Oil Shale Processing as a Source of Aquatic Pollution: Monitoring of the Biologic Effects in Caged and Feral Freshwater Fish

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The biologic effects of the oil shale industry on caged rainbow trout (*Oncorhynchus mykiss*) as well as on feral perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) were studied in the River Narva in northeast Estonia. The River Narva passes the oil shale mining and processing area and thus receives elevated amounts of polycyclic aromatic hydrocarbons (PAHs), heavy metals, and sulfates. The effects of the chemical load were monitored by measuring cytochrome P4501A (CYP1A)-dependent monooxygenase (MO) activities [7-ethoxyresorufin *O*-deethylase and aryl hydrocarbon hydroxylase (AHH)] as well as conjugation enzyme activities [glutathione *S*-transferase (GST) and UDP-glucuronosyltransferase] in the liver of fish. CYP1A induction was further studied by detecting the amount and occurrence of the CYP1A protein. Histopathology of tissues (liver, kidney, spleen, and intestine) and the percentage of micronuclei in fish erythrocytes were also determined. Selected PAHs and heavy metals (Cd, Cu, Hg, and Pb) were measured from fish muscle and liver. In spite of the significant accumulation of PAHs, there was no induction of MO activities in any studied fish species. When compared to reference samples, AHH activities were even decreased in feral fish at some of the exposed sites. Detection of CYP1A protein content and the distribution of the CYP1A enzyme by immunohistochemistry also did not show extensive CYP1A induction. Instead, GST activities were significantly increased at exposed sites. Detection of histopathology did not reveal major changes in the morphology of tissues. The micronucleus test also did not show any evidence of genotoxicity. Thus, from the parameters studied, GST activity was most affected. The lack of catalytic CYP1A induction in spite of the heavy loading of PAHs was not studied but has been attributed to the elevated content of other compounds such as heavy metals, some of which can act as inhibitors for MOs. Another possible explanation of this lack of induction is that through adaptation processes the fish could have lost some of their sensitivity to PAHs. Either complex pollution caused by oil shale processing masked part of the harmful effects measured in this study, or oil shale industry did not have any severe effects on fish in the River Narva. Our study illustrates the difficulties in estimating risk in cases where there are numerous various contaminants affecting the biota.
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Estonian oil shale is of Middle Ordovician origin, and its basin is the largest commercially exploited oil shale deposit in the world. Since 1916, 872 million tons of oil shale have been excavated (*1*); at present, approximately 80% of its yield is used as a fuel for electric thermal power plants (TPPs). During the recent decades of increased economic activity in Estonia, intensive and uncontrolled development in the oil shale industry has brought serious ecologic problems to the northeast part of the country.

The River Narva receives its pollution mainly from drainage water from two oil shale mines (Sirgala and Narva) and by leachate from the oil shale ash plateaus of the Estonian and Baltic TPPs. The average river runoff is 400 m^3/sec and the drainage area is 56,200 km^2. Approximately 250 million m^3 water was pumped out of the mines in 1995 (*2*) and was discharged directly or without proper purification into natural bodies of water. Some water pollution is received from the Slantsy oil shale processing plant in Russia. The main pollutants in the River Narva are sulfates, chlorides, oil products, heavy metals, and polycyclic aromatic hydrocarbons (PAHs) (*3–8*). Approximately 0.8–1.2 million m^3 highly alkaline waste water per year enters the Narva (*7*). This influx is formed by leachate from the ash field of the TPPs.

Northeast Estonia also receives pollution from TPPs via air (*6*). A total of 102 kg PAHs is emitted annually: 4.2 kg is benzo(*a*)pyrene (*7*). PAHs will be deposited by dry deposition or washed out by precipitation. Within a radius of up to 1–10 km around the Baltic TPP the daily benzo(*a*)pyrene deposition load is in the range of 30–87 ng/m^2, and is 9–19 ng/m^2 on the northern coast of Lake Peipsi (*9*). In the oil shale fly ash there are up to 1 mg/kg Hg, 2 mg/kg Cd, and 165 mg/kg Pb (*7*).

The part of the River Narva that runs downstream from the town of Narva is an important river lamprey spawning site. It was also an important spawning area for salmon, although it has lost its significance. Approximately 80,000 people use the water from the River Narva for drinking. Indeed, there is a risk for aquatic life as well as a risk for human health.

Little attention has been paid to the effects of pollution connected with oil shale mining, combustion, thermal processing, and waste management on fish. Some studies in the Estonian oil shale basin (*10,11*) have shown that biomarkers of environmental stress (e.g., content of blood electrolytes and frequencies of micronuclei in erythrocytes) detected in rainbow trout caged in the drainage water of an oil shale mine differ from control fish. The pollution caused by leaching of the oil shale has been reported at oil shale areas in the United States as well (*12–15*). Recently, a fish liver cell line (PLHC-1) has been used to monitor the fish-specific effects of lipophilic contaminants in the sediments from the River Narva (*16*).

The leachate of oil shale ash plateaus of Estonian TPPs is acutely toxic for fish. This is mainly due to the extreme alkalinity (pH ~13). Ninety-six-hour median lethal concentrations of leachate from ash plateaus of TPPs for common carp (*Cyprinus carpio*) and pike-perch (*Stizostedion lucioperca*) were 4% and 1%, respectively (*17*).

Complex mixtures of pollutants make it difficult to determine the most suitable methods for monitoring the aquatic contamination. Cytochrome P4501A (CYP1A)-dependent monooxygenase (MO) activities in fish tissues have been used to detect PAH-type pollution [reviewed by Payne et al. (*18*), Di Giulio et al. (*19*), and Tuvikene et al. (*20*)]. MOs represent the first step of biotransformation in xenobiotic metabolism (*21*). In

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the second phase, endogenous molecules such as glutathione and glucuronic acid can be joined with the metabolites (*22,23*). Xenobiotic metabolism in fish has been regarded as an early warning sign for selected aquatic contaminants. Inhibition of biotransformation reactions can enhance toxicity and bioaccumulation of lipophilic xenobiotics (*24*). More serious effects of pollution can be seen as changes in cellular and tissue morphology (*25*). Vacuolization and different lesions have been observed in the tissues of fish collected from heavily polluted areas (*19,26,27*). Further, genetic effects (e.g., the occurrence of DNA strand breaks and micronuclei) caused by contamination with polycyclic or halogenated hydrocarbons reveal those changes that can lead to mutagenicity, carcinogenicity, teratogenicity, and later to population changes (*19,28–31*).

