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Heavy Metals Resistant Rotifers from a Chromium Contaminated Wastewater can Help in Environmental Clean-up

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Abstract.- Rotifers, isolated from an industrial wastewater of tanneries showed tolerance against Cd^{2+} (0.37 mg ml^{-1}), Cr^{6+} (2.6 mg ml^{-1}), Pb^{2+} (5.5 mg ml^{-1}) and Cu^{2+} (2.2 mg ml^{-1}). Chromium, copper and cadmium processing capability of rotifers was assessed to examine the suitability of rotifers as bioremediator of industrial wastewater. *Philodina roseola* decreased 40% Cr^{6+}, 67% Cu^{2+} and 76% Cd^{2+} from the medium each containing 10 µg ml^{-1} of the metal within two days of inoculation. At the end of 8 days of culturing, the decrease for Cr^{6+}, Cu^{2+}, and Cd^{2+} in the metal containing medium was 88%, 77%, and 89%, respectively. The decline in concentration of Cr^{6+}, Cu^{2+} and Cd^{2+} in metal containing media by *Notommata copeus* was 82%, 61% and 97%, respectively after two days of inoculation and 96%, 81% and 99% after 8 days. The resistance of rotifers against heavy metals and their uptake ability can be exploited for metal detoxification and environmental clean-up operations.

Keywords: Heavy metal resistance, bioremediation, *Philodina roseola*, *Notommata copeus.*

INTRODUCTION

Pollution by heavy metals affects the diversity of soil biota, their abundance and activity (Brookes, 1995; Mhatre and Pankhurst, 1997). When metals are discharged with effluent, they may result in severe contamination of downstream ecosystems (Nriagu and Pacyna, 1988). In an aquatic environment, metals occur both in the dissolved or soluble fraction and in particulate matter. Elevated levels of metal ions are generally toxic and cause major damage to cells (Inouhe *et al*., 1996).

The environmental chemistry of chromium has been widely studied (Richard and Bourg, 1991; Kotas and Stasicka, 2000). The nature and behaviour of Cr in wastewater depends on the physicochemical conditions of the effluents originating from various industrial sources (Kotas and Stasicka, 2000). The toxicity, mobility and bioavailability of Cr depend fundamentally on its chemical form. Chromium in the environment might be present mainly as Cr^{3+} and Cr^{6+}. Cr^{6+} is highly

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soluble and about 300 times more toxic than Cr^{3+}. On the other hand, Cr^{3+} precipitates at the average pH of natural waters. Chromium is found in wastewater released by industry such as chromium plating, metal cleaning and processing, wood preservation, alloy making, tanning of hides and occurs naturally at high concentration in ultramafic rocks. Tannery wastewaters contain mainly Cr^{3+} (Rutland, 1991; Ellis *et al*., 2002), though other heavy metals are also present.

Cadmium (Cd) occurs in nature primarily in association with lead and zinc ores. It is extensively used in the industry for a number of applications including electroplating, protection against corrosion and stabilizing plastic. The wastewater of these industries may contain cadmium ranging from 10mM to 100 mM (Shuttleworth and Unz, 1988). Cd is of particular concern because it is a strong mutagen and carcinogen, being very slowly excreted from the body, with a half-life of about 20 years (Giaginis *et al*., 2006; Martelli *et al*., 2006). Cd causes an indirect oxidative stress which might be related to its mutagenic and carcinogenic actions (Adamis *et al*., 2003).

Copper is rarely found in natural water, but is found in man polluted environments. Any copper present normally originates from industrial effluents, seepage, water from refuse dumps, pesticides or corrosive water that has come into contact with fitting and pipes containing copper (Udom *et al*., 2004; Andrews and Sutherland, 2004). Trace amounts of copper are essential for life, but it also catalyzes the synthesis of reactive oxygen species, leading to severe damage to cytoplasmic constituents through oxidation of proteins, cleavage of DNA and RNA, and lipid peroxidation (Garcia *et al*., 2002).

Lead is a non-essential metal with no biological role in microorganisms, animals and plants (Bruins *et al*., 2000). It contaminates surface water mainly from anthropogenic sources (96%), particularly from combustion of leaded fuels, pyrometallurgical non-ferrous metal production and coal combustion (Monterroso *et al*., 2003; Andrews and Sutherland, 2004). The most serious effects of lead are related to impacts on central nervous system (Goyer, 1993).

