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Daphnia Pulex Toxicity Testing of Ethylenediaminetetraacetic Acid Tetrasodium Salt Dihydrate and the Wastewater Effluent from Extraction of Rhodium using Emulsion Liquid Membranes

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ABSTRACT: Ecotoxicity of rhodium (Rh) from the model of mining waste water side stream was examined in this paper. Rh was extracted from the model of the mining waste water using an emulsion liquid membrane (ELM). The extractions were done at pH of 1.87 and pH 2.92 and 41.6 % and 46.2 % of Rh was extracted respectively. The side streams of pH of 1.87 and pH 2.92 after the extractions were examined for ecotoxicity using *Daphnia pulex* in the acute toxicity testing. All test organisms died after 48 hours of exposure to the side stream effluent with the original pH = 1.87, while 10-60 % survival rates were observed at the mining spent effluent with original pH of 2.92 if the strength of the effluent ranged from 12.5 to 62.5 %. Results for ethylenediaminetetraacetic acid tetrasodium salt dehydrate (EDTA) showed that an increase in concentration of EDTA in the test concentrations lead to the death of *D. pulex*. It was concluded that the very dilute water side streams are toxic hence the discharge of the spent Rh side-stream should thus be discouraged and prevented at all costs.

Key words: *Daphnia pulex*, Emulsion liquid membrane, Ethylenediaminetetraacetic acid tetrasodium salt dehydrate, Rhodium

INTRODUCTION

Membranes have historically been viewed as the semipermeable barriers between two adjacent phases (Kislik, 2010). From the operational point of view, membranes are easy to apply at industrial scale for the following reasons (Kislik, 2009): the underlying scientific concepts are relatively simple, their operation and scale-up is uncomplicated to achieve, they are environmentally-friendly, and low-cost from the energy point of view. This type of semipermeable barrier can consist of polymer films and liquids (Kislik, 2010). If the membrane system is based on a liquid matrix, then it is called a liquid membrane. Such systems involve the contacting of an immiscible organic liquid (named the diluent) with a feed phase i.e. the treated side-stream (Bartsch and Way, 1996; Noble and Way, 1987). During the interphase contact, the metal or pollutant of interest is extracted from the feed phase into the diluent and finally sequestered inside separate aqueous phase called the stripping phase (Bartsch and Way, 1996; Noble and Way, 1987). A specific example of the liquid membrane is the emulsion liquid membrane (ELM). ELM separation technique was invented by Li in 1968 (Kargari *et al.,* 2004; Patnaik, 1995). The ELM has been used in the extraction of metals such as as copper, zinc, nickel and cadmium (Fouad and Bart, 2008) from mining refinery waste water. In this study the ELM was used for the extraction of Rh. Ethylene-diaminetetraacetic acid tetrasodium salt dihydrate (EDTA) is a mono- to tetradentate ligand depending on the pH of the aqueous phase (Kari and Giger, 1996; Repo *et al.,* 2011). EDTA is used to prevent precipitation of metals at high pH through chelation (Kołodyńska *et al.,* 2008). This should include Rh as it is precipitated if the pH of the aqueous phase increases above 4.00 (Barbosa *et al.,* 2007). EDTA may affect the results of metal toxicity which will be considered in data evaluation from the *Daphnia pulex (D. pulex)* acute test (Bergers and de Groot, 1994).The presence of EDTA is necessary in any toxicity test based on the fact that some metals, e.g. Rh are unstable in solution at neutral pH values where the test needs to be performed due to the organism's requirements. *D. pulex* is a Cladocera crustacean belonging to Arthropoda phylum, subphylum Crustacea, Branchiopoda class, and subclass Phyllopoda (Christie *et al.,* 2011; Liu *et al.,* 2014). They are commonly known as water fleas (Liu

