THE IMMUNOMODULATORY EFFECTS OF CHLORPYRIFOS AND MALATHION ON THE INNATE IMMUNE SYSTEM AND DISEASE RESISTANCE IN JUVENILE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

by

Walter Che Ming Leung
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Approval

Name: **Walter Che Ming Leung**

Degree: **Master of Environmental Toxicology**

Title of Project: **The immunomodulatory effects of chlorpyrifos and malathion on the innate immune system and disease resistance in juvenile rainbow trout, *Oncorhynchus mykiss*.**

Examining Committee:

Chair: **Dr. Gordon L. Rintoul, Assistant Professor**

_____

Dr. Chris J. Kennedy, Professor
Senior Supervisor
Department of Biological Sciences, S.F.U.

_____

Dr. Russell A. Nicholson, Associate Professor
Supervisor
Department of Biological Sciences, S.F.U.

_____

Dr. Peter S. Ross, Research Scientist
Supervisor
Institute of Ocean Sciences, Fisheries and Oceans Canada

_____

Dr. Francis C.P. Law, Professor
Department of Biological Sciences, S.F.U.
Public Examiner

Date Defended/Approved: Dec-02-2009

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Abstract

The immunomodulation of the current use pesticides (CUPs) chlorpyrifos and malathion in rainbow trout was examined following exposure to environmentally relevant sub-lethal concentrations. Malathion exposure (28 d) at 2.68, 6.7, and 13.4 µg/L caused increased leukocyte phagocytic activity which returned to baseline values after a 14 d post exposure recovery period. Increases in fish mortality challenged with *Listonella anguillarum* was observed in trout exposed to 1.34 µg/L of malathion after the recovery period. Chlorpyrifos exposure resulted in a reduction in serum lysozyme and relative leukocyte proportions in the head kidney (at 1.3 µg/L), but no change in resistance to *L. anguillarum*. Results indicate that the CUPs chlorpyrifos and malathion are immunomodulatory through measures of individual immune parameters, however, results from the disease challenge studies make the ecological significance of these changes unclear. More research is needed to link immunomodulatory effects at the cellular level to whole organism effects.

Keywords: Immunotoxicology; Rainbow trout; Chlorpyrifos; Malathion

Dedication

I would like to dedicate this work to my parents.

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I would like to deeply thank my senior supervisor Dr. Kennedy for his advice, support, and guidance throughout my MET studies, and providing me an opportunity to work on a challenging but rewarding project that has prepared me well for future endeavours. I would also like to thank my committee members Dr. Nicholson and Dr. Ross for providing technical advice and valuable comments along my study. Special thanks to Dr. Law for leading me to the MET program and providing me research opportunities after my undergraduate degree.

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Finally, on a personal note, I must express my enduring gratitude to my parents, brothers, and my buddies, Emma, Josh, David, Wei, Howie, and Allen for always being there for me during my journey at SFU.

Life is beautiful thanks to you people.

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List of Acronyms

ACH_{50}	Alternative Complement Activity
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
CUPs	Current Use Pesticides
DCHF-DA	2′,7′-Dichlorofluorescein Diacetate
$DiOC_6(3)$	3,3-Dihexyloxacarbocyanine
DMSO	Dimethyl Sulfoxide
EGTA	Ethylene Glycol Tetraacetic Acid
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GVB	Gelatin Veronal Buffer
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HEWL	Hen Egg White Lysozyme
IHNV	Infectious Hematopoietic Necrosis Virus
K_{OW}	Octanol Water Partition Coefficient
LC_{50}	Lethal Concentration 50
LFV	Lower Fraser Valley
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex

OPs	Organophosphorus insecticides
OD	Optical Density
PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate Buffer Solution
PCB	Polychlorinated Biphenyl
PCP	Pest Control Product
PMA	Phorbol Myristate Acetate
PMRA	Pest Management Regulatory Agency
PS	Peptone Saline
RaRBC	Rabbit Red Blood Cells
RBA	Respiratory Burst Activity
SEM	Standard Error of the Mean
SI	Stimulation Index
sRPMI-1640	Roswell Park Memorial Institute Solution Supplemented
SSC	Side Scatter
sHBSS	Hank’s Balanced Salt Solution Supplemented
TSA	Tryptic Soy Agar
USEPA	United States Environmental Protection Agency

Introduction

Teleost Immune System

The primary function of the immune system in fish is for protection against infectious or neoplastic diseases. As such, it is imperative that the immune system operate at an optimum level where its activity is neither suppressed, which can cause susceptibility to viral, bacterial or parasitic infections and cancers, or enhanced, which may evolve into allergies and autoimmunities (Colosio et al., 1999).

Teleosts are the earliest class of vertebrates that possess both innate (non-specific) and adaptive (acquired) immune systems as are found in higher vertebrates (Whyte, 2007). Fish have evolved a complex system of innate defence mechanisms that are both constitutive and responsive in the prevention of invasion of bacteria and viruses (Ellis, 2001). Innate defence mechanisms are fast-acting and are comprised of both humoral and cellular factors that act synergistically with the components of the adaptive immune system to clear infections. Innate responses are especially important to fish because of an evolutionary status that has resulted in less efficient adaptive immune capabilities compared to higher vertebrates (Anderson and Zeeman, 1995; Magnadottir, 2006; Whyte, 2007). Pathogens can trigger rapid responses of innate immune components due to the ability of immune cells (leukocytes) and circulating immuno proteins to recognize macromolecules and macromolecular complexes that are generally not found in multi cellular organisms, but found in bacteria and viruses. These include

polysaccharides proteins, lipopolysaccharide, peptidoglycans, bacterial and double stranded viral RNA (Magnadottir, 2006).

External barriers such as the skin, gills, and mucus comprise the first line of defence in teleosts, preventing the entrance of potential pathogens. Bacterial challenge using fish with mucus removed have shown that direct injection of bacteria into the body or bath challenges can increase susceptibility to pathogens (Ellis, 2001). Once pathogens are successful in penetrating this first line of defence, they encounter a large repertoire of circulating immuno-proteins including complement, transferrins, anti-proteases, haemolysin, lysozyme, interferon, C-reactive proteins, and anti-bacterial peptides (Whyte, 2007). Among these, proteins such as lysozyme and complement are found in the external mucus layer to aid physical barriers in combating pathogens (Rice, 2000).

Lysozyme and complement proteins can directly inactivate pathogens and activate other components of the immune system. In the immunotoxicological literature, quantification of their titer and/or activity has been commonly used as indicators of immunocompetence, and therefore, is described in more detail here. Lysozyme is a ubiquitous enzyme found in mucus, lymphoid tissue, plasma, and in other body fluids of freshwater and marine water fish (Saurabh and Sahoo, 2008). Lysozyme hydrolyses the constituents of the peptidoglycan layer of bacterial cell walls (Ellis, 1999). It is bactericidal to various gram-negative bacterial fish pathogens, including the important marine pathogen, *Listonella anguillarum* (Grinde, 1989). The antibacterial activity of lyzosyme can be potentiated with histone-derived peptides which have no activity on their own (Patrzykat et al., 2001), a fact which exemplifies the interacting nature of these and other immune system proteins. Lysozyme can also activate phagocytes by binding to

them and promoting their phagocytic ability (Surabh and Sahoo, 2008). The relevance of lysozyme in disease resistance has been investigated using strains of fish that varied in lysozyme activity; correlations with varying disease resistance status (Roed et al., 2002, Lund et al., 1995, Balfry and Iwama, 2004). This suggests the possibility of using serum lysozyme activity for selection in order to improve the survival of fish challenged with pathogens.

Complement is an evolutionarily-conserved component of the innate immune system found across different classes of vertebrates like lysozyme. Complement is comprised of a group of 35 serum proteins that play a role in host defence against infection. Complement proteins can be classified into classical pathway (antibody-dependent), and alternative pathway proteins (microbe membrane-dependent) based on their mechanism of activation (Holland and Lambris, 2002). Once activated, a reaction cascade of these proteins leads to the formation of a polymerized membrane attack complex that results in the destruction of pathogens by creating pores on their surfaces. In addition, complement protein complexes formed during cascade pathways can modulate inflammatory reactions (account for the protective response of an organism to remove injurious stimuli) by acting on the major cellular effectors of the innate immune system (Rice, 2000). For example, C3a, C4a, and C5a are proteins of complement systems that promote phagocytosis, chemotaxis (mobility of macrophages) and respiratory burst activity in leucocytes; C3b proteins can also attract phagocytes (Boshra et al., 2006). Complement protein activities are higher in fish serum compared with those of mammals, suggesting a more important role in the innate immunity of fish (Yano, 1996).

The major cellular effectors of the innate immune system can be categorized as phagocytic cells (leucocytes), which include granulocytes (neutrophils), monocytes/macrophages, and non-specific cyototoxic cells (Magnadottir, 2006). These cells have recognition pattern receptors on their surfaces that are activated upon contact with pathogens that exhibit ligands recognized by these receptors (Whyte, 2007). This leads to the phagocytosis of the pathogen and the release of signals in the form of cytokine and chemokine molecules which attract and activate other phagocytic cells, resulting in an influx of neutrophils and monocytes to an area of infection (Secombes, 2001). Phagocytic cells can be additionally activated by humoral proteins such as complement protiens, resulting in an enhanced ability to engulf tissue debris and invading microbes (Rice, 2000).