Rotifers are the smallest of the metazoan, hardly observable with the naked eye and are abundant in aquatic environments. They are important in aquatic food chains, zooplanktonic profiles, biogeochemical cycles, animal diversity and adaptations to environmental stress (Meglitsch, 1967). Rotifers are useful as models in ecotoxicology because they often play a key role in the dynamics of fresh water and coastal marine ecosystems (Wallace and Snell, 1991).

The toxicity of heavy metals has been assessed using toxicity tests with rotifers. As early as the 1970's, Schaefer and Pipes (1973) used short term mortality tests with *Philodina acuticornis* and *Philodina roseola*, respectively, to evaluate the toxicity of heavy metals. Snell and his coworkers have provided body of information on the tolerance of rotifers to heavy metals and anthropogenic stresses, besides using them for rapid toxicity assessment (Snell and Persoone, 1989a, b; Snell and Janssen, 1997; Snell, 1998).

The aim of the present study was to evaluate the role of rotifers in wastewater treatment plants containing heavy metals such as Cd^{2+}, Pb^{2+} and Cr^{6+} in their effluent. This laboratory has already emphasized the role of various micro-organisms in wastewater treatment involving successive use of various categories of micro-organisms (Dar and Shakoori, 1999; Rehman and Shakoori, 2001; Shakoori and Muneer, 2002; Shakoori *et al*., 2004; Rehman *et al*., 2007, 2008).

MATERIALS AND METHODS

Collection of water samples

Wastewater samples were collected from the tanneries in the Kasur district, an area close to Lahore. Samples were collected in the sterilized screw-capped bottles and transferred to the laboratory where they were kept at room temperature. Physicochemical parameters of wastewater *viz*., temperature (ºC), pH, dissolved oxygen (mg l^{-1}), chromium (µg ml^{-1}), copper (µg ml^{-1}), cadmium (µg ml^{-1}), and lead (µg ml^{-1}) were measured (APHA, 1989). Samples were inoculated in Bold Basal medium (Nichols and Bold, 1965) containing some boiled wheat grains.

Culturing and identification of rotifers

For isolation of rotifers, 100 µl of the effluent was inoculated in liquid medium (100 ml) containing 6-8 soaked wheat grains. Increase in number of rotifers was noted after 3 days. Small drops of the culture were placed on parafilm strip, which was placed on glass slide and observed under a compound microscope for desired organisms. The rotifers, picked by this drop method, were used for further studies. Identification of the acoelomate organisms was done by observing their body shape, morphological features and behaviour (Meglitsch, 1967; Barnes, 1974; APHA, 1989).

Determination of optimum temperature and pH

For determination of optimum temperature for growth, 20 rotifers were inoculated in Bold Basal Medium containing some wheat grains in 3 sets, each of 3 flasks, and incubated at 20, 25 and 30 ºC for 8 days. Number of individuals was counted and a graph was plotted between temperature and the number of individuals. For determination of optimum pH, Bold Basal medium (100ml) in 3 sets, each of 3 flasks, with pH set at 6.0, 7.0, 8.0, was inoculated with 20 individuals in each flask for 8 days. The growth was estimated by counting.

Resistance to heavy metals

The rotifer culture was divided into four parts in separate flasks. Each of the cultures was treated with increasing concentration of the four metal ions *viz.*, Cu^{2+}, Cr^{6+}, Pb^{2+} and Cd^{2+} which were gradually added to the medium to check the resistance of rotifers against these heavy metals. The cultures were incubated at optimum pH and temperature. The number of rotifers was counted every day in all the cultures containing different metal ions for determination of variation in the number of individuals. Counting was done according to Haq *et al*. (1998). At least three counts were done to get a mean of every reading. A control was run containing rotifers but without addition of any metal ion.

Heavy metal processing

For determination of heavy metal processing efficiency of rotifers, rotifers were grown on Bold Basal medium with wheat grains in different flasks containing 10 µg ml^{-1} Cr^{6+}, 10µg ml^{-1} Cd^{2+}, and 10 µg ml^{-1} Cu^{2+}, at optimum pH and temperature. Control culture was also run for each metal containing the same concentration as in the treated one (10 µg ml^{-1}) but without the rotifers. The heavy metal processing capability of rotifers was checked by estimation of the amount of heavy metal ions in the medium after 2, 4, 6 and 8 days of culturing. For the estimation of heavy metals, the AA1275 atomic absorption spectrophotometer (Varian, USA) was used.

