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Effects of Exposure of *Oncorhynchus mykiss* to the Water-Accommodated Fraction of Petroleum Hydrocarbons

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Residual hydrocarbons arising from navigation and the transport of petroleum are the most common pollutants in the marine environment. Such inputs, which occur chronically, and at low concentrations, represent a high grade of danger for marine organisms given the solutional capabilities of the lighter fractions and their teratogenic capabilities (Betton 1994; Peña-Méndez 1999; Gagnon and Holdway 2000).

The cause/effect relationship between pollution and lesions of fishes and other organisms have proven difficult to establish (ICES 1986). It is for this reason that close interaction between laboratory research and practical applications needs to be established (Wester et al. 1994). In this context, histopathology of liver, gills, and skin of fishes has been a useful tool as an effective indicator in the evaluation of environmental quality in coastal and estuarine waters.

In the present research, experimental evaluation was made of the effects produced by increasing concentrations of the water-accommodated fraction of petroleum hydrocarbons on the salmon *Oncorhynchus mykiss* in smolt and juvenile stages based on macroscopic observations, and also on histological sections made from gills, skin, and livers of juveniles exposed to increasing concentrations of the toxicants administered at sublethal levels over extended time periods.

Selection of this fish species was made based on its ease of maintenance and handling in the laboratory. Also, it was a species for which a good deal of biological information was available, including its previous use in ecotoxicological testing. Skin and gills were chosen for observation as these were the tissues most immediately and directly exposed to pollution in fishes. The liver served as an integrator of biochemical and physiological functions. Also this organ carries out key functions in the excretion of xenobiotics (Geneser 1993). The analyses of sensitivity in *Oncorhynchus mykiss* were carried out with the underlying hypothesis that the results obtained could be extrapolated to other fish species, and that the results might be used as a tool in environmental monitoring programs.

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MATERIALS AND METHODS

Smolt and juveniles of *Oncorhyncus mykiss* (rainbow trout) were obtained from Piscicultura Polcura, VIII Región, Chile. Smolt sizes ranged from 6 to 8 cm in length and 2 to 3 g in weight. Juveniles measured an average of 18.87 ± 0.22 cm, with weight of 63.1 ± 3.33 g. Petroleum sensitivity testing was carried out at Bioassay laboratory, Universidad de Concepción. Test individuals were transported to the laboratory in 120 L plastic tanks at a density of 120 indiv./m^3 with constant aeration. They were acclimated at the laboratory, for a two week period in temperature-controlled glass aquaria (12 ± 1°C), with constant aeration and daily feeding according to their life stage. Excess food was cleaned from the bottom of the aquaria every day. Water was changed every 48 hr.

Toxicity testing was carried out using diesel fuel 2-D for boats engine. A stock toxicant solution was prepared by adding 300 ml of the fuel to 700 ml distilled water and agitated for 20 hr, to obtain the water-accommodated fraction of petroleum hydrocarbons (WAF). The concentration of total hydrocarbons in the WAF was 11.6 mg L^{-1}. This and all other hydrocarbon determinations were made using a Hewlett Packard gas chromatograph, series 5890 equipped with a HP series 5972 selective mass detector, following methods outlined by (Reish and Oshida 1986)

Seawater concentrations of WAF used in static toxicity testing with smolt of *O. mykiss* included 0; 0.06; 0.11; 0.18; 0.31; and 0.52 mg L^{-1}. liters of each concentration were distributed into two-liter glass containers into each which were distributed six randomly chosen smolt. Four replicates were carried out for each toxicant concentration and the control. The assay was carried out for 96 h under the same conditions as acclimation of the fish in the laboratory. Both water and the water-WAF mixture were changed every 48 hr.

Range of sublethal concentrations of the WAF was determined by exposing juvenile *O. mykiss* to 0 (control); 0.043; 0.070; 0.120; 0.230; 0.350 ;0.430; 0.870 and 2 mg L^{-1}. Four forty-liters glass containers with 3 fish each were used for every experimental concentration. Following methods proposed by Wedemeyer and Yasutake (1977). Assay conditions were the same as those used in acclimation, i.e., temperature-controlled (12±1°C), with constant aeration and daily feeding according to their life stage. Glass container's water was changed, for the same conditions every 48 hr. Times of exposure to the toxicant were 7, 18, and 30 days.

