Morvan *et al.*, 1996; Le Morvan-Rocher *et al.*, 1995). However, other researchers have found that the effect of temperature on innate immunity is in the same order as compared to specific immunity (Bly *et al.*, 1997; Nikoskelainena *et al.*, 2004). Nonetheless, granulocyte numbers appear to remain constant in varying temperatures, while lymphocyte numbers are reduced at lower temperatures, and non-specific immune functions also seem to be relatively temperature independent compared to specific immune functions (Bly and Clem, 1992; Ellis, 2001).

**1.5: Aquatic toxicology**

**1.5.1: Overview**

The majority of ecotoxicological research has focused attention on the aquatic environment because it is the ultimate sink for many environmental pollutants, due to either direct discharges or hydrologic and atmospheric processes (O'Halloran *et al.*, 1998a; Vos *et al.*, 1989; Zelikoff, 1994). Data obtained in aquatic toxicological testing provides a sensitive indicator of the state of the environment and early warning signs of the hazards posed by environmental pollutants. In addition, the use of lower vertebrates is providing knowledge of the fundamental principles that underlie toxicity in all species (Pritchard, 1993). Many aquatic toxicology studies still involve the use of acute tests limited to a study period of a few days at most and use high concentrations, with mortality as the endpoint i.e. LD$_{50}$ or LC$_{50}$ values (the dose or concentration that is lethal to 50% of the test population) (Adams and Rowland, 2004). However, the concentrations used in acute toxicity tests are highly unrealistic and inherent difficulties are encountered in extrapolating from controlled laboratory conditions to the real environment. Furthermore, these tests offer no information
on the risk of long-term (chronic), low-level and environmentally-relevant exposures and they also overlook factors such as multiple routes of exposure, the bioavailability of toxicants in the presence of sediment and other aquatic organisms, synergism of a mixture of toxicants, carcinogenicity, and multigenerational effects and life-stage sensitivities (ANZECC and ARMCANZ, 2000; Warne, 1998).

Presently, these LC$_{50}$ values along with assessment factors (AF) (i.e. safety factors or application factors that use arbitrary values of 10, 100 or 1000 to predict the chronic effects of a chemical from acute data), are still highly used by regulators to assess the “risk” of a chemical from acute test data whenever chronic data is absent (Adams and Rowland, 2004; ANZECC and ARMCANZ, 2000). However, during the past decade there has been a major research drive to develop sublethal and chronic bioassays that use NOEC (no observable effect concentration) and LOEC (lowest observable effect concentration) values to describe effects. This is leading to the evolution of aquatic toxicology into a multidisciplinary field, which uses long-term, low-level exposure studies investigating sublethal, multigenerational, multi-species and community effects (ANZECC and ARMCANZ, 2000). The inclusion of such tests provides a more realistic identification and characterisation of the chemical hazard (ANZECC and ARMCANZ, 2000; O'Halloran, 1996).

1.5.2: Aquatic toxicology in Australia

In Australia, water quality guidelines are set by the Australian and New Zealand Environment and Conservation Council (ANZECC) (Ball et al., 2001). Many of the guideline values have been derived from toxicity data from exotic species, which has raised concerns that the information used may not be adequate to provide protection to the wide-ranging diversity of Australian aquatic ecosystems (ANZECC and ARMCANZ, 2000). The guidelines recommend the use of site-specific information to overcome inadequacies in environmental
variation, however due to scarcity in local data, the same guidelines apply the assumption that “Australian biota, as a whole, appears as sensitive as biota from elsewhere” (ANZECC and ARMCANZ, 2000). This assumption is based on a limited number of studies in freshwater native fish that have shown that local species have similar LD$_{50}$ values to some pollutants (Johnston et al., 1990; Skidmore and Firth, 1983; Sunderam et al., 1992). However, some experiments using local species have reported that they are more sensitive to certain chemicals (Allen and Bacher, 1986; Davies et al., 1994), highlighting the need for more testing of local species.

1.5.3: Biomarkers (Biomonitoring)

Biomarkers used in environmental toxicology act as early-warning signals by reflecting adverse biological responses towards anthropogenic environmental pollutants, before effects on the population and community are apparent. Furthermore, biomarkers provide information on both the potential mechanism of action and the biological effects of pollutants, rather than a mere quantification of their environmental levels (Van der Oost et al., 2003).

Immune system parameters may potentially be used as biomarkers in fish and many are applied in high intensity fish farming, particularly to measure the efficiency of vaccines, immunostimulants and the adverse effects of antibiotic treatments (Anderson and Zeeman, 1995; Wester et al., 1994). However, the use of fish immune markers in ecological risk assessment has still not reached full maturity because the immune system can be influenced by a large variety of stressors, which implies that immunological biomarkers may be useful and sensitive but often non-specific (Van der Oost et al., 2003). Additionally, the relationships between immunotoxic pollutants and fish diseases, as well as the ecological significance of such effects in the field, remain unclear (Weeks et al., 1992). Regardless, the field continues to grow due to the commercial interests of aquaculturalists and the
fundamental physiological role of the immune system, which implies that its impairment could lead to a devastating outbreak of disease in stocks or ecosystems (Wester et al., 1994).

1.6: Immunotoxicology

Immunotoxicology is the study of the deleterious effects of xenobiotics on the immune system. However, immunotoxicity may occur as a result of compounds acting directly on immune tissue or as a result of a systemic stress response that leads to immunosuppression (i.e. direct and indirect immunotoxicity, respectively). The immune system is a host’s last line of defence against infectious agents and neoplasms (Luster et al., 1988). It is a complex network of cells in a constant state of proliferation and differentiation, which is highly regulated by numerous soluble factors such as antigens, mitogens, cytokines, hormones and neurotransmitters. The immune system is highly integrated with other organs and their functions, including the central nervous system (CNS). These characteristics make the immune system extremely vulnerable to insult from xenobiotics and pioneers in the field of immunotoxicology have documented several findings that have highlighted the immune system as an important target organ in toxicology (Dean and Murray, 2001).

Firstly, immunocompetent cells are required for host resistance to disease and consequently, exposure to immunotoxins can result in an increased incidence of infections, neoplasms, allergies or autoimmune dysfunctions. Chemical agents that target cellular proliferation will affect immunocompetence, as the immune system requires continued cellular proliferation and differentiation for self-renewal. The physiology of the immune system is better understood than many target organ systems and the mechanism of cytotoxicity may be better determined. Many assays that assess immunotoxicity can be completed using small volumes of blood or lymphoid tissue. Finally, a more appropriate risk extrapolation to humans may be achieved by confirming experimental data from animals with in vitro immunotoxicity assays.
using human blood leukocytes (Dean and Murray, 2001; Murray and Thomas, 1992; Weeks et al., 1992).

The use of immune endpoints in the risk assessment of a chemical has some complications. The ultimate endpoint in immunotoxicology is death due to disease, whether it is bacterial, viral or cancer. Immunotoxicology assumes that there is a threshold of exposure below which a change in the immune system may be observed, but there is no effect on the health status of the organism. This theory is supported by the fact that immunodeficient individuals may live healthily if infecting agents are absent, and healthy individuals may become ill if they receive a large enough dose of infecting agent. Additionally, the immune system has functions that may be maintained by a number of different cell types, known as “functional redundancy” e.g. cytotoxic T-cells and NK cells are both involved in the eradication of tumour cells (Luster et al., 1994). In some cases, the immune system in one compartment can become increasingly active to compensate for immunosuppression in another part of the immune system. Hormetic dose-response relationships have also been observed for some parameters, in that low doses of immunotoxins may cause an enhanced activity, while higher doses result in immunosuppression (Rice and Weeks, 1991; Zelikoff et al., 1995).

Due to the above-mentioned complications, one immunological assay is insufficient to provide an adequate immunotoxic assessment of a chemical. Therefore, the United States National Toxicology Program (NTP) established and validated a testing battery of assays and a tiered approach to evaluate the potential immunotoxicity of chemicals in humans (Luster et al., 1988). The first tier of assays comprises a number of histopathology parameters (i.e. organ weights, general haematology parameters, serum Ig levels etc) and a limited number of functional assays (i.e. flow cytometry identification of immune cell subpopulations). A larger
number of *ex vivo/in vitro* and *in vivo* tests are only employed if there was an indication of immunotoxicity in the first tier of testing (Vos et al., 1989).

From these pioneering studies Luster and co-workers (1988; 1994) made a number of important conclusions that are now fundamental principles of immunotoxicological assessment. Firstly, a good correlation existed between immune function and host resistance, in that resistance would not be altered without one or more immune assays being altered. Nevertheless, immune changes could occur without alterations in host resistance due to the functional reserve of the immune system. No single assay could fully identify an immunotoxin although some were good indicators (e.g. flow cytometric enumeration of immune cell surface markers had an individual concordance level >70%). However, when a combination of 3 tests was used (including cell surface markers and plaque forming cell assay) a 100% concordance level was reached. The ability to resist infection was dependent on the degree of immune damage and the dose of the infecting agent. Finally, most immune function-host resistance relationships followed a linear model rather than a linear-quadratic (threshold-like) model.

### 1.7: Fish immunotoxicology

There has been a large amount of research attention devoted to using fish species to assess biological and biochemical responses to environmental contaminants because fish possess a number of ideal characteristics. Firstly, fish are practical because they are numerous and ubiquitous in the environment, they live intimately with aquatic ecosystems and are an important link in the food chain. As such, fish may be continually exposed to chemicals by different routes, for long periods of time and also bioaccumulate and biomagnify toxins from their environment to extremely high levels (Anderson and Zeeman, 1995; Zelikoff, 1994). Similarities between mammalian and fish immune functions mean that fish can be used to
indicate possible effects of toxicants on humans, and also for comparative studies where mechanisms of immunotoxicity are being assessed. Comparative studies can also identify toxicants that are harmful to the immune systems of all organisms, as well as chemicals that may cause a greater environmental stress by damaging multiple animal phyla (Siwicki et al., 1998; Zelikoff et al., 2000).

Fish have a number of advantages over rat and mouse immunotoxicological models. Larger fish species are available, which provides a large number of immune cells for study and although a continual supply of clean water is required, they are less expensive to purchase and maintain than their mammalian counterparts. They are amenable to laboratory and field studies and therefore can be exposed to toxicants in well-defined laboratory conditions and also under more “natural conditions”. They are more diverse phylogenically than mammals and provide more alternative models for study. Finally, results can provide evolutionary reference points for other vertebrate studies (Zelikoff, 1994). However, between different fish species there is considerable variation in both the basic physiological features and their responsiveness in certain assays (Van der Oost et al., 2003). This requires that researchers first optimise each immune assay to the conditions that best suit the species in the study.

1.7.1: Assays used in fish immunotoxicology

The assays available for immunotoxicology studies in fish are adapted from mammalian studies and are only limited by the availability of some research tools, such as monoclonal antibodies raised against immune cell surface markers (Anderson and Zeeman, 1995; Zelikoff et al., 2002). Simple measurements of immune system integrity are often analysed in other toxicological studies, but are not the primary focus of these studies. Haematocrit and leukocrit values, as well as gross morphological data such as immune organ weights, are quick and easy methods that can indicate immunotoxicity (Blaxhall, 1972). Differential cell
counts more specifically enumerate the effect of toxicants on immune cell subpopulations, however they can be tedious to perform and are subjective. The use of vital stains (i.e. dyes that are excluded by living cells such as trypan blue), in conjunction with haemocytometer counts, can give important information on the concentration of cells in a suspension and the percentage that are viable (Anderson and Zeeman, 1995).

Of the functional assays used in fish immunotoxicology, phagocytic parameters appear to be the most studied. As one of the first lines of internal defence, and the fact that ectothermic animals rely on their non-specific immune functions more so than mammals, phagocyte function has long been of great interest in fish immunology and the subject has received the attention of reviewers (Secombes, 1994; Secombes and Fletcher, 1992). Although there have been numerous methods described to quantify phagocytic function, they can be broadly classified as engulfment assays or respiratory burst assays. Fish phagocytes will engulf a number of biological and non-biological particles including (fluorescent) latex beads, carbon particles, stained yeast cells and bacteria (Thuvander et al., 1992). Enumeration of the percentage of cells with particles, along with the number of particles per cell, may be achieved with microscopy counting or through flow cytometry. Respiratory burst is measured through the use of fluorophores or dyes such as luminol (i.e. chemiluminescence (CL) assay), dihydrorhodamine 123 or nitroblue tetrazolium (NBT assay) (Secombes, 1994; Verburg-van Kemenade, 1992). These compounds are reduced by the reactive oxygen species (ROS) that are produced during respiratory burst, and can be quantified in a photometer, flow cytometer or via microscopy. After activation, immune cells also become more “sticky” due to the appearance of adherence molecules on the surface of cells. The adherence of leukocytes to bacteria, glass, antigen-labellelatex beads and red blood cells (RBCs) has also been used as a measure of immune functional integrity.
Another important functional assay is the induction of lymphocytes to proliferate by chemical mediators (i.e. mitogens), termed mitogenesis or lymphoproliferation (DeKoning and Kaattari, 1991). Lymphoproliferation occurs after exposure to mitogens (i.e. cellular products from various plant and bacteria), which specifically stimulate either T-cells (e.g. concanavalin A, ConA and phytohemagglutinin A, PHA), B-cells (e.g. lipopolysaccharide, LPS) or both (e.g. pokeweed mitogen, PWM) (Dean et al., 1994). The measurement of proliferation can be simply calculated through counting the increase in cell concentration, using a haemocytometer, or through more sophisticated methods that measure the uptake of radiolabelled DNA precursors i.e. $^3$H-thymidine (Anderson and Zeeman, 1995).

The ability of lymphocytes to produce antibodies against an antigen is often measured using the plaque-forming cell (PFC) assay. Sheep RBCs (sRBCs) or specific antigens (e.g. O-antigen) are injected into the fish and the lymphocytes are harvested after a pre-determined number of days. The lymphocytes are then mixed with sRBC, either with or without specific antigen labelling and mounted on a microscope slide (Rijkers et al., 1980a; Smith et al., 1999). During the incubation period, lymphocytes secrete antibodies that bind to the antigens on sRBC. The PFC assay also requires that complement systems of the host are working, as it acts secondarily, with complement proteins binding to the antigen-antibody complexes on the sRBCs, resulting in their lysis and the formation of a plaque (Anderson and Zeeman, 1995; Rijkers et al., 1980a).

Measurements of circulating antibodies may be used to show immunosuppression of humoral immune functions, however fish immunologists more commonly measure antibody titres to assess the effectiveness of vaccines. Quantification of circulating antibodies can be achieved by a range of techniques including agglutination of particulates, precipitation by complexing with antigen, activation of complement/complement fixation, fluorescent antibody techniques,
enzyme linked immunosorbent assays (ELISA) and radioimmunoassays (Davidson and Secombes, 1992; Smith, 1992). The method used depends on what the researcher is aiming to identify, as some assays measure the total immunoglobulin in the serum, while others are only reactive to certain epitopes of the immunoglobulin (Anderson and Zeeman, 1995).

Disease challenge models investigate and compare the survival rate of fish from treated and control groups after exposure to pathogens. These models represent the ultimate endpoint in immunotoxicological studies as they investigate the integrity of the intact immune system and provide clear evidence that the chemical exposure is altering resistance to disease (Pottinger and Day, 1999). However, apparent difficulties occur due to the exposure protocol of the virulent pathogens through injection or bathing. Injecting fish with pathogens is not a “natural” route of exposure, but allows for a controlled dose. Bathing fish mimics a more natural exposure route, but calculations of the pathogen dose may be inaccurate. Furthermore, tight quarantine measures are required by laboratories to ensure that pathogens are not released into stock populations (Anderson and Zeeman, 1995).

1.8: Fish immunomodulators

Many known mammalian immunotoxins, such as metals, heavy metals, organometallic compounds, pesticides and aromatic hydrocarbons, are common aquatic contaminants (Zelikoff et al., 2000). The tiered system used to identify immunotoxins in mammalian systems has also identified a large number of environmental chemicals with a potential to impair components of the immune system in aquatic animals (Zelikoff et al., 2002). Non-specific, antibody- and cell-mediated immunity can all be targeted by certain pollutants (Vos et al., 1989; Zelikoff, 1998). There is a large amount of circumstantial evidence to link pollution with disease outbreaks in wild populations, however the causal relationship between specific chemical pollutants and disease has not been clearly defined (Grinwis et al., 2000a;
Watermann and Kranz, 1992). Nonetheless, it is widely accepted that immunosuppression does increase the incidence of disease in fish (Zelikoff, 1994). Pesticides, heavy metals, polycyclic aromatic hydrocarbons (PAHs) and surfactants have been the most frequently examined toxicants, whereas dioxins, furans and polychlorinated biphenyls (PCBs) have been investigated less often (Bols et al., 2001). The following is a brief summary of some of the immunotoxicological studies conducted in fish.

1.8.1: Metals, heavy metals and organometallic compounds

Metal, heavy metal and organometal contamination of aquatic ecosystems is common, due to industrial discharges, municipal waste, surface runoff, damage and weathering of vessel protective paints, ocean dumping, and fallout from the atmosphere (O'Halloran et al., 1998a; Zelikoff, 1994). In mammals, heavy metals are considered the most potent inorganic immunotoxins, causing reduced resistance to infection and developing tumours. Some metals are essential in biological processes (i.e. co-factors in enzymes), however they may cause toxicity at high doses (Zelikoff, 1993). In both mammalian and fish experiments, heavy metals may be immunosuppressive at high doses, however, many cause stimulation of some immune functions at lower doses (Low and Sin, 1998; Zelikoff and Cohen, 1996). Chronic sublethal doses result in bioaccumulation in fish and a decreased resistance to infection (Zelikoff and Cohen, 1996). There is a large body of literature concerning the immunotoxicity of metal compounds in fish species and modulation of fish immune functions has been shown with exposures to copper, aluminium, cadmium, arsenic, chromium, lead, nickel, zinc and mercury (Zelikoff, 1993; Zelikoff and Thomas, 1998). The effects of metal compounds on aquatic organisms have been extensively reviewed by a number of authors (Anderson and Zeeman, 1995; Zeeman and Brindley, 1981; Zelikoff, 1993; Zelikoff and Thomas, 1998).
1.8.2: Organotins

Organotin contamination of harbours and marinas has been a significant environmental concern, due to their disruption of endocrine and reproductive functions and immunotoxic properties at very low concentrations (Vos et al., 2000). In mammals, they have been known for their specific reduction of T-lymphocytes through apoptotic mechanisms (Raffray and Cohen, 1991). The majority of environmental organotin pollution is from biocidal antifouling paints that leach TBT to protect ship hulls from algal and mollusc growth. Nevertheless, organotins enter both freshwater and marine environments through treated woods, run-off from landfill, sewage and industrial discharges (Fent, 1996; O'Halloran et al., 1998a). Once in the aquatic environment, they are bioaccumulated and biomagnified by invertebrates, fish and aquatic mammals and can reach extremely high levels in the tissues of these organisms (Focardi et al., 1999; Schwaiger et al., 1992; Tsuda et al., 1988). The immunotoxic effects of TBT in aquatic organisms appear to be similar to those observed in mammals. A comprehensive review of the TBT and DBT literature is presented in chapter 3 of this thesis.

1.8.3: Pesticides

Pesticides represent a large group of toxic chemicals use to control pest species. Organochlorine (OC), organophosphate (OP) and carbamate pesticides are common aquatic pollutants and studies have demonstrated immunosuppression in mammals (Voccia et al., 1999). They enter water bodies through intentional application, run-off from farms, aerial drift and accidental and illegal release (Zelikoff, 1994). Some of them are persistent environmental contaminants and are readily bioaccumulated and biomagnified by aquatic organisms. The primary mode of action of most pesticides is to disrupt the CNS of the organism, although many are also carcinogenic, teratogenic and/or immunotoxic. The OC pesticides have been linked to increased disease incidences in feral fish populations and have shown immunotoxicity in laboratory experiments (Zelikoff, 1994). All OC pesticides, except
for endosulfan, have been banned for use in Australia since 1997 due to their environmental persistence. Chapter 4 reviews the literature on endosulfan, which is the last OC pesticide that is still widely used in Australia. A number of OP pesticides have displayed immunotoxicity in laboratory studies including malathion, methylparathion and trichlorphon. Chapter 5 reviews the literature on chlorpyrifos, the most-highly used OP pesticide in developed countries.

1.8.4: Aromatic hydrocarbons

Aromatic hydrocarbons include a large number of compounds that occur naturally but are also produced in industrial processes. Some are persistent environmental contaminants (i.e. dioxins and PCBs) and can bioaccumulate and biomagnify in aquatic organisms, while others are broken-down by biological metabolism (i.e. polycyclic aromatic hydrocarbons, PAHs) (O'Halloran et al., 1998a). They are important environmental pollutants with immunotoxic properties, but were not studied in this thesis. This section gives a brief overview of the literature concerning their immunotoxicity but more extensive reviews are available (Anderson and Zeeman, 1995; Bols et al., 2001; Kerkvliet, 2002; O'Halloran et al., 1998a; Zelikoff, 1994).

Halogenated Aromatic Hydrocarbons (HAHs) are a large class of chemicals that includes dioxins (e.g. polychlorinated dibenzodioxin, PCDD), polychlorinated dibenzofuran (PCDF), PCBs and polybrominated biphenyls (PBBs). These chemicals are potent toxins and have numerous effects including hepatotoxicity, teratogenicity, carcinogenicity, neurotoxicity and immunotoxicity (Dean and Murray, 2001). Some of the HAH congeners are stable in the environment, resist metabolic detoxification and accumulate in the fat of fish and other animals to extremely high levels (Zelikoff, 1994).
Due to their long half-lives, dioxins are widely disseminated environmental HAHs that are immunotoxic (Bols et al., 2001). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been the most widely studied dioxin and the immune system is a sensitive target of its toxicity (Kerkvliet, 2002). It is now known that TCDD and other HAHs exert their toxic effects through binding of the aryl hydrocarbon (Ah) receptor, which is involved in the regulation of gene expression (Denison et al., 2002). In mammals, TCDD exposure induces thymic atrophy (De Heer et al., 1994; Vos and Van Loveren, 1995), suppresses both humoral and cell-mediated immune functions and reduces the host’s resistance to infections and tumours (Kerkvliet, 2002). Although only a couple of studies have investigated immunotoxicity of TCDD in fish, it appears these species were somewhat resistant to the toxic effects (Grinwis et al., 2000b; Spitsbergen et al., 1986). Fish exposed to TCDD did display some signs of immunotoxicity such as reduced mitogenesis and thymus atrophy, however this was at levels nearing the lethal dose for mice (Spitsbergen et al., 1986).

PCBs are structurally related to dioxins and are also very persistent, accumulating in the food chain due to their lipophilic nature (Duffy et al., 2002; Safe, 1994). In mammals, exposure to PCBs depresses both humoral and cell-mediated immune responses possibly through a similar mechanism to TCDD i.e. Ah receptor signal transduction (Yoo et al., 1997; Zelikoff, 1994). There have been limited studies using fish, however they have shown that exposure to PCBs suppresses humoral responses, reduces macrophage production of ROS, causes thymic atrophy and decreases resistance to infection challenges (Arkoosh et al., 1994b; Duffy et al., 2002; Grinwis et al., 2001; Snarski, 1982).

PAHs are a class of compounds that are widely dispersed throughout the environment, but do not persist because biological processes can degrade them (Dean et al., 1994). The PAHs that have received the most research attention are 7, 12 dimethylbenzo(a)anthracene (DMBA),
benzo(a)pyrene (BaP) and 3-methylcholanthrene (3MC). In mammals, they cause immunosuppression of antibody production to both T-dependent and T-independent antigens. PAHs appear to primarily effect B-cell maturation and function but may also target T-helper cell regulation to suppress humoral immunity (Dean and Murray, 2001). Some PAHs are carcinogenic and researchers have implicated their potent immunotoxic properties as the cause (Zelikoff, 1994). Laboratory studies using a number of fish species have reported that BaP caused suppression of plaque forming cells, lymphoproliferation, macrophage activity, other innate immune functions and host resistance (Arkoosh et al., 1994b; Carlson et al., 2002; Carlson et al., 2004; Lemaire-Gony et al., 1995; Smith et al., 1999; Walczak et al., 1987). The PAH, 3MC, appears to cause immunotoxicity through the induction of apoptotic mechanisms in lymphocytes and macrophages (Reynaud et al., 2001; Reynaud et al., 2003). In vivo exposure of fish to DMBA resulted in reduced immune organ cellularity (Hart et al., 1998), a reduced number of primary and secondary PFCs (Arkoosh et al., 1994b), reduced phagocytic activity and a marked reduction in NCC activity, which appeared particularly sensitive (Seeley and Weeks-Perkins, 1997).

Phenols are widely disseminated aquatic contaminants that are by-products of many industrial processes (Roszell and Anderson, 1996). Particular research attention has been focused on chlorophenols that are formed in the chlorine bleaching of wood pulp and is one of the toxicants in pulp mill effluent. Studies in fish have reported that phenols caused suppression of macrophage and NK activities (Anderson and Brubacher, 1993; Roszell and Anderson, 1993; Roszell and Anderson, 1994; Voccia et al., 1994).

1.8.5: Antibiotics and immunostimulants

Antibiotics are used in aquaculture to treat outbreaks of infections and as a prophylactic during husbandry and introduction of new stocks. However, the use of antibiotics has
negative aspects including the risk of developing resistant pathogens, hepatotoxicity, hypersensitivity and immunosuppression. Antibiotic-induced hypersensitivity and immunosuppression has been demonstrated in humans, other mammals, birds and fish (Zelikoff, 1994). Antibiotics, such as oxolinic acid, streptomycin and tetracycline analogues have been implicated as immunotoxins in fish (Colorni et al., 1998; Lunden and Bylund, 2000; Rijkers et al., 1980b). High in vitro and in vivo doses of tetracycline analogues (i.e. oxytetracycline, doxycycline) have an immunosuppressive effect on humoral cellular and neutrophil responses (Grondel et al., 1985; Rijkers et al., 1980b; Siwicki et al., 1989).

A number of natural products and synthetic chemicals have been shown to enhance certain immune functions in both mammals and fish. These have been collectively named immunostimulants and fish farmers have focused a large amount of research attention on these products as potential prophylactics (Sakai, 1997). Chapter 6 contains a comprehensive review of the studies conducted in this field.

1.8.6: Polluted sites and chemical mixtures

Industrial and domestic activities that contaminate the aquatic environment mostly result in sites polluted by complex mixtures of chemicals that pose many problems for the appropriate risk assessment and remediation methods of the area. The interaction of chemicals present in mixtures may act in synergism to increase toxicity or attenuate toxic responses, which complicates the use of data from individual chemical testing. Analysis of the toxic effects of site-specific pollution is achieved through comparing the responses of fish from the polluted site with fish from control (reference) sites and by exposing fish to contaminated sediment in the laboratory (O'Halloran et al., 1998a; Pulsford et al., 1995). An investigation into the immunotoxic effects of chemical mixtures and polluted sites was beyond the scope of this thesis. However, it is hoped that future projects would use the assays optimised in this thesis.
to assess polluted sites and complex chemical mixtures. The following section gives a brief overview of how immunotoxicity studies have been used to assess polluted sites.

A number of studies in the USA and Europe have compared the immune functions of fish from contaminated environments (i.e. PCBs and PAHs) to those from “clean” control sites. They have reported that fish from contaminated sites show signs of immunotoxicity including histological changes in the spleen and reduced splenic somatic indices (Koponen et al., 2001; Pulsford et al., 1995), suppression of NCC activities (Faisal et al., 1991b; Rice et al., 1996), decreased T- and B-cell mitogenesis (Faisal and Huggett, 1993; Faisal et al., 1991a), suppressed humoral responses (Arkoosh et al., 1991), decreased numbers of circulating lymphocytes (Pulsford et al., 1995) and inhibition of macrophage functions such as phagocytosis (Weeks and Warinner, 1984), chemotaxis (Weeks and Warinner, 1986), ROS production and oxidative burst (Rice et al., 1996; Warinner et al., 1988). Some studies have also reported increases in circulating phagocytes (Pulsford et al., 1995) and enhanced pinocytosis (Weeks et al., 1987). It is worth noting that in some studies suppression of immune functions was reversed when fish were placed in clean water for a specified time, although macrophage activity recovered slower than other functions (Faisal and Huggett, 1993; Faisal et al., 1991a; Faisal et al., 1991b).

Laboratory studies investigating sediments experimentally contaminated with PAHs have reported reduced hydrogen peroxide production of leukocytes but a number of other parameters (i.e. ROS production, total serum protein and serum lysozyme activity) were not altered (Hutchinson et al., 2003). Conversely, in an experiment using an outdoor mesocosm system experimentally contaminated with creosote, the authors reported elevated oxidative burst, phagocytic function and circulating B-cells, while other immune functions were unchanged (Karrow et al., 2001).
Crude oil is a mixture of volatile long-chain hydrocarbons with traces of aromatic hydrocarbons and heavy metals (Anderson and Zeeman, 1995). There have been limited studies concerning the immunotoxicity of crude oil, as most studies have focused on the most toxic components of crude oil e.g. PAHs. However, a study that exposed winter flounder (Pseudopleuronectes americanus) to crude oil in sediment reported that treated fish had reduced numbers of hepatic melano-macrophage centres (i.e. lymph-node like structure in fish) (Payne and Fancey, 1989). Tahir and co-workers (1993) exposed dab (Limanda limanda) to sediments experimentally contaminated with increasing concentrations of diesel oil-based drilling mud and reported that low concentrations appeared to stimulate immune functions, while higher concentrations suppressed immune parameters. Rainbow trout that received an i.p. injection of an extract from diesel oil-based drilling mud, also appeared to have increased T-cell mitogenic responses at low doses, but displayed reduced serum lysozyme concentrations at the lowest dose, while there was no change in serum Ig concentrations and complement activity (Tahir and Secombès, 1995).

Sewage sludge & treated effluents are the bacterial biomasses of sewage treatment plants and the treated waste of human populations, respectively. They are discharged into aquatic ecosystems and may contain traces of pesticides, aromatic hydrocarbons and heavy metals, as well as a high load of microorganisms and nutrients. Fish exposed to sewage sludge in the laboratory have displayed some indications of immunotoxicity including changes to head kidney histology, inhibition of ROS and bactericidal activities and a decreased resistance to fungal diseases (Bucher and Hofer, 1993; Secombès et al., 1991; Secombès et al., 1992). Rainbow trout experimentally exposed to sewage treatment plant effluents displayed an immune response that was suggestive of chronic inflammation (Hoeger et al., 2004). However, in situ field experiments have not supported the laboratory evidence that suggests
that sewage sludge can cause immunosuppression. Plaice (*Pleuronectes platessa*) caught along a sewage sludge gradient showed that phagocytes from fish located closer to the dumping site had higher bactericidal activity but other immune functions were not affected. Fish exposed to high levels of sewage sludge also displayed higher levels of serum Ig and lysozyme, but anti-protease concentrations were not altered. These findings were contradictory to laboratory results and the researchers attributed these changes to the high-load of microorganisms within the effluent (Secombes *et al.*, 1995).

**Pulp mill effluents** (PME) contain a complex mixture of a variety of organic and inorganic compounds and may include varying concentrations of heavy metals, PCDDs, PCDFs, and pentachlorophenol, depending on the treatment of effluent before discharge (Fatima *et al.*, 2001; Wallis *et al.*, 1994). Many of these chemicals have been investigated individually in laboratories but a number of studies have also aimed to assess the immunotoxicity of the mixture of chemicals, in both laboratory and field experiments. Fish exposed to PME under laboratory conditions have displayed depressed leukocrit values (McLeay and Gordon, 1977) and suppressed PFCs (Fatima *et al.*, 2001). Groups that have investigated the effects of various types of PME, as well as effluents that received primary and secondary treatment, have reported that all effluents caused immunosuppression in fish including primary and secondary treated effluents (Aaltonen *et al.*, 2000a; Aaltonen *et al.*, 2000b; Aaltonen *et al.*, 1997). Voccia and co-workers (1994) exposed rainbow trout to effluents from both a bisulphate mill (chlorine-free effluent) and a kraft mill (80% chlorate) and reported that the chlorine-free effluent was immunosuppressive, while the kraft mill effluent stimulated immune response.

Field studies have aimed to determine the detrimental effects of pulp mill effluents on aquatic ecosystems and monitor the effects of introducing new processes in the treatment of effluents
within pulp mill plants. The introduction of elemental chlorine-free bleaching technologies and activated sludge treatment of effluents at the mills are methods employed to reduce the level of contaminants in effluents. There have been reports of immune dysfunction in macrophages (Fournier et al., 1998), serum IgM concentration (Jokinen et al., 1995; Soimasuo et al., 1995) and lymphocyte numbers (Anderson et al., 1988) from fish collected from sites contaminated with bleached kraft mill effluent. However, changes in the bleaching process together with the installation of the secondary-treatment systems (i.e. activated sludge) returned fish immune parameters to levels comparable to reference sites (Karels et al., 2001; Karels et al., 1998; Soimasuo et al., 2000).

1.9: Summary of Chapter 1

The immune system of fish share many similarities with their mammalian counterparts. The structure and function of immune systems from numerous fish species have been characterised and exploited for use in studies investigating the immunomodulation of xenobiotics. Laboratory studies have demonstrated a link between immunosuppression and a reduced resistance to infections and neoplasms. Similar studies have shown that immunostimulants can increase fish immune defence systems and help protect them from disease. The identification of immunotoxins in ecotoxicology (or immunostimulants in aquaculture) requires the use of a tiered battery of tests, similar to that used in mammalian testing. This is due to the functional redundancy of the immune system and the fact that one immunological assay has been insufficient in determining immunomodulators in other species. The use of immunotoxicology as a tool for ERA is still in development, however evidence from field studies indicates that immune dysfunction is correlated with levels of pollution. Since immune functions appear to be sensitive to a variety of stressors, i.e. temperature changes, handling stress, prolonged anaesthesia and reduced water quality in temporary holding facilities, these conditions need to be standardised to reduce the variability
of results. Further complications arise from individual variation of immune responses, which may be due to the genetic diversity of wild fish populations.

ANZECC and ARMCANZ (2000) have recommended the use of site-specific information for determining appropriate trigger values for water quality guidelines. At present, there is a slowly growing body of knowledge concerning the general toxicology of pollutants in large freshwater Australian fish, while more recently, most investigations have employed the use of sublethal assays in the search for biomarkers. A common native species for ecotoxicological data gathering in Australia is yet to be identified and the methodologies standardised. To date, the immune functions of Australian freshwater fish have only been characterised in silver perch and crimson-spotted rainbowfish, and *in vitro* data on the immunotoxicology of a few pollutants have also been investigated. This thesis aims to expand the knowledge of the immune systems of Australian freshwater fish, and employ this knowledge in studies concerning the immunomodulation of xenobiotics relevant to the Australian environment and aquaculture industry.
Chapter 2: Methodology

2.1: Fish procurement, handling and maintenance

2.1.1: Species selection

2.1.1.1: Crimson-spotted rainbowfish (*Melanotaenia fluviatilis*)

Class: Actinopterygii (ray-finned fishes) Order: Atheriniformes (silver sides)

Family: Melanotaeniidae

Crimson-spotted rainbowfish are also known as jewel fish, freshwater sunfish or pink ear. They are a small ornate fish found in the mid and lower regions of the Murray and Murrumbidgee Rivers. The species is not widely distributed in the Darling river but is found in some areas and populations are also found in some coastal rivers (Lake, 1967). Crimson-spotted rainbowfish are classified as non-threatened, common and widespread (Koehn and Morrison, 1990; MDBMC, 2002a). Crimson-spotted rainbowfish have been inbred and grown for six generations at the Key Centre for Toxicology at RMIT University. The original stock was collected from the Murray River in 1990 and since then they have been used for a number of ecotoxicological tests to determine their potential as a biomarker species. Due to this breeding program they have advantages as a test species as they are genetically inbred, well adjusted to laboratory conditions and have been studied extensively in the past. This reduces experimental variation from two key factors; stress from handling and maintenance, and genetic variation. Native species acquired from aquaculturalists are commonly out-bred by farmers who seasonally introduce genetics from wild fish. Large numbers of cultured fish are used for stocking of waterways and out-breeding is used to add genetic diversity to depleted populations in the wild. However, genetic diversity can add to individual variation in experiments and is undesirable.
2.1.1.2: Silver perch (Bidyanus bidyanus)

Class: Actinopterygii (ray-finned fishes) Order: Perciformes (perch-likes)

Family: Terapontidae (Grunters or tigerperches)

Silver perch is recognised as one of four important economic and recreational species of the Murray-Darling Basin, along with Murray cod, golden perch and native freshwater catfish. They are also known as black bream, grunter, or bidyan and once occurred throughout the Murray-Darling system except at high altitudes (Lake, 1967). In recent times, silver perch have been listed under the Victorian flora and fauna guarantee act as critically endangered (MDBMC, 2002a). They grow rapidly on commercial pellets, tolerate a wide range of temperatures for growth outdoors and do not cannibalise each other, unlike Murray cod and golden perch. Due to these qualities, silver perch have long been identified as an ideal species for aquaculture (Rowland et al., 1983). Past research on the species has shown greater tolerance to ammonia and nitrite compared to rainbow trout, but comparisons to other Australian natives have not been made (Frances et al., 1998; Frances et al., 2000). Silver perch have been used as a test species in a least one ecotoxicological experiment involving endosulfan (Sunderam et al., 1992). Previous research on the immune functions of silver perch has been conducted within our research group at the Key Centre for Toxicology, RMIT University (O'Halloran et al., 1996a; O'Halloran et al., 1996b; O'Halloran et al., 1998b).

2.1.1.3: Golden perch (Macquaria ambigua)

Class: Actinopterygii (ray-finned fishes) Order: Perciformes (perch-likes)

Family: Percichthyidae (Temperate perches)

Golden perch is also known as Murray perch, callop and yellowbelly. Wild golden perch are sold at the Victoria market (Melbourne, Vic, Australia) as Murray perch for $A12-18/kg. They are a favoured angling species and can grow to over 20 kg but are commonly caught at 4.5 kg (NFA, 2003). Golden perch are able to adapt to a wide range of conditions in culture,
as their native habitat undergoes large fluctuations in temperature and water flow. These changes in temperature are necessary for spawning to occur and flooding is a key trigger as the floodplains provide a smorgasbord of zooplankton for young fish (Lake, 1967). Golden perch has disappeared in regions upstream from major dams, such as the Wyangala Dam on the Lachlan River, Yarrawonga weir on the Murray River and the Burrinjuck Dam on the Murrumbidgee River. However, their status and distribution is still listed as widespread and common (MDBMC, 2002a). Golden perch breeding and reproduction has been well known for decades and has been studied extensively in programs for restocking (Lake, 1967; Rowland et al., 1983). Past research into golden perch has involved life-cycle and habitat requirements (Anderson et al., 1992b; Gehrke, 1990a; Gehrke, 1990b; Gehrke, 1991; Gehrke, 1994b; Gehrke et al., 1993; Ingram, 1993; Mallen-Cooper, 1994; Musyl and Keenan, 1992), production and feeding studies (Anderson and Braley, 1993; Collins and Anderson, 1995; Collins and Anderson, 1997; Collins and Anderson, 1999) and the presence of insulin-like growth factor-I in their blood (Anderson et al., 1993). To date, no immunological studies have been conducted using golden perch but it has been a test species in limited ecotoxicology studies (Sunderam et al., 1992).

2.1.1.4: Murray cod (Macqualla peeli peelii)

Class: Actinopterygii (ray-finned fishes) Order: Perciformes (perch-likes)

Family: Percichthyidae (Temperate perches)

Murray cod is the largest and best-known Australian freshwater species. Its distribution and abundance has declined in the past 50 years due to the construction of dams, changes to river flows and temperatures, and increased siltation of streams. The species is now fragmented and in low abundance and has been listed under the Victorian flora and fauna guarantee act as “vulnerable” (MDBMC, 2002a). Research programs involving Murray cod have focused on aquaculture solutions and the ecology of the species. Aquaculture research has included;
spawning (Rowland, 1988; Wyse, 1973), rearing (Cadwallader and Gooley, 1981; De Silva et al., 2002; Gunasekera et al., 2000) and diet (De Silva et al., 2000; Gunasekera et al., 2000; Turchini et al., 2003). Ecological studies have included, behavioural patterns and habitat requirements (Gehrke, 1994a; Humphries et al., 2002; Mallen-Cooper, 1993; Reynolds, 1984).

2.1.2: Wet-laboratory water

All fish were held in the wet-laboratory facility at RMIT-University (Bundoora, Vic, Australia). The laboratory was a flow-through design that was supplied with carbon-filtered water, pre-heated to 19-20°C. This temperature could be raised in certain sections of the laboratory through six inline heaters. The water had an oxygen concentration 7.6-7.8 mg/L, a pH of 6.8-7.2 and conductivity of 100-120 μS.

2.1.3: Handling and maintenance

2.1.3.1: Crimson-spotted rainbowfish (*Melanotaenia fluviatilis*)

Crimson-spotted rainbowfish used in these studies were obtained from the Ecotoxicology research group at RMIT University (Bundoora, Vic, Australia) at about 2 years of age. Two weeks before experiments were conducted, groups of four fish were randomly acclimatized in a 35L aquarium at 25±1°C with a flow rate of approximately 7-10 L/hr. Each day rainbowfish were fed a diet of commercially available fish flakes (Saga fish flakes) and frozen brine shrimp (Posaqua, both obtained from MAS Imports, Coburg, Vic, Australia). Their tanks were cleaned at least three times a week and daily during experiments. Fish were acclimatised in groups of 4-5 in 35 L tanks at least 2 weeks before *in vivo* experiments.
2.1.3.2: Silver perch (*Bidyanus bidyanus*)

Silver perch fingerlings were purchased from Glenwaters native fish farm (Glenburn, Vic, Australia) and transported to the laboratory (1 hr) in a water-filled, oxygenated bag. They were then transferred to 500L or 1000L plastic tubs (RELN, Ingleburn, NSW, Australia) receiving a constant supply of aeration and fresh carbon filtered water at 22±1°C (<150L/hr). They were fed commercially available pellets (Ridley Inc., Pakenham, Vic, Australia) daily and the tanks were cleaned at least three times a week. Fish were grown to an appropriate size (50g) before use. Fish were acclimatised for at least 2 weeks before being used for *in vitro* experiments and were sampled from the same tank. Silver perch would not acclimatise in small groups and exposure tanks for *in vivo* experiments.

2.1.3.3: Golden perch (*Macquaria ambigua*)

Large golden perch (i.e. 150-200g) were purchased from Beauford native fish farm (Beauford, Vic, Australia) and transported back to the laboratory (1.5 hr) in aerated water buckets. Golden perch fingerlings were purchased from Glenwaters native fish farm (Glenburn, Vic, Australia) and transported for 1 hr in an oxygenated bag. Fingerlings were then transferred to 1000L plastic tubs (RELN, Ingleburn, NSW, Australia) or 1-2 larger fish were salt bathed and transferred into 75L glass aquaria receiving aeration and a constant supply of fresh carbon filtered aquaria water at 22±1°C (i.e. <150 L/hr and <13-15 L/hr for the large tub and glass aquaria respectively). Golden perch fingerlings were fed blackworms (*Lumbriculus variegatus*, Mal Davidson, Beechworth, Vic, Australia) and finely chopped ox heart, while larger fish were fed yabbies (*Cherax destructor*), ox heart and fillets of common carp. Fish were fed every second day and aquaria were cleaned in the days between feeding. Fish were not used until they had grown to an appropriate size (>30g) and were acclimatised for at least two weeks before *in vitro* experiments.
2.1.3.4: Murray Cod (*Maccullochella peelii*)

Murray cod (150-250 g) were purchased from Australian Aquaculture Products (Euroa, Vic, Australia) and transported to the laboratory (2 hr) in an aerated 1000L transportable tank on the back of a utility vehicle. Individual fish were then transferred to 40 L tanks receiving aeration and a constant supply (5L/hr) of fresh carbon filtered aquaria water at 20±1°C. The remaining fish were kept in a 1000L plastic tank (RELN, Ingleburn, NSW, Australia) receiving 150 L/hr of fresh carbon filtered aquaria water at 20±1°C. Murray cod were fed a commercially available pellet every second day (Skretting Australia, Cambridge, Tasmania, Australia) and tanks were cleaned on the days between feeding. They were acclimatised in tanks for four weeks before conducting *in vivo* or *in vitro* experiments.

