



Comparison of single and joint effects of Zn and Cu in continuous flow and batch reactors

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Comparison of single and joint effects of Zn and Cu in continuous flow and batch reactors

Sema Sevinç Şengör, Petros Gikas, James G Moberly, Brent M Peyton and Timothy R Ginn

Abstract

BACKGROUND: Microbial behavior in batch reactors may be different from that in continuous flow reactors, which is expected to affect microbial response to heavy metal exposure. Four parallel continuous flow reactors and batch growth tests were used to investigate the single and joint toxicity of Zn and Cu to *Artrobacter* sp. JM018.

RESULTS: The results indicated that Cu is more toxic than Zn under all conditions. In the batch reactors, all Zn concentrations showed a stimulatory effect on microbial growth. However in the continuous system, $125 \mu\text{mol L}^{-1}$ Zn exposure produced inhibition. In the case of mixed Zn and Cu exposures in the batch system, the presence of Zn reduced the severity of Cu inhibition, with a net impact of reduced growth in all cases, whereas in the continuous system microbial growth and substrate utilization rates sharply decreased and ceased.

CONCLUSION: The results clearly showed that growth in batch reactors underestimated significantly the heavy metal inhibition, compared with the continuous system. Therefore, the results of batch reactor tests should not be used directly when heavy metal inhibition is to be interpreted for continuous flow systems.

Keywords: copper; zinc; joint toxicity; *Artrobacter* sp. JM018; growth inhibition; batch reactor; continuous reactor

INTRODUCTION

Heavy metals are defined as metals with above 5 g cm^{-3} density, where the transition elements from V (excluding Sc and Ti) to As, from Zn (excluding Y) to Sb, from La to Po, as well as the actinides and lanthanides are referred to as

heavy metals.¹ Heavy metal toxicity has been observed in various aqueous systems, including rivers, lakes and marine waters. The presence of heavy metals in the aquatic environment may be due to natural (e.g. leakage from natural minerals) or anthropogenic (e.g. industrial wastewaters) sources. Often, the presence of heavy metals destabilizes the performance of wastewater treatment facilities, with negative impact on the quality of the aquatic environment. Heavy metal toxicity has been studied in various microbial

systems,^{1–3} and concentrations of heavy metals above critical thresholds may be toxic, mutagenic and carcinogenic

to a range of biotic species including microorganisms.^{1,2} However, it is well documented that trace amounts of ‘essential’ heavy metals that are often limiting in natural systems (such as Fe, Zn, Ni, Cu, Co) act as growth

stimulants,^{5–8} and are utilized by microorganisms in required biochemical pathways. Among these metals, Cu is chemically highly reactive and plays a significant role in the respiratory chain of many microorganisms. On the other hand, Zn constitutes an important component of a variety of enzymes and DNA-binding proteins, where life seems not to

be possible without its existence.¹ However, increased concentrations of Zn, Cu and other heavy metals have been known to interfere with the essential ions within the cellular sites and block important functional groups of biochemical molecules such as enzymes, polynucleotides, and

essential nutrient transport systems.¹ In general, above trace levels, heavy metals are found to increase lag time and reduce growth rates and yields.^{9–13,5,6,14,15}

Joint effects of Cu and Zn to microorganisms

Although aquatic microorganisms are usually exposed to combinations of heavy metals, most toxicity studies focus on exposure to and effects of single heavy metals. Metals in mixtures are known to interact with biological systems in ways that can significantly alter the toxicity of the individual compounds.^{16,3,4} The combined effect of more than one heavy metal can be greater than the summation

