Int. J. Aquat. Biol. (2016) 4(5): 330-339
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Genotoxic evaluation of the Ergene River, Turkey, on mosquito fish, *Gambussia affinis* (Baird and Girard, 1853) using the piscine micronucleus assay

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Abstract: The Ergene River is located in the Thrace Region of Turkey and is polluted by industrial and municipal waste. In the present study, we investigated the genotoxic and cytotoxic effects of water samples on *Gambussia affinis* in vivo using the piscine micronucleus (MN) test. Fish were exposed to 50, 100, 150 and 300 mL L^{-1} of water samples for 24 hrs, and MN, nuclear abnormalities (NA), polychromatic-normachromatic erythrocytes (PCEs/NCEs), and apoptotic erythrocytes were evaluated. In addition, water samples were analysed to determine the concentrations of the heavy metals. The results showed that MN, NA, and apoptosis frequencies significantly increase at all concentrations compared to the control. A significant correlation was found between genotoxicity endpoints and the concentration of water samples. The PCE-NCE ratio was significantly decreased at all treatments. The metal content of river water was not associated with the increase in the seasonal frequency of genotoxicity endpoints. The results indicated that the Ergene River has genotoxic and cytotoxic effects on erythrocytes of *G. affinis* in an in-vivo piscine MN test that could be due to organic and inorganic effluents.

Article history:
Received 5 May 2016
Accepted 11 October 2016
Available online 25 October 2016

Keywords:
Genotoxicity
River
In-vivo micronucleus test
Apoptosis
Nuclear abnormalities

Introduction

One of the main problems of the industrial areas is pollution of water resources. If the industrial regions are near agricultural areas and cities, industrial effluents, agricultural runoff and urban contaminants are released into the environment and contaminate water sources. Hence, these water bodies need to be evaluated for cytotoxic and genotoxic effects of pollutants on aquatic organisms (Valko et al., 2005). Genotoxic contaminants may interact with DNA and induce mutations, chromosomal alterations, birth defects, and cancer in vertebrates (Nigro et al., 2002; Frenzilli et al., 2004). Genetic alteration and mutations may be induced by many contaminants in the aquatic ecosystems (De Flora et al., 1993). For monitoring the effects of aquatic ecosystems' pollution, fish are often suitable models because of their ability to metabolize xenobiotics and bioaccumulate pollutants (Al-Sabti and Metcalfe, 1995). The observed genotoxic effects in fishes' tissues are related to industrial, agricultural, and domestic activities (Rajaguru et al., 2003; Ergene et al., 2007; Summak et al., 2010; Osman et al., 2011; Radic et al., 2013).

The piscine micronucleus (MN) test is a common method to assess of the impact of pollution on aquatic ecosystems. MN, nuclear lesions, and binuclei are the most frequent parameters as bio-indicators of the genotoxic effects in fish species (Osman and Kloas, 2010), and the MN test is considered one of the most useful methods for evaluating genotoxicity in aquatic systems (De Lemos et al., 2001; Andrade et al., 2004; da Silva Souza and Fontanetti, 2006). Micronuclei are chromosome fragments or whole chromosomes that lag at cell division due to the lack of a centromere, damage, or a defect in cytokinesis that are formed by both clastogenic and aneugenic compounds (Heddle

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et al., 1991). Binucleated cells, blebbed, lobed, and notched nuclei are different forms of morphological nuclear abnormalities (NAs) described in fish erythrocytes (Carrasco et al., 1990). These abnormalities are considered to be indicators of the genotoxic damage (Ergene et al., 2007).

It is important to assess cytotoxic effect of pollutants beside genotoxic effects on living organisms. The cytotoxic effect of chemicals are assessed using the polychromatic erythrocyte-normachromatic erythrocyte (PCE-NCE) ratio, which reflects inhibition of cell division and maturation of nucleated erythropoietic cells (Kirkland, 1990). Measuring the PCE-NCE ratio is an approved method in erythrocytes of fishes (Kirkland, 1990; Randalli and Farrell, 1992; Cavas, 2008). Also, it has been employed to visualize DNA degradation due to apoptosis (Olive et al., 1993; Singh, 2000; Osman et al., 2012), which is a form of nuclear destruction in which the nucleus disintegrates and nuclear fragments are formed (Lawrence and Hemingway, 2003). If the damage produced reaches a high level, it can lead to cell apoptosis (Hao et al., 2009; Osman et al., 2012).

