

**Assessing the Effect of Leachate of Copper Slag from the ISASMELT process on Cell growth and Proximate Components in Microalgae, *Chlorella vulgaris* (Beijerinck)**

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**Abstract**

Dumping or disposal of huge quantities of slag (containing significant amounts of heavy metals) generated during matte smelting and refining of copper is likely to cause environmental and space problems. Detailed chemical studies of copper slags indicated their long-term stability and poor leachability of heavy metals. Since chemical analyses alone are insufficient for quantifying biological impact, the effect of copper slag leachate (CSL) on growth and proximate components in marine microalgae, *Chlorella vulgaris* (Beijerinck) were assessed under laboratory conditions. An inhibitory effect of CSL on cell growth (14.3%) was well below the defined criteria of 50% under algal growth inhibition test. Cellular concentrations of the total protein (TP) in CSL exposed cultures were significantly higher than controls ( $P < 0.05$ ). Although the cells of *C. vulgaris* exposed to CSL accumulated slightly elevated levels of the total lipid (TL) compared to those in control, but the differences were insignificant ( $P > 0.05$ ). Exposure of *C. vulgaris* to 100% CSL did not induce stress proteins as evidenced by the SDS-PAGE. Further studies assessing the changes in the growth rate and lipid profile would help in complete understanding of toxic nature of CSL in microalgae.

**Keywords:** Copper slag, algal growth inhibition test, total protein, total lipid, SDS-PAGE

## **Introduction**

The generation of waste materials from different mineral industries over the past few decades has touched an all time high as a sequel to increased production of metals from the mineral ores owing to sudden spurt in industrial development. Generally, the glassy materials left over when metals are pyro-metallurgically extracted from ores which have been considered as waste are termed 'slags'. Limited landfill space with concomitant increase in waste disposal costs has prompted the reuse of metal slags. Over the past few years, possibilities of reuse of metal industry slags (steel slag, fly ash, bottom ash and blast furnace slag) in various applications such as embankments, roads, pavements and alternative materials has been explored (Vazquez et al. 1991; Kamon and Katsumi 1994; Hartlén, Carling, and Nagasaka 1997; Kamon 1997; Sarsby 2000; Mroueh, Laine-Ylijoki, and Eskola, 2000). One such by-product obtained during matte smelting and refining of copper is generally referred to as copper slag.

The world copper production is currently about 15.32 million tonnes (International Copper Study Group 2010). Owing to rapid industrialization, the demand for copper is expected to increase in the near future. Assuming that 2.2 tonnes of slag are generated for each tonne of copper processed (Gorai, Jana, and Premchand 2003), it has been estimated that ~34 million tonnes of slag are generated annually from world copper production. Copper slags are suitable for a variety of applications such as for manufacture of cement, in aggregates, landfill, ballast, abrasives, roofing granules, glass, tiles, bituminous pavements, etc (see review by Gorai, Jana, and Premchand 2003). The possible utilization of copper slag as filler material for the preparation of composite materials and preparation of added-value products such as abrasive tools, cutting tools and railroad ballast has been recently evaluated by (Biswas and Satapathy 2010). Relatively higher specific gravity and density of spent copper slag compared to normal sand has led to novel attempts to re-use copper slag as material for reclaiming land from the sea in island nations such as Singapore (Lim and Chu 2006).

The main ecological concern associated with the utilization of metal-bearing waste in earthworks, however is the subsequent leaching of heavy metals into soil and water. Reducing characteristics of the metal slag mobilizes some of the heavy metals and its ions in waste materials into the surrounding environment (Van der Sloot 1991; Zevenbergen and Hoppe 1991; Comans et al. 1991). Furthermore, possible implications of leached metal ions from slag into the sediments and surrounding seawater resulting in metal bioaccumulation in the marine food chain hinders the large-scale application. Copper slag has been excluded from the listed hazardous waste category of United States Environmental Protection Agency (USEPA) and it also does not feature in the hazardous

waste category of the hazardous waste management rules of the Ministry of Environment and Forests, India (MoEF). However, it is not clear whether a concentration limit-based classification specified for other waste materials specified by the MoEF would also be applicable to copper slag if it is to be used as a reclamation material (Shanmuganathan et al . 2008).

The composition of the slag depends both on the smelting process as well as the composition of the copper concentrates used. Statistical analysis of the chemical composition of 28 copper slags from United States (US), Canada and Chile revealed that a large variation in the concentration of the heavy metals (Shen and Forssberg 2003; Gorai, Jana, and Premchand 2003). The stability and leachate toxicity studies of spent copper slag using toxicity characteristic leaching procedure (TCLP) of USEPA (Federal Register 1990) indicated that the test values were far below the prescribed limits (Lim and Chu 2006). Also, poor leachability of the metal ions reflecting the long-term stability of spent copper slag from the ISASMELT process has been demonstrated recently by Shanmuganathan et al. (2008). In another study, four different tests (multiple extraction procedure using seawater, pressure leaching, mechanical activation combined with pressure leaching as well as leaching with seawater after mechanical activation) of copper slag revealed that the leachability of metal ions into solution was highest when leached with seawater but was below the prescribed limits of USEPA 40CFR Part 261 (Shanmuganathan et al. 2010; unpublished results).