2.2: Fish manipulations

The *in vivo* experiments of chapter 3, 4 and 5, involved exposing the fish to the test chemicals via intraperitoneal injection. Prior to the start of the exposure protocol, fish were acclimatised in the exposure tanks for at least 2-4 weeks. Before receiving an injection they were anaesthetised by bathing for ≤ 3 mins in MS 222 (3-aminobenzoic acid ethyl ester methanesulfonate salt) (Sigma Chemical co, St Louis, MO, USA) in carbonate buffered aquaria water. Effective concentrations for golden perch, rainbow fish and Murray cod were 150-250 mg/L. Silver perch were more sensitive to MS 222, i.e. they needed less exposure to MS222 (80-100 mg/L) to be effectively anaesthetised. Fish were back in their recovery tank of clean aquaria water within 5 min. Fish undergoing lethal surgery were anaesthetised prior to sacrifice by cranial fracture. Blood was immediately collected via caudal vein puncture, which aids to reduce the volume of blood in the head kidney and spleen.
2.3: Sample isolation methods

2.3.1: Blood and serum collection

Murray cod, silver perch and golden perch were bled via the caudal vein using a 26 gauge, ¼ inch needle with a 1 or 3 mL heparinized syringe (CSL, Melbourne, Vic, Australia) (figure 2.1). Rainbowfish blood was collected by severing the tail and drawing from the caudal vein using a 75 μL heparinized capillary tube. To obtain serum, the blood was placed in microfuge tubes and centrifuged at 800g for 15 mins at 4°C. The serum was then aspirated from the top of the sample.

Figure 2.1: Collection of blood from the caudal vein of Murray cod

2.3.2: Head kidney

Fish were anaesthetised, killed, their weight quickly recorded and blood taken. The head kidney was removed, weighed and placed on ice in 2mL RPMI 1640 (with 20mM N-(2hydroxyethyl)piperazine-N-2-ethane sulfonic acid (HEPES), 300 mg/L glutamine and 100 μg/mL gentamycin sulfate) (Sigma Chemical Co., St Louis, MO, USA). RPMI 1640 plus bicarbonate, HEPES and gentamycin will be referred to as RPMI in this text. Cells were isolated from the head kidney tissue by repeated gentle passage through a 3 mL syringe. The
The cell suspension was then filtered into a 50 mL (Greiner, Frickenhausen, Germany) or 10 mL tube (Sarstedt Pty Ltd, Technology Park, SA, Australia) through a 250 μm mesh (Allied Screen Fabrics, Sydney, NSW, Australia) to remove large clumps of connective tissue.

The cell suspension was washed in 10 mL or 50 mL ice cold RPMI and centrifuged at 400 g for 7 mins at 4°C. To remove the red blood cells from the sample, the pellet was resuspended in 4 mL RPMI and layered onto 3 mL of histopaque (Sigma Chemical Co., St Louis, MO, USA) for density gradient centrifugation. If samples appeared to have a high concentration of erythrocytes present, they were diluted in higher volumes of RPMI (i.e. 8, 12 or 16 mL of RPMI) and 4 mL layered over of 3 mL histopaque.

The samples were then centrifuged at 400g for 30 mins at room temperature, which resulted in a white buffy coat of immune cells floating on top of the histopaque (figure 2.2). The immune cells were collected with a transfer pipette, washed in at least 10 mL of ice cold tissue culture media (TCM) i.e. RPMI 1640 (Sigma Chemical Co., St Louis, MO, USA) with 10% fetal calf serum (FCS) (CSL, Parkville, Vic, Australia), 50 μM 2-mercaptoethanol (2-ME, BDH Chemicals, Poole, UK), and 100 μg/mL gentamycin sulfate (Sigma Chemical Co., St Louis, MO, USA) and centrifuged at 400g for 7 mins at 4°C. This washing step was repeated to remove any residual histopaque. The isolated head kidney cells were then resuspended in TCM and placed on ice.
2.3.3: Spleen

Spleens were removed, weighed and placed in 5 mL RPMI 1640 on ice. They were then washed three times in sterile RPMI. The spleens were flushed with RPMI through a bent 26-gauge needle and 3mL syringe, which disrupts the capsule, teases out the splenocytes and removes blood clots. The cells were then excised from the capsule by gently pressing and stroking it with the needle. The cell and tissue mixture was passed through a sterile nylon 250 μm mesh (Allied Screen Fabrics, Sydney, NSW, Australia) into a 50 mL or 10 mL tube (Griener, Frickenhausen, Germany), washed in 50 mL or 10 mL ice cold RPMI and centrifuged at 400g for 7 mins at 4°C. The cell pellet was resuspended in 4 mL RPMI and layered onto 3 mL of histopaque for density gradient centrifugation. The samples were centrifuged at 400g for 30 mins at room temperature. The immune cells were collected from the Buffy layer (figure 2.2) with a transfer pipette and washed at least twice in 10 mL of ice cold TCM to remove residual histopaque. The isolated spleen cells were then resuspended in TCM and placed on ice.

![Figure 2.2: The appearance of samples after centrifugation.](image)
2.3.4: Cell concentration and viability - trypan blue exclusion analysis

A minimum sample of cells (5 μL) was taken from each cell suspension and added to 895 μL of TCM and 100 μL of trypan blue (2% w/v trypan blue in 0.9% w/v saline, Sigma Chemical Co., St Louis, MO, USA). Cell concentration and viability was determined using a haemocytometer (Improved Neubauer Brightline, Hausser Scientific, Blue Bell, Pa, Germany) on a compound microscope (x 400 magnification) with trypan blue dye exclusion being the indicator of viability. Trypan blue penetrates non-viable cells with compromised cell membranes, causing the cytoplasm to appear blue. Cell viability of fish immune cells was usually >95%, and is similar to previous reports of immune cells isolated from other fish species (O'Halloran 1996, Rosenberg-wiser and Avtalion, 1982, Dunier et al., 1995). The cells were then diluted in TCM to the desired cell concentration for subsequent assays.

2.4: Flow cytometric analysis of phagocytic function

2.4.1: Background

Flow cytometry is a technique that uses the light scattering properties of cells to categorise them into different subpopulations based on size, granularity and/or their emission of fluorescent signals. The flow cytometer achieves this by passing cells in a continuous single file inside a fluid stream upon which a laser is focused (e.g. 488nm argon laser). Scattered laser light is detected in photomultiplier tubes (PMTs) positioned forward of the laser path (i.e. termed “forward scatter”, FS) and at right angles (i.e. termed “side scatter”, SS) (figure 2.3). Larger cells take a longer time to pass through the laser path and therefore produce a greater FS signal. Cells with more cytoplasmic granules and particles (i.e. granulocytes) scatter more light at a 90° angle and therefore produced a greater SS signal.
Flow cytometers can also identify fluorescent signals of varying wavelengths, allowing further analysis of individual cell characteristics by using fluorescent dyes and latex beads or fluorescent-labelled antibodies. Fluorescence is detected in PMTs that are located at right angles to the laser path and have band pass filters to measure specific wavelengths. Some of the cell characteristics that can be described by this technology are; cell surface markers, growth cycle changes, cell viability and phagocytic activity. This enables quantification as well as physical separation of different cell subpopulations, via a cell-sorting function of the flow cytometer.

Flow cytometry is commonly used in immunology and pathology for the diagnosis of diseases and as a research tool for the biomedical sciences. In immunological studies involving carp, (Koumans-Van Diepen et al., 1994b) channel catfish (Eellsaesser et al., 1985) and salmonids (Morgan et al., 1993), flow cytometers were used to identify and quantify different immune cell subpopulations. The technique has also been used to analyse the phagocytic function of fish macrophages and granulocytes (Thuvander et al., 1992; Thuvander et al., 1987). The power of simultaneously analysing many different cell characteristics provides ecotoxicology
researchers a more rapid and less subjective alternative to manual methods currently used in the field of cytology.

Phagocytosis is a primitive defence mechanism, conserved in both vertebrates and invertebrates. The function plays an important role because it is the first step in a cascade of events that include; the destruction of the invading organism, antigen processing and presentation, and the regulation of the immune response through the secretion of cytokines (Neumann et al., 2001; Siwicki et al., 1998). Phagocytic ability has also been proposed as a biomarker for the detection of toxicants in aquatic toxicology (Siwicki et al., 1998).

In this study, flow cytometry was used to measure the phagocytic activity of head kidney cells from four different native Australian species of fish. Their phagocytic activity was quantified by flow cytometry (EPICS Elite II, Coulter, Hialeah, FL, USA) after an incubation period with fluorescent (fluorescein isothiocyanate, FITC) latex beads (1 μm, Polysciences Inc., Warrington, PA, USA). Cells that engulfed FITC beads had a peak light emission at 520nm (measured in PMT2), while the majority of unengulfed beads were excluded from the analysis by the use of the “live gating” option available in the Epics Elite Expo32 software (Coulter, Hialeah, FL, USA). This live gating option enables analysis of events that relate only to the cells, without the need for physical separation of the much smaller unengulfed beads from the cell suspension prior to flow cytometry. There was a background fluorescence (i.e. ~10% of the granulocyte-gated counts) caused by unengulfed beads passing the laser in close proximity to cells. Correction for this background effect was made during experiments by the use of negative controls, which were an identical set of samples that were incubated under the same conditions as the test samples but without beads. Beads were then added to the negative control samples immediately before analysis on the flow cytometer (i.e. t = 0), and thus represented a sample where no phagocytosis had occurred (figure 2.4).
The phagocytosis data collected in this assay was expressed as the percentage of granulocytes emitting fluorescence (i.e. FITC+ve events) at 520 nm, while the analysis of mean voltage output of FITC +ve events provided an indication of the number of beads engulfed per cell. Cell viability can be monitored through a change in the cell structure (i.e. events in the debris-gated region) and by the exclusion of the fluorescent vital dye propidium iodide (PI, 1 μg/mL, Sigma Chemical Co., St Louis, MO, USA), which like trypan blue enters cells with compromised membranes. Once inside the cells, PI binds to DNA and is detected by the emission of fluorescence at 617nm. The majority of PI fluorescence is found in the “debris-gated” region, which exhibited low FS (i.e. smaller) and high SS (i.e. more granular) characteristics due to the non-viable cells losing their structural integrity shortly after their membranes become permeable to PI. The following section is a description of the development and optimisation of assay conditions for each species, which was a crucial step prior to the measurement of phagocytic ability of head kidney cells following in vitro and in vivo exposure to environmental pollutants and immunostimulants.
Figure 2.4: The forward scatter (FS) vs side scatter (SS) of Murray cod head kidney cells a) before (negative control) and b) after incubation for 48 hr at 20ºC with FITC-latex beads. The FITC “signal” measured in PMT 2 shows c) negative control with a single peak representing unengulfed beads (background fluorescence) and d) multiple peaks after 48 hr incubation (at 20ºC) with beads, representing phagocytes with multiple beads internalised. Colours represent: Granulocytes, blue; Lymphocytes, pink; Debris, yellow; FITC +ve, green; PI +ve, red.
2.4.2: Optimisation and Validation

2.4.2.1 Crimson-spotted rainbowfish (*Melanotaenia fluviatilis*)

Crimson-spotted rainbowfish were sacrificed and their head kidney cells isolated as described in section 2.3. The following experiments were performed to optimise the quantification of phagocytic function in rainbowfish using flow cytometry.

**Incubation time and validation**

Phagocytic activity can vary between different species, therefore the time taken to reach peak phagocytosis was investigated for each species. An incubation temperature of 25°C was chosen for all rainbowfish experiments, as this was optimum temperature for mitogenesis, as previously reported by O’Halloran (1996). Crimson-spotted rainbowfish head kidney cells (1x10^6 cells/mL) were incubated with 2.5x10^7 FITC-latex beads (1:25 cell to bead ratio) in TCM (1 mL) in an atmosphere of 95% air / 5% CO₂ (special mix balanced in air, Linde Gas, Sydney, NSW, Australia). Samples were analysed on the flow cytometer (EPICS Elite II, Coulter, Hialeah, FL, USA) at the start of the incubation period (i.e. t = 0 hr) and then every 2 hr period for 24 hr, in order to determine the minimum time required for phagocytes to reach peak fluorescence. Immediately after flow cytometric analysis, a small amount of sample was pipetted into a haemocytometer and granulocytes (with and without engulfed beads) within a specified area were counted under a light microscope. Results were expressed as percentage phagocytosis; which for the manual method was the number of cells with beads / total granulocyte count x 100; while for the flow cytometry results were calculated as the number of FITC positive granulocytes / 10,000 events x 100. The number of FITC +ve lymphocytes were also measured to determine if there was non-specific uptake of beads by non-target cells. The data indicates that lymphocytes did not pinocytose the beads and that the increase in the FITC signal was due to phagocytosis.
The percentage of rainbowfish head kidney granulocytes with FITC beads increased over the 24 hr incubation period, with peak phagocytosis being reached at approximately 20 hr (figure 2.5). The measurements of percentage phagocytosis using flow cytometry were lower than the values generated from enumeration under the light microscope. It is difficult to prevent subjectivity in a manual enumeration method, especially when this requires positive identification of a particular cell type. Damaged cells or granulocytes that were difficult to positively identify under the light microscope were not included in the total granulocyte count, thereby increasing the likelihood that a ‘counted’ granulocyte would be actively phagocytosing latex beads, and possibly resulting in an overestimation of activity. Conversely, with flow cytometry, there is no such subjectivity and the total event count included any cell or ‘other event’ that possessed the characteristics that ‘allowed’ them to fall within the granulocyte gated region. This may have resulted in an underestimation of activity. Regardless, the flow cytometry method follows has similar kinetics (figure 2.5) and correlates well ($r^2 = 92.33\%$, figure 2.6) to light microscope enumeration method and was therefore considered a valid method for the quantification of phagocytosis.

![Graph showing phagocytosis over time](image)

**Figure 2.5**: Phagocytosis of FITC latex beads by rainbowfish head kidney cells over a 24 hr period. Analysed by flow cytometer and haemocytometer. The values represent the mean ± se of duplicate samples from one fish.
Flow cytometer analysis of % phagocytosis

Microscope analysis of % phagocytosis

Figure 2.6: Regression analysis of flow cytometer and light microscope methods for assessing phagocytosis.

\[ r^2 = 0.923 \]

Optimum cell:latex bead ratio

In this assay, unengulfed beads passing the laser in close proximity to cells caused background fluorescence and interfered with the light scattering properties of cells. Therefore, as the number of beads per cell was increased, the background fluorescence increased and subpopulation counts decrease. Increasing the number of beads in the assay also increases the incidence of bead clumps, which leads to an increase in the FITC mean voltage. The optimum cell:bead ratio was therefore investigated to determine the number of beads per cell required to achieve maximum phagocytosis, without the significant reduction of cell counts.

Rainbowfish head kidney cells at 1x10^6 cells/mL, were incubated with various bead concentrations (12.5, 25, 50 and 100x10^6 beads/mL) to provide cell:bead ratios of 1:12.5, 1:25, 1:50 and 1:100, respectively. The samples were incubated in an atmosphere of 95% air/5% CO₂ at 25°C for 20 hr and were then analysed on the flow cytometer to determine the cell:bead ratio that would produce the highest fluorescence without interfering with cell counts. Increasing the number of beads per cell above 25 caused no significant increase in
FITC positive events in the granulocyte subpopulation (i.e. phagocytic activity) (figure 2.7). At 1:100 mean FITC voltage was dramatically increased by bead clumps (i.e. >5 beads), which were highly fluorescent and could not be gated out due to their size (data not shown). A cell:bead ratio of 1:50 and 1:100 caused a reduction in the cell counts in the lymphocyte and granulocyte subpopulations. The reduction of events in the subpopulation-gated regions is due a higher proportion of events falling outside these regions because of interference in the light-scattering characteristics of cells by unengulfed beads in close proximity. Therefore, the optimum cell:bead ratio for maximising fluorescence while minimising the effect on cell counts was determined to be 1:25.

![Figure 2.7: The effect of cell:bead ratio on phagocytic function of rainbowfish head kidney cells.](image)

The values represent the mean ± se of triplicate samples from one fish.
Figure 2.8: The effect of cell:bead ratio on cell counts of rainbowfish head kidney cells. The values represent the mean ± se of triplicate samples from one fish.

**Standardised phagocytosis protocol for crimson-spotted rainbowfish**

Crimson-spotted rainbowfish head kidney cells (1x10⁶ cells/mL) were incubated with 2.5x10⁷ FITC latex beads in TCM in 95% air/5% CO₂ at 25°C for 20 hr and were then analysed with the flow cytometer. Negative controls (i.e. cells suspension of 1x10⁶ cells/mL incubated with the samples) were analysed immediately following the addition of 2.5x10⁷ FITC latex beads. Counting gates were placed around two subpopulations representing; granulocytes (i.e. large granular cells with high FS and SS) and lymphocytes (i.e. smaller cells with lower FS and SS). Debris (i.e. PI positive events smaller that the lymphocytes with lower FS but higher SS) was also gated so that the toxic effects of the test chemicals could be further quantitated. A total of 10,000 events per sample was collected and stored electronically for future analysis.
2.4.2.2: Silver perch (*Bidyanus bidyanus*)

Silver perch were sacrificed and head kidney cells isolated as described in section 2.3. The following experiments were performed to optimise conditions of the phagocytosis assay for silver perch. An incubation temperature of 25°C was chosen for all experiments, as this was optimum temperature for silver perch mitogenesis, as previously reported by O’Halloran (1996).

**Incubation time**

Silver perch head kidney cells (1x10⁶ cells/mL) were incubated with 2.5x10⁷ FITC-latex beads (1:25 ratio). Samples were analysed with the flow cytometer at the start of the incubation period (i.e. t = 0 hr) and then every 2 hr period for 24 hr in order to determine the minimum time required for peak fluorescence. Silver perch head kidney cells reached peak phagocytosis at 18 hr (figure 2.9a), however the number of granulocytes and lymphocytes in the sample was reduced after 16 hr of incubation. Lymphocytes numbers were reduced more rapidly with a reduction in counts apparent after only 2 hr of incubation. The optimal duration for silver perch phagocytosis was determined to be 18 hr.

![Figure 2.9: a) Phagocytosis of FITC latex beads and b) subpopulation and debris counts of silver perch head kidney cells over a 24 hr period.](image)

The values represent the mean ± se of triplicate samples from one fish.
Cell concentration and bead ratio

Cell concentrations of $1 \times 10^6$ and $2 \times 10^6$ cells/mL were incubated for 18 hr with latex beads at the three different cell: bead ratios of 1:12, 1:25 and 1:50 in order to determine the optimum cell:bead ratio for measuring silver perch phagocytosis. Over the range investigated, the percentage phagocytosis did not differ between 1:12 and 1:25 beads per cell (figure 2.10). However, at a cell:bead ratio of 1:50, a large amount of background fluorescence occurred which interfered with the measurement of phagocytosis. There was no effect on granulocyte numbers, however lymphocytes numbers displayed a marginal linear decrease as the ratio was increased (figure 2.11). Therefore, a ratio of either 1:25 or 1:50 was deemed appropriate for silver perch phagocytosis and 1:25 was chosen for the standardised protocol to keep lymphocytes counts higher and background fluorescence and variation to a minimum.

![Figure 2.10: The effect of cell:bead ratio on the phagocytic function of silver perch head kidney cells (at $1 \times 10^6$ cells/mL). The values represent the mean ± se of triplicate samples from one fish.](image)

**Figure 2.10:** The effect of cell:bead ratio on the phagocytic function of silver perch head kidney cells (at $1 \times 10^6$ cells/mL). The values represent the mean ± se of triplicate samples from one fish.
Figure 2.11: The effect of cell: bead ratio on cell counts of silver perch head kidney cells (1x10$^6$ cells/mL). The values represent the mean ± se of triplicate samples from one fish.

Investigation of tissue culture media additions

Due to concerns in the decline of granulocyte and lymphocytes numbers during overnight incubation (refer to figure 2.9b), a study was conducted to optimise the chemical characteristics of the media used in the assay. Silver perch head kidney cells (1x10$^6$ cells/mL) were incubated with 2.5x10$^7$ FITC latex beads in TCM with a range of pH (7.25, 7.35 and 7.45 adjusted with 1M HCL and 1M NaOH) and 2-mercaptoethanol (2-ME) concentrations (25, 50 and 100 µM). Samples were incubated for 18 hr in an atmosphere of 95% air/5% CO$_2$. The treatments caused no significant improvement in phagocytic activity, however a pH of 7.35 with 50 µM of 2-mercaptoethanol was deemed to be optimal for phagocytosis.

There were no significant changes in the subpopulation counts but there was a reduction in lymphocyte numbers when there was either, a high pH (i.e. 7.45) and low 2-ME concentration (i.e. 25 µM) or a low pH (i.e. 7.25) and a high 2-ME concentration (figure 12c). There was no significant reduction in granulocyte counts, however at 100 µM of 2-ME counts were marginally lower and more variable (figure 2.12b). There were also reduced granulocyte counts at a high pH (i.e. 7.45) and low 2-ME concentration (i.e. 25 µM).
Figure 2.12: The effect of pH and 2-mercaptoethanol (2-ME) on the phagocytic activity and subpopulation counts of silver perch head kidney cells. a) Phagocytosis b) Granulocytes c) Lymphocytes d) Debris. The values represent the mean ± se of triplicate samples from one fish.
It was deemed from these results that the optimum pH and 2-ME concentration for both cell integrity and phagocytic activity was 7.35 and 50 µM, although slight variation of these variables should not cause a significant change in results.

*Standardised phagocytosis protocol for Silver perch*

Silver perch head kidney cells (1x10^6 cells/mL) were incubated with 2.5x10^7 FITC latex beads, in TCM (pH 7.35) with 50 µM 2-ME in an atmosphere of 95% air/5% CO_2 at 25°C for 18 hr and were then analysed on the flow cytometer. Negative controls (i.e. cells suspension of 1x10^6 cells/mL incubated with the samples) were analysed immediately following the addition of 2.5x10^7 FITC latex beads. Counting gates were placed around two subpopulations representing; granulocytes and lymphocytes. Debris was also gated so that the toxic effects of the test chemicals could be quantitated. A total of 10,000 events per sample was collected and stored electronically for future analysis.

*2.4.2.3: Golden perch (Macquaria ambigua)*

Golden perch were sacrificed and their head kidney cells isolated as described in section 2.3. The following experiments were performed to optimise conditions of the phagocytosis assay for golden perch. The optimum temperature for mitogenic responses (i.e. 20°C) was used for all incubations.

*Incubation time*

Golden perch head kidney cells (1x10^6 cells/mL) were incubated in 1mL of TCM with 2.5x10^7 FITC latex beads. Samples were analysed on the flow cytometer at the start of the incubation period (i.e. t=0 hr) and then every 2 hr for 24 hr, in order to determine the minimum time to reach peak fluorescence (maximum phagocytosis). Golden perch head kidney phagocytic activity increased with time, reaching a maximum at 22 hr (figure 2.13).
Figure 2.13: Phagocytosis of FITC beads by golden perch head kidney cells over a 24 hr period. The values represent the mean ± se of triplicate samples from one fish.

**Cell concentration and bead ratio**

Golden perch head kidney cells (1x10^6 cells/mL) in 1mL of TCM were incubated for 20 hr with latex beads at the five different cell: bead ratios of 1:6, 1:12, 1:25, 1:50 and 1:100, in order to determine the optimum ratio for measuring golden perch phagocytosis. There was no difference in phagocytic activity for cell bead ratios of 1:12, 1:25 and 1:50. However, a cell:bead ratio of 1:25 reduced the lymphocyte counts, while a cell:bead ratio of 1:50 also begins to reduce the number of granulocyte events recorded (figure 2.15). Therefore, 1:12 was deemed the appropriate cell bead ratio to use in the standardised protocol.

**Standardised phagocytosis protocol for Golden perch**

Golden perch head kidney cells (1x10^6 cells/mL) were incubated with 1.2x10^7 FITC latex beads, in TCM in 95% air/5% CO₂ at 20°C for 22 hr and were then analysed on the flow cytometer. Negative controls (i.e. cells suspension of 1x10^6 cells/mL incubated with the samples) were analysed immediately following the addition of 1.2x10^7 FITC latex beads.
Counting gates were placed around two subpopulations representing granulocytes and lymphocytes. Debris was also gated so that the toxic effects of the test chemicals could be quantitated. A total of 10,000 events per sample was collected and stored electronically for future analysis.

Figure 2.14: The effect of cell:bead ratio on the phagocytic activity of golden perch head kidney cells. The values represent the mean ± se of triplicate samples from one fish.

Figure 2.15: The effect of cell:bead ratio on subpopulation counts of golden perch head kidney cells. The values represent the mean ± se of triplicate samples from one fish.
2.4.2.4: Murray cod (*Maccullochella peelii peelii*)

Murray cod were sacrificed and their head kidney cells isolated as described in section 2.3. The following experiments were performed to optimise conditions of the phagocytosis assay for Murray cod.

**Incubation time and temperature**

Murray cod head kidney cells (1x10^6 cells/mL) were incubated in 1mL of TCM with 0, 1.2 or 2.5 x 10^7 FITC latex beads at 15, 20 and 25°C. In order to determine the optimum time and temperature for phagocytic activity, samples were analysed on days 2, 3, 5 and 7 using the flow cytometer. Three different temperatures were tested in these experiments due to data from the mitogenesis assays that showed optimum activity at 15°C (section 2.6.2.2). Although the subpopulation counts of the other native fish were reduced after overnight culture, incubation periods were extended to 7 d in an attempt to increase the sensitivity of phagocytic activity measurements. The maximum phagocytic activity occurred at 20°C increasing linearly from 15% to a maximum of 25% by 7 d. Percentage phagocytosis also increased linearly at 15°C but was slower to begin and suboptimal at all time points compared to 20°C. Activity at 25°C was also suboptimal at all time points compared to 20°C. A cell:bead ratio of 1:25 increases the phagocytic activity at day 3, 5 and 7 (figure 2.16b). Granulocytes were generally more robust than lymphocytes and marginal numbers were lost only at 25°C. Lymphocytes survived well at cooler temperatures and after 7 d at 15°C, 70% of the lymphocytes remained (figure 2.17b). Therefore, it was determined that the optimal time and temperature for Murray cod phagocytosis was 2 d at 20°C.
Figure 2.16: Effect of incubation temperature on phagocytosis of FITC beads by Murray cod head kidney cells over a 7 day period at a cell:bead ratio of a) 1:12.5 b) 1:25. The values represent the mean ± se of triplicate samples from one fish.
Figure 2.17: The effect of incubation time and temperature on subpopulation and debris counts. a) Granulocytes, b) Lymphocytes, c) Debris. The values represent the mean ± se of triplicate samples from one fish.
**Cell:Bead ratio**

Murray cod head kidney cells (1x10⁶ cells/mL) in 1mL of TCM were incubated for 2, 3, 5 and 7 d at 20°C with FITC latex beads at four different cell: bead ratios (1:6, 1:12, 1:25 and 1:50). There was a linear increase in FITC +ve events for all cell:bead ratios except 1:50 when background fluorescence begins to interfere with the detection of beads engulfed by granulocytes (figure 2.18). Figure 2.19 shows cells incubated at 20°C for 3 d at the five different ratios. There was no difference in the counts of granulocyte and debris subpopulations, but from 1:25 and 1:50 cells per bead there was a progressive decline in the lymphocyte subpopulation counts (figure 2.19). From these results it was determined that a ratio of 1:25 would be adequate to measure both maximal phagocytosis and lymphocyte counts.

![Figure 2.18: The effect of cell:bead ratio on the phagocytosis of FITC latex beads by Murray cod head kidney cells.](image)

Cells were incubated at 20°C for 2, 3, 5 and 7 d. The values represent the mean ± se of triplicate samples from one fish.
**Figure 2.19: The effect of cell:bead ratio on subpopulation counts of Murray cod head kidney cells.**
a) Granulocytes and b) Lymphocytes and debris. Cells were incubated for 3 d at 20°C. Values represent the mean ± se of triplicate samples from one fish.

**Standardised protocol for Murray cod**

As it was of interest to enumerate lymphocytes following exposure to chemicals, it was determined that for this assay conditions should also support lymphocytes. The conditions for maximum lymphocyte counts and maximum phagocytic activity occurred under 2 experimental conditions, at 2 d and 20°C as well as at 5 d and 15°C. Under these conditions lymphocyte counts were approximately 2500 and phagocytosis was 15%. Although a higher phagocytic activity occurred as time progressed, lymphocytes became non-viable in the process. Therefore it was decided that the most effective incubation time and temperature was 2 d at 20°C.

Murray cod head kidney cells (1x10⁶ cells/mL) were incubated with 2.5x10⁷ FITC latex beads. Duplicate (or triplicate) samples were incubated in TCM with 95% air/5% CO₂ at 20°C for 2 d and were then analysed on the flow cytometer. Negative controls (i.e. cells suspension of 1x10⁶ cells/mL incubated with the test samples) were analysed immediately following the addition of 2.5x10⁷ FITC latex beads. Counting gates were placed around two
subpopulations representing; granulocytes and lymphocytes. Debris was also gated so that the toxic effects of the test chemicals could be quantitated. A total of 10,000 events per sample was collected and stored electronically for future analysis.

2.5: Lysozyme assay

2.5.1: Background

Lysozyme (N-acetylmuramidase glycanohydralase, E.C. 3.2.1.17) is one of three hydrolyase enzymes that have a defensive role in the circulatory system. Lysozyme is conserved in all vertebrates and has also been isolated from invertebrates, insects, bacteria and viruses (Grinde et al., 1988). It is extremely well characterised, with all major properties identified including; amino acid sequences, conformations, nucleotide sequences, function and molecular weight (12-15 kDa in fish). Lysozyme attacks structures containing β1-4 linked N-acetylmuramamide and N-acetylglucosamine, which are components of the cell walls of gram negative bacteria (Alexander and Ingram, 1992). In fish, it is found in the blood, mucus and lymphomyeloid tissue, highlighting its role in fish innate immune systems, which is increasingly important as their specific immune system is slower and less developed in comparison to mammals (Ginde et al., 1988). Variation has been found between species and with individuals of the same species, and according to season, sex, maturity and past exposure to pathogens (Ginde et al., 1988).

Lysozyme activity can be measured spectrophotometrically as the enzyme clears a solution of Micrococcus lysodeikticus (0.74 mg/mL, Sigma Chemical Co., St Louis, MO, USA) from a 96 well flat-bottom plate. The amount of lysozyme present in the sample was quantitated using a standard lysozyme purified from hen egg white (i.e. HEWL 0 μg/mL – 20 μg/mL at a pH of 5.8). Lysozyme was chosen as an indicator of immunomodulation due to the easy and rapid assay method and its importance in the innate defence of fish. It also has the advantage
of being non-lethal to the fish, as blood samples may be taken non-destructively via the caudal vein of live animals. This allows the monitoring of immunomodulating effects of chemicals over a period of time.

2.5.2: Optimisation of the lysozyme assay

The effect of pH

Observations in other species of fish have shown that there are differences in the optimum pH for maximum lysozyme activity and that isozymes of the enzyme exist (Watts et al., 2002). Lysozyme activity was tested over the range of pH 5.0-6.2, at 0.2 unit intervals, for each of the four native species. This was achieved using 1 M HCl to vary the pH of the phosphate/citrate buffer containing the *Micrococcus lysodeikticus* (i.e. 1 M phosphate/citrate with 0.9% sodium chloride and 0.75 mg/L *M. lysodeikticus*). *M. lysodeikticus* solution (175 μL of varying pH) was added to 25 μL of fish serum (collected as described in section 2.3) in a flat bottom 96 well plate. The absorbance in each well was measured at 450 nm every 30 seconds for 10 mins on a DIAS plate reader (Dynatech Laboratories Inc., Chantilly, Va, USA) and the data collected using Biolinx assay management software (V2.1, Dynatech Laboratories Inc., Chantilly, Va, USA). The four Australian native fish had an optimal lysozyme activity at a lower pH compared to that of hen egg white lysozyme, which has an optimal activity at pH 5.8. Optimum pH for all the species was about 5.4, although for Murray cod and silver perch had a broader optimal range of 5.4 - 5.8 (figure 2.20). Consequently, a pH of 5.4 was used for golden perch and rainbowfish, while for Murray cod and silver perch a pH of 5.6 was used.
Figure 2.20: The effect of pH on Australian freshwater fish lysozyme activity. Values represent the mean ± se of triplicate samples from one fish. Serum was pooled from 6 fish for rainbowfish results.

**Lability**

The effect of freezing Murray cod serum was investigated to be sure that storage would not significantly affect lysozyme activity. This test was conducted only on Murray cod lysozyme because the need for extended storage became apparent in the extensive in vivo studies conducted in this species. Murray cod serum samples were analysed on the day of collection without freezing and after 7 and 14 d at –20°C. The activity of the enzyme was marginally reduced after freezing but storage over two weeks did not result in a significant reduction in activity (figure 2.21).

Figure 2.21: The effect of storage at -20°C on Murray cod serum lysozyme activity. Values represent the mean ± se of quadruplicate samples from one fish.
2.5.3: Standardised lysozyme protocols for Australian native fish

In vitro

50 μL of treatment or phosphate/citrate buffer was added to a 96 well plate in triplicates or quadruplicates and 25 μL of fish serum was added and incubated for 2 min. 125 μL (1.05 mg/mL) of bacterial solution was added with the optimum pH for the species used (i.e. 5.4 for golden perch and rainbowfish and 5.6 for Murray cod and silver perch). Absorbance in each well was measured at 450 nm every 30 seconds for 10 mins on a DIAS plate reader and the data collected using Biolinx assay management software.

In vivo

25 μL of fish serum was added to a 96 well plate and then 175 μL (0.75 mg/mL) of bacterial solution was added at the optimum pH for the species being tested. Absorbance in each well was measured at 450 nm every 30 seconds for 10 mins on a DIAS plate reader and the data collected using Biolinx assay management software.

2.6: Mitogenesis Assay

2.6.1: Background

Mitogen-stimulated proliferation of lymphocytes is an immune function that has been observed in many vertebrates, including fish species such as Chinook salmon (Arkoosh et al., 1994b), trout (DeKoning and Kaattari, 1991; Etlinger et al., 1976), European carp (Capsi et al., 1984; Koumans-Van Diepen et al., 1994a; Rosenberg-Wiser and Avtalion, 1982), channel catfish (Luft et al., 1991), English sole (Arkoosh et al., 1994a) and red drum (LoPresto et al., 1995). Previous research by our laboratory has measured mitogenesis with a varying degree of success in Australian native fish such as sand flathead (Platycephalus bassensis) (O'Halloran, 1996), crimson-spotted rainbowfish (Barry et al., 1995) and silver perch (O'Halloran et al., 1996b; O'Halloran et al., 1998b).
Lymphoproliferation was included in the panel of tests used for the assessment of immunotoxic chemicals by the national toxicology program in the United States (see section 1.6) (Luster et al., 1994). It has been used in aquatic toxicology to show toxicant-induced lymphosuppression in many fish species including; carp (Siwicki et al., 2000), catfish (Albergoni and Viola, 1995), Chinook salmon (Arkoosh et al., 1994b), dab (Tahir and Secombes, 1995), rainbow trout (O'Halloran et al., 1998b; Siwicki et al., 2000; Spitsbergen et al., 1986), spot (Faisal and Huggett, 1993; Faisal et al., 1991a) and silver perch (O'Halloran et al., 1998b).

Lymphoproliferation is similar to the lymphocytic response to antigens that are presented by macrophages, however it does not require the action of macrophages and occurs rapidly in response to agents conserved in many foreign organisms such as bacteria (i.e. lipopolysaccharide) and plants (i.e. phytohemagglutinin, PHA). It is commonly measured through the amount of tritiated thymidine (i.e. radiolabelled DNA subunit) that is incorporated into the lymphocytes as they grow and divide in response to the mitogen.

2.6.2: Optimisation

Teleost species appear to require a wide range of optimal culture conditions for the measurement of mitogenesis (DeKoning and Kaattari, 1991; Rosenberg-Wiser and Avtalion, 1982). Variables including mitogen concentration, incubation time, temperature, tritium exposure time and cell concentration were investigated to formulate a standardised protocol for use in subsequent toxicity studies. These optimisation studies focused on Murray cod and golden perch mitogenesis because standardised protocols for rainbowfish and silver perch have been previously described by our group (O’Halloran, 1996).
2.6.2.1: **Golden perch (Macquaria ambigu**a)

Golden perch were sacrificed and head kidney cells isolated as described in section 2.2 and 2.3.

**Mitogens**

A number of biological agents have mitogenic properties. This means they stimulate proliferation of lymphocytes that have had no prior exposure to the agent. The discovery of the plant lectin phytohemagglutinin (PHA) mitogenic activity, led to the discovery of many more mitogens from many different sections of the animal kingdom (Sharma, 1981). The mitogens and concentration ranges used in the study of all native species include:

- Phytohemagglutinin (PHA) (0, 0.156, 0.312, 0.625, 1.25, 2.5, 5.0 and 10.0 μg/mL)
- Concanavalin A (ConA) (0, 0.312, 0.625, 1.25, 2.5, 5.0, 10.0 and 20.0 μg/mL)
- Lipopolysaccharide (LPS) (0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0 and 40 μg/mL) and
- Pokeweed mitogen (PWM) (0, 0.78, 0.156, 0.312, 0.625, 1.25, 2.5, 5.0 μg/mL).

**Incubation time and temperature**

Temperature and incubation time were first investigated to determine the maximum responses to mitogens. Golden perch head kidney cells (2.5x10^6 cells/mL) were incubated for 3, 5 and 7 d at 15, 20 and 25°C in 96 well plates. The cells were cultured in a total volume of 200 μL of TCM with the aforementioned concentrations of mitogens, in an atmosphere of 95% air/5% CO₂ (Linde Gas, Sydney, NSW, Australia). The cells were pulsed with tritiated thymidine (Amersham International, Amersham, UK) at 0.5 μCi/well for 24 hr before harvesting onto glass fibre paper using a semi-automatic cell harvester (Skatron, Lier, Norway). Figure 2.22 shows the dose-response curve of Golden perch head kidney cells in response to PHA at various temperatures. Mitogenesis is represented by the radioactivity incorporated into the cells DNA during proliferation (i.e. counts per minute, CPM). The optimum temperature for
culture of golden perch lymphocytes was 20°C (figure 2.22). The best mitogenic response occurred at 5 d when basal proliferation was lower compared to 3 d when it is undesirably high (figure 2.23). Peak responses are also reduced if the incubation period is extended to 7 d (figure 2.23).

Figure 2.22: The effect of temperature on the mitogenic response to phytohemagglutinin (PHA) of golden perch head kidney cells (2.5x10^5 cells/well) after 5 d incubation. Values represent the mean ± se of triplicate samples from one fish.

Figure 2.23: The effect of incubation period (at 20°C) on the mitogenic response to phytohemagglutinin (PHA) of golden perch head kidney cells (2.5x10^5 cells/well). Values represent the mean ± se of triplicate samples from one fish.
Tritiated-thymidine exposure period

Due to the weak mitogenic response shown by Golden perch lymphocytes, a study was conducted to determine whether additional incubation time with the tritiated thymidine would increase the sensitivity of the assay. Golden perch head kidney cells (2.5 x 10⁶ cells/mL) were incubated for 3 and 5 d at 20°C with 0.5 μCi/well tritiated thymidine being added at 24 hr and 48 hr before harvesting cells. The addition of the tritiated thymidine for an extra 24 hr increased both the counts per min and the difference in the basal and peak mitogenic responses (i.e. proliferation index) (figure 2.24). Therefore, the Golden perch standardised mitogenesis protocol used a 48 hr tritium exposure period.

![Figure 2.24: Tritiated thymidine exposure time and incorporation into proliferating golden perch head kidney cells (2.5x10⁵ cells/well) stimulated with phytohemagglutinin (PHA). Values represent the mean ± se of triplicate samples from one fish.](image)

Cell concentration

Golden perch head kidney cells at 1.0, 2.5 and 5 x10⁵ cells/well were incubated with PHA, ConA, LPS and PWM for 5 d at 20°C, with the aim of determining the cell concentration that would produce an optimal mitogenic response. Golden perch head kidney cells at a concentration of 5.0x10⁵ cells/well produced a clear mitogenic response (figure 2.25). A cell concentration of 2.5x10⁵ cells/well displayed a weakened mitogenic response and there was
no response observed with a cell concentration of $1.0 \times 10^5$ cells/mL. Cell concentrations greater than $5.0 \times 10^5$ were not investigated, as cell yields would not be sufficient to fulfil assay requirements, especially in the cases of large in vitro experiments and where chemical exposure causes immunosuppression leading to low cell yields.

Figure 2.25: The effect of cell concentration on the mitogenic responses of golden perch head kidney cells. Cells were incubated at three concentrations for 5 d at 20°C. a) phytohemagglutinin (PHA), b) poke weed mitogen (PWM), c) Lipopolysaccharide (LPS) and d) Concanavalin A (Con A). Values represent the mean ± se of triplicate samples from one fish.
**Standardised protocol for Golden perch mitogenesis**

Golden perch head kidney cells (5.0x10^5 cells/well) were incubated for 5 d at 20°C with LPS (0.65 – 40 μg/mL), PHA (0.156 – 10 μg/mL), ConA (0.625 – 20 μg/mL) and PWM (0.78 – 5 μg/mL) in at total volume of 200 μL in 96 well plates (Sarsted Australia, PTY. LTD., Technology Park, SA, Australia), in an atmosphere of 95 % air/5% CO₂. Cells were pulsed by adding 0.5 μCi/well of tritiated thymidine (Amersham International, Amersham, UK) 48 hr prior to harvesting onto glass fibre mats using a semi-automatic 12 well harvester (Skatron, Lier, Norway). Tritiated thymidine incorporation (i.e. proliferation) was quantitated using a liquid scintillation counter (LKB, Wallac, Turku, Finland) and ACSII scintillation fluid (Amersham International, Amersham, UK)

2.6.2.2: Murray cod (*Maccullochella peelii peelii*)

**Mitogens**

The concentration of each mitogen investigated was unchanged from the Golden perch protocol (see section 2.6.2.1)

**Incubation time and temperature**

Temperature and incubation period were first investigated to determine the maximum responses to mitogens, focusing on the temperature range that gave the best response from Golden perch cells. Murray cod head kidney cells (2.5 x 10^5 cells/well) were incubated in 96 wells plates, for 3, 5 and 7 d at 15, 20 and 25°C in TCM with an atmosphere of 5% CO₂. Cells were pulsed with tritiated thymidine (0.5 μCi/well) 24 hr prior to cell harvesting. Figure 2.26 shows the response of Murray cod head kidney cells to PHA, which elicited the highest mitogenic activity of the four mitogens tested. Peak mitogenesis was observed at 15°C and the incubation period yielding greatest sensitivity was 5 d. At 3 d basal proliferation was found to be undesirably high as it was for Golden perch at 20°C. Figure 2.27 shows the
mitogenic response of all mitogens at the optimum temperature and incubation period of 15°C for 5 d. Although only small responses were observed for the other three mitogens this could be due to assay conditions. Therefore, cell concentration, thymidine exposure time and the media was varied in attempts to increase the response level.

Figure 2.26: The response of Murray cod head kidney cells to phytohemagglutinin (PHA) at three temperatures for a) 3 d, b) 5 d, c) 7 d and d) at the optimum temperature of 15°C. Values represent the mean ± se of triplicate samples from one fish.
Figure 2.27: The proliferative response of Murray cod head kidney cells to 4 mitogens. The maximum concentrations of the mitogens (i.e. 1) were 20, 10, 5 and 40 μg/mL for Concanavalin A (Con A), phytohemagglutinin (PHA), poke weed mitogen (PWM) and Lipopolysaccharide (LPS), respectively. Values represent the mean ± se of triplicate samples from one fish.

**Cell concentration and tritiated thymidine exposure period**

Murray cod head kidney cells (1.0, 2.5, 5.0 and 10.0x10^5 cells/well) were incubated with PHA for 5 d at 15°C, in order to determine the cell concentration that would produce an optimal mitogenic response. Additional plates were produced to investigate the effect of extending tritium exposure period. Although mitogenic response from this fish was low, increasing the cell concentration resulted in an increase of both the peak and basal counts per minute (figure 2.28). Therefore, 5.0x10^6 cells/mL was chosen as the appropriate cell concentration as using cells at 10.0x10^6 cells/mL did not increase sensitivity of the assay. Extending the exposure time to tritiated thymidine did not present any significant increase in the sensitivity of the assay i.e. there was no increase in the proliferation index due to an equal increase in both basal and peak mitogenesis (figure 2.28). Therefore, the standardised protocol used a 24 hr exposure period to tritium.
Figure 2.28: The effect of tritiated thymidine exposure time and cell concentration on the phytohemagglutinin (PHA)-stimulated mitogenesis of Murray cod head kidney cells. 
a) 1.0x10^6 cell/mL b) 2.5x10^6 cell/mL c) 5.0x10^6 cell/mL d) 10x10^6 cell/mL. Values represent the mean ± se of triplicate samples from one fish.
**Media conditions**

**Osmolarity**

Other researchers have used media with modified osmolarity to optimise the response of lymphocytes (Scapigliati *et al.*, 2002). The mitogenic response of Murray cod head kidney cells was investigated at various osmolarities, with the aim of further increasing the response. Murray cod head kidney cells (5.0x10^5 cells/mL) were cultured in TCM at various osmolarities of 190, 220, 250, 280, 310, 340 and 360 mOsm. Cells were incubated for 5 d at 15°C and were pulsed with tritiated thymidine (0.5 μCi/well) 24 hr prior to harvesting. Murray cod head kidney cells displayed a broad range of tolerance, however, the optimum range for Murray cod mitogenic response was between 250 and 340 mOsm (figure 2.29). Culturing cells outside this osmolarity range resulted in a decrease in tritiated thymidine incorporation in both the basal and stimulated cells. Media for subsequent tests contained an osmolarity of approximately 310 mOsm.

