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Micronucleus Test, Nuclear Abnormalities and Accumulation of Cu and Cd on *Gambusia affinis* (Baird & Girard, 1853)

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Abstract

In the present work the induction of micronuclei (MNi) and nuclear abnormalities (NAs) in erythrocytes and Cu and Cd accumulation in whole body of *Gambusia affinis* were studied. Fish were exposed to two different Cu and Cd concentrations, 0.1 ppm and 1 ppm, for 1 and 2 weeks periods and to Cu-Cd combination (0.1 ppm Cu + 0.1 ppm Cd) for 2 weeks period using a semi-static renewal system. Micronucleus and nuclear abnormality analysis were carried out on peripheral blood erythrocytes. When fishes were exposed Cu and Cd in combination, Cu accumulation was increased compared to their singly (0.1 ppm) exposed concentrations. Micronucleus and nuclear abnormality analysis tests revealed that, although Cu and Cd did not significantly increase micronuclei frequency, nuclear abnormalities were significantly induced compared to control groups.

Keywords: *Gambusia affinis,* nuclear abnormalities, heavy metal accumulation, Cu, Cd.

Cu ve Cd'un *Gambusia affinis* (Baird ve Girard, 1853)'te Birikimi, Mikronukleus Testi ve Nükleer Anormalliklerin Oluşumu

Özet

Bu çalışmada Cu ve Cd'un *Gambusia affinis*'te birikimi, mikronukleus ve nükleer anormalliklerin oluşumu çalışılmıştır. Balıklar 0.1 ppm ve 1 ppm Cu ve Cd konsantrasyonları ile bir ve iki hafta, 0.1 ppm Cu + 0.1 ppm Cd karışım konsantrasyonu ile iki hafta yarı statik sistemde muamele edilmişlerdir. Mikronukleus ve nükleer anormallikler periferik kan eritrositlerinde analiz edilmiştir. Cu ve Cd'un birlikte uygulandığı balıklarda, Cu birikimi tek başına (0.1 ppm) uygulandığı gruba göre artış göstermiştir. Mikronukleus ve nükleer anormallik test sonuçları, Cu ve Cd'un balık eritrositlerinde mikronukleus frekansını anlamlı arttırmamasına rağmen nükleer anormallikleri kontrole göre anlamlı arttırdığını göstermektedir.

Anahtar Kelimeler: *Gambusia affinis*, nükleer anormallikler, ağır metal birikimi, Cu, Cd.

Introduction

In last decades, environmental contamination by metals has received significant attention especially with respect to effects on aquatic ecosystems. Rivers and lakes, directly influenced by mining, metal smelting and other industrial activities; have been contaminated by many potentially toxic trace metals such as Cadmium (Cd) and Copper (Cu). (Pacyna *et al.*, 1995; Mohapatra and Rengarajan, 1996; Eiseler, 1998; Kalay, 1996; Kargın and Çoğun, 1999).

Fish require Cu as micronutrients (Watanabe *et al.*, 1997) and can obtain these metals from either water or their diet (Handy, 1996; Eiseler, 1998). However it may become toxic when present in high concentrations in the environment. Copper toxicity to aquatic biota is related primarily to dissolved cupric ion (Cu^{+2}) and possibly to some hydroxyl complexes.

Cd is a ubiquitous toxicant which has been recognized as one of the most deleterious non-essential heavy metals (Stoeppler, 1991). Cd is typically found at very low (i.e. parts per billion) concentrations in rivers, lakes and ponds (Lugowska, 2007; Donson, 1992; Hollis *et al.*, 1999). Cd is among the most toxic metals in the aquatic environment, possesses no known biological role and exhibits high toxicity if allowed to accumulate in metabolically-active tissues (Sorensen, 1991). In fish, Cd can damage gills (Voyer *et al.*, 1975; Eiseler, 1998; Canlı and Kargın, 1995) result in skeletal deformities

(Muramoto, 1981), and disturb calcium balance (Wicklund-Glynn *et al*., 1994; Hollis *et al*., 1999). Cd can also damage fish energy production metabolisms (Smet and Blust, 2001).