Observations of macroscopic damage to individuals in each treatment was made following criteria proposed by ICES, International Council for the Exploration of the Sea (1986). Histological observations were carried out by obtaining samples of liver, skin and gills from all specimens exposed to all concentrations of toxicant samples were treated by standard histological methods (Humason 1962). Sections were cut to 5-6 μm, and stained with hematoxylin and eosin for observations of microscopic alterations comparing toxicant-exposed tissues with

unexposed controls. Histological preparations were analyzed and photographed using a Nikon Model SC light microscope.

Observations were tallied and results were compared using the PROBIT software and Fisher's nonparametric analysis.

RESULTS AND DISCUSSION

The LC_{50} at 4 days of 0.46 mg L^{-1} for smolt was about three times less that of the juveniles, which was LC_{50} 18 days at 1.74 mg L^{-1}. No direct relationship between LC_{50} and organism size could be demonstrated, although more tolerance for the toxicant was observed in test fishes of comparatively larger size. Similar results were obtained by Grahl-Nielsen (1987), who proposed that the LC_{50} depended mainly on the developmental state of the individual, and level of toxicant which could be accumulated in its tissues. In the sublethal toxicity assay was not possible to obtain a NOEC value because in all concentration was observed some effect.

Presence of mucosity on the fishes was the first macroscopic manifestation of negative effects of the toxicant. At lower concentrations of WAF (i.e., 0.043 mg L^{-1}) this symptom became increasingly evident in the fishes with increase in exposure time (i.e.,18 and 30 days). Other negative effects of the toxicant in the fishes was the loss of scales, and occurrence of skin and fin ulceration. About 40% of the individuals presented irritation at the base of the pectoral fin, and about 10% had lesions between the tail and adipose fin. Increase in the loss of scales and appearance of skin lesions showed major alterations that with increased with time of exposure to the WAF which was not avoided by secretion of mucus.

One of the most obvious symptoms of stress in WAF-exposed fishes was growing discoloration of the gills over 7, 18 and 30 days as compared with the controls. Discoloration of the liver was likewise observed over this period. During this period, the gills underwent disintegration of filaments, and the liver became flaccid to the touch. Macroscopic alterations in the fishes was accompanied by histological alterations in the skin, liver and gills relative to the controls (Table 1).

Table 1. Percentage of specimens showing gill, skin or liver damage at a microscopic level.

	7 days	18 days	30 days
Control	0	0	100**
0.0043	17	40*	100**
0.35	25	44*	100**
0.43	25	62.5*	100**
0.87	42*	100**	100**

*Significance level at $p< 0.05$, ** $p<0.01$

Sections of skin from the controls showed clear stratification of epidermis, basal membrane, compact dermis, hypodermis, and subcutaneous muscle (Fig.1A). In

contrast sections of skin from the toxicant-stressed fishes (0.043 mg L^{-1} at 30 days). Showed deterioration of the basal membrane, increase in thickness of the dermis, cellular alteration of the hypodermis, and disintegration of subcutaneous muscle (Fig.1B). Similarly, sections of the gills from control organisms showed clearly defined lamellae and branchial filaments (Fig.1C) whereas exposed organisms (0.043 and 0.350 mg L^{-1} at 7, 18, and 30 days) showed separation of the epithelium at the base of the lamellae, damage to branchial filaments and secondary lamellae, associated with hypertrophy and laminar telangiectasia (Fig.1D). Similar structural anomalies of the interlamellar region in branchial tissue were observed by Khan (1999) in *Margariscus margarita* inhabiting a stillwater pond contaminated with diesel fuel.

Histological sections of livers from organisms exposed to different WAF levels for different time periods (Fig.1E) showed cellular alterations related to loss of cellular shape in hepatocytes, and differences in quantities of cytoplasmic components (Fig.1F). This may be attributable to a functional change of the hepatocytes. This alteration was shown by a considerable variation in the cytoplasm, where the quantity of lipids present, in the form of vacuoles, increased with increase in time of exposure and amount of WAF to which the fishes were exposed. Lipid vacuoles are limited in number in healthy cells, but have been observed to increase notably under pathologic conditions (Geneser,1993).

Hepatic alteration in fishes exposed to WAF was related to the biochemical activity carried out in this organ. Alteration in the lysosomal activity of the hepatic cell may produce increased permeability of the lysosomal membrane. Since all membranes are structured with double phospholipid layers, action of the toxicant in the case studied may favor incorporation of hydrocarbons into the lipids, provoking escape of cytosol containing digestive enzymes (Capuzzo 1981; Moore 1985; De Robertis 1995).

