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Arsenic Induced Liver Hyperplasia and Kidney Fibrosis in Rainbow Trout (*Oncorhynchus mykiss*) by Microinjection Technique: A Sensitive Animal Bioassay for Environmental Metal-Toxicity

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Arsenic is a common aquatic environmental toxicant and has been associated with arsenic-related blackfoot disease as well as cancer to the human lung, skin and liver (IARC 1980; Gilman and Swierenga 1985; Chen and Wang 1990; Leonard 1991). Also arsenic was accorded as priority for study and protection (UNEP/FAO/IAEA 1995) by UNEP's consultation on the carcinogenic and mutagenic aquatic pollutants in the Mediterranean.

Uniikely to most human carcinogens, experimentation by arsenic according to rodent carcinogenicity bioassays has failed to induce tumours and so was unable to prove the carcinogenicity or any precarcinogenic stages (by arsenic compounds themselves) (Woo and Arcos 1985; Snow 1992). On the contrary studies have shown that arsenic was able to increase the carcinogenic response to rats when exposed to benzo (a)purene (Ishinishi et al. 1977). Therefore arsenic is concerned, until now, as an indirect or co-carcinogenic agent (Snow 1992). Furthermore, arsenic has not been studied in fish carcinogenesis bioassays (Masahito *et al.* 1988; Metcalfe 1989; Snow 1992; Bailey *et al.* 1996).

In the present study a sixth-month term fish bioassay was conducted according to the rainbow trout carcinogenicity bioassay. The purpose of this study was i) to investigate the possibility of any carcinogenic action by arsenic *in vivo,* starting the exposure from a concentration lower than that usually measured in the aquatic environment and ii) to observe in detail the toxic symptoms over a 6 month test period.

MATERIALS AND METHODS

In our sixth month bioassay that was conducted in order to investigate the role of arsenic in fish carcinogenesis, we utilized the rainbow trout, *Oncorhynchus mykiss,* at the early life stages of development. The rainbow trout were obtained from a commercial supplier (Giannetas S.A., Trout Hatchery Station, Ioannina, Greece) at the eyed stage of development (eye pigments visible through the egg's chorion). Embryos were held in the laboratory at 10-11°C for approximately one week until the completion of hatching. Once all the trout were in the sac-fry stage

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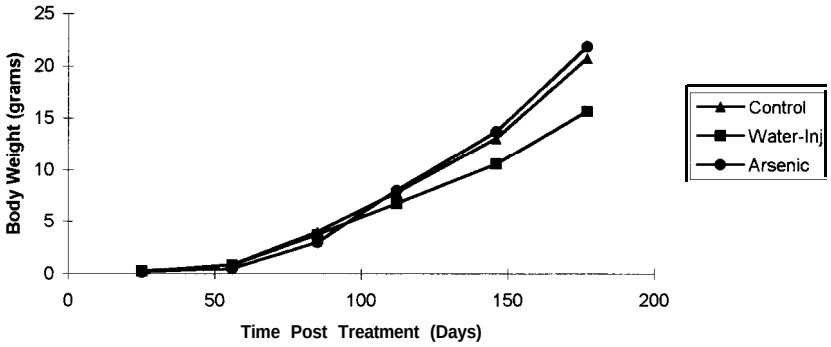

Figure 1. The rainbow trout growth rate in a 6 month test period in all groups.

of development they were separated into four groups (control, water, $AsCl_3$+water and Sham-injected) and placed into hatchery boxes for the 4 day long microinjection period.