![Figure 2.29: Proliferation profiles of Murray cod head kidney cells incubated at various different osmolarities. a) tissue culture media diluted with water b) tissue culture media adjusted with 30 mM NaCl. Values represent the mean±se of triplicate samples from one fish.](image-url)
**Serum**

The use of homologous serum supplements has been reported to greatly increase the mitogenic responses of trout (DeKoning and Kaattari, 1991) and has been used to aid in the mitogenesis of carp lymphocytes (Rosenberg-Wiser and Avtalion, 1982). The use of homologous serum for optimising the mitogen-stimulated proliferation of lymphocytes isolated from spleen and head kidney was investigated.

Murray cod head kidney cells (5.0x10^6 cells/mL) were cultured in TCM with 10 % FCS, no serum, 10% fresh Murray cod serum (MCS) and 10% heat-treated MCS. Cells were incubated for 5 d at 15°C and were pulsed with tritiated thymidine (0.5 μCi/well) 24 hr prior to harvesting.

Heat-treated Murray cod serum greatly increased both the basal and PHA-stimulated proliferation of Murray cod head kidney cells, however, it could not be said that mitogenic responses were improved. Therefore, the standardised protocols used 10% heat-treated FCS because fish serum did not increase mitogenesis and FCS batches would be less variable in composition than serum collected from fish. Cells did not proliferate without serum and the fish serum required heat-treatment to be effective but did not improve mitogenesis (figure 2.30).
Figure 2.30: Proliferation profiles of Murray cod head kidney cells incubated with various serum supplements i.e. fetal calf serum (FCS) or Murray cod serum (MCS). Values represent the mean±se of triplicate samples from one fish.

 Mercaptoethanol concentration (2-ME)

Various concentrations of 2-ME were tested to find the optimum concentration for the lymphocyte proliferative assay. Murray cod head kidney cells (5.0×10⁶ cells/mL) were cultured in TCM with various concentrations of 2-ME (i.e. 0, 25, 50 and 100 μM). Cells were incubated for 5 d at 15°C and were pulsed with tritiated thymidine (0.5 μCi/well) 24 hr prior to harvesting. Figure 2.31 illustrates that the addition of 2-ME did not aid in the mitogenic response of Murray cod head kidney cells. Peak responses were lower for all groups with the supplement.
Figure 2.31: The effect of 2-mercaptoethanol (2-ME) on the mitogenic response of Murray cod head kidney cells. Values represent the mean±se of triplicate samples from one fish.

**HEPES**

The removal of HEPES before culturing of lymphocytes was investigated, with the aim of increasing lymphoproliferation. Standard cell isolation methods described in section 2.3 were used, except that HEPES free sample used HEPES free media for the washing steps after density gradient centrifugation. Murray cod head kidney cells (5.0x10⁶ cells/mL) were cultured in TCM with and without HEPES. Cells were incubated for 5 d at 15°C and were pulsed with tritiated thymidine (0.5 μCi/well) 24 hr prior to harvesting. The removal of HEPES, prior to cell culture, marginally increased the mitogenic response of Murray cod head kidney cells (figure 2.32).
Figure 2.32: The effect of N-(2-hydroxyethyl)piperazine-N-2-ethane sulfonic acid (HEPES, 20 mM) on the mitogenic response of Murray cod head kidney cells. Values represent the mean ± se of triplicate samples from one fish.

**Standardised protocol for Murray cod mitogenesis**

Murray cod head kidney cells (5.0x10^5 cells/well) were incubated for 5 d at 15°C with PHA (10–2.5 μg/mL), in 96 well plates, with HEPES free TCM (200 μL at 310 mOsm with no 2-ME and 10% heat-treated FCS) and an atmosphere of 95% air/5% CO_2_. Cells were pulsed by adding 0.5 μCi/well of tritiated thymidine (Amersham International, Amersham, UK) 24 hr prior to harvesting onto glass fibre mats using a semi-automatic 12 well harvester (Skatron, Lier, Norway). Tritiated thymidine incorporation into newly divided cells (i.e. proliferation) was quantitated using a liquid scintillation counter (LKB, Wallac, Turku, Finland) and ACSII scintillation fluid (Amersham International, Amersham, UK)
2.7: Conclusions

Four Australian native fish were chosen for this study. Murray cod, golden perch and silver perch were chosen due to their economical and ecological value within the Murray-Darling Basin. Crimson-spotted rainbowfish were selected due to their past use in ecotoxicological studies and their inbred genetics. All Australian native fish adapted well to aquarium conditions. There were no deaths due to transport and handling, while the fish displayed no signs of disease during captivity. However, silver perch did not acclimatise to exposure tanks and there was an unacceptable mortality rate in the untreated control groups.

Variation in the amount of anaesthesia required to immobilise the fish was observed. Silver perch and rainbowfish were the most sensitive to the anaesthetic MS 222 needing 150 mg/L for induction. Murray cod and Golden perch needed approximately 200-250 mg/L to be effective within 2 min. The cell isolation method previously used by O’Halloran and co-workers (1996; 1998b) was adequate in isolating cells from blood, head kidney and spleen of all species. The head kidney often retained some debris that was not separated by histopaque or 54% percoll. This debris was reduced by removing as much connective tissue as possible at the beginning of the isolation process.

Flow cytometry was a useful tool, not only in measuring phagocytosis but also for the monitoring of cell subpopulations in the optimisation of culture conditions. The use of flow cytometry correlated very well with the traditional method of microscopy. Standardised protocols were established for all species and were used to investigate the effects of in vitro and in vivo exposures to environmental pollutants and immunostimulants.

Lysozyme activity was found in all species as expected. Assay incubation temperature was unable to be optimised, as the available plate-reader did not have the facilities. All species
showed optimal lysozyme activity at pH 5.4-5.6, which is lower than the optimum for hen egg white lysozyme of 5.8.

Standardised protocols for measuring immune function have been adapted for all native species used in this study. Crimson-spotted rainbowfish and silver perch protocols were previously formulated by O'Halloran (1996). This investigation successfully optimised the conditions required for lymphoproliferation of golden perch and Murray cod head kidney cells. Traditional supplements, 2-ME and HEPES did not aid in the mitogenic responses of Murray cod lymphocytes. Further investigations into the suitability of the traditional lymphocyte media, RPMI 1640, as well as other parameters could be conducted in the future to further improve the sensitivity of the assay.

2.8: Summary

The following is a summary of the extensive optimisation studies conducted in this chapter.

- Australian freshwater fish species adapt well to aquarium conditions, although Murray cod are more suitable as test species in immuno-ecotoxicological assessments.

- Isolation of immunocytes could be achieved with techniques similar to those used in other mammalian and fish species.

- Assays that have been previously used in the immunotoxicological assessment of chemicals in mammals and exotic fish species, have been adapted and standardised for use in Australian freshwater fish.
Mitogenesis, lysozyme and phagocytic function were chosen as the immunological parameters used in this project for the assessment of immunomodulating properties of chemicals.

Table 2.1: Summary of the optimised conditions for the immune function assays

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<th>Mitogenesis</th>
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Chapter 3: The effect of organotin exposures on the immune system of Australian freshwater fish

3.1: Introduction: Tributyltin (Cas No. 1461-22-9) and Dibutyltin (Cas No. 683-18-1)

The biocidal activity of tributyltin (TBT) against fungi was first discovered during the early 1950s, however its use as a biocidal agent was not seriously developed until the 1960s when TBT was used in industrial biocides, agricultural chemicals, wood preservatives and marine antifouling paints (Clark et al., 1988; Gitlitz and Moran, 1984). Currently, TBT-based antifouling marine paints are still the most widely used, replacing copper, mercury and lead-based paints, due to the fact they are effective over a longer period of time (Eisler, 2000). However, this has caused great concern as TBT is released directly into the marine environment (Clark et al., 1988) and the pollutant is highly toxic to aquatic organisms (Fent, 1996). Further concerns arise from human exposure to detectable levels of TBT in dairy, meat and fish products (Kannan et al., 1995a; Kannan et al., 1995b).

TBT is extremely toxic to aquatic organisms and has been described as the most toxic substance deliberately introduced into the aquatic environment (Maguire, 1987). TBT has been shown to cause a range of disruptions to the nervous, reproductive and immune systems of aquatic organisms and studies have found that concentrations as low as 1-2 ng/L (i.e. parts per trillion, ppTR) can cause adverse effects in some organisms (Fent, 1996). Globally, control of TBT use began in the early 1980s, while restrictions were implemented in Australia during 1989-1990. Although restrictions have been in place for over a decade, there are still some “hot spots” of TBT contamination in marine environments and there continues to be evidence of its effects in marine organisms (Rees et al., 2001).
Dibutyltin (DBT) is the primary metabolite of TBT and is also used in the plastics industry as a stabiliser for polyvinylchloride (PVC) and other chlorine-containing materials (Boyer, 1989). It has also been used as a deworming agent in poultry, and hence poultry products have contained measurable levels of DBT (Epstein et al., 1991). These chemicals are not regarded as highly problematic within the Murray-Darling basin. However, this study investigated TBT and DBT as model immunotoxicants in Australian freshwater fish of the Murray-Darling basin, as they are both known for their immunotoxicity in mammals and aquatic organisms (Boyer, 1989).

**3.2: Structure and physical properties of TBT and DBT**

TBT compounds are organic derivatives of tetravalent tin. Specifically, TBT chloride (figure 3.1a) has the molecular formula of $\text{C}_{12}\text{H}_{27}\text{ClSn}$ (WHO, 1990). In pure water TBT chloride disassociates to form $\text{TBT}^+$, which behaves like a simple monoprotic acid and is highly lipophilic (Maguire, 1987; WHO, 1990). In this literature review, the term TBT is used for a range of TBT species that may occur in the aquatic environment, though most studies have involved the use of TBT chloride (TBTCl) or TBT oxide (TBTO) (Maguire, 1987).

DBT dichloride (figure 3.1b) has the molecular formula of $\text{C}_8\text{H}_{18}\text{Cl}_2\text{Sn}$, and is also lipophilic. In this literature review, the term DBT is used for a range of DBT species that may occur in the aquatic environment, however most studies have involved the use of DBT dichloride.
3.3: Mechanism of action of TBT and DBT

TBT toxicity occurs by several separate modes of action, including (but not limited to) alterations in energy production (uncoupling of oxidative phosphorylation), endocrine disruption, inhibition of cytochrome P450 systems, inhibition of Na⁺/K⁺/Ca²⁺ ATPase ion pumps and inhibition of heme metabolism (Meador, 2000).

TBT binds to the mitochondrial membrane of cells and produces an oligomycin-like inhibition of coupled respiration. Specifically, TBT passes into the outer mitochondrial membrane where it acts as a Cl⁻/OH⁻ exchanger. This results in a pH reduction within the mitochondria, which is necessary to drive the synthesis of ATP. The ultimate outcome is a reduced energy supply to the cell, which is displayed in the form of decreased DNA, RNA and protein synthesis (Boyer, 1989).

TBT also inhibits the action of the sarco-endoplasmic reticular Ca²⁺-ATPase-type pumps (SERCAs) and plasma membrane Ca²⁺-ATPase-type pumps (PMCAs). The SERCA pumps control cytosolic Ca²⁺ levels by translocating Ca²⁺ into the endoplasmic reticulum for storage.
and the PMCA pumps Ca\textsuperscript{2+} out of the cell to the extracellular fluids. The result of their inhibition is activation of Ca\textsuperscript{2+} influx pathways, leading to massive accumulation of cytosolic Ca\textsuperscript{2+} and subsequent death of the cell by apoptosis (Chow et al., 1992; Corsini et al., 1997; Kass and Orrenius, 1999). Several thymocyte studies have shown classic apoptotic events leading to the death of thymocytes and include (but are not limited to) an increase in intracellular Ca\textsuperscript{2+} concentration, cytochrome c release from mitochondria, caspase activation and chromatin condensation (Aw et al., 1990; Chow et al., 1992; Stridh et al., 1999; Yu et al., 2000).

The mechanism of action for endocrine disruption is not clearly understood, although the weight of evidence suggests that it occurs via two mechanisms i.e. 1) a neurotoxic action resulting in the abnormal release of neuropeptides controlling reproductive processes and/or 2) inhibition of enzymes that metabolise gonadotrophins (Oberdorster and McClellan-Green, 2002). Experimental animals exposed to TBT displayed an increase in endogenous levels of androgens and a decrease in estrogens levels, resulting in the masculinization of females and reproductive failure (LeBlanc and McLachlan, 2000; Vos et al., 2000).

### 3.4: The toxicokinetics of TBT and DBT

TBT has a relatively poor absorption from oral (20-50% depending on the vehicle) and dermal doses (i.e. 10%), however sufficient amounts can be absorbed to induce systemic toxicity in rats and rabbits (Krigman and Silverman, 1984). After exposure to TBT and DBT, the chemicals are rapidly and widely distributed to the peripheral compartments with the highest concentrations found in the kidney and liver (Boyer, 1989; WHO, 1990).

In mammals and fish, TBT is rapidly hydroxylated by cytochrome P450 dependent monooxygenase systems, which results in the successive removal of the butyl chains leaving
the products DBT and monobutyltin (MBT) (Krigman and Silverman, 1984). The rate of TBT elimination differs with different tissues and estimates for biological half-lives in mammals range from 23 to 30 d (WHO, 1990). The metabolites of DBT, MBT and inorganic tin are excreted mainly in the faeces, while up to 80% of the parent compound may also be excreted in the faeces following oral doses (Krigman and Silverman, 1984).

3.5: The mammalian toxicity data of TBT and DBT

3.5.1: Acute, subchronic and chronic data

TBT compounds are considered moderately toxic via both ingestion and dermal absorption. The reported LD$_{50}$ values range from 44-234 mg/kg for a single oral exposure in rats, mice and rabbits. Intraperitoneal exposure results in lower LD$_{50}$ values, which reflects the lower rates of absorption from oral and dermal routes. High acute doses have also been reported to cause alterations in blood lipid levels, the endocrine system, liver, spleen and transient deficits in brain development (WHO, 1990). Both chronic and subchronic studies have reported damage or changes to the endocrine glands, hormone and haemoglobin concentrations, spleen, thymus, liver and bile ducts (Boyer, 1989; Krajnc et al., 1984; Wester et al., 1990b). Due to a lack of human and animal data, regulatory bodies could not determine whether TBT and DBT were carcinogenic (USEPA, 1997). Davis and co-workers (1987) reported that there was no evidence of TBT-induced mutagenicity or genotoxicity in a range of test systems.

3.5.2: Endocrine disruptions

TBT clearly disrupts endocrine systems in mammals. Rats exposed to TBT showed an increased weight of the adrenal gland, focal necrosis in the thyroid gland and atrophy of adrenocorticotrophin hormone-producing cells (Funahashi et al., 1980). Additionally, there have been reports of decreases in levels of the metabolism controlling hormones such as
thyroid stimulating hormone and thyroxine and an increase in serum concentrations of the
gonadotrophin, luteinising hormone and the stress-related corticosterones, which are also
immunosuppressive (Krajnc et al., 1984; Wester et al., 1990b).

3.5.3: Immunotoxicity

Both TBT and DBT are well-documented immunotoxins and possess potent thymolytic
properties in rats. Immunotoxicity has been observed after an acute dose and with lower
subchronic and chronic doses, while rats were clearly the most sensitive mammalian species
tested (Boyer, 1989). TBT and DBT exposure caused atrophy of the thymus and of the
thymus-dependent areas in the spleen and lymph nodes (Funahashi et al., 1980; Krajnc et al.,
1984; Pieters et al., 1994a; Seinen and Willems, 1976). Both chemicals cause a suppression
of T cell mediated immune responses, which has been associated with a marked reduction of
rapidly proliferating immature thymoblasts (Pieters et al., 1994b; Snoeij et al., 1988).

In vitro studies by Raffray and Cohen (1991) demonstrated that thymocytes died as a result of
organotin-induced apoptosis, which specifically targeted immature thymocytes. Furthermore,
ex vivo studies supported this conclusion and showed that the mechanism was independent of
protein synthesis and did not require fully conserved cellular energetics (Raffray et al., 1993).
The exact mechanism of organotin-induced apoptosis has received much discussion in the
literature over the years (Pieters et al., 1994a). Recent findings indicate that the process
involves the inhibition of SERCAs and PMCAs pumps, which control intracellular levels of
Ca$^{2+}$ (see section 3.3) (Kass and Orrenius, 1999).

In addition to TBT and DBT’s thymolytic properties, in vitro studies have also shown that
TBT and DBT can inhibit the function of natural killer cells (NK), polymorphonuclear
leukocytes and neutrophils (Arakawa and Wada, 1984; Elferink et al., 1986; Vos et al., 1985;
Vos et al., 1984; Whalen et al., 1999). Moreover, it has been suggested that TBT’s inhibition
of NK tumour surveillance *in vivo* may be a non-genotoxic mechanism of carcinogenesis, as rats exposed to the organotin displayed an increase in neoplastic diseases (Vos *et al.*, 1984).

### 3.6: Sources of aquatic contamination by organotins

Although TBT has various uses, “hot spots” in the environment have been found in areas of high boating activity, indicating that TBT-based antifouling paints were the major source of environmental contamination (Clark *et al.*, 1988; Fent, 1996; Maguire, 1987). As TBT use escalated during the 1970s and 1980s, there were growing concerns that the new paints were having a detrimental effect on the marine environment and aquaculture industries, especially the oyster industry (Alzeiu and Heral, 1984). TBT and DBT may also enter the aquatic environment through sewage as leachate from PVC pipes and effluent from pulp mills, but the levels released were not significant compared to contamination from marine paints (Hoch, 2001).

Accurate estimation figures concerning the consumption and use of organotins is somewhat lacking, but the principle applications can be addressed. Fent (1996) estimated that the use of tri-substituted organotins for biocides was 8,000 tonnes/year, but the use of di- and mono-substituted derivatives as plastic stabilizers was much greater, at 27,000 tonnes/year. Production of TBT-based antifouling paints was estimated at approximately 4,000 tonnes in 1998 by the European Union (Greenpeace, 1999).

### 3.7: The fate of organotins in the aquatic environment

When dispersed into the water from antifouling paints, the exact structure of the TBT compound is thought to vary depending on the paint product. However, it is likely an equilibrium mixture of speciation products exists i.e. hydrated cations, chloride, carbonate and hydroxide (Clark *et al.*, 1988; Fent, 1996).
TBT is more dense than water and highly lipophilic (Meador, 2000). Consequently, it adsorbs strongly to particulate matter and is deposited in sediments or is found in the surface microlayer, but does not readily volatise from water surfaces (Gitlitz and Moran, 1984; Maguire, 1987; WHO, 1990). Once deposited in the sediment, TBT is available to marine organisms through the action of filter feeders or when resuspended into the water column (Langston and Burt, 1991).

Breaking of the Sn–C bond can occur by a number of different processes, including ultraviolet irradiation, biological cleavage, chemical cleavage, gamma irradiation and thermal cleavage. The two main processes that are likely to occur in the natural environment are chemical and biological cleavage (Blunden and Chapman, 1982). The half-life of TBT in aquatic environments has received considerable attention and markedly different results have been reported. Some studies have reported half-lives of 6 d – 6 weeks, whereas other studies have reported half–lives as long as 1-2 years. Measured TBT levels at marinas have shown a half-life of 60-90 d (at 5°C) (Matthiessen et al., 1995).

### 3.8: Environmental levels of organotins

Since TBT and DBT are effective at very low concentrations, problems have arisen in their accurate detection and measurement, especially at levels below 20 ng/L. Although analytical techniques have improved over the years, variations may occur in measurements between laboratories (Readman and Mee, 1991). In the 1980s, reports of the water concentrations of TBT in and nearby harbours and marinas ranged from 10 – 4000 ng/L (pptr) (Alzieu et al., 1991; Ritsema et al., 1991). Surveys of sediment contamination have found TBT concentrations in the range of several hundred parts per billion (i.e. ppb or μg/L) to low parts per million (ppm or mg/L) (Fent, 1996; Meador, 2000). Since the introduction of restrictions,
some reports have indicated that organotin levels have fallen (Waite et al., 1991). Nevertheless, TBT is still detectable in significant quantities in the sediment of many enclosed bays and marinas (Valkirs et al., 1991). In addition, imposex frequency in some areas has still not declined 10-15 years after the advent of regulations (Gibson and Wilson, 2003).

There have been very few studies investigating the contamination of TBT and DBT in Australian waters, especially in recent years. Most recently, a study of TBT contamination along coastal Western Australia reported high levels of TBT in marine sediment (i.e. 0.001 - 1.35 μg TBT/g) and mussel tissue (i.e. 0.003 – 0.32 μg TBT/g) that correlated with areas of high boating activity (Burt and Ebell, 1995). In 1993, a survey of organotin levels in Victorian coastal waters found TBT levels of 1.25, 0.212 and 0.803 ppb, and DBT levels of 0.092, 0.040 and 0.262 ppb in Port Phillip bay, Westernport bay and the Gippsland lakes, respectively (Daly and Fabris, 1993). In NSW, Sydney Harbour had levels of 0.220 ppb TBT and 0.051 ppb DBT and Georges River had lower levels of 0.1 ppb TBT and 0.04 ppb DBT (Bately et al., 1989). There are no studies reported in the literature concerning organotin contamination of the Murray-Darling Basin.

3.9: Regulation of TBT

The regulation of TBT in Australia and other developed nations began in the 1980s. The legislation introduced in Australia during 1989-1990 included a total ban on organotin-based paints for vessels less than 25 m in length, a ban on the retail sale of paints containing organotins, the introduction of a permit system for vessels greater than 25 m in length and a maximum leaching rate of 5 μg cm\(^{-2}\)day\(^{-1}\) (Gibson and Wilson, 2003). However, environmental levels of TBT still persist in the marine environment even after the
introduction of worldwide restrictions and further restrictions targeting large vessels are being introduced by some countries (Victorian EPA, 2000).

3.10: Organotin levels in aquatic organisms

Organotins can be readily bioaccumulated in fish due to their lipophilic nature. Food chain accumulation by a number of different aquatic species has been demonstrated (Boyer, 1989). Reported BCFs for fish range from 400 for rainbow trout (*Oncorhynchus mykiss*) (Martin et al., 1989) to 11,000 for red sea bream (*Pagrus major*) (Yamada and Takayanagi, 1992). Fish also biomagnify TBT but the degree of biomagnification may depend on the exposure concentration. Fish that were fed lower concentrations of TBT accumulated more than fish fed a higher concentration in their diet. This may also mean that laboratory measurements of the biomagnification and assimilation efficiency of TBT may underestimate actual field values, if highly contaminated feed is used (Ikeda and Yamada, 2003)

Importantly, it was discovered that salmon reared in sea pens coated with TBT had accumulated TBT levels of 0.28-0.90 μg TBT/g in their flesh. TBT was introduced into the market place in the USA, when it was discovered that the flesh of salmon sold to consumers had levels of 0.081-0.20 μg TBT/g (Short and Thrower, 1986). A survey of fish from market places in Australia, Papua New Guinea and Solomon Islands also detected TBT at low levels in tissue samples from fish available at markets (Kannan et al., 1995c).

3.11: The effects of TBT and DBT on aquatic organisms

The adverse effects of TBT on non-target species was first noticed during the 1970s, when commercial oysters (*Crassostrea gigas*) displayed signs of shell deformations and reduced population numbers (Alzeiu and Heral, 1984; Alzieu, 1991). Since then, reports have found that TBT is 10 – 100 times more acutely toxic to fish than DBT (de Vries et al., 1991; Wester
and Canton, 1987). The acute 96 hr LD$_{50}$ values for TBT have been reported for many freshwater fish and range from 6 to 24 μg/L. Aquatic invertebrates are more sensitive to TBT than fish, with reported LC$_{50}$ values below 5 μg/L (Maguire, 1987).

In addition to the acute lethal effects of TBT and DBT, many sublethal mechanisms of toxicity have been reported. Inhibition of cytochrome P450 metabolism (Fent and Stegeman, 1993), histopathological changes (Wester and Canton, 1987), endocrine disruption and reproductive inhibition (Vos et al., 2000), as well as immunotoxicity have all been reported in aquatic organisms.

### 3.11.1: Immunotoxicity

Numerous studies have reported TBT and DBT as immunotoxins in fish and our group has previously reported immunotoxicity in rainbow trout and in the Australian freshwater native silver perch (*Bidyanus bidyanus*) (O'Halloran et al., 1998b). As in mammals, TBT exposure has been reported to reduce host resistance and cause atrophy of fish thymuses.

*In vitro* studies using isolated spleen and head kidney cells from juvenile rainbow trout have shown that TBT and DBT suppress both LPS and ConA-stimulated lymphoproliferation at concentrations ≥50 ppb. Flow cytometric analysis showed dose-dependent changes in cell light-scattering properties, which correlated with the inhibition of mitogenesis. The results also indicated that DBT was the more potent immunotoxin of the two chemicals (O'Halloran et al., 1998b).

TBT can also either suppress or increase the phagocytic activity of fish macrophages depending on the dose given (as measured by chemiluminescence (CL)) (Rice and Weeks, 1989; Wishkovsky et al., 1989). In a series of *in vitro* and *in vivo* studies, these researchers...
found that TBT stimulated macrophage CL by inducing calcium influx into the cell, which in turn results in a cascade of events including an increase in phospholipase activity, followed by arachidonate release and an increase of lipoxygenase activity. These events cause a temporary rise in reactive oxygen species, which is observed as an increase in CL when fish are exposed to low concentrations of TBT (Rice and Weeks, 1991).

The immunotoxicity of in vivo TBT exposure in channel catfish (Ictalurus punctatus) and rainbow trout (Oncorhynchus mykiss) have been previously reported. Catfish dosed with 1.0 mg/kg TBT displayed a suppressed NK activity (at 3 and 7 d) and a suppressed phagocyte oxidative burst (3 d) after exposure. Haematological examination of the fish showed an increase in circulating monocytes and neutrophils, but there was a decrease in haematocrit values and lymphocytes. Additionally, humoral responses were suppressed in all groups dosed with 0.01, 0.1 or 1.0 mg/kg (Rice et al., 1995). In vivo studies with rainbow trout bathed in 4.0 μg/L for 28 d have reported a dose-related lymphocytic depletion, a marked proliferation of reticuloendothelial cells, increased erythrophagia in the spleen and severe lesions within epithelia of the gills and pseudobranch epithelial cells (Schwaiger et al., 1992).

Intensive field experiments involving a number of different chemicals linked TBT pollution with an increased prevalence of lymphocystis virus infections in the flounder (Platichthys flesus) along the Dutch coastline (Grinwis et al., 2000a). Experiments have also shown that TBT induces apoptosis in invertebrate haemocytes, in a similar manner to mammalian thymocytes (Cima and Ballarin, 1998). Cytoskeletal alterations of clam haemocytes induced by TBT led to significantly reduced amoebocytic, phagocytic and lysosomal activity (Cima et al., 1999).
3.12: Aim of the study

To date, there has been little ecotoxicological data reported on the Australian freshwater fish species investigated in this study. This is surprising considering the importance of these species in the economy and ecology of the Murray-Darling basin, especially in the case of the three large species. The three large fish species investigated in this study are widely disseminated within the basin, hold positions near the top of the aquatic food web, their numbers have declined dramatically since settlement and they have current commercial industries, which are growing.

The aim of this study was to use the organotins as model immunotoxicants to assess the suitability and efficiency of newly optimised immunotoxicity assays for the four freshwater native fish. Their reported immunotoxicity in other fish species, and the past experience within our research group at RMIT, makes the organotins excellent candidates for model immunotoxins for a number of reasons. Firstly, due to a high probability that immunosuppression will be found, it allows the testing of the assays to determine if they are working appropriately. Secondly, it allows for the assessment of the sensitivity of each immune assay, i.e. the most sensitive assay would be the one that can measure an altered immune system at lowest concentration. Finally, comparisons can be made with other species that have been tested in our laboratory (i.e. rainbow trout and silver perch) (O'Halloran, 1996). It is especially important to compare the results of native species with exotic species, such as rainbow trout, as the majority of the regulatory limits imposed in Australia are based on northern hemisphere species. Information obtained in these experiments also will also help determine the most suitable fish species for immuno-ecotoxicological testing of chemicals that enter the Murray-Darling basin.
3.13: Methods

3.13.1: Spleen and head kidney sampling

Native fish (i.e. rainbowfish, silver perch, golden perch and Murray cod) were housed and maintained as described in section 2.1. Fish were anaesthetised and killed as described in section 2.2. For the *in vivo* experiments, fish weight was recorded immediately prior to injection and then after the 14 d study period, before tissue sampling. The percentage of body weight gained or lost was calculated as: (pre-exposure – post-exposure weight) / pre-exposure weight x 100.

Immune tissues were sampled as described in section 2.3. Somatic indices were calculated as: (organ weight/body weight) x 100.

3.13.2: Cell isolation

The methods for the isolation of fish immune cells is described in detail in section 2.3. Briefly, cells were disrupted from the spleen and head kidney tissue and passed through a 250 μm nylon mesh. Red blood cells were separated from the cell suspension by density gradient centrifugation. The immune cells were washed twice, counted (with a haemocytometer in the presence of trypan blue) and diluted to the desired concentration. Cell yield was calculated as total number of cells collected / organ weight (g).

3.13.3: Exposure protocols

*In vitro exposures*

TBT (chloride), DBT (dichloride) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Concentrated stock solutions were dissolved in dimethyl sulfoxide (i.e. DMSO, BDH Chemicals, Poole, UK) for each concentration level and then diluted 1:100 in TCM for addition to the incubation as one quarter of the final incubation volume, resulting in a final
DMSO concentration of 0.25% v/v (i.e. 1:400 dilution). The final incubation concentrations of TBT and DBT tested for Murray cod were 0, 2.5, 50 and 500 μg/L (ppb). The final concentrations of TBT and DBT tested for rainbowfish, silver perch and golden perch were 0, 0.025, 0.25 and 2.5 mg/L (ppm).

**In vivo exposures**

Fresh stock solutions of TBT and DBT were diluted in PEG (polyethylene glycol) at 125 mg/mL and were kept at 4°C for no longer than 1 week. On the day of use, the stock was diluted 1:100 in PEG to give the high dose solution of 1.25 mg/mL. This was serially diluted 1:5 in PEG to attain the medium and low dose solutions of 0.25 and 0.05 mg/mL. Fish were dosed with 2 mL/kg, achieving exposures of 0.1, 0.5 and 2.5 mg/kg.

*In vivo* exposure studies were not conducted in silver perch because they would not acclimatise in exposure tanks, which resulted in an unacceptable frequency of mortalities in the control groups. Golden perch were not used for *in vivo* exposure studies because sufficient numbers were unavailable. At the commencement of the rainbowfish *in vivo* exposure study, 2 fish were each given a single i.p. injection of organotin at a high, medium or low concentration plus 2 fish were injected with the solvent and 2 fish were anaesthetised and weighed but not injected (i.e. untreated control). This was replicated on the next day to achieve a minimum sample size of 4 fish for each of the exposure and control groups (table 3.1). Although 10 fish were processed on each day, due to the small size of rainbowfish head kidneys and because the mitogenesis assay was not performed, the time taken to process the tissue was not significantly lengthened.
Table 3.1: Organotin in vivo exposure protocol for rainbowfish.

<table>
<thead>
<tr>
<th>Day of study</th>
<th>TBT Organotin treatment (mg/kg)</th>
<th>DBT Organotin treatment (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated control</td>
<td>0 (Solvent control)</td>
</tr>
<tr>
<td>Day 1-14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Day 2-15</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* One fish from the group survived for sampling

At the commencement of Murray cod in vivo exposure periods, three fish were given a single i.p. injection of organotin at a high, medium or low concentration, plus another fish was treated as either a solvent or untreated control (i.e. anaesthetised and weighed but not injected). This was replicated on at least 4 different days to achieve a minimum sample size of 4 fish for each of the exposure groups (table 3.2). This study design was necessary, as 4 fish was the maximum number that could be optimally processed on each day. Processing more than 4 fish in a day resulted in the need for extra centrifuge spins, delaying the time taken before the cells could be cultured, which would compromise immune function (particularly lymphocyte mitogenesis). For the organotin in vivo experiments, 2 extra fish were exposed to a lethal dose of TBT at 12.5 mg/kg (i.e. resulting in n=6) and 4 extra fish were exposed to a lethal dose of DBT at 2.5 mg/kg (i.e. resulting in n=8) (table 3.2).

Controls from the organotin and pesticide experiments (i.e. chapters 3-5) were combined resulting in a sample size of 7 (one fish died before sampling) for the solvent and 8 for the untreated control groups. Although combining controls from the organotin and pesticide in vivo exposure studies may have introduced a co-variant (i.e. different days of sampling), this variation was kept to a minimum as the conditions and procedures in the experiments did not change between sampling days. This assertion is also supported by the relatively low inter-day variation found in the control results for the various parameters measured (table 3.3). The
benefit of combining the control fish data was that the variance within the control group was reduced and the statistical power of the experiments was increased. On day 14 after exposure, the fish were sacrificed and immune assays were performed.

<table>
<thead>
<tr>
<th>Day of study</th>
<th>TBT</th>
<th>Organotin treatment (mg/kg)</th>
<th>DBT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>0 (Solvent control)</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Day 1-14</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 2-15</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 5-19</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 6-20</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Died before sampling. * One fish from the group survived for sampling

<table>
<thead>
<tr>
<th>Day of study</th>
<th>Untreated</th>
<th>DBT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0 (Solvent control)</td>
<td>0.1</td>
</tr>
<tr>
<td>Day 1-14</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 2-15</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 5-19</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 6-20</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3.3: Inter-day variation of immune parameters from Murray cod untreated controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometry</td>
<td>\</td>
</tr>
<tr>
<td>Phagocytosis (% (FITC +ve Granulocytes)</td>
<td>10.0%</td>
</tr>
<tr>
<td>Beads per cell (Mean Voltage)</td>
<td>20.7%</td>
</tr>
<tr>
<td>Granulocytes (Counts per 10,000 events)</td>
<td>4.9%</td>
</tr>
<tr>
<td>Lymphocytes (Counts per 10,000 events)</td>
<td>6.0%</td>
</tr>
<tr>
<td>Lysozyme activity</td>
<td>14.0%</td>
</tr>
<tr>
<td>Mitogenesis (Proliferation index)</td>
<td>8.2%</td>
</tr>
</tbody>
</table>
3.13.4: Phagocytosis

Standardised protocols for the phagocytosis assay are described in detail in section 2.4.2. Briefly, triplicate samples were assayed and the final concentration of head kidney cells in each assay was $1.0 \times 10^6$ cells/mL. The optimum cell:bead ratio was used for each species i.e. 1:12 (golden perch) or 1:25 (all other fish). This was achieved by adding FITC beads ($1 \times 10^9$ beads/mL) to the tubes at either 12 or 25 $\mu$L/mL of head kidney cell suspension (i.e. 1:12, 1:25, respectively). For the *in vitro* assays, cells were initially at a higher stock concentration (i.e. $1.33 \times 10^6$ cells/mL) and consequently 750 $\mu$L/mL of the cell suspension was added to the samples. The organotin intermediate stock solutions were then added at 250 $\mu$L/mL, resulting in a DMSO concentration of 0.25% v/v. Identical negative control tubes were prepared for all tests, however the beads were not added until immediately before flow cytometric analysis.

Cells were cultured under the optimum conditions for the species being tested, with tubes placed on ice at the end of the incubation period, prior to flow cytometry. Just before analysis (i.e. 2-5 min), propidium iodide (PI) at 1 $\mu$g/mL was added to each tube. 10,000 events were collected for each sample and gates were set up in regions representing granulocytes, lymphocytes and debris. Data was saved in listmode files for future analysis. The percentage of FITC +ve events of the total population (including debris events i.e. FITC+ve total) and in the granulocyte-gated region (i.e. FITC +ve granulocytes), as well as number of events in each gated region was expressed as a percentage of untreated control values.

3.13.5: Mitogenesis

Standardised protocols for the mitogenesis assay are described in detail in section 2.6.2.2. Briefly, triplicate samples were assayed and the final concentration of head kidney cells in every assay was $5.0 \times 10^5$ cells/mL in a final volume of 200 $\mu$L. Prior to cell addition, 100 $\mu$L of PHA was added to the wells of a 96 well plate at concentrations of 0, 2.5, 5 and 10 $\mu$g/mL.
For the *in vitro* assays, 50 μL of the treatments (or DMSO) were first added to the appropriate wells before 50 μL of cells were added at twice (i.e. 1x10^7 cells/mL) the stock concentration used in the *in vivo* assay (i.e. 5x10^6 cells/mL). For the *in vivo* assays, 100 μL of cells from the treated fish was added after the addition of PHA.

All plates were cultured under the optimum conditions for the species tested. Tritiated thymidine (1 μCi/well) was added to the wells 48 or 24 hr before harvesting, for Murray cod and all other species respectively. The cells in each well were harvested onto fibreglass discs using a semi-automatic cell harvester and the radioactivity (i.e. CPM) of each disc was analysed in a scintillation counter. Proliferation index was calculated as Peak CPM (i.e. 2.5 μg/mL PHA) / basal CPM (i.e. 0 PHA) and then expressed as percentage of the untreated control values.

### 3.13.6: Lysozyme

Standardised protocols for the lysozyme assay are described in detail in section 2.5.3. Briefly, all tests were performed in quadruplicate and 25 μL of fish serum was added to the wells of a flat-bottom 96 well plate. For the *in vitro* assays, 50 μL of the treatment or buffer was added to the serum and then 125 μL of the bacterial solution was added (i.e. 1.05 mg/mL at the optimum pH). For the *in vivo* assays, 175 μL of the bacterial solution (i.e. 0.75 mg/mL at the optimum pH) was added to the wells with serum. The plates were acclimatised to room temperature for 2 min, then the absorbance in each well was measured at 450 nm every 30 seconds for 10 mins on a DIAS plate reader. The data was collected using the Biolinx assay management software and saved on file for future analysis. Lysozyme activity was calculated from a hen egg white standard curve (i.e. 0 - 20 μg/mL) and results were then expressed as percentage of the untreated control.
3.13.7: Statistics and data presentation

Statistics were performed using the computer package SPSS 11.0 (SPSS Inc., Chicago, Illinois, USA) and the power analysis was conducted using Minitab 13.2 (Minitab Inc, Pennsylvania State University, PA, USA). A power study was conducted prior to the exposure studies, with $1-\beta=0.8$, $\alpha = 0.05$ and $\sigma=13$. The results showed that a $n$ of 4 was able to detect a significant difference of 25% and a $n$ of 6 was able to detect a significant difference of 20%.

Analyses were performed on raw data, which was first analysed for linear relationships and checked for normal distribution using standard error of skewness. Data was transformed if the distribution was not normal prior to ANOVA. Analysis of variance (i.e. one-way ANOVA) was performed and Tukey’s compromise post hoc test was conducted to determine homogenous subsets. Somatic index data was analysed using analysis of convariance (ANCOVA).

Values from the untreated control fish were used to standardise the data and results from the exposure groups are presented in the figures and tables as a percentage of control (i.e. untreated animals). The groups designated “0” dose represent the solvent controls, which are also expressed as a percentage of the untreated control. Analysis of untreated and solvent controls with a one-way ANOVA showed that these groups were not significantly different.

3.13.8: Environmental relevance and justification of doses

Due to their lipophilic nature, organotins tend to bioaccumulate in the tissues of aquatic organisms. BCFs have been reported in the range of between 400-11,000, although no figures are available for the species investigated in this study. Based on bioaccumulation studies in rainbow trout (Fent and Stegeman, 1993; Schwaiger et al., 1992), it was previously calculated
by our group that an *in vivo* exposure of 0.1, 0.5, 2.5 and 12.5 mg/kg represents a water borne TBT concentrations of 0.04, 0.2, 1 and 5 ppb respectively (O'Halloran, 1996). Concentrations of over 1 ppb TBT have been detected in Australian marine environments (Daly and Fabris, 1993), although levels in freshwater environments are generally lower i.e. 0.1 ppb (Bately *et al.*, 1989). The doses that were previously employed to test the immunotoxicity of TBT in rainbow trout were used in this study so that comparisons could be made between exotic and native species. The *in vitro* concentrations used in this study reflect the concentrations that have been found in the tissues of other species exposed to TBT, although the *in vitro* dose of 2.5 mg/L may be considered to be higher than an environmentally-relevant dose. Therefore, in the experiments involving Murray cod, the more appropriate levels of 2.5-500 μg/L were used, which were also the same concentrations previously investigated by our group in rainbow trout.

Intraperitoneal injection has been chosen as the route of administration by many researchers (Arkoosh *et al.*, 1994b; Lemaire-Gony *et al.*, 1995; Rice *et al.*, 1995; Spitsbergen *et al.*, 1986; Tahir and Secombes, 1995) because it ensures that fish receive the desired dose and avoids variation in uptake and assimilation encountered when attempting to administer doses via the water or diet. Furthermore, lower concentrations may be used and the toxicants are metabolised to some degree before excretion, which reduces the hazard of handling the chemicals and reduces the contamination of wastewater.
3.14: Results

3.14.1: The effect of \textit{in vitro} organotin exposure on the phagocytosis of head kidney cells from four Australian native fish

This study investigated the \textit{in vitro} effect of TBT and DBT on the subpopulations and the phagocytic activity of head kidney cells from four freshwater Australian fish (i.e. crimson-spotted rainbowfish, silver perch, golden perch and Murray cod) (figures 3.2 – 3.9). The results show that both TBT and DBT displayed some effects in all species, but between species there were some interesting differences in the tissues and functions targeted by the chemicals.

In the experiments using rainbowfish head kidney cells, granulocytes appeared to be the main targets of organotin toxicity (figures 3.2 and 3.3). Both TBT and DBT treatment caused a dose-dependent reduction in the number of granulocytes with beads (i.e. FITC +ve), reaching statistical significance at $\geq 0.25$ mg/L (figure 3.2a and 3.3a). Granulocytes treated with 0.025 mg/L DBT had decreased mean voltage by 40% compared to control, indicating an inhibition of phagocytosis that caused the granulocytes engaged in phagocytosis to have fewer beads per cell and therefore a lower mean voltage of FITC fluorescence (figure 3.3a). Counts in the granulocyte-gated region were reduced by 30% in samples treated with 0.25 mg/L TBT and DBT, and in samples treated with 2.5 mg/L granulocyte counts were significantly reduced by 90% by both organotins (figures 3.2b and 3.3b). The reduction of granulocytes in the samples also caused a decrease in cell concentration within the samples, which was noticeable in a lengthening of the amount of time to collect 10,000 events (data not shown).
**Figure 3.2:** The effect of *in vitro* TBT exposure on rainbowfish head kidney cells. a) Phagocytosis (i.e. FITC +ve granulocytes) and number of beads per cell (i.e. mean voltage) b) Granulocytes and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=6) and values represent mean ± se of 5-6 fish. Untreated control values were 20±3% for FITC +ve granulocytes, 8±1 volts for mean voltage, 3350±300 events for granulocytes and 3050±600 events for lymphocytes. * denotes significantly different from the solvent control (p<0.05).† denotes linear relationship (p<0.05).

**Figure 3.3:** The effect of *in vitro* DBT exposure on rainbowfish head kidney cells. a) Phagocytosis (i.e. FITC +ve granulocytes) and number of beads per cell (i.e. mean voltage) b) Granulocytes and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=6) and values represent mean ± se of 5-6 fish. Untreated control values were; 20±3% for FITC +ve granulocytes, 8±1 volts for mean voltage, 3350±300 events for granulocytes and 3050±600 events for lymphocytes. * denotes significantly different from the solvent control (p<0.05).
Silver perch head kidney cells were the least sensitive to organotin treatment, i.e. only their lymphocytes were reduced at the highest concentration of TBT and the medium concentration of DBT. There was no significant change in the phagocytic activity or mean voltage in any of the treated samples (figures 3.4 and 3.5). TBT at 2.5 mg/L caused a significant decrease in the number of lymphocytes, while DBT caused a dose-dependent reduction of lymphocytes that reach statistical significance at $\geq 0.25$ mg/L. Additionally, counts in the granulocyte-gated region were significantly reduced by 35% in samples exposed to 2.5 mg/L DBT.

Golden perch head kidney cells were the most sensitive of the species tested, i.e. they were affected at the lowest concentrations. Figure 3.6a shows that TBT treatment caused no significant change in the number of beads per cell but there was a dose-dependent reduction in the number of granulocytes with beads, which reached statistical significance at $\geq 0.25$ mg/L. DBT treatment caused a significant decrease in the number of granulocytes with beads at 0.25 mg/L, while 2.5 mg/L DBT cause a reduction of 40% of control but this did not reach statistical significance due to some variation in the data (figure 3.7a). There was no significant change in the mean voltage of DBT treated FITC +ve granulocytes but there was a significant increase in mean voltage of granulocyte gated region from the 2.5 mg/L TBT treatment (figures 3.6a and 3.7a).

