Journal of Animal and Veterinary Advances 9 (23): 2890-2899, 2010
ISSN: 1680-5593

Histopathological and Genotoxic Effects of Pollution on *Anguilla anguilla* in the Gediz River (Turkey)

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Abstract: The aim of the present study was to determine heavy metal accumulation in water, sediment and some tissues of *Anguilla anguilla* along with determining histopathological and genotoxic effects of accumulation on these tissues by using light microscopy. Water, sediment and fish tissue samples taken from different sites of 3 different study areas were studied and the order of accumulation of metals was Fe>Pb>Mn>Co>Zn>Ni>Cr>Cu>Cd in water, Fe>Co>Mn>Pb>Zn>Ni>Cr>Cu>Cd in sediment and Cd>Mn>Cu>Cr>Ni>Fe>Zn>Co>Pb, Cd>Pb>Ni>Cr>Mn>Cu>Fe>Co>Zn and Cr>Ni>Zn>Cd>Pb>Mn>Cu>Fe>Co in liver, muscle and gill of fish, respectively. In histopathological studies, a decrease in the length of primary and secondary lamellae of gills, fusion in secondary lamellae, cellular proliferation, clavate lamellae formation and necrosis were observed. In liver tissue, dilation of sinusoid, increase in the number of erythrocytes, ruptured hepatocytes, decrease in glycogen accumulation and vacuolization were observed. In muscle tissue, necrosis, cellular dissolution and loss of striatation in muscle fibers were found. It was observed that pollution of water had no genotoxic effect on *Anguilla anguilla*.

Key words: Bioaccumulation, water, sediment, fish, histopatology, micronucleus

INTRODUCTION

Environmental pollution came into being when urban life started and has increased parallel to the industrial development since then. Especially in the second half of the 20th century, increasing environmental pollution due to rapid population growth caused natural resources to become more polluted as a result of which destruction of ecosystem became a much more acute issue. As the aquatic environment that forms a part of the ecosystem is used for discharge and disposal of used water and other wastes, it has become the most polluted part when air and soil are also considered.

These pollutant elements affecting the natural balance can be grouped as follows: Organic materials, industrial wastes, petroleum derivatives, artificial agricultural fertilizers, detergants, radioactivity, pesticides, inorganic salts, artificial organic chemical materials and waste heat. According to this classification, heavy metals are among industrial wastes and some pesticides and they have reached to a level threatening the ecological balance (Gumgum *et al*., 1994; Solomons and Forstner, 1984; Leland *et al*., 1978; Hammond and Beliles, 1980). Although, heavy metals are trace components of aquatic environment, their natural levels and accumulations in organisms can be different. The term heavy metal covers all the metals and metalloids in the nature. In the era, the areas of use for heavy metals are increasing parallel to the development in industry and improvement in life standards. Pest control is an important factor contributing to the increase.

Heavy metals are considered to be among important pollutants of aquatic ecosystems as they do not degrade or decompose in the nature but tend to accumulate in aquatic organisms (Veena *et al*., 1997; Kalay and Canli, 2000).

Heavy metals may accumulate in tissues of aquatic animals when such animals are exposed to high levels of heavy metals (Vinodhini and Narayanan, 2009).

With regard to metal pollution, it is defined that gills and kidney tubules are the first organs exposed to heavy metals and gills and digestion system are the regions where heavy metals are received and accumulated while gills and liver are the regions where heavy metals are stored (Thophon *et al*., 2003; Olojo *et al*., 2005). It is known that heavy metals have toxic effects even at places away from the source of pollution as they have the ability of biological accumulation (Barlas, 1997). Toxic

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substances may knock down immune system, reproduction system, nervous system and endocrine system in animals and these effects can be at organ, tissue and cell level (Geeraerts and Belpaire, 2009). Fish are susceptible to acute and chronic environmental changes and they show a classical stress response.

This stress response covers the changes in plasma glucocorticoids and cathecolamines. Environmental changes may cause hypoxia, metabolic acidosis and alkalosis, hypotension and hypoglysemia (Wendelaar, 1997; Fabbri *et al*., 1998). Gill tissue is an organ having a large surface and separating blood from water in fish and is very susceptible to changes in concentrations of the variables (ph, salinity, temperatue, ammonia, heavy metals etc.) in the environment. These variables affect the structural integrity of the gill and cause morphological changes.

