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THE INFLUENCE OF SINGLE AND COMBINED EFFECTS OF Zn, Cu AND TEMPERATURE ON MICROBIAL GROWTH

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ABSTRACT

The purpose of the present study is to investigate the single and joint effect of zinc and copper to the growth pattern of the metal tolerant species of *Arthrobacter* sp. JM018. The results showed that, both, Zn and Cu at concentrations between 1 to 10 μM stimulated the growth of the above microorganism at 35 °C. Stimulation was reduced with the increase of Zn concentration, while the opposite phenomenon was observed for copper. On the other hand, similar concentrations of joint Zn and Cu resulted to slight growth inhibition, indicating antagonism between the studied heavy metals. Experiments with the same microorganism at 20 °C and 35 °C, at metal free and 10 μM Zn, indicated that the stimulatory effect of zinc was significantly more pronounced at lower temperatures. The latter is indicative of the strong role of temperature on the expression of heavy metals to microorganisms.

Keywords: microorganisms, heavy metal, copper, zinc, temperature, batch reactor, *Arthrobacter* sp., joint toxicity, growth stimulation, growth inhibition

1. Introduction

Heavy metal contamination is a global environmental problem. The release of metals into the ecosystem either from naturally occurring sources or from anthropogenic activities poses a serious threat to public health due to their persistence, biomagnification and accumulation within the food chain. Arsenic, copper, cadmium, lead, chromium, nickel mercury and zinc are among the most commonly encountered heavy metals in polluted water (Kalavrouziotis *et al.*, 2009). Although trace amounts of many heavy metals act as micronutrients to the microorganisms (Gikas and Romanos 2006; Burgess *et al.*, 1999; Bruins *et al.*, 2000), high concentrations are known to be toxic due to their interference with essential biochemical pathways (Nies, 1999). Metal availability, mobility, and hence, toxicity to aquatic microorganisms are strongly affected by the speciation of the heavy metals in water, soil and sediment systems (Tessier and Turner, 1995; Hagarova *et al.*, 2012).

Microbial bioremediation has been emerged as an alternative technology to reduce heavy metal concentrations to acceptable levels in the ecosystem and enhance the removal of metals from aqueous systems (Groudev *et al.*, 2010; Kumar *et al.*, 2010). To understand the behavior of complex aquatic and biogeochemical ecosystems, it is vital to study effects of heavy metals to native microorganisms. Of primarily importance in the environmental detoxification process is the ability of certain microbial stains to tolerate increasing concentrations of heavy metals (Gikas, 2008). The latter process may affect the growth characteristics of the microorganisms and even the cellular morphology (Chakravarty and

Banerjee 2008). Studies investigating such changes have been demonstrated for the acidophilic heterotroph *Acidocella* sp. GS19h strain (Chakravarty *et al.*, 2007), for *Acidiphilium symbioticum* H8 (Chakravarty and Banerjee, 2008) and for *Pseudomonas aeruginosa* strain 4EA (Naik and Dubey, 2011), due to exposure to heavy metals. Besides morphological changes, reduction/adaptation of bacterial distribution, diversity and reduction enzyme expression profiles of various bacterial isolates due to the effect of heavy metals have recently been investigated (Jose *et al.*, 2011).

