CHEMICAL MIXTURE IMMUNOTOXICITY TO RAINBOW TROUT

by

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ABSTRACT

Several studies were conducted, and their results compiled into this thesis to explore the utility of incorporating immunotoxicological endpoints into field studies for ecotoxicological risk assessment. In the following chapters, the hazard of chemical mixture exposure to rainbow trout was assessed using a panel of recently developed immune assays. Chapter one contains a review the risk assessment process, and discusses the relevance and placement of immunological endpoints in the framework for ecotoxicological risk assessment. Fish immunotoxicological endpoints that show the greatest potential as biomarkers for ecoxoticological risk assessment are emphasized in the discussion.

Chapter two contains results from an outdoor creosote microcosm field study. In this study, several immune parameters were evaluated in rainbow trout (*Oncorhynchus mykiss*) after they had been exposed for 28 days in microcosms dosed initially with liquid creosote concentrations of 0, 5, 9, 17, 31, 56 and 100 *u*l/l. The most noticeable changes were concentration-dependent reductions in pronephros leukocyte oxidative burst and the number of sIg^+ peripheral blood leukocytes. Plasma lysozyme levels were reduced, while pronephros leukocyte phagocytic activity was enhanced marginally across creosote concentrations. Blastogenesis in response to lipopolysaccharide (LPS) was slightly impaired in head kidney leukocyte cultures prepared from creosote-exposed fish, whereas blastogenesis in response to phytohaemagglutinin (PHA) and concanavalin A (ConA) was unaffected. Overall the results suggest that creosote has the potential to alter some innate immune functions in rainbow trout. Polycyclic aromatic hydrocarbons (PAHs), a major constituent of liquid creosote, are the suspected immune altering agents. The LOEC or the immune responses measured in this study was 17 *u*l/l using nominal creosote concentrations, representing a total PAH concentration of 611.63 ng/l in the water.

Chapter three includes results from a second outdoor microcosm study, conducted in order to re-evaluate the immunotoxic effects of liquid creosote to rainbow trout (*Oncorhynchus mykiss*). During this study, two separate experiments were conducted. The first experiment was designed to monitor the kinetic effects of exposure to creosote; caged fish were sampled on days 7, 14, 21, and 28 from microcosms initially dosed with 0, 3, and 10 *u*l creosote. A second experiment was designed to monitor immune parameters after 37 d of exposure in microcosms initially dosed with 0.3, 1, 3, 10, and 30 *u*l creosote. Two replicate control microcosms were included in the 37 d exposure to evaluate variability across microcosms. Pronephros leukocytes were monitored in both experiments for phagocytic activity, oxidative burst, and surface immunoglobulin-positive (SIg^+) B cell counts. Serum lysozyme activity was also measured for the 28 d kinetic experiment. During the kinetic study, oxidative burst was progressively inhibited in fish exposed to 3 and 10 *u*l creosote, returning to control levels by day 28. Phagocytic activity was initially stimulated after seven days of exposure, returning to control levels by day 28. Although control SIg^+ B cell counts were quite variable across sampling days, SIg^+ B cell counts were also elevated after seven days of exposure in creosote exposed fish. These cell numbers decreased significantly during the remainder of the study. Lysozyme activity appeared to be unaffected by creosote exposure. After the 37 d of exposure, oxidative burst was suppressed in creosote exposed fish; phagocytic activity was enhanced. No detectable change was observed for SIg^+ B cell counts. The overall results confirm that creosote has the potential to alter certain measured immune parameters. Modulation of the measured immune parameters varied with the duration of exposure. Polycyclic aromatic hydrocarbons (PAHs), a major constituent of liquid creosote, are the suspected immune- altering agents.

The final chapter contains results from a field study, used to assess whether or not exposure to sites within the Hamilton Harbour, which are known to be highly contaminated with polycyclic aromatic hydrocarbons (PAHs), heavy metals, and sewage

treatment plant (STP) effluent, could affect immune function in fish. Caged rainbow trout (*Oncorhynchus mykiss*) were sampled after 7, 14, and 21 days of exposure from five sites within the Harbour, and a Lake Ontario reference site. The Harbour sites were selected for their proximity to either highly contaminated sediments or industrial and municipal discharges. Pronephros leukocytes from fish at the six sites were evaluated for phagocytic activity, oxidative burst, and the number of surface immunoglobulin-positive B cells. Serum lysozyme activity was also measured. Fish from the Harbour sites showed a greater reduction in pronephros leukocyte phagocytic activity over the course of the study when compared to the Lake Ontario reference fish; pronephros leukocyte oxidative burst was also reduced at two of the Harbour sites. Although B cell counts did not change throughout the duration of the study, overall counts were lower at two of the Harbour sites when compared to the Lake Ontario site. Serum lysozyme activity increased at site 4 and at the Lake Ontario reference site over the 21 days of exposure. Lysozyme activity was elevated at sites 5 and 6, when compared to Lake Ontario fish lysozyme activity, and did not change throughout the course of the study. These results indicate that several immune parameters were altered in fish during exposure at various sites within the Harbour. The role of chemical and physical stressors at these sites in the observed immunomodulation is discussed.

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LIST OF ABBREVIATIONS

ANF	α-napthoflavone
APC	antigen presenting cell
BaP	benzo(a)pyrene
BSA	bovine serum albumin
CMI	cell mediated immunity
ConA	concanavalin A
CPM	counts per minute
CTL	cytotoxic T lymphocyte
DCFHDA	dichlorofluorescin diacetate
DCM	dichloromethane
DMBA	7, 12-dimethylbenz[a]anthracene
DTH	delayed type hypersensitivity
EC	effective concentration
ELISA	enzyme-linked immunosorbent assay
EROD	ethoxyresorufin-*o*-deethylase
EPA	Environmental Protection Agency
GPC	gell permeation chromatography
HMI	humoral mediated immunity
H_2O_2	hydrogen peroxide
HPLC	high pressure liquid chromatography
HR	host resistance
I	phagocytic index
IFN	interferon
IL	interleukin
IP	intraperitoneal
LOEC	lowest observed effect concentration
LPS	lipopolysaccharide
3MC	3-methylcholanthrene
MFO	mixed function oxidase
MLR	mixed leukocyte reaction
MMC	melanomacrophage centres
MS-222	methane tricainesulfonate
NCC	natural cytotoxic cell
NF	net fluorescence

NK cell	natural killer cell
NOEC	no observed effect concentration
4-NP	4-nonylphenol
NWRI	National Water Research Institute
PBS	phosphate buffered saline
PFC	plaque-forming cells
PTK	protein tyrosine kinase
P450	cytochrome P450 monooxygenase
PAH	polycyclic aromatic hydrocarbon
PBL	peripheral blood leukocyte
PCB	polychlorinated biphenyl
PCDF	polychlorinated dibenzofurans
PHA	phytohaemagglutinin
PMA	phorbol 12-myristate 13-acetate
RAP	remedial action plan
RBF	round bottom flask
sIg^+ leukocyte	B lymphocytes expressing IgM receptors
SOD	superoxide dismutase
sRBCs	sheep red blood cells
STP	sewage treatment plant
TBTO	bis(tri-*n*-butyltin)oxide
4-t-OP	4-tert-octylphenol

INTRODUCTION

I.1 GENERAL INTRODUCTION AND STATEMENT OF THE PROBLEM

The field of immunotoxicology has been growing exponentially over the past two decades due to an increasing awareness that certain drugs, chemicals, and biologicals disrupt normal immune function. Chemical toxicity to the immune system may result in immunosuppression, eventually reducing host resistance to infectious diseases. On the other hand, an immune response targeting either a xenobiotic, or host proteins altered by the xenobiotic, may result in the development of allergy or autoimmunity. Despite the endless number of publications describing the immunosuppressive and sensitizing effects caused by xenobiotics, the use of immunotoxicity data for risk assessment has received little attention, rarely progressing past the "hazard identification stage" until recent years (Luster et al., 1995). A number of factors may account for this. Redundancy and compensatory mechanisms, built into the immune system, have made it difficult for immunotoxicologists to reach a consensus as to which immunological endpoint(s) should be given priority. In addition to this, immunotoxicity testing has often been conducted with little concern for its association with other toxicological endpoints, and its predictive value in terms of disease susceptibility and population health (Luster et al., 1995).

During the late 1980s the National Toxicology Program (NTP) devised a criteria for evaluating immunotoxicity in mice using a multi-tiered approach. More recently, Luster and Kimber (1996) reported that certain combinations of assays from the NTP testing panel could be used to predict immunotoxicity with a level of concordance of greater than 90%. These assays are currently used to screen compounds for immunotoxicity, and may constitute the first tier of the multi-tiered testing panel. A number of these assays have been accepted by regulatory agencies through out Europe and the U.S.A., in an attempt to harmonize immunotoxicity testing internationally (Hastings, 1998), and are now

being incorporated into the human risk assessment process (Van Loveren et al., 1998). Immunotoxicological endpoints are also being cautiously reviewed by environmental toxicologists for their potential use in ecotoxicological risk assessment. An awareness of neuro-endocrine-immune crosstalk (Savino and Dardenne, 1995), and an increasing concern about the presence of endocrine disrupting chemicals in our environment will likely add to the popularity of this discipline in the years to come.

Recently, both for practical and ethical reasons, a great deal of emphasis has been placed on developing alternative models for higher vertebrates in immunotoxicological studies. Fish may represent ideal candidates for a number of reasons. First, fish are already being utilized extensively in ecotoxicological risk assessment to predict effects at higher trophic levels. Second, the fish immune system is comprised of cells that are functionally analogous to those cells of their mammalian counterparts. These immunologically competent cells participate in the lysis of tumor cells, allograft rejection, hypersensitivity reactions, antibody production, mitogenic stimulation, antigen presentation, cytokine secretion, and phagocytosis, accompanied by the production of reactive oxygen intermediates (Zelikoff, 1998). Third, numerous examples cited by Zelikoff (1994), suggest that mammalian and fish immune systems appear to be compromised by stressors in similar ways. Data are, however, still needed to compare the sensitivity of the fish immune system, and the immunotoxic mechanisms of action to that of our mammalian counterparts (Karol, 1998).

In order for fish immunotoxicological endpoints to be incorporated into an ecotoxicological risk assessment process, concentration-response relationships (EC_{50} and LOEC) must first be established. Once established, the ecological relevance of these relationships can be explored by linking them to responses at higher levels of biological organization. Aquatic toxicologists have recognized the limitations of conducting laboratory toxicity tests for the extrapolation of effects at higher levels of biological organization. It is for this reason that microcosm exposure studies are being used to

generate ecosystem response data in aquatic ecotoxicological risk assessment. It is felt that they may potentially bridge the gap between laboratory and full-scale field studies. In this way, toxicological endpoints can be generated under more realistic conditions (Thompson et al., 1993), and an opportunity is provided to study stressor effects at entire population and community levels which are not seen in single-species laboratory studies (Liber et al.,1992).

I.2 THESIS OBJECTIVES AND OUTLINE

The objectives of this thesis are to:

1. Review the risk assessment process and discuss where immunotoxicology fits into the framework for ecotoxicological risk assessment. Emphasis will be placed on fish immunotoxicological endpoints.

2. Incorporate a panel of recently developed immunotoxicity tests into an outdoor microcosm study to evaluate the immunotoxicity of liquid creosote to rainbow trout after 28 d of exposure.

3. Provide insight to the kinetics of creosote immunomodulation, as well as validate the results obtained from objective 2, by monitoring fish immune parameters at weekly intervals and extending fish exposure to 37 d during a subsequent microcosm field season.

4. Incorporate the panel of immunotoxicity tests from objectives two and three into a field study to determine whether or not exposure to various organic and inorganic contaminants is sufficient to alter immune parameters in rainbow trout caged at various sites within the Hamilton Harbour.

This thesis is composed of four main chapters, which are followed by a general conclusion. Chapter one contains a review of the risk assessment process, and discusses where fish immunotoxicology fits into the framework for ecotoxicological risk assessment. Chapter two contains the results from a 28 d rainbow trout immunotoxicity study conducted in outdoor microcosms treated with liquid creosote. Chapter three reviews a subsequent creosote microcosm study, monitoring rainbow trout immune

parameters during 28 d, and after 37 d of exposure. Chapter four contains the immunotoxicity results from a 21 d field study at various sites within the Hamilton Harbour.

CHAPTER 1

IMMUNOTOXICOLOGY AND ECOTOXICOLOGICAL RISK ASSESSMENT

1.1 ECOTOXICOLOGICAL RISK ASSESSMENT

Ecotoxicological risk assessment is a means of identifying and quantifying risks to non-human biota, and determining the acceptability of those risks (Suter, 1995). Risk is defined in this context, as the probability that a chemical will have an adverse toxicological effect (i.e. pose a hazard) on the ecosystem. The framework for ecotoxicological risk assessment proposed by the U.S. Environmental Protection Agency in 1992, and reviewed by Solomon et al. (1996) is summarized in figure 1.1. In this framework, risk assessment is comprised of three steps: hazard identification; risk analysis; and risk characterization. In human health risk assessment, it is sufficient to identify a hazard by establishing that exposure to a potentially toxic chemical may occur. Hazard identification is more complex in an ecotoxicological risk assessment, in that it is necessary to define the sources of chemical exposure, describe the exposed environment, and choose the appropriate endpoints for the assessment (Suter, 1995). The analysis phase of the risk assessment involves characterizing the exposure to a chemical (i.e. identify the exposure concentration based on prior knowledge of the physical and chemical properties of the compound, its fate, transport, and exposure routes), and identifying the ecological effects that resulted from this exposure (i.e. establishing the dose-response relationships). The risk characterization phase involves integrating the exposure and effects assessments to estimate the risk posed by exposure to a chemical. Uncertainties in the assessment process should also be addressed in this phase (Suter, 1995).

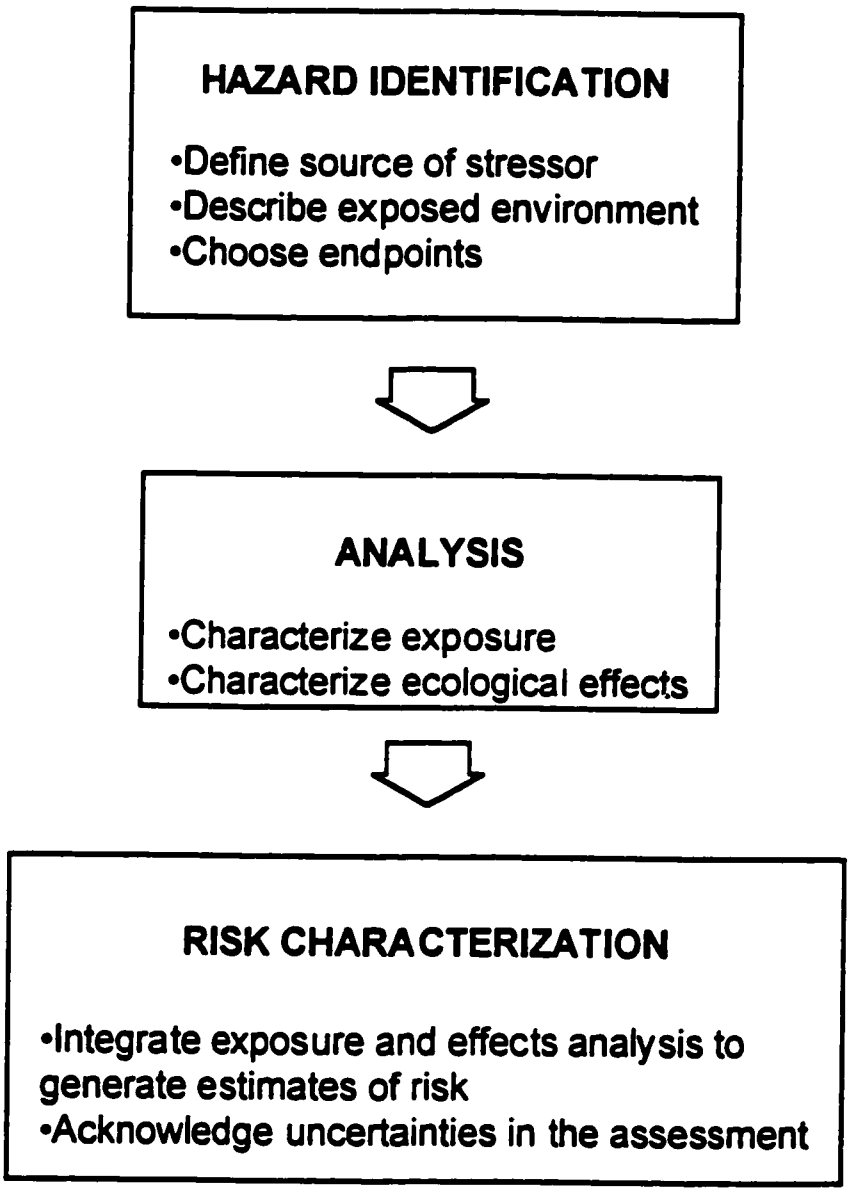

Figure 1.1: Framework of an ecotoxicogical risk assessment. (Adapted from Solomon et al., 1996)

1.2 IMMUNOTOXICOLOGICAL ASSESSMENT ENDPOINTS

1.2.1 Host resistance studies

The selection of assessment endpoints is critical to the risk assessment. They must be unambiguous, sensitive, quantifiable, and have both social and ecological relevance (Suter, 1995). From a risk assessment perspective, the most ecologically relevant immunological endpoint is decreased host resistance (HR) to infection (Figure 1.2). Great efforts were made by the NTP to develop pathogen models, under controlled laboratory conditions, for detecting altered HR in rodents. A panel of pathogen models was selected based on relative sensitivity, reproducibility, relevance as human pathogens, and specificity to various compartments of the immune system (Table 1.1) (Thomas, 1998). Recently, the utility of these assays in human risk assessment was demonstrated by extrapolating from rat to human the health risk of exposure to bis(tri-*n*-butyltin)oxide (TBTO) (Van Loveren et al., 1998).

Unfortunately, very little progress has been made with regard to using fish HR as an endpoint in ecotoxicological risk assessment. A number of promising candidate pathogen models have been identified and are currently being used, to a limited degree, to assess fish HR in laboratory studies (Table 1.2). However, mammalian research has taught us that tumor, viral, bacterial, and parasitic models are required to accurately assess HR (Table 1.1). Selection of an appropriate HR model is dependent on both the exposure route and immunological profiles already obtained for an immunotoxicant (Thomas and Sherwood, 1996). *A. salmonicida* and *V. anguillarum* may not, for example, be good models for evaluating TBTO immunotoxicity in fish because a major route of TBTO uptake occurs by ingestion (Rice et al., 1995), and TBTO primarily targets cell-mediated immunity (Van Loveren et al., 1998). Additionally, a number of reports suggest that a sublethal bacterial infection can actually provide some resistance to chemically related immunosuppression (Luebke et al., 1997). Clearly, researchers have a great deal more to learn about the pathogenesis of some of these models, and mechanisms of host

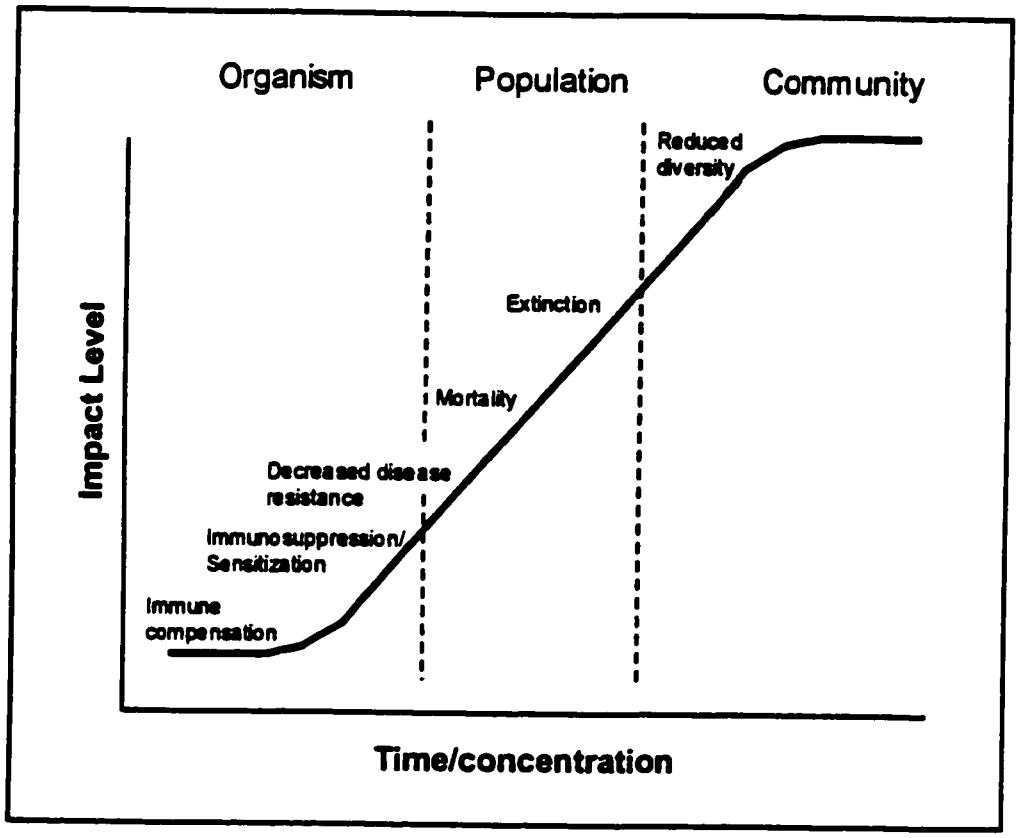

Figure 1.2: Impact level at the organism, population and community levels in response to exposure to an immunotoxic compound. As exposure duration and concentration increase, the level of impact increases from immunomodulation to increased susceptibility to disease at the organism level, followed by mortality and possible extinction at the population level.

Table 1.1: Current NTP testing panel for evaluating immunotoxicity in mice (Luster et al., 1992)

Tier 1

- Hematology
- Weights: body, spleen, thymus, kidney, liver
- Cellularity: spleen, bone marrow
- Histology of lymphoid organs
- IgM antibody plaque-forming cells (PFCs)
- Lymphocyte blastogenesis
  - T cell mitogens (PHA, Con A)
  - T cell mixed leukocyte response (MLR)
  - B cell mitogen (LPS)
- Natural killer (NK) cell activity

Tier 2

- Quantify splenocyte phenotypes (B and T cell surface markers)
- Enumerate IgG antibody PFC response
- Mixed leukocyte reaction (MLR)
- Cytotoxic T lymphocyte (CTL) cytolysis or delayed type hypersensitivity
- Macrophage clearance
- Host resistance models
  - Syngeneic tumor cells
    - PYB6 sarcoma (tumor incidence)
    - B16F10 melanoma (lung burden)
  - Bacterial models
    - *Listeria monocytogenes* (morbidity)
    - *Streptococcus species* (morbidity)
  - Viral models
    - Influenza (morbidity)
  - Parasite models
    - *Plasmodium yoelii* (parasitemia)

Table 1.2: Candidate pathogen models for evaluating altered host resistance in fish

Microorganism	Agent	Immune response	Reference
Vibrio anguillarum	bacteria	cellular and humoral immunity phagocytic cells	Evelyn, 1996
Yersinia ruckeri	bacteria	cellular immunity	Evelyn,1996
Aeromonas salmonicida	bacteria	cellular and humoral immunity phagocytic cells	Loghothetis and Austin, 1994 Evelyn, 1996
Ichthyophthirius multifiliis	protozoa	NCC, humoral immunity	Dickerson and Clark, 1998
Trypanosoma danilewskyi	protozoa	humoral immunity	Wang and Belosevic, 1994
Infectious hematopoietic necrosis virus	virus	cellular and Humoral immunity	La Patra et al., 1997

protection before standardized testing methodologies can be developed. Once a panel of models has been validated, HR studies will undoubtedly prove to be an invaluable tool for establishing disease causality in fish immunotoxicology.

1.2.2 Immune assays

Another approach to evaluating chemical-induced immunomodulation is to use molecular and immune function assays as biomarkers of chemical exposure and effect. The NTP developed a multi-tiered testing panel of assays in the 1980s to evaluate immunotoxicity in mice (Table 1.1). The advantage of utilizing these assays in risk assessment is that they can be used to quantify immune parameters in populations at risk more readily than measuring changes in the frequency or severity of clinical diseases (Luster et al., 1993). Additionally, immune assays detect subtle immunomodulatory changes that may be tolerated by the host due to functional redundancy and compensatory mechanisms built into the immune system (Luster and Kimber, 1996). Immune assays may therefore, be useful tools for predicting increased host susceptibility to disease.

Luster et al. (1995) utilized the NTP rodent data base to establish a relationship between immune assay and HR endpoints. They showed that there was a good correlation between these endpoints and that, while no one test was effective at predicting altered HR, thymus or spleen/ body weight ratios and assays measuring NK cell activity, delayed type hypersensitivity (DTH), plaque-forming cells (PFC), lymphoproliferative response to T cell mitogens, and surface marker expression were the best predictors of changes in HR (>70% concordance). On the other hand, leukocyte counts and lymphoproliferative response to LPS were poor predictors of altered HR. Certain combinations of these assays was shown to greatly improve the predictability of altered HR. Combining the PFC assay with either spleen/ body weight ratios or one of the following assays; cytotoxic T lymphocyte activity (CTL), NK cell activity, proliferative response to T cell mitogens, and surface marker expression improved HR predictability to

>75%. Pairwise combinations of the NK cell activity assay with assays measuring DTH, proliferative response to T cell mitogens, CTL, and surface marker expression improved predictability to >77%. Lastly, pairwise combinations of mixed leukocyte response (MLR) with DTH, thymus/body weight ratio, and CTL improved HR predictability to >80 %.

How can these results be interpreted in the context of fish immunotoxicology? First, although such an evaluation is also overdue for fish immunological endpoints, it is likely that a few sensitive immune assays is sufficient for the initial screening chemicals for potential immunotoxicity. Second, a correlation between HR and immune assays provides evidence to support the use of immunological assays as biomarkers in risk assessment and environmental effects monitoring programs. This is critical, since the logistics of immunizing fish in field contaminant exposure studies limits the use of HR and assays that specifically evaluate a fish immune response (Rice et al., 1996). HR and more in depth mechanistic studies should be conducted in a controlled laboratory setting after the initial screening process has occurred, since confounding variables, such stress related to handling and temperature changes, can greatly influence immune parameters (Wester et al., 1994). In depth studies will help identify the immunolodulating compound(s), define their mechanism(s) of action, and establish a relationship between the measured immune parameters and increased susceptibility to disease. Third, immune assays can be incorporated into existing field and laboratory toxicity testing protocols to correlate immune parameters with more ecologically relevant endpoints such as, increased susceptibility to disease, reproductive success, and mortality. Many of the assays originally developed for rodents have already been adapted for use in fish (Luebke et al., 1997). The remainder of this discussion will focus on assays that show the greatest potential as biomarkers for ecoxoticological risk assessment.

A. Plaque-forming Assay

The most sensitive assay for measuring chemical immunomodulation in mammals is the PFC assay (Karol, 1998). This assay quantifies the number of plasma cells in a given

leukocyte population, following antigenic sensitization, to measure a humoral immune response. Since processing of the antigen by antigen presenting cells (APCs), T cell activation, and B cell proliferation and differentiation are all involved in this reaction, the assay is sensitive to subtle changes in various compartments of the immune system. This assay has also been modified to evaluate fish humoral immune response. Arkoosh et al. (1994) used a version of the assay to evaluate spleen and pronephros leukocyte PFC response in chinook salmon (*Oncorhynchus tshawytscha*) exposed to contaminated sites near Puget Sound, WA. The advantage of using this assay in field studies is that a humoral immune response to a specific antigen can be evaluated within 2 weeks of sensitization (Anderson and Zeeman, 1995). At least four to six weeks are required before detectable levels of antibodies can be detected by an enzyme-linked immunosorbent assay (ELISA); often serum antibodies are not measured until at least 12 weeks post vaccination (Angell Killie and Jorgensen, 1994). PFC assays are however, very labour intensive, and must be initiated the day on which fish are processed. Temple et al. (1993) conducted a comparison of these two techniques, promoting the ELISA technique. Unfortunately, both techniques require immunization prior to chemical exposure, and the logistics of certain field studies may not allow for such a design (Rice et al., 1996).

B. Leukocyte phenotypic markers

Flow cytometric quantification of lymphocyte populations, based on their cell surface marker expression, is another technique, derived from mammalian immunology, that is being adapted for use in fish immunotoxicology research (Dunier et al., 1994). Evaluating phenotypic markers is considered one of the most sensitive predictors of immunotoxicity in mammals (Vandebriel et al., 1995). However, there are certain limitations to this technique, since immunosuppressive effects can occur with certain compounds without obvious changes in cell phenotypes (Hastings, 1998). In addition to quantifying immune cells of different lineage, flow cytometry is also used in mammalian immunotoxicology to

measure cell differentiation (Lai et al., 1998), cell signaling events, oxidative damage (Bruchiel et al., 1997) and, more recently, intracellular cytokine levels (Mascher et al., 1999). Flow cytometric technology is limited by the commercial availability of molecular probes. Unfortunately, antibodies recognizing membrane immunoglobulin are currently the only unequivocal cell surface markers available for quantifying fish lymphocyte populations (Miller et al., 1998). As more probes become available, phenotypic labeling, along with other flow cytometric techniques, will be a valuable tools for screening chemicals that are immunotoxic to fish.

C. Macrophage function

Macrophage function has proven to be a sensitive marker of chemical-induced immunomodulation in fish, and is likely the most frequently cited endpoint in the literature (Fournier et al., 1998; Luebke et al., 1997; Rice et al., 1996; Seeley and Weeks-Perkins, 1997; and Zelikoff, 1998). Macrophages have important roles in both specific (antigen processing and presentation) and non-specific (phagocytosis and destruction) defense (Wester et al., 1994). Assays of macrophage function include measuring chemotaxis, phagocytosis, and pinocytosis (Wester et al, 1994). Phagocyte function can also be evaluated by measuring the oxygen-dependent (i.e. reactive oxygen and nitrogen species), and oxygen-independent (i.e. lysozyme) phagocyte killing mechanisms (Secombes, 1996). Unfortunately, it is still not clear how to interpret the functional changes in these assays, and how they relate to either an immune response or altered HR. Generally, a single functional assay is used to evaluate macrophage function. However, macrophage function is highly dependent on the cell's stage of development (Lewis, 1995). Hence, a change in the phagocytic activity may simply reflect a shift in the cell's development, and have no bearing on the outcome of HR. These assays have great value as an initial screening test for fish immunotoxicity, however, in depth studies should consider a number of different endpoints in order to evaluate macrophage development and how it relates to altered HR to disease (Lewis, 1995).

D. Natural cytotoxic cell assay

NK cell function is also a highly sensitive endpoint for evaluating cell-mediated immunity in rodents; often these cells are the first to succomb to chemical-induced immunotoxicity (Karol, 1998). NK cells naturally kill tumour cells, parasites, and bacterial and viral infected cells (Karol, 1998). Their function is assessed by measuring their ability to lyse tumor cells; K562 in humans and YAC-1 for rodents. Functionally analogous cells, called natural cytotoxic cells (NCC), have also been identified in fish (Luebke et al., 1997). Using the same endpoint and target cells, researchers have shown that fish NCCs are also useful for evaluating immunotoxicity in fish (Luebke et al.,1997; Rice et al. ,1996; and Seeley and Weeks-Perkins, 1997).

E. Lymphoproliferation assays

Lymphoproliferative assays are being used extensively as screening tools in fish immunotoxicology studies to evaluate T and B lymphocyte proliferation (Dunier et al., 1994; Luebke et al, 1998; Zelikoff, 1998) despite an awareness that these assays lack sensitivity (Vandebriel et al., 1995), are not good predictors of altered HR (Luster et al., 1995), and are often not optomized using the appropriate mitogen concentrations (Vandebriel et al., 1995). Although proliferative assays may be useful for mechanistic and in depth studies, they are not reliable or sensitive enough for the initial screening process. Vandebriel et al. (1995) have suggested using either the PFC or MLR assays instead for evaluating cellular proliferation.

1.2.3 Immune biomarkers and microcosm studies

The greatest concern associated with using immunological biomarkers in risk assessment is the uncertainty associated with predicting effects at the population and community levels. No where, is this more evident than in using fish as sentinel species in field studies. Establishing causality to population changes in field studies is susceptible to bias and misinterpretation because if the complexity of ecosystems (Luebke et al., 1997).

Questions that need to be addressed include; how the duration of exposure, chemical mixtures and genetic heterogeneity affect fish immunity, what are the kinetics of immunomodulation and the high risk groups within a population (i.e. early- life- stages) (Colborn, 1996). There is evidence to suggest that laboratory-based immunotoxicological studies can be used to predict effects in feral fish populations (Zelikoff, 1997). However, field studies are the only way to test laboratory derived causality hypotheses at population levels (Luebke et al., 1997).

Microcosm studies may help to establish a relationship between immunomodulation and effects at the population and community levels. Although microcosms do not behave identically to natural ecosystems, they have proven their utility in impact assessments because they simulate conditions more typical of the natural environment (Liber et al., 1992). The advantages and limitations to using microcosms and mesocosms to simulate field conditions are listed in table 1.3. From an immunotoxicological perspective, microcosms offer an opportunity for researchers to predict effects at the population level by monitoring temporal immunomodulation. They increase the practicality of using fish HR and immune response assays under simulated field conditions, and allow for correlations to be made between immune parameters and other more ecologically relevant endpoints such as, reproductive success, and mortality. Mesocosm studies have recently been used to establish a link between TBTO contaminated water/sediment, liver tumor occurrence, and a lymphocystis viral infection in flounder (*Platichthys flesus*).

Table 1.3: Advantages and limitations of simulated microcosm field studies (Adapted from Graney et al., 1995).

Advantages	Limitations
The test system contains a functional ecosystem	Difficult to establish a realistic fish community with predator prey relationships
Can monitor test system over time	High cost to design and maintain
Concentration-response relationships can be established	Divergence of the replicated system occurs with time
Can replicate treatment groups	Scaling factors must be carefully considered
More realistic environmental conditions	
Effects on multiple species can be monitored simultaneously	
Species interactions can be investigated	

1.3 CONCLUSIONS

A great deal of progress has been made in the field of immunotoxicology over the last two decades. Some of the lesions learned from mammalian immunotoxicological studies are also likely applicable to fish research (i.e. it is most likely that several sensitive immune assays are adequate for identifying hazardous chemicals). Currently, there exists a number of established immune assays for evaluating fish immune function which can be used to identify hazardous chemicals. These assays can also be used as assessment endpoints in risk assessment, provided a relationship with increased disease susceptibility is established using laboratory-based HR studies. Microcosm studies may provide an opportunity to combine immune assessment endpoints under more realistic conditions to predict effects at the population and community levels, and establish relationships with other more ecologically relevant assessment endpoints.

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CHAPTER 2

CHARACTERIZING THE IMMUNOTOXICITY OF CREOSOTE TO RAINBOW TROUT (*Oncorhynchus mykiss*): A MICROCOSM STUDY

2.1 ABSTRACT

Several immune parameters were evaluated in rainbow trout (*Oncorhynchus mykiss*) after they had been exposed for 28 days in microcosms dosed initially with liquid creosote concentrations of 0, 5, 9, 17, 31, 56 and 100 *ul*/l. The most noticeable changes were concentration-dependent reductions in pronephros leukocyte oxidative burst and the number of sIg^+ peripheral blood leukocytes. Plasma lysozyme levels were reduced, while pronephros leukocyte phagocytic activity was enhanced marginally across creosote concentrations. Blastogenesis in response to lipopolysaccharide (LPS) was slightly impaired in head kidney leukocyte cultures prepared from creosote-exposed fish, whereas blastogenesis in response to phytohaemagglutinin (PHA) and concanavalin A (ConA) was unaffected. Overall the results suggest that creosote has the potential to alter some innate immune functions in rainbow trout. Polycyclic aromatic hydrocarbons (PAHs), a major constituent of liquid creosote, are the suspected immune altering agents. The LOEC or the immune responses measured in this study was 17 *ul*/l using nominal creosote concentrations, representing a total PAH concentration of 611.63 ng/l in the water.

Key words: polycyclic aromatic hydrocarbons (PAHs); (*Oncorhynchus mykiss*); immunotoxicity; microcosm

2.2 INTRODUCTION

Numerous laboratory and field studies have provided evidence of altered immune function in fish exposed to PAHs in water and contaminated sediment (Weeks and Warriner, 1986; Payne and Fancey, 1989; Secombes et al., 1991; Faisal and Huggett, 1993; Arkoosh et al., 1994; Lemaire-Gony et al., 1995; Arkoosh et al., 1996. PAH-mediated alteration of the immune system is suspected of predisposing fish to clinical diseases (Dunier and Siwicki, 1993). This hypothesis has been based largely on indirect evidence, including the increased occurrence of skin lesions, fin erosion and neoplasia in fish at PAH contaminated sites (Weeks and Warriner, 1986; Dunier and Siwicki, 1993). Concentration-response relationships have not, however, been clearly demonstrated. In order for immunotoxicology to be incorporated into a risk assessment process, concentration-response relationships for immunological endpoints (EC_{50} and LOEC) must first be established. Once established, the ecological relevance of these relationships can be explored by linking them to responses at higher levels of biological organization.

In recent years researchers have recognized the limitations of conducting laboratory toxicity tests for the extrapolation of effects at higher levels of biological organization. Microcosm exposure studies are being used to generate ecosystem response data in aquatic risk assessment because they can potentially bridge the gap between laboratory and full-scale field studies. Toxicological endpoints can be generated under more realistic conditions (Thompson et al., 1993), and an opportunity is provided to study stressor effects at entire population and community levels which are not seen in single-species laboratory studies (Liber et al.,1992).

In this study, the immunotoxicity of creosote to rainbow trout (*Oncorhynchus mykiss*) was studied in outdoor microcosms treated with liquid creosote. Immune parameters including, pronephros leukocyte mitogenic response, phagocytic activity and oxidative burst, and peripheral blood surface immunoglobulin-positive B cell counts and lysozyme activity were monitored in fish after 28 d of exposure in creosote treated microcosms.

Phagocytic and B lymphocyte cells are known to play a key role in regulating teleost immune response through phagocytosis/endocytosis, antigen processing and presentation, and the secretion of cytokines (Verburg-van Kemenade et al., 1995). B lymphocytes also differentiate into plasma cells, the main effector cells in humoral defense. Lysozyme may act as an opsonin for phagocytic activity, and has been shown to specifically cleave peptidoglycans forming the cell wall of gram positive bacteria, resulting in osmolysis (Ellis, 1990). Liquid creosote, a coal tar distillate, is used mainly as a wood preservative for railway ties and marine pilings. Although it is a complex mixture of over 300 compounds, seventeen polycyclic aromatic hydrocarbons (PAHs) account for 63% of its volume (CEPA, 1994), making it a suitable candidate for PAH mixture studies.

2.3 MATERIALS AND METHODS

A. Microcosm

Seven outdoor microcosms were constructed of steel and lined with a PVC liner with a depth of 1.05m and a surface area of 11.95 m^2 . Gravel was back filled around the microcosms to help moderate temperature fluctuations. Sifted sediment was added to the microcosms in plastic trays to provide 50% coverage of the floor. The microcosms were filled with 12,000 l of water from a well-fed irrigation pond and circulated for 3-4 wk prior to dosing to achieve homogeneity. The microcosms were dosed with liquid creosote by sub-surface injection into a stream of water being pumped at a rate of 1,360 l/h such that the initial creosote concentrations were 0, 5, 9, 17, 31, 56 and 100 *u*l/l. Dosing of the microcosms was based on a series of graded creosote doses with no replication, commonly referred to as a regression approach. Liber et al. (1992) and Thompson et al. (1993) have discussed the advantages and disadvantages of this experimental design. The microcosms remained static throughout the duration of the study, and were exposed to natural sunlight and precipitation. Fish were not added to the microcosms until 103 d post dosing (October 14) to ensure sublethal exposure, steady state conditions, and an optimal temperature profile for rainbow trout. The photoperiod for the duration of the rainbow trout study was determined by ambient light which had a light: dark cycle of 10:14 h.

B. Experiment

Female rainbow trout (≅100 g) were obtained in early October from Rainbow Springs Hatchery, Thamesford, ON. The fish were acclimated for two wk in the irrigation pond at the microcosm site prior to exposure. Fish (n= 120) were weighed, tagged and allocated to each of the seven microcosms on days 103 to 108 after creosote dosing. A total of 15 fish were exposed at each creosote concentration. In the control microcosm, the sample size was doubled in order to better characterize natural population variability. Initiation of exposure was staggered by adding a new cage containing three fish to each

concentration on each of the first 5 d of the study. The cages were constructed from nylon netting supported by circular plastic tubing (internal diameter 40 cm, length 800cm, mesh size 1.5 cm). Fish were fed commercial trout chow at a ration of 2 % body weight/d. Temperature, pH, dissolved oxygen, alkalinity, and water hardness profiles were routinely monitored during the acclimation period and throughout the duration of the study. After the 28 d exposure, one cage was removed from each microcosm, two from the control group. The distance among cages within each microcosm appeared sufficient to prevent stress to the remaining caged fish during this process. Fish were immediately anaesthetized with methane tricainesulfonate (MS-222), weighed and sampled for peripheral blood from the caudal vein using heparinized vacutainers.

C. Preparation of pronephros and peripheral blood leukocyte suspensions

Peripheral blood was centrifuged at 200 X g for 10 min at 4°C. Aliquots of plasma were frozen at -20°C for lysozyme analysis. The leukocyte buffy coat was collected, diluted to 7 ml with Ca^{2+} and Mg^{2+} free HBSS (10 U heparin/ml, pH 7.4), and centrifuged through 3 ml of Histopaque-1077 (d=1.077, Sigma Chemical Company, St Louis, MO, U.S.A) at 400 g for 30 min at 9°C. Leukocytes were collected at the interface, washed three times with HBSS, and resuspended in $NaHCO_3$ free RPMI 1640 medium + 25 *u*M HEPES + L-glutamine (pH 7.4, Gibco, Burlington, ON, Canada) supplemented with 10 % fetal calf serum (Gibco), 50 U/ml penicillin, and 50 *ug*/ml streptomycin (Gibco). Total leukocyte count and viability was determined by trypan blue exclusion.

Single cell suspensions of pronephros leukocytes were prepared by pressing tissue through a 100 *um* stainless steel mesh with the flat end of a syringe plunger over a plastic petri disk containing chilled Ca^{2+} and Mg^{2+} free HBSS (10 U heparin/ml, pH 7.4). Pronephros leukocytes were prepared in a similar manner to peripheral blood leukocytes.

D. Pronephros leukocyte oxidative burst

The oxidative burst assay was conducted according to Brousseau et al. (1999) using a Coulter EPICS XL-MCL flow cytometer. Cell suspensions were adjusted to 10^6 cells/ml

in 2 ml of PBS supplemented with 0.5% (w/v) glucose. Leukocytes are incubated with 4 *u*M of 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes Inc., Eugene, OR, U.S.A) for 15 min in the dark at 18°C. DCFH-DA is incorporated into the hydrophobic lipid regions of the cell. Phorbol myristate acetate (PMA) (Molecular Probes Inc., Eugene, OR, U.S.A) was added at a final concentration of 100 ng/ml to a 1 ml aliquot of each sample to activate the cells. The release of hydrogen peroxide (H_2O_2) within the cells oxidizes DCFH to 2',7'-dichlorofluorescin (DCF) which fluoresces at 530 nm. The net fluorescence (NF_{60}) is proportional to the net amount of H_2O_2 generated over a given time (60 min). Net fluorescence values were normalized across days by expressing them as a percent of the control mean for each day.

