Pakistan J. Zool., vol. 39(4), pp. 259-264, 2007.

Potential Use of a Ciliate, *Vorticella microstoma*, Surviving in Lead Containing Industrial Effluents in Waste Water Treatment

ABDUL REHMAN, FARAH RAUF SHAKOORI AND ABDUL RAUF SHAKOORI*

Department of Microbiology and Molecular Genetics (AR) and School of Biological Sciences (ARS), University of the Punjab, New Campus, Lahore 54590, Pakistan and Department of Zoology, GC University, Lahore, Pakistan (FRS)

Abstract.- The capability of a lead tolerating ciliate *Vorticella microstoma* isolated from industrial wastewater of tanneries of Kasur, to reduce the concentration of lead ions in the medium was determined and its potential use as bioremediator of wastewater was evaluated. The ciliate tolerated 150µg/ml of lead, and showed optimum growth at 25°C and at pH 7.5. The protozoan culture grown in medium containing 10 µg/ml of lead could reduce 92% of Pb^{2+} from the medium after 48 hours, 96% after 72 hours and 98% after 96 hours of incubation. On acid digestion of the ciliate it was found that it accumulated 92% of Pb^{2+} ions from the medium. The heavy metal uptake ability of *Vorticella microstoma* can be exploited for metal detoxification and environmental clean-up operations.

Key words: Heavy metal resistance, metal uptake, bioremediation**,** *Vorticella microstoma,* lead.

INTRODUCTION

Heavy metals are the most abundant pollutants in the sewage and in wastewater (Hong *et al.,* 1996) and are one of the main causes of water and soil pollution (Nriagu and Pacyna, 1988). Anthropogenic use of metals has resulted in the extensive contamination of freshwater ecosystems that has had harmful effects on fauna. Human activities, such as mining operations and the discharge of industrial wastes, have resulted in the accumulation of metals in the environment and eventually are accumulated through the food chain, leading to serious ecological and health problems (Cheng, 2003). Concentration of lead in atmosphere is of serious environmental concern (Zelikoff *et al.,* 1988).

Lead contamination in surface water mainly comes from anthropogenic sources (96%), particularly from combustion of leaded fuels, pyrometallurgical non-ferrous metal production and coal combustion. Lead in natural waters may be in the form of organic lead complexes originally from the fuel of ever growing automobile population and subsequent break down of tetraethyl lead (Andrews and Sutherland, 2004; Monterroso *et al.,* 2003). The most serious effects of lead are related to impacts on central nervous system (Goyer, 1993). It is considered a non-essential metal with no biological role in microorganisms, animals and plants (Bruins *et al.,* 2000).

*Correspondence author: Email; arshak@brain.net.pk

0030-9923/2007/0004-0259 $ 8.00/0

Parker (1979) reported a LC_{50} value of 45,000 µgPb/l to the marine ciliate *Uronema marinum*. A concentration of 6.98 mg/l of Pb killed 65% of the individuals, but caused the disappearance of only one species, *Chilodonella uncinata* (Madoni *et al.,* 1996). The 24-h LC_{50} value of lead was 10.78 mg/l for *Spirostomum teres* (Twagilimana *et al.,* 1998).

Recent advances have been made in understanding metal-microbe interaction and their application for metal detoxification. Microbial metal bioremediation is an efficient strategy due to its low cost, high efficiency and eco-friendly nature. Ciliate protozoa are cosmopolitan eukaryotic micro-organisms adapted for life in soils and aquatic ecosystems. They are believed to be important grazers of bacteria and other microorganisms and in some artificial ecosystems such as activated sludge wastewater treatment plants, ciliates significantly improve effluent quality (Nicolau *et al*., 2001; Curds, 1982).

One of the objectives of this study was to evaluate the survival of protozoa in media containing Pb^{2+} and determine the uptake of lead by

these organisms. 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*., 2005, 2006).

MATERIALS AND METHODS

Sample collection

Wastewater samples from a tannery effluent were collected from Kasur in screw capped sterile bottles. The pH and temperature of these samples were also recorded at the time of collection. The samples were inoculated in Bold-basal salt medium in 100 ml conical flasks (Haq *et al.,* 1998). A large number of bacteria, yeast, algae, and various protozoa were present in the original wastewater sample.