TBT and DBT treatment resulted in a dose-dependent reduction in the number of lymphocytes. This reached statistical significance at $\geq 0.25$ mg/L for TBT and $\geq 0.025$ mg/L for DBT treated samples (figures 3.6b and 3.7b). Granulocytes were significantly reduced by both organotins at doses of 2.5 mg/L but were unaffected by doses of 0.25 mg/L (figures 3.6b and 3.7b).
Figure 3.4: The effect of *in vitro* TBT exposure on silver perch head kidney cells. a) Phagocytosis (i.e. FITC +ve) and number of beads per cell (i.e. mean voltage) b) granulocyte and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated control values were; 37±2% for FITC +ve granulocytes; 216±31 volts for mean voltage; 2000±150 events for granulocytes and 1400±150 events for lymphocytes. * denotes significantly different from the solvent control (p<0.05).

Figure 3.5: The effect of *in vitro* DBT exposure on silver perch head kidney cells. a) Phagocytosis (i.e. FITC +ve) and number of beads per cell (i.e. mean voltage) b) granulocyte and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated control values were; 37±2% for FITC +ve granulocytes; 216±31 volts for mean voltage; 2000±150 events for granulocytes and 1400±150 events for lymphocytes. * denotes significantly different from the solvent control (p<0.05). † denotes linear relationship (p<0.05).
Figure 3.6: The effect of *in vitro* TBT exposure on golden perch head kidney cells. a) Phagocytosis (FITC +ve) and number of beads per cell (mean voltage) b) granulocyte and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=7) and values represent mean ± se of 7 fish. Untreated control values were; 17±2% for FITC +ve granulocytes; 31±3 volts for mean voltage; 4100±250 events for granulocytes and 2200±250 events for lymphocytes. * denotes significantly different from the solvent control (p<0.05).† denotes linear relationship (p<0.05).

Figure 3.7: The effect of *in vitro* DBT exposure on golden perch head kidney cells. a) Phagocytosis (FITC +ve) and number of beads per cell (mean voltage) b) granulocyte and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=7) and values represent mean ± se of 7 fish. Untreated control values were; 17±2% for FITC +ve granulocytes; 31±3 volts for mean voltage; 4100±250 events for granulocytes and 2200±250 events for lymphocytes. * denotes significantly different from the solvent control (p<0.05).† denotes linear relationship (p<0.05).
Both organotins at 500 μg/L significantly suppressed the number of Murray cod head kidney granulocytes with beads. This occurred in a dose-dependent manner for DBT (figure 3.9a), but for TBT there appeared to be a slight elevation in the number of granulocytes with beads at 50 μg/L (i.e. FITC +ve granulocytes) before a significant reduction in the 500 μg/L TBT-treated group (figure 3.8a). The mean voltage of FITC +ve granulocytes appeared to be increased by 50 and 500 μg/L TBT, but there was some variation between fish and this was not statistically significant. DBT treatment at 50 μg/L significantly increased mean voltage before reducing it to 34% of control levels at 500 μg/L. Murray cod head kidney subpopulations appeared to be of similar sensitivity to both organotins i.e. 500 μg/L significantly reduced their numbers by similar levels, although lymphocytes appeared to be reduced by a slightly greater amount at 50 μg/L, suggesting that they may be more sensitive than granulocytes (figure 3.8b and 3.9b).
Figure 3.8: The effect of *in vitro* TBT exposure on Murray cod head kidney cells. a) Phagocytosis (i.e. FITC +ve granulocytes) and number of beads per cell (i.e. mean voltage) b) Granulocytes and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated control values were; 21±2% for FITC +ve granulocytes; 108±9 volts for mean voltage; 6900±300 events for granulocytes and 1400±100 events for lymphocytes. * denotes significantly different from the solvent control (p<0.05).

Figure 3.9: The effect of *in vitro* DBT exposure on Murray cod head kidney cells. a) Phagocytosis and number of beads per cell b) Granulocytes and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated control values were; 21±2% for FITC +ve granulocytes; 108±9 volts for mean voltage; 6900±300 events for granulocytes and 1400±100 for lymphocytes. * denotes significantly different from the solvent control (p<0.05). † denotes linear relationship (p<0.05).
3.14.2: The effect of *in vitro* organotin exposures on Murray cod immune parameters

**Mitogenesis**

At all of the TBT concentrations tested, there was a reduction in both the basal and PHA-stimulated CPM of Murray cod head kidney lymphocytes (figure 3.10). At 500 $\mu$g/L there was a significant reduction in the basal and PHA-stimulated incorporation of tritiated-thymidine. However, due to a large variation no statistically significant differences were found in the proliferation indices from the fish exposed to TBT. Cell cultures exposed to 2.5 $\mu$g/L DBT had a very similar profile to control wells, while 50 $\mu$g/L appeared to increase mitogenesis (figure 3.11). Treatment with 500 $\mu$g/L DBT caused a significant reduction in basal and PHA-stimulated incorporation of tritiated-thymidine, which was similar to the reduction caused by TBT. Both TBT and DBT exposures, did not significantly reduce the proliferation index of Murray cod head kidney lymphocytes.

![Graphs of lymphoproliferation profiles and proliferation index of Murray cod head kidney cells](image)

**Figure 3.10:** The effect of *in vitro* TBT on the a) lymphoproliferation profiles and b) the proliferation index of Murray cod head kidney cells. Proliferation data is expressed as percentage of untreated control (i.e. $1.29 \pm 0.11$, n=4) and values represent the mean ± se of 4 fish. * denotes significantly different from the solvent control (p<0.05).
Figure 3.11: The effect of *in vitro* DBT on the a) lymphoproliferation profiles and b) the proliferation index of Murray cod head kidney cells. Proliferation data is expressed as percentage of untreated control (i.e. 1.29±0.11, n=4) and values represent the mean ± se of 4 fish. * denotes significantly different from the solvent control (p<0.05).


**Body and organ weights**

There was a single death in the group that received a single i.p. injection of 2.5 mg/kg TBT. Most rainbowfish in this experiment lost some body weight during the study period and there was a large amount of variation in this parameter (table 3.4). Although there was no significant difference between the exposure groups and the solvent control group, the rainbowfish exposed to 2.5 mg/kg lost an average of 10% body weight, which was much greater than fish exposed to the lower concentrations of TBT.

Head kidney somatic indices were not significantly different in the rainbowfish dosed with TBT, however a large amount of variation was seen within these groups (table 3.4).
There was a large amount of variation in head kidney cell yields, with the exception of the solvent dosed group (table 3.4). Consequently, no significant difference in the head kidney cell yields of these groups was evident.

Table 3.4: The effect of in vivo organotin exposure on rainbowfish body and organ weights.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Organotin concentration (mg/kg)</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in body weight (%)</td>
<td></td>
<td>-6.1±1.4</td>
<td>-0.4±1.5</td>
<td>-2.9±0.7</td>
<td>-10.2±3.0</td>
</tr>
<tr>
<td>Head kidney Somatic index</td>
<td></td>
<td>0.30±0.04</td>
<td>0.29±0.10</td>
<td>0.64±0.23</td>
<td>0.35±0.04</td>
</tr>
<tr>
<td>Head kidney Cell yield a</td>
<td></td>
<td>1170±59</td>
<td>2049±874</td>
<td>788±270</td>
<td>1388±283</td>
</tr>
</tbody>
</table>

*a Cell yield data is expressed cells/g of head kidney tissue. Values represent the mean ± se of 4 fish (except 2.5 mg/kg, n=3).

Phagocytosis

TBT exposure caused a dose-dependent reduction in the percentage of granulocytes with beads, statistical significance being reached at ≥0.5 mg/kg. There was no significant difference in the number of beads per granulocyte, although there appeared to be a 20% increase at the highest dose (figure 3.12a). There was no change in the number of granulocytes in any of the TBT treated groups and there was very little variation between fish. Conversely, TBT exposure appeared to reduce the number lymphocytes in all groups, however there was also a large variation between individuals, which meant the groups were not statistically different (figure 3.12b).
Figure 3.12: The effect of in vivo TBT exposure on rainbowfish head kidney cells. a) Phagocytosis (i.e. FITC +ve granulocytes) and number of beads per cell (i.e. mean voltage) b) granulocyte and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish (except 2.5 mg/kg, n=3). Control values were 18±2% FITC +ve granulocytes, 27±2 volts for mean voltage, 6750±350 events for granulocytes and 1200±200 events for lymphocytes. * denotes significantly different from the solvent control (p<0.05). † denotes linear relationship (p<0.05).

3.14.4: The effect of in vivo organotin exposure on Murray cod immune parameters

Body and organ weights

A single i.p. injection of TBT and DBT at 12.5 mg/kg (i.e. 52.5 and 63.1 mmol/kg, respectively) was lethal to the Murray cod, with all fish dying within 96 hr for TBT (n=6), while the single fish exposed to DBT at this dose died within 120 hr. More surprisingly, only 2 of 8 fish exposed to DBT at 2.5 mg/kg (i.e. 12.62 mmol/kg) survived the study period, whereas all fish exposed to TBT at this concentration survived (table 3.5). This result is contrary to the major of findings in other species, which have shown TBT to be the more acutely toxic of the two organotins (de Vries et al., 1991; Wester and Canton, 1987). This result indicates that Murray cod are more sensitive to organotins than rainbow trout, which survived a dose of 12.5 mg/kg TBT (O'Halloran, 1996). Murray cod that received a single i.p.
injection of TBT and DBT did not significantly change in body weight compared to controls over the study period (table 3.5).

*In vivo* organotin exposure did not significantly change the somatic indices of Murray cod spleens or head kidneys (table 3.5). The 2 fish that survived the 2.5 mg/kg DBT dose appeared to have reduced splenic and head kidney somatic indices, but there were insufficient survivors for this to reach statistical significance.

Murray cod dosed with 0.1 and 0.5 mg/kg TBT had significantly lower head kidney cell yields compared to the solvent control and fish dosed with 2.5 mg/kg had significantly lower splenic cell yields (table 3.5). Exposure to DBT had no significant effect on the cell yield of either the head kidney or the spleen, although the 2 Murray cod that survived the 2.5 mg/kg injections appeared to have lower splenic cell yields (table 3.6).
Table 3.5: The effect of *in vivo* organotin exposure on Murray cod body and organ weights.

<table>
<thead>
<tr>
<th>Chemical &amp; Parameter</th>
<th>Organotin concentration (mg/kg)</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>2.5</th>
<th>12.5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mortality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBT</td>
<td></td>
<td>0/7 fish, 0%</td>
<td>0/4 fish, 0%</td>
<td>0/4 fish, 0%</td>
<td>0/4 fish, 0%</td>
<td>6/6 fish, 100%</td>
</tr>
<tr>
<td>DBT</td>
<td></td>
<td>0/7 fish, 0%</td>
<td>0/4 fish, 0%</td>
<td>0/4 fish, 0%</td>
<td>6/8 fish, 75%</td>
<td>1/1 fish, 100%</td>
</tr>
<tr>
<td><strong>Change in body weight (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBT</td>
<td></td>
<td>-2.63±2.3</td>
<td>1.38±2.0</td>
<td>3.67±3.1</td>
<td>1.56±2.2</td>
<td></td>
</tr>
<tr>
<td>DBT</td>
<td></td>
<td>-3.02±1.9</td>
<td>-0.67±3.3</td>
<td>-4.67±3.1</td>
<td>-4.83±0.2</td>
<td></td>
</tr>
<tr>
<td><strong>Spleen Somatic index</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TBT</td>
<td></td>
<td>0.057±0.014</td>
<td>0.069±0.012</td>
<td>0.052±0.008</td>
<td>0.048±0.005</td>
<td></td>
</tr>
<tr>
<td>DBT</td>
<td></td>
<td>0.073±0.014</td>
<td>0.057±0.010</td>
<td>0.064±0.010</td>
<td>0.037±0.008</td>
<td></td>
</tr>
<tr>
<td><strong>Head kidney Somatic index</strong></td>
<td></td>
<td></td>
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<tr>
<td>TBT</td>
<td></td>
<td>0.274±0.037</td>
<td>0.284±0.025</td>
<td>0.207±0.019</td>
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<tr>
<td>DBT</td>
<td></td>
<td>0.259±0.034</td>
<td>0.233±0.021</td>
<td>0.230±0.031</td>
<td>0.195±0.023</td>
<td></td>
</tr>
<tr>
<td><strong>Spleen Cell yield a</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBT</td>
<td></td>
<td>932±185</td>
<td>812±118</td>
<td>1173±145</td>
<td>278±148*</td>
<td></td>
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<tr>
<td>DBT</td>
<td></td>
<td>892±156</td>
<td>1787±544</td>
<td>1549±407</td>
<td>413±207</td>
<td></td>
</tr>
<tr>
<td><strong>Head kidney Cell yield a</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBT</td>
<td></td>
<td>630±31</td>
<td>292±24*</td>
<td>310±72*</td>
<td>561±108</td>
<td></td>
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<tr>
<td>DBT</td>
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<td>727±100</td>
<td>535±52</td>
<td>517±140</td>
<td>754±333</td>
<td></td>
</tr>
</tbody>
</table>

*a* Cell yield data is expressed cells/g of head kidney tissue. Values represent the mean ± se of 4-7 fish (except DBT 2.5 mg/kg n=2). * denotes significantly different from the solvent control (p<0.05).

**Lysozyme**

Serum lysozyme activity was not significantly different from controls after an *in vivo* dose of TBT or DBT (figure 3.13). Compared to the control, Murray cod receiving 0.1 mg/kg of the organotins appeared to have slightly elevated lysozyme activity and the fish that received 2.5 mg/kg appeared to have lower lysozyme activity. The groups receiving 0.1 and 2.5 mg/kg DBT were statistically different from each other (figure 3.13).
Figure 3.13: The effect of *in vivo* organotin exposure on Murray cod serum lysozyme activity. Data is expressed as percentage of untreated control lysozyme activity (i.e. 7.12±1.02 μg/mL, n=7, one sample was lost), calculated from a hen egg white lysozyme standard curve. Values represent the mean ± se of 4-6 fish (except DBT 2.5 mg/kg, n=2, one solvent control sample was lost). Letters denote homogenous subsets (p<0.05).

**Mitogenesis**

Murray cod head kidney cells from fish dosed with 2.5 mg/kg TBT had a significantly lower proliferation index compared to the solvent control, however this was caused by an increase in basal proliferation rather than a decrease in peak proliferation (figure 3.14). Fish exposed to 0.5 mg/kg also had a slightly higher basal CPM, resulting in a lower proliferation index, while those dosed with 0.1 mg/kg had a lower peak CPM. The reason for this pattern is unclear, however these results indicate that TBT is disrupting the normal mitogenesis of Murray cod head kidney lymphocytes *in vivo* (figure 3.14). Exposure to 0.1 and 0.5 mg/kg DBT did not result in any significant changes in the proliferation index or the basal and PHA-stimulated incorporation of tritiated-thymidine into Murray cod head kidney lymphocytes (figure 3.15). There was some variation in the 0.5 mg/kg group that was caused by one fish with a proliferation index 171% of the control, while the other 3 fish had proliferation indices of 77-88% of the control. The two fish that survived the 2.5 mg/kg dose had very low proliferation indices, as their basal CPM was higher than their PHA-stimulated CPM. Unfortunately, there were an inadequate number of survivors in this group to reach statistical significance and
more importantly, it has been estimated that 2.5 mg DBT/kg is over the LD₅₀ for Murray cod (figure 3.15).

**Phagocytosis**

*In vivo* exposure to TBT resulted in some changes in the phagocytic activity and subpopulation numbers of Murray cod head kidney cells (figure 3.16). Fish exposed to 0.1 and 0.5 mg/kg TBT had an increased percentage of granulocytes with beads, which was statistically significant at 0.5 mg/kg (figure 3.16a). Although the mean voltage was not significantly different from controls, there was a significant rise in the granularity (i.e. SS increase) of Murray cod head kidney granulocytes in fish dosed with 0.1 and 0.5 mg/kg (figure 3.16b). This indicates that granulocytes in these exposed fish have more particles within their cytoplasm but they are unlikely to be beads as mean voltage data did not significantly change. Both SS and FITC +ve events returned to control levels in fish dosed with 2.5 mg/kg. There was no significant change in the number of events in the debris-gated region, which reflects a uniform cell isolation technique between the sample groups (figure 3.16d). There was also no significant change in the number of non-viable cells, although in the 0.1 and 0.5 mg/kg groups there were less than 80% of control. The reason for this is unclear but may reflect variation in staining by PI (figure 3.16d).
Figure 3.14: The effect of \textit{in vivo} TBT exposure on a) the lymphoproliferation profile and b) proliferative index of Murray cod head kidney cells. Proliferation is expressed as percentage of untreated control (i.e. 1.46±0.12, n=8) and values represent the mean ± se of 4-7 fish. * denotes significantly different from the solvent control (p<0.05).

Figure 3.15: The effect of \textit{in vivo} DBT exposure on a) the lymphoproliferation profile and b) proliferative index of Murray cod head kidney cells. Proliferation is expressed as percentage of untreated control (i.e. 1.46±0.12, n=8) and values represent the mean ± se of 4-7 fish (except DBT 2.5 mg/kg, n=2). No statistically significant differences were found.
Figure 3.16: Murray cod head kidney phagocytic function and subpopulation counts following in vivo exposure to TBT. a) Phagocytosis (i.e. FITC +ve), b) number of beads per cell (i.e. mean voltage and side scatter (SS) increase), c) granulocytes and lymphocyte subpopulation counts, d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control samples (n=8) and values represent mean ± se of 4-7 fish. Untreated control values were; 10±1% for FITC +ve total; 25±4% for FITC +ve granulocytes; 130±27 volts for mean voltage; 123±14 volts for SS increase; 4050±200 events for granulocytes; 3350±200 events for lymphocytes; 1600±150 for debris and 800±150 for PI +ve. * denotes significantly different from the solvent control (p<0.05). † denotes linear relationship (p<0.05).
Murray cod that were dosed with 2.5 mg/kg TBT displayed a significant reduction in the number of head kidney lymphocytes (figure 3.16c). Concomitantly, there was a rise in granulocyte counts, as the data are comprised of a proportional count of 10,000 events from the head kidney. This rise was not statistically significant, but was expected as any specific in vivo toxicity towards one subpopulation means that more cells of another subpopulation would be counted in the 10,000 event sample. Cell yield data also helps confirm there was no rise in cell numbers within the head kidney. The head kidney cell yields were significantly lower for fish in the 0.1 and 0.5 mg/kg group and splenic cell yields were significantly lower in the 2.5 mg/kg group, thus it can be concluded that these are due to the reduction in lymphocyte populations.

DBT showed clear signs of immunotoxic action in the head kidney cells of exposed Murray cod (figure 3.17). There was a dose-dependent reduction in the number of lymphocytes in Murray cod head kidneys that was statistically different from the control at 2.5 mg/kg. There was also a concomitant dose-dependent increase in granulocytes, which reached statistical significance at 0.5 mg/kg (figure 3.17c). This rise in granulocyte numbers was similar to the one observed in the TBT experiments and, as explained previously, is caused by a specific toxicity towards the lymphocyte subpopulation of the head kidney.

In vivo DBT exposure also resulted in a dose-dependent decrease in the number of granulocytes with beads, which reached statistical significance at 2.5 mg/kg (figure 3.17a). This may indicate that DBT exposure is inhibiting the phagocytosis of Murray cod granulocytes, however it must also be considered that there was a concomitant rise in the number of granulocytes (figure 3.17c). There was also a significance increase in mean voltage in fish exposed to 2.5 mg/kg DBT. This indicates that of the lower number of FITC +ve granulocytes in the sample, a higher intensity of fluorescence is emitted due to a larger
number of beads being held together within each cell (figure 3.17b). This suggests that the cells which are least affected by DBT exposure are the more robust cells, which are also more active in phagocytosis. There was no significant difference in the number of PI +ve events (i.e. non-viable cells), however there was large variation between fish. Conversely, there were significantly lower numbers of debris-gated events in the highest dosed group. This result probably reflects the fact that there was more granulocyte in those samples, which are more robust and survive the incubation period better than lymphocytes (figure 3.17d).
Figure 3.17: Murray cod head kidney phagocytic function and subpopulation counts following *in vivo* exposure to DBT. a) Phagocytosis (FITC +ve), b) number of beads per cell (i.e. mean voltage and side scatter (SS) increase), c) Granulocytes and lymphocyte subpopulation counts, d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control samples (n=8) and values represent mean ± se of 4-7 fish (except DBT 2.5 mg/kg, n=2). Untreated control values were; 10±2% for FITC +ve total; 25±4% for FITC +ve granulocytes; 130±27 volts for mean voltage; 123±14 volts for SS increase; 4050±200 events for granulocytes; 3350±200 events for lymphocytes; 1600±150 events for debris and 800±150 for PI +ve. * denotes significantly different from the solvent control (p<0.05). † denotes linear relationship (p<0.05).
3.15: Discussion

The findings of the present study are that TBT and DBT are immunotoxic in the four Australian native fish crimson-spotted rainbowfish, silver perch, golden perch and Murray cod. The results indicated that the lymphocyte population was the main target of organotin exposure, however granulocytes and their phagocytic function were also affected.

The flow cytometric assay for phagocytosis demonstrated that in vitro exposure to TBT and DBT resulted in a marked reduction in lymphocyte populations in all species except rainbowfish (figures 3.2-3.9). In rainbowfish, granulocytes were reduced and there was a concomitant increase in lymphocyte counts (figures 3.2 and 3.3). This result may reflect differences in species, however other factors must also be considered. Firstly, the rainbowfish were of a markedly older age despite being in excellent health. The rainbowfish used in these experiments were two year-old ex-breeding stocks obtained from the Department of Biology and Environmental Biology, at RMIT and as such, they were considerably older compared to the other species, which were approximately one year old. In addition, there were numerous technical difficulties associated with the use of small fish and obtaining cell suspensions from such small immune organs. Sterile cultures were much harder to achieve when working with rainbowfish and this was one of reasons that the mitogenesis assay was unsuccessful in this species. Although rainbowfish cultures were visibly free from contamination after the incubation period, it was considered that a low level of microbial presence might influence the assay and stimulate the lymphocytes within the samples. When using rainbowfish head kidney cells, complications in the flow cytometry assay were encountered when enumerating dead cells within the “debris” region due to their particularly small size and proximity to background noise events and unengulfed beads, which are omitted from the counts. Furthermore, samples dosed with high concentrations of organotins had lower cell concentrations at the end of the incubation period, which led to a lengthening of sampling
time. This indicates that not all dead cells appeared in the “debris” gate region and that extreme toxicity may cause many dead cells to be “gated-out”. It is likely that this reduction in cell concentration and difficulties in enumerating debris resulted in the increase of the lymphocyte counts. Nonetheless, the rainbowfish lymphocytes were surprisingly robust in this assay and did not change their light-scattering profile, even at the highest dose. Other conceivable possibilities for the increase in lymphocyte counts could be due to; (a) an organotin-induced stimulation of lymphoproliferation, although this would seem very unlikely; (b) contamination of the lymphocyte-gated region by dead granulocytes, but this would also mean that these cells had decreased their SS characteristics, which is unlikely as dead cells, especially apoptotic cells, usually increase their side-scattering of light due to events such as DNA fragmentation; (c) bacterial contamination of the samples may have occurred but in the in vitro experiments this would have affected all samples equally. Nevertheless, the significant reduction in the number of granulocytes in rainbowfish at lower organotin concentrations was also observed at the highest organotin dose in Murray cod and golden perch. In addition, there were also significant reductions in the number of granulocytes with beads at 0.5 mg/L organotin for Murray cod and at 0.25 mg/L organotin for golden perch.

The increase in mean voltage in rainbowfish samples treated with the organotins at ≥0.25 mg/L is a phenomena that occurred in other assays with reduced counts in the granulocyte-gated region and indicates that either the granulocytes with a greater number of beads are more robust than those with fewer beads, or there was an increase in background counts of bead clumps that fluoresce at a higher intensity (figures 3.2 and 3.3). Alternatively, the lipophilic organotins may be affecting membrane integrity resulting in a non-specific uptake of beads. It should also be kept in mind that the mean voltage of control samples was the average fluorescence intensity of 3000 events, whereas the mean voltage of the 2.5 mg/L
treated group is the average fluorescence intensity of only 400 events. Mean voltage was most useful in reporting inhibition of phagocytosis in situations when toxicity did not occur.

In summary, environmentally relevant in vitro doses of TBT and DBT caused suppression of phagocytic functions and toxicity of immune cell subpopulations in the four Australian freshwater fish. Additionally, there were some interesting differences between species and specific toxicities directed towards certain immunocyte subpopulations. In general, lymphocytes appeared to be the more susceptible subpopulation, but Murray cod granulocytes appeared to be almost as sensitive, while rainbowfish lymphocytes were surprisingly robust. In all species except silver perch, the number of FITC +ve granulocytes was also suppressed, suggesting that TBT and DBT can directly target granulocyte phagocytosis.

Overall, golden perch was the most sensitive species to in vitro DBT exposure, with a significant reduction in lymphocytes occurring with exposures to 0.025 mg/L (figure 3.7) and a significant reduction in the number of granulocytes engulfing beads with exposure to 0.25 mg/L DBT (figure 3.8). It was also the most sensitive species to TBT exposure with a significant reduction in lymphocytes and granulocytes with beads at 0.25 mg/L (figure 3.7). It should be noted that more fish (i.e. n=7) were used in these experiments, adding to the statistical power of this experiment.

Rainbowfish appeared more sensitive to TBT than Murray cod, while Murray cod appeared more sensitive to DBT. Rainbowfish were the most sensitive of the species tested in relation to effects of TBT on granulocyte numbers and function with suppression of phagocytosis at 0.25 mg/L TBT, compared to 0.5 mg/L TBT in Murray cod leukocytes (figure 3.2). Conversely, the phagocytic activity of Murray cod head kidney leukocytes showed a significant reduction at 0.5 mg/L DBT (figure 3.9), while rainbowfish showed a significant
decrease in the percentage of granulocytes with beads only at the highest DBT dose of 2.5 mg/L (figure 3.3). Of the species tested, silver perch were the least sensitive to TBT, with their granulocytes showing no change in numbers or activity at the highest dose (figure 3.4). However, their lymphocytes were significantly reduced by DBT at 0.25 mg/L, and by TBT at 2.5 mg/L (figures 3.4 and 3.5). The order of sensitivity to TBT was golden perch > rainbowfish > Murray cod > silver perch, while for DBT the order of sensitivity was golden perch > Murray cod > rainbowfish > silver perch. DBT appeared to be more toxic to lymphocytes than TBT, which has been previously reported in other fish (i.e. rainbow trout and silver perch) (O'Halloran et al., 1998b) and in mammalian studies (Snoeij et al., 1987).

Murray cod serum lysozyme was unaffected by in vitro exposures to the organotins but the lymphoproliferation of head kidney lymphocytes was significantly reduced by both TBT and DBT with in vitro exposures of 500 μg/L (figure 3.10 and 3.11). Previous reports from our group have shown that in vitro doses of TBT and DBT reduced the PHA-stimulated proliferation of head kidney lymphocytes isolated from both rainbow trout and silver perch (O'Halloran et al., 1997; O'Halloran et al., 1998b). In those experiments, 50 μg/L TBT (but not DBT) significantly reduced proliferation in the head kidney, whereas 50 μg/L DBT significantly inhibited proliferation in spleen derived lymphocytes (O'Halloran et al., 1997; O'Halloran et al., 1998b). Murray cod head kidney lymphocytes may be less sensitive to in vitro exposures of TBT. However, it should be noted that the mitogen-induced proliferative responses observed in the aforementioned rainbow trout and silver perch assays was higher than in the studies reported here, which may allow for a greater sensitivity in toxicant-induced lymphosuppression.

A number of technical problems were encountered with the in vivo exposure of crimson-spotted rainbowfish due to the small size of the fish. The primary difficulty was the lack of
immune cells and serum that could be obtained from a fish. Sterile removal of head kidneys was also more difficult to perform in this species, due to the close proximity of the oesophagus. If the oesophageus was inadvertently cut during the procedure, the risk of bacterial contamination from the digestive tract flora was raised. The amount of serum that could be collected from the rainbowfish was limited (30-50 μL maximum) and it also had a tendency to coagulate, causing the loss of many samples and thus, the lysozyme assay was not completed in this species. The mitogenesis assay was also unsuccessful in this species because it required a larger number of cells compared to the phagocytosis assay, and the pooling of cells from different animals compounded the risk of contamination of the tissue cultures. The phagocytosis assay was successful because comparatively few cells were needed and the pooling of cells was unnecessary, while incubation times were shorter, reducing the chance of contamination.

TBT and DBT were quite toxic to rainbowfish with a dose of 12.5 mg/kg being lethal, while one fish also died when dosed with 2.5 mg/kg TBT group (table 3.4). Therefore, it was concluded that the rainbowfish LD₅₀ for an i.p. dose of TBT and DBT is between 2.5 and 12.5 mg/kg. It also indicates than TBT is more toxic to rainbowfish than DBT, as previously reported in studies involving other species (de Vries et al., 1991; Wester and Canton, 1987). In vivo exposure to ≥0.5 mg/kg TBT caused a reduction in the number of rainbowfish head kidney granulocytes engulfing beads. There also appeared to be a marked reduction in the number of lymphocytes, especially in the group dosed with 0.5 mg/kg, where lymphocytes counts were approximately 50% of control levels (figure 3.12). A reduction in lymphocyte numbers has been previously reported following in vivo TBT exposure in catfish (Rice et al., 1995) and rainbow trout (Schwaiger et al., 1992). Rice et al. (1995) also reported that in vivo TBT exposure can reduce the oxidative burst of catfish macrophages. The present study supports this finding that TBT can also inhibit the function of fish phagocytes.
In comparison to rainbowfish, Murray cod were much easier to work with in these experiments. They are a sizable fish that will accept a simple pellet meal, acclimatise well individually in tanks and provide an excess of immune cells and serum for experiments. They are also the top predator of the Murray-Darling basin and are becoming more available from fish farmers due to an increase in their aquaculture. Because of these qualities, they are an ideal species for ecotoxicological assessment of chemicals that are released into Australian freshwater environments.

Murray cod that were dosed with 12.5 mg/kg (52.5 mmol/kg) TBT all died within 96 hr of injection and the 1 fish dosed with 12.5 mg/kg (63.1 mmol/kg) DBT died within 120 hr. In addition, 6 of 8 fish dosed with 2.5 mg/kg (12.62 mmol/kg) DBT did not survive the 14 d study period, surviving an average 9.4 d (table 3.5). This suggests that DBT was more toxic to Murray cod than TBT, which was not expected as other studies have reported the opposite (de Vries et al., 1991; Wester and Canton, 1987), including the rainbowfish study in this chapter. This finding warrants more research to determine whether DBT is more toxic than TBT due to a species-specific mechanism. The highest sublethal dose previously used by our group to study organotin immunotoxicity in rainbow trout was 12.5 mg/kg. These results also demonstrate that Murray cod is more sensitive to organotins than trout, a commonly used test species in ecotoxicological risk assessments. Consequently, further investigation is needed as the present Murray cod findings imply that the species used for the risk assessment of Australian freshwater ecotoxicology may need to be reviewed.

Organotin doses of up to 2.5 mg/kg had no effect on the body weight or splenic and head kidney somatic indices of Murray cod. However, there were some significant reductions in the cell yields from the spleen (i.e. at 0.1 and 0.5 mg/kg) and the head kidney (i.e. at 2.5
Thymic atrophy and reduced numbers of lymphocytes have been a commonly reported effect of TBT and DBT in both fish and mammals (Boyer, 1989; Fent, 1996; Rice et al., 1995; Snoeij et al., 1988). This study found that, although there was no significant reduction in somatic indices of immune organs, there was a depleted immune population within these organs. It also appears that the function of lymphocytes may be altered by *in vivo* exposure to TBT, whereas DBT had no effect on lymphoproliferation with *in vivo* doses up to 0.5 mg/kg. TBT changed the proliferation profile of Murray cod head kidney lymphocytes, with 0.1 mg/kg slightly reducing peak CPM and 0.5 and 2.5 mg/kg increasing basal CPM (figures 3.14 and 3.15). The highest dose of TBT significantly reduced the proliferation index of Murray cod lymphocytes. This was not through the reduction of peak CPM but because of a rise in basal CPM and may have been due the introduction of an antigen that stimulated basal proliferation. Nevertheless, *in vivo* TBT exposure appeared to disrupt the lymphoproliferation of Murray cod head kidney lymphocytes but the results are difficult to interpret due to the inter-fish variation in mitogenic responses. Although the biological significance of the results of the *in vivo* experiment is somewhat equivocal, the assay showed excellent promise in the *in vitro* exposure experiments. Therefore, due to the significance of *in vivo* exposure data, it was decided that this assay would still be employed for both the *in vitro* and *in vivo* immunotoxic assessments of pesticides, immunostimulants and algal toxins, as reported in later chapters.

The flow cytometric analysis of head kidney cell subpopulations and the function of their granulocytes showed much greater sensitivity and interesting results. Most striking of the results was the effect of *in vitro* and *in vivo* DBT exposure on subpopulation counts and granulocyte phagocytosis of FITC beads (figure 3.17). There was a clear dose-dependent reduction in the number of lymphocytes and a concomitant dose-dependent increase in the number of granulocytes. Although the data of only two fish was available, the cell yield data
shows markedly reduced splenic cell yields at the highest dose of DBT (i.e. a lymphocyte population), and therefore, it was concluded that DBT reduced lymphocyte numbers rather than increased granulocyte numbers. This was also the expected result from in vivo DBT exposure, as the literature agrees that DBT is a potent thymolytic compound that reduces the number of T-lymphocytes in test animals (Fent, 1996; Pieters et al., 1994a; Snoeij et al., 1988). Previous experiments have also found that DBT is more effective at killing lymphocytes than TBT (O'Halloran et al., 1998b; Snoeij et al., 1988). In this in vivo study, TBT also reduced the number of lymphocytes but only at the highest dose of 2.5 mg/kg.

In addition to DBT’s effect on the subpopulation counts in Murray cod head kidneys, it appears to inhibit the ability of granulocytes to phagocytose FITC beads (figure 3.17). However, the fact that there was a concomitant rise in events within the granulocyte region, led to re-analysis of the data, as it was considered that rather than inhibiting phagocytosis, the extra events within the granulocyte-gated region may be reducing the percentage of FITC +ve granulocytes due to the appearance of granulocytes that were not actively engulfing beads. Re-analysis determined that this was not the case because the raw number of cells with beads (i.e. not expressed as a percentage of the granulocyte population) was also reduced in a dose dependent manner (figure 3.18) indicating that true inhibition of phagocytosis was occurring.
Conversely, in vivo TBT exposure resulted in a significant rise in the phagocytic activity of Murray cod granulocytes. Fish dosed with 0.5 mg/kg had significant increases in the percentage of active granulocytes and their SS characteristics (figure 3.16). This indicates that not only were there more granulocytes involved in engulfing beads, but also that the granulocyte population as a whole had more particulate matter in their cytoplasm. More beads may cause this effect however, it might also show that in vivo exposure to TBT changed the morphology of the granulocytes, perhaps by activating them, which could also make the cells more granular. The fact that the mean voltage did not rise significantly suggests that the latter scenario may be the case. Other in vitro and in vivo studies in both fish and rats have reported that high doses of TBT suppressed phagocytic function, while lower doses enhanced the activity. Rice and Weeks (1989) reported that in vitro doses of TBT increased the phagocytic functions (i.e. chemiluminescence) of macrophages from the toadfish before causing immunosuppression at higher doses. Long term in vivo studies in rats also reported that TBT at a high doses suppressed macrophage function, while lower doses increased phagocytosis (Vos et al., 1985). Another interesting observation is that the in vitro and in
vivo Murray cod phagocytosis data concurred for the flow cytometry assay. *In vitro* TBT exposure at 50 μg/L caused a slight rise in the number of granulocytes with beads followed by suppression at 500 μg/L, while *in vivo* TBT exposure caused a significant rise in the number of granulocytes with beads at 0.5 mg/kg followed by a return to control levels at 2.5 mg/kg. Moreover, *in vitro* and *in vivo* DBT exposure both resulted in a dose-dependent decrease in the number of granulocytes with beads.

In conclusion, these assays along with the general somatic index and cell yield data, provided a clear indication of the immunotoxic properties of TBT and DBT, both *in vitro* and *in vivo*. *In vivo* experiments with the larger Murray cod were more successful than those with small rainbowfish, due to better cell yields and easy handling, exposure and dissection. *In vivo* experiments were not performed in the two intermediate-sized species because; large numbers of golden perch were unavailable due a relatively small aquaculture industry, while silver perch would not acclimatise to small exposure tanks, as they preferred to school in larger numbers and became stressed in smaller groups, with these stress factors resulting in an unacceptable rate of mortality in the control groups.

During the *in vivo* experiments with Murray cod and rainbowfish, considerable inter-fish variation was observed, especially in the cell yield, body and organ weight data, however these parameters still provided useful additional information for the functional assays. The lysozyme assay did not show any significant results from *in vitro* or *in vivo* experiments but this is the first time the possible effect of TBT on lysozyme activity has been reported. It is also a quick and easy assay that provides additional immunotoxicology data with serum that is abundantly available. It was initially anticipated to develop an ELISA assay to assess serum IgM levels in native fish as part of this doctoral thesis. Unfortunately, the rabbit-anti-fish immunoglobulin polyclonal antibodies that were experimentally raised were lost due to
freezer breakdown. The lymphoproliferation assay provided useful *in vitro* data, but inter-fish variation in lymphocyte responses reduced the sensitivity of the assay for *in vivo* experiments. The flow cytometry assessments were exceptionally sensitive and provided a variety of different parameters to analyse. The use of flow cytometry was originally developed to assess the phagocytosis in the four native fish species and to replace a microscopic assay to measure the phagocytic ability of head kidney granulocytes (section 2.4.2). However, it proved to be far more useful, by not only removing subjectivity of microscopy counting, but also by providing information on a number of different parameters such as relative proportions of lymphocytes and granulocytes and non-viable cells. The flow cytometry assay also appeared to have the least amount of biological variation compared to the other assays, and was very useful in the immunotoxicological assessment of other pesticides, immunostimulants and algal toxins, as reported in the later chapters.
Chapter 4: The effect of endosulfan exposure on the immune functions of Australian freshwater fish

4.1: Introduction: Endosulfan (CAS No. 115-29-7)

Endosulfan was first registered in Australia during the 1960s for a wide variety of uses including home garden use (now banned), protection of fruit and vegetable crops and non-food crops such as cotton (NRA, 1998). Currently, 70% of endosulfan usage in Australia is for the protection of cotton crops from the native budworm *Helicoverpa punctigera*. It is used in rotation with other insecticides and is applied to crops early in the season, as it is ineffective against some resistant species such as the cotton bollworm (*Helicoverpa armigera*). In earlier years, endosulfan was applied up to 6 times a season but since July 1999, legislation has restricted its use to twice a season. Endosulfan is still in use as it preserves some beneficial predator insects and reduces the flaring of secondary pests, such as mites and aphids. The other major use for endosulfan is the protection of vegetable crops, which accounts for 20% of the total usage. The remaining 10% of endosulfan usage is on oilseeds, fruit crops and ornamentals (NRA, 1998).

Endosulfan is the last cyclodiene organochlorine insecticide still available for use in Australia, as it has a much lower persistence compared to its relatives such as dichlorodiphenyl-trichloroethane (DDT), aldrin and dieldrin. It has been allocated the highest risk ranking of all the cotton pesticides in Australia, due to its high annual usage and high toxicity to aquatic organisms (Nowak *et al*., 1995). Because of endosulfan’s high environmental risk, the ecotoxicology of endosulfan has been studied extensively in many countries. There is also a large body of literature concerning the effects of endosulfan on fish because of their specific sensitivity to the chemical. However, there have been comparatively few studies into endosulfan’s potential to cause immunotoxicity in fish and mammals.
4.2: Structure

Endosulfan (6,7,8,9,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-
benzodioxa-thiepin-3-oxide) is an organochlorine cyclodiene chemical (figure 4.1). Technical
grade endosulfan consists of α and β stereoisomers in a ratio of 7:3 and a purity of 94-96% 
(Naqvi and Vaishnavi, 1993).

Figure 4.1: The structure of endosulfan isomers a) α-endosulfan and b) β-endosulfan 
(NRA, 1998)

4.3: Mode of action

Endosulfan, like other cyclodiene pesticides, targets the γ-aminobutyric acid 
(GABA)/chloride (Cl⁻) ionophore complex and the glycine receptor. Of the two classes of 
GABA receptor, endosulfan targets the GABA_A receptors, which are coupled to Cl⁻ channels, 
while GABA_B receptors (coupled to Ca^{2+} and K⁺ channels via GTP binding proteins) are not 
targeted. Within the GABA subunits there are multiple allosteric binding sites for GABA, 
benzodiazepines and picrotoxinin-like convulsants (Eldefrawi and Eldefrawi, 1989). This 
receptor is the primary mediator of inhibitory neurotransmission in the brain and regulates 
neurotransmitter release (Costa, 1987; Kamijima and Casida, 2000). Endosulfan acts as an 
antagonist by binding at a non-competitive blocking site, stabilizing non-conducting
formations of the Cl\textsuperscript{-} channel and effectively inhibiting GABA-induced Cl\textsuperscript{-} uptake (Kamijima and Casida, 2000; Nagata and Narahashi, 1994; Vale et al., 2003).

In addition, endosulfan also binds to the glycine receptor, which is a pentameric protein that also regulates Cl\textsuperscript{-} flux and motoneuronal output, but is modulated by glycine. Blockage of the GABA\textsubscript{A} and glycine-gated Cl\textsuperscript{-} channels results in a reduction of neuronal inhibition and thus, promotion of neurotransmitter release. This causes hyperexcitability of the CNS leading to convulsions and death (Bloomquist, 2003).

4.4: Toxicokinetics

Following oral administration, endosulfan is absorbed slowly but incompletely with some of the parent compound being excreted in the faeces (Maier-Bode, 1968). Metabolism in animals occurs via oxidation and hydrolysis and results in sulfate, diol, hydroxyether, lactone, ether and hydroxy endosulfan carboxylic acid derivatives. Unlike other organochlorine pesticides, endosulfan does not persist \textit{in vivo} because it is quickly metabolised and eliminated (Smith, 1991). The toxicity of endosulfan is dependent on the presence of the sulfur atom and its removal significantly decreases its toxicity (Knauf and Schulze, 1973). After acute over-exposure, high endosulfan concentrations can temporarily be found in the liver and the spleen, while lower residue levels are found in muscle and kidney tissue (NRA, 1998). The biological half-life in rats is 1.2 – 7 d and in the zebra fish (\textit{Brachydanio rerio}) it is 2.9-5.6 d (Cecilia et al., 1992). Endosulfan is excreted primarily via the faeces (approximately 80\% of an oral dose), although a small amount may be found as metabolites in the urine soon after exposure (NRA, 1998). Fish rapidly excrete the chemical once they are placed in clean water (Cecilia et al., 1992).
4.5: Toxicity data

The signs of acute endosulfan poisoning are similar between species and include hyperactivity, tremors, and convulsions followed by death (Gupta, 1976; WHO, 1984). Technical grade endosulfan oral LD$_{50}$ values range between 7 and 121 mg/kg and vary due to differences in species, gender, solvent vehicle, nutritional status and formulation (Naqvi and Vaishnavi, 1993; WHO, 1984). Intraperitoneal LD$_{50}$ values in the mouse vary from 6.9 to 13.5 mg/kg but the highest tolerated oral dose was reported to be 15 mg/kg, with 100% mortality occurring in mice receiving a 20 mg/kg dose (Deema et al., 1966; Gupta, 1976; Smith, 1991).

A 2 year study reviewed by the FAO/WHO (1968) joint committee indicated there was a no-effect level (NOEL) of 50 ppm (2.5 mg/kg/d) of endosulfan in rats, but that 100 ppm (5.0 mg/kg/d) reduced their growth and survival rate. In a three generation rat study, endosulfan showed no effect on reproduction parameters at levels up to 2.5 mg/kg/d (FAO/WHO, 1968). However, in male rats degeneration of the seminiferous tubules and testes was observed at oral doses of 10 mg/kg/d for 15 d (Gupta and Chandra, 1977). Doses above 5 mg/kg/d on day 6-14 of gestation decreased the survival of rat dams and increased the rate of resorption and skeletal abnormalities of foetuses (Gupta et al., 1978). Endosulfan shows no signs of carcinogenicity in experimental animals (NRA, 1998)

4.5.1: Immunotoxicity

Banerjee and Hussain (1987) investigated the effects of dietary endosulfan on a number of cellular and humoral immune parameters in rats. No suppression of immunity was observed at 10 ppm (i.e. 0.5 mg/kg/d). They reported that endosulfan exposure in rats resulted in a significant decrease in IgG, IgM (above 30 ppm) and gamma-globulin (at 50 ppm) plasma levels. They also observed a dose-dependent decrease in both the leucocyte and macrophage
migration inhibition test, which was significantly reduced above 30 ppm. Cockerels that received dietary endosulfan (25-100 ppm), displayed a decrease in haemagglutinin titres against bovine serum albumin even at the lowest dose. In contrast, Akay and co-workers (1999) found no changes in white blood counts from rats exposed to endosulfan at 0.006 mg/kg/d, which is 100 times the acceptable daily intake (ADI). Researchers investigating the effect of \textit{in vitro} endosulfan exposure on sheep peripheral leukocytes have reported that endosulfan inhibited the migration of monocytes and the mitogenic responses of lymphocytes at concentrations of $10^{-4}$M (Pistl \textit{et al.}, 2003; Pistl \textit{et al.}, 2001).

