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Monitoring DNA Damage in Gills of Freshwater Mussels (*Anodonta anatina*) Exposed to Heavy Metals

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ABSTRACT

Study was carried out to investigate the genotoxic effect of different levels of heavy metals on gill cells of freshwater mussels (*Anodonta anatina*), a sentinel species in aquatic environment. Freshwater mussels were exposed to none (0µg L^{-1}), low (120 µg L^{-1}), medium (240 µg L^{-1}) and high (360 µg L^{-1}) levels of lead (Pb), chromium (Cr) and copper (Cu) alone and in combinations (Pb + Cr + Cu) for 15 days under laboratory conditions. Gill cells of mussels were used to determine the DNA damage by comet assay. The tail DNA (%), comet tail length and olive tail moment (OTM) were the parameters selected to detect DNA damage. Low doses (120 µg L^{-1}) of each metal induced significantly higher levels of DNA strands breaks as compared to medium dose (240 µg L^{-1}) and very low levels of DNA damaged was observed at high dose (360 µg L^{-1}). Cu and Pb showed significantly higher value of % of tail DNA (56.74±1.81, 47.36±1.23) and comet tail length (41.30±0.758, 49.15±1.90), respectively, as compared to Cr and combined metal exposure (Pb + Cu + Cr). The lowest levels of DNA damage for all the parameters were observed in combined metal treatment. Genotoxic effect of metals on freshwater mussels is very important to assess the aquatic health and could be suggested as biomarker. It is concluded that the Cu and Pb induced more DNA damage as compared to Cr and combined metal exposure (Pb + Cu + Cr). Moreover, our results showed that the low dose treatment of metals have more genotoxic effect as compared to the medium and high doses.

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Authors' Contributions
MS conceived and designed the study and wrote the article. MS performed the experimental work. NAQ and ASC statistically analyzed the data. MNK supervised the work.

Key words
DNA damage, Freshwater mussels, Heavy metals, Comet assay.

INTRODUCTION

Freshwater ecosystems such as lakes and rivers are very essential resources because they contribute in biodiversity, regulation of climate, managing floods and to meet the demands of drinking water (Ra *et al*., 2011; Hansen, 2012). The biodiversity of freshwater ponds are mainly depends on the variety of free floating organisms such as phytoplankton and zooplankton (Sohail *et al*., 2014). Unfortunately, freshwater system are continually polluted due to increasing anthropogenic activities such as urbanization, industrialization, agricultural expansion, and manipulation of mineral resources which cause a severe damage especially in less develop countries (Thevenon *et al*., 2013; Zan *et al*., 2012). Among the other harms, the risks of toxic metals in freshwater bodies cause adverse effect to the health of ecosystem (UNEP, 2011). The exposure to these metals can also induce harmful effects in organisms such as interruption in homeostasis and destruction in their DNA (Tsangaris *et al*., 2010).

Few of the metals such as copper and zinc are required for metabolic processal though could be lethal at higher concentrations. Some metals prove dangerous even at very minute concentrations (*e.g*., mercury, cadmium, and lead) and are suggested as harmful metals for aquatic life because of their perseverance as describe by Turkmen *et al*. (2008). Lead is one of the more common and persistent environmental pollutants. Lead has no valuable function for the living organism and is said to be non-essential metal (Johannesson, 2002). It can also accumulate in the tissues and it interferes with the other bio elements such as Ca, Zn and Cu which cause a variety of serious disorders (Berrahal *et al*., 2011).

Many invertebrates found in aquatic ecosystem are sensitive to contaminants and could accumulate heavy metals form their surrounding water (Reinecke *et al*., 2003). Soegianto *et al*. (1999) reported that the concentration of copper in unpolluted water is less than 5 ppb and it may reach almost about 3 ppm in highly polluted environment (Parry and Pipe, 2004). The constantly increasing

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concentration of copper in aquatic environment is a serious danger to the aquatic organisms. Though, copper is vital for the proper functioning of living organisms. For example, it may be a cofactor for the enzyme action, but high concentration may prove toxic to an organism when exposed chronically to an aquatic environment (Gaetke and Chow, 2003).