For this reason, gills are thought to be indicators of water pollution (Ortiz *et al*., 1999; Bhagwant and Elahee, 2002; Wood *et al*., 2002; Koca *et al*., 2005, 2008). Liver plays an important role in protecting inner homeostasis in vertebrae. The higly dynamic nature of the liver and its regulation in many metabolic and physiological processes makes this organ a valuable model to study (Segner, 1998). Muscle tissue forms a major part of the body weight of fish when compared to other vertebrae (Fabbri *et al*., 1998) and economically it is very valuable.

Histological examination of tissues is a useful method to determine both the effects of environmental variables and also types of indicators in biological survey programs. Many studies have reported that not death but major damage in organs is observed in animals that are exposed to heavy metals, Polycyclic Aromatic Hydrocarbons (PAH) and pesticides even in trace amounts.

As a result, it is known that metals accumulate on sediment surface in bentic living things, planctonic organisms and other living things through food chain and aquatic organisms and human beings are affected negatively as a result of the said accumulation. For this reason, toxicological bioaccumulation and pathological researches of metals being discharged to aquatic environments have a great importance for the presence of biological life and protection of nature (Davies *et al*., 1991; Srivastava *et al*., 1994; Ankley *et al*., 1996; Klavins *et al*., 2000; Gonzales *et al*., 2000; Singh, 2001). In aquatic organisms, various cytogenetic methods such as chromosomal aberrations, sister chromatid exchange and micronucleus formation are used to test genotoxicity of chemicals (Al-Sabti ,1986). Micronucleus test is a commonly used method in fish species as it is a simple, reliable and sensitive test (Jiraungkoorskul *et al*., 2007). Micronucleus is composed of small chromatic fragments generated as a result of chromosomal breakage following clastogenic effect and full chromosomes not migrating at anaphase as a result of a aneugenic effect (Heddle *et al*., 1991).

The efficacy of this test system as an indicator of structural genomic damage has long been established and micronucleus test has been used as a measure of genotoxic stress in fish under both laboratory and field conditions (Al-Sabti, 2000; Grisolia and Starling, 2001; Cavas and Ergene-Gozukara, 2003; Koca *et al*., 2005, 2008).

MATERIALS AND METHODS

Study area: The Gediz river is 401 km long and lies in the west of Turkey. Its basin is 17.500 km^2. It is used for irrigation, fishing and supporting dam lakes. Its pollution resources are sand and gravel mines, leather industry, residential wastes and wastes of organized industrial zones. The study was conducted in three different sites (Fig. 1):

Site 1: The area polluted mainly by downtown wastes
Site 2: Muradiye settlement area
Site 3: Menemen settlement area

Analitical procedure: The study was conducted at three stations. Using Nansen Sampler, water samples were collected from a depth of 0.5 m below the surface into clean polyethylene bottles. The water temperature, electrical conductivity, dissolved oxygen and pH were measured *in situ*. To acidify the samples, 1 mL of 0.5% HNO_3 was added. Fish were killed and then kept on ice until used for laboratory tests. Approximately, 4 g of the epaxial muscle on the dorsal surface of the fish, the entire liver and two gill racers were dissected from each sample, washed using ice-cold distilled water, dried in filter paper, weighed, packed in polyethylene bags and frozen at -30°C until analysis (Bernhard, 1976).

The samples were preserved and analysed according to the American Public Health Association and the American Water Works Association standards (APHA-AWWA-WPCF, 1996) After weighing, 5 mL of 65% nitric acid was added to samples for digestion in a CEM Mars 5 microwave digestion system. The advantages of microwave digestion are the shorter times