During the microinjection phase the temperature was kept at 7-8°C to slow down developement. The microinjection procedure is as follows: approximately 10 sac-fry every time were placed in a dip net and anaesthetised with isoamyl alcohol (Carlos Erba), 1ml/l, for less than 45 seconds. They were then transferred from the dip net to a dry circular filter paper (radius 7.5cm) quickly, where the fry were immobilised by adhesion to the filter paper. The Hamilton push-button repeating dispenser model (BP 2000) with a Hamilton microlitre syringe (25µl capacity) was used for the microinjections in order to dispense the solutions in 0.5µl (±1%) volumes. The syringe needle was inserted right into the yolk-sac in an interior-posterior direction. The arsenate trichloride ($AsC1_3$) (Merck Chemical Co.), was diluted in deionized water in a concentration of 20ng As/sac-fry. Once all the sac fry were injected, the were placed in a small well aerated recovering nursing aquarium tank (0.6 x 0.2 x 0.2m). The microinjection procedure for each batch took less than 1 min. Once the fry had resumed normal activity, they were placed back into the hatchery boxes. After completion of this procedure and once the fry started to feed on commercial food the hatchery boxes were removed and the fish were fin clipped and reared all together at the approximately same density in the 3 raceways made of fiberglass (1.30m x 0.4m x 0.3m).

After the single injection in the sac-fry the fingerlings were raised at a water temperature of 12-15°C for 6 months, reaching a weight of approximately 22 grams (Fig. 1.). The stocking densities of the tanks were kept constant. Fish were fed *ad libitum* with a commercial trout diet (Trouvit Co, IOANNINA, GREECE). The diet contained 53% crude protein, 13% crude fat, 0.6% crude fiber, 12% ash,

9% moisture. Fish were raised in 95% recycled fresh water obtained from an intensive aquaculture farm (pH 7.3-7.8, hardness 45mg $CaCO_3$ temperature 19-22°C).

According to the test protocol fish were weighed, counted and grossly examined for possible external abnormalities during development and growth every 30 days post treatment for 6 months. When fish were 6 months old they were sacrificed by an overdose of Isoamyl Alcohol. Fish were preserved in Bouin's fixative. The liver, kidney, spleen, stomach and gastric caeca were histologically examined. The specimens were embedded in paraffin, sectioned at a thickness of 4-5 pm and stained with hematoxylin and eosin (H&E). All specimens were surveyed by cutting 5-6 sections from the centre of each paraffin block which then were examined microscopically.

Our statistical analysis was performed with the aid of the computer program SPSS (V.8). Analysis of Variance (ANOVA), x^2 test and student's t test were applied (Zarr 1984).

RESULTS AND DISCUSSION

The use of the microinjection technique offers three main advantages : i) a rapid procedure, ii) a uniform dosing of the metal solution to the individual test organism without any loss and, iii) the use of nanogram quantities, similar to levels in which they occur in the environment (Black 1988; Kotsanis and Metcalfe 1991; Kotsanis and Iliopoulou-Georgudaki 1997).

Table 1. Percent survival of rainbow trout to swim-up stage after a single injection of 5µl of test compound solution or control solution directly into the yolk-sac.

Injected Compound	Concentration	% Survival 4 days	7 days	28days
Control[b]	-	100% 50/50	100% 50/50	88%[d] 44/50
Sham-Injected[a]	-	90% 45/50	88% 44/50	- -
Water[b]	0.5µl	81.3% 61/75	78.6% 59/75	70.6%[c] 53/75
$AsCl_3$+water[b]	21 ng As/sac-fry	41.4% 58/140	40% 56/140	35.7%[c] 50/140

a Group kept for 7 days
b Even though mortalities ceased for nearly a week some fish died in the next 7 - 28 days in the inoculated and noninoculated groups.
c Frequencies are significantly different $X^2(p<0.05)$
d Frequency significantly different from treated groups $X^2(p<0.05)$

During the microinjection phase the majority of the mortalities among the inoculated fry were observed during the first 24-48 hours post treatment in the deionized water-injected group (0.5 μl/sac-fry) while in the $AsCl_3$+water group (21ng As/sac-fry) they were observed during the first 24-72 hours. Mortalities seized for almost a week but continued throughout the "swim up" thus after 28 days post treatment a 29.4% and 64.3% mortality was observed in the water and $AsCl_3$+water group respectively (Table 1). Control fish showed no mortality during the microinjection phase but during the "swim up" stage a 12% mortality was observed. Sham-injected (only inoculated) had a 12% mortality during the microinjection phase.

