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Characterizing the immunotoxicity of creosote to rainbow trout (*Oncorhynchus mykiss*): a microcosm study

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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 μl/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 of the immune responses measured in this study was 17 μl/l using nominal creosote concentrations, representing a total PAH concentration of 611.63 ng/l in the water.

Keywords: Polycyclic aromatic hydrocarbons (PAHs); *Oncorhynchus mykiss*; Immunotoxicity; Microcosm

1. 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

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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 establish 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. Innate immune parameters have been monitored in fish after 28 days of exposure in creosote treated microcosms. 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, 17 polycyclic aromatic hydrocarbons (PAHs) account for 63% of its volume (CEPA, 1994), making it a suitable candidate for PAH mixture studies.

2. Materials and methods

2.1. Microcosm

Seven outdoor microcosms were constructed of steel lined with a PVC liner with a depth of 1.05 m 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 12000 l of water from a well-fed irrigation pond and circulated for 3–4 weeks 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 1360 l/h such that the initial creosote concentrations were 0, 5, 9, 17, 31, 56 and 100 μl/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 days 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.

2.2. Experiment

Female rainbow trout ($\cong$ 100 g) were obtained in early October from Rainbow Springs Hatchery, Thamesford, ON. The fish were acclimated for 2 weeks 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 103–108 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 days of the study. The cages were constructed from nylon netting supported by circular plastic tubing (internal diameter 40 cm, length 800 cm, mesh size 1.5 cm). Fish were fed commercial trout chow at a ration of 2% body weight/day. 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-day 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.

2.3. Preparation of pronephros and peripheral blood leukocyte suspensions

Peripheral blood was centrifuged at $200 \times g$ 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, USA) at $400 \times g$ 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 μM HEPES + L-glutamine (pH 7.4, Gibco, Burlington, ON, Canada) supplemented with 10% fetal calf serum (Gibco), 50 U/ml penicillin, and 50 μg/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 μm 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.

2.4. Pronephros leukocyte oxidative burst

The oxidative burst assay was conducted according to Brousseau et al. (1998) using a Coulter EPICS XL-MCL flow cytometer. Cell suspensions were adjusted to 106 cells/ml in 2 ml of PBS supplemented with 0.5% (w/v) glucose. Leukocytes are incubated with 4 μM of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes Inc., Eugene, OR, USA) 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, USA) 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_{t60}) 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).

2.5. Pronephros leukocyte phagocytosis

Leukocyte phagocytic activity was assayed by flow cytometry according to Brousseau et al. (1998) using fluorescent latex beads (1.03 μM diameter, Molecular Probes). Cell suspensions were adjusted to 106 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 $100 \times g$ through a gradient mixture of 3% bovine serum albumin (GIBCO) and RPMI 1640 medium and resuspended in 500 μl Isoflow solution (Coulter Corp., Hialeah, FL, USA). For each leukocyte preparation, 10000 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.

2.6. Pronephros lymphocyte proliferation

The lymphocyte proliferation assay was conducted according to Brousseau et al. (1998). Aliquots of 5×10^6 cells in 100 μl of RPMI medium supplemented with 4% FCS + 50 U/ml penicillin, and 50 μg/ml streptomycin were added in triplicate to a 96 well culture plate for each mitogen and control. The cells were then incubated for 72 h at 18°C with 100 μl 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 μg/ml, respectively. [^{3}H]Thymidine (ICN Biomedicals, St-Laurent, QU, Canada) at 0.5 μCi 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 mean incorporation by the control 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 control cultures to give a stimulation index. When all fish were considered, the incorporation mean by the control cultures was 235 ± 141 CPM ($n = 65$) with a range from 67 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.

2.7. 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 μl 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 μl RPMI 1640 in 1 ml of RPMI 1640 medium for 45 min on ice. Cells were then washed three times, and incubated with 300 μl 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.

2.8. 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 μl 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 μg/ml equivalent of hen egg white lysozyme activity.

2.9. 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).

2.10. PAH analysis

Grab samples (1 l) were taken from each microcosm at 114 days 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 $\times$ 0.25 mm SPB-5 column with a stationary phase thickness of 0.25 μm 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 days 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 days of the microcosm study to represent sediment steady state concentrations.