of the individual effects of each metal (synergy) or vice versa (antagonism), or may be equal to the sum of the individual effects of each metal (additive effect).^{5,17} The interaction of microorganisms with combinations of heavy metals, especially in synergistic situations, should be taken into account when establishing tolerance levels of heavy metals concentrations in environmental systems.⁵ The impact of Zn and Cu alone and in binary mixtures on *V. fischeri* was studied by Utgikar *et al.*,¹⁷ who reported that the toxic effects of binary mixtures of copper and zinc were substantially higher than those of individual metals, indicating synergistic interactions between the two metals. Their study also showed that these metals probably exerted different toxicity/inhibition mechanisms exhibited as differences in the qualitative nature of responses, as well as different functional dependencies of the toxic effect on the concentration of Zn and Cu. Lin *et al.*¹⁸ and Cabrero *et al.*¹⁹ studied both the individual and combined effects of copper and zinc on activated sludge microorganisms. They observed that the microorganisms exhibited different kinetic responses to Cu and Zn, while Cu inhibited microbial growth more severely than Zn. Both of the aforementioned studies showed that when Cu and Zn coexisted in batch microbial cultures, their combined effect was additive. The study by Aston *et al.*³ on single and combined toxicities of Cu and Zn to *Acidithiobacillus caldus* strain BC13 showed a less than additive response when Cu and Zn were present in binary mixtures, and reported a higher Cu tolerance compared with individual Zn exposure, in contrast to the previous work by Watkin *et al.*²⁰ on *A. caldus* strain KU. Franklin *et al.*²¹ studied the individual and combined effects of Cu and Zn on the cell division rate of alga *Chlorella* sp. They found that the cellular uptake of Cu was far greater than that of Zn. Additional growth inhibition bioassays were also conducted to measure intracellular and extracellular (membrane-bound) metal concentrations to determine the impact of each metal. The toxicity of combinations showed a less than additive response of Cu and Zn, which was probably due to competitive binding of these metals at the cell surface. Their results showed that when algal cells were exposed to equitoxic mixtures of Cu and Zn, both extra- and intracellular Zn concentrations were reduced compared with the individual effect of Zn. This suggested that Cu and Zn shared common uptake and transport sites on *Chlorella* sp. and that Cu outcompeted Zn for cell binding, which resulted in the inhibition of Zn uptake in the presence of Cu, and subsequent reduction in growth inhibition.²¹

Batch versus continuous growth systems

The primary disadvantage of the studies cited above is that they measure short-term inhibitory responses in batch systems. A number of studies have demonstrated that such short-term batch assays may not reflect the responses observed in continuous flow systems after prolonged exposure to heavy metals.^{22–24} Often, continuous flow systems approximate more closely the natural environment, such as a pond with inflow and outflow streams, and allow long-term exposure of the microorganism to the pollutant, due to its continual input of pollutants and nutrients.²⁵ Tripathi *et al.*²⁵ studied the individual inhibition of Zn and Cu in batch and semicontinuous cultures to the cyanobacteria *Anabaena doliolum*. They observed that the semicontinuous culture was greater than two-fold more sensitive than the batch culture, which was associated with the greater accumulation of the metals within the microorganism. Hu *et al.*²² studied nitrification inhibition by individual loads of Cu, Zn, Ni and Cd in batch and continuous flow reactors. Their short-term batch assays significantly underestimated the observed inhibition in the

continuous reactors, where the inhibition was attributed to the slow kinetics of Zn, Ni and Cd internalization and was exacerbated due to the continuous exposure of the biomass to the heavy metal in the continuous system. Sen *et al.*²³ studied the removal of Cr(VI) using *Fusarium solani* in batch and continuous systems. They found that the continuous reactor performed better than the batch system, as the first operated for a longer time achieving higher Cr(VI) removal, compared with the batch mode of operation. On the other hand, a study by Caravelli and Zaritzky²⁶ showed a *Sphaerotilus natans* batch system with high concentrations of initial Cr(VI) exhibiting higher Cr(VI) reduction in terms of both rate and efficient use of energy source, compared with continuous systems. The studies by Chao and Chen²⁷ and Chen *et al.*²⁴ compared the relative toxicity relationships for various heavy metals using batch and continuous tests on the alga *Selenastrum capricornutum*. Chen *et al.*²⁴ showed that their continuous tests resulted in a higher degree of inhibition compared with batch tests, except for the Cd–Zn and Ni–Cd mixtures. From the studies cited above, it is clear that to predict the behavior of microorganisms in the presence of heavy metals in real systems (such as aquatic systems, soils and biological wastewater treatment plants), the long-term exposure of microorganisms to the pollutants at continuous flow conditions need to be taken into account.