In phagocytosis, engulfed pathogens are surrounded by the phagocyte membrane and internalized into acidic phagosomal vesicles. Phagosomal vesicles are then fused with lysosomes containing NADPH oxidases that convert molecular oxygen into toxic oxygen-derived molecules in a process termed respiratory burst (Janeway et al., 2001). The toxic products include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical (OH^-) and hypohalite (OCl^-). Respiratory burst activity is essential for destroying ingested microorganisms and clearing infections when bacteria or fungi are engulfed by leucocytes (Boesen et al., 2001). Deficiencies in NADPH oxidase result in the inability of phagocytes to undergo respiratory burst, and can cause chronic granulomatous disease in humans, a condition where patients become prone to bacterial and fungal infections.

The importance of phagocytosis as a defensive mechanism is also evident from its conserved nature in all metazoan organisms (Neumann et al., 2001). Not only does phagocytosis destroy invading organisms, it also serves the important function of antigen processing and presentation to lymphocytes which activates adaptive immunity and links the innate and adaptive immune compartments, as well as the regulation of the immune response through the secretion of cytokines (Neumann et al., 2001). The importance of phagocytic function is well recognized in the field of immunotoxicology and has been used in many studies as an indicator of immunological health status, and as an immunotoxic marker (Fournier et al., 2000; Zelikoff et al., 2000).

As in mammals, the teleost adaptive immune response is responsible for the production of antibodies that recognize discrete epitopes (macromolecules recognized by the immune systems) on invading pathogens which aid in their removal (Carlson and Zelikoff, 2004). For example, antibodies have been demonstrated to contribute to protection against *L. anguillarum* (Ellis, 1999). Upon a second exposure to the same pathogen, a greater number of antigen-specific lymphocytes will trigger a faster response (Arkoosh and Kaattari, 1991) to clear subsequent infections. Rainbow trout that survived a primary challenge experiment with *Yersinia ruckerii* had a much higher survival rate upon a second challenge compared to challenge-naïve trout, suggesting the involvement of adaptive immunity. The induction of various genes that link the innate and adaptive immune responses were also observed (Raida and Buchmann, 2009), revealing a concerted effort between the adaptive and innate immune system to clear infections. However, this arm of the immune system was not assessed in the current study as

examinations of selected innate parameters do not allow additional assessment of the adaptive immune parameters logistically.

Immunomodulation in fish

It is well known that endogenous factors such as stress, life stage, and genetic factors can affect the immune response of fish (Schreck, 1996). As well, the immune system in fish can also serve as a sensitive indicator of environmental change, including the presence of xenobiotics, toxins, and other alterations in natural water quality parameters (Burnett, 2005). Environmental factors including photoperiod, temperature, pH, oxygen level, particulates and salinity can also modify immune responses (Bowden, 2008). Xenobiotics encompassing a wide range of chemical classes including pesticides, organics, including halogenated hydrocarbons, and metals have shown to be immunomodulatory in fish and other aquatic organisms, causing stimulating and suppressive effects on both innate and adaptive immune responses. The immunomodulatory actions of these xenobiotics commonly result in the suppression or enhancement of respiratory burst and phagocytic activities, the production of antibody forming cells, and mitogen-stimulated T and B lymphocyte proliferation (reviewed in Dunier and Siwicki, 1993; Rice, 2000; Galloway and Handy, 2003; Carlson and Zelikoff, 2004; Burnett, 2005; Reynaud and Deschaux, 2006). Mixtures of xenobiotics present in effluents of pulp mill (van den Heuvel et al., 2005) and sewage (Salo et al., 2007) were also shown to be immunomodulatory in fish.

Disease challenge studies have shown that these xenobiotic-induced immunomodulatory effects on individual various immune parameters can be linked to the increased susceptibility of fish to bacterial infections, a fact that indicates an overall

negative impact on the immunocompetence of these organisms. For example, Pacific herring (*Clupea pallasii*) exposed to ppb levels of water-soluble hydrocarbons for 57 d had suppressed macrophage activity, and reduced pathogen resistance to *L. anguillarum* (Kennedy and Farrell, 2007). Fish exposed to a pyrethroid (permerthrin) for 2 d had increased *Yersinia ruckerii*-induced mortality (Zelikoff, 2000). An organophosphate (OP) pesticide, malathion, also increased the mortality of Japanese medaka exposed to *Y. ruckerii* following of exposures of 14 d and 21 d (Beaman et al., 1999). The infectious hematopoietic necrosis virus (IHNV) when concurrently exposed with the pyrethroid esfenvalerate, led to higher mortality in juvenile chinook salmon than those exposed to IHNV alone (Eder et al., 2004).

Laboratory demonstrations of chemically-induced immune system modulation and susceptibility to bacterial infections have also been observed under field conditions, although these studies are limited in number. Salmonids from the Puget Sound area, WA, exposed to PAH/PCB had increased susceptibility to bacterial infection following laboratory challenges to *L. anguillarum*, compared to salmon sampled from reference hatcheries (Arkoosh et al., 1998). Taken together, lab and field studies demonstrate that the fish immune system can be sensitive to sub-lethal exposures of toxicants and may render fish more susceptible to diseases, a fact which may translate to higher population effects in teleosts living in contaminated environments.

Malathion and Chlorpyrifos

In British Columbia, the heavy use of pesticides has become a growing concern due to their contamination of important salmon habitats through intentional application, runoff from agricultural fields and aerial drift or accidental contamination events. In

2003, over 4 million kg of pesticides were sold province-wide (Enkon, 2003). Among the vast array of pesticides used in B.C., two organophosphorous insecticides (OPs), malathion and chlorpyrifos, are of interest from a regulatory standpoint. These pesticides are listed by the Pest Management Regulatory Agency (PMRA) in Canada as being of national concern and have been nominated with other pesticides to be screened under the national pesticide research fund by the PMRA in 2007 due to their reported sublethal toxicities in various species. Moreover, malathion is on Environment Canada's 1998 list of toxic substances used in the lower Fraser/Georgia Basin. Chlorpyrifos is listed on the Fisheries and Oceans Canada priority pesticide list, and the US National Oceanic and Atmospheric Administration pesticides of potential concern list (Verrin et al., 2004).

Malathion (Pest Control Product – PCP #25638) and chlorpyrifos (Pest Control Product – PCP #25823) are both broad spectrum OP insecticides with 4252 kg and 3444 kg sold, respectively, in the Georgia Basin area in 2003 (Enkon, 2003). Malathion has been registered in Canada for use since 1953 and is mainly used for pest control on crops (alfafa, canola, grass feedlots, etc) and mosquito and fly control. Chlorpyrifos was registered in 1970 to control pests on a wide range of fruits and vegetables and is also used to eradicate mosquitoes and termites.

OPs is a group of pesticide that has the added advantage of more specific targeting of the insect nervous system over the mammalian nervous system (Boelsterli, 2003) thus reducing the potential toxicity to humans. This relies on the principle that in insects, the relatively unreactive thionate (P=S) group of malathion and chlorpyrifos (Figure 1) can be converted to the more reactive oxon (P=O) group through bioactivation by insect cytochromes (CYPs). The resulting oxon group is much more polarized than

thionate and makes the phosphorus atom electrophilic. The electrophilic phosphorus in the center of malathion or chlorpyrifos can then attack the active serine hydroxyl group of acetylcholine esterase (AChE) to form a stable covalent bond between the insecticide and the enzyme (Boelsterli, 2003). The enzyme becomes inactivated and cannot be hydrolyzed to re-establish the active serine hydroxyl group. Thus the inactivation of AChE is irreversible and lead to the buildup of acetylcholine which ultimately causes neurotoxicities. Because of the conversion of thionate to oxons, the bioactivated compound becomes approximately 1000-fold more reactive as anti-AChE agents than their parent phosphorothionates (Thompson and Richardson, 2004). In contrast, malathion and chlorpyrifos are not bioactivated in the mammalian system, instead they are detoxified by carboxylesterases and other esterases (Boelsterli, 2003) thus rendering less harm to mammals than insect when exposed to the same dose.

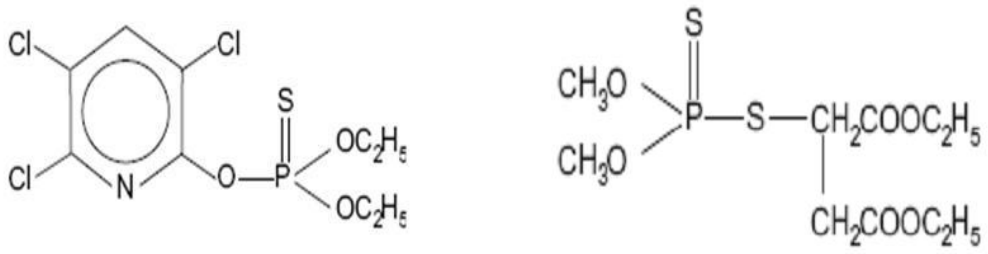

Figure 1 Chemical structure of chlorpyrifos (left) and malathion (right).

The use of OPs has gradually replaced the organochlorine-based pesticides as they are less persistent in the environment, more selective to target organisms, and more rapidly biodegrade to less harmful compounds (Blakley et al., 1999). Nevertheless, both of these pesticides are acutely toxic to non-target species such as fish at low

concentrations. The average 96-h LC_{50} value for rainbow trout is 130 µg/L for malathion and 13 µg/L for chlorpyrifos (USEPA, 2007).