2.11. 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 $p \leq 0.05$.

3. Results

3.1. Water chemistry and physical profiles

The mean temperature, dissolved oxygen, pH, hardness and alkalinity for the irrigation pond during the 2-week 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-day study (Fig. 1A). The increase in dissolved oxygen corresponded with the temperature profile (Fig. 1B). The pH profile in Fig. 1C remained constant at 8.0 ± 0.4 (mean $\pm$ SD). Water hardness and alkalinity were 226 ± 21 mg/l $CaCO_3$ (mean $\pm$ SD) and 125 ± 17 mg/l (mean $\pm$ 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 days after the addition of creosote are illustrated in Fig. 2A and B, 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 linear relationships with the nominal creosote concentrations on 114 days (Table 1, Fig. 3).

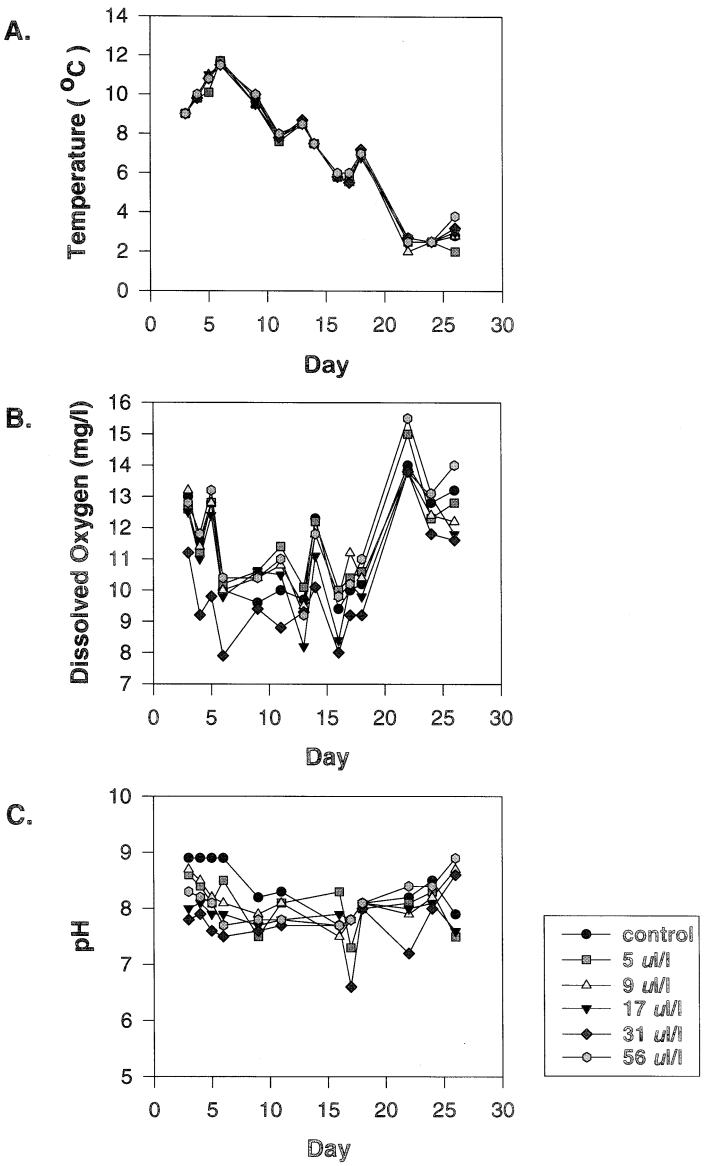

Fig. 1. Temperature (A), dissolved oxygen (B), and pH (C) profiles for the control and creosote-treated microcosms during the 28 day study.