Although limited studies have examined the inhibition of metals in continuous and batch systems, batch versus continuous reactors have not been compared to evaluate the individual and combined effects of heavy metals on the growth of microbial cultures. Therefore, the aim of this study was to investigate the individual and joint effects of two heavy metals on the rate and extent of growth of a monoculture in continuous or batch mode. We examined the effects of Zn and Cu on *Arthrobacter* sp. JM018, where *Arthrobacter* species are considered to be ubiquitous²⁸ and have the ability to adjust membrane characteristics to successfully survive in extreme environments,²⁹ in a continuous flow reactor versus classical batch growth, which is the first attempt to the authors' knowledge. It is true that mixed microbial populations (often coexisting in microbial consortia) are encountered in environmental systems. However, mixed microbial populations are not ideal to study the mechanisms of the effects of heavy metals on microbial behavior, as the observed phenomena can be attributed to either actual changes in microbial metabolism, or to shift in microbial populations.^{30–31} Therefore, in order to avoid interference of population shifting with microbial behavior in continuous and batch systems, the present study was carried out on a heavy metal tolerant monoculture, growing under sterile conditions.

MATERIALS AND METHODS

Microorganism, growth media and inoculum preparation

Arthrobacter sp. JM018 was isolated from sediment samples in heavy metal contaminated Coeur d'Alene River, Idaho, where the site was contaminated with high levels of Zn (0.75% mass) and Pb (0.5% mass)^{32,4} and provided a unique habitat for cultivation of heavy metal tolerant organisms. 16S rRNA gene clone-libraries and microarrays from the sediment samples taken at that time indicated that *Arthrobacter* sp. were present in the microbial community.^{33,4} After isolation, *Arthrobacter* was grown on a modified formulation of metal toxicity medium (MTM) to decrease metal complexation and precipitation.⁹ The MTM was prepared by dissolving the following in 1 L of distilled water: 0.9 g, C₆H₁₂O₆; 0.06 g, Na₂SO₄; 0.02 g, NaHCO₃; 0.004 g, NaH₂PO₄;

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 to sterilize before inoculation. Stock solutions of $10 \text{ mmol L}^{-1} \text{ ZnCl}_2$ and $10 \text{ mmol L}^{-1} \text{ CuCl}_2$ were prepared by dissolving 500 mL ZnCl_2 and CuCl_2 in deionized water, acidifying with three drops of concentrated hydrochloric acid to pH 1.5, and filter sterilizing ($0.2 \mu\text{m}$) into sterile MTM to minimize complexation or precipitation at high temperature and to keep the solution stable over time. For these experiments, a 5% by volume inoculum was taken from cultures unexposed to metals from late exponential/early stationary phase cells. Cell growth was monitored using optical density measurements at 600 nm.

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 to 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 ($0.2 \mu\text{m}$) Cu or Zn stock solutions to give final concentrations of 50, 100 and $150 \mu\text{mol L}^{-1}$ Zn and Cu. Serum bottles were incubated at 25°C and shaken at 100 rpm. Samples were taken at regular intervals for cell growth (optical density, OD at 600 nm) and metal analyses.

CSTR experiments

In the continuous flow experiments, four 330 mL reactors were used. All four reactors were initially run under metal-free conditions until steady state condition was achieved. Upon reaching steady state, copper and zinc were introduced into each of the reactors as follows: $125 \mu\text{mol L}^{-1}$ Zn and $40 \mu\text{mol L}^{-1}$ Cu in reactor 2, $125 \mu\text{mol L}^{-1}$ Zn in reactor 3, $40 \mu\text{mol L}^{-1}$ Cu in reactor 4, and the first reactor was used as control (no Zn or Cu addition). The reactors were operated with a flow rate of 0.5 mL min^{-1} , resulting in a residence time of 11 h. At regular intervals, culture suspensions were withdrawn from the reactors to measure optical density, pH and glucose concentration.

