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Techniques for Assessment of Heavy Metal Toxicity Using *Acanthamoeba* sp, a Small, Naked and Free-Living Amoeba

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1. Introduction

Heavy metals are natural component of earth crust that cannot be degraded and destroyed but accumulate through food chain and produce potential human health risks and ecological disturbances (Loka et al., 1990). Other than occur naturally, their quantity in the environment increased due to anthropogenic activities (Greger, 1999). Heavy metals are toxic to most organisms at specific concentrations; and often cause serious upset both at cellular and genomic levels of cells. Various organisms have been employed as indicator of the metal contamination; they are mainly multicellular organisms such as fishes and bivalves (Shazili et al., 2004) based on their suitability to accumulate the metals from the environment. Some of these organisms have ability to balance between bioaccumulation and depuration of contaminants in the their body (Chin, 2005), therefore the measurement of the metals in these organisms becomes inaccurate. This chapter describes the use of a single cell organism, *Acanthamoeba,* a small, naked and free-living amoeba to assess the toxicity of heavy metals that are commonly present in our aquatic ecosystem.

2. Why *Acanthamoeba*?

Acanthamoeba is an amoeba genus, a type of small, naked and free-living amoebae. This amoeba genus occupies in most habitats such as in soil, air, dust and water. In their natural environment, these free-living organisms feed on bacteria and any particulate matters; some of them, however can infect brain, eyes, skin, and lungs of a variety of mammals, including humans (Cabral & Cabral, 2003).

The interest arose to investigate the toxicity effects of heavy metals using free-living amoebae is due to their ubiquitous in the environment as well as the amoeba cells are considered as the closest cell towards the human diploid cells, since both of them are eukaryotes. The advantage is free-living amoebae are single cell organisms, so any changes in the environment can directly affect the amoebae. Moreover, the trophozoites of amoebae are not covered by a cell wall so they are more sensitive to these environmental changes, hence they are suitable candidates for hazard prediction and environmental risk assessment

due to heavy metal pollution. Heavy metals that were investigated in the present study were commonly occur in our environment and potentially can impose hazards to human's health such as lead, zinc, cadmium and mercury.

Acanthamoeba sp used in this study was a local species, isolated from water at Setiu Wetlands, Terengganu, Malaysia and labeled as *Acanthamoeba* sp. (SW isolate). The amoeba was maintained axenically in Polypeptone medium at 30°C. The cytotoxicity and genotoxicity potential of heavy metals on the amoeba involved determination of the amoeba viability, IC_{50} of the metals (the concentrations that could inhibit the 50% growth of *Acanthamoeba*), observation on the changes on their ultra-cellular structure, the amoeba membrane permeability, mode of cell death and DNA damage due to exposure of the amoeba to the metals.

3. Experimental approach

Assessements for the metal's toxicity on *Acanthamoeba* are summarized in two categories; the cytotoxicity and Genotoxcity studies. The cytotoxicity study involved determination of amoeba viability and IC_{50} values, observation on changes in the amoeba morphology, membrane permeability and determination of mode of cell death. In the genotocity study, the main focus was observation on the DNA damage after amoeba exposure to the metals.

3.1 Cytotoxicity study

Mercury (mercury chlorides), cadmium (cadmium dichloride), lead (lead nitrate) and zinc (zinc sulphate) were first prepared as stock solutions by dissolving appropriate amount of these metals (in powder form) in polypeptone media to give 100 ppm for mercury and cadmium, 500 ppm for zinc and 50 ppm for lead.

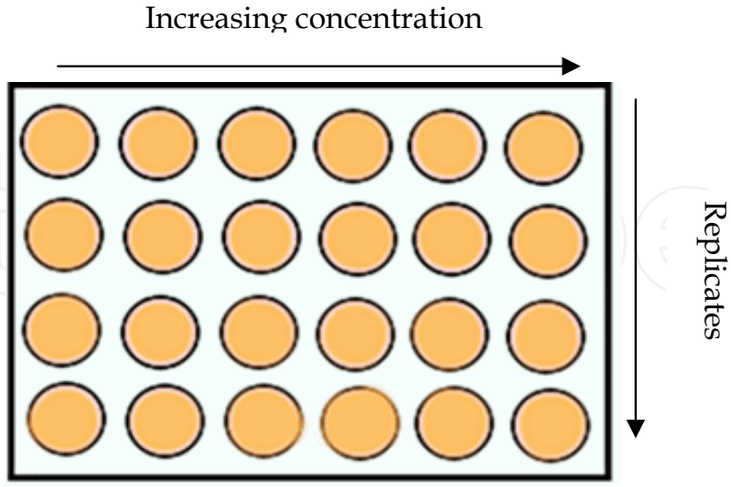

Fig. 1. Experimental design in 24-well plate for treatment of heavy metals on *Acanthamoeba*.

