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Genotoxic Effects and LC_{50} Value of NaOCl on *Orthrias angorae* (Steindachner 1897)

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Abstract Studies show that different organisms used as bio-indicators have indicated several genotoxic and mutagenic effects of disinfected waters. In this study, the 96 h LC_{50} mean value of NaOCl for *Orthrias angorae* was calculated to be 0.5509 mg/L. The results showed that NaOCl is highly toxic to *O. angorae* specimens. Statistical analysis demonstrated a significant increase in micronuclei after the induction of 0.5 mg/L NaOCl concentration after 36 h. The same increase has been reported for 0.37 and 0.5 mg/L NaOCl concentrations after 72 h. Even though the MN frequency of 0.37 mg/L was similar after 36 and 72 h, only 72 h micronuclei frequency was statistically significant. The 72 h MN frequency of the negative control group was smaller than 36 h MN frequency of the negative control group. This discrepancy has led to 72 h MN frequency being statistically significant. MN frequency of 0.25 mg/L NaOCl concentration was insignificant when compared to negative test groups. The benzene treatment also caused a significant increase ($p < 0.01$) in the frequency of micronucleated erythrocytes.

Keywords Sodium hypochlorite · Micronuclei · *Orthrias angorae* · LC_{50} value · Peripheral erythrocyte

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Water is disinfected in order to utilize both waste water and surface water as drinking and utility water and to prevent the spread of pathogens (Monarca et al. 2000). Chlorination is widely used in the disinfection of drinking and utility water worldwide (Gustavino et al. 2005; Hidalgo et al. 2002; Monarca et al. 2004, 2000). Used for this aim, sodium hypochlorite (NaOCl) is a solution containing active chlorine gas (Cl_2) by 12.5%–25% (Emmanuel et al. 2004). Dilute solution has very stable hypochlorite ions and is a strong chemical oxidant, and free chlorine does not occur in nature. NaOCl is an agent, utilized as a multipurpose disinfectant for more than a 100 years, which rapidly responds to various microorganisms. As an antiseptic, sodium hypochlorite has a wide spectrum of effects covering bacteria, mycobacteria, spores, viruses, algae and even protozoans (Hidalgo et al. 2002). Due to its high features of biocide, NaOCl can be successfully applied in the household, industrial, medical and scientific exercises as well as disinfection of water (Emmanuel et al. 2004). Though sodium hypochlorite is a widely used substance for disinfection, it may cause the emergence of toxic, mutagenic/carcinogenic products for human and aquatic organisms (Monarca et al. 2000). Epidemiological studies have demonstrated that consumption of drinking water containing chlorine increases the risk of gastrointestinal and urinary cancers (Monarca et al. 2004). When sodium hypochlorite is added to water or waste water, the solution easily reacts on biological materials containing nucleotide bases and proteins, causing the generation of many volatile and non-volatile disinfection by-products (DBP) which are usually lipophilic, stable and toxic for the aquatic environment (Crebelli et al. 2005; Emmanuel et al. 2004). These organic halogenated compounds have subsequently been identified, such as haloalkenes, haloacetic acids, haloacetonitriles, haloketones and haloaldehydes (Monarca

et al. 2004). These components, known to be mutagenic and carcinogenic, originate from the interaction of NaOCl with organic materials such as humic acid and fulvic acid that exist in surface water (Crebelli et al. 2005; Guzzella et al. 2004). GC/MS (gas chromatography/mass spectrometry) analyses conducted after chlorination revealed that the DBP generation is at very high levels in NaOCl-disinfected water. It has been observed that water disinfected by chlorine-containing disinfectants performs genotoxic activity in plants, mussels and fish (Monarca et al. 2004).

Since environmental pollution is at issue by way of disinfection, it is needed to check disinfected water in terms of toxic and genotoxic effects (Hutchinson et al. 1998). Certain difficulties may occur while conducting chemical analyses, long-term carcinogenicity tests and epidemiological studies. Compared to the others, short-term mutagenicity tests are faster and cheaper tests that can predict carcinogenic activity. In recent years, researchers have started to use the micronucleus (MN) test as an alternative way to traditional chromosomal aberration analysis. The micronucleus test has been successfully applied in fish, salamanders and molluscs, as an index of exposure to genotoxic pollutants of drinking water (Monarca et al. 2004). Short-term mutagenicity tests, mainly the AMES test, are widely adopted in chlorine-containing drinking water (Monarca et al. 2004). However, it is particularly important to carry out this examination with tests in vivo (Hutchinson et al. 1998). Especially micronuclei tests are quite sensitive indicating the genotoxity and quality of water at studies in situ. The measurement of micronuclei frequency in the tests in vivo made in aquatic animals and plants for this aim is extensively used as an indicator of the clastogenic effects of water (Monarca et al. 2004).

