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Heavy Metal Uptake by *Euplotes mutabilis* and its Possible Use in Bioremediation of Industrial Wastewater

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Abstract A ciliate protozoan, *Euplotes mutabilis*, isolated from heavy metal laden industrial wastewater, has been shown to tolerate multiple heavy metals thus suggesting its significance in bioremediation of industrial effluents. This ciliate tolerated Zn^{2+} up to 33 μg/mL, Cd^{2+} up to 22 μg/mL and Ni^{2+} up to 18 μg/mL. The ciliate could uptake 85% Zn^{2+}, 84% of Cd^{2+} and 87% of Ni^{2+} after 96 h of inoculation of growth medium containing 10 μg/mL of Zn^{2+} and 5 μg/mL of Cd^{2+} and Ni^{2+}, with actively growing ciliates. After 6 days of incubation the ciliate removed 87% Cd^{2+}, 92% Ni^{2+}, and 93% Zn^{2+} from the wastewater. The heavy metal uptake capability of *Euplotes mutabilis* may be employed for metal detoxification operations.

Keywords Bioremediation · Metal tolerance · Metal uptake · *Euplotes mutabilis* · Wastewater

Uncontrolled discharge of heavy metal containing wastewaters to the environment can be detrimental to humans, animals and plants. Cadmium (Cd) is one of the most dangerous heavy metals both to human health and aquatic ecosystems. The toxicity of Cd has also been well documented in selective types of almost all major phyla of eukaryotes (Unger and Roesijadi 1996; Coeurdassier et al. 2004). Cd is carcinogenic, embryotoxic, teratogenic and mutagenic and may cause hyperglycemia, reduced immunopotency and anemia due to its interference with iron metabolism.

Nickel, a major environmental pollutant, is known for its clastogenic, toxic, and carcinogenic potential (Ross 1995; Hartwig and Schwerdtle 2002). The carcinogenic potential of nickel compounds depends largely on their solubility. The particulate nickel compounds like Ni_3S_2 or NiO are strong carcinogens, whereas the soluble nickel (II) salts exert weaker effects (Dunnick et al. 1995). This may be due to differences in bioavailability. Water soluble nickel salts are taken up only slowly by cells, while particulate nickel compounds are phagocytosed and, due to the low pH, are gradually dissolved in lysosomes, yielding high concentrations of nickel ions in the nucleus (Costa et al. 1981).

Zinc is a major inorganic pollutant, which has inhibitory and stimulating effects on the growth along with accumulation in plants (Kumar 1989). Seedling growth and enzyme activities have been found inhibited by zinc in *Phaseolus aureus* cv. R-851 (Veer 1989). Zinc inhibits transporter-mediated glutamate uptake (Vandenberg et al. 1998), and depending on concentration, can inhibit or potentiate glycine receptors (Han and Wu 1999). It is also known that zinc is toxic to neurons. Studies in animal models suggest that endogenous zinc mediates neurodegeneration resulting from ischemia (Koh et al. 1996) and seizure (Suh et al. 1996). It has been suggested that increased intracellular zinc may result in mitochondrial impairment and generation of reactive oxygen species (Dineley et al. 2003).

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In view of the hazardous effects of heavy metal, their removal from the contaminated environment has been a challenge for a long time (Honjoh et al. 1997). Because traditional clean up processes of heavy metal contaminated soils and waters are expensive and practical only in small areas (Moffat 1995), new cost effective technologies that include the use of microorganisms, biomass, and live plants need to be investigated (Gardea-Torresdey et al. 1996; Miller 1996; Ebbs and Kochian 1997).

Shakoori et al. (2004) have reported 99% and 48% reduction of Zn^{2+} and Cr^{6+} by *Vorticella microstoma* after 96 h, respectively from the medium. These microorganisms actively contribute to the amelioration of the effluent quality, since the majority of them feed upon dispersed bacteria (Madoni 2000). Heavy metal uptake processes by biological cells are known under the general term of biosorption. These phenomena include both passive adsorption of heavy metals to the cell walls and metabolically mediated uptake by the cells (Gadd 1990).