Isolation and culturing of protozoan

For isolation of protozoa, antibiotics, *i.e*. ampicillin (25 µg/ml), chloramphenicol (35 µg/ml) and gentamicin (25 µg/ml), were used to prevent growth of bacteria. Algae were excluded by keeping the culture in semidarkness. Yeast was excluded by absence of any organic substance in the medium. Culture was plated to YEPD medium and no growth appeared on the fungal medium. Axenic culture of protozoa was made according to Shakoori *et al.* (2004).

One hundred milliliters of Bold-basal salt medium, in 250 mL conical flasks, was inoculated under aseptic conditions with 10µL of inoculum containing 40-50 ciliates. The cultures were maintained in the laboratory for one week at room temperature (25-27°C). The growth of *Vorticella* was observed in the cultures by counting the number of ciliates at regular intervals.

Determination of growth curves in different media

The growth curves of *Vorticella* were determined in different media *i.e.* LB (2 % (w/v) proteose peptone and 0.1% Bacto yeast extract), molasses medium (1% aqueous solution of molasses), wheat and rice grain medium (1 boiled rice and wheat grain in 10mL of distilled water) and Bold-basal salt medium [$NaNO_3$ (0.25g/l), $CaCl_2.H_2O$ (0.025g/l), $MgSO_4.7H_2O$ (0.075g/l), K_2HPO_4 (0.075g/l), KH_2PO_4 (0.175g/l), NaCl (0.025g/l), EDTA (0.05g/l), KOH (0.031g/l), $FeSO_4.7H_2O$ (0.04g/l), H_2SO_4 (0.001,L/l), H_3BO_3 (0.01142g/l), $ZnSO_4.7H_2O$ (0.00881g/l), $MnCl_2.4H_2O$ (0.00144g/l), MoO_3 (0.00071g/l), $CuSO_4.5H_2O$ (0.00157g/l) and $Co(NO_3).6H_2O$ (0.00049g/l)], diluted 1:1000 with distilled water, for 8 days. Glucose as carbon source was only added as 1g/L in Bold-basal salt medium. The pH of each medium was adjusted at 7.5. No metal ions were added in these media. The growth of culture was checked by counting the number of protozoan cells in the medium as described earlier (Haq *et al.,* 1998).

Resistance to lead ions

Resistance of *Vorticella* to $Pb^{\square 2+}$ was checked by addition of Pb $(NO_3)_2$ in the 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 treatment with $Pb^{\square 2+}$ the concentration in the medium on the first day was 5 µg/ml of $Pb^{\square 2+}$ with an increase of 5 µg/ml of $Pb^{\square 2+}$ every day for 30 days. Although the death of protozoa is confirmed by the lysis of the cell, movement was considered to be a vital sign of life. When the protozoa became inactive, no more metal was added.

Determination of optimum growth conditions

For optimum growth of the ciliate, two parameters *i.e.* temperature and pH were considered. For determination of optimum temperature, Bold-basal salt medium (50 ml) in five 250 ml conical flasks was inoculated with 40 protozoan cells and incubated at different temperatures *viz*., 20°C, 25°C, 30°C and 37°C for 8 days. Ciliate cells were observed under microscope after staining with neutral red (1%) and their growth was assessed by counting under light microscope at 100X magnification.

For ascertaining optimum pH, Bold-basal salt medium (50 ml) in seven 250 ml conical flasks, each with different pH *viz*., 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, was inoculated with 40 protozoan cells and incubated at 25°C for 8 days. Growth was determined by counting the *Vorticella* population at each time point.

Growth curves of ciliate

The effect of Pb ions on growth of the culture

was checked by counting the number of protozoan cells in the medium. The cells were grown in the salt medium, to which lead was added at a concentration of 1µg/mL per day for eight days. At least three counts were taken 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.

Biosorption of metals by the ciliate

The metal processing capability of *Vorticella* was checked by adding $Pb^{\square 2+}$ at a concentration of 10.0 µg/ml of $Pb^{\square 2+}$ in the culture medium. The control culture medium also contained Pb^{2+} at a concentration of 10 µg/ml but was without the ciliates. The cultures were incubated for 6 days and from each medium (control and treated) 5 mL culture was taken out under sterilized conditions after 0, 48, 72 and 96 hours, respectively. The cultures were spun down at 3000 rpm for 15 min and the supernatants were used for the estimation of Pb^{2+} by atomic absorption spectrophotometer (Varian, U.S.A) at wavelength 217.0 nm. The amount of $Pb^{\square 2+}$ in the supernatants was determined using standard curve. The percentage reduction in the amount of Pb^{2+} in the medium was calculated.

