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Effects of Four Heavy Metals on Cell Morphology and Survival Rate of the Ciliate *Bresslauides* sp.

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ABSTRACT.– Heavy metals are highly toxic chemicals often contaminating several ecosystems and causing detrimental effects on organisms living in such polluted environments. To evaluate the toxicity of heavy metals on eukaryotic microbes, a ciliate *Bresslauides* sp. was isolated from a canal located in Chulalongkorn University. A clonal culture of this ciliate was established and subsequently used to examine detailed morphological features of the isolated organism using light and scanning electron microscopy. The ciliate culture in exponential phase was then tested with several chosen concentrations of soluble compounds of copper, lead, zinc, and cadmium under laboratory conditions. The survival rate was documented after 24 hr incubation. Based on the median lethal concentration (LC_{50}), the result indicated that cadmium was the most toxic metal with an LC_{50} of 0.09 mg/l, followed by copper (1.91 mg/l), zinc (3.66 mg/l), and lead (10.03 mg/l), respectively. *Bresslauides* sp. showed its highest sensitivity to Cd but considerable tolerance to Pb. Microscopic examination of cells treated with heavy metals at the LC_{50} concentration revealed cytological alterations, displaying intracellular vacuolarization, morphological deformities, and cellular lysis. This investigation not only reports for the first time the acute toxicity of the four metals to this ciliate genus, but also reveals the potential utilization of this isolate as a biological indicator for Cd-polluted environments.

KEY WORDS: Bioindicator; bioremediation; ciliate; pollutant; protozoa

INTRODUCTION

Heavy metals constitute highly harmful chemicals often contaminating several ecosystems and causing detrimental effects on organisms living in such polluted environments. Even though some metals are required for biological processes, others are hazardous to living creatures even at minimal concentrations (Beveridge et al., 1997). Unlike other compounds, heavy metals cannot be degraded by microbes and therefore may accumulate through the food chains and food webs (Martín-González et al., 2006). As a consequence, the chemicals persist in the biological and ecological systems, posing both acute and chronic effects depending on the types of metals and the organisms they affect (Nriagu & Pacyna, 1988).

Toxic effects of heavy metals have been reported in several groups of organisms. In plants, exposure to Cu, Cd, and Cr could inhibit root growth and induce nuclear abnormalities and chromosomal aberrations (Hemachandra & Pathiratne, 2015). Mercury was demonstrated to increase mortality and induction of teratogenicity in red sea bream embryos and larvae (Huang et al., 2011). In addition, growth rates in freshwater algae could be affected by Cd, Cu, and Zn (Magdaleno et al., 2014) and mixtures of these three metals also interfered with the reproductive rate and life expectancy of the freshwater rotifer *Brachionus calyciflorus* (Xu et al., 2014). Furthermore, subcellular deformities at the

ultrastructural level were documented in ciliates incubated with heavy metals (Martín-González et al., 2005; Nilsson, 2003).

Ciliates are unicellular microbial eukaryotes comprising an important group of primary consumers (Uchida et al., 1997). Their widespread distribution as major constituents in every environment makes this particular group of microbes an appropriate model organism for monitoring the effects of both physical and chemical factors on biological communities (Madoni, 1993). Moreover, their quick reaction to both toxic and non-toxic chemicals has been extensively reported, since they exhibit a short generation time and possess a delicate cellular membrane with no external wall covering (Gertler et al., 2010; Gutiérrez et al., 2003; Forge et al., 1993; Martín-González et al., 2006; Twagilimana et al., 1998). Unlike bacteria and fungi, lacking a cell wall makes the cytoplasmic membrane of ciliates directly exposed to any contaminants [e.g., heavy metals, pesticides, insecticides, etc.], leading to a rapid response to such pollutants (Gutiérrez et al., 2009). Furthermore, comparative genomic studies demonstrated that two ciliate model organisms, *Paramecium* and *Tetrahymena* share more orthologs with humans than any other non-ciliated microbial model organisms (Dessen et al., 2001; Turkewitz et al., 2002). This strengthens the use of ciliates as a bioindicator to reflect the effects of toxic chemicals on higher eukaryotes, including humans.