The health of aquatic ecosystem has been monitored through the chemical analysis of pollutant level in soft tissues of bivalves (Goldberg, 1986; Cantillo, 1998). As being sessile, mussels are ecologically very important as they can bioaccumulate a broad range of varying type of contaminants from aquatic ecosystems (Kimbrough *et al*., 2008). Freshwater mussels are good candidates for evaluating the aquatic health as their soft tissues are continuously exposing to ambient water and due to their filter feeding habit they can assess the toxicity even when the level of pollutants are changing slightly (Blackmore and Wang, 2003; Viarengo and Canesi, 1991). Bivalves have huge survival rate, are easy to keep in the laboratory conditions, and are able to bear greater concentrations of variety of pollutants (Sarkar *et al*., 2008; Zhou *et al*., 2008).

Freshwater mussels are usually manipulated for determining genotoxicity (Binelli *et al*., 2010; Rocher *et al*., 2006; Parolini *et al*., 2011). The soft tissues such as in muscular foot, mantle, digestive tract and gills are generally used as target organs to determine the aquatic pollution. Due to continuous exposure to water, gill tissue of fresh water mussels is suggested to study pollutants in water (Makala and Oikari, 1990; Stambuk *et al*., 2008). Many studies have shown that the gill cells are more vulnerable than the haemocytes for the genotoxic related contaminants (Bourgeault *et al*., 2010; Vincent- Hubert *et al*., 2011).

It is essential to locate the early warning response at molecular level to protect the aquatic ecosystem from sever damages (Kalpaxis *et al*., 2004). DNA strand breakage is considered to be one of the primary signals of environmental deterioration (Binelli *et al*., 2007; Klobucar *et al*., 2008) and this technique has become well known to evaluate the genotoxic effect of contamination on organismic and population level (Depledge, 1998). Comet assay is a quick method to assess the genotoxicity in aquatic organism due to environmental pollutants (Jha, 2008). This is said to be one of the commonly used methods for contaminant biomonitoring in aquatic animals related to genotoxicity (Chen *et al*., 2007; Picado *et al*., 2007).

The aim of this study was to evaluate the effect of trace metals on gills of freshwater mussels with reference to the DNA damage. Secondly, the effect of different doses of various metals on DNA damaging was also investigated in this study.

MATERIALS AND METHODS

Animal collection and placement

Freshwater mussels (*Anodonta anatina*) of average size (64.5±2 g) were collected from an unpolluted freshwater pond. The temperature of pond water at the time of collection was approximately 20.5±1.5°C. Mussels were directly carried to the fish hatchery in cool plastic bags and placed in large rectangular cemented tanks with filtered pond water. Animals were fed with the green algae collected from the same fish pond and acclimatized for 10 days before used for experimentation. Mussels were kept at 16.8±1.2°C and water was changed daily during acclimatized period.

Experimental design

Five freshwater mussels were exposed to none T_0 (0µg L^{-1}), low T_1 (120µg L^{-1}), medium T_2 (240µg L^{-1}) and high T_3 (360 µg L^{-1}) doses of lead, copper and chromium in glass tanks (24"x18"x24") in triplicate design (3 tanks as per treatment) for 15 days. Primary stock solutions of Pb, Cu and Cr were prepared in distilled water by using Pb (NO_3), Cr (NO_3). $9H_2O$ and $CuSO_4$. $5H_2O$, respectively, and further diluted to achieve the required concentration for exposure. The water was changed after every 5 days with the renewal of each chemical. The temperature and pH of aquarium water was 17.6±1.3°C and 7.15±0.15, respectively. The mussels were sacrificed at the end of experiment and gills were collected for estimation of DNA damage.

Isolation of gill cells

Mussels shells were washed with distilled water to remove the unwanted particles before those were opened with the help of a scalpel and small steel rod. The gills were then excised carefully and rinsed in cold phosphate buffer solution (PBS) under low light to eliminate the risk of UV-induced DNA damage. The isolation of gill cells were carried out by a mechanical process by mincing the chopped gills in a cold PBS with the help of sterilised sharp scalpel blades for 15 min. Then suspension was passed through a 40µm sieve to remove the large particles and the filtrate was diluted in 1ml PBS. The cell viability in PBS was assessed by using the trypan blue exclusion test and it was >85% in all the cases.

Comet assay

The protocol of comet assay was used according to Singh *et al*. (1988). Ethidium bromide (2 µg/ml) was used for staining the slide and then examined with fluorescence microscope at magnification power of x400. The scoring of microscopic images was carried out by using computer software Comet IV. Three slides were prepared from each

sample and the images of 20 cells were scored from each slide. Olive tail moment (product of tail length and the fraction of total DNA in the tail), comet tail length and amount of DNA in comet tail were the parameters assessed for DNA damaging.