Two distinct leukocyte populations exhibiting oxidative burst were detected from pronephros tissue samples using flow cytometry. In order to derive the net fluorescence intensity, a gate was drawn around the larger more granular cells which represented the major leukocyte population. This population was assumed to be representative of the residing macrophage population within the pronephros as they are the most predominant phagocytic leukocyte population (Manning, 1994).

E. Pronephros leukocyte phagocytosis

Leukocyte phagocytic activity was assayed by flow cytometry according to Brousseau et al. (1999) using fluorescent latex beads (1.03 *u*M diameter, Molecular Probes). Cell suspensions were adjusted to 10^6 cells/ml in 1 ml Leibovitz L-15 culture medium (GIBCO) and incubated for 30 min at 18°C. Negative controls were incubated in PBS with 1 % paraformaldehyde. Leukocyte suspensions were incubated with 10^8 beads for 18 h at 20°C. The cells were then centrifuged for 5 min at 100 X g through a gradient mixture of 3 % bovine serum albumin (GIBCO) and RPMI 1640 medium and resuspended in 500 *u*l Isoflow solution (Coulter Corp., Hialeah, FL, U.S.A.). For each leukocyte preparation, 10,000 cells were analyzed. The number of cells with three or more beads was recorded, and defined the proportion of phagocytic cells. A note also was made of the mean fluorescence in the

phagocytic population, which was a measure of total bead uptake and an indication of the phagocytic activity of the phagocytic cells. The two sets of data were used to calculate a phagocytic index (I). This was the percentage of phagocytic cells multiplied by the mean total fluorescence in the phagocytic cells. Values were normalized across days by expressing them as a percent of the control mean for each day.

F. Pronephros lymphocyte proliferation

The lymphocyte proliferation assay was conducted according to Brousseau et al. (1999). Aliquots of 5 X 10^6 cells in 100 ul of RPMI medium supplemented with 4% FCS + 50 U/ml penicillin, and 50 ug/ml streptomycin were added in triplicate to a 96 well culture plate for each mitogen. The cells were then incubated for 72 h at 18°C with 100 ul of the mitogens: lipopolysaccharide (LPS), concanavalin A (Con A), phytohaemagglutinin (PHA) or RPMI 1640 medium as a control. Final mitogen concentrations were 100, 10, and 5 ug/ml respectively. [3H]-thymidine (ICN Biomedicals, St-Laurent, QU, Canada) at 0.5 uCi was added to each well and incubated for 18 h. The plates were then frozen at -20°C until the end of the experiment. Cell harvesting involved thawing the plates, and collecting the cells onto a glass microfiber filter using a 1295-001 LKB Wallac cell harvester. Radioactivity was measured on a 1205 LKB Wallec betaplate liquid scintillation counter and expressed as counts/min (CPM). For each fish, the mean incorporation by mitogen-treated cultures as CPM was compared to the spontaneous mean incorporation by non-stimulated cultures. When the incorporation was significantly higher in mitogen-treated cultures as judged by T test ($p<0.05$), the fish was reported as responding to the mitogen. For those cultures that responded to the mitogen, the mean incorporation was divided by the incorporation mean for the non-stimulated cultures to give a stimulation index. When all fish were considered, the incorporation mean by the control cultures was 235 CPM ± 141(n=65) with a range from 67 CPM to 541 CPM. The mean incorporation was not significantly different between control (non-mitogen treated) cultures from fish exposed and not exposed to creosote. As well, for further statistical

analysis (see below), the change in CPM (mean incorporation for the control cultures subtracted from the incorporation mean of the responding mitogen-treated cultures) was normalized across days by expressing the change in CPM as a percent of the daily control mean change in CPM.

G. Peripheral blood surface immunoglobulin-positive (sIg^+) leukocyte marking

The number of surface immunoglobulin-positive (sIg^+) leukocytes was determined by flow cytometry according to Dunier et al., 1994. Leukocytes at 10^6 cells/ml were incubated with 100 *ul* of supernatant from hybridoma culture 1-14 (1:100), a known B cell marker, of De Luca et al. (1983) (Courtesy of N.W. Miller) or 100 *ul* RPMI 1640 in 1 ml of RPMI 1640 medium for 45 min on ice. Cells were then washed three times, and incubated with 300 *ul* of goat-anti-mouse FITC (1:100, GIBCO) in 1 ml RPMI 1640 medium for 30 min in the dark on ice. After three washings the percent of surface immunoglobulin-positive (sIg^+) leukocytes was determined using flow cytometry. The percentage of sIg^+ leukocytes was normalized across days by expressing the values as a percent of the control mean for each day.

H. Plasma lysozyme activity

Plasma lysozyme activity was measured according to Marc et al. (1995) with slight modifications. The assay measures a lysozyme-induced decrease in the optical density of a 1.25 mg/ml (*Micrococcus lysideikticus*) (Sigma) PBS (pH 7.5) suspension at 410 nm. Optical densities were measured over a 10 min incubation period with 10 *ul* of fish plasma using a Microplate EL311 autoreader. A standard curve was made with lyophilized hen egg white lysozyme (Sigma). The results were expressed as *ug*/ml equivalent of hen egg white lysozyme activity.

I. Plasma cortisol analysis

Concentrations of cortisol in plasma were determined with a commercial radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA, USA, 07-221102). The characteristics of the assay were previously described by Hontela et al. (1995).

J. PAH analysis

Grab samples (1l) were taken from each microcosm on 114 d and preserved with 80 g/l sodium thiosulphate. PAH analytes were liquid extracted into HPLC grade methylene chloride and dried with an excess of anhydrous sodium sulphate (Sigma). Samples were concentrated under vacuum and resuspended in 2 ml of HPLC grade iso-octane. Samples were spiked with an internal standard (bromonapthalene) to determine the efficiency of recovery. PAH concentrations were determined with a Varian 3400 gas chromatograph equipped with a Varian Saturn II ion trap mass spectrometer. Samples were injected onto a 30 m X 0.25 mm SPB-5 column with a stationary phase thickness of 0.25 *um* at 300 °C under splitless conditions. The transfer line and manifold temperatures were held constant at 260 and 250 °C respectively. PAHs were mass scanned at 45-550 m/z.

Water and sediment total PAH concentrations up to 84 d were generously provided by Bestari et al. (1998). All results were expressed using creosote nominal concentrations, and total PAH concentrations in the water and sediment as dose surrogates. Sediment total PAH concentrations are presented as the geometric mean of measured PAH concentrations on 28, 56, and 84 d of the microcosm study to represent sediment steady state concentrations.

K. Statistical analysis

An analysis of variance (ANOVA) using a general linear model followed by regression analysis was used to analyse the data (SYSTAT 5.0). Dunnett's test for comparisons was used to detect significant differences across concentrations and to derive the lowest-observed-effect (LOEC) and no-observed-effect (NOEC) concentration

endpoints. All data were tested for compliance to the assumptions of normality and variance homogeneity. Data sets, which violated these assumptions, were transformed using a log or square root transformation. Significance was determined at $p \leq 0.05$.

2.4 RESULTS

A. Water chemistry and physical profiles

The mean temperature, dissolved oxygen, pH, hardness and alkalinity for the irrigation pond during the 2 wk acclimation period were: 14 °C, 12 mg/l, 8.6, 223 mg/l and 110 mg/l, respectively. A temperature decrease of 10 °C was observed over the duration of the 28 d study (Figure 2.1A), and an increase in dissolved oxygen corresponded with the temperature profile (Figure 2.1B). The pH in Figure 2.1C remained constant at 8.0 ± 0.4 (mean ± SD). Water hardness and alkalinity were 226 ± 21 mg/l $CaCO_3$ (mean ± SD) and 125 ± 17 mg/l (mean ± SD) respectively. Nitrate and nitrite levels were all less than the detection limits of 0.1 and 0.08 mg/l.

Total PAH concentrations in the water and sediment total PAH concentrations up to 84 d after the addition of creosote are illustrated in Figures 2.2A and 2.2B, respectively. Total PAH concentrations were not corrected for the 80% extraction recovery. While numerous PAHs were detected in the water and sediment phases, fluorene, anthracene, fluoranthrene, and pyrene were the only water borne PAHs to exhibit significant curvilinear relationships with the nominal creosote concentrations on 114 d (Table 2.1, Figure 2.3).

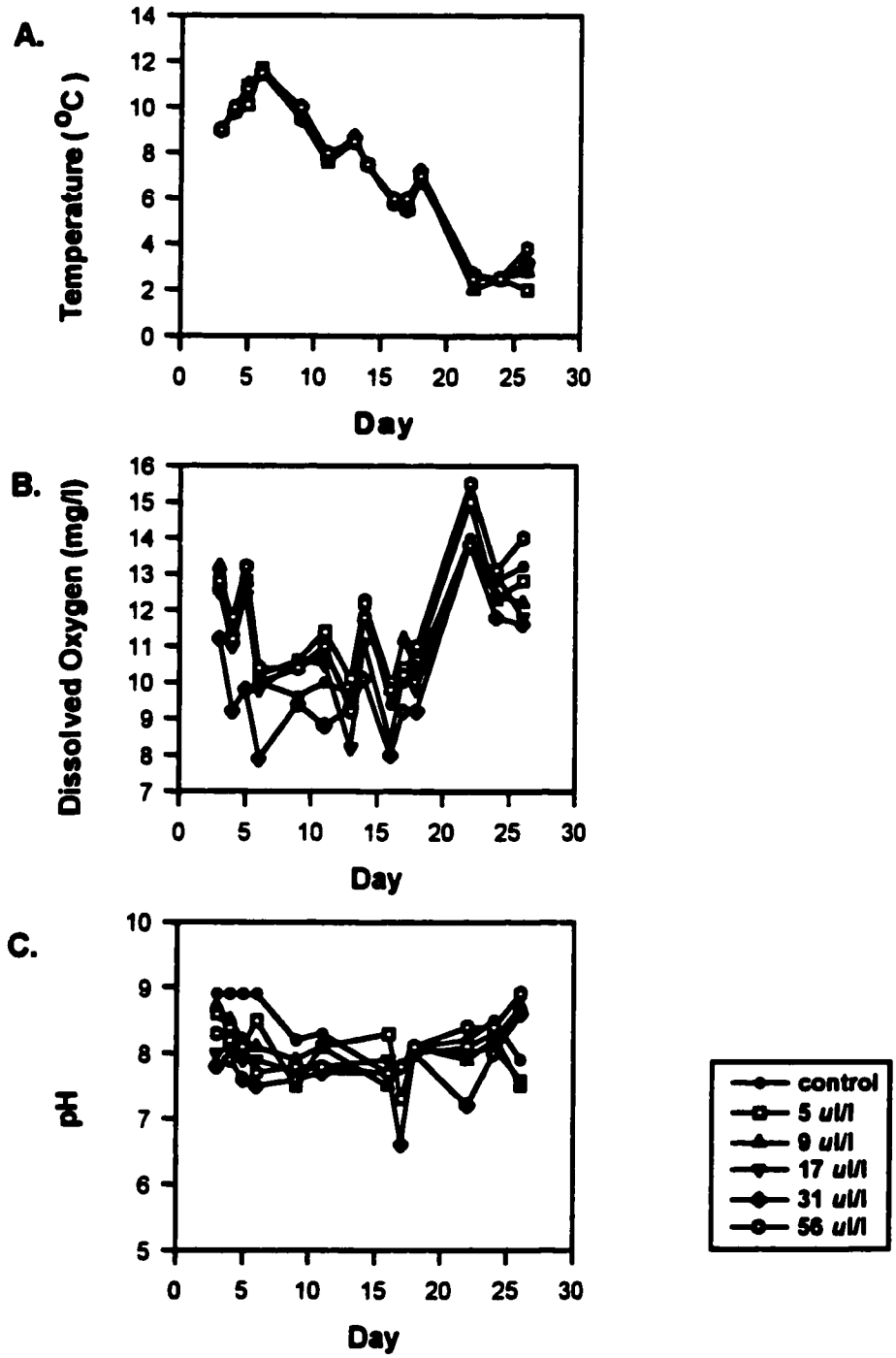

Figure 2.1: Temperature (A), dissolved oxygen (B), and pH (C) profiles for the control and creosote-treated microcosms during the 28 d study.

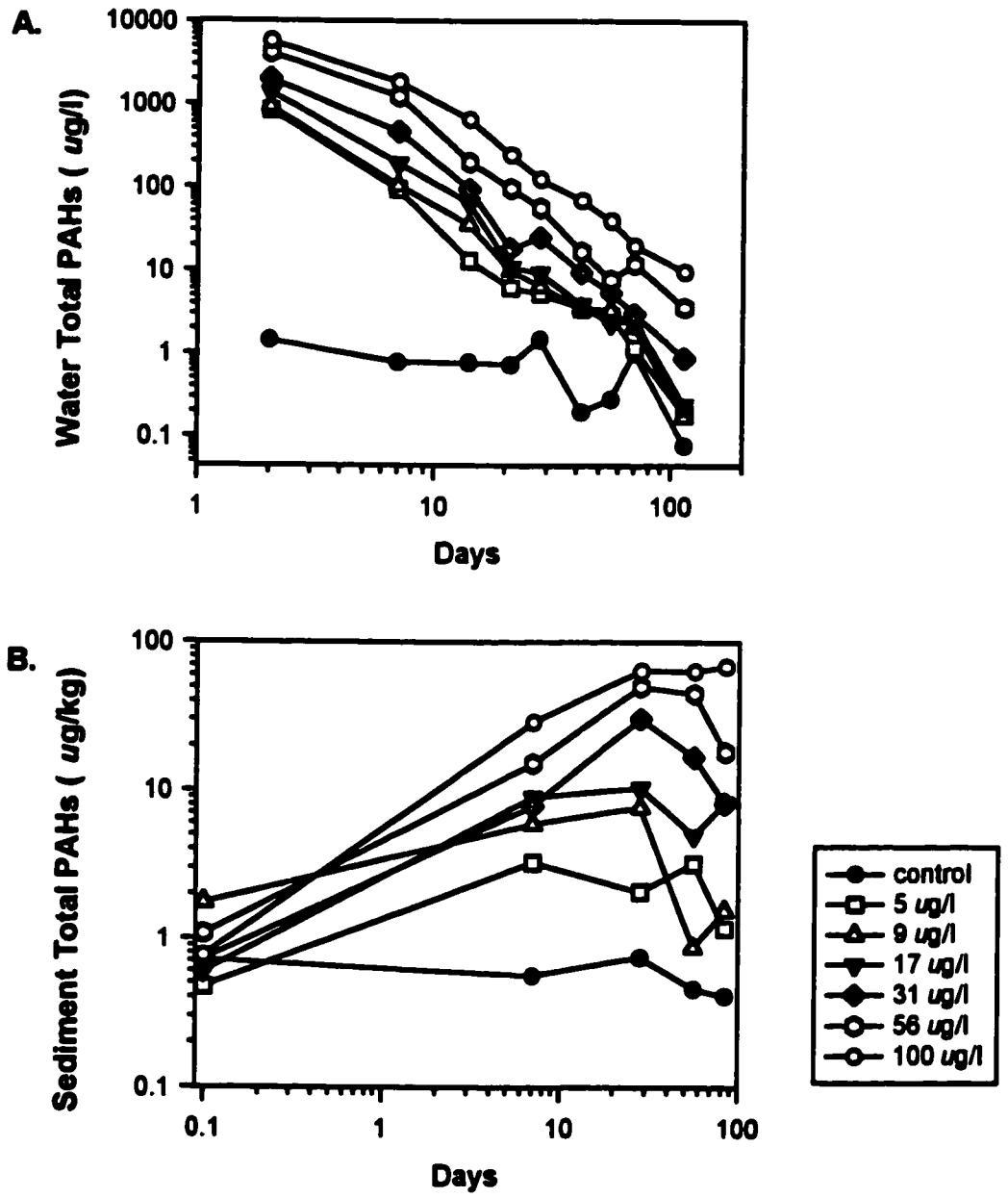

Figure 2.2: Change in total PAH concentrations in the water (A), and sediment (B) with time after dosing microcosms with creosote. The first sediment sample was taken on the day of dosing; the first water sample, on the second day after dosing. Fish were added to the microcosms 103 days after dosing.

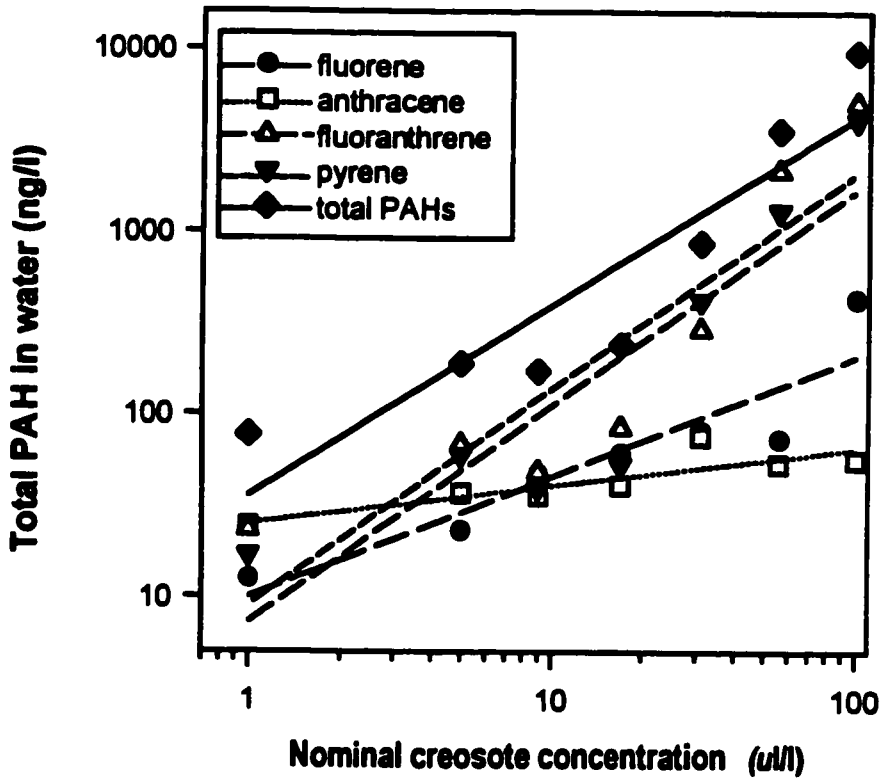

Figure 2.3: Relationship between PAH water concentration and nominal creosote concentration 114 d after creosote dosing. Lines represent the linear regression equation y=a+bx through each data set($p \leq 0.01$) (See Table 2.1 as well).

Table 2.1: PAH concentrations for control and creosote-treated microcosms on day 15 of the rainbow trout study.[a]

PAH (ng/l)	Creosote (*u* l/l) 1	5	9	17	31	56	100	F ratio
Fluorene *	12.60	23.10	47.20	61.30	81.60	73.60	430.10	28.70
Phenanthrene	25.10	38.50	54.90	42.40	79.00	45.80	59.40	
Anthracene *	24.20	36.80	35.60	40.60	75.50	53.80	56.40	15.41
Fluoranthrene *	23.30	67.60	48.10	83.90	291.40	2159.20	5076.40	21.70
Pyrene *	17.10	59.90	39.50	53.20	420.50	1307.90	4122.20	23.35
Benzo(a)anthracene	nd	137.00	25.02	88.42	505.62	105.85	170.69	
Chrysene	nd	nd	nd	nd	162.40	67.28	348.90	
Benzo(b)fluoranthene	nd	36.82	63.52	105.57	607.45	120.20	179.72	
Benzo(k)fluoranthene	nd	89.91	67.37	110.98	639.05	122.90	188.74	
Benzo(a)pyrene	nd	53.09	nd	nd	178.20	13.46	49.63	
Indo(1,2,3-cd)pyrene	nd	76.21	nd	25.26	89.54	25.12	nd	
Total detectable PAHs	102.30	618.93	381.21	611.63	3130.26	4095.11	10682.18	

* Significant linear relationship with nominal creosote concentrations ($p \leq 0.01$)

[a] Naphthalene, acenapthene, dibenzo(a,h)anthracene, and benzo(g,h,i)perylene were not detected in the water

(nd)= not detected

B. Weight change and mortality

Rainbow trout in the 56 ul/l concentration gained less weight than control fish, although, the difference across concentrations was not statistically significant (Figure 2.4). Liver weights increased slightly in the 31 and 56 ul/l concentrations but were also not significant. Increased liver weights combined with reduced weight gain led to an overall increase in the liver to body weight ratio (Figure 2.5). A significant linear relationship was observed using creosote nominal ($y= 0.40+3.20\log x$), F-ratio=7.83, $p<0.01$, $R^2=0.11$), water total PAH ($y= 0.55+0.16\log x$), F-ratio=10.29, $p<0.01$, $R^2=0.14$), and sediment total PAH concentrations ($y= 0.88+0.12\log x$), F-ratio=7.54, $p<0.01$, $R^2=0.11$).

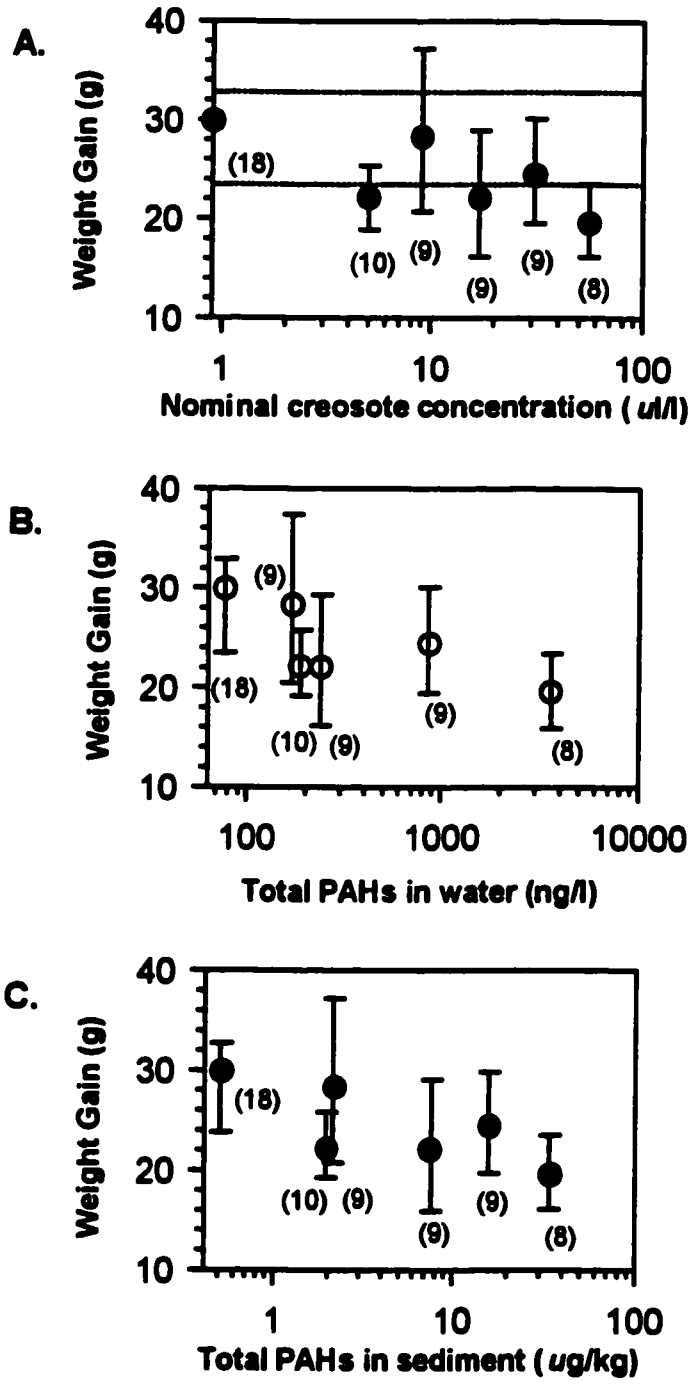

Figure 2.4: Rainbow trout mean weight gain (with 95% confidence intervals) with respect to nominal creosote concentrations (A), total PAH concentrations in the water (B), and sediment (C) during the 28 d microcosm study. Sediment concentrations are presented as the geometric mean of measured PAH concentrations on 28, 56, and 84 d of the microcosm study. Sample size is indicated as (n).

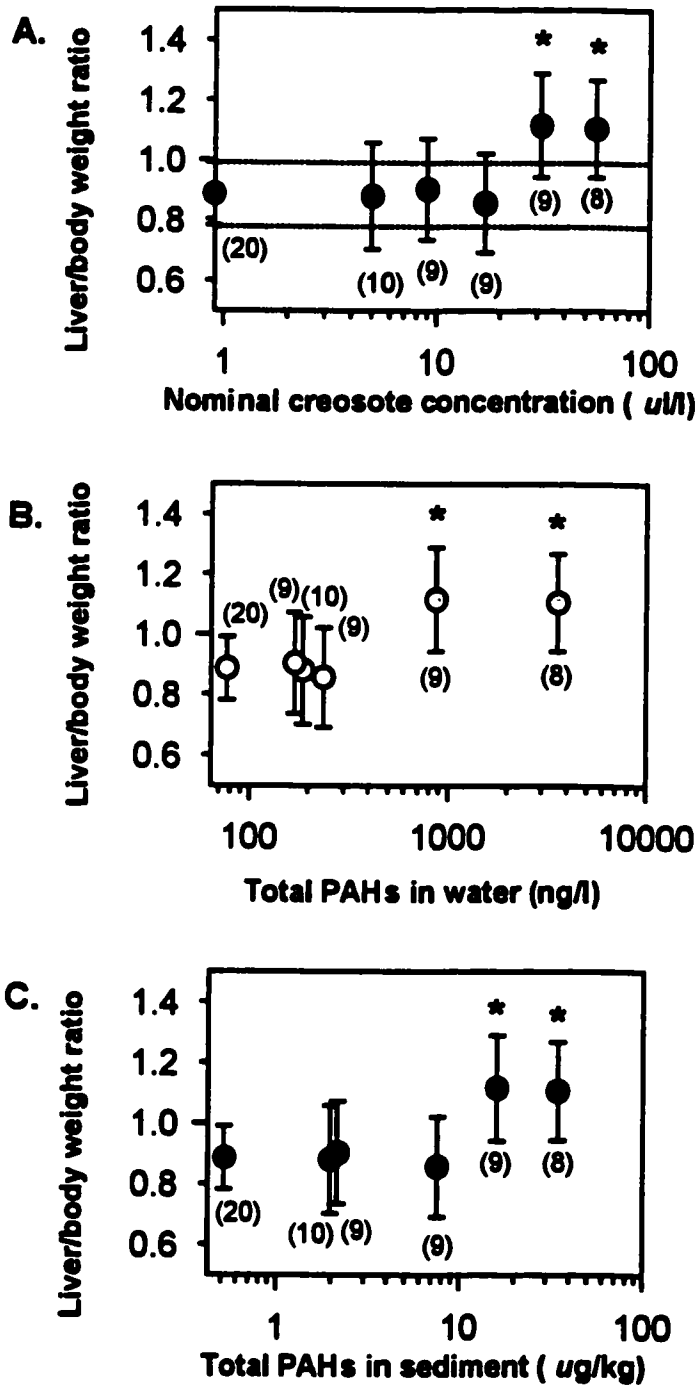

Figure 2.5: Liver to body weight ratio of individual fish exposed to creosote during the 28 d microcosm study. Dose surrogates include nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on 28, 56, and 84 d of the microcosm study. Results are expressed as mean liver to body weight ratio with 95% confidence interval. Means with significant differences from the control mean ($p<0.05$) are indicated by *. Sample size is indicated as (n).

The LOEC was 31 ul/l using nominal creosote concentrations, representing a total PAH concentration of 3130.26 ng/l in the water. Others observed an increase in EROD activity (Whyte, 1998) and PAH bile metabolites (Lewis, 1997) in the fish from the creosote microcosms, which indicates that the fish took up and metabolized PAHs during the 28 day exposure. Fish mortality occurred, but except for the highest creosote concentration (100 ul/l), this showed no relationship to the nominal creosote dose. At 100 ul/l, all fish died within the first 3 d of exposure. However, at 0, 5, 9, 17, 31 and 56 ul/l creosote, percent mortalities of 23, 13, 40, 40, 27, and 47 were seen over the 28 days of exposure. Mortalities in the control group, and a substantial amount of the treatment losses, appeared to be due to fish becoming entangled in the mesh cages.

C. Oxidative burst

Creosote exposure significantly reduced leukocyte oxidative burst in a concentration-response dependent fashion (Figure 2.6A-C). This concentration-response relationship followed a significant linear response using creosote nominal (log y= log (2.82-0.42x), F-ratio=29.37, $p<0.01$, $R^2=0.32$), water total PAH (log y= log (2.26-0.48x), F-ratio=27.74, $p<0.01$, $R^2=0.31$), and sediment total PAH concentrations (log y= log (1.98-0.36x), F-ratio=31.64, $p<0.01$, $R^2=0.33$). The LOEC was 17 *ul/l* using nominal creosote concentrations, representing a total PAH concentration of 611.63 ng/l in the water.

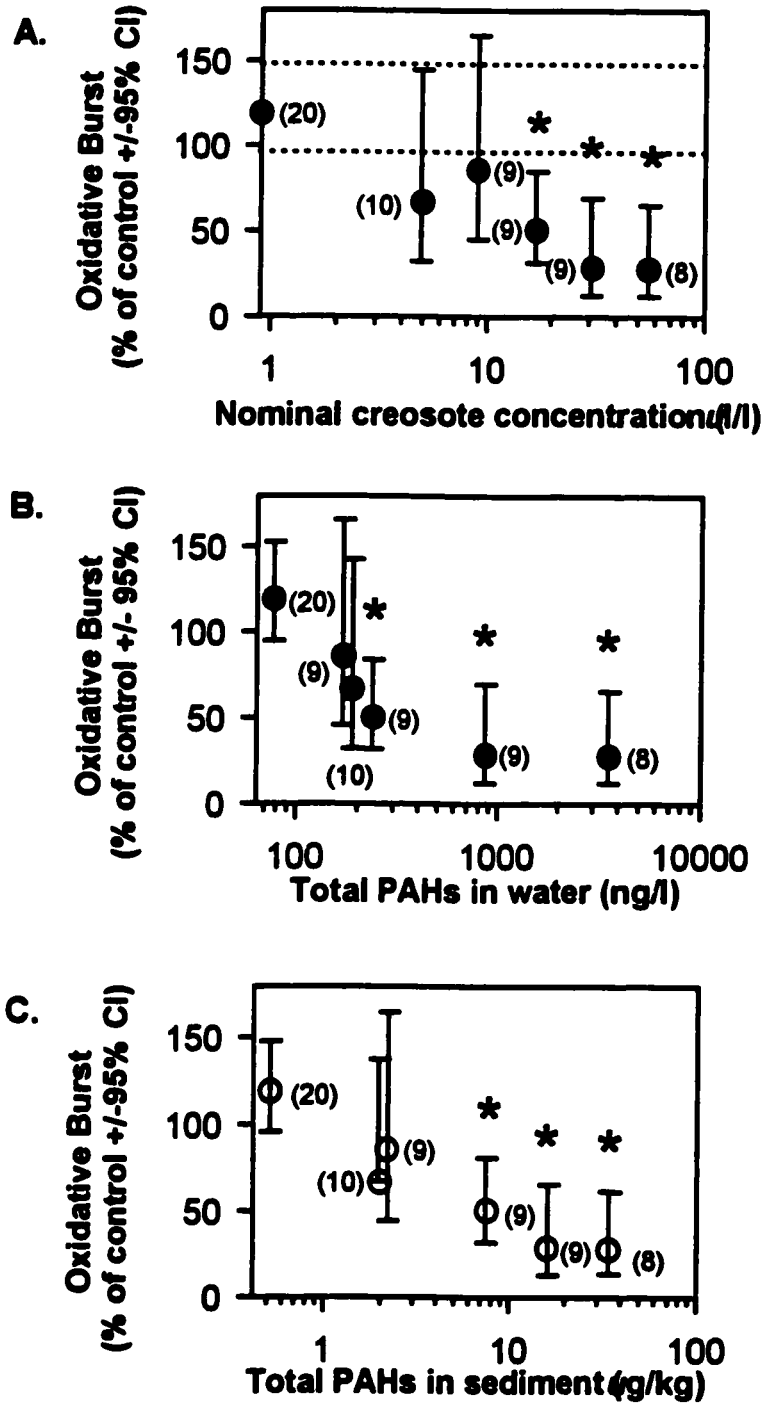

Figure 2.6: Oxidative burst of pronephros leukocytes from rainbow trout exposed in vivo to liquid creosote for 28 d. Dose surrogates include nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on 28, 56, and 84 d of the microcosm study. The net fluorescence for each fish was normalized as a percent of the daily control mean net fluorescence. Results are expressed as mean percent net fluorescence with 95% confidence interval. Means with significant differences from the control mean ($p<0.05$) are indicated by *. Sample size is indicated as (n).

D. Phagocytosis

The phagocytic activity of head kidney leukocytes was influenced by creosote exposure, but the extent depended on the phagocytic parameter under consideration. The number of beads engulfed by phagocytic cells, as measured by the total mean fluorescence, appeared to be slightly stimulated by creosote exposure; however, this response could not be statistically validated. A significant stimulatory trend was determined for the percent of phagocytic cells actively engulfing beads across creosote concentrations. While no one concentration was different from the control group, a significant linear relationship was observed across nominal creosote concentrations $\sqrt{}$ y= log (9.61+0.93x), F-ratio=4.53, p=0.03, R^2=0.07 and sediment total PAH concentrations $\sqrt{}$ y= log(10.17+0.69x), F-ratio 4.45, p=0.04, R^2=0.07. Finally, the phagocytic index, which included both of the above values, increased significantly across creosote concentrations compared to control values (Figure 2.7A-C). This response also followed a significant linear relationship using creosote nominal (log y= log(1.91+0.12x), F-ratio=10.42, p<0.01, R^2=0.12), water total PAH (log y= log(1.84+0.08x), F-ratio=4.92, p=0.03, R^2=0.07), and sediment total PAH concentrations (log y= log (2.00+0.08x), F-ratio=7.03, p=0.01, R^2=0.10). The LOEC was 17 ul/l using nominal creosote concentrations. The phagocytic index was, however, not significantly stimulated at 31 ul/l concentration.

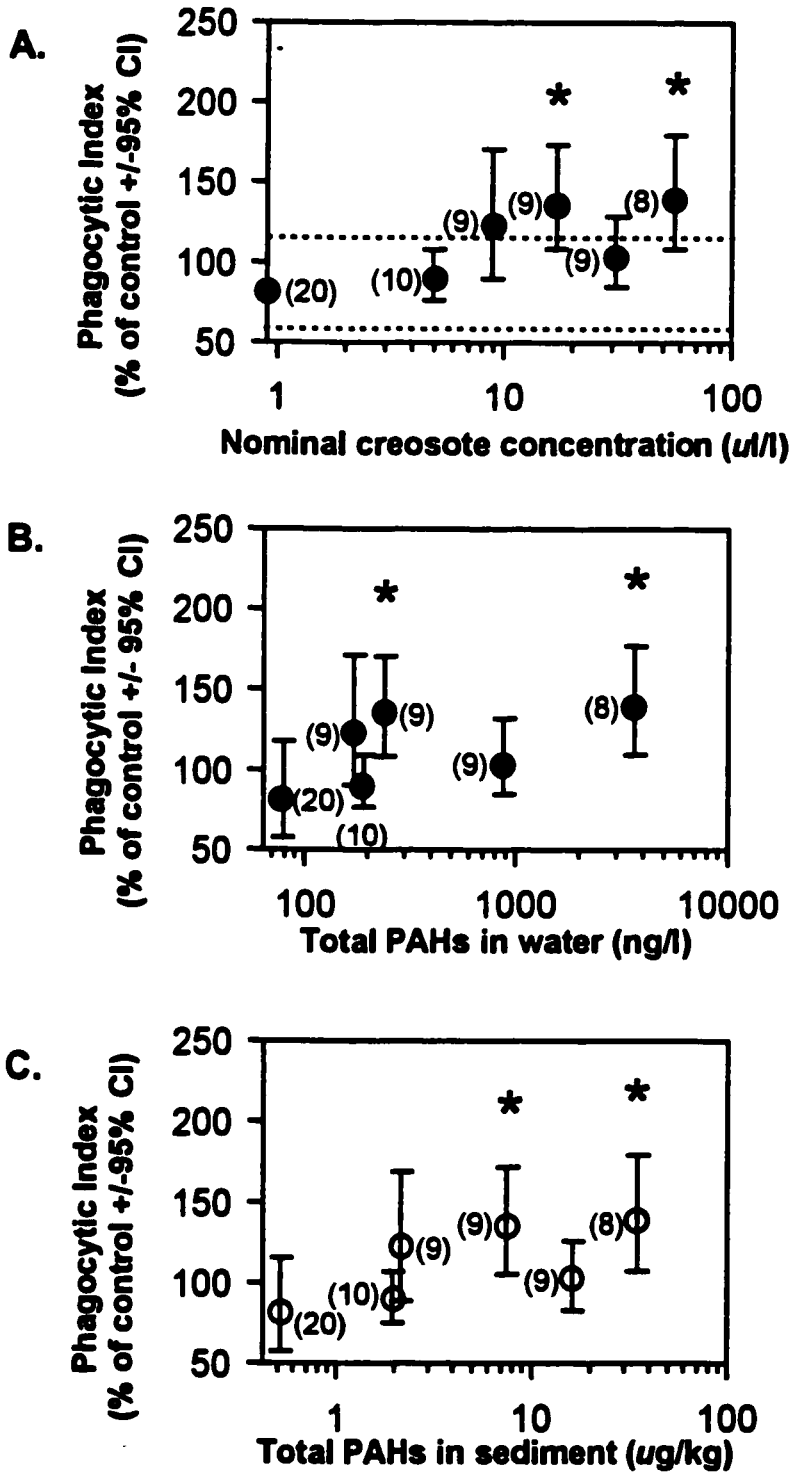

Figure 2.7: Phagocytic index of pronephros leukocytes from rainbow trout exposed in vivo to liquid creosote for 28 d. Dose surrogates include nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on 28, 56, and 84 d of the microcosm study. The phagocytic index for each fish was normalized as a percent of the daily control mean phagocytic index. Results are expressed as mean percent net fluorescence with 95% confidence interval. Means with significant differences from the control mean ($p<0.05$) are indicated by *. Sample size is indicated as (n).

E. Lymphocyte proliferation

Exposure of fish to creosote for 28 days had only a slight effect on the ability of their head kidney lymphocytes to undergo blastogenesis in vitro. In the control group (n=20), the percentages of fish responding positively to ConA, PHA, and LPS were 90, 90 and 95 % respectively. For all the creosote exposed fish (n=45), the percentages of fish responding positively to ConA, PHA, and LPS were 82, 96 and 96 %, respectively. The magnitude of the mitogen stimulation index varied widely between fish, with a range from 2 to 24 fold for the control group and 2 to 20 fold for the creosote-exposed fish. When the incorporation of [H^3]-thymidine into DNA was normalized as a percent of the control change in CPM, the results were not statistically different across creosote concentrations for ConA and PHA treated cultures, but were for LPS. The responses to LPS showed a linear relationship with creosote nominal (log y= log (2.12-0.31x), F-ratio=17.06, $p<0.01$, $R^2=0.23$), water total PAH (log y= log (2.47-0.27x), F-ratio=16.04, $p<0.01$, $R^2=.22$), and sediment PAH concentrations (log y= log (1.93-0.23x), F-ratio=17.02, $p<0.01$, $R^2=0.23$) (Figure 2.8A-C). The reduction in incorporation was only significant at the 56 *ul/l* creosote concentration.

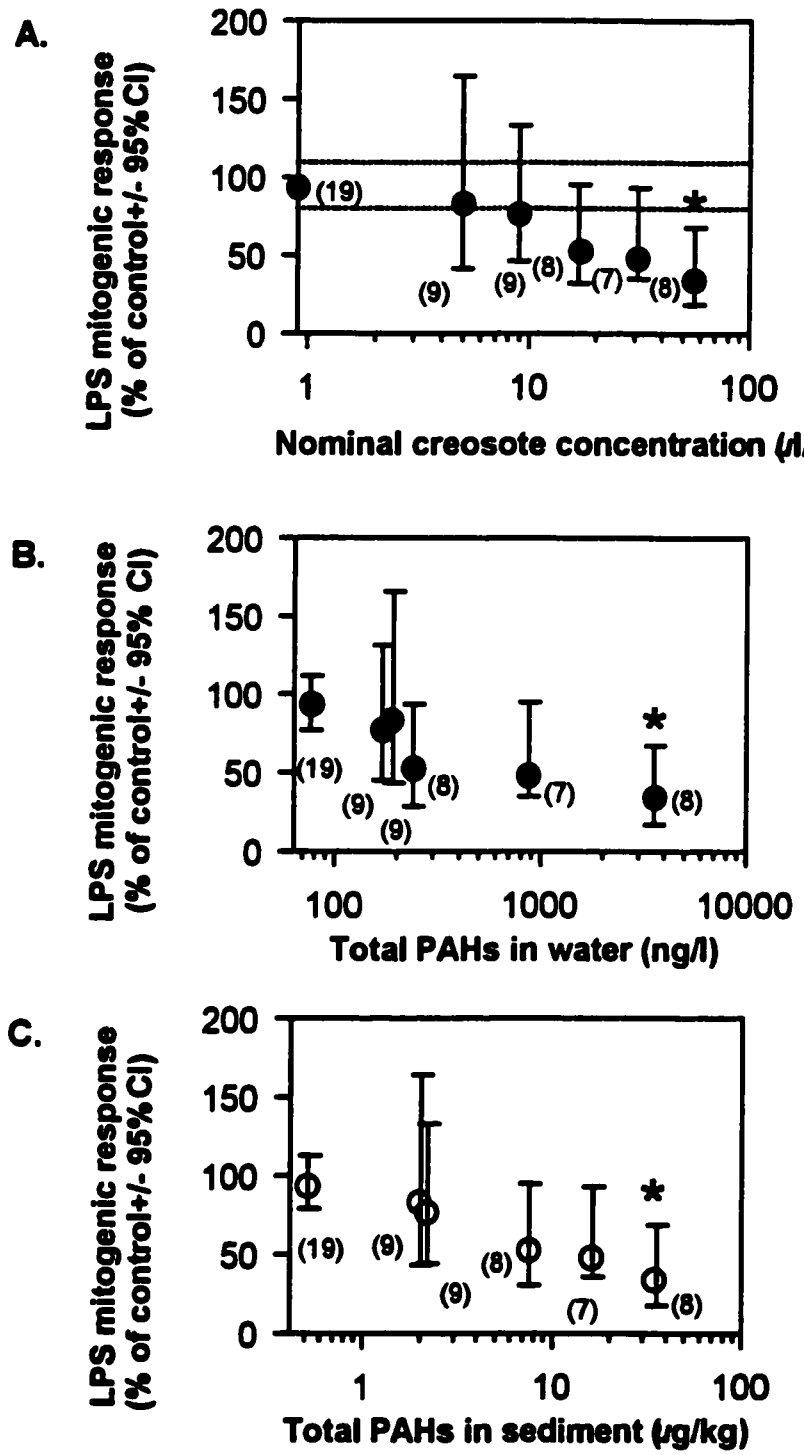

Figure 2.8: Blastogenic response of pronephros leukocytes from rainbow trout exposed in vivo to liquid creosote for 28 d. Dose surrogates include nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on 28, 56, and 84 d of the microcosm study. Blastogenic activity (change in CPM) for each fish was normalized as a percent of the daily control mean change in CPM. Results are expressed as mean percent mitogenic response with 95% confidence interval. Means with significant differences from the control mean ($p<0.05$) are indicated by *. Sample size is indicated as (n).