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et al., 2014) and they inhabit lakes, eutrophic ponds and rockpods (Chen and Stillman, 2012; Hebert, 1978). *Daphnia spp.* is very sensitive environmental changes (Liu *et al.,* 2014) and they have the ability to physiologically, morphologically and behaviourally adapt to the environmental changes (Connelly *et al.,* 2009; Hunter and Pyle, 2004; Mirza and Pyle, 2009). The ease of laboratory culture, sexual and asexual (parthenogenetic) reproduction make *Daphnia spp.* a model organism in ecotoxicology (Chen and Stillman, 2012; Christie *et al.,* 2011) and in monitoring of the levels of water pollution (Liu *et al.,* 2014). *D. pulex* has been successfully used in various studies to assess the toxicity of metals such as cadmium, zinc, nickel and arsenic (Caumette *et al.,* 2012; Clifford and McGeer, 2010; Kozlova *et al.,* 2009). In a different study the toxicity of copper, lead, zinc and asenic from fresh water was successfully assessed using Daphnia (Caumette *et al.,* 2012; Theegala, Suleiman, and Carriere, 2007).

At the same time, mammalian (including human) toxicity of EDTA is low due to its low absorptivity and this chemical compound is not a carcinogen (Kari and Giger, 1996; Sorvari and Sillanpää, 1996). Thus even though the toxicity of spent side-stream after the ELM extraction process will be affected by the EDTA, if this influence is quantified and taken into account, a general estimate about the Rh toxicity to environmental compartments it is discharged into should be feasible. The preliminary toxicity assessment of the Rh spent side-stream is performed in this study using the acute *D. pulex* toxicity test. There is insufficient data in literature ti indicate that Rh is toxic to human beings. Some studies have shown the sensitizing activity to humans of the hexachlororhodiate ($RhCl_6^{3-}$) (Bingham *et al.,* 2001) and the cytogenetic of rhodium Chloride (RhCl (III)) has been reported (Migliore *et al.,* 2002). The appropriateness of the acute testing is that most metal refinery operations aim to work on the zero-discharge principle, i.e. any spillage of the Rh-bearing materials and side-streams into the environmental will be the result of industrial accidents. The aim of the study was to investigate the ecotoxicity of Rh which remains un-extracted in the ELM technology of metal waste water processing using*D. pulex.* The present study is the first to address rhodium toxicity using *D. pulex.* The principle of the test method is as follows: The daphnids are exposed to the test substance added to water at a range of concentrations for 48 hours. This is because daphnids are highly sensitive and have a short generation time. Under identical test conditions and an adequate range of test substance concentrations, different concentrations of a test substance exert different average degrees of effect on the swimming ability of *Daphnia spp*. Different concentrations result in different percentages of Daphnia being no longer capable of swimming at the end of the test. Immobility of daphnids is used as the end point for toxicity testing. The concentrations causing zero or 100 % immobilizations are derived directly from the test observations whereas the 48-hour EC_{50} is determined by calculation if possible (He *et al.,* 2012; Ton *et al.,* 2012).

