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Toxic Effects in Fish and the Mutagenic Capacity of Water from the Sava River in Yugoslavia

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Xenobiotics are known to cause enhancement of the activities of mixed function oxygenases (MFO) in the livers of exposed fish (for a review see: BEND & JAMES, 1978). This induction has been used both as a tool for the detection of the presence of xenobiotics in a body of water and as an early, badly needed sublethal biological indicator of a given concentration of a toxic substance (PAYNE & PENROSE 1975; AHOKAS et al. 1976; KURELEC et al. 1977). Equally useful is the "induct-test", a method used in our laboratory recently, consisting of i/p application of hexane extracts of water samples to young experimental carps and measurement of MFO as a response (KURELEC et al. 1980). The ultimate carcinogens formed by action of MFO have been shown definitivelly to cause neoplasia in fish (STICH & ACTON 1976; MEARNS & SHERWOOD 1977; HARSHBARGER 1977).

Fish, animals that are at the top of the food web, can consequentlly be used as indicator organisms in screening for carcinogens in water, when they are thus topically exposed. The use of a combination of MFO induction and the *Salmonella* test for the detection of mutagenic/carcinogenic substances has been shown to be useful even as a tool for detection of polluted sites in a water body (PAYNE 1976; KURELEC et al. 1977; 1979).

In order to assess the biological effects of substances in the water of the Sava River, the life artery of Yugoslavia, where 8 million people live and an important part of industry is located, we measured their early toxic effects - the induction of benzo(a)pyrene monooxygenase (BPMO) activity in natural fish population as well as their late effects - the appearance of tumors in fish of the Sava River, the induction potential of Sava waters in experimental fish by "induct-test" and the mutagenic capacity of Sava water extracts as recorded in a bacterial short-term test.

MATERIAL AND METHODS

Chemicals: Benzo(a)pyrene was obtained from Roth (Karlsruhe, F.R. Germany), NADP, NADPH, glucose-6-phosphate and dimethyl sulphoxide (DMSO) from Serva (Heidelberg, F.R. Germany), n-hexane, spectroflourometric grade, was from Merck (Darmstadt, F.R. Germany) and 7,8-benzoflavone from Aldrich (Milwaukee, USA). All other chemicals used were of analytical grade.

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Animals : carp, one year old, weighing 10-15 g, F_1 - sister generation, were used for detection of hexan extractable xenobiotics from water samples using the "induct-test" (KURELEC et al. 1980). Carp, two year old, weighing 100-150 g, F_1 - sister generation were used in experiments involving exposure to Sava River water in aluminium cages. Fish specimens, caught by sport-fishermen during their official games, were used for estimation of the level of liver BPMO activity in the natural population.

Epidemiological study of frequency of neoplasia in fish : Data were collected by fish pathologists by direct observations and necropsies of fish catches during official fishing competitions. Over 70% of neoplasms classified by both the International Classification of Diseases of WHO (Nos 140-239) and the Union Internationale le Cancer could be detected in this way. Some competitions were intentionally organized on certain stretches polluted by known quantity and type of pollutants. The number of specimen were recorded routinely.

BPMO activity level in natural population of fish : Fish of five species from two locations were used for measurements of their liver BPMO activities. Postmitochondrial fractions of liver were prepared according to PAYNE & PENROSE (1975) with minor modifications described previously (KURELEC et al. 1977). Proteins were determined by the method of LOWRY et al. (1951). BPMO activity is expressed in arbitrary units using the quinine sulphate standard of 0.001 mg/ml as 1000 a.u. per mg of protein per 15 min of incubation time (KURELEC et al. 1977). 10^{-4} M of 7,8-benzoflavone were added in experimental design in 10/ul of DMSO to the duplicates of the standard assay.

Cage exposure of carp : Fourteen specimen of two-year old carp were put in aluminium metal cage 1x1x1 m, instaled in the River Sava. On the 5th, 10th and 140th day, five, five and four specimen were taken out, respectively, and their liver BPMO was determined. Control group of carps from the same experimental group was held in laboratory conditions (basins, 0.80 x 0.40 x 0.40 m, dechlorinated water flow with 16 volume changes per day) and five specimen were analysed in the same way on the 10th and 20th day. The muscle slices were examined for their organoleptic characteristics by boiling and baking.