Statistical analysis

Observations were made and all the experiments run in triplicate. At least three separate flasks were maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

RESULTS

Physico-chemical characteristics of wastewater

Table I shows physico-chemical characteristics of wastewater collected from the ponds. The pond had chromium concentration of 2.1 µg ml^{-1} , cadmium 1.76 µg ml^{-1}, lead 0.2 µg ml^{-1}, and copper 0.7µg ml^{-1}.

Wastewater samples from tannery effluents were observed for the presence of various micro-organisms. The metazoan micro-organisms found in the samples were some rotifers *viz.*, *Philodina roseola, Notommata copeus, Platyias patulus, Testudinella truncate* and *Brachionus rubens* and a gastrotrich *Chaetonotus brevispinosus*. The appearance of various metals and pollutant resistant micro-organisms, in ponds constantly receiving toxic industrial effluents, showed a high capacity of the microorganisms to evolve in response to xenobiotic stress.

Table I.- Physicochemical parameters of wastewater collected from tannery effluents from Kasur district.

Parameters	
Temperature (ºC)	21.66±0.47*
pH	8.86±0.04
Dissolved Oxygen (mg l^{-1})	1.68±0.01
Chromium(µg ml^{-1})	2.10±0.08
Cadmium (µg ml^{-1})	1.76±0.09
Lead (µg ml^{-1})	0.20±0.04
Copper (µg ml^{-1})	0.70±0.16

*Means ± Standard deviation.

Growth curves

Philodina roseola and *Notommata copeus* were selected for study of optimum growth conditions, resistance to other heavy metals and their capability to process toxic metals. *Philodina roseola* showed optimum growth at 20 ºC and pH 8.0, whereas *Notommata copeus* showed optimum growth at 30 ºC and pH 8.0 (Figs. 1, 2).

Metal resistant rotifers

The chromium resistant rotifers also showed resistance against other heavy metals *viz.*, Cu^{2+}, Pb^{2+} and Cd^{2+} (Table II). *Philodina roseola* and *Notommata copeus* could tolerate 5.5 mg ml^{-1} of Pb^{2+}, 2.6 mg ml^{-1} of Cr^{6+}, 2.2 mg ml^{-1} of Cu^{2+} and 0.37 mg ml^{-1} of Cd^{2+}. At these doses the population of *Philodina roseola* was decreased 50% in Cr^{6+}, 57% in Cu^{2+}, 58% in Pb^{2+} and 60% in Cd^{2+}, whereas for *Notommata copeus* this decline was 46%, 58%, 50%, and 71%, respectively. The gradation of heavy metal toxicity would be Cd^{2+}>Cu^{2+} >Cr^{6+}>Pb^{2+} for both the rotifers.

Table II.- Survival and growth of *Philodina roseola* and *Notommata copeus* (indicated by Number ml^{-1}) in media containing different metal ions and without metal ions.

Medium containing heavy metals	*Philodina roseola* Control (n=3)	*Philodina roseola* Treated (n=3)	*Notommata copeus* Control (n=3)	*Notommata copeus* Treated (n=3)
Cu^{2+} (2.2 mg ml^{-1})[a]	700± 10.96^{b}	300± 12.00	600± 06.50	250 ± 03.60^{***}
Cr^{6+} (2.6 mg ml^{-1})	600± 10.12	300± 09.45^{***}	600± 03.60	325± 9.29^{***}
Pb^{2+} (5.5 mg ml^{-1})	600± 05.86	250± 11.35^{***}	750± 05.56	375± 5.01^{***}
Cd^{2+} (0.37 mg ml^{-1})	750± 05.29	300± 09.70^{***}	700± 04.72	200± 3.51^{***}

[a] For treatment with Cu^{2+}($CuSO_4.5H_2O$) the concentration of Cu^{2+} in the medium on the first day was 50µg ml^{-1} with an increase of 50µg ml^{-1} every day for 45 days. For treatment with Cr^{6+} ($K_2Cr_2O_7$) the concentration of Cr^{6+} in the medium on the first day was 50µg ml^{-1} with the increase of 50 µg ml^{-1} every day for 52 days. For treatment with Pb^{2+} [$Pb(NO_3)_2$] the concentration of Pb^{2+} in the medium on the first day was 100µg ml^{-1} with the increase of 100 µg ml^{-1} every day for 55 days. For treatment with Cd^{2+} ($CdCl_2$) the concentration of Cd^{2+} in the medium on the first day was 5µg ml^{-1} with the increase of 5 µg ml^{-1}every day for 74 days. These concentrations of different metals were selected, based on our experience with other micro-organisms studied in the industrial waste. The dose was administered until population was reduced by 50%.
[b]Means ± SEM., Student's 't' test, ***P<0.001.