Some metals can cause nuclear abnormalities. The formation of nuclear abnormalities described by Carrasco *et al*. (1990) have been reported in fish erythrocytes, as a consequence of exposure to environmental and chemical contaminants of cytotoxic, genotoxic, mutagenic or carcinogenic action. However, the mechanisms responsible for such abnormalities have not been described yet. Micronuclei are formed during cellular division, reflecting mutagenic effects by loss of chromosomal fragments or whole chromosomes that are not included in the main nucleus following anaphase. The micronucleus test in fish has potential of detecting clastogenic and aneugenic effect of environmental agents in aqueous media. Since teleost erythrocytes are nucleated, MNi have been scored in fish erythrocytes as a measure of clastogenic activity (Al-Sabti and Metcalfe, 1995).

The formation of morphological nuclear alterations (NAs), was first described in fish erythrocytes by Carrasco *et al*. (1990). NAs including blebbed, lobed and notched nuclei and binucleated cells, have been used by several authors as possible indicators of genotoxicity (Ayllon and Garcia-Vazquez, 2000; Çavaş and Ergene, 2003; 2005a, 2005b, Da Silva Souz and Fontanetti, 2006). Although the mechanism responsible for NAs has not been fully explained, these abnormalities are considered to be indicators of genotoxic damage and therefore, they may complement the scoring of micronuclei in routine genotoxicity surveys.

Cu and Cd were extensively studied in various toxicological investigations (Arkhipchuk and Garanko, 2005; Ravera, 1984) and tested in genotoxicity assays, with sometimes contradictory results (Straus, 2003; Brooks *et al*., 2004).

The response of aquatic organisms exposed to several metals simultaneously requires consideration of the interactions between their effects on the organisms. This additive action of more than one metal on target organisms should be taken into consideration when developing toxicologically relevant water quality criteria.

Since *G. affinis* are widespread and use for biological control of mosquitoes and also one of the most used animals for many experiment tests, easy to obtain and accommodate laboratory conditions, this study was undertaken to determinate nuclear abnormalities, MNi and accumulation of heavy metals (Scott and Chambers, 1996).

In the present study, we aimed to investigate genotoxic effects of two metals, Cu and Cd on *G. affinis* and their accumulation upon exposure to the metals at two concentrations, individually and in combination.

Materials and Methods

Fish Employed

Mosquito fish (*G. affinis*) were obtained from the Güllapoğlu Lake, Edirne Turkey in May 2008 [Güllapoğlu Lake water: Ca^{2+} 40 mg/L, pH 8.0, 14°C] and held in flowing dechlorinated i.e. activated carbon filtered) Edirne city tap water. Fish were held in at 21°C, 12 h : 12 h light:dark regime in 100 L continuously aerated glass aquarium tanks for 2 weeks before the experiments. Water parameters during the experiment remained constant as follows; dissolved oxygen 9.8±2.5 mg/L, temperature 20±1°C, pH 8.1±0.4 and conductivity 779±11 µhos. They were fed approximately 3% of their body weight per day with Pınar carp bait (Pınar Bait: Zn 70 mg/kg, Mg 25 mg/kg, Mn 25 mg/kg, Fe 2 mg/kg, Co 0.2 mg/kg, wet weight).

For exposure, 100 fish were randomly transferred to six glass aquarium (16 or 17 fish to each aquarium, 50 x 50 x 100 cm, 100 L) exposure tanks which were operated with semi-static renewal with continuous aerationed.

Doses and Times for Sample Collection

Metal salts used for preparation of stock solution were $CdSO_4.8H_2O$ (CAS 7790-84-3, purity >98%, Merck) and $CuSO_4.5H_2O$ (CAS 7758-99-8, purity >99%, Merck). Stock solutions of 1000 mg/L volumes were obtained by dissolving the Cd or Cu salt in dechlorinated (activated carbon filtered) tap water. The required volume of stock solution was added to the respective experimental aquaria to achieve the desired concentrations of 0.1 and 1.0 ppm.

Dechlorinated water was changed once in every two days during the experiment period. *G. affinis* were exposed to Cu (0.1 and 1 ppm), Cd (0.1 and 1 ppm) or Cu-Cd mix (Cu+Cd, 0.1 ppm each) for 1 and 2 weeks using a semi-static renewal system. A group was kept in an aquarium filled with only filtered tap water as a negative control. Cyclophosphamide (CAS 6055-19-2) was used as positive control at a concentration of 5 mg/L (for one week). Prior to the experiments, the aquaria were filled with the respective solutions of Cd or Cu for seven days for initial adsorption of metals onto inner tank surfaces. Before sampling processes, clove oil (55 mg/L concentration) was used as anaesthesia (Cho and Heath, 2001).