The objective of this study was to evaluate the survival of a ciliate protozoan, *Euplotes mutabilis*, in media containing the heavy metals Cd^{2+}, Zn^{2+} and Ni^{2+} and to determine the efficiency of uptake of these metals by the ciliate. A number of authors have already emphasized the role of protozoa in wastewater treatment plants (Fernandez-Leborans et al. 1998; Shakoori et al. 2004; Rehman et al. 2006, 2008). The aim of this study was to demonstrate the significance and efficiency of *Euplotes mutabilis* in remediation of industrial wastewater contaminated with metal ions, particularly Cd^{2+}, Zn^{2+} and Ni^{2+}.

Materials and Methods

For the present study already established axenic culture of *Euplotes mutabilis* from this laboratory was used. One hundred milliliters of Bold-basal salt medium [$NaNO_3$ (0.25 g/L), $CaCl_2 \cdot H_2O$ (0.025 g/L), $MgSO_4 \cdot 7H_2O$ (0.075 g/L), K_2HPO_4 (0.075 g/L), KH_2PO_4 (0.175 g/L), NaCl (0.0025 g/L), EDTA (0.05 g/L), KOH (0.031 g/L), $FeSO_4 \cdot 7H_2O$ (0.04 g/L), H_2SO_4 (0.001 L/L), H_3BO_3 (0.01142 g/L), $ZnSO_4 \cdot 7H_2O$ (0.00881 g/L), $MnCl_2 \cdot 4H_2O$ (0.00144 g/L), MoO_3 (0.00071 g/L), $CuSO_4 \cdot 5H_2O$ (0.00157 g/L) and $Co(NO_3) \cdot 6H_2O$ (0.00049 g/L)], diluted 1:1000 with distilled water in 250 mL conical flasks were inoculated under aseptic conditions with 10 µL of inoculum containing 40–50 ciliates. Glucose as carbon source was added as 1 g/L in Bold-basal salt medium (Shakoori et al. 2004; Rehman et al. 2006). The cultures were maintained in the laboratory at room temperature (25–27°C). The pH of the medium was adjusted to 7.5. The growth of *E. mutabilis* was observed in the cultures by counting the number of ciliates at regular intervals.

The effect of different metal ions on growth of the culture was analyzed by counting the number of protozoan cells in the medium. The cells were grown in the salt medium, to which Cd^{2+}, Ni^{2+} and Zn^{2+}, ions were added at a concentration of 1 µg/mL per day for 8 days. At least three counts were taken every day to get a mean of every reading. The growth was compared with that of the control culture, which contained no added metal ions. The activity, shape and size of the protozoans were also noted. The size was measured with an ocular micrometer after restricting the movement of the ciliates by putting the culture in methylcellulose and staining with 1% neutral red.

Resistance of *E. mutabilis* to three metal ions i.e. Cd^{2+}, Ni^{2+} and Zn^{2+} was checked by addition of the respective metal salts viz., $CdCl_2 \cdot H_2O$, $NiCl_2$ and $ZnSO_4 \cdot 7H_2O$ to Bold-basal salt medium. Metal ions were sterilized separately and added to the medium when the temperature of the salt medium was slightly less than 50°C. For Cd^{2+}, Ni^{2+} and Zn^{2+}, the concentration in the medium on the first day was 1 µg/mL with an increase of 1 µg/mL every day for 33 days for Zn^{2+}, 22 days for Cd^{2+} and 18 days for Ni^{2+}. Although the death of protozoa is confirmed by the lysis of the cell, movement is considered to be a vital sign of life. When the protozoa became inactive, no more metal was added.