Temperature is also a determined factor that affects the growth of microorganisms (Lee *et al.*, 2011; Guo *et al.*, 2010), as well as the toxicity of heavy metals (Cathum *et al.*, 2005). Although biochemical reaction rates may roughly double with temperature increase by 10 °C (Rittman and McCarty 2001), microorganisms function at an optimum performance at a specific temperature range (Prescott *et al.*, 2002). Temperature has been shown to affect the reduction rate of Cr(VI) (and hence chromium toxicity to *Escherichia coli* (Shen and Wang, 1994)), while in general, the optimum Cr(VI) resistance and reduction in microorganisms has been determined to be between 30-36 °C (Shen and Wang, 1994; Ishibachi *et al.*, 1990; Wang and Xiao, 1995; Krauter *et al.*, 1996; Vaiopoulou and Gikas 2012). Bioaccumulation of heavy metals has been determined to maximize at 25 °C and 30 °C for Cu(II) and Cd(II), respectively, by *Pseudomonas putida* (Uslu *et al.*, 2011) and at 30 °C, 25 °C and 30 °C for Cd(II), Pb(II) and Cu(II), respectively, by *Rhizophus arrhizus* (Uslu *et al.*, 2003). On the other hand, a study on the effect of physical and physiological factors on heavy metal sorption by *Bacillus subtilis* and *Bacillaceae* sp. showed that a relatively high temperature (45 °C) was optimum for Ag(I), Cr(III) and Pb(II) sorption by the above bacterial strains (Fosso-Kankeu *et al.*, 2010). Experiments conducted to examine the effect of heavy metal bioleaching by sulfur oxidation from sewage sludge has also been shown to highly depend on the process temperature, affecting the variation of growth rates of the bacterial species with pH (Tyagi *et al.*, 1994). Activity of ammonia oxidizing bacteria (AOB) to simultaneous variations in Zn concentration, temperature (23-33 °C) and AOB concentration was studied by Lee *et al.* (Lee *et al.*, 2011), where temperature was observed to have a significant effect on the lag time and ammonia oxidation rate at AOB concentrations below 2.0×10^7 copies/mL.

While limited studies have focused on the impact of temperature on microorganisms under heavy metal exposure; to the authors' knowledge, the effects of temperature on the tolerance of microorganisms exposed to single and combined heavy metals have not yet been investigated. The individual and joint effects of Zn and Cu on the rate and extent of growth of a monoculture *Artrobacter* sp. JM018 (a heavy metal tolerant species) in a continuous flow reactor versus classical batch growth was reported by Sengor *et al.* (2012). In the present study, the effects of temperature on the individual and combined presence of Zn and Cu on the growth patterns of *Arthrobacter* sp. JM018 were tested using 1, 5, and 10 µM Zn, Cu, and 1:1 mol/mol (Zn/Cu) mixtures at 35 °C, in batch reactors. Comparative batch growth experiments were also conducted on *Pseudomonas* sp. and *Arthrobacter* sp. in the presence of metal free growth medium at 35 °C and at ambient temperature (20 °C), and in the presence of 0.01mM, 0.05 mM and 0.1 mM Zn at 20 °C. Microbes from the studied genera may exhibit relatively high tolerance to heavy metals as they have been isolated from a variety of heavy metal contaminated sites (Moberly *et al.*, 2010; Zhang *et al.*, 2004; Mongodin *et al.*, 2006).

2. Materials and methods

2.1. Microorganisms, growth media and inoculum preparation

Arthrobacter sp. JM018 and *Pseudomonas* sp. were isolated from sediment samples from Coeur d'Alene River, Idaho, USA, where the site was contaminated with high levels of Zn (0.75% mass) and Pb (0.5% mass) (Moberly *et al.*, 2009; 2010) and provided a unique habitat for growth of heavy metal tolerant organisms. 16S rRNA gene clone-libraries and microarrays from the sediments samples taken at the time of collection indicated that *Arthrobacter* sp. were present in the microbial community (Moberly *et al.*, 2010; Barua 2007). After isolation, *Arthrobacter* and *Pseudomonas* were grown on a modified formulation of metal toxicity medium (MTM) to decrease metal complexation and precipitation (Sani *et al.*, 2001). The MTM was prepared by dissolving the following in one liter of distilled water: 0.9 g,

$C_6H_{12}O_6$; 0.06 g, Na_2SO_4; 0.02 g, $NaHCO_3$; 0.004 g, NaH_2PO_4; 0.016 g, NH_4Cl; and 0.02 g, yeast extract. Buffer capacity of the MTM was maintained with the addition of PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)], at a concentration of 1.73 g l^{-1}. The medium was autoclaved in serum bottles for sterilization before inoculation. Stock solutions of 10 mM $ZnCl_2$ and 10 mM $CuCl_2$ were prepared in deionized water and acidified with 3 drops of concentrated hydrochloric acid to pH 1.5, and filtered through 0.2 μm membrane filter for sterilization (autoclave of heavy metal solution was avoided to prevent complexation or precipitation at high temperature and keep the solution stable with time). A 5% by volume inoculum was taken from batch cultures unexposed to metals from the late exponential/early stationary cell growth phase. Cell growth was monitored by measuring optical density (O.D.) at 600 nm using a Genesys™ 10 Series Spectrophotometer (Thermo Electron Corporation).