Chemical analysis of leachate alone however, does not generate requisite information on its possible environmental impact because certain metals adhere to particles or organic compounds forming weak metal-complexes, which on dissolution could act either as a nutrient or toxicant depending upon its concentration in solution. Therefore, absolute metal concentrations do not always reflect the extent to which the aquatic organisms are affected (Wong, Kwan, and Tam 1979). Unfortunately, no research delineating the inhibitory effects of copper slag leachate (CSL) on marine flora and fauna has been carried out. Stress effects of environmental contaminants are easily and rapidly manifested in unicellular organisms (Jochem 2000). Microalgae form the base of the aquatic food chain and are more sensitive to contaminants compared to other test organisms (Joubert 1983; Wong and Coulture 1986; Lewis 1995; Bailey and Young 1997; Nancharaiyah, Rajadurai, and Venugopalan 2007). Algal tests are rapid, simple and cost effective (Arensberg, Hemmingson, and Nyholm 1995; Pun, Cheung, and Wong 1995) and reproducible due to the usage of high cell densities and clonal cultures (Mirenda and Hall 1992; Stauber 1995). Furthermore, the use of algal toxicity tests is becoming more common in many countries where toxicity tests with fish are

increasingly being restricted by animal ethics legislation (Stauber 1995). Therefore, microalgae are routinely used as model organisms for assessing the effects of environmental contaminants.

Against this background, an experimental study was conducted to assess the effect of CSL on growth and proximate components in common marine microalgae, *Chlorella vulgaris*. Further, the possible induction of stress proteins in *C. vulgaris* treated with CSL was also assessed. It is anticipated that the outcome of the study may form the basis for future reuse of spent copper slag as material for reclaiming land from sea.

## **Materials and Methods**

### **Microalgal culture**

Batch cultures of *C. vulgaris* (Beijerinck) maintained at National Institute of Oceanography (NIO) Goa (India) were used for the experiments. Microalgal cultures used were uni-algal but non-axenic. Cells were grown (250 ml Erlenmeyer flasks) in an autotrophic medium (Liang, Sarkany, and Cui 2009) (filtered double distilled water, 1000 ml; NaCl 0.025 g; NaNO<sub>3</sub> 0.25 g, K<sub>2</sub>HPO<sub>4</sub> 0.175 g, MgSO<sub>4</sub> 0.075g, NH<sub>4</sub>Cl 0.05 g, CaCl<sub>2</sub> 0.025 g, FeCl<sub>3</sub> 0.003 g, final pH 7) with continuous light cycle (2800 lumen using fluorescent lamps), air supply (200 ml min<sup>-1</sup>) at 25±1 °C. Cultures were shaken occasionally to avoid clumping. In order to prevent the growth of bacteria, medium was supplemented with filtered antibiotic solutions of chloramphenicol, 2 mg L<sup>-1</sup>; streptomycin, 3 mg L<sup>-1</sup> and tetracycline, 3 mg L<sup>-1</sup>. Prior experimental trials indicated these concentrations completely arrested the growth of bacteria. Initial cells densities enumerated with haemocytometer were found to be 4.2±0.4×10<sup>6</sup> cells ml<sup>-1</sup>.

### **Test material and preparation of leachate**

Fresh copper slag sample was obtained in clean polythene bags from one of the copper smelter plants in India which adopts the ISASMELT process of MIM Technologies, Australia. Extraction procedures followed in this process have been described by Shanmuganathan et al. (2008). The test material was highly abrasive, shiny black in colour and had a crystalline appearance. The chemical composition (wt.%) of the representative slag sample as measured using the Toxicity Characteristic Leaching Procedure (TCLP) followed by inductive coupled plasma-atomic absorption spectrophotometer (ICP-AAS) and flame atomic absorption spectrophotometer (FAAS) by (Shanmuganathan et al. 2008) is presented in Table 1.

For preparation of copper slag leachate (CSL), 100 g of dried slag was mixed in 1000 ml of growth medium and kept in an orbital shaker incubator (150 rpm) for 24 hrs. This main purpose of this procedure was to carry out the mechanical activation of the slag. The mixture was then filtered through Whatman filter paper No.1 (0.45  $\mu\text{m}$ ) and the supernatant solution was considered as CSL stock solution (100%). Different dilutions of CSL were prepared from this stock solution.