3.2. Weight change and mortality

Rainbow trout in the 56 μl/l concentration gained less weight than control fish, although, the difference across concentrations was not statistically significant (Fig. 4). Liver weights increased slightly in the 31 and 56 μl/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 (Fig. 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 ($y = 0.88 + 0.12 \log x$),

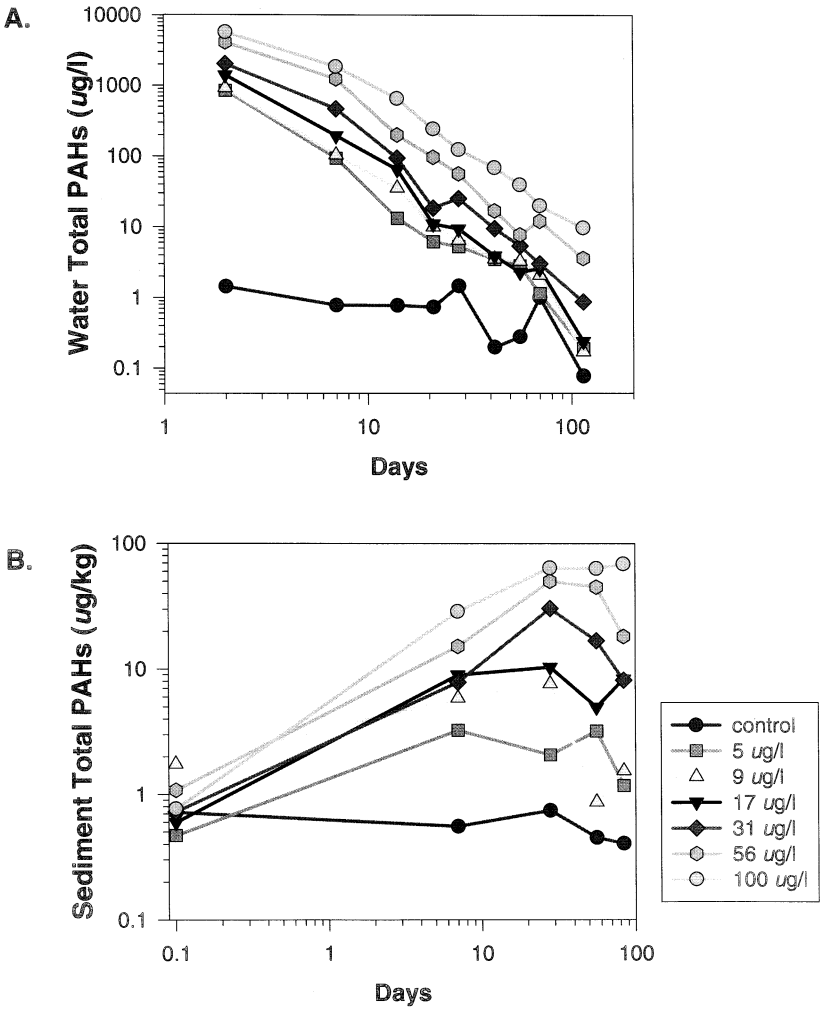

Fig. 2. Change in total PAH concentrations in the water (A), and sediment (B) with time after dosing microcosms with creosote. The first sediment sample was taken on the day of dosing; the first water sample, on the second after dosing. Fish were added to the microcosms 103 days after dosing.

F-ratio $= 7.54$, $p < 0.01$, $R^2 = 0.11$). The LOEC was 31 μl/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 μl/l), this showed no relationship to the nominal creosote dose. At 100 μl/l, all fish died within the first 3 days of exposure. However, at 0, 5, 9, 17, 31 and 56 μl/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.

3.3. Oxidative burst

Creosote exposure significantly reduced leukocyte oxidative burst in a concentration–response

Table 1
PAH concentrations for control and creosote-treated microcosms on day 15 of the rainbow trout study

PAH (ng/l)	Creosote (μl/l)							F-ratio
	1	5	9	17	31	56	100	
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	

Naphthalene, acenapthene, dibenzo(a,h)anthracene, and benzo(g,h,I)perylene were not detected in the water. nd, not detected.
* Significant linear relationship with nominal creosote concentrations ($p \leq 0.01$).

dependent fashion (Fig. 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 μl/l using nominal creosote concentrations, representing a total PAH concentration of 611.63 ng/l in the water.

3.4. 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 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 (Fig. 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 ($\log y = \log(1.84 + 0.08\text{x})$, 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 μl/l using nominal creosote concentrations. The phagocytic index was however, not significantly stimulated at 31 μl/l concentration.