Metal Accumulation Analysis

Levels of Cu and Cd were analyzed in bodies of five test animals from each dose group (0.1 ppm 1.0 ppm) to determine the extent of Cu and /or Cd accumulation due to water-borne metal exposure. At the end of the exposure periods, 5 fishes from each tank were sacrificed. Body weights, total lengths and dorsal lengths of all animals were measured. All bodies were then weighed and digested by (1:1) concentrated nitric/perchloric acids (Merck) in closed tubes at 80-100°C until the content of tubes turned to a yellow clear liquid (Güner, 2007, 2008).

Cd and Cu values in each fish were determined by flame atomic absorption spectrophotometer (Unicom 929 AA). The standards used to make calibration cue were 1, 3, 4 and 5 mg/L.

The Nuclear Abnormalities (NA) and Micronucleus Test (MNT)

Peripheral blood samples were obtained from the caudal vein of the specimens and smeared on clean slides. After fixation in pure ethanol for 20 min, slides were left to air-dry and then the smears were stained with 10% Giemsa solution for 25 min. Observations were made using an Olympus BH2 research microscope.

Five slides were prepared from each fish. 1000 erythrocytes were scored from each slide under 1000-fold magnification to determine the frequency of notched nuclei, lobed nuclei, budding, fragmenting and also micronucleated cells, which was calculated as per 1000 cells (‰).

NAs were classified according to Carrasco *et al*. (1990). Blebbed nuclei present a relatively small evagination of the nuclear membrane, which contains euchromatin. Evaginations larger than the blebbed nuclei which could have several lobes were classified as lobed nuclei. Nuclei with vacuoles and appreciable depth into a nucleus that does not contain nuclear material were recorded as notched nuclei.

Statistical Analysis

The SNK test was used to compare Cu and Cd concentrations among Cu Cd treatment groups. As metal accumulation was not significantly different from each other, and two-way Anova analysis was performed followed by a SNK test as a post hoc test. Groups were considered to be significantly different from each other if $P<0.05$. All analyses were performed using an SPSS program. Partial correlation coefficients between body metal contents (0.1 ppm heavy metal doses) and body parameters (total length, weight and dorsal length) were calculated (all parameters exposed log transform).

The frequencies of MNi and NAs were expressed per 1000 cells (‰). The statistical significance of the differences in mean values, between exposure and control groups, were determined with the Student's t-test at $P<0.05$ level.

Results

Body Parameter Analysis

Total lengths for all specimens used for all experiments were measured as to be 23.919±3.485 mm. Total weights were 0.4±0.11 g (Table 1).

No mortality was observed in the control and the experimental groups at the end of the second week. Cd values in the control groups were below detectable limits. Cu levels of control group didn't change at the end of first and second weeks. According to the control groups, not only different doses of Cu groups, but also different doses of Cd groups significant accumulation was observed.

Results of Cu and Cd Accumulation

Single or combined exposure of Cu and Cd metals significantly increased Cu and Cd accumulation in whole body of test animals compared to control. When fishes were exposed Cu and Cd in combination, Cu accumulation was increased compared to their singly (0.1 ppm) exposed concentrations. The highest amount of Cd and Cu were detected in animals at the end of the second week.

A negative correlation was observed between body mass and metal accumulation for both Cu and Cd, but Cd showed more negative correlation than Cu between body parameters (Table 2).

Cu and Cd groups showed a similar pattern of accumulation, with a faster initial rate of accumulation in body (Figure 1 and 2).

Results of Nuclear Abnormalities and Micronucleus Test

The frequency of NAs and MNi induction is shown in Table 3. Cu and Cd concentrations significantly induced NAs at all treatment groups ($P<0.001$). Among the abnormalities observed lobbed nuclei were the most frequent. MNi formation was not significantly induced except 1.0 ppm Cu for 2 weeks exposure.