The test concentration of $AsCl_3$ was chosen after an extensive preliminary test assay at which the sac-fry were weighed prior the treatment and showed to have approximately a constant weight of 0.095 grams (±0.003gr) at a temperature of 10°C. The LD_{50} (96h) obtained in this assay for the microinjection of $AsCl_3$ was 0.29mg As/Kg (0.02-3.9mg As/Kg) after a single microinjection of the metal solution directly into the yolk-sac. It should be emphasized, that for the estimation of the LD_{50} in the preliminary test we had subtracted the mortalities recorded in the control-water injected group, which was approximately 20%.

Table 2. Incidence of kidney and liver lesions at the 6 months' necropsy for rainbow trout treated as control, or with Water, $AsCl_3$ in Water (21ngAs/sac fry).

Treatment		Visual Survey		Final Survey (Visual + Histological)	
		Kidney	Liver	Kidney	Liver
Control[a]	(n=40)	0/40 0 %	0/40 0 %	0/40 0 %	0/40 0 %
Water[a]	(n=40)	0/40 0 %	0/40 0 %	0/40 0 %	0/40 0 %
$AsCl_3$[a]	(n=48)	19/48 39.5%	4/48 8.3%	21/48 43.7%	6/48 12.5%

$AsCl_3$: Refers to the group microinjected only once with an aqueous solution.
a : Incidence was shown to be statistically different.

At the end of the six month test period, the animals were sacrificed. At the necropsy, kidney lesions were observed grossly and histologically in the $AsC1_3$+Water group. The incidence of these lesions were (19/48) 39.6% in the arsenic treated fish. Some lesions were observed grossly in the liver of 4 specimens [4/48, (8.3%)] but no lesions were observed grossly in the spleen, stomach and gastric caeca. The kidney lesions were usually observed at the

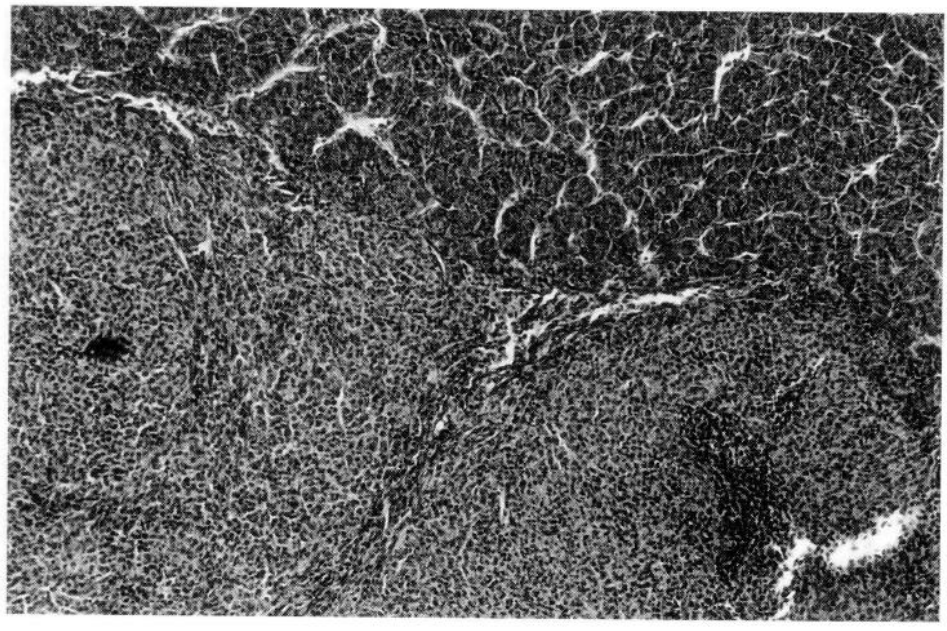

Figure 2a. Light microscopy of a hyperplastic lesion in the liver of a 6 months rainbow trout treated initially with 21ngAs/sac-fry, (H&E, x16).