3.5. 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 Con A, PHA, and LPS were 90, 90 and 95%, respectively. For all the creosote exposed fish ($n = 45$), the percentages of fish responding positively to Con A, PHA, and LPS were 82, 96 and 96%, respectively. The

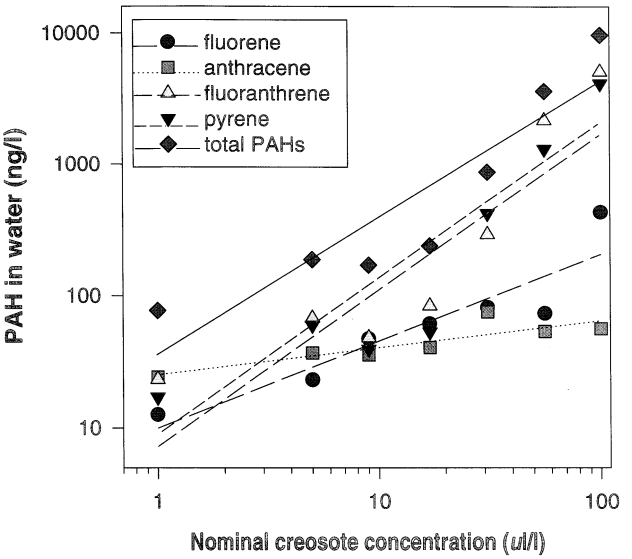

Fig. 3. Relationship between PAH water concentration and nominal creosote concentration 114 days after creosote dosing. Lines represent the linear regression equation $y = a + bx$ through each data set ($p \leq 0.01$) (see Table 1 also).

magnitude of the mitogen stimulation index varied widely between fish, with a range from 2- to 24-fold for the control group and 2–20-fold for the creosote-exposed fish. When the incorporation of [^{3}H]thymidine into DNA was normalized as a percent of the control change in CPM, the results were not statistically different across creosote concentrations for Con A 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, $p < 0.01$, $R2 = 0.22$), and sediment PAH concentrations ($\log y = \log (1.93 - 0.23x)$, F-ratio = 17.02, $p < 0.01$, $R^2 = 0.23$) (Fig. 8A–C). The reduction in incorporation was only significant at the 56 μl/l creosote concentration.

3.6. sIg$^+$ leukocyte marking

Peripheral blood sIg$^+$ leukocyte counts were reduced in fish exposed to creosote compared to control fish (Fig. 9A–C). This response showed a linear response using creosote nominal concentrations ($py = \log(10.75 - 2.52x)$, F-ratio = 16.39, $p < 0.01$, $R^2 = 0.25$), water total PAH concentrations ($py = \log(13.66 - 2.20x)$, F-ratio = 15.17, $p < 0.01$, $R^2 = 0.24$), and sediment total PAH concentrations ($py = \log(9.28 - 1.96x)$, F-ratio = 18.34, $p < 0.01$, $R^2 = 0.27$). The LOEC for reduced percent sIg$^+$ leukocyte was 17 μl/l. Although the percent of sIg$^+$ leukocytes at the 5 and 31 μl/l concentrations was reduced, the response was not statistically significant.

3.7. Plasma lysozyme activity

Plasma lysozyme concentrations were reduced across creosote concentrations (Fig. 10A–C). The control mean lysozyme concentration ± SE was 2.0 + 0.2 μg/ml. Lysozyme concentrations were significantly reduced at the 5 and 56 μl/l 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, $p < 0.01$, $R^2 = 0.16$).

3.8. 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).

4. 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 microcosms after 28 days of exposure using nominal creosote, water total PAH and sediment total PAH concentrations. The LOEC was shown to be 17 μl/l using nominal creosote concentrations, representing 611.63 ng/l total PAHs in water.