2.2. Batch Experiments

Batch experiments were conducted in duplicates and under sterile conditions in 500 mL serum bottles sealed with butyl rubber septa. 100 mL of medium was added in each bottle and autoclaved at 121 °C for 20 min. After cooling to 25 °C, 5% v/v inoculum was added. Serum bottles were supplemented with filter sterilized (0.2 μm) Cu or Zn stock solutions to give final (single and combined) concentrations of 1, 5 and 10 µM of Zn and/or Cu. Serum bottles were incubated at 20 or 35 °C and where continuously shaken at 100 rpm. Samples were taken at regular intervals for cell growth (O.D. at 600nm) and metals analyses. Microbial growth was monitored by measuring the O.D. of each sample withdrawn at regular intervals from the reactors. Zinc and copper concentrations were monitored using the U.S. Environmental Protection Agency approved colorimetric ZincoVer5® reagent method (620nm) (Hach Method 8009 (Sani *et al.*, 2003); and porphyrin method (Hach Method 8506, Loveland, Co Sani *et al.*, 2001). Calibration standards for both metals were prepared from serially diluted stock solutions of 10 mM $ZnCl_2$ and 10 mM $CuCl_2$. Triplicate samples were obtained for O.D. and heavy metal concentration measurements. Theoretical limits of quantification were 0.75 and 0.2 μM for Zn and Cu, respectively. The Student's *t*-distribution, with a 0.05 level of significance, was employed to reject the statistically extreme values of O.D. and heavy metal concentrations.

3. Results and discussion

3.1. Exposure of Arthrobacter sp. to Zn and Cu at 35 °C

Figure 1 shows changes in O.D. (at 600 nm) with incubation time for *Arthrobacter* sp. JM018 grown at 35 °C, under batch conditions, at single 1, 5, and 10 µM Zn and Cu concentrations, and under combinations of 1/1, 5/5, and 10/10 µMCu/µMZn respectively. The corresponding maximum specific growth rates have been calculated and shown in Figure2. According to the data presented in Figure 2, zing stimulated the growth of *Arthrobacter* at all studied concentrations, however the stimulation effect was decreased from 19%, at 1µM Zn, to 2%, at 10 µM Zn concentration. Exposure of *Arthrobacter* sp. JM018 to 50, 100 and 150 µM Zn concentrations has been studied by Sengor *et al.* (2012), where all of the Zn concentrations tested in this range showed an increasing stimulation with increasing Zn concentration. The difference in this observed stimulation effects may be due to the effect of 35 °C cultivation temperature, compared to room temperature (23 °C) in the previous study(Sengor *et al.*, 2012), as the same growth medium and conditions were used in both studies for the same bacterial species. On the other hand, the response of *Arthrobacter* sp. JM018 to 0-250 µM Zn at pH range between 6-8 studied by Moberly *et al.* (2010), showed inhibition, with an exception of a small stimulatory effect at pH 6 and 10 µM Zn. The observed differences could be due to the cultivation temperature or due to differences in the organic substrate and possibly to the inoculum history. Apart from *Arthrobacter* sp., stimulatory effect of Zn have also been observed for the growth of activated sludge up to 40 mg l^{-1} (610 µM) Zn (Lin *et al.*, 2003) and for 1 mg l^{-1} (15 µM) Zn (Cabrero *et al.*, 1998); and for the growth of *Shewanella* isolates MB4 and FB 18 for up to 25 µM Zn (Toes *et al.*, 2008).

