Acute Toxicity of Some Heavy Metals to the Freshwater Amoebae *Vahlkampfia ustina*

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Abstract

In-vitro toxic effects of nickel (Ni), copper (Cu), cadmium (Cd), chromium III (Cr), iron (Fe), zinc (Zn), cobalt (Co) and arsenic (As) towards the freshwater amoebae *Vahlkampfia ustina* were studied. The maintained *Vahlkampfia ustina* amoebae were exposed to different concentrations (0.01, 0.1, 1, 3, 5, 7 and 9 mg/l) of the tested heavy metals for different contact times (1, 10, 24, 48, 72 and 96 h). The average median lethal concentrations (LC_{50}) of nickel, copper, cadmium, chromium, iron, zinc, cobalt and arsenic on *Vahlkampfia ustina* amoebae were 4.9050, 1.0372, 3.3258, 2.6643, 0.6804, 5.7099, 7.8285 and 7.8487 mg/l, respectively. Statistically, the interaction between the tested heavy metals, used concentrations and different contact times had a significant toxic effect on *Vahlkampfia ustina* amoebae. In descending order, the highest lethal effects of tested heavy metals on *Vahlkampfia ustina* amoebae were as follows: Fe > Cu > Ni> Cd > Zn > Co > As.

1. Introduction

Freshwater ecosystem is one of the environments most seriously affected by pollution. Water pollution may be of biological and/or chemical origin. Biological contaminants come from sewers and failed septic system, boat toilets, animals and other sources. The chemical contamination may be due to organic or inorganic substances. The organic chemical pollutants include fertilizers in runoff from agricultural fields and seeping of petroleum from tankers. Inorganic pollutants include metals, acid rain caused by volcanic discharges, acid pollution of lakes by runoff from acid soils and chemical wastes (byproduct) of factories. The hazards of these pollutants are not only their high toxicity, but also their longevity as most of them persist in the environment, often unchanged for a long period, and can reach human food and drinks (Allen, 1995).

Metals exert biological effects that can be beneficial or harmful. Many metals such as Fe, Cu, Co, Mn, Zn, and Cr are essential for human health, although they can also cause harmful effects at high doses. Other metals such as Hg, Pb, Cd, and As aren't essential for human health and they are toxic even in low concentrations. However whether a metal in the environment can cause an adverse effect to the ecosystem and human health depending on exposure and bioavailability on how much of the metal enters the body and reaches the critical target organs (Caussy et al., 2003). Heavy metals are common pollutants of sewage, particularly where

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there is an input of industrial wastes. Freshwater environments can get polluted with heavy metals through discharged final effluents of waste treatment plants. Heavy metals are toxic to most micro-organisms at certain concentrations (Lester, 1983).

The contamination of aquatic environment by heavy metals is greatly concerned because of the presence of these residues in varying quantities in different compartments of the aquatic ecosystem. Heavy metals severely affected the growth, morphology and metabolism of micro-organisms through functional disturbance, protein denaturation or the destruction of the integrity of cell membranes (Leita et al., 1995).

Freshwater amoebae are unicellular sarcodines occurring naturally in the aquatic habitats and moist soil. They are common and important organisms of ecological communities within different substrates and biofilms (Anderson, 2000). They are particularly numerous in soils, but are also abundant in freshwater and marine water habitats. They move by means of the so-called amoeboid movement and don't have constant body form (Rogerson and Patterson, 2000).

To our knowledge only scarce data were published concerning the use of freshwater amoebae as bioindicators for chemical toxicity (Fernandez-Leporans and Herrero, 2000 and Walochnik et al., 2002, Al-Herrawy and Hikal, 2005). Therefore, the objective of this study isolation, identification, purification and maintenance of some predominant strains of freshwater amoebae to be used as test organisms. Also preliminary assessment of toxic effects of some heavy metals to the isolated and purified strains of freshwater amoebae.