The present study was conducted to determine the acute LC_{50} value of NaOCl and whether NaOCl can elicit a genotoxic effect at the chromosomal level as evidenced by the MN formation in peripheral erythrocytes of *Orthrias angorae*.

Materials and Methods

The fish species selected in this study, *O. angorae*, popularly known as Angora loach (Froese and Pauly 2008), belongs to the family *Balitoridae*. The fish were caught by electrofishing from the Kura-Aras river basin (Lat 38°30′E, long 44°84′N) in the Bolukbasi region in eastern Turkey. A total of 60 fish were acclimatized for a week in a 500-L tank with well-aerated water at 18–20°C. They were fed every 2 days with appropriate pelleted food which was withdrawn 24 h before the experiments. The fish were divided into five test groups. Each group of fish was transferred to a 30-L aquarium, and 1 L of water was added for each gram of fish. The temperature ranged from 18 to 20°C during experimentation. The pH of the water ranged from 7.2 to 7.3, which was slightly higher than natural. Dissolved oxygen was 8–9 mg/L. Total hardness ($CaCO_3$) of the test water was 150–170 mg/L.

Each set of 10 fish specimens was exposed to a different concentration of NaOCl (0.25, 0.37, 0.50, 0.75 and 1 mg/L) for 96 h. No fish was used more than once for a given experiment (Pandey et al. 2005). The negative control group was kept in non-chlorinated tap water at the same conditions as the test groups (Cavas and Ergene Gozukara 2003; Finney 1971; Pandey et al. 2005). Exposure time was 24, 48, 72, and 96 h, and mortality was recorded from the start of the experiment. The experiment was replicated twice under the same conditions. Mean mortality from a particular dose and its replicate was calculated. LC_{50} values were calculated from the data obtained in acute toxicity bioassays, by Finney's method of "probit analysis" (Finney 1971).

A total of 25 fish were used to determine MN frequency. NaOCl was added at three concentrations (0.25, 0.37 and 0.50 mg/L) to 15-L aquaria containing five specimens each. The negative control group was housed in a 15-L aquarium of tap water, and blood samples were obtained after 24, 36, 48 and 96 h and the 6th day. Benzene, at a concentration of 10 mg/L was used as a positive control (Cavas and Ergene Gozukara 2003). Blood samples for the smears were collected from a caudal vessel 24, 36, 48 and 96 h and 6 days after beginning treatment with NaOCl (Gustavino et al. 2005). Five fine blood smears were prepared for each fish. They were fixed with the pure ethanol for 20 min on the slides. The prepared slides were left to be dried in the open air and then stained for 15 min with the Giemsa solution diluted (1:10) in the phosphate buffer (Bahari et al. 1994; Cavas and Ergene Gozukara 2003; Pandey et al. 2005). The experiment was replicated under the same conditions.

Slides were examined under a light microscope with immersion oil. A total of one thousand erythrocytes were analyzed from each fish. The frequency of micronucleated cells per 1,000 erythrocytes was determined for each fish. The number of MN was expressed as per thousand of erythrocytes (Arkhipchuk and Garanko 2005).

Particles, being separate from the main nucleus and having a rounded structure in erythrocytes with a regular nucleus in the preparation are evaluated as micronuclei. MN could provide this differentiation and the nucleus was controlled by microscrew so as not to confuse dye particles and MN.

The statistical significance of the differences in mean values between the treatment and control groups was determined with the ANOVA (Sanchez-Galan et al. 2001).

Results and Discussion

The 96 h LC_{50} mean value of NaOCl (95% confidence limit) for *O. angorae* was calculated to be 0.5509 mg/L. The mortality rate of the control group was zero (Table 1). The results showed that NaOCl is highly toxic to *O. angorae* specimens. Figure 1 shows the relationship between the concentration of the NaOCl and the percentage of fish mortality by probit analysis methods (Finney 1971). It is clear from Table 1 and Fig. 1 that as the concentration of NaOCl increases, fish mortality proportionally increases. The erythrocytes of *O. angorae* were generally observed as round with a centrally located nucleus and considerable cytoplasm around it (Fig. 2)

The results of the micronucleus test are summarized in Table 2.