In this respect, the principal components of the WAF, which were aromatics (1,2,4 trimethylbenzene, 1-methyl-3-(1-methylethyl)-benzene, naphthalene, 2-propylben-zene, 2-methylnaphthalene, 2,6-dimethylnaphthalene, 2-3-6-trimethyl-naphthalene) have been identified as carcinogenic xenobiotics, capable of solublizing lipidic components of membranes and thus increasing the sensibility of the test organism to the pollutant (Whittle and Mackie 1976; Hardy et al. 1974; NRC 1983). Similar observations on vacuolization and accumulation of lipids have been reported in flounders which inhabited hydrocarbon-polluted waters (Khan 1998), and sewage effluent (Leonardi and Tarifeno 1996).

The marked discoloration of the gills observed in the present study was accompanied by deformations at the microscopic level. Although Flores (1992) attributed discoloration in the gills of *O. mykiss* to reduced absorption of oxygen, the deformations mentioned above, attributable to the toxic agent, may have been responsible for the discoloration. Since all systems were heavily aerated, Flores supposition may be discarded. However, the microscopic deformations may also

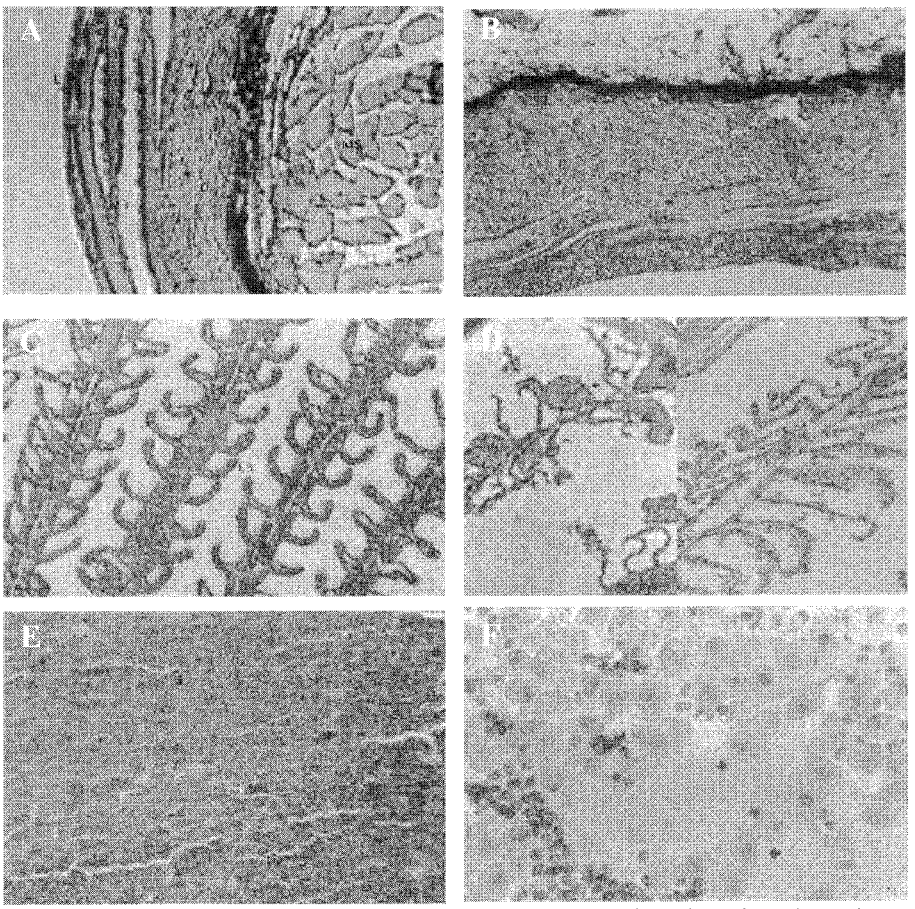

Figure 1. Control (left column) and hydrocarbon-exposed (right column) section of tissue from *Oncorhyncus mykiss*. Bar scale = 100 um. (A) Skin of control. (B) Skin of fish exposed to 0.043 mg/L WAS for 30 days. (C) Gill of control. (D) Gill of hydrocarbon-exposed fish to show damage to branchial filaments. (E) Liver of control. (F) Liver of fish exposed to hydrocarbons, to show alteration related to the loss of cellular form in hepatocytes and cytoplasmic components.

have been accompanied by interference in the physiological functioning of the gills.