For determining uptake of heavy metals by *E. mutabilis*, the ciliates were grown by inoculating 100 mL of Bold-basal medium in three 250 mL conical flasks with 10 µL of original laboratory culture (40 ± 2 cells) at 25°C. Zinc was added at a concentration of 10 µg/mL in the medium containing ciliate cells but cadmium and nickel each was added at a concentration of 5 µg/mL separately. The control culture medium, containing the same metal concentrations as the treated samples, was maintained without any ciliates. All cultures were incubated for 6 days, at which time 5 mL samples were removed under sterile conditions after 0, 48, 72, 96 h. The cultures were centrifuged at 350*g* for 15 min and the supernatants were used to estimate Cd^{2+}, Ni^{2+} and Zn^{2+} concentrations by atomic absorption spectrophotometer (Varian, U.S.A) at wavelengths 228.8, 232.0 and 213.9 nm, respectively. The amount of metal in the supernatants was determined using standard curves, which were prepared by taking various known concentrations of $CdCl_2 \cdot H_2O$, $NiCl_2$ and $ZnSO_4 \cdot 7H_2O$ in the medium. The percentage decrease in the amount of Cd^{2+}, Ni^{2+} and Zn^{2+} in the medium was calculated.

The efficacy of ciliates to remove Cd^{2+}, Ni^{2+} and Zn^{2+} from industrial effluents was determined at lab-scale for which two plastic containers one containing 10 L of industrial effluent (temperature, 30.0°C; pH, 7.7; dissolved oxygen, 0.0123 ± 0.03 g/L; Cd^{2+} 0.0160 ± 0.03 mg/L, Ni^{2+} 0.0154 ± 0.03 mg/L and Zn^{2+} 0.0119 ± 0.01 mg/L), with 1.5 L of 72 h grown *E. mutabilis* culture, and the second containing 10 L of industrial effluent with no *E. mutabilis* culture. In both containers concentration of Cd^{2+}, Ni^{2+} and Zn^{2+} was maintained at 5 μg/mL. For this purpose concentration of each metal ion was measured in industrial effluent and additional amount of metal salts were added to the containers to make up the concentration of each metal to 5 μg/mL. After 6 days of incubation at room temperature (25 ± 2°C) three samples from each container were taken, centrifuged to separate the cells, and supernatants used to estimate the amount of Cd^{2+}, Ni^{2+} and Zn^{2+} ions. The quantity removed by the ciliates was then calculated with reference to 5 μg/mL.

All values are an average of three replicates and have been shown as Mean ± SEM. For determining significance of differences between the control and the experimental treatments, Student's "*t*" test was applied.

Results and Discussion

Figure 1 shows growth of *E. mutabilis* in media with and without metal ions. The growth of ciliate, which is indicated by the number of cells/mL, was affected by the presence of metal ions in the culture media. The *E. mutabilis* control culture contained 0.039×10^3 cells/mL on day 1, which increased to 2.105×10^3 cells/mL after 8 days (54-fold increase). However, in the presence of Cd^{2+} (8 μg/mL) the number increased from 0.175×10^3 to 0.942×10^3 cells/mL in 8 days (5.38-fold increase). In the presence of Zn^{2+} (8 μg/mL) the number of cells increased from 0.039×10^3 to 1.555×10^3 cells/mL (40-fold increase), whereas the number of cells increased from 0.200×10^3 to 0.850×10^3 cells/mL in the presence of Ni^{2+} (8 μg/mL) in 8 days (4.25-fold increase). The addition of metal ions in the medium resulted in slower growth and delayed cell division (Fig. 1).

Maximum number of *E. mutabilis* cells in each metal containing medium was achieved on day 8 except for nickel containing medium, where it was achieved on day 5. The maximum *E. mutabilis* cells for control (2,385.00 ± 1.00) were obtained in 7 days and the maximum number of protozoan cells in Cd^{2+}, Zn^{2+}, and Ni^{2+} containing medium was 942.25 ± 0.50, 1,555.00 ± 1.00, and 966.66 ± 1.53, respectively. Growth rate of *E. mutabilis* was slower in all metal ions but the slowest rate was observed in the presence of Cd^{2+} and Ni^{2+} ions.