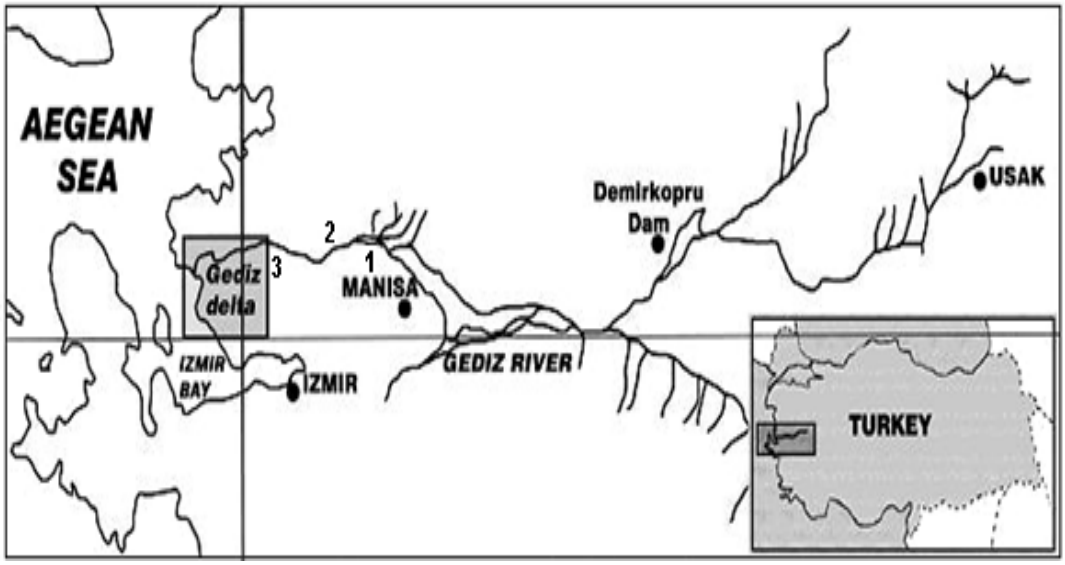

Fig. 1: Sampling sites of the Gediz river

and minimal loss of volatile compounds (Gulmini *et al*., 1994). After digestion, the samples were cooled down to room temperature and diluted to 25 mL with 2.5% HNO_3. The Cd, Co, Cu, Ni, Pb, Mn, Cr, Fe and Zn in samples were determined by means of a Varian Terra Liberty II inductively coupled plasma atomic emission spectrometer. The concentrations of heavy metals were expressed as micrograms per gram of wet weight tissue.

Histological procedures: For light microscope analyses, the gill, liver and muscle tissues from *Anguila anguila* were fixed in both buffered neutral formalin and Saint-Marie fixative (+4°C) (Tuckett and Morris-Kay, 1988), dehydrated in graded ethanol series, cleared in xylene and embedded in paraffin.

Five-micrometer-thick gill, liver and muscle tissue slices cut by means of a rotary microtome (Leica RM 2145) were dehydrated and stained with Mayer's Haematoxylin-Eosin (H and E), Gomori trichrom, Masson trichrom and Periodic Acid Shiff-Haematoxylin (PAS-H) stain (Bancroft and Cook, 1994). The sections were examined and photographed using an Olympus BX 51 microscope. Erythrocytes count of the eel did not show any micronuclues or nucleus abnormalities.

Preparing blood preparations for gentoxicity test: A drop of blood from gills was smeared on slide and air dried. After fixation in methanol for 20 min the slide was stained with a 5% Giemsa solution and mounted with entellan. Preparations were studied by a single observer. In each fish, 1000 cells were counted at 1000× magnification.

RESULTS AND DISCUSSION

Analitical result: The Gediz river is exposed to agricultural waste, industrial waste and residential waste. In the present study, it was aimed to show the pollution in Gediz river with various parameters. Physicochemical measurements would reveal heavy metal toxicity and the extent of the damage on tissue and organs of *Anguilla anguilla* that has been consumed. According to the results obtained form the study in the Gediz river, ammonium was found as 0.050-1.610 mg L^{-1}, orthophosphate and nitrit was found as 0.010-0.58 mg L^{-1} and nitrite was found as 0.043-1.118. Other measurements on water quality are shown in Table 1.

The amounts of cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead and zinc were measured in sediment, water and fish samples and mean values and standart deviations are shown in Table 2-4. Accordingly, accumulations in water, sediment, muscle, liver and gill follow the following sequences, respectively: Fe>Pb> Mn>Co>Zn>Ni>Cr>Cu>Cd, Fe>Co>Mn>Pb>Zn>Ni>Cr> Cu>Cd, Cd>Mn>Cu>Cr>Ni>Fe>Zn>Co>Pb, Cd> Pb>Ni> Cr>Mn>Cu>Fe>Co>Zn Cr>Ni>Zn>Cd>Pb>Mn>Cu>Fe >Co.