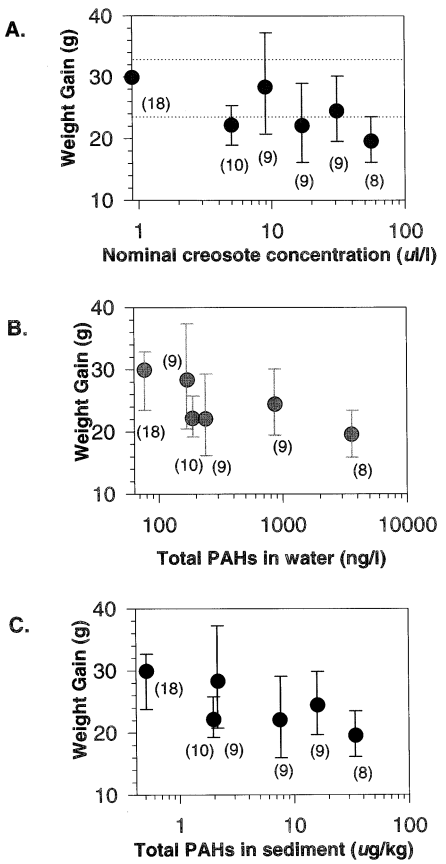

Fig. 4. Relationship between rainbow trout mean weight gain (with 95% confidence intervals) and nominal creosote concentrations (A), total PAH concentrations in the water (B), and sediment (C) during the 28 day microcosm study. Sediment concentrations are presented as the geometric mean of measured PAH concentrations on days 28, 56, and 84 of the microcosm study. Sample size is indicated as (n).

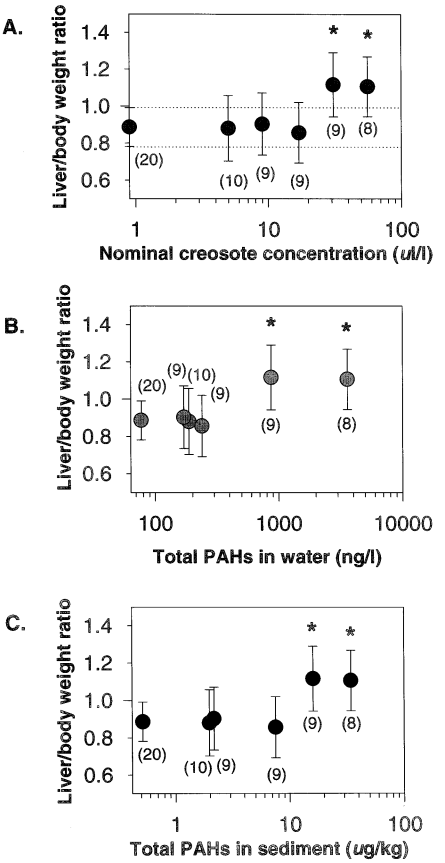

Fig. 5. Liver to body weight ratio of individual fish exposed to creosote during the 28 day microcosm study. Dose surrogates included nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on days 28, 56, and 84 of the microcosm study. Results are expressed as mean liver to body weight ratio with the 95% confidence interval. Means with significant differences from the control mean ($p < 0.05$) are indicated by *. Sample size is indicated as (n).

Reduced pronephros leukocyte oxidative burst 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

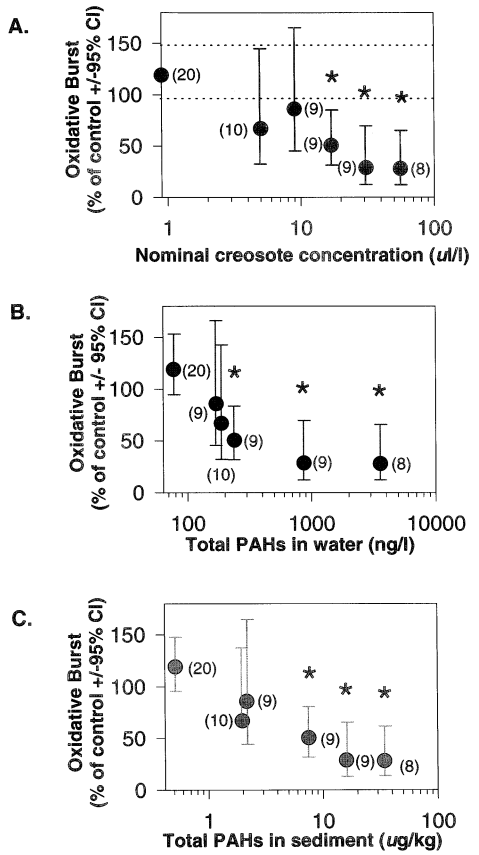

Fig. 6. Oxidative burst of pronephros leukocytes from rainbow trout exposed in vivo to liquid creosote for 28 days. Dose surrogates included nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on days 28, 56, and 84 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 the 95% confidence interval. Means with significant differences from the control mean ($p < 0.05$) are indicated by *. Sample size is indicated as (n).