In this study, a set of methods was used to reveal the biologic effects of oil shale processing on caged fish [rainbow trout (*Oncorhynchus mykiss*)] and feral fish [perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*)] in the River Narva in northesast Estonia, where the river reveals the pollution gradient toward the Baltic Sea (*16,32*). The main aim was to detect which of the methods would be the best for future biomonitoring purposes. CYP1A induction was detected by measuring the catalytic activities of MOs, and by analyzing the CYP1A protein amount in fish liver and CYP1A distribution in various tissues to detect whether the enzyme protein was present and whether it was catalytically active. Xenobiotic metabolism was further studied by analyzing hepatic conjugation enzyme activities. Morphologic changes in different tissues (liver, kidney, spleen, and intestine) were detected and the percentage of micronuclei in fish blood was calculated to express the genotoxicity of the pollutants. The chemical contents of nine PAHs as U.S. Environmental Protection Agency priority pollutants (*33*) and four heavy metals were analyzed to determine the tissue contents of these pollutants, which were believed to be abundant at the study areas.

Materials and Methods

Chemicals. Benzo(*a*)pyrene, 5-bromo-4-chloro-3-indolylphosphate, 1-chloro-2,4-dinitrobenzene (CDNB), glutathione (reduced form), nitroblue tetrazolium, uridine 5′-diphosphoglucuronic acid, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxyresorufin was purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and *p*-nitrophenol from Fluka AG (Buchs SG, Buchs, Switzerland). The level 1 peroxidase antiperoxidase detection system and the AEC chromogen system were from Signet Laboratories, Inc. (Dedham, MA). All other chemicals were of analytical grade.

Caging studies. Immature 1-summer-old (0+) rainbow trout (*O. mykiss*) were obtained from a fish farm in Roosna-Alliku (northern Estonia). A 3-week caging experiment was conducted during the fall in the River Narva (Figure 1). The three caging sites were *a*) 25 km upstream from the town of Narva (Mustajõgi, the proposed reference area), the source of drinking water for the town; *b*) near the ash plateaus of Baltic TPP (5 km upstream from the town; the Narva water reservoir); and *c*) 5 km downstream from the town (Riigiküla).

In the cages made of soft net, the maximum density of fish was < 3 kg/m^3. There were 8–11 fish in the cages. The fish were transported straight from the fish farm to the caging sites. The cages were placed 0.5 m under the surface of the water. Fish were not fed during the caging. Abnormal mortality was not observed during the experiment (10% of the fish died at the Riigiküla site). The rainbow trout weighed 44 ± 10 g (mean ± standard deviation) and were 16 ± 1 cm in length. There were no significant differences between the groups in these parameters.

The physicochemical characteristics of River Narva water at Mustajõgi at the end of the caging were as follows: temperature 6.5°C, pH 8.2, conductivity 0.33 mS/cm, and dissolved oxygen 9.5 mg/L. The respective parameters for Baltic TPP were 10.5°C, 8.0, 0.26 mS/cm, and 10.5 mg/L; for Riigiküla the parameters were 4.4°C, 7.9, 0.22 mS/cm, and 10.0 mg/L.

Feral fish. In the fall, perch (*Pe. fluviatilis*) and roach (*R. rutilus*) were caught from the study sites in the River Narva (Mustajõgi, Baltic TPP, and Riigiküla). Baltic TPP and Riigiküla are isolated from each other by a hydroelectric dam situated in the town of Narva. The fish were caught with gill nets within 36 hr. The nets were set in areas with a relatively slow current. The gill nets were checked two to three times, and undamaged fish were collected into cages to wait for further processing.

There were 5–10 perch and 19–21 roach per study area. The wet weights of the perch and roach were 100 ± 89 g and 113 ± 48 g, respectively. The mean lengths were 19 ± 5 cm and 21 ± 3 cm, respectively. There were no significant differences between the groups in total length or total weight. Age was estimated from length using gender-specific age-length curves constructed for both species from each study area. All of the fish fit the category of 4–6 years of age (*34*).

During sampling, the physicochemical characteristics of the river for the Mustajõgi site were as follows: temperature 11.5°C, pH 8.0, conductivity 0.30 mS/cm, and dissolved oxygen 9.3 mg/L. The same parameters for the Baltic TPP site were 11.1°C, 7.6, 0.29 mS/cm, and 7.6 mg/L; for Riigiküla they were 10.5°C, 7.9, 0.32 mS/cm, and 9.1 mg/L, respectively.

Collection and processing of samples. In the field, fish were stunned by a blow to the head and were examined for the presence of gross external lesions and severe parasite infections. Total weight and length were measured immediately. Blood samples were taken with a heparinized capillar straight from the bulbus arteriosus and smear slides were prepared for the micronucleus test. The abdominal cavity of the fish was opened and the gall bladder was removed intact. The liver, spleen, trunk kidney, and intestine were excised and representative portions were cut and placed into 10% neutral buffered formalin for histopathologic examination. White

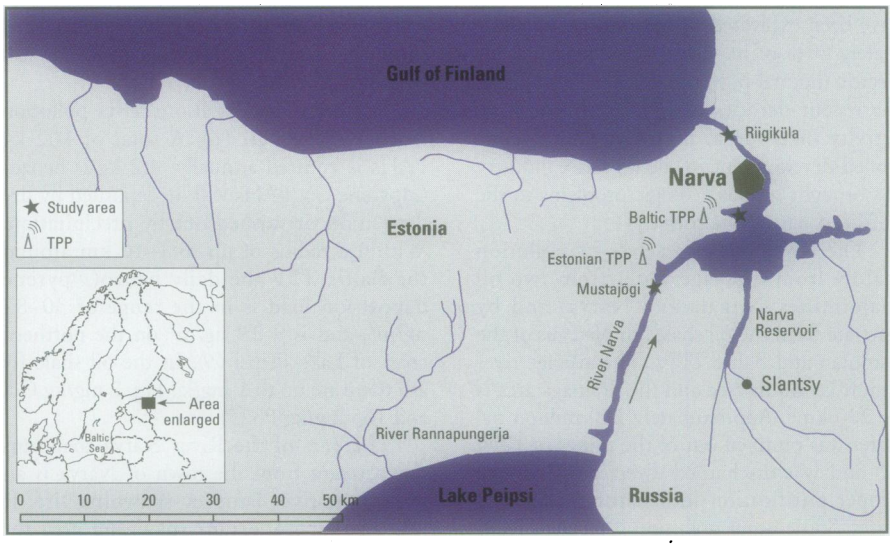

Figure 1. Study areas (Mustajõgi as reference, Baltic thermal power plant and Riigiküla as exposed) in the River Narva (northeast Estonia).

muscle samples were cut dorsolaterally above the horizontal plane of the fish. Liver samples for enzyme analyses as well as liver and muscle samples for chemical analyses were stored in liquid nitrogen. The subcellular fractions were prepared within 10 days of sampling, as described by Tuvikene et al. (*20*). All microsomal and postmicrosomal fractions were stored at -80°C. The air-dried micronucleus preparations were fixed with absolute methanol (5 min).