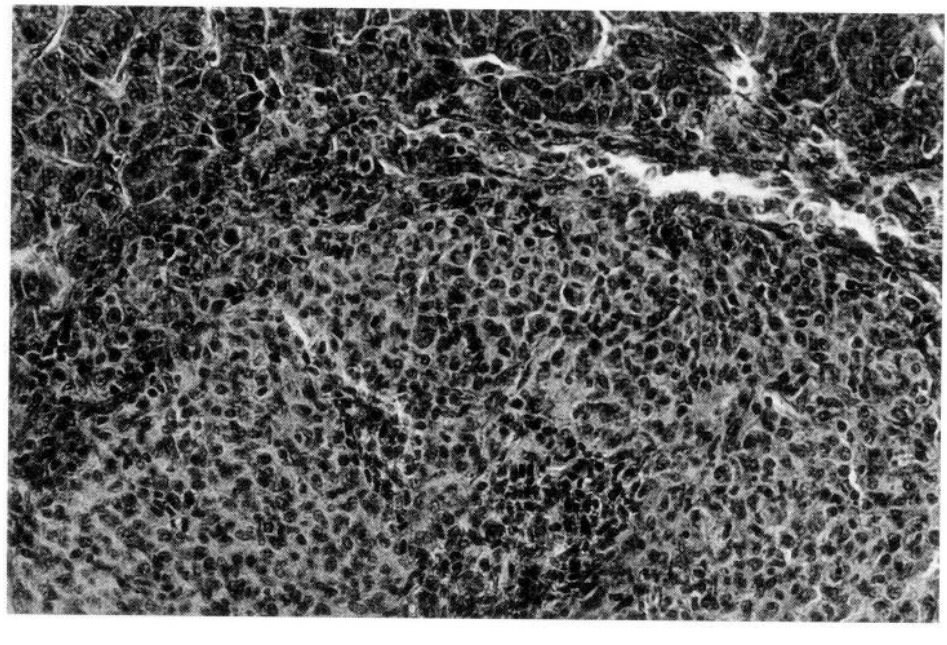

Figure 2b. Light microscopy of the same hyperplastic lesion at a higher magnification in the liver of a 6 months rainbow trout treated initially with 21ngAs/sac-fry, (H&E, x40).

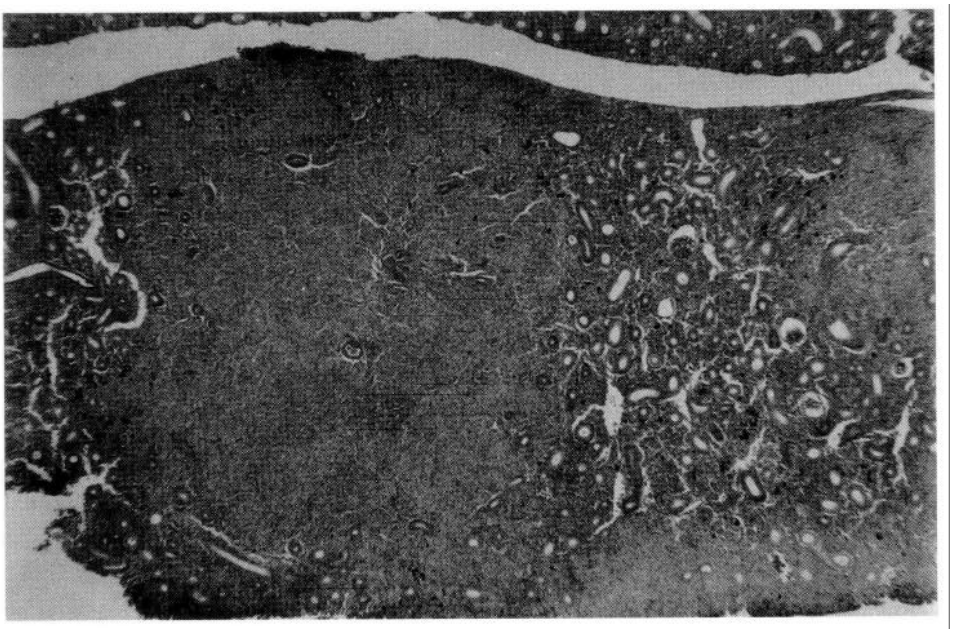

Figure 3a. Light microscopy of a large area of kidney fibrosis of a 6 months rainbow trout treated initially with 21 ngAs/sac-fry, (H&E, x16)

