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Stimulation of Enzymatic Defense Mechanisms and Appearance of Liver Damage in Juvenile Trout (*Oncorhynchus mykiss*) Exposed to Water-Accommodated Trace Petroleum Residues

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The prevention of petroleum spills which produced major visual impacts became a prime concern of the Intergovernmental Oceanographic Commission (IOC) beginning in the 1950's. Subsequently, concern was also shown for the toxic effects on biota produced by the use of oil dispersants used in cleanup of oil spills (Fischer and Foss 1993). Chronic, low level inputs of petroleum residues to the environment are less visible, and although they do not produce widespread alarm, these may nevertheless produce toxic effects in organisms which are difficult to evaluate. Petroleum residues enter aquatic environments in small but regular quantities from diverse sources such as tanker operations, drydocking, marine terminals, bilge and fuel oils, municipal and industrial wastes, use of outboard motors, and others, contributing to often severe alterations in the aquatic biota in impacted environments (Betton 1994; Khan 1998). Pollutant-induced death of organisms is rarely immediate, but depends on the homeostatic-assimilative capacity of a given species for dealing with sublethal effects of toxicants. Early detection of pollutant effects involves definition and evaluation of sublethal toxic effects which may ultimately contribute to mortality (Rudolph et al. 2001).

Inducible mixed function oxidases (MFO) form the principal enzyme system involved in transformation and detoxification of xenobiotic substances. This transformation involves conversion/metabolism into more polar, hydrophilic compounds in order to facilitate their excretion (Clark 1997). Routine clinical analysis using transaminases as indicators in response to toxicity is used to evaluate effects of pesticides (Begun and Vijayaraghavan 1995; Sivakumari et al. 1997), pulp and paper mill effluent (Jeney et al. 1996), and effects of ammonia on some freshwater teleosts (Kumar and Mini 1997). This analysis is based on the hypothesis that there exists a measurable, consistent correlation between enzymatic activity in serum and degree of contamination or the extent of tissue damage (Goíc and Chamorro 1994).

In this study, we present results on the effects of a water-accommodated petroleum hydrocarbons (WAF) on activation of the multifunction oxidase systems (MFO) in the trout, *Oncorhynchus mykiss,* as a measure of toxicity.

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Transaminase activity and concomitant histological effects of the WAF observed in the fishes are also included. This study was a part of our long-term efforts to evaluate effects of potential pollutants on the health of juvenile trout, in a multidisciplinary effort to predict and diagnose, and finally evaluate negative effects of such pollutants. The experimental study includes a test species which not only serves as a useful model, but also represents an important commercial resource as this trout and its marine salmonid relatives are currently cultivated in Chile.

MATERIALS AND METHODS

Juvenile rainbow trout were obtained from a culture system (Piscicultura Polcura, VIII Region, Chile) having a mean length of 18.87± 0.22 cm and mean weight of 63.11 ± 3.33 g. Fish were acclimated for two weeks in 120 l aquaria with 2 μm filtered water at 11°C ± 1, and aerated with oil-free compressed air. The water, was changed completely every 48 h. The trout were maintained in a 16 h light:8 h dark photoperiod and fed every other day at a rate of 1% of the body weight.

The petroleum hydrocarbon fraction accommodated to saturation in water (WAF) was prepared using "2-D" diesel fuel. About 0.3 liters of 2-D were added to about 0.7 liters of water and agitated using a stirring device for 20 h at 18 ± 1°C. After this period, the water phase was separated by siphoning and a portion retained for analysis and the remaining used in fish tests. For these tests, dilutions of the saturated WAF were made which gave 0.043, 0.12, 0.23, 0.35, 0.43, 0.87, and 1.74 mg total hydrocarbons per liter as determined by analysis (see below). Each hydrocarbon concentration as well as pollutant-free (control) water was distributed into 40 l glass aquarium, each containing four test fish. Four replicates were carried out for each concentration and the control group (16 individuals per concentration). All systems were maintained under the same conditions as in the acclimation phase. Water and water-hydrocarbon treatments were renewed every 48 h.