Histological results

Gills: A decrease in the length of primary and secondary lamella of gills (Fig. 2a, b), loss in secondary lamellae (Fig. 2b), sporadic separation in secondary lamellar epithelium, clavate lamellae formation (Fig. 2c) and accumulation in blood cells due to impaired circulation as

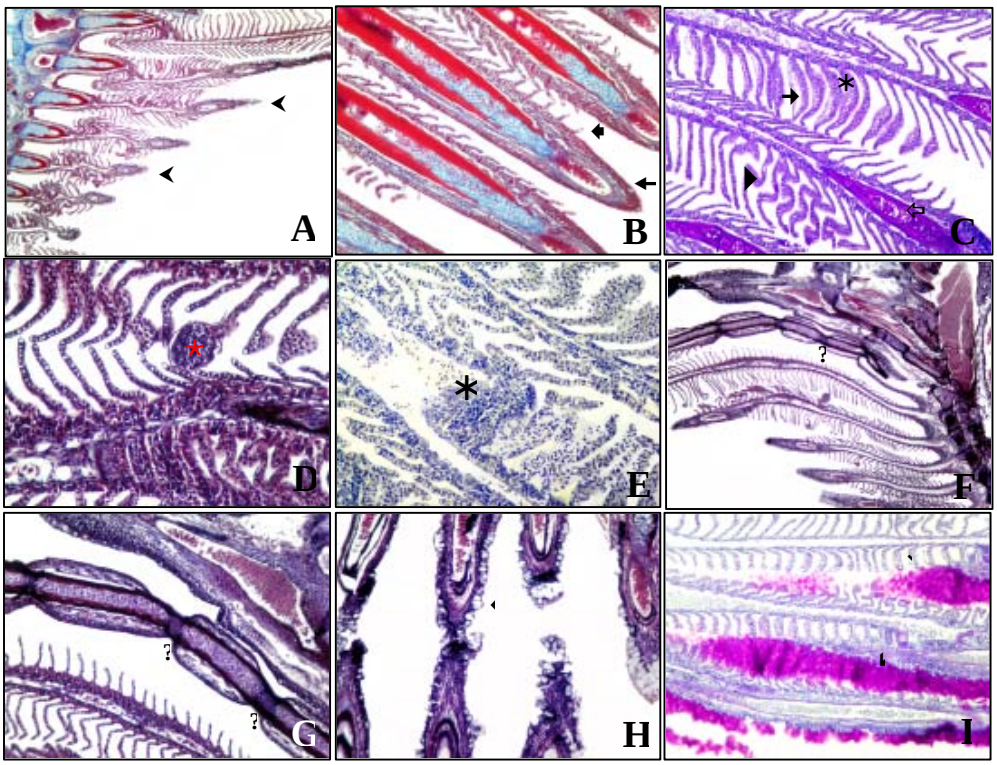

Fig. 2: The observed changes in gills of *A. anguilla* A: ➤: Decrease of the mean length of primary lamellae, B: ➠: Decrease of the mean length of secondary lamellae, ➞: The lost of secondary lamellae C: ➜: Epithelial separating in secondary lamellae, ▸: Clavate lamellae formation ⇨: Degeneration of cartilage of primer lamellae, D:✳: Accumulation of blood cells in dilatationed sinusoids, E: ❋: Hyperplasia F, G: Δ: Breaking of primer lamels, H, I:➞: Mucous cells in breaking primary lamellae regions. A: Masson trichrome, x4, B: Masson trichrome, x10, C: PAS+H, x20, D: Gomori trichrome, x20, E: H and E, x20, F: Gomori trichrome, x4, G,H: Gomori trichrome, x20, I: PAS+H, x10

Table 1: The physical and chemical parameters of Gediz river water

Parameters	Site 1	Site 2	Site 3
Temperature (°C)	16±8.5	14± 8.4	15±9.5
pH	8.34±0.03	8.22±0.06	7.90±0.05
Electrical Conductivity (EC)	1460±1.251	1670±1.250	1830 ±10.25
Dissolved Oxygen (DO)	10.50±0.561	11.72±0.346	12.75±0.421
i-PO_4^*	0.100±0.003	0.300±0.002	0.580±0.006
NH_3-N	1.610±0.200	0.050±0.500	0.089±0.010
NO_2-N	1.118±0.371	1.050±0.061	0.043±0.0171
NO_3-N	0.050±0.075	0.040±0.461	0.030±0.037
K	24.730±0.326	23.60±0.361	15.65±0.041
Na	18.65±1.425	12.55±0.375	21.74±0.441
Ca	3.580±0.003	3.710±0.004	7.850±0.006