Estimation of lead ions in the ciliate

For further confirmation of metal uptake by the protozoans, the ciliates were grown in two 250 mL conical flasks containing 100 mL of Bold-basal medium, to which Pb^{2+} (10 µg/mL) was added separately and incubated at 25°C. After 96 hours, 10 ml culture was taken, cells were pelleted, washed three times in saline solution and acid digested (H_2SO_4: HNO_3, 1:1) at 100°C. Metal contents of the digest were measured by Atomic Absorption Spectrophotometer (AAS) at wavelength 217.0 nm. All the experiments were done in triplicate. Amount of lead uptake by ciliate cells was calculated in µg/mL by using standard curve.

Statistical analysis

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

RESULTS

Physicochemical characteristics of wastewater

The appearance of various metal resistant micro-organisms in ponds constantly receiving toxic industrial effluents showed a high capacity to evolve in response to xenobiotic stress. The temperature of ponds harboring the ciliates ranged between 19ºC to 25ºC and pH ranged between 7.86 and 8.53. These ponds had $Pb^{\square 2+}$ ranging between 0.25 ±0.04 and 1.01 ±0.004 µg/ml.

Growth curves in different media

The most suitable temperature for lead resistant ciliate was found to be 25°C and maximum growth for *Vorticella microstoma* was observed at pH 7.5. The growth curve pattern of *Vorticella* was obtained by counting the number of cells in the culture every day for 8 days. There was a gradual increase in the number of cells in each culturing medium. The number of cells increased from 80 to 6080 cells/ml in LB medium, from 80 to 4620 cells/ml in 1% Molasses medium, from 100 to 6620 cells/ml in Wheat and Rice medium and from 240 to 8100 cells/ml in Bold-basal salt medium. A large number of media have been tried and reported for the growth of protozoa. Generally it is tedious to grow protozoa in the laboratory due to special organic supplements needed in the medium for their growth (Weekers and Vogels 1994). In this study *Vorticella* has been successfully grown in the Bold-basal salt medium. The growth curves are shown in Figure 1.

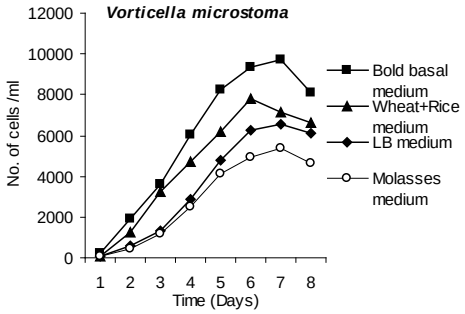

Fig. 1. Growth curves of *Vorticella microstoma* in different media containing no metal ions.

Lead resistant ciliate

Vorticella microstoma was found to be resistant to $Pb^{\square 2+}$ at a concentration of 150 µg/mL. There was apparently no reduction in the size of *V. microstoma* cells. Movement, which is a vital sign of life, was taken as a parameter of metal toxicity. The presence of Pb $(NO_3)_2$ (150 µg/mL) did not have any significant effect on the movement of ciliates.

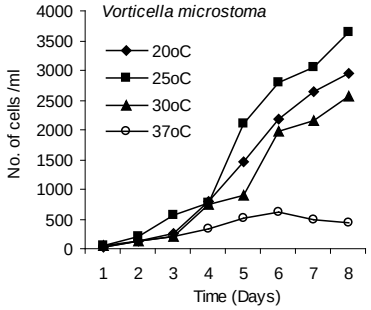

Fig. 2. Effect of temperature on the growth of *Vorticella microstoma* in Bold-basal medium.

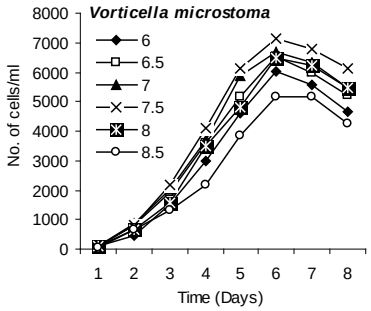

Fig. 3. Effect of pH on the growth of *Vorticella microstoma* in Bold-basal medium.

Optimum growth conditions

The most suitable temperature for lead resistant ciliate was found to be 25°C (Fig. 2) and maximum growth for *Vorticella microstoma* was observed at pH 7.5 (Fig. 3).