The heavy use of OPs in B.C. has resulted in the detection of these chemicals in farm ditches of the Lower Fraser Valley (LFV), an area with the highest concentration of farms in Canada (Wan et al., 2004). In the LFV, four different OPs have been detected in samples: azinphos-methyl, chlorpyrifos, diazinon, and dimethoate at concentrations between 0.2 and 1.2 µg/L among a complex mixture of several classes of pesticides (Wan et al., 2004). Specifically, chlorpyrifos was detected at a concentration of 0.13 µg/L from October to November in 2003, and 0.02 µg/L May to June in 2004. The former concentration is roughly equivalent to $1/100^{th}$ of the acute 96-h LC_{50} value in rainbow trout determined from USEPA's Ecotox database (USEPA, 2007). In light of an acute to chronic toxicity ratio value of 8 established for chlorpyrifos (Giesey et al., 1999), and a common conversion factor of 10 to adjust for extrapolation of sub-lethal effects from LC_{50} values (equals to $1/80^{th}$ of 96-h LC_{50} when both factors are considered) (Health Canada, 2008), this surveyed concentration suggests an environmentally realistic probability of chronic adverse sub-lethal effects in rainbow trout particularly if exposure continues for long durations.

Although malathion was not detected in this pesticide survey (Wan et al., 2004), concentrations as high as 1 µg/L have been detected in Texas, and peak concentrations measured at 11.2 µg/L in storm water run-off in a Lagoon in Ventura County, California has been recorded (reviewed in McCarthy and Fuiman, 2008). These authors subsequently selected 0, 1, and 10 µg/L of malathion as environmentally relevant levels of exposure for their study which matches the levels used in the current study. The

average rainbow trout 96-h LC_{50} values for malathion from USEPA's ECOTOX database is 130 µg/L (USEPA, 2007), by applying an uncertainty factor of 10 for adjusting acute to chronic exposure and another factor of 10 for adjusting lethal to sublethal effects, a dose response concentrations investigating chronic sublethal effect of malathion to trout covering 1.3 µg/L (1% of 96-h LC_{50}) would also be environmentally relevant, and was used in this study.

Immunomodulatory potential of malathion and chlorpyrifos

Previous research indicates that malathion may cause immunomodulation in both mammalian and fish models. For example, at low non-cholinergic doses (300-fold concentrations below that which can affect plasma cholinesterase), malathion activated both the humoral immune response and macrophage function (Rodgers and Ellefson, 1992). Malathion caused changes in several immune parameters and increased the susceptibility of Japanese medaka (*Oryzias latipes*) to *Yersinia ruckerii* (Beaman et al., 1999). A 7 and 14 d-exposure to sublethal concentrations of malathion (0.2 mg/L and 0.8 mg/L) caused a concentration-dependent suppression of antibody forming cell numbers, while a 21 d-exposure to 0.1 mg/L and 0.3 mg/L increased the susceptibility of medaka to *Y. ruckerii*. Malathion's ability to impact adaptive immunity is also supported by chronic studies that showed a reduction of antibody titer in channel catfish (*Ictalurus punctatus*) (Plumb and Areechon, 1990), and in several species of Indian carp (Dash et al., 2000).

There is no current evidence that chlorpyrifos exposure leads to increased disease susceptibility in aquatic animals. However, individual immune parameters such as head kidney immune cell counts and phagocytosis were suppressed in Nile tilapia (*Oreochromis niloticus*) after a 96-h exposure at 20% (0.21 mg/L) and 40% (0.42 mg/L)

of the tilapia 96-h LC_{50} (Giron-Perez et al., 2006), and also after a 3 month exposure to 1 µg/L (Holladay et al., 1996). Moreover, modulation of transcription of cytokine concentrations that regulate immune responses have also occurred in chlorpyrifos-exposed chinook salmon after 96-h at concentratiosn as low as 1.2 µg/L (Eder et al., 2004; Eder et al., 2008).

Objectives of Study

There are two major research objectives of the present study. The first is to characterize the immunomodulatory effect(s) of malathion and chlorpyrifos on juvenile rainbow trout (*Onchorhynchus mykiss*), a locally relevant species, and to determine the extent and recovery of immunomodulation in these fish. The second objective is to determine if these pesticides can increase susceptibility to bacterial infections in these fish. To establish and better characterize any immunotoxicity caused by either pesticide in trout, a chronic exposure study (28 d) including a recovery period (14 d post 28 d exposure) was conducted to assess whether the immunotoxic effects are reversible upon withdrawal of exposure or delayed toxic effect is observed.

Two complementary experiments were performed to achieve these research objectives. Experiment 1 included flow cytometric-based and microplate-based assays to assess possible alterations in several innate immune responses that were described earlier, namely serum lysozyme activity, alternative complement activity, phagocytosis, and respiratory burst activity.

Experiment 2 involved a standardized *in vivo* challenge of rainbow trout with the pathogen, *Listonella anguillarum* following pesticide exposure. This study attempted to provide a definitive answer on whether either of these pesticides has the ability to affect

disease resistance in rainbow trout. *L. anguillarum* is a gram negative marine bacteria that is the causative agent for vibriosis, a disease responsible for causing mortality in both farmed and wild salmonid stocks. The disease is characterized by hemorrhagic septicaemia, with clinical signs of red spots on the ventral and lateral areas of the fish, swollen and dark skin lesions that ulcerate, and the release of blood exudates in addition to corneal lesions (Crosa et al., 1995). Since salmonids species are susceptible to vibriosis, and all the immune responses measured in Experiment 1 are essential in combating this disease in salmonids, *L. anguillarum* was deemed a suitable bacterial agent for this study.

Materials and Methods

Experimental Fish

Juvenile rainbow trout (average weight 25.8 g, range 20.1-30.0 g) were obtained from Miracle Spring Hatchery (Mission, B.C.) and were maintained in an indoor flow through system at Simon Fraser University. Fish were acclimated for 5 d prior to fin clipping for identification purposes in the disease challenge studies, and subsequently acclimated for another 7 d prior to any exposure to chemicals. Fish were held in 150 L fibreglass tanks supplied with filtered, de-chlorinated municipal tap water pH 6.8 under a 12 h light : 12 h dark photoperiod, and at approximately 12 °C. The flow rate of water in each tank was 1.5 L/min. Trout were fed *ad libitum* with commercial salmon pellets (Ewos Pacifica, Surrey, BC) every other day with cleaning of excess food and waste 30 min after feeding. All work with animals was conducted in accordance with Canadian Council of Animal Care (CCAC) guidelines, under an approved SFU Animal Care protocol.

Pesticide Exposure

Analytical grade chlorpyrifos (*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinol)-phosphorothionate; 99.2% purity) and all other chemicals, unless otherwise stated, were purchased from Sigma (Oakville, ON, Canada). Technical grade malathion (95% purity; *O,O* -dimethyl-*S*-(1,2-dicarbethoxy)ethyl phosphorodithioate) was received as a gift from Dr. P. Belton.

Pesticides were solubilized in reagent grade acetone to form stock solutions used for dosing; the final concentration of acetone in exposure tanks was 0.00013 % (v/v). Chemical solutions were transferred to marriotte bottles and mixed with flow through de-chlorinated municipal tap water, and flow rate of the bottles adjusted to produce final constant nominal chlorpyrifos concentrations of 0.13, 0.26, 0.65 and 1.3 µg/L, and malathion concentrations in tanks of 1.34, 2.68, 6.7, and 13.4 µg/L which represent 1, 2, 5, and 10 % of rainbow trout 96-h LC_{50} values, respectively. Monitoring of flow rates of mariotte bottles were conducted twice per day with necessary adjustments of the bottles as required, and chemicals in the bottles were renewed every 48 h. The duration of pesticide exposure was 28 d with an additional 14 d recovery period (post-28 d exposure).

Each treatment for each pesticide was done in duplicate tanks. Trout (n = 40) were randomly assigned to tanks following acclimation, and were fin clipped, resulting in different clip patterns for each set of replicate tanks. Because of the labour intensive and time sensitive procedures of the immune assays, and the fact that it was not possible to prepare and renew chemical solutions for all 8 marriotte bottles on sampling or the following assay days, fish (n = 6) from only one of the two duplicate tanks across the full spectrum of treatments were sampled per day. The start dates for chemical exposure in duplicate tanks were staggered for 5 days with the sampling on the second set of treatment tanks repeated 5 days later. On each sampling day, additional fish (n = 12) from each tanks were used for the host resistance study in addition to the 6 fish sampled for immune assay. Thus a total of 18 fish were randomly removed from each tank on each sampling day.

Blood Sampling

At the end of the 28 d exposure and 14 d post exposure periods, 6 fish from each tank (n = 12 fish/treatment) were removed, weighed, sacrificed and sampled for blood. Blood samples were drawn by caudal venipuncture using non-heparinized syringes after anaesthetisation with 0.3 g/L of tricaine methanesulfonate (MS-222, Syndel laboratories) buffered with 0.3 g/L sodium bicarbonate. To collect serum, whole blood was allowed to clot for 1 hour in eppendorf tubes at room temperature followed by 5 h at 4 ºC and later centrifuged at 2000 x *g* for 5 min at 4 ºC (Amar et al., 2004). All serum samples were preserved at -80 ºC prior to analysis for lysozyme and alternative complement activity.