The heavy metals, in the present study, have significantly hampered the growth of the ciliate cells. When the

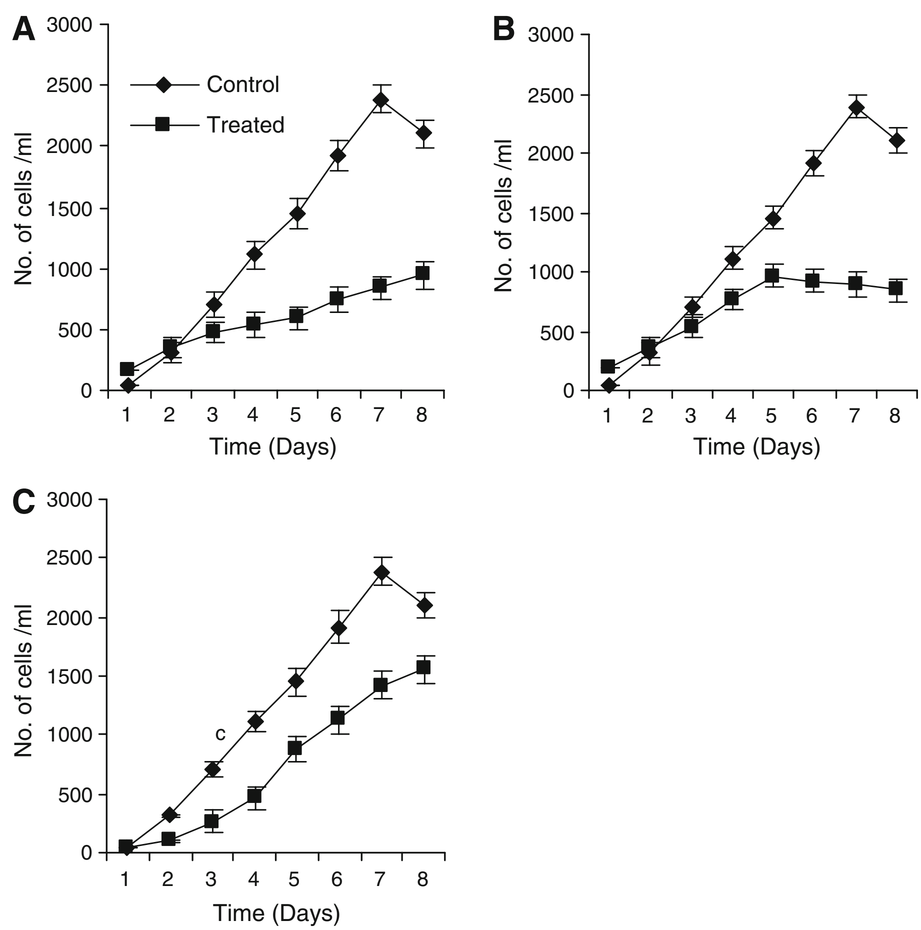

Fig. 1 Growth curves of *Euplotes mutabilis* in Cd^{2+} (**a**), Ni^{2+} (**b**) and Zn^{2+} (**c**) containing medium. Control cultures did not contain any metal ions