Statistical analysis demonstrated a significant increase in micronuclei after the induction of 0.5 mg/L NaOCl concentration after 36 h. The same increase has been reported for 0.37 and 0.5 mg/L NaOCl concentrations after 72 h. Even though the MN frequency of 0.37 mg/L was similar after 36 and 72 h, only 72 h micronuclei frequency was statistically significant. The 72 h MN frequency of the negative control group was smaller than 36 h MN frequency of the negative control group. This discrepancy has led to 72 h MN frequency being statistically significant. MN frequency of 0.25 mg/L NaOCl concentration was insignificant when compared to all negative test groups. The benzene treatment also caused a significant increase ($p < 0.01$) in the frequency of micronucleated erythrocytes. The result concludes that 0.25 mg/L NaOCl concentration is not mutagenic for *O. angorae.*

Table 1 Acute 96-h toxicity of NaOCl in *O. angorae* specimens

Point	Concentrations (mg/L)	95% confidence limits	G	Chi-squared
LC 10	0.3687	0.4337–0.3134	0.0875	7.922
LC 20	0.4233	0.4825–0.3713		
LC 30	0.4680	0.5234–0.4184		
LC 40	0.5093	0.5628–0.4609		
LC 50	0.5509	0.6049–0.5017		
LC 60	0.5958	0.6537–0.5430		
LC 70	0.6485	0.7154–0.5878		
LC 80	0.7169	0.8018–0.5878		
LC 90	0.8230	0.9458–0.7161		

Control group (theoretical spontaneous response rate) = 0.0000

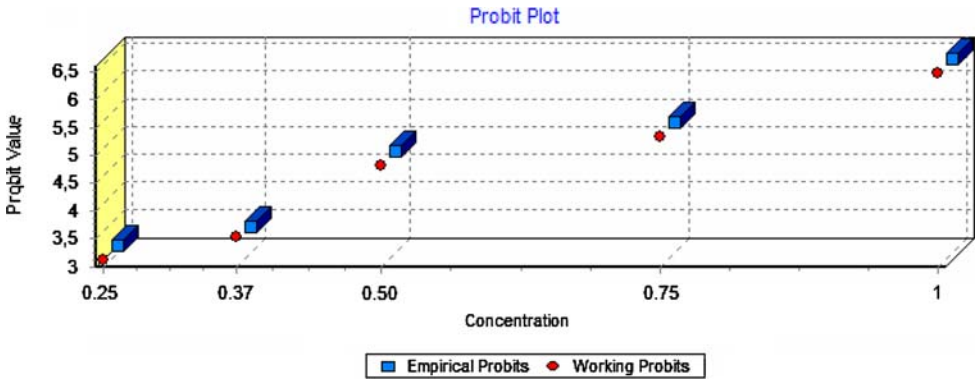

Fig. 1 Plot of adjusted and predicted regression line of NaOCl in *O. angorae*. The erythrocytes of *O. angorae* were generally observed as round with a centrally located nucleus and considerable cytoplasm around it (Fig. 2).

Table 2 MN frequency in erythrocytes of *O. angorae* exposed to different concentrations of NaOCl

Treatment	MN frequency (mean ± SD)		
	36 h	72 h	Day 6
Negative control	1 ± 1.225^{ns}	0.6 ± 0.548^{ns}	1.6 ± 0.894^{ns}
Positive control (10 mg/L benzene)	10.2 ± 0.837*	9.4 ± 1.140*	9.6 ± 0.548*
0.25 mg/L	1.6 ± 0.548^{ns}	1.4 ± 0.548^{ns}	1.8 ± 0.447^{ns}
0.37 mg/L	2.2 ± 0.447^{ns}	2.2 ± 0.447*	1.8 ± 0.447^{ns}
0.50 mg/L	2.6 ± 0.548*	2.4 ± 0.894*	2.2 ± 1.095^{ns}