The cytotoxicity test on amoebae was conducted in 24-well plates. The amoebae used were taken from confluent growth cultures to ensure only healthy amoebae were used in the experiment. To get various concentration of metals, an appropriate volume of each metal

stock solution was transferred into 24-wells containing 10^4 amoebae/mL following $M_1V_1 = M_2V_2$ formula. For cadmium and mercury, six different concentrations with three replicates of metal solutions (0 ppm, 4 ppm, 8 ppm, 12 ppm, 16 ppm, 20 ppm) were tested on *Acanthamoeba* sp. The final volume in each well of 24-well plates was 1000 µL. Then, the plates were sealed with parafilm and incubated for 72 h at 30°C. Similar techniques were applied for getting various concentrations of zinc and lead in toxicity test on *Acanthamoeba*. Concentrations of lead used were 0 ppm, 6 ppm, 12 ppm, 18 ppm, 24 ppm and 30 ppm whereas concentrations for zinc were 0 ppm, 30 ppm, 60 ppm, 90 ppm, 120 ppm and 180 ppm.

3.1.1 Determination of amoeba viability and IC_{50} of metals against *Acanthamoeba*

The cytotoxicity of metals on the amoebae was investigated using the eosin dye assay to evaluate the cell viability. The technique was described by Wright et al., (1988) for determination of viable cells for *Entamoeba histolytica.* Eosin is a synthetic and acidic dye attracted to the tissue elements of a basic nature such as cytoplasm of cells (Sims, 1998). The principle of this assay was based on the release of eosin dye by viable trophozoites cytoplasm after being incubated with 0.1 M NaOH solution. In this assay, the floating trophozoites were considered as dead cells and thus were discarded leaving only the viable trophozoites attached to the 24 well plate surfaces.

Briefly, after incubation, the culture medium of the amoeba cultures in 24-well plates was discarded using a micropipette. The plates were then immediately washed with sodium chloride solution (0.9%) at 37°C. This procedure was completed quickly and the plates were not allowed to cool in order to prevent the detachment of amoebae. Then, the plates were allowed to dry at room temperature and the amoebae were fixed with methanol and allowed to dry. Next, the plates were stained with aqueous eosin (0.5%) for 15 min. Stained plates were washed once with tap water and then twice with distilled water and allowed to dry again. Later 200 µL of 0.1 M sodium hydroxide solution was added into each well to dissolve the protein and release the dye. The resulting solution was later transferred into 96-well plate. The optical density of the solution in each well was determined at 490 nm using a microplate reader. The percentage of inhibition of amoeba growth was calculated from the optical density of the treated wells relative to control wells multiply by 100. A dose-response curve representing percentage of inhibition and metal concentration was plotted. The IC_{50} values of the metals against *Acanthamoeba* were derived from this curve.

3.1.2 Visualization of changes in the amoeba morphology

To visualize the morphology of amoebae in detail after treatment with heavy metals, the amoebae were harvested and processed for scanning electron microscopy following techniques by Nakisah et al. (2008).

The amoeba was cultured on a glass slide in 6 well plate containing heavy metals for 72 h at 30°C. Then, the culture was fixed with warm glutaraldehyde in PBS (2.5% Glutaraldehyde v/v) for 2 h after the removal of all medium. After that, the amoebae on the cover slide was washed 3 times for 10 min each with PBS buffer. The amoebae were later post-fixed with 1% osmium tetraoxide at room temperature for 1 h. The amoebae then were subjected to dehydration process using a graded series of ethanol from 30% 50%, 60%, 70%, 80%, 90%, 95% (for 10 min each) and 100% (twice for 10 min each). The amoebae were later critical

point dried before the cover slip containing amoebae was attached to aluminum stub and coated with gold dust. The sample was then viewed under Scanning Electron Microscopy (JEOL JSM-6360LA, analytical SEM).

3.1.3 Membrane permeability study by AOPI staining

In membrane permeability study, fluorescence dyes of acridine orange (AO) and propidium iodide (PI) staining were used to examine the integrity of *Acanthamoeba* membrane after the amoebae (10^4 cells/mL) were treated with the metals (at their IC_{50} concentrations). The experiments including controls were done in 15 mL culture flasks for 72 h at 30°C. After that, the amoebae were harvested by centrifugation at 3000 rpm for 15 min. The supernatant then was discarded and the pellets from each treatment were washed once with PBS and re-centrifuged at the same speed for 5 min. The final pellets obtained were re-suspended in 100 µL AO/PI solution (The AOPI solution was prepared by adding 2 µL of acridine orange (1 mg/mL) and 2 µL propidium iodide (1 mg/mL) in 996 µL PBS). The cell suspensions were incubated for 10 min in the dark since both dyes were light sensitive. The *Acanthamoeba* cell suspension was placed onto a slide and covered with a cover slip. The slide was then viewed under fluorescence microscopy in the dark condition

3.1.4 Determination of apoptosis type of cell death by DNA laddering assay

DNA laddering assay was employed to determine the mode of cell death after the amoebae were exposed to IC_{50} concentrations of the metals. Amoebae (10^4 cells/mL) were incubated in polypeptone media at 30°C in the presence and absence of heavy metals for 72 h. Amoebae were collected by centrifugation at 3000 rpm for 15 min. The amoeba DNA was extracted using Dneasy Kit (Qiagen) according to the manufacturer's protocol. The concentration of DNA was first determined from the OD_{260} using a spectrophotometer (Beckman, USA). The integrity of DNA then was inspected by running the samples on a 1.2 % of agarose gel at 20 volts and visualized using Ethidium bromide.

$$\text{DNA concentration} = A_{260} \times 0.05\ \mu g/\mu L \times D,$$

Where A_{260} is the absorbance measured at 260 wavelength and D is the dilution factor.