The water of the Ergene River, in the Thrace region of Turkey, is mostly used for agricultural purposes although it runs in an industrial area. Pollution of the River Ergene continues as a consequence of increasing agricultural, industrial, and domestic effluents. For this reason, continuous monitoring of its water quality is necessary, and therefore, many data have been regularly recorded on the status of this river. However, there are no known data available regarding the genotoxic and cytotoxic effects of pollutants on its aquatic organisms using the in-vivo piscine MN assay or other genotoxicity techniques.

Therefore, we investigated the cytotoxic and genotoxic effects of the water of the Ergene River on mosquito fish, *Gambussia affinis* under laboratory conditions using the piscine MN test. MN, NAs, apoptosis, and the PCE-NCE ratio are determined in erythrocytes after exposure of *G. affinis* to the Ergene River water sampled from two stations.

Materials and Methods

Study area and water samples: The Ergene River is located in the middle of the Thrace Region of Turkey. Industrial, agricultural, and domestic regions are located along the river, and consequently a large volume of the industrial, agricultural, and domestic wastes contaminate this river. The Ergene River origins from the Istranca Mountain in Tekirdag City. It drians to the Saroz Gulf and then to the Aegean Sea after conneting to the Meric River. Its length is 283 km with a basin of 11,000 km^2, and its water potential is 1.71 billion m^3 per year (domestic waste water, 230,000 m^3/day; industrial waste water, 330,000 m^3/day).

Two stations, including station 1, Cerkezkoy, 41°17'00"N, 28°00´00"E and station 2, Muratli, 41°10'27"N-27°30'31"E, were selected along the Ergene River. Both sampling stations were located near the industrial and residential areas. The water samples were collected in September, December, March, and June, 2014.

Chemical analyses of water samples: Water samples were taken manually with a horizontal water bottle and kept in dark containers at low temperature before transporting to the laboratory. Measurements were done within one day. For measuring dissolved metals, the samples were filtered through a 0.45 µm membrane filter and acidified with HNO_3 (pH<2). Element quantification was achieved by inductively coupled plasma mass spectrometry (ICP-MS) according to EPA 2008. Average values of three replicates were taken for each measurement.

Test organisms: *Gambusia affinis* (Cyprinodontiformes; Poeciliidae) were collected from the Balkan Campus (41°38'45"N, 26°37'21"E) in Edirne, Turkey, by a fish trap. The fish were transferred to the laboratory and kept in continuously aerated glass aquaria (100 L) for 2 weeks before the experiment. Dissolved oxygen, pH and temperature of the aquarium water were monitored daily. The weight and length of the specimens were 0.14±0.1 (mean±SD) g and 23.9±3.5 (mean±SD) mm, respectively.

Experimental design: Before the experiment, fish

were kept in an aquarium (100 L) at 20-21°C. Fish were then introduced into aquaria containing dechlorinated tap water (as control) and four different concentrations of Ergene River's water samples (50, 100, 150 and 300 mL L^{-1}) for 24 hours exposure periods. Five specimens were tested at each treatment with three replicates.

Analysis of micronuclei, nuclear abnormalities and apoptosis: Fish erythrocytes were analysed for micronuclei, NAs (notched, lobed, and blebbed nuclei), PCE-NCE ratio, micro- and bi-nucleated cells and apoptosis. Slides were prepared according to Ueda et al. (1992). Briefly, peripheral blood samples were obtained from the caudal vein of the specimens and smeared on clean slides. Cells were dried overnight, fixed with absolute methanol for 5-10 min, and stained with acridine orange (0.01 g/100 mL) in Sorensen's phosphate buffer. Three slides were prepared from each fish, and 2000 cells were observed from different slides for each fish. The frequency of the abnormalities was determined in erythrocytes under 100X magnification in a fluorescence BX51 microscope according to Carrasco et al. (1990). The slides were coded and randomized prior to scoring for MN, NA, apoptosis, and PCE-NCE ratios.