2. Materials and Methods

2.1. Test Organism

Freshwater amoebae have been concentrated and isolated from the collected Nile water samples according to the method of Al-Herrawy et al. (2013). The isolated freshwater amoebae were identified morphologically according to Page (1988). The identified strains of amoebae were cultured mono-xenically on non-nutrient (NN) agar plates previously seeded with 100 μl *Escherichia coli* that were used as a source of food for the growth of free-living amoebae according to the method of Al-Herrawy et al. (2014). Isolated and purified freshwater amoebae were identified on the bases of both trophozoite and cyst morphology and physiology (Pussard and pones, 1977).

2.2. Heavy Metals Used

Nickel (Ni) in the form of Nickel (Π) chloride anhydrous, Copper (Cu) in the form of copper sulphate pentahydrate, Cadmium (Cd) in the form of cadmium chloride trihydrate,

Trivalent Chromium (Cr) form of chromium trioxide, Iron (Fe) in the form of ferric chloride anhydrous, Zinc (Zn) in the form of zinc sulfate monohydrate, Cobalt (Co) in the form of cobalt chloride hexahydrate and Arsenic (As) in the form of sodium arsenate were used. All of the selected metals were dissolved in distilled water. Stock solutions of these metal compounds were prepared on the basis of molecular weight of metal contents where 1 ml from stock solution contained 1 mg from the metal.

2.3. Toxicity Test Procedures

A short-term static relative sensitivity toxicity test was used in the present work according to duration, method of adding test solutions and purpose of the experiments (American Public Health Association, 1998). The isolated test organisms (freshwater amoebae) were separately exposed to duplicate containers of each experimental concentration used. A control sample including amoebae alone was presented with each experiment. From the control amoebae sample, the percentage of positivity (death) was calculated (Al-Herrawy and Hikal, 2005).

2.4. Mass Production of Selected Test Organisms

The selected strains of freshwater amoebae (resulting from previous isolation, purification and identification steps) were used as test organisms. They were separately cultured on GS agar plates and incubated at 30°C for 3-5 days. The produced enormous numbers of freshwater amoebae were collected from the agar surface as they were still in the trophic stage. The collected trophozoites were washed 3 times with distilled water and centrifuged at 500 xg for 5 min. The supernatant was discarded while sediments were re-suspended in 2 ml distilled water and counted using Sedgwick Rafter counting cell (Al-Herrawy and Hikal, 2005).

2.5. Preparation of Selected Toxicant Concentrations

Stock solutions of the 8 selected heavy metals were separately used for the preparation of desired different concentrations. Three basic preliminary concentrations (1, 0.1 and 0.01 mg/l) were prepared and tested for each toxicant. According to the obtained results from the three preliminary tested concentrations, other ascending (3, 5, 7 and 9 mg/l) or descending (0.007, 0.004 and 0.001 mg/l) concentrations were prepared and used for 1, 10, 24, 48 and 96 h exposure periods (Hikal, 2005).

2.6. Determination of Median Lethal Concentrations (48 and 96-hr LC_{50})

Median lethal concentration (LC_{50}) was defined as the

concentration at which 50% of the test organisms died within a definite time of exposure to the toxicant. Amoebae were equally distributed into Petri dishes each containing one of the previously prepared concentrations of each metal. The mean of three replicates of each metal concentration was calculated. A petri dish containing amoebae only with distilled water was used as a control. After 96 h of exposure, treated amoebae were examined microscopically to detect metal toxicity through loss of movement, rounding and encystations. When stained with 1% vital stains (e.g. trepan blue), living amoebae didn't take stains, while the dead ones stained with blue color.

2.7. Statistical Analysis

The obtained data were subjected to analysis of variance (ANOVA) according to Snedcor and Cochran (1990). Least significant differences (LSD) were used to compare between means of treatments according to Waller and Duncan (1969) at probability 5% and 1%. Data were statistically analyzed using "MSTATC" computer program V. 2.1 (1985).