In this study, the analysis of sensitivity of *O. mykiss* to WAF showed that long term exposure to the sublethal concentrations of hydrocarbons could be as deleterious for the organisms as periodic exposure to the higher concentrations. This is an important consideration, as waste waters usually carry low, but constant levels of hydrocarbons into the aquatic environment.

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REFERENCES

Anderson JW, Neff JM, Cox BA, Tatem HE, Hightower GM (1987) Characteristics of dispersions and water-soluble extracts of crude and refined oils and their toxicity to estuarine crustaceans and fish. Mar. Biol. 27: 75-88

Betton CI (1994) Oils and hydrocarbons. *In:* Calow P (Eds.) Handbook of ecotoxicology. Volumen II, pp 244-263

Capuzzo J (1981) Predicting pollution effects in the marine environment. Oceanus 24(1): 25-33

De Robertis E (1995) Biología Celular y Molecular. El Ateneo (Ed.) 469 pp

Flores M (1991) Cultivo de trucha arco iris (*Oncorhynchus mykiss*) en piscicultura de agua dulce. Tesis. Universidad Católica Ssma. Concepción. 66 pp.

Galbraith L, Burns CW (1997) The toxic effects of rubber contaminants on microbial food webs and zooplankton of two lakes of different trophic status. Hidrobiologia 353:29-38

Geneser F (1993) Histología. Editorial Médica Panamericana.768 pp

Grahl-Nielsen O (1987) Hydrocarbons and phenols in discharge water from offshore operations. Fate of the hydrocarbons in the recipient. Sarsia 72:75-82

Hardy R, Mackie KJ, McIntyre AD (1974) Discrimination in the assimilation of n-alkanes in fish. Nature 252:577-578

Humason GL (1962) Animal tissue techniques. Freeman WH & Company (Eds.) San Francisco. 468 pp

ICES, International Council for the Exploration of the Sea (1986) Report of the ICES Workshop on the use of pathology in studies of the effects of contaminations. Project CM/ICES E . P401-464

Khan RA (1999) Study of Pearl dace (*Margariscus margarita*) inhabiting a stillwater pond contaminated with diesel fuel. Bull Environ Contam Toxicol 62:638-645

Khan RA (1998) Influence of petroleum at a refinery terminal on feral winter flounder, *Pleuronectes americanus*. Bull Environ Contam Toxicol 61:770-777

Leonardi M, Tarifeño E (1996) Effects of de la descarga de aguas servidas por un emisario submarino en los lenguados, *Paralichthys microps* (Günther, 1881) y *Paralichthys adspersus* (Steindachner, 1867) in the Concepción Bay, Chile: Experimentals evidences. Rev Biol Mar, Valparaíso 31(1): 23-44

Moore M (1985) Cellular responses to pollution. Mar Pollution. 16(4):604-608

National Research Council (NRC) (1983) Polycyclic aromatic hydrocarbons in the aquatic environment: Formation, sources, fate and effects on aquatic biota. NRCC 18981, P106-107. NRCC/CNRC, Ottawa

Peña-Méndez EM, Astorga-España MS, García-Montelongo FG (1999) Polycyclic

aromatic hydrocarbons and n-alkanos in the intertidal limpet *Petella crenata* from the coast of Trenerife, Canary Islands. Bull Environ Contam Toxicol 63:665-672

Wedemeyer GA, Yasutake WT (1977) Clinical methods for the assesment o effects o environmental stress on fish health. Thechnical Paper of the US. Fish and Wild Life Service United State Departament of the Interior. Fish and Wild Life Service. United State Departament of the interior. Fish and Wild Life Service. Washington DC

Wester PW, Vethaak AD, Muiswinkel WB (1994) Fish as biomarkers in immunotoxicology. Toxicology 86:213-232

Whittle, KJ, Mackie PR (1976) Hydrocarbons in marine zooplankton and fish, part II. In Effects of pollutants in aquatic organisms. Soc For Exp Biol Seminar Series(2):85-105 Cambridge University Press, Cambridge

Wilkinson L (1988) SYSTAT: the system for statistic. SYSTAT Inc Evaston, JL,620 pp.