*Chemical parameters concentration are mg L^{-} Sample: N:8

Table 2: Heavy metal concentrations of the water samples collected from Gediz river (means±SD, μg mL^{-1})

Metals	Site 1	Site 2	Site 3
Cd	0.005±0.002	0.003±0.0002	0.005±0.004
Co	0.030±0.098	0.067±0.0070	0.060±0.860
Cr	0.018±0.006	0.016±0.0110	0.001±0.008
Cu	0.008±0.006	0.009±0.0130	0.006±0.009
Fe	0.232±0.307	0.308±0.3240	0.279±0.379
Mn	0.130±0.140	0.127±0.1190	0.015±0.129
Ni	0.020±0.140	0.018±0.0200	0.010±0.014
Pb	0.143±0.095	0.160±0.0820	0.170±0.090
Zn	0.050±0.123	0.023±0.0210	0.024±0.010

Table 3: Heavy metal concentrations of the sediment samples collected from Gediz rver (means±SD, μg g^{-1})

Metals	Site 1	Site 2	Site 3
Cd	2.218±2.99700	4.910±0.0600	2.346±2.2940
Co	79.786±255.170	87.601±18.116	907.125±208.351
Cr	35.673±11.1730	84.601±22.617	75.930±18.871
Cu	145.506±40.7530	44.211±10.001	45.150±11.610
Fe	489.601±218.890	150.207±2.2670	159.018±271.117
Mn	83.490±25.7500	519.987±81.031	472.572±116.73
Ni	116.760±45.4130	94.720±21.071	99.740±13.501
Pb	3.178±1.10270	127.364±30.520	135.251±31.613
Zn	107.671±26.3610	115.860±0.5843	120.52±10.1140

Table 4: Heavy metal concentrations of the *Anguilla anguilla* samples collected from Gediz river (means±SD, μg g^{-1})

	Anguilla anguilla organs		
Metals	Muscle	Liver	Gill
Cd	1.2067±1.278	2.071±0.0060	0.0650±0.001
Co	0.0276±0.007	0.0375±0.017	0.0355±0.010
Cu	0.3761±0.045	0.3030±0.074	0.3545±0.004
Ni	0.0781±0.006	0.5950±0.021	1.4851±1.236
Pb	0.0032±0.003	0.9610±0.002	1.0315±1.115
Zn	0.0028±0.003	0.0561±0.056	5.355 ± 0.360
Cr	0.390±0.8710	1.260±1.1500	1.970±0.4500
Mn	0.984±0.0710	0.432±0.0150	0.782±0.0062
Fe	0.044±0.0290	0.167±0.0740	0.067±0.0060

a result of capillary dilatation (Fig. 2d) were detected in gill tissue. Moreover, rupture of capillaries and erythrocyte releases were observed in secondary lamellae (Fig. 2e). Cartilage cells were deformed in some primary lamellar epithelium (Fig. 2c).

While integrity of secondary lamellae was impaired as a result of the necrosis and exfoliation observed in secondary lamellar epithelium, adhesion was observed due to hyperplasia (Fig. 2c, e). Some primary lamellae broke up at one or more points as a result of articulation (Fig. 2f, g) and many mucous cells were observed at the broken part (Fig. 2h, i).

Liver: In liver, altered paranchymal cell positioning, deterioration in cell membrane and necrotic areas were observed (Fig. 3a, b). In hepatocytes, intracellular glycogen storage in the form of different sizes of particles (Fig. 3c), vacuolization, alteration in the position of nucleus (Fig. 3d) and picnotic nucleus (Fig. 3a, d) were detected.