Concentration of hydrocarbons in the WAF was determined using a Hewlett-Packard Series 5890 gas chromatograph with column temperature 150°C, injector temperature 250°C and detector temperature 290°C. A Hewlett-Packard series 5972 mass selective detector was used for identification with a scan range of 50-550 mass units, initial temperature 100°C, initial time 3 min, final temperature 260°, final time 10 min, temp. increment 10°C/min, injector temp. 250°C, and detector temp. 280°C. A C-21 hydrocarbon standard was used at a concentration of 0.3 mg/ml.

Blood samples were obtained from test fishes by caudal puncture after 7, 18, and 30 days exposure to hydrocarbons. Each trout was narcotized with benzocaine for a period not exceeding two minutes to facilitate sampling. Blood plasma was separated by centrifugation and stored at -20°C for later analyses (Wedemeyer

and Yasutake 1977). Data on transaminase activity was obtained from at least six individuals per treatment taking into account that coefficients of variation in salmonid hematologic parameters usually range between 10 and 15% (Wedemeyer and Yasutake 1977). The concentrations of glutamic pyruvic transaminase (GTP), glutamic oxalacetic transaminase (GOT) and gamma glutamic transferase (γ-GT) were determined kinetically at 340 nm using the BioSystems Kit and expressed in enzymatic units per liter.

Multifunction oxidase (EROD activity) in the microsomal fraction of hepatic tissue was analyzed as resorufin dealkylation in both control and hydrocarbon-exposed individuals after 7, 18, and 30 days of exposure. Microsomes were obtained by differential centrifugation at 100.000 x g of tissue macerate. This test quantified the transformation of 7-ethoxyresorufin into resorufin by ethoxy resorufin o-deethylase (EROD) mediated by P488 dealkylation (Lubert et al. 1985). The reaction mixture included 2.21 ml 50 mM Tris-HCl, 25 mM HgCl (pH 7.5), 10 µl microsomal protein, 10 µl NADPH, and 10 µl 0.41 mM 7-ethoxyresorufin (reaction substrate) and 2 g hepatic tissue pool. The samples were read by fluorimetry with excitation at 522 nm and emission at 586 nm. EROD activity was expressed in picomoles of resorufin/min/mg microsomal protein. Protein concentration in the microsomal fraction was determined by the methods of Lowry et al. (1951). This analysis was carried out on some individuals from all treatments and control.

Fishes from all treatment groups and controls at 7, 18, and 30 days were macroscopically examined, and sampled to observe histological conditions in gills, liver, and skin. Tissue samples were preserved in 10% formalin and prepared for microscopic examination using standard techniques of paraffin embedding, sectioning and staining with hematoxylin and eosin (Humanson 1962).

The SYSTAT statistical package (Wilkinson et al. 1992) was used to analyze the data. The non-parametric test of Dunnett was used for data comparison of enzymatic activity.

RESULTS AND DISCUSSION

Concentration of total hydrocarbons in the stock WAF was about 11.58 mg/l. Although the highest concentration of hydrocarbons in the present study was about 1.74 mg/l, at no time throughout the experiment was there any surface hydrocarbon film (reflective sheen) observed in any of the experimental systems. The principal hydrocarbons detected in the WAF were aromatics between C-8 and C-26 (Figure 1), which may act as mutagens and carcinogens at low concentrations when test organisms are exposed for extended periods of time (Arinc and Sen 1999).