\textit{In vitro} studies, using Jurkat cells as a model (i.e. a human T-cell leukemic cell line), reported that endosulfan induced apoptosis, which may have direct relevance to loss of T-cells and thymocytes \textit{in vivo}. After 48 hr, endosulfan (50 μM) caused an increase in both DNA fragmentation and annexin-V binding. The authors also suggested a role for mitochondrial dysfunction and oxidative stress in endosulfan toxicity (Kannan \textit{et al.}, 2000).

Inhibition of the GABAergic system by known GABA$_A$ antagonists results in immunosuppression, which may partly explain reports of endosulfan’s immunosuppression \textit{in vivo} (Devoino \textit{et al.}, 1994). Devoino and co-workers (1994) discovered that blockage of the GABA receptor with bicuculline and picrotoxin (0.5-2 mg/kg) resulted in reduced B- and T-cell responses. These chemicals have a similar mechanism of action as endosulfan, binding at a non-competitive site of the GABA/Cl$^-$ ionophore complex.

Chemicals can theoretically alter immune functions through endocrine disruption. Organochlorine pesticides act as estrogen receptor antagonists causing disruption to intercellular signalling and ultimately reproductive failure (Hodges \textit{et al.}, 2000). \textit{In vitro} and \textit{in vivo} assays that screen chemicals for estrogenic properties have demonstrated that
endosulfan has estrogenic properties that are comparable to DDT (Shelby et al., 1996; Soto et al., 1994). Additional investigations concerning the intracellular mechanism involved have demonstrated that endosulfan can enhance STAT3-mediated transcription activity. Presumably, this mechanism could have implications for the immune system as the main function of STAT3 is as a signal transducer for the IL-6 family of cytokines including leukaemia inhibitory factor and leptin (Sekine et al., 2004). Leukaemia inhibitory factor has a role in differentiation of various cell types including granulocytes, monocytes and corticotrophic cells (Metcalf et al., 1988; Stefana et al., 1996). Furthermore, estrogen receptors have been identified on the cell surface of human myeloid monocytic cell lines suggesting that endosulfan may be able to exert effects on precursor immunocytes by signalling them to differentiate (Cutolo et al., 2001).

4.6: Sources of environmental contamination

Endosulfan enters the environment through agricultural runoff, leaching and, in some countries, the direct application to water bodies to treat pests (Wan, 1989). In Australia, endosulfan is mainly used on the cotton crops of northern NSW and southern Queensland. The focus on its environmental effects has been intense due to its high rate of use, frequency of detection in sampling programs and high toxicity to fish (Peterson and Bately, 1993). It is applied at a rate of 3 – 3.5 kg/ha per year and 400t was applied to crops during 1992 in NSW alone (Sunderam et al., 1992). Endosulfan has often been suspected as the cause of fish kills and most endosulfan contamination is a result of drift from crops being sprayed either by air or by boom sprayers (Nowak et al., 1995). Australian surveys have shown that seasons with higher rainfalls resulted in higher levels of residues in fish. This was due not only to pesticide runoff, but also due to terrestrial food sources entering the system and sediment layers containing the pesticide being resuspended into the water column (Nowak and Julli, 1991). The same survey reported that residues in fish were found during seasons without endosulfan
use (i.e. winter), and it is now recognised that endosulfan does persist in sediment layers and is released slowly into the water producing low levels of contamination for many months (Peterson and Bately, 1993)

4.7: Endosulfan in the environment and its fate

Endosulfan readily binds to soil with a half-life of up to 2 years and has often been detected at hazardous waste sites by environmental protection agencies (Naqvi and Vaishnavi, 1993). Endosulfan does not persist in the water column and has a half life of 3-7 d (Sunderam et al., 1992). However, it readily binds to aquatic sediments and may persist there, with a half-life of 1-6 months. Its degradation in water produces endosulfan diol and the rate of endosulfan hydrolysis is mostly dependent on the pH (Naqvi and Vaishnavi, 1993; Peterson and Bately, 1991). In natural waters, endosulfan would not be readily degraded by photolysis, as turbidity blocks light from the sediment layers where endosulfan resides (Peterson and Bately, 1991).

4.8: Endosulfan levels found in fish

Residues in fish predators have been reported, but were not higher than residues found in fish in the same area, leading the authors to conclude that endosulfan does not biomagnify (Matthiessen et al., 1982). However, many aquatic animals have shown that they can accumulate the compound to significant levels during exposure periods (Naqvi and Vaishnavi, 1993). Studies in fish have reported BCFs of 2,755 for marine species (striped mullet, *Mugil cephalus*) (Schimmel et al., 1977) and 2,650 for freshwater fish (zebra fish) (Cecilia et al., 1992), although BCFs as high as 11,500 have been also been reported (yellow tetra, *Hyphessobrycon bifasciatus*) (Jonsson and Toledo, 1993).

In an Australian study of endosulfan contamination in the NSW cotton field areas, levels of endosulfan in live carp caught in waters near cotton crops were found to be elevated. Fish
from a fish kill site had an average of 19.9 mg/kg (wet weight) of endosulfan in their livers and 7.8 mg/kg in their gill tissue. In areas not identified as fish kill sites, endosulfan was still detected in carp livers with an average level of 0.4 mg/kg (Nowak et al., 1995).

4.9: Aquatic ecotoxicology

Toxicokinetic studies have reported that endosulfan biotransformation pathways are similar in both fish and mammals, with the principal metabolite being the sulfate, and the final detoxification products consisting of the alcohol and ether (Rao and Murty, 1982). Generally, endosulfan can be readily detoxified and excreted by fish, however they tend to accumulate the chemical during times of ongoing exposure (Matthiessen et al., 1982). The fat content of fish appears to be an important factor for the amount of endosulfan that is accumulated. Fish with more fatty tissue tend to accumulate more endosulfan compared to lean fish and may be protected, to some extent, from its toxic effects (Matthiessen et al., 1982).

Endosulfan is very toxic to fish, even compared to other organochlorine pesticides such as DDT (Joshi and Rege, 1980; Nowak et al., 1995). Signs of acute endosulfan poisoning include frequent jumping and erratic movement followed by convulsions (Gopal et al., 1981; Haider and Inbaraj, 1986; Pandey, 1988). The 96 hr LC$_{50}$ values vary from 0.014 μg/L for the harlequin fish (Rasbora heteromorpha) to 14 μg/L for catfish (Clarias batrachus). The 96 hr LC$_{50}$ values for Australian fish are 2.2 μg/L for the native firetail gudgeon (Hypseleotris gallii), 2.4 μg/L for the eastern rainbowfish (Melanotaenia duboulayi) and silver perch (Bidyanus bidyanus), 0.5 μg/L for golden perch (Macquaria ambigua) and 0.2 μg/L for bony bream (Nematolosa erebi) (Sunderam et al., 1992). The LC$_{50}$ values for exotic species that inhabit the Murray-Darling basin are 3.1 μg/L for mosquitofish (Gambusia affinis), 0.1 μg/L for European carp (Cyprinus carpio) and 1.6 μg/L for rainbow trout (Oncorhynchus mykiss) (Sunderam et al., 1992). Factors that are known to influence endosulfan toxicity include
temperature, salinity, life-cycle stage and bioassay procedure (i.e. static vs. flow through water systems). Endosulfan toxicity increases with temperature and it is more toxic to fish than invertebrates, and younger fish are more susceptible than adults (Naqvi and Vaishnavi, 1993).

Metabolic and respiratory interferences have been commonly reported in fish exposed to lethal and sublethal doses of endosulfan. Researchers have reported ATPase inhibition, hyperglycaemia (but a reduced glucose absorption rate in the intestines), glycogenolysis, reduced or elevated oxygen consumption and thyroid hormone disruption (Dalela et al., 1978; Gopal et al., 1981; Murty and Devi, 1982; Reddy and Gomathy, 1977; Sastry and Siddiqui, 1982; Singh and Srivastava, 1981). Both chronic and subchronic endosulfan exposure of fish resulted in a decrease of alkaline phosphatase activity, resulting in impaired carbohydrate metabolism (Verma et al., 1981). Additionally, sublethal endosulfan exposure resulted in a lowering of metabolic rate, while a lethal dose markedly increased metabolic rate (Reddy and Gomathy, 1977). In fish, endosulfan exposure induces EROD, aldrin epoxidase and aryl hydrocarbon hydroxylase activities in trout hepatocytes (Jensen et al., 1991). A number of researchers have also observed changes in the liver histology of fish exposed to endosulfan (Amminikutty and Rege, 1977; Cecilia et al., 1992; Rao et al., 1980).

Endosulfan has been shown to affect endocrine function and corticosteroidogenic cells, causing adrenocorticotropic hormone release and subsequent corticosteroid release (Pandey, 1986). In addition to corticosteroids ability to modulate metabolism, they are immunosuppressive and anti-inflammatory and are therefore relevant to immunotoxicological studies. Endocrine disruption is also thought to be the cause of a number of reproductive dysfunctions observed in fish (Khillare and Wah, 1987; Kulshrestha and Arora, 1984; Pandey, 1988).
4.9.1: Immunotoxicity

There have been relatively few studies concerning the immunotoxicity of endosulfan in fish. *In vitro* studies with endosulfan have reported suppression of lymphocyte blastogenesis. B-cells mitogenesis (i.e. LPS-stimulated) was significantly suppressed at concentrations above 1 ppm, while T-cell mitogenesis (i.e. PHA-stimulated) was less sensitive and was suppressed at the highest dose of 10 ppm (O'Halloran et al., 1996b). Abidi and Srivastava (1988) reported that bloch (*Channa punctatus*) exposed to endosulfan showed a dose-dependent increase in the number of erythrocytes and leukocytes, haemoglobin concentration and packed blood volume. In field experiments at a lake sprayed with endosulfan in Botswana, researchers found an increase in fish blood cell counts. In most cases these levels returned to normal before spraying had ceased and returned to pre-spraying levels within six months (Matthiessen, 1981).

4.10: Aim of the study

The aim of this study was to investigate the effects of endosulfan exposure on the immune functions of fish native to the Murray-Darling basin (i.e. crimson-spotted rainbowfish, silver perch, golden perch and Murray cod).

4.11: Methods

4.11.1: Spleen and head kidney sampling

Native fish (i.e. rainbowfish, silver perch, golden perch and Murray cod) were housed and maintained as described in section 2.1. Fish were anaesthetised and sacrificed as described in section 2.2. For the *in vivo* experiments fish weight was recorded immediately before injection and then after the 14 d exposure period prior to tissue sampling. The percentage of
body weight gained or lost was calculated as: (pre-exposure – post-exposure weight) / pre-exposure weight x 100.

Immune tissues were sampled as described in section 2.3. Somatic indices were calculated as: (organ weight/body weight) x 100.

4.11.2: Cell isolation

The method for the isolation of fish immune cells is described in detail in section 2.3 and also briefly described in section 3.13.2. Cell yield was calculated as the total number of cells collected / organ weight (g).

4.11.3: Exposure protocols

*In vitro exposures*

Technical grade endosulfan (Hoechst, Melbourne, Australia) was dissolved in DMSO (BDH Chemicals, Poole, UK) to obtain a high intermediate stock solution of 4 g/L. This was serially diluted 1:10 to achieve medium and low intermediate stock solutions of 0.4 and 0.04 g/L. For each assay, the intermediate stock solutions were diluted 1:100 in media before a further 1:4 addition to the final test volume, resulting in a final DMSO concentration of 0.25% v/v (i.e. 1:400 dilution). For the phagocytosis assay 250 μL of treatment was added to incubation tubes and 750 μL of cell suspension (1.33x10⁶ cells/mL). In the lymphoproliferation assay 50 μL of the chemical treatment was added to a final test volume of 200 μL and the lysozyme assay received 50 μL of the pesticide with 25 μL of serum and 125 μL of the *M. lysodeikticus* solution (1.05 mg/mL). The final concentrations of endosulfan tested in each assay were 0.1, 1 and 10 mg/L (ppm).
In vivo

Stock solutions of technical grade endosulfan were dissolved in PEG (polyethylene glycol) at 2.5 g/mL. On the day of use, the stock was diluted 1:100 to give a high concentration of 25 mg/mL. This was serially diluted 1:5 to attain concentrations of 0.04, 0.2, 1 and 5 mg/mL. Fish were dosed with 2 mL/kg, achieving exposures of 0.08 (Murray cod only), 0.4, 2, 10 (lethal to Murray cod but not rainbowfish) and 50 (lethal to all fish) mg/kg.

The endosulfan in vivo exposure protocols for rainbowfish and Murray cod are is shown in table 4.1 and 4.2. Protocols were performed as described in detail for the organotins (section 3.13.3). In vivo exposure studies were not conducted in silver perch because they would not acclimatise in exposure tanks, which resulted in an unacceptable frequency of mortalities in the control groups. Golden perch were not used for in vivo exposure studies because sufficient numbers were unavailable.

Table 4.1: Endosulfan in vivo exposure protocol for rainbowfish.

<table>
<thead>
<tr>
<th>Day of study</th>
<th>Untreated control</th>
<th>0 (Solvent control)</th>
<th>0.04</th>
<th>2</th>
<th>10</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1-14</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Day 2-15</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* Died before sampling.

Table 4.2: Endosulfan in vivo exposure protocol for Murray cod

<table>
<thead>
<tr>
<th>Day of study</th>
<th>Untreated control</th>
<th>0 (Solvent control)</th>
<th>0.08</th>
<th>0.4</th>
<th>2</th>
<th>10</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1-14</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 2-15</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 5-19</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 6-20</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Died before sampling.
This dose range was chosen to determine the lethal concentration of endosulfan in Murray cod and rainbowfish at high doses, however the lower concentrations reflect concentrations reported in the tissue of fish from contaminated areas (see section 4.8). The reasons an i.p. injection was chosen as the route of administration are discussed in section 3.13.8.

4.11.4: Phagocytosis
Standardised protocols for the phagocytosis assay are described in detail in section 2.4.2 and the methods used in this experiment are the same as the organotin study described in section 3.13.4.

4.11.5: Mitogenesis
Standardised protocols for the mitogenesis assay are described in detail in section 2.6.2.2 and the methods used in this experiment are the same as the organotin study described in section 3.13.5.

4.11.6: Lysozyme
Standardised protocols for the lysozyme assay are described in detail in section 2.5.3 and the methods used in this experiment are the same as the organotin study described in section 3.13.5.

4.11.7: Statistics
Statistics were performed using the same methods described in the section 3.13.7.
4.12: Results

4.12.1: The effect of *in vitro* endosulfan exposure on the phagocytosis of head kidney cells from four Australian fish

The effect of *in vitro* endosulfan exposure on the phagocytic function and subpopulation numbers of head kidney cells was investigated in four native Australian fish, i.e. crimson-spotted rainbowfish, silver perch, golden perch and Murray cod (figures 4.2-4.6). In rainbowfish, both the number of phagocytically active (FITC +ve) granulocytes and their activity in terms of the number of beads ingested (i.e. mean voltage of FITC +ve cells) were reduced to approximately 60% of control at the highest endosulfan concentration of 10 mg/L (figure 4.2a). Granulocyte numbers were not significantly changed at any of the doses investigated, while lymphocyte counts were slightly, but not significantly, elevated at the highest dose (figure 4.2b). The concentrations of endosulfan studied in this experiment had no significant effect on the phagocytic function or subpopulation numbers of silver perch head kidney cells (figure 4.3).
Figure 4.2: The effect of *in vitro* endosulfan exposure on rainbowfish head kidney cells. 
a) Phagocytosis (FITC +ve granulocytes) and number of beads per cell (mean voltage) b) 
Granulocyte and lymphocyte subpopulation counts. Data is expressed as percentage of 
untreated control samples (n=6) and values represent mean ± se of 4-6 fish. Untreated control 
values were 20±3% for FITC +ve granulocytes, 8±1 volts for mean voltage, 3350±300 events 
for granulocytes and 3000±650 events for lymphocytes. No statistically significant differences 
were found.

Figure 4.3: The effect of *in vitro* endosulfan exposure on silver perch head kidney cells. 
a) Phagocytosis (FITC +ve granulocytes) and number of beads per cell (mean voltage) b) 
Granulocytes and lymphocyte subpopulation counts. Data is expressed as percentage of 
untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated control 
values were 37±2% for FITC +ve granulocytes, 216±31 volts for mean voltage, 2000±150 
events for granulocytes and 1400±150 events for lymphocytes. No statistically significant 
differences were found.
Golden perch head kidney cells exposed to 10 mg/L endosulfan exhibited a significant increase in the percentage of cells actively phagocytosing beads (i.e. FITC +ve granulocyte events), however there was no change in the average number of beads per cell (i.e. mean voltage) (figure 4.4). At 1 mg/L, counts were elevated by 10%, while 10 mg/L significantly increased these counts to 50% above control levels (Figure 4.4a). In earlier experiments that attempted to use serum as an opsonin to increase activity, it was observed that increases in bead clumping led to an increase in the granulocyte counts and mean voltage (data not shown). Therefore, microscopic examination of the samples was also performed but did not indicate an increase in the number of bead clumps or an elevation of granulocyte counts. Therefore this result indicates that samples treated with 10 mg/L resulted in more granulocytes engaged in phagocytosis but the number of beads per granulocyte did not increase. There was no change in the number of granulocytes or lymphocytes (Figure 4.4b).

Murray cod head kidney cells produced unexpected findings following in vitro endosulfan exposure. The results indicated that endosulfan specifically killed Murray cod head kidney granulocytes as there was a distinct lack of events in the granulocyte gated region at 10 mg/L that could only be explained by endosulfan-induced toxicity towards granulocytes (figure 4.5c). This effect was also seen in rainbowfish head kidney cells exposed to the organotins (see figure 3.2 and 3.3) but is in contrast to the majority of in vitro exposure experiments where lymphocytes were the more sensitive subpopulation. There was also a large increase in PI +ve and the “debris” gated events, indicating that a large number of non-viable cells were present in the sample, which may have come from viable cells that would have been situated in the granulocyte gated region (figure 4.5d). Samples also took longer to collect 10,000 events on the flow cytometer, suggesting that cell numbers were reduced (data not shown). The large increase in the number of FITC +ve granulocytes and their mean voltage suggests either: 1) active granulocytes with beads were more robust than granulocytes not engulfing
beads; or 2) there was an increase in the background counts of beads within the granulocyte gate (figure 4.5a and b). The likelihood is that both these factors contributed to the rise in FITC +ve events and mean voltage.

Figure 4.4: The effect of *in vitro* endosulfan exposure on golden perch head kidney cells. 

**a) Phagocytosis (FITC +ve granulocytes) and number of beads per cell (mean voltage) b) Granulocytes and lymphocyte subpopulation counts.** Data is expressed as percentage of untreated control samples (n=7) and values represent mean ± se of 5-7 fish. Untreated control values were 17±2% for FITC +ve granulocytes, 30±3 volts for mean voltage, 4100±200 events for granulocytes and 2150±250 events for lymphocytes. * denotes significantly different from control (p<0.05).
Figure 4.5: The effect of in vitro endosulfan exposure on Murray cod head kidney cells. 

a) Phagocytosis (FITC +ve) b) number of beads per cell (mean voltage and side scatter (SS) increase) c) Granulocytes and lymphocyte subpopulation counts d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated controls values were; 14±1 for FITC+ve total, 20±2% for FITC +ve granulocytes, 108±10 volts for mean voltage, 113±12 volts for SS increase, 6900±300 for granulocytes, 1400±100 for lymphocytes, 1200±140 events for debris and 6±1% for PI+ve * denotes significantly different from control (p<0.05).
4.12.2: The effect of \emph{in vitro} endosulfan exposure on Murray cod immune functions

\textit{Lysozyme}

Murray cod serum lysozyme activity was not significantly different following \emph{in vitro} exposure to endosulfan (table 4.3).

\textit{Lymphoproliferation}

\emph{In vitro} exposure to 10 mg/L endosulfan caused a reduction in the mean CPM of peak mitogenesis (table 4.3). The proliferative index was not altered over the concentration range because both basal and peak proliferations were reduced equally at the highest concentration. Endosulfan at 10 mg/L may have been directly toxic to the T-cells of the Murray cod head kidney. However, results from the flow cytometer demonstrated that the granulocyte population was more sensitive that the lymphocyte population, therefore this result could reflect a reduction in granulocytes, which could also be the reason why the bell shape profile was maintained.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Parameter & Endosulfan concentration (mg/L) & \\
\hline
       & 0     & 0.1 & 1.0 & 10.0 \hline
Lysozyme & 99.2±1.2 & 105.4±3.5 & 95.6±5.3 & 84.9±6.6 \hline
Proliferation index & 110.7±12.0 & 96.2±2.9 & 96.2±6.1 & 106.4±16.2 \hline
Peak CPM & 1219±307 & 1113±289 & 1239±239 & 622±300 \hline
\end{tabular}
\caption{The effect of \emph{in vitro} endosulfan exposure on the activity of Murray cod serum lysozyme and mitogenesis.}
\end{table}

*Data is expressed as percentage of untreated control samples and values represent mean ± se of 4 fish. Control values were 10.09 ± 1.49 µg/mL HEWL equivalent for lysozyme and 1.29±0.11 for proliferation index. CPM = counts per minute. No statistically significant differences were found.*
4.12.3: The effect of *in vivo* endosulfan exposures on rainbowfish immune parameters

Rainbowfish were treated *in vivo* with endosulfan and responses analysed to provide a comparison to Murray cod, the larger fish of interest in this study. Rainbowfish dosed with 50 mg/kg endosulfan exhibited 100% mortality within 48 hr (data not shown), however all fish survived the 14 d study period when dosed with 10 mg/kg. Therefore, it can be concluded that the LD$_{50}$ for an i.p. injection of endosulfan is between 10 and 50 mg/kg.

The body weight of the rainbowfish did not significantly change following exposure to endosulfan (table 4.4). Fish dosed with 2 mg/kg lost 10% of their body weight on average, but this was not significantly different from the solvent controls, which lost an average of over 5% of their body weight during the study period (table 4.4). Unexpectedly, 3 of the 4 fish in the 10 mg/kg group gained weight, but the group exhibited a large variation in this parameter as one fish lost approximately 20% of their body weight, while another gained 20% (table 4.4).

Head kidney somatic indices were lower in all treated groups, but this was more pronounced in the 0.4 and 2 mg/kg group (table 4.4). The variation within the 0.4 mg/kg group was greater due to two normal-sized fish with remarkably small head kidneys. Rainbowfish dosed with 10 mg/kg had somatic indices that were only slightly below control levels (table 4.4). There was an elevated cell yield in rainbowfish exposed to 0.4 mg/kg, resulting from the two fish with small head kidneys that still produced relatively normal cell yields (table 4.4). Fish dosed with 2 and 10 mg/kg displayed cell yields almost identical to controls and there was very little variation within these groups (table 4.4).
Table 4.4: Effect of *in vivo* endosulfan exposure on rainbowfish body and organ weights

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0</th>
<th>0.4</th>
<th>2</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in body weight (%)</td>
<td>-6.27±4.29</td>
<td>-4.27±2.30</td>
<td>-10.95±3.37</td>
<td>2.69±8.93</td>
</tr>
<tr>
<td>Head kidney Somatic index</td>
<td>0.36±0.06</td>
<td>0.22±0.09</td>
<td>0.24±0.02</td>
<td>0.30±0.05</td>
</tr>
<tr>
<td>Head kidney Cell yield a</td>
<td>681±125</td>
<td>1332±429</td>
<td>711±109</td>
<td>668±117</td>
</tr>
</tbody>
</table>

*a* Cell yield data is expressed cells x 10⁶/mg of head kidney tissue. Values represent the mean ± se of 4 fish. No statistically significant differences were found.

*In vivo* endosulfan exposure of rainbowfish at 0.4 and 2 mg/kg caused a slight but insignificant reduction in the percentage of active phagocytes, which returned to control levels at 10 mg/kg (figure 4.6a). There was a significant decrease in the number of lymphocytes in rainbowfish dosed at 10 mg/kg.

**Figure 4.6:** The effect of *in vivo* endosulfan exposure on rainbowfish head kidney cells. a) Phagocytosis (FITC + ve) and number of beads per cell (mean voltage) b) Granulocytes and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=4) i.e. 18±1% for FITC +ve granulocytes, 47±5 volts for mean voltage, 6300±350 events for granulocytes and 800±150 for lymphocytes. Values represent mean ± se of 4 fish. * denotes significantly different from the control (p<0.05).
4.12.4: The effect of *in vivo* endosulfan exposures on Murray cod immune parameters

**Body and organ weight changes**

Murray cod dosed with 50 mg/kg died within 18 hr of exposure. One fish dosed with 10 mg/kg survived the 14 d period, however 3 others died between 36-48 hr after exposure. Therefore, it can be concluded that the 96 hr LD$_{50}$ for Murray cod following an i.p. injection of endosulfan is between 2 and 10 mg/kg. The single fish that survived the 14 d period following the 10 mg/kg dose was pale, did not feed, lost body weight (15% b.w.) and was inactive but had no obvious signs of neurotoxicity. Fish exposed to 2 mg/kg lost some body weight but did not display any other signs of toxicity or stress (i.e. immobile, discoloured and not feeding). Murray cod dosed with 0.08 and 0.4 mg/kg did not show any signs of stress and fed well and the group exposed to 0.08 mg/kg gained an average of 5% body weight during the 14 d study period (table 4.5).

*In vivo* endosulfan exposure did not significantly lower the somatic indices of either the spleen or head kidney of Murray cod (table 4.5). The sole fish that survived the 10 mg/kg dose had an extremely small spleen, very dark in colour; at this near lethal dose this fish could be suffering from chemical-induced stress. Conversely, the head kidney of this fish was of a relatively normal size and yielded a higher number of cells per gram of head kidney tissue compared to other groups (table 4.5).
Head kidney cell yields were unchanged by *in vivo* endosulfan exposure (table 4.5). The fish that survived the 10 mg/kg dose had an elevated head kidney cell yield but this could not be referred to as typical without more data. Splenic cell yields were also not significantly different from the control (table 4.5).

**Lysozyme**

*In vivo* endosulfan exposure of Murray cod had no significant effect on serum lysozyme activity (table 4.5).

**Table 4.5: The effect of *in vivo* endosulfan exposure on Murray cod body and organ weights and lysozyme activity**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Endosulfan concentration (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Mortality</td>
<td>0/7</td>
</tr>
<tr>
<td>Change in Body weight (%)</td>
<td>-3.02±1.90</td>
</tr>
<tr>
<td>Spleen Somatic index</td>
<td>0.057±0.014</td>
</tr>
<tr>
<td>Head kidney Somatic index</td>
<td>0.259±0.034</td>
</tr>
<tr>
<td>Spleen Cell yield a</td>
<td>892±156</td>
</tr>
<tr>
<td>Head kidney Cell yield a</td>
<td>727±100</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>101±5%</td>
</tr>
</tbody>
</table>

*a* Cell yield data is expressed cells/g of head kidney tissue. Values represent the mean ± se of 4-7 fish (except 10 mg/kg n=1). Lysozyme data is expressed as percentage of control (i.e. 7.12±0.93 μg/mL HEWL equivalent) and values represent mean ± se of 4-6 (one solvent control lost (coagulated)) fish. No statistically significant differences were found.
Phagocytosis

*In vivo* exposure of Murray cod had no significant effect on the phagocytic activity of Murray cod head kidney cells (figure 4.7a). There was a large variation in the number of beads per cell for Murray cod dosed with 0.08 and 0.4 mg/kg (figure 4.7b). Granulocyte numbers were unchanged in all the groups and there was minimal variation. The number of lymphocytes in fish exposed to TBT was not significantly different from the solvent controls (figure 4.7c). Head kidney cell cultures from fish in the 0.08 mg/kg group also displayed a significant increase in the number of non-viable cells and there was an increase in the number of debris-gated events, but due to large variation in this parameter this increase was not statistically significant (figure 4.7d).

Mitogenesis

*In vivo* endosulfan exposure had a significant suppressive effect on the proliferation of Murray cod head kidney cells, even at the lowest dose of 0.08 mg/kg (figure 4.8a). Fish dosed with 2 mg/kg had a lower proliferation in both the presence and absence of mitogen, while the proliferation index was reduced to 84% of the untreated control (figure 4.8b). The sole survivor from the 10 mg/kg group had extremely low basal and peak proliferation values of 148 and 419 CPM, respectively.
Figure 4.7: Murray cod head kidney phagocytic function and subpopulation counts following in vivo exposure to endosulfan: a) Phagocytosis (FITC +ve) b) number of beads per cell (mean voltage and side scatter (SS) increase) c) Granulocytes and lymphocyte subpopulation counts d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control samples (n=8) and values represent mean ± se of 4-7 fish. Untreated control values were; 10±2% for FITC +ve total; 25±4% for FITC +ve granulocytes; 130±30 volts for mean voltage; 123±14 for side-scatter increase; 4050±200 for granulocytes; 3350±200 for lymphocytes; 1600±150 for debris and 800±150 for PI +ve. * denotes significantly different from solvent control (p<0.05).
Figure 4.8: The effect of *in vivo* endosulfan exposure on: a) the lymphoproliferation profile and b) proliferative index of Murray cod head kidney cells. Proliferation data is expressed at percentage of untreated control (i.e. 1.46±0.12, n=8) and values represent the mean ± se of 4-7 fish. * denotes significantly different from solvent control (p<0.05)

4.13: Discussion

Comparison of the effect of *in vitro* endosulfan exposure on the phagocytosis of head kidney cells from four different Australian species showed contrasting results. High endosulfan concentrations (≥ 10 mg/L) caused a suppression of rainbowfish phagocytosis, while the same concentration significantly increased the phagocytosis of golden perch (figures 4.2 and 4.4). It is possible that the increase in golden perch granulocyte activity is an endosulfan-induced differentiation of immature granulocytes exerted through its endocrine disrupting properties. Endosulfan has been reported to act on estrogen receptors and STAT3 pathways (i.e. IL-6 signal transducer) in a similar manner to leukaemia inhibiting factor which induces differentiation of granulocytes and macrophages (Metcalf *et al*., 1988; Sekine *et al*., 2004).
Although estrogen receptors have not been identified on the surface of fish leukocytes, they have recently been identified on human myeloid monocytic cells (Cutolo et al., 2001).

Golden perch lymphocytes were slightly sensitive to in vitro endosulfan, while in Murray cod the granulocyte subpopulation appeared to be sensitive at 10 mg/L (figure 3.4 and 3.5). Silver perch head kidney cells were unaffected by all of the in vitro endosulfan concentrations investigated in these experiments (figure 3.3). Therefore, it is difficult to rank the order of sensitivity for these species, except to say that silver perch was the least sensitive and all other species showed some immune-related sensitivity at the highest dose of 10 mg/L.

In vitro endosulfan exposure at 10 mg/L was not toxic to Murray cod lymphocytes, as measured by flow cytometry in the phagocytosis assay (figure 4.5c). However, this dose did reduce the proliferation of Murray cod head kidney lymphocytes in the presence and absence of mitogen. This suggests that a high dose of endosulfan may suppress T-cell proliferation without altering lymphocyte counts, possibly through its toxicity directed towards the granulocyte subpopulation, as was shown by flow cytometry. Granulocytes modulate/stimulate lymphocyte function through the secretion of cytokines and this project found that mixed cell cultures (i.e. head kidney) performed better in the mitogenesis assay compared to cultures containing only lymphocytes (i.e. spleen). This was attributed to the support received through cytokine signalling and endosulfan-induced toxicity towards granulocytes may have eliminated this support.

In vivo endosulfan exposure did not have a major effect on the body weight, organ weights or immune functions of rainbowfish (table 4.4). There was an increase in cell yield from the head kidney at the lowest dose, while the highest dose caused a reduction in the number of lymphocytes from this organ. Murray cod were clearly more sensitive to acute in vivo endosulfan exposure than rainbowfish. Three of the four Murray cod died after exposure to
endosulfan at 10 mg/kg, whereas all rainbowfish survived this dose (table 4.5). Immunotoxicity was evident in Murray cod exposed to endosulfan, but a dose-response relationship was not observed. Fish dosed with 0.08 mg/kg had significantly lower proliferation indices and a significant increase in non-viable cells. Higher doses resulted in a decreased proliferation, but to a lesser extent, and both basal and PHA-stimulated mitogenesis was lower in the 2 and 10 mg/kg groups (figure 4.8).

Endosulfan targets the GABA\textsubscript{A} receptors that are present in the brain. This suggests that if endosulfan possesses any immunotoxic properties it would very likely be through an indirect mechanism, via affects on the neuro-immuno-endocrine system. Indeed, other known inhibitors of the GABA/Cl\textsuperscript{-} ionophore complex also caused immunosuppression in mice (Devoino et al., 1994). The exact mechanism was not investigated by these researchers, however Pandey (1986) has reported that endosulfan also disrupts corticosteroidogenic and catecholamine-secreting cells causing an increase in the secretion of immunosuppressive corticosteroids. Therefore, endosulfan may exert its effects through a stress-like response, causing a release of immunosuppressing hormones such as cortisol. The response of a fish to circulating cortisol may also vary depending on a number of factors such as temperature, age and nutritional status.

Overall, there are some indications that endosulfan is able to modulate immune functions of Australian native fish. However, the results differ markedly between the different species investigated in this study. Ultimately, there may be other factors that could play a more important role in determining the outcome. Factors such as fish age and diet were some obvious differences between species in these experiments. The rainbowfish used in this experiment were relatively older than the other species and, by necessity, each fish species
were maintained on different diets. Further experiments are required to conclude whether diet and age do affect the toxicity of endosulfan.

In conclusion, endosulfan was highly toxic to Murray cod and rainbowfish, with its acute toxicity being more severe than the immunotoxic responses observed. Therefore, if ingested in one high dose endosulfan is more likely to kill Murray cod before it exerts serious immunosuppressive effects. However, endosulfan persists in the sediment of Australian rivers and can accumulate in sediment dwelling organisms and their predators. Murray cod being the top predators of the Murray-Darling basin may be continuously exposed to low levels of contamination throughout the year via their diet and water. In times of high rainfall, they may also be exposed through eating terrestrial invertebrates that enter the waterways, which may be contaminated with endosulfan (Nowak and Julli, 1991). Therefore, chronic feeding studies should be conducted to determine if low long-term oral doses of endosulfan result in any impacts on immune system function. While, studies reported here indicate that endosulfan is only likely to directly affect immune cells at high concentrations, mechanisms of in vivo immunosuppression could be further investigated by measuring the release of corticosteroids.
Chapter 5: The effect of chlorpyrifos exposure on the immune functions of Australian freshwater fish.

5.1: Introduction: Chlorpyrifos (CAS No. 2921-88-2)

Chlorpyrifos is a broad-spectrum organophosphate (OP) insecticide that has been registered for use in Australia since 1965 and is used globally for crop protection and pest control. The application of organophosphate pesticides to crops escalated in the 1970’s when they began replacing organochlorine pesticides, which have longer persistence in aquatic and terrestrial environments (Barron and Woodburn, 1995). Chlorpyrifos is effective against a wide range of arthropod and insect pests including Coleoptera, Diptera, Homoptera and Lepidoptera species. The majority of chlorpyrifos used in Australia is for the control of termites in new and existing buildings, but it is also used for the protection of crops such as cotton, sugarcane, vegetables and fruit. Its application is particularly suitable for integrated pest management, as it is relatively less harmful to beneficial species compared to other pesticides (Childers et al., 2001). Furthermore, it is an important tool in rotation with other chemicals in pest resistance management programs. In 2000, there were 164 registered products containing chlorpyrifos (NRA, 2000). When it first entered the market it was primarily used to reduce mosquito numbers in wetlands, but it is now used minimally for that purpose in Australia due to its toxicity against non-target aquatic species (Kamrin, 2000).

5.2: Structure and physical properties

Chlorpyrifos (O,O-Diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) belongs to a group of organophosphorus compounds known as phosphorothioates (figure 5.1).
Figure 5.1: The chemical structure of chlorpyrifos.

5.3: Mode of action

Chlorpyrifos exerts its primary action through inhibition of acetylcholinesterase enzymes (AchE), although the parent compound is a relatively weak anticholinesterase. Chlorpyrifos is activated in the liver by microsomal cytochrome P450’s to an active oxon (phosphate ester) metabolite (Sultatos, 1991). In humans, CYP 1A2, 2B6 and 2C19 are specifically involved in producing this oxon metabolite, which is approximately 3 orders of magnitude more potent than chlorpyrifos as an anticholinesterase (Buratti et al., 2002; Vittozzi et al., 2001). Primarily, chlorpyrifos oxon reversibly phosphorylates the active site of AchE, inhibiting the breakdown of the neuronal messenger acetylcholine (ACh). This causes an accumulation of ACh at synapses and neuromuscular junctions resulting in hyperexcitability of the nervous system (Costa et al., 2003). Although the reaction is reversible and AchE can be reactivated, the rate of reactivation after organophosphate binding is much slower compared to ACh. Additionally, there is a time-dependent loss of ability to be reactivated that occurs when AchE interacts with organophosphate pesticides. This “aging” process occurs when one of the chlorpyrifos ethyl groups is cleaved and a charged monosubstituted phosphoric acid residue is left in the active site of AchE (Moretto and Johnson, 1986).

Studies investigating chlorpyrifos-induced neurotoxicity and immunotoxicity have led to the conclusion that AchE inhibition is not the primary or only target in these conditions. The
exact mechanisms of action remains unclear, however a number of alternative targets have been identified and include neuropathy target esterase (Glynn et al., 1998), carboxylesterase, butyrylcholinesterase, nicotinic acetylcholine receptors (Katz et al., 1997), cannabinoid receptors (Quistad et al., 2002) and the aryl hydrocarbon receptor (Long et al., 2003). Chlorpyrifos can bind to and/or phosphorylate these proteins, most of which are involved in the regulation of some homeostatic processes. Chlorpyrifos interaction with these targets has been shown to cause a range of other biochemical and physiological effects such as, modification of specific transcription factor expression, Ca\textsuperscript{2+} flux and secretion of cytokines (see section 5.5.3: immunotoxicity).

### 5.4: Toxicokinetics

Chlorpyrifos is rapidly absorbed following oral exposure and it is quickly bound to macromolecules and transported to the liver. Essentially, all the dose undergoes first pass metabolism before entering the systemic circulation (Nakatsugawa, 1992). The plasma half-life of chlorpyrifos in rats is 17 hr, however in fish it is markedly longer at 4.5 d (Racke, 1993).

The esterases that metabolise OPs are classified as A-esterases (i.e. arylesterase and paraoxonase), and are not inhibited by chlorpyrifos oxon, as they are able to hydrolyse the diethyl thiophosphate group of the oxon metabolite. Paraoxonase activity varies between species and administration of this enzyme protects the host from chlorpyrifos poisoning (Li et al., 1995). There is also a direct correlation between LD\textsubscript{50} values and its activity (Costa et al., 2003). B-esterases (carboxylesterases and cholinesterases) are inhibited by chlorpyrifos oxon as they bind the substrate but are unable to hydrolyse it.
Chlorpyrifos metabolites or their glucuronide conjugates are rapidly cleared from tissues and are excreted primarily via the urine. A smaller amount (10-15%) of chlorpyrifos is excreted in the faeces unchanged and low concentrations of chlorpyrifos remain in tissues for several days (Risher and Navarro, 1997; Smith et al., 1966).

5.5: Chlorpyrifos toxicity data

5.5.1: Acute, subchronic and chronic

Oral LD$_{50}$ values for chlorpyrifos in mammals ranges from 96 mg/kg for rats to over 2000 mg/kg for rabbits. In comparison to rats, the oral LD$_{50}$ for rabbits is higher, due to a 7-fold greater activity of the detoxifying enzyme paraoxonase (Costa et al., 1986). Cholinesterase inhibition is the most sensitive and consistent indicator of OP exposure (Carr and Chambers, 1996). Subchronic dietary studies established a NOEL for brain cholinesterase inhibition at 1.0 mg/kg/d, while lower doses (i.e. 0.1 mg/kg/d) caused inhibition of plasma and RBC cholinesterases. Chronic dietary studies reported a NOEL of 0.7 mg/kg/d for brain, plasma and RBC cholinesterase inhibition, but doses up to 32 mg/kg/d, did not reduce survival rates or increase the incidence of cancer (NRA, 2000). Chlorpyrifos does not interact with mammalian genetic material and long-term exposure studies in animals have provided no evidence that chlorpyrifos may cause cancer in humans (Gollapudi et al., 1995). The effects of chlorpyrifos in young and developing animals have indicated that infants and children are not considered to be at an increased risk from chlorpyrifos (Breslin et al., 1996; McCollister et al., 1974).

5.5.2: Neurotoxicity

In addition to AchE inhibition, OP insecticides may also target other proteins leading to the development of a different set of pathological conditions namely OP-induced delayed neurotoxicity (OPIDN) (Moretto and Johnson, 1986). The exact mechanism of OPIDN is still
being investigated, but it is known to involve the inhibition of neuropathy target esterase (NTE), which leads to degeneration of axonal neurons (Richards et al., 1999). NTE is an esterase found in neurons and lymphocytes and has serine esterase activity. Inhibition requires that a negatively charged species be bound to the catalytic serine residue (i.e. “aging”), much like cholinesterases, except that the process is more rapid with NTE. The exact function of NTE is unknown but it is thought that the non-esterase activity of NTE, which is sensitive to OP-mediated aging, may be important in axonal maintenance (Glynn et al., 1998).

### 5.5.3: Immunotoxicity

A number of studies have reported OP-induced immunotoxicity in mammals. In general, most have reported stimulation of immune functions at lower doses and suppressed immune functions with exposure to acute doses sufficient to cause neurotoxicity or AchE inhibition (Galloway and Handy, 2003). The exact molecular mechanism and targets of chlorpyrifos in the immune system remains unclear. Theoretically, immune related targets might be any of the serine hydrolase class enzymes, which are important in complement and thrombin processes, or acetylcholine receptors (AchR) that exist on the surface of lymphocytes. Additionally, organophosphates are reactive and may cause damage through direct oxidative damage to membranes (Galloway and Handy, 2003).

In rats, chlorpyrifos exposure causes hypothermia followed by a fever, which has been shown to be due to the release of the pro-inflammatory cytokine TNF-α (Rowsey and Gordon, 1999). Blakley and associates (1999) dosed rats with 5.0 mg/kg twice a week for 1 month and reported a reduction in T-cell mitogenesis, but no effect on B-cell mitogenesis or phagocytic functions. In addition, neonatal rats that were exposed to low levels of chlorpyrifos displayed impaired T-cell mitogenesis in adulthood (Navarro et al., 2001). Human subjects who
showed flu-like symptoms and had been chronically exposed to chlorpyrifos displayed a
decrease in their T-cell mitogenic activity and an increase in circulating multi-organ
to autoantibodies. Additionally, they had increased CD26 expression (also known as dipeptidyl-
peptidase IV, which plays a role in T-lymphocyte activation) and a decrease in CD5
expression (mature T-lymphocytes) (Thrasher et al., 2002). As a known target of
chlorpyrifos, NTE is also found in lymphocytes and its inhibition has been used as a
biomonitoring tool (Maroni and Bleecker, 1986). It’s function in the nervous and immune
system is unknown, but as it is thought to be a “house-keeping” molecule within the brain, it
is likely to have a similar function in lymphocytes (Glynn et al., 1998).

Lymphocytes posses a non-neuronal cholinergic system that includes ACh, choline
acetyltransferase (ChAT, i.e. ACh synthesising enzyme) and both nicotinic and muscarinic
acetylcholine receptors (nAChR and mAChR, respectively) (Kawashima and Fujii, 2000).
This immuno-cholinergic system plays a role in regulation and stimulation, and agonists of
nAChR and mAChR cause a variety of functional and biochemical effects including an
increase in intracellular Ca\(^{2+}\), up-regulation of c-fos mRNA (AP-1 transcription factor
protein), stimulation of NO production and an increase in IL-2 mRNA (Kawashima and Fujii,
2003a). Stimulation of T- and B-cells with mitogens results in an enhanced synthesis and
release of ACh and up-regulation in the expression of ChAT and mAChR mRNAs. This
evidence indicates that the immune system is at least in part under the control of a non-
neuronal cholinergic system (Kawashima and Fujii, 2003b). Chlorpyrifos and chlorpyrifos
oxon are both able to bind to activated nAChR from skeletal muscle, an action that is
independent of AchE inhibition. Katz and co-workers (1997) showed that chlorpyrifos at 0.3
mM inhibited the binding of a known nAChR ligand ([\(^3\)H]-thienyl-cyclohexylpiperidine) by
52%. This concentration is below the concentration required for AchE inhibition and results
in desensitisation of the receptor. The researchers suggest that binding of chlorpyrifos to the
receptor occurs at an allosteric site distinct from the binding site of ACh. The desensitisation of nAChR on lymphocytes has not been investigated, but could have implications for the cells’ ability to respond to intercellular signalling.