MATERIALS & METHODS

The EDTA and the standardised Rh, Kerosene, Polyisobutylene (PIB), and trioctyl amine (TOA) were purchased from Sigma Aldrich (Johannesburg, South Africa), Saarchem brand HNO_3 was purchased from Merck Chemicals Pty. Ltd. (Merck, Port Elizabeth, South Africa), SPAN 80 and TBP from Fluka Analytical (Johannesburg, South Africa). An Olympus UCMAD3 microscope mounted with an Olympus ultra 20 soft imaging system UTVX-2 was used for all microscopic work (Institute for Water Research, Rhodes University, South Africa). All glassware was purchased from Sigma-Aldrich (Johannesburg, South Africa). The *D. pulex,* for the 48 hour acute *D. pulex* toxicity test was purchased from the Institute for Water Research at Rhodes University (Grahamstown, South Africa). For the acute 48-hour toxicity testing, the *D. pulex* was cultivated in the dilution water described in ISO 6341 in the temperature of 20 ± 2°C in the light rhythm of 16 hours light and 8 hours dark. The green alga *Chlorella* sp. was used as a food source. The cultures were prepared in 2 L containers, with an initial population density of one daphnid per 100 ml. The neonates were separated and used for toxicity determination. The organisms were not fed during the experiments. The MilliQ water used in this chapter was prepared by reverse osmosis, using a Milli-RO® 15 water purification system (Millipore®, Bedford, MA, USA), consisting of a Super-C carbon cartridge, two Ion-X® ion-exchange cartridges and an Organex-Q® cartridge. The water was filtered through a 0.22 µm Millipak® stack filter (Millipore®, Bedford, MA, USA). Preliminary experiments indicates that Rh was unstable in solutions when the pH of the aqueous phase increased above 3.14. On the other hand, the *D. pulex* acute toxicity test is conducted at neutral pH values. Thus the first partial task in the toxicity testing was to stabilise the concentrations of Rh in its aqueous solutions around pH 7. For this purpose, solutions of EDTA (purchased from Sigma-Aldrich, Johannesburg, South Africa) in Milli Q water with the EDTA concentrations ranging from 0 to 3 g/L were prepared by dissolving between 0.0000 and 0.7500 g of EDTA in 100 ml of 0.01 M HCl and transferred into a 250 ml volumetric flask. The volume was made up to mark with 0.01 M HCl to make a concentration of 3 g/L EDTA. About 50 ml of this

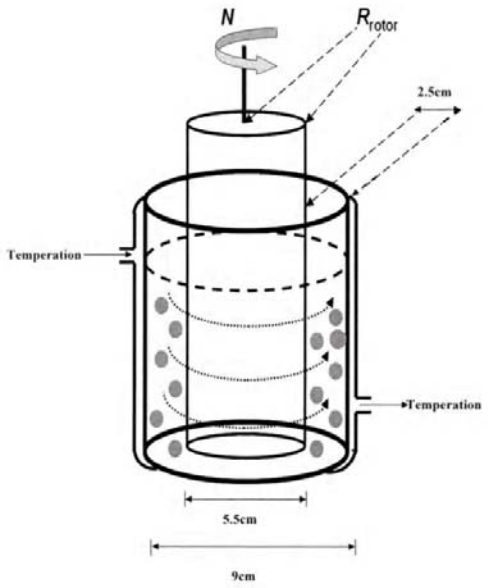

Fig. 1. The TVC. The diameters of internal and external cylinders and the thickness of the external cylinder are shown in the diagram. The membrane globule (●) are dispersed in the feed phase of the system via the rotation of the inner cylinder of the TVC. The dotted arrow around the internal cylinder represent the stirring pattern due to the rotation of the inner cylinder, and point out the even distribution of energy in the entire volume of the ELM-side stream mixture

solution was put into the Erlenmeyer flask and 5 ml of 1000 mg/L standardised Rh solution was added.

The solution was stored at 20 ± 2°C for 72 hours and ICP readings were then taken from this solution for Rh concentration, and any precipitation was observed visually. Another solution was prepared in the same fashion as stated above, but the pH of the final solution was adjusted to 8.4 with 2.5 M NaOH. The solution was incubated at 20 ± 2 °C for up to 3 days. Precipitation and Rh concentrations were assayed periodically over the 72-hour period. No precipitation was observed in any of the solutions and the Rh concentration remained stable within 20 % of the initial value only if the concentration of EDTA was equal to 3 g/L. Therefore the solution prepared in this manner was considered suitable for use in the acute *D. pulex* test. In this experiment, the diluent with extractant contained 5 g PIB which was dissolved in 100 mL of Kerosene inside a 250 mL volumetric flask. To it, 30 mL of toluene (Johannesburg, Sigma-Aldrich, South Africa was added, together with 2.5390 g of TOA and 12.4936 g of SPAN 80. Both TOA and SPAN 80 were weighed out analytically on the PA214 balance and completely dissolved in the kerosene-toluene mixture. Then Kerosene was used to make up the volume to 250 mL to obtain the final ELM concentrations as follows: 20.000 g/L (m/v) of PIB, 10.156 g/L (m/v) TOA and 49.972 g/L (m/v) of SPAN 80.