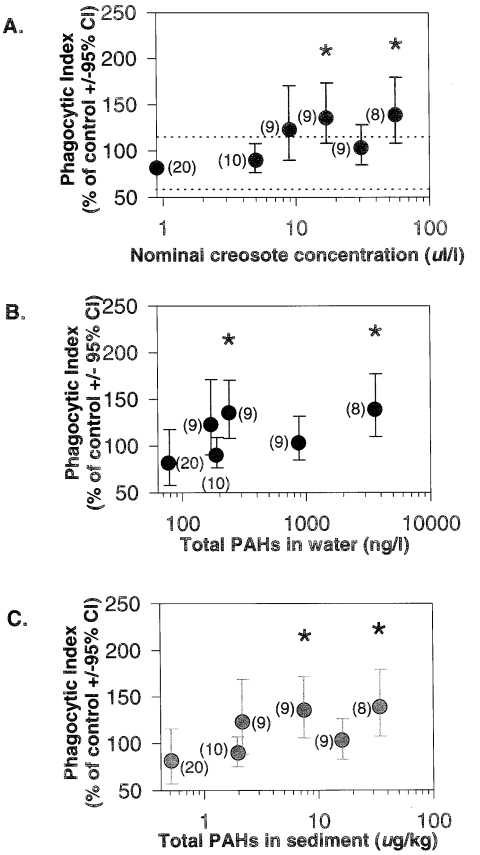

Fig. 7. Phagocytic index of pronephros leukocytes from rainbow trout exposed in vivo to liquid creosote for 28 days. Dose surrogates included nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on days 28, 56, and 84 of the microcosm study. The phagocytic index for each fish was normalized as a percent of the daily control mean fluorescence index. Results are expressed as mean percent phagocytic index with the 95% confidence interval. Means with significant differences from the control mean ($p < 0.05$) are indicated by *. Sample size is indicated as (n).

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 μl/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 activ-

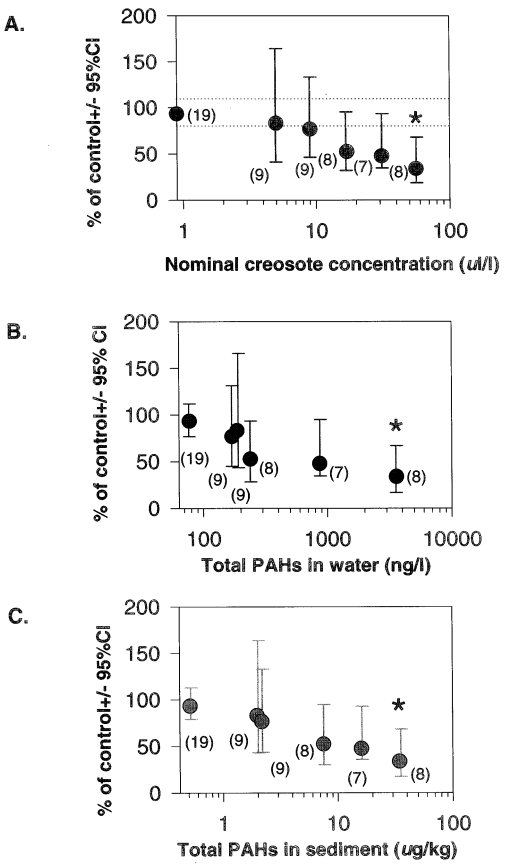

Fig. 8. Blastogenic response of pronephros lymphocytes to LPS from rainbow trout exposed to liquid creosote in vivo for 28 days. Dose surrogates included nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on days 28, 56, and 84 of the mesocosm 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 of control with the 95% confidence interval. Means with significant differences from the control mean ($p < 0.05$) are indicated by *. Sample size is indicated as (n).