Head Kidney Leukocyte Sampling

Head kidney leucocyte isolations were obtained through a procedure of Crippen et al., (2001). Head kidneys were aseptically extracted, and with a sterile syringe plunger, the organs were pushed through a nylon mesh (75 µm) along with 2 mL of ice-cold supplemented Hank's Balanced Salt Solution (sHBSS) supplemented with 10 IU/mL heparin, 1% penicillin-streptomycin solution (P/S), 15 mM HEPES, 2% foetal calf serum (FCS; Gibco) in 15 mL Falcon tubes. Cold de-ionized water (9 mL) was added and the cell suspensions were mixed by gentle inversion for 20 s to remove erythrocytes. To restore isotonic equilibrium, 1 mL of 10 x phosphate buffer solution (PBS) was added immediately with gentle inversion. Cells were placed on ice for 10 min before removing settled debris. The cell suspension was centrifuged for 400 x *g* for 5 min at 4 ºC, followed by two washes with 4 mL of sHBSS before final re-suspension and adjustment of the cell concentration to 1 x 10^7 viable cells/mL in supplemented RPMI-1640 medium (sRPMI; containing 1 % P/S, 7.5% FCS, and 15 mM HEPES). Viability and density were

examined using trypan blue exclusion. Live cells with intact cell membranes exclude trypan blue dye which allows for a visual inspection that distinguishes live cells with clear cytoplasm from dead cells with blue cytoplasm under magnification.

Phagocytosis Assay

Phagocytosis was assayed by flow cytometry based on the methods outlined in Brousseau et al., (1998) and Karrow et al., (1999). Cells were incubated with fluorescent latex beads (Fluorescein isothiocyanate, or FITC). Following incubation, the suspensions comprising cells and microspheres was layered over a density gradient. By centrifugation, the cells are pelleted, whereas non-phagocytosed beads stay at the surface of the gradient. Cells are then analyzed by flow cytometry where the intensity of the fluorescence is related to the number of microspheres phagocytosed by phagocytes.

Duplicate leucocyte samples were assayed for each fish using 1 x 10^6 isolated head kidney cells incubated at room temperature in either 1 mL of sRPMI (live cells) or 1% paraformaldehyde (fixed cells as negative controls that do not phagocytose beads but may fluoresce due to unspecific binding of beads to the cell surface) for 30 min. Cells were then centrifuged at 500 x *g* for 5 min at 18 °C and cell pellets were re-suspended in 100 µL of sRPMI and transferred to 96-well culture microplate. To each well, 100 µL latex beads (1.0 µm) were added and incubated with the cell suspension adjusted to a ratio of 100 beads to each cell at 18 °C for 18 h.

At the end of the incubation period, cell suspensions were carefully layered over a 3% Bovine Serum Albumin (BSA) gradient in phosphate-based saline (PBS) and centrifuged at 100 x *g* for 10 min to remove non-ingested beads. Cells were resuspended

in 1 mL PBS and analyzed by flow cytometer to quantify the number of beads engulfed (FACS ARIA, Becton Dickinson).

Voltage settings on the flow cytometer for forward and side scatter were established with samples with cells only, and the settings for fluorescence were established with a suspension of beads in sRPMI. The fluorescence histograms of cell number versus fluorescence intensity were analyzed, with a gate drawn based on the forward and side scatter profiles to include the larger, more granular cells which represented granulocytes (Karrow et al., 2001), excluding free beads, cell debris, and lymphocytes for 10,000 events. The percentage of phagocytes containing at least three beads (activated phagocytes) were determined by creating a gate of bead positive cells based on the fluorescence histogram (fluorescence values for cells with 3 beads triples the level of cells with 1 bead). The percent of cells that phagocytosed beads was calculated by subtracting percent bead-positive fixed cells from percent bead-positive live cells. Values were normalized across days by expressing them as a percent of the control mean for each day.

Oxidative Burst Assays

The oxidative burst assay was carried out as per Karrow et al., (1999) and Chilmonczyk et al., (1999). Immune cells are incubated with probes that diffuse into cells where nonspecific esterases cleave off the acetate moiety of the probe releasing the intact substrate. Upon cell activation with stimulating agent (e.g., phorbol myristate acetate (PMA)) the hydrogen peroxide (H_2O_2) generated will oxidize the nonfluorescent probe. The resulting fluorescence measured on the flow cytometer is proportional to the intensity of the oxidative burst.

Duplicate samples were prepared from each fish by dispensing 100 µL of head kidney leucocyte suspensions containing approximately 1 x 10^7 cells/mL in sRPMI into 96-well culture plates, topped up to 200 µL by the addition of 100 µL of sRPMI and stored overnight on ice at 4 °C. Prior to the assay, cells were transferred to 5 mL culture glass tubes containing 780 µL of sRPMI. DCHF-DA probe was added to the cells (5 µM, final concentration) and incubated for 15 min at room temperature in the dark to allow the probe to diffuse into the cells. Each of the duplicate tubes was either activated with 10 µL of PMA (100 ng/mL, final concentration) in DMSO to stimulate the release of hydrogen peroxide in the cells or remained unstimulated with the addition of 10 µL of DMSO. After 60 min of incubation at room temperature in the dark, each sample was measured on a flow cytometer for fluorescence. A total of 10,000 events were collected for each tube and the mean fluorescence intensity (MFI) from the fluorescence histograms of cell number versus fluorescence intensity was collected. Data were expressed as a stimulation index (SI) where SI = MFI of stimulated sample / MFI of unstimulated sample. The stimulation indices were normalized across days by expressing the data as a percent of the control mean for each day.

Partial Relative Leukocyte Differential Counts

The relative differential for lymphocytes and granulocytes was performed using flow cytometry based on the method developed by Inoue et al., (2002), with slight modifications. This method differentiates granulocytes from lymphocytes population base on their different membrane structure complexity. Larger amounts of membrane structures found in the granulocytes result in a higher uptake of a dye and higher

fluorescent emission upon staining with the naturally fluorescent lipophilic dye 3,3-dihexyloxacarbocyanine ($DiOC_6(3)$; Invitrogen) (Inoue et al., 2002).

A stock solution of $DiOC_6(3)$ was prepared in ethanol at 500 µg/mL, and working solution was prepared by diluting the stock 10-fold with HBSS just before staining. 1 x 10^6 head kidney leucocytes (100 µL of 1 x 10^7 cells/mL in sRPMI was topped up with 880 µL of HBSS supplemented with 15 mM HEPES and 1% P/S) in 5 mL glass culture tubes and stored overnight on ice at 4 °C.

The next day, 10 µL of the $DiOC_6(3)$ working solution was added to each culture tube. After gentle vortexing of the suspension, the sample was incubated at room temperature for 10 min in the dark and again placed on ice until analysis by flow cytometery within 1 h. Two main populations were discernable on the FITC vs SSC plot; a grouping characterized by lower FITC and SSC which was expected to be lymphocytes and thrombocytes, plus a grouping with higher FITC and SSC properties which were predicted to be granulocytes (Shelley et al., 2009). Gates were drawn around the lymphocyte/thrombocytes and granulocyte populations to determine proportions of each cell type relative to total leukocytes.

Lysozyme

A turbidometric method was used to determine lysozyme activity (Demer and Baynes, 1997). 0.1 M phosphate/citrate buffer (pH 5.8) was prepared by adding 0.1 M citric acid to 0.1 M sodium hydrophosphate. A series of hen egg white lysozyme (HEWL) standards were prepared fresh daily from a frozen aliquot of a standard solution (1 mg/mL) in the phosphate/citrate buffer to give final concentrations of 0, 2.5, 5, 10, 20 µg/mL. 25 µL of undiluted serum and standards were added to cells of a flat bottomed

96-well assay plates in triplicate, followed by the immediate addition of 175 µL of 0.075% (w/v) of *M. lysodeikticus* bacterial substrate solutions also prepared in the same buffer.

The plate was shaken for 5 s in a multiscan spectrophotometer (Bio-Tek PowerWave 340) to allow mixing of standards and substrates, and the change in turbidity was immediately measured every 15 s for 10 min at 450 nm in a negative kinetics mode. Lysozyme activity for each sample was determined from the change of absorbance over 10 min based on the HEWL standard curve with results expressed in µg/mL HEWL equivalents.

Alternative Complement Activity

Serum alternative complement activity was assayed as per Panigrahi et al., (2005), where activity is determined through the measurement of rabbit red blood cells (RaRBC) hemolysis.

RaRBC in Alsevers buffer (Hemostat) were pelleted by centrifugation at 1500 x *g* for 10 min at 4 ºC, and then washed three times with 0.01M of EGTA-Mg-GVB buffer (pH 7) (10 mM $MgCl_2$ and 10 mM EGTA in GVB^{2+}) by centrifuging at 400 x *g* for 5 min at 4 ºC (Wu et al., 2007). Erythrocyte density was adjusted to 2 x 10^8 cells/mL using the same buffer. Serum samples were thawed on ice and diluted 1:100 in the buffer. A series of diluted serum were adjusted to a final volume of 0.25 mL with the appropriate addition of buffer and mixed with 0.1 mL of the adjusted RaRBC in 15 mL Falcon tubes.

A 100% hemolysis control (0.1 ml RaRBC with 3.4 ml of distilled water) and a cell blank control (0.1 mL RaRBC with 0.25 mL buffer) were included. Test mixtures were incubated at 20 ºC for 90 min on a shaker set at 75 rpm. Lysis of RaRBC was

terminated by the addition of 3.15 mL of iced 0.85 % NaCl solution. Unlysed RaRBC were pelleted by centrifugation at 1600 x *g* for 10 min at 4 ºC and the optical density (O.D.) of the supernatants was measured at 414 nm using a spectrophotometer.