### **Experiment I: effect of CSL on cell growth**

The effect of different concentrations of CSL on cell growth in *C. vulgaris* was assessed by performing algal growth inhibition test (Stauber 1995). This test determines the concentration of contaminant (CSL) which reduces the growth of microalgae by 50%. Assay flasks (in triplicate) were setup containing fresh medium and the CSL stock in different concentrations of 10–100%. Re-suspended cultures ( $4.2 \pm 0.4 \times 10^6$  cells  $\text{ml}^{-1}$ ) were inoculated in the assay flasks and maintained at  $26 \pm 1$  °C under continuous light condition (2800 lumen) and air supply (200  $\text{ml min}^{-1}$ ). The pH of the medium after the addition of CSL was 6.7. Assay flasks were gently agitated daily to avoid clumping of cells. Similarly, control assay tanks were setup but without CSL. Cell growth (cells  $\text{ml}^{-1}$ ) in treatment and control assay flasks was measured at the end of 2, 3, 6, 7 days of exposure (DoE) using haemocytometer. To allow for any possible circadian rhythm in cell division time, the cultures were sampled approximately at the same time of the day (1100 hrs).

### **Experiment II: effect of CSL on cellular proximate composition**

Cellular concentrations of proximate components such as total protein (TP) and total lipid (TL) in *C. vulgaris* treated with CSL and control were determined. Exposure doses chosen for assessing the sub-lethal effects of CSL on the TP and TL were based on results obtained from experiment I.

The cellular fractions of the TP in *C. vulgaris* control as well as treatment flasks were extracted as described by Weis, Verde, and Reynolds (2002). A known volume of microalgae was centrifuged and the microalgal pellet obtained. To this pellet, 2 ml of extraction buffer (100mM Tris, 10mM EDTA and 100mM NaCl; final pH 7.4) was added and re-suspended. Pre-treated (1N HCl and distilled water) glass beads (dia, 4 mm) were added to the suspension and vortexed for 1 m and then placed on ice for an equal time period. This process was repeated for at least 15 times for maximum extraction of proteins from cells. The suspension was then centrifuged at  $13000 \times g$  for 5 min and the

resulting supernatant aspirated. Concentrations of the TP in the supernatant were then assayed following the procedure suggested by Lowry et al. (1951).

Cellular fractions of the TL in *C. vulgaris* grown in treatment and control flasks were extracted following the procedure as described by Liang, Sarkany, and Cui (2009) with a slight modification. A known volume of microalgae suspension was centrifuged and the wet pellet was dried at 70 °C in a hot air oven for 20 min. The resultant biomass was then suspended in 5 ml of 100% methanol and glass beads (pre-washed in 1N HCl and distilled water) of 4 mm diameter were added and vortexed for 15 min at room temperature. The process was repeated 10 times for maximum lipid extraction from cells. The pooled methanolic extracts were treated with equal volume of n-hexane. The combined n-hexane extract was then collected in a pre-weighed container and is allowed to evaporate. Concentration of the TL was then determined by the following formula:

$$TL = F_w - I_w$$

Where  $F_w$  is the final weight of the container after the hexane evaporation and  $I_w$  is the initial weight of the container.

### **Experiment III: effect of CSL in the induction of stress proteins:**

The effect of CSL in inducing the production of stress proteins by *C. vulgaris* was assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) as as described by Laemmli (1970) with a slight modification (10% gel). The protein extracts of cells treated with 100% CSL and control (0% CSL) at the end of 7 DoE were subjected to SDS-PAGE. Marker used for comparing protein bands was medium Mwt (19-117Kda) marker from Fermentas (#SM0441). The gel was stained using the silver staining procedure and the marker band alone was stained using the Coomassie brilliant blue staining procedure.

### **Statistical Analysis**

The significant differences in the TP and TL levels between the control and treatments were analysed by analysis of variance (ANOVA). A comparative *post hoc* assessment of cellular concentrations of the TP and TL between control and CSL treated cultures was made with Newman-Keul's multiple comparison test with 95% confidence interval (Zar 1999). Significance in all statistical tests was judged at a ' $P \leq 0.05$ ' level. All statistical analyses were performed using GraphPad Prism Software (Graph Pad, San Diego, CA, USA).

## Results

### Experiment I: effect of CSL on cell growth

Measured cell growth of *C. vulgaris* in treatment and control assay flasks at different exposure periods of 2, 3, 6 and 7-DoE is presented in Table 2. In control, cell growth of  $1.33\pm 0.16$ ,  $5.82\pm 0.33$ ,  $10.92\pm 0.62$  and  $15.46\pm 0.5\times 10^6$  cells  $\text{ml}^{-1}$  at the end of 2, 3, 6 and 7-DoE, respectively was recorded. The pH of the medium over a 7 day period after addition of CSL decreased to 6.5 from an initial pH of 6.7. At 3-DoE, a small perceptible decrement (14.3%) in cell growth was witnessed in treatment flasks with  $\geq 60\%$  CSL concentration ( $4.96\pm 0.04\times 10^6$  cells  $\text{ml}^{-1}$ ) when compared to control ( $5.82\pm 0.33\times 10^6$  cells  $\text{ml}^{-1}$ ). Beyond 3-DoE, no further change in the pattern of decrease in cell densities was recorded in treatment flasks containing  $>60\%$  CSL concentration. Since the observable inhibitory effect of CSL of cell growth in *C. vulgaris* was well below 50%, effective concentration ( $\text{EC}_{50}$ ) values could not be determined. This decrement at higher concentrations was deemed to be insignificant since the growth of *C. vulgaris* was not reduced by 50%.