3.2 Genotoxicity study by alkaline comet assay

The analysis of DNA damage in heavy metal-treated *Acanthamoeba* was performed by Alkaline Comet Assay (Collins, 2004). This assay is also known as the alkaline version of the single cell electrophoresis that is used for a wide range of applications including DNA damage and repair studies, genetic toxicology, radiation biology, and environmental monitoring (Cotelle and Ferard, 1999). In this study, the analysis of the DNA damage in *Acanthamoeba* sp was performed after 2 h treatment with heavy metals (at their IC_{25} concentrations) in 15 mL culture flasks. Lower concentration of metals is essential in comet assay in order to avoid false positive results on the DNA damage. After treatment, the alkaline Comet assay described by Collins (2004) and employed by Nakisah et al. (2009) for *Acanthamoeba* was followed.

First, the amoebae were harvested and centrifuged at 1500 rpm for 7 minutes. The supernatant was discarded and the pellet was washed with Ca^{2+} and Mg^{2+} free PBS and was centrifuged again. After that, the pellet was mixed thoroughly with 80 µL 0.7% low melting

agarose (LMA) and placed above the hardened first layer of 0.6% normal melting agarose (NMA) that was prepared earlier. Following that, the cover slip was placed on the agar and the slides were left on ice to solidify the LMA for 5 min. The cover slip then was removed and the gel was again coated with 200 µL of 0.5% LMA as the third layer to prevent the nuclear DNA from escaping during the cell lysing process.

The cells were incubated in alkaline lysis buffer for one hour at 4°C. Next, the slides were placed in horizontal electrophoresis tank containing electrophoresis buffer (pH>13). The electrophoresis was carried out at 25 V/cm and 300 mA for 5 min. After that, the slides were neutralized with neutralizing buffer of Tris-HCl (pH 7.5) three times, 5 min each. The slides were then stained with 20µg/mL ethidium bromide and were left overnight at 4°C before viewed under fluorescence using 590 nm filter.

3.2.1 Comet scoring

Three slides were used for each treatment and 50 cells were counted from each slide. Comets were scored from 0-4 as described by Collins (2004) for detecting DNA damage in individual cells. To compare the significance among groups in alkaline comet assay, Kruskal Wallis test was done. A p value of <0.05 was considered statistically significant.

4. Results & discussion

Evidences on the toxic effect of the four metals (cadmium, mercury, lead and zinc) on *Acanthamoeba* sp are described and highlighted in this chapter.

4.1 Determination of amoeba viability and IC_{50}

The cytotoxic effects of the four metals against *A.polyphaga* after 72 h of treatment are shown in Fig. 2. Based on eosin dye assay, there were significant increases of inhibition of amoeba population after exposure to increased concentration of metals ($p< 0.05$). The IC_{50} values of the metals against *Acanthamoeba* sp with comparison with data for a reference amoeba (*A. polyphaga* CCAP 1501/3A) are shown in Table 1. Based on IC_{50} value obtained in the present study, *Acanthamoeba* spsp (SW) is more tolerate to these metals then *A. polyphaga* (CCAP 1501/3A). This observation indicates the former *Acanthamoeba* which was a local species, and was isolated from the environment can adapt with higher concentration of the metal presents in the environment.

Metal	*Acanthamoeba* spp	IC_{50} values(ppm)
Mercury	*A. polyphaga* (CCAP1501/3A)	2.2
	Acanthamoeba sp (SW isolate)	4.0
Cadmium	*A.polyphaga* (CCAP 1501/3A	3.6
	Acanthamoeba sp (SW isolate)	7.0
Lead	*A. polyphaga* (CCAP1501/3A)	8.6
	Acanthamoeba sp (SW isolate)	9.0
Zinc	*A. polyphaga* (CCAP1501/3A	42
	Acanthamoeba sp(SW isolate),	36

Table 1. The IC_{50} values of heavy metals against *Acanthamoeba* sp (SW isolate) in comparison with *A. polyphaga* (CCAP1501/3A).