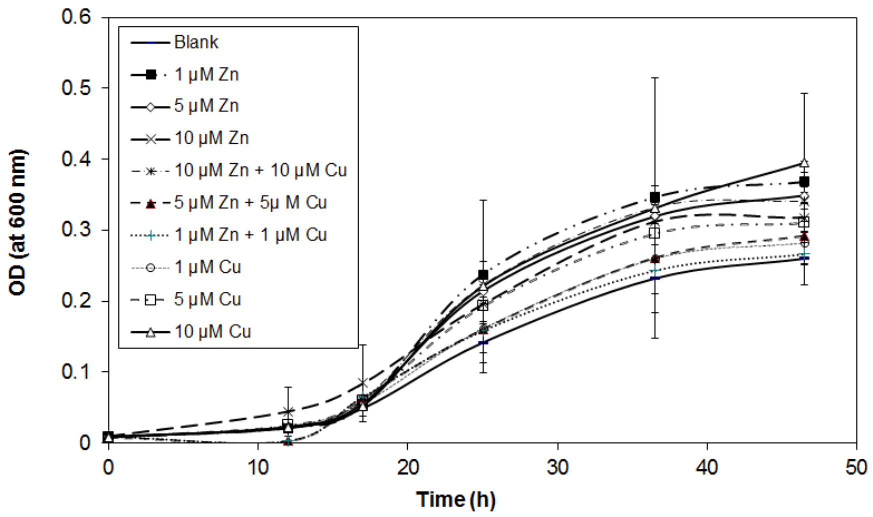

Figure 1. Optical density versus incubation time for *Arthrobacter* sp. growing under batch conditions at metal free, and at the presence of single 1, 5, and 10 µM Zn and Cu concentrations, and under 1/1, 5/5, and 10/10 µMCu/µMZn combinations at 35 °C. Error bars are 95 % confidence intervals.

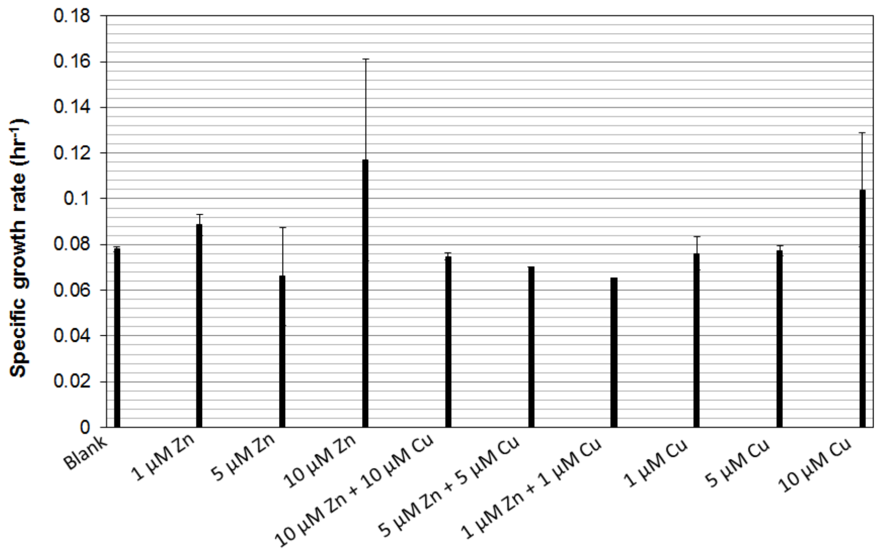

Figure 2. Specific growth rates (h^{-1}) of *Arthrobacter* sp., to metal free, and to the presence of single 1, 5, and 10 µM Zn and Cu concentrations, and to 1/1, 5/5, and 10/10 µMCu/µMZn combinations at 35 °C. Error bars are 95 % confidence intervals.