Bresslauides is a genus of ciliates found in both terrestrial and freshwater habitats. The organism belongs to the class Colpodea and is closely related to *Colpoda*, a well-studied model organism that has been subjected to many biological studies, especially several aspects of toxicity assessment (Díaz et al., 2006; Forge et al., 1993; Rico et al., 2009). However, toxicological studies of such nature have never been undertaken on *Bresslauides*. It is possible that *Bresslauides* may react on toxic chemicals in the same way as its phylogenetically close relative, *Colpoda*. Therefore, in the present study, we examine, for the first time not only in Thailand but also in the world, the acute toxicity of four heavy metals (cadmium, copper, lead, and zinc) to *Bresslauides* sp. isolated from a canal located in Chulalongkorn University and reveal its potential utilization as a biological indicator for heavy metals.

MATERIALS AND METHODS

Isolation and cultivation of *Bresslauides*

The ciliate *Bresslauides* sp. used in this research was collected from a canal located in Chulalongkorn University, Bangkok, Thailand [13°44'25.7"N, 100°31'41.2"E] using a 20-µm mesh size plankton net. A clonal culture of *Bresslauides* sp. was established and maintained at room temperature in autoclaved hay infusion medium (boiled 5 g of hay/1 liter of distilled water) with autoclaved yeast powder as food source.

Morphological examination

To examine morphological features of the isolated ciliate, cells of *Bresslauides* sp. were observed and photomicrographed using a BH-2 Olympus light microscope connected to a Canon EOS 7D digital camera (Fig. 1). Cells were also prepared for scanning electron microscopy to examine detailed taxonomic characters. The culture was fixed at room temperature for one hour with 4% OsO_4, at a final concentration of 1%. The fixed cells were then washed through a 10-µm polycarbonate membrane