NAs were classified according to Carrasco et al. (1990). Erythrocytes that had a small evagination of the nuclear membrane were classified as blebbed nuclei. Erythrocytes with evaginations larger than those of blebbed nuclei and with several lobes were classified as lobed nuclei. Nuclei with vacuoles and an appreciable depth into a nucleus that did not contain nuclear material were recorded as notched nuclei. Micronuclei were considered as circular, non-refractive, small chromatin bodies showing the same staining pattern as the main nucleus (Al-Sabti and Metcalfe, 1995). Micronuclei with one-third or one-fifth's the diameter of the main nucleus with same colour, refraction, and texture of the main nucleus, were counted as micronuclei. Unmature erythrocytes containing ribosomes in their cytoplasm were considered PCEs. Mature erythrocytes without ribosomes in their cytoplasm were considered NCEs. PCEs stain differently from NCEs because of the RNA in the cytoplasm that reflects acridine orange with a reddish colour. Decreases in the proportion of PCE to NCE were considered as indicators of induced cytotoxicity (Suzuki et al., 1989). PCE frequency was calculated based on Pacheo and Santos (2002) as:

PCE frequency (%)=[No.PCEs/(No.PCEs+NCEs)]x100.

The condensed or fragmented erythrocytes were considered apoptotic cells.

Statistical analysis: Student's t-test was used to compare differences between two groups. Multiple comparisions were performed using one-way analiysis of variance. Correlations between total heavy metal content and frequencies of MN, PCE/NCE, and NAs were analysed using linear regression. Pearson correlation coefficients were calculated to compare concentration increases with genotoxicity endpoints and the relation amoung genotoxicity endpoints; MN, NA, PCE/NCE, apoptosis. In all cases, $P \leq 0.05$ was considered as the accepted significance level. Statistic analysis were performed using SPSS software (version 22).

Results

Mean values of genotoxicity and cytotoxicity endpoints at both stations and the significance of comparisions with the negative control ($P \leq 0.01$) are shown in Table 1. The results showed that MN and NAs were significantly increased due to a genotoxic effect. The frequencies of the observed parameters among seasons are not included in Table 1 as the endpoints showed no correlation. Fish in treatments of the station 1 were alive after 24 hrs exposure period, whereas all fish of the tratments at station 2 died during the spring and summer. The frequencies of genotoxicity endpoints were not significantly different among seasons. The results of the poly-chromatic and normachromatic cell and apoptotic cells are shown in Figure 1.

The correlation between concentrations of tested river water samples and MN, NA, and apoptosis are shown in Figure 2. Water samples taken from station 1 had a significant correlation between concentration

Table 1. Frequencies of MN, PCE/NCE, total NA and apoptosis after exposure to 50,100,150 and 300 mL L^{-1} concentrations of the Ergene River water in erythrocytes of *Gambusia affinis*.

Group	Station	Parameters			
		MN	PCE/NCE	Total NA	Apoptosis
(-) Control		0.38±0.09	2.18±0.08	11.36±2.34	24.53±3.36
(+) Control		5.46±0.4***	0.16±0.02***	26.47±1.38***	242.01±4.37***
50 mL/L	1	1.20±0.68	0.65±0.11***	10.47±2.34	115.92±30.34*
	2	0.98±0.26*	0.72±0.08***	10.58±1.38	79.855±30.19*
100 mL/L	1	3.41±0.87**	0.45±0.09***	17.73±1.86	173.59±30.12**
	2	2.26±0.99*	0.46±0.11***	25.20±4.08*	141.07±45.27*
150 mL/L	1	4.66±0.61***	0.30±0.09***	19.74±2.09*	216.80±30.26***
	2	3.64±6.06***	0.30±0.08***	19.61±1.44	183.97±36.37**
300 mL/L	1	5.32±0.51***	0.25±0.03***	23.67±0.86**	259.34±15.04***
	2	5.56±1.04**	0.18±0.07***	24.83±0.33*	251.42±38.22***

Values marked with *, ** and *** represent *P* values of $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively, in relation to the negative control using t-test. Values presented as Mean±SE; MN: micronucleus; PCE/NCE: polychromatic erythrocyte/normachromatic erythrocyte; NA: nuclear abnormalities (notched, lobed and budding nuclei); cyclophosphamide (5 mg L^{-1}): positive control.