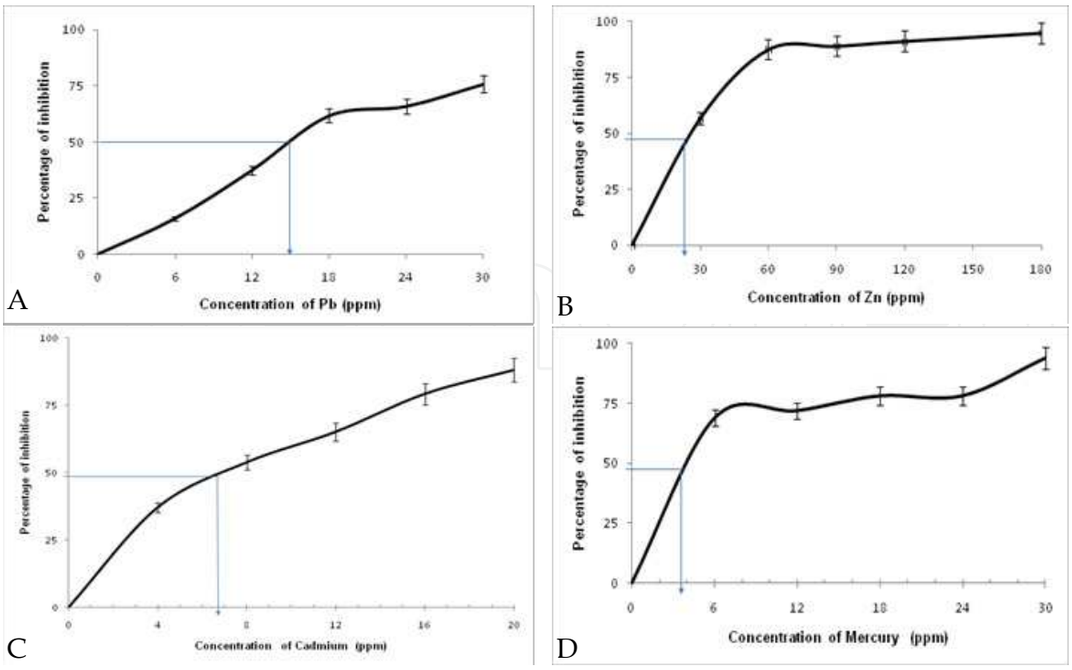

Fig. 2. Percentage of inhibition of *Acanthamoeba* sp. (SW isolate) exposed to (A) Lead, (B) zinc, (C) Cadmium and (D) Mercury. Data presented are means of three replicates. The IC_{50} values are derived from the dose-response curve for each metal.

4.2 Visualization of changes in the amoeba morphology

Details toxicity of the four metals on the amoeba can be visualised under scanning electron microscopy. Severe damage on the amoeba morphology as compared with controls is apparent (Fig. 3). Tiny projections on the surface of *Acanthamoeba* (called acanthopodia), the prominent characteristics of the genus, were almost completely lost. This structure is crucial for the amoeba to move or adhere to the substratum. Therefore, the presence of the metal ions in the amoeba media or in the environment will affect the biology and movement of this amoeba.

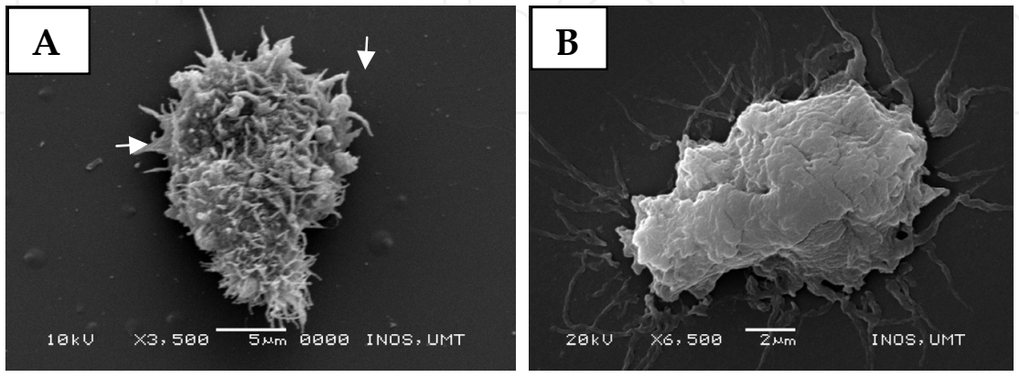

Fig. 3. Scanning electron microscopy of trophozoite of *Acanthamoeba* sp (SW isolate). (A) In control and (B) after treatment with IC_{50} of lead. Acanthopodia (arrows) are prominent on the surface of control *Acanthamoeba* but these structures are lost in lead-treated amoeba.

4.3 Membrane permeability study by AOPI staining

The integrity of *Acanthamoeba* membrane was affected after being exposed to the metals (Fig.4). Under fluorescence microscopy, the heavy metals-treated *Acanthamoeba* were stained orange or green with orange granules observed in their cells, in contrast with control *Acanthamoeba* which were stained green. Chromatin condensation observed as green to red fluorescence precipitates in the cytoplasm of treated *Acanthamoeba* resulted from disintegration of nuclear envelope followed by nuclear fragmentation. The live *Acanthamoeba* only allows acridine orange dye when at low concentration to enter the cells, but not for propidium iodide, thus they stained green. By this technique, apoptotic and necrotic cells can also be visualised.