The growth curve pattern of *Vorticella* was obtained by counting the number of cells in the culture every day for 8 days. There was a gradual increase in the number of cells in control and treated flasks. The number of cells in control increased from 42±02.51 to 2866.33±01.53 cells/ml after 8 days of culturing showing thereby 68 fold increase. The number of cells increased from 2501.53 to 2125±02.51 cells/ml (50 fold increase) in Pb^{2+} containing medium. Maximum number of *Vorticella* cells for control (without metal) and in $Pb^{\square 2+}$ containing medium was achieved on day 8. Growth rate of *Vorticella* was slower in $Pb^{\square 2+}$ containing Bold-basal medium. The reduction in the cell population was 26% (Fig.4).

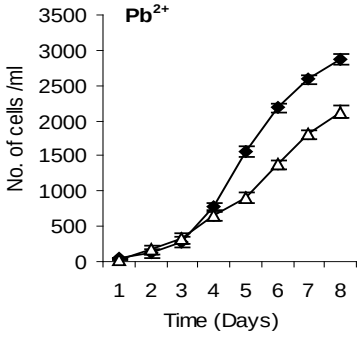

Fig.4. Growth curves of *Vorticella microstoma* in $Pb^{\square 2+}$ containing medium. Control culture did not contain $Pb^{\square 2+}$ ions.

Metal uptake ability

Vorticella microstoma could efficiently process $Pb^{\square 2+}$ from the medium. The protozoan culture grown in medium containing lead (10.0 µg/ml) could reduce 92% of lead from the medium after 48 hours, 96% after 72hours and 98% after 96 hours, respectively (Fig.5). The amount of lead estimated after acid digestion was 9.2 µg/ml for *Vorticella microstoma.* The percent bioaccumulation was 92% for Pb^{2+} (Fig.6).

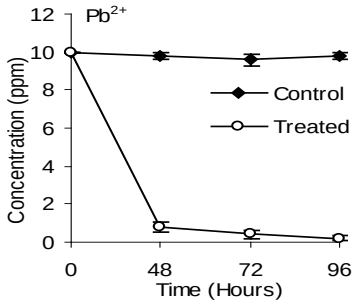

Fig. 5. Uptake of $Pb^{\square 2+}$ by *Vorticella microstoma* growing in $Pb^{\square 2+}$ containing medium. The control did not contain cells of the isolate.

DISCUSSION

Microorganisms have a high affinity for metals and can accumulate both heavy and toxic metals by a variety of mechanisms (Shuttleworth and Unz, 1993; Ledin, 2000; Shakoori and Muneer; 2002; Pas *et al.,* 2004). These have been used to remove metals from polluted industrial and

domestic effluents on a large scale. Microorganisms have a high surface area-to volume ratio because of their small size and therefore provide a large contact area that can interact with metals in the surrounding environment (Ledin, 2000).

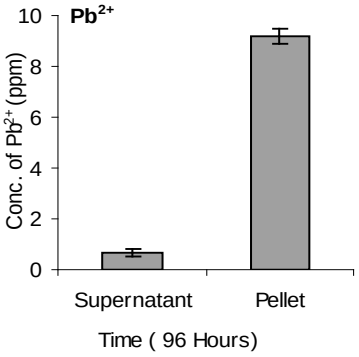

Fig. 6. Amount of lead accumulated by *Vorticella microstoma* (pellet) and in the medium (supernatant) after 96 hours of incubation.

In the present study *Vorticella microstoma* was found to be resistant to Pb^{2+} at a concentration of 150 µg/mL. This resistance concentration is much higher as compared to original lead concentration found in the wastewaters which indicates that these protozoans have evolved some resistant mechanism to cope xenobiotics stress. Metal resistant protozoa have also been reported in wastewaters and metal-polluted environments (Madoni *et al.,* 1996 Haq *et al.,* 2000: Shakoori *et al.,* 2004). Madoni *et al.* (1996) found that *Aspidisca cicada* showed a 70% survival in the presence of 6.98 mg/l of Pb. It was observed that the average velocity of *Euplotes aediculatus* cells decreased as the concentration of cadmium increased (Salvado *et al.,* 1997).

Shakoori *et al.* (2004) reported a very high level of metal resistance in *Vorticella microstoma*. The ciliate was found to tolerate Pb^{2+} and Cr^{6+} at a concentration of 550 and 260 µg/ml, respectively. The ciliate showed remarkable potential to remove metal ions from the culture medium. The concentration of Cr^{6+} was reduced 48% after 192 hour in a culture medium containing Cr^{6+} (100 µg/ml) (Shakoori *et al.,* 2004). Frequent occurrence of ciliates in wastewater or industrial effluents indicates that they are able to withstand the heavy metal contaminated environment. This property makes protozoa excellent candidate for exploitation in metal detoxification and bioremediation (Haq *et al.,* 2000; Rehman *et al.,* 2005). *Halteria grandinella* and *Euplotes aediculatus* are excellent and convenient bioindicator for evaluating the toxicity of waters wastewaters polluted by heavy metals (Madoni and Romeo, 2006).