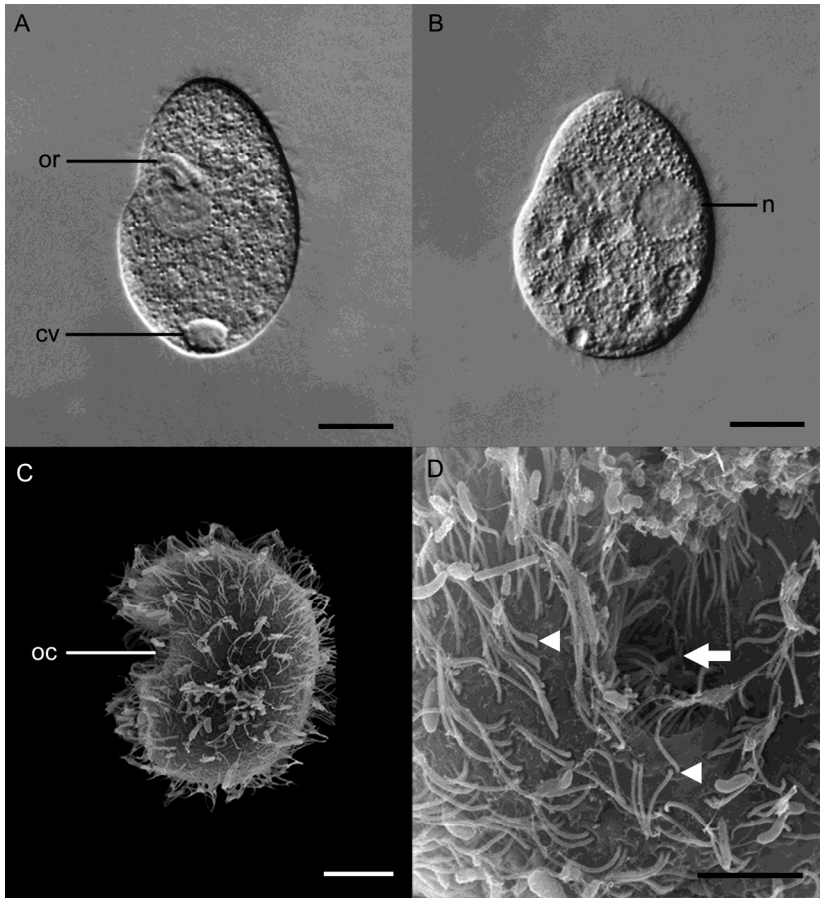

FIGURE 1. Light micrographs (A and B) and scanning electron micrographs (C and D) of *Bresslauides* sp. used in this study. A. A ciliate cell showing a ventral oral region (or) and posterior contractile vacuole (cv). B. A focal plane of the ciliate demonstrating a single rounded macronucleus (n). C. Lateral view of *Bresslauides* sp. showing an oral cavity (oc) and dorsal curvature of the kidney-shaped cell. D. A higher magnification view of the oral area (arrow) and somatic cilia arranged in dikinetid pattern (arrowheads) of the isolated *Bresslauides* sp. Scale bars: A and B = 20 μm; C = 10 μm; D = 5 μm.

filter (Millipore Corp., Billerica, MA, USA) with distilled water twice. Dehydration was performed in a graded series of ethanol [10%, 20%, 30%, 50%, 70%, 85%, 95%, and 100%] and finally dried with liquid CO_2 in an Autosamdri-814 critical point dryer (Samdri® 780, Tousimis). The filter was then affixed on brass stubs and coated with a 20 nm thick layer of gold using an SCD040 Balzers sputter coater (Liechtenstein). The processed cells were examined under a JEOL JSM-5410 LV scanning electron microscope. SEM images of the ciliates were shown on a black background using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA) (Fig. 1). Cytological examination using light and scanning electron microscopy was performed to establish taxonomic identification based on morphological features described in Foissner (1993).

Determination of heavy metal toxicity

Evaluation of the 50% lethal concentration

To examine the toxicity of the heavy metals — Cd, Cu, Pb, and Zn — hay infusion medium was used to make stocks of soluble compounds (1 mg/ml) of $CdCl_2$, $CuSO_4$, $Pb(NO_3)_2$, and $ZnSO_4$, respectively. These stocks were subsequently used to prepare 7 different two-fold metal concentrations for testing the isolated ciliate. The lowest concentration tested would show no effect on ciliate survival and the highest concentration would account for greater than 50% mortality rate. Culture in the exponential phase of *Bresslauides* sp. was used once cell counts reached about 1,000 cells/ml. To conduct the experiment, the culture was distributed into sterile 24-well microtiter plates (500 µl/well). For each metal concentration, the same volumes were transferred into each well to make up the required final (1X) concentrations. Four replicates of each concentration were performed and hay infusion medium with no metals was added as negative control in the same manner. All experiments were done at room temperature for 24 hr and three independent assays were run.

After 24 hr incubation, total and living cell numbers [i.e., live cells that swim actively] were determined by directly counting under observation in a stereo microscope. Briefly, twenty microliters were pipetted from each well. Since *Bresslauides* sp., is an active swimmer rather than a glider, gentle agitation was performed to ensure uniform distribution of cells before sampling. Numbers of cells in three different sampling times were counted and averaged for each well. The same counting was repeated for every replicate, metal concentration, and independent bioassay. Percentage of cell viability was calculated using the following formula: living cells in the treatment/total cells in the control × 100. The concentration that produces lethality in 50% of the ciliate population (LC_{50}) was used as the endpoint for toxicity assessment and this was calculated by Probit analysis with 95% confidence limits using the Statplus software (2009).

Cytological observation

To examine the toxic effect of the four heavy metals on cell morphology, *Bresslauides* cell culture was separately exposed to hay infusion medium mixed with the soluble compound of each heavy metal at the LC_{50} concentration. Briefly, 500 µl of cell culture were distributed into the 24-well plate. The two-fold LC_{50} concentrations of each metal were prepared and then transferred into the cell-containing wells (500 µl/well) to make up the final LC_{50} concentrations. After inoculation, cells were immediately observed and captured with light microscopy apparatus mentioned above.