F. Sig^+ leukocyte marking

Peripheral blood Sig^+ leukocyte counts were reduced in fish exposed to creosote compared to the control fish (Figure 2.9 A-C). This response showed a linear response using creosote nominal concentrations ($\sqrt{y}$=log (10.75-2.52x), F-ratio= 16.39, $p<0.01$, R^2= 0.25), water total PAH concentrations ($\sqrt{y}$= log(13.66-2.20x), F-ratio=15.17, $p<0.01$, R^2=0.24), and sediment total PAH concentrations ($\sqrt{y}$= log (9.28-1.96x), F-ratio=18.34, $p<0.01$, R^2=0.27). The LOEC for reduced percent sig^+ leukocyte was 17 *ul*. Although the percent of sig^+ leukocytes at the 5 and 31 *ul* concentrations was reduced, the response was not statistically significant.

G. Plasma lysozyme activity

Plasma lysozyme concentrations were reduced across creosote concentrations (Figure 2.10A-C). The control mean lysozyme concentration ± SE was 2.0 ± 0.2 *ug*/ml. Lysozyme concentrations were significantly reduced at the 5 and 56 *ul* creosote concentrations showing a linear relationship using creosote nominal concentrations (y=1.73-0.41x, F-ratio=9.19, $p<0.02$, R^2=0.12), total PAH concentrations (y=2.25-0.37x, F-ratio=11.06, $p<0.02$, R^2=0.14), and sediment PAH concentrations (y=1.55-0.26x, F-ratio=14.39, $p<0.01$, R^2=0.16).

H. Plasma cortisol levels

Plasma cortisol levels were not significantly different across creosote concentrations. For all of the fish sampled during the study, the overall mean plasma cortisol concentration was 2.04 ng/ml ±1.95 (mean±SD), which is in the range commonly found for resting rainbow trout (Woodward and Strange, 1987).

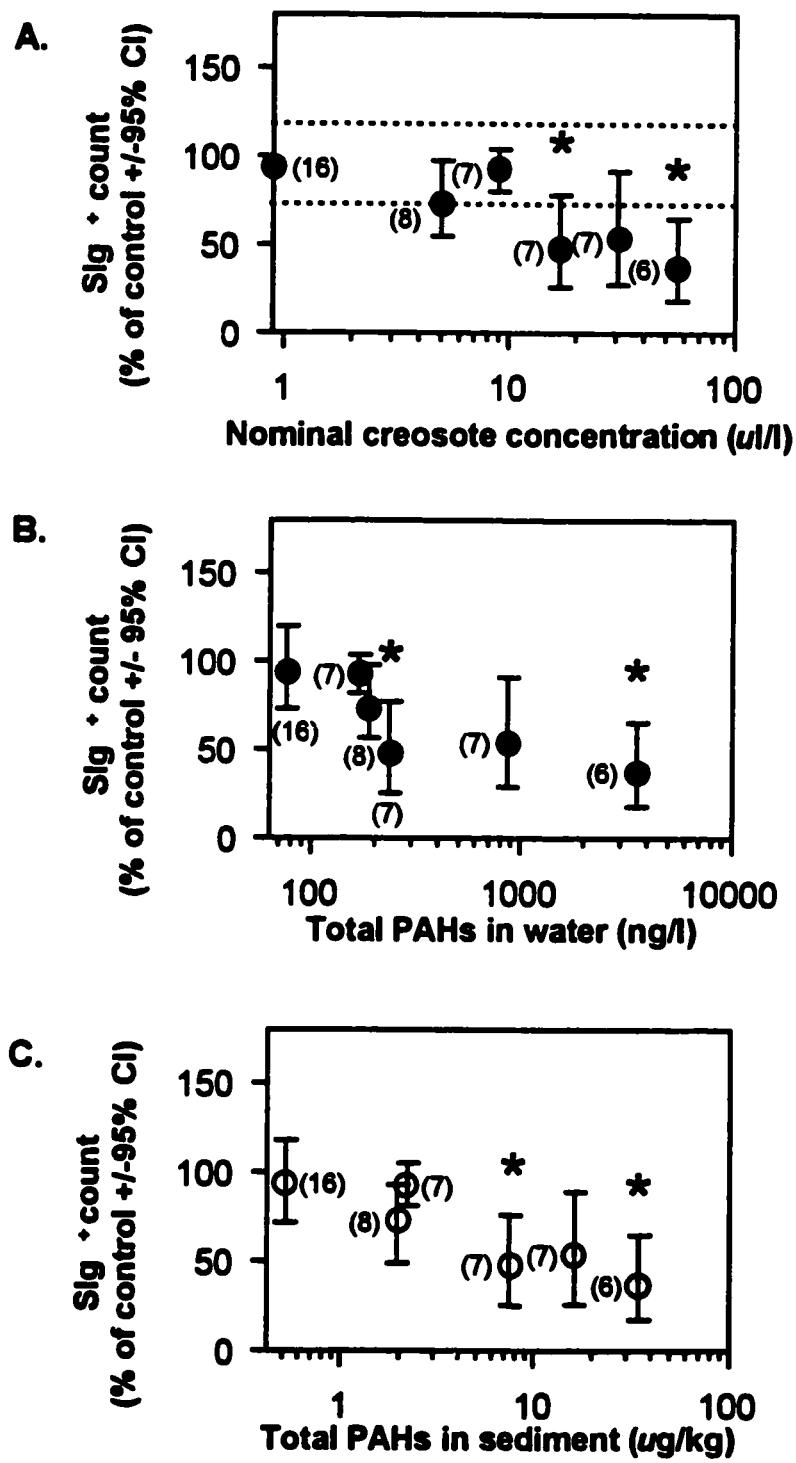

Figure 2.9: Percent of Sig+ peripheral blood lymphocytes from rainbow trout exposed in vivo to liquid creosote for 28 d. Dose surrogates include nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on 28, 56, and 84 d of the microcosm study. Sig+ counts for each fish were normalized as a percent of the daily control mean count. Results are expressed as mean percent count with 95% confidence interval. Means with significant differences from the control mean ($p<0.05$) are indicated by *. Sample size is indicated as (n).

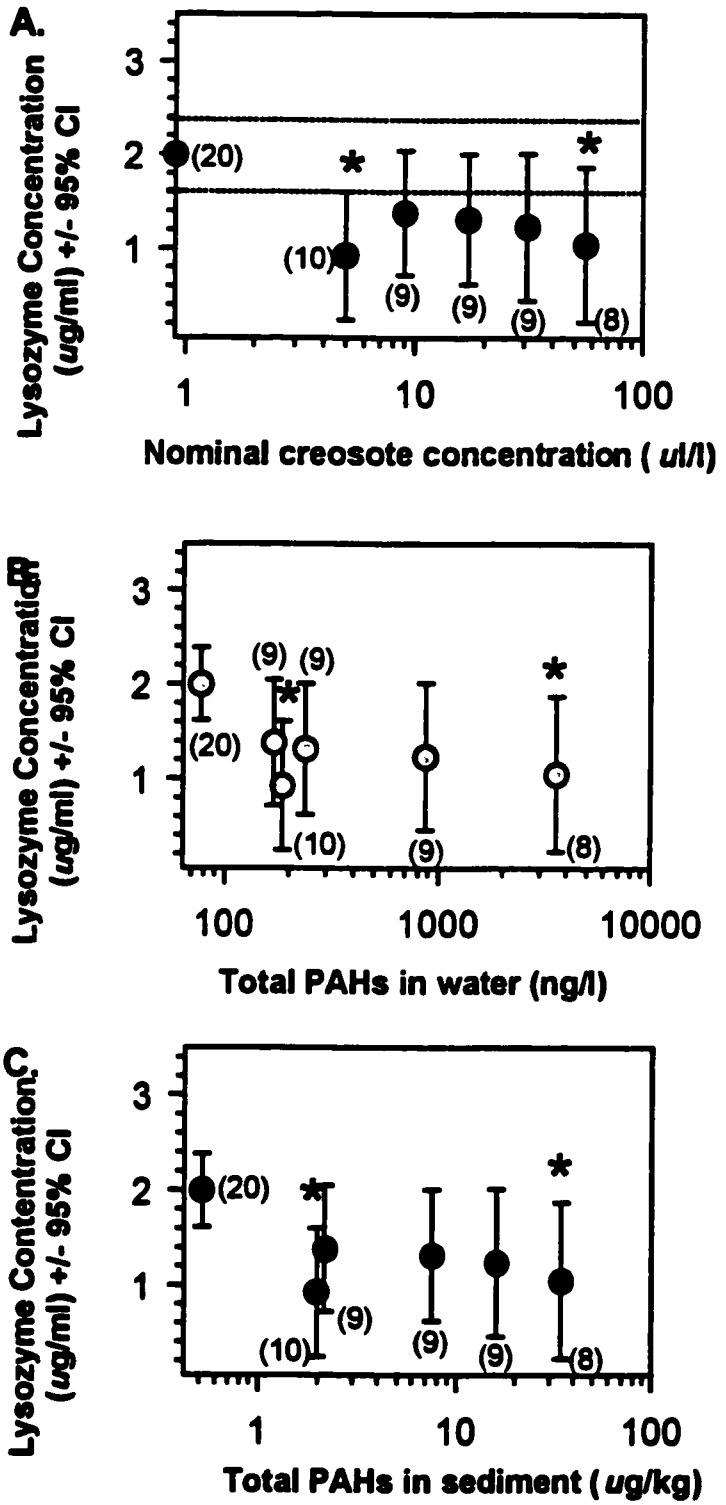

Figure 2.10: Lysozyme in plasma from rainbow trout exposed to liquid creosote for 28 d. Dose surrogates include nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on 28, 56, and 84 d of the microcosm study. Results are expressed as mean ug/ml equivalents of hen egg white lysozyme with 95% confidence interval. Means with significant differences from the control mean ($p<0.05$) are indicated by *. Sample size is indicated as (n).

2.5 DISCUSSION

In this study, immunological techniques were successfully incorporated into a creosote exposure study in order to characterize immunotoxicity to rainbow trout. Suppression of pronephros leukocyte oxidative burst, as measured by intracellular H_2O_2 levels, proved to be a sensitive indicator of exposure to liquid creosote. A concentration-response relationship was evident in the mesocosms after 28 d of exposure using nominal creosote, water total PAH and sediment total PAH concentrations. The LOEC was shown to be 17 *ul*/l using nominal creosote concentrations, representing 611.63 ng/l total PAHs in water.

Reduced pronephros leukocyte oxidative burst, as measured by the reduction of ferricytochrome c, has also been reported in dab (*Limanda limanda*) exposed to sewage sludge containing PAH and hydrocarbon contaminants (Secombes et al., 1997). Splenic macrophage oxidative burst in European sea bass (*Dicentrarchus labrax*) was also reduced 14 h after benzo(a)pyrene injection; however, pronephros macrophage oxidative burst was stimulated (Lemaire-Gony et al., 1995). It was suggested that benzo(a)pyrene oxyradical metabolites may have accounted for the increased H_2O_2 in pronephros macrophages, since the rate of benzo(a)pyrene metabolism was higher in pronephros than in splenic macrophages the day after injection. Exposure duration may also account for the stimulated oxidative burst seen in the pronephros macrophages in this study. One should also note that different techniques used to measure respiratory burst must be taken into consideration when comparing studies. Intracellular H_2O_2 concentrations measured in our work, for example, do not necessarily parallel extracellular H_2O_2 concentrations (Ward, 1992).

Pronephros leukocyte phagocytic activity was also modulated by creosote exposure. A concentration-dependent increase in the phagocytic index was observed across microcosms, with mean values reaching a LOEC around the 17 ul/l nominal creosote concentration, representing 611.63 ng/l total PAHs in water. The increased phagocytic

index is largely due to an increase in the percentage of phagocytic cells rather than enhanced phagocytic activity. The increase in the percent phagocytic cells in the head kidney could have been due to an increase in the number of phagocytic cells, a decrease in other cell types, or a combination of both. Weeks and Warriner (1986) reported suppressed pronephros phagocytic activity in spot (*Leiostomus xanthurus*) and hogchoker (*Trinectes maculatus*) exposed to Elizabeth River sediments which were shown to contain total PAH concentrations as high as 13,000 ug/g. Lemaire-Gony et al. (1995) also reported suppressed splenic macrophage phagocytic activity in European sea bass intraperitoneally dosed with 20 mg/kg benzo(a)pyrene. Pronephros macrophage phagocytic activity appeared slightly enhanced in these fish, although the response was not statistically significant. Increased phagocytic activity has also been reported in American plaice (*Hippoglossoides platessoides*) exposed to sediments contaminated with PAHs, PCBs and PCDFs (Lacroix et al., 1997) and in mammalian studies using DMBA (Dean et al., 1986). In the Dean et al. (1986) study, researchers suggested that resident intraperitoneal macrophages were activated by DMBA.

Macrophages play a key role in regulating teleost immune response through antigen presentation, phagocytosis, and the secretion of cytokines (Verburg-van Kemenade et al., 1995). PAH induced changes in macrophage function could contribute to an altered an immune response. This change may be sufficient to reduce host resistance to clinical disease (Dean et al., 1986). Blanton et al. (1988) reported that decreased IL-1 production by murine spleen macrophages exposed to benzo(a)pyrene resulted in reduced levels of IL-2 production by splenocytes. Ladics et al. (1992) reported that splenic macrophages were target sites of benzo(a)pyrene toxicity resulting in the suppression of splenic humoral immunity. Increased amounts of benzo(a)pyrene metabolites were observed in splenic macrophages but not in neutrophils, T cells, or B cells. It is suspected that reactive benzo(a)pyrene metabolites produced by hepatocytes and macrophages may bind to nucleophilic target sites, impairing the ability of macrophages to respond to an

immunological challenge. Rainbow trout in this study did metabolize PAHs because bile PAH metabolites were elevated in fish from the creosote microcosms (Lewis, 1997). Therefore, the production of PAH metabolites may have contributed to the immune-altering effects of creosote by acting on macrophage function.

The concentration-dependent reduction in the number of B lymphocytes in peripheral blood has several possible explanations. One possible cause is an impairment in B lymphocyte proliferation in the kidney, as the response to LPS by pronephros lymphocytes from creosote-exposed fish was slightly reduced. Another possible explanation is a decrease in the ability of developing B lymphocytes to express surface immunoglobulin IgM. Thirdly, a decrease in the number of peripheral blood B lymphocytes could represent a shift in the leukocyte traffic, resulting from recruitment to other tissues. Namaware and Baker (1996) reported that stress-induced lymphocytopenia may be due to leukocyte trafficking, resulting from changes in the adhesive interaction between white blood cells and various tissue stromata. Finally, these possibilities may be operating in combination to reduce the number of peripheral B lymphocytes in fish from the creosote microcosms.

Creosote exposure appeared to have little effect on lymphocyte blastogenesis, as head kidney leukocyte cultures prepared from control and creosote-exposed fish responded similarly to PHA and Con A, while the response to LPS was only slightly impaired in cultures from creosote-treated fish. In studies by others on several different fish species, the effect of PAH exposure on lymphocyte blastogenesis has varied considerably. Spot pronephros lymphocyte proliferation in response to Con A was significantly inhibited at sites along the Elizabeth River containing high concentrations of benzo(a)pyrene (Faisal and Huggett, 1993). Inhibition of lymphocyte proliferation was reversed in benzo(a)pyrene and benzo(a)pyrene- 7,8 dihydrodiol exposed lymphocytes by α-naphthaflavone, a potent cytochrome P450 inhibitor, suggesting that immunosuppression involved cytochrome P450 dependent metabolic pathways. On the

other hand, Faisal et al. (1991) reported pronephros lymphocyte proliferation in response to LPS was stimulated in spot exposed to Puget Sound PAH-contaminated sediments. These results are in contrast to the results with the English sole. Arkoosh et al. (1996) reported that, in response to Con A, spleen leukocytes from English Sole at sites along the Elizabeth River showed increased proliferation, whereas the LPS induced proliferation of splenocytes from the English sole was not affected during laboratory exposure of the fish to Puget Sound PAH-contaminated sediments. From these results and the current study, the value of lymphocyte blastogenesis in assessing the risk of creosote exposure to the immune system of fish appears to be questionable. Part of the problem appears to be due to the variability associated with the lymphocyte mitogenic assay, which might be overcome in the future by improvements to the assay. As well, an effect on lymphocyte proliferation might be dependent on route and duration of PAH exposure (Arkoosh et al., 1996).

Plasma lysozyme activity was also suppressed after 28 d of exposure to creosote. A significant reduction at the 5 *u*l concentration may be due to elevated levels of PAHs at this concentration. While a concentration-response to creosote was evident, plasma lysozyme activity did not appear to be as sensitive to the toxic response as respiratory burst. Lysozyme activity has been used previously in fish studies as an indicator of exposure to various organic pollutants. Secombes et al. (1991) reported that dab serum lysozyme levels were not affected by exposure to sewage sludge for 12 weeks; however, pronephros oxygen free radical production was reduced. More recently, Secombes et al. (1997) showed elevated lysozyme levels in plaice caught along a sewage sludge gradient. In contrast, they found that dab exposed to oil-contaminated sediments for 2-4 weeks had decreased serum lysozyme activity. Tahir and Secombes (1995) also reported suppressed lysozyme levels after 6 weeks in rainbow trout injected with 0.6 ml/kg oil-based drilling mud extract. An eight week time trial using 2.4 ml/kg of the same extract also appeared to reduce serum lysozyme levels. This trend, however,

was not statistically validated. Lysozyme has been shown to specifically cleave peptidoglycans forming the cell wall of gram positive bacteria, resulting in osmolysis. It has also been suggested that lysozyme may also act as an opsonin for phagocytic activity (Ellis, 1990). The reduction in lysozyme levels observed in this creosote study, indicate that these fish may be at a higher risk of developing bacterial infections.

Harper et al. (1996) reported that immunosuppression resulting from PAH exposure was primarily associated with compounds containing four or more benzene rings. Although the majority of the waterborne PAHs measured in this creosote study also contain four or more rings, only pyrene and fluoranthene were shown to exhibit a significant linear relationship with nominal creosote concentrations using current detection limits. This may implicate them as the primary immunomodulating agents found in creosote. A net toxic response, however, may be due to the combined effects of all PAHs, as well as other chemicals, found in creosote. Water and sediment PAH concentrations indicated that the PAHs partitioned rapidly into the sediment and that the microcosms were at equilibrium prior to the rainbow trout study. This more accurately reflects an exposure scenario found in the natural environment than is typically seen in laboratory-based exposure studies.

The physical profiles of the microcosm study also modelled realistic conditions that are generally controlled for in laboratory-based studies. This is illustrated by the rapid decline in temperature that was observed throughout the study and that is characteristic to much of Canada in the fall. Low environmental temperatures are known to induce immunosuppression in fish (Le Morvan-Rocher et al. 1995), and may have enhanced the changes in immunological parameters of fish exposed to creosote compared to control fish. Moreover, Ottinger and Kaattari (1997) reported that rainbow trout leukocytes were more sensitive to aflatoxin B1 induced immunosuppression at this time of year compared to studies that were conducted during the spring. Temperature and photoperiod are both

controlled in laboratory-based studies but are an integrated part of microcosm and field studies, and may contribute to the overall toxicity of a compound(s).

2.6 CONCLUSION

Rainbow trout immune parameters were modified by environmentally realistic concentrations of liquid creosote, with both stimulatory and suppressive effects being observed. Although some endpoints appeared more sensitive than others, concentration-response relationships were observed for pronephros leukocyte respiratory burst, phagocytic index, and lymphocyte proliferation to LPS, as well as for peripheral blood B-cell marking and lysozyme activity. These correlations may be due to the complex integration of the immune system as a whole. Changes in one branch of the immune system are often accompanied by alterations in another (Tahir and Secombes, 1995). Although the underlying mechanism(s) of action for PAH immunotoxicity are unclear to date, the results from this study clearly indicate that environmental concentrations of PAHs can impair fish immune parameters, possibly to a degree where resistance to disease is compromised.

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CHAPTER 3

EFFECT OF CREOSOTE EXPOSURE TIME ON RAINBOW TROUT (*Oncorhynchus mykiss*) IMMUNE PARAMETERS: A MICROCOSM STUDY

3.1 ABSTRACT

An outdoor microcosm study was conducted to re-evaluate the immunotoxic effects of liquid creosote to rainbow trout (*Oncorhynchus mykiss*). During this study, two separate experiments were conducted. The first experiment was designed to monitor the kinetic effects of exposure to creosote; caged fish were sampled on days 7, 14, 21, and 28 from microcosms initially dosed with 0, 3, and 10 *u*l/l creosote. A second experiment was designed to monitor immune parameters after 37 d of exposure in microcosms initially dosed with 0.3, 1, 3, 10, and 30 *u*l/l creosote. Two replicate control microcosms were included in the 37 d exposure to evaluate variability across microcosms. Pronephros leukocytes were monitored in both experiments for phagocytic activity, oxidative burst, and surface immunoglobulin-positive (Sig^+) B cell counts. Serum lysozyme activity was also measured for the 28 d kinetic experiment. During the kinetic study, oxidative burst was progressively inhibited in fish exposed to 3 and 10 *u*l/l creosote, returning to control levels by day 28. Phagocytic activity was initially stimulated after seven days of exposure, returning to control levels by day 28. Although control Sig^+ B cell counts were quite variable across sampling days, Sig^+ B cell counts were also elevated after seven days of exposure in creosote exposed fish. These cell numbers decreased significantly during the remainder of the study. Lysozyme activity appeared to be unaffected by creosote exposure. After the 37 d of exposure, oxidative burst was suppressed in creosote exposed fish; phagocytic activity was enhanced. No detectable change was observed for Sig^+ B cell counts. The overall results confirm that creosote has the potential to alter certain measured immune parameters. Modulation of the measured immune parameters varied with the duration of exposure. Polycyclic

aromatic hydrocarbons (PAHs), a major constituent of liquid creosote, are the suspected immune- altering agents.

Key words: creosote, (*Oncorhynchus mykiss*); immunotoxicity; kinetics, microcosm

3.2 INTRODUCTION

Previously, we reported that certain immune parameters were affected in rainbow trout (*Oncorhynchus mykiss*) after 28 d of exposure in creosote-treated microcosms. Liquid creosote, a coal tar distillate that has been used extensively as a wood preservative for railway ties and marine pilings, was used to treat the water in this study. Since seventeen polycyclic aromatic hydrocarbons (PAHs) account for 63% of the volume of liquid creosote (CEPA, 1994), it is likely that a number of these compounds contribute to the immunomodulating potency of liquid creosote. Evidence of altered immune function in fish exposed to PAHs in water and contaminated sediment is extensive (Weeks and Warriner, 1986; Payne and Fancey, 1989; Secombes et al., 1991; Arkoosh et al., 1994; Lemaire-Gony et al., 1995; Arkoosh et al., 1996). Faisal and Huggett (1993) reported suppressed lymphocyte proliferation in Spot (*Leiostomus xanthurus*) sampled from a site along the Elizabeth River, Virginia, having total sediment PAH concentrations as high as 570 mg/kg dry weight. It is speculated that exposure to certain PAHs may predispose fish to clinical diseases (Weeks and Warriner, 1986; Dunier and Siwicki, 1993).

The advantage of using microcosm studies to generate immunotoxicological data is that they allow researchers to identify toxicological endpoints under more realistic conditions (Thompson et al., 1993). They also provide an opportunity to investigate stressor effects at the population and/or community level which may not be apparent in single-species laboratory studies (Liber et al.,1992). To date, concentration-response and kinetic relationships have not been clearly demonstrated, and need to be explored in more detail in order for immunotoxicology to be incorporated into an environmental risk assessment process. Once established, the ecological relevance of these relationships can be explored by linking them to responses at higher levels of biological organization.

In this manuscript, a second microcosm study was conducted during the following year to re-evaluate the previously published immunotoxic effects of liquid creosote to the rainbow trout immune system (Karrow et al., 1999). During this study, two separate experiments were conducted. The first experiment was designed to monitor the kinetics of certain fish immune parameters during a 28 d creosote exposure by sampling caged fish from the microcosms on 7, 14, 21, and 28 d of the experiment. A second experiment was designed to monitor fish immune parameters after 37 d of exposure in creosote-treated microcosms. Pronephros leukocytes were monitored in both experiments for phagocytic activity, oxidative burst, and surface immunoglobulin-positive (SIg^+) B cell counts. Serum lysozyme activity was also measured during the kinetic study. Phagocytic cells and B lymphocytes are known to play a key role in regulating teleost immune response through phagocytosis/endocytosis, antigen processing and presentation, and the secretion of various cytokines (Verburg-van Kemenade et al., 1995). Phagocytic cells also exhibit oxidative burst, a process by which antibacterial oxygen radicals are produced. Following antigenic stimulation, B lymphocytes differentiate into plasma cells, the main effector cells in humoral defense. Lysozyme may act as an opsonin for phagocytic activity, and has been shown to specifically cleave peptidoglycans forming the cell wall of gram positive bacteria, resulting in osmolysis (Ellis, 1990).

3.3 MATERIALS AND METHODS

A. Microcosm

Details of the microcosm construction are outlined in Karrow et al. (1999). The microcosms were filled with 12 000 l of water from a well-fed irrigation pond and circulated for 21-28 d prior to dosing to achieve homogeneity. Dosing of the microcosms for the 28 d kinetic experiment was achieved by a series of 10 sub-surface injections, at four day intervals, into a stream of water such that the creosote nominal concentrations after the last injection were 0, 3, and 10 *u*l/l. Fish were not added to these microcosms until 80 d post dosing (October 10) to ensure sublethal exposure, steady state conditions, and an optimal temperature profile for rainbow trout (Fig 3.1A).

Dosing of the microcosms for the 37 d experiment was achieved by a single sub-surface injection into a stream of water, such that the initial creosote concentrations were 0.3, 1, 3, 10, and 30 *u*l/l. Dosing of the microcosms was based on a series of graded creosote doses with no replication, commonly referred to as a regression approach. Liber et al. (1992) and Thompson et al. (1993) have discussed the advantages and disadvantages of this experimental design. When compared to an ANOVA design, the regression approach offers both economic advantages, and the ability to interpolate within the range of concentrations.

Two replicate controls were also included in the 37 d experiment to evaluate variability between microcosms. The microcosms remained static throughout the duration of the study, and were exposed to natural sunlight and precipitation. Fish were not added to these microcosms until 74 d post dosing (October 4) for the reasons described above (Fig. 3.1B). Ambient light, with a photoperiod of approximately 10 h light: 14 h dark was used throughout the study.

B. Experiment

Female rainbow trout (≅100 g) were obtained in early September from Rainbow Springs Hatchery, Thamesford, ON. The fish were acclimated for 14 d in the irrigation pond at the microcosm site prior to exposure. Fish (n=120) for the 28 d kinetic experiment were allocated to each of the three microcosms on day 80 after creosote dosing ceased. A total of 40 fish were exposed in each microcosm however, fish (n=10) were divided into four separate cages to facilitate sampling on 7, 14, 21 and 28 d (Fig. 3.1A). The floating cages, measuring 60 cm long, 60 cm wide, and 60 cm deep, were constructed from nylon netting (mesh size 0.8 cm) supported by a wood frame. All fish were fed commercial trout chow at a ration of 2 % body weight/d. On the respective sampling days, one cage was removed from each concentration, then replaced by a new cage of fish to ensure that the biomass remained constant within each microcosm throughout the duration of the study. The distance between cages within each microcosm was sufficient to prevent stress to the remaining caged fish during sampling. After removal, fish were immediately anaesthetized with methane tricainesulfonate (MS-222), weighed and sampled for peripheral blood from the caudal vein using non-heparinized vacutainers. After bleeding, fish were euthanized by cervical dislocation and dissected to obtain samples of pronephros tissue. The preparation of pronephros leukocyte suspensions and serum was previously reported by Karrow et al. (1999). Briefly, peripheral blood was centrifuged, and aliquots of serum were frozen at -20°C for lysozyme analysis. Single cell suspensions of leukocytes were prepared by pressing pronephros tissue through a 100 *um* stainless steel mesh with the flat end of a syringe. The leukocyte suspension was then diluted, and centrifuged through a Histopaque-1077 (d=1.077, Sigma Chemical Company, St Louis, MO, U.S.A) density gradient. Leukocytes were collected at the interface, washed thrice, and resuspended in $NaHCO_3$ free RPMI 1640 medium + 25 *uM* HEPES + L-glutamine (pH 7.4, Gibco, Burlington, ON, Canada) supplemented with 10 % fetal calf serum (Gibco), 50 U/ml penicillin, and 50 *ug*/ml streptomycin (Gibco). Total leukocyte count and

viability was determined by trypan blue exclusion. Immune assays were initiated within 24 h of sampling, as suggested by Rice et al. (1996), to reduce the time constraints associated with incorporating immunological assays into field studies.

Fish (n= 112) for the 37 d experiment were weighed, tagged and allocated to each of the seven microcosms on days 74 and 77 after creosote dosing. Initial mean ± SD weights were 125.54 +/- 21.05 g. A total of 16 fish were exposed in each microcosm. Initiation of exposure was staggered over three days by adding a cage containing eight fish to each microcosm on the first (cage replicate one) and third (cage replicate two) days of the study (Fig. 3.1B). Staggering the exposure was necessary, since it was not logistically possible to sample all of the fish on one day. Six fish were sampled from each of the seven treatments on each of two replicate sampling days (12 fish in total per concentration). The cages, previously described, were partitioned into four separate compartments with aged plywood, such that each cage contained eight fish. Fish were fed as described above. Temperature, pH, dissolved oxygen, and alkalinity were routinely monitored during the acclimation period and throughout the duration of the study. After the 37 d of exposure, one cage was removed from each concentration on days 111 and 114 post dosing. The sampled fish were immediately anaesthetized with MS-222, and sampled for peripheral blood and pronephros tissue.

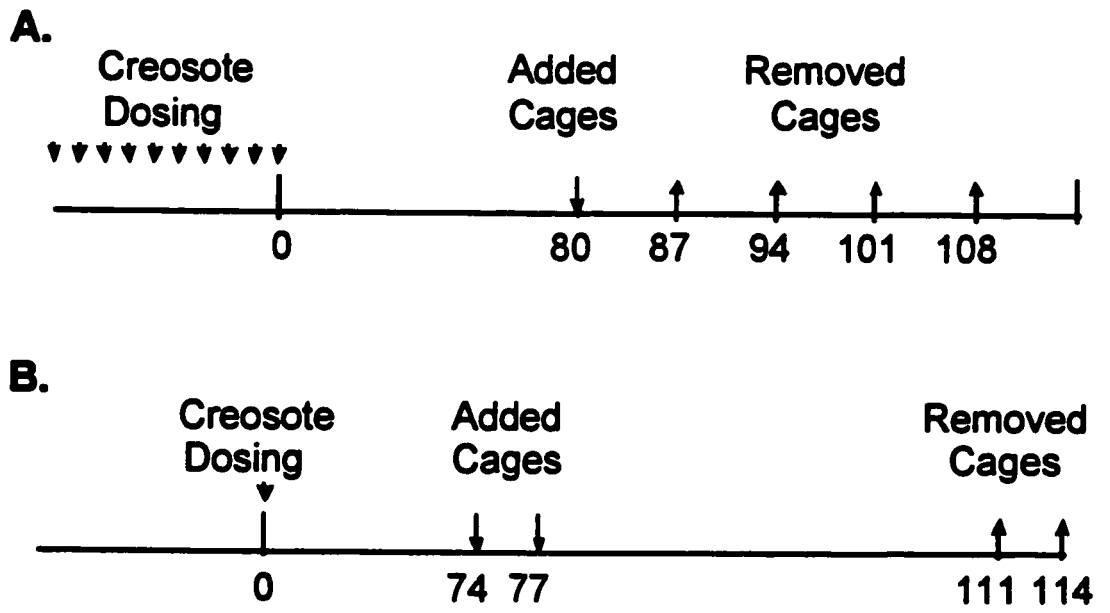

Figure 3.1. Temporal design of the two creosote immunotoxicity experiments. The microcosms for the 28 d kinetic exposure (A) were dosed with a series of 10 sub-surface injections at four day intervals. Caged trout were added to the microcosms 80 d after dosing and removed on days 87, 94, 101, and 108 post dosing. Microcosms for the 37 d exposure were dosed with a single sub-surface injection (B). Fish cages were added on days 74, and 77 post dosing then removed on days 111 and 114, respectively.

C. Pronephros leukocyte oxidative burst

The methodology for measuring leukocyte oxidative burst by flow cytometry (Coulter EPICS XL-MCL) was previously reported by Karrow et al. (1999). Briefly, cell suspensions were adjusted to 10^6 cells/ml, incubated with 4 uM of 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes Inc., Eugene, OR, U.S.A) for 15 min in the dark at 18°C, then activated with phorbol myristate acetate (PMA) (Molecular Probes Inc., Eugene, OR, U.S.A) at a final concentration of 100 ng/ml. The release of hydrogen peroxide (H_2O_2) within the cells oxidizes DCFH to 2',7'-dichlorofluorescin (DCF) which fluoresces at 530 nm. The net fluorescence (NF_{530}) is proportional to the net amount of H_2O_2 generated over a given time (60 min).

Two distinct leukocyte populations exhibiting oxidative burst were detected from pronephros tissue samples using flow cytometry. In order to derive the net fluorescence intensity, a gate was drawn around the larger more granular cells, which represented the major leukocyte population. This population was assumed to be representative of the residing macrophage population within the pronephros as they are the most predominant phagocytic leukocyte population (Manning, 1994).

D. Pronephros leukocyte phagocytosis

The methodology for measuring leukocyte phagocytic activity by flow cytometry was previously reported by Karrow et al. (1999). Briefly, leukocyte suspensions (1 x 10^6 cells/ml) were incubated with 10^8 fluorescent latex beads (1.03 *u*M diameter, Molecular Probes) for 18 h at 20°C. Negative controls were incubated in PBS with 1 % paraformaldehyde. Cells were washed by density gradient centrifugation through a mixture of 3 % BSA (GIBCO) and RPMI 1640 medium then analyzed by flow cytometry to determine the percent of cells engulfing three or more beads within a gated region drawn around the macrophage population. The mean fluorescence, which is a measure of the total bead uptake per cell; and the phagocytic index (I), where I is equal to the mean fluorescence times the number of cells engulfing three or more beads in 10,000 cells were also determined.

E. Pronephros immunoglobulin-positive (sIg$^+$) leukocyte marking

SIg$^+$ leukocyte marking was previously reported by Karrow et al. (1999). Pronephros leukocytes (10^6 cells/ml) were incubated with 100 *u*l of ascites of the monoclonal antibody mouse-anti-trout 1-14 (1:100), a known B cell marker, of De Luca et al. (1983) (Courtesy of N.W. Miller) or 100 *u*l RPMI in 1 ml of RPMI 1640 medium for 45 min on ice. Cells were washed then labelled with goat-anti-mouse FITC (1:100, GIBCO). After another series of washes, the number of surface immunoglobulin-positive (sIg$^+$) leukocytes in 10,000 cells was then determined using flow cytometry by counting fluorescing cells within a gated

region drawn around the less granular and fluorescent population of cells to exclude phagocytic cells.

F. Serum lysozyme activity

Lysozyme activity was only measured in samples from the 28 d kinetic experiment. The 38 d exposure samples were damaged during storage. Measurement of lysozyme activity was reported by Karrow et al. (1999). The assay measures a lysozyme-induced decrease in optical density of a 1.25 mg/ml (*Micrococcus lysideikticus*) (Sigma) PBS (pH 7.5) suspension at 410 nm. Optical densities were measured over a 10 min incubation period using a Microplate EL311 autoreader, after the addition of 10 *ul* of fish plasma. The slope of the change in optical density was then converted to hen egg white lysozyme equivalents using lyophilized hen egg white lysozyme (Sigma) as a standard.

G. PAH analysis

Total water and sediment PAH concentrations up to 56 d were provided by Bestari et al. (1998a). Extraction and analysis of water samples for PAHs is described in detail by Bestari et al. (1998b). Grab samples (1l) of water were taken from each microcosm on 84 d and 80 d for the 37 d and 28 d time study respectively, then preserved with 80 g/l sodium thiosulphate. PAH analytes were liquid extracted into HPLC grade dichloromethane (DCM) and dried with an excess of anhydrous sodium sulphate (Sigma). Samples were concentrated under vacuum and resuspended in 2 ml of iso-octane. High-performance liquid chromatography analysis was conducted on the extracts using a Supelcosil LC-PAH reverse phase column (5 *um*, 4.6 mm X 25 cm)(Sigma-Aldrich, Mississuaga, ON) with a C_{18} Supelguard LC-PAH precolumn guard in a Shimadzu LC-10AD liquid chromatography solvent delivery module. The module was equipped with SIL-10A autoinjector, SCL-10A system controller, four-channel membrane degassing unit, RF-10A spectrofluorometric detector, and EZCHROM chromatographic data system. An acetonitrite/water mobile carrier was used to establish a 45 min solvent gradient, with an additional 15 min allotted to reach the initial gradient. The gradient programming was as

follows: Initial acetonitrile:water (40:60) ran for 5 min at a flow rate of 1.5 ml/min. The acetonitrile concentration was then gradually increased to 100% over 30 min, then maintained at the highest concentration for 15 min. Variable excitation and emission wavelength programming was utilized in order to maximize detection of the individual PAHs. Extract injection volumes were 2- 10 *ul*.

H. Statistical analysis

A two factorial ANOVA (factors=day, concentration) was used to analyse the data from the 28 d kinetic experiment. Tukey's post Hoc test was used to make pairwise comparisons of the treatment groups at each sampling time, and the sampling times within each treatment group. Regression analysis was used to identify both linear and quadratic trends over the duration of the study at each concentration. Regression slopes from the 3 and 10 *ul*/l concentrations were compared to the regression slope from the control microcosm according to Sokal and Rohlf (1981). All data were tested for compliance to the assumptions of normality and variance homogeneity. Data sets, which violated these assumptions, were transformed using a log transformation. Significance was determined at $p \leq 0.05$.

A factorial analysis of variance (ANOVA) (factor=concentration), with two staggered cage replicates per concentration and sub-sampling (fish within cages), was used to analyse the data from the 37 d experiment. The effect of staggered cages was controlled, by blocking the cage replicates to identify linear and quadratic trends over the range of concentrations (SAS). Dunnett's test for comparisons was used to detect significant differences across concentrations and to derive the lowest-observed-effect (LOEC).

3.4 RESULTS

A. Water chemistry and physical profiles

The mean ± SD temperature, dissolved oxygen, pH, and alkalinity for the water source (irrigation pond) during the 2 wk acclimation period were; 15 ± 3 °C, 9.1± 0.3 mg/l, 8.4 ± 0.4, and 118 ± 21mg/l respectively. A temperature decrease of 14 °C was observed over the duration of the study (Fig. 3.2A). On the last day of sampling, the mean ± SD dissolved oxygen concentration for the 37 d study microcosms was 9.3 ± 0.1 mg/l. Dissolved oxygen concentrations in the 0, 3, 10 *u*l creosote microcosms for day 28 of the kinetic experiment were; 8.3, 9.4, and 7.2 mg/l respectively. The pH profile in Fig. 3.2B was variable but no pattern was observed across concentrations. The mean ± SD pH was 7.9 ± 0.4; alkalinity was 87.5 ± 26.5 mg/l $CaCO_3$, and total kjeldah nitrogen was 0.46 ± 0.07 mg/l.

Total water and sediment PAH concentrations, up to 56 d after microcosms were dosed with creosote, were measured in both experiments (Fig. 3.3A-B, 3.4A-B). Total PAH concentrations were not corrected for the 80% extraction recovery. While numerous PAHs were detected in the water, phenanthrene, acenaphthene, fluoranthene, fluorene, and pyrene all were shown to increase across nominal creosote concentration on day 80 of the 28 d kinetic experiment (Table 3.1). Acenaphthene and fluoranthene were the only waterborne PAHs to exhibit significant linear relationships with the nominal creosote concentrations on day 84 of the 37 d experiment (Table 3.1). Fluorene and pyrene were also detected in the higher creosote concentrations, though the relationship with nominal creosote concentration was not significant.

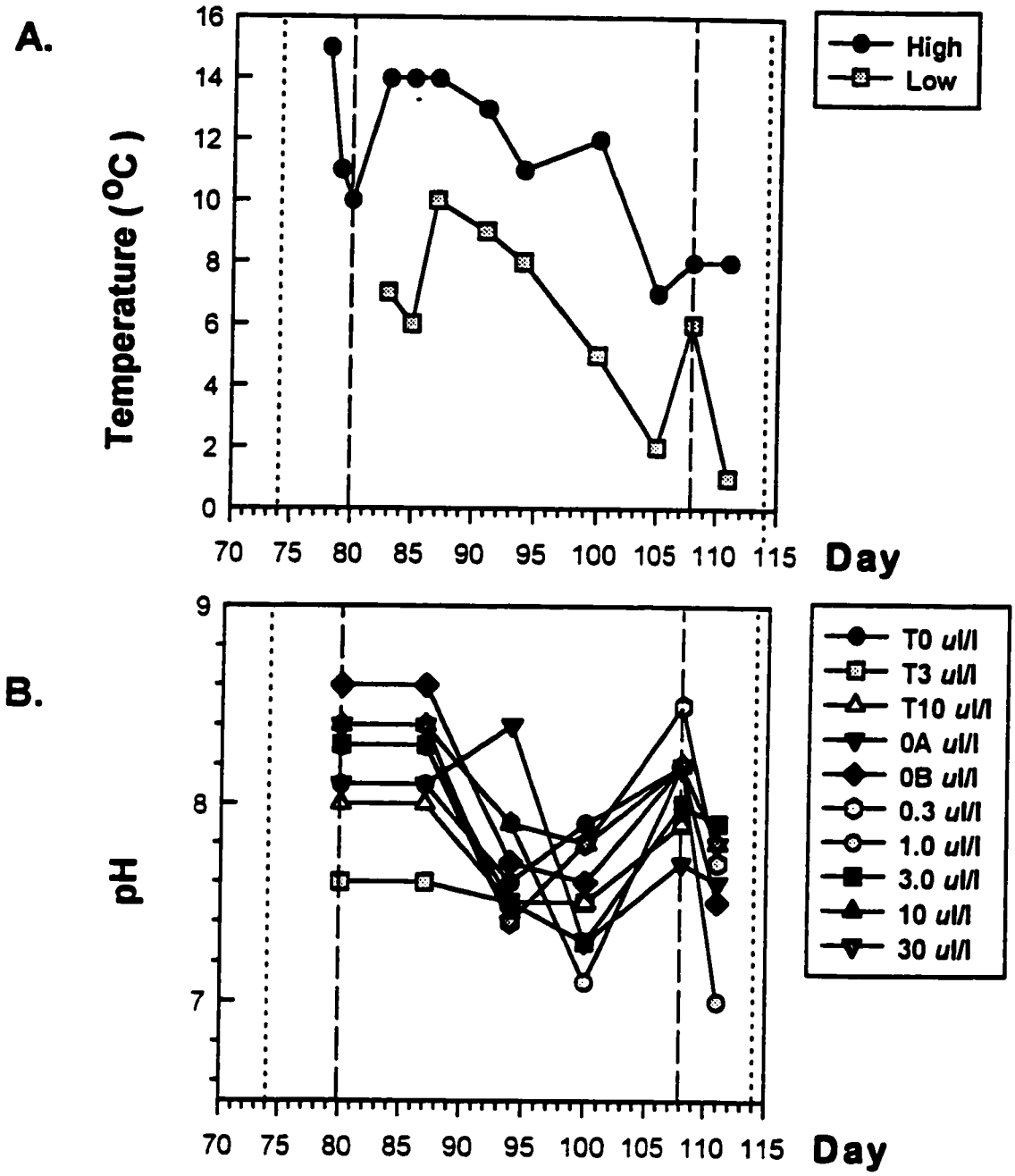

Figure 3.2: Daily high and low temperature (A), and pH (B) profiles for controls and creosote-treated microcosms during the 37 d (fine dotted lines) and 28 d kinetic experiments (course dotted lines).