Metal processing ability

The metal processing efficiency of rotifers (*Philodina roseola* and *Notommata copeus*) is shown in Figure 3. *Philodina roseola could* decrease 40% Cr^{6+}, 67% Cu^{2+} and 76% Cd^{2+} from the medium within two days of inoculation of metal containing media, whereas in control media the decline in metal concentration was 4%, 10% and 14%, respectively. However, at the end of 8 days of culturing, this decrease for Cr^{6+}, Cu^{2+}, and Cd^{2+} in metal containing medium was 88%, 77% and 89%, respectively, as against 8%, 12% and 9% decrease, respectively, in the control medium. *Notommata copeus* has proven to be a better accumulator of heavy metals. The decrease in concentration of Cr^{6+}, Cu^{2+} and Cd^{2+} in metal containing media was 82%, 61% and 97% , respectively, after 2 days, and 96%, 81% and 99%, respectively, after 8 days of culturing.

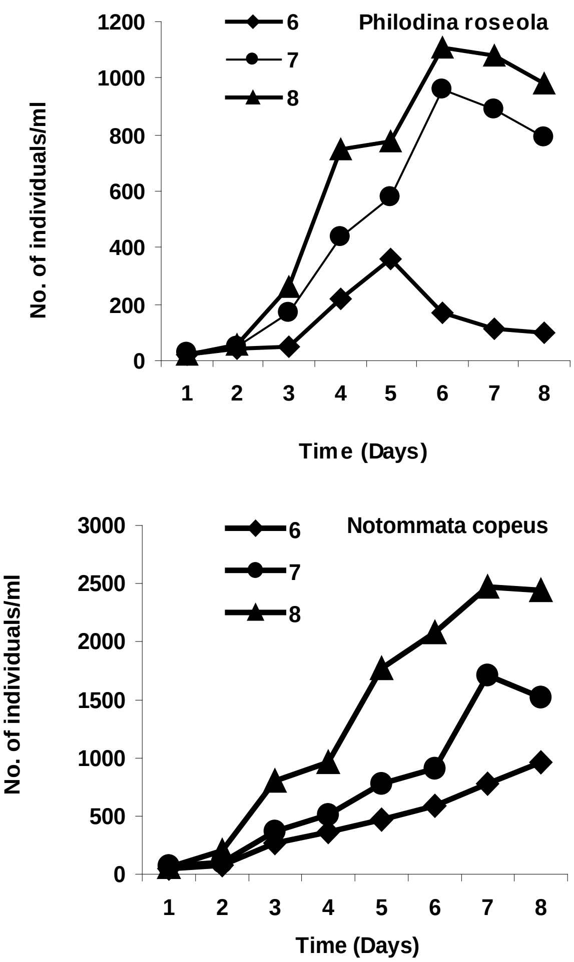


Fig. 1. Growth curves of rotifers, *Philodina roseola* (left) and *Notommata copeus* (right) at different pH *viz*., 6, 7 and 8.

DISCUSSION

When biological methods of heavy metal removal are compared with conventional physical and chemical methods, such as precipitation, adsorption, electrolysis, and reverse osmosis, several potential advantages are apparent (Wilde and Benemann, 1993). These include the use of naturally abundant renewable biomaterials that can be produced comparatively cheaply, the ability to treat large volumes of waste water due to rapid adsorption, and the reduction of metals to very low levels. Biological methods using organisms such as bacteria (Yilmaz and Ensari, 2005), algae (Rehman and Shakoori, 2001), yeasts (Dar and Shakoori, 1999), and plant root tissues (Chen *et al*., 1996) have been reported to remove heavy metals from aqueous solutions. However, metal resistance studies are rare in rotifers and gastrotrichs. The ASTM guidelines strongly advocate the use of rotifers in the toxicity testing (ASTM, 1991). As compared with bacteria or yeast, these microscopic animals are phylogenetically closer to higher animals, which are to be protected against the hazards of heavy metal ions present in the ecosystems.