3. Results

3.1. Isolation of Free-Living Amoebae from Nile River Water
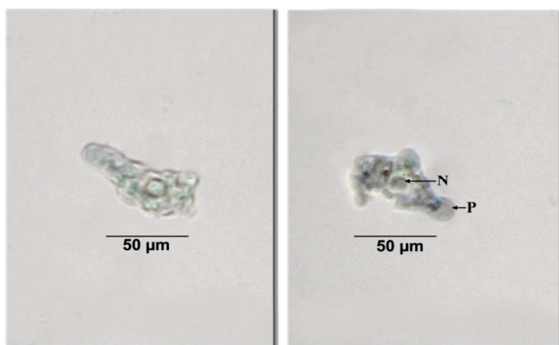
(A)
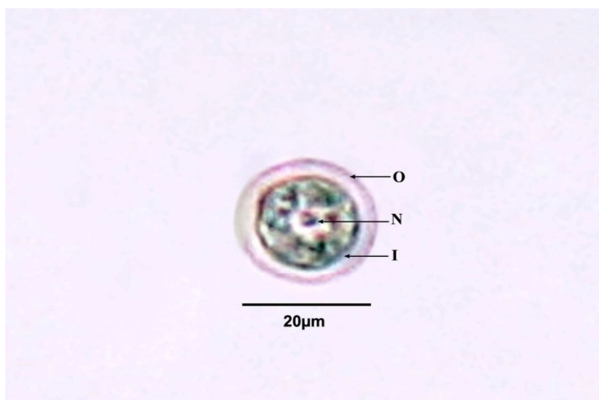
(B)

Fig. 1. *Vahlkampfia ustina* amoebae. A) Trophic form. N: nucleus P: pseudopodia B) Cyst form. O: outer cyst wall I: inner cyst wall N: nucleus

Identification of the collected freshwater amoebae (from Nile river) revealed the isolation of a pure strain of *Vahlkampfia ustina*. The trophic stage measured 20-50 μm in length and 5 μm in width with the nucleus measuring 5 μm in diameter. The cyst was smooth walled structure without exit pores, round to slightly oval in shape, and measured 10-15 μm in diameter {Figure 1 (a,b)}.

3.2. Lethal Effect of Tested Heavy Metals on *Vahlkampfia ustina*
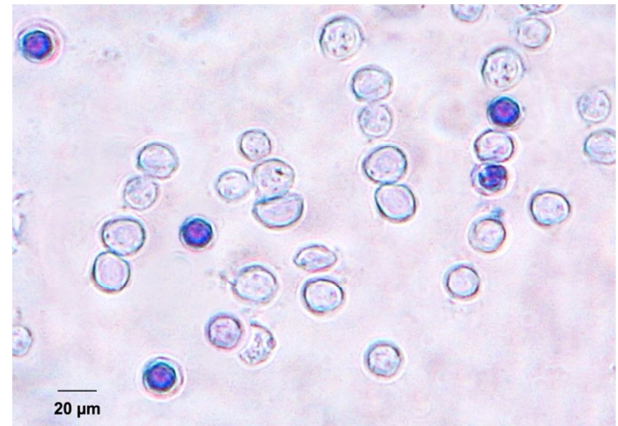

Fig. 2. Cysts of *Vahlkampfia ustina* stained with trepan blue. Dead cysts appeared blue while alive cysts appeared unstained.

Mortality values, corresponding to loss of viability, were estimated by loss of the ability of amoebae to multiply on sub-culturing. Also dead amoebae took the blue color when stained with trepan blue stain, while living amoebae were not stained with this vital stain (Fig.2). The test organism (*Vahlkampfia ustina*) was used to study the lethal effects of the 8 previously mentioned heavy metals (Ni, Cu, Cd, Cr, Fe, Zn, Co and As) in different doses (0.01, 0.1, 1, 3, 5, 7 and 9 mg/l) and different contact times (1, 10, 24, 48, 72 and 96 h). Results of toxicity test with amoebae were summarized in Table (1) and figures 3,4. The acute toxicity to *Vahlkampfia ustina* increased with the increase in metal concentrations.