Based on the present data, addition of copper resulted to growth stimulation of *Arthrobacter* with maximum stimulation of 20% at 10 µM Cu. Similar stimulatory effects of Cu have also been observed for exopolyphosphatase (PPX) activity of *A. ferrooxidans* up to 1-2 µM Cu, where inhibition in the PXX activity occurred for Cu concentrations greater than 5 µM (Alvarez and Jerez, 2004), and up to 10 µM Cu for the archaeon *Sulfolobus metallicus* (Remonsellez *et al.*, 2006). Zn was also observed to stimulate the PXX activity of *A. ferrooxidans* at 1-2 µM concentrations; however, this stimulation effect was only half compared to that of exposure to Cu (Alvarez and Jerez, 2004). Stimulation effect of Zn on the PXX activity was also seen for *S. metallicus* in the micromolar range, similarly lower than of the Cu effect (Remonsellez *et al.*, 2006), both consistent with our observations with *Arthrobacter* sp. Stimulation by Cu was also observed for the superoxide dismutase (SOD) activity of the strain N6 of the yeast *Cryptococcus* sp. when the cells were grown in the presence of 10 mM $CuSO_4$, and the stimulation effect was remarkably enhanced in the presence of 10 mM $CuSO_4$ (Abe *et al.*, 2001).

On the other hand, addition of mixture of zinc and copper resulted to slight growth inhibition, which was measured to vary between 2%, at 10 µM Zn + 10 µM Cu, to 15%, at 1 µM Zn + 10 µM Cu. From the collected data, it looks that zinc and copper have a strong antagonistic effect to the growth of *Arhtrobacter*, as the phenomenon is reversed from growth stimulation at the presence of single Zn or Cu to growth inhibition at joint presence of both metals. However, it should be taken into account that the total concentration of metals (Zn + Cu) was double in the case of joint concentrations, and this may be one of the reasons for the observed growth inhibition.

3.2. Exposure of Arthrobacter sp. to Zn at 20 and 35 °C

Growth curves and specific growth rates for *Arthrobacter* sp. growing in the presence of metal free growth media at 35 °C and 20 °C, and in the presence of 10 µM Zn at the same temperatures are shown in Figure 3. The relative specific growth rates are shown in Figure 4. A significant increase of 92% of specific growth rate for *Arthrobacter* is observed with the increase of temperature form 20 to 35 °C. On the other hand, the stimulatory effects of zinc to the growth of *Arthrobacter* are obviously more pronounced at 20 °C, compared with 35 °C, as in the first case it is observed stimulation by 61% at 10µM Zn, while in the second case the same concentration of zinc has almost inert effect to growth. Temperature may have significant influence over the function and cell structure of the microorganisms, where higher temperature, up to an optimum value, would increase the metabolic activity and energy of the system (Prescott *et al.*, 2002), perhaps promoting the stimulation effect of the heavy metal. Fosso-Kankeu *et al.* (2010) observed higher removal efficiency of Ag(I), Cr(III) and Pb(II) from aqueous solutions at a higher temperature (45 °C) irrespectively of the type of the studied microorganism. The latter is similar to the trend observed by Goyal *et al.* (2003) for biosorption of Cr(VI) by *Saccharomyces cerevisae*. Although Fosso-Kankeu *et al.* (2010) showed a positive correlation between temperature increase and heavy metal biosorption, the degree of variation of the metal uptake was different among various heavy metals. However, in our study we have observed that zinc stimulation is more pronounced at lower temperatures.

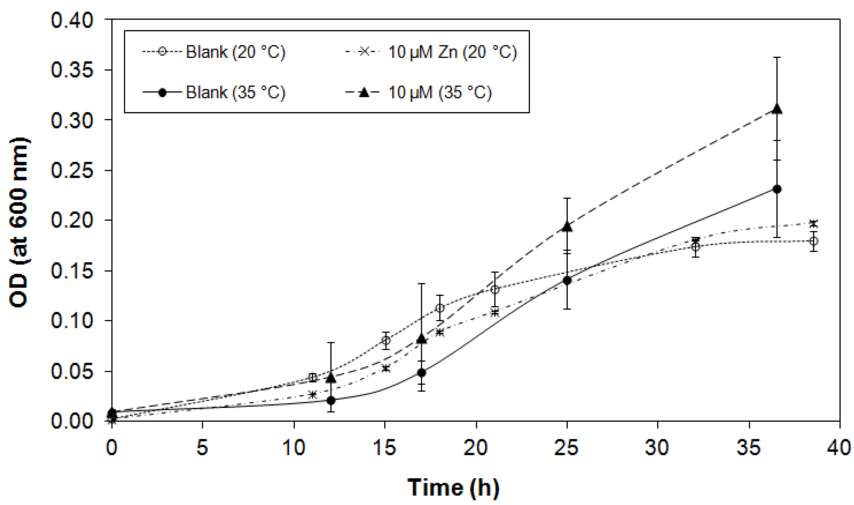

Figure 3. Optical density versus incubation time for *Arthrobacter* sp. growing in the presence of metal free growth medium and in the presence of 10 µM at 35 ºC and at 20 ºC at metal free and in the presence of 10 µM Zn. Error bars are 95 % confidence intervals.