RESULTS AND DISCUSSION

The isolated *Bresslauides* sp. showed a kidney-shaped appearance with a conspicuous curvature on the dorsal side (Fig. 1). Cells possess a large rounded macronucleus and are covered with cilia arranged in a dikinetid pattern. A wide oral region at the mid-ventral area can be easily observed and the cell dimensions of this isolate are 30-55 µm (40.35±6.13 µm, N = 100) in width and 40-72.5 µm (53.6±6.87 µm, N = 100) in length (Fig. 1).

Microscopic examination of cells treated with heavy metals at the LC_{50} concentration revealed cytological alterations (Fig. 2). Heavy metal-exposed cells exhibited intracellular vacuolarization, morphological

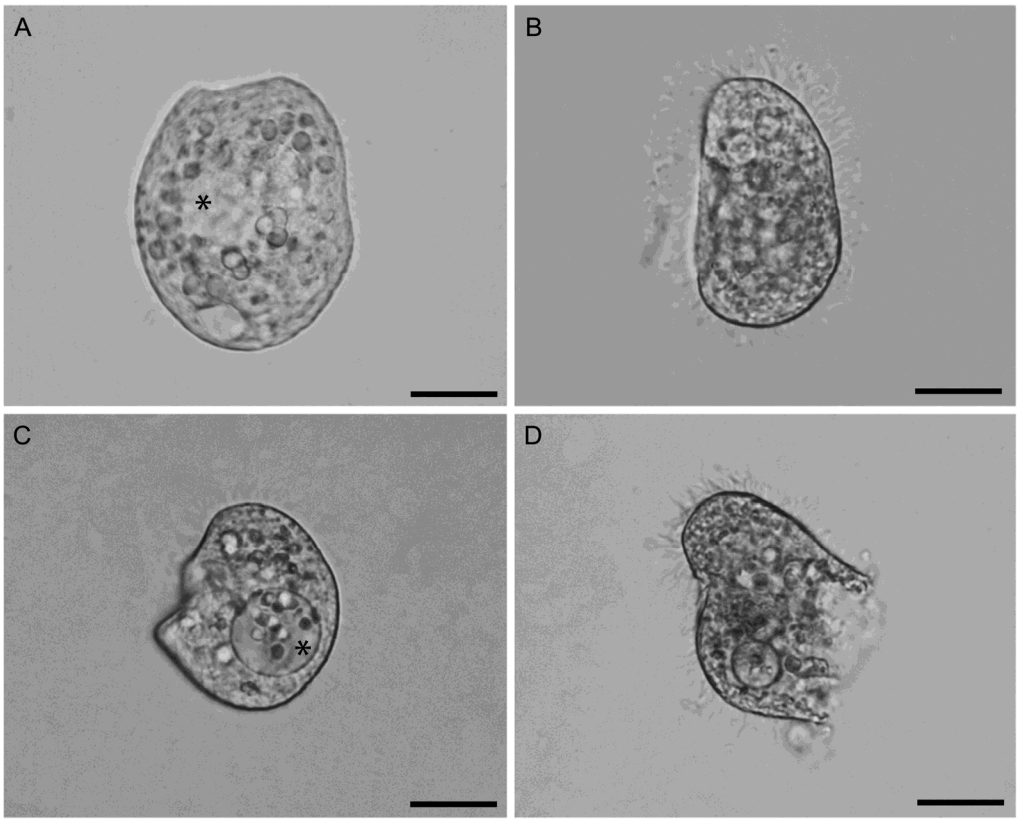

FIGURE 2. Light micrographs of *Bresslauides* sp. showing cells treated with Cu (A), Pb (B), Zn (C), and Cd (D) at the median lethal concentrations of each metal. After exposure to heavy metals, cells exhibited vacuolar formation (*) (A and C), cytological deformity (B), and cellular rupture (D). Scale bars: 20 μm.