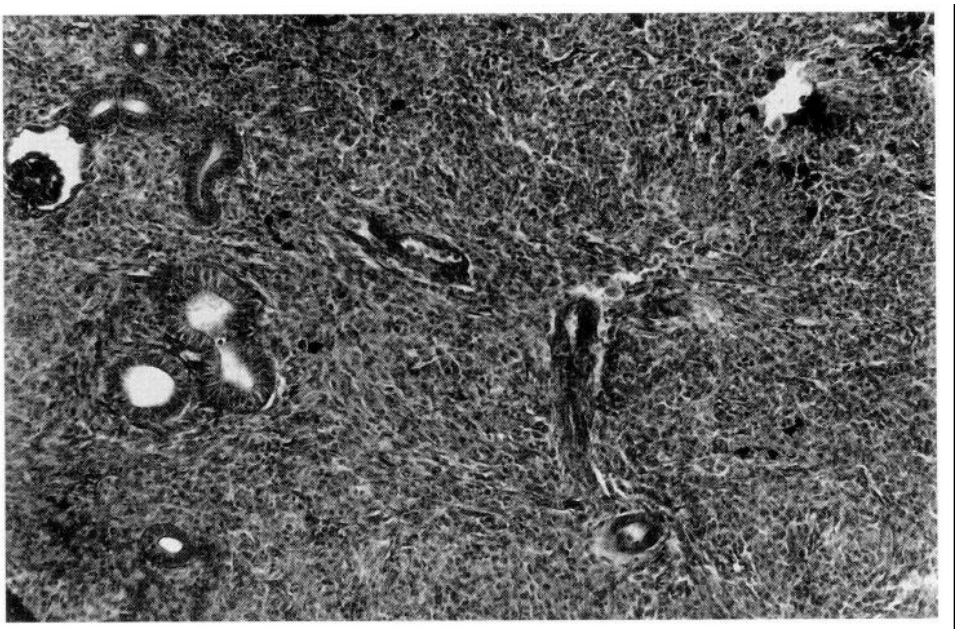

Figure 3b. Light microscopy of the same lesion at a higher magnification, of a 6 months rainbow trout treated initially with 21ngAs/sac-fry, (H&E, x40).

posterior part of the kidney while on the liver were observed on the dorsal surface. These lesions, both on the kidney and liver, were round, white and their size was relatively large 5-10mm. It should be noted that multiple lesions were observed grossly on some kidneys. No grossly visible lesions were noticed in the control and the water-injected groups (Table 2).

Table 3. A detail histopathological description of the liver and kidney lesions at the 6 months necropsy for rainbow trout treated as controls, or with water, $AsCl_3$ in water (21ngAs/sac fry)

Histopathological Interpretation	Treatment		
	Control n=40	Water n=40	Arsenic n=48
Nonneoplastic Proliferative Lesions	0/40 0%	0/40 0%	4/48 8.3%[a]
Fibromas in Kidney	0/40 0%	0/40 0%	9/48 19%[a]
Granulomas in Kidney	0/40 0%	0/40 0%	12/48 25%[a]
Granulomas in Liver	0/40 0%	0/40 0%	2/48 4.2%[a]

a : Frequencies were significantly different from control groups ($p<0.05$).

The histological analysis (Table 3.) showed that the liver lesions observed in the arsenic group were interpreted as nonneoplastic proliferative lesions, whereas an extensive proliferation of hepatocytes and cholangiocytes was noticed in an unorganized manner since there is no evidence of hepatocytic cords, in the area where necrosis had occured (Fig. 2a., 2b.). The kidney lesions observed in the arsenic group were large areas of fibrosis, whereas an extensive proliferation of fibrotic tissue and a regenerative proliferation of glomerular cells was noticed (Fig.3a., 3b.).

In addition the other cellular alterations observed in the arsenic treated fish were mainly extensive necrosis in the kidney, involving principally the second proximal segments. Also other inflammatory lesions such as granulomas were observed and were basically multifocal lesions in liver and mostly in kidney consisting of several cell types, but primarily histiocyte-like cells with large, foamy cytoplasm and a small vesicular nucleus distributed among a network of fibrocytes and capillaries (Table 3).