Muscle: In muscle tissue, cellular dissolution in interfibrillar area, myocyte nuclei release, necrosis (Fig. 4a) and alteration in the formation of muscle fibrils (Fig. 4b) were observed. Losses were seen in the endomysium layer around the muscle fibers (Fig. 4c). Erythrocyte counts in ells did not reveal micronucleus or any nucleus abnormality. Various researchers have reported that the Gediz river is faced with an intensive pollution risk (Akcay *et al*., 2003; Parlak *et al*., 2006). In general, nitrite has to be <0.001 mg L^{-1} in fresh water. High levels of nitrite indicate an intensive industrial amd microbiological pollution (FAO/WHO, 1989). In the study, nitrite was found as 1.160 mg L^{-1} in site 1 where city waste waters are thought to be abundant. Excessive nitrite is the result of using intensive inorganic agricultural fertilizer.

It has to be below 1 mg L^{-1}. In the present study, nitrite level varied between 0.030 and 0.050 mg L^{-1} (Table 1). Level of ammonium concentration has to be <0.2 mg L^{-1} in surface water (FAO/WHO, 1989) and ammounium concentrations above the said level indicates organic pollution. In the study, it was 1.610 mg L^{-1} in site 1. Orthophosphate has to be 0.30 mg L^{-1} in natural waters. In the Gediz river, its level was detected as 0.58 mg L^{-1}. According to the results on water quality, site 1 where city waste water was abundant was measured as the most polluted site.

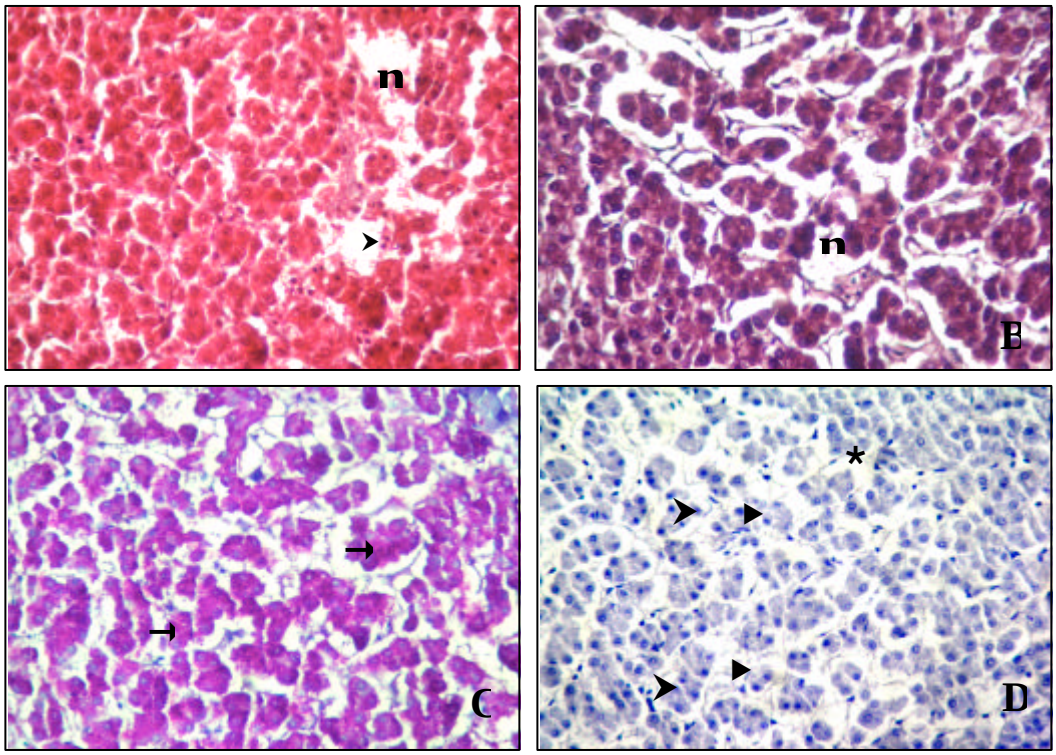

Fig. 3: The observed changes in liver of *A. anguilla*. A, B: n: Changes of cells configuration, Necrosis, C: →: Stored glycogen in hepatocytes, D: ▸: Intracellular vacuolization, ✱: Hepatocytes with excantric and ➤: Picnotic nucleus. A: Masson trichrome, x40, B:Gomori trichrome, x40, C: PAS+H, x40, D: H and E, x20