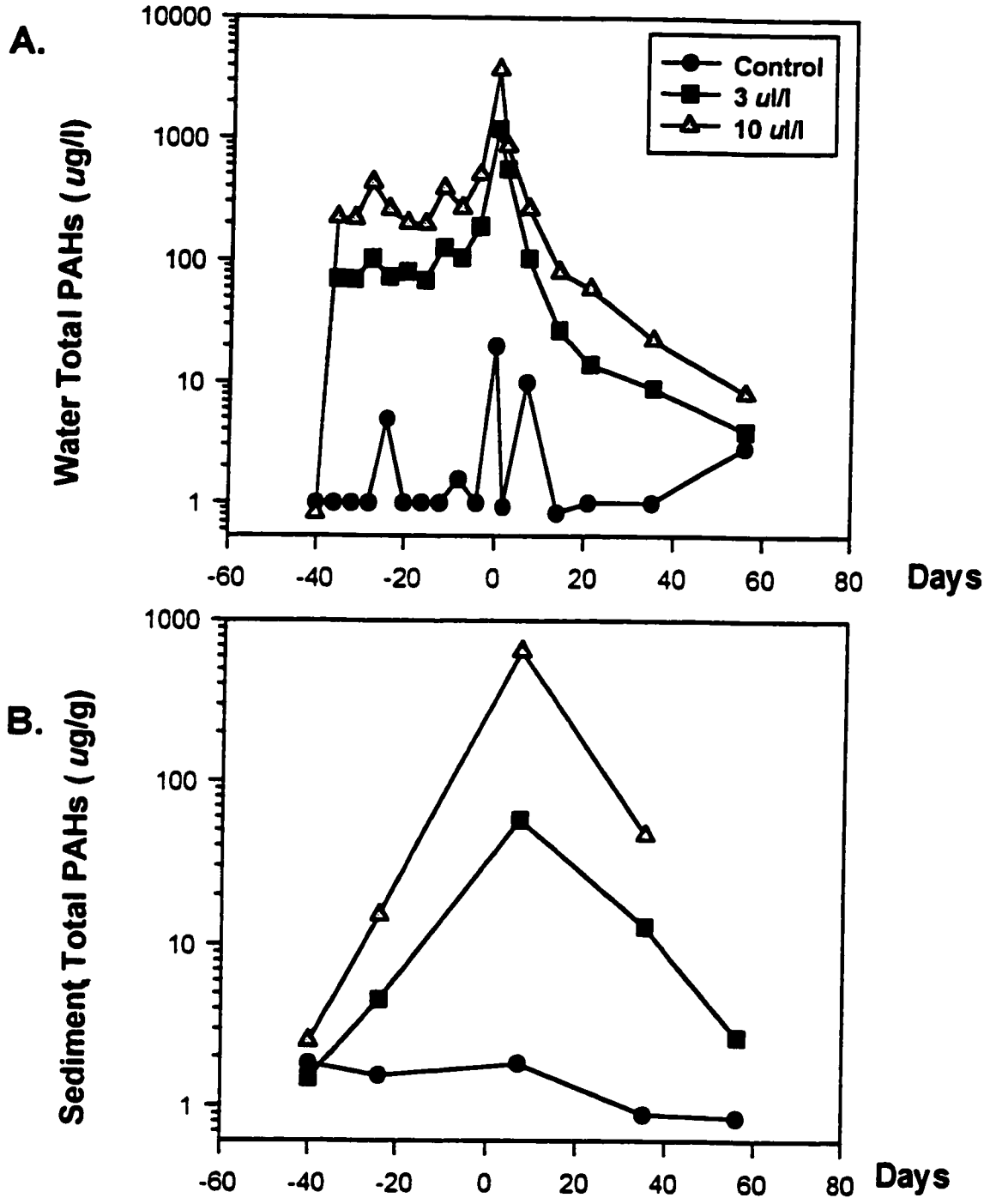

Figure 3.3: Change in total PAH concentrations in the water (A), and sediment (B) during and after repeated dosing of microcosms with creosote for the 28 d kinetic experiment. The first sediment sample was taken on the first day of dosing; the first water sample, within minutes of dosing. Fish were added to the microcosms 80 days after dosing. Day 0 represents the last day of dosing.

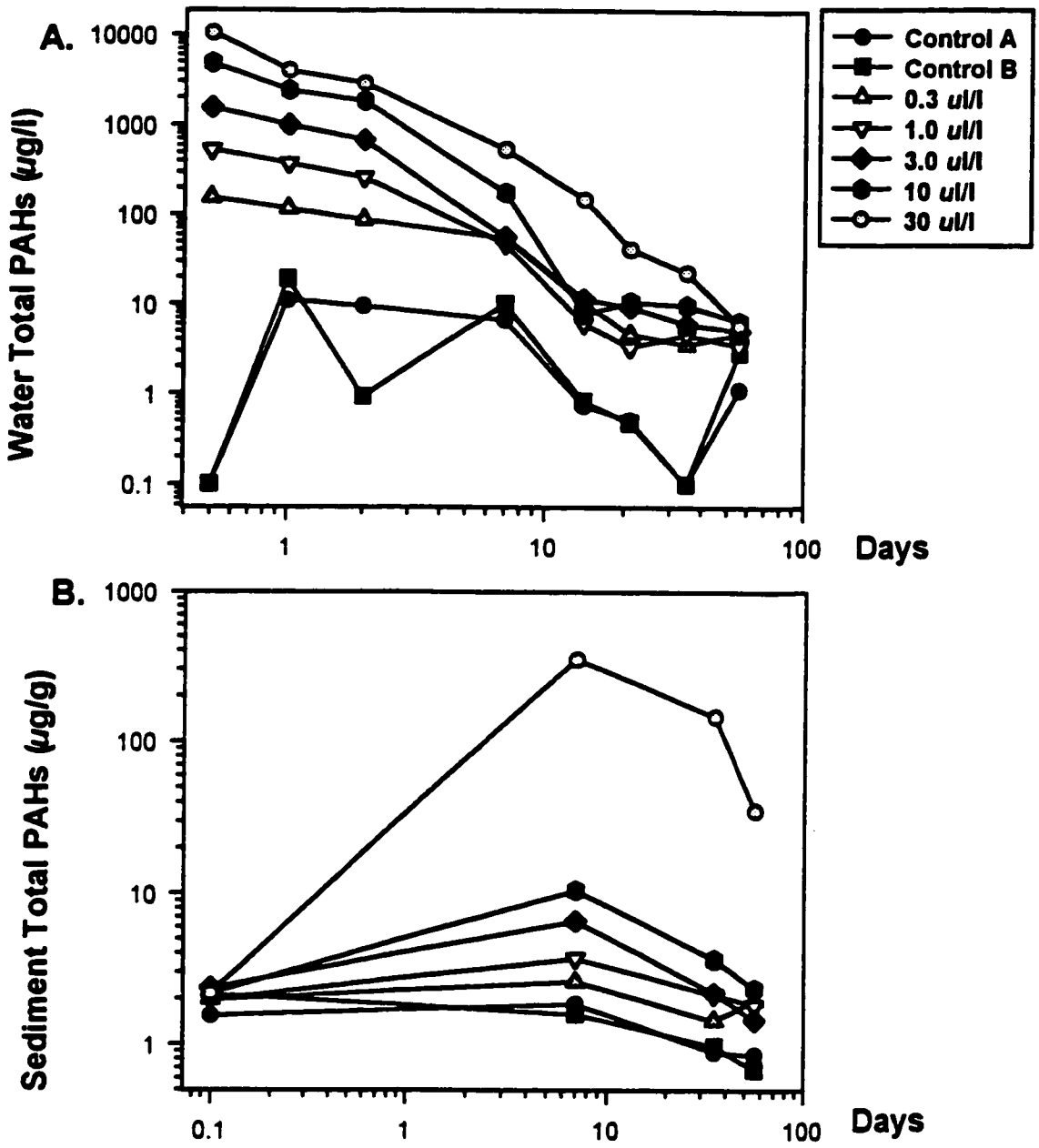

Figure 3.4: Change in total PAH concentrations in the water (A), and sediment (B) after dosing microcosms with creosote for the 37 d experiment. The first sediment sample was taken on the day of dosing; the first water sample, within minutes of dosing. Fish were added to the microcosms 74 and 77 days after dosing.

Table 3.1: PAH extraction recoveries and water concentrations for control and creosote-treated microcosms on day 84 and 80 of the 37 d study, and 28 d time study respectively*

Nominal Creosote Concentration (u l/l)	% Recovery	37 d creosote exposure study										28 d creosote time study		
		0A	0B	0.3	1	3	10	30	Slope	Y-intercept	F ratio	0	3	10
PAH (ng/l)														
Acenapthene[a]	42.80	35.81	nd	42.45	66.55	110.87	189.11	214.76	2.23	72.61	171.86	37.04	69.14	230.83
Fluorene	52.00	nd	nd	nd	nd	161.67	185.76	763.86				nd	103.84	213.87
Phenanthrene	65.90	113.05	152.92	92.40	99.05	154.00	183.93	94.29				54.04	133.04	190.37
Anthracene	58.00	nd	nd	nd	nd	16.86	16.97	13.44				nd	19.88	20.42
Fluoranthene[a]	80.50	nd	98.52	nd	139.30	142.03	400.23	701.10	2.31	156.67	16.52	nd	465.61	571.46
Pyrene	76.90	nd	nd	202.97	nd	nd	nd	634.11				nd	297.69	437.71
Benzo(a)anthracene	72.80	nd	nd	nd	nd	nd	6.19	8.01				nd	8.48	12.93
Chrysene	63.40	nd	nd	nd	nd	nd	nd	nd				nd	nd	293.96
Benzo(b)fluoranthene	73.40	nd	nd	nd	nd	19.61	71.37	71.03				nd	45.96	47.09
Benzo(k)fluoranthene	71.80	0.60	3.39	nd	nd	2.63	17.16	19.84				nd	15.23	11.92
Benzo(a)pyrene	61.70	nd	nd	11.78	nd	nd	nd	nd				nd	nd	nd
Total detectable PAHs	67.02	149.46	252.83	349.60	298.90	607.67	1050.72	2520.44	2.61	452.89	52.77	91.08	1158.87	2030.56

* Significant linear relationship with nominal creosote concentrations ($p \leq 0.05$)

[a] Naphthalene, dibenzo(a,h)anthracene, indo(1,2,3-cd)pyrene, and benzo(g,h,i)perylene were not detected in the water

(nd)= not detected

B. Weight change and mortality

Although no detectable change in final fish weights and liver weights was observed throughout the course of the 28 d kinetic experiment, the ratio of these two measurements showed a significant increase across creosote treatments. Liver/body weight ratios increased at the 3 *u*l/l nominal creosote concentration ($p=0.012$) and at the 10 *u*l/l concentration ($p=0.001$) when compared to the control group. The liver/body weight ratio for fish sampled on day 14 at the 10 *u*l/l concentration was significantly greater than the control fish liver/body weight ratio measured at the respective sampling time. A significant decreasing linear trend was also measured during the course of the study at the 10 *u*l/l concentration ($\log y = \log (0.62-0.04x)$, $p \leq 0.03$). The slope of this relationship was not significantly different from the slope of the control group (Fig. 3.5). No mortalities were observed at any of the concentrations during the 28 d kinetic experiment.

The two control microcosms in the 37 d creosote experiment were not significantly different from one another for all measured parameters, the exception being mortality. Hence, replicates for each of the control microcosms were pooled for statistical analysis. Fish weight gain during the 37 d experiment was highly variable; the pooled mean across concentrations $\pm$ SD was 6.37 ± 15.81 g. No significant changes in weight gain or liver to body weight ratio were observed across concentrations. Fish mortality occurred at all concentrations, showing no relationship with creosote nominal concentration. Percent mortalities of 25, 31, 13, 13, 38, and 44 were seen over the 37 days of exposure at 0A, 0.3, 1.0, 3.0, 10, and 30 *u*l/l of creosote. Mortalities were not observed in the second control microcosm (0B).

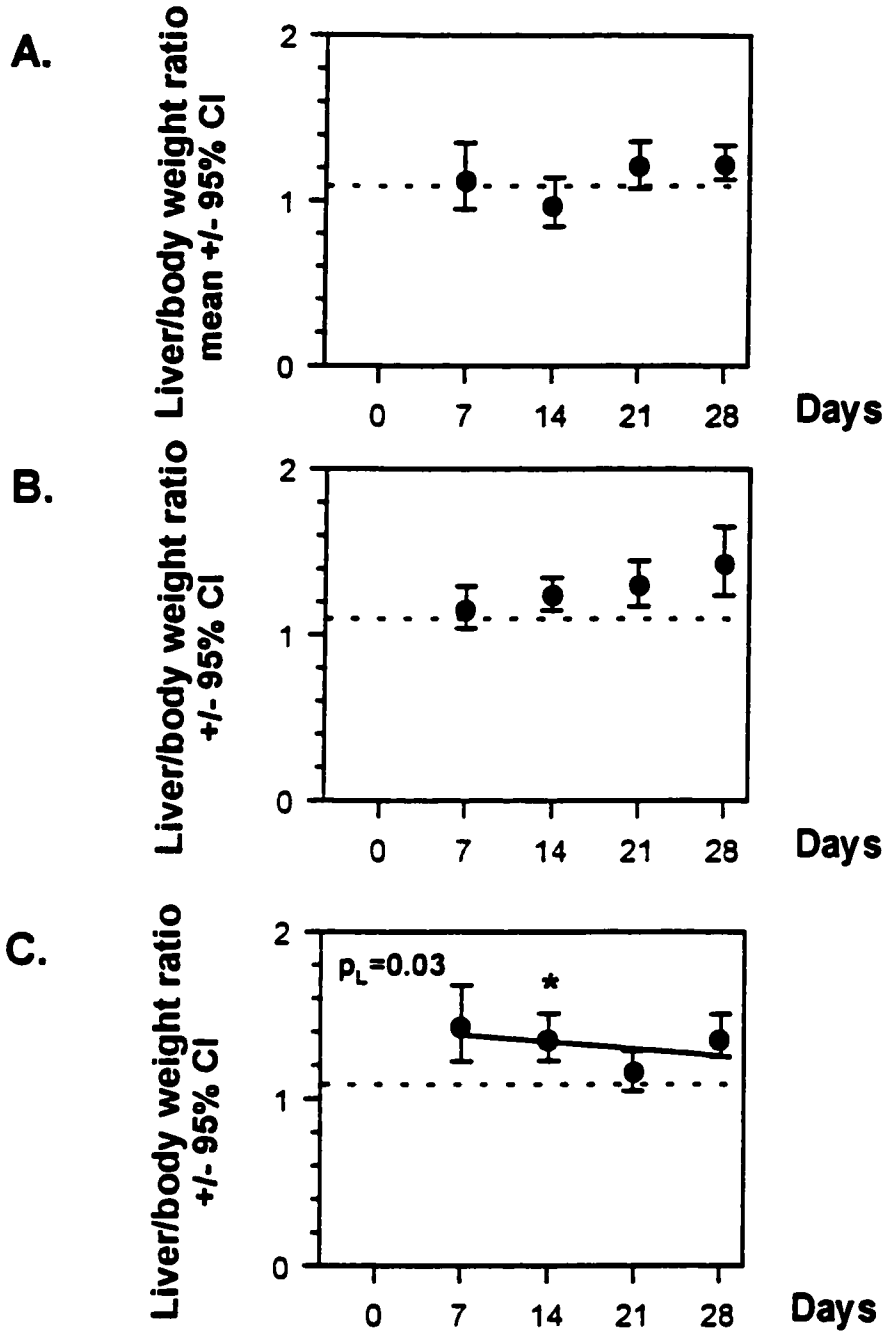

Figure 3.5: Liver to body weight ratio of fish from the control (A), 3 *u*l/l (B), and 10 *u*l/l (C) creosote-treated microcosms during the 28 d exposure. Results are expressed as the means with 95 % confidence intervals. Sampling groups which are significantly different from the control group at the respective sampling time ($p<0.05$) are indicated by *. The line represents the linear regression equation y= a+bx through the data set. The level of significance for the linear regression is defined by (p_L). The dotted line represents the baseline mean on day 0.

C. Oxidative burst

Creosote exposure reduced pronephros leukocyte oxidative burst in both experiments. This reduction in oxidative burst was dependent on the duration of creosote exposure, as seen in the 28 d kinetic experiment (Fig. 3.6). Overall, oxidative burst was significantly inhibited in fish exposed to 3 *u*l/l creosote when compared to the control ($p=0.036$) and the 10 *u*l/l creosote concentrations ($p<0.001$). The response across treatment groups was lower on day 14 when compared to day 7 ($p=0.001$), indicating that macrophage oxidative burst may vary with respect to time. Oxidative burst was most severely inhibited during weeks 2-3 of the experiment, returning to near control levels by day 28. This is illustrated by the quadratic ($\log y = \log(5.94-0.23+0.0067x^2)$, $p \leq 0.02$) relationship fit to the data set from 3 *u*l/l creosote treatment group. Similar trends were also observed in the 10 *u*l/l creosote concentration, showing a quadratic ($\log y = \log (5.59-0.23x+0.0064x^2)$, $p<0.02$) relationship over the 28 d. A significant quadratic ($\log y = \log(5.15-0.04x+0.00094x^2)$, $p=0.02$) relationship was also observed across nominal creosote concentrations after 37 d of exposure (Fig. 3.7). None of these individual concentrations were significantly different from the replicate control groups.

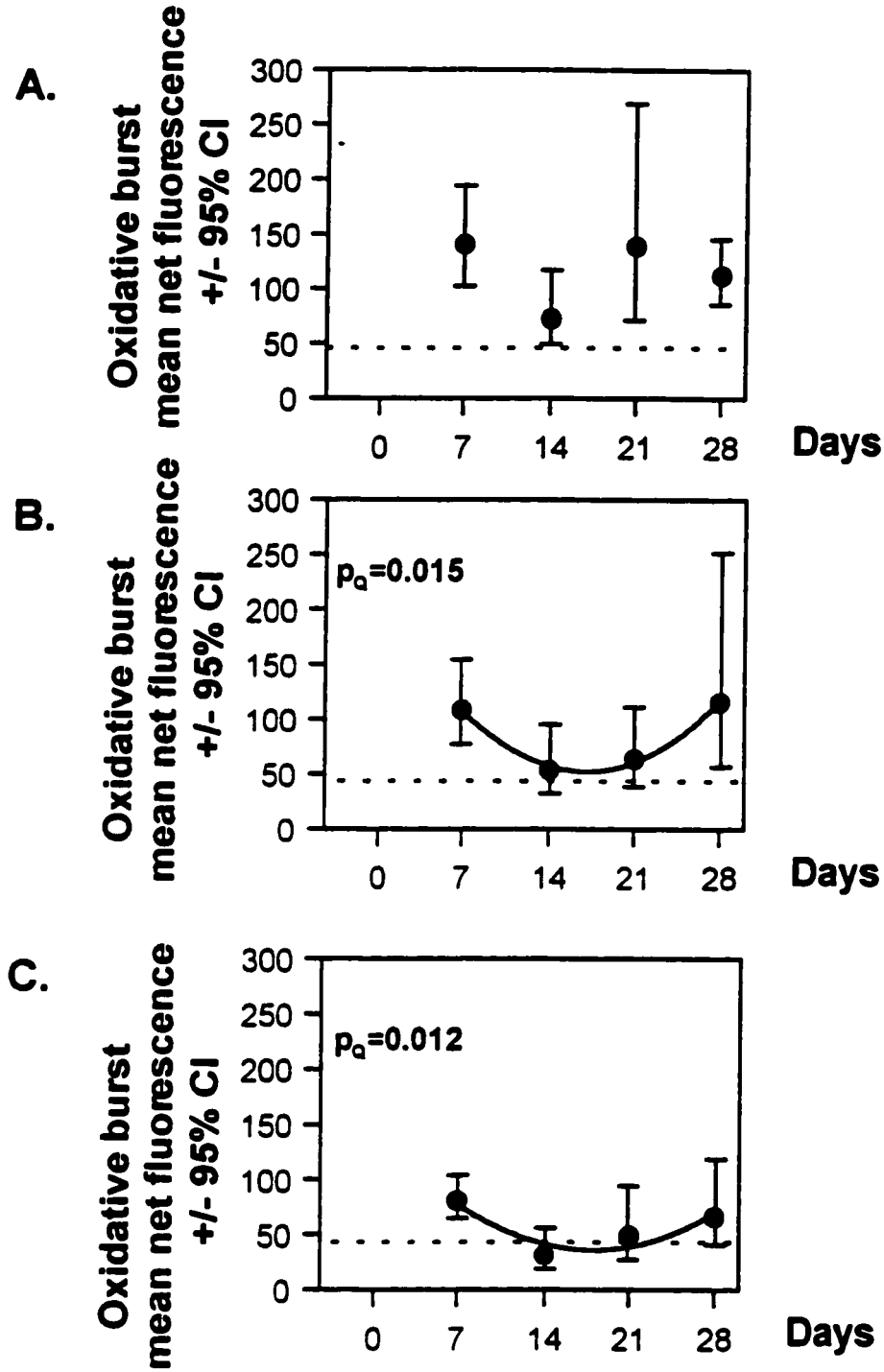

Figure 3.6: Oxidative burst of pronephros leukocytes from fish in the control (A), 3 *u*M (B), and 10 *u*M (C) creosote-treated microcosms during the 28 d exposure. Results are expressed as the means with 95 % confidence intervals. The lines represent the polynomial regression equation $y= a+bx+b^2x^2$ for each data set. The level of significance for the polynomial regressions is defined by (p_Q). The dotted line represents the baseline mean on day 0.

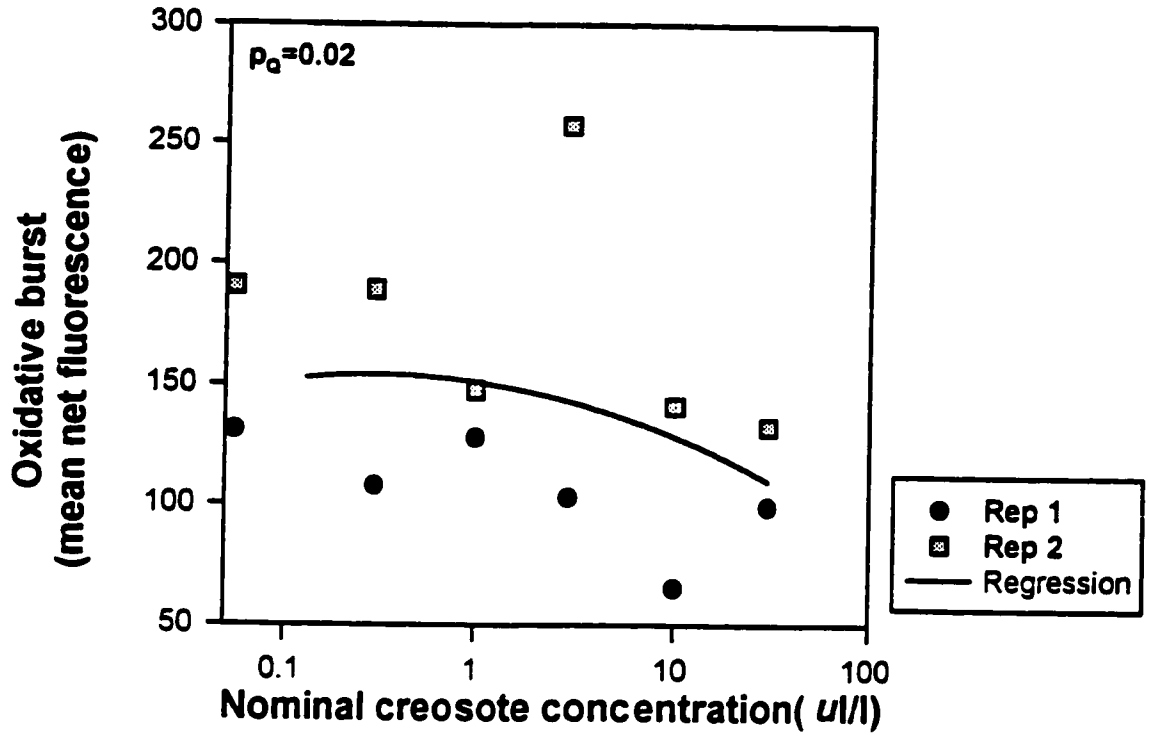

Figure 3.7: Oxidative burst of pronephros leukocytes from rainbow trout exposed to liquid creosote for 37 d in outdoor microcosms. Results are expressed as the mean of six fish for each replicate cage at each concentration. The line represents the polynomial regression equation $y= a+bx+b^2x^2$ through the data set. The level of significance for the polynomial regression is defined by (p_Q).

D. Phagocytosis

The phagocytic activity of pronephros leukocytes was stimulated by creosote exposure, but the extent was dependent on the parameter under consideration, and the duration of exposure. In general, stimulation was highest on day 7, gradually returning to control levels by day 28. The number of beads engulfed by phagocytic cells, as measured by the total mean fluorescence, increased across nominal creosote concentrations. Pairwise comparisons of the treatment groups to the control microcosm revealed an apparent increase at the 3 *u*M concentration ($p=0.06$), and a significant increase at the 10 *u*M concentration ($p=0.023$). Pairwise comparisons across time indicate that phagocytic activity measured on day 28 in the 10 *u*M microcosm was significantly reduced compared to the day 7 response ($p=0.02$), indicating that phagocytic activity decreased with respect to duration of exposure (Fig.3.8A-C).

A similar stimulatory trend was also observed for the percent of phagocytic cells across all creosote concentrations, again decreasing with respect to duration of exposure (Fig.3.9A-C). Pairwise comparisons of the treatment groups revealed that the response in the 3 *u*M concentration was significantly greater than the control group ($p<0.001$). The response in the 10 *u*M concentration was also greater than the response observed in both the 3 *u*M ($p=0.005$) and control microcosms ($p<0.001$). A more detailed examination of this concentration response relationship showed that the percent of phagocytic cells in fish exposed at the 10 *u*M concentration was significantly higher than the control response on days 7 ($p<0.001$), 14 ($p=0.002$), and 21 ($p=0.043$). Differences were not observed on day 28. A significant linear decrease over time was observed in the control group ($\log y = \log(2.78-0.09x)$, $p=0.04$) (Fig.3.9A), and the 10 *u*M concentration ($\log y = \log(3.62-0.09x)$, $p<0.05$)(Fig.3.9C). Although slope of the response at 10 *u*M appeared steeper than the control slope, it was not significantly different.

Trends for the phagocytic index were similar to those observed for the percent of phagocytic cells. Pairwise comparisons of the treatment groups revealed a significant

increase in phagocytic response at the 3 *u*l/l concentration when compared to the control group ($p=0.002$). The response at the 10 *u*l/l concentration was greater than the response observed in both the 3 *u*l/l ($p=0.005$) and control microcosms ($p<0.001$). The percent of phagocytic cells in fish exposed at the 10 *u*l/l concentration was significantly higher than the control response on days 7 ($p=0.001$), and 14 ($p=0.001$). Differences were not observed on either day 21 or day 28. A significant quadratic ($\log y = \log(11.22-0.15x+0.0038x^2)$, $p=0.01$) relationship was observed for the control group over time; the linear trend for the 10 *u*l/l creosote concentration was $\log y = \log(12.03-0.11x)$ ($p<0.05$). Although the slopes appear to increase across concentration, they are not significantly different from the control slope (Fig. 10A-C).

Phagocytic activity was also stimulated after 37 d of creosote exposure. The total mean fluorescence was increased across nominal creosote concentrations, though the difference is not statistically significant (Fig.3.11A). A stimulatory trend was observed for the percent of phagocytic cells across creosote concentrations, showing a significant quadratic relationship ($\log y = \log(10.31+0.093x-0.0028x^2$, $p<0.01$) (Fig.3.11B). Dunnetts test for comparisons was used to detect significant differences at the 3 and 10 *u*l/l concentrations compared to the control. Finally, the phagocytic index increased in a significant quadratic ($\log y = \log(10.31+0.093x-0.0028x^2$, $p<0.01$) fashion across creosote nominal concentrations (Fig. 3.11C). Again, Dunnetts test for comparisons was used to detect significant differences at the 3 and 10 *u*l/l concentrations compared to the control.

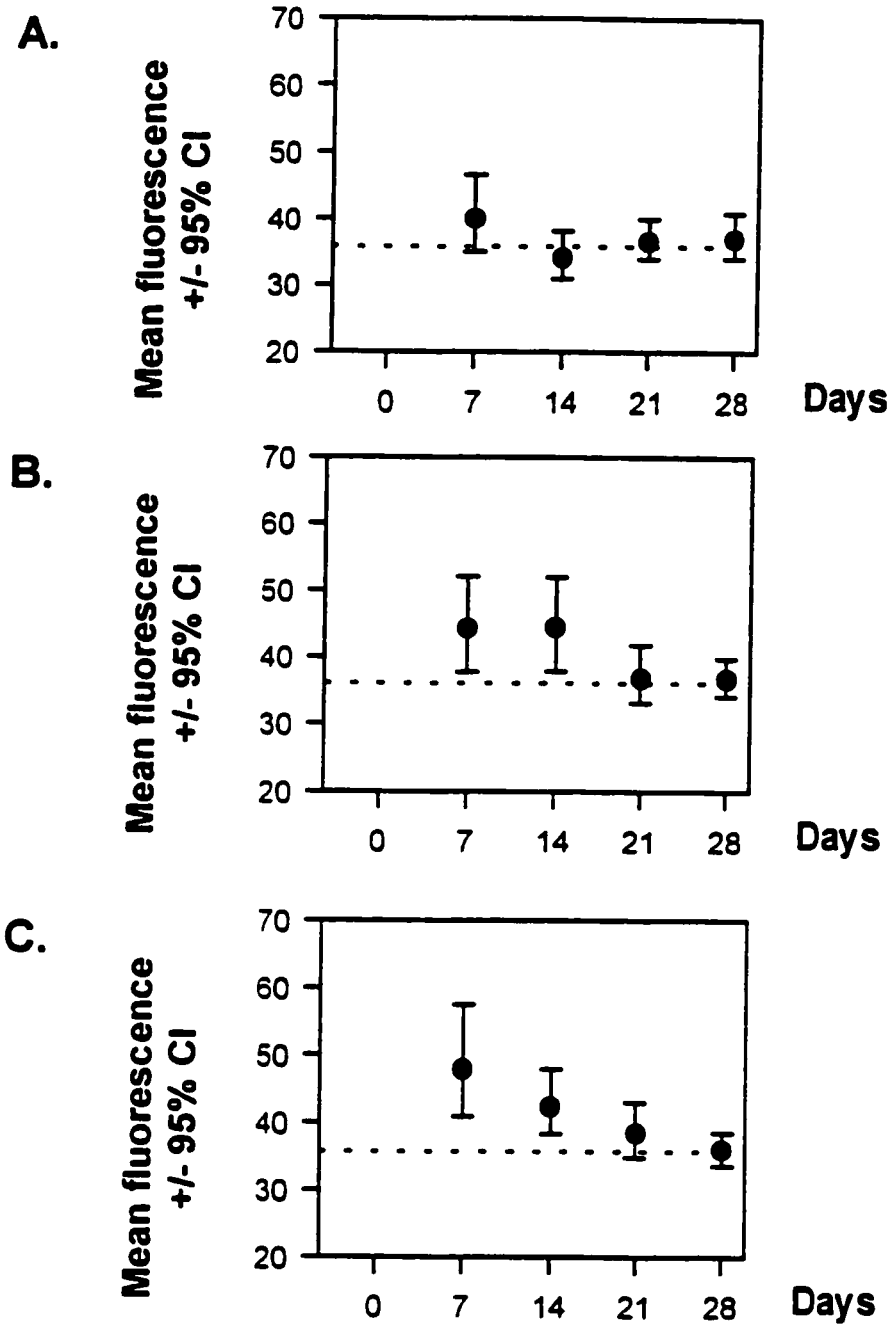

Figure 3.8: Phagocytic activity as measured by the total mean fluorescence of pronephros leukocytes from fish in the control (A), 3 *ul/l* (B), and 10 *ul/l* (C) creosote-treated microcosms during the 28 d exposure. Results are expressed as the means with 95 % confidence intervals. The dotted line represents the baseline mean on day 0.

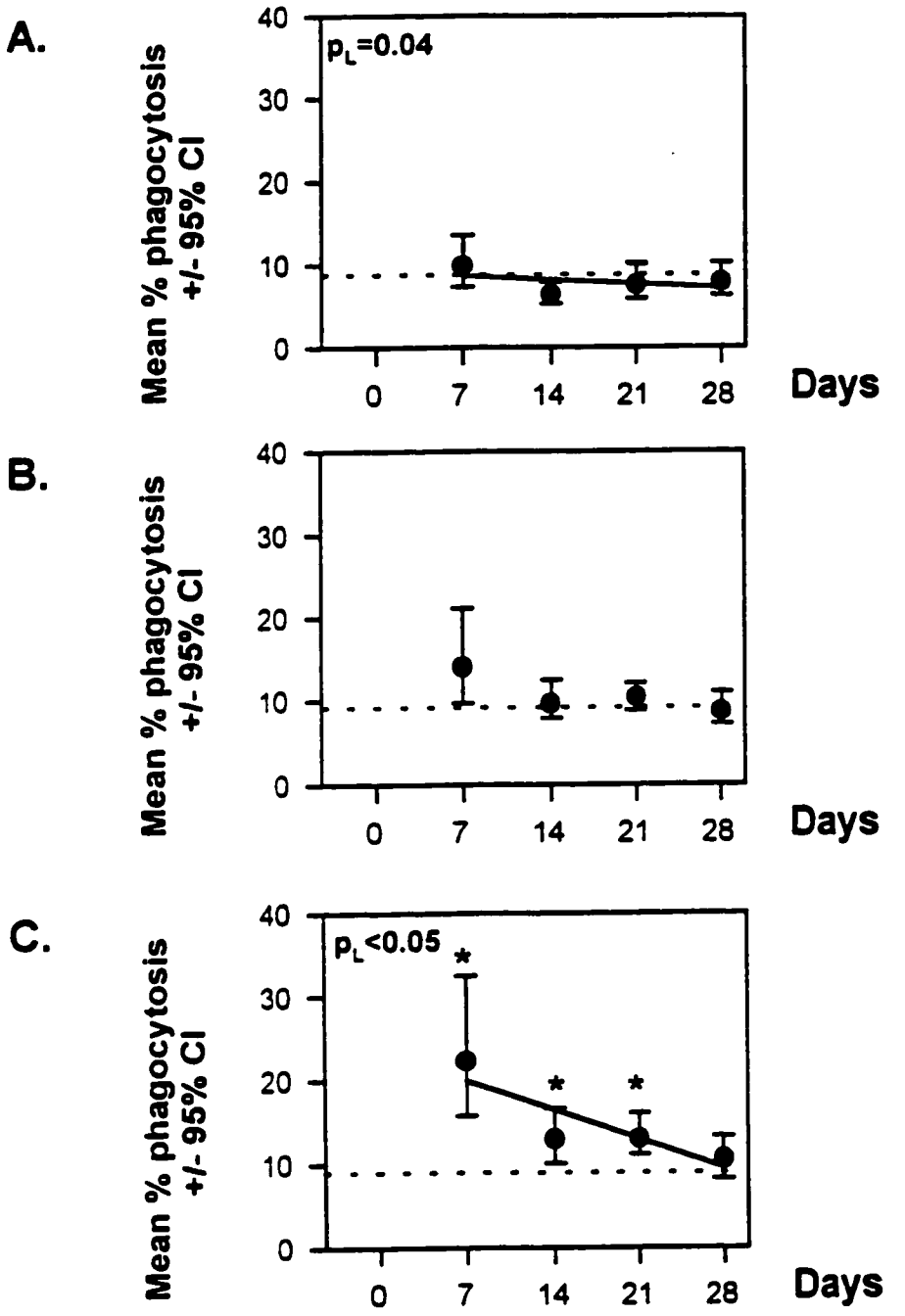

Figure 3.9: Phagocytic activity as measured by the percent of pronephros leukocyte phagocytic cells from fish in the control (A), 3 *ul*/l (B), and 10 *ul*/l (C) creosote-treated microcosms during the 28 d exposure. Results are expressed as the means with 95 % confidence intervals. Sampling groups which are significantly different from the control group at the respective sampling time ($p<0.05$) are indicated by *. The lines represent the linear regression equation $y= a+bx$ through each data set. The level of significance for the linear regressions is defined by (p_L). The dotted line represents the baseline mean on day 0.

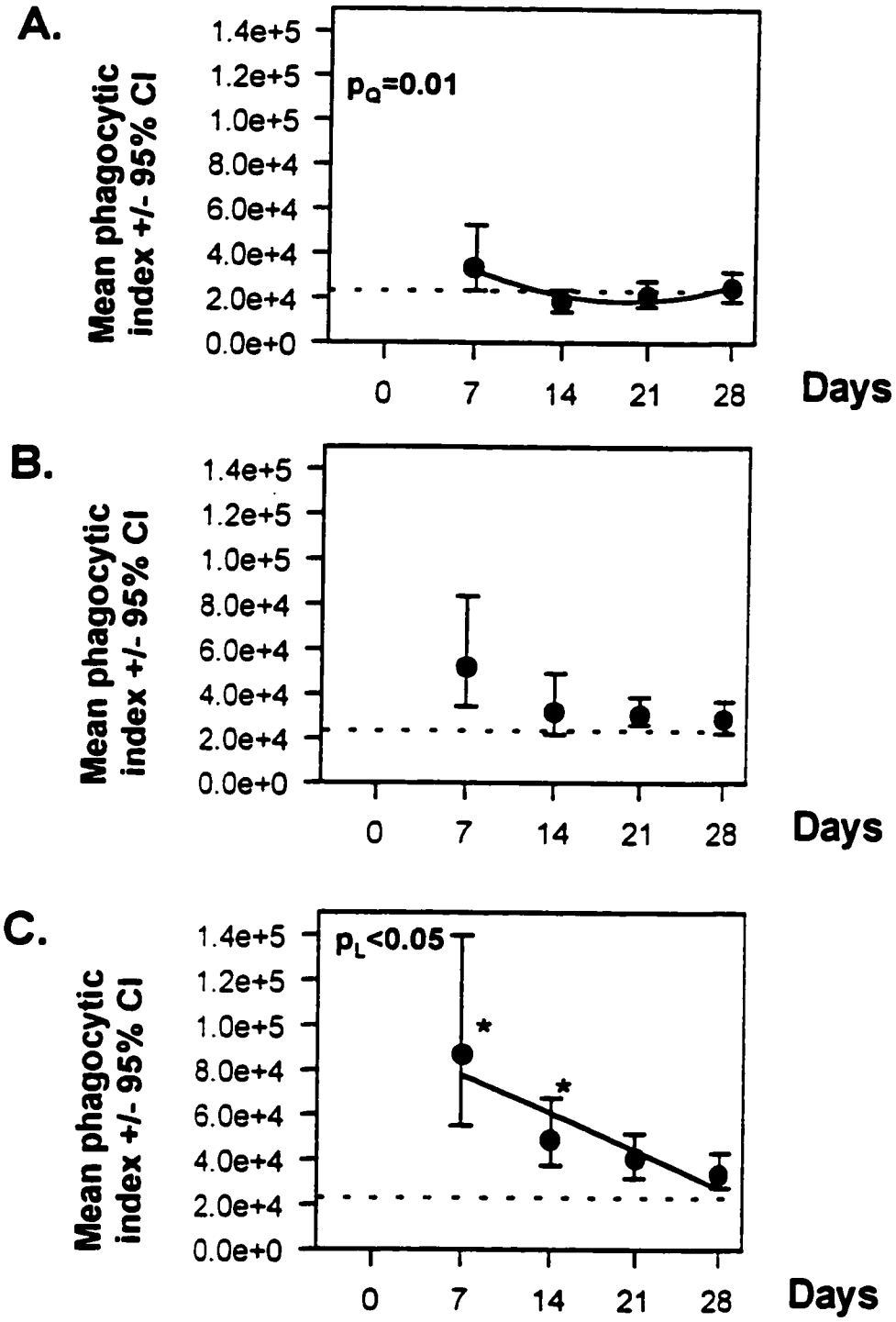

Figure 3.10: Phagocytic activity as measured by the phagocytic index of pronephros leukocyte phagocytic cells from fish in the control (A), 3 *ul/l* (B), and 10 *ul/l* (C) creosote-treated microcosms during the 28 d exposure. Results are expressed as the means with 95 % confidence intervals. Sampling groups which are significantly different from the control group at the respective sampling time ($p<0.05$) are indicated by *. The lines represent the linear regression equation $y= a+bx$ or polynomial regression equation $y= a+bx+b^2x^2$ through the data sets. The level of significance for the linear and polynomial regressions is defined by (p_L) and (p_Q) respectively. The dotted line represents the baseline mean on day 0.