Analytical techniques

Microbial growth was monitored by measuring the optical density of each sample withdrawn at regular intervals from the reactors using the Genesys™ 10 Series Spectrophotometer (Thermo Electron Corporation) at 600 nm. Glucose concentration was measured using the Glucose (HK) Assay Kit (SIGMA). Zinc and copper concentrations were monitored using the US Environmental Protection Agency approved colorimetric (spectroscopic) ZincoVer5® reagent method (620 nm) (Hach Method 8009;¹⁰ and colorimetric (spectroscopic) porphyrin method (Hach Method 8506, Loveland, CO¹⁵). Both methods were modified to utilize small sample volumes. Calibration standards for both metals were prepared from serially diluted stock solutions of $10 \text{ mmol L}^{-1} \text{ ZnCl}_2$ and $10 \text{ mmol L}^{-1} \text{ CuCl}_2$. Theoretical limits of quantification were $0.75 \pm 0.08 \mu\text{mol L}^{-1}$ for Zn and $1.3 \pm 0.2 \mu\text{mol L}^{-1}$ for Cu.

RESULTS AND DISCUSSION

Batch experiments

Optical density at 600 nm (which is linearly correlated with biomass concentration) versus incubation time graphs for *Arthrobacter* sp.

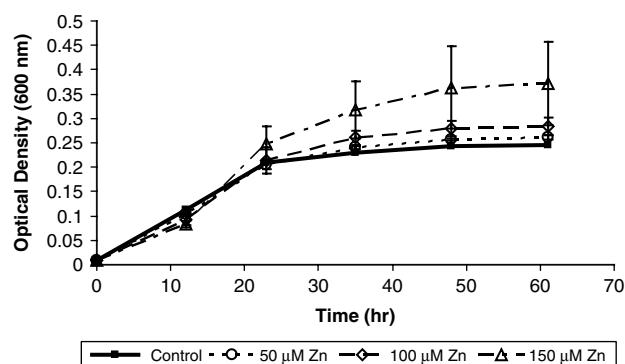


Figure 1. Optical density versus incubation time for *Arthrobacter* sp. growing under metal-free batch conditions, and in the presence of 50, 100, and $150 \mu\text{mol L}^{-1}$ Zn concentrations. The points are the average of duplicates, and error bars indicate \pm standard deviation of the mean ($n = 2$). Error bars smaller than the symbol are not shown.

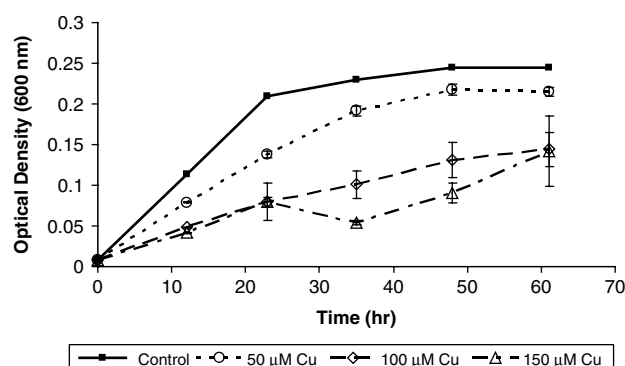


Figure 2. Biomass versus time graph for *Arthrobacter* sp. growing under metal-free batch conditions, and in the presence of 50, 100, and $150 \mu\text{mol L}^{-1}$ Cu concentrations. The points are the average of duplicates, and error bars indicate \pm standard deviation of the mean ($n = 2$). Error bars smaller than the symbol are not shown.