Table 1. Median lethal concentrations (48 and 96-h LC$_{50}$) of tested heavy metals on *Vahlkampfia ustina*

Metals	LC 50 (mg/l)		
	Vahlkampfia ustina		
	48 hr	96 hr	Mean
Ni	5.454000	4.356000	4.905000
Cu	1.515000	0.559400	1.037200
Cd	5.543100	1.108500	3.325800
Cr	4.621100	0.707400	2.664250
Fe	1.293100	0.067830	0.680465
Zn	6.083200	5.336600	5.709900
Co	8.469600	7.187300	7.828450
As	8.368300	7.329100	7.848700
	Time (A)	Metals (B)	(AB)
LSD 5%	0.32420	0.64840	0.91700
LSD 1%	0.43590	0.87170	1.23300

The numbers of living cells were significantly decreased in

all metals compared with control (P<0.01). The number of living cells was decreased as the concentration of the metal ions increased (P<0.01).
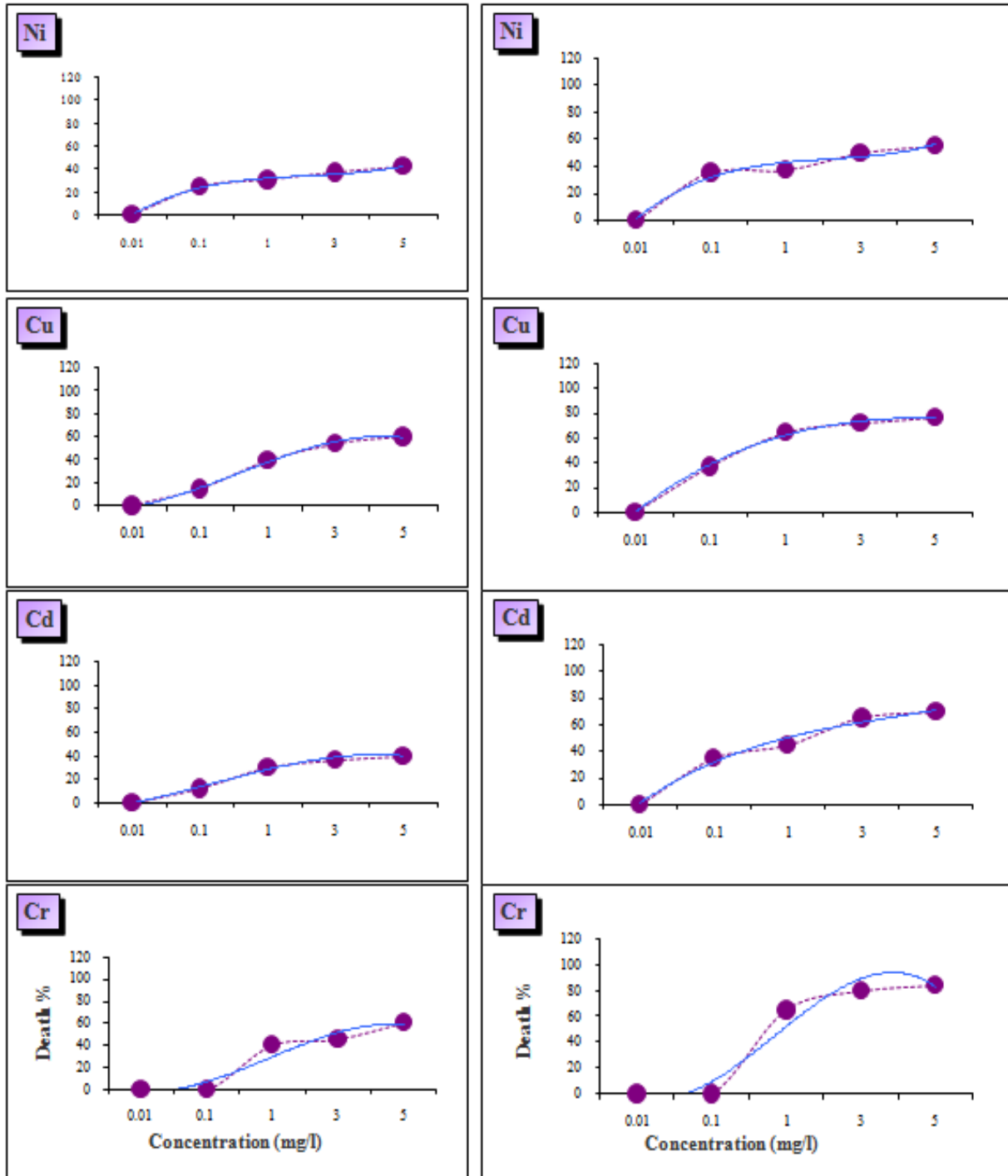