### Experiment II: effect of CSL on TP and TL concentrations

Results of experiment I indicated that the reduction in cell growth first occurred in assay flasks containing 60% CSL. Therefore, exposure doses of 60% and 100% CSL along with control (0% CSL) were chosen for estimating the effect of CSL on the TP and TL concentrations.

The measured levels of the TP in *C. vulgaris* in control and treatment flasks increased progressively with the DoE (Figure 1). The increment was concomitant with the increase in cell densities. Cellular concentrations of the TP in *C. vulgaris* grown in treatment (60 and 100% CSL) and control (0% CSL) exhibited significant differences (ANOVA). Measured levels of the TP in *C. vulgaris* grown in 60 and 100% CSL were always significantly higher than the levels recorded in control ( $P < 0.05$ ). At 7-DoE, cellular concentrations of the TP were  $189.85\pm 1$  and  $223.55\pm 1.19$   $\mu\text{g ml}^{-1}$  in control and treatment (60% CSL) flasks, respectively. Measured levels of the TP in *C. vulgaris* treated with 100% CSL ( $225.1\pm 1.80$   $\mu\text{g ml}^{-1}$ ) did not significantly differ from those exposed to 60% CSL ( $P > 0.05$ ).

In general, the measured cellular concentrations of the TL in control and treatment flasks increased progressively with the DoE and pattern of increment was in line with rise in cell numbers over the exposure periods (Figure 2). However, the TL levels of *C. vulgaris* grown in 60% and 100% CSL were higher compared to control from 4-DoE onwards. Although there were differences in the TL

levels in *C. vulgaris* harvested from control and treatment flasks, the differences were found insignificant ( $P>0.05$ ). Measured levels of TL in control were  $4.57\pm 0.05$  and  $9.34\pm 0.07$  mg ml<sup>-1</sup> at 4-DoE and 7-DoE, respectively. Concentrations of the TL in *C. vulgaris* treated with 60% CSL (4-DoE,  $7.55\pm 0.09$ ; 7-DoE,  $18.37\pm 0.29$  mg ml<sup>-1</sup>) and 100% CSL (4-DoE,  $7.53\pm 0.07$ ; 7-DoE,  $18.28\pm 0.46$  mg ml<sup>-1</sup>) were relatively higher compared to control (0% CSL). However, the differences in the TL levels extracted from cells exposed to 60 and 100% CSL were insignificant.

### **Effect of CSL on induction of stress proteins**

To assess the induction of stress proteins, SDS-PAGE analysis of proteins extracted from cells exposed to 100% CSL at the end of 7-DoE was performed. The bands formed in SDS-PAGE (Figure 3) revealed that a dose of 100% CSL did not induce production any stress proteins in *C. vulgaris*. However, elevated (enhanced) levels of the TP were discernible at higher concentrations of CSL **(Result of experiments II)**.

### **Discussion**

Copper slag refers to the non-ferrous glassy material produced during pyro-metallurgical production of copper from copper ores. Dumping or disposal of such huge quantities of slag is likely to cause space and environmental problems as the reducing capacities of metal slags tend to mobilize metal ions when in contact with a solution (Van der Sloot 1991; Comans et al. 1991). Further, chemical analysis alone does generate sufficient information regarding eco-toxicity of copper slag. Therefore, bioassay studies with copper slag need to be performed to evaluate the sublethal effects. In the present study, we evaluated the potential effects of the leachate of copper slag obtained from the ISASMELT process copper plant on cell growth and proximate composition in marine microalgae, *C. vulgaris*.

Microalgae form the base of the food chain in the respective aquatic environments and contribute to the primary productivity of the ecosystem. Unicellular algae are highly sensitive to a wide range of toxicants and are known to respond to a variety of industrial wastes (Gorai, Jana, and Premchand 2003; Shen and Frossberg 2003). Further, their ability to grow in very small test volumes over short durations, make them ideal test organisms in eco-toxicity studies. In the present study, *Chlorella vulgaris*, common unicellular microalgae present in both marine and freshwater ecosystems was used as test species to assess the effect of CSL. The algal inhibition test involves the exposure of algae to differing concentrations of the test substance and then observing as to which concentration of the test substance decreases the cell density of the cultures by more than 50% (Stauber 1995). Because of



their high cell densities and clonal nature of cultures, algal inhibition tests are generally very reproducible to a variety of test substances (Mirenda and Hall 1992; Stauber 1995).