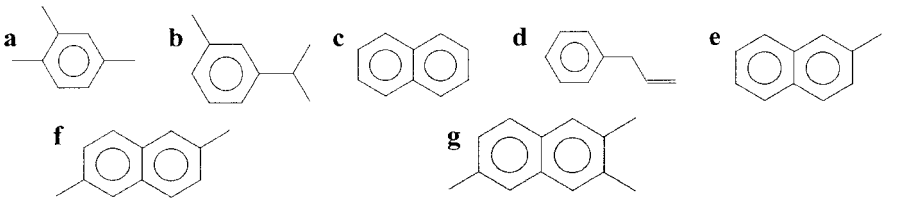

Figure 1. Principal aromatic hydrocarbons present in the water accommodated fraction (WAF) of 2-D diesel fuel used in present experimentation **a)** trimethylbenzene MW=121, **b)** 1-methyl-3-(1-methyl)-benzene MW=134, **c)** naphthalene MW=128, **d)** 2-propanyl-benzene MW=118, **e)** 2-methyl-naphthalene MW =142, **f)** 2-6-dimethyl naphthalene MW=156, **g)** 2,3,6-trimethyl-naphthalene MW=170.

Analysis of transaminase activity showed significant differences only with GOT (Table 1). The concentration of GPT fluctuated between 18 and 21 U/l with controls about 15 U/l . Similarly, the concentration of γ-GT in controls was about 5.4 U/l and in experimental individuals was 3.6 to 5.0 U/l, and were not be significantly different than the controls ($p > 0.05$).

After seven days exposure of the trout to WAF hydrocarbons, the GOT levels were five times higher than those of the control fish (Table 1). The control group was sampled by the same periods as the experimental groups, i.e., 7, 18, 30 days. No significantly differences were found, thus the same average value of 40.1 ± 27.2, was reported for each period. During the 18 day exposure period, the GOT activity showed an increase similar to that observed over seven days for experimental systems containing 0.12 and 0.23 mgl^{-1} hydrocarbons. In trout exposed to 0.35 mgl^{-1} WAF hydrocarbons, the GOT was elevated seven times that of the controls. With hydrocarbons as high as 1.74 mgl^{-1} this enzymatic activity decreased significantly. At 30 days of exposure to 0.043, 0.43, and 0.87 mgl^{-1} hydrocarbons, the GOT activity did not exceed 143 ± 40.26 U/l and was not significantly different from organisms subjected to nominally distinct concentrations ($F= 0.38$, $p>0.05$), although all were significantly different from the controls ($F=35.43$, $p< 0.001$, Table 1).

The liver, being the principal site for the metabolism of toxic materials absorbed by the gastrointestinal tract, is particularly sensitive to injury by xenobiotic substances (Zakrewsky 1991). Analysis of the trout blood plasma showed significant stimulation of glutamic oxalacetic transaminase (GOT). This transaminase is synthesized in the liver, and its presence in the plasma is indicative of liver damage, presumably as a response to the aromatic hydrocarbons present in the WAF (Begun and Vijayaraghavan 1995).

Table 1. Glutamic oxalacetic transaminase (GOT) activity in blood serum of *O. mykiss* (U/l) after three time periods of exposure to different WAF hydrocarbon concentrations (mgl^{-1}).

7	Control	0.43 (mgl^{-1})			
days	40.1	208.5			
	±27.2	±62.6			
	(n=6)	(n = 6)			
18	Control	0.12 (mgl^{-1})	0.23 (mgl^{-1})	0.35 (mgl^{-1})	1.74 (mgl^{-1})
days	40.1	219.9	220.9	272.2	128.9
	±27.2	±59.5	±63.2	±47.1	±69.6
		(n = 7)	(n = 8)	(n = 6)	(n = 7)
30	Control	0.043 (mgl^{-1})	0.43 (mgl^{-1})	0.87 (mgl^{-1})	
days	40.1	143.5	116.7	127.1	
	±27.2	±40.3	±65.1	±48.4	
		(n = 6)	(n = 8)	(n = 8)	