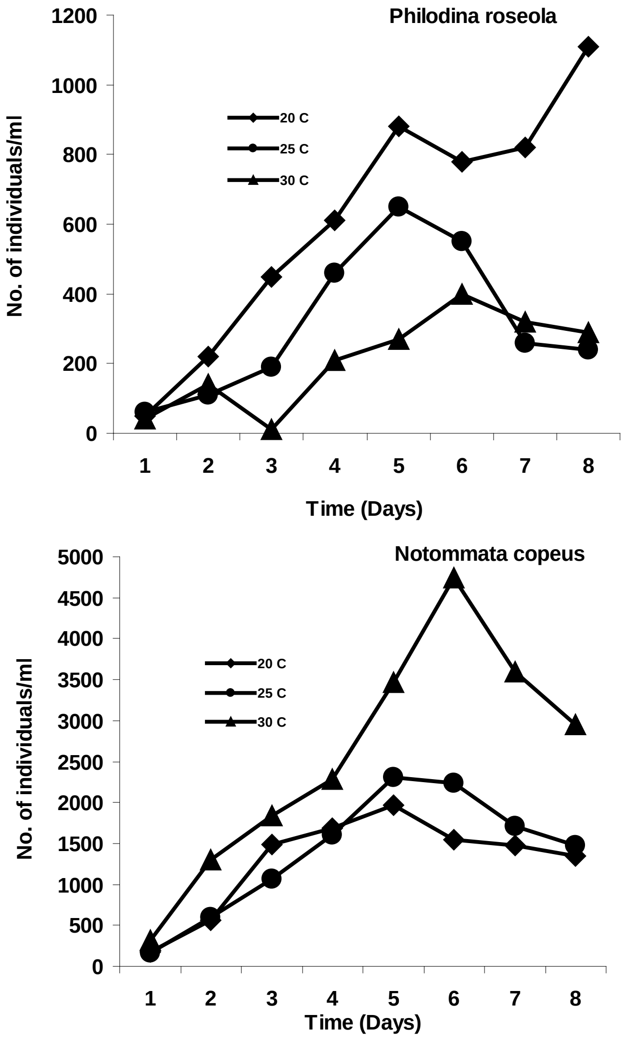


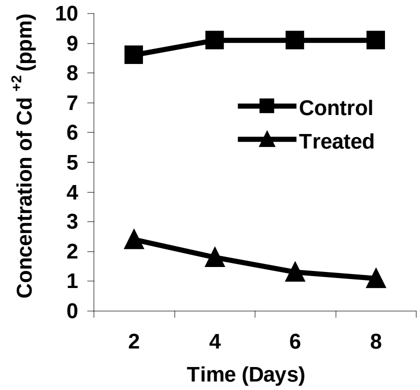
Fig. 2. Growth curves of rotifers, *Philodina roseola* (left) and *Notommata copeus* (right) at different temperatures *viz.*, 20ºC, 25ºC and 30ºC.

Environmental contamination with toxic heavy metal ions in the industrial wastes is one of the major concerns of developing countries, like Pakistan. Industrial effluents loaded with toxic Cr^{6+} are released in the environment without treatment in developing countries. Chemical detoxication of chromium requires aluminum salts, which means adding more metals to the environment. The presence of metal resistant rotifers in industrial effluents carrying highly toxic Cr^{6+} metal ions frequently used in tanning industry has indicated adaptation of these organisms to environment containing toxic metals (Haq *et al*., 2001). In a previous study we have reported that some forms of algae are very sensitive to Cr^{6+} but this is not the case with rotifers.

A vast variety of microorganisms including the metazoan microorganisms have developed strategies to survive and proliferate successfully in polluted environments. Some organisms take metals, some organisms sequester them for their own use, and some bind them in nontoxic forms. Some organisms chelate metals; some organisms reduce metals; others oxidize them. All this activity suggests that these metals play a key role in the growth of microorganisms and their cycling of major nutrients (Morel and Price, 2003).

Bioremediation of heavy metals using microorganisms has received a great attention in recent years for its potential application in industry, as it is nondestructive, cheap and economical. Rehman *et al*. (2007) reported that *Candida tropicalis* culture grown in the medium containing Cu^{2+} (100 mg l^{-1}) could reduce 74% of copper from the medium after 96 hours of incubation. The yeast was also capable of decreasing Cu^{2+} ions by 16%, 20%, 29%, 43%, 46%, 55% and 68% from the medium after 6, 12, 18, 24, 30, 48 and 72 hours, respectively. *C. tropicalis* was observed to remove 64% copper from the industrial wastewater after 4 and 74% after 8 days.