Statistical analysis

Analysis of variance (ANOVA) was applied on data to analyse the variation among comet parameters for different doses of heavy metals. Tukey pairwise comparison test was used to assess the difference between control and treatment groups and statistical significance was defined at $p<0.05$. Minitab 17 software and Microsoft excel were used for all the statistical analysis.

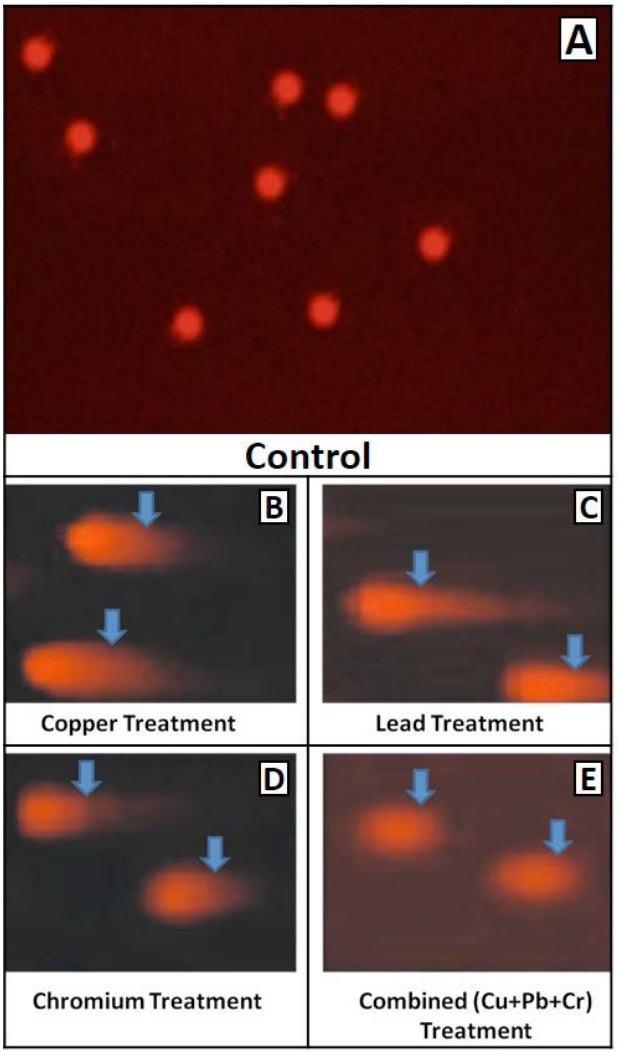

Fig. 1. The image of comets by fluorescent microscope from the gills of freshwater mussels (*Anodonta anatina*) collected from the control (A) and treated tanks (B to E).

RESULTS

There were significant differences between treatment groups at all concentration as compared to controls except the combined treatment at low and medium dose and Cr alone at higher dose (Table I). The control levels were the same for all metals so had same value as mentioned in Table I. The value of tail DNA percentage, tail length and olive tail moment decreased from lower to higher doses of metals but still the values at high doses were significantly higher than the controls except the chromium and combined treatments which returned to the control level. The comet image of gill cells of freshwater mussels collected from the control tank showed no damages in DNA (Fig. 1A) whereas damage was observed in all treatment groups of various heavy metals (Fig. 1B-E).

For % of tail DNA highest values were observed in Cu-exposed mussels (56.74±1.81) as compared to Pb (47.36±1.23), Cr (43.0±1.31) and combined treatment (25.94±1.16) at low dose (120 µg L^{-1}).The least value of tail DNA was noticed at high dose (360 µg L^{-1}) such as for Cu (20.89±0.55), Pb (10.88+0.73), Cr (7.94±0.71) and combined (8.16±0.98) treatments. There were no significant differences between the Pb and Cr at low concentration (120 µg L^{-1}) but both were significantly different from Cu and combined treatment (Pb+Cu+Cr) at the same concentration. The values in combined metal exposure at medium concentration (240 µg L^{-1}) was significantly lower than all the other treatments at the same concentration ($p<0.05$) (Table I).

In case of comet tail length, significantly higher values were observed in Pb-exposed mussels (49.15±1.90) followed by Cu (41.30±0.75) Cr (26.6±0.78) and combined treatment (18.20±1.13) at low dose. Trend for the same parameter in all metals at low dose was Pb > Cu > Cr > Pb + Cu + Cr (Table I). As usual the value of comet tail length was also decreasing with increasing the concentration of metals. The lowest values of tail length were found at higher dose (360 µg L^{-1}) of all metals exposure such as Cu (16.05±0.73), Pb (9.00±0.67), Cr (8.30±0.54) and combined-metal exposure (7.20±0.53) (Table I).