ns: non significant ($p > 0.05$); * $p < 0.001$

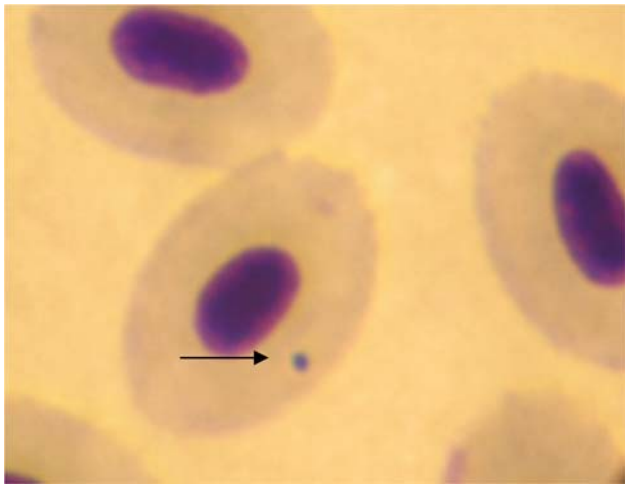

Fig. 2 Erythrocytes of *O. angorae*. Arrow: A micronucleated erythrocyte. (magnification 1,000×)

Benzene, a well-known genotoxic agent, was used as the positive control in our experiments. It caused a significant increase in the MN frequency. Benzene-induced MN formation in fish erythrocytes has been previously reported by Al Sabti (Al Sabti and Metcalfe 1995; Cavas and Ergene Gozukara 2003; Pandey et al. 2005). Contamination of drinking water and insufficient hygiene in developing countries results in diarrhoea in 4 billion people and the deaths of 2.2 million people, the majority of which are children below 5 years old, every year (Clasen and Edmondson 2006).

Contaminated water causes epidemic diseases like typhoid fever, hepatitis A, polio and cholera besides diarrhoea. To prevent these kinds of water-based disease, chlorinating water has been efficiently used as a measure for almost 100 years. Studies show that drinking chlorinated water affects the proliferation and growth system very well besides resulting in urinary and gastric cancer (Guzzella et al. 2004; Ohe et al. 2004).

In this study, the LC_{50} value of NaOCl in *O. angorae* was found to be 0.5509 mg/L. This value showed that NaOCl, even at quite low doses, results in defects in cell division at chromosomal aberration level (Fig. 2).

Orthrias angorae is a species (Froese and Pauly 2008) which is nourished with sediments and found rarely compared with other *Balitoridae* family members. *Orthrias angorae* has been used for the first time to determine the acute toxicity of NaOCl in this study.

For the determination process of the LC_{50} value, a control group has been established and death rates for 24, 48, 72 and 96 h have been recorded. For other studies, a time period of 96 h has also been included. Studies on toxic values of NaOCl are very rare. Göksu et al. (2002) have determined the 24-h acute LC_{50} value of NaOCl in *Pincdata radiata* as 1.75 mg/L, which is much higher than our value. The reason for this discrepancy might be the oysters' ability to develop a defence system against this kind of chemical and the shortness of the time used.

Another difficulty faced while determining the LC_{50} value is the decrease in the amount of chemical and the application time. In future studies, controlled application of this kind of substance, which has the volatilisation feature, should be included. In this study, while establishing the correlation between the substance quantity and death rate, no relation between time and death rate was observed. The reason for this might be due to the volatilisation of this kind of chemical due to ventilation and losing its effect.

In this study, benzene (Al Sabti 1986) has been used as a positive control. The highest dose of NaOCl has resulted in a lower MN frequency than benzene on the 6th day. MN frequencies depending on the dose induced by NaOCl and time are shown in Table 2. While there is a direct correlation between dose increase and MN frequency, no such relation could be found between time and MN frequency. In benzene application, the same event has been observed and more MN has been reported at the 36th h compared to the 6th day.

Gustavino et al. (2005), showed the mutagenic activity of NaOCl in *Cyprinus carpio* with the Comet test and MN test.

Guzzella et al. (2004) determined the in vitro mutagenic effect of three disinfectants including NaOCl with the salmonella mutagenity test, microtox and mutatox methods. Bolognesi et al. (2004) determined that NaOCl increases the MN frequency in *Dreisenna polymorfa*.

On the contrary to the above-mentioned studies, Crebelli et al. (2005) stated that NaOCl does not show mutagenic activity in *Allium cepa* with the anaphase aberration test and in *Tredescentia* with the micronucleus test.

Finally, the results of our study can be summarized as follows:

1. In order to determine the acute toxic effect of NaOCl, the 96 h LC_{50} mean value of NaOCl for *O. angorae* was calculated to be 0.5509 mg/L.
2. There is an increase in micronucleus formation together with the material dosage increase. As the dosage of NaOCl increases abnormality formation within the cells, it may be concluded that this material may have a genotoxic effect.

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