The results showed that combined use of Cu and Cd is more effective than their individual use. Although combined use of Cu and Cd did not

Table 1. Same Descriptive Statistics (Total length, weight and dorsal length) of Mosquito fish

	N	Mean	Std. Deviation	Minimum	Maximum
Total length (mm)	110	23.919	3.485	16.490	45.140
Dorsal length (mm)	110	4.386	0.768	2,680	8.250
Total Weight (g)	110	0.135	0.110	0,010	0.900

Table 2. Partial correlation coefficients of Cu and Cd levels and body parameters (all parameters exposed log transform)

	Total Length	Dorsal Length	Total Weight	Cu	Cd
Total length	1	0.8875*	0.9488*	-0.2649	-0.6892*
	P= .	P= 0.000	P= 0.000	P= 0.182	P=0.000
Dorsal length		1	0.9395*	-0.4459	-0.7016*
		P= .	P= .000	P=0.020	P= .000
Total Weight			1	-0.4144	-0.7522*
			P= .	P=0.032	P= .000
Cu				1	0.3853*
				P= .	P= 0.047
Cd					1

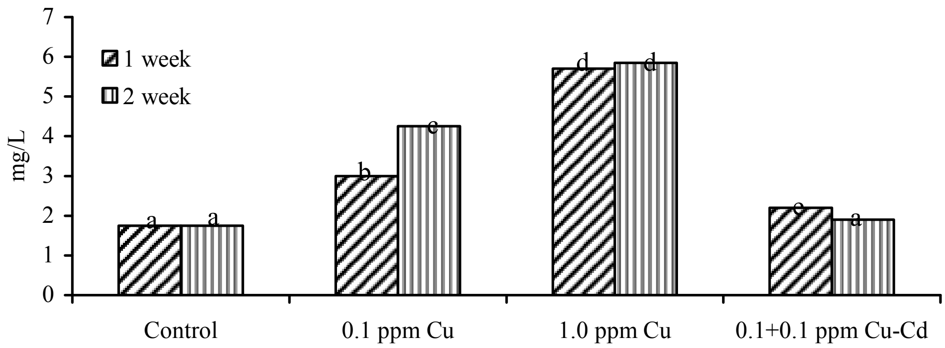

Figure 1. Cu accumulation in *Gambusia affinis* after different concentrations of Cu for one and two weeks exposure period. a, b, c, d, e letter show differences between groups (P<0.01).

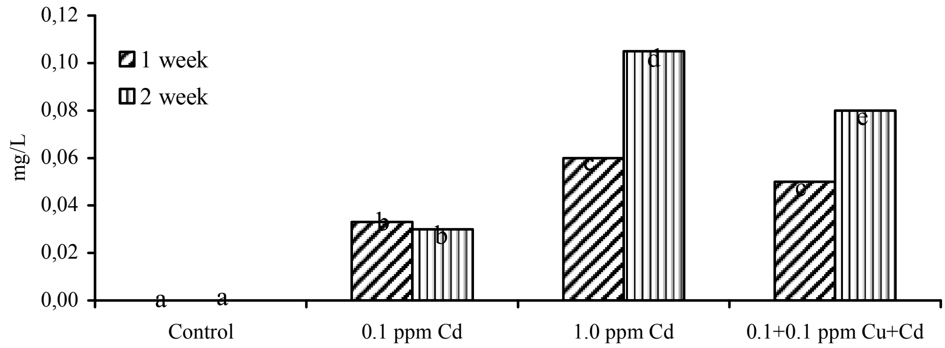

Figure 2. Accumulation of Cd in *Gambusia affinis* after different concentrations of Cd for one and two weeks exposure period. a, b, c, d, e letter show differences between groups (P<0.01).

Table 3. Frequency of notched, lobed, notched, budding, fragmenting nuclei and micronucleated erythrocytes in specimens of *G. affinis* exposed to different heavy metal treatments and time (one, two week)