Comparative batch growth experiments were also conducted for *Pseudomonas* sp. For the *Pseudomonas*, the microbial yields also showed higher growth at metal-free medium at 35 °C, followed by the metal-free medium at 20 °C and by 10 µM Zn at 20 °C (data not shown). The growth curves showed that there was increase of lag time from 8 and 15 hours between growth at metal-free (at 20 °C) and at 10 µM Zn concentration (at 20 °C), respectively, whereas at metal free growth at 35 °C the lag time was observed to be zero (Gikas *et al.*, 2009). Increase in lag time with the decrease in temperature has also been observed by Lee *et al.* (2011) for the activity of ammonia oxidizing bacteria. The latter may be attributed to the fact that the biochemical reactions are accelerated until an optimum temperature value (Prescott *et al.*, 2002), which may reduce the lag time required for the synthesis of enzymes, substrates, acclimation to the heavy metals, etc. prior to the start of the succeeding phase. The latter results, comparing the growth behavior of the two bacterial monocultures (*Pseudomonas* sp., *Arthrobacter* sp.) tested under these concentration ranges of Zn, possibly indicates different mechanisms of Zn toxicity by the two microbial species

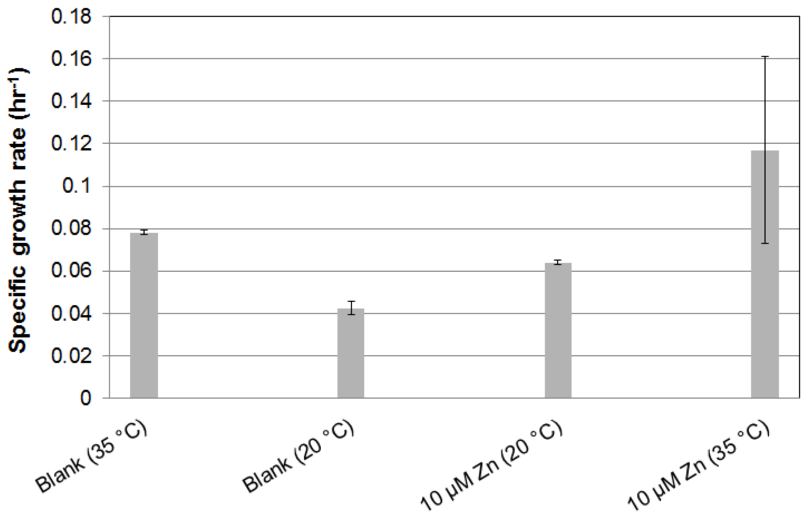

Figure 4. Specific growth rate (h^{-1}) of *Arthrobacter* sp., at metal free growth medium at 35 °C and 20 °C, and at10 µM Zn at 35 °C and 20 °C. Error bars are 95 % confidence intervals.

4. Conclusions

In the present study, batch tests were carried out to evaluate the individual and combined effects of Zn and Cu on the growth patterns of *Arthrobacter* sp. JM018 at 35 °C. The results showed that in all of the Zn and Cu concentrations exhibited a stimulatory effect on JM018 growth; however, the stimulatory effects of zinc were faded out with the increase of Zn concentration from 1µM to 10µM, while the opposite phenomenon was observed for copper. On the other hand, joint concentrations of Zn and Cu resulted to growth inhibition. Experiments with *Arthrobacter* growth at different temperatures with and without the presence of zinc, indicated that the stimulatory effects of zinc was significantly higher at lower temperatures. The latter is indicative of the strong role of temperature on the expression of heavy metals to microorganisms.

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