### **Effect of CSL on cell growth**

In the present study, a small decrement in cell growth (14.3%) was observed in the assay flasks treated with  $\geq 60\%$  CSL. This reduction in cell density is considered to be insignificant as the decrement is  $< 50\%$ . The decrement however might be due to stress exerted by the metal ions present in CSL solution upon the cells of the microalgae. Unfortunately, no studies assessing the toxic effect of leached metal ions from slags on microalgae are available for comparison. However, studies concerning the individual metal ion solutions affecting normal growth pattern in microalgae have been conducted. About 22% reduction in the growth and biomass of *C. vulgaris* accompanied by reduction in chlorophyll content following an exposure to  $80\mu\text{M}$  concentration of  $\text{Cu}^{2+}$  ions in solution has been documented by Lupi, Fernandes, and Sa'-Correia (1998). Progressive increase in  $\text{Fe}^{3+}$  in the medium from 3mM to 6mM resulting in significant decrease in the cells numbers of *C. vulgaris* has been demonstrated (Liu, Wang, and Zhou 2007). Such a decrement in the cell numbers due to increased iron content of the medium can most likely be due to blocking of the intake of other nutrients into the cell by the binding of iron to the cell nutrient transport systems. Though copper slag obtained from ISASMELT process contains ore of iron and silicon, the effect of increased silicon ions in the solution has been showed no effect on the growth of *Chlorella vulgaris* (Exley et al. 1993). Recently, experimental studies of Qian et al. (2009) have shown that increased concentrations of Cd and Cu ions in solution resulted in reduction of cell growth and chlorophyll production in *C. vulgaris*. A study comparing the toxicity of low copper concentrations with volcanic ash leachate additions (between  $2 \times 10^{-7}$  and  $4 \times 10^{-9}$  M copper) and copper nitrate solutions (between  $10^{-5}$  and  $10^{-7}$  M copper) in *Anabaena flos-aquae* has been carried out (McKnight, Feder, and Stiles 1981). This study indicated that growth in *A. flos-aquae* was inhibited by other metal ions rather than Cu; most probably by a synergistic effect of elevated concentrations of several trace metals, or by toxic organic compounds present in the leachate.

Therefore, it can be deduced that the CSL although not effectively decreasing the growth by 50%, does confer metal stress upon the microalgal cells  $\geq 60\%$  concentration, leading to a perceptible decrease in the cell numbers. Occurrence of insignificant growth reduction in *C. vulgaris* at 50% leachate concentration following exposure to two different landfill leachates in Hong Kong has been reported (Cheung, Chu, and Wong 1993). Therefore, it appears that the inhibitory effect on the microalgal cells is dependent on the metal composition of leachate and is highly species-specific.

Although algal inhibition tests provide basic information on the reduction in cell densities at a particular leachate concentration, assessing the growth rates of individual treatment concentrations would provide better insights into the correlation studies between reduction in cell densities and difference in growth rates (Nyholm 1985). Also further studies looking into the effect of CSL on the chlorophyll concentration is required as CSL may or may not inhibit the production of chlorophyll at the same time causing the production of smaller sized cells.

### **Effect of CSL on proximate components**

The TP levels in *C. vulgaris* treated with 60 and 100% CSL were significantly higher when compared to control (0% CSL). This was not unexpected as proteins constitute the major component of microalgal biomass (Liang, Sarkany, and Cui 2009). Previous studies have shown that under stress conditions, microalgae produce more protein than those in stress-free conditions (Maxwell et al. 1994; Honjoh et al. 1995; Pietryczuk, Piotrowska, and Czerpak 2008). Higher concentrations of (2E)-dodecane-1, 12-dioic acid resulting in increased production of water soluble proteins in *C. vulgaris* along with induction of certain newer types of proteins has been documented by Pietryczuk, Piotrowska, and Czerpak (2008).

A study by (Choudhary et al. 2007) documented that over a concentration gradient of 0.05–0.20 mg/L of lead, copper and zinc, the eukaryotic microalgae *Spirulina platensis* exhibited increased production of proline amino acid. The proteins quantified in this study were mainly water soluble proteins, as they were extracted with the help of the extraction buffer and bead shearing. An accumulation of significant amounts of the TP in *C. vulgaris* treated with 60 and 100% CSL is in agreement with previous studies of (Pietryczuk, Piotrowska, and Czerpak 2008). Further research however, confirming which metal ion(s), from a pool of various metal ions in the CSL, causes the increment in the TP levels is required. Based on the results of the present study, it can only be hypothesized that the increment in TP levels with increasing CSL concentrations could be due to the fact that the cell produces the proteins to combat metal stress by binding to the latter in the form of organometallic complexes, thereby facilitating the cytoplasmic concentration of these ions and preventing their toxic effect.

In the present study, accumulation of TL increased with the progress in DoE and increase in CSL concentration. In contrast, the extended presence of iron in the medium did not result in enhanced accumulation of neutral lipids by cells of *C. vulgaris* in spite of increase in the total cell numbers (Liu, Wang, and Zhou 2007). *Chlorella* is considered to be an excellent form of storage molecules

such as pigments, starch, single cell protein and other glycolipids, trehalose lipids and most importantly biodiesel. Further, the production of lipids and their subsequent storage are also affected by certain external factors namely temperature, light, nutrient availability and most importantly, metal ions present in the solution (Xu, Miao, and Wu 2006). Concomitant increase in the TL levels with increasing CSL concentration might be due to the production of lipids to bind to the metal ions, thereby preventing its entry into the cytoplasm. Further studies to understand the changes in lipid profiles associated with increased cellular lipid production on exposure to CSL are necessary.