The value of olive tail moment (OTM) also varied among the different doses of metals. Cu and Pb exposure showed significantly higher value of OTM (13.48±0.44, 12.91±0.45, respectively) at the low doses ($p<0.05$) and again the Cr and combined treatment showed low value (6.94±0.38, 3.89±0.20, respectively) at the same dose. The value of OTM was significantly higher (7.75±0.16) in Cu exposure at the medium dose as compared to other metals exposure at the same dose. There were no significant differences found between Pb and Cr at medium dose and Cr and combined metal treatment at the high dose ($p>0.05$)

(Table I). Cr induced low DNA damage than Cu and Pb but higher than the combined metal treatment at all the doses for all the parameters (Table I).

Table I.- Effect of different doses of Pb, Cu, Cr individually and in combination on mean % tail DNA, comet tail length, olive tail moment.

Dose metals	T_0	T_1	T_2	T_3
Tail DNA (%)				
Pb	1.34± 0.28^{g}	47.36± 1.23^{b}	26.89± 0.97^{d}	10.88+ 0.73^{f}
Cu	1.34± 0.28^{g}	56.74± 1.81^{a}	30.03± 0.42^{cd}	20.89± 0.55^{e}
Cr	1.34± 0.28^{g}	43.05± 1.31^{b}	33.86± 1.71^{c}	7.94± 0.71^{f}
Pb+Cu+Cr	1.34± 0.28^{g}	25.94± 1.16^{d}	19.60± 1.27^{e}	8.16± 0.98^{f}
Comet Tail length				
Pb	3.45± 0.23^{g}	49.15± 1.90^{a}	21.95± 0.89^{d}	9.00± 0.67^{f}
Cu	3.45± 0.23^{g}	41.30± 0.75^{b}	18.05± 0.58^{de}	16.05± 0.73^{e}
Cr	3.45± 0.23^{g}	26.65± 0.78^{c}	17.40± 1.16^{e}	8.30± 0.54^{f}
Pb+Cu+Cr	3.45± 0.23^{g}	18.20± 1.13^{de}	8.75± 0.57^{f}	7.20± 0.53^{fg}
Oil Tail moment				
Pb	0.17± 0.03^{h}	12.91^{a}± 0.45	3.87± 0.18^{cd}	1.63^{fg}± 0.09
Cu	0.17± 0.03^{h}	13.48^{a}± 0.44	7.75± 0.16^{b}	3.38^{de}± 0.10
Cr	0.17± 0.03^{h}	6.94^{b}± 0.38	4.85± 0.41^{c}	1.30^{gh}± 0.11
Pb+Cu+Cr	0.17± 0.03^{h}	3.89^{cd}± 0.20	2.49± 0.18^{ef}	1.12^{gh}± 0.13

*Any means (±S.E.M) in row and column with different superscripts are significantly different ($p<0.05$).
T_0, none (0µg L^{-1}); T_1, low (120µg L^{-1}); T_2, medium (240µg L^{-1}) and T_3, high (360 µg L^{-1}).

DISCUSSION

Cu and Pb both induced high DNA damage in gill cells as compared to Cr and combined exposure of metals. Pb showed slightly higher value for the tail length than Cu at low dose as difference in means was 7.85±1.92 (n=20) but in case of % of tail DNA Cu shows more value than Pb and mean difference was 9.38±2.01 (n=20) at the same dose. For the OTM at the same dose the little difference was observed in between Cu and Pb but again more values were observed in Cu-exposed mussels and difference in means was 0.92±0.65 (n=20). The combined metals exposure induced very low damaging in DNA as showed in all observed parameters. Vincent-Hubert *et al*. (2011) compared the haemocytes and gills of mussels for comet assay study and concluded that the gills have more sensitivity for the contaminants as compared to the haemocytes.

Our results resemble to previous study where the mussels were exposed to the Nano and ionic forms of copper and significant difference was observed in copper exposed mussel as compared to controls and it was also stated that the higher damage was observed in mussel exposed to ionic form of Cu (Gomes *et al*., 2013).