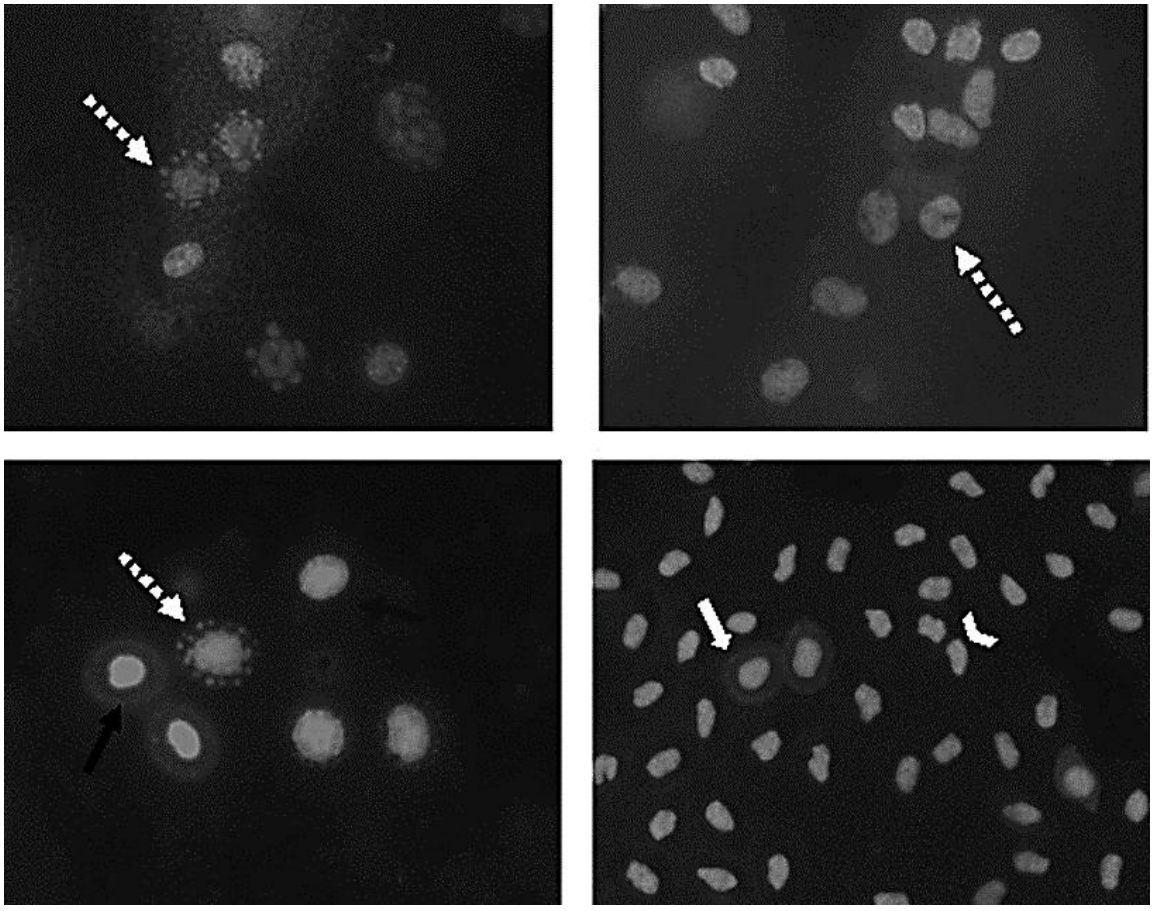

Figure 1. Apoptotic cells, polychromatic and normachromatic erythrocytes of *Gambusia affinis* stained with Acridine Orange (black arrow: normal erythrocyte; datted arrow: apoptotic erythrocyte; white arrow: polychromatic erythrocyte; arrowhead: normachromatic erythrocyte).

and MN (R=0.898*, *P*=0.038), NA (R=0.909*, *P*=0.032), and apoptosis (0.899*, *P*=0.038) in *G. affinis* erythrocytes under laboratory conditions. These significant correlations were also obtained with the water samples of station 2 between concentration and MN (R=0.987**, *P*=0.002) and apoptosis (R=0.945*, *P*=0.015). Apoptosis was showed a significant correlation with NA (R=0.906,

Table 2. Physical and chemical properties of mean values of water samples collected at different seasons from the Ergene River and their limit values (Class I) according to Turkish Water Pollution Control Regulation (2008).