Induction of BPMO in experimental fish after i/p application of hexane extracts ("induct-test") : Twenty five liters of Sava water sampled at 7 locations upstreams from Zagreb (location 0, 1a, 1b, 2, 3, 4 and 5), was extracted with 120 ml of n-hexane using glass separatory funnels. The hexane extracts were reduced (protecting from light) to the volume of 3 ml with a Rotavapour (Büchi, Switzerland) at 35°C. One third of the total volume were used for i/p application to the experimental carp, divided to four animals receiving an equivalent of 2 l of water, and the remaining 2/3 (representing 16 l of water) were used for the mutagenicity testing. BPMO activity was measured in the postmitochondrial fraction of carp liver two days after i/p application of hexane extracts.

Detection of BPMO inhibitors in water extracts: Hexane extracts of 1 l of Sava water applied in 20 µl of DMSO were checked for their inhibition effect in the standard BPMO assay, using the livers of benzo-a-pyrene (B(a)P) - pretreated one year old carp (with a single dose of 30 mg B(a)P per kg body weight, after 2 days of exposure). The inhibition is expressed as a percentage of inhibition in the activity of standardly induced activity of BPMO.

Mutagenicity testing of water extracts with Salmonella typhimurium TA 100: Testing for mutagenicity was performed with the Ames test (AMES et al. 1975) using the liver postmitochondrial fraction from Aroclor 1254 induced rats and the tester strain of Salmonella typhimurium TA 100. Hexane extracts of Sava River water were applied in DMSO.

RESULTS

Activity of BPMO in natural population of fish in the Sava River

The fish caught in the Sava River have activities of BPMO which are significantly higher than are the activities of either the fish from our experimental pool or from a fish farm (Table 1).

TABLE 1

Activity of BPMO in natural population of fish in the Sava River

Species	No. of specimen	BPMO (in a.u.)	%inhibition by 7,8-BF
Chub (Leuciscus cephalus)	7	329.0 ± 225.1*	90**
Barbel (Barbus barbus)	2	224.4 186.2	91
Podust, Croat. (Chondrostoma nasus)	4	118.5 ± 50.2	75
Buborak, Croat. (Vimba vimba El.)	3	278.3 ± 241.9	82
Carp (Cyprinus carpio)	4	463.5 ± 159.9	81
Carp from farm (1.250 g)	3	29.0 ± 7.9	0
Experimental carp (12 g)	7	20.1 ± 13.4	0
Carps induced by B(a)P	3	342.5 ± 64.3	79

*mean ± standard deviation, ** mean

The efficiency of inhibition of these high BPMO activities in the presence of 10^{-4} M 7,8-benzoflavone, the specific inhibitor of their activity (WIEBEL et al. 1974) in the in vitro assay is similar to the efficiency of inhibition of the BPMO in experimental carps induced by i/p application of B(a)P. Thus, at least in carps from the River Sava this high BPMO level was induced. There is no similar direct evidence with other species from the Sava River, since there are no corresponding data for the natural level of BPMO for these other species, but the similarity in the efficiency of inhibition by benzoflavone in these fish strongly suggests that their BPMO were induced like those in carps.

Activity of BPMO in farm carps exposed to Sava River water at location 5

Experimental carps exposed to Sava River water exhibit a dose response of BPMO activity with respect to time of exposure (Table 2).

TABLE 2

Induction of BPMO in farm carps exposed to Sava River water at location 5

Days of exposure	No. of specimens	BPMO (in a.u.)
0	12	13.4 ± 5.8*
5	5	49.2 ± 31.9
10	5	69.9 ± 36.8
140	4	298.0 ± 96.0
Controls 10	5	13.8 ± 7.2
Controls 20	5	14.1 ± 6.1

*mean ± standard deviation

During this exposure, carps have fed, grown and behaved in the same way as the control group. The organoleptic characteristics of the exposed fish were not different from the characteristics of control group.

Induction of BPMO in experimental carp after i/p application of water extracts of Sava River

Hexane extracts from 2 l of water, applied i/p to the young carp ("induct-test") did not induce BPMO. At the same time, hexane extracts added to the in vitro system for detection of BPMO activity, using post-mitochondrial fractions of the livers of carps induced by B(a)P, inhibit the activity of this enzyme (Table 3).