The condition factor (CF) was calculated from the total weight and total length of fish according to the formula $CF = [W(g) \times L(cm)^{-3}] \times 100$. The liver somatic index (LSI; the percentage weight of detached liver of the total fish weight) was also calculated.

Extraction and chemical analyses of PAHs. For extraction, a sample [3 g wet weight (ww), 2–4 fish from each species per site] of pooled tissues were ground and boiled with a mixture of ethanol and potassium hydroxide. The homogeneous mass was cooled and diluted by deionized water and then extracted by diethyl ether.

PAH analyses were carried out with high-performance liquid chromatography with fluorometric detection (model 1311; Nauchpribor, Minsk, Belarus) according to the method of Trapido and Palm (*35*). A method based on the Schpol'skii effect, a specific fluorescence emission spectra in frozen hexane at 77°K (*36*), was also applied for quantitative determination of benzo(*a*)pyrene. The detection limit was 2 ng/sample.

Chemical analyses of heavy metal. Preparation of heavy metal samples (2–4 pooled tissues from each fish species per site; 0.5–2.5 g) for total Cd, Cu, Hg, and Pb measurements was performed according to the Finnish standards (*37*).

Pb, Cd, and Cu were determined by using differential pulse anodic stripping voltammetry. The voltammograms were obtained with an Autolab system (Ecochemie, Utrecht, The Netherlands) attached to a 633 VA stand (Metrohm, Henrisau, Switzerland). The detection limits in measuring solution were as follows: Pb, 10^{-9} mol/L; Cd, 10^{-10} mol/L; and Cu, 10^{-9} mol/L.

Hg was measured by a cold vapor method using a Varian SpectrAA 250 Plus atomic absorption spectrophotometer (Varian Australia PTY, Mulgrave, Australia) equipped with a VGA-77 vapor generation accessory. The detection limit for Hg was 10^{-8} mol/kg.

Enzyme and protein analyses. The enzyme and protein analyses of the studied fish were conducted as described in Tuvikene et al. (*20*). The hepatic monooxygenase activities were measured from the microsomal fractions, using 7-ethoxyresorufin and benzo(*a*)pyrene as substrates. The deethylation of 7-ethoxyresorufin (EROD) was measured with a Shimadzu fluorescence spectrophotometer (RF-5001PC; Shimadzu Corporation, Kyoto, Japan) in a kinetic reaction with resorufin as reference (*38*). The amount of hydroxylated benzo(*a*)-pyrene (AHH) was measured with a Perkin-Elmer MPF-43A spectrofluorometer (Perkin-Elmer, Norwalk, CT) using 3-hydroxybenzo (*a*)pyrene as reference (*39*). Microsomal UDP-glucuronosyltransferase (UDP-GT) activity was measured spectrophotometrically (Shimadzu UV-240; Shimadzu) using *p*-nitrophenol as the aglycone (*40*). Cytosolic glutathione *S*-transferase (GST) activity was analyzed with a Perkin-Elmer Lambda 2 UV/VIS spectrophotometer (Perkin-Elmer and Co., GmbH, Überlingen, Germany) with CDNB as the substrate (*41*). The enzyme analyses were carried out at 18°C. The protein content in microsomal and supernatant fractions was measured according to the method of Bradford (*42*).

Immunoblotting. We randomly selected 2–4 samples from each fish species from different study areas to screen CYP1A protein content. Microsomal protein samples (75 µg from rainbow trout and 150 µg from perch and roach) and CYP1A standards [purified CYP1A1 from scup (*Stenostomus chrysops*)] were analyzed by denaturing gel electrophoresis on 6–15% acrylamide gradient gels. Proteins were electrophoretically transferred onto 0.2 µm nitrocellulose, incubated with monoclonal antibody (MAb) 1-12-3 (anti-scup CYP1A1) (*43*) at 10 µg/ml and then with goat antimouse immunoglobulin G linked to alkaline phosphatase (1/5,000 dilution). Color was developed by enhanced chemiluminescence as directed for the Schleicher and Schuell rad-free chemiluminescence detection kit (Schleicher and Schuell, Keene, NH). Values for CYP1A equivalents were determined from the integrated optical density of the MAb 1-12-3 cross-reactive proteins relative to that of scup CYP1A1 standards.

Immunohistochemistry. Three to five randomly selected tissue samples from each fish species from different study areas were processed and embedded in paraffin. Standard 5-µm sections were deparaffined and hydrated, during which they were incubated in 3% H_2O_2 to block endogenous peroxidase (*44*). Sections were then analyzed for P4501A content using MAb 1-12-3 on scup CYP1A1 (*43*). CYP1A in tissues was detected by an indirect peroxidase labeling method (*45*). Sections of liver from uninduced and from β-naphthoflavone-induced rainbow trout were included in each set as negative and positive controls for the staining method.

Histopathology. We histologically examined 5–7 perch and 3–7 roach per site. Thin sections (5 µm) from paraffin-embedded tissue samples (liver, kidney, spleen, and intestine) were cut and stained with hematoxylin and eosin for light microscopic examination. Apart from tissues that appeared normal, the following morphologic changes were categorized: degenerative changes (cell swelling, cell hypertrophy, hydropic vacuolation, hyalinization, nuclear pleomorphism, and megalocytic hepatosis), necrotic changes (coagulative necrosis and caseous necrosis), acute inflammation (caused by bacterial toxins and viruses), abnormal growth and cell proliferation (as compensatory or protective response), preneoplastic focus of cellular alteration, and neoplasms. Abnormal structural changes were classified by their appearance and intensity (focal, moderate, diffuse).