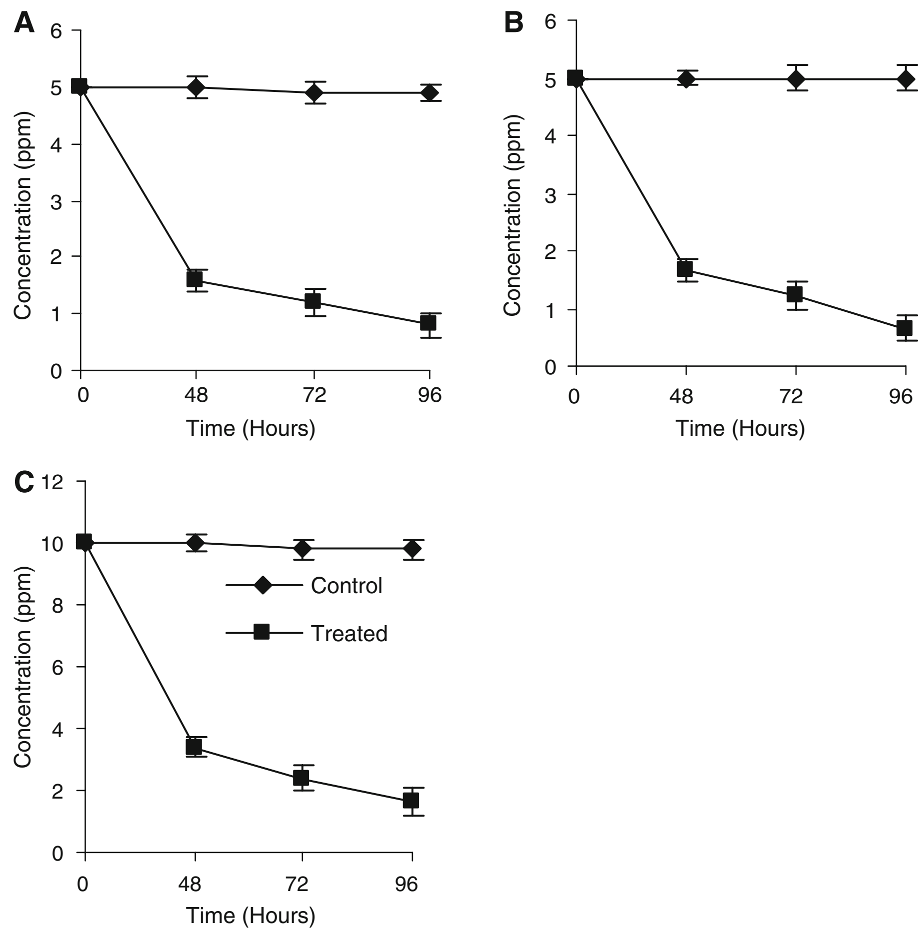

Fig. 2 Uptake of Cd^{2+} (**a**), Ni^{2+} (**b**) and Zn^{2+} (**c**) by *Euplotes mutabilis* growing in Cd^{2+}, Ni^{2+} and Zn^{2+} containing medium. The control did not contain cells of the isolate

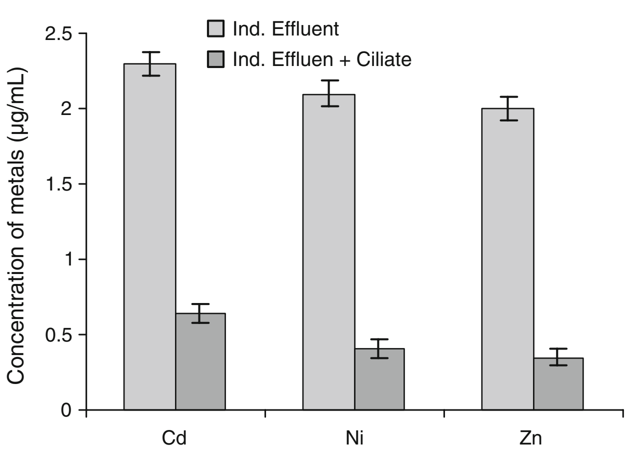

Fig. 3 Concentration of Cd^{2+}, Ni^{2+} and Zn^{2+} in 10 L of industrial effluent with and without *Euplotes mutabilis* after 6 days of incubation at room temperature. The initial concentration was 5 μg/mL of each metal

cell populations of metal-treated cultures were compared with those of the corresponding control culture on day 8, it was observed that the cadmium-treated culture had 45% lower cell numbers when compared with the control culture. In the presence of Zn^{2+} ions, this decrease was 26%, and for Ni^{2+} it was 59% as compared with the control. The order of resistance, in terms of reduction in the cellular population, was $Zn^{2+} > Cd^{2+} > Ni^{2+}$. Metal resistant protozoa have been reported in wastewaters and metal-polluted environments (Shakoori et al. 2004; Madoni and Romeo 2006; Rehman et al. 2007).