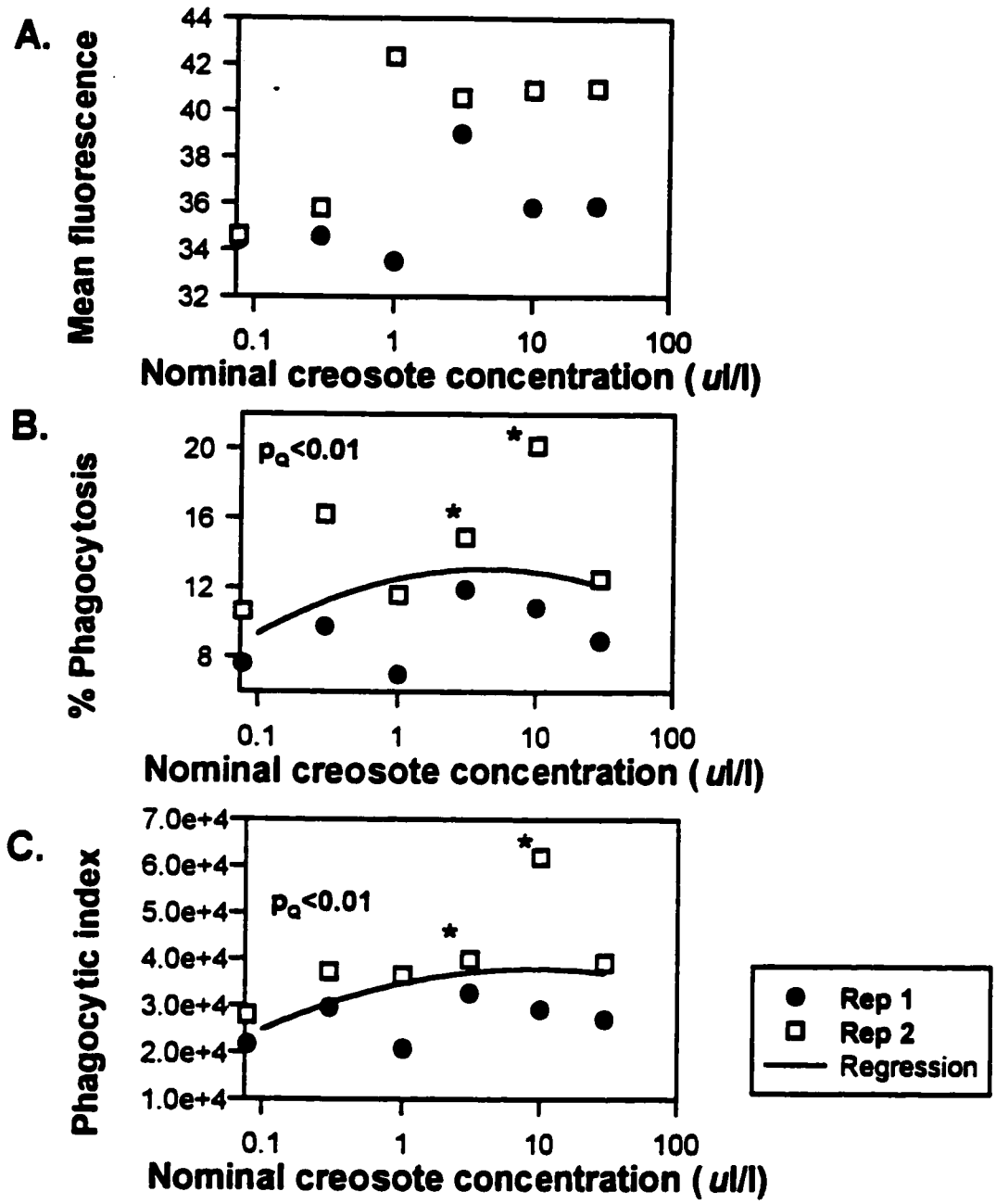

Figure 3.11: Phagocytic activity of pronephros leukocytes from rainbow trout exposed to liquid creosote for 37 d in outdoor microcosms. Phagocytic activity is expressed as the total mean fluorescence (A), percent of phagocytic cells (B), and phagocytic index (C). Results are presented as the mean of six fish for each replicate cage at each concentration. Concentrations which are significantly different from the control ($p<0.05$) are indicated by *. The lines represent the polynomial regression equation $y= a+bx+b^2x^2$ through each data set. The level of significance for the linear and polynomial regressions is defined by (p_L) and (p_Q) respectively.

E. Sig^+ leukocyte marking

Sig^+ leukocyte counts were also influenced by creosote exposure. Sig^+ leukocyte numbers in the creosote exposed fish initially increased, then gradually declined by day 28, until they were at, or less than day 0 baseline levels (Fig. 3.12). Fish Sig^+ leukocyte numbers at the 10 *ul*/l concentration were significantly elevated on day 7 with respect to the control fish ($p=0.02$). Differences were not detected across creosote concentration at any of the other sampling times. Pairwise comparisons of the sampling times within each treatment group indicate that fish Sig^+ leukocyte numbers may vary during exposure. Control fish cell numbers, for example, were significantly elevated on day 21 when compared to day 7 values ($p=0.003$). A significant quadratic relationship was observed for this group of fish over the 28d ($\log y = \log(4.52+0.21x-0.0055x^2$, $p<0.01$) (Fig. 3.12A). On the other hand, fish cell numbers in the 3 *ul*/l treatment group gradually decreased during the study. Cell numbers on day 28 were significantly lower than day 7 ($p=0.002$), 14 ($p<0.001$), and 21 ($p=0.007$) sampling times. A significant decreasing quadratic relationship ($\log y = \log(5.60 +0.13x-0.005x^2$, $p<0.01$) was measured over the 28 d in the 3 *ul*/l treatment group (Fig. 3.12 B). Fish cell numbers in the 10 *ul*/l treatment group were also lower on day 28 when compared to counts measured on day 7 from the same treatment group ($p=0.010$) (Fig. 3.12 C). No change in pronephros Sig^+ leukocyte counts was detected across creosote concentrations after 37 d of exposure (Fig. 3.13).

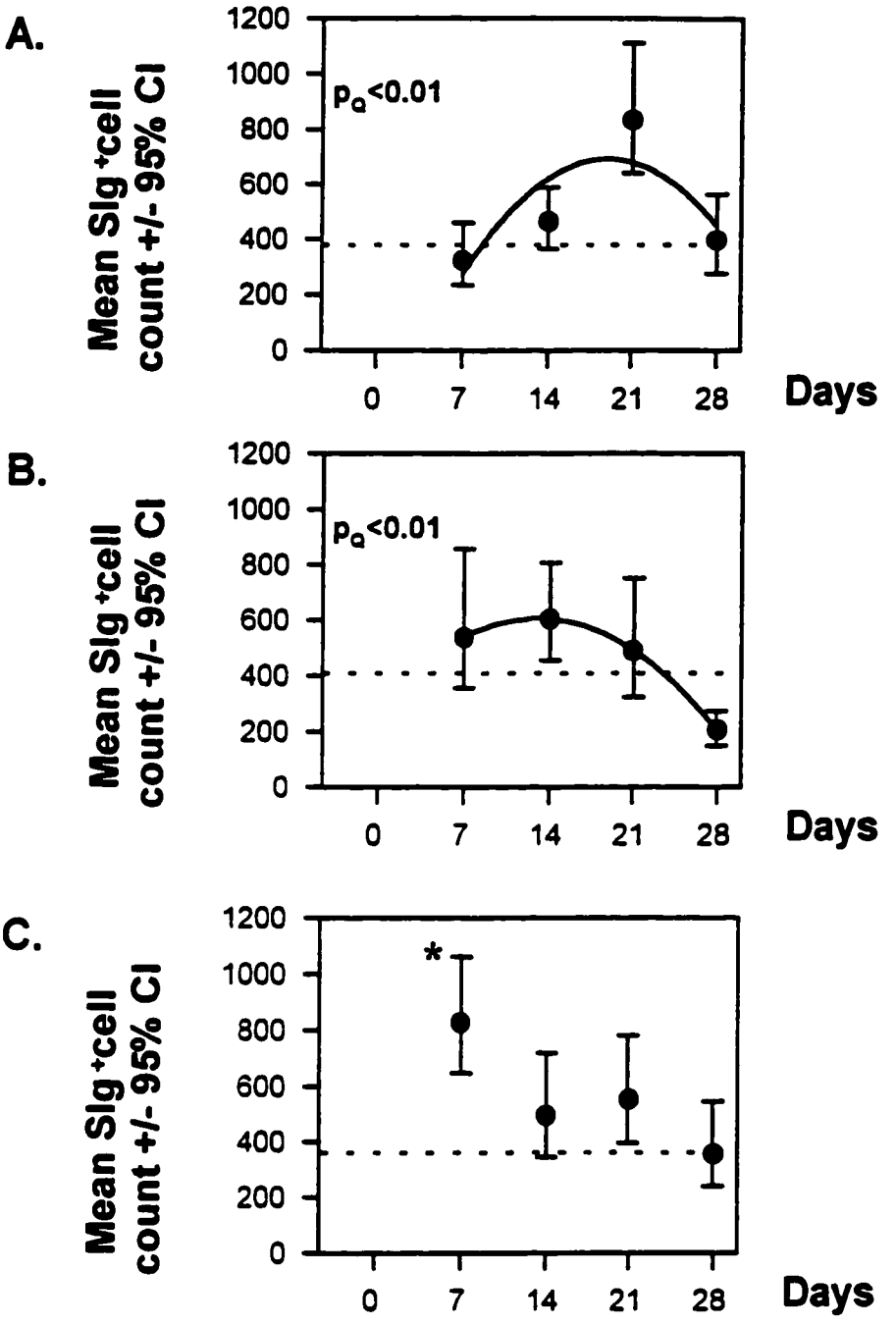

Figure 3.12: Slg+ pronephros leukocyte counts per 10,000 cells from fish in the control (A), 3 *ul*/l (B), and 10 *ul*/l (C) creosote-treated microcosms during the 28 d exposure. Results are expressed as the means with 95 % confidence intervals. Sampling groups which are significantly different from the control group at the respective sampling time ($p<0.05$) are indicated by *. Lines represent the polynomial regression equation $y= a+bx+b^2x^2$ through each data set. The level of significance for the polynomial regressions is defined by (p_Q). The dotted line represents the baseline mean on day 0.

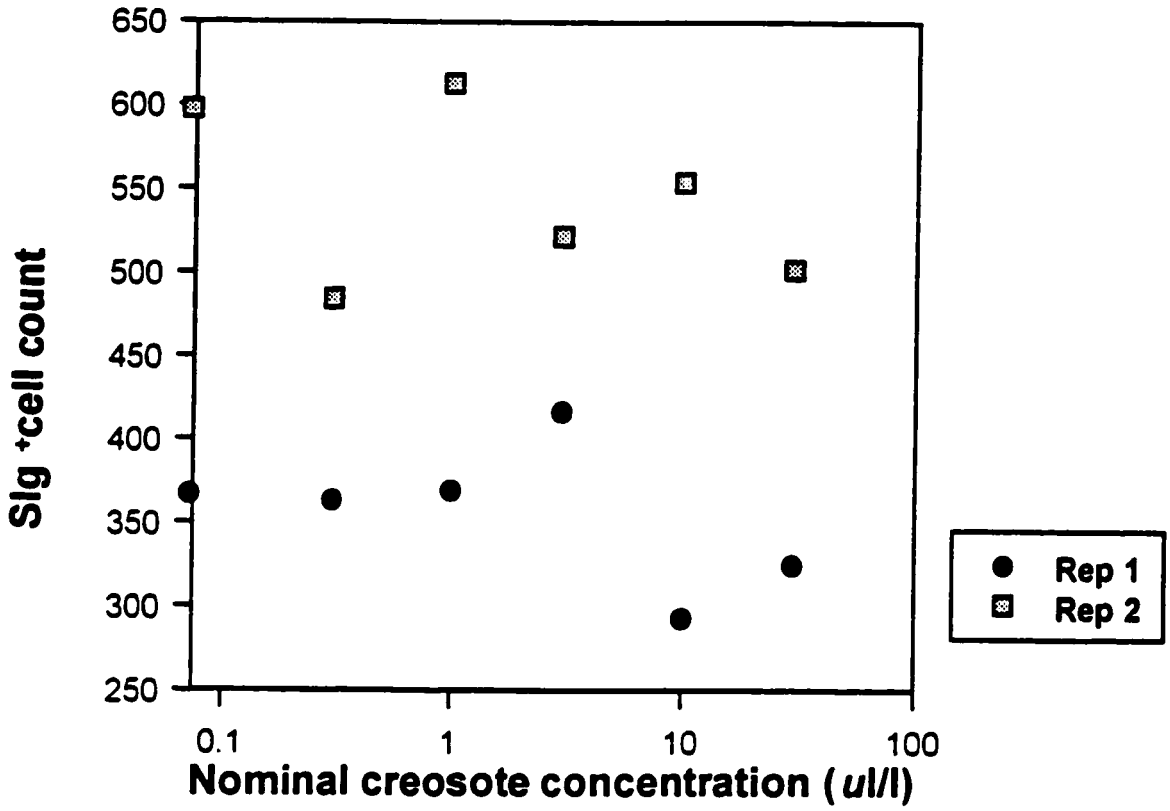

Figure 3.13: Sig+ pronephros leukocyte counts per 10,000 cells from rainbow trout exposed to liquid creosote for 37 d in outdoor microcosms. The results are expressed as the mean of six fish for each replicate cage at each concentration.

F. Serum lysozyme activity

Serum lysozyme activity did not appear to be significantly influenced by creosote exposure. Lysozyme activity was slightly elevated in all treatment groups on day 7 of the 28 d study (Fig. 3.14). Lysozyme activity measured on day 7 was significantly higher than levels measured on day 21 ($p=0.013$) and day 28 ($p=0.019$). Although no significant differences were observed across creosote concentrations, a significant linear relationship was observed over the duration of the study at the 10 *u*l/l creosote concentration ($y= 48.22-3.32x$, $p=0.02$).

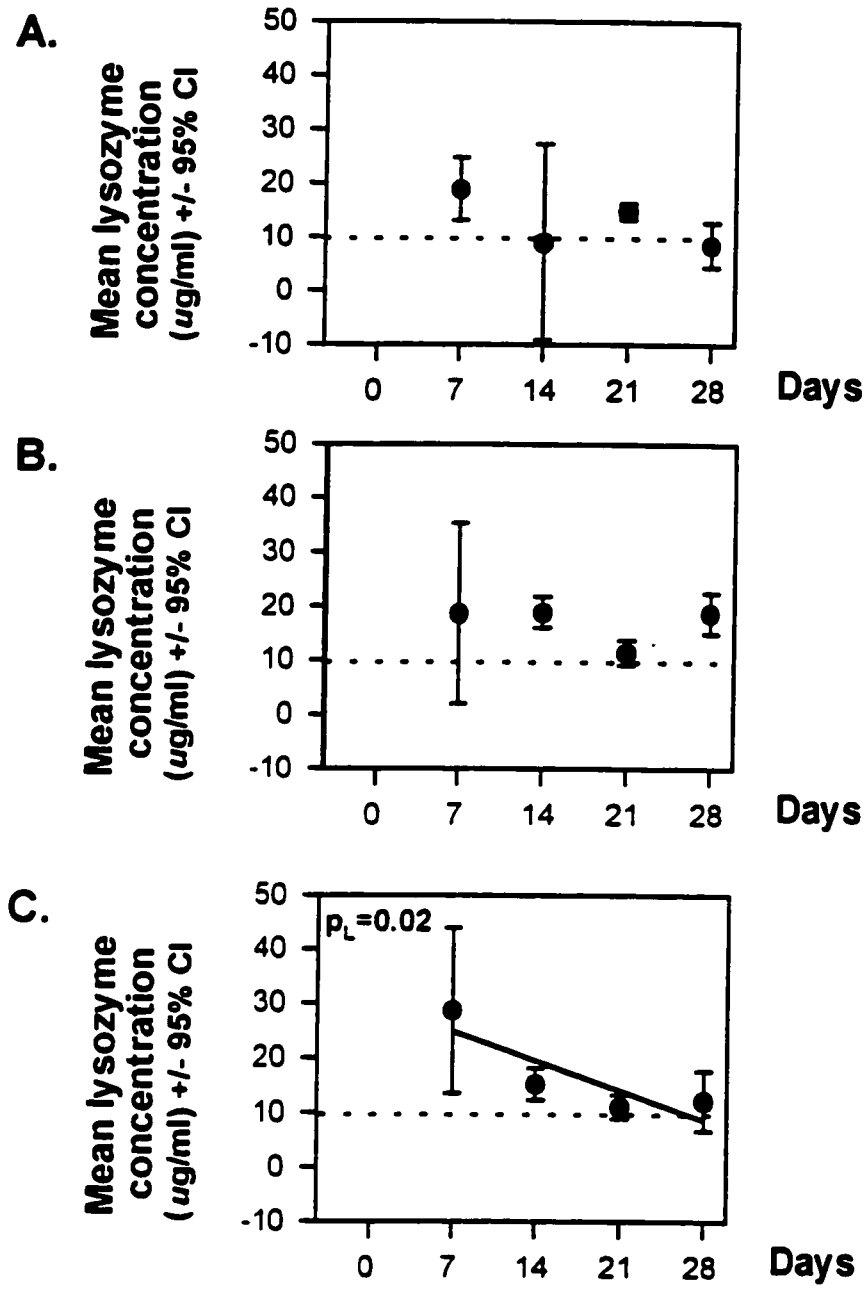

Figure 3.14: Lysozyme concentration in the serum of rainbow trout in the control (A), 3 *ul*/l (B), and 10 *ul*/l (C) creosote-treated microcosms during the 28 d exposure. Results are expressed as the means with 95 % confidence intervals. The line represents the linear regression equation y= a+bx through the data set. The level of significance for the linear regression is defined by (p_L). The dotted line represents the baseline mean on day 0.

3.5 DISCUSSION

Many of the results from the following experiments support previous observations reported by Karrow et al. (1999). In both cases, certain immune parameters were altered in fish exposed to liquid creosote. The data in the current study however, suggests that creosote-induced immunomodulation is, in addition to being concentration dependent, also dependent on the duration of exposure.

A. Pronephros leukocyte oxidative burst

Results from the kinetic experiment indicate that pronephros leukocyte oxidative burst is a temporal dependent event, making it difficult to interpret comparisons made across treatment groups throughout time. Both of the present experiments show that pronephros leukocyte oxidative burst is significantly inhibited by creosote exposure, confirming observations make previously by Karrow et al., (1999). We have also observed suppressed pronephros leukocyte oxidative burst in rainbow trout treated i.p. with 7,12-dimethylbenz(a)anthracene (DMBA) (unpublished data). Since different dosing regimes were utilized in the present creosote experiments, and differences in individual and combined water PAH concentrations are apparent (Table 3.1), we are limited in our ability to compare the results from each of these studies. Results from the 28 d experiment show that oxidative burst was most severely inhibited during weeks 1-3 of exposure, returning to near control levels by day 28. Oxidative burst was also inhibited in fish that were exposed to liquid creosote for 37 d, although the response at any individual concentration was not statistically significant. Detecting this overall reduction after 37 d, when differences were not evident on day 28 of the kinetic experiment, is likely attributed to the larger number of treatment groups and robustness of the trend analysis.

It is unclear why the production of H_2O_2 is inhibited in pronephros leukocytes, and why the magnitude of the response is dependent on the duration of exposure. One possible explanation is that creosote concentrations in the water were reduced to below

effect levels during the exposure. However, others have also reported time-dependent, and tissue variable differences in fish immune parameters. Secombes et al. (1997) noted that relatively few immune parameters measured from dab (*Limanda limanda*) exposed to oil-contaminated sediments were consistent across concentration and sampling times. Oxidative burst was, for example, unaffected after two weeks of exposure, but significantly inhibited after four weeks. This response was only observed at the lowest sediment dilution. Lemaire-Gony et al. (1995) reported that splenic macrophage oxidative burst in European sea bass (*Dicentrarchus labrax*) was reduced after benzo(a)pyrene injection, while oxidative burst from pronephros macrophages was stimulated. The authors suggested that benzo(a)pyrene oxyradical metabolites may have accounted for the increased H_2O_2 in pronephros macrophages, since benzo(a)pyrene was metabolized at a higher rate in pronephros macrophages than in splenic macrophages. A recent study by Marionnet et al. (1997) has confirmed that PAHs can under go metabolic activation in spleen and pronephros tissues, though tissue differences appeared to be dependent on the detection method. Exposure duration may have accounted for the stimulated pronephros macrophage oxidative burst reported in the Lemaire-Gony et al. (1995) study, since fish were sacrificed within 24 h of dosing.

Although it is unclear how PAHs induce immunotoxicity at the cellular and molecular level, one hypothesis is that reactive PAH metabolites bind to susceptible proteins which, in turn, alters cell signaling pathways (Davila et al., 1997). PAH metabolites have been shown to bind to protein tyrosine kinases (PTKs) and Ca^{2+}-ATPases, thereby disrupting Ca^{2+} signals within mammalian lymphocytes (Krieger et al., 1995; Davila et al., 1997; Zhao et al., 1997). Metabolic activation is also a prerequisite for the induction of contact hypersensitivity to PAHs. Reactive PAH metabolites reportedly bind to proteins, forming immunogenic hapten-protein conjugates which activate specific T cells that mediate contact hypersensitivity (Anderson et al., 1995). Recently, Zhao and Ramos (1998) reported that benzo(a)pyrene inhibited *c-fos* and *c-jun* transcription factor gene

expression in primary hepatocyte cultures. These nuclear factors are also found in lymphocytes, and are essential for inducing the transcription of specific cytokine genes. Since pretreatment of the hepatocytes with ∝ naphthoflavone, an inhibitor of cytochrome P450, prevented benzo(a)pyrene modulation, Zhao and Ramos (1998) hypothesized that the mechanism(s) may have involved reactive benzo(a)pyrene metabolites. Benzo(a)pyrene has also been reported to alter the expression and synthesis of certain immunoregulatory gene products including, IL-2, IFNγ, and IL-2 receptor mRNA (Vandebriel et al., 1998).

Although lymphocytes are the predominant model used in these mechanistic studies, there is no evidence to suggest that phagocytic cells are not equally susceptible to the deregulated molecular events caused by PAHs. Data supporting this hypothesis has recently been published. Fabiani et al. (1998) reported that a mixture of PAHs increased human monocyte NADPH oxidase activity after 12 and 24 hr of incubation. Other enzymes, such as protein kinase C, superoxide dismutase (SOD), peroxidase, catalase, and glutathione peroxidase, that are also involved in regulating respiratory burst in macrophages, may also directly or indirectly be affected by PAHs.

The hypothesis that PAH metabolites induce immunotoxicity in fish models, by binding to and disrupting the normal function of cellular proteins, has not been addressed. Recent experiments indicate that fish leukocytes possess cytochrome P450, capable of metabolically activating PAHs (Marionnet et al. (1997). In addition to this, currently identified signal transduction pathways in fish appear homologous with mammalian counterparts (Bernstein et al., 1998). This implies that molecular events, observed during PAH deregulated cell signaling, may also occur in fish. Since the generation of superoxides from fish leukocytes is also mediated by NADPH oxidase (Secombes, 1996), it is tempting to speculate that enzymes responsible for respiratory burst in fish may also be modulated by PAHs.

B. Pronephros leukocyte phagocytosis

Phagocytic activity measured in the present experiments was significantly stimulated by creosote exposure, verifying previously reported observations (Karrow et al., 1999). Similar responses were observed in fish injected i.p. with DMBA in our laboratory (unpublished data). Modulated phagocytic activity in creosote exposed fish however, appears to be dependent on the duration of exposure. Increased phagocytic activity was attributed to an increase in leukocyte phagocytizing capacity (mean fluorescence), as well as an increase in the percent of phagocytic cells. Temporal changes in the phagocytic index were more significantly influenced by the percent of phagocytic cells. Results from 37 d study identified the LOEC as 3 *u*M using nominal creosote concentrations. This value was considerably lower the endpoint reported previously (Karrow et al., 1999). However, it is difficult to make an accurate comparison of these two endpoints, since the duration of exposure, concentration of PAHs in the water, and the physical profile of the water was different in both studies.

Reports of fish phagocytic activity being influenced by PAH exposure appears to vary greatly. Weeks and Warriner (1986) reported suppressed pronephros phagocytic activity in spot (*Leiostomus xanthurus*) and hogchoker (*Trinectes maculatus*) exposed to Elizabeth River sediments which contain total PAH concentrations as high as 13,000 ug/g. Lemaire-Gony et al. (1995) also reported suppressed splenic macrophage phagocytic activity in European sea bass intraperitoneally dosed with 20 mg/kg benzo(a)pyrene. Although pronephros macrophage phagocytic activity appeared slightly enhanced in these fish, the response was not statistically significant. Increased phagocytic activity has also been reported in American plaice (*Hippoglossoides platessoides*) exposed to sediments contaminated with PAHs, PCBs and PCDFs (Lacroix et al., 1997) and in mammalian studies using 7, 12-dimethylbenz[a]anthracene (DMBA) (Dean et al., 1986). In the Dean et al. (1986) study, researchers suggested that resident intraperitoneal macrophages may have been activated by DMBA.

A review by Lewis (1995a) provided significant insight to the variable phagocytic responses reported in immunotoxicology reports. Lewis reported that one of the major problems with test methods cited in the literature is that they generally evaluate a single macrophage function, and rarely evaluate macrophage development. Diethylstilbestrol for example, has been shown to alter resident macrophages in mice, such that they are directed to an inflammatory stage of development. Characteristically, the macrophages have increased phagocytic activity but, because they are not completely cytolytically activated, have decreased host resistance to microorganisms. This paradox between increased phagocytic function and decreased host resistance was not understood until it was determined that these inflammatory macrophages were unable to respond to inflammatory signals to become cytolytically activated. Fish exposed to creosote in the present study also exhibited increased phagocytic activity and decreased cytolytic activity, as measured by the production of hydrogen peroxide in response to PMA. This change in macrophage function may also represent a shift toward an inflammatory stage of development. Evaluating macrophage tumor cell cytolysis is required, however, to test this hypothesis (Lewis, 1995b). Increased numbers of inflammatory macrophages in the pronephros may represent a nonspecific immune response directed at PAH-mediated tissue damage, or PAH-protein conjugates. The gradual decline in phagocytic activity, observed through out the kinetic experiment, may be attributed to either decreasing creosote concentrations in the water during exposure, or to compensatory mechanisms attempting to regain systemic homeostasis.

C. Macrophage function and fish health

Macrophages are cardinal effector cells, aiding in the clearance or lysis of tumors, bacteria, parasites and viruses. They participate in wound healing and in a number of normal metabolic functions for the purpose of maintaining homeostasis (Lewis, 1995a). Modulated macrophage activity has been reported to impair tumor cell killing (Lewis, 1995b), and resistance to *Listeria monocytogenes and Streptococcus pneumoniae* in

mammals (Vandebriel et al., 1995). Inflammatory macrophages have also been implicated in the etiology of inflammatory pulmonary toxicity and hepatotoxicity (Laskin and Pendino, 1995). Although we have observed modulated macrophage immune function in fish exposed to creosote, it is premature to conclude that modulation is sufficient to impair fish health without conduction host resistance studies. This will allow us to establish a relationship between functional changes in the immune system and the development of clinical disease (Karol, 1998).

D. Pronephros Sig^+ leukocyte marking

The number of Sig^+ B cells in pronephros tissue was also influenced in fish exposed to creosote. Although no detectable change was observed after 37 d of exposure, the number of Sig^+ B cells, from fish exposed during the 28 d experiment, was shown to vary greatly among creosote exposed fish, and the control fish. Sig^+ B cell counts for the control fish gradually increased throughout the experiment, then returned to near day 0 baseline levels by day 28. The creosote exposed fish Sig^+ B cell counts however, initially increased, then progressively declined throughout the study to day 0 levels, or below. Previously, we reported that pronephros lymphocyte proliferation to the B cell mitogen, lipopolyssacharide (LPS), and peripheral blood Sig^+ B cell counts were reduced in fish exposed to creosote for 28 d (Karrow et al., 1999). A number of possible explanations may account for the initial increase observed on day 7 of the kinetic study. First, an apparent increase in Sig^+ B cells may be attributed to a decrease in either T cells or Sig^- pre-B cells. DMBA-induced apoptosis of a pre-B cell line has been reported by Yamaguchi et al. (1997). The gradual return to control levels suggests that either creosote levels were reduced to below effect levels during exposure, or that lymphocyte(s) returned to homeostasis via compensatory mechanisms. Second, increased Sig^+ B cell numbers may be attributed to T cell activation, since certain T cell cytokines have been reported to stimulate the recruitment and proliferation of B lymphocytes (Sikorski et al., 1996). A subsequent decrease may follow, since activated

Sig^+ B cells undergo apoptosis in the absence of antigenic selection signals (MacLennan, 1998). Initial tissue specific increases, followed by reductions in plasma cell numbers, have also been reported by Slifka and Ahmed (1998). They suggested that the decrease in plasma cell numbers may be due to the selection of high affinity plasma cells and the apoptotic loss of low affinity cells. Third, it is also possible that we may be witnessing a shift in the leukocyte traffic to other tissues as a result of stress (Schreck, 1996).

E. Serum lysozyme activity

Lastly, in this study we also showed that serum lysozyme levels were also temporally dependent. Levels were not however, affected by creosote exposure over the duration of the 28 d experiment. Lysozyme concentrations peaked on day 7 in the 10 *u*l/l creosote concentration, returning to baseline levels by day 21. This trend was not measured in either the control or 3 *u*l/l creosote microcosms. Previously, we observed reduced plasma lysozyme levels in fish exposed to creosote after 28 d (Karrow et al., 1999). Secombes et al. (1997) reported that decreased serum lysozyme activity in dab exposed to oil-contaminated sediments, was the only consistent immune parameter in their testing panel. Our results using rainbow trout do not support this. Tahir and Secombes (1995) also reported suppressed lysozyme levels after 6 weeks in rainbow trout injected with 0.6 ml/kg oil-based drilling mud extract. It is possible that the initial increase in lysozyme levels, and other measured immune parameters observed during the kinetic 28 d exposure study, may be attributed to elevated levels of stress hormones, such as cortisol, released during the initial stages of exposure (Secombes et al., 1997). Levels of cortisol increase within seconds, following handling stress; lysozyme levels increase within minutes (Demers et al., 1997). Our contrasting results measured over the two field seasons may be credited to the different concentration ranges of creosote used in each study. In the previous study, plasma lysozyme levels were reduced by creosote exposure at the 56 *u*l/l nominal creosote concentration. Levels were also reduced at 5 *u*l/l, though we did not attribute this reduction to creosote exposure, since concentrations

between were unaffected. If an initial stimulation in lysozyme activity occurred during the previous study, it was not detected because changes were not monitored over time. This emphasizes the importance of monitoring immune parameters during exposure.

F. Water chemistry and physical profiles

A number of PAHs were identified in the water from both experiments. Pyrene and fluoranthene were implicated as the primary immunomodulating agents found in creosote because their concentration was linear with respect to nominal creosote concentrations (Karrow et al., 1999). In the 37 d experiment, a linear relationship for fluoranthene was again found; pyrene was however, only detected in the 0.3 and 30 *ul/l* creosote concentrations. In addition to fluoranthene and pyrene, chrysene was also measured in water samples from the 28 d kinetic study. All three of these compounds showed an incremental increase with respect to creosote concentration. Recently chrysene and pyrene were reported to alter humoral immunity in adult deer mice (Peden-Adams et al, 1999). It is likely that these PAHs contributed to the modulation of leukocyte oxidative burst, phagocytic activity, and SIg^+ cell counts; however, a net toxic response may be due to the combined effects of all PAHs, as well as other chemicals, found in creosote. The water and sediment PAH concentrations provided by Bestari et al. (1998a) indicated that the PAHs partitioned rapidly into the sediment and that the microcosms were at equilibrium prior to the rainbow trout study. This more accurately reflects an exposure scenario found in the natural environment than is typically seen in laboratory-based exposure studies.

As mentioned in our last report (Karrow et al., 1999), the immunosuppressive effects of temperature may have enhanced changes in immunological parameters which we are attributing to creosote exposure. Again, the temperature dropped 14°C during both experiments. This may have contributed to some of the variation measured in the control group during the 28 d kinetic study, and to the differences observed between staggered

replicates in the 37 d experiment. This decline in temperature is typical during the fall for this geographic location, and is an integrated part of microcosm and field studies.

3.6 CONCLUSION

This study confirmed that environmentally realistic concentrations of liquid creosote modulate rainbow trout immune parameters, with both stimulatory and suppressive effects being observed. In addition to this, modulation was dependent on the duration of exposure, showing an initial stimulatory or inhibitory response, then returning to near control levels after 28 d of exposure. Pronephros leukocyte oxidative burst, phagocytic activity, and SIg^+ B cell counts were all sensitive indicators of exposure to creosote; serum lysozyme levels, on the other hand, were not. The paradox between macrophage oxidative burst and phagocytic activity is perhaps the most interesting observation made during this study. This may be due to the complex integration of the immune system as a whole, and should be investigated in more detail. Although the mechanisms of PAH immunotoxicity are still being investigated, the results from this study indicate that environmental concentrations of PAHs can alter fish immune parameters. The apparent ability of these parameters to recover to near control levels during exposure emphasizes the need to monitor immune parameters during exposure.

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CHAPTER 4

EFFECT OF EXPOSURE TO VARIOUS SITES WITHIN THE HAMILTON HARBOUR ON (*Oncorhynchus mykiss*) IMMUNE PARAMETERS

4.1 ABSTRACT

A field study was used to assess whether or not exposure to sites within the Hamilton Harbour, which are known to be highly contaminated with polycyclic aromatic hydrocarbons (PAHs), heavy metals, and sewage treatment plant (STP) effluent, could affect immune function in fish. Caged rainbow trout (*Oncorhynchus mykiss*) were sampled after 7, 14. and 21 days of exposure from five sites within the Harbour, and a Lake Ontario reference site. The Harbour sites were selected for their proximity to either highly contaminated sediments or industrial and municipal discharges. Pronephros leukocytes from fish at the six sites were evaluated for phagocytic activity, oxidative burst, and the number of surface immunoglobulin-positive B cells. Serum lysozyme activity was also measured. Fish from the Harbour sites showed a greater reduction in pronephros leukocyte phagocytic activity over the course of the study when compared to the Lake Ontario reference fish; pronephros leukocyte oxidative burst was also reduced at two of the Harbour sites. Although B cell counts did not change throughout the duration of the study, overall counts were lower at two of the Harbour sites when compared to the Lake Ontario site. Serum lysozyme activity increased at site 4 and at the Lake Ontario reference site over the 21 days of exposure. Lysozyme activity was elevated at sites 5 and 6, when compared to Lake Ontario fish lysozyme activity, and did not change throughout the course of the study. These results indicate that several immune parameters were altered in fish during exposure at various sites within the Harbour. The role of chemical and physical stressors at these sites in the observed immunomodulation are discussed.

Key words: (*Oncorhynchus mykiss*); Hamilton Harbour; immunotoxicity; multiple stressors

4.2 INTRODUCTION

Numerous classes of pollutants including, heavy metals (O'Neill, 1981a,b; Zelikoff et al. 1997), aromatic hydrocarbons (Anderson et al., 1984; Arkoosh et al., 1994), and pesticides (Dunier et al, 1994a; Rice et al., 1995) have immunomodulating properties which may potentially increase the risk of disease to fish populations. Little, however, is known about the immunotoxicity of chemical mixtures to fish in marine environments (Arkoosh et al., 1996; Aaltonen et al., 1997), and even less is known about exposure in fresh-water systems (Rice et al., 1996).

Hamilton Harbour is a suitable site for assessing the impact of exposure to multiple contaminants in fresh-water on the fish immune system. It is geographically isolated from the west end of Lake Ontario by the Burlington Shipping Canal, and receives spatially heterogeneous contaminant loading from two steel industries, numerous municipal waste treatment facilities (STPs), as well as urban and rural runoff from the surrounding watershed. It was designated an "Area of Concern" by the International Joint Commission in 1985 because of the high levels of heavy metals, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs). Reduced input loading of suspended solids and contaminants over the past 10 years can be accredited to the implementation of the Remedial Action Plan (RAP): however, it has been suggested that this improved water quality may cause sediment-bound contaminants to partition back into the water column, increasing their bioavilibilty to fish and wildlife (Ling et al., 1993).

Adverse effects on fish exposed to sediments from the Hamilton Harbour have been reported in several studies (Krantzberg and Boyd, 1992; Balch et al., 1995). Mortalities in laboratory-based tests on rainbow trout sacfry and juvenile fathead minnows (*Pimephales promelas*) were largely attributed to anoxia and ammonia toxicity at some sites. Exposure to sediments from the central region of the Harbour resulted in reduced growth and the accumulation of various organic and heavy metal contaminants (Krantzberg and Boyd, 1992).

Reports of skin and liver tumours are also higher in Harbour-dwelling white sucker (*Catostomus commersoni*) and brown bullhead (*Ictalurus nebulosus*) populations compared to Lake Ontario (Cairns and Fitzimmons, 1988; Hayes et al., 1990). Studies with rainbow trout suggest that this may, in part, be attributed to the high levels of PAHs found in the sediments (Balch et al., 1995). Although the mechanism(s) of PAH-induced tumour formation remain unclear, it is of interest to note that those PAHs which are carcinogenic are also potent immunotoxicants (Zelikoff, 1994), and that natural cytotoxic cells, from feral fish exposed to PAH-contaminated sediments, were unable to recognize and destroy tumor target cells (Faisal et al., 1991). In order for PAHs to become carcinogens they must be metabolically activated by cytochrome P450. These reactive metabolites can subsequently bind to macromolecules such as proteins, RNA, and DNA, and may result in altered protein function, cell damage, and events leading to oncogene expression (Di Giulio et al., 1995). Evidence of this occurring in fish exposed to PAHs is best supported by reports of DNA adducts in fish livers (Tuvikene, 1995). DNA adduct formation and subsequent mutations have been correlated to the initiation of cancer (Davila et al., 1997). It is also speculated that liver parasites may be credited for the etiology of white sucker neoplasms from contaminated areas in the Great Lakes (Smith et al., 1994). Since PAH-induced immunomodulation is suspected of predisposing fish to disease (Dunier and Siwicki, 1993), this would most likely be manifested as an increase in recurrent infections, certain cancers (Luster et al., 1994), and decreased resistance to parasitism.

The present study sought to assess whether or not exposure of fish to various sites in the Harbour was sufficient to alter several immune parameters. These sites were selected for their proximity to either highly contaminated sediments, or various industrial and municipal discharges (Fig. 4.1). Caged rainbow trout were sampled after 7, 14, and 21 days of exposure at five Harbour sites and a Lake Ontario reference site (site 1). The Harbour sites included; the Burlington STP plume (site 2), a Harbour reference site (site 3), a site containing heavily PAH-contaminated sediment known as Randle Reef (site 4),

and two sites within the Windermere basin. One of the Windermere basin sites was situated near the outflow of the Red Hill Creek (site 5); the other near the Dofasco boatslip (site 6). The Hamilton Sewage Treatment Plant discharges into Red Hill Creek about 1 km upstream of the Windermere Basin. Sediments within and surrounding the Dofasco boatslip are highly contaminated with heavy metals, PAHs and PCBs. Pronephros leukocytes were evaluated for phagocytic activity. Phagocytic cells contribute to the regulation of an immune response through antigen processing and presentation, and the secretion of cytokines (Verburg-van Kemenade et al., 1995). The production of antibacterial oxygen radicals, specifically hydrogen peroxide, was also measured in this same population of cells. Surface immunoglobulin-positive B cell counts were measured in the pronephros tissue by cell surface marking. B lymphocytes play a key role in regulating an immune response, and can differentiate into plasma cells, the main effector cells in humoral defense. Lastly, serum lysozyme activity was measured. It is believed that lysozyme may act as an opsonin for phagocytic activity, and has been shown to damage the cell wall of gram positive bacteria, resulting in osmolysis (Ellis, 1990).

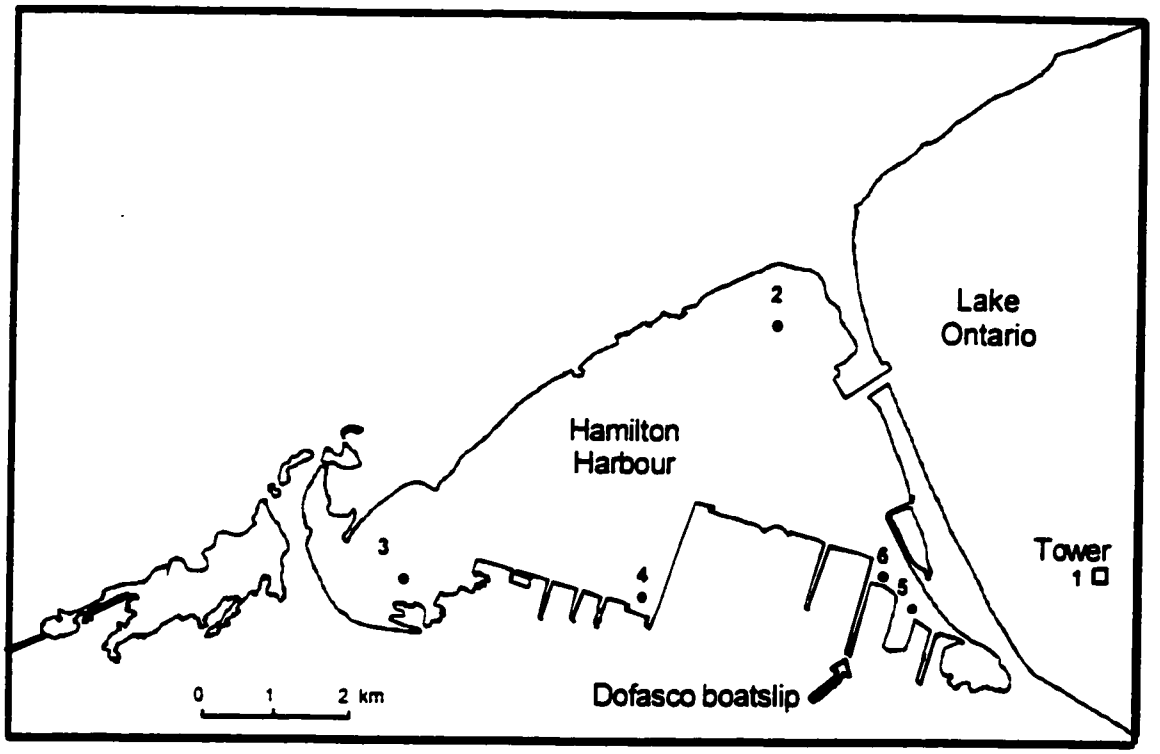

Figure 4.1: Site locations for the sampling of caged of fish in Hamilton Harbour and Lake Ontario. The sites are identified by numbers; 1 (Lake Ontario), 2 (Burlington STP), 3 (Lax), 4 (Rankle Reef) , 5 (Windermere basin), and 6 (Dofasco boatslip).

4.3 MATERIALS AND METHODS

A. Experiment

Female rainbow trout (60-100 g) were obtained in late April 1997 from Rainbow Springs Hatchery, Thamesford, ON, and acclimated for 14 d in the National Water Research Institute (NWRI) wet-lab using dechlorinated Burlington city water (hardness 131 mg/l $CaCO_3$, pH 8.05, conductivity 328 *us*/cm). Fish were transported to the six sites in bags containing oxygenated water that were contained in insulated coolers. They were then transferred to plastic cages, constructed out of laundry baskets crimped together with plastic ties, at each site for exposure. The caged fish were suspended in the water column at a depth of about 3 m. Three cages of fish (n=5 per cage) were added to each site on the first day of the study.

A second group of three cages per site was to be deployed on the third day to facilitate the sampling 10 fish per site at each time interval; with the manpower available, a maximum of 30 fish could be processed per sampling day. Bad weather, however, delayed the deployment of the second batch of cages by an additional two days. An extra cage containing 5 fish was placed at each site to determine the accumulation of PAHs by whole-body tissues.