The piscine erythrocyte micronucleus test. The methanol-fixed preparations were processed further by dying with 5% Giemsa (Sigma, St. Louis, MO) for 10 min. The excess color was washed with distilled water and the slides were mounted with Permount SP-15-500 (Fisher Chemical, Fair Lawn, NJ) and covered with a cover slip. The preparations were dried for at least 24 hr before the counting of micronuclei. One thousand erythrocytes per slide were counted for micronuclei detection under the light microscope (× 400). To ensure the results, the micronuclei were further observed under an oil immersion lens (× 1,000). The micronuclei and other nuclei lesions were scored according to the characteristics presented in Carrasco et al. (*46*).

Statistical data processing. The SPSS/PC+ (SPSS, Chicago, IL) software was used for statistical data processing. Because the studied material consisted of groups that varied in size, the assumption of equal variances was first tested. The data were further tested with a nonparametric Kruskal-Wallis (K-W) one-way analysis of variance, as well as with the nonparametric Mann-Whitney test (M-W).

Results

Contents of PAHs. In the muscle of caged rainbow trout and feral perch, the total content of PAHs increased downstream toward the Baltic Sea (Tables 1 and 2). The accumulation was higher in perch (up to 6.5-fold) than in rainbow trout. The accumulation of total PAHs in the muscle of feral roach was also higher (up to 8.5-fold) than in rainbow trout (Tables 1 and 3), but did not show the same trend as in perch and rainbow trout.

Feral fish liver accumulated more PAHs than muscle (Tables 2 and 3). There were no major differences in PAH profiles between study sites, fish species, or fish tissues. The dominating compounds were pyrene (45–62 and 47–53% of the total PAHs in muscle and liver, respectively) and phenanthrene (9–26 and 14–28%). The contents of fluoranthene,

benz(*a*)anthracene, and chrysene were also remarkable. The percentage of 3-, 4-, and 5-ring compounds of total PAHs built up 10–27, 62–87, and 1–5%, respectively.

Contents of heavy metals. There were no exact trends between study sites in total heavy metal concentrations of fish (Table 4). The content of Hg in muscle was higher in feral fish than in caged rainbow trout. Moreover, feral fish muscle and liver showed almost the same amount of Hg. There were no marked differences in Cd or Pb contents of muscle between caged and feral fish. Feral fish liver contained approximately one order of magnitude more Cd than muscle. The liver also seemed to be the site of Cu accumulation; the content of Cu in the liver of feral fish was 15- to 140-fold higher than in muscle.

General physiologic indices. There were no statistical differences in CF values between different sites within each fish species (K-W *p*-values 0.733, 0.162, and 0.715 for rainbow trout, perch, and roach, respectively). There were also no statistical differences in LSI values (K-W *p*-values 0.194, 0.065, and 0.171 for rainbow trout, perch, and roach, respectively) (Table 5). However, the mean LSI of perch caught from Riigiküla was 36% higher than the value of reference fish.

CYP1A induction and conjugation activities. There were no significant differences in MO activities in caged rainbow trout (K-W *p*-values 0.771 and 0.933 for AHH and EROD, respectively) (Figure 2). In feral fish, however, AHH and EROD activities differed in most cases (K-W *p*-values 0.026 and 0.163 and 0.002 and 0.076 in perch and roach, respectively) (Figure 2). The trend was toward decreased values at exposed areas. At Baltic TPP, AHH activity decreased 55 and 33% in perch and roach, respectively, as compared to Mustjõgi. At the same site, EROD activity in perch decreased 5% and decreased 74% in roach.

The amount of CYP1A protein did not always follow EROD induction: thus, CYP1A protein content was not necessarily increased in those individual samples where EROD activity was elevated. CYP1A content in perch showed a trend toward elevated values at exposed areas (1.9-fold at Baltic TPP, 2.4-fold at Riigiküla) when compared to

Table 1. Representative PAHs (ng/g dry weight) in pooled muscle samples of rainbow trout (2–4 fish per site) caged in the River Narva.

	Study site		
		Baltic	
PAH	Mustajõgi	TPP	Riigiküla
3-ring			
Phenanthrene	69	197.7	139.7
Anthracene	2.6	5.4	2.8
4-ring			
Fluoranthene	36.5	105	90.8
Pyrene	231.3	417.6	555.1
Benzo(*a*)anthracene	17.9	23.4	36.4
Chrysene	47.2	77.4	51.1
5-ring			
Benzo(*e*)pyrene + benzo(*b*)fluoranthene	12.4	5.4	10.7
Benzo(*a*)pyrene	4.2	7.7	3.8
Total	421	840	890

Abbreviations: PAH, polycylic aromatic hydrocarbon; TPP, thermal power plant.

Table 2. Representative PAHs (ng/g dry weight) in pooled muscle and liver samples of feral perch (2–4 fish per site) in the River Narva.

	Study site		
		Baltic	
Tissue/PAH	Mustajõgi	TPP	Riigiküla
Muscle			
3-ring			
Phenanthrene	705.9	326.7	770.8
Anthracene	26.5	12.3	21.3
4-ring			
Fluoranthene	299.5	313.3	479.2
Pyrene	1,240.2	1,970.0	2,416.7
Benzo(*a*)anthracene	109.3	170.0	300.0
Chrysene	268.6	580.0	737.5
5-ring			
Benzo(*e*)pyrene + benzo(*b*)fluoranthene	88.2	93.3	80.4
Benzo(*a*)pyrene	10.8	12.3	14.5
Total	2,749	3,478	4,820
Liver			
3-ring			
Phenanthrene	182.3	1,103.3	2,750.0
Anthracene	5.9	34.7	106.7
4-ring			
Fluoranthene	106.9	980.0	1326.7
Pyrene	596.7	4,160.0	5,333.3
Benzo(*a*)anthracene	47.2	476.6	516.7
Chrysene	173.8	993.3	1,270.0
5-ring			
Benzo(*e*)pyrene + benzo(*b*)fluoranthene	45.9	115.7	90.0
Benzo(*a*)pyrene	6.9	34.3	47.3
Total	1,166	7,898	11,441

Abbreviations: PAH, polycylic aromatic hydrocarbon; TPP, thermal power plant.