The studies of As toxic effects on fish and especially on trout embryos are very limited (Mance 1990). No teratogenic response was reported from a 28-day LC50

of 0.54 mg/l toxicity testing (U.S. EPA 1980) while in another study exposing trout embryos to As(III) skeletal malformations at concentrations as low as 0.25 mg/l was observed (Birge *et al.* 1983). Other studies of acute toxicity testing involving aqueous immersion of rainbow trout fry, showed that when trout were exposed to arsenic trioxide for 21 days at a concentration of 2.6 mg/l at a temperature of 15°C no effect on growth was observed (Mance 1992). Dietary studies showed that, when juvenile rainbow trout were fed semi-purified diets containing graded levels of disodium arsenate heptahydrate (DSA) for 12-24 weeks under standard laboratory conditions, no demonstrable gallbladder lesions or any other toxic effects of arsenic exposure was observed for concentrations lower than 0.281 mg As/Kg body weight/day. While for concentrations between 0.281-0.525 mg As/Kg body weight/day gallbladder lesions were seen. This was believed to be associated with these levels, including to inflammation with fibrosis of the gallbladder wall (Cockell and Bettger 1991; Cockell *et al.* 1991). In addition, field studies in the Puget Sound water body revealed that the significantly elevated concentrations of arsenic found in the sediments could have contributed to various liver diseases observed especially in species with restricted territorial habits as the bottom-dwelling fish (Malin *et al.* 1987).

In general, the pathogenesis of nonneoplastic proliferative lesions is known to play a key role in liver carcinogenesis (Metcalfe 1989; Boorman et al. 1997). Hinton and colleagues (1988) for example, who had exposed early life stages of the medaka (*Oryzias latipes*) to DENA (N-nitrosodiethylamine) have shown the sensitivity of early life stages to a single dose of a potential carcinogen, inducing degenerative and nonneoplastic proliferative lesions commonly observed after carcinogen exposure. In the present trout assay a single injection of 0.21mgAs/Kg into the yolk-sac was capable to induce similar lesions. The liver hyperplastic lesions observed could progress towards a neoplastic stage since restorative proliferation could promote the evolution of initiated cells to progress to a malignant or benign neoplasm (Peraino *et al.* 1984; Farber and Sarma 1987).

In conclusion, a single exposure of rainbow trout to arsenic induced degenarative and inflammatory lesions (necrosis, granulomas, fibrosis) and nonneoplastic proliferative lesions (liver hyperplasia). Our results strongly support that the trout model shows to be very sensitive to metals and is capable of evaluating the bioactivity of metals.

Hopefully our findings will strengthen the knowledge about the bioactivity of arsenic and promote further experimentation on metal carcinogenicity testing since there is insufficient data available concerning metal-related neoplastic diseases in aquatic laboratory and field studies.

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REFERENCES

Bailey GS, Williams DE, Hendricks JD, (1996) Fish models for environmetal carcinogenesis: the rainbow trout. Environ Health Prespect 104(1): 5-21

Birge WJ, Black JA, Westerman, AG, Ramey BA, (1983) Fish and amphibian embryos - A model system for evaluating teratogenicity. Fund Appl Toxicol 3 : 237-242

Black JJ, (1988) Carcinogenicity tests with rainbow trout embryos : A review. Aquatic Toxicology 11:129-142

Boorman GA, Botts S, Bunton TE, Fournie JW, Harshbarger JC, Hawkins WE, Hinton DE, Jokinen MP, Okihiro MS, Wolfe MJ, (1997) Diagnostic criteria for degenerative, inflammatory, proliferative nonneoplastic and neoplastic liver lesions in medaka (Oryzias latipes): consensus of a National Toxicology Program Pathology Working group. Toxicol Pathol 25(2): 202-210

Chen CJ, Wang CJ, (1990) Ecological correlation between arsenic level in well water and age-adjusted mortality from malignant neoplasms. Cancer Res 50:5470-5474