Experimental results were plotted on a log-log scale as % hemolysis vs volumes of sera added on Excel (Microsoft). The volume yielding 50% hemolysis was determined by excel and used for the calculation of the ACH_{50} as follows:

ACH_{50} value = 1/k times (reciprocal of the serum dilution) times 0.5

Where k is the amount of serum added giving 50% lysis and 0.5 is a correction factor because the current assay is performed on half scale of the original method (Yano, 1992). 1 unit of ACH_{50} is the amount of serum capable of lysing 4 x 10^7 RaRBC at 20 ºC in EGTA-Mg-GVB buffer in a total volume of 0.7 mL.

Disease Challenge Study

The pesticide-exposed fish were experimentally infected with the marine pathogen *L. anguillarum*, via immersion challenges immediately following the 28-d pesticide exposures and 14-d recovery period. This bacterial pathogen was selected for the disease challenges because the fish were reared strictly in freshwater and therefore were assumed to be immunologically naïve to the pathogen. *L. anguillarum* is infectious and lethal in freshwater, and there is precedent for its use as a model pathogen for disease challenges in other freshwater studies (Balfry et al., 2001; Wood et al., 1996). Mortality data therefore would provide information on the effect of pesticides on innate immunity with possible correlations between disease resistance results (i.e., mortality) and the results of the various *ex vivo* immune tests performed on the fish.

Primary isolates of *L. anguillarum* (Pacific Biological Station, Nanaimo, British Columbia, isolate number 2004-124, serotype 01) were used for all the disease challenges. *L. anguillarum* cells were grown for 18 h on tryptic soy agar (TSA supplemented with 1.0% NaCl) at 20 °C, and harvested into sterile peptone-saline (PS; 0.1% peptone and 0.85% NaCl). The suspension was diluted and absorbance measured at 540 nm. An estimate of the concentration of cells in the suspension was calculated based on the assumption that 1 OD_{540} contained 10^9 cells/mL. The actual concentration of cells was subsequently determined by drop plating serial dilutions (5×25 µL aliquots) of the suspension onto TSA plates, incubating them overnight at 20 °C, and counting the number of colony forming units.

The average challenge dose was determined to be 1.59×10^8 cfu/mL (final doses ranged from 1.12×10^8 cfu/mL to 2.13×10^8 cfu/mL). In preliminary trials this dose of *Listonella* caused 60–70% mortality in fish, a level used in previous host resistance studies (Balfry et al., 2001).

Challenges were performed by pooling 12 fish per pesticide group into a large bucket containing 14 L of aerated peptonesaline (P-S), and immediately adding 1 L of the challenge inoculums (*L. anguillarum* in P-S). After 10 min of exposure, the water and fish from the container were transferred to a 150 L fibreglass tank with flowing dechlorinated municipal water at 12.7±0.6 °C (mean±S.E.M.). Mortalities were monitored and recorded three times per day for 14 days, with fish from different treatment groups identified based on fin clips. All fish that died were individually bagged and frozen at −80 °C, for later necropsy and verification of fin clip. The spleen was aseptically swabbed and streaked onto TSA plates from approximately 25% of these fish. The fish were

assumed to have died of vibriosis caused by *L. anguillarum*, following the observation of non-pigment producing, circular, cream-coloured colonies appearing as a pure culture on the TSA plates. The colonies were subsequently smeared onto glass slides and Gram stained. Pure cultures of Gram negative, rod-shaped, curved bacteria were observed for the final confirmation of cause of death.

Statistical Analysis and Calculations

All statistical analyses were conducted using Prism® (Graphpad). All data sets were tested for normality of residuals (D'Agostino-Pearson test) and homogeneity of variance (Bartlett's test). In most cases, these two conditions were met, and subsequent analysis of variance (ANOVA) was used to indicate the presence of significant differences between control and treatment means, followed by Dunnett's test to identify the significant means ($p < 0.05$) from the control means. If the normality or the variance tests were failed, then a non-parametric Kruskal-Walis one way ANOVA was performed followed by pair-wise multiple comparisons using the Dunn's test ($p < 0.05$). Data from replicates tanks were pooled after the confirmation of insignificant replicate tanks variability using a two-way ANOVA analysis, where treatment groups and the duplicate tank were tested as two independent variables. This yielded an $N = 12$ for each data set. All results were expressed as mean ± standard error of the mean.

For disease challenge studies, the survival functions of fish in all treatment groups were plotted using the Kaplan-Meier method. Differences in mortality among the treatment groups and the control group were examined by Log-Rank test (Kleinbaum, 2005). When a significant difference ($p < 0.05$) was detected among mortalities curves, the Log-Rank test with Bonferroni-adjusted probabilities for multiple comparisons were

performed to identify individual treatment group that differed from the control group (significance denoted at $p < 0.0125$ as 4 groups are compared to the control group simultaneously). Median time to death for fish in each group was calculated by the statistical software.

Results

28 d malathion exposure and recovery

No mortality was observed in any of the concentrations tested for either pesticide. In addition, trout exhibited normal feeding behaviour at all concentrations throughout the experiment and there was no significant differences in change of body weight of fish among treatment groups at the end of the exposure period. Cell viability values for all *ex-vivo* assays were always greater than 95%**.**

Respiratory burst activities were not affected by malathion exposure at any concentration (Table 1). Also, the activities of serum lysozyme were not affected by malathion exposure at the end of the 28 d exposure period or during the recovery period (Table 1). No significant differences in serum ACH_{50} activity were observed between control fish and those treated with malathion (Table 1).

Malathion exposure caused significant changes in the phagocytic responses of immune cells (Figure 2). After a 28 d exposure to the three highest concentrations of malathion (2.68, 6.7 and 13.4 µg/L), a larger proportion of trout leucocytes ingested beads. The levels of ingestion of three or more fluorescent beads in these groups were 165 ± 17.7 ($p < 0.05$), 171 ± 19.5 ($p < 0.05$), and 166 ± 14.2 % ($p < 0.01$) of controls. After a 14 d recovery period, the percentage of phagocytic cells (cells which ingested beads) from fish in the three highest concentrations did not differ statistically significantly from the control group for ingestion of three beads or more.

In order to confirm that the increase in the percentage of phagocytic cells was due to the stimulation of the cells and not to a change in the proportion of phagocytic cells, the percentages of phagocytic cells in each exposure group were determined through gating of the phagocytic cell populations (distinctive group of cells that exhibited high side scatter and forward scatter values). There were no differences in the proportion of phagocytic cells in the total populations of cells across treatment groups; this indicates that the increase seen was due to the response of individual cells.

Kaplan-Meier estimates of the survival during disease challenge, together with cumulative mortality are illustrated in Fig. 5. The median survival times for the various groups of control (control fish from both pesticide exposed and from both 28 d exposure and 14 d post exposure) fish were 5 to 6 days and the final cumulative mortality for the control groups ranged from 69.6 to 76.9 %.

Mortality curves were not significantly different among the fish exposed to malathion for 28 d. However, following a 14 d recovery period, there was a statistically significant increase (17%) in mortality among the fish exposed to the lowest malathion concentration 1.3 µg/L ($p = 0.0034$), with a median day until death of 4.5 days. Fish exposed to 2.6, 6.7, and 13.4 µg/L of malathion did not reach statistical significance and the median day until death were either 5 or 6 days.

28 day chlorpyrifos exposure and recovery

Chlorpyrifos exposure resulted in no change in the phagocytic activities of any treatment group compared to controls (Figure 2). The percentages of cells that ingested beads and respiratory burst activities were not affected by chlorpyrifos exposure (Table 1). Serum ACH_{50} activities were not significantly different between control and

chlorpyrifos-exposed groups (Table 1). Following *L. anguillarum* challenge, mortality was not significantly different between control and exposed groups in ether the 28 d or 14 d recovery periods (Fig. 5).

The proportion of granulocytes in the population of total leucocytes in head kidney decreased with fish exposed to the highest concentration (1.3 µg/L) at 73.4 ± 4.94 % of control ($p < 0.05$) (Fig. 3). This decrease in granulocyte proportion continued through the 14 d recovery period in fish exposed to the highest concentration (78.0 ± 3.21 % of control; $p < 0.01$). Chlorpyrifos exposure did not induce a change in lysozyme activity in fish exposed for 28 d, however, during the 14 d post exposure period, fish previously exposed to the highest chlorpyrifos concentration (1.3 µg/L) showed suppressed lysozyme activity (3.24 ± 0.325 µg/mL) compared to the control values (6.57 ± 1.23 µg/mL; $p < 0.05$) (Fig. 4).