Table 3. Representative PAHs (ng/g dry weight) in pooled muscle and liver samples of feral roach (2–4 fish per site) in River Narva.

	Study site		
		Baltic	
Tissue/PAH	Mustajõgi	TPP	Riigiküla
Muscle			
3-ring			
Phenanthrene	765.0	303.3	690.8
Anthracene	15.5	11.5	21.8
4-ring			
Fluoranthene	465.0	202.9	328.1
Pyrene	1,645.0	698.6	1,255.5
Benzo(*a*)anthracene	170.0	71.8	78.9
Chrysene	485.0	145.5	244.5
5-ring			
Benzo(*e*)pyrene + benzo(*b*)fluoranthene	39.0	14.4	27.8
Benzo(*a*)pyrene	20.0	12.9	14.5
Total	3,605	1,461	2,662
Liver			
3-ring			
Phenanthrene	392.8	796.5	–
Anthracene	14.0	18.8	–
4-ring			
Fluoranthene	210.1	36.9	–
Pyrene	990.0	1,500.0	–
Benzo(*a*)anthracene	127.6	180.9	–
Chrysene	188.3	316.6	–
5-ring			
Benzo(*a*)pyrene	18.5	32.7	–
Total	1,941	2,882	–

Abbreviations: –, not analyzed; PAH, polycylic aromatic hydrocarbon; TPP, thermal power plant.

Table 4. Heavy metal contents (mg/kg dry weight) in pooled fish muscle/liver samples (2–4 fish per site) from the River Narva.

	Study site		
		Baltic	
Metal/fish	Mustajõgi	TPP	Riigiküla
Mercury			
Rainbow trout	0.30/–	0.13/–	0.26/–
Perch	0.70/0.36	0.86/0.47	0.37/–
Roach	0.38/0.41	0.54/0.35	0.53/0.42
Cadmium			
Rainbow trout	0.01/–	0.02/–	0.01/–
Perch	0.01/0.22	0.02/–	0.04/–
Roach	0.04/0.12	0.01/0.15	0.02/0.14
Lead			
Rainbow trout	0.24/–	0.29/–	0.12/–
Perch	0.38/0.66	0.09/0.82	0.28/–
Roach	0.02/0.97	0.23/0.55	0.14/1.40
Copper			
Rainbow trout	1.40/–	1.45/–	1.10/–
Perch	1.22/170.5	0.93/8.0	1.10/–
Roach	3.25/64.90	1.49/57.80	0.95/14.40

Abbreviations: –, not analyzed; TPP, thermal power plant.

Table 5. Condition factor and liver somatic index in caged and feral fish from the River Narva.

Site	CF[a,b]	LSI[b]	*n*
Caged rainbow trout			
Mustajõgi	1.06 ± 0.05	1.06 ± 0.17	11
Baltic TPP	1.05 ± 0.07	1.17 ± 0.35	8
Riigiküla	1.04 ± 0.07	1.22 ± 0.22	8
K-W	0.733	0.194	
Feral perch			
Mustajõgi	1.25 ± 0.16	1.16 ± 0.18	5
Baltic TPP	1.10 ± 0.08	1.14 ± 0.24	10
Riigiküla	1.20 ± 0.16	1.58 ± 0.38	6
K-W	0.162	0.065	
Feral roach			
Mustajõgi	1.18 ± 0.13	1.32 ± 0.28	19
Baltic TPP	1.15 ± 0.11	1.21 ± 0.23	21
K-W	0.715	0.171	

Abbreviations: CF, condition factor; K-W, Kruskal-Wallis test; LSI, liver somatic index (percentage weight of liver per total fish weight); *n*, number of fish; TPP, thermal power plant.
[a] $W(g)/L(cm)^3 \times 100$. [b] Mean ± standard deviation.

Table 6. CYP1A protein content (pmol/mg) and respective EROD activity (pmol/min/mg) in selected feral perch and roach from the River Narva.

Fish/site	CYP1A[a]	EROD[a]	*n*
Perch			
Mustajõgi	2.12 ± 0.25	39.4 ± 5.8	3
Baltic TPP	2.63 ± 0.64	31.2 ± 24.2	3
Riigiküla	5.05 ± 0.94	46.7 ± 21.0	2
Roach			
Mustajõgi	1.98 ± 2.51	11.4 ± 21.0	4
Baltic TPP	2.95 ± 2.33	1.98 ± 2.0	4

Abbreviations: CYP1A, cytochrome P4501A; EROD, 7-ethoxyresorufin *O*-deethylase; *n*, number of fish; TPP, thermal power plant.
[a] Mean ± standard deviation.

Mustjõgi (Table 6) (K-W p-value, no statistical significance). EROD activity in the same samples showed 0.79- and 1.2-fold changes at Baltic TPP and Riigiküla, respectively. In roach, CYP1A content was 1.4-fold at Baltic TPP and EROD activity was 0.7-fold as compared to Mustjõgi.

Immunohistochemical staining with MAb 1-12-3 showed CYP1A in vascular endothelial cells of spleen in few exposed roach (data not shown). There was a weak CYP1A staining in liver or perch as well as in the vascular endothelium of intestine in few roach at exposed sites (data not shown).

GST activities in both caged and feral fish differed slightly between sampling sites (K-W p-value 0.008 in rainbow trout and 0.031 in roach) (Figure 2). Baltic TPP fish showed increased activities. The increase of GST activity was 19% in rainbow trout and 28% in roach as compared to Mustajõgi. GST activity in perch was elevated by 5% at Baltic TPP and by 59% at Riigiküla, although statistical significance between the groups was not clear (K-W p-value 0.052).

UDP-GT activities did not differ markedly (K-W p-value 0.206 in rainbow trout, 0.068 in perch, and 0.223 in roach) (Figure 2). The UDP-GT activity of perch, however, was 35% lower at Baltic TPP as compared to Mustjõgi (reference site).