After exposure, fish were transferred back into bags containing oxygenated water, and transported in coolers to the National Water Research Institute wet-lab, where they were immediately processed. Transportation back to the laboratory took about 30-60 min. Fish were anaesthetized in the wet-lab with methane tricainesulfonate (MS-222), weighed, measured, and then bled from the caudal vein into nonheparinized vacutainers. Fish were euthanized by cervical dislocation and pronephros tissue was removed by dissection. The preparation of pronephros leukocyte suspensions and serum was previously described by Karrow et al. (1999). Single cell suspensions of pronephros leukocytes were prepared by pressing tissue through a 100 *um* stainless steel mesh with the flat end of a syringe plunger over a petri dish containing chilled Ca^{2+} and Mg^{2+} free HBSS (10 U heparin/ml, pH

7.4). The leukocyte suspension was then diluted to 7 ml with Ca^{2+} and Mg^{2+} free HBSS (10 U heparin/ml, pH 7.4), and centrifuged through 3 ml of Histopaque-1077 (d=1.077, Sigma Chemical Company, St Louis, MO, U.S.A) at 400 g for 30 min at 9°C. Leukocytes were collected at the interface, washed three times with HBSS, and resuspended in $NaHCO_3$ free RPMI 1640 medium + 25 *u*M HEPES + L-glutamine (pH 7.4, Gibco, Burlington, ON, Canada) supplemented with 10 % fetal calf serum (Gibco), 50 U/ml penicillin, and 50 *ug*/ml streptomycin (Gibco). Total leukocyte count and viability was determined by trypan blue exclusion. Cell viability was greater than 90 % in all samples. Immune assays were initiated within 24 h of sampling as suggested by Rice et al. (1996) to reduce the time constraints associated with incorporating immunological assays into field studies.

B. Pronephros leukocyte oxidative burst

The oxidative burst assay, previously described by Karrow et al. (1999), was modified for use on a fluorometric plate reader (Millipore Cytofluor 2350 plate reader) using 96-well plates. Triplicate control and sample cell suspensions (100 *u*l, 1 x 10^5 cells/well) were incubated with 4 *u*M of 2,7 dichlorofluorescin diacetate (DCFH-DA)(Molecular Probes Inc., Eugene, OR, U.S.A.) for 15 min in the dark at 20°C. Phorbol myristate acetate (PMA) (Molecular Probes Inc.. Eugene, OR, U.S.A.) was added at a final concentration of 100 ng/ml to the sample wells to activate the cells. The release of hydrogen peroxide (H_2O_2) within the cells oxidizes DCFH to 2',7'-dichlorofluorescin (DCF), which fluoresces at 530 nm. The total fluorescence was measured at 30 min intervals for 120 min using an excitation filter 530 nm and emission filter 590 nm. The net fluorescence (NF_{t120}) for each sample was measured as the slope of the change in fluorescence minus the slope of the change in fluorescence for the control wells.

C. Pronephros leukocyte phagocytosis

The methodology for measuring leukocyte phagocytic activity by flow cytometry was previously described by Karrow et al. (1999). Briefly, leukocyte suspensions (1 x 10^6 cells/ml) in 1 ml Leibovitz L-15 culture medium (GIBCO) were incubated with 10^8

fluorescent latex beads (1.03 *u*M diameter, Molecular Probes) for 18 h at 20°C. Negative controls were incubated in PBS with 1 % paraformaldehyde. Cells were washed by density gradient centrifugation through a mixture of 3 % BSA (GIBCO) and RPMI 1640 medium, then resuspended in 500 *ul* of Isoflow solution (Coulter Corp.. Hialeah, FL, U.S.A.). The cell suspensions were then analyzed by flow cytometry to determine the percent of cells engulfing three or more beads within the gated region drawn around the more granular and fluorescent cells to exclude lymphocytes. The mean fluorescence, which is a measure of the total bead uptake per cell, and the phagocytic index (I), where I is equal to the mean fluorescence times the number of cells engulfing three or more beads in 10 000 cells were also measured for each sample.

D. Pronephros immunoglobulin-positive (sIg$^+$) leukocyte marking

Sig$^+$ leukocyte marking was previously described by Karrow et al. (1999). Pronephros leukocytes (10^6 cells/ml) were incubated with 100 *ul* of ascites of the monoclonal antibody mouse-anti-trout 1-14 (1:100), a known B cell marker, of De Luca et al. (1983) (Courtesy of N.W. Miller) or 100 *ul* RPMI in 1 ml of RPMI 1640 medium for 45 min on ice. Cells were washed thrice, then incubated in the dark with goat-anti-mouse FITC (1:100. GIBCO). After three more washes, the number of surface immunoglobulin-positive (sIg$^-$) leukocytes in 10,000 leukocytes was then determined using flow cytometry by counting fluorescing cells within a gated region drawn around the less granular and fluorescent population of cells to exclude phagocytic cells.

E. Serum lysozyme activity

Measurement of lysozyme activity was described by Karrow et al. (1999). The assay measures a lysozyme-induced decrease in optical density of a 1.25 mg/ml *Micrococcus lysideikticus* (Sigma) PBS (pH7.5) suspension at 410 nm. Optical densities were measured over a 10 min incubation period with 10 *ul* of fish serum using a Microplate EL311 autoreader. The slope of the change in optical density was then converted to hen egg white lysozyme equivalents using lyophilized hen egg white lysozyme (Sigma) as a

white lysozyme equivalents using lyophilized hen egg white lysozyme (Sigma) as a standard.

F. Analysis of PAHs in the water, tissue and sediment

The extraction and cleanup of water samples for PAHs was described by Carey et al. (1988). Grab samples (1l) of water were taken on the last day of the study, spiked with a surrogate standard (EPA 525/525.1 Internal Standards Mix 525, Sigma-Aldrich, Oakville, ON) then extracted thrice with 100 ml of dichloromethane (DCM), concentrated under vacuum, then partitioned into 1 ml iso-octane for analysis. GC-MSD analysis was performed with a Hewlett-Packard (HP) 5972 MSD interfaced to an HP5890 Series II GC. Sample injection was made, in the splitless mode, by the HP7673A autosampler onto a 30 m x 0.25 mm ID x 0.25 m DB-5 fused silica capillary column (Chromatographic Specialties Inc., Brockville, ON). Injection port and detector interface temperatures were 265 °C and 285 °C, respectively. The GC oven temperature program was 80 °C initially, held at 80 °C for 3 min, increased to 180 °C at 60 °C/min, then increased to 280 °C at 30 °C/min, and held at 280 °C for 22 min. Helium carrier gas pressure was 7 psi. The electron energy and electron multiplier voltage were 70 eV and 2000 V, respectively. Quantitative analysis of 17 PAHs was performed by comparing the ion peak areas to an external PAH calibration standard (EPA 610 PAH Mixture, Sigma-Aldrich in Oakville, ON). Quantitation of the internal surrogate spike standard was used to determine the recovery of the procedure.

Extraction and cleanup of whole fish tissue for PAHs was performed by mixing 10 g of previously thawed homogenized fish tissue (5 pooled fish samples) with anhydrous sodium sulphate. After the addition of a surrogate standard (EPA 525/525.1 Internal Standards Mix 525, Sigma-Aldrich, Oakville, ON), PAHs were extracted from the tissue into DCM three times with a polytron homogenizer. Each time, the supernatant was decanted through Celite (Fisher Scientific, Toronto, ON) into a round bottom flask (RBF). The combined sample extract was dried through anhydrous sodium sulphate, concentrated under vacuum, then partitioned into 1:1 hexane in DCM for gel permeation chromatography (GPC). Sample

extracts were eluted with 1:1 hexane in DCM through a glass chromatography column (1.9 cm ID) filled to a length of 50 cm with pre-swelled Bio-Beads S-X3. After GPC, the extract was concentrated under vacuum, partitioned into 3 ml of iso-octane, then cleaned up by silica gel column chromatography, using about 8 cm of 3% deactivated silica gel (VWR-Canlab, Mississauga, ON) topped with 2.5 cm of anhydrous sodium sulphate in a glass chromatography column (12 mm x 350 mm). Elution of the sample through silica gel was accomplished with 40 ml of hexane (fraction 1) and 60 ml of 1:1 hexane in DCM (fraction 2). Collected fractions were evaporated down, partitioned into iso-octane and adjusted to 10 ml. Both fractions were analyzed by selected ion monitoring GC-MSD for their PAH content.

Extraction and cleanup of sediments for PAHs was achieved by homogenizing 10g of dried sediment sample with 3-4 ml of organic free water, adding a surrogate standard (EPA 525/525.1 Internal Standards Mix 525, Sigma-Aldrich, Oakville, ON), and extracting thrice into 100 ml of 1:1 acetone in hexane by sonification. Each time, the supernatant was decanted into a RBF through a filter funnel containing 5 cm of Celite. The combined sample extract was concentrated, back extracted thrice with 100 ml of organic free water, concentrated under vacuum, partitioned into about 3 ml of iso-octane, and passed through silica gel column for analysis by GC-MS as described above.

G. Measurement of total ammonia in water samples

Water ammonia concentrations were determined by TRAACS-800 automated colorimetric phenate procedure, specific to the determination of ammonia nitrogen concentrations up to 1.0 mg/L. Briefly, the addition of sodium phenoxide (VWR-Canlab, Mississauga, ON) to a solution of ammonium salts, in the presence of sodium hypochlorite (VWR-Canlab, Mississauga, ON) reacts, via the Berthelot reaction, to form a blue-coloured compound which can be measured spectrophotometrically at the 630 nm (NLET, 1994).

H. Statistics

A randomized complete block design (staggered cage replicates representing the block), two factorial analysis of variance (ANOVA) (factors=day,site) was used to analyzed the

data. When variation between cage replicates exceeded the variation within cages, the analysis was carried out on the means. Tukey's Post Hoc test was used to make pairwise comparisons of the data. In addition, a linear regression analysis was used to identify linear trends across time at each site. Again, when variation between cage replicates exceeded the variation within cages, the regression analysis was carried out on the means. The slopes of the regressions were compared with the slope of the Lake Ontario reference site according to Sokal and Rohlf (1981). All data were tested for compliance to the assumptions of normality and homogeneity of variance. Data sets, which violated these assumptions, were transformed using a log or square root transformation. Significance was determined at $p < 0.05$ unless otherwise indicated.

4.4 RESULTS

A. Water chemistry and physical profiles

A temperature increase of less than 5 °C was observed across all sites during the 21 d study (Fig. 4.2A). Water temperature at the Lake Ontario reference site was lower than at the Harbour sites. Dissolved oxygen concentrations changed little during the study, and were well above 7 mg/l at all sites, the exception being site 5 on 21 d, where oxygen concentrations dipped to 5.5 mg/l (Fig. 4.2B). Oxygen concentrations were lower at all Harbour sites compared to the Lake Ontario reference site. Free ion content, as measured by conductivity, was considerably higher at all the Harbour sites when compared to the Lake Ontario reference site (Fig. 4.2C). The pH profile decreased slightly at all sites during the study and was considerably lower at sites 5 and 6 (Fig. 4.2D). Total water ammonia levels, pH, and temperature were all used to calculate unionized ammonia concentrations during the study (Fig. 4.3). Unionized ammonia levels measured at the Harbour sites were clearly much higher than levels measured at the Lake Ontario reference site. With the exception of sites 1 and 3, unionized ammonia levels measured on the first day of exposure at all sites. exceeded the safe limit of 40 *ug*/l (Hurvitz et al., 1997). In general, ammonia levels were reduced as the water temperature increased, such that levels were below 30 *ug*/l at most of the sites by day 21. Ammonia levels at site 2 and 6 however, did not follow this trend. Ammonia levels initially decreased, then gradually increased to 31 *ug*/l by day 21.

Total and individual PAH concentrations in the water and sediment are summarized in Tables 4.1 and 4.2. Extraction recoveries for all PAHs were in the 70-95% range. Sample concentrations were not adjusted for the recoveries. The Lake Ontario site contained the second highest concentration of PAHs in the water column; napthalene and phenanthrene were the predominant PAHs (Table 4.1). Analysis of a second water sample has subsequently confirmed the total aqueous PAH levels at site 1, though the PAH profile was different (Appendix 3, table C1).

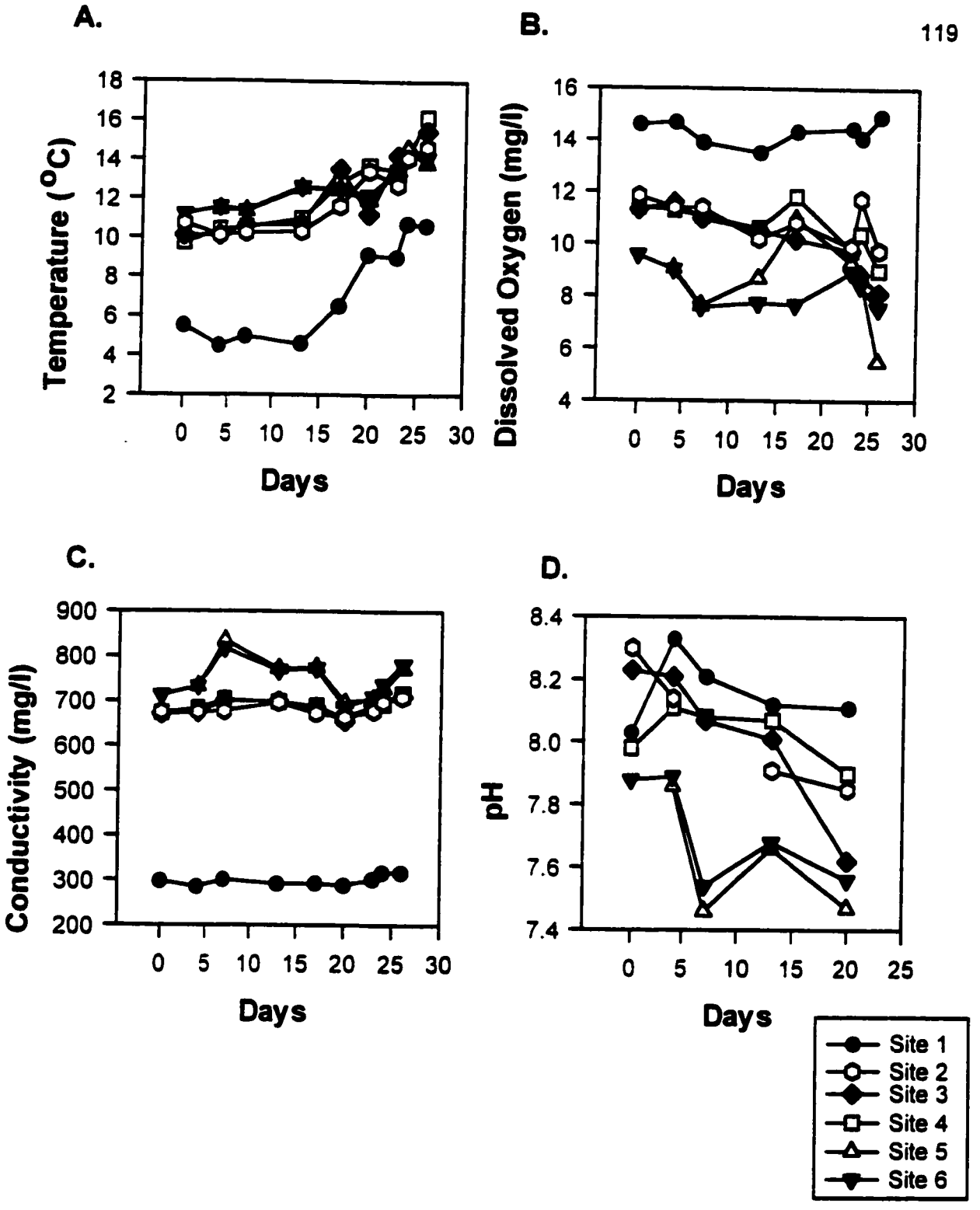

Figure 4.2: Temperature (A), dissolved oxygen (B), conductivity (C), and pH (D) profiles during the 21 d Hamilton Harbour study. Sites are labeled as; 1 (Lake Ontario), 2 (Burlington STP), 3 (Lax), 4 (Randle Reef), 5 (Windermere basin), and 6 (Dofasco boatslip).

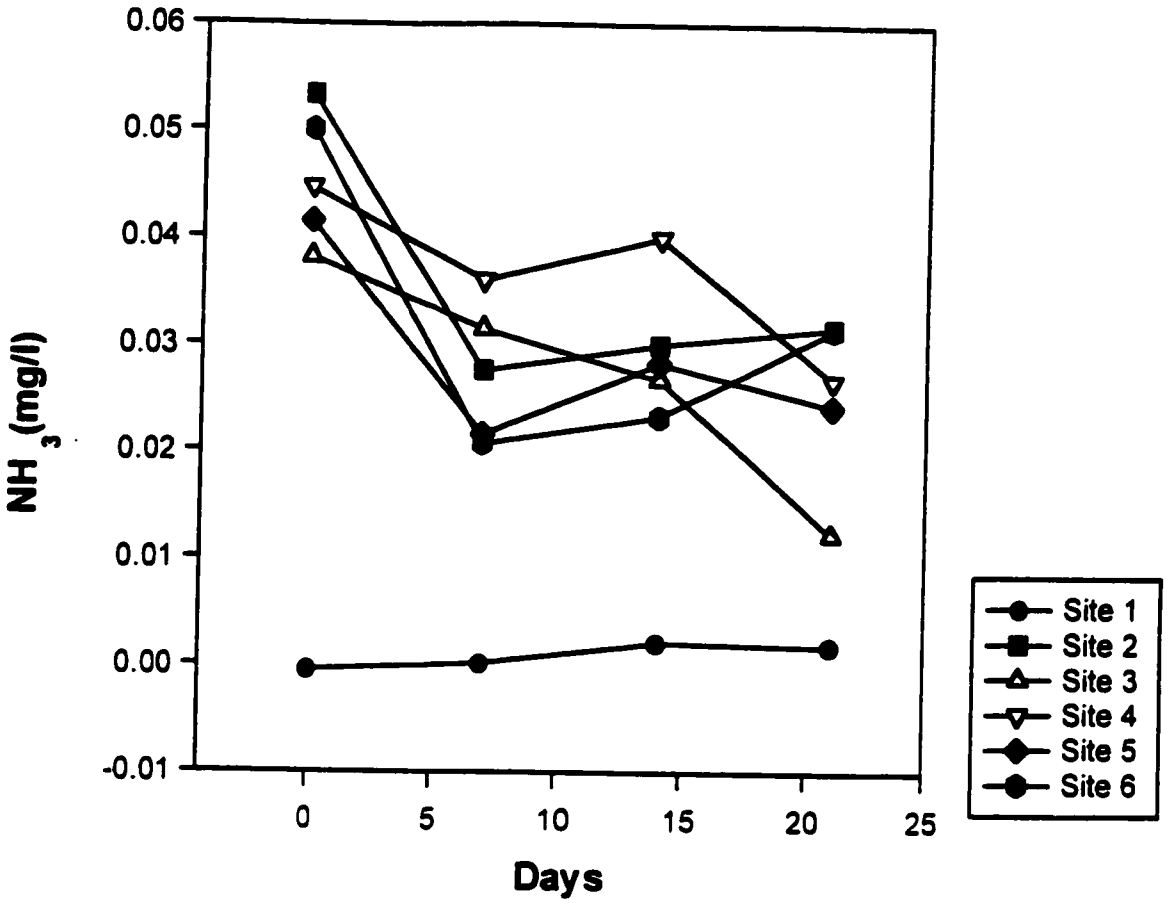

Figure 4.3: Unionized ammonia concentrations measured during the Hamilton Harbour 21 d study. Sites are labeled as; 1 (Lake Ontario), 2 (Burlington STP), 3 (Lax), 4 (Randle Reef), 5 (Windermere basin), and 6 (Dofasco boatslip).

Table 4.1: Water PAH concentrations for the Lake Ontario reference and Hamilton Harbour sites.[a]

PAH (*u* g/l)	Sites 1	2	3	4	5	6
Naphthalene	0.036	0.003	0.002	0.010	0.003	0.003
Acenaphthylene	nd	nd	nd	nd	nd	nd
Acenaphthene	0.004	0.007	0.008	0.005	0.007	0.006
Fluorene	0.005	nd	nd	nd	nd	nd
Phenanthrene	0.015	0.003	nd	nd	0.002	nd
Anthracene	nd	nd	nd	nd	0.015	nd
Fluoranthene	0.003	nd	nd	nd	nd	nd
Pyrene	nd	nd	0.002	0.002	0.004	0.002
Benzo[a]anthracene	0.005	0.006	0.005	0.005	0.004	0.008
Chrysene	nd	nd	nd	nd	0.005	nd
Total	0.068	0.019	0.017	0.022	0.040	0.019

[a] Sites 1 (Lake Ontario), 2 (Burlington), 3 (Lax), 4 (Randall's Reef), 5 (Windermere), 6 (Dofasco). Benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(a)pyrene perylene, indo(1,2,3-cd)pyrene, dibenzo(a,h)anthracene, and benzo(g,h,l)perylene were not detected in the water. (nd)= not detected

Table 4.2 summarizes sediment PAH concentrations at each site. Sites 3, 4, 5, and 6 contained total sediment PAH concentrations that were 7, 190, 21, and 33 fold higher than the Lake Ontario reference site. Total sediment PAH concentrations were lower at site 2 compared to the Lake Ontario site. Naphthalene was measured in whole fish tissue (0.057 *u*g/g wet weight) at site 4. PAHs were not detected in samples from any of the other sites, likely due to the rapid rate at which they are biotransformed in fish (Ariese et al., 1993).

B. Condition factor, mortality, liver/body weight ratio, and behaviour

In general, fish samples from sites 2 and 3 appeared lethargic, and more easily succumbed to the anesthetic compared to the Lake Ontario reference fish; fish from site 4 were also lethargic on day 7, but appeared hyperactive on day 21. Fish from sites 5 and 6 always appeared hyperactive. No fish mortalities were recorded for the duration of the harbour study; however, ventral surface skin abrasions and fin erosion were observed on some of the fish from various sites. While no apparent pattern of abrasion development was observed over the duration of the study, the percentage fish with abrasions from sites 3, 4, 5, and 6 was 17, 30, 30, and 20 % respectively; 23 % of the fish at site 5, and 10% of the Lake Ontario fish had abrasions on sampling day 21. Measured immune parameters from individual fish did not correlate with the presence of skin abrasions.

Fish condition factor (weight/length x 100) was significantly lower at site 2 when compared to the Lake Ontario reference site ($p<0.05$). Harbour sites 4 and 6 were also affected throughout the course of the study. Fish condition factors from both of these sites appeared to increase on day 7 with respect to day 0 baseline values. This was followed by a gradual decline in fish condition, resulting in a significant decreasing linear trend ($p<0.01$) over the course of the study. The measured slope of the regression line fit to data from each of these sites was significantly different from the slope of the

regression fit to the Lake Ontario data ($p<0.05$)(Fig. 4.4). No change in liver/body weight ratio was observed at any of the harbour sites.

Table 4.2: Sediment PAH concentrations for the Lake Ontario reference and Hamilton Harbour sites.[a]

PAH (*u* g/g dry weight)	Sites 1	2	3	4	5	6
Naphthalene	0.01	0.01	0.15	2.00	0.50	2.15
Acenaphthylene	nd	0.01	0.08	1.74	0.57	0.61
Acenaphthene	0.01	nd	0.02	1.47	0.14	0.19
Fluorene	0.01	nd	0.04	1.82	0.16	0.22
Phenanthrene	0.09	0.02	0.34	14.70	1.08	1.35
Anthracene	0.02	0.01	0.08	4.80	0.40	0.37
Fluoranthene	0.16	0.06	0.85	26.06	1.88	3.91
Pyrene	0.14	0.05	0.77	22.28	1.59	3.66
Benzo[a]anthracene	0.05	0.03	0.42	9.90	1.05	2.30
Chrysene	0.08	0.05	0.46	8.23	1.28	2.34
Benzo[b]fluoranthene	0.14	0.16	1.25	46.81	3.60	5.34
Benzo[e]pyrene	0.05	0.05	0.49	7.47	1.83	2.01
Benzo[a]pyrene	0.07	0.05	0.52	13.26	1.93	2.05
Perylene	0.03	0.08	0.25	4.21	0.68	0.88
Indeno[1,2,3-cd]pyrene	0.06	0.07	0.68	11.01	1.90	2.19
Dibenzo[a,h]anthracene	0.01	0.01	0.11	1.52	0.38	0.46
Benzo[g,h,i]perylene	0.05	0.05	0.50	8.96	1.69	1.71
Total	0.97	0.69	7.00	186.23	20.65	31.73

[a] Sites 1 (Lake Ontario), 2 (Burlington), 3 (Lax), 4 (Randall's Reef), 5 (Windermere), 6 (Dofasco). (nd)= not detected

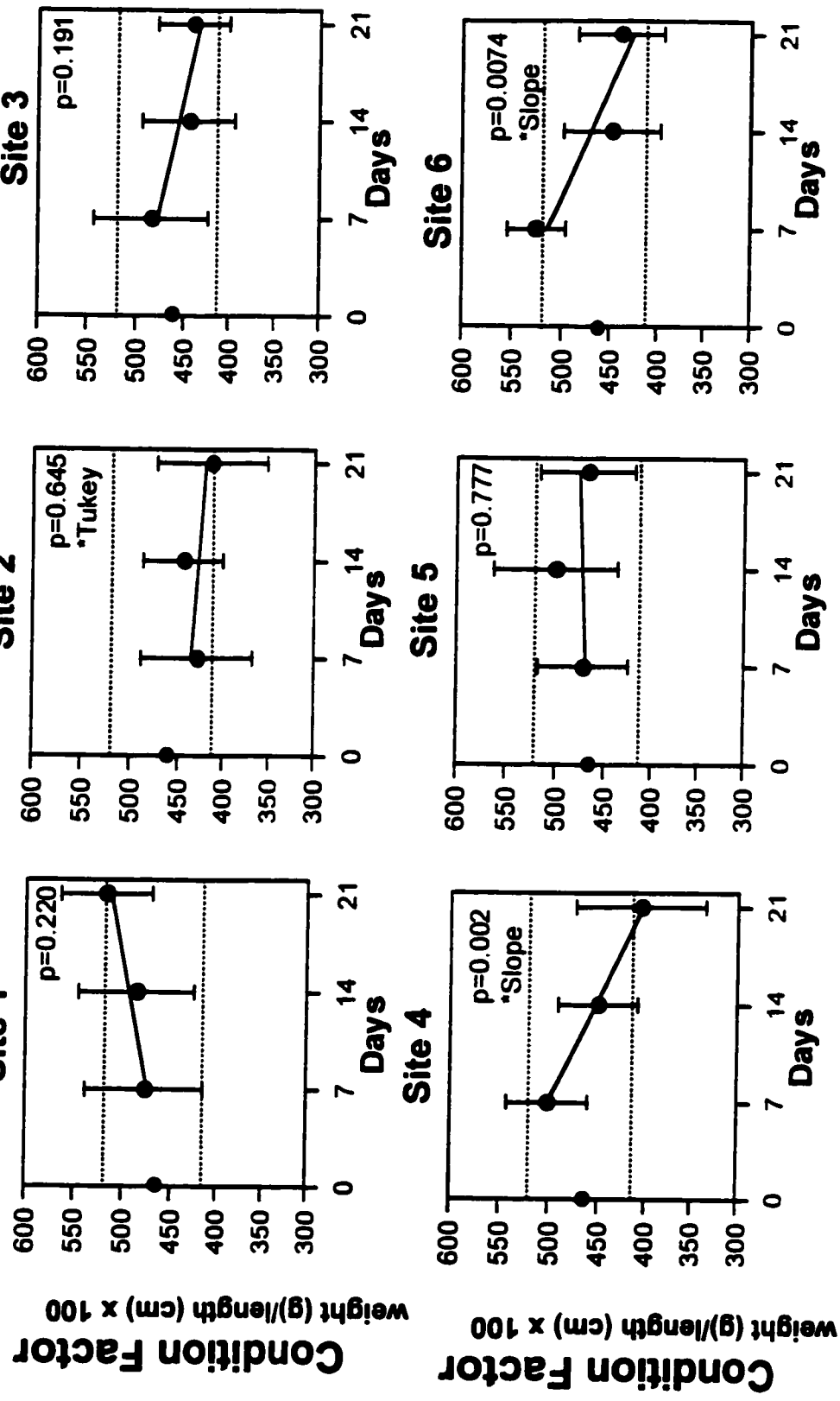

Figure 4.4: Mean rainbow trout condition factors (with 95% confidence intervals) measured on 0, 7, 14, and 21 d of the Hamilton Harbour study. Sites are labeled as; 1 (Lake Ontario), 2 (Burlington STP), 3 (Lax), 4 (Randle Reef), 5 (Windermere basin), and 6 (Dofasco boatslip). The results are expressed as the means of ten fish on each sampling day. Solid lines represent the linear regression equation y= a+bx through each data set accompanied by their respective p value. Sites which are significantly different from the Lake Ontario reference site ($p<0.05$) using Tukey's post Hoc test are indicated by (*Tukey). Slopes which are significantly different from the Lake Ontario site ($p<0.05$) are indicated by (*Slope).

C. Oxidative Burst

Pronephros leukocyte oxidative burst was significantly inhibited in fish during the 21 d exposure at sites 2 and 3 within the Harbour when compared to the Lake Ontario reference site. The decline in oxidative burst followed a linear relationship over time at both sites ($p<0.01$); an apparent, but non-significant decrease was also observed at site 4. A comparison of the slopes of the regression line through the cage means at sites 2 and 3 was also significantly different from the regression slope through the Lake Ontario cage means ($p<0.05$)(Fig 4.5).

D. Phagocytosis

The percent of pronephros leukocytes engulfing three or more beads was reduced during exposure in fish caged at a number of the Harbour sites when compared to the Lake Ontario reference site. Significant decreasing linear relationships were observed throughout the course of the study at sites 3, 4, and 6 ($p<0.05$), and at site 5 ($p<0.01$). The slope of the regression through the cage means from each of these sites was also significantly different from the Lake Ontario control response slope ($p<0.05$)(Fig 4.6).

The mean fluorescence of pronephros leukocytes was also reduced during exposure in fish sampled from sites 2 and 3 when compared to the Lake Ontario reference site. A significant linear decrease in fluorescence was observed at both these sites over the 21 days of exposure ($p<0.05$); apparent, but non-significant decreases were observed at sites 4, and 6. The measured slope of the regression line through the cage means at sites 2 and 3 was also significantly different from the regression slope through the cage means at the Lake Ontario reference slope($p<0.05$); differences at sites 4, and 6 were again apparent, but non-significant (Fig 4.7).

Similar responses were observed then the phagocytosis data were calculated using the phagocytic index (Fig 4.8). Significant decreasing linear relationships were observed during the study at sites 3, 5, and 6 ($p<0.05$). The regression slopes of the

cage means at all sites, including sites 2 and 4, were significantly different from the regression slope through the cage means of the Lake Ontario reference site ($p<0.05$).

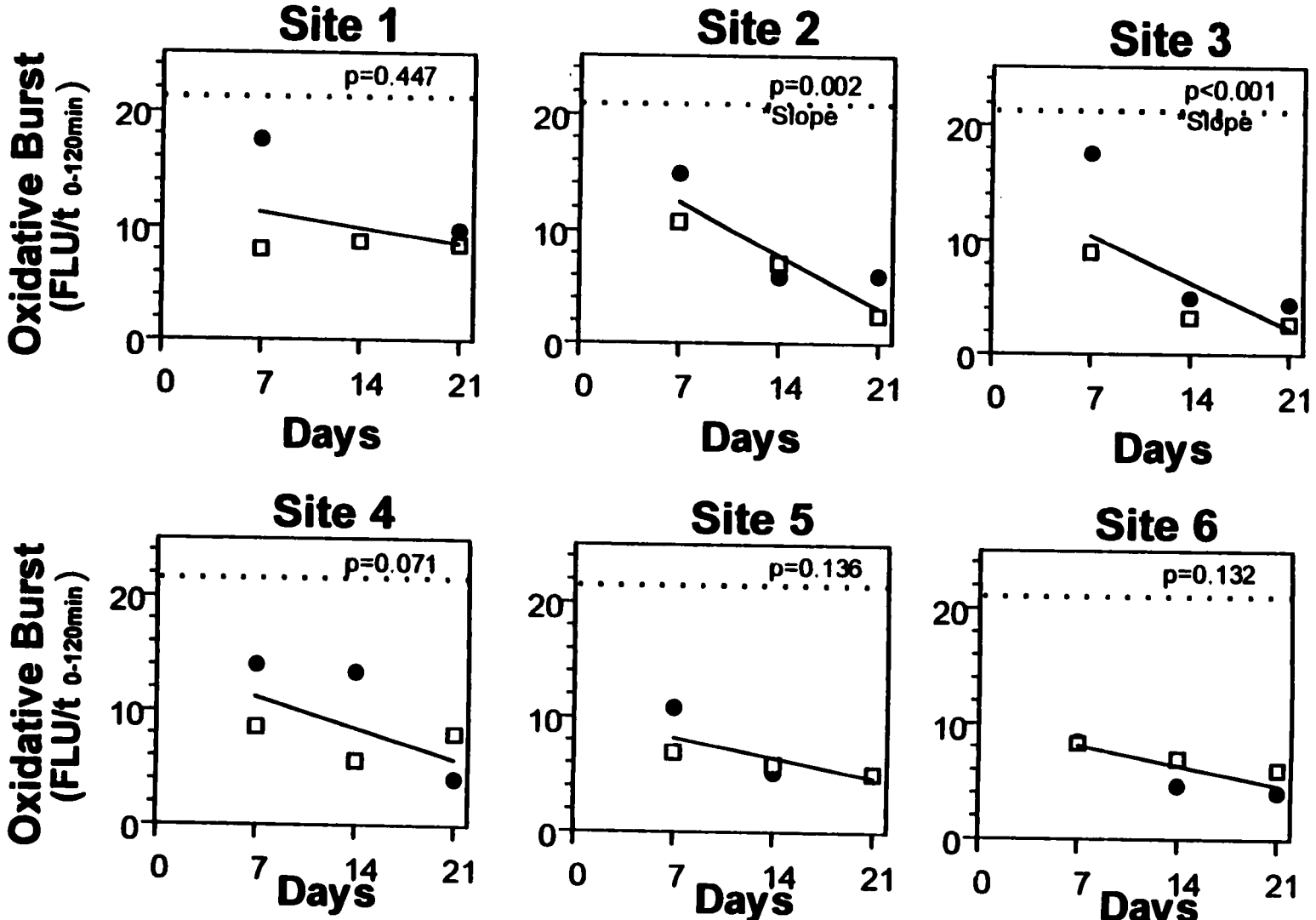

Figure 4.5: Oxidative burst of pronephros leukocytes from rainbow trout sampled on 0, 7, 14, and 21d of the Hamilton Harbour. Sites are labeled as; 1 (Lake Ontario), 2 (Burlington STP), 3 (Lax), 4 (Randle Reef), 5 (Windermere basin), and 6 (Dofasco boatslip). The results are expressed as the mean of five fish for each replicate cage at each sampling time. Solid lines represent the linear regression equation y= a+bx through the cage means accompanied by their respective p value. The dotted line represents the baseline mean on day 0. Slopes which are significantly different from the Lake Ontario site ($p<0.05$) are indicated by (*Slope).

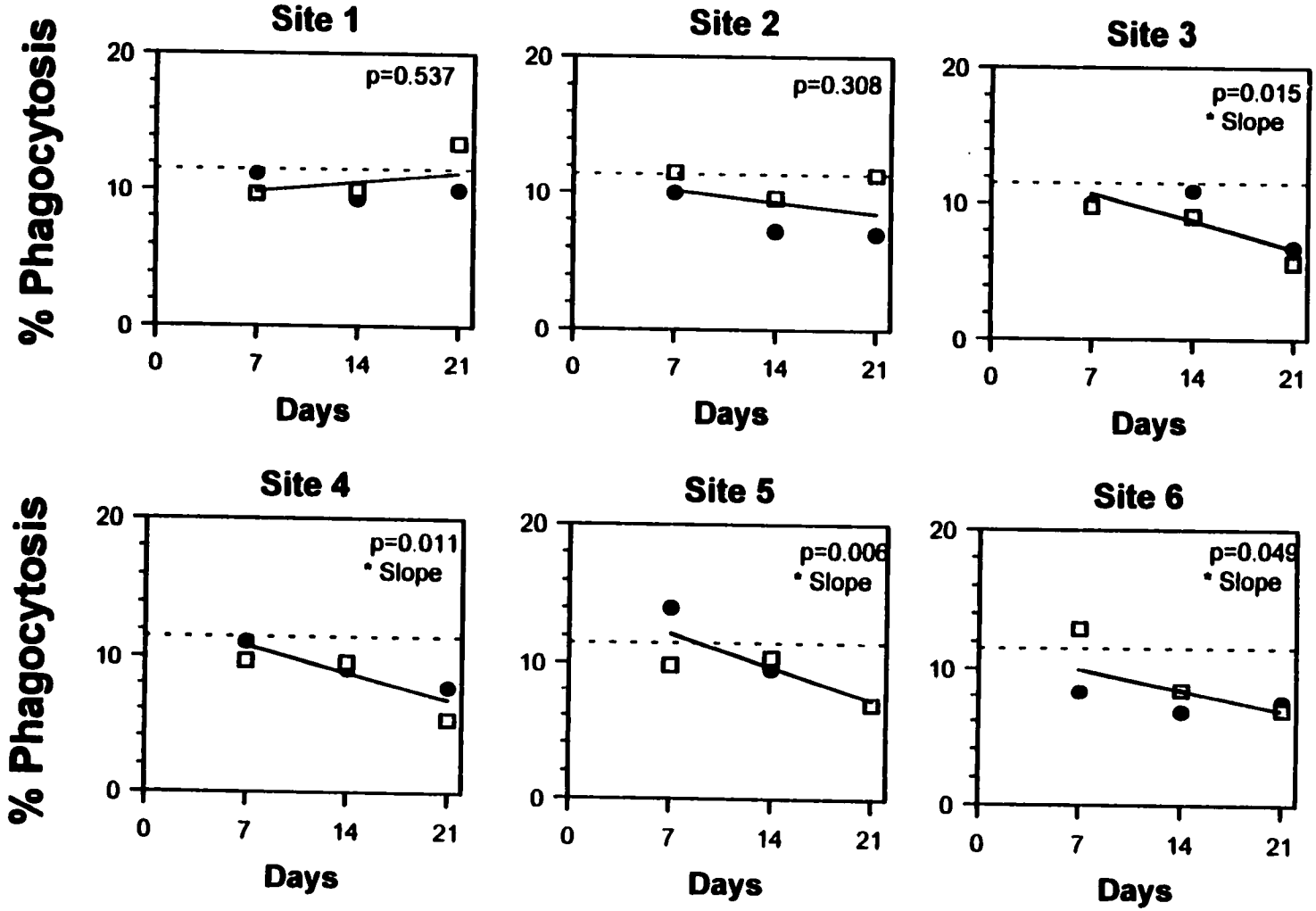

Figure 4.6: Percent of phagocytic cells engulfing three or more beads of pronephros leukocytes from rainbow trout sampled on 0, 7, 14, and 21d of the Hamilton Harbour. Sites are labeled as; 1 (Lake Ontario), 2 (Burlington STP), 3 (Lax), 4 (Randle Reef), 5 (Windermere basin), and 6 (Dofasco boatslip). The results are expressed as the mean of five fish for each replicate cage at each sampling time. Solid lines represent the linear regression equation y= a+bx through the cage means accompanied by their respective p value. The dotted line represents the baseline mean on day 0. Slopes which are significantly different from the Lake Ontario site ($p<0.05$) are indicated by (*Slope).

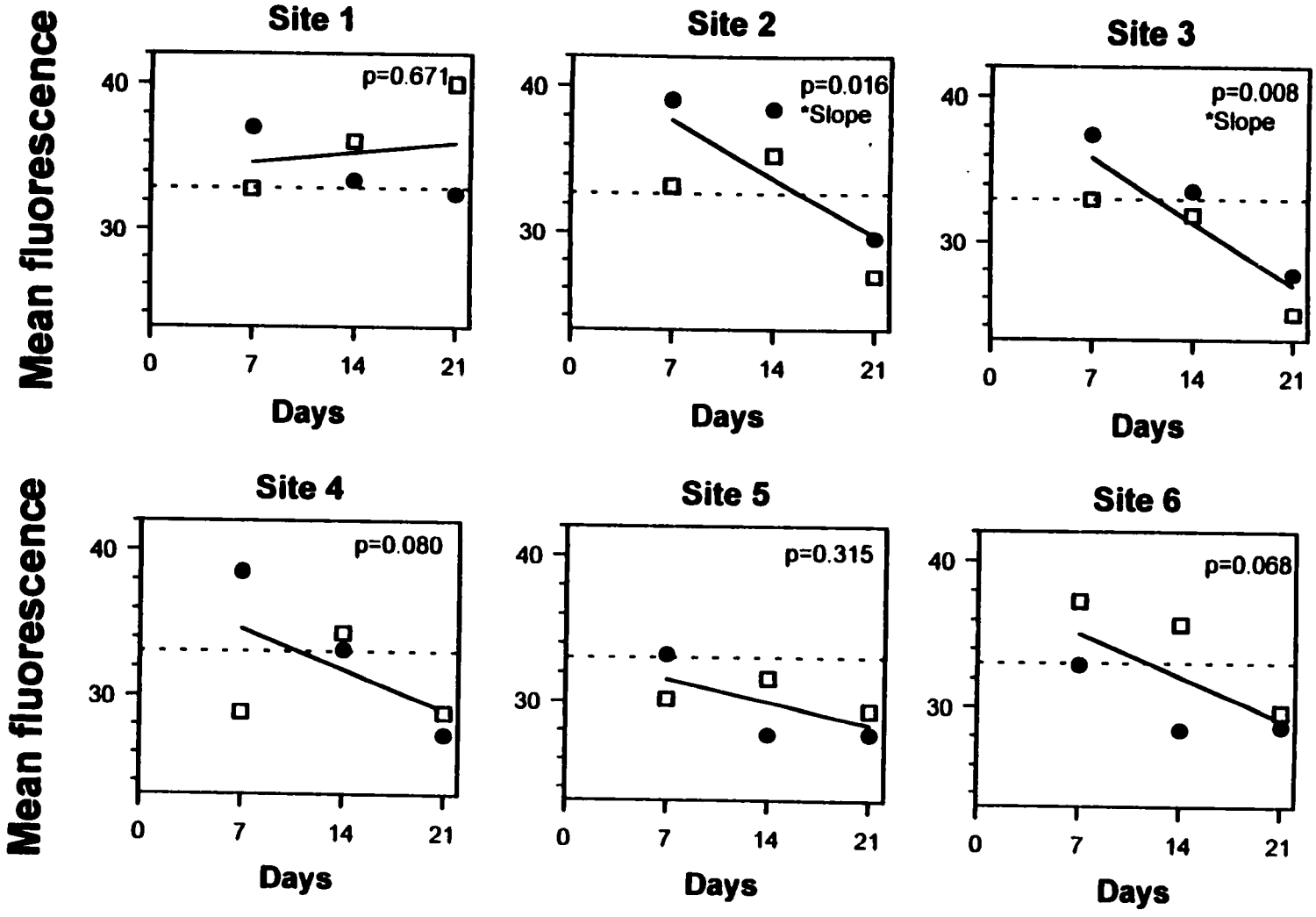

Figure 4.7: Mean fluorescence of phagocytic cells engulfing three or more beads of pronephros leukocytes from rainbow trout sampled on 0, 7, 14, and 21d of the Hamilton Harbour. Sites are labeled as; 1 (Lake Ontario), 2 (Burlington STP), 3 (Lax), 4 (Randle Reef), 5 (Windermere basin), and 6 (Dofasco boatslip). The results are expressed as the mean of five fish for each replicate cage at each sampling time. Solid lines represent the linear regression equation $y = a+bx$ through the cage means accompanied by their respective p value. The dotted line represents the baseline mean on day 0. Slopes which are significantly different from the Lake Ontario site ($p<0.05$) are indicated by (*Slope).