Cockell KA, Bettger WJ, (1991) Investigations on the gallbladder pathology associated with dietary exposure to disodium arsenate heptahydrate in juvenile rainbow trout (Oncorhynchus mykiss).Toxicology 77(3):233-48

Cockell KA, Hilton JW, Bettger WJ, (1991) Chronic toxicity to dietary disodium arsenate heptahydrate to juvenile rainbow trout (Oncorhynchus mykiss). Arch Environ Contam Toxicol 21(4): 518-527

Farber E, Sarma DSR, (1987) Biology of disease. Hepatocarcinogenesis: A dynamic cellular perspective. Lab Invest 56: 4-22

Gilman JPW, Swierenga SHH, (1985) Inorganic carcinogenesis. In: Searle CE (ed) Chemical Carcinogenesis, Second Ed, American Cancer Society Monograph, Washington, D.C., pp 577-630

Hinton DE, Couch JA, Swee JT, Courtney LA, (1987) Cytological changes during progression of neoplasia in selected fish species. Aquatic Toxicology 11: 77-112

IARC (1980) Some metals and metallic compounds. In: IARC Monographs on the Evaluation on the Carcinogenic Risk to Humans, Suppl. 7, Lyon.

Ishinishi N, Kodama Y, Nobutomo K, Hisanaga A, (1977) Prelimenary experimental study on carcinogenicity of arsenic trioxide in rat lung. Environ Health Perspect 19:191-196

Kotsanis N, Metcalfe CD, (1991) Enhancement of hepatocarcinogenesis in rainbow trout with carbon tetrachloride. Bull Environ Contam Toxicol 46:879-886

Kotsanis N, Iliopoulou-Georgudaki J, (1997) Toxic effects of cadmium at early stages of rainbow trout (Oncorhynchus mykiss). Toxicol And Environ Chem 62:197-205

Leonard A, (1991) Arsenic In: Merian E (ed) Metals and Their Compounds in the Environment,VCH, New York, pp 751-773

Malins DC, McCain BB, Brown DW, Chan SL, Myers MS, Landahl JT, Prohaska PG, Friedman AJ, Rhodes LD, Burrows DG, Gronlund WD, Hodgins HO, (1984) Chemical pollutants in sediments and diseases in bottom-dwelling fish in Puget Sound. Washington Environ Sci Technol 18:705-713

Mance G, (1990) Toxicity of metals to freshwater fish. In: Mance G (ed.) Pollution threat of heavy metals in aquatic environments. Pollution Monitoring Series, Elsevier Applied Science London and New York, pp 22-126

McGeachy SM, Dixon DG, (1989) The impact of temperature on the acute toxicity of arsenate and arsenite to rainbow trout (Oncorhyncus mykiss). Ecotoxicol Environ Safety 178(1):86-93

Masahito P, Ishikawa T., Sugano H, (1988) Fish tumours and their importance in cancer research. Jpn J Cancer Res 79:545-555

Metcalfe CD, (1989) Tests for predicting carcinogenicity in fish. CRC Critical Reviews in Aquatic Sciences 1:111-112

Peraino C, Staffeldt EF, Carnes BA, Ludeman VA, Blomguist VA, Vessselinovitch SD, (1984) Characterization of histochemically detactable altered hepatic foci and their relationship to hepatic tumorigenesis in rats treated once with diethylnitrosamine or benzo(a)purene within one day after birth. Cancer Res 44: 3340-3349

Snow ET, (1992) Metal Carcinogenesis: Mechanistic Implications. Pharmac Ther 53:31-65

UNEP/FAO/IAEA, (1995) Assessment of the state of pollution in the Mediterranean Sea by carcinogenic, mutagenic and teratogenic substances. MAP Techn Report Series No.92 Athens

U.S. Environmental Protection Agency, (1980) Ambient water quality criteria for arsenic. U.S.EPA Rep. 440/5-80-201 pp 205

Woo Yin-Tak, Arcos JC, (1985) Environmental Chemicals. In: Sontag JM (ed) Carcinogens in Industry and the Environment, pp 167-212

Zarr JH, (1984) Biostatistical Analysis. Prentice Hall, New Jersey