Histopathology and the micronucleus test. Histopathologic detection of liver, kidney, spleen, and intestine of perch and roach did not reveal any major morphologic changes. At Mustajõgi, 13 feral fish (7 roach and 6 perch) were histologically examined. In one roach liver, a focal nonspecific necrotic lesion was discovered. However, all of the other livers appeared to be in good condition with no parasitic infections. The most prominent lesions found in this study were melano-macrophage centers (MMCs), which were found in the spleen of two roach (diffuse) and one perch (moderate) at this site. The Baltic TPP catch site consisted of 14 fish (7 roach and 7 perch). Extensive development of MMCs was observed in the liver of one perch. Nevertheless, all of the other liver and kidney tissue samples at this site appeared to be undamaged. In the spleen, however, five of seven perch examined had moderate to diffuse appearance of MMCs. In addition, one roach had a high density of MMCs in the spleen. The catch of the third study site, Riigiküla, consisted only of eight fish (five perch and three roach). In one roach, diffuse MMC abundance in both liver and spleen was detected. Also, there were slight parasite infections in the liver of two perch.

Of all of the fish examined in this study, no signs of nuclear pleomorphism, megalocytic hepatosis, or hydropic vacuolation of hepatocytes were observed. Further, neither preneoplastic focus of cellular alteration nor neoplasms were found. The structure of the anterior intestine of the fish appeared to be intact and, except in one roach at the Baltic TPP site, no inflammations were found.

The micronucleus test did not reveal any significant differences between study sites. In all cases the micronuclei frequency was low (Mustajõgi 1.0 and 0%, Baltic TPP 1.3 and 0.9%, and Riigiküla 0.3 and 1.0% for roach and perch, respectively). In addition to few micronuclei, few blebbed, lobed, and notched nuclei and few binucleated erythrocytes were seen.

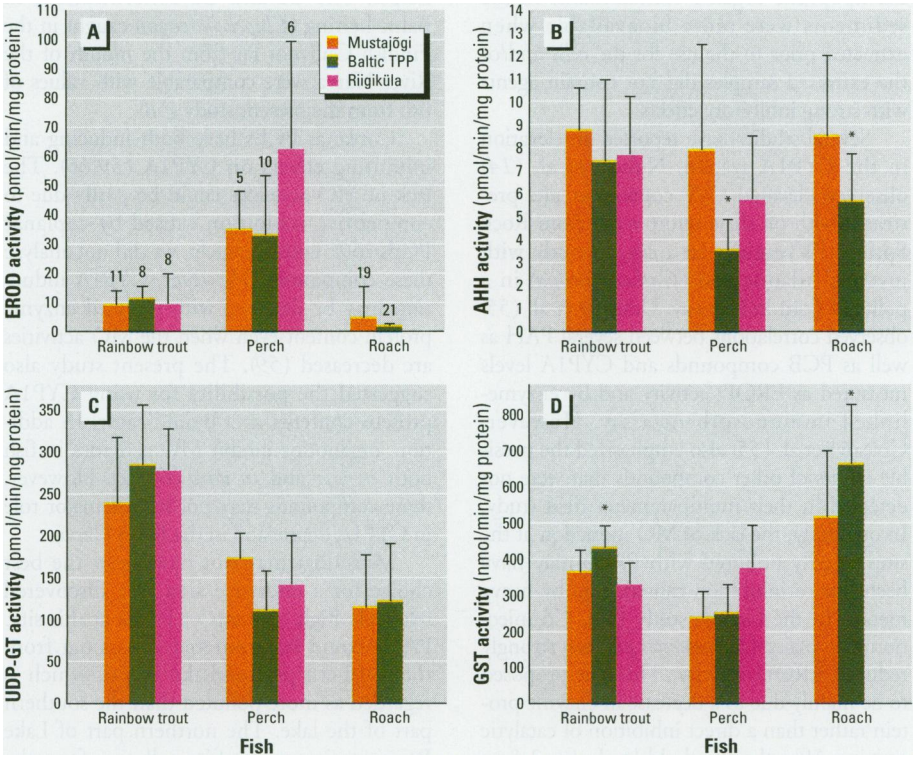

Figure 2. Responses of cytochrome P4501A-dependent monooxygenase activities and conjugation enzyme in rainbow trout, perch, and roach caged or caught from the River Narva. The Mustajõgi site is the reference site, and Baltic TPP and Riigiküla are exposed sites. (*A*) 7-Ethoxyresorufin *O*-deethylase (EROD; mean ± standard deviation); the number of each type of fish is listed for each site. (*B*) Aryl hydrocarbon hydroxlase (AHH). (*C*) UDP-glucuronosyltransferase (UDP-GT). (*D*) Glutathione *S*-transferase (GST).
*Significantly different from reference (Mann-Whitney p-value < 0.05).

Discussion

The oil shale mining and processing area in northeast Estonia is heavily polluted with PAHs and also with other compounds. In our study this complex mixture of pollutants seemed to cause only a few measurable biologic effects in fish, among them an increase in GST activities and slight changes in CYP1A. When the few changes detected were compared to the chemical composition in the study area, the biologic parameters seemed to underestimate the chemical loading.

Accumulation of PAHs and CYP1A induction. According to Neff (*47*), the relative concentration of PAHs in aquatic ecosystems is generally highest in the sediments, intermediate in aquatic biota, and lowest in the water column. Based on this knowledge the accumulation of PAHs in fish from the River Narva was higher than would have been expected from the content of PAHs in water or sediment. Total PAH contents ranged from 42 ng/L at Mustajõgi to 1,373 ng/L at Riigiküla (*48*) and from 52 ng/g dry weight (dw) at Mustajõgi to 744 ng/g dw at Riigiküla (*16*) in water and sediments, respectively. The total PAH content in rainbow trout muscle ranged from 421 to 890 ng/g dw in the River Narva. In the Tuvikene et al. (*20*) study, which was conducted in south Estonia, the total content of PAHs in rainbow trout caged for 3 weeks ranged from 20 to 165 ng/g dw in the River Suur Emajõgi and up to 738 ng/g dw in the harbor area of Lake Võrtsjärv. In the present study, the dominating compounds in fish tissues were pyrene, chrysene, and fluoranthrene. Benzo(*a*)pyrene, which is regarded as highly carcinogenic among PAH compounds, showed higher content in fish tissues collected from the River Narva than in fish tissues from other Estonian bodies of water (*3,49*).