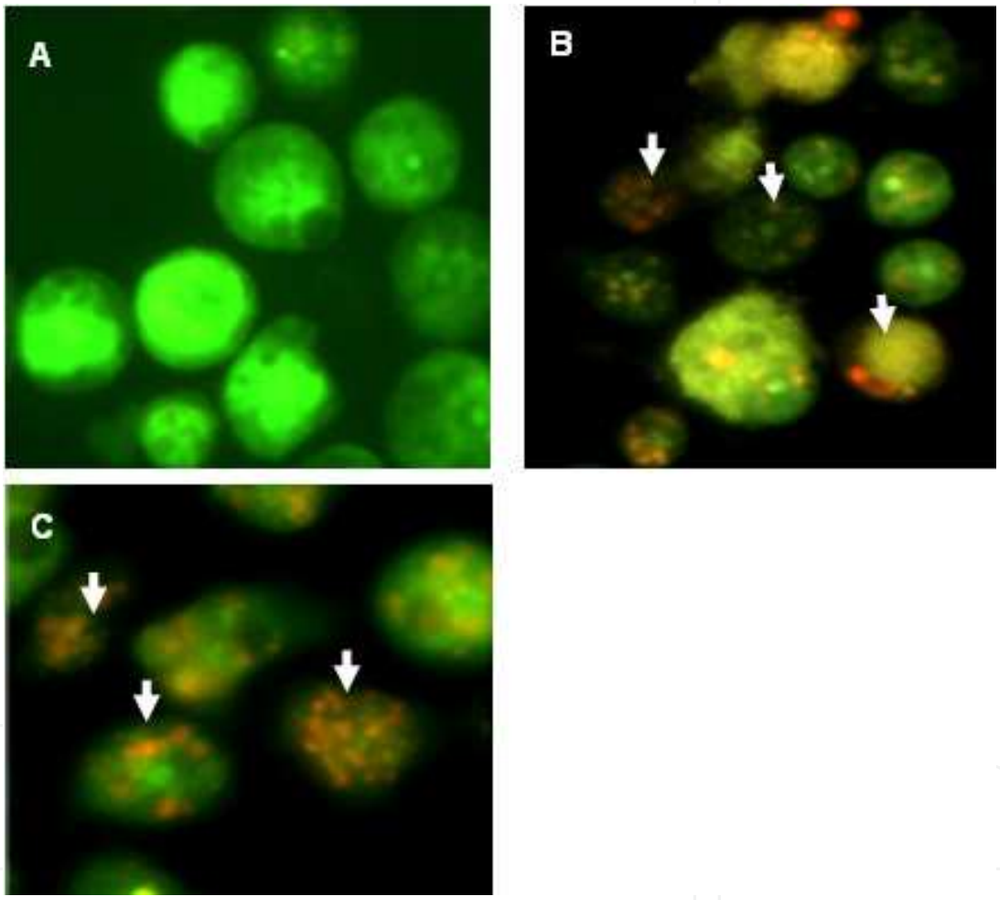

Fig. 4. Fluorescence microscopy of *Acanthamoeba* sp. (SW isolate). (A) Untreated amoebae stained with AOPI dyes display green fluorescence (B) Amoeba stained with AOPI after 72h exposure to cadmium. (C) Amoeba cells stained with AOPI after exposure to mercury. Arrowheads indicate damage on the amoeba membrane. Orange granules inside the cells in B and C are damaged lysosomes. (Magnification X400).

4.4 Determination of apoptosis type of cell death by DNA laddering assay

DNA laddering is one of the more reliable techniques presently employed to study apoptosis both *in vitro* and *in vivo*. Results showed that all metals cause inter-nucleosomal

DNA cleavage in *Acanthamoeba* as shown in the DNA laddering assay to suggest apoptotic type of cell death was induced in this amoeba when exposed to the metals (Fig 5). In contrast, there was no evidence of internucleosomal cleavage or ladder pattern observed from control amoeba. Interestingly, there are two DNA fragments were observed in amoeba treated with Pb and Zn but only one fragment observed in Hg and Cd-treated amoeba. Different mechanism probably involved for Pb and Zn while inducing apoptosis in this amoeba which deserves further analysis and study. Apoptosis occurs in cells injured by certain levels of toxic agent. It is also crucial process for eliminating cancer cells (Guchelaar et al., 1997). From this study, IC_{50} value for the metals used was a sufficient amount to create a toxic environment to the amoeba cells. Morphological estimation for apoptosis is based on cell characteristics such as chromatin condensation, formation of apoptotic bodies from one cell (each having a fragmented piece of nucleus surrounded by a viable cell membrane), shrinkage of cytoplasm, and blabbing of plasma membrane with an irregular outline (Searler et al., 1982).