deformities, and cellular rupture (Fig. 2). It has been suggested that heavy metals can induce membrane alterations, resulting in osmotic disorganization which ultimately leads to cellular vacuolarization (Smith, 1983). Furthermore, several studies have shown that heavy metals, such as Cd, Cu, and Pb, have strong effects on the organization of actin and tubulin (Eun et al., 2000; Liu et al., 2009; Olabarrieta et al., 2001). These two proteins are the main components of cytoskeletons and function as cellular scaffolds, maintaining cell shape. *Bresslauides* cells treated with the metals clearly lost their morphological integrity and became round almost immediately after exposure. In addition, shortly after exposing the organisms to the heavy metals, cellular rupture was observed for a majority of cells, confirming severe toxicity of the metals to the organism (Fig. 2). This may be a result of the accumulation of metals on the plasma membrane, causing destruction of their integrity and ultimately leading to cellular lysis (Madoni & Romeo, 2006).

Our *Bresslauides* isolate showed varying degrees of sensitivity to the four heavy metals tested and the percentages of cell viabilities are illustrated in Fig. 3. The order of heavy metal toxicity to *Bresslauides* sp.

was Cd > Cu > Zn > Pb. This order of toxicity was also observed by Martín-González et al. (2006) when the same metals - excluding Pb - were used to treat three freshwater ciliate species — *Drepanomonas revoluta*, *Euplotes* sp., and *Uronema nigricans*. In general, a decrease in cell survival was observed in all metals, when the metal concentrations were increased (Fig. 3). This response was especially obvious when the cells were treated with Cd. In addition, based on the mean lethal concentration values after 24-hr exposure (24-hr LC_{50}), Cd was shown to be the most toxic metal to *Bresslauides* sp. with the LC_{50} being 0.09 mg/l. The toxicity of this metal is further shown by the fact that only trivial concentrations, ranging from 0.06-0.12 mg/l, were used on the organisms. Furthermore, the Cd response of this *Bresslauides* strain placed its degree of sensitivity only after *Halteria grandinella* (0.07 mg/l) which exhibited an LC_{50} of 0.02 mg/l less than *Bresslauides* and is the most Cd susceptible ciliate among all species reported so far (Madoni & Romeo, 2006).

A study by Forge et al. (1993) reported a 24-hr LC_{50} of Cu and Zn for *Colpoda steinii* at 0.25 and 0.85 mg/l, respectively. However, all other isolates belonging to the genus *Colpoda* showed higher LC_{50} values of Cu and Zn, ranging from 5.4 to 8.1 mg/l for the former and from 33.9 to 147.4 mg/l for the latter (Díaz et al., 2006; Forge et al., 1993; Rico et al., 2009). In contrast, our examined *Bresslauides* sp. demonstrated a much lower LC_{50} of 1.91 mg/l for Cu and 3.66 mg/l for Zn. The results suggest that our studied ciliate has a high degree of sensitivity to these two metals and support the use of this *Bresslauides* sp. as a bioindicator for Cu and Zn contamination. Previous studies showed the adverse effects of Cu as well as mercury and nickel on feeding rates of *Euplotes mutabilis* (Al-Rasheid & Sleigh, 1994) and growth of *Tetrahymena pyriformis* (Nicolau et al., 1999). In addition, some studies reported the utilization of Cu as a controlling agent for algal bloom (Haughey et al., 2000). Over-use of Cu-containing chemicals undeniably leads to hazardous effects on algal producers and ciliate consumers, ultimately destroying the ecological balance at both trophic levels (Le Jeune et al., 2007).

Among all tested metals, Pb demonstrated the lowest degree of toxicity to *Bresslauides* sp. with the LC_{50} reaching 10.03 mg/l. Data on the sensitivity/resistivity of this metal have not been previously reported in any ciliate members of the class Colpodea and are documented here for the first time. This high level of resistance is by far greater than that of any other freshwater ciliate tested for this metal with the reported LC_{50} values ranging from 0.12 mg/l in *Dexiotricha granulosa* and *Halteria grandinella* to a maximum of 5 mg/l in *Opercularia minima* (Madoni et al., 1992, 1994, 1996; Madoni & Romeo, 2006). Even though, of all ciliates examined so far, a marine species of *Uronema marinum* demonstrated the greatest tolerance to Pb (45 mg/l), our *Bresslauides* sp. was approximately 2-100 times more resistant to Pb when considering the freshwater ciliate species (Parker, 1979). It has long been known that freshwater ciliates are more susceptible to some heavy metals than marine forms (Parker, 1979). Our experiment provides the first line of evidence in regards to a freshwater ciliate with the second highest Pb resistance of all ciliates studied so far. Besides, among all metals tested, Zn and Pb are known to be elements with lower toxicities when compared to the other two, Cd and Cu. This same scenario was also documented in