Chlorpyrifos’ interference of transcription factors (i.e. AP-1 and SP-1) may also have a significant effect on the activated immune system, which is in a high state of proliferation and differentiation. AP-1 (Fos/Jun) proteins have a major role in T-lymphocyte development and function. Specifically, they are proteins that bind to promoter regions of DNA and increase the expression of cytokines and FAS ligand (i.e. CD95L), which are important in cellular signalling, apoptotic control and immune homeostasis. AP-1 has a binding site in the promoter region of Fas ligand and up-regulates its expression in activated T-cells, while SP-1 has been implicated in maintaining basal levels of Fas ligand within T-cells (Li-Weber and Krammer, 2003). Dysfunction of the Fas/Fas ligand system leads to severe diseases and mice with a defective gene for Fas ligand display strong lymphoproliferative disorders and autoimmune diseases (Brunner et al., 2003).

5.6: Sources of environmental contamination

The NRA has reported that importation of chlorpyrifos into Australia for the 1996-97 financial year approached 1000 tonnes (NRA, 2000). The use of chlorpyrifos as a termiticide poses the greatest risk to aquatic environments due to the fact that a high rate of application is required i.e. over 500 kg/ha compared to less than 5 kg/ha for crops. Modern construction techniques often results in the simultaneous spraying of large sections of new estates with up to 1000 kg/ha of chlorpyrifos (NRA, 2000). Heavy fish kills in adjacent waterways have been predicted and observed.
The Queensland government have published reports of environmental chlorpyrifos levels that achieved 100 times the lethal dose for fish, along with fish kills containing up to 1,000 individuals. A higher rate of incidences occur in Queensland due to heavier rainfall in the tropical and subtropical climates and the vicinity of new residential estates to waterways (Boyd et al., 2002).

5.7: Chlorpyrifos in the environment and its fate

Degradation of chlorpyrifos (and eventual mineralisation) occurs via photolysis, hydrolysis and metabolism by biota. The significance of photolysis in the degradation of chlorpyrifos still remains unclear, as hydrolysis is usually occurring simultaneously. The time chlorpyrifos remains in an environmental compartment is dependent on many environmental variables. After being applied to plant foliage, the half-life of chlorpyrifos is less than 1-7 d, with most of the dose volatising. Soil surface half-lives are generally a few days to a couple of weeks, whereas subsurface half-lives are longer 1-2 months. After entering the aquatic environment, chlorpyrifos concentrations quickly decline as the hydrophobic chemical partitions into sediments where slow to moderate degradation occurs (half-life 1-16 d) (Racke, 1993). The movement and timing of chlorpyrifos through various environmental compartments has significant implications for laboratory studies. Repeat-pulse studies reflect the environmental reality more closely than chronic studies, due to the fact that chlorpyrifos entering the water column only remains there for less than 24 hr.

5.8: Environmental levels of chlorpyrifos

Chlorpyrifos contamination of waterways resulting from agricultural applications has generally been below 1 μg/L (NRA, 2000). General pesticide monitoring programs in agricultural areas in the USA, France, Canada and Australia rarely find chlorpyrifos in surface waters (Racke, 1993). The low persistence of chlorpyrifos in the aquatic environment is due
to its tendency to volatise from the surface or to partition to the sedimentary layer. Chlorpyrifos does not readily leach from soils, which limits the amount of runoff from farms. However, surface runoff may deliver high concentrations of chlorpyrifos to riverine systems if unpredictable storms occur soon after treatment. This is likely to be a particular problem in cotton growing areas where chlorpyrifos is used in high volumes at a time when summer storms may occur (Boyd et al., 2002).

Interestingly, some of the highest levels of chlorpyrifos detected in surface waters have been from Australian reports. There have been reports of surface water levels reaching over 4 µg/L and, on rare occasions, levels reaching 25 µg/L were found in rice farming areas (Boyd et al., 2002). Passive samplers accumulated chlorpyrifos to levels as high as 26 µg/L in a monitoring program during 1998, which was thought to be a result of high pulses entering the rivers. Persistent contamination occurs in certain areas during spring where concentrations between 0.01 and 0.1 µg/L prevail through September and October with levels occasionally approaching 20 µg/L (NRA, 2000). Mathematical predications indicate that excessive spray drift can give rise to levels of contamination that are capable of killing fish (NRA, 2000).

5.9: Chlorpyrifos levels in fish

Due to its lipophilic nature, fish are able to absorb and bioconcentrate chlorpyrifos to moderate or high levels and variable bioconcentration factors (BCF) between 100-5100 have been reported (Racke, 1993). A BCF of 1374 has been recorded for rainbow trout (Murphy and Lutenske, 1986), 1673 for fathead minnow (Jarvinen et al., 1983) and 745 for oysters (Thacker et al., 1992). Experiments that applied a higher concentration of chlorpyrifos reported a larger BCF, while field experiments conducted in small lakes still reported high BCFs of 1333-4667, despite the low levels of chlorpyrifos exposure (NRA, 2000). In most experiments equilibrium was quickly achieved and residues depurated rapidly in clean water,
with typical half-lives of about 2 d. Chlorpyrifos is quickly metabolised and detoxified by fish and aquatic invertebrates and subsequently does not remain in the food chain (Barron and Woodburn, 1995).

Chlorpyrifos tissue concentrations from field and semi-field studies are less commonly reported than BCFs from laboratory studies. However, in Australia, the Queensland government has documented incidences of chlorpyrifos contamination in canals and waterways and concentrations of chlorpyrifos up to 14 mg/kg have been found in the tissue of fish from contaminated waters (NRA, 2000).

5.10: Aquatic ecotoxicology

Extensive testing has shown chlorpyrifos to be highly toxic to fish, aquatic arthropods, shellfish and algae (figure 5.3) (NRA, 2000). Acute LC$_{50}$ values for freshwater and marine fish have been between 2-520 µg/L, but were generally below 100 µg/L (figure 5.2). The bluegill sunfish has been the most sensitive species with an LD$_{50}$ of around 2 µg/L, while mosquitofish were the least sensitive with an LD$_{50}$ of 520 µg/L (USEPA, 1999). A 96 hr LC$_{50}$ value of 24.6 mg/L has been reported for crimson-spotted rainbowfish and the only other Australian fish species tested for chlorpyrifos sensitivity was Galaxias maculatus, which had a reported LC$_{50}$ of <4.0 mg/L (Warne et al., 1998). For invertebrates, acute LC$_{50}$ values are typically in the 0.1-10 µg/L range and algal endpoints are typically above 100 µg/L (NRA, 2000). Chronic exposure of aquatic organisms generally results in NOECs of <1.0 µg/L. Rainbow trout studies reported a NOEC of 0.5 µg/L, while there was complete mortality at 2-3 µg/L (USEPA, 1999).
5.11: Aim of the study

The aim of this study was to investigate the immunotoxic properties of chlorpyrifos in freshwater Australian fish native to the Murray-Darling Basin. This addressed the paucity of data available in native species for organophosphate pesticides and adds to the body of literature available on the immunotoxic properties of chlorpyrifos in teleosts.

5.12: Methods

5.12.1: Spleen and head kidney sampling

Native fish (i.e. rainbowfish, silver perch, golden perch and Murray cod) were housed and maintained as described in section 2.1. Fish were anaesthetised and killed as described in section 2.2. For the in vivo experiments, fish weight was recorded immediately before injection and then after the 14 d study period, before tissue sampling. The percentage of body weight gained or lost was calculated as: (pre-exposure – post-exposure weight) / pre-exposure weight x 100.

Immune tissues were sampled as described in section 2.3. Somatic indices were calculated as: (organ weight (g)/body weight (g)) x 100.

5.12.2: Cell isolation

The methods for the isolation of fish immune cells are described in detail in section 2.3 and is also briefly described in the organotin study section 3.13.2. Cell yield was calculated as; total number of cells collected / organ weight (g).
5.12.3: Exposure protocols

**In vitro exposures**

Chlorpyrifos was purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA). Concentrated stock solutions were dissolved in dimethyl sulfoxide (i.e. DMSO, BDH Chemicals, Poole, UK) for each concentration level and then diluted 1:100 in TCM for addition to the incubation as one quarter of the final incubation volume, resulting in a final DMSO concentration of 0.25% v/v (i.e. 1:400 dilution). The final concentrations of chlorpyrifos tested were 0, 0.1, 1 and 10 mg/L (i.e. ppm).

**In vivo exposures**

Fresh stock solutions of chlorpyrifos were diluted in PEG (polyethylene glycol) at 2.5 g/mL and were kept at 4°C for no more than 1 week. On the day of use, the stock was diluted 1:100 in PEG to give a high concentration of 25 mg/mL. This was serially diluted 1:5 in PEG to attain medium and low concentrations of 5 and 1 mg/mL. Fish were dosed with 2 mL/kg, achieving exposures of 2, 10 and 50 mg/kg.

The chlorpyrifos in vivo exposure protocols for rainbowfish and Murray cod are shown in table 5.1 and 5.2. Controls were combined and protocols were performed as described for the organotins (section 3.13.3).

**Table 5.1: Chlorpyrifos in vivo exposure protocol for rainbowfish**

<table>
<thead>
<tr>
<th>Day of study</th>
<th>Untreated control</th>
<th>0 (Solvent control)</th>
<th>2</th>
<th>10</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1-14</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Day 2-15</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
This dose range was chosen in an attempt to determine the lethal concentration of chlorpyrifos in Murray cod and rainbow fish at high doses, however the lower concentrations reflect concentrations reported in the tissue of fish from contaminated areas (see section 5.9). The reason for using an i.p. injection as the route of administration is discussed in section 3.13.8

### 5.12.4: Phagocytosis

Standardised protocols for the phagocytosis assay are described in detail in section 2.4.2 and the methods used in this experiment are the same as the organotin study described in section 3.13.4.

### 5.12.5: Mitogenesis

Standardised protocols for the mitogenesis assay are described in detail in section 2.6.2.2 and the methods used in this experiment are the same as the organotin study described in section 3.13.5.

### 5.12.6: Lysozyme

Standardised protocols for the lysozyme assay are described in detail in section 2.5.3 and the methods used in this experiment are the same as the organotin study described in section 3.13.5.

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### Table 5.2: Chlorpyrifos in vivo exposure protocol for Murray cod

<table>
<thead>
<tr>
<th>Day of study</th>
<th>Untreated control</th>
<th>0 (Solvent control)</th>
<th>2</th>
<th>10</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1-14</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 2-15</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 5-19</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 6-20</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
5.12.7: Statistics

Statistics were performed using the same methods described in the section 3.13.7.

5.13: Results

5.13.1: The effect of \textit{in vitro} chlorpyrifos exposure on the phagocytosis of head kidney cells from four species of Australian native fish

In all species studied, \textit{in vitro} chlorpyrifos treatment did not have a significant effect on either the number of cells with beads (i.e. FITC +ve) or the number beads per cell (i.e. mean FITC voltage) (figure 5.2a – 5.5a).

Golden perch and Murray cod lymphocytes showed a slight linear decrease in lymphocyte numbers following \textit{in vitro} chlorpyrifos exposure but this only significantly reduced in Murray cod exposed to 50 mg/kg (figures 5.4b & 5.5c). In addition, \textit{in vitro} chlorpyrifos exposure at 10 mg/L caused a variable 50\% increase in the number of non-viable Murray cod head kidney cells (PI +ve), suggesting that this concentration may have been toxic to some of the samples (figure 5.5d). In contrast, rainbowfish and silver perch did not show any significant change in head kidney subpopulation numbers (figure 5.2b and 5.3 b). Comparison of the four species shows that lymphocyte populations were the most likely targets \textit{in vitro}, however high concentrations only significantly reduce their numbers in one of the four species. Silver perch and rainbowfish, were the least sensitive species and displayed no signs of toxicity from \textit{in vitro} chlorpyrifos exposure.
**Figure 5.2:** The effect of *in vitro* chlorpyrifos exposure on rainbowfish head kidney cells. 

a) Phagocytosis (FITC +ve) and number of beads per cell (mean voltage)  
b) Granulocytes and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=6) and values represent mean ± se of 4-6 fish. Untreated control values were 20±3% for FITC +ve granulocytes, 8±1 volts for mean voltage, 3350±300 events for granulocytes and 3050±600 events for lymphocytes. No statistically significant differences were found.

**Figure 5.3:** The effect of *in vitro* chlorpyrifos exposure on silver perch head kidney cells. 

a) Phagocytosis (FITC +ve) and number of beads per cell (mean voltage)  
b) Granulocytes and lymphocyte subpopulation counts. Data expressed as percentage of untreated control samples and (n=4) represent mean ± se of 4 fish. Untreated control values were; 37±2% for FITC +ve granulocytes; 216±31 volts for mean voltage; 2000±150 events for granulocytes and 1400±150 events for lymphocytes. No statistically significant differences were found.
Figure 5.4: The effect of *in vitro* chlorpyrifos exposure on golden perch head kidney cells. a) Phagocytosis (FITC +ve) and number of beads per cell (mean voltage) b) Granulocytes and lymphocyte subpopulation counts. Data expressed as percentage of untreated control samples (n=7) and represent mean ± se of 5-7 fish. Untreated controls values were 17±2% for FITC +ve granulocytes, 30±3 volts for mean voltage, 4100±200 for granulocytes and 2130±250 for lymphocytes. No statistically significant differences were found.
Figure 5.5: The effect of \textit{in vitro} chlorpyrifos exposure on Murray cod head kidney cells. 

a) Phagocytosis (FITC +ve) b) number of beads per cell (mean voltage & side scatter (SS) increase) c) Granulocytes and lymphocyte subpopulation counts d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated controls values were; 14±1 for FITC+ve total, 20±2% for FITC +ve granulocytes, 108±10 volts for mean voltage, 113±12 volts for SS increase, 6900±300 for granulocytes, 1400±100 for lymphocytes, 1200±140 events for debris and 6±1% for PI+ve. * denotes significantly different from solvent control (p<0.05).
5.13.2: The effect of *in vitro* chlorpyrifos exposures on Murray cod immune functions

**Mitogenesis**

*In vitro* exposure of Murray cod head kidney lymphocytes to chlorpyrifos had no significant effect on T-cell mitogenesis, which is stimulated by PHA (table 5.3). Therefore Murray cod T-cell proliferation was unaffected by *in vitro* chlorpyrifos exposures up to 10 mg/L.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chlorpyrifos concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Proliferation index*</td>
<td>110.7±12.0</td>
</tr>
<tr>
<td>Peak CPM*</td>
<td>1219±307</td>
</tr>
</tbody>
</table>

*Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated control values 1.29±0.11 for proliferation index. No statistically significant differences were found.*

5.13.3: The effect of *in vivo* chlorpyrifos exposure on rainbowfish immune parameters

In the present study rainbowfish were treated (by i.p. injection) with chlorpyrifos up to 50 mg/kg. After 14 days all fish (including the solvent control) lost weight, while in the 10 mg/kg group there was large variation as one fish lost 10% of their body weight and one fish gained 10% of their body weight (table 5.4). Consequently, chlorpyrifos treated fish did not exhibit a significant difference in body weight change compared to controls. Chlorpyrifos treated rainbowfish displayed a dose-dependent reduction in the somatic indices of head kidneys, while cell yields appeared to be reduced at all doses (table 5.4).
Table 5.4: The effect of *in vivo* chlorpyrifos exposure on rainbowfish body and organ weights

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chlorpyrifos concentration (mg/kg)</th>
<th>0</th>
<th>2</th>
<th>10</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in body weight (%)</td>
<td></td>
<td>-6.31±2.03</td>
<td>-8.70±3.10</td>
<td>-0.36±4.81</td>
<td>-3.98±2.66</td>
</tr>
<tr>
<td>Head kidney Somatic index</td>
<td></td>
<td>2.36±0.36</td>
<td>2.03±0.19</td>
<td>1.47±0.19</td>
<td>0.94±0.50†</td>
</tr>
<tr>
<td>Head kidney Cell yield a</td>
<td></td>
<td>3.08±1.10</td>
<td>1.54±0.41</td>
<td>1.91±0.49</td>
<td>1.34±0.51</td>
</tr>
</tbody>
</table>

*a* Cell yield data is expressed cells x 10⁶/mg of head kidney tissue. Values represent the mean ± se of 4 fish (except for 50 mg/kg where n=3). † denotes linear relationship (p<0.05).

None of the chlorpyrifos treatments affected the phagocytic function of rainbowfish head kidney cells (figure 5.6). There appeared to be an increase in lymphocyte numbers at 2 and 10 mg/kg, however the variation in response prevented this from reaching statistically significance. Granulocyte numbers did not significantly change in response to chlorpyrifos at any of the concentration tested (figure 5.6b).

![Figure 5.6: The effect of *in vivo* chlorpyrifos exposure on rainbowfish head kidney cells. a) Phagocytosis and number of beads per cell b) Granulocyte and lymphocyte subpopulation counts. Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated control values were 38±1% for FITC +ve granulocytes, 70±6 volts for mean voltage, 5259±350 events for granulocytes and 800±200 events for lymphocytes. No statistically significant differences were found.](image-url)
5.13.4: The effect of *in vivo* chlorpyrifos exposure on Murray cod immune parameters

**Body and organ weight changes**

Murray cod dosed with 50 mg/kg displayed obvious signs of neurotoxicity and ceased feeding, however all fish survived the 14 d exposure period. Signs of neurotoxicity included discolouration (pale), and tremors and spasms followed by short periods of unconsciousness. During tissue sampling, the flesh of the high-dosage fish felt rigid to touch and they had clearly lost body weight. Fish from lower dose groups showed no signs of neurotoxicity but also lost some body weight. Chlorpyrifos exposure caused a linear reduction in the percentage body weight of Murray cod (figure 5.7).

Chlorpyrifos caused a dose-dependent reduction in splenic somatic index of Murray cod (figure 5.8a). Spleens from fish dosed with 50 mg/kg were visibly smaller and darker compared to the other dosage groups, which may be attributed to haemorrhaging. In contrast to the spleen, chlorpyrifos caused no significant effect on the somatic indices of Murray cod head kidney (figure 5.8b).

![Figure 5.7: The effect of *in vivo* chlorpyrifos exposure on the body weight of Murray cod. Values represent the mean ± se of 4-7 fish. † denotes a linear relationship (p<0.05).](image-url)
Figure 5.8: The effect of in vivo chlorpyrifos exposure on Murray cod somatic indices. a) Spleen and b) Head kidney. Values represent the mean ± se of 4-7 fish. † denotes a linear relationship p<0.05

Cell yields

There was an average reduction in the cell yields from both the spleens and head kidneys, however large variations between fish were also apparent (figure 5.9). This may be due to the many steps performed in the isolation process, which can lead to cell loss, but this parameter can still be used qualitatively, for comparative purposes. In both the spleen and the head kidney, there was a general reduction in cell yield as the dose was increased. The slight increase in spleen cell yields from 10 – 50 mg/kg, is likely to be a reflection of the fact that fish dosed with 50 mg/kg had a markedly reduced spleen weight and cell yield is represented in the figure as number of cells per gram of tissue.
Figure 5.9: The effect of in vivo chlorpyrifos exposure on the cell yields of Murray cod spleen and head kidney. Values represent the mean ± se of 4-7 fish. No statistically significant differences were found.

**Lysozyme**

The lysozyme activity of Murray cod exposed to chlorpyrifos was not significantly different from the solvent controls (figure 5.10). It is worth noting that the large amount of variation seen in the 50 and 10 mg/kg groups was due to a single fish in each group that had markedly elevated lysozyme activity. This variation may illustrate the range of individual responses that can occur in genetically outbred species.

Figure 5.10: Murray cod serum lysozyme concentration following a single i.p. injection of chlorpyrifos 14 d prior to tissue sampling. Data is expressed as percentage of untreated control (i.e. 6.90±0.98 μg/mL HEWL equivalent, n=8). Values represent the mean ± se of 4-6 fish (one solvent control was lost). No statistically significant differences were found.
**Phagocytosis**

The effect of *in vivo* chlorpyrifos exposure on the phagocytic activity and subpopulation numbers of Murray cod head kidney cells is shown in figure 5.11. Murray cod exposed to *in vivo* concentrations chlorpyrifos showed no significant changes in any of the parameters measured. However, there are some trends worth noting. Mean voltage and side scatter (SS) increase were both decreased at 10 and 50 mg/kg (figure 5.11b). These parameters are measures of the number of beads per phagocyte and the increase in SS resulting from increased granularity of the cells cytoplasm. Although not significantly different, these results may indicate that high doses of chlorpyrifos reduced the rate of phagocytosis by Murray cod head kidney granulocytes but did not affect the number of cells engaged in phagocytosis (i.e. no change in FITC +ve granulocytes).

Subpopulation counts displayed a trend where lymphocytes counts were reduced, while granulocyte counts were increased as a result of *in vivo* chlorpyrifos exposure (figure 5.11c). It is likely that the increase in granulocyte counts were a result of the proportional flow cytometric data where the selective toxicity towards lymphocytes caused an artefactual increase in granulocyte counts (as seen in the organotin studies). This is supported by the somatic index results that showed a greater decrease in splenic somatic index (i.e. containing mainly lymphocyte populations) compared to the head kidney somatic index (i.e. containing a mixed population of cell types). Debris counts were lower in the 2 and 10 mg/kg dosed group, which may be due to better cell preparations that contained less debris at the beginning of incubation or the higher proportion of “more robust” granulocytes in the sample. The difference could also be due to the unintentionally gating out of debris events, as they are of a small size and occur near the forward scatter limit and the “live” gate that excludes unengulfed beads (see section 2.4).
Figure 5.11: Murray cod head kidney phagocytic function and subpopulation counts following *in vivo* exposure to chlorpyrifos. a) Phagocytosis (FITC +ve) b) number of beads per cell (Mean voltage and side scatter (SS) increase) c) Granulocytes and lymphocyte subpopulation counts d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control samples (n=8) and values represent mean ± se of 4-7 fish. Untreated control values were; 10±12% for FITC +ve total; 25±4% for FITC +ve granulocytes; 130±27 volts for mean voltage; 123±14 volts for side-scatter increase; 4050±200 for granulocytes; 3350±200 for lymphocytes; 1600±150 for debris and 800±150 for PI +ve. No statistically significant differences were found.
**Lymphoproliferation**

Fish dosed with chlorpyrifos showed a reduction in their basal and peak responses of lymphocytes to PHA (figure 5.12a). Doses of 50 mg/kg caused a severe reduction in lymphocyte proliferation both in the presence and absence of mitogen and a significant reduction in proliferation index was observed (figure 5.12b). These results clearly show that Murray cod T-lymphocytes were affected by high *in vivo* doses of chlorpyrifos.

**Figure 5.12:** The effect of *in vivo* chlorpyrifos exposure on a) the lymphoproliferation profile and b) proliferative index of Murray cod head kidney cells. Proliferation data is expressed as percentage of solvent control (i.e. 1.37±0.10, n=7) and values represent the mean ± se of 4-7 fish. * denotes significantly different from solvent control.
5.14: Discussion

The head kidney cells from all of the Australian fish examined showed little direct sensitivity to chlorpyrifos, as demonstrated by the in vitro exposures of 0.1-10 mg/L. Murray cod lymphocytes exhibited a slight sensitivity towards increasing doses of chlorpyrifos (figure 5.5). Past research by our group has shown that in vitro malathion exposure caused severe suppression of silver perch mitogenesis at 10 mg/L (O'Halloran et al., 1996b). Not surprisingly, these data indicate that silver perch head kidney cells were more sensitive to malathion than chlorpyrifos, particularly as silver perch appeared to be the least sensitive species in the present study. Overall, the order of sensitivity of these fish species to chlorpyrifos as demonstrated in the flow cytometric phagocytosis assay was; Murray cod > golden perch > rainbowfish = silver perch. Nevertheless, there was little difference between the species due to a lack of toxicity.

Lymphoproliferation was also unaffected by in vitro exposure to chlorpyrifos at concentrations up to 10 mg/L (table 5.3). In rats, exposure to chlorpyrifos at 5.0 mg/kg for 28 d showed that T-lymphocytes are a more sensitive subpopulation to chlorpyrifos exposure in comparison to B-cells or phagocytic cells (Blakley et al., 1999). This effect may be due to the fact that T-lymphocytes possess cholinergic receptors that have been implicated in immune function and regulation and that chlorpyrifos has been shown to bind and desensitise these receptors. Although it is likely that Murray cod possess nAChRs, these non-neuronal cholinergic systems are yet to be identified in Australian fish, however they have been characterised in more primitive fish such as electric ray (Katz et al., 1997). Nonetheless, the in vitro concentrations investigated in this study did not have a direct effect on Murray cod T-lymphocyte proliferation. Although levels above 10 mg/L may not be reached within the head kidney tissue of fish and may not be environmentally relevant, higher in vitro doses
could be investigated to determine if higher concentrations can cause T-cell inhibition or cytotoxicity.

_in vivo_ chlorpyrifos treatment of Australian fish was performed using crimson-spotted rainbowfish and Murray cod. No fish died following _in vivo_ chlorpyrifos exposures up to 50 mg/kg. There was a linear reduction of Murray cod body weight but this was not observed in the rainbowfish (figure 5.7). There were indications that the rainbowfish controls were stressed to some degree, as the group lost weight (table 5.4) The effects of chlorpyrifos exposure were clearly apparent in the Murray cod dosed with 50 mg/kg, which displayed obvious signs of neurotoxicity, including; discolouration (pale appearance), a change in the posture of pectoral fins (protruding forwards instead of being held by the body), cessation of feeding, terrors and spasms followed by a short period of unconsciousness. In addition, when sampling tissue from these fish it was noted that the flesh was rigid to touch. Splenic somatic indices were reduced in a dose-dependent manner and cell yields were also reduced at 10 and 50 mg/kg but this was highly variable between individuals. These gross morphology results from Murray cod suggest that lymphocytes are more sensitive to chlorpyrifos, as the spleen (i.e. containing a cell population that is >90% lymphocytes) was more affected than the head kidney (containing a mixed population of granulocytes 60-70% and lymphocytes 20-30%). The dose-dependent reduction of head kidney somatic indices in rainbowfish might suggest that granulocyte populations are also sensitive in this species, especially as the percentage of lymphocytes was lower in these fish at only around 10% of the head kidney cell population.

Chlorpyrifos showed very little immunotoxicity in rainbowfish exposed to _in vivo_ doses. _In vivo_ chlorpyrifos-treated fish had no change in granulocyte counts but lymphocyte counts in all treated groups were elevated compared to controls (figure 5.6). This result is hard to interpret, especially in light of the somatic index results, which suggests chlorpyrifos induced
atrophy of rainbowfish head kidneys and toxicity towards the cell population. It is worth noting that control fish had a low percentage of lymphocytes (10%) in their flow cytometry counts and an increase of 50% equals a 5% change in flow cytometric counts. It could be interpreted that chlorpyrifos is reducing both subpopulations of rainbowfish head kidney cells and that granulocytes are being depleted at a greater rate. This might cause a slight rise in lymphocyte counts that is significant only because their numbers were low in the beginning.

The results from the subpopulation counts of Murray cod head kidney cells after in vivo chlorpyrifos exposure were more expected. They displayed a very slight reduction in numbers of lymphocytes, with a concomitant increase in granulocyte numbers (figure 5.11). This was expected due the flow cytometry data consisting of a proportional count of 10,000 events, as previously observed in the effects of in vivo DBT exposure on Murray cod head kidney cells (see section 3.14.4). By referring to the organ somatic indices and cell yield data, it is possible to confirm that this result is likely to be a slight selective toxicity to head kidney lymphocytes. In addition, this concurs with mammalian immunotoxicity testing of chlorpyrifos, which reported a selective toxicity to T-lymphocyte populations (Blakley et al., 1999).

The phagocytic activity of rainbowfish head kidney cells was unchanged by in vivo chlorpyrifos exposure. Murray cod dosed with chlorpyrifos displayed a reduction in the number of beads per cell (mean voltage) and side scatter, which is a measure of the increased granularity of the phagocytes cytoplasm as a result of bead engulfment. The reduction in both of these parameters indicate that phagocytes engaged in engulfing beads have, on average, a lower rate of bead uptake leading to the lower number of beads per cell upon sampling.
The lysozyme activity of Murray cod was not significantly changed by \textit{in vivo} chlorpyrifos exposures (figure 5.10). One fish in the highest dose group had a lysozyme activity 2.5 times higher than the average control. If this was an individual response to chlorpyrifos, it would probably be a reaction to cellular damage resulting from a very high chlorpyrifos dose rather than a specific effect. The effect of \textit{in vivo} chlorpyrifos exposure on the lysozyme activity of rainbowfish could not be investigated, as adequate quantities (25 μL) of serum could not be obtained from these small fish.

\textit{In vivo} exposure of Murray cod to chlorpyrifos (via i.p. injection) led to a reduction in the basal and PHA-induced proliferation at all concentrations (figure 5.12). PHA specifically stimulates T-lymphocytes and these results show that chlorpyrifos can suppress Murray cod T-cell mitogenesis at high doses. The effect of \textit{in vivo} chlorpyrifos exposure on lymphoproliferation of rainbowfish head kidney cells was not performed due to technical difficulties previously described (see section 3.15). However, it was unfortunate that this assay could not be performed in rainbowfish, as it would have been interesting to compare the chlorpyrifos-induced effects on mitogenesis with that found in Murray cod.

The fact that chlorpyrifos had no significant effect until it was at very high concentrations indicates that it is highly unlikely to be an environmental immunotoxin. Concentrations of chlorpyrifos that resulted in T-cell immunosuppression in Murray cod also caused obvious signs of severe neurotoxicity, which would probably be of greater significance than the immunosuppression observed. With such obvious signs of neurotoxicity, it is likely that the observed immunosuppression is a result of a disruption of the general homeostasis of the fish and a stress response via the HPI axis, rather than chlorpyrifos exerting selective toxicity towards lymphocytes. Additional evidence for this comes from the \textit{in vitro} results that showed chlorpyrifos had no direct effect on lymphocytes even at similarly high doses.
Environmental levels of chlorpyrifos measured in fish flesh have been reported at over 10 mg/kg in Queensland, Australia (Boyd et al., 2002; NRA, 2000). Murray cod dosed at this concentration showed a slight suppression of somatic index, cell yields and the phagocytic rate of granulocytes, however these results did not reach statistical significance. *In vivo* doses of chlorpyrifos above 2 mg/kg appeared to decrease the number of beads per cell, which indicates a reduction in the rate of phagocytosis. However, the *in vivo* inhibition of phagocytosis is not through the direct action of chlorpyrifos on phagocytes, as no effects are seen *in vitro*. If chlorpyrifos does stimulate phagocytic activity of Murray cod granulocytes, it is probably through an indirect mechanism involving the hypothalamo-pituitary-interrenal axis.

This study has indicated that chlorpyrifos is not likely to cause immunosuppression in Australian freshwater fish. However, a number of other experiments should be conducted to complete the picture. This study used a single i.p. injection of chlorpyrifos followed by tissue sampling 14 d later. However, chlorpyrifos is a fast acting chemical that does not persist in fish and may exert its effects in a shorter time period. Some effects may occur in days or may be seen in a matter of hours. *In vivo* studies that examined earlier tissue sampling time points of 1 d and/or 4 d would investigate this aspect. Chronic, continual chlorpyrifos exposure studies are not environmentally relevant to fish. This is because once chlorpyrifos enters the water it quickly partitions to the sediment, resulting in a pulse exposure to fish in the habitat. Repeat-pulse exposure experiments would reflect the environmental picture more closely and would demonstrate whether chlorpyrifos can cause any cumulative effects. Feeding studies are also not relevant to benthic fish that feed from sediment layers.
Before chlorpyrifos is classified as not immunotoxic in Australian fish, the effect of chlorpyrifos on young and developing fish should be explored. Neonatal rats that were exposed to chlorpyrifos displayed reduced T-cell mitogenesis once they had developed into adults (Navarro et al., 2001). Indeed, much of the current research involving chlorpyrifos involves its effect on the developing brain. It has now been demonstrated that chlorpyrifos can interact directly with developing neural cells to inhibit replication and neuritic outgrowth (Song et al., 1998). The implications of this for aquatic species have not yet been explored, but it could lead to a range of biochemical and physiological disruptions that may lead to immunosuppression in adult fish.
Chapter 6: The effect of immunostimulant exposure on the immune functions of Murray cod

6.1: Introduction

Immunostimulants are a growing group of both natural and synthetic compounds that stimulate the non-specific immune functions of a host and ultimately, increase their resistance to pathogens. They work primarily to stimulate phagocytes and natural killer cells, increasing bactericidal activities and the release of complement, lysozyme and cytokines. These agents differ from vaccines that increase host protection through the production of antigen-specific memory B-cells and also differ from chemotherapies that act directly on the invading organism (e.g. antibiotics). Immunostimulants are often used as adjuvants to enhance vaccine antibody titres and chemotherapy efficiencies. When used alone the protection is often short lived and may only appear in a specific window of time (Anderson, 1992). Interest in their application has been inspired, in part, by the development of resistant pathogens to chemotherapies and the inability to develop vaccines for some diseases (Sakai, 1999). The types of compounds that have been reported to enhance non-specific immune function include bacteria and bacterial products, complex carbohydrates (e.g. β-glucan), nutritional factors (e.g. vitamins), animal extracts, cytokines, lectins, plant extracts and synthetic drugs (e.g. levamisole) (Cook et al., 2003). This study investigated the immunomodulatory effects of the synthetic drug, levamisole and the complex carbohydrate, β(1→3)-glucan (sourced from barley) on Murray cod immune functions.

6.2: Levamisole (CAS no. 16595-80-5)

Levamisole was first introduced in 1966 as an anthelmintic that acts on the nicotinic acetylcholine receptors on the surface of nematodes (Köhler, 2001). Soon after its
immunostimulating properties were observed as a side effect in treated animals (Renoux, 1978). In the late 1970’s, interest in the drug was high as many researchers were reporting that levamisole could also stimulate immune functions that were depressed in patients afflicted with chronic inflammations (Alsaran et al., 2001; Zulman et al., 1978). Animal studies also reported anti-metastatic properties and levamisole has been used for the last 25 years in the treatment of cancer (Wiebke et al., 2003).

6.2.1: Structure of levamisole

Levamisole (L-2,3,5,6-tetrahydro-6-phenyl-imidazo [2,1-b] thiozole) is a synthetic imidazo-thiazole derivative and levo-isomer of tetramisole (figure 6.1a).

![Figure 6.1: The structure of levamisole and its metabolites. a) Levamisole (L-2,3,5,6-tetrahydro-6-phenyl-imidazo[2,1-b]thiozole) b) PDT 6-phenyl-2,3-dihydroimidazo [2,1-b] thiazole and c) OMPI (L-2-oxo-3-(2-mercaptoethyl)-5-phenyl-imidazolidine) (Hanson et al., 1991).](image)

6.2.2: Toxicokinetics of levamisole

Levamisole is a fast and short acting drug, which is absorbed rapidly from the gastrointestinal tract or from any other administration site (Stogaus and King, 1995). It has a wide distribution and access to the peripheral compartment, with the highest concentrations of the
drug found in the liver and the kidney (Graziani and De Martin, 1977a; Pereda et al., 2002). The half-life of levamisole in humans is 4 hr and the drug is completely eliminated in 2 days (Graziani and De Martin, 1977b; Symoens and Rosenthal, 1977).

Levamisole is quickly and extensively metabolised by 4 principal pathways in the liver, with less than 5% of the parent drug being excreted (Graziani and De Martin, 1977a). Most levamisole (95%) is eliminated after biotransformation in the liver but a small portion of the parent compound may be passed by the kidneys or in the faeces (Stogaus and King, 1995).

6.2.3: Toxicity data

Levamisole is generally considered safe and a single dose of 2.5 mg/kg has no adverse effects in humans (Graziani and De Martin, 1977b). In rats, oral dosages up to 100 mg/kg do not appear to cause mortality, while fertility and the number of birth defects in rats and rabbits appear unaffected by levamisole (Renoux, 1980). Chronic studies have shown that levamisole doses of 2.5 mg/kg given for 12-18 months did not cause any carcinogenicity in rats and dogs. The intraperitoneal administration of a high dose (25 mg/kg/d) to mice for 1 month did not change the haematology of the animals, however, there was an observed immunosuppression of mitogenesis (Renoux and Renoux, 1977). Levamisole is not considered teratogenic or genotoxic (Renoux, 1980).

6.2.4: Levamisole mechanism of action

6.2.4.1: Immunomodulation of levamisole at the cellular level

In mammals, levamisole has been reported to increase immune functions of T-cells and phagocytes (Van Wauwe and Janssen, 1991). Levamisole’s ability to enhance macrophage activities is critically dose-dependent and numerous enhanced functions have been reported such as phagocytosis, chemotaxis, random migration, adherence, intracellular killing, IgG and
C3 binding (Renoux, 1980). It also induces the differentiation of immature spleen and lymph node cells into functional T-cells. Levamisole’s ability to increase the number of reactive T-cells has also been shown through the stimulation of DNA synthesis by murine splenocytes in the presence of the drug, without any added mitogen or antigen (Metaye et al., 1990). *In vitro* tests show an enhancement of PHA-induced mitogenesis, however, B-cell enriched spleen cells were not stimulated by levamisole (Renoux, 1980). The stimulation of B-cells is thought to be indirect, via T-cell signalling, and has a narrow range of effectiveness, with high doses causing suppression (Symoens and Rosenthal, 1977; Van Wauwe and Janssen, 1991). The reduction of spleen weights in treated mice indicates that there is activation rather than proliferation of immunocompetent cells (Renoux, 1980).

Although levamisole has some clear immunostimulatory properties there have been reported observations that have implications for its effectiveness. In human patients and rodent models, levamisole appears to work better in immunosuppressed subjects which could be a prerequisite for its effectiveness (Beic et al., 1996). Levamisole also inhibits immune responses at high doses or following long exposure periods. Rheumatoid arthritis patients that received long term doses of levamisole showed reduced levels of IgG and IgM in their circulation (Van Wauwe and Janssen, 1991).

### 6.2.4.2: Immunomodulation of levamisole at the molecular level

Levamisole stimulates parasympathetic and sympathetic ganglia and also inhibits the re-uptake of norepinephrine in sympathetic ganglia (Renoux, 1980; Symoens and Rosenthal, 1977). It potentiates the acetylcholine-evoked responses of both α3β2 and α3β4 human neuronal nicotinic receptors (Levandoski et al., 2003). However, it can either potentiate or inhibit responses depending on the concentrations of both levamisole and acetylcholine. Levamisole inhibition occurs through open channel block and potentiation is through a
reversible non-competitive mechanism. The evidence suggests that levamisole may bind to a pseudo-site on the receptor and does not block the acetylcholine binding to the receptor (Levandoski et al., 2003).

Lymphocytes and mononuclear leukocytes possess nicotinic acetylcholine receptors that regulate their proliferation and function (Kawashima and Fujii, 2003b; Skok et al., 2003). Studies have shown that levamisole exerts cholinergic effects on leukocytes resulting in increased intracellular cyclic nucleotides (i.e. cAMP and cGMP), which is a clear indication that the cell has been signalled to respond (Hadden et al., 1975). It is likely that the parasympathetic nervous system plays a role in immune-neurohumoral cross-talk and that levamisole could modulate immune function through binding of nicotinic receptors in the lymphocytic cholinergic system.

Compared to controls, rats fed levamisole (18 mg/kg) have lower levels of free plasma corticosteroid, which is an endogenous immunosuppressant. This indicates that levamisole either inhibits the synthesis of corticosteroid or increases its metabolism, which may result in an increased immune response (Stogaus and King, 1995). Levamisole also specifically inhibits alkaline phosphatase (ALP), a non-specific protein phosphatase, which removes phosphate groups from molecules to activate/deactivate enzymes, but whose function in vivo is not well known (Soory and Suchak, 2003; Van Belle, 1976). It has also been shown that levamisole and a number of other inhibitors of ALP all enhanced in vitro mitogenesis suggesting that ALP has a negative role in lymphocyte proliferation (Metaye et al., 1990).

6.2.6: Effects on fish immunology

Investigations into the immunomodulating properties of levamisole in fish began in the mid 1980’s as aquaculturalists looked to new forms of disease management. Researchers
discovered that levamisole could aid in the non-specific immune response when given alone and also aid the specific immune response when used as an adjuvant (Anderson, 1992). The immunostimulating properties of levamisole have been demonstrated in a number of fish species both in vitro and in vivo. However, specific effects and effective doses vary between studies and immunosuppression is commonly reported at high doses (Sakai, 1999).

It has been demonstrated that levamisole can stimulate teleost immune responses in vitro. Siwicki and co-workers (1990) reported that spleen cells cultured with levamisole had enhanced phagocytic activity, respiratory burst and an increased number of plaque forming and antibody producing cells against Yersinia ruckeri O-antigen. Other studies have reported an increase in natural cytotoxic activity in seabream (Sparus aurata L.) after the head kidney was exposed to levamisole in vitro (Cuesta et al., 2002). In contrast, Mulero et al. (1998a) found that isolated leukocytes exposed to levamisole showed no signs of phagocytic stimulation and that respiratory burst (measured by NBT reaction) was inhibited at some concentrations.

Siwicki and Studnika (1989; 1987) reported that carp injected with levamisole showed enhanced phagocytic and myeloperoxidase activity in neutrophils, increased leucocyte numbers and serum lysozyme levels. Rainbow trout injected with levamisole showed increased protection against Vibrio anguillarum, which was a result of the enhancement of non-specific immune responses such as phagocytic activity, chemiluminescence responses of leukocytes and NK cell activities. Serum complement concentration also increased in levamisole-injected rainbow trout (Kajita et al., 1990).

Many studies have investigated the use of levamisole as an adjuvant, either alone or in combination with traditional adjuvants (i.e. Freund’s). The majority of studies have found an
increased resistance to pathogens through an increase in antibody response and non-specific defence functions. In addition, *V.anguillarum* vaccination of Atlantic salmon with levamisole showed no histopathologies in the gills, skin, head kidney and spleen of exposed fish. Compared to *V.anguillarum* treated fish alone, levamisole treated fish also had significantly higher antibody titres (Morrison *et al.*, 2001). Coho salmon (*Oncorhynchus kisutch*) injected with levamisole mixed with modified Freund’s complete adjuvant showed increased resistance to *Aeromonas salmonicida* compared to fish that were treated with Freund’s adjuvant alone (Olivier *et al.*, 1985). Midtlyng and co-workers (1996) have reported that furunculosis vaccine administered with levamisole as a adjuvant to pre-smolt Atlantic salmon increased protection compared to unvaccinated fish, for up to six weeks. Bathing rainbow trout for 30 min in levamisole solution 5 μg/mL before a 2 min bath in *A. salmonicida* O-antigen bacterin elevated both the non-specific defence mechanisms and specific antibody titres (Jeney and Anderson, 1993a).

The immunostimulatory effects of dietary levamisole have also been investigated, as oral administration is the most preferred method of exposure by fish farmers. Mulero and co-workers (1998b) fed levamisole to gilthead seabream (*Sparus aurata* L.) and found an increase in growth, phagocytic activity, serum complement and disease resistance. The fish also displayed increased natural cytotoxic activity after they were fed levamisole (up to 300 mg/kg) for 10 d (Cuesta *et al.*, 2002). Oral administration of levamisole to carp (*Cyprinus carpio*) increased the number of leukocytes, serum lysozyme activities and stimulated phagocyte function (Siwicki, 1989). The effect of levamisole on the non-specific immunity of carp has been investigated. Carp fed levamisole over a 12-week period had elevated lysozyme levels in their serum (Alexander and Ingram, 1992). Sahoo and Mukherjee (2002) reported that immunocompromised (i.e. aflatoxin exposed) rohu (*Labeo rohita*) provided with...
dietary levamisole increased specific immunity and disease resistance but did not benefit healthy fish.

Carp immersed in a levamisole bath (10 μg/mL, 24 h) showed enhanced resistance against *Aeromonas hydrophilia*, and its phagocytes increased chemotactic ability, phagocytic activity and chemiluminescence (Baba *et al.*, 1993). Bathing Atlantic salmon (*Salmo salar*) in doses of 1.25, 2.5 and 5 ppm (μg/mL) increased their resistance to *Paramoeba* sp., the cause of amoebic gill disease. Fish that had previous exposure to *Paramoeba* species benefited most and the effect was detectable from 2-3 weeks post-treatment (Findlay *et al.*, 2000).

Although the immunostimulatory action of levamisole has been reported in fish, optimum doses vary between species and should be carefully considered. Rainbow trout injected with high doses (5 mg/kg) of levamisole did not show stimulated chemiluminescence responses of head kidney leukocytes, compared with fish injected with the optimum dose 0.1–0.5 mg/kg (Kajita *et al.*, 1990). Rainbow trout splenocytes immunised with *Y. ruckeri* O-antigen and cultured with 50 μg/mL of levamisole did not result in stimulation of antibody forming cells and that antibody production was completely suppressed by high doses of levamisole (Siwicki *et al.*, 1990).