Measured parameters	Station 1	Station 2	Limit values
pH	7.9	8.1	6.5-8.5
Temperature (°C)	22	24	25
Al (µg l^{-1})	204.583	347.474	300
Cr (µg l^{-1})	10.034	15.330	20
Mn (µg l^{-1})	210.662	304.774	100
Fe (µg l^{-1})	187.383	232.163	300
Co (µg l^{-1})	1.539	1.656	10
Ni (µg l^{-1})	8.991	13.531	20
Cu (µg l^{-1})	25.707	30.857	1000
Zn (µg l^{-1})	57.168	28.272	200
As (µg l^{-1})	14.502	18.486	20
Cd (µg l^{-1})	0.804	1.136	3
Ba (µg l^{-1})	55.854	74.861	1000
Pb (µg l^{-1})	3.192	2.248	10

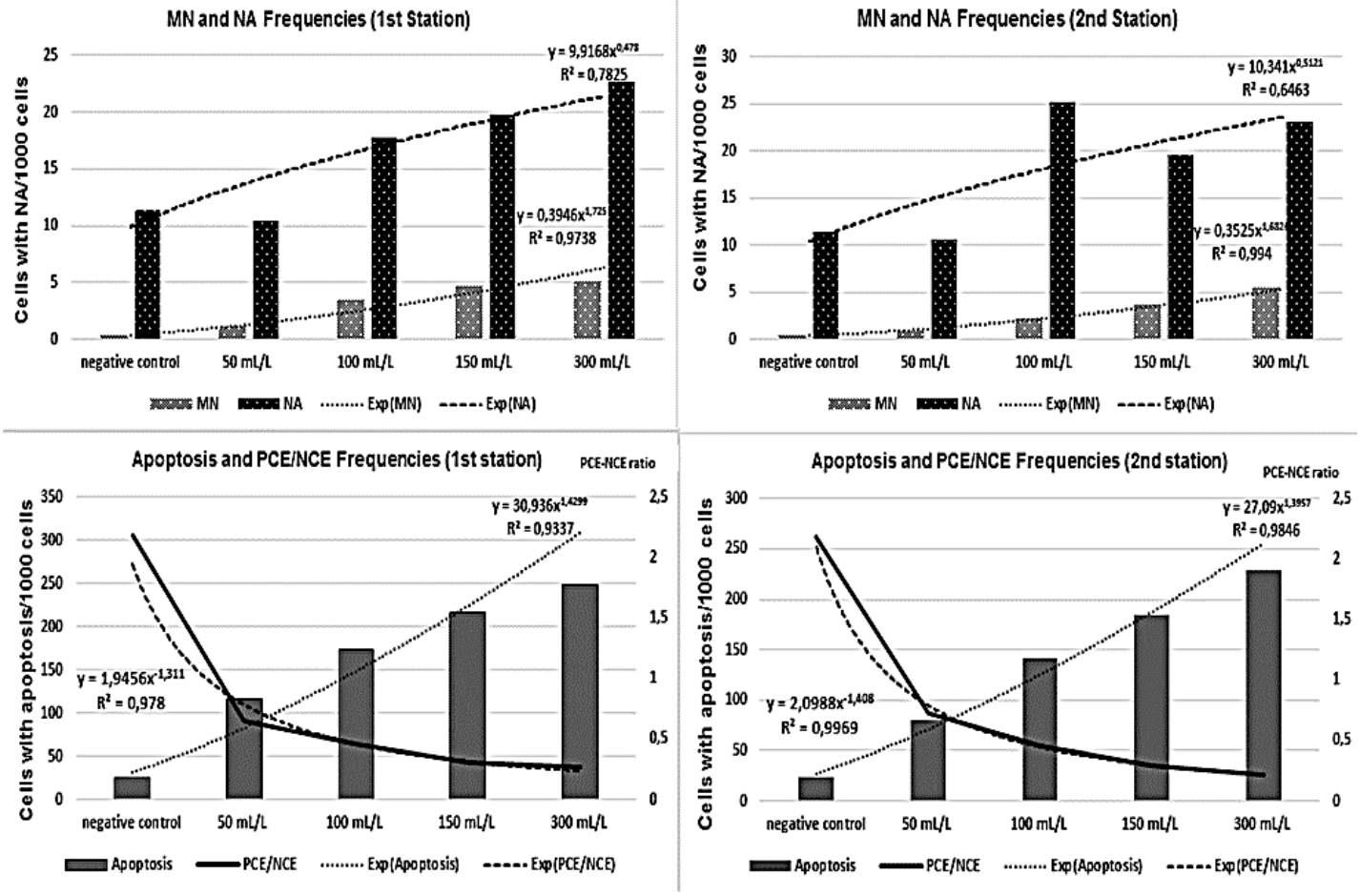

Figure 2. Frequencies of MN, NA, PCE/NCE, and apoptosis after exposure of the Ergene River water on *Gambussia affinis*.