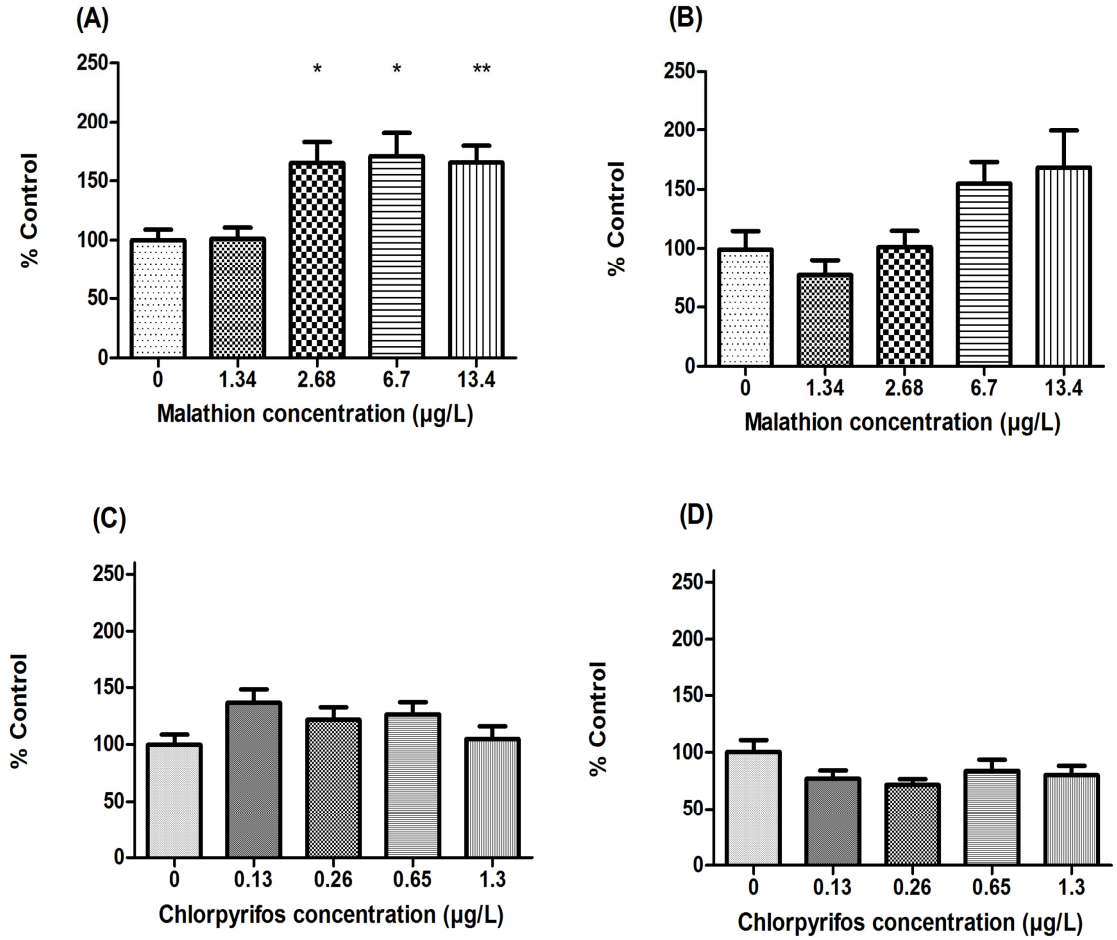

Figure 2 Phagocytosis of fluorescent beads. Percentage of head kidney leucocytes capable of phagocytosing three beads or more expressed as percentage control values: (A) after 28 d (B) after recovery period to malathion exposure (C) after 28 d (D) after recovery period to chlorpyrifos exposure. Each bar represents mean ± S.E. of 12 fish. * denotes a significant difference from controls ($p < 0.05$) ** ($p < 0.01$).

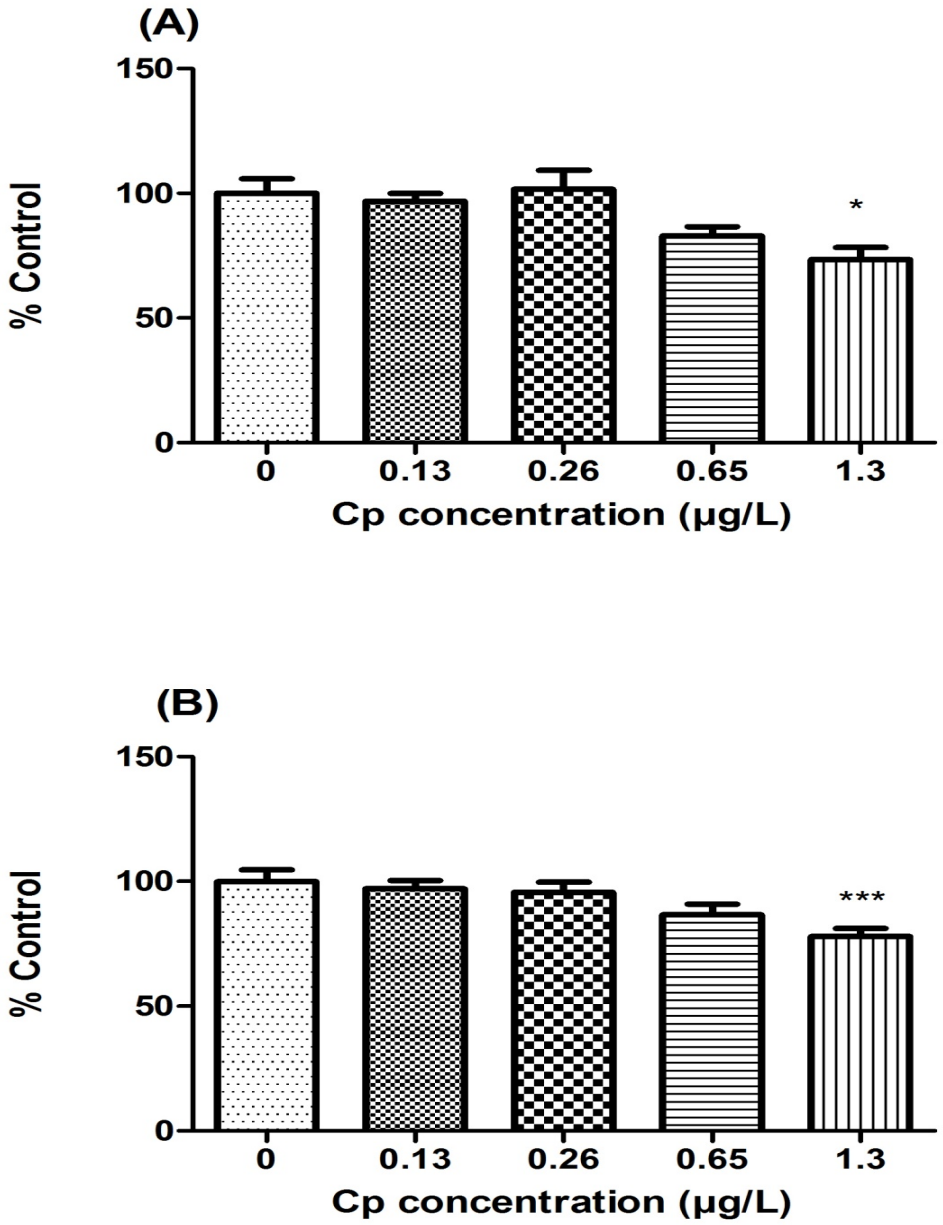

Figure 3 The proportion of neutrophils in head kidney of rainbow trout expressed as percentage of control values: (A) after 28 d (B) after recovery period exposure to chlorpyrifos. Each bar represents mean ± S.E. of 12 fish. * denotes a significant difference from controls ($p < 0.05$) *** ($p < 0.001$).

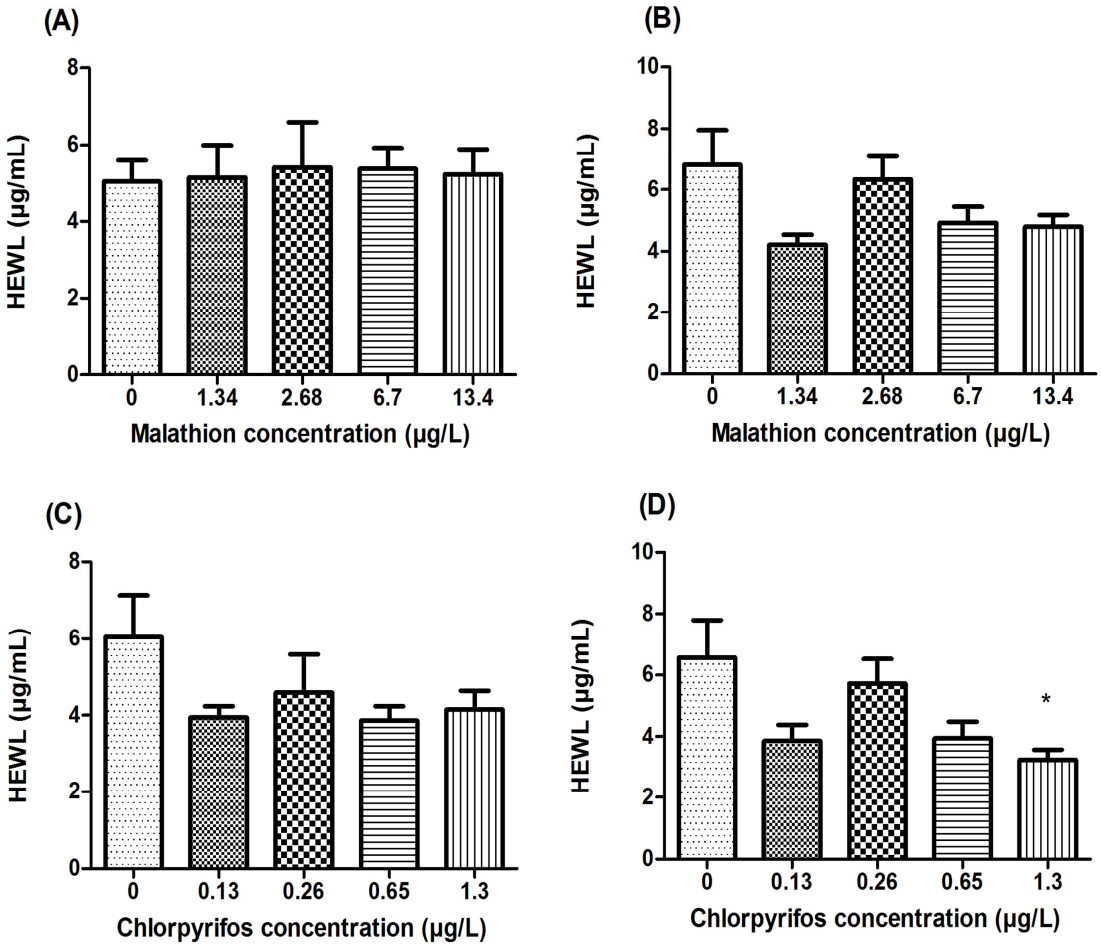

Figure 4 Serum lysozyme activity expressed as hen egg white lysozyme equivalent: (A) after 28 d (B) after recovery period to malathion exposure (C) after 28 d (D) after recovery period to chlorpyrifos exposure. Each bar represents mean ± S.E. of 12 fish. * denotes a significant difference from controls ($p < 0.05$).