The EROD activity was determined at 7, 18, and 30 days for fishes exposed to different concentration. The EROD values were averaged because not significant differences were found. The hydrocarbons concentration ranges were 0.043-0.43; 0.12-0.23; 0.043-0.35 mgl^{-1}. It was not possible to perform the analysis of EROD at 0.87 - 1.74 mgl^{-1}, because there was not enough material collected. The WAF induced an increase in EROD activity which was significantly higher than in controls after exposures of seven days ($t = 2.8$, $p = 0.05$), 18 days ($t=6.42$, $p < 0.05$) and 30 days ($t=4.1$, $p<0.05$). The EROD activity increased 5X after seven days exposure, 10X after 18 days, and 20X after 30 days, independently of the hydrocarbon concentration in which they were maintained (Table 2)

Table 2. Ethoxy resorufin o-deethylase (EROD) activity in the liver microsomal fraction of *O. mykiss* after three time periods of exposure at different WAF hydrocarbon concentrations.

	Control	7 days	18 days	30 days
WAF (mgl^{-1})	0	0.043 – 0.43	0.12 – 0.23	0.043 – 0.35
EROD	18.02	94.64	189.59	367.04
(pmol/min/mg)	±7.35	±19.90	± 52.05	± 53.75
	(n = 8)	(n = 6)	(n = 6)	(n = 6)

The detoxifying activity of the MFO system in the liver, as measured by the EROD activity, was stimulated in the fishes. Intensity of this activity was directly related to the time of exposure of the fishes to the WAF hydrocarbons. Levine et

al. (1994) obtained similar results in studies of *Dorosoma cepedianum* using benzo-a-pyrene as a toxicant. Both Tables 1 and 2 suggest that the enzymatic effects were related to time of exposure rather than to hydrocarbon concentration.

Fishes exposed to WAF hydrocarbons demonstrated macroscopic lesions such as loss of scales, erosion of caudal fin and skin damage on the fin rays (Fig. 2A,B), discoloration of hepatic and gill tissues. At the histological level, juveniles at both seven and 30 days showed loss of cellular shape in hepatocytes, separation of epithelia from the basal lamellae and hyperplasia in gill filaments. Damage was also observed in secondary lamellae, attributable to hypertrophy and telangiectasis (Rudolph et al. 2001)

Macroscopic and histological observations of liver, gill, and skin alterations in the trout studied were similar to those described for flatfishes exposed to environmental pollutants (Myers et al. 1998). Our observations noted degenerative lesions of branchial and hepatic tissues similar to those described by other authors for demersal and benthic fishes exposed to chemicals and wastewaters (Bucke et al. 1993; Vethaak 1993; Leonardi and Tarifeño 1996).

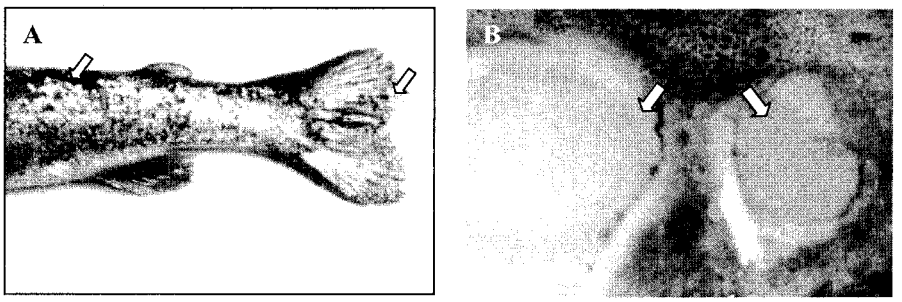

Figure 2. (A) Macroscopic lesions on the skin and fins of *O. mykiss* after exposure to 0.043 mg/l WAF hydrocarbons for 30 days and (B) Exposure to 0.043 mg/l WAF hydrocarbons for 7 days (from 400 x).

We conclude that ingress of low concentrations of hydrocarbons in waters with restricted circulation present a serious risk for their resident organisms. Damage to organisms exposed to WAF hydrocarbons appeared more related to the time of exposure than to the concentration of these toxicants.

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