In the present study, total PAH concentrations ranged from 1,461 to 4,820 ng/g dw in muscle and from 1,166 to 11,441 ng/g dw in the liver of feral fish. Similar or even higher concentrations of PAHs in fish have been discovered in the Arabian Gulf (*50*). In that

study, concentrations of total PAHs from whole fish of mullet (*Arias* spp.) and minnow (*Siganus canaliculatus*) were 3.6 and 2.7 mg/g ww, respectively. Khan et al. (*50*) also studied the accumulation and elimination of PAHs and reported that the mullet absorbed high levels of PAHs and reached a steady-state equilibrium after approximately 2 weeks. The transfer of oil-exposed minnows to clean water did not cause marked loss of the absorbed PAHs with high molecular mass for up to 2 weeks. This is not in accordance with the knowledge that PAHs with high molecular mass are metabolized more rapidly than the PAHs with low molecular mass (*51*). The lack of rapid metabolism of PAHs seemed to occur in our study as well.

Our fish material consisted of species with different backgrounds. Rainbow trout and perch are carnivores, whereas roach are herbivores. Rainbow trout were not fed during the caging, although they may have ingested particles from the water that flows through the cages. The food of carnivores may contain animals in which the chemicals have already accumulated in greater amounts than in the food of herbivores. The caged fish had similar background (e.g., age, genetic background), whereas the feral fish represented different states of sexual maturity and ages (4–6 years of age). These differences may have some impact on the responses, as described by Palm and Krause (*31*) and Otto and Moon (*52*).

PAH contents were higher in feral fish than in caged rainbow trout. This difference can be explained by the different exposure time. Rainbow trout were introduced to the study area for 3 weeks. Perch and roach, however, had been continuously accumulating pollutants during their lifetime. This may lead to a certain level of adaptation to the pollutants. Further, the content of PAHs was higher in perch and roach liver than in muscle at the exposed areas. The rate of pollutant distribution to specific tissues was determined, e.g., by the regional blood flow through each tissue. Therefore, the organs that have a high blood flow (e.g., the liver and the kidney) tend to accumulate xenobiotics most readily (*53*).

In spite of high concentrations of PAHs, there was no induction in MO activities in any studied fish species. In a previous study (*20*) rainbow trout from the same stock as we used for the present study were caged in waters upstream from the River Narva in winter and spring 1994. EROD and AHH activities in rainbow trout for reference sites were similar in that study as in the current study, but showed clear induction of CYP1A in more polluted sites. It can be concluded that rainbow trout were able to reflect the pollution with catalytic CYP1A induction, at least in some conditions. In feral fish AHH activities showed decreased trends at some exposed sites. Van der Oost et al. (*54*) reported similar observations in feral roach from Amsterdam lakes. In that study none of the CYP dependent indicators (total CYP, total CYP1A, EROD) were induced in roach from the polluted sites, and the CYP also was inhibited in fish from the more polluted areas. The authors suggested that the pollutant levels in the studied lakes were too low to cause a significant induction of hepatic MO enzymes or that the control fish were exposed to MO-inducing xenobiotics, which were not measured. In this study the reference values of EROD for roach were slightly lower than in our study, 1.6 ± 1.6 and 4.1 ± 9.8 pmol/min/mg protein, respectively. In our study the fish from the reference site were probably also slightly exposed to MO xenobiotics.

The effects of River Narva sediment extracts have been studied with a fish liver cell line (PLHC-1) (*16*). In that *in vitro* study the sediment extracts caused both CYP1A induction and cytotoxicity in the cells. This finding is somewhat controversial to the lack of evidence of catalytic CYP1A induction *in vivo* in the present study. The cells may be more sensitive as bioindicators than fish and/or the sediments may have contained more biologically active compounds than the overlying waters or fish tissues. It is also possible that the lipophilic organic contaminants in the sediments were more bioavailable when extracted prior to the use for the cells and/or the extracted samples did not contain agents with strong inhibitory effects.

Several studies have reported an elevation in the CYP1A system. Nelson et al. (*14*) observed elevated CYP content in rats pretreated with oil shale retort water from Rock Springs, Wyoming. In a caging study with juvenile Atlantic cod (*Gadus morhua*) in a polluted fjord in Norway, Goksøyr et al. (*55*) observed correlations between several PAH as well as PCB compounds and CYP1A levels measured as EROD activity and by enzyme-linked immunosorbent assay. However, Goksøyr et al. (*55*) also emphasized the possible effects of other compounds that were not detected in their multiparameter field study. In our study, the lack of MO induction at the sites heavily polluted with PAHs may have been due to inhibition caused, e.g., by heavy metals. In the George study (*56*), Cd injection into plaice (*Pleuronectes platessa*) strongly reduced EROD activity. This was proposed to be mainly due to a decrease in enzyme protein rather than a direct inhibition of catalytic activity. After the threshold level of > 2.4 µg Cd/g liver, the EROD activity began to decrease significantly. In our study, the Cd content was approximately one order of magnitude lower; however, because of a chronic exposure this Cd amount may have had some inhibitory effect on MO activities. Viarengo et al. (*57*) observed that the EROD activity in bass (*Dicentrarchus labrax*) liver, which had previously been induced by *in vitro* treatment with β-naphthoflavone or benzo(*a*)pyrene, was significantly inhibited by nano- to micromolar concentrations of ionic Cu or Hg or methylmercury, whereas treatments with mixtures of these compounds had an additive inhibitory effect.

There were not many differences in heavy metal contents between the fish species studied. Only Hg accumulated more in feral fish than in caged rainbow trout. Heavy metal contents in fish did not differ dramatically as compared to contents from neighboring bodies of water. According to Hödrejärv et al. (*4*), the content of heavy metal (HM) in the muscles of roach caught upstream of the Lake Peipsi study area was 0.12, 0.07, 2.2, and 0.1 mg/kg dw, respectively, for Hg, Cd, Pb, and Cu. From the same place the numbers for perch were in the ranges of 0.05–0.2, 0.05–0.11, 0.1–2.1, and 0.1–0.2. Lake Peipsi is a relatively clean lake in terms of HM; they come mainly with air pollution from oil shale processing areas. When these values are compared to values in the present study, fish from the River Narva had higher Hg and Cu concentrations, Cd was almost the same, and Pb content was even higher in fish from Lake Peipsi. The Cu and Cd concentrations in Baltic herring (*Clupea harengus*) caught in the Finnish Gulf not far from the mouth of the River Narva were comparable with values of fish from the present study (*58*).