The first model mining side-stream was a solution of 7 mg/L of Rh (purchased from Sigma-Aldrich, Johannesburg, South Africa) which was prepared in 0.01 M HCl (pH 1.87) by dilution of the standardised Rh solution with initial concentration of 999 ± 5 mg/L. A second model mining side-stream was prepared in the same fashion, but 0.001 M HCl (pH = 2.92) was used as the solvent. The model mining side-streams containing Rh were mixed with the optimised ELM and both were put into the Taylor-vortex column (TVC) (Figure 1) in the ratio 1:5 (10 ml ELM : 50 ml the model mining side-stream). The solutions were then stirred in the Taylor vortex column using a speed of 250 rpm for 30 minutes. The ELM/feed phase mixture was then taken out of the column and placed in a conical flask. It was allowed to stand at 21 ± 2 °C until phase separation was observed between the spent model mining side-stream and the loaded ELM.

The spent mining effluent was always put aside and stored at 5 ± 2 °C until the ICP mass balance analyses and the toxicity testing could be conducted. The procedure was repeated until 250 ml of the spent side-stream was collected for both model mining side-streams. All samples were always analysed within 72 hours of production. Approximately 1 g; 1.5 g and 3 g of Ethylenediaminetetraacetic acid were weighed analytically using the PA1214 analytical balance (Pioneer™, Ohaus Corporation, Johannesburg, South

Africa)and placed in 1000 ml volumetric flasks. They were then dissolved in 500 ml of MilliQ water. Each volumetric flask was then filled to the mark to make 1 g/L, 1.5 g/L and 3 g/L of EDTA respectively. The pH was adjusted using 5 M hydrochloric acid (HCl) up to a pH of around 4. All glassware was purchased from the Sigma-Aldrich (Johannesburg, South Africa). The test concentration was made by pippeting 25ml of the 1g/L EDTA stock solution into a 200 ml volumetric flask. The solution was made up to the mark using the growth medium to make a concentration of 12.5 % of the stock solution. This mixture was hand shaken for mixing.This procedure was repeated using 50 ml, 100 ml, 125 ml, 175 ml and 200 ml of the stock solution making it up to 200ml using the growth medium to make up test concentrations of 25 %, 50% 62.5 %, 87.5 % and 100 % respectively.

a) Each test concentration was divided into four batches of 50 ml each and poured into 100 ml beakers. 5 *daphnids* were put into each solution.

b) The *daphnids*were exposed for 48 hours. The *daphnid*survival was inspected after 24 hours of exposure and again after 48 hours

The above procedure was repeated using stock solution of 1.5 g/L and 3 g/L of EDTA.

For each of the model mining side-streams, 200 ml of the spent effluent was brought to room temperature and transferred into a 1000 ml acid-washed glass beaker. Then 1 g of EDTA was added to each of the solutions and salt crystals were completely dissolved. The volume was made up to approximately 900 ml with Milli Q water and pH was adjusted to 6.8 using 2.5 M NaOH. The solutions were then transferred into the acid-washed 1000 ml volumetric flask and the volume was made up to the mark with Milli Q water. Each of the two solutions had a final pH of around 6.90. Both solutions were then stored at 5 ± 2 ° C for 120 hours. They were examined under optical microscope at magnification of 400 x for any traces of crystals forming and ICP readings were taken to confirm the concentration. No crystal formation was observed and the Rh concentration remained within 20 % of the initial value.The test concentration of both mining effluents and the EDTA solution, 25 ml of each of the stock solutions (see previous section) was pipetted into an acid-washed 200 ml volumetric flask. All three solutions were then made up to the mark using the *D. pulex* growth medium to make a concentration of 12.5 % of the stock solution. The resulting mixtures were hand-shaken to achieve complete content's mixing and then further subdivided into four batches of 50 ml each. Each of the 50 ml aliquots was poured into a separate 100 ml beaker and 5 Daphnids were put into each solution. This procedure was repeated using 25 %, 50 %, 62.5 %, 87.5 % and 100 % of each of the spent mining effluent solutions. Next all daphnids were exposed to the test solutions for 48 hours. All toxicity experiments were performed in triplicate.