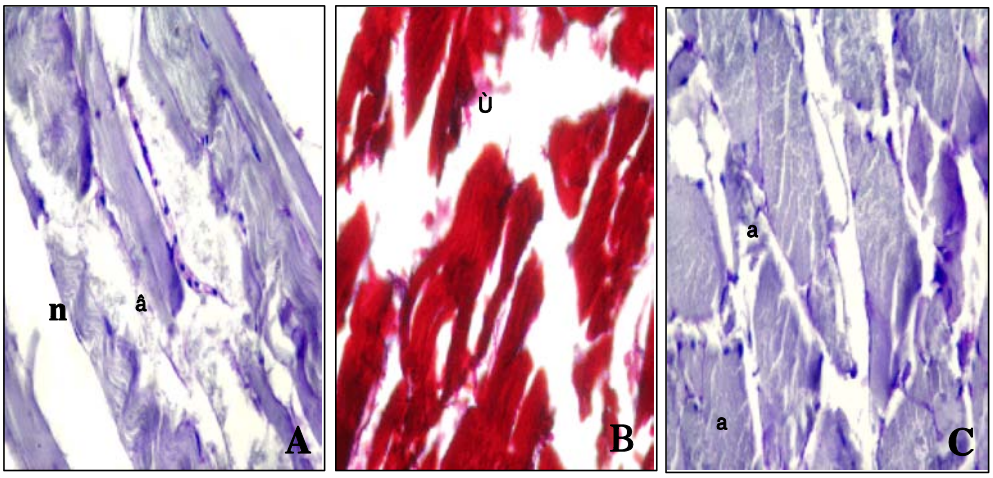

Fig. 4: The observed changes in muscle of *A. anguilla*. A:✳: Dissolution in sarcoplasm and releases nuclei of myocyte, n: necrosis, B:✱: Disorganization of the intermyofibrillary network, C: ⇨: Loss of endomysium layer. A: PAS+H, x40, B: Masson trichrome, x20, C: PAS+H, x40

Table 5: Heavy metal concentrations in the liver and muscle in other studies

		Metals				
Habitat /fish/tissue	Organs	Cd	Cu	Mn	Pb	Zn
Bacuta, Spain (Usero *et al*., 2003)	Liver	0.480	23.40	4.50	0.60	37.00
(*A.anguilla*)	Muscle	0.038	0.70	14.10	0.09	11.40
Wildecosystems (Linde *et al*., 2004)	Liver	1.410	10.50	1.90	-	-
Spain (*A.anguilla*)	Muscle	0.060	0.29	-	0.10	-
Comargue, France (Oliveira Ribeiro *et al*., 2005)	Liver	0.160	19.00	1.93	0.16	53.75
(*A.anguilla*)						
Koyceoiz lake (Yilmaz, 2009)	Liver	0.430	73.91	11.04	1.63	199.32
Turkey (*A.anguilla*)	Muscle	0.160	2.21	3.14	1.16	106.71
FAO/WHO (1989) limits		0.500	30.00	-	0.50	40.00
EU limits		0.100	10.00	-	0.10	-
Range of international standards		0.200	100.00		0.5-10	40-100

Not including international standars

Today, heavy metals are used in many areas (Industrial etc.). These heavy metals are discharged to water and cause accumulation in sediment and aquatic living things. Consequently, increase of heavy metal in aquatic environment diminishes some very sensitive species or causes accumulation in tissues and organs of tolerant species and thus reaches to upper trophic levels. In this study, it was detected that heavy metals accumulate in sediment in different concentrations. Co, Mn and Pb concentrations were found to be the most intensive ones (Table 3). Accumulation is high in all three stations. In other studies, Cr concentration has been recorded as the highest one (Akcay *et al*., 2003; Parlak *et al*., 2006). In studies on tissues of *Anguilla anguilla*, it has been reported that accumulation is high especially in liver tissue (Oliveira Ribeiro *et al*., 2005, Usero *et al*., 2003; Yilmaz, 2009) (Table 5). While Cu and Mn concentrations are high in these studies, Cd accumulation was found to be the most intensive one in the study. In general, it has been reported that the least accumulation is in muscle tissue while the highest accumulation is in liver and gills. The study supports these findings (Melgar *et al*., 1997).

Toxic environmental conditions cause two types of structural changes in the tissues of living things. One of them is the direct toxic effect due to degeneration and necrosis while the other one is the defence responses of the living things (Bhagwant and Elahee, 2002). Tissues such as liver and gills are metabolically active tissues and the studies conducted indicate that these tissues accumulate high levels of heavy metals (Allen, 1995).