Bolognesi *et al*. (1999) also showed similar results where the mussels were exposed to different concentrations of Cu and high levels of DNA damage were observed at low concentration of Cu (40 µg L^{-1}). Al-Subiai *et al*. (2011) reported the effect of Cu on marine mussels and suggested that the high dose of Cu was toxic to animal and 100% mortality was observed at 100 µg L^{-1} which is contradictory to our results where no mortality was observed in freshwater mussels at any dose of copper. Our finding are in line with a previous study where mussels were exposed to low and high doses of polycyclic aromatic hydrocarbon and higher levels of DNA strands breaks were observed in mussels which were exposed to the low doses of polycyclic aromatic hydrocarbon (Large *et al*., 2002). Freshwater mussels were exposed to various doses of Pb to evaluate the genotoxic effects and significantly high DNA strands breaks was observed at low dose 50 µg L^{-1} of lead and very little DNA damage was found at high dose of 500 µg L^{-1} (Black *et al*., 1996).

Cr and combined treatments (Pb + Cu + Cr) showed the sametrends; the low level of DNA breaks was observed as the doses were increased. The combined exposure of metals showed very low value in DNA damage than the metals individually which showed some synchronized effects of metals. It is unclear why the combined treatment of heavy metal induced low level of DNA damage but one of the previous study also showed the similar results where the mussels were exposed to the a combination of Cu, Cd and Hg and low level of DNA damage were observed in mussels that were exposed to the combination of metals, studied by Bolognesi *et al*. (1999). Heavy metals have been shown to induce changes in the metabolism of organism and enhanced the reactive oxygen species which generated oxidative stress and induced DNA damage (Gaetke and Chow, 2003). It is previously mentioned that the metals and nanoparticles have direct effect on DNA in

nucleus and occasionally during cell division which can cause DNA damage (Bhatt and Tripathi, 2011; Singh *et al*., 2009; Karlsson, 2010).

Many previous studies had been conducted on freshwater and marine mussels to assess the genotoxic effect of heavy metals and found deleterious effects. Cr is the major industrial discharge that put adverse effects on animal tissues (Wahlberg and Skog, 1965). The large concentration of Cr was observed in aquatic environment and it can greatly accumulate in tissues of aquatic animals which may cause defects at molecular level (Fatima and Usmani, 2013; Taweel *et al*., 2011). The sufficient amount of lead also accumulated in aquatic animals even though at minute levels in aquatic environment (Vinodhini and Narayan, 2008; Abdel-Baki *et al*., 2011). Pb is noxious even in very small concentration because Pb can mimic other essential elements such as calcium, magnesium and zinc which inflict deleterious effects on enzyme activity (Jennette, 1981) and also prove carcinogenic (Fracasso *et al*., 2002). The genotoxic effects of Pb on freshwater mussels were also reported by Black *et al*. (1996). Emmanouil *et al*. (2007) reported that the Cr has more adverse effects and damaged DNA strands at the tissue concentration of ≥2.70 µg/g wet weight under laboratory conditions.

Cr has been significantly correlated with the DNA strand breaks in the mussels collected from the wild (Rank *et al*., 2005). Many previous studies reported the significantly higher levels of DNA damage in aquatic organism collected from contaminated water with metals (Frenzilli *et al*., 2001; Nacci *et al*., 2002; Steinert *et al*., 1998). This is in accordance with our findings where the DNA damage detected with the Comet assay seem to be correlated with the comparative heavy metal concentrations in the surrounding environment. Al-Subiai *et al*. (2011) reported the genotoxic effects of Cu on the DNA strand breaks of bivalve mussels.

Induction of DNA damaging has also been reported in numerous other studies where animals were exposed to different heavy metals such as mercury, cadmium, Cr, Cu and Pb (Hartmann and Speit, 1994; Hayashi *et al*., 2000). Several previous studies also reported that the 50% or higher level of DNA strand breaks in cells of aquatic animals including mussels were linked to the different chemicals exposed in laboratory or in polluted environment (Frenzilli *et al*., 2001, 2004; Regoli *et al*., 2005; Machella *et al*., 2006; Gorbi *et al*., 2008). Heavy metals are harmful for aquatic life and in present study the specific effect of metals at the specific concentrations was evaluated on sub-cellular level of bivalves.

CONCLUSION

It is concluded that the Cu and Pb induced higher levels of DNA damage compared to Cr and combined metal exposure. Furthermore our findings showed that the low dose (120µg L^{-1}) of metal concentrations had more genotoxic effect compared to the medium (240 µg L^{-1}) and high (360 µg L^{-1}) doses. Gills are suggested to be the best target organ to assess the genotoxic effects and the freshwater mussels (*Anodonta anatina*) are considered as one of the key species for bio-monitoring studies.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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