RESULTS & DISCUSSION

Results are outlined in Tables 1-3. From Table 1 and 2 it can be seen that the EDTA was not toxic to *D. pulex* for lower test concentrations at concentrations of 1 g/L and 1.5 g/L. However there was some considerable die off at the highest concentration of 35 % and 25 % at 1 g/L and 1.5 g/L respectively. At higher concentration of 3 g/L EDTA, the data clearly shows that the addition of 3 g/L of EDTA led to minor toxicity to *D. pulex* as only the undiluted solution led to complete die-off of the Daphnids (see Table 3 for details) It can be concluded from these results that the increase in concentration of EDTA in the test concentrations leads to the death of *D. pulex*. Results for the EDTA solution and the spent mining effluents are shown in Tables 4 and 5. The daphnids were well adapted to the test conditions as 100 % survival was observed in the control experiment. All test organisms died after 48 hours of exposure to the mining effluent with the original pH = 1.87, while 10-60 % survival rates were observed at the mining spent effluent with original pH of 2.92 if the strength of the effluent ranged from 12.5 to 62.5 % (see Tables 4 and 5). A maximum

Table 1. The percentage survival of *D. magna* after 24 hours and 48 hours after exposure to 1 g/L EDTA

Test concentration (%)	% survival after 24hrs	% survival after 48hrs
12.5	100	100
25	100	100
50	95	95
62.5	100	100
87.5	100	90
100	100	65
control	100	100

Table 2. The percentage survival of *D. magna* after 24 hours and 48 hours after exposure to 1.5 g/L EDTA

Test concentration (%)	% survival after 24hrs	% survival after 48hrs
12.5	90	90
25	100	100
50	100	100
62.5	90	95
87.5	100	90
100	100	75
control	100	100

Table 3. The percentage survival of *D. magna* after 24 hours and 48 hours after exposure to 3 g/L EDTA

Test concentration (%)	% survival after 24hrs	% survival after 48hrs
12.5	100	100
25	100	100
50	95	95
62.5	95	80
87.5	75	5
100	75	0
Control	100	100

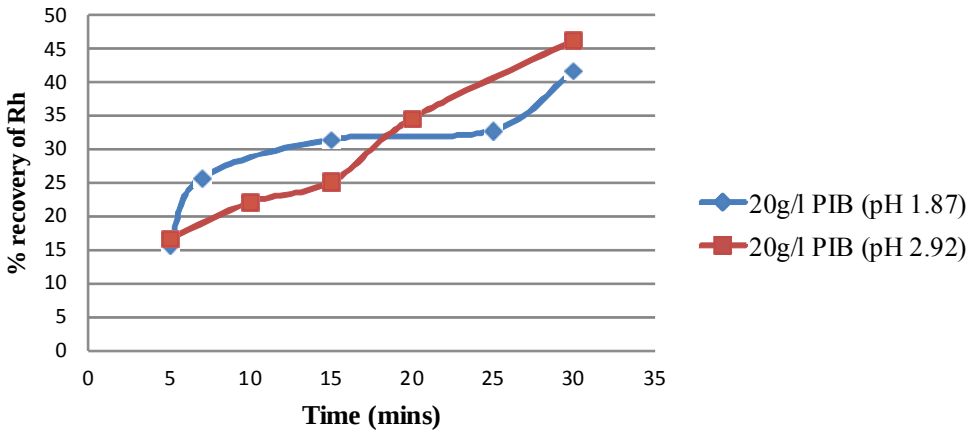

Fig. 2. Percentage recovery of Rh from the aquoes phase of the ELM containing 20 g/L PIB after demulsification at pH 1.87 and pH 2.92

recovery of 41.7 % of Rh was extracted after stirring in the TVC for 30 minutes at pH = 1.87 and 46.2 % was extracted at pH 2.92 as shown in Fig. 2.