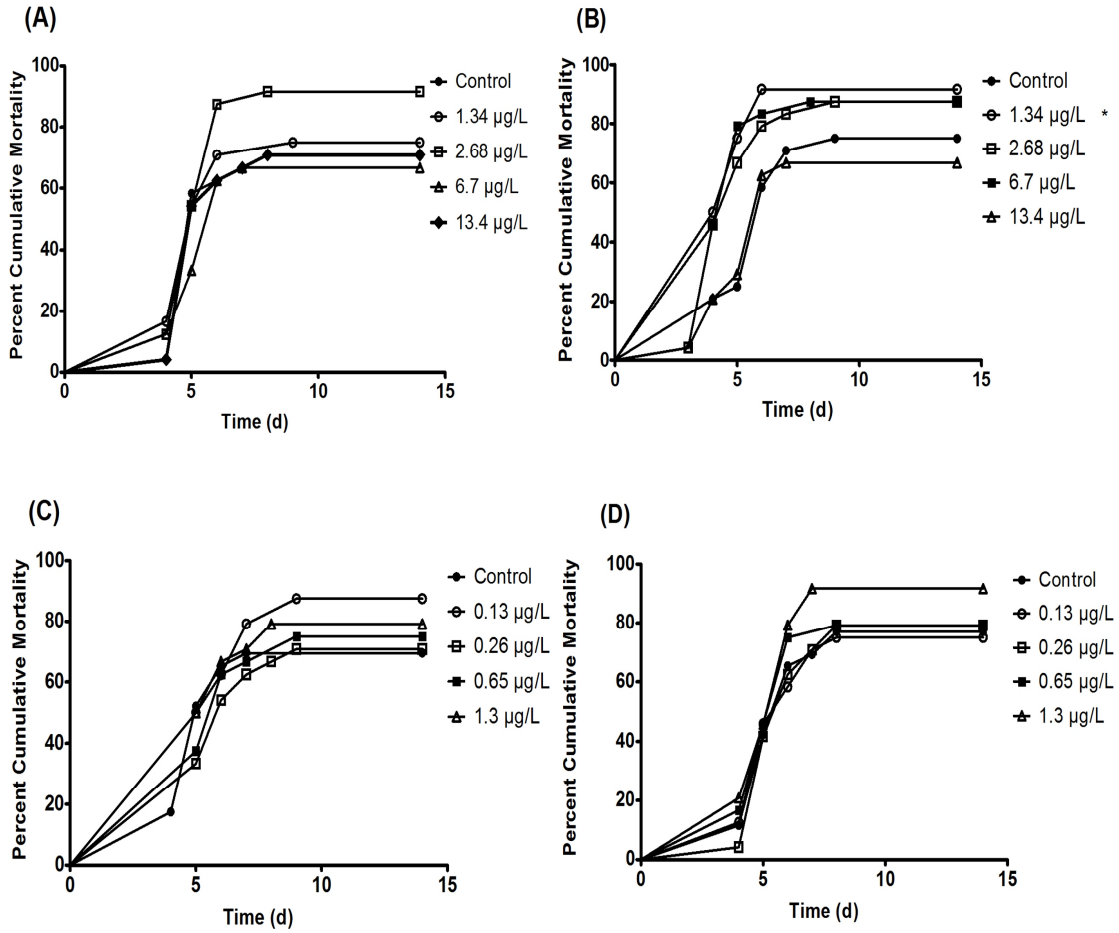

Figure 5 Mortality of rainbow trout following challenge with *L. anguillarum* : (A) after 28 d (B) after recovery period to malathion exposure (C) after 28 d (D) after recovery period to chlorpyrifos exposure. * denotes a significant difference from controls ($p < 0.0125$).

Table 1 Respiratory burst activity of rainbow trout expressed as stimulation index and then expressed as percentage control values. Serum alternative complement activity expressed as unit of $ACH_{50.}$ Each value represents the mean ± S.E. of 12 fish.

Pesticide Concentration (µg/L)	Respiratory Burst Activity (relative to control) %	Serum Alternative Complement Activity %
Malathion – 28 day exposure		
Control	100±12	289±22
1.34	207±47	290±11
2.68	194±27	274±16
6.7	198±39	293±22
13.4	202±57	269±24
p-value	0.085	0.875
Malathion – 14 day recovery		
Control	100±12	311±24
1.34	86±14	267±17
2.68	166±43	289±23
6.7	119±37	294±23
13.4	159±54	256±13
p-value	0.445	0.285
Chlorpyrifos – 28 day exposure		
Control	100±24	341±31
0.13	76±17	349±26
0.26	80±17	352±28
0.65	73±18	332±25
1.3	70±11	346±30
p-value	0.741	0.988
Chlorpyrifos – 14 day recovery		
Control	100±14	380±25
0.13	215±36	349±29
0.26	335±208	346±21
0.65	277±118	351±24
1.3	246±76	365±12
p-value	0.413	0.834

Discussion

The objective of this study was to investigate potential immunomodulatory effects of subchronic chlorpyrifos and malathion exposures in juvenile rainbow trout. To capture concentration-response relationships, a range of four test concentrations for each pesticide was selected, based on their respective averaged 96-h LC_{50} values from the US EPA's ECOTOX database (USEPA, 2007) that corresponded to 1, 2, 5, and 10 % of this value. Concentrations of chlorpyrifos equivalent to 1% of its 96-h LC_{50} (13 µg/L), and concentrations of malathion equivalent to 1 up to 10% of its 96-h LC_{50} (134 µg/L) have been detected in the environment in Canada and the United States, respectively. No mortality was observed in any of test concentrations for either pesticide. This was unexpected, as the duration of exposure (28 d) was considerably longer than 96-h. As such, these concentrations were termed sub-lethal, and due to environmental data, considered realistic environmental concentrations. All concentrations tested for both pesticides were well below their respective drinking water quality guideline in British Columbia (B.C.) (90 µg/L for chlorpyrifos and 190 µg/L for malathion). Unfortunately, Canadian water quality guidelines for the protection of aquatic species for malathion and chlorpyrifos do not exist (CCME, 2009).

A flow-through exposure system was adopted in this study as the best method to deliver close to nominal concentrations. High density polyethylene mariotte bottles (to reduce adsorption of pesticides) were used to achieve test concentrations and maintain a constant delivery of pesticides during the 28 d exposure. Based on the flow rate of

water, tank size, and bottle composition selected, full pesticide renewal was achieved approximately every 1.5 h, thus minimizing pesticide loss. Importantly, physical disturbance and additional stress posed on fish from renewing water in each tank on a more frequent basis were avoided with this flow through system.

Because of its high log Kow value (5.1), chlorpyrifos has a propensity for leaving the aqueous phase of the system, binding to organic material, and accumulating in aquatic sediments (Giesey, 1999). Thus, some chemical may have been lost by binding to waste material in the tanks, despite daily cleaning. However, such loss was considered minimal when compared to that which may have occurred in a static renewal system (pesticides renewed every 1.5 h in flow through versus 12-24 h for static renewal) due to the shorter residence time of pesticide in the tank. Malathion has a much lower log Kow value (2.75) (Tomlin, 1994), thus is unlikely to bind to organic matter. The half-life for malathion in water is short and highly dependent on the water pH value (Wolfe et al., 1976). At 10ºC (close to the water temperature of 12ºC in the present study), and pH 7, the half life of malathion is approximately 37 d. Since water pH was close to 7 in the tanks (6.8), and the fact that pesticide stocks in mariotte bottles were renewed every 48 h, minimal loss due to hydrolysis of malathion in water was expected. Therefore, in both cases, nominal pesticide concentrations were likely close to those achieved in tanks.

Immunomodulations by Malathion

In the present study, a measure of both innate immunological parameters and the functioning of the immune system as a whole through a disease challenge following pesticide exposure were made. Serum lysozyme, alternative complement activity (ACH_{50}), and respiratory burst activity (RBA) were not affected by a subchronic

malathion exposure. However, an increase in the percentage of head kidney cells that phagocytosed was seen following a 28 d exposure, with significant increases over controls observed in the three highest concentrations tested. This increase could be due to an increase in the number of phagocytic cells, a decrease of cells in other cell types, or a combination of both (Karrow et al., 1999). In the current study, gates were drawn to include phagocytic (granulocyte and macrophage) populations such that only the phagocytic cell populations were assessed for phagocytic activity. Since no difference in the percentage of these cell populations were observed across treatment groups, the increase in percentage of cells that phagocytosed was likely due to change in cell function and not from a change in the overall composition of head kidney cell suspensions (i.e., higher proportion of phagocytic cells relative to non-phagocytic cells). Interestingly, this increase of cells that phagocytosed returned to baseline values following a 14 d recovery period.