Fig. 3. Lethal concentrations (48, 96-h LC50) of Ni, Cu, Cd and Cr on *Valkampfia ustina*
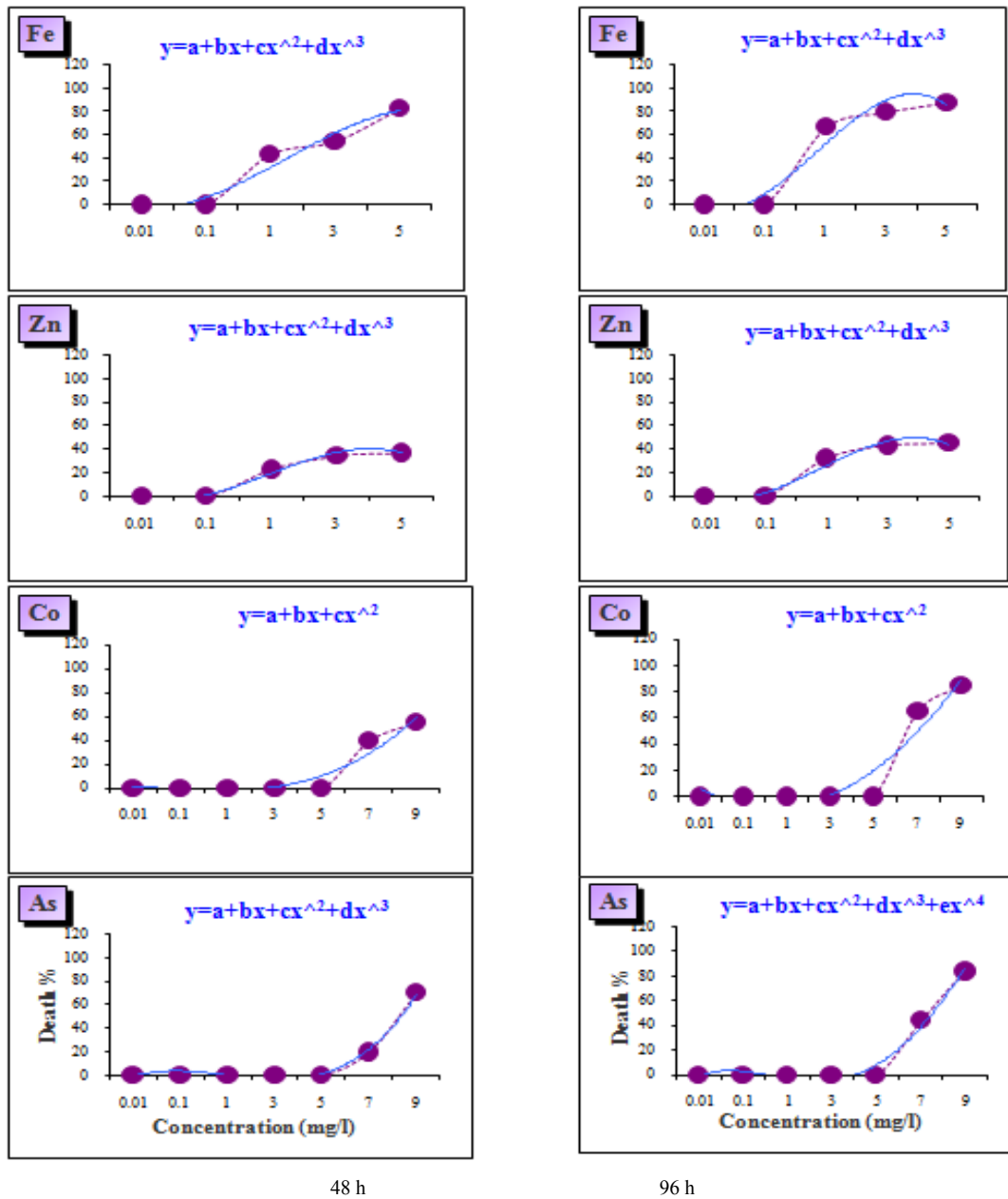
Fig. 4. Lethal concentrations (48, 96-h LC50) of Fe, Zn, Co and As on *Valkampfia ustina*