			Notched	Lobed	Bud	Fragmenting	MN	Total Nuclear abnormalities (-MN)
Control			2.8±0.73	5.2±1.2	1.0±0.4	1.0±0.4	0.2±0.2	10±2.07
W_1	0.1 ppm	Cu	5.8±1.06***	10.8±1.01*	4.6±1.16**	1.8±0.58	0.0	23.0±1.54***
		Cd	10.6±3.04***	12.0 ±2.16***	5.2±2.08***	2.2±0.86	0.6±2.4	31.2±6.06***
	1.0 ppm	Cu	4.8±0.96	13.0±1.37***	2.0±089	0.8±0.58	0.2±0.2	20.6±1.77***
		Cd	5.6±1.36*	10.2±1.93**	3.8±0.58**	2.6±0.24*	0.8±0.58	22.2±1.31***
W_2	0.1 ppm	Cu	7.8±1.42***	9.8±1.90**	2.0±1.04	0.0	0.8±0.2	19.6±3.65***
		Cd	6.8±1.49**	9.4±1.50*	2.6±0.50	2.0±0.83	0.6±0.4	20.8±3.61***
	1.0 ppm	Cu	4.0±0.83	13.0±2.21***	4.4±0.67***	0.4±0.24	1.6±0.67*	21.8±1.95***
		Cd	6.4±1.24*	12.4±1.6***	4.0±0.83**	0.2±0.2	0.6±0.6	23.0±2.79***
	0.1+0.1 Cu-Cd		15.4±2.6***	23.2±4.85***	7.6±0.74***	2.0±0.89	0.8±0.37	48.0±8.44***

W_1 and W_2 weeks,* P<0.05, **P<0.01 and ***P<0.001

significantly induce MNi formation, NAs were slightly induced compared to their total effect of singly exposure ($P<0.001$). One and two weeks of exposure periods showed no relation between the groups.

Discussion

When a toxic substance is introduced into the aquatic environment three main steps can be identified before a response is produced from an aquatic organism (Tao *et al*., 1999). i) chemical and physico-chemical processes, in which the substance interacts with other constituents of the water and becomes available to the organism; ii) physiological processes, including absorption, transport, distribution, metabolic transformation, accumulation, and iii) excretion and intoxication processes, including combination with receptors. In the present study the experiment conditions were stable to avoid differences of metal accumulations of fishes.

Cu is essential for several aquatic species such as *Cyprinus carpio* and *Oncorhynchus mykiss* (Eiseler, 1998). However it may become toxic when present in high enough concentrations in the environment. In this study, 0.1 and 1.0 ppm Cu concentrations were studied on whole body accumulation. No mortality was observed in control and the experiment groups and also water parameters during the experiment remained constant. Results showed that, Cu levels of fishes were increased with both of time and doses. At the other hand, Cd was a non-essential, extremely toxic trace element and readily accumulated in the tissues of many aquatic organisms such as fishes and crayfish (Güner, 2007, 2008). In this study, the value of Cd accumulation in the all body on dose 0.1 ppm group were not changed according to first and second week exposure period although Cd accumulation after 1 ppm exposure increased after two weeks exposure period. Similar results were obtained in other studies. Hollis *et al* 2001 stated that after Cd exposure, Cd accumulation in a time dependent fashion to 2 times (whole body) of Rainbow trout. Also it was shown that Cd and Cu accumulated in organs of rainbow trout (Handy, 1993) and dogfish after metal exposure (De Boeck *et al*., 2010).

Mixture of Cd and Cu affected metal accumulation in different ways. Pelgrom *et al*. (1994) stated that Cd and Cu exposure have a complex interaction mechanism as it was concluded e.g. from the significantly decreased whole body Cd-content of Cu/Cd-co-exposed fish compared to the Cd-content of Cd-exposed fish (Pelgrom *et al*., 1994). But in the present study Cd content was increased in Cu/ Cd-co-exposed fish compared to the Cd-content of singly exposed fish. Several factors may affect accumulation of metal when co-exposed as whole body water, calcium and sodium content. The other reason of observed differences might be the heavy metal accumulation and body size. In the present study it was observed that Cd accumulation has a negative correlation with the length of test animals. Cd accumulation increased while the body length decreased. This might be the reason for different accumulation levels of whole body Cd content of Cu/Cd co-exposed fish. Moreover, co-exposure of Cu/Cd significantly decreased whole body Cu content. Also these results showed an obvious complex interaction mechanism of Cu/Cd co-exposure experiments. The negative relationships between heavy metal levels in the tissues and fish sizes were generally supported in the literature (Vinikour *et al*., 1980; Bowles *et al*., 2001; Besser *et al*., 2001)

Nussey *et al*. (2000) showed that accumulation of metals (Cr, Mn, Ni, and Pb) decreased with an increase in the length of fish *Labeo umbratus*. The accumulation of Zn, Cu and Mn in tissues of fish (Lethrinidae) captured from the Arabian Gulf was affected by the sex (Al-Yousuf *et al*., 2000). Patterns of heavy metal bioconcentration with age, sex or body mass can influence, to the extent of masking, observed trends in biomagnification. A primary reason for a decrease in metal concentration with size is related to new tissues being incorporated at a greater rate than metals can be actively transported into the tissues to establish a steady-state concentration (dilution by growth) (Vinikour *et al*., 1980). In the present study, it was found that the relation between Cu and Cd levels of body and body parameters (weight, total and dorsal length) were negative.