Uptake of metals by living cells has become one of the most attractive means for bioremediation of industrial wastes and other metal polluted environments. 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 (Gadd, 1990). Metal bioaccumulation has been reported as the main mechanism of resistance to heavy metals in ciliates (Martin-Gonzalez *et al.,* 1999, 2006). The biosorption term has been used, in the present study, to indicate that the metal was removed by one or more of these processes.

Rehman *et al.* (2005) reported that *Stylonychia* could efficiently uptake Pb^{2+} from the medium. The protozoan culture grown in medium containing lead (10 µg/ml) could reduce 80% of lead from the medium after 48 hours, 82% after 72 hours and 86% after 96 hours, respectively. In the present investigation *Vorticella microstoma* showed fairly high capability to uptake Pb^{2+} from the environment and the percent bioaccumulation of lead was 98%.

Rehman *et al.* (2006) reported that *Tachysoma pellionella* culture grown in the medium containing Cr^{6+} (10 µg/ml) could reduce 77% Cr^{6+} from the medium after 48 hours, 85% after 72 hours and 92% after 96 hours, respectively. It could also reduce 68% Pb^{2+} after 48 hours, 80% after 72 hours, and 88% after 96 hours from the medium containing Pb^{2+} at a concentration of 10 µg/ml. The presence of metal resistant ciliate in industrial effluents carrying highly toxic metal ions has indicated adaptation of these organisms to environment containing toxic metals. In this study we have reported the isolation of *Vorticella microstoma* which is resistant to highly toxic metal ions and may be employed for metal detoxification operations.

REFERENCES

ANDREWS, S. AND SUTHERLAND, R. A., 2004. Cu, Pb and

Zn contamination in Nuuanu watershed, Oahu, Hawaii. *Sci. Total Environ*., **324:** 173-182.

BRUINS, M.R., KAPIL, S. AND OEHMEI, F.W., 2000. Microbial resistance to metals in the environment. *Ecotoxicol. environ. Saf*., **45:** 198-207.

CHENG, S., 2003. Heavy metals in plants and phyoremediation. *Environ. Sci. Pollut. Res. Inter.*, **10:** 335-340.

CURDS, C. R., 1982. The ecology and role of protozoa in aerobic sewage treatment processes. *Annu. Rev. Microbiol*., **36:** 27-46.

FERNANDEZ-LEBORANS, G., HERRERO, O.Y. AND NOVILLO A., 1998. Toxicity and bioaccumulation of lead in marine protozoa communities. *Ecotoxicol. environ. Saf.*, **39:** 172-180

GADD, G.M., 1990. Heavy metal accumulation by bacteria and other microorganisms. *Experientia*, **13:** 273-280.

GOYER, R.A., 1993. Lead toxicity: Current concerns. *Environ. Hlth. Perspect*., **100:** 177-187.

HAQ, R.U., QAZI J.I. AND SHAKOORI, A.R., 1998. Growth and survival of protozoa isolated from a tannery effluent. *Folia Microbiol.*, **43:** 109-112.

HAQ, R.U., REHMAN, A. AND SHAKOORI, A.R., 2000. Effect of dichromate on population and growth of various protozoa isolated from industrial effluents. *Folia Microbiol.*, **45:** 275-278.

HONG, Y.C., AZAD, H.R. AND COOKSEY, D.A., 1996. A chromosomal locus required for copper resistance, competitivefitness and cytochrome c biogenesis in *Pseudomonas fluorescens. Proc. natl. Acad. Sci. USA*, **93:** 7315-7320.

LEDIN, M., 2000. Accumulation of metals by microorganisms-processes and importance for soil systems. *Earth-Sci. Rev*., **51:** 1-31.

MADONI, P., DAVOLI, D., GORBI, G. AND VESCOVI, L., 1996. Toxic effect of heavy metals on the activated sludge protozoan community. *Wat. Res*., **30:**135-141.

MADONI, P. AND ROMEO, M.G., 2006. Acute toxicity of heavy metals towards freshwater ciliated protists. *Environ. Pollut.*, **141:** 1-7.