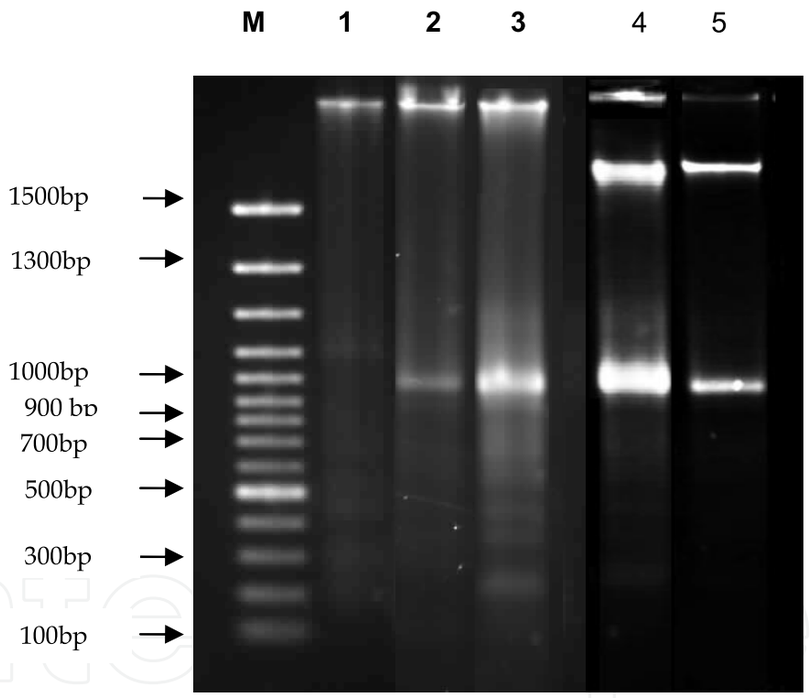

Fig. 5. Agarose gel electrophoresis of genomic DNA of *Acanthamoeba* sp (SW isolate). Explanation for lanes: M-marker, 1-*Acanthamoeba* (control) shows no ladder pattern, 2-amoeba exposed to IC_{50} of cadmium, 3-amoeba exposed to IC_{50} of mercury, 4-amoeba exposed to IC_{50} of lead and 5- amoeba exposed to IC_{50} of zinc. DNA fragmentation at 1000 bp (white arrows) is observed in all metal-treated amoebae, and fragmentation at >1500 bp is observed only in Pb and Zinc-treated amoebae (arrowheads).

DNA fragmentation observed was also reported in other study for humans exposed to lead (Danadevi et al., 2003). Such evidence may be due to a direct effect of lead on the DNA structure, oxidative mechanisms (Stohs & Bagchi, 1995) or indirectly due to another

mechanism involving the activation of caspases in the process of cell death (Saleh et al., 2003). Although most reports on apoptosis are for multicellular organisms, apoptosis to occur in unicellular organisms is also possible (Gordeeva et al., 2004).

Zinc has been reported to prevent apoptosis, an effect assumed to be due to the ability of the metal ion to inhibit a Ca^{2+}/Mg^{2+}-dependent endonuclease (Cohen & Duke, 1984; Waring et al., 1990). However, it is also reported that zinc failed to inhibit the formation of apoptotic cells (Cohen et al., 1992). At higher concentration of zinc, when methallothionien is induced, the level of oxidant decreases and therefore causes oxidative damage to DNA by some unknown mechanism.

4.5 Genotoxicity of metals on the amoeba genome

The toxicity of the metals used in this study not only affects the amoebae at the cellular level, but also affect the amoeba's DNA. The scoring of DNA damage was done manually based on the comet length and intensity of the tail and categorizing cells into five classes based on extent of migration of DNA with strands breakage during electrophoresis (Collins, 2004). In this way, the percentage of DNA damage in the comet tail and their score of the tail can be determined. DNA damage was categorized into five classes (as score 0, 1, 2, 3 and 4) during electrophoresis (Fig. 6). The damage in the DNA with scores 4 and 3 are considered very severe and irreversible, compared with scores 2, 1 and 0 (intact DNA). The data obtained were graphically expressed in histograms by plotting frequency of comets and the corresponding DNA damage measurement for each metal.

Assessments of genotoxic effect of mercury, cadmium, zinc and lead on *Acanthamoeba* are illustrated in Figs. 7 and 8. The DNA damage with scores 4 and 3 were only observed in mercury and cadmium-treated *Acanthamoeba* to indicate the degree of toxicity of the two metals imposed on this amoeba. On the other hand, in lead and zinc-treated amoebae, only the DNA damage at score 1 and 2 were observed.

DNA damage of the amoeba cells is due to inadequacy in tolerance capacity of the phytochelatin to arrest the metal ions and forming extensive double-strand breaks. Several investigations have concluded that based on the characteristic appearance of the comets, apoptotic cells can be distinguished from necrotic cells in the alkaline comet assay (Olive et al., 1993: Fairbairn et al., 1996: Kizilian et al., 1999). Apoptotic cells were concluded to form comets with large fan-like tails and small heads while necrotic cells were concluded to form comets with relatively large heads and narrow tails of varying length.

Alkaline comet assay DNA electrophoresis is sensitive in detecting DNA single strand breaks and alkali-labile damage in individual cells (Gedik et al., 1992; Singh et al., 1988). In addition, the visual classification system should be regarded as a reliable way of measuring DNA migration in the comet assay because it is a common practice that only one investigator analyze the samples within one investigation (Forchhammer, 2008). As comet assay is a very sensitive method for detection of DNA damage, a low dose level would be expected to give an acceptable effect compared to high dose that might produce false cytotoxic effect and to avoid only comet with only score 4 found (Hartmann et al., 2003).