P=0.034), MN (R=0.97, *P*=0.006), and PCE/NCE (R=-0.89, *P*=0.043) for samples from station 1 and MN (R=0.972, *P*=0.006) and PCE/NCE (R=-0.874, *P*=0.05) for samples of station 2.

The results of the metal and non-metal analysis of water samples in both stations are shown in Table 2. The concentration of metals was not significantly different between stations except Cr (*P*=0.03) and Rb (*P*=0.03). Table 2 also shows the limit values of pollutants established by the Turkish Water Pollution Control Regulation (2004). The physical and chemical properties of the water samples were

under or near Class I limit values according to the Turkish Water Pollution Control Regulation (2004) (Ministry of Environmental and Urban Planning (MEUP), 2004). There were no correlations between stations for metal concentration and abnormalities. Pearson's correlation showed no significant correlation concentrations and NA frequencies or MN.

Discussion

The results showed a higher incidence of MN and NAs in erythrocytes of *G. affinis* exposed to different concentrations of the Ergene River water under laboratory conditions. Similar results were obtained in environmental toxicology studies with such frequencies being significantly elevated with an increasing pollution gradient (Osman et al., 2011). The researchers observed that micronuclei are induced in the fishes inhabiting polluted sites compared to those of clean river systems (Al-Sabti and Hardig, 1990; Pietripiana et al., 2002; Bagdonas et al., 2003; Rodriguez-Cea et al., 2003; Bombail et al., 2001; Cavas and Ergene-Gozukara, 2005). It is known that MN formation as well as the induction of NA are considered to be the consequence of genotoxic events in fish (Metcalfe, 1988; Pacheo and Santos, 2002). MN and NA frequencies observed in the present study indicate that the Ergene River water has a potential genotoxic effect on its fish fauna.

The Ergene River basin is located near an industrial region including textile chemistry, domestic metal and mine industries and factory effluents and may contain metals, such as Cu, Cr, Ni, Cd, Pb and Zn (Chino et al., 1991; Aonghusa and Gray, 2002; Cavas and Ergene-Gozukara, 2003; Manzoor et al., 2006). It is known that metals found in polluted water have a potential genotoxic effect on aquatic organisms (Hei and Filipic, 2004; Barbosa et al., 2010; Summak et al., 2010). Toxic metals and their interactions with other compounds present in effluents may have genotoxic effects on living organisms, and metals in a mixture may have additive chronic toxicity compared to their individual effects (Enserink et al., 2003). Guner and Muranli (2011) showed that nuclear abnormalities were significantly induced in erythrocytes of *G. affinis* when fish were exposed to Cu and Cd in combination.

In the present study, there was no significant correlation between metal concentrations in water samples and MN or NA frequencies. As shown by the metal analyses of water for both stations 1 and 2 (Class I quality water, according to Turkish Water Pollution Control Regulation (2004)), metal concentrations were found under limit concentrations MEUP, 2004). In addition, we found no correlation between metal concentration and MN, PCE/NCE, or NAs. The results are in agreement with Ergene et al. (2007) that found no significant correlations between metal concentrations and genotoxicity endpoints in erythrocytes of Nile tilapia (*Oreochromis niloticus*) exposed to Berdan River water for different time periods (Ergene et al., 2007). Their result also revealed that MN and NA frequencies were significantly increased, although the chemicals properties of the Berdan River within the limit values according to the Turkish Water Pollution Control Regulation (Ergene et al., 2007).

An increase of MN, PCE/NCE, and NAs may indicate the presence of large quantities of organic and inorganic pollutants in river water due to industrial, domestic and agricultural runoff (Osman and Kloas, 2010; Summak et al., 2010). It is known that genetic aberrations cannot be attributed to a single agent due to the constitution of a complex mixture (Donbak et al., 2005). Municipal effluents are recognized as a major source of many environmental contaminants, including polyaromatic hydrocarbons, pesticides, steroids, and metals (Gagne et al., 2006). White and Rasmussen (1998) noted that despite the noteworthy genotoxicity of some industrial wastewater, domestic wastewater constitutes a greater genotoxic hazard to aquatic systems and their associated biota (White and Rasmussen, 1998). An ecological risk assessment of polluted waters is difficult due to antagonistic or synergistic effects of a mixture of contaminants. Our

results are in accordance with the other investigations that MN and NA frequencies increased as the river water concentrations increased; this may have been due to other genotoxic chemicals and organic pollutants present in the Ergene River water.