Levamisole’s immunostimulating effect has been demonstrated in fish, and the drug can be easily administered via different routes. Results have shown, however, that the effects of levamisole are dose and time dependent and that low doses may result in no effect and high doses are likely to cause immunosuppression. In addition, it may take time for the effects of levamisole to appear and the duration of immunostimulation is likely to be in a specific window of time (Mulero *et al.*, 1998b).
6.3: β-Glucans (CAS no. 9037-91-6)

β-glucans are a large class of carbohydrate biopolymers that are ubiquitous in environmental microflora, occur in potential pathogens and are structurally distinct from carbohydrates found in the cell wall of animals (Anderson, 1992). Consequently, they are ideal recognition molecules and animal immune systems have a natural predisposition to react to them (Lowe et al., 2001). The class contains many structurally different compounds and includes amylose (α1→4 linked), glycogen (α1→4 linked and α1→6 branches) and cellulose (β1→4 linked).

β-glucans are water-soluble endospermic cell wall polysaccharides of grains such as barley and oats and also occur in the cell walls of yeast and fungi. Many β-glucans have immunomodulating activities, however this varies depending on their structural parameters, which may differ in solubility, molecular mass and branching frequency. They have been found to lower blood cholesterol, glucose and insulin levels, however the mechanism by which they affect these levels is not well understood (Delaney et al., 2003). In addition, β-glucans have been used to treat infectious diseases and have been studied in clinical trials for the treatment of cancer (Chihara, 1992)

6.3.1: Structure of β-Glucans

Chemically, β-glucans are a heterogeneous group of polysaccharides consisting of a chain of β-D-glucopyranosyl monomers with 1→3 or 1→4 links (figure 6.2) (Colleoni-Sirghie et al., 2003). There are no distinguishable differences in the chemical composition of oat and barley β-glucans (Delaney et al., 2003).
6.3.2: Toxicokinetics of β-glucans

Limited investigations concerning the toxicokinetics of β-glucans have shown that they are rapidly cleared to the Kupffer cells of the liver, where they remain for 4-6 weeks (Suda et al., 1996; Yoshida et al., 1996). The plasma half-life of β-glucan is 1.4-1.8 mins and the majority of the compound is associated with the cell-free fraction of plasma (Yoshida et al., 1996). Studies have reported that (1→3)-β−glucan is not readily incorporated into the host cells for degradation and is excluded from the body even after modification of biological responses (Suda et al., 1996).

Mammals lack the enzymes necessary to hydrolyse β-glucans and therefore, they are broken-down by oxidative degradation into their monomer units. Cells of macrophage linage have a major role to play in β-glucan metabolism, as they have the capability to produce reactive oxygen and nitrogen species needed for oxidative degradation (Suda et al., 1996).

6.3.3: β-glucan toxicity data

There is limited data concerning the toxicity of β-glucans as they are considered to be relatively safe. Nonetheless, toxicity testing of soluble β–glucans involving a number of species and dosage regimes have reported no toxicity at high doses but observed
immunomodulation including, splenomegaly and an increase in the number of lymphocytes (Delaney et al., 2003; Williams et al., 1988).

6.3.4: Mechanism of action

β-glucans exert their effects through macrophages, neutrophils, monocytes and NK cells that possess α₃β₂ integrin receptors that specifically bind β-glucans (Czop and Austen, 1985). This integrin is a heterodimer also known as membrane attack complex of complement (Mac-1), complement receptor type 3 (CR3), or CD11b/CD18 and has a dual function. As Mac-1, it serves as an adhesion molecule to mediate the migration of leukocytes from the capillaries to surrounding tissue via generation of a high-affinity binding site for intercellular adhesion molecule-1. As CR3, it stimulates phagocytic and degranulation responses to microorganisms or immune complexes opsonized with modified fragments of the complement 3 protein (iC3b). CR3 is involved in the cytotoxic responses of the cell and requires ligation of the receptor at two distinct sites (figure 6.3a). One site is for iC3b fragments and a second site binds β-glucans and both are contained in the α-chain of CD11b molecule (figure 6.3b) (Xia et al., 1999). Phagocytosis and cytotoxic degranulation occurs, when both these sites are bound to iC3b-coated microorganisms with surface polysaccharides that have affinity for the lectin site on CR3 (figure 6.3). β-glucans prime CR3 of neutrophils, macrophages and NK cells for cytotoxic killing of iC3b-opsonized tumour cells that otherwise trigger no killing due to a lack of recognition polysaccharides on the surface of tumour cells (Xia et al., 1999).

CR3 is yet to be definitively identified in fish, however there have been reports suggesting its presence on the surface of neutrophils and macrophages from different species. β-glucan specifically binds to receptors on the surface of Atlantic salmon macrophages (Engstad and Robertsen, 1993) and channel catfish neutrophils (Ainsworth, 1994). These fish also have receptors that actively take up yeast cell wall preparations (i.e. zymosan), which are
specifically inhibited by β-glucan (Engstad and Robertsen, 1994). Injections of β-glucan up-regulated a surface protein that was inhibited by carp anti-C3 antibodies, Mg$^{2+}$ depletion and mAb for murine CR3 (Nakao et al., 1998). In addition, Mulero and associates (2001) have produced a mAb against a 140 kDa fish cell-surface receptor which they thought was an integrin. Their receptor was involved in adhesion and aggregation and their antibody inhibited chemiluminescence of head kidney phagocytes.

Another major effect of β-glucans binding to receptors on macrophages, neutrophils and NK cells is that they respond by producing chemical mediators such as leukotrienes, cytokines, prostaglandins and nitric oxide (Abel and Czop, 1992; Ljungman et al., 1998; Vetvicka et al., 1996; Yoshioka et al., 1998). Cells such as lymphocytes, hepatocytes, vascular endothelial cells and fibroblasts respond by maturing, differentiating, proliferating and secreting more chemical mediators including acute-phase proteins (figure 6.4) (Chihara, 1992).

![Figure 6.3: a) Proposed mechanism for neutrophil CR3-dependent cytotoxic activation by iC3b-opsonized yeast. Based on functional data, both the lectin site and the I-domain of CR3 must be bound simultaneously to β-glucan and iC3b, respectively, for stimulation. b) Schematic representation of CR3 showing its intertwined two-chain structure and the major domains of CD11b (Ross et al., 1999).](image)
Figure 6.4: Macrophage signalling after exposure to β-glucan. IL = interleukins; APPIF = acute phase protein inducer; VDHIF = vascular dilation and haemorrhage inducer; CSF = colony stimulating factor; MIF = migration inhibitory factor; GAF = glucocorticoid antagonizing factor; SAA = serum amyloid A; IPA = plasminogen activator inducer; VPF = vascular permeability factor; CTL = cytotoxic T lymphocytes; NK = natural killer cells (modified from Chihara, 1992).

6.3.5: Effects on aquatic species immunology

There have been many studies reporting immunostimulation by different types of β-glucan in different aquatic species (Sakai, 1999). The effects in fish appear remarkably similar to mammals, with changes in serum proteins and phagocyte activities observed upon treatment of fish (Robertsen, 1999). The existence of CR3 on the surface of fish leukocytes is not yet established but the cytokines and mechanisms involved are beginning to be identified in fish and the activation of fish macrophages has been reviewed (Secombes, 1994).
In vitro effects

Reduction of the dyes nitroblue tetrazolium (i.e. NBT assay) and luminol (i.e. CL assay) is an indicator of the amount of reactive oxygen species (ROS, superanion) produced by macrophages. Studies have shown that macrophages exposed in vitro to β-glucan are stimulated to produce ROS (table 6.1). Vazzana and associates (2003) used the CL assay to show that after β-glucan exposure, peritoneal and head kidney macrophages are stimulated to a greater extent than spleen and blood macrophages. Some studies observed that β-glucan did not increase the bactericidal activity of the macrophages (i.e. measured by phagocytosis of A. salmonicida) even though ROS production was increased. In addition, there have been reports that high doses of β-glucan can lead to either inhibition or exhaustion of ROS production (Castro et al., 1999; Jørgensen and Robertsen, 1995; Tahir and Secombes, 1996).

Both β-glucan and LPS can induce the production of extracellular lysozyme by Atlantic salmon macrophage cultures in vitro. Increased lysozyme concentrations were found in the supernatants of fish macrophage cultures exposed to microbial polysaccharide but not in exposed hepatocyte cultures (Lunde and Robertsen, 1997). Hepatocytes respond to β-glucans in vitro by producing acute-phase proteins but many require the presence of other cytokines such as macrophage activating factor (Jørgensen et al., 2000).
Table 6.1: Summary of studies reporting \textit{in vitro} β-glucan immunostimulation.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Species</th>
<th>Exposure</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantic Salmon</td>
<td>0.1-50 μg/mL</td>
<td>RB ↑ (↓ high dose)</td>
<td>(Jørgensen and Robertsen, 1995)</td>
</tr>
<tr>
<td>(\textit{Salmo salar})</td>
<td>1-7 d</td>
<td>Killing → Lysozyme ↑</td>
<td>(Lunde and Robertsen, 1997)</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td></td>
<td>(Sveinbjörnsson and Seljelid, 1994)</td>
</tr>
<tr>
<td>Turbot</td>
<td>0.5-500 μg/mL</td>
<td>RB ↑ (↓ high dose)</td>
<td>(Castro \textit{et al.}, 1999)</td>
</tr>
<tr>
<td>(\textit{Psetta maxima})</td>
<td>1-6 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gilthead seabream</td>
<td>0.5-500 μg/mL</td>
<td>RB ↑ (↓ high dose)</td>
<td>(Castro \textit{et al.}, 1999)</td>
</tr>
<tr>
<td>(\textit{Sparus aurata})</td>
<td>1-6 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snapper</td>
<td>0.001-1 % v/v</td>
<td>NBT ↑</td>
<td>(Cook \textit{et al.}, 2001)</td>
</tr>
<tr>
<td>(\textit{Pagrus auratus})</td>
<td>0-90 min &amp; 1-3 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dab</td>
<td>0.125-8 μg/mL</td>
<td>RB ↑ (↓ high dose)</td>
<td>(Tahir and Secombes, 1996)</td>
</tr>
<tr>
<td>(\textit{Limanda limanda})</td>
<td>24 &amp; 48 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue gourami,</td>
<td>0.01-10 ng/mL</td>
<td>Phagocytosis ↑</td>
<td>(Fock \textit{et al.}, 2001)</td>
</tr>
<tr>
<td>(\textit{Trichogaster trichopterus})</td>
<td>12 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sea bass</td>
<td>1 mg/mL</td>
<td>CL ↑</td>
<td>(Vazzana \textit{et al.}, 2003)</td>
</tr>
<tr>
<td>(\textit{Dicentrarchus labra})</td>
<td>0-50 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}modified from Robertson (1999) and Sakai (1999)

RB = respiratory burst, CL = chemiluminescence assay, NBT = nitroblue tetrazolium assay
Killing = \textit{A. salmonicida} engulfment
↑ = increase  ↓ = decrease  → = no change

\textit{In vivo effects}

\textit{Intraperitoneal dosage route}

It has been demonstrated in numerous experiments that an i.p. injection of β-glucan given to fish results in increased resistance to a number of bacterial infections, with the duration of protection being 1–4 weeks after a single injection (Robertsen, 1999). More recently, there have been reports that an i.p. injection of β-glucan protects rainbow trout from infectious haematopoietic necrosis virus and white spot syndrome virus (Chang \textit{et al.}, 2003; Lapatra \textit{et al.}, 1998; Namikoshi \textit{et al.}, 2004). Most investigations concerning \textit{in vivo} β-glucan immunomodulation have focused on the stimulation of macrophages (measured by NBT assay and CL assay) and protection of fish from bacterial challenge by injection with specific pathogens. A number of studies have reported an increased protection to bacterial infection
after an i.p. injection of β-glucan (table 6.2) (Robertsen, 1999). The data indicates that β-glucans from a number of sources can protect commercial aquatic species from different types of bacterial pathogens that are relevant to aquaculture. Only one reported study failed to increase fish immunity following β-glucan exposure (Midtlyng et al., 1996).

Table 6.2: Investigations reporting increased protection to bacterial and viral pathogens following an intraperitoneal injection of β-glucan

<table>
<thead>
<tr>
<th>Species</th>
<th>Pathogen</th>
<th>Glucan type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantic salmon (Salmo Salar)</td>
<td>V. anguillarum</td>
<td>Yeast glucan</td>
<td>(Robertsen et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>V. salmonicida</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y. ruckeri</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brook trout (Salvelinus fontinalis)</td>
<td>A. salmonicida</td>
<td>Yeast glucan</td>
<td>(Anderson and Siwicki, 1994)</td>
</tr>
<tr>
<td>Carp (Cyprinus carpio)</td>
<td>E.tarda</td>
<td>Lentinan, Schizophyllan, Scleroglucan</td>
<td>(Yano et al., 1989)</td>
</tr>
<tr>
<td>Channel catfish (Ictalurus punctatus)</td>
<td>E. ictaluri</td>
<td>Yeast glucan</td>
<td>(Chen and Ainsworth, 1992)</td>
</tr>
<tr>
<td>Coho salmon (Oncorhynchus kisutch)</td>
<td>A. salmonicida</td>
<td>Schizophyllan</td>
<td>(Nikl et al., 1991)</td>
</tr>
<tr>
<td>Grass carp (Ctenopharyngodon idellus)</td>
<td>A. hydrophila</td>
<td>Scleroglucan</td>
<td>(Wang and Wang, 1997)</td>
</tr>
<tr>
<td>Tilapia (Tilapia aureus)</td>
<td>A. hydrophila</td>
<td>Scleroglucan</td>
<td>(Wang and Wang, 1997)</td>
</tr>
<tr>
<td>Yellowtail (Seriola quinquergadiata)</td>
<td>Streptococcus sp.</td>
<td>Scleroglucan, Schizophyllan</td>
<td>(Matsuyama et al., 1992)</td>
</tr>
<tr>
<td>Striped snakeheads (Channa striata)</td>
<td>A. invadans</td>
<td>Yeast glucan</td>
<td>(Miles et al., 2001)</td>
</tr>
<tr>
<td>Rainbow trout (Oncorhynchus mykiss)</td>
<td>IHNVb</td>
<td>Yeast glucan</td>
<td>(Lapatra et al., 1998)</td>
</tr>
<tr>
<td>Grass prawns (Penaeus monodon)</td>
<td>WSSVc (Oral)</td>
<td>Schizophyllan</td>
<td>(Chang et al., 2003)</td>
</tr>
<tr>
<td>Kuruma prawn (Penaeus japonicus)</td>
<td>WSSVc</td>
<td>Yeast glucan</td>
<td>(Namikoshi et al., 2004)</td>
</tr>
</tbody>
</table>

\( ^{a} \text{modified from Robertsen (1999) and Sakai (1999)} \\
\( ^{b} \text{infectious haematopoietic necrosis virus} \\
\( ^{c} \text{White spot syndrome virus} \)

Tilapia and grass carp showed an increased resistance to *Aeromonas hydrophila* and increased their number of NBT positive cells following injection with β-glucans derived from a number
of sources (Wang and Wang, 1997). Jorgensen and associates (1993), injected Atlantic salmon with β-glucan and recorded the dynamics of leukocyte numbers over a number of days. They found an accumulation of macrophages, neutrophil and thrombocytes in the peritoneum that peak at 2 d after the injection and then declined rapidly but stayed elevated (up to ten times) compared to controls. There have also been reports that immunostimulants trigger neutrophils to produce adhesion proteins (integrins such as Mac-1) that facilitate the migration to the site of injury (Anderson et al., 1992a). Rainbow trout exposed with an i.p. injection β-glucan also showed an increased number of glass-adherent neutrophils (NBT positive cells), with elevated phagocytic activity (Jeney and Anderson, 1993b). They also showed that exposing the fish by immersion had similar effects.

Lysozyme and complement activity was also influenced by the i.p. administration of β-glucan (Engstad et al., 1992; Jørgensen et al., 1993). Lysozyme and complement activity in serum may increase 2- to 5-fold in response to an injection of fungal β-glucans. However, lysozyme and complement-mediated haemolytic activity show different kinetics with respect to the appearance in time after injection of antigen (yeast) and therefore it appears that lysozyme and complement are induced through different mechanisms (Robertsen, 1999). Santarem and co-workers (1997) showed that β-glucan alone increased serum lysozyme concentrations of exposed turbot but required co-administration of O-antigen to increase phagocytic activity. Atlantic salmon injected with yeast β-glucan displayed increased C3 complement components in the serum (Lunde and Robertsen, 1997) and also showed induced serum amyloid A (SAA) transcripts in the liver (Jorgensen et al., 2000). Furthermore, supernatants from salmon macrophages stimulated with β-glucan induced SAA transcripts in cultured primary salmon hepatocytes suggesting that cytokines are involved (Jorgensen et al., 2000). These observations support the hypothesis that microbial polysaccharides up-regulate the non-specific defence of fish by stimulating the production of acute-phase proteins by hepatocytes.
**Oral dosage route**

Although oral exposure is the preferred route of administration for fish farmers, the protection obtained may often be relatively low compared to injection and individual variation in response to immunostimulants may be large (Robertsen, 1999). There has been a report that oral administration of β-glucan resulted in no additional protection to pathogens (Toranzo et al., 1995). However, the majority of studies have reported increased macrophage activities, lymphoproliferation, antibody titres and/or resistance to pathogens after feeding fish diets supplemented with β-glucan (table 6.3) (Cook et al., 2003; Couso et al., 2003; Nikl et al., 1993; Siwicki, 1994; Verlhac et al., 1998; Yoshida et al., 1995). Commercial β-glucan supplements (i.e. EcoActiva™, Bio-resources Division, Carlton and United Breweries, Melbourne) are now available for the addition to fish food pellets (Cook et al., 2003).

### Table 6.3: Studies reporting immunostimulation following oral β-glucan exposure

<table>
<thead>
<tr>
<th>Fish</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainbow trout</td>
<td>Antibody titre ↑</td>
<td>(Verlhac et al., 1998)</td>
</tr>
<tr>
<td>(Oncorhynchus mykiss)</td>
<td>Mitogenesis ↑</td>
<td>(Siwicki, 1994)</td>
</tr>
<tr>
<td></td>
<td>Furunculosis resistance ↑</td>
<td>(Jeney et al., 1997)</td>
</tr>
<tr>
<td>Chinook salmon</td>
<td><em>A. salmonicida</em> resistance ↑</td>
<td>(Nikl et al., 1993)</td>
</tr>
<tr>
<td>(Oncorhynchus tshawytscha)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snapper</td>
<td>Growth (winter) ↑</td>
<td>(Cook et al., 2003)</td>
</tr>
<tr>
<td>(Pagrus auratus)</td>
<td>NBT ↑</td>
<td></td>
</tr>
<tr>
<td>African catfish</td>
<td>NBT ↑</td>
<td>(Yoshida et al., 1995)</td>
</tr>
<tr>
<td>(Clarias gariepinus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gilthead seabream</td>
<td>Pasteurellosis resistance ↑</td>
<td>(Couso et al., 2003)</td>
</tr>
<tr>
<td>(Sparus aurata)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carp</td>
<td><em>A. hydrophila</em> resistance ↑</td>
<td>(Sahoo and Mukherjee, 2002)</td>
</tr>
<tr>
<td>(Cyprinus carpio)</td>
<td>Specific immune functions ↑</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-specific immunity ↑</td>
<td></td>
</tr>
<tr>
<td>Juvenile prawns</td>
<td>Growth ↑</td>
<td>(Lopez et al., 2003)</td>
</tr>
<tr>
<td>(Litopenaeus vannamei)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grass prawns</td>
<td>WSSV resistance ↑</td>
<td>(Chang et al., 2003)</td>
</tr>
<tr>
<td>(Penaeus monodon)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prawns (Penaeus</td>
<td>Vibriosis resistance ↑</td>
<td>(Scholz et al., 1999)</td>
</tr>
<tr>
<td>vannamei)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

↑ = increase  
* = modified from Robertsen (1999) and Sakai (1999)

NBT = nitroblue tetrazolium

WSSV = White spot syndrome virus
6.4: Aim of the study

Murray cod were the focus of this study because their aquaculture industry is a young but productive industry that is predicted to grow rapidly. Furthermore, Murray cod are grown in large intensive indoor systems with very high stock densities, which is often accompanied by an increased incidence of diseases. Aquaculturists are interested in a cheap and easily administered prophylactic agent that could improve the health of fish and production. The purpose of this study was to determine if levamisole or β-glucan could enhance Murray cod immune functions. The in vivo testing was an attempt to determine an appropriate dosing regime of Murray cod for these agents. The in vitro testing aids in setting these levels and also gives some insight into the mechanisms of action.

Rainbowfish were not studied in this chapter because they do not have an aquaculture industry that requires the use of immunostimulants. Silver perch were not amenable to in vivo exposures and were not studied in this chapter. Golden perch were not studied because their aquaculture industry is not yet established and a supply of golden perch was unavailable.

6.5: Methods:

6.5.1: Spleen and Head kidney Sampling

Murray cod were housed and maintained as described in section 2.1. Fish were anaesthetised and killed as described in section 2.2. For the in vivo experiments fish weight was recorded immediately before injection and then after the exposure 14 d period before tissue sampling. The percentage of body weight gained or lost was then calculated as: (pre-exposure – post-exposure weight) / pre-exposure weight x 100.

Immune tissues were sampled as described in section 2.3. Somatic indices were calculated as: (organ weight/body weight) x 100.
6.5.2: Cell isolation

The methods for the isolation of Murray cod immune cells is described in detail in section 2.3 and also briefly described in the organotin study section 3.13.2. Cell yield was calculated as: total number of cells collected / organ weight (g).

6.5.3: Exposure protocols

In vitro exposures

Levamisole and β-glucan were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA). Concentrated stock solutions were dissolved in saline (i.e. DMSO, BDH Chemicals, Poole, UK) for each concentration level and then diluted 1:100 in TCM for addition to the incubation as one quarter of the final incubation volume (i.e. 1:400 dilution). The final concentrations tested were 0 (i.e. saline), 0.25, 2.5 and 25 μg/mL and 0 (i.e. saline), 0.1, 1 and 10 μg/mL for levamisole and β-glucan, respectively.

In vivo exposures

Levamisole

Fresh stock solutions of levamisole were diluted in saline at 250 mg/mL and were kept at 4°C for no more than 1 week. On the day of use, the stock was diluted 1:100 to give a high concentration of 2.5 mg/mL. This was serially diluted 1:10 to attain a medium and low concentration of 0.25 and .025 mg/mL. Fish were dosed with 2 mL/kg, achieving exposures of 0.05, 0.5 and 5 mg/kg.

β-glucan

β-glucan stock solution of 50 mg/mL was dissolved in saline by heating to 80°C while stirring. On the day of use, this was diluted 1:100 to attain a high concentration of 0.5
mg/mL. This was serially diluted 1:10 to attain a medium and low concentration of 0.05 and 0.005 mg/mL. Fish were dosed with 2 mL/kg achieving exposures of 0.01, 0.1 and 1 mg/kg.

The immunostimulant *in vivo* exposure protocols for Murray cod are shown in table 6.4.Protocols were performed as described in section 3.13.3., except that saline was used as the solvent control and controls from just these two experiments were combined to resulting in a sample size of 4 for the untreated control and all groups in the mitogenesis assay. Levamisole doses were repeated on day 9-23 because a plate was dropped during the processing of the mitogenesis assay resulting in an extra fish in the sample for the phagocytosis assay. Therefore, in the phagocytosis results there was a sample size of 5 fish for saline control groups for both immunostimulants and levamisole treatment groups also had a sample size of 5 fish. The untreated control and the β-glucan exposure groups had a sample size of 4 fish.

<table>
<thead>
<tr>
<th>Day of study</th>
<th>Levamisole treatment (mg/kg)</th>
<th>0 (Saline control)</th>
<th>0.05</th>
<th>0.5</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1-14</td>
<td>Untreated control</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 2-15</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 5-19</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 6-20</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 9-23*</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day of study</th>
<th>β-glucan treatment (mg/kg)</th>
<th>0 (Saline control)</th>
<th>0.01</th>
<th>0.1</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1-14</td>
<td>Untreated control</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 2-15</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 5-19</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Day 6-20</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Repeated experiment due to lost sample in mitogenesis assay
6.5.4: Phagocytosis

Standardised protocols for the phagocytosis assay are described in detail in section 2.4.2 and the methods used in this experiment are the same as the organotin study described in section 3.13.4.

6.5.5: Mitogenesis

Standardised protocols for the mitogenesis assay are described in detail in section 2.6.2.2 and the methods used in this experiment are the same as the organotin study described in section 3.13.5.

6.5.6: Lysozyme

Standardised protocols for the lysozyme assay are described in detail in section 2.5.3 and the methods used in this experiment are the same as the organotin study described in section 3.13.5.

6.5.7: Statistics

Statistics were performed using the same methods described in the section 3.13.7.
6.6: Results: In vitro exposures

6.6.1: The effect of in vitro levamisole exposure on Murray cod immune parameters

Phagocytosis

Incubating Murray cod head kidney cells with levamisole resulted in no significant change in the percentage of phagocytic granulocytes (i.e. percentage of FITC +ve granulocytes), although there was a significant increase in the activity of these cells with an increase in the number of beads per cell (i.e. mean voltage) at 2.5 μg/kg (figure 6.5). The treatment did not appear to be cytotoxic as increasing the levamisole dose did not alter the number of events in each subpopulation and PI +ve and debris events did not increase. There was large variation in the phagocytosis of granulocytes dosed at 25 μg/mL, which may reflect variation of granulocyte response between individuals.

Mitogenesis

In vitro exposure to levamisole had no significant effect on the lymphoproliferation of Murray cod head kidney cells (figure 6.6). However, it is worth noting that all cultures exposed to in vitro concentrations of levamisole displayed a lower mean basal and peak CPM.
Figure 6.5: Phagocytic function and subpopulation counts of Murray cod head kidney immune cells after *in vitro* exposure to levamisole. a) Phagocytosis (FITC +ve) b) number of beads per cell (i.e. mean voltage and side scatter (SS) increase c) Granulocytes and lymphocyte subpopulation counts d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated control values were 9±1% for FITC +ve total, 18±2% for FITC +ve granulocytes, 144±42 volts for mean voltage, 141±51 volts for SS increase, 5550±350 events for granulocytes, 2550±250 events for lymphocytes, 1350±100 events for debris and 8±1% for PI +ve. * denotes significantly different from the solvent control (p<0.05).
Figure 6.6: The effect of in vitro levamisole exposure on Murray cod head kidney lymphocytes a) lymphoproliferation profiles and b) proliferation index. Proliferation index is expressed as percentage of untreated control (i.e. 1.11±0.03, n=4) and values represent mean±se of 4 fish. No statistically significant differences were found.

6.6.2: The effect of in vitro β-glucan exposure on Murray cod immune parameters

Phagocytosis

In vitro β-glucan exposure resulted in a dose-dependent increase in the number of beads per granulocyte (i.e. mean voltage) (figure 6.7b). There was no significant change in the number of events in the subpopulation gated-regions, however there was a linear decrease in the number of non-viable (PI +ve) cells and debris gate. The incubation of Murray cod head kidney with β-glucan did not significantly increase the percentage of granulocytes with beads, however samples dosed at 10 μg/mL increased to almost 120% of control (figure 6.7a).
Figure 6.7: Phagocytic function and subpopulation counts of Murray cod head kidney immune cells after in vitro exposure to β-glucan. a) Phagocytosis (FITC +ve) b) number of beads per cell (mean voltage and side scatter (SS) increase c) Granulocytes and lymphocyte subpopulation counts d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control (n=4) samples and values represent mean ± se of 4 fish. Untreated control values were 9±1% for FITC +ve total, 18±2% for FITC +ve granulocytes, 144±42 volts for mean voltage, 141±51 volts for SS increase, 5550±350 events for granulocytes, 2550±250 events for lymphocytes, 1400±100 events for debris and 8±1% for PI +ve. † denotes linear relationship (p<0.05).
Mitogenesis

*In vitro* β-glucan exposure resulted in a dose-dependent increase in the proliferation index of Murray cod head kidney cells (figure 6.8b). In addition, β-glucan also shifted the optimal concentration of PHA from 5 to 10 μg/mL and it is possible that higher concentrations of PHA may have led to greater stimulatory effect (figure 6.8 a).

![Graph showing in vitro effect of β-glucan on Murray cod head kidney lymphocytes.](image)

**Figure 6.8:** The *in vitro* effect of β-glucan on Murray cod head kidney lymphocytes a) lymphoproliferation profiles and b) proliferation index. Proliferation index is expressed as percentage of control (i.e. 1.11±0.03, n=4) and values represent mean±se of 4 fish. † denotes linear relationship (p<0.05).
6.7: Results: *In vivo* exposures

6.7.1: The effect of *in vivo* levamisole exposure on Murray cod immune parameters

*Body and organ weight and lysozyme*

Murray cod exposed to levamisole grew less over the 14 day period than controls and although individual variation was large, fish dosed with 5 mg/kg grew significantly less than the saline control (table 6.5). There was a dose-dependent increase in the splenic somatic index of Murray cod exposed to levamisole and fish dosed with 5 mg/kg had significantly higher somatic indices compared to saline control fish (table 6.5). There was a small but insignificant rise in the average somatic indices of the head kidneys at 0.05 and 0.5 mg/kg. This returned to control levels at 5 mg/kg (table 6.5). There was no significant change in the cell yields from either the spleen or the head kidney when exposed to levamisole. There was large variation in the cell yields of all groups (table 6.5).

Serum lysozyme concentration did not change after *in vivo* exposure to levamisole (table 6.5). In the 0.5 mg/kg and saline group, two fish with higher lysozyme activity (~15 μg/mL) increased the variation. The average lysozyme concentration of Murray cod used in these experiments was found to be 6-9 μg/mL.

Table 6.5: The effect of *in vivo* levamisole exposure on Murray cod body and organ weights.

<table>
<thead>
<tr>
<th>Chemical &amp; Parameter</th>
<th>Levamisole concentration (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Change in body weight (%)</td>
<td>1.57±3.88</td>
</tr>
<tr>
<td>Spleen Somatic index</td>
<td>0.053±0.012</td>
</tr>
<tr>
<td>Head kidney Somatic index</td>
<td>0.200±0.016</td>
</tr>
<tr>
<td>Spleen Cell yield</td>
<td>209±54</td>
</tr>
<tr>
<td>Head kidney Cell yield</td>
<td>227±71</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>113.9±28.7</td>
</tr>
</tbody>
</table>

* denotes significantly different from the saline control (p<0.05). † denotes linear relationship (p<0.05)
**Phagocytosis**

In *vivo* exposure of levamisole to Murray cod did not significantly change the phagocytic function of Murray cod head kidney cells (figure 6.9a). However, in contrast to the *in vitro* studies, there was a reduction in the number of beads per cells (mean voltage) and cell granularity (SS) exposed to levamisole (figure 6.9b). This was not a dose-dependent relationship and did not reach statistical significance, but may be of interest as the data suggest that *in vivo* exposure levamisole may have inhibited the phagocytic rate of head kidney granulocytes. There was no significant change in the number of granulocytes or lymphocytes, debris gated events or non-viable cells (figure 6.9c and d). Fish dosed with 0.05 mg/kg appeared to have reduced numbers of debris-gated events and non-viable cells but the reason for this is unclear. This result may indicate overall “cleaner” cell preparations for that group (figure 6.9d).

**Mitogenesis**

Exposing Murray cod with levamisole did not significantly increase the lymphoproliferation of head kidney cells. However, as with *in vitro* observations both basal and peak CPM were marginally lower in fish exposed to levamisole (figure 6.10). Fish treated with 0.5 mg/kg and 5 mg/kg appear to have stronger proliferative response than the 0.05 mg/kg group, however a large amount of individual variation in the low dose group may have contributed to this (figure 6.10b)
Figure 6.9: Murray cod head kidney phagocytic function and subpopulation counts following *in vivo* exposure to levamisole. a) Phagocytosis (FITC +ve), b) number of beads per cell (mean voltage and side scatter (SS) increase), c) Granulocytes and lymphocyte subpopulation counts, d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 5 fish. Untreated control values were; 8±2% for FITC +ve total; 19±3% for FITC +ve granulocytes; 177±43 volts for mean voltage; 139±34 volts for side-scatter increase; 4000±450 events for granulocytes; 3000±130 events for lymphocytes; 1800±150 events for debris and 1138±204 events for PI +ve. No statistically significant differences were found.
Figure 6.10: The effect of *in vivo* levamisole exposure on Murray cod head kidney lymphocytes a) lymphoproliferation profiles and b) proliferation index. Proliferation index is expressed as percentage of control (i.e. 1.46±0.12, n=4) and values represent mean±se of 4 fish. No statistically significant differences were found.

6.7.2: The effect of *in vivo* β-glucan exposure on Murray cod immune parameters.

*Body and organ weights*

In contrast to levamisole treated fish, those dosed with β-glucan showed increase in their body weight over the study period, however this was not statistically significant from the solvent control (table 6.6). Fish dosed with β-glucan gained over 6-7% of their body weight on average, whereas the control fish did not gain or lose any average weight during the 14 d post-exposure period. β-glucan exposure did not significantly change the somatic indices of the spleen or head kidney. There was a variable increase at 1 mg/kg but this did not reach statistical significance (table 6.6).

There were large individual variations in the cell yields of both the head kidney and the spleen (table 6.6). The cell yields of fish exposed to β-glucan were not significantly different from the saline controls.
**Lysozyme activity**

treatment did not significantly change the serum lysozyme concentration of Murray cod (table 6.6). A single fish caused the large variation in both the saline and 0.01 mg/kg groups with almost twice the lysozyme activity.

Table 6.6: The effect of *in vivo* β-glucan exposure on Murray cod body and organ weights

<table>
<thead>
<tr>
<th>Chemical &amp; Parameter</th>
<th>β-glucan concentration (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Change in body weight (%)</td>
<td>2.63±3.21</td>
</tr>
<tr>
<td>Spleen Somatic index</td>
<td>0.029±0.003</td>
</tr>
<tr>
<td>Head kidney Somatic index</td>
<td>0.221±0.012</td>
</tr>
<tr>
<td>Spleen Cell yield a</td>
<td>789±200</td>
</tr>
<tr>
<td>Head kidney Cell yield a</td>
<td>1099±282</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>114±29</td>
</tr>
</tbody>
</table>

a Cell yield data is expressed cells/g of head kidney tissue. Lysozyme data is expressed at percentage of untreated control (i.e. 7.91 mg/mL HEWL equivalent, n=4). Values represent the mean ± se of 4 fish (except saline control, n=5). No statistically significant differences were found.
**Phagocytosis**

There was no significant change in the phagocytic functions of Murray cod head kidney cells (figure 6.11). There was however an increase in the number of granulocytes to over 120% and a corresponding decrease in the number of lymphocytes to approximately 80%. Somatic indices and cell yield data suggests that there is a recruitment of granulocytes to the head kidney in response to β-glucan. It is difficult to interpret if this is a result of recruitment of large granular cells to the head kidney or if the treatment is selectively reducing the number of lymphocytes. However, there was no significant difference in the somatic indices of either the head kidneys or spleen and there appeared to be a rise in cell yield of head kidney in the 0.01 and 0.1 mg/kg group. Therefore it is more likely that this result indicates a rise in granulocyte numbers within the head kidney.

**Mitogenesis**

*In vivo* β-glucan treatment of Murray cod had no significant effect on the mitogenic activity of their head kidney cells. There was an insignificant reduction of proliferative index at 1 mg/kg however this was due to a higher than normal basal response of lymphocytes from fish exposed to 1 mg/kg (figure 6.12).
Figure 6.11: Murray cod head kidney phagocytic function and subpopulation counts following in vivo exposure to β-glucan. a) Phagocytosis (FITC +ve), b) number of beads per cell (Mean voltage and side scatter (SS) increase), c) Granulocytes and lymphocyte subpopulation counts, d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish (except saline control, n=5). Untreated control values were; 8±2% for FITC +ve total; 19±3% for FITC +ve granulocytes; 177±43 volts for mean voltage; 139±34 volts for side-scatter increase; 4000±500 for granulocytes; 3000±100 for lymphocytes; 1800±150 for debris and 11±2% events for PI +ve. No statistically significant differences found.
Figure 6.12: The effect of *in vivo* β-glucan exposure on Murray cod head kidney lymphocytes a) lymphoproliferation profiles and b) proliferation index. Proliferation index is expressed at percentage of control (i.e. 1.46±0.12, n=4) and values represent mean±se of 4 fish. No statistically significant differences found.

### 6.8: Discussion

Both levamisole and β-glucan showed immunostimulatory properties *in vitro*. They both increased phagocyte rate of granulocytes in the phagocytosis assay, which has proved to be the most sensitive parameter in the flow cytometric assay (figure 6.5 and 6.7). Even though different methodologies have been applied, this result concurs with the many studies that have found an enhanced phagocytic activity in other aquatic species following *in vitro* exposure to β-glucan and levamisole (Castro *et al.*, 1999; Cook *et al.*, 2001; Siwicki, 1989; Sritunyalucksana *et al.*, 1999; Sveinbjornsson and Seljelid, 1994). Additionally, β-glucan reduced the number of non-viable (PI +ve) events and frequency of events in the debris gate (figure 6.7). Over the 48 hr incubation period some lymphocytes die. Decreased debris and PI fluorescence indicates that β-glucan is also an agent that slows the general degradation of lymphocytes in culture. This protective effect may be of use to tissue culture applications but it remains to be seen if this effect is via some other mechanism or through macrophage
signalling and therefore only useful for cultures with a mixture of lymphocytes and granulocytes.

Murray cod head kidney leukocytes exposed in vitro to β-glucan at 10 μg/mL also significantly increased their proliferation index and also changed the optimal PHA concentration from 5 to 10 μg/mL (figure 6.8). The mechanism involved is likely to be through β-glucan primed macrophages releasing cytokines (Ljungman et al., 1998), which enhance mitogenesis but this remains to be investigated. Comparing mitogenic activity of mixed lymphocyte/phagocyte population to the response of pure lymphocyte populations would indicate if macrophage signalling is involved.

In vivo exposure of Murray cod to levamisole resulted in no change of the functional immune parameters measured. Levamisole exposure did lead to a dose-dependent increase in splenic somatic index but there was also a significant decrease in the body weight of fish exposed to 5 mg/kg (table 6.5). Reduced growth following levamisole exposure is of a high interest to fish farmers but it remains to be seen if this effect occurs during chronic dosages or if it is a specific acute response to levamisole. The possibility remains that levamisole may induce the immune system in a manner that demands energy requirements that would otherwise be used for growth. However, further investigations are required as fish are very susceptible to a variety of stressors, which could result in weight loss.

Exposure to in vivo doses of β-glucan resulted in no statistically significant differences in the phagocytosis assay, however there was one noteworthy trend. In fish exposed to β-glucan at 0.01 and 0.1 mg/kg there was an increased number of granulocytes and decreased the number of lymphocytes (figure 6.11). As flow cytometric counts are proportional, the result reflects either the stimulation of granulocytes or the suppression of lymphocytes (possibly both). It is
more likely, that the decrease in lymphocyte counts is due to a stimulation and recruitment of
granulocytes to the head kidney. This would agree with the findings of Jorgensen et al. (1993), who reported an increase in the number of neutrophils in the head kidney 3 weeks
post β-glucan exposure. β-glucan also showed a general immunostimulatory effect on the
gross parameters measured. In addition, there was an increase in the splenic somatic indices
and cell yields suggesting the recruitment of cells rather than the suppression of lymphocyte
numbers. However, as individual variation was high, the results were not statistically
significant and more fish need to be tested to determine biological significance. Although
there was no pronounced immunostimulation in vivo the results suggest that β-glucan may aid
in increasing immune responses by stimulating granulocytes and even the growth of Murray
cod may be enhanced.

Murray cod serum lysozyme concentration was not changed by any of the treatments used in
this study (table 6.5 and 6.6). Researchers have reported enhanced lysozyme activities in carp
following both i.p. and oral exposure (Alexander and Ingram, 1992; Siwicki, 1989). Other
researchers have also observed an increase in lysozyme activity following exposure to in vivo
β-glucan. This was not seen in this study and may be due to the relatively short exposure
period used in this study compared to others. For instance, Alexander and Ingram (1992) fed
their fish levamisole everyday for 12 weeks prior to lysozyme measurement and this study use
a single i.p. injection of levamisole 2 weeks before sampling.

The lack of pronounced in vivo immunostimulation by β-glucan and levamisole may be
explained by the hypothesis that immunostimulants (especially levamisole) work better on
immunosuppressed subjects. Cook and associates (2003), found that the feeding of β-glucan
supplemented food to snapper was only effective in significantly increasing the immune
response of fish held in “winter” conditions and Sahoo and Mukherjee (2001) reported that β-
glucan was only effective in immunosuppressed (aflatoxin-induced) rohu. Other studies have shown similar results and in humans levamisole is renowned to work only in immunocompromised patients (Van Wauwe and Janssen, 1991). These results may indicate that the conditions in which the Murray cod were not stressful to the fish. Observation of the fish also supports this as all fish used in the study acclimatised well and were feeding daily. Out of approximately 200 Murray cod only 4 failed to acclimatise, would not eat and looked chronically stressed (i.e. not feeding, discoloured and immobile). These fish were not used in any study. Future studies should be conducted on immunosuppressed Murray cod to determine if detrimental conditions can be reversed with immunostimulatory subjects. The stressor involved could be a number of different variables including a chemical immunosuppressor or an environmental immunosuppressor such as low temperatures, transport or crowding.

Concentration and timing of dose also needs greater investigation and may be the reason that minimal immunostimulation was observed in vivo. Although studies have reported immunostimulation using concentrations of β-glucan within the range used in this study (Robertson, 1994), some other researchers have using concentrations much higher (i.e. up to 50 mg/kg) (Engstad and Robertson, 1995; Jørgensen et al., 1993). β-glucan exposure at higher concentrations should be investigated, as the highest concentrations did show some immunostimulation and higher doses are unlikely to cause any side effects. Higher doses of levamisole may begin to show signs of toxicity as the highest dose caused a significant reduction in growth and also caused insignificant but interesting effect of reducing the number of beads per granulocyte and granularity (i.e. mean voltage and side scatter).

Timing experiments would show at what interval β-glucan and levamisole exert their maximum effects. Many studies investigating immunostimulants have found stimulatory
effects from 2 d – 6 weeks post exposure. This investigation chose a 14 day exposure period
but the maximum effects of the immunostimulants may have been earlier or later that the
sampling point.

There is no doubt a future exists for immunostimulants in Australia freshwater aquaculture,
especially as the industry grows and farming becomes more intensive and moves to indoor
systems. Finding a cost effective and simple agent would be of great benefit to the industry.
β-glucan supplements (e.g. EcoActiva\textsuperscript{TM}) for the addition to feeds and diets that contain
glucans have been available for some time and the products are marketed as
immunostimulatory (Cook et al., 2001; Robertsen, 1994). The challenge is in determining the
appropriate agent, dose regimes and timing, for each species. Because the effects of
immunostimulants are relatively short-lasting, treatment of fish may be reserved for specific
times of the year (i.e. during winter or transport) when immune functions are suppressed. If
an effective and inexpensive agent can be determined, it may be used throughout the year as a
general prophylactic. The other challenge is to determine the most cost-effective route of
administration. Although an i.p. injection generally gives a more uniform dose and response,
it is labour intensive and is not the preferred method of administration for fish farmers.
Supplementation of feed with immunostimulants is an attractive option to fish farmers as they
can easily switch feeds, with no need to change major operational procedures. Levamisole
and β-glucan did show some signs of immunostimulation in Murray cod. Longer term feeding
experiments involving more fish and emulating fish farming practices should be the next step
for the investigation into these immunostimulants.
Chapter 7: The effect of *in vitro* cyanobacterial exposure on the immune functions of Murray Cod

7.1: Introduction

Cyanobacteria are primitive photosynthetic prokaryotes that are found in freshwater and marine environments. They form green scums on lakes and rivers and have been known for their toxicity for at least 1,000 years. One of the first written accounts came from a Chinese General Zhu Ge-Ling, who reported mortality in his troops after they drank from a river that was green (NTP, 2003). The first case reported in the scientific literature occurred in 1878 in Australia, when George Francis reported a “Poisonous Australian lake” in *Nature* (Francis, 1878). To date, approximately 2000 strains of cyanobacteria have been identified. They produce a wide range of polyketide and polypeptide secondary metabolites with a variety of biological activities including antibiotic, algicide, cytotoxic, enzyme inhibition and immunomodulation. Some of these metabolites have been identified as neurotoxic alkaloids and hepatotoxic polypeptides and polyketides (NTP, 2000; Schembri *et al.*, 2001). Cyanotoxins have been classified into three classes based on their toxic effects and structure. Cyclic peptides such as microcystin (MC-LR) and nodularin are hepatotoxic, alkaloids such as anatoxin (ATX) and saxitoxin (STX) are neurotoxic, and cylindropermopsin (CYN) is a structurally distinct alkaloid with hepatotoxic and cytotoxic properties (Ohtani *et al.*, 1992). The effect of cyanobacterial toxins on the immune system has also been investigated, with both immunostimulation and immunosuppression being reported (Mundt *et al.*, 1991; Palíková *et al.*, 1998).
7.2: Structure of cyanobacterial toxins

7.2.1: Microcystin-LR (MC-LR)

The cyclic peptide MC-LR has a molecular mass of about 1,000 kDa and contains a leucine in the X position, arginine in the Z position, and methyl groups in both R positions (figure 7.1). The chemical structure of microcystins (MCs) include an unusual aromatic amino acid, Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), containing a substituted phenyldecadienoic acid (Neilan et al., 1999; NTP, 2000). Sixty-five isoforms have been identified that vary in degree of methylation, epimerisation, peptide sequence and toxicity. The toxicity of MCs is dependent on specific known structural variations, i.e. methylation of the β-amino acid (figure 7.1 R₁ and R₂) is conserved in all toxic microcystins and in the hepatotoxic pentapeptides nodularin and motuporin (Neilan et al., 1999). Polyketide synthases/peptide synthetases complexes are responsible for the production of MC-LR and the 55 kb gene cluster encoding for microcystin synthetase has been isolated and sequenced from a number of different cyanobacterial genera (Kaebernick et al., 2001; Neilan et al., 1999).