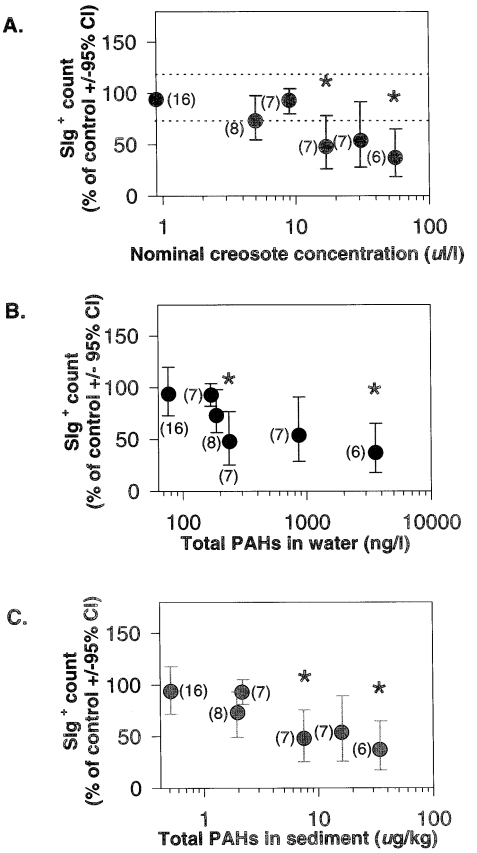

Fig. 9. Percent of SIg^+ peripheral blood lymphocytes from rainbow trout exposed in vivo to liquid creosote for 28 days. Dose surrogates included nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on days 28, 56, and 84 of the mesocosm study. SIg^+ lymphocyte counts for each fish were normalized as a percent of the daily control mean count. Results are expressed as mean percent count with the 95% confidence interval. Sample size is indicated as (n).

ity in spot (*Leiostomus xanthurus*) and hogchoker (*Trinectes maculatus*) exposed to Elizabeth River sediments which were shown to contain total PAH concentrations as high as 13000 μg/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.

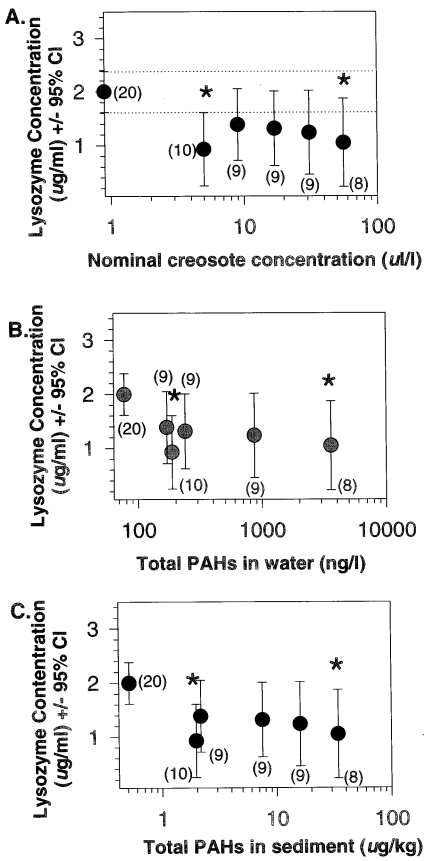

Fig. 10. Lysozyme in plasma of rainbow trout exposed to liquid creosote for 28 days. Dose surrogates included nominal creosote concentrations (A), and total PAH concentrations in the water (B), and sediment (C). Sediment concentrations are presented as the geometric mean of measured PAH concentrations on days 28, 56, and 84 of the mesocosm study. Results are expressed as mean μg/ml equivalents of hen egg white lysozyme with the 95% confidence interval. Sample size is indicated as (n).

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. 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, 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. Narnaware 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 days of exposure to creosote. A significant reduction at the 5 μl/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 8 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 implicates 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).

5. 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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