Ciliates are usually found in polluted wastewaters containing less than 10 μg/mL concentrations of toxic metal ions (Shakoori et al. 2004). The ciliate, *E. mutabilis*, can survive very easily in such polluted waters. The metal removal efficiency of *E. mutabilis* is greater than 80% in such metal contaminated wastewaters (Rehman et al. 2006, 2008) and these ciliates are excellent and convenient bio-indicator for evaluating the toxicity of wastewaters polluted by heavy metals (Madoni and Romeo 2006).

E. mutabilis was found to resist Zn^{2+} up to a concentration of 33 μg/mL, Cd^{2+} 22 μg/mL and Ni^{2+} 18 μg/mL. No reduction in the size of *E. mutabilis* cells was observed. Movement was taken as a parameter of effect on growth rate. The hypotrich, *Euplotes patella*, showed the lowest sensitivity for both nickel and most of other tested metals. *E. patella* can resist Ni concentration up to 10 mg/L [24 h LC_{50s}] (Madoni 2000).

Figure 2 shows the removal of heavy metal ions from the medium by *E. mutabilis* growing in medium containing cadmium (5 μg/mL) could decrease 68% of cadmium from the medium after 48 h, 76% after 72 h and 84% after 96 h, respectively (2a). Likewise, the ciliate decreased 67% nickel from the medium containing 5 μg/mL nickel after 48 h, 76% after 72 h and 87% after 96 h (2b). It could also remove 66% of zinc after 48 h, 76% after 72 h and 85% after 96 h, respectively from the medium containing 10 μg/mL of zinc (2c).

Figure 3 shows the ability of ciliate, *E. mutabilis*, to remove Cd^{2+}, Ni^{2+} and Zn^{2+} from contaminated industrial effluents. *E. mutabilis* was observed to remove 87% Cd^{2+} from the wastewater after 6 days. *E. mutabilis* could also remove 92% Ni^{2+} and 93% Zn^{2+} from the wastewater after 6 days of incubation. In comparison, the microbial flora alone of the industrial effluent was able to decrease only 54% Cd^{2+}, 58% Ni^{2+} and 60% Zn^{2+} after 6 days of incubation at room temperature.

Rehman et al. (2006) reported that *E. mutabilis* grown in the medium containing Cu^{2+} (5 μg/mL) could reduce 60% of copper from the medium after 48 h, 82% after 72 h and 95% after 96 h. It could also reduce 67% Hg^{2+} after 48 h, 75% after 72 h, and 82% after 96 h from the medium containing Hg^{2+} at a concentration of 10 μg/mL. In one of the reports from this laboratory, the live *E. mutabilis* could remove 81% Pb^{2+} and 84% Cr^{6+} from the medium after 96 h of incubation whereas killed organisms could remove only negligible quantity of heavy metal from the medium (Rehman et al. 2008). In the present study *E. mutabilis* could remove 84% Cd^{2+}, 85% Zn^{2+}, and 87% Ni^{2+} from the medium after 96 h of incubation. This clearly indicates that the ciliates actively take up the heavy metals. Metal bioaccumulation has also been reported to be the main mechanism of resistance to heavy metals in ciliates by others (Martin-Gonzalez et al. 2006; Diaz et al. 2006).

In this study we are reporting the multiple heavy metal uptake potential of *Euplotes mutabilis* which is resistant to highly toxic metal ions and may be employed for metal detoxification operations.

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