4. Discussion

In an aquatic environment, metals occur both in the dissolved or soluble fraction and in particulate matter. It has been suggested that they may block enzyme systems or interfere with some essential cellular metabolites of bacteria and protozoa (Morgan and Lackey, 1958). Metals also act on the cell membrane and interfere with cytoplasmic or nuclear functions after entry into the cell (Larsen and Nilsson, 1983). To our knowledge only scarce data were published concerning the toxicity of heavy metals to freshwater amoebae.

In the present work, Nickel is one of the major elemental constituents of the earth, constituting about 2% by weight.

Nickel is present in significant concentrations in industrial and municipal discharges, particularly in steel mill and electroplating wastes (Snodgrass, 1980). Nickel and its compounds cause a variety of cancer in rodents and are listed as possible causative agents for occupational or environmental cancer in man, but its toxicity toward aquatic biota is not well known (Dunnick et al., 1995).

Ni showed severe toxic effect on *Vahlkampfia ustina,* The 48 and 96-hr LC_{50} values for *Vahlkampfia* were 5.454000 and 4.356000 mg/l, respectively. Higher lethal concentrations were obtained by Al-Rasheid and Sleigh (1994) as they found that the survival rate of *Euplotes mutabilis* (ciliated protozoa) after 1 hr in 2 mg/l nickel was 58%. Only 42% survived after 1 hr in 4 mg/l nickel solution. The calculated 1-hr LC_{50} for

nickel at 20°C was 3.9 mg/l. There was a significant decrease in particle uptake in the surviving cells compared to the control, however there was no significant difference between the two concentrations. Also, Larsen and Nilsson (1983) found that in an organic medium nickel inhibited the movement of the ciliate *Tetrahymena pyriformis* at a concentration of 352 mg/l, which also reduced the rate of endocytosis.

Madoni (2000) also found large differences in sensitivity of 12 freshwater ciliates to nickel. *Spirostomum teres* showed the highest sensitivity (0.17 mg Ni^{-1}, 24-hr LC_{50}), while *Euplotes patella* was the most tolerant species (7.7 mg Ni^{-1}, 24-hr LC_{50}).