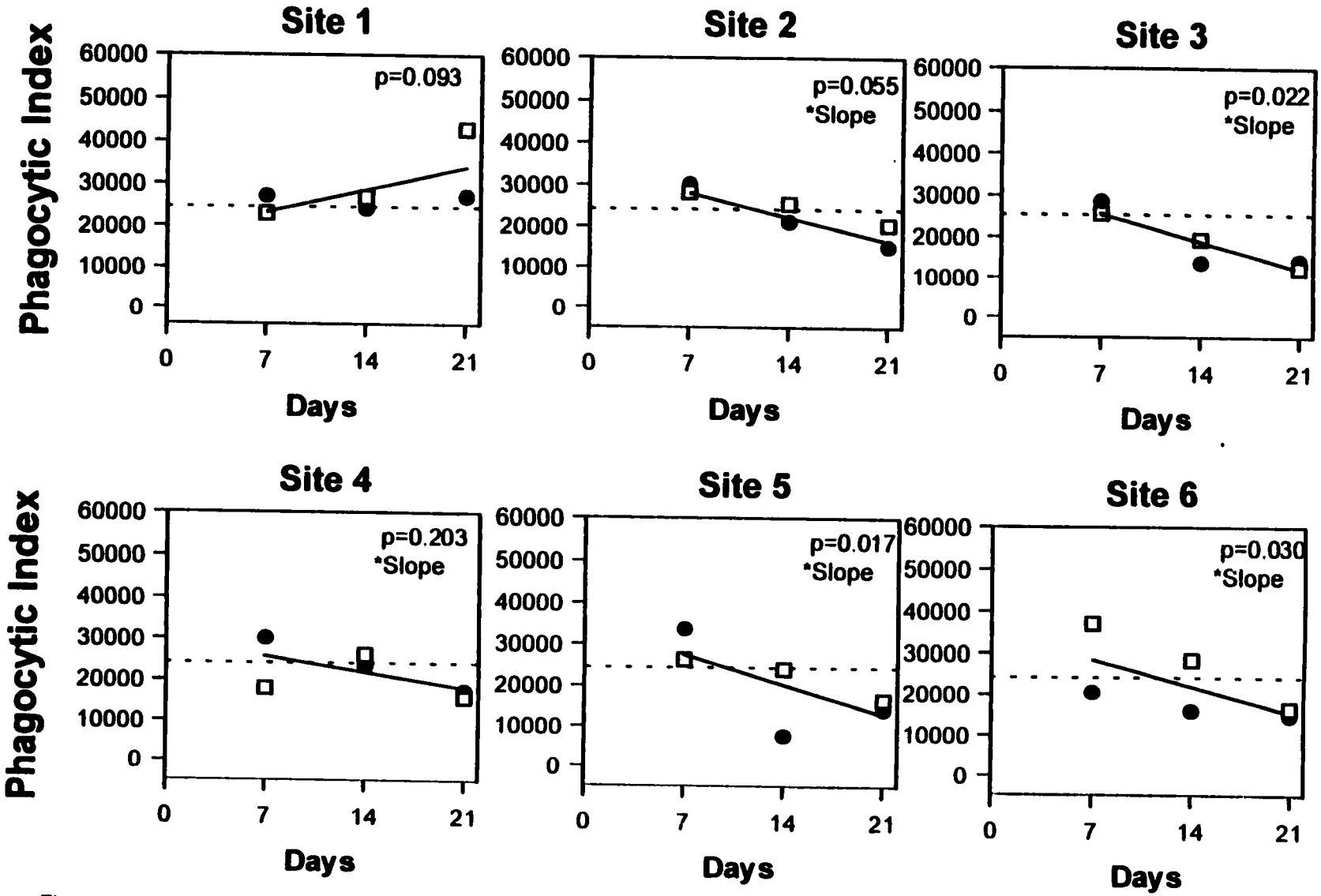

Figure 4.8: Phagocytic index of pronephros leukocytes from rainbow trout sampled on 0, 7, 14, and 21d of the Hamilton Harbour. Sites are labeled as; 1 (Lake Ontario), 2 (Burlington STP), 3 (Lax), 4 (Randle Reef), 5 (Windermere basin), and 6 (Dofasco boatslip). The results are expressed as the mean of five fish for each replicate cage at each sampling time. Solid lines represent the linear regression equation y= a+bx through the cage means accompanied by their respective p value. The dotted line represents the baseline mean on day 0. Slopes which are significantly different from the Lake Ontario site ($p<0.05$) are indicated by (*Slope).

E. SIg^+ leukocyte marking

Pronephros sIg^+ leukocyte counts measured at sites 3 and 6 were significantly lower than those measured at the Lake Ontario site ($p<0.05$). Counts at sites 2, 4, and 6 also appeared lower than the Lake Ontario reference site, but were not statistically significant. A significant change in sIg^+ leukocyte counts was not observed at any of the sites during the course of the study.

F. Serum lysozyme activity

Serum lysozyme concentrations were significantly higher at sites 5 and 6 than at the Lake Ontario reference site ($p<0.01$). Within the Harbour, lysozyme concentrations at site 6 were significantly higher than levels measured at sites 2, 3, and 4 (Fig. 4.10). Sites 1 and 4 showed a significant linear increase in lysozyme activity during exposure. A comparison of the regression slope through the fish serum lysozyme levels at each site, revealed that site 6 was significantly different than the Lake Ontario ($p<0.05$).

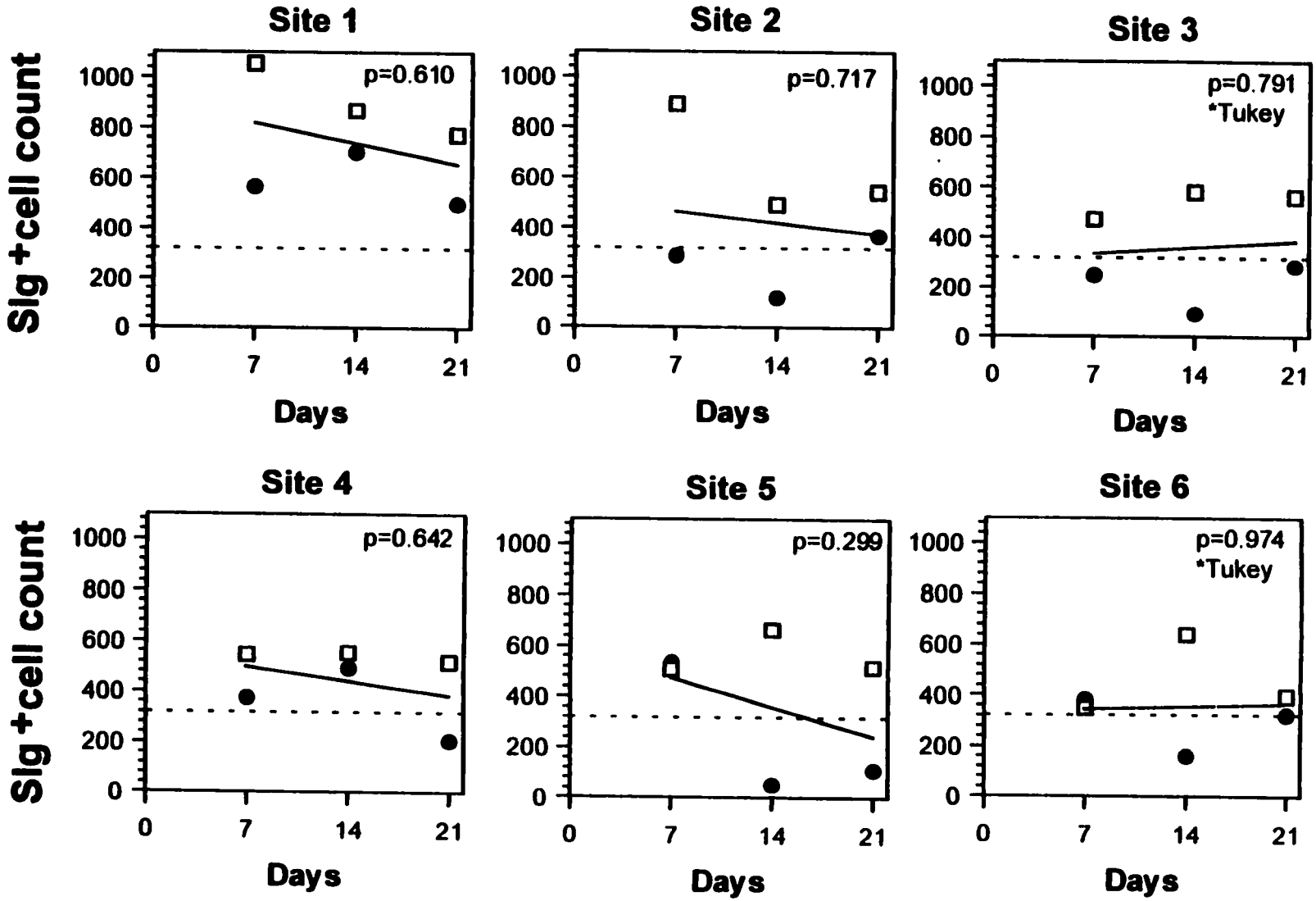

Figure 4.9: Percent of Sig+ lymphocytes from rainbow trout sampled on 0, 7, 14, and 21d of the Hamilton Harbour. Sites are labeled as; 1 (Lake Ontario), 2 (Burlington STP), 3 (Lax), 4 (Randle Reef), 5 (Windermere basin), and 6 (Dofasco boatslip). The results are expressed as the mean of five fish for each replicate cage at each sampling time. Solid lines represent the linear regression equation y= a+bx through the cage means accompanied by their respective p value. The dotted line represents the baseline mean on day 0. Sites which are significantly different from the Lake Ontario site ($p<0.05$) are indicated by (*Tukey).

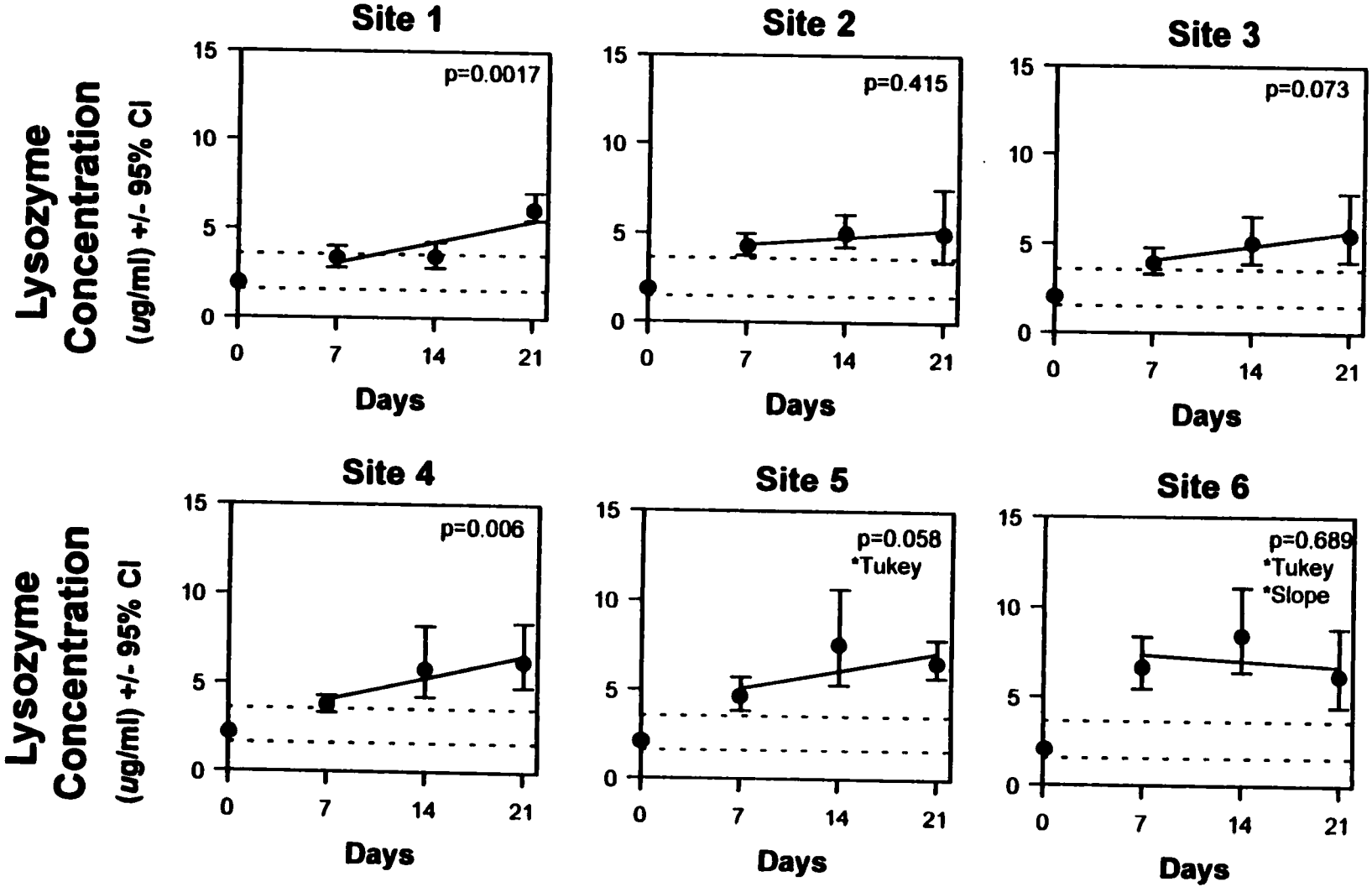

Figure 4.10: Mean (with 95% confidence intervals) lysozyme concentrations in serum from rainbow trout sampled on 0, 7, 14, and 21d of the Hamilton Harbour. Sites are labeled as; 1 (Lake Ontario), 2 (Burlington STP), 3 (Lax), 4 (Randle Reef), 5 (Windermere basin), and 6 (Dofasco boatslip). The results are expressed as the means of ten fish on each sampling day. Solid lines represent the linear regression equation y= a+bx through the cage means accompanied by their respective p value. Sites which are significantly different from the Lake Ontario site ($p<0.05$) are indicated by (*Tukey). Slopes which are significantly different from the Lake Ontario site ($p<0.05$) are indicated by (*Slope).

4.5 DISCUSSION

Exposure of caged rainbow trout over 21 d at various sites within Hamilton Harbour altered several immune parameters over time. Some of the immunological parameters were however, more sensitive than others (Table 4.3). When compared to the Lake Ontario fish, the oxidative burst of pronephros leukocytes, pronephros B lymphocyte counts and serum lysozyme levels were only affected at two of the Harbour sites. The phagocytic index of pronephros leukocytes, on the other hand, was significantly inhibited at all, emphasizing the sensitivity of this parameter for assessing the impact of stressors on the fish immune system (Fournier et al., 1998). The reduced phagocytic index was attributed to a decrease in the percent of phagocytic cells at site 5, and a reduction in phagocytic activity at site 2. The inhibited phagocytic index at sites 3, 4, and 6 was due to a combination of both phagocytic parameters.

Modulation of the selected immune parameters at various sites within the Harbour is likely attributable to multiple stressors, because numerous immunotoxic contaminants have been identified within the Harbour water and sediments. Heavy metal concentrations were not measured in the present study. However, levels of Cr, Cu, Pb, and Zn in the Harbour sediment have been reported to be as high as 500, 160, 700 and 4 500 *ug/g*, respectively, within the central basin of the Harbour (Krantzberg and Boyd, 1992). It has been suggested that complexation of metals with either iron or sulfur compounds may restrict the bioavailability of these compounds to fish. However, Krantzberg and Boyd (1992) showed that 21 d laboratory exposed fathead minnows (*Pimephales promelas*) and mayfly nymphs (*Hexagenia limbata*) to sediments from sites centrally located within the Harbour, generally resulted in higher tissue residues of As, Cd, Cr, Cu, Fe, Pb, Ni, and Zn than in control organisms. Organisms exposed to sediment from the Windermere channel did not exceed control tissue. Numerous examples of metal-induced immunomodulation in fish have been reported. Dunier (1994b) for example, demonstrated that Cu and Zn suppressed carp spleen and pronephros macrophage

activity at 12.5 and 3 mg/l respectively. Cu (0.3 mg/l) also suppressed lymphocyte blastogenic response to phytohaemagglutinin (PHA). Zelikoff (1994) reported that trout peritoneal macrophage activity, as measured by hydrogen peroxide and superoxide production, was reduced after 30 days of exposure to 2 *ug*/l of Cd. Though most researchers have reported an immunosuppressive response, low doses may also be immunostimulatory, possibly due to stress and subsequent hyperactivity (Anderson, 1996).

Table 4.3: Summary of immune parameters, from fish caged at various sites within the Hamilton Harbour, which were significantly different from the Lake Ontario reference fish.

Parameter	Sites 2	3	4	5	6
Oxidative Burst	+	+			
Phagocytosis					
% phagocytosis		+	+	+	+
mean total fluorescence	+	+			
phagocytic index	+	+	+	+	+
SIg^+ leukocyte count		+			+
Lysozyme activity				+	+

(+)= significantly different from the control ($p<0.05$)

PAHs may have also contributed to the immunosuppression observed at various sites within the Harbour, more specifically sites 3, 4, 5 and 6. Previous studies conducted in our laboratory, and in other laboratories, indicate that PAHs modulate immune parameters in fish. Sewage sludge containing PAHs and hydrocarbon contaminants has been shown to reduce pronephros leukocyte oxidative burst and serum lysozyme activity in dab (*Limanda limanda*) (Secombes et al., 1997). Inhibited oxidative burst and stimulated phagocytic activity from pronephros leukocytes, reduced numbers of peripheral blood SIg^+ B cells, and decreased plasma lysozyme activity were observed in rainbow trout exposed to creosote contaminated sediments, containing PAHs (Karrow et al., 1999). Reports of suppressed phagocytic activity were also made by Weeks and Warriner (1986) in spot (*Leiostomus xanthurus*) and hogchoker (*Trinectes maculatus*) pronephros leukocytes exposed to PAH contaminated sediments from the Elizabeth River, VA. Lemaire-Gony et al. (1995) reported reduced oxidative burst in European sea bass (*Dicentrarchus labrax*) splenic macrophages after intraperitoneal dosing with benzo(a)pyrene. Lastly, a study by Faisal and Huggett (1993) showed that spot pronephros lymphocyte proliferation in response to the mitogen, concanavalin A, was significantly inhibited at sites along the Elizabeth River.

In the present study, PAH concentrations in the water did not correlate with immunomodulatory responses measured at the various sites. Total PAH concentrations at the Lake Ontario reference site, for example, were higher than those measured at most of the Harbour sites. These PAHs appear to have little or no immunomodulatory effects in trout at the concentrations measured, since immunosuppression was not observed at this site using our panel of immune assays. Initially, we suspected that the water sample had some how been contaminated by the boat exhaust during sampling. However, repeated sampling has subsequently confirmed that PAHs are present in the water column at site 1, though the PAH profile appears variable. The apparent source(s) of these PAHs are unknown. A major expressway is located nearby, and the prevailing

wind from the Harbour's steel mills is, however, over the Lake. It is unlikely that sources would include sediment-bound PAHs partitioning back into the water column, since Lake Ontario sediment PAH concentrations were less than 1.0 *ug*/g dry weight.

Immunosuppression appeared to be more pronounced at sites which had PAH contaminated sediments, even though the degree to which immunosuppression occurred did not parallel sediment PAH concentrations. The number of suppressed immune parameters at sites 3, 5 and 6, for example, was greater than the number observed at site 4, where sediment PAH concentrations were the highest (Table 4.3). This suggests that other compounds may also be contributing to the immunosuppression observed at these sites. Results from Parrott et al. (1998) reported that the induction of trout hepatic mixed function oxidase (MFO), in these same fish, was greater at sites containing PAH contaminated sediments, though induction at sites 5 and 6 was greater than at site 4. It is believed that metabolic activation is required before certain PAHs can target the immune system. PAH metabolites have been shown to bind to protein tyrosine kinases (PTKs) and Ca^{2+}-ATPases, thereby disrupting Ca^{2+} signals within lymphocytes, possibly resulting in apoptosis (Krieger et al., 1995; Davila et al., 1997; Zhao et al., 1997). Faisal and Huggett (1993) also showed that spot pronephros lymphocyte proliferation was reversed in benzo(a)pyrene and benzo(a)pyrene- 7,8 dihydrodiol exposed lymphocytes by α-naphthaflavone, a potent cytochrome P450 inhibitor, suggesting that immunosuppression in fish caused by certain PAHs may also involve cytochrome P450 dependent pathways. The higher 7-ethoxyresorufin-*O*-deethylase (EROD) induction at sites 5, and 6 may be attributed to PAH derivatives, other Ah-receptor binding compounds (i.e. PCBs) not measured in this study, and/or the interaction of certain heavy metals (Lemaire-Gony et al., 1995). Sediment PCB and Cu concentrations have been documented greater than 2000 ng/g, and within the range of 151-225 *ug*/g, respectively at sites 5, and 6 (RAP, 1992). Lemaire-Gony et al. (1995) reported that long-term Cd exposure modified the induction of hepatic EROD activity by benzo(a)pyrene. EROD activity was induced by a

factor of 11 and 21 for Cd and benzo(a)pyrene respectively; a combination of the two compounds resulted in a 34 fold induction. This induction of MFO by chemical mixtures could potentially increase the risk of PAH immunotoxicity by increasing the rate at which reactive PAH metabolites are produced.

Alkylphenols were also identified at a number of the Harbour sites, in water, sediment and fish tissues (Gamble et al., 1998). The source of these compounds in the aquatic environment has previously been linked to sewage treatment plant effluents (Bennie et al., 1997). 4-nonylphenol (4-NP) water concentrations at sites 5 and 6 were 0.984 and 0.858 *ug/l*; fish tissue concentrations were 0.054 and 0.068 *ug/g* wet weight respectively. Although little is known about the immunomodulatory effects of alkylphenols, a recent study by Rice et al. (1998) showed that dietary 4-NP (10 ppm) and arochlor 1254 (10 ppm) enhanced channel catfish, (*Ictalurus punctatus*) head kidney neutrophil oxidative burst activity and reduced the percent of B cells. 4-NP was also reported to enhance aroclor 1254 induction of gut and liver CYP1A.

Unionized ammonia levels in the Harbour may have also been high enough to modulate rainbow trout immune parameters during the 21 d study. Initially, ammonia levels were greater than 40 *ug/l* at all Harbour sites, with the exception of site 3. These levels however, declined during exposure. A sufficient change in ammonia levels during the first week of the study may account for some of the variation between cages at each site, since the deployment of cages was staggered by five days. Hurvitz et al. (1997) reported that long term exposure of trout to unionized ammonia (60-80 *ug/l*) was sufficient to modulate an immune response. Although they reported that trout antibody titers, following vaccination with (*Streptococcus imiae),* were similar to control fish, resistance to streptococcus infection was significantly reduced. This is indicative of modulated cellular or innate immunity. Plasma lysozyme activity was also inhibited in rainbow trout by acute exposure to unionized ammonia as reported by Mock and Peters (1990). In light of the high ammonia concentrations found in the Harbour, channel

catfish, being more ammonia tolerant than rainbow trout, may be a more suitable test species for future Harbour studies.

In addition to the numerous chemical stressors, fish were also subject to a number of physical stressors, which may have modulated the immune system. High levels of microorganisms associated with certain sewage treatment plants may ,for example, modulate fish immune parameters (Secombes et al., 1997). In addition to this, dissolved oxygen concentration, water temperature, and pH measurements from the Lake Ontario reference site were considerably different from the Harbour site measurements, emphasizing the difficulty in finding suitable reference sites for studies of this scale. These water quality parameters may have influenced the respective fish immune parameters measured at each site. Hardie et al. (1994), for example, reported that macrophage activating factor induced the up-regulation of oxidative burst measured in rainbow trout pronephros macrophages at 14 °C but not at 7 °C. Thus, it appears as though T cell function may be inhibited at the range of temperatures measured at the Lake Ontario site. Raymond et al. (1998) reported that Medaka (*megalobathracus japonicus*) immune parameters were affected during exposure to low pH ground water contaminated with organic and heavy metal contaminants. As well, fish from sites 4 and 6 showed a significant linear decrease in their condition factors over 21 d, indicating that the energy expendature to maintain homeostasis may have been greater at these sites. This may seriously affect fish health, as cellular and humoral disease resistance is very energy demanding (Schreck, 1996). Although no correlation was observed with immunological assays, the high number skin abrasions observed at a number of sites suggests that Harbour fish were more stressed during exposure than the Lake Ontario reference fish. Fish behaviour may be linked to the presence of these abrasions, as hyperactive fish from sites 4, 5, and 6 had the highest percentage of abrasions; lethargic fish from sites 2 and 3 had few, if any, abrasions. These hyperactive fish may have been in contact with the cage material more often, resulting in physical trauma to the skin.

Increased activity may have also contributed to the decrease in fish condition observed at sites 4 and 6. Whether or not this behavioural modification is attributed to chemical and/or physical stressors. remains to be determined, as other stressors such as noise and turbulence, generated from shipping traffic at these sites, may have acted as confounding variables. Stress associated with the handling and transportation of fish has also been well documented, and can influence hormone plasma protein levels within minutes. Levels of cortisol and catecholamines can increase within 30 s of netting; serum complement and lysozyme levels can increase within 10 min (Demers et al., 1997). With this in mind, it is possible that serum lysozyme levels increased during transport back to the laboratory. All fish were however, processed in a similar manner. Sampling stress did not likely influence the oxidative burst, phagocytosis, and Slg^+ B cell assays, since cells were allowed to recover over night in culture media before analysis.

Although we have not shown that an immune response is impaired in this study, it is clear that exposure to sites within the Hamilton harbour can modulate phagocytic function, B cell numbers. and lysozyme activity, possibly to the degree whereby host resistance to infection is compromised. More information is required before a relationship between these functional changes in the immune system and the development of clinical disease can be established (Karol, 1998). In a follow up study, researchers may wish to consider antigenic challenge prior to exposure. Thus, a change in the following immune parameters could be directly linked to a specific immune response.

The identification of the immunomodulating compound(s) in this study, may prove to be challenging. A study conducted by Lemaire-Gony et al. (1995) emphasized the complexity of interpreting responses associated with multiple chemical stressors. Exposure of European sea bass (*Dicentrarchus labrax*) to Cd (40 ug/l) and benzo(a)pyrene (20 mg/kg) in combination, inhibited the oxidative burst from splenic macrophages. This response was attributed to benzo(a)pyrene rather than Cd exposure. Kidney macrophage oxidative burst on the other hand, was enhanced by Cd

and benzo(a)pyrene alone, and also when the two toxicants were combined. Splenic and kidney macrophage phagocytosis, however, appeared to be synergistically inhibited by the combination of these two chemicals. This example emphasizes that interpretation of immunotoxicity may depend on a number of variables, including the type of immune parameter being measured, tissue, species, and potential interaction with other systems (i.e. neuroendocrine and hepatic systems).

The goal of this study was to assess whether or not exposure to a number of sites within the Hamilton Harbour was sufficient to alter immune parameters in fish. We have shown that several immune parameters were modulated, not only with respect to duration of exposure, but also when compared to the Lake Ontario reference site. Future studies may wish to focus on the Windermere Basin, since immune effects were severe at sites 5 and 6. For logistic reasons, may also be more useful to restrict sampling to 21 days, as altered immune parameters were the most pronounced after this duration of exposure. By restricting sampling to a smaller region, fish sample sizes could be increased at each site. Cage replicates could also be incorporated into the study, without having to complicate the study design by staggering exposure. Recommended assays would include phagocytic activity and oxidative burst, because of their sensitivity. Challenge by vaccination should also be considered to determine whether or not exposure is sufficient to modulate an immune response. B cell counts and serum immunoglobulin levels could be used to measure a change in immune response, provided the sample size is increased to accommodate the large fish to fish variability.

4.6 CONCLUSION

The results from this study indicate that immune parameters were modified in rainbow trout caged at various sites within the Hamilton Harbour. Modification of some of these parameters appeared to be more pronounced with the duration of exposure. Although identification of the stressors remains to be determined, immunomodulation at various sites within the Harbour may be attributed to multiple stressors, since numerous immunotoxic contaminants have been identified within the Harbour water and sediments.

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SUMMARY AND GENERAL CONCLUSIONS

The studies compiled in the thesis were designed to assess the immunotoxicity of chemical mixtures to fish. A panel of recently developed immune assays was incorporated into two creosote microcosm studies, and a field study conducted in the Hamilton Harbour, to assess the hazard of exposure to chemical mixtures. Attempts were made to establish concentration-response relationships, and monitor immune parameters during exposure.

The results from these studies demonstrated the potential that immune parameters offer as bioindicators of exposure and effect when assessing chemical mixture toxicity in the field. Concentration-response relationships were established for liquid creosote using the panel of immune assays. Although several of the responses were reproducible over two field seasons, observations from the kinetic study suggested that they were temporally dependent. The biological significance of these temporal changes remains to be determined, however, such changes must be considered by ecotoxicological risk assessors when assessing the health risks of exposure to chemical mixtures.

Results from the Hamilton Harbour study showed that exposure to various chemical and physical stressors in the Harbour was sufficient to adversely affect measured immune parameters. Again, modulation of the immune parameters was dependent on the duration of exposure. Unfortunately, it was not possible to establish causality to these responses since the assays are not able differentiate among various chemical and physical stressors. In the future, controlled laboratory studies and field studies conducted in pollutant specific areas may help to answer some of these questions.

Several of the immune assays in the testing panel were repeatedly more sensitive than others. For example, blastogenic response to the mitogens, PHA, Con A, and LPS, in the first microcosm study, was extremely variable. This assay was subsequently removed from the testing panel for the Hamilton Harbour and second creosote studies. B

cell marking was also variable across sampling days. Although this assay was a useful biomarker of exposure, it is important to note that phenotypic markers can not evaluate changes in the functional immune system. It is also difficult to distinguish between proliferative responses and leukocyte trafficking using this assay. Increasing the sample size may help to improve the sensitivity of this assay. Measuring serum or plasma lysozyme activity has advantages in that it is easily performed and economical. However, this assay consistantly did not detect the subtle immunological changes that were measured using several of the other immune assays. Measuring lysozyme activity may require modified sampling techniques to optimized the sensitivity of this assay, since the stress associated with sampling can immediately alter the response. Leukocyte oxidative burst and phagocytic activity assays were the most promising assays in the test panel for use in field studies. In addition to providing reproducible results, they were consistently sensitive to subtle changes that occurred during exposure. It is important to note that, although these assays measure functional changes in the innate immune system, they do not evaluate a change in immune response. Their utility as preliminary screening assays for hazard identification is promising, but, until a relationship is established between these functional changes and disease resistance, they will continue to provide little insight to effects occurring at the population level.

It is extremely difficult to extrapolate from changes observed in the laboratory and field to effects occurring at the population level. There is a great deal of immunological genetic diversity at the population level, which ensures the survival of a species. Ecotoxicological risk assessors currently recognize the importance of protecting sensitive species, but are willing to accept losses of certain high risk groups within a species, without an understanding of the long term implications that may be associated with this reduction in genetic diversity. Pathogenic challenge studies should help to identify high risk groups, and predict the outcome of their loss on the entire population. Microcosm studies may provide the means for measuring such a response at the entire

population level. They may also be useful for establishing relationships between functional changes at the organism level, and disease resistance at the population level.

Table A1: Summary of rainbow trout parameters measured after 28 d of exposure to liquid creosote during the 1995 microcosm field study.

Rep	Concn µ l/l	%Phage	Phagemean	Phage ant*mean	Burst 10	B cell count	LPS control	LPS mean	LPS Diff.	Lysozyme ug/ml	Liver weight (g)	Initial weight (g)	Final weight (g)	Weight Diff (g)	Liver/wt X 100
1	0	8.12	42.5	28537	66.31	-	302.5	3092.7	1780	1.499	2.16	130.30	206.60	76.30	1.09
1	0	6.77	46.7	18418	326.85	-	466.9	3046.4	3460	1.496	1.71	98.00	125.70	27.70	1.36
1	0	11.81	42.2	40258	148.95	-	540.6	2757.8	2217	1.502	1.20	111.60	131.40	19.80	0.92
1	0	7.94	28.5	17827	62.74	-	291.3	2531.0	2240	1.496	0.91	93.60	87.50	-6.10	0.69
2	0	10.24	30.8	23701	105.58	112.06	228.3	2803.7	2674	1.496	1.23	109.00	119.50	10.50	1.03
2	0	17.82	52.1	51373	102.83	43.62	238.8	2418.7	2180	1.5	0.77	84.50	87.50	3.00	0.78
2	0	4.65	39.8	16202	381.94	166.34	308.8	4463.3	4146	1.48	0.88	107.60	126.00	17.40	0.70
2	0	14.09	33.1	30230	121.46	76.06	382.9	2547.2	2154	1.48	1.08	87.40	100.50	3.10	1.05
3	0	6.87	27.6	15325	144.56	17.08	449.7	2680.9	2231	1.501	1.89	136.10	121.10	-14.00	1.32
3	0	16.37	27.1	31269	111.81	178.74	238.4	5408.3	5570	1.497	0.85	86.30	99.00	12.70	0.97
3	0	17.76	26.6	36286	93.13	188.66	561.9	2150.7	1589	1.499	0.83	83.80	94.50	0.70	0.87
3	0	9.36	27	17852	80.39	43.52	147.4	3486.5	3321	1.503	1.10	113.00	132.20	19.20	0.83
4	0	8.80	28.4	23877	74.83	49.86	-	-	-	1.498	0.78	86.60	96.80	10.80	0.82
4	0	9.54	22.9	18477	121.84	105	78.0	483.2	404	1.496	1.11	107.70	124.90	17.20	0.89
4	0	13.62	25.1	28960	89.66	138.5	74.0	310.8	237	1.499	0.80	105.40	113.90	8.50	0.70
4	0	16.64	28.9	37151	143.91	109.51	81.3	465.4	384	1.496	0.43	111.20	120.60	9.40	0.36
5	0	23.88	57	108644	51.89	78.01	307.5	3125.8	2818	1.501	1.34	148.20	141.60	-6.60	0.95
5	0	14.21	36.3	37978	80.67	122.46	213.7	2186.3	1973	1.5	1.14	124.60	130.20	5.60	0.87
5	0	7.85	58.1	31644	191.58	93.6	285.4	1622.1	1227	1.502	1.16	130.10	131.00	0.90	0.89
5	0	8.07	57.5	33210	115.6	105.75	312.9	1652.1	1189	1.503	0.85	108.60	110.90	2.30	0.77
1	5	12.34	28.2	23585	34.66	-	368.9	7348.3	6886	1.496	1.31	123.40	117.10	-6.30	1.12
2	5	8.88	48	33732	376.83	77.74	-	-	-	1.478	1.58	133.60	148.40	14.80	1.05
3	5	12.80	31.3	25622	36.28	27.83	116.1	880.4	774	1.498	0.85	78.70	80.00	0.30	0.89
4	5	7.80	30.8	18729	74.83	78.06	114.4	1256.7	1141	1.496	0.89	122.50	129.10	6.60	0.83
5	5	17.44	42.4	56364	140.18	76.05	280.4	2287.8	2007	1.5	0.79	100.60	96.80	-4.80	0.83
5	5	17.50	26.5	36346	82.31	66.56	182.3	788.6	636	1.502	0.76	108.80	113.00	4.20	0.68
5	5	24.08	33.1	56243	21.26	66.66	223.1	1568.3	1345	1.502	1.48	120.00	133.40	13.40	1.11
6	5	12.67	68.2	71414	14.77	57.51	199.6	1856.3	1657	1.5	1.19	104.00	97.00	-7.00	1.23
6	5	13.61	33.4	36620	66.89	100.43	128.4	1438.4	1310	1.503	0.83	111.60	103.40	-8.20	0.81
5	5	14.77	43.4	40478	46.36	103.97	168.8	888.8	620	1.498	1.19	104.30	122.40	18.10	0.97
1	9	27.66	28.1	58033	30.83	-	134.6	824.4	690	1.5	0.79	96.00	103.00	8.00	0.77
1	9	16.26	28.3	30214	67.71	-	146.3	1092.0	946	1.499	2.04	130.90	164.10	27.20	1.24
2	9	13.38	30.6	32188	78.6	76.97	167.8	2481.1	2303	1.501	0.84	109.60	104.40	-5.40	0.80
2	9	13.67	36.6	31288	60.69	134.32	389.0	3838.1	3449	1.502	0.83	96.60	90.00	-6.60	1.04
3	9	22.54	34	41609	362.7	84.77	528.9	1858.9	1330	1.499	0.81	84.40	97.10	12.70	0.83
3	9	13.28	26.9	26912	221.42	67.7	400.5	3388.8	2988	1.5	1.43	114.80	118.80	4.00	1.20
4	9	7.77	31.8	19798	71.89	121.22	101.7	474.2	372	1.496	0.84	111.60	128.00	16.40	0.73
4	9	9.15	26.9	20899	119.66	148.14	110.6	1098.5	988	1.497	0.74	109.60	124.60	15.00	0.69
5	9	33.62	70.5	157471	28.64	78.66	392.8	1800.8	1408	1.5	1.02	101.30	109.30	8.10	0.88
1	17	18.21	28.2	47182	16.77	-	162.6	2698.7	1826	1.501	1.36	113.50	126.00	12.50	1.07
1	17	19.34	32.4	36188	22.08	-	131.3	772.0	641	1.499	1.52	117.50	128.30	8.80	1.20
2	17	14.17	46.4	46182	64.62	60.36	172.3	1083.2	911	1.505	0.88	103.70	111.20	7.50	0.88
2	17	13.63	43.8	36283	66	36.06	448.0	5097.6	4652	1.503	0.92	104.00	104.00	0.00	0.86
3	17	10.03	28.6	21865	66.46	56.29	138.9	1894.9	1656	1.501	0.71	115.50	114.80	-0.70	0.62
3	17	10.24	28	20097	66.1	28.41	232.9	2141.2	1808	1.498	0.92	131.20	131.10	-0.10	0.71
4	17	17.84	48	46160	46.43	13.14	107.5	174.4	67	1.436	0.61	127.50	115.90	-11.60	0.53
4	17	9.10	37.1	30846	76.69	66.32	-	-	-	1.496	0.86	97.00	98.70	1.70	0.88
5	17	22.73	67	109877	70.42	57.36	241.1	1656.9	1616	1.499	0.86	96.30	101.30	5.00	0.85
1	31	11.33	27.6	20943	29.9	-	-	-	-	1.499	1.38	102.10	114.50	12.40	1.20
1	31	10.12	26.6	23549	15.51	-	120.2	488.7	348	1.507	1.76	112.10	116.40	4.30	1.53
2	31	16.27	48.8	56542	56.46	62.79	-	-	-	1.5	0.87	103.50	105.30	1.80	0.82
2	31	12.48	42.5	40386	114.34	67.28	163.1	1752.4	1589	1.498	1.32	102.50	114.00	11.50	1.16
3	31	14.08	28.4	28434	28.28	56.81	161.6	1813.6	1352	1.5	1.28	103.80	119.80	16.00	1.04
3	31	16.48	33.5	34218	4.38	-	465.7	3626.0	3160	1.496	1.05	110.00	113.40	3.40	0.82
4	31	12.36	23.1	24888	71.82	66.49	88.6	466.7	370	1.493	0.88	119.70	116.80	-2.80	0.84
4	31	9.17	33.6	23843	17.58	17.68	78.1	179.9	102	1.498	1.18	116.80	112.20	-4.60	1.05
5	31	13.13	30.6	36388	127.93	32.53	223.4	1346.5	1122	1.502	1.54	109.80	111.60	1.80	1.38
1	88	11.11	32.2	24249	16.44	-	115.1	561.8	447	1.503	1.05	115.10	118.70	3.60	0.88
1	88	10.28	36	27778	26.43	-	116.5	316.7	203	1.499	1.35	110.60	109.80	-1.80	1.14
2	88	9.83	36.8	31497	64.51	66.37	269.5	1616.0	1327	1.501	1.48	117.60	123.30	5.70	1.16
2	88	8.16	48.3	33366	71.67	28.36	200.6	2388.2	2186	1.503	1.62	117.40	113.20	-4.20	1.43
3	88	18.60	33.9	46463	67.71	78.67	183.1	2425.9	2233	1.499	1.08	126.60	127.80	1.10	0.85
3	88	20.10	36.4	47753	21.88	77.06	186.0	1141.6	947	1.498	0.88	78.70	82.10	2.40	1.07
4	88	17.56	38.6	48430	6	63.61	86.9	317.0	250	1.491	1.05	88.40	81.50	-6.90	1.27
4	88	13.83	38.4	46076	16.97	8.48	86.3	191.9	108	1.462	1.17	114.60	113.00	-1.60	1.05

Table A2: Summary of rainbow trout parameters measured after 7, 14, 21, and 28 d of exposure to liquid creosote during the 1996 microcosm field study.