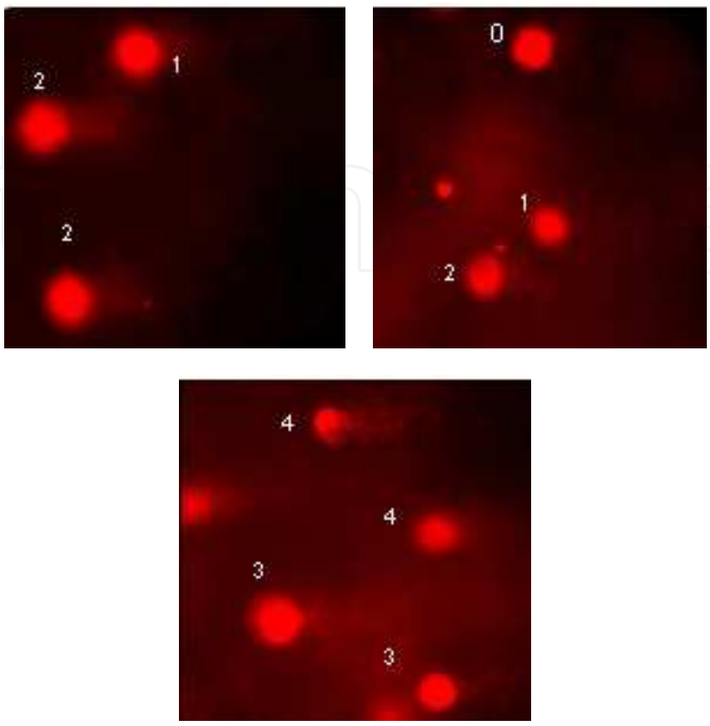

Fig. 6. Cells of *Acanthamoeba* sp. with different scores of the DNA damage following Collins's descriptions after 2 h treatment with the metals. Two hundred of cells on a slide were counted and data obtained are presented in Fig. 7 and Fig. 8.

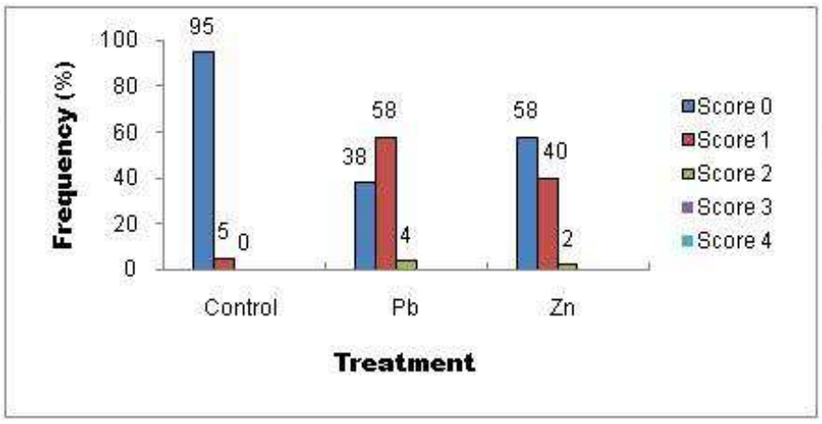

Fig. 7. Frequency of DNA damage scoring of the *Acanthamoeba* sp. (SW isolate) after treated with IC_{25} of lead and zinc. Each data are significantly different among group ($p < 0.05$)(Kruskal-Wallis test).

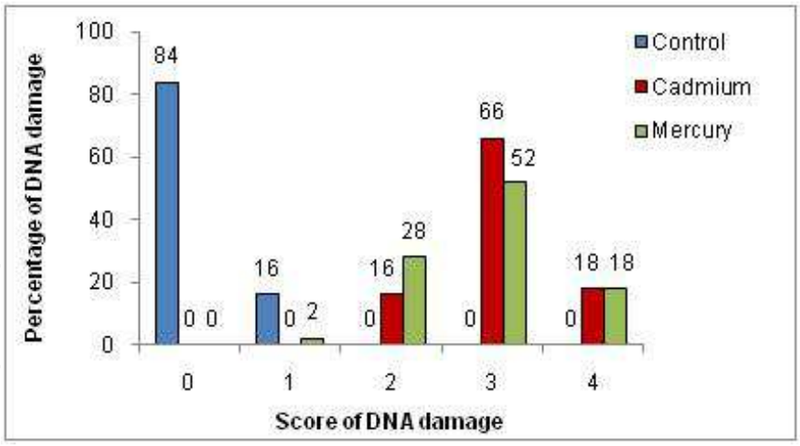

Fig. 8. DNA damage scoring following 2 h treatment with IC_{25} of cadmium and mercury on *Acanthamoeba* sp. (SW isolate) assessed by alkaline comet assay. Each data are significantly different among group ($p < 0.05$)(Kruskal-Wallis test).

In the present study, cadmium is observed to be the most toxic to metals to the amoeba followed by mercury, lead and zinc. The results obtained in a part reflect the chemical and physical properties of the elements and their positions in the Periodic Table. The increase in atomic number (number of protons) from the upper right corner of the periodic table to the lower left corner is reflected in the formulas of the oxygen acids of the elements in their highest states of oxidation. The smallest atoms group only three oxygen atoms about themselves while the larger atoms coordinate a tetrahedron of four oxygen atoms (Holden, 2001).

This study was aimed to investigate the effect on the excess of the metals in the environment to living organisms such as amoeba. Maximum permissible concentration in the environment for all metals used in the present study is shown in Table 2.