Copper is essential micronutrient required for growth and metabolism but become potentially toxic in excess amounts (Cairns et al., 1980; Bogaerts et al., 2001). Cu can interact with radicals, oxygen in particular. These radicals also cause Cu to become toxic (Aksoy et al., 2004). In the present work, the mean LC_{50} value for *Vahlkampfia* was (1.0372 mg/l) This result was in accordance with that of Madoni et al. (1996) as they also found that the LC_{50} values of copper were 0.31- 2.05 mg/l for protozoa in an activated sludge community. On the contrary, other workers found that there was almost no mortality for the ciliate *Tetrahymena pyriformis*, but growth and grazing were still heavily inhibited by exposure to 145 mg/l copper (Nicolau et al., 1999). Again, Yamaguchi et al. (1973) observed complete inhibition of growth of *Tetrahymena pyriformis* in axenic cultures at 63.5 mg/l. In contrast, Piccinni et al. (1987) observed no effect on growth following addition of 10 mg/l copper to axenic cultures of *Tetrahymena pyriformis*. Nilsson (1981) stated that the addition of 100 mg/l copper to the normal 2% proteose peptone medium was tolerated by *Tetrahymena* and stimulation of phagocytosis occurred. Unz and Shuttleworth (1996) reported that concentrations higher than 63.5 µg/l copper inhibited the growth of filamentous micro-organisms, while Dilek and Yetis (1992) found that up to 10 mg/l Cu concentration did not affect micro-organism kinetics in the activated sludge.

Cadmium is one of the most toxic bivalent heavy metals. The mechanisms of its toxicity are related to the capacity for association with thiol groups of proteins (Fernandez-Leborans and Novillo, 1994). In the present work, The mean LC_{50} value was (3.3258 mg/l) for *Vahlkampfia.* Treating shortly freshwater *Amoeba proteus* with a high concentration of cadmium, Ord and Al-Altia (1979) observed significant changes in the fine organization of mitochondria, endoplasmic reticulum and nuclei, as well. In an ultrastructural study of *Tetrahymena* cells exposed to 10 µM Cd for 2 days, Krawczynska et al. (1989) noticed increase in number of vacuoles and lipid droplets in the cytoplasm similarly to that described by Dunlop and Chapman (1981)

and Pyne et al. (1983). Also observations by Martin-Gonzalez et al. (2005) showed that Cd at the concentrations from 0.5 to 2 mg/l induced similar ultrastructural alterations in the vegetative cells of the ciliates *Colpoda steinii*, *Cyrtolophosis elongate* and *Drepanomonas revolute*. Also about 5-10% of exposed vegetative cells were transformed into resting cysts which have no detectable metabolism and surrounded by a complex resting cyst wall composed of several biochemically and ultrastructurally distinct layers (Gutierrez and Martin-Gonzalez, 2002; Gutierrez et al., 2003).

Hexavalent chromium {Cr(VI)} contamination in the environment is a result of the extensive use of chromate and dichromate in numerous industries including alloy manufacturing, electroplating dyes and pigments manufacturing wood preserving, leather tanning, power plants, and nuclear facilities (Langard, 1980; Patterson, 1985; Riley et al., 1992; James, 1996; Barnhart, 1997). Chronic human exposure to Cr(VI), even at low concentrations, can lead to several detrimental health impacts including carcinogenesis and mutagenesis (Yassi and Nieboer, 1988; World Health Organization, 1990; National Toxicological Program, 1991; Hayes, 1997).

In the present work, the mean LC_{50} value of Cr was 2.66425 mg/l for *Vahlkampfia* amoebae. These values agreed with those by Madoni *et al*. (1996) in which 26.2 mg/l of Cr (VI) lowered the protozoan density by only 8%, whilst 68.8 mg/l of Cr (VI) raised the mortality to 42%. They also found that the very low toxicity of chromium could be ascribed to the change in the valency state of Cr from the hexavalent to the less toxic and soluble trivalent form.

In the present work, the mean LC_{50} value for iron reached 0.680465 mg/l.

Cobalt is one of the essential trace elements for humans. It enters the composition of vitamin B_{12}, as a main cofactor. This element accumulates in the core structure of micro-organisms (Aksoy et al., 2004). Cobalt ions bind to sulphydril (-SH) groups and form complexes, leading to toxic effects on living cells (Nilsson, 1999). In the present work, the mean LC_{50} value was 7.82845. Ermolli et al. (2001) reported that 0.475 mM Cobalt produced a toxic effect on hacat human keratinocytes and that this effect could only be observed after 4 hr. Aksoy (2004) found that the lethal effect of Co on *Entamoeba histolytica* was presented at lower concentrations (0.05 mM) and was increased dose-dependently. It was also observed that *Entamoeba histolytica* cysts maintained their vitality, though at a minimal level at all the three concentrations (0.05, 0.1 and 0.2 mM) at the end of the 7th hr. No living parasite remained in the culture medium at the end of the 24th hr. Co at low concentrations has a slowly developing lethal effect on *Entamoeba*

histolytica cysts.