It is crucial to assess genotoxicity and cytotoxicity of the environmental pollutants on aquatic organisms. In fish, there are several types of nuclear lesions whose origin is not still understood (Ayllon and Garcia-Vazquez, 2000). Nuclear abnormalities have been used by some authors as a signal of cytogenetic damage in fish species (Pacheco and Santos, 1997; Metcalfe, 1988). As a result of the present study Cd and Cu did not significantly induce MN, but significantly induced total nuclear abnormalities at different concentration and exposure times. As one would expect from its essentiality for living organisms, Cu did not induce significant MNi increase in brown trout and European minnow (*Phoxinus phoxinus*) (Sanchez-Galan *et al*., 1999)and crucian carp (*Carassius auratus gibelio* Bloch.) (Arkhipchuk and Garanko, 2005). Ayllon and Garcia Vazquez (2000) reported that intraperitoneal injection of Cd induced a significant increase of the NAs, but not MNi, in both minnow and mollie. However, according to Ayllon and Garcia Vazquez (2000) Cd did not induce MNi formation., according to Sanchez-Galan *et al*. (1999, 2001) Cd induced MNi in both brown trout (*Salmo trutta*), European minnow (*Phoxinus phoxinus*) and in eel (*Anguilla anguilla*). Similar results were obtained in other fish species such as *Tilapia s*p. (Manna and Sadhukhan, 1986;

Chandra and Khuda-Bukhsh, 2004), brown trout (Sanchez-Galan *et al.*, 1999) and crucian carp (Arkhipchuk and Garanko, 2005). Different results can be obtained about effects of heavy metals on different species and the contradictory results may be due to different sensitivity of the species after exposure.

In the present study, Althogh 0.1 ppm and 1.0 ppm Cu and Cd concentrations significantly induced total nuclear abnormalities, NAs are not related to neither metal concentration nor exposure period. Furthermore; total nuclear abnormalities were increased slightly after Cd/Cu (0.1+0.1 ppm) co exposure. Also, in the present study Cu and Cd did not significantly induced MNi formation except 1.0 ppm Cu for two weeks exposure period. There was no association between MNi induction and metal concentration and exposure period.

Similarly, Carrasco *et al.* (1990) did not find a significant association between the variations including MNi and the levels of chemical pollution in sediments or fish tissues, pointing out another weakness of the micronucleus test in fish species. It is possible that lack of sensitivity is due to the low and variable frequency of MNi existing in wild fish. This problem could be solved by analyzing a cell type with a high mitotic index, an essential condition for the MNi formation (Heddle *et al*., 1991).

This species of fish (*G. affinis*) may protect itself from toxic subtances at early stages of exposure, possibly by a defence mechanism. And this defence mechanism provided this species with resistance abilities that no mortality was observed during experiment periods, even at high concentrations and exposure periods. That was, maybe the reason of non-relation between NAs, MNi and concentration and exposure period.

Also our results are in accordance with the results of Castano *et al.* (1998) who found, after intraperitoneal injection on rainbow trout, that Cd was accumulated in all tissues although the metal did not significantly induce MNi formation. In the present study 1.0 ppm Cd was accumulated in whole body although the metal did not significantly induced MNi formation.

Çavas and Garanko (2005) observed that Cu and Cd increased the micronucleus and binucleus frequencies in cells of gill and liver tissues of three fish species; Common carp, Prussian carp and Peppered cory, whereas in most cases no significant increase was found in peripheral blood erythrocytes. These differences in the erythrocyte micronucleus frequencies are generally thought to be related to cell kinetics and replacement. This also may explain our results that individual and combination exposure of fishes to heavy metals led to an accumulation in the body but this accumulation did not show increased MNi frequency in peripheral blood erythrocytes.

The results of the present study showed that Cu and Cd did not induce MNi but induced NAs when used alone and in combination. *G. affinis* accumulated Cu and Cd in whole body and co-exposure of Cu and Cd increased accumulation of Cd.

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