Figure 3 shows the percentange of Rh left in the side stream effluent and percentage mass balance of Rh after extraction after extraction using the ELMs. There is a knowledge gap on the extraction of Rh using ELMs from the literature hence these results were compared to those of solvent extraction of Rh. The results are comparable to the studies on solvent extraction using kerosene as the diluent and tri-iso-octylamine as the extractant(Lee *et al.*, 2009). In their study the extracted Rh was 36 %. However in their study the non-Newtonian modifier PIB was not used. Masss balance is a concept based on the principle of conservation of matter i.e matter is neither created nor destroyed. It is a concept which is widely used in the manufacturing processes in the pharmaceutical industries (Baertschi *et al.*, 2013). The concept can be simplified by the equation 1;

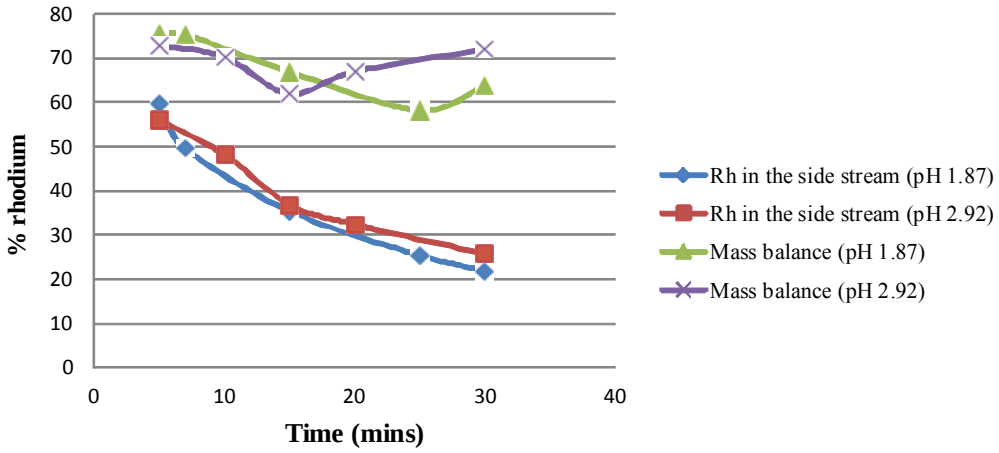

Fig. 3. Percentange of Rh left in the side stream effluent and percentage mass balance of Rh after extraction after extraction using the ELMs

Table 4. The percentage survival of *D. pulex* after 24 hours and 48 hours after exposure to aqueous phase effluent of mining side-streams made by a solution of Rh at pH = 1.87 after extraction of Rh using the ELM

Test concentration (%)	% survival after 24 hrs	% survival after 48 hrs
12.5	85	0
25	90	0
50	70	0
62.5	45	0
87.5	15	0
100	0	0
Control	100	100

Table 5. The percentage survival of *D. pulex* after 24 hours and 48 hours after exposure to aqueous phase effluent of mining side-streams made by a solution of Rh at pH = 2.92 after extraction of Rh using the ELM

Test concentration (%)	% survival after 24 hrs	% survival after 48 hrs
12.5	100	65
25	90	45
50	65	20
62.5	45	10
87.5	15	0
100	0	0
Control	100	100

Input = output (1)

Mass balance was calculated using the Equation (2) below:

(2)

$$\% \ Mass \ Balance = \frac{(C_{elm} \times V_{elm} + C_{ww} \times V_{ww})}{C_{in} \times V_{in}} \times 100$$