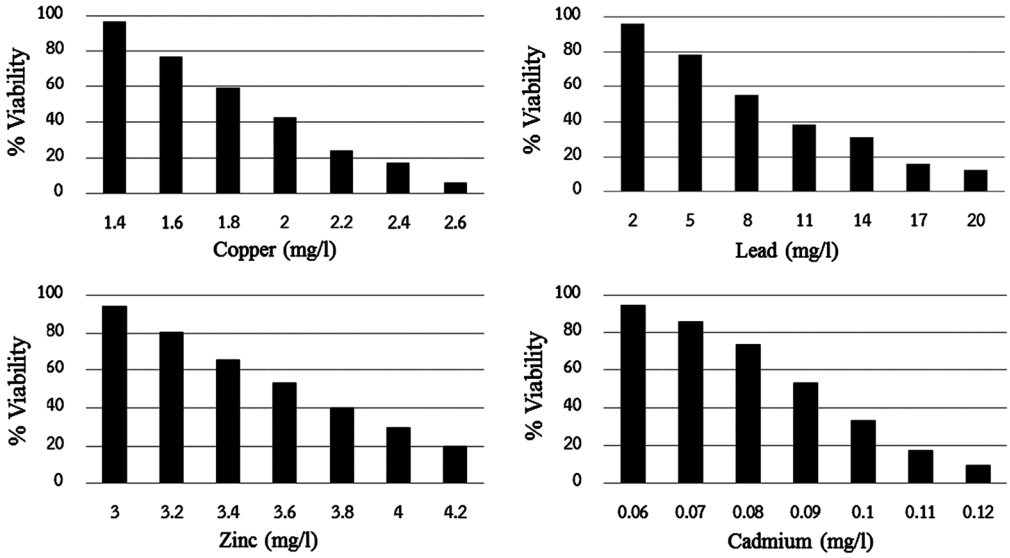

FIGURE 3. Mean percent viability values documented for *Bresslauides* sp. after 24-hr exposure to different concentrations of heavy metal ions.

studies by Madoni et al. (1992, 1994) and in agreement with our investigation that demonstrated a lesser degree of susceptibility of *Bresslauides* to Pb. Rehman et al. (2005, 2008) showed that *Stylonychia mytilus* could biologically absorb > 85% of Pb from the culture medium after 96-hr inoculation. The high level of Pb resistance observed in our *Bresslauides* isolate may imply its potential utilization as a bioremediator for Pb-contaminated wastewater.

CONCLUSION

In this study, we examined toxicity of four heavy metals to a freshwater ciliate *Bresslauides* in laboratory conditions. As opposed to its close relative *Colpoda*, this ciliate has never been subjected to this kind of study and we expanded the data in this particular aspect for the first time. Cadmium was the most hazardous chemical to the tested ciliate, followed by copper, zinc, and lead, respectively. This has proven to be useful in terms of employing this *Bresslauides* isolate as a bioindicator for Cd, Cu, and Zn. Revelation of *Bresslauides*' sensitivity to Cd in this study has emphasized the importance of examining toxic effects of heavy metals on diverse genera of ciliates since a better candidate that can serve as a good bioindicator is awaiting our discovery. However, *Bresslauides*' property as Cd bioindicator will be confirmed, if the isolate is subjected to test with an actual Cd-polluted wastewater derived from industrial effluents. In addition, our work revealed that *Bresslauides* has the highest Pb tolerance of any known freshwater ciliate and may potentially be employed as a bioremediator for biologically removing this particular metal from polluted water. Such bioremediation ability of *Bresslauides* needs to be further investigated both under laboratory and field conditions.

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