![Figure 7.1: The general chemical structure of microcystin (cyclo-(D-Ala₁-X²-D-MeAsp³-Z⁵-Adda⁵-D-Glu⁶-Mdha⁷)]. X and Z are variable amino acids L-leucine and L-alanine respectively; R¹ and R² are both CH₃ in MC-LR (Neilan et al., 1999).](Image)
The toxin is produced by a number of cyanobacterial species, but the most toxic and widely studied is *Microcystis aeruginosa*. *Nodularia* and *Osillatoria* species also produce significant amounts of the toxin, while the gene necessary for the synthesis of the peptide has been found in a genetically diverse range of microcystin-producing and non-toxic cyanobacterial species (Neilan *et al*., 1999).

**7.2.2: Cylindrospermopsin (CYN)**

Cylindrospermopsin was first identified by Ohtani *et al.* (1992) when it was isolated from laboratory cultures of *Cylindrospermopsis raciborskii*. At that time, the toxin was found to belong to a new class of tricyclic guanidine alkaloids. It possesses potent hepatotoxic and cytotoxic activity, which is dependent on the pyrimidine ring (figure 7.2) (Griffiths and Saker, 2003). Schembri and associates (2001) showed that the ability to produce CYN was linked to the presence of specific polyketide synthases and peptide synthetases genes, and these genetic markers have since been used to accurately identify toxic and non-toxic strains of cyanobacteria (Fergusson and Saint, 2003).

![Chemical structure of cylindrospermopsin](image)

**Figure 7.2: The chemical structure of cylindrospermopsin.**

**7.3: Mechanism of Action**

**7.3.1: Microcystin-LR**

MC-LR is actively taken into hepatocytes via the bile acid transport system where the majority of toxicity occurs. They are potent inhibitors of the protein phosphatases type 1 and 2A (*IC*<sub>50</sub> ≈ 0.1 nM) and cause damage in hepatocytes by the hyperphosphorylation of DNA...
and microfilaments, including cytokeratins. MC-LR binds to the phosphatases in two steps. Firstly, the toxin binds rapidly to the enzyme, reversibly inhibiting its catalytic activity. Secondly, the \( N \)-methyldehydroalanine (Mdha) portion of the molecule covalently binds a nucleophilic site on the phosphatase in an irreversible process. This covalent binding suggests that MC-LR tends to accumulate in the liver as a MC-LR-phosphatase complex, which can lead to problems in accurately detecting accumulated toxin in the organ (Williams et al., 1997). MC-LR also stimulates phospholipase \( A_2 \) and cyclooxygenase in hepatocytes, while in macrophages it induces the release of pro-inflammatory cytokines (Rocha et al., 2000).

### 7.3.2: Cylindrospermopsis

The toxic mechanism of CYN is largely unknown and appears to involve a number of pathways. CYN-induced inhibition of protein synthesis was confirmed by a globulin synthesis assay using a rabbit reticulocyte cell-free system (Terao et al., 1994) and is a sensitive, early indicator of cellular responses to CYN (Froscio et al., 2003). Protein synthesis inhibition is irreversible and the parent compound is responsible, as inhibiting cytochrome P450 function does not reduce the inhibition of protein synthesis (Froscio et al., 2003). Conversely, other researchers have reported that activation of CYN by CYPs is a necessary step for maximum toxicity as CYP inhibitors offer some protection against CYN induced toxicity (Norris et al., 2002). Cellular disruption commences with the detachment of ribosomes from the membranes of the rough endoplasmic reticulum. CYN is highly toxic regardless if it is injected or given orally (Seawright et al., 1999). In both rats and mice, CYN reduces glutathione stores not through detoxification consumption, but by inhibiting glutathione synthesis (Norris et al., 2002; Runnegar et al., 1995).
CYN has guanidine and sulfate groups that are able to covalently bind to DNA and RNA and cause breakages (Shen et al., 2002). Humpage (2000) suggested that CYN caused cytogenetic damage by two mechanisms. They proposed that CYN could break DNA, while also inhibiting spindle formation causing the loss of whole chromosomes.

7.4: Immunotoxicity in mammals

A number of studies have reported immunotoxicity from both extracts of MC-LR producing cyanobacteria and pure MC-LR. Low doses (10^{-12} to 10^{-9}) of MC-LR significantly enhanced human polymorphonuclear leukocyte adherence (Hernandez et al., 2000) and rat macrophages stimulated \textit{in vitro} with pure MC-LR (0.1, 0.3 and 1.0 \mu g/mL), displayed a dose-dependent increase in their secretion of interleukin-1\beta (IL-1\beta) and tumour necrosis factor--\alpha (TNF--\alpha) (Rocha et al., 2000). Mice exposed to extracts of 4 cyanobacterial species (\textit{Nodularia spumigena}, \textit{Synechocystis aquatilis}, \textit{Oscillatoria redekei} and \textit{Microcystis aeruginosa}) displayed a dose–dependent immunosuppression of mitogenesis and plaque-forming cells and the cyanobacterial strains \textit{Limnothrix redekei}, \textit{Oscillatoria tenuis} and \textit{Synechocystis aquatilius} each inhibited the lymphoproliferation of human lymphocytes (Mundt et al., 1991; Mundt et al., 2001). Additionally, low doses of \textit{Nodularia spumigena} extract (0.2 mg/mouse) initially stimulated plaque-forming cells before suppressing their function at higher doses (0.4 mg/mouse) (Mundt et al., 1991).

Subchronic studies involving mice exposed orally with 150 \mu g kg^{-1} MC-LR for 28 d, showed the number of leukocytes increased due to lymphocytosis, but the number of polymorphonuclear leukocytes was unchanged. A dose of 50 \mu g/kg caused no changes and both treatment groups showed no alterations in number of erythrocytes, haemoglobin concentration and haematocrit (Heinze, 1999).
Immunotoxicological studies into the effects of CYN are limited. However, mice repeatedly exposed to CYN resulted lymphophagocytosis in the spleen and thymus, which is indicative of an immunotoxic response (Shaw et al., 2000)

7.6: Cyanobacterial toxins in the aquatic environment

7.6.1: Monitoring

Cyanobacteria are commonly monitored during phytoplankton testing of drinking water supplies, although they are not the main focus of monitoring programs and there is commonly no specific management response. In Finland, automated testing of Nodularia populations in the Baltic Sea occurs through samplers installed on ferries. In New Zealand, China, Thailand, South Africa, Latvia (for the Baltic Sea), some states of Australia, Brazil and Germany, there have been co-ordinated efforts to monitor cyanobacteria populations and cyanotoxin concentrations (Jones and Chorus, 2001). In a study of 75 water bodies in Florida, USA, 88 of the 167 samples showed significant concentrations of toxic cyanobacteria (i.e. Microcystis, Cylindrospermopsis and Anabaena) (Carson, 2000). Studies performed in and around the Murray-Darling Basin, Australia, have reported similar results. Samples were taken from 231 algal bloom sites and of those, 92 (42%) were acutely toxic in mice following an i.p. injection (Baker and Humpage, 1994). Detection of toxic cyanobacterial blooms was traditionally achieved through morphological studies and extraction of the toxin with subsequent HPLC or GC analysis. More recently, molecular techniques continue to be developed to aid in the rapid detection of toxic blooms (Baker et al., 2001; Fergusson and Saint, 2003).

7.6.2: Distribution and occurrence

In recent years, the occurrences of harmful and toxic phytoplankton blooms have increased in frequency, intensity and geographic distribution, and have had a wide range of social, economic and environmental impacts. They occur in eutrophic aquatic habitats mainly as a
result of pollution and the proportion of cyanobacterial blooms which are toxic has been estimated in a number of surveys worldwide to be as high as 70% (Chorus et al., 2000; Humpage and Falconer, 1999). Livestock mortalities have been attributed to the drinking of water supplies contaminated with cyanotoxins (Thomas et al., 1998). Toxic blooms have also caused severe economic losses in fisheries, tourism and aquaculture. The problem in aquaculture is significant as many cultured stocks are bivalve shellfish, crustaceans and fin fish larvae that are filter feeders and can accumulate toxic cyanobacteria (Magalhães et al., 2003).

7.7: The effect of cyanobacterial toxins on aquatic organisms

7.7.1: Toxicokinetics

There is a danger of human exposure to cyanobacterial toxins through the consumption of contaminated fish and shellfish (Magalhaes et al., 2001). Consequently, studies into the bioaccumulation of cyanotoxins in fish and shellfish have been comprehensive. Fish are generally more tolerant to algal toxins than mammals and can accumulate them over time. In addition, exposure may occur in some water bodies for several months of the year, which can lead to high concentrations of toxins in tissues and an increased risk of harmful exposure to humans who may consume these fish (Carson, 2000).

In fish, MC-LR is absorbed rapidly from the GIT and a small amount can also be absorbed via the gills (Zambrano and Canelo, 1996). Once in the systemic circulation the mechanisms involved is the same as for mammals. MC-LR is quickly transported to the liver where it is taken up into hepatocytes by the bile acid transport system and covalently binds to protein phosphatase 1 and 2A (Fischer et al., 2000; Tencalla and Dietrich, 1997).
Cylindrospermopsin has been shown to accumulate in the tissues of the Redclaw crayfish (*Cherax quadricarinatus*) and rainbowfish (*Melanotaenia eachamensis*) demonstrating that CYN human exposure could occur from the consumption of farm-raised freshwater aquatic foods (Saker and Eaglesham, 1999).

### 7.7.2: Aquatic Toxicology

Aquatic toxicity testing of MC has been conducted in numerous invertebrates and a few fish. In contrast, there has been only one study investigating the effects of cylindrospermopsin in aquatic organisms (table 7.1).

#### Table 7.1: A summary of cyanotoxin toxicity data from experiments on aquatic organisms.

<table>
<thead>
<tr>
<th>Species</th>
<th>Toxin</th>
<th>LD(_{50})</th>
<th>Other reported toxicities</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brine shrimp (<em>Artemia salina</em>)</td>
<td>CYN</td>
<td>8.1 (\mu g/mL) (24 hr) 0.71 (\mu g/mL) (72 hr)</td>
<td>None</td>
<td>(Metcalf <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td>Brine shrimp (<em>Artemia salina</em>)</td>
<td>MC-RR</td>
<td>5.0 (\mu g/mL) (24 hr)</td>
<td>None</td>
<td>(Kiviranta <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>Freshwater Protozoan (<em>Tetrahymena pyriformis</em>)</td>
<td>MC-LR</td>
<td>232 (\mu g/mL) (24 hr)</td>
<td>Growth rates IC(_{50}) = 160 (\mu g/mL) Respiration</td>
<td>(Ward and Codd, 1999)</td>
</tr>
<tr>
<td>Copepod (<em>Diaptomus birgei</em>)</td>
<td>MC-LR</td>
<td>0.45-1.0 (\mu g/mL) (48 hr)</td>
<td>None</td>
<td>(DeMott <em>et al.</em>, 1991)</td>
</tr>
<tr>
<td><em>Daphnia pulicaria</em></td>
<td>MC-LR</td>
<td>21.4 (\mu g/mL) (48 hr) 11.6 (\mu g/mL) (48 hr) 9.6 (\mu g/mL) (48 hr)</td>
<td>None</td>
<td>(DeMott <em>et al.</em>, 1991)</td>
</tr>
<tr>
<td><em>Daphnia hyalina</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Daphnia pulex</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Carp</em> (<em>Cyprinus carpio</em>)</td>
<td>MC-LR</td>
<td>550 (\mu g/kg) (i.p. 7 d)</td>
<td>Sublethal hepatic damage and gill damage</td>
<td>(Råbergh <em>et al.</em>, 1991) (Carbis <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td><em>Rainbow trout</em> (<em>Oncorhynchus mykiss</em>)</td>
<td>MC-LR</td>
<td>&lt;400 (\mu g/kg) (No mortalities) 550 (\mu g/kg) (100 % mortality) 1000 (\mu g/kg) (100% mortality)</td>
<td>Hepatic and renal damage</td>
<td>(Kotak <em>et al.</em>, 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30-40 mg/kg (12-36 hr)</td>
<td></td>
<td>(Tencalla <em>et al.</em>, 1994) (Phillips <em>et al.</em>, 1985) (Fischer <em>et al.</em>, 2000)</td>
</tr>
<tr>
<td><em>Atlantic Salmon</em> (<em>Salmo salar</em>)</td>
<td>MC-LR</td>
<td>550 (\mu g/kg) (x 3 over 9 d. 36 day exp. (few mortalities)</td>
<td>None</td>
<td>(Andersen <em>et al.</em>, 1993)</td>
</tr>
</tbody>
</table>

MC-X = *Microcystis* extract
Numerous studies have shown analogous but not identical pathological changes, in fish when compared to mammals, with the main target organ being the liver (Carbis et al., 1997; Fischer et al., 2000; Råbergh et al., 1991; Tencalla and Dietrich, 1997). MC-LR has also been implicated as the cause of “netpen liver disease”, which has afflicted Atlantic salmon in the northeast Pacific (Andersen et al., 1993; Williams et al., 1997). Some studies have recorded gill damage, indicating that absorption of MC-LR also occurs via the gills (Carbis et al., 1997; Zambrano and Canelo, 1996). In addition, Zambrano and Canelo (1996) investigated the effects of microcystin on the activity of Na\(^+/\)K\(^+\) pumps from gill microsomes. They found that microcystin could inhibit the aspartic dephosphorylation step of the sodium pump enzymes, which could lead to an inhibition of gill function. To date, no studies on the acute effects of CYN in fish have been reported in the literature.

7.7.3: Immunotoxicity in aquatic organisms

Palíková et al. (1998), investigated the in vivo effect of pure microcystin and the biomass of blue-green algae on a number of immunological parameters in carp and silver carp. They observed a reduction in total leukocyte counts and leukocrit values, however there were species differences in the types of cells affected. In the carp, cytotoxic T-cells and slg\(^+\) B-cells were reduced, whereas in the silver carp, mainly neutrophilic myelocytes and metamyelocytes were affected which resulted in a reduction of phagocytic activity. The biomass of blue-green algae affected immunological parameters to a greater extent than pure microcystin. There have been no studies on the effects of CYN on teleost immune functions.

7.8: Aim

The aim of these preliminary experiments was to determine if in vitro exposures to MC-LR and CYN were immunotoxic to Murray cod head kidney cells. In vivo studies were not conducted because sufficient amounts of cyanotoxins were unavailable.
7.9: Methods

7.9.1: Spleen and Head kidney Sampling
Murray cod were housed and maintained as described in section 2.1. Fish were anaesthetised and killed as described in section 2.2.

7.9.2: Exposure protocol
Microcystin-LR (MC-LR) was purchased from Sapphire Biosciences (Crows Nest, NSW, Australia). Cylindrospermopsin was obtained from Carolyn Haskard, (Australian Water Quality Centre, Salisbury, SA, Australia). Stock solutions were dissolved in saline at 2 mg/mL for MC-LR and 3 mg/mL for cylindrospermopsin. These were diluted 1:100 for MC-LR and 1:150 for cylindrospermopsin to obtain intermediate stock solutions of 20 μg/mL (high concentration). These were serially diluted 1:10 to obtain 2 μg/mL (medium) and 0.2 μg/mL (low) stocks. For all assays, the intermediate stocks were diluted 1:4 resulting in final concentrations of 0 (i.e. saline), 0.05, 0.5 and 5 μg/mL.

7.9.3: Cell isolation
The methods for the isolation of Murray Cod immune cells is described in detail in section 2.3.

7.9.4: Phagocytosis
Standardised protocols for the phagocytosis assay are described in detail in section 2.4.2 and the methods used in this experiment are the same as the in vitro organotin study described in section 3.13.4.
7.9.5: Mitogenesis

Standardised protocols for the mitogenesis assay are described in detail in section 2.6.2.2. and the methods used in this experiment are the same as the *in vitro* organotin study described in section 3.13.5.

7.9.6: Statistics

Statistics were performed using the same methods described in section 3.13.7.

7.10: Results

7.10.1: The effect of *in vitro* MC-LR exposure on Murray cod immune functions

*Phagocytosis*

Culturing MC-LR with head kidney cells resulted in a dose-dependent increase in the percentage of FITC-positive granulocytes and in the mean voltage of the FITC fluorescence (*p*<0.05) (figure 7.3). This indicates that MC-LR stimulates phagocytes by both increasing the numbers of cells engaged in phagocytosis and the number of beads engulfed per cell. At the concentrations tested there was no toxicity observed in either the granulocyte or lymphocyte subpopulations.

*Mitogenesis*

MC-LR did not significantly affect either the basal or PHA-stimulated responses of lymphocytes or the proliferation index of lymphocytes (figure 7.4).
Figure 7.3: Phagocytic function and subpopulation counts of Murray cod head kidney immune cells after in vitro exposure to microcystin-LR (MC-LR). a) Phagocytosis (FITC +ve) b) number of beads per cell (mean voltage and side scatter (SS) increase c) Granulocytes and lymphocyte subpopulation counts d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of control samples and values represent mean ± se of 4 fish. Control values were 9±1% for FITC +ve total, 18±2% for FITC +ve granulocytes, 144±42 volts for mean voltage, 141±51 volts for SS increase, 5550±350 events for granulocytes, 2550±250 events for lymphocytes, 1350±100 events for debris and 8±1% for PI +ve. † denotes linear relationship (p<0.05).
Figure 7.4: The effect of *in vitro* microcystin-LR (MC-LR) exposure on Murray cod head kidney lymphocytes a) lymphoproliferation profiles and b) proliferation index. Proliferation index is expressed at percentage of untreated control (i.e. 1.13±0.04) and values represent mean±se of 4 fish. No statistically significant differences found.

### 7.10.2: The effect of *in vitro* cylindrospermopsin exposure on Murray cod immune functions

**Phagocytosis**

Cylindrospermopsin at 0.05 μg/mL significantly increased the percentage of granulocytes with beads and also elevated the average number of beads per cells. It did not affect the numbers of events in the granulocyte and lymphocyte gated-regions or the number of non-viable cells and debris-gated events (figure 7.5).

**Mitogenesis**

In the lymphoproliferation assay there was a small but insignificant increase in lymphocyte proliferation at 0.05 μg/mL (figure 7.5). The basal and peak CPM of lymphocytes exposed to 5 μg/mL cylindrospermopsin was consistently lower than all other groups (figure 7.6a).
Figure 7.5: The effect of *in vitro* cylindrospermopsin exposure on subpopulations and phagocytic activity of Murray cod head kidney cells. a) Phagocytosis (FITC +ve) b) number of beads per cell (mean voltage and side scatter (SS) increase c) Granulocytes and lymphocyte subpopulation counts d) debris counts and non-viable cells (PI +ve). Data is expressed as percentage of untreated control samples (n=4) and values represent mean ± se of 4 fish. Untreated control values were 9±1% for FITC +ve total, 18±2% for FITC +ve granulocytes, 144±42 volts for mean voltage, 141±51 volts for SS increase, 5550±350 events for granulocytes, 2550±250 events for lymphocytes, 1350±100 events for debris and 8±1% for PI +ve. * denotes significantly different from the solvent control (p<0.05).
7.11: Discussion

There was evidence that both MC-LR and CYN increased the phagocytic activity of Murray cod granulocytes (figure 7.3 and 7.5). Further studies would be needed to elucidate the mechanism, but the observations of the present study concur with the findings of Hernandez and associates (2000) in mammals. They found that MC-LR (and nodularin) significantly increased the early spontaneous adherence of human peripheral polymorphonuclear leukocytes. The mechanism of this process can be further explained by observations of Rocha and co-workers (2000), who recorded an increased release of the pro-inflammatory cytokines, IL-1β and TNF-α by rat phagocytes in response to MC-LR and nodularin exposure. IL-1β and TNF-α both upregulate Fc-receptor-mediated phagocytosis by human polymorphonuclear cells (Bergman et al., 2002) and therefore an increase in the secretion of these cytokines would increase phagocytic activity.
The release of TNF-α has been shown to be a key step in the development of microcystin-induced shock. Rocha and co-workers (2000) showed that in the presence of microcystin rodent macrophages produced TNF-α and IL-β in a dose-dependent manner. They subsequently showed that protection from microcystin toxicity could be achieved by blocking the action of TNF-α with specific antibodies directed against the cytokine. In addition, it has been suggested that TNF-α is also involved in MC-LR induced tumour promotion in rat liver (Fujiki and Suganuma, 1994). Further studies measuring cytokine secretion from cyanobacteria-exposed fish immunocytes would determine if this were the case. Similar studies would also determine whether CYN exposure also resulted in secretion of the same cytokines and at the same concentrations.

The concentrations of cyanotoxins used in these in vitro experiments were below cytotoxic levels and no change was observed in any of the subpopulations numbers. Further studies using higher concentrations of the cyanotoxins should be completed to ascertain what concentrations cause cytotoxicity. Concentrations of MC-LR above 5 μg/mL should be tested to determine if higher doses continue to stimulate the granulocytes, or whether toxicity is observed. It is also possible that concentrations of CYN below 0.05 mg/mL could result in greater stimulation of phagocytes. Concentrations of CYN above 5 μg/mL should also be tested to determine if higher doses of CYN results in significant immunosuppression or cytotoxicity.

There was no significant change in proliferation of Murray cod lymphocytes when incubated with either MC-LR or CYN. However, CYN at 5 μg/mL caused a slight reduction in both basal and PHA-induced CPM, which suggests an inhibition of tritiated-thymidine uptake.
(figure 7.4a). However, this was not statistically significant due to a large individual variation in Murray cod responses, but warrants further investigation.

These results indicate that acute exposure to MC-LR and CYN can modulate Murray cod head kidney cell responses. They also suggest that in vitro CYN displays a greater degree of immunomodulation compared to MC-LR, which is known to be more toxic. As shown from previous studies in humans and rodents, one of the most prominent effects is the increase in phagocytic activity. As foreign polypeptides, it is likely that Murray cod antigen presenting cells are able to recognise these proteins through innate immunological systems, and may respond via the release of cytokines which could lead to imbalances in homeostasis. Other researchers have observed immunosuppression in mammals and fish but have found different cell targets in different species (Palíková et al., 1998). These findings show that MC-LR and CYN have moderate immunomodulating properties after an acute in vitro dose. In vivo studies on the chronic effects of cyanotoxins would add further information on the long-term effects of these toxins on fish immune function and may be more relevant to some situations where fish are exposed to cyanotoxins for several months in a year.
Chapter 8: Overview and Conclusions

8.1: Motivation of the present study

The Murray-Darling basin is the largest river system in Australia with significant economic, social, recreational and cultural value. The fish species of the basin are unique to the system, however, populations have declined dramatically since settlement due to habitat destruction and pollution from anthropological activities. ANZECC and ARMCANZ (2000) have set water quality guidelines, which are largely based on tests from exotic species, as data on the acute toxicity of many chemicals on native fish is still not available. Ecotoxicological testing involving native fish have investigated the acute effects of some chemicals, although recently most investigations have also included the sublethal effects of xenobiotics on physiological systems.

The immune system protects its host from infections and neoplasm, while immunocompetent cells are vulnerable to chemical insult because they require constant proliferation and differentiation for renewal. It is therefore an important and sensitive target organ system, and consequently a tiered battery of tests has been validated for the assessment of immunotoxins. This tiered system has identified a number of xenobiotics that have potential to cause immunomodulation in fish. Nevertheless, very little attention has been given to the effect of chemicals on the immune function of Australian fish and there is also a lack of information on the immunology of native freshwater fish. Therefore, this study aimed to address the paucity of information concerning the immunology of particular Australian freshwater fish by optimised and exploiting immune assays to investigate the modulation of immune functions by environmental pollutants and therapeutic immunostimulants. This final chapter discusses the methods and summarises the findings of this study.
8.2: Appraisal of Methods

8.2.1: Selection of test organism

8.2.1.1: Use of small fish

Many research groups investigating the immunotoxicity of environmental pollutants have used small fish, such as medaka (Anderson and Brubacher, 1993; Wester et al., 1990a; Zelikoff et al., 2002), guppy (Wester and Canton, 1987; Wester et al., 1990a) and zebra fish (Rougier et al., 1996; Rougier et al., 1994). These studies have found that small fish have a number of advantages including a short life cycle and higher breeding rates (which is advantageous in generational and life-cycle toxicity testing), an ability to use large numbers (which increases statistical power) and reduced equipment and maintenance costs.

This project investigated the small Murray-Darling native crimson-spotted rainbowfish, which has been studied for over ten years at the Key Centre for Toxicology, RMIT. Previous studies at RMIT have investigated this species as a potential sentinel species using reproductive endpoints and they have also been used in multigenerational and limited immunotoxicology studies. The rainbowfish used in this study had been inbred for six generations, were extremely well acclimatised to laboratory conditions and were in excellent condition. Consequently, variability in results from genetics and handling stress should have been reduced to a minimum. Nevertheless, technical difficulties with immunotoxicity assessment in these fish were encountered because of their small body and organ sizes.

The primary difficulty was a lack of immune tissue and serum that could be obtained from a fish of this size (i.e. <12 g). Assays that required higher cell concentrations could not be completed without pooling cells from different animals, which also increased the opportunity for cell cultures to become contaminated. This was a common issue for cell cultures from the head kidneys, because it is located in close proximity to the oesophagus, which is a potential
source of microbial contamination if it is severed. The lysozyme assay was unsuccessful due to a minimal volume of serum that could be collected from a single fish, as well as excessive blood clotting when attempting to collect blood into heparinized capillary tubes.

Although rainbowfish were supposedly well acclimatised to laboratory conditions a large amount of variation was still seen in general parameters after in vivo exposures. In general, control fish in these experiments lost weight that was comparable to treated fish, which may be attributed to stress from territorial and feeding dominance by larger, dominant males in certain exposure groups. There was also a large variation in the size of fish, with the smallest weighing 5 g and the largest weighing over 10 g. This is a likely source of variation seen in parameters such as weight change, somatic indices and cell yields and may have also contributed to some variation in the phagocytosis assay. It should also be emphasised that these fish were ex-breeding stocks that were considerably older (2 y) than the larger fish used in this study (i.e. 1 y), especially considering the shorter life cycle of rainbowfish (i.e. about 3-4 y). Although studies have shown that adult fish have a greater capacity to mount an immune response than juvenile fish (Hrubec et al., 2004) and that juvenile fish immune functions are more susceptible to chemical insult (Duffy et al., 2002), no studies have compared the immune function of the different age classes of adult fish.

8.2.1.2: Use of farmed native Australian fish

Investigations concerning fish immunology and immunotoxicology have mainly been focused on cultured fish species such as rainbow trout, European carp, channel catfish and various species of salmon (O'Halloran et al., 1998a). These are commonly used because of their high economic value, large body of literature concerning their immunology and the availability of various research tools for some of these species (i.e. monoclonal antibodies to label cell surface markers, genetic libraries and molecular probes). Additionally, laboratory conditions
for their optimal health and growth are well-defined, including culture temperature, nutrition and handling protocols. Endogenous influences on immune function, such as breeding cycles and circadian rhythms may also be defined. Compared to wild fish, farmed fish have the advantage of being easily acquired and maintained in laboratories. Stress from handling during experiments may also be reduced, as cultured fish have been handled from a young age. However, it should be emphasised that farmed fish are kept in “unnatural” conditions, especially those grown with indoor systems and/or fed artificial pellet diets. These conditions are optimised to promote accelerated growth rates but should be considered when extrapolating the toxicological responses of cultured fish to wild populations.

This thesis investigated immune functions of three cultured freshwater Australian fish, the golden perch, silver perch and Murray cod. Procurement of cultured freshwater native fish at a desired size (i.e. 100 g) from a local supplier was challenging because much of the industry is in the warmer parts of northern NSW and Queensland. In addition, many fish farmers demanded a high price per kilogram for intermediate sized fish and fish were always supplied at a bigger size than ordered.

Golden perch were procured early in the project and large yearling fish of 150-250 g were obtained from a fish farmer who grew them in outdoor ponds. Most of these fish adapted well to laboratory conditions, but would only accept live food (i.e. small yabbies or fish) or thawed meats (i.e. carp fillets or ox heart). Dominance was also a problem if fish were held in tanks with more than one golden perch, and the submissive fish appeared chronically stressed (i.e. not feeding, discoloured and immobile). Some of the golden perch held by themselves did not acclimatise, would not feed, appeared stressed (i.e. immobile and discoloured) and were not used. Unfortunately, the fish farmer supplying golden perch went out of business during the research project and another local source of large golden perch could not be found. The
golden perch aquaculture industry has not progressed in Victoria, due to the previously described difficulties in feeding and slow growth rates in outdoor systems. Fingerling sized (i.e. <10 g) golden perch were acquired to continue work on *in vitro* mitogenesis assays and *in vivo* exposures. However, due to similar problems with dominance and feeding, the fingerling fish were not used as insufficient numbers reached a suitable size during the 18 months they were grown in the laboratory. It should be noted that all the results reported in this thesis, including the optimisation studies and *in vitro* testing of the organotins and pesticides, used the larger yearling fish.

Silver perch used in this study were either purchased as yearlings of an appropriate size (i.e. 100 g) from fish farmers or quickly raised in the laboratory from fingerling size (i.e. <10 g). They adapted well in large tanks with many individuals and readily accepted a commercial pellet diet. Some dominance behaviour occurred and a pecking order was evident, with larger fish appearing to eat more food, grow faster and bite at the tails and fins of smaller fish. Compared to the other two native fish, silver perch were more sensitive to the anaesthetic MS222, with lower doses being more effective in a quicker time. In addition, they were also more susceptible to handling stress and would not acclimatise in smaller exposure tanks with lower group numbers, resulting in unacceptably high mortalities in the untreated and solvent controls. Consequently, *in vivo* exposure of silver perch to TBT was attempted but unsuccessful and focus was turned to the more aquaria-amenable species. If *in vivo* exposures of silver perch were to be reattempted, it is recommended that larger tanks and sample sizes are used or that fish are tagged and placed in a large tank with a larger group of fish and sufficient water flow to minimise uptake of excreted compounds by other fish.

Yearling over-wintered (i.e. held at lower temperature to reduce growth) Murray cod of 150-200 g in size were procured from a fish farmer who grew them using indoor systems. They
had been weaned onto a commercial pellet and gained weight quickly while being held. They adapted very well to large tanks with many individuals, but were required to be held at a high density to prevent territorial behaviour. Individual Murray cod adapted extremely well to exposure tanks, and only 4 out of over 200 fish showed signs of stress such as a lack of feeding and weight loss (not used in experiments). Well-acclimatised Murray cod displayed aggressive feeding and territorial behaviour, which included attacking the siphon used to clean the tanks. The only perceivable problem with the Murray cod was that they were supplied at a larger than agreed size and smaller fish would have been more desirable for the size of the exposure tanks. Nevertheless, this was not a major problem, due to the flow-through design of the tanks in the laboratory, and water exchange rates were increased to accommodate these larger fish.

Murray cod are an excellent species for ecotoxicological testing because they are the top(61,518),(962,801)

8.2.2: Measurement of immune function

The immune system protects its host from disease and is a highly complex and integrated system. Numerous assays have been developed to measure the activity of its various components such as the innate, humoral and cell-mediated functions, while the integrity of the entire system is assessed through disease challenge models. The assessment of immunotoxins
in mammals has been achieved via a tiered system, which was developed and validated by the National Toxicology Program (Luster et al., 1988). The first tier involves simple measurements, while the second tier investigates immunomodulation in functional assays. Tier 3 tests involve the assessment of the integrity of the entire immune system through host resistance models. The tiered battery of tests is intended to determine the immunotoxicity of chemicals to whole organisms (i.e. in vivo exposures), however, in vitro exposures of primary cell cultures offer valuable insights into the mechanism of action of toxic chemicals.

This study investigated a number of tier 1 tests (i.e. lymphoid organ weights and cellularity, somatic index and lysozyme activity) and a limited number of tier 2 tests (i.e. lymphoproliferation and phagocyte function) to assess the effects of chemicals on the immunity of Australian freshwater fish. Tier 3 tests (i.e. host resistance assays) were not conducted, as the focus of this study was to develop simple screening tests in native freshwater species and the special pathogen containment facilities required for host resistance testing were not available.

The measurement of lymphoid organ weights, somatic index and cellularity are the most basic parameters that can be used to screen chemicals for in vivo immunotoxicity. Although these parameters display variation between individuals in most fish studies, this study reported significant changes in native freshwater fish exposed to certain xenobiotics. Furthermore, the information obtained from these parameters offer insights into the mechanism of action of chemicals when used in combination with the other functional assays used in this study.

The lysozyme assay is a simple and rapid measurement of the innate bactericidal function of fish serum. The optimal pH was identified for the four native fish investigated in this study, but unfortunately, assay temperatures could not be optimised due to a lack of this function in
the plate reader used. The lysozyme activity of the native fish investigated was relatively low compared to some other fish species reported in the literature (i.e. bluefin tuna 20-50 μg/mL) (Watts et al., 2002). Golden perch had the highest serum lysozyme activity, however, this probably reflects the older age and larger size of the fish used in the optimisation study rather than species-specific differences. Significant differences were seen between two groups of silver perch that were either reared in ponds and transported a long distance to the laboratories or had been reared from fingerlings within the laboratory (data not shown).

Pollutants such as heavy metals have been reported to increase fish serum lysozyme activity (Low and Sin, 1998) and diesel oil was reported to suppress lysozyme activity (Tahir and Secombes, 1995). In this study, the lysozyme assay appeared to be the least sensitive and little change was seen in this parameter when fish immunocytes were exposed to in vitro and in vivo doses of xenobiotics. This may have been due to variation in the responses of individual fish, the low activity of native fish lysozyme or the mode of action of the chemicals tested. Inter-fish variation was seen in some groups and appeared to increase in a dose-dependent manner, which may also suggest that there are subgroups of responders and non-responders (figure 5.10).

Nevertheless, lysozyme function may not have been targeted by the chemicals investigated in this study as previously reported for other environmental contaminants such as PAHs and PCBs (Hutchinson et al., 2003) and cadmium (Zelikoff et al., 1995). Reports of the stimulation of lysozyme activity is most common after exposure to microbes, vaccines and immunostimulants (i.e. β-glucan) (Engstad et al., 1992; Koskela et al., 2004; Matsuyama et al., 1992; Paulsen et al., 2001). This study did not observe an immunostimulant-induced increase in lysozyme activity, but the assay could be a useful tool in the future for native fish farmers to monitor the effectiveness of vaccines and immunotherapies where a change in
serum lysozyme might be expected. However, it has been demonstrated that inter-fish variability is an issue and large test groups are recommended.

The lymphoproliferation assay was optimised for head kidney lymphocytes of Murray cod and golden perch. Our group had previously studied the mitogenesis assay for rainbowfish and silver perch, so optimisation was not re-examined in this study (O'Halloran, 1996). As previously reported by our group, the native fish lymphocyte responses to mitogenic stimuli were lower compared to other exotic species, such as rainbow trout (O'Halloran, 1996). Additionally, the assay shows a high variation in response between fish, while responses from splenic lymphocytes were very variable and unreliable, and thus were not used to test for immunotoxicity. Information from flow cytometry suggested that the cell-culture media was not fully supporting the growth of lymphocytes, as there was a gradual loss over the incubation period. However, the head kidney cell cultures from golden perch and Murray cod gave a consistent, day-to-day, bell-shaped curve when stimulated with PHA, and therefore this was selected to determine the immunotoxicity of the chemicals in Murray cod.

While exposure to high doses of organotins suppressed PHA-stimulated lymphoproliferation, it became apparent when using other chemicals that this assay lacked some sensitivity, due to the low mitogen-induced response measured. If future researchers were to use this assay, it is recommended that the cell culture conditions be re-examined and further optimised. Although this thesis investigated numerous conditions for the optimisation of the mitogenesis assay, no significant improvements in response were observed. However, experiments with β-glucan demonstrated that 10 μg/mL aided in the mitogenesis and increased the proliferation index of Murray cod head kidney lymphocytes and might be considered as a supplement in any future investigations.
The flow cytometric analysis of phagocytosis was used to replace tedious and subjective microscopy counting that was previously used by our group to quantify phagocytic activity. The flow cytometry phagocytosis assay correlated well with the method of microscopy counting and was deemed suitable to assay the phagocytic function of head kidney granulocytes. The flow cytometer also has the advantage of offering additional data on the integrity of head kidney subpopulations and increases the sensitivity of phagocytic assays, although care is needed in the calibration of the machine and interpretation of subpopulation results.

The alignment of the laser and the voltages of the detectors (PMTs) need to be set before commencement of experiments, and calibration of the machine needs to be assessed daily with calibration beads (i.e. Flow-check beads, Beckman-Coulter, Fullerton, CA, USA). It should be noted that the data collected from the flow cytometer are proportional counts of 10,000 events, therefore specific toxicity towards one cell subpopulation during in vivo exposures results in a perceived increase in the other subpopulation (and visa versa). Furthermore, a high level of cytotoxicity and reduced cell concentration during in vitro exposures can lead to an increase in parameters such as mean voltage. The mean voltage of FITC +ve events describes the number of beads ingested by granulocytes during phagocytosis because the intensity of the fluorescent signal increases when more beads pass the laser together. It is a sensitive measure of granulocyte activity and phagocytic rate, however, in the presence of low cell viability and reduced cell concentration, mean voltage becomes an inaccurate measurement due to a rise in the proportion of bead “clumps” within the granulocyte-gated region. Nevertheless, with these implications in mind, the flow cytometric analysis of phagocytosis was the most sensitive and useful technique used in this study, as it simultaneously obtained information on many different parameters of the head kidney cell cultures.
8.3: Conclusions

Suitable native test species

- Murray cod was identified as a freshwater native Australian species that is extremely well suited to immuno-ecotoxicology testing of chemicals in laboratory situations. They are readily available from fish farmers, adapt extremely well to tanks, are robust to handling and stress, feed on a standard commercial pellet diet and a large amount of tissue can be obtained from one fish. They also have a high ecological, economic, recreational and cultural value.

Development of immune assays

- Functional immune assays that have been previously used in mammals and other fish species were successfully adapted to four freshwater Australian fish. Immune responses were generally consistent, although lower when compared to exotic species, such as rainbow trout.

- The previously identified immunotoxins, TBT and DBT, were used to test the efficiency and sensitivity of the assays. These experiments demonstrated that all of the functional assays showed some immunomodulation by TBT and DBT, although there were differences in the sensitivity of assays and degree of immunosuppression. Of the three functional assays, the phagocytosis assay appeared to be the most sensitive, followed by the mitogenesis assay, while the lysozyme assay was the least sensitive, but this may also have been due to the mechanism of action of the organotins.
Organotin exposures

- *In vitro* exposures to organotins appeared to target lymphocyte subpopulations in the three large freshwater fish, however in rainbowfish, granulocytes were the most sensitive subpopulation. Furthermore, the phagocytic activity of head kidney granulocytes was suppressed in golden perch, Murray cod and rainbowfish. High *in vitro* concentrations of organotins also dramatically reduced the mitogenesis of Murray cod head kidney cells. Of the native fish species, silver perch head kidney cells were the least sensitive to *in vitro* exposures of TBT and DBT.

- The organotins were acutely toxic to the native species at concentrations that have been previously used by our group to assess immunotoxicity in rainbow trout. Rainbowfish exposed to *in vivo* doses of TBT had suppressed phagocytic activities and reduced head kidney lymphocyte numbers. Conversely, TBT at the lowest and medium doses appeared to stimulate phagocytic function of Murray cod, while the highest dose began to adversely affect lymphocyte subpopulation numbers and mitogenesis. DBT was clearly immunotoxic in Murray cod causing dose-dependent reductions in phagocytic activity and lymphocyte numbers. As previously reported in other studies, DBT was the more potent immunotoxin in these native fish. More information (i.e. BCFs for Australian fish and organotin levels in the Murray-Darling Basin) is required to determine if concentrations of organotins would reach sufficient levels in the Australian environment to cause immunotoxicity in freshwater native fish.

Endosulfan exposures

- Only the highest *in vitro* doses of endosulfan resulted in significant alterations of immune functions. *In vivo* doses of endosulfan were highly toxic to Murray cod and rainbowfish following i.p. exposure: 50 mg/kg was lethal to all rainbowfish dosed, whereas 10 mg/kg
killed 75% of the Murray cod that received this dose. Murray cod lymphocytes displayed significantly reduced mitogenic responses at the two lowest doses, however, this returned to control levels at the highest dose. In general, acute doses of endosulfan were highly toxic to native freshwater fish, and in the field, this acute toxicity is likely to be more severe than the immunotoxic responses observed in these experiments. However, endosulfan has some ability to bioaccumulate, therefore, it is possible that chronic exposure to low levels could result in immunotoxicity.

**Chlorpyrifos exposures**

- *In vitro* doses of chlorpyrifos up to 50 mg/L caused no significant changes in the phagocytosis of the four native fish and did not affect the lysozyme activity or lymphoproliferation of Murray cod. *In vivo* doses of 50 mg/kg caused clear signs of neurotoxicity in Murray cod, but this was not observed in rainbowfish. There were dose-dependent reductions in the head kidney somatic index of rainbowfish and in splenic somatic index and body weight change of Murray cod. The head kidney lymphocytes from Murray cod dosed with 50 mg/kg had significantly lower proliferation compared to solvent controls. Therefore, chlorpyrifos is unlikely to be an environmental immunotoxin as immunosuppression only occurred at high concentrations where neurotoxicity was also apparent.

**Immunostimulant exposure**

- Both the immunostimulants investigated in this study showed some immunostimulatory properties *in vitro*. There was a significant increase in the phagocytic activity of Murray cod head kidney cells exposed to levamisole at 2.5 μg/mL. There was also a dose-related increase in the phagocytic activity of Murray cod head kidney granulocytes exposed to β-glucan. β-glucan reduced counts of non-viable cells (PI +ve) and debris events and
appeared to increase mitogenic responses. Unfortunately, *in vivo* doses of the immunostimulants did not show strong signs of stimulating immune functions under the dosage protocol used in this study. There was a significant dose-related increase in the splenic somatic index of Murray cod that received levamisole, while granulocyte counts were elevated in fish dosed with \( \beta \)-glucan. The lack of functional stimulation *in vivo* may be due to the timing or dosage regime used in the experiments. Higher concentrations and longer study periods should be explored as some researchers have used \( \beta \)-glucan at up to 50 times greater than the highest concentration investigated in this study, with no apparent adverse consequences.

**Cyanobacteria toxin exposures**

- The *in vitro* study on the immunotoxicity of cyanobacteria toxins in Murray cod head kidney cells showed that the toxins were able to cause some immunomodulation. Microcystin-LR caused a dose-dependent increase in the percentage of granulocytes and number of beads per granulocytes, while cylindrospermopsin exposure resulted in significant increases in the phagocytic activity and the number of beads per phagocyte. These results suggest that further studies into the immunotoxicity of cyanobacteria toxins are warranted.

**Future directions**

- In future studies, more fish in each group would be beneficial in the statistical analysis of the results. This is because of the inter-fish variation, which reduced the statistical power of the *in vivo* experiments. However, increasing the number of fish per group has logistical challenges because prolonging the time cells are out of culture can reduce their responses. The number of fish processed in a day needs careful consideration and will depend on the size of the fish, the number of immune cells required for the assays and the
number of assays conducted. The processing of more than 4 large Murray cod per day was not recommended due to the time it takes to process immunocytes for the assays.

- Chronic and/or multiple repeat-pulse experiments should be conducted in the future using the xenobiotics investigated in these studies, especially for the more persistent environmental pollutants, such as the organotins and endosulfan.

- Although immunomodulation has been demonstrated in these four Australian native fish, the use of immunological endpoints still needs much development before it can be used as a biomonitoring tool for Australian ecological risk assessments. Therefore, in future studies, the relationship between immune dysfunction and disease needs to be established in these species. The immune function of wild fish needs to be studied in many locations over a long time period to establish geographical and seasonal variations. Finally, appropriate assays and methodologies for field experiments need to be established, particularly for testing in remote areas some distance from laboratories.

**Final statement**

- This project has been invaluable in increasing the knowledge of immune functions in a number of highly-valued native fish species that could be considered for immunoeccotoxicological testing in Australia. Furthermore, it has also demonstrated that immune functional assays can be exploited to assess the immunomodulation of xenobiotics in Australian freshwater species.
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