Where C_{elm} and is the concentration of Rh recovered in the stripping phase of the ELM and the volume of the stripping phase after demulsification respectively and and are the concentrations of Rh remaining in the side stream and the volume of the side stream left after extraction respectively and and are the initial concentration of Rh and initial volume of the side stream before extraction. Mass balance was calculated using equation 2 which took into consideration of the extracted Rh and Rh in the remaining side stream after extraction. Hence the mass balance equation 2 can be simplified as follows

$$Mass\ balance = Rh_{ww} + Rh_{extracted} \quad (3)$$

Where represents percentage of Rh remaining in the waste water side stream after extraction. The mass balance was 63.7 % and 71.9 % at pH 1.87 and pH 2.92 respectively as it can be see from Figure 2 above. To account for all the rhodium initially used in the beggining of extraction process based on equation 1 that states that input should be equal to output, equation 4 was used

$$Input\left(Rh_{(INITIAL)}\right) = Output\left(Mass\ balance + Rh_{(trapped)}\right) \quad (4)$$

Where represents the initial concentration in percentage of Rh used before extraction and represents Rh trapped in the organic phase of the ELM after extraction process. Substituting equation 3 in equation 4, equation 5 is formed.

$$Rh_{(INITIAL)} = Rh_{ww} + Rh_{extracted} + Rh_{(trapped)} \quad (5)$$

Rhodium left in the side stream and trapped in the ELM may be calculated using equation 6 below which is the re-arranged equation 5

$$Rh_{ww} = Rh_{(INITIAL)} - Rh_{extracted} - Rh_{(trapped)} \quad (6)$$

As it can be see from Figure 2 that at pH 1.87 and pH 2.92, 22 % and 25.7 % of the Rh was left in the side stream after extractiom respectively. Using equation 3, the Rh trapped in the ELM is 36.8 % for extraction at pH 1.87 and 28.1 for extraction at pH 2.92. The concentration of Rh trapped in the ELM and left in the waste water was 58.3 % and 53.8 % for pH 1.87 and 2.92 respectively. This is equivalent to 4.1 mg/L and 3.8 mg/L for pH 1.87 and 2.92 respectively using equation 7 below.

$$\frac{Rh_{extracted} + Rh_{(trapped)}}{Rh_i} \times C_i \quad (7)$$

Where C_i is the initial concentration of Rh which was 7 mg/L. The amount of Rh left in the side stream after extraction (see section 3.2 above) may have resulted in the observed die off. The other studies revealed that other heavy metals like copper, mecury, zinc, cadmium, iron and manganese are also toxic to *daphnia* (Sorvari and Sillanpää, 1996). In addition some organic components of the ELM, most probably toluene, the molecules carried over into the spent mining effluent may have also contributed to the observed die off. This was probably observed to a higher extent at pH of 1.87 than at pH of 2.92, providing an explanation of the results in Tables 4 and 5. It has been reported that the presence of EDTA reduces the toxicity of the metal (Sorvari and Sillanpää, 1996). This is because the toxicity of the metal is related to the free uncomplexed ions (Foster and Morel, 1982). However in the study of the complexation of the metals with EDTA and DTPA, it was concluded that the concentration of free ions is not the only reason for the explanation of toxicity to daphnia, this was because metals like mercury still showed high toxicity levels even after chelation. The results also showed that there was no significant difference in the toxicity of metals chelated using EDTA and DTPA except for mercury and iron. In the current study we found that the increase of EDTA for chelation of the metal may result in the die off of daphnia, hence a minimum amount of EDTA which will not cause die off should be used. Other studies have reported that the presence of EDTA may introduce errors in the study of the ecotoxicity of metals (Bergers and de Groot, 1994; Huebert and Shay, 1992). In the current study 1 g/L of EDTA was used because of the minimum die-off observed.

CONCLUSION

Considering that the side stream was diluted before toxicity testing and there was a considerable die off, it can be concluded that the very dilute water side streams are toxic aquatic life. Therefore the discharge of the spent Rh side-stream should thus be discouraged and prevented at all costs. It can be concluded that some components of the ELM carried over into the stripping phase and might have contributed to the observed die off. Rh compounds should be treated as highly carcinogenic and toxic.

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