### **Effect of CSL on the induction of stress proteins**

Aquatic organisms are equipped with diverse strategies to maintain equilibrium with the surrounding environment in response to changes in nutrients, temperature, pH or metal stress. Under metal induced stress, organisms are often confronted with a problem of selecting metal ions needed for their growth and at the same time excluding those which may aggravate the stress. Further, the maintenance of selected ions at optimal intracellular concentrations is necessary (Cobett and Goldsbrough 2002). The task of selection and sequestering of wanted and unwanted ions are mediated by a specialised group of polypeptides called as stress proteins. Induction of stress proteins as a biomarker for determining the effective concentration of a contaminant that alters the metabolic process in an organism has been postulated by (Bierkens, Maes, and Plaetse 1998) and the same hypothesis was applied in the present study.

In the present study, analysis of protein bands in SDS-PAGE revealed that induction of stress proteins did not occur in 100% CSL treated *C. vulgaris*; rather the same proteins are produced albeit at higher concentrations. *Chlorella* spp. has been reported to produce stress proteins on exposure to heavy metal or xenobiotic contaminants (Yoshida et al. 2006; Pietryczuk, Piotrowska, and Czerpak 2008). A Cadmium (Cd) concentrations of 100 mg L<sup>-1</sup> induced the algae *C. sorokinian* to produce newer Cd binding proteins (Yoshida et al. 2006). A concentration of 10<sup>-3</sup> M of (2E)-dodecane-1, 12-dioic acid, induced *C. vulgaris* to produce stress proteins (Pietryczuk, Piotrowska, and Czerpak 2008).

Interestingly, CSL did not induce the production of stress proteins in *C. vulgaris* in the present study. Although elevated TP levels were recorded at 60 and 100% CSL concentrations, no stress proteins were produced as evidenced by analysis of protein bands in SDS-PAGE (Figure 3). Therefore, it does appear that CSL evokes a response in *C. vulgaris* to produce elevated levels of proteins to combat stress rather than inducing the production of stress proteins. It can be

hypothesized that the cells of *C. vulgaris* produces proteins to bind to metal ions as organometallic peptide complexes, thereby preventing or neutralizing the toxic effect of the same in the cytoplasm. Further studies with leachate from copper slags of varying metal compositions are required to re-validate the applicability of stress protein induction as an indicator of metal toxicity.

## **Conclusion**

In conclusion, algal growth inhibition tests revealed that leachate of copper slag from the ISASMELT process is non-toxic to *C. vulgaris*. Elevated levels of the TP and the TL in treatment flasks of 60 and 100% CSL when compared to control were discernible. Further, CSL did not induce production of stress proteins. The conclusions drawn here have to be interpreted with some caution as the copper slag used was from one particular copper extraction process. Further studies assessing the effects of CSL with varying metal composition focussing changes in growth rate, lipid profile and leachate purification with protein-metal speciation are required for providing greater insights into the eco-toxicity of copper slag.

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**Table 1.** Chemical composition (%) of the representative copper slag sample used in the study (Shanmuganathan et al., 2008).

Elements	Concentration in slag (%)
As	0.0037
Bi	0.0016
Cd	0.00094
Cr	0.0059
Cu	0.4
Fe	52.2
Pb	0.0323
Ni	0.0053
Si	37.78
S	0.53
Zn	0.1687

**Table 2.** Growth in copper slag exposed cells of *C. vulgaris* (cells $\pm$ S.D $\times 10^6$  ml $^{-1}$ ) at the end of different days of exposure (DoE).

Days of Exposure (DoE)	CSL Concentrations										
	Control (0%)	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
2	1.33 $\pm$ 0.10	1.11 $\pm$ 0.09	1.14 $\pm$ 0.05	1.13 $\pm$ 0.07	1.10 $\pm$ 0.08	1.14 $\pm$ 0.10	1.13 $\pm$ 0.11	1.14 $\pm$ 0.10	1.12 $\pm$ 0.09	1.14 $\pm$ 0.09	1.14 $\pm$ 0.08
3	5.82 $\pm$ 0.33	5.71 $\pm$ 0.09	5.74 $\pm$ 0.10	5.72 $\pm$ 0.13	5.80 $\pm$ 0.09	5.72 $\pm$ 0.11	4.96 $\pm$ 0.12 <sup>a</sup>	4.97 $\pm$ 0.10 <sup>a</sup>	4.95 $\pm$ 0.09 <sup>a</sup>	4.95 $\pm$ 0.10 <sup>a</sup>	4.95 $\pm$ 0.10 <sup>a</sup>
6	10.92 $\pm$ 0.62	10.93 $\pm$ 0.14	10.86 $\pm$ 0.15	10.86 $\pm$ 0.16	11.30 $\pm$ 0.44	11.11 $\pm$ 0.20	9.39 $\pm$ 0.12 <sup>b</sup>	9.43 $\pm$ 0.11 <sup>b</sup>	9.46 $\pm$ 0.19 <sup>b</sup>	9.49 $\pm$ 0.19 <sup>b</sup>	9.47 $\pm$ 0.21 <sup>b</sup>
7	15.46 $\pm$ 0.40	15.38 $\pm$ 0.14	15.18 $\pm$ 0.29	15.20 $\pm$ 0.28	15.21 $\pm$ 0.24	15.29 $\pm$ 0.21	12.62 $\pm$ 0.29 <sup>c</sup>	12.60 $\pm$ 0.25 <sup>c</sup>	12.53 $\pm$ 0.20 <sup>c</sup>	12.52 $\pm$ 0.18 <sup>c</sup>	12.56 $\pm$ 0.18 <sup>c</sup>