In fish, gills are the widest surfaces in contact with aquatic environment and thus, toxic materials cause damage in gills. These damages are edema, excessive hypertrophia and hyperplasia in lamellar epithelial cells and chloride cells. The aim of these alterations in the tissue is to minimize gill surface and consequently respiratory surface (Mallatt, 1985; Koca *et al*., 2005, 2008) and also to increase diffusion distance. As a consequence, respiratory function of gills is decreased

and general health of the fish is affected which may even cause death (Thophon *et al*., 2003). Moreover, it has been reported that copper (Mason *et al*., 2002; Figueiredo-Fernandes *et al*., 2007), aluminum (Birchall *et al*., 1989), cadmium (Randi *et al*., 1996), lead (Olojo *et al*., 2005) and nickel (Athikesavan *et al*., 2006) cause separation in lamellar epithelium, proliferation, lamellae adhesion and lamellar aneurism.

The results obtained in the study are consistent with the literature and gills were observed to be the most affected organ.

In the study, mucous cells were observed especially at the tips of primary lamellae and between secondary lamellae. Mucous generated by these cells is a very functional material with respect to respiration, ionic and osmotic regulation, reproduction, secretion, resistance against disease, communication, nest making and protection (Shephard, 1994). Mucous secretion in fish prevents penetration of chemical toxicant and harmful materials by inceasing the diffusion distance in gills. Moreover, by causing hypoxia through making oxygene intake difficult (Nero *et al*., 2006), it impairs swimming capacity and behaviour of the fish. In a study, Thophon *et al*. (2003) reports that acute toxic action of Cd first targets gill lamellae and kidney tubules but toxic effects observed in gills is less compared to the ones in kidney and livers. Hepatocellular degeneration, vacuolization, sinusoidal dilation and red blood cell accumulation that we observed in the liver of eels (*Anguilla anguilla*) is similar to the findings obtained at liver exposed to Cu (Figueiredo-Fernandes *et al*., 2007) and Pb (Olojo *et al*., 2005).

In histological and cytological (TEM) studies exposed glass-eels and young yellow eels to cadmimum-nitrate for a short time and they observed edema in gills, lamellae adhesion, rupture, necrosis, fibrosis in liver and disturbance in hepatocytes (Triebskorn *et al*., 2008). These findings are similar to the findings. In a study conducted on eels, it is shown that Cd, Pb and Cu accumulate in liver while Mercury (Hg) accumulates in muscle (Linde *et al*., 2004).

As eels have a long life (8-12 years), pollutants may accumulate in high concentrations. Thus, even little-polluted waters are potentially harmful (Jacques, 1990). Exposure to even trace amount of heavy metals may affect not only its physiology but also its growth, reproduction and migration.

As a result, heavy metals can be a serious ecological risk for fish, eels that have only a single reproduction chance during a long and difficult life span are getting diminished as a result of changing environmental conditions and pollution. Fish provide a suitable model for monitoring aquatic genotoxicity and wastewater quality because of their ability to metabolize xenobiotics and accumulate pollutants (Grisolia and Cordeiro, 2000). Micronucleus assay has been used successfully in various fish species (Grisolia and Starling, 2001; Koca *et al*., 2008; Matsumoto *et al*., 2006; Ergene *et al*., 2007).

CONCLUSION

In this study, no micronucleus formation has been observed in genotoxicity (Micronucleus test) studies conducted on some fish species caught from polluted waters. There are two possible explanations of such results: firstly, some fish species such as *Genyonemus lineatus* (Carrasco *et al*., 1990) and *Phoxinus phoxsinus* (Sanchez-Galan *et al*., 1998) have lower susceptibility to pollutants Secondly, fish have a very effective micronuclei removal system that prevents the increase in the level of micronuclei in peripheral blood (Zuniga *et al*., 1996; Zuniga-Gonzalez *et al*., 2000). In the study, not observing micronucleus and any nucleus abnormality in erythrocytes of *Anguilla anguilla* despite the pollution in the Gediz river suggests that these fish may have one or both of the systems stated. In some in situ studies conducted on eels, some researchers have reported that these fish (*Anguilla anguilla*) are not a suitable material to determine genotoxicity of water (Sanchez-Galan *et al*., 2001; Rodriguez-Cea *et al*., 2003).

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