TABLE 3

Induction of BPMO in experimental carps after i/p application of water extracts

Location No.	BPMO activity (in a.u.)* December, 1978		September, 1979		December, 1979	
1b	10.5 ± 2.1	(-)**	16.7 ± 5.5	(0)**	17.5 ± 3.0	(70)
2	9.0 ± 1.1	(-)	22.0 ± 7.5	(33)	21.3 ± 6.4	(94)
3	9.5 ± 0.7	(-)	24.0 ± 13.2	(49)	7.3 ± 1.7	(-)
4	12.0 ± 4.2	(-)	19.3 ± 7.8	(47)	6.8 ± 3.6	(-)
5	-	-	28.0 ± 10.6	(63)	7.3 ± 3.3	(-)
6	-	-	22.3 ± 11.5	(62)	10.0 ± 3.5	(-)
0	-	-	19.5 ± 12.3	(0)	-	-
Control carp			26.5 ± 17.6	(0)		
B(a)P induced carp			164.8 ± 57.5	(82)		
Hex. oil-induced carp***			124.8 ± 36.7	(89)		

* all experiments were done with 4 specimens. Values present mean ± standard deviation. **In parentheses are given percents of inhibitions of B(a)P-induced BPMO acitivity in vitro (means) caused by the addition of hexane extracts of 1 l of Sava River water (dissolved immediately before testing in 20 µl of DMSO). ***hexane extracts of water experimentally polluted with Lybian crude oil were given i/p (1500 mg/kg body weight) to experimental carp.

Only the water from location 0 (corresponding to the spring of the Sava River) did not contain these hexane-extractable inhibitory substances. At that location hexane extracts of water also do not contain the xenobiotics which can induce BPMO. It holds also for the single sample (September, 1979) from the location 1b. At all other locations the inhibitory substances were present in the hexane extracts (see Table 3), and consequently the BPMO activities estimated in experimental fish were false, i.e. activities that were strongly influenced by simultaneous presence of inhibitors.

Mutagenicity of water extracts with Salmonella typhymurium TA 100

Hexane extracts of Sava water from seven locations were tested for their mutagenic potency. Extracts of 1 l of water per plate were examined with Salmonella typhimurium TA 100 either with or without the addition of the postmitochondrial fraction of the livers from rats treated with Aroclor 1254. From the Table 4 it is obvious that only the water at location 1a is highly mutagenic.

The waters from other locations are either not mutagenic, or only slightly so. The fact that waters from location 1b., located at a distance only 2 km downstream from 1a., do not exhibit the mutagenic activity could be explained neither by speed of the processes of biodegradation, nor by the dilution of the active substance since there is no additional source of water at that segment of the Sava River.

TABLE 4

Mutagenicity of water extracts with Salmonella typhimurium

Location		Relative mutagenicity* + S - 9 Mix	- S - 9 Mix
1a		10 +	0
1b		1 +	0
2		-	0
3		-	0
4		-	0
5		1 +	0
6		1 +	0
Controls**	Control plates	127 ± 6	115 ± 4
	20 µl DMSO/plate	128 ± 3	112 ± 7
	5 µg B(a)P/plate	407 ± 7	-
	10 µg B(a)P/plate	756 ± 3	-
	90 µg MC/plate	5780 ± 226	-
	1 µg B(a)P-oxid/plate	-	1135 ± 54

*one + corresponds to a 30% increase in number of his^+ revertants over control.** values are expressed in number of his^+ revertants of Salmonella typhimurium TA 100 per plate.

Frequency of neoplasm in fish from Sava River

Twenty one species of Sava River fish, represented by 198133 specimens, were examined. A total of 2085 fish were necropsied. The greatest number of any species examined were bleak (Alburnus alburnus) 44670, and the smallest, 23, of brown trout (Salmo trutta m. fario). One hundred sixteen fish were found to be diseased, the diseased individuals comprising, on the average, 5.56% of their species numbers necropsied. Bleak had a high disease incidence of 24.74%. Diseases noted were viral and bacterial diseases, and infections with four species of helminth parasites. In no instance were neoplasms detected (Table 5).