Concn u l/l	TIME (d)	Bcell count	Burst	%Phago	Phagomean	Phago cnt*mean	Lysozyme ug/ml	Final Weight (g)	Liver weight (g)	LiverWt, X 100
0	0	382	19.43	41.40	35.50	86768	.	106.50	1.00	0.94
0	0	421	67.36	10.23	37.40	33292	-6.20	142.30	1.50	1.05
0	0	373	95.67	6.88	35.00	16788	1.69	100.00	1.90	1.90
0	0	210	92.66	5.85	34.10	17602	8.64	117.60	1.14	0.97
0	0	389	58.17	3.53	65.80	60385	25.76	79.90	1.00	1.25
0	0	176	19.37	4.25	28.20	9448	-1.48	134.70	1.60	1.19
0	0	849	34.54	18.26	36.30	27271	19.74	129.40	1.10	0.85
0	0	626	37.13	6.68	36.30	16836	10.45	106.50	1.20	1.13
0	0	.	51.24	10.74	31.40	11982	14.80	142.30	1.30	0.91
0	0	.	.	6.93	28.30	13737	13.00	100.00	1.00	1.00
0	7	872	119.97	18.18	55.50	68496	8.78	113.60	1.00	0.88
0	7	468	432.69	8.10	37.20	27300	12.67	123.50	1.30	1.05
0	7	255	111.48	12.54	44.70	43956	17.43	178.40	1.90	1.07
0	7	228	145.97	6.31	27.90	14284	20.22	178.40	1.20	0.67
0	7	336	145.51	9.48	33.60	24434	22.33	139.30	1.30	0.93
0	7	258	148.97	7.59	34.40	23930	19.37	117.60	1.60	1.36
0	7	346	118.35	15.05	43.40	54820	12.61	138.90	2.30	1.66
0	7	337	88.44	15.19	55.00	78754	13.71	131.90	1.70	1.29
0	7	139	158.99	11.32	40.10	44118	24.99	181.60	2.70	1.49
0	7	407	98.74	4.23	37.80	15056	37.03	133.00	1.60	1.20
3	7	761	167.2	30.05	44.60	118561	7.87	155.00	1.50	0.97
3	7	1044	140.04	13.83	37.20	40472	14.83	90.60	0.90	0.99
3	7	316	86.09	15.21	45.00	58771	28.63	145.00	1.70	1.17
3	7	1221	110.51	27.29	75.60	155929	38.85	95.00	1.20	1.26
3	7	191	246.83	3.60	46.90	17071	-5.72	106.00	1.10	1.04
3	7	678	57.58	10.91	43.50	39011	-0.96	126.00	1.40	1.11
3	7	304	162.64	14.66	48.80	63373	5.06	130.00	1.40	1.08
3	7	295	114.33	14.55	43.40	56155	71.51	168.00	1.80	1.07
3	7	586	79.94	16.48	35.20	45799	23.45	95.00	1.30	1.37
3	7	1005	49.37	12.85	33.90	31182	2.79	124.00	2.00	1.61
10	7	897	115.89	19.30	67.10	92790	9.09	82.00	1.10	1.34
10	7	584	53.61	37.18	63.40	179509	17.90	102.00	1.10	1.08
10	7	1293	64.53	34.77	56.90	137077	33.07	156.00	2.10	1.35
10	7	546	126.25	18.89	42.30	70390	71.09	92.60	1.00	1.08
10	7	1114	91.02	10.46	33.20	30758	13.80	120.00	2.10	1.75
10	7	624	100.31	43.42	58.20	207915	17.59	105.20	2.20	2.09
10	7	916	53.97	11.62	33.20	33516	57.49	142.00	1.80	1.27
10	7	682	88.16	17.69	45.20	71872	14.48	137.80	1.90	1.38
10	7	1437	111.28	37.34	49.20	149269	12.76	156.10	2.20	1.41
10	7	656	51.29	18.75	43.90	65865	39.93	133.80	2.50	1.87
0	14	340	48.06	5.47	27.30	13549	21.24	120.00	1.90	1.58
0	14	276	128.2	4.60	32.80	14100	-3.03	174.30	1.80	1.03
0	14	334	34.22	7.52	34.30	25406	10.81	119.20	1.30	1.09
0	14	569	54.99	9.01	38.70	28222	11.80	116.50	1.10	0.94
0	14	664	26.43	6.93	31.10	16776	12.12	140.60	1.30	0.92
0	14	480	120.17	7.93	36.80	17239	9.21	125.00	1.20	0.96
0	14	599	158.83	10.27	41.50	35904	44.09	140.40	1.00	0.71
0	14	792	148.18	4.33	28.60	9707	-33.47	152.90	1.10	0.72
0	14	419	104.71	6.03	31.70	18101	.	113.20	1.10	0.97
0	14	439	45.43	4.68	42.90	20864	.	196.80	2.00	1.02
3	14	377	55.21	4.88	30.70	13016	18.44	132.60	1.60	1.21
3	14	846	20.38	13.54	50.50	61608	19.55	112.60	1.20	1.07
3	14	440	34.42	7.00	36.80	23408	21.11	130.50	1.70	1.30
3	14	497	56.39	13.39	49.50	47348	17.02	130.20	1.80	1.38
3	14	808	107.19	9.39	45.30	26427	27.52	152.00	1.60	1.05
3	14	651	86.45	6.85	45.20	27277	18.38	130.00	1.50	1.15
3	14	505	156.37	14.71	35.70	13635	14.33	201.20	2.20	1.09
3	14	1183	11.28	9.33	40.30	31610	17.30	124.00	1.80	1.45
3	14	359	122.53	10.57	62.10	67021	16.18	110.00	1.50	1.36
3	14	848	45.45	12.68	58.10	61195	.	178.00	2.50	1.40
10	14	917	9.79	20.10	45.60	84376	.	112.00	1.50	1.34
10	14	242	59.91	7.91	38.20	28433	14.12	132.00	1.80	1.36
10	14	991	43.93	10.76	35.70	31494	12.89	127.00	2.00	1.57
10	14	416	28.16	8.16	43.30	34262	10.64	159.00	1.80	1.13
10	14	614	81.29	13.49	45.70	45459	14.43	89.60	1.10	1.23
10	14	284	13.63	12.92	40.20	49354	18.63	113.70	1.90	1.67
10	14	704	13.87	22.20	62.10	116526	19.18	142.00	1.50	1.06
10	14	246	35.62	12.34	45.50	54058	17.14	168.20	2.30	1.37
10	14	521	28.78	18.96	39.30	61112	.	159.40	2.00	1.25
10	14	620	82.36	11.12	34.20	34691	.	148.60	2.40	1.62
0	21	721	106.74	6.06	39.40	19426	13.63	93.10	1.30	1.40
0	21	1121	536.16	15.78	33.80	36484	15.26	101.50	1.20	1.18
0	21	854	115.61	10.39	43.50	38022	16.47	117.20	1.10	0.94
0	21	353	445.99	4.48	30.50	12161	18.32	130.30	1.80	1.38

Table A2: (con't)

Concn u/l	TIME (d)	Bcell count	Burst	%Phago	Phagomean	Phago cnt*mean	Lysozyme ug/ml	Final Weight (g)	Liver weight (g)	LiverWt, X 100
0	21	871	92.61	8.54	32.30	18385	13.73	117.50	1.10	0.94
0	21	1170	93.67	8.24	38.70	22547	13.68	106.50	1.20	1.13
0	21	1086	187.29	7.39	34.80	15709	.	93.30	1.00	1.07
0	21	823	150.56	6.26	35.00	16566	14.21	141.80	2.00	1.41
0	21	917	180.51	4.15	38.60	14149	16.51	147.90	2.00	1.35
0	21	.	20.08	10.54	42.40	37441	11.91	120.60	1.70	1.41
3	21	341	19.86	12.54	33.20	32180	8.61	160.60	2.20	1.37
3	21	634	59.82	12.87	38.40	39947	16.30	113.20	1.40	1.24
3	21	365	53.59	9.50	41.30	34389	11.71	128.90	2.20	1.71
3	21	857	57.61	11.34	30.40	26071	9.63	131.30	1.90	1.45
3	21	658	100.79	8.48	35.20	22600	9.78	137.20	1.50	1.09
3	21	127	29.53	10.95	44.00	41417	8.39	109.10	1.80	1.65
3	21	348	189.19	8.90	37.70	28471	8.79	140.20	1.80	1.28
3	21	640	56.55	13.62	32.50	35828	10.95	101.70	1.10	1.08
3	21	917	151.23	6.86	30.80	16473	12.36	149.40	1.70	1.14
3	21	708	.	10.96	49.30	42550	17.71	109.10	1.30	1.19
10	21	267	59.29	15.96	38.40	50426	10.22	126.40	1.40	1.11
10	21	443	41.09	15.04	47.00	53634	6.54	100.60	1.10	1.09
10	21	461	17.92	16.13	51.60	70288	11.34	94.70	1.30	1.37
10	21	786	17.18	15.57	41.50	50776	7.94	121.40	1.60	1.32
10	21	847	75.88	8.52	35.60	24756	13.75	126.70	1.60	1.26
10	21	281	14.01	8.19	31.20	22452	10.40	96.10	1.30	1.35
10	21	603	153.77	10.37	35.10	33109	11.35	122.90	1.20	0.98
10	21	1081	45.98	13.46	41.30	44013	11.01	152.40	1.60	1.05
10	21	923	213.69	14.15	34.40	36693	11.26	157.50	1.90	1.21
10	21	439	66.59	17.67	33.80	44127	17.49	131.10	1.20	0.92
0	28	755	76.16	5.49	35.50	17058	10.94	109.60	1.40	1.28
0	28	211	73.24	12.40	38.70	41666	3.27	99.50	1.20	1.21
0	28	1137	94.71	7.88	31.90	21534	11.24	146.10	2.00	1.35
0	28	368	87.02	5.44	33.50	16720	6.17	109.70	1.20	1.09
0	28	291	166.63	6.94	33.30	20293	11.27	172.20	2.00	1.16
0	28	341	167.26	12.68	36.00	37142	-0.99	169.20	1.80	1.06
0	28	471	194.75	6.68	34.00	17614	6.92	113.90	1.70	1.49
0	28	390	123.64	5.60	40.30	19342	20.95	95.20	1.10	1.16
0	28	271	88.46	8.16	51.30	34264	8.40	151.80	1.80	1.19
0	28	302	119.78	11.16	39.50	39327	8.35	141.80	1.80	1.27
3	28	142	94.75	13.61	36.20	42660	12.12	115.40	1.30	1.13
3	28	348	246.54	6.84	33.30	21016	17.16	152.10	1.70	1.12
3	28	141	29.56	8.96	33.30	27695	15.45	119.70	1.80	1.50
3	28	179	182.29	12.33	40.50	44374	28.30	135.20	2.40	1.78
3	28	463	113.73	8.52	34.60	24995	16.31	99.40	1.60	1.61
3	28	252	178.87	10.44	39.80	38769	17.29	163.50	2.40	1.47
3	28	229	58.63	7.83	45.90	34098	12.15	65.90	0.70	1.06
3	28	123	606.14	4.30	34.50	13934	23.98	122.10	2.30	1.88
3	28	182	280.07	10.40	36.70	33065	21.35	150.40	2.60	1.73
3	28	189	17.35	7.82	34.50	24723	23.55	154.90	2.00	1.29
10	28	281	37.11	14.19	35.50	43240	6.05	110.40	1.60	1.45
10	28	247	79.73	14.03	31.00	39226	-0.38	76.90	1.10	1.43
10	28	1033	21.52	11.66	35.60	33518	3.54	132.90	2.20	1.66
10	28	596	172.4	7.24	39.30	24002	6.05	122.70	1.50	1.22
10	28	397	164.64	7.27	32.80	20307	17.73	146.50	1.70	1.16
10	28	242	18.37	12.92	36.00	43156	16.62	116.60	1.30	1.11
10	28	118	47.54	9.39	36.30	32264	15.86	102.00	1.40	1.37
10	28	290	139.38	7.94	35.40	24268	17.60	134.20	2.20	1.64
10	28	528	81.43	8.07	39.50	27291	18.11	171.20	2.10	1.23
10	28	453	95.2	19.79	40.90	71185	20.79	149.80	2.00	1.34

Table A3: Summary of rainbow trout parameters measured after 37 d of exposure to liquid creosote during the 1996 microcosm field study.

Rep	Concn u l/l	Burst	%Phago	Phago cnt*mean	Phagomean	B cell count	Initial weight (g)	Final weight (g)	Weight Diff (g)	Liver weight (g)	Liver/wt, X 100
1	0.3	134.721	3.66	10330	29.2	225	149.6	166.3	16.7	1.8	1.07
1	0.3	36.234	13.80	31008	25.5	770	119.5	104.5	-15.0	1.6	1.53
1	0.3	124.431	8.95	25548	33.4	418	134.9	148.5	13.6	1.9	1.28
1	0.3	74.95	9.44	23886	29.1	387	.	117.6	.	2	1.70
1	0.3	163.555	14.24	62008	52.6	154	.	166.4	.	1.5	0.89
1	0.3	112.518	8.11	24714	37.6	226	.	132.2	.	2.5	1.89
1	1	134.736	5.21	12573	28.3	424	139.6	167.6	28.0	1.9	1.13
1	1	174.02	5.49	15038	31.3	392	105.1	127.6	22.5	1.8	1.41
1	1	101.14	10.03	39526	50.1	384	122.1	152.7	30.6	1.5	0.98
1	1	72.697	8.91	22931	28.3	219	98.4	126.1	27.7	1.6	1.27
1	1	132.519	8.99	25833	32.7	279	76.9	86.6	9.7	1.3	1.50
1	1	152.213	3.23	8592	30.3	537	.	134.8	.	2.6	1.93
1	3	113.94	7.62	20107	32.7	775	139	161.4	22.4	2	1.24
1	3	63.372	14.65	58373	46.4	185	98.8	90.5	-8.3	1.3	1.44
1	3	61.93	7.72	25317	40	235	139.3	166.6	27.3	1.7	1.02
1	3	192.781	10.70	28043	32.7	553	132.6	114.3	-18.3	1.6	1.40
1	3	128.516	8.53	28068	45.8	254	119.6	140.7	21.1	1.7	1.21
1	3	60.509	22.12	.	38.4	496	.	90.5	.	1.3	1.44
1	10	57.03	15.35	40538	38.1	280	150	147.6	-2.4	1.5	1.02
1	10	92.84	8.89	20811	31.5	204	142.2	165.3	23.1	1.8	1.09
1	10	80.46	10.84	32548	41.5	104	97.6	117.5	19.9	1.4	1.19
1	10	32.419	8.54	22954	34.2	605	136.4	138.2	1.8	1.6	1.16
1	30	103.617	8.21	25097	37	310	109.1	113.8	4.7	1.7	1.49
1	30	68.837	10.49	30503	32.7	428	139.6	136.1	-3.5	1.6	1.18
1	30	125.41	8.15	25680	38	236	157.7	150.3	-7.4	2	1.33
1	0a	135.11	5.85	16412	39.8	255	146.4	150.5	4.1	1.7	1.13
1	0a	75.024	15.32	52086	42	656	103	118.7	15.7	1.2	1.01
1	0a	137.24	6.75	17289	29.5	789	96.1	110.5	14.4	2.3	2.08
1	0a	109.37	5.02	13790	32.6	172	148.6	173.3	24.7	1.6	0.92
1	0a	217.22	5.90	15668	30.9	188	114.2	131.2	17.0	1.5	1.14
1	0a	95.988	12.30	32528	38.2	683	.	118.7	.	1.2	1.01
1	0b	173.781	5.32	15804	32.1	376	161.9	175.6	13.7	2.7	1.54
1	0b	80.61	7.48	18840	34.8	308	120	123.6	3.6	1.4	1.13
1	0b	161.912	3.97	10780	31.3	225	126.4	146.8	20.4	1.6	1.09
1	0b	202.2	4.27	12107	32.2	118	94.5	92.8	-1.7	1.7	1.83
1	0b	91.788	9.34	26749	34.2	347	127.2	116.3	-10.9	1.4	1.20
1	0b	91.972	9.79	28314	35.1	311	119.2	119.2	0.0	1.5	1.26
2	0.3	124.322	21.80	28218	30.7	567	109.7	102.1	-7.6	1	0.98
2	0.3	141.037	14.51	38747	32.7	416	98.1	92.2	-5.9	1.3	1.41
2	0.3	188.203	11.56	27155	30.4	462	152.8	112.1	-40.7	1.9	1.69
2	0.3	347.457	22.40	63908	43.7	783	112.8	122.7	9.9	1.5	1.22
2	0.3	176.783	10.56	33598	41.5	238	128.9	137.1	8.2	2.5	1.82
2	0.3	156.62	16.33	35865	35.7	419	137.5	156.6	19.1	1.5	0.96
2	1	198.556	7.84	22645	34.7	984	134.8	159	24.2	1.7	1.07
2	1	149.99	14.00	37783	38.6	729	143.7	125.4	-18.3	2.2	1.75
2	1	151.907	12.44	31526	36.3	751	122.6	138.3	15.7	1.7	1.23
2	1	133.7	7.94	44808	62.6	312	91	72	-19.0	1.2	1.67
2	1	79.548	14.68	42899	35.6	439	116.3	133.6	17.3	2.1	1.57
2	1	171.76	12.64	39854	46.2	481	136.1	122.1	-14.0	2	1.64
2	3	157.83	17.90	46017	34.7	308	110.7	132.9	22.2	1.5	1.13
2	3	191.254	11.83	45831	49.8	915	157.7	161.5	23.8	2	1.10
2	3	408.45	6.09	20551	39.6	275	114	134.6	20.6	1.5	1.11
2	3	573.11	7.76	30562	42.7	287	118.2	102.6	-15.6	1.7	1.66
2	3	156.877	24.05	.	42.1	454	115	111	-4.0	1.5	1.35
2	3	58.5	21.61	58103	34.5	912	.	180.2	.	1.9	1.05
2	10	79.411	28.70	58083	30	1383	175.8	195.6	19.8	2.5	1.28
2	10	104.151	12.16	42682	40.5	394	117.3	131.9	14.6	1.9	1.44
2	10	47.051	16.60	41415	36.2	354	108.4	119.7	11.3	1.5	1.25
2	10	200.48	10.07	38339	45.7	354	128.1	118.7	-9.4	1.8	1.52
2	10	282.53	24.94	82769	36.7	530	130.4	140.9	10.5	2	1.42
2	10	152.25	28.61	108332	58.4	310	101.1	98.8	-2.3	1.8	1.82
2	30	68.678	10.61	33586	41.3	299	140.5	151.3	10.8	2	1.32
2	30	220.55	11.23	34488	37	554	105.5	114.2	8.7	1.8	1.58
2	30	111.358	12.62	40117	41.1	439	134.1	130.6	-3.5	1.3	1.00
2	30	139.584	12.32	39555	42.1	610	152.1	154.1	2.0	2.2	1.43
2	30	121.008	15.75	48206	43.4	607	153.2	136.1	-17.1	2.3	1.69
2	0a	344.42	5.88	23523	44.7	621	153.3	131.3	-22.0	2.9	2.21
2	0a	289.54	6.76	17207	30.3	381	136.4	149.5	13.1	1.6	1.07
2	0a	135.698	13.36	35770	35.4	706	143.4	167.3	23.9	1.6	0.96
2	0a	99.499	11.60	25208	31.5	466	126.5	145.2	18.7	1.6	1.10
2	0a	162.94	9.69	22725	29.3	526	122.1	135.6	13.5	1.3	0.96
2	0b	98.119	12.00	27891	32	405	108.2	138.7	30.5	2	1.46
2	0b	.	11.03	30054	40.9	899	134.3	130	-4.3	1	0.77
2	0b	144.161	13.13	30715	33.3	1254	151.8	175.6	23.8	1.8	1.03
2	0b	160.23	13.11	35812	34	495	146.7	128.1	-18.6	2.1	1.64
2	0b	250.521	7.05	21095	34.3	335	102.8	102.3	-0.5	1.8	1.76
2	0b	203.774	13.27	39508	35	480	78.1	88.1	10.0	1.5	1.70

Table A4: Summary of rainbow trout data collected during the 21 d Hamilton Harbour filed study. The sites are identified by numbers: 0 (lab control), 1 (Lake Ontario control site), 2 (Burlington sewage treatment plant), 3 (Lax), 4 (Randle reef), 5 (Windermere basin), and 6 (Dofasco bootslip).

Rep	Time (d)	Site #	Scell count	Phagomean	%Phago	Phago cnt*mean	Burst	Lysozyme ug/ml	Final weight (g)	Final length (mm)	Liver weight (g)	Liver/wt, X 100	CondFact g/cmx100
1	0	0	487	34.60	12.14	28021	19.087	5.48	101	210	0.93	0.92	480.95
1	0	0	327	23.20	13.49	19790	14.251	4.06	91.2	195	0.86	0.94	46.77
1	0	0	426	25.40	8.00	14816	35.683	3.32	113.3	220	1.14	1.01	51.50
1	0	0	285	33.80	11.82	24552	19.51	3.11	91.7	200	0.87	0.95	45.85
1	0	0	273	34.80	15.49	34883	20.984	1.15	88.5	205	0.86	0.97	43.17
1	0	0	336	36.20	13.51	32291	12.171	2.65	118.5	220	1.08	0.91	53.86
1	0	0	329	27.50	14.73	29120	23.019	1.17	85.1	190	0.75	0.88	44.79
1	0	0	301	30.50	11.18	20452	24.079	1.49	85.8	190	0.67	1.02	34.63
1	0	0	396	31.40	10.35	19112	25.56	3.82	105	205	0.86	0.80	51.22
1	0	0	.	33.30	6.98	14688	29.773	1.43	85.9	190	0.86	1.00	45.21
1	7	0	383	46.00	9.83	34119	10.573	8.02	127.7	210	1.48	1.16	60.81
1	7	0	122	41.70	12.42	35607	17.85	8.61	136.2	220	1.34	0.98	61.91
1	7	0	236	37.20	11.06	24349	6.707	7.98	112.3	210	0.9	0.80	53.48
1	7	0	603	33.20	9.95	21261	7.154	6.92	85.1	185	0.49	0.75	35.19
1	7	0	411	33.30	13.60	29916	8.591	7.54	55.2	170	0.6	1.09	32.47
1	7	1	1251	28.70	16.68	24800	13.986	4.03	116.4	220	1.03	0.88	52.91
1	7	1	242	38.30	8.93	29046	19.167	2.42	88	195	0.81	0.92	45.13
1	7	1	401	43.80	9.57	32040	21.93	3.37	149.7	230	1.42	0.95	65.09
1	7	1	485	33.90	11.01	22893	16.156	3.83	59.9	175	0.6	1.00	34.23
1	7	1	930	40.50	15.18	38023	.	.	79.2	185	0.59	0.74	42.81
1	7	2	270	42.60	11.35	32552	19.497	5.10	88	195	0.88	1.00	45.13
1	7	2	319	32.70	10.85	25330	11.719	4.40	51.3	170	0.46	0.90	30.18
1	7	2	186	36.60	9.46	26481	12.46	5.13	84.6	190	0.96	1.13	44.53
1	7	2	176	34.80	7.49	21846	14.44	5.19	85.1	190	0.73	0.86	44.79
1	7	2	603	49.00	11.53	45456	17.864	4.90	83	190	1.01	1.22	43.68
1	7	3	396	45.40	20.40	52391	12.796	5.89	84.2	195	0.88	1.05	43.18
1	7	3	228	38.80	8.48	21179	15.009	4.38	103.7	210	0.93	0.90	46.38
1	7	3	217	30.00	9.19	20059	14.804	4.94	79.9	190	0.7	0.88	42.05
1	7	3	187	36.50	6.32	20397	33.426	4.34	132.2	220	1.06	0.80	60.09
1	7	4	258	.	7.74	.	6.636	4.27	106	210	0.99	0.93	50.48
1	7	4	289	50.30	17.80	63068	8.888	4.60	104	205	0.84	0.81	50.73
1	7	4	517	42.40	15.03	46289	11.472	3.67	109	205	1.13	1.04	53.17
1	7	4	615	30.40	10.00	23186	19.868	4.21	103	205	0.98	0.95	50.24
1	7	4	732	31.10	8.18	18124	19.112	3.86	92.3	200	0.85	0.92	46.15
1	7	5	529	31.50	14.29	32901	9.229	8.60	81.7	200	0.66	0.81	40.85
1	7	5	437	39.70	11.08	33884	13.877	4.48	112.7	210	0.96	0.85	53.67
1	7	5	412	30.40	13.76	28222	8.926	4.67	112.6	210	0.99	0.88	53.62
1	7	5	699	28.40	21.28	43480	12.763	7.06	108.3	210	1.25	1.15	51.57
1	7	5	563	36.30	11.74	30320	10.322	3.03	65.7	180	0.56	0.85	36.50
1	7	6	337	24.60	5.02	9820	13.326	7.97	104.1	215	0.73	0.70	48.42
1	7	6	258	36.00	10.80	30553	9.421	8.49	126	220	1.11	0.88	57.27
1	7	6	263	33.20	6.81	17831	10.476	5.18	108.6	215	1.07	0.99	50.51
1	7	6	325	41.30	9.59	27829	8.033	4.16	104.5	210	0.81	0.78	48.76
1	7	6	822	29.50	10.87	17173	4.118	8.42	99.6	205	0.69	0.69	48.68
2	7	0	286	28.10	8.44	15685	10.34	4.86	100.6	200	1.16	1.17	50.30
2	7	0	420	30.60	10.36	23789	19.303	3.93	118.9	210	1.08	0.92	56.62
2	7	0	209	35.10	9.78	27156	23.758	6.35	112.9	215	0.93	0.82	52.51
2	7	0	376	28.20	6.71	13164	8.286	7.78	127.6	210	1.33	1.04	60.76
2	7	0	713	28.00	8.00	16189	40.93	4.48	97.8	205	0.68	0.70	47.71
2	7	1	726	28.80	5.61	12799	13.751	2.48	98	200	1.04	1.06	48.00
2	7	1	615	40.60	7.75	24848	5.464	4.19	100.4	210	0.93	0.93	47.81
2	7	1	2042	34.90	15.80	28806	14.863	3.61	107	205	0.93	0.87	52.20
2	7	1	1104	30.30	10.56	23419	3.228	5.39	104.6	210	0.95	0.91	48.81
2	7	1	953	29.60	7.37	12786	5.866	2.62	67.2	180	0.84	1.25	37.33
2	7	2	712	31.50	11.29	23429	10.938	3.22	91.7	215	0.88	0.96	42.65
2	7	2	753	39.40	16.55	46767	4.147	4.64	108.9	205	1.06	0.97	53.12
2	7	2	912	31.50	9.48	22140	20.162	3.36	111.5	220	0.94	0.84	50.68
2	7	2	1267	30.70	9.94	20854	14.536	3.43	54	175	0.54	1.00	30.86
2	7	3	1080	22.60	9.38	12054	4.218	2.54	63.2	180	0.44	0.70	35.11
2	7	3	.	28.10	7.44	16278	10.549	3.20	98	195	1.09	1.11	50.28
2	7	3	288	40.60	8.86	31325	13.34	4.60	116.6	210	1.29	1.10	56.62
2	7	3	739	33.60	10.62	28367	13.434	3.31	113.4	210	1.38	1.22	54.00
2	7	3	770	39.20	12.81	39139	8.693	3.46	87.3	195	0.82	0.94	44.77
2	7	4	1146	29.80	7.61	17072	6.643	3.41	116	220	1.39	1.20	52.73
2	7	4	886	29.70	11.31	26781	11.246	4.64	144.3	230	1.39	0.96	62.74
2	7	4	.	26.60	11.90	23518	7.56	2.96	78.4	190	0.68	0.87	41.26
2	7	4	946	28.30	11.15	23370	6.907	3.44	122.2	220	1.1	0.90	56.56
2	7	4	358	.	8.16	.	5.077	3.52	83.8	185	0.86	1.03	45.30
2	7	4	1568	29.80	8.84	16808	.	.	80.4	185	0.55	0.68	43.46
2	7	5	934	29.40	10.91	25532	8.577	3.89	131.2	230	1.07	0.82	57.04
2	7	5	217	26.30	7.79	16329	8.407	3.66	89	190	0.89	1.00	46.64
2	7	5	288	38.50	17.16	51848	7.937	4.87	96	205	0.95	0.99	46.83
2	7	5	1151	25.30	5.89	10842	6.887	3.95	68.6	180	0.64	0.93	38.11
2	7	5	289	31.20	10.59	28899	3.98	4.32	93.2	200	0.99	1.06	46.60
2	7	5	.	.	.	.	.	.	58.8	175	0.55	0.94	33.60
2	7	6	533	30.10	13.11	28037	8.58	9.39	124.3	210	1.02	0.82	58.19
2	7	6	446	35.80	10.95	30819	9.4	4.56	100.4	210	0.79	0.79	47.81
2	7	6	509	35.60	16.82	42467	7.617	5.62	114.9	210	1.21	1.05	54.71
2	7	6	162	44.90	11.95	43389	17.899	9.80	109	205	1.37	1.27	52.68
2	7	6	284	40.10	12.55	40605	3.632	5.79	117.8	210	1.01	0.86	56.10
1	14	0	403	28.30	9.77	19071	1.919	5.79	132.6	220	1.59	1.20	60.27
1	14	0	645	29.00	12.19	21055	5.458	6.39	71	175	0.5	0.70	40.57
1	14	0	578	29.30	6.93	14773	11.84	11.00	105.3	205	1.08	1.03	51.37
1	14	0	204	.	8.37	.	2.81	5.61	120.9	215	1.15	0.95	56.23
1	14	0	129	34.60	7.16	18280	10.479	10.65	100.6	210	1.1	1.09	48.00

Table A4: (cont'd)

Rep	Time (d)	Site #	Bacil count	Phagemean	%Phage	Phage ml^mean	Bacid	Lysozyme ug/ml	Final weight (g)	Final length (mm)	Liver weight (g)	Liver/wt X 100	CondFact g/mm^100
1	14	1	828	26.80	6.12	10755	8.281	2.63	71.4	185	1	1.40	36.69
1	14	1	288	41.70	8.43	28866	14.436	4.13	111.7	210	0.93	0.83	53.19
1	14	1	1176	27.70	8.69	16223	8.389	2.82	91.4	200	1.15	1.28	46.70
1	14	1	336	36.80	11.96	33818	7.62	4.80	86	200	0.8	0.84	47.50
1	14	1	1143	36.00	13.21	31886	8.014	2.09	82.6	185	1.05	1.27	42.31
1	14	2	235	32.80	8.54	15846	5.249	5.52	90	200	0.85	1.08	45.00
1	14	2	185	.	3.85	.	13.889	4.44	81.6	185	0.71	0.87	41.85
1	14	2	182	37.40	8.32	20748	8.321	3.37	77.8	180	0.53	0.68	40.86
1	14	2	138	40.10	7.51	27083	7.83	8.13	128.4	220	1.35	1.07	54.88
1	14	2	185	43.80	13.08	42362	4.869	4.50	87.8	185	0.88	0.88	38.66
1	14	3	316	.	11.54	.	17.014	5.27	107.1	215	0.82	0.77	49.81
1	14	3	175	31.00	8.88	23066	2.017	8.84	88.3	185	1.28	1.45	46.79
1	14	3	253	.	21.13	.	17.788	4.87	80.3	185	0.89	0.88	41.16
1	14	3	276	38.40	12.02	26302	4.614	11.08	108.2	205	1.52	1.40	52.78
1	14	3	110	33.40	5.97	18165	12.887	3.70	88.3	210	0.88	0.89	47.29
1	14	4	282	37.20	9.40	30167	12.008	8.77	100.1	205	1.16	1.16	46.83
1	14	4	280	36.40	10.49	25274	5.723	4.12	77.4	180	0.72	0.93	40.74
1	14	4	588	32.10	8.73	20714	47.18	3.87	72.7	185	0.66	0.80	38.30
1	14	4	642	28.80	7.89	14305	8.719	6.60	68.7	180	0.57	0.87	32.61
1	14	4	638	36.40	9.01	27046	15.364	2.31	86.4	200	1.01	1.09	47.70
1	14	5	221	28.20	7.38	14784	2.479	3.63	116.6	220	1.03	0.94	52.50
1	14	5	184	28.20	8.88	23349	10.716	10.12	110.7	225	1.39	1.26	49.20
1	14	5	325	.	7.76	.	8.671	8.88	83.3	180	0.82	0.88	43.84
1	14	5	360	.	10.43	.	8.032	8.05	138.6	230	1.24	0.91	58.48
1	14	5	184	.	16.58	.	6.787	8.14	88.8	180	0.67	0.88	38.74
1	14	6	174	23.80	3.74	6648	8.888	18.13	108.8	220	1.44	1.36	48.66
1	14	6	187	26.30	4.70	10234	10.36	8.52	113	210	1.18	1.04	53.61
1	14	6	210	31.00	11.57	22780	3.014	8.72	75.2	180	0.7	0.93	38.66
1	14	6	185	30.50	7.73	18567	3.404	6.30	72.8	180	0.68	0.91	38.32
1	14	6	138	30.70	8.78	18939	2.074	5.38	48.8	170	0.45	0.83	28.28
2	14	0	564	27.30	6.76	11193	8.383	3.87	101.6	200	0.91	0.88	50.78
2	14	0	731	31.40	7.68	17588	10.614	3.46	121.8	220	1.01	0.83	56.36
2	14	0	851	23.40	8.83	12836	5.722	3.78	70.7	175	0.73	1.03	40.40
2	14	0	848	31.10	10.40	16334	3.677	8.72	84.7	170	0.82	1.13	32.18
2	14	0	176	50.60	16.36	42277	2.848	8.88	88.4	185	0.88	0.85	36.97
2	14	1	1006	43.20	10.12	33367	4.415	4.49	141.3	230	1.35	0.98	61.43
2	14	1	788	35.80	7.76	21422	8.482	4.01	128	210	1.17	0.91	60.86
2	14	1	572	36.80	8.03	28028	11.866	4.04	92.7	185	0.82	0.89	47.54
2	14	1	821	28.30	12.08	20849	11.316	2.36	88.7	180	0.89	1.28	38.68
2	14	1	1271	34.00	11.34	32586	10.222	4.84	110	215	1.02	0.83	51.16
2	14	2	1280	38.10	8.82	26006	10.813	5.80	111.6	210	1.04	0.85	53.14
2	14	2	382	33.00	8.85	25372	11.838	8.84	81.7	180	0.77	0.84	43.00
2	14	2	.	38.10	8.13	24384	14.178	4.25	87.8	180	0.89	0.87	37.87
2	14	2	1643	32.70	8.83	24880	8.313	4.88	88	205	1.27	1.30	47.60
2	14	2	383	33.10	12.24	28536	3.884	4.35	85.8	200	0.71	0.83	42.80
2	14	3	888	28.80	10.96	18467	3.21	3.81	84.2	180	0.88	0.87	36.87
2	14	3	882	34.70	8.79	23882	2.122	3.49	100.1	200	0.82	0.82	50.05
2	14	3	888	30.00	12.09	23880	2.846	3.83	80.5	175	0.48	0.81	34.57
2	14	3	436	33.70	8.28	19144	3.031	5.72	109.9	210	0.93	0.85	52.33
2	14	3	432	36.80	5.85	14861	5.883	6.81	81.1	180	0.87	0.83	33.84
2	14	4	891	36.80	10.03	27887	10.407	8.40	105	210	1.46	1.38	50.00
2	14	4	404	36.00	8.28	22880	3.704	6.38	85	185	0.74	0.87	43.88
2	14	4	387	36.00	14.21	40878	8.172	11.38	102.2	205	1.35	1.32	48.85
2	14	4	378	36.40	7.87	24822	8.258	5.84	88.5	205	0.83	0.94	48.05
2	14	4	884	28.30	8.54	15124	4.421	5.77	103.1	210	0.82	0.83	48.10
2	14	5	883	32.80	8.12	23088	4.636	8.40	88.8	210	0.88	0.88	47.57
2	14	5	380	36.80	10.28	27465	5.44	.	138.8	230	1.11	0.81	58.38
2	14	5	778	32.10	12.61	22174	8.743	4.83	88.8	205	0.84	0.74	42.34
2	14	5	624	24.80	11.04	23801	4.818	13.02	132.1	225	1.52	1.15	58.71
2	14	6	888	34.30	16.89	50803	11.229	8.88	88.7	210	1.33	1.33	47.48
2	14	6	635	38.10	3.46	12881	8.104	10.37	102.8	210	1.88	1.85	48.88
2	14	6	621	36.80	10.48	28868	3.372	5.82	88.8	200	0.85	0.88	44.30
2	14	6	633	32.10	8.88	23827	8.537	8.77	83.8	200	1.08	1.18	48.85
2	14	6	328	36.80	7.82	24887	12.818	12.42	105.4	210	1.53	1.45	50.18
1	21	0	385	28.40	4.88	8812	8.187	4.84	148	240	1.48	1.00	88.83
1	21	0	448	28.10	4.17	8303	8.141	1.82	121.8	215	0.83	0.78	58.66
1	21	0	245	24.80	4.88	10115	5.338	8.02	118.4	220	1.03	0.88	52.81
1	21	0	188	38.80	12.78	36228	8.142	4.71	110.3	220	0.87	0.78	50.14
1	21	0	283	41.70	12.54	38806	8.137	5.71	80.8	185	0.81	0.75	41.44
1	21	1	748	28.80	8.41	18341	13.869	5.10	80.4	200	0.75	0.83	46.20
1	21	1	1087	27.10	12.83	27834	11.887	5.10	116	220	0.36	0.33	52.73
1	21	1	187	33.80	7.82	23788	4.878	5.75	132	225	1.22	0.82	58.67
1	21	1	228	40.40	11.88	38863	11.888	7.11	128	225	0.88	0.78	57.33
1	21	2	848	37.30	7.35	19837	8.281	3.58	83.2	185	0.82	0.75	42.87
1	21	2	381	28.10	7.79	14108	4.248	5.09	87.7	210	0.72	0.74	46.52
1	21	2	588	27.80	7.75	16256	8.438	2.52	108.1	220	0.84	0.78	48.14
1	21	2	134	27.20	5.51	11870	5.347	5.85	114	225	0.97	0.85	50.67
1	21	3	227	26.50	5.83	11388	4.281	7.38	82.8	180	0.85	0.91	45.88
1	21	3	887	28.80	5.83	15088	8.45	4.08	78.8	205	1.02	1.33	37.37
1	21	3	241	28.00	8.83	10870	3.707	5.84	118.4	225	0.9	0.77	51.73
1	21	3	312	23.80	8.88	17162	8.088	8.10	88	210	1.85	2.08	42.38
1	21	3	238	31.40	7.87	16366	2.732	3.82	100.4	215	0.72	0.72	46.70
1	21	4	385	28.20	4.82	10361	7.381	8.38	88.2	180	0.6	0.88	38.89
1	21	4	144	32.30	12.88	30200	4.888	7.88	132.2	225	1.48	1.12	58.78
1	21	4	177	27.80	5.81	11881	2.721	5.78	78	200	0.57	0.73	38.00
1	21	4	181	23.80	8.73	16832	3.278	8.82	82.1	180	0.89	0.88	34.80

Table A4: (con't)

Rep	Time (d)	Site #	Bcell count	Phagomean	%Phago	Phago cnt*mean	Burst	Lysozyme ug/ml	Final weight (g)	Final length (mm)	Liver weight (g)	LiverWt. X 100	CondFact g/cmx100
1	21	4	306	27.20	9.94	18548	3.668	6.13	131.8	230	1.02	0.77	57.30
1	21	5	161	28.30	6.58	15212	8.528	7.63	78.3	190	0.78	1.00	41.21
1	21	5	370	25.70	3.03	9812	2.503	4.99	63.9	180	0.56	0.88	35.50
1	21	5	134	29.60	4.30	11756	3.889	6.56	101.2	215	1.02	1.01	47.07
1	21	5	188	.	11.12	.	0.273	7.82	83.6	195	0.65	0.78	42.67
1	21	5	189	27.30	16.38	38003	8.333	8.96	103	215	0.95	0.92	47.91
1	21	6	264	28.70	4.67	10856	7.152	5.16	77.1	200	0.64	0.83	38.55
1	21	6	241	31.40	11.10	16875	2.357	4.56	108.7	215	0.8	0.74	50.58
1	21	6	651	24.60	7.29	10843	5.483	4.36	79	205	0.69	0.87	38.54
1	21	6	474	30.80	7.99	18880	5.888	13.33	108.6	220	0.95	0.87	49.36
1	21	6	157	27.80	7.86	17239	1.982	5.43	61.1	170	0.61	1.00	35.94
2	21	0	361	22.00	16.60	22767	4.022	8.61	102.1	215	1	0.98	47.49
2	21	0	94	20.80	7.43	11147	1.237	12.13	122.3	210	1.13	0.92	58.24
2	21	0	257	25.70	12.18	19802	8.204	9.51	88.5	180	0.8	0.90	38.94
2	21	0	262	32.00	9.81	20874	2.894	12.36	131.4	220	1.23	0.94	58.73
2	21	0	307	22.90	7.57	12389	4.003	9.88	78.5	190	0.73	0.93	41.32
2	21	1	1049	36.30	12.66	37449	6.053	6.70	85.6	200	0.74	0.86	42.80
2	21	1	365	38.40	10.18	34482	12.47	6.10	138.2	235	1.05	0.76	58.61
2	21	1	566	35.90	15.75	44313	9.618	8.85	117.3	215	0.97	0.83	54.56
2	21	1	604	49.40	13.68	50279	13.978	5.21	106.7	215	0.85	0.80	48.63
2	21	1	1089	40.00	16.13	48004	4.349	6.27	84.8	185	1.59	1.88	46.84
2	21	2	1662	22.90	13.28	22142	4.716	5.88	46.5	155	0.42	0.92	28.35
2	21	2	617	24.10	11.05	17768	2.488	3.81	74	200	0.52	0.70	37.00
2	21	2	274	26.90	8.31	20120	12.184	16.02	99.8	210	0.92	0.92	47.52
2	21	2	271	27.90	13.54	19856	0.924	5.14	67.7	185	0.44	0.65	36.58
2	21	2	279	32.60	11.55	22837	0.728	4.07	57.8	180	0.4	0.69	32.11
2	21	3	680	25.40	9.93	21195	10.408	6.83	88.3	200	1.08	1.22	44.15
2	21	3	355	27.20	7.91	14184	1.357	2.06	71.3	190	0.62	0.87	37.53
2	21	3	528	24.00	4.14	8157	.	11.69	83.4	195	0.74	0.89	42.77
2	21	3	316	26.40	6.61	13469	1.213	4.58	105.2	210	0.78	0.74	50.10
2	21	3	942	21.30	2.81	4734	2.747	6.88	82	220	0.63	0.77	37.27
2	21	4	516	27.00	5.29	11603	6.751	5.80	63.8	185	0.57	0.89	34.48
2	21	4	303	28.20	1.28	4355	10.544	8.43	73	195	0.82	1.12	37.44
2	21	4	727	33.10	7.16	22943	11.357	6.91	84	200	0.96	1.14	42.00
2	21	4	453	26.40	7.15	17381	4.324	9.37	80	185	0.85	1.42	32.43
2	21	4	570	28.50	11.84	22827	0.103	2.28	52.8	162	0.39	0.74	32.59
2	21	5	387	27.60	5.32	12559	2.852	7.05	118.4	200	1.2	1.01	59.20
2	21	5	585	32.50	9.36	23880	5.911	5.14	94.3	205	0.78	0.83	46.00
2	21	5	407	33.60	8.45	19141	7.342	4.49	91	205	0.64	0.70	44.39
2	21	5	305	23.50	3.89	7135	5.26	8.47	95.8	205	0.83	0.87	46.73
2	21	5	987	29.60	9.95	19694	5.278	6.32	123.8	220	1.03	0.83	58.27
2	21	6	621	31.00	6.41	17347	7.176	12.40	108.8	215	0.99	0.93	49.67
2	21	6	640	27.50	4.93	12267	4.193	5.56	71.9	200	1.25	1.74	35.95
2	21	6	217	29.50	6.06	14813	7.181	7.58	81.2	195	0.73	0.90	41.64
2	21	6	158	31.90	8.87	18417	6.162	2.83	110.8	215	0.88	0.79	51.53
2	21	6	372	28.60	9.12	18873	5.909	7.74	92.8	200	0.69	0.74	46.40

APPENDIX 2

Table B1: Summary of statistical analysis of the raw data, excluding controls, from the 28 d creosote study conducted in 1995

Variable	p	Linear Probability	Quadratic Probability
%phagocytosis	0.027	0.627	0.395
Mean fluorescence	0.223	0.194	0.546
Count*mean	0.002	0.229	0.735
Burst	0.03	0.014	0.823
Bcell	0.249	0.106	0.804
Weight difference	0.022	0.351	0.527
Liver/weight	0.298	0.003	0.471
LPS diff.	0.207	0.014	0.341

Table C1: Confirmed water PAH concentrations for the Lake Ontario reference site.[a]

PAH (*u* g/l)	
Naphthalene	0.023
Acenaphthylene	nd
Acenaphthene	nd
Fluorene	nd
Phenanthrene	nd
Anthracene	nd
Fluoranthene	nd
Pyrene	nd
Benzo[a]anthracene	nd
Chrysene	nd
Total	0.023

[a] nd= not detected