There is limited information on malathion's ability to modulate the phagocytic activities of fish leucocytes. Malathion has been shown to suppress phagocytosis in marine invertebrates. In lobster (*Homarus americanus*) (De Guise et al., 2004), concentrations of malathion at 6-7 fold lower than the 96-h LC_{50} value for this species significantly suppressed phagocytosis following both acute (1-3 days), and sub-acute (1-3 weeks) exposure.

The difference in the effect on phagocytosis (suppression in lobster/stimulation in trout) caused by malathion exposure, may be species-specific, due to a variety of factors including pesticide toxicokinetics. In mammals, macrophages are known cellular targets for malathion, and administration of low doses of malathion to mice can induce

peritoneal mast cell degranulation which leads to a subsequent augmentation of leukocyte function and enhances macrophage phagocytosis (Rodgers et al., 1996; Rodgers and Ellefson, 1992, Rodgers and Xiong, 1997), results which are in line with the observation in the present study.

Phagocytic activity may also have been enhanced as a result of repair responses to damage in body surface, gills, and other tissues of trout caused by direct malathion toxicity. Gill structural lesions were found after the acute exposure of catfish (*Heteropneustes fossilis*) to malathion (33% of 96-h LC_{50}/4 mg/L of 11.7 mg/L) along with a marked increase in leukocyte populations in the gills (Dutta et al., 1996; Dutta et al., 1998). Histopathological damage in the gills were also evident in mosquito fish (*Gambusia affinis*) exposed to concentrations of malathion at 5 and 10 % of its 96-h LC_{50} for 10, 20, and 30 days (Cengiz and Unlu, 2003) with the degree of injury being both concentration- and time-dependent; the concentrations and duration of exposure used were similar to the present study. Damage to gills from exposure to pollutants has been associated with inflammatory responses including increased granulocyte numbers in primary gill filaments, increased MHC II expression (Hoeger et al., 2005), and increased head kidney phagocyte chemiluminescence response (which measures RBA) (Muhvich et al., 1995). Immune assays in combination with the histopatholgical examinations of gills may provide a better understanding of this phenomenon and help link sublethal immunomodulation to tissue damage caused by pollutants in futures studies.

Another cause for the observed stimulation of this innate immune parameter may be due to a compensatory response from chemical-induced suppression of the adaptive immune system (Fatima et al., 2001; Karrow et al., 1999) where increases in innate

immune responses are observed with depressed adaptive immune responses. Although not measured in the current study, adaptive immune responses are susceptible to malathion suppression particularly with antibody cell formation. For example, subchronic exposure of Japanese medaka to malathion resulted in concentration-dependent reductions in the number of antibody plaque forming cells, which corresponded to an increased susceptibility to *Yersinia ruckerii* (Beaman et al., 1999). In other studies, chronic (30-d) malathion exposure led to decreased agglutination antibody titers against the formalin-killed bacteria *Edwardsiella ictaluri* in channel catfish (Plumb and Areechon, 1990) and decreased antibody titers against bovine serum albumin antigen in Indian major carp (Dash et al., 2000).

Malathion induced immunomodulation provides an example illustrating that it may be difficult to characterize/predict the risk of environmentally relevant concentration of chemical to fish based on selected immune parameters measured. Extrapolating results from cellular or humoral assays to the whole organism or ecological levels has been recognized as a challenge in aquatic toxicological studies (Bols et al., 2001).

While current experimental evidence suggests an enhancement of an innate immune parameter in trout, literature data suggest possible suppression of adaptive immune parameters. It is logistically impossible to measure all relevant immune parameters or to select *a priori* the ones which may be affected. The current study complemented the use of a panel of innate immune responses with a disease challenge in order to assess the overall health and functionality of the immune system of chemically exposed-fish. Fish exposed to each study pesticide were subsequently exposed to *L. anguillarum* in order to evaluate complex interactions of immune system components in a

systematic manner (Kollner et al., 2002) which can provide the most definitive tool for examining a compound's immunotoxic potential, even when it is now proven difficult to interpret the results from individual immune parameter measurements.

In this study, disease challenge data that examined the ecologically relevant endpoint of mortality, demonstrated that a statistical increase in susceptibility (17% over controls) of trout to the pathogen in the lowest concentration tested (1% of 96-h LC_{50}) occurred during the recovery period, but not immediately following exposure. Thus, there is a lack of correlation between the stimulation observed in the phagocytosis assay and the response of fish exposed to *L. anguillarum*, suggesting that qualitative changes in phagocytosis may not always be critical for whole organism defence against this particular bacterium. Similar findings have been reported where pesticide-induced increases in phagocytic and respiratory activities failed to correlate to trout host disease resistance to *L.anguillarum* (Shelley et al., 2009). In terms of the overall immunotoxicity of malathion, the lack of a concentration- response relationship between increased susceptibility to the bacterium and malathion exposure makes it difficult to conclude that a sub-chronic exposure of malathion results in a compromised immune system in trout, although malathion has been reported to cause a concentration-dependent increase susceptibility of Japanese medaka to *Y. ruckerii* (Beaman et al., 1999).

The cause of pesticide-induced increase in susceptibility to bacterial challenge after a recovery period, but not immediately after the exposure period in the group exposed to the lowest concentration of malathion is unclear. Examples of delayed toxicity in fish are scarce partly because of the limited immunotoxic studies that incorporate recovery periods. However, a similar example is shown with esfenvalerate (a

pyrethroid) exposed-chinook salmon where an increased mortality rate of approximately 20% was observed after 4 d exposures and an additional 7 d recovery period (Eder et al., 2008). Rainbow trout exposed to 1% of the 96-h LC_{50} (1% of 17.1 µg/L) value of chlorothalonil exhibited increases in respiratory burst and phagocytic activities after a 14 d recovery period, but not immediately after a 28 d, suggesting a delayed immune modulation caused by the pesticide (Shelley et al., 2009).

In terms of environmental relevance, malathion-exposed Japanese medaka demonstrated a concentration-dependent increase susceptibility to disease challenge (Beaman et al., 1999) with immunomodulating effects observed at 1.3 µg/L malathion. Combined with the observation that suppression of phagocytosis in lobsters occurred at 5 µg/L (DeGuise et al., 2004), and the increase of phagocytic population in the head kidney from the present study occurring at 2.68, 6.7, and 13.4 µg/L, these studies indicate that malathion may modulate the immunes system in various aquatic animals at environmentally relevant concentrations. However, malathion's effect on the overall immunocompetence in fish may be species-dependent.

Immunomodulations by Chlorpyrifos

In contrast to malathion, chlorpyrifos suppressed two of the immune parameters measured, but did not alter disease susceptibility of fish against a *L. anguillarum* bacterial infection. Serum lysozyme activity was suppressed at the highest concentration tested (10% of 96-h LC_{50}) after the 14-d recovery period, but not after the initial 28-d exposure. The same group of fish exhibited significant decrease in the proportion of granulocytes in head kidney cell preparations after 28 d exposure, which persisted to the 14 day recovery period. The decrease of granulocytes in the head kidney leucocyte population numbers

may explain the suppression of lysozyme activity because lysozyme is released into the circulation from the lysosomes of granulocytes, monocytes, and macrophages with the highest level found in fish head kidneys (Saurabh and Sahoo, 2008). The observation of suppression occurring after a recovery period for both of these parameters measured provides an example that innate immune responses induced by pesticide exposures may be slow to return to control levels even after a recovery period.

Reports on chlorpyrifos induced change in phagocytic activities vary, results from this study, and *in vitro* testing on four different Australian fish species (Harford et al., 2005) showed a lack of immunomodulation, but experiments on Nile tilapia (Giron-Perez et al., 2006; Holladay et al., 1996) demonstrated suppression in phagocytosis. This suggests that chlorpyrifos induced phagocytosis change may be species-specific.

The present disease challenge study did not reveal any change in the susceptibility of trout to *L. anguillarum*, thus the changes in the two parameters by chlorpyrifos may not be the most important defence mechanism in combating *L. anguillarum*. Concurrent exposure of chlorpyrifos and infectious hematopoietic necrosis virus (IHNV) in juvenile chinook salmon did not cause higher mortality rates from the virus, whereas esfenvalerate exposure provided a synergistic effect with IHNV causing elevated mortality rates (Clifford et al., 2005). Exposure of chlorpyrifos alone to chinook salmon also did not increase susceptibility to IHNV challenge (Eder et al., 2008). Thus, the overall immunotoxic potential of chlorpyrifos to fish remains to be confirmed.

The lack of a chlorpyrifos-induced decrease in host resistance observed here further supports the idea that alterations in immune parameters are not always good indicators of overall immune competence, thus highlighting the importance of performing

disease challenges in immunotoxicological studies concurrent with individual parameter measurements (Hoeger et al., 2004; Hamoutene et al., 2008).

Conclusions

Rainbow trout immune parameters were modified by environmentally realistic concentrations of pesticides with both stimulatory and suppressive effects. Head kidney granulocytes percentage and serum lysozyme activites were affected by chlorpyrifos exposure but resulted in no change of host resistance to *L. anguillarum*. Increased phagocytic cells were observed in trout exposed to three highest concentrations of malathion, but did not correspond to chemical induced mortality in disease challenge study. Fish exposed to the lowest concentration of malathion tested did not show any alteration of innate immune parameters measured, but after a recovery period a slight increase of mortality was observed in the disease challenge study.

This study has demonstrated that using changes (or lack of changes) of certain immune parameters to predict chemical induced suppression of host resistance can be a complicated task. Disease challenge study may remain the most informative deterministic tool of identifying immunotoxicity of the many current used pesticides in Canada.

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