TABLE 5

Frequency of neoplasia in fish from the River Sava

Common name	Latin name	No. of specimens examined	necropsied	diseased	neoplasms	*diseased %
Brown trout	Salmo trutta m. fario	23	23		0	
Carp	Cyprinus carpio	1560	87	5	0	5.75
Goldfish	Carassius carassus	7330	119	2	0	1.01
Chub	Leuciscus cephalus	5865	91		0	
Roach	Rutilus rutilus	23500	264	6	0	2.27
Rudd	Scardinius erithrophtalmus	33830	368	4	0	1.09
Platnica, Croat.	Rutilus pigus virgo	32360	175	3	0	1.71
Podust, Croat.	Chondrostoma nasus	6321	62		0	
Barbel	Barbus barbus	4289	93		0	
Gudgeon	Gobio gobio	3932	20		0	
Bream	Abramus brama	20020	290		0	
Orfe	Leuciscus idus	6150			0	
Bleak	Alburnus alburnus	44670	380	94	0	24.74
Tench	Tinca tinca	2450			0	
Čikov, Croat.	Misqurnus fossilis	320			0	
Wels	Silurus glanis	80			0	
Pumpkinseed	Lepomis gibbosus	1245			0	
Perch	Perca fluviatilis	4518	48		0	
Bullhead	Cottus gobio	106			0	
Pike	Esox lucius	520	35		0	
Grass carp	Ctenopharyngodon idella	544	30	2	0	6.67
Total number of specimens:		198133	2085	116	0	5.56

*%are calculated from necropsied fish only, and comprise parasitic (Ligula intestinalis, Diplostomum spathaceum, Acantocephala, Bothriocephalus govkongensis), viral (Epithelioma papulosum cyprini) and bacterial diseases (Erythrodermatitis cyprini).

DISCUSSION

The five species living in the investigated segment of the Sava River are highly induced with respect to their liver BPMO activities. Obviously, this is the consequence of the reaction to the xenobiotics present in this water, as can be concluded from the exposure experiment, where uninduced experimental carps were exposed, and their dose versus time of exposure - response was observed. The activities of BPMO from fish livers of natural populations could be inhibited in vitro by 7,8-benzoflavone in a similar manner, as the activities of BPMO from experimental fish treated i/p with B(a)P. In fish, only cytochrome P 448 -dependent mixed function oxydases are inducible (KURELEC et al. 1980). Benzoflavone inhibits selectively only this type of mixed function oxydases (WIEBEL et al. 1974). Thus, the inhibition of BPMO from fish living in the Sava River by benzoflavone should be interpreted as to be caused by the xenobiotica of the methylchloranthrene type, i.e. type II of inducers.

At the same time, the i/p application of hexane extracts of Sava River water downstream from location 1 failed to induce liver BPMO. This finding disagrees with our previous experience showing a direct relationship between the amount of xenobiotica in water samples and the degree of BPMO induction in fish treated with i/p with hexane extracts of water (KURELEC et al. 1980). This disagreement could be reasonably explained by the finding that hexane-extractable substances from Sava River waters are inhibitory to the activity of BPMO. AHOKAS et al. (1976) observed that BPMO in pike (Essox lucius L), chronically exposed to Lake Vatianjyrvi water, which is heavily polluted by mixed effluents from both pulp and chemical factories and human communities, was inhibited. They suggested the use of this finding (i.e. inhibition) as an indicator of the specific type of pollution as opposed to the pollution caused by petroleum products which have been found to cause the induction of BPMO (PAYNE & PENROSE, 1975). The type of pollution in Sava River water is similar to the type of pollution described in Lake Vatianjyrvi, but the reaction of all five species from the Sava River tested in our experiment was positive. Obviously, these "inhibitors of BPMO" do not exert their effects under natural conditions. On the other hand, their nature, specially their hexane-extractibility, prevents the use of water-hexane extracts, either in the method of measurement of induction of BPMO in the livers of experimental fish after i/p application or in the Ames' test. In order to establish the presence of mutagenic substances in complex types of environmental samples, the possible presence of BPMO inhibitors should be assessed, either by enzymological assay, as in this case, or by including the test samples as "internal standards" in experimental design when standard carcinogens are tested. However, presence of BPMO inhibitors might be used, per se, as indicator of specific types of pollution in environmental quality studies.

There is a good correlation between the quality of water and the state of induction of BPMO activity, but no correlation to low water quality was observed with frequency of neoplasms in the wild population of fish. Thus, a population of aquatic organisms though to be "at highest

carcinogenic risk", i.e. fish, do not exhibit the expected neoplasms, and the statement that "surface waters containing fish which reproduce normaly can be regarded as healthy water" - and the further stipulation that it should be suitable for preparation of drinking water by natural method only (POELS et al. 1978) - must be viewed guardedly, for a hexane extract of a few liters of Sava River water contains sufficient mutagenic substances to yield significant increases in the number of revertants in the Ames test for mutagenicity, and the presence of these harmful substances neither affects the reproduction of the fish, nor increases the apparent incidence to tumors. Thus, monitoring of tumor frequency in fish, and their use as the sole sentinel organism for detection of water-borne mutagens and/or carcinogens, does not appear to be reasonable.

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