Zinc is an essential trace element in living organisms. The regulation of the intracellular concentration of Zn is of great importance. A deficiency in Zn affects proper functioning of various organs (Goyer, 1991) and disturbs cell development, differentiation and cell division (Vallee and Falchuk, 1993). Excess amounts of Zn doesn't seem to have any adverse long time effects in humans (Vallee and Falchuk, 1993) but whereas some reports indicate that Zn may inhibit apoptosis (Zalewski et al., 1991), others suggest that Zn actually induces apoptotic cell deaths (Haase et al., 2001) and propose Zn as a potential cytotoxic agent in treatment of thyroid cancer (Litaka et al., 2001). Inhalation of Zn causes metal fume fever (Lander and Hoagland, 1998).

In the present work, the mean LC_{50} value for *Vahlkampfia* (5.70990 mg/l). Nicolau et al. (1999) declared that zinc caused inhibition of growth, grazing and high mortality of *Tetrahymena pyriformis* above 45 mg/l. At a concentration of 15 mg/l zinc inhibited growth by more than 50% and inhibited grazing capacity almost completely, but did not induce mortality. Madoni et al. (1996) found LC_{50} values of 0.57 50.0 mg/l of zinc for protozoa in an activated sludge community. The LC_{50} of Zn was not determined by Nicolau et al. (1999), but protozoa mortality rates around 50% after 24-hr of exposure was determined for higher doses above 45 mg/l zinc. Zinc affected *Tetrahymena pyriformis*, causing more than 50% decrease in ATP (Adenosine-5- Triphosphate) levels at 15 mg/l and inhibition of ACP (Acid Phosphates) activity in the cultures at 45 mg/l (Nicolau et al., 2004).

Arsenic is a naturally occurring metal in aquatic ecosystem, but its levels are increasing due to pollution. The background levels of most unpolluted freshwaters are below 1µg/l of arsenic (Moore and Ramamoorthy, 1984; Blanck et al., 1989). Arsenic is used for the manufacture of agricultural chemicals and synthesis of various inorganic and organic compounds. Arsenic is not known to be essential for any animals, and it can cause cancer, heart, liver and other neurological disorders (Phillips, 1990). It isn't among the very toxic substances, as its toxicity starts at concentrations higher than 1 mg/l (Dojlido and Best, 1993).

In the present work, the mean LC_{50} value of As for *Vahlkampfia ustina* amoebae was 7.8487 mg/l. (Tisler and Zagorc-Koncan , 2002) found that the acute arsenic toxicity to *Vibrio fischeri* increased slowly with increasing concentrations. The 30-min EC_{50} value for this luminescent bacterium reached 72.4 µg/l. They also recorded that the 96-hr LC_{50} values of arsenic reached 28.1 mg/l for Zebra fish and 15.3 mg/l for rainbow trout. The median lethal concentration for rainbow trout was 13.0 mg/l which is similar to the previous data (Bartell *et al.*, 1992). The LC_{50}

values of arsenic for different fish species were between 0.05 and 59 mg/l depending on age, species, and test conditions (Moore and Ramamoorthy, 1984; Crompton, 1997).

In conclusion, the highest lethal effect of tested heavy metals, for *Vahlkampfia* amoebae, in descending order was as follows: Fe > Cr > Cu > Ni > Cd > Zn > Co > As However, further investigations are necessary to insure that *Vahlkampfia* amoebae can be used as a test organism for toxicity bioassay tests.

5. Conclusion

Vahlkampfia ustina could be used as sensitive and convenient early warning bioindicators for the detection of toxicity of waters polluted with heavy metals.

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