MARTIN-GONZALEZ, A., DIAS, S. AND GUTIERREZ, J.C., 1999. The use of protists in ecotoxicology: applications and perspectives. *Recent Res. Develop. Microbiol*., **3:** 93-111.

MARTIN-GONZALEZ, A., DIAS, S., BORNIQUEL, S., GALLEGO, A. AND GUTIERREZ, J.C., 2006. Cytotoxicity and bioaccumulation of heavy metals by ciliated protozoa isolated from urban wastewater treatment plants. *Res. Microbiol.*, **157:** 108-118.

MONTERROSO, P., PATO, P., PEREIRA, E., VALE, C. AND DUARTE, A. C., 2003. Distribution and accumulation of metals (Cu, Cd, Zn and Pb) in sediments of a lagoon on the northwestern coast of Portugal. *Mar. Pollut. Bull*., **46:** 1200-1205.

NICOLAU, A., DIAS N, MOTA, M. AND LIMA, N., 2001. Trends in the use of protozoa in the assessment of wastewater. *Res. Microbiol*., **152:** 621-630.

NRIAGU, J.O. AND PACYNA, J.M., 1988. Quantitative assessment of world wide contamination of air, water and soils by trace metals. *Nature*, **333:** 134-139.

PARKER, J. G., 1979. Toxic effects of heavy metals upon cultures of *Uronema marinum* (Ciliophora: Uronematidae). *Mar. Biol.*, **54:** 17-24.

PAS, M., MILACIC, R., DRASLAR, K., POLLAK, N. AND RASPOR, P., 2004. Uptake of chromium (III) and chromium (VI) compounds in the yeast cell structure. *Biometals*, **17:** 25-33.

REHMAN, A., ASHRAF, S., QAZI, J. I. AND SHAKOORI, A. R., 2005. Uptake of lead by a ciliate, *Stylonychia mytilus*, isolated from industrial effluents: potential use in bioremediation of wastewater. *Bull. environ. Contam. Toxicol.*, **75:** 290-296.

REHMAN, A., SHAKOORI, F. R. AND SHAKOORI, A. R., 2006. Uptake of heavy metals by a ciliate, *Tachysoma pellionella*, isolated from industrial effluents and its potential use in bioremediation of toxic wastewater. *Bull. environ. Contam. Toxicol.*, **77:** 469-476.

SALVADO, H., GRACIA, M. P., AMIGO, J. M. AND RIUS, M., 1997. Effects of cadmium on growth and motility in *Euplotes aediculatus* isolated from activated sludge. *Bull. environ. Contam. Toxicol*., **58:** 838-844.

SHAKOORI, A. R. AND MUNEER, B., 2002. Copper resistant bacteria from industrial effluents and their role in remediation of heavy metals in wastewater. *Folia Microbiol.*, **47:** 43-50.

SHAKOORI, A. R., REHMAN, A. AND HAQ, R. U., 2004. Multiple metal resistance in the ciliate protozoan, *Vorticella microstoma*, isolated from industrial effluents and its potential in bioremediation of toxic wastes. *Bull. environ. Contam. Toxicol.*, **72:** 1046-1051.

SHUTTLEWORTH, K. L. AND UNZ, R. F., 1993. Sorption of heavy metals to the filamentous bacterium *Thiothrix* strain Al. *Appl. environ. Microbiol*., **59:** 1274-1282.

TWAGILIMANA, L., BOHATIER, J., GROLIERE, C-A., BONNEMOY, F. AND SARGOS, D., 1998. A new low-cost microbiotest with the protozoan *Spirostomum teres*: culture conditions and assessment of sensitivity of the ciliate to 14 pure chemicals. *Ecotoxicol. environ. Saf.*, **41:** 231-244.

WEEKERS, P. H. H. AND VOGELS, G. D., 1994. Axenic cultivation of the free living soil amoeba, *Acanthamoeba castellanii* and *Harmanella vermiformes* in a chemostat. *J. Microbiol. Meth*., **19:**13-18.

YILMAZ, E.I., 2003. Metal tolerance and biosorption capacity of *Bacillus circulans* strain EB1. *Res. Microbiol*., **154:** 409-41.

ZELIKOFF, J. T., LI, J. H., HARWIG, A., WANG, X. W., COSTA, M. AND ROSSMAN, T. G., 1988. Genetic toxicology of lead compounds. *Carcinogenesis* (Lond.), **9:** 1727-1732.

(Received 13 January 2007, revised 6 February 2007)