The results showed a significant negative correlation between PCE/NCE ratio and apoptotic cell frequency; the PCE-NCE ratio significantly decreased at all treatments. Homeostasis is provided by erythropoiesis and destruction of erythrocytes. New erythrocytes are continuously entering the circulatory system, and defeat erythrocytes are destroyed at the same rate (Randalli and Farrell, 1992). Assessment of the PCE-NCE ratio can provide evidence of exposure to toxic substances. It is known that PCEs are immature erythrocytes that have ribosomes in the cytoplasm, and that NCEs are mature erythrocytes that lack ribosomes in the cytoplasm (Kirkland, 1990). Inhibition of maturation of nucleated erythropoietic cells causes a decrease in the PCE-NCE ratio. Reductions in the proportion of PCE/NCE are considered to be indicators of mutagen-induced cytotoxicity (Suzuki et al., 1989). The results of the present study indicated that Ergene River water has cytotoxic effects due to a significant reduction in the PCE-NCE ratio, and this reduction is strongly correlated with the induction of apoptosis.

Although water quality monitoring studies involves measurements of physical-chemical parameters, biological monitoring has become essential as it reveals the harmful effects of noxious chemicals and can indicate risk to the environment and human health (Ternjej et al., 2013). In the present study, we did not perform detailed chemical analyses of the Ergene River water, since the Ministry of Environmental and Urban Planning (MEUP) investigates the Ergene River on a regular basis. Based on the criteria of dissolved oxygen, chemical oxygen demand, biological oxygen demand, sulphate, chlorine, sulphur, and sodium, class IV water quality for this river is considered (MEUP, 2014). A high concentration of sulphate as reported for this river, strongly interferes with the biogeochemical cycling of iron and phosphorus and can lead to eutrophication, which further enhances toxicity (Lamers et al., 2002; Geurts et al., 2009). It is known that sulphates present an ecotoxicological risk (Mihaljevic et al., 2011; Stankovic et al., 2011). In addition to the potential mutational effect related to the complex mixtures, there are other possible problems for resident organisms that could produce alarming indices of organic wastes and result in the death of various fish species (Batzias and Siontorou, 2006). In the present study, death of fish after exposure to river water taken during spring and summer may be due to low concentrations of chemical and biological oxygen demand, sulphur, and sulphate concentrations that has been indicated in Class IV water quality (MEUP, 2014).

In the present study, the polynucleated erythrocytes of *G. affinis* were the most frequent abnormalities with exposure to higher concentrations of riverine water. Polynucleated cells were found to cause apoptosis of the cells due to DNA degradation. Apoptosis is a form of nuclear destruction in which the nucleus disintegrates and nuclear fragments are formed; apoptosis occurs both naturally and in response to chemically-induced cellular damage (Lawrence and Hemingway, 2003). The results also showed a positive correlation between apoptosis and concentration and between apoptosis and the other genotoxicity endpoints i.e. MN and NA. The apoptotic effect of water pollution on fish erythrocytes has been previously investigated (Omar et al., 2012; Osman et al., 2012; Walia et al., 2013). Metals, such as iron, copper, and zinc lead to DNA fragmentation (Razzaque, 2007), and fragmentation patterns may involve more than one mechanism leading to cell death and inducing apoptosis and/or necrotic cell death (Razzaque, 2007; Omar et al., 2012). Similarly, in the present study the high incidence of MN, NA, and polynucleated cells that could cause cell death showed that the Ergene River has a genotoxic effect, leading to cytotoxicity and apoptosis.

The effects of chemical interactions and the influence of complex matrices on toxicity cannot be

determined from chemical test alone (Polard et al., 2011; Radic et al., 2013). In the present study, we concluded that genotoxic and cytotoxic effects of the Ergene River may be due to chemical interactions and these interactions and toxicity mechanisms cannot be predicted by solely measuring physical, chemical, organic, and inorganic components of the water.

Ethical Approval: All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (TUHDYEK-2013/22).

Acknowledgements

This study was funded by The Scientific and Technological Research Council of Turkey-TUBITAK (1919B011300445).

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