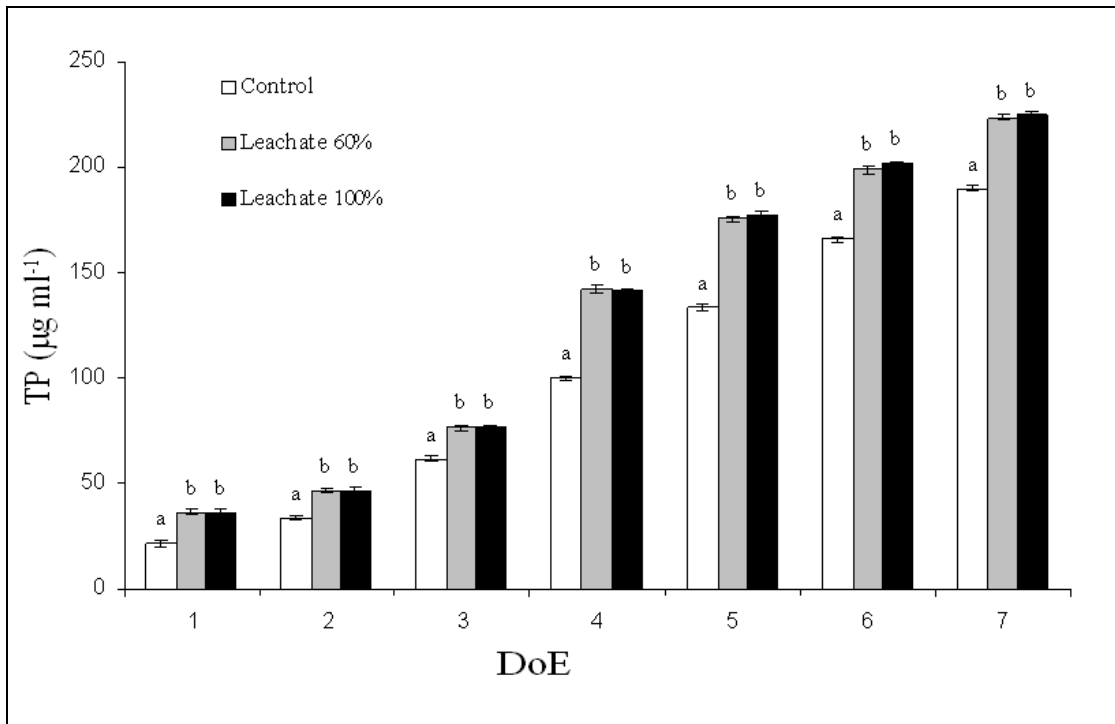
*Values in rows with same superscript represent decrease in cell growth when compared to control*