Coplanar PCBs have both inducing and inhibiting effects on CYP1A (*59,60*). The lack of MO activities could be partly due to competitive inhibition caused by coplanar PCBs (*60*). Unfortunately, we did not analyze these compounds. However, CYP1A induction may be detected with elevated enzyme protein content even when the MO activities are decreased (*59*). The present study also suggested the possibility for using CYP1A protein contents as a bioindicator. In addition, organotins inhibit MO activities in fish both *in vivo* and *in vitro* (*61,62*). However, these compounds may not have a major role in CYP1A depression in the River Narva.

Mustajõgi may not have been the best choice for a reference area. We discovered relatively high contents of PAHs at this site. PAHs could have entered Mustajõgi from the northern part of Lake Peipsi, which is regarded as more polluted than the southern part of the lake. The northern part of Lake Peipsi receives most of its pollution from the River Rannapungerja, which brings some drainage waters from oil shale mines. Part of the pollution is believed to enter the area via air. There is a PAH contamination problem in the entire northeast portion of Estonia.

Induction of glutathione conjugation. GST activity, a conjugation enzyme activity, showed a trend toward increased values in all studied species at exposed sites. Elevated levels of GST activities indicated a defense response to chemical or oxidative stress in the cells (*54*). The maximum induction of GST activity was reached in feral perch (1.6-fold as compared to reference).

There is evidence that different organic and inorganic pollutants are able to enhance GST activities in fish. Di Giulio et al. (*19*) studied the effects of Black Rock Harbor (Long Island Sound, CT) sediments polluted with PAHs and PCBs on the biotransformation of channel catfish. They found significantly elevated activity of liver GST. At its highest, GST activity was 1.3-fold after 14 days of exposure. Willett et al. (*63*) found elevated cytosolic GST activity in Atlantic croaker from PAH- and PCB-contaminated sites at the liver PAH content that was comparable to that in feral perch and roach in the current study. A number of studies have also failed to detect significant increases in hepatic GST activities of several fish species exposed to PAHs in the laboratory (*56*) or caged or collected from the contaminated sites (*20*).

Rodrígues-Ariza et al. (*64*) observed a significant increase in EROD and cytosolic GST (even 1.5-fold) in *Mugil* sp. in response to the high levels of organic xenobiotics. These fish also contained high concentrations of metals such as Fe and Cu. Liver Cu content of *Mugil* sp. was comparable to Cu content of fish livers from the River Narva. Moreover, according to Martínez-Lara et al. (*65*), copper (II) treatment significantly increased GST activity after a 2-day exposure, and the activity decreased at high doses. On the contrary, George (*66*) failed to find elevated GST activity in *Pl. platessa* after Cd injection (up to 1 mg Cd/kg). Thus, the interpretation of the cause for increased GST activities in the River Narva is difficult.

Unspecific physiologic indices. In the present study, the overall CF of fish and the LSI were not seriously affected by the pollutants. For example, the liver enlargement possibly expressed the increased metabolism of xenobiotics (*67*). According to Everaarts et al. (*68*), the LSI of sunfish and hardhead catfish was increased at PAH-contaminated areas. Theodorakis et al. (*69*) reported that treatment with PAH-rich sediment increased the LSI of bluegill sunfish 2-fold as compared to controls. On the contrary, Van der Oost et al. (*70*) did not observe any enlargement of the fish liver at areas polluted with PCBs, organochlorine pesticides, and PAHs. Based on our data, the nonspecific parameters failed to reveal any effects of the oil shale industry.

Histopathology and the micronucleus test. The pollutants of the oil shale industry contain several known carcinogens, such as PAHs, nitroaromatic and aminoaromatic compounds, and heavy metals (*71*). These compounds may act as carcinogens in different ways. PAHs can induce CYP1A in fish and thus accelerate the disposition of hydrocarbons, but also enhance the formation of carcinogenic derivatives of PAHs (*72*). Pb, instead, may not act as CYP1A inducer but can be a promoter of carcinogenesis (*72*).

The genotoxicity of emissions from the oil shale industry has been studied using the Ames *Salmonella*/microsome assay (*73*). Ambient air and spent oil shale ash samples proved only weakly mutagenic, and samples derived from both drinking water and waste river water were not mutagenic at all. We detected the frequency of micronuclei in fish blood as a measure for genotoxicity but did not find clear evidence of enhanced micronuclei formation at exposed areas. All values were within limits (0–1.3%) mainly regarded as control levels [≤ 1%, (*74,75*); < 0.8%, (*76*)].

Micronuclei are small intracytoplasmic pieces of chromatin that result from impaired mitoses of chromosome breakage. The piscine micronucleus test has been proposed as a potentially rapid and inexpensive *in situ* biologic indicator of chemical contamination in wild fish.

According to previous studies in Estonia, the average number of micronucleated erythrocytes in rainbow trout collected from the Narva Fish Farm (near Baltic TPP) was 2.27% and was 0.05% in material from the South Estonian Fish Farm (*31*). Micronuclei levels in fish blood can change according to fish species. Micronuclei of roach and perch from Lake Peipsi were 2.5 and 1.5%, respectively (*31*). However, the piscine micronucleus test has been criticized for its lack of sensitivity to the presence and effects of some genotoxic chemicals because no definitive or consistent correlation between the measured contamination and the frequency of micronuclei was detected (*46*).

Some teleost fish living in areas contaminated with aromatic hydrocarbons, chlorinated hydrocarbons, pesticides, and/or metals in the sediments have increased frequencies of histologically identifiable diseases, including liver neoplasms (*27*). In our study, histopathology in the tissues of perch and roach from the River Narva did not reveal any major morphologic alterations. A nonspecific necrotic lesion, cystic parenchymal degeneration, found in one roach liver at Mustajõgi possibly resulted from a variety of agents (*77*). However, there was only one visible cystic formation in the observed section of the liver. MMCs were seen in the spleen of perch at the Baltic TPP site. MMCs in fish vary in number and size with such factors as age, starvation, environmental stress, and disease (*78,79*). In this study, all the sampled fish were 5 ± 1 year old with no gross signs of nutritional deficiency or health problems. Therefore, the abundance of MMCs could be evidence of environmental stress at the study area. Inflammation is a common protective response to tissue injury. Chronic inflammation without evident bacterial, viral, or parasitic infection, as seen in one roach from Baltic TPP, could be interpreted as a defensive reaction of fish to foreign materials (*80*).

The number of fish detected by means of histopathology was small. Therefore, the present findings serve more as a prescreening material than as distinct evidence of morphologic changes.

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