The survival, growth and heavy metal processing efficiency of rotifers observed in this study is indicative of a greater extent of adaptability, evolution and tolerance of these organisms in increasingly polluted ecosystem. Resistance and processing of rotifers, *Philodina roseola* and *Notommata copeus* against very high concentration



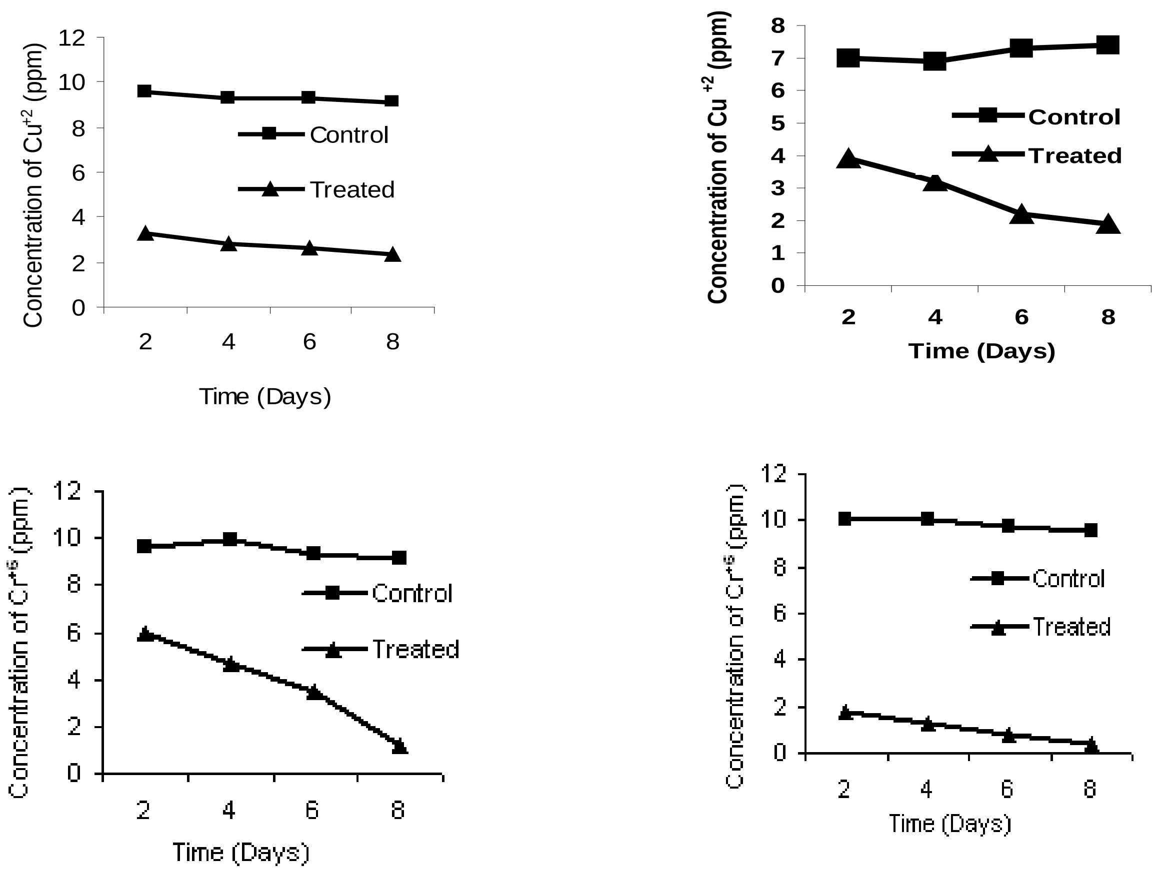


Fig. 3. Concentrations of heavy metals in different medium in which *Philodina roseola* (left) and *Notommata copeus* (right) were growing. The estimations were done at different time period.

of Cr^{6+}, Cu^{2+} and Cd^{2+} has shown the capability of these organisms to survive in the environment loaded with heavy stresses of metals. Such studies can help in understanding the role of metazoan microorganisms in waste processing and metal resistance.

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