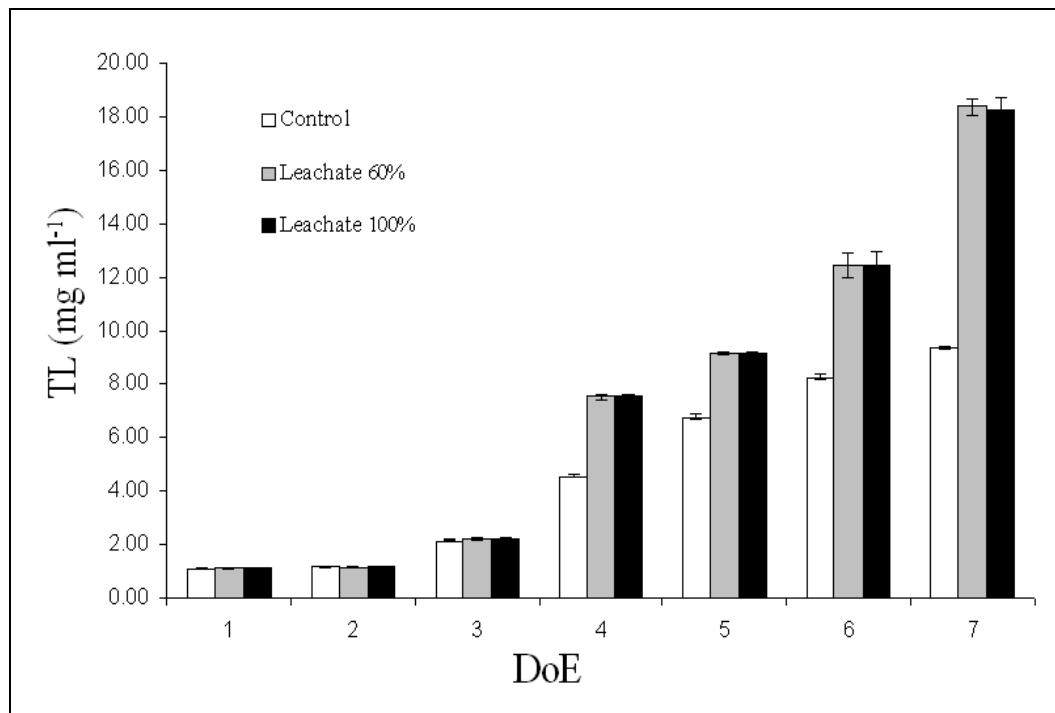
### Legends to Figures

Fig.1. Changes in the concentrations of total protein (TP) in *C. vulgaris* ( $\mu\text{g ml}^{-1}$ ) on exposure to 60% and 100% CSL. Values with different superscripts are statistically significant ( $P < 0.05$ ). Vertical bars represent standard deviation ( $n=3$ ).

Fig.2. Changes in the concentrations of total lipid (TL) in *C. vulgaris* ( $\text{mg ml}^{-1}$ ) on exposure to 60% and 100% CSL. Values are statistically insignificant ( $P > 0.05$ ). Vertical bars represent standard deviation ( $n=3$ ).

Fig.3. Protein bands formed by SDS-PAGE. L1 is lane containing protein bands of 100% CSL, L2 is lane containing protein bands of control (0% CSL) and L3 is marker protein (stained with Coomassie brilliant blue staining).

**Fig. 1**

**Fig. 2**

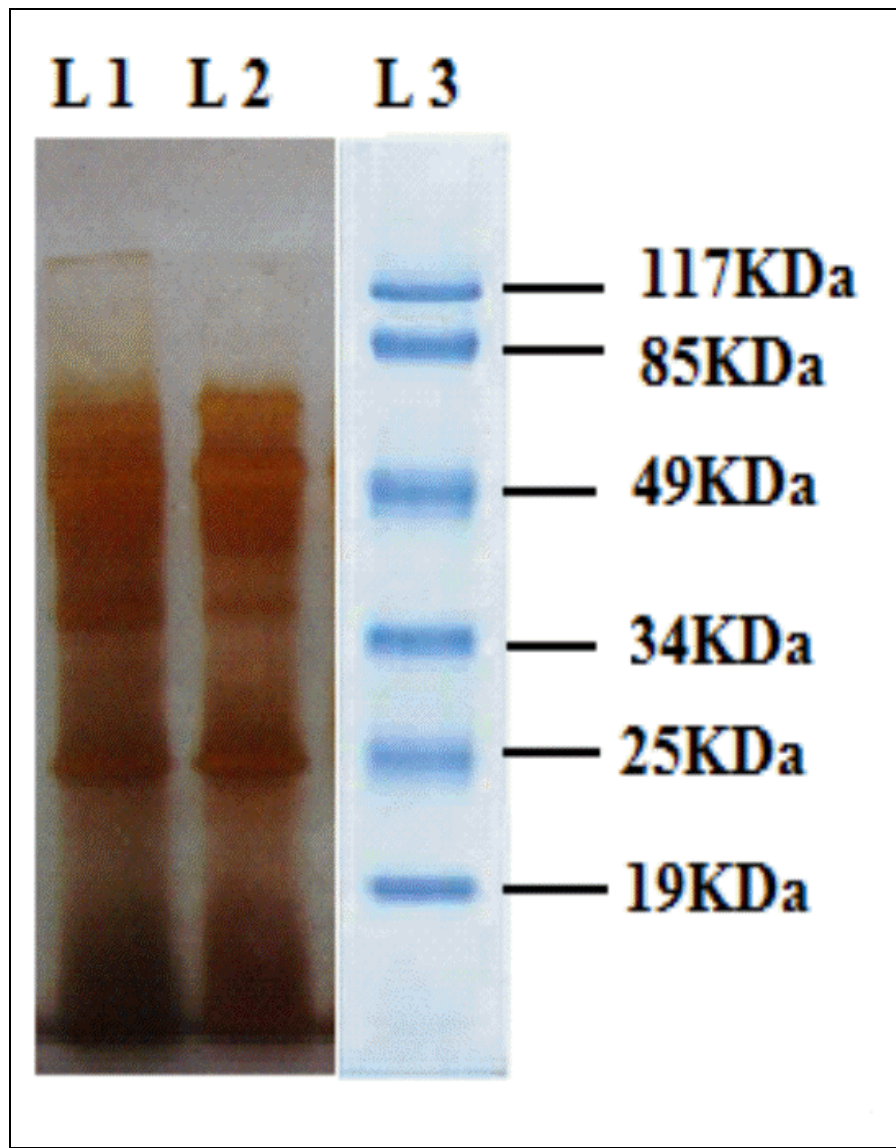


Fig. 3