Heavy metal	**Max conc. in air (mg/m3)**	**Max. conc. in sludge (soil) (mg/ Kg or ppm)**	**Max. conc. in drinking water (mg/l)**	**Max conc. in H_20 supporting aquatic life (mg/l or ppm)**
Cd	0.1-0.2	85	0.005	0.008
Pb	-	420	0.01	0.0058
Zn	1.5	7500	5.0	0.0766
Hg	-	< 1	0.002	0.05
Ca	5.00	Tolerable	50	Tolerable >50
Ag	0.01	-	0.0	0.1

Table 2. United State Environmental Protection Agency (USEPA) maximum contamination levels for heavy metal concentration in air, soil and water with World Health Organization (WHO) guidelines (Duruibe *et al*., 2007).

Heavy metal cations enter the amoeba cells thus gave a physiological or toxic effect to the amoebae therefore affect the growth of the amoeba population. The presence of heavy metals in high concentration in the culture medium of amoebae in this study creates unfavorable or harsh condition to the amoebae to live, therefore promotes the conversion of amoeba trophozoites into cysts stage. Cysts are non-motile inactive stage in the life cycle of protozoa. There is no any form of reproduction occurs in cyst stage of *Acanthamoeba* and as a result no growth or increasing in number of cells in the population was observed.

In higher organisms, lead toxicity can affect their organ system as well as at molecular level. Proposed mechanisms for toxicity involve fundamental biochemical processes. These include lead's ability to inhibit or mimic the actions of calcium which can affect calcium-dependent or related processed to interact with proteins including sulfhydryl, amine, phosphate and carboxyl groups which result in denaturation and inactivation of enzymes and disruption of cell organelle membrane integrity and cell division (Ochiai, 1987). Similar disturbance might occur to the amoebae when exposed to the four metals used but such study was not conducted in the present study.

Heavy metals are known to disturb biochemistry process and also inactivated the enzyme systems in humans (Wiley et al., 2008), as well as in free living amoebae. Heavy metals ions combine with proteins, often with the sulfhydryl groups and inactivated the protein. They may also precipitate the cell proteins. Heavy metals such as cadmium and mercury ions being more hydrophobic than amoebae cell membranes, thus they can cross the cell membranes easily (Wiley et al., 2008). In eukaryote cell membrane, the major lipids are phosphoglycerides, sphingolipids and cholesterols. These lipids appear to participate in a variety of cellular processes, including allowing transportation through the membrane.

Zinc plays a vital role in the biosynthesis of nucleic acids, RNA polymerase and DNA polymerase thus involved in the healing processes of tissues in mammals. However, zinc has been shown to exert adverse reproductive, biochemical, physiological and behavioral effects on a variety aquatic organism (Sirover & Loeb, 1976). The toxicity of zinc to such organisms is influenced by many factors such as the temperature, hardness and pH of the water. Previous study reported that zinc inhibited the activity of DNA polymerase-1 activity in *E. coli* (Miyaki et al., 1977).

All experiments in this study were done using the trophozoites, active stage of amoebae. Trophozoites stage is more sensitive to heavy metals treatments due to absence of cell wall (Diaz et al., 2006). Since amoebae are eukaryotic cells, therefore in many aspects, their morphology and physiology are similar to the cells of multicellular animals, including human. Hence, the cytotoxicity effects of cadmium, mercury, lead and zinc on amoebae can be used for estimating the cytotoxic effects for humans as well.

5. Conclusion

Toxicity of heavy metals (such as zinc, lead, cadmium and mercury) in the environment can be assessed using *Acanthamoeba* sp, a type of free-living amoebae. Morphological observation, cell viability, IC_{50} values, type of cell death and the DNA damage in *Acanthamoeba* are tools to reveal the hazard properties of the metals to living organisms.

6. Acknowledgment

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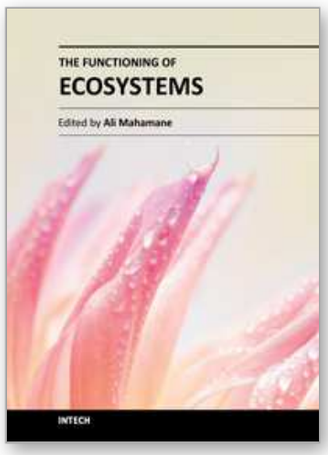

The Functioning of Ecosystems

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The ecosystems present a great diversity worldwide and use various functionalities according to ecologic regions. In this new context of variability and climatic changes, these ecosystems undergo notable modifications amplified by domestic uses of which it was subjected to. Indeed the ecosystems render diverse services to humanity from their composition and structure but the tolerable levels are unknown. The preservation of these ecosystemic services needs a clear understanding of their complexity. The role of the research is not only to characterise the ecosystems but also to clearly define the tolerable usage levels. Their characterisation proves to be important not only for the local populations that use it but also for the conservation of biodiversity. Hence, the measurement, management and protection of ecosystems need innovative and diverse methods. For all these reasons, the aim of this book is to bring out a general view on the biogeochemical cycles, the ecological imprints, the mathematical models and theories applicable to many situations.

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