JM018 growing under batch conditions at single 50, 100, and $150 \mu\text{mol L}^{-1}$ Zn and Cu concentrations, and under combinations of 50/50, 100/100, and $150/150 \mu\text{mol L}^{-1}$ Cu/ $\mu\text{mol L}^{-1}$ Zn, respectively, are given in Figs 1 to 3, respectively. Figure 1 shows that, compared with the control (metal free cultivation), all of the Zn concentrations showed a stimulatory effect on the extent of JM018 growth. It is observed that stimulation increases with Zn concentration, for the range of concentrations checked. The response of *Arthrobacter* sp. JM018 to combinations of pH (6–8) and Zn concentrations (0 – $250 \mu\text{mol L}^{-1}$) was studied by Moberly *et al.*⁴ It was observed that with the exception of a small stimulatory effect at pH 6 and $10 \mu\text{mol L}^{-1}$ Zn, increases in both pH and Zn had a negative effect on specific growth rate and cell yield, showing a lower Zn tolerance than the present study. This difference may be attributed to the difference in the organic substrate used or possibly inoculum history.

The stimulatory effect of Zn at concentrations up to 40 mg/L ($610 \mu\text{mol L}^{-1}$)¹⁸ and with 1 mg L^{-1} ($15 \mu\text{mol L}^{-1}$)¹⁹ has also been reported for the growth of activated sludge; and up to $25 \mu\text{mol L}^{-1}$ Zn on the growth of both *Shewanella* isolates MB4 and FB18.³⁴ On the other hand Tripathi *et al.*²⁵, Amor *et al.*³⁵, and Benko-Coker and Ekundayo³⁶ observed inhibition even at concentrations as low as $2.5 \mu\text{mol L}^{-1}$ Zn on the alga *A. doliolum*, $50 \mu\text{mol L}^{-1}$ on *Bacillus* sp., and $10 \mu\text{mol L}^{-1}$ Zn on *Pseudomonas* sp., respectively. The

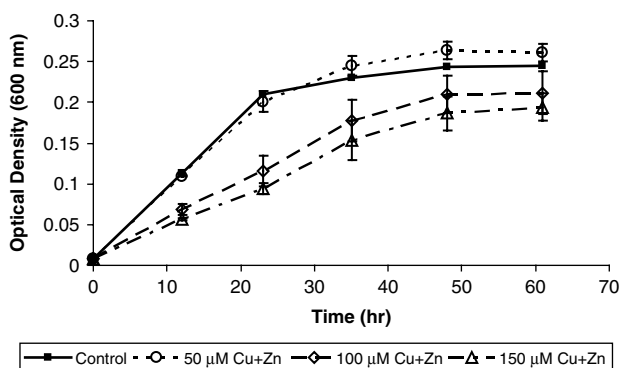


Figure 3. Biomass versus time graph for *Arthrobacter* sp. growing under metal-free batch conditions, and in the presence of combined 50, 100, and 150 $\mu\text{mol L}^{-1}$ Zn and Cu concentrations. The points are the average of duplicates, and error bars indicate \pm standard deviation of the mean ($n = 2$). Error bars smaller than the symbol are not shown.

no-observable-effect concentration (NOEC), lowest-observable-effect concentration (LOEC) and median effective concentration (EC_{50}) for Zn exposure of the alga *Chlorella* sp. after 72 h was shown to be 0.31 $\mu\text{mol L}^{-1}$, 0.57 $\mu\text{mol L}^{-1}$ and 1.4 $\mu\text{mol L}^{-1}$, respectively;²¹ and the EC_{50} value for the aerobic cell growth of *S. putrefaciens* CN32 was observed to be 300 $\mu\text{mol L}^{-1}$; whereas for other respiration conditions it was observed to be 210, 280, and 49 $\mu\text{mol L}^{-1}$, for hematite, ferric citrate, and nitrate reduction, respectively.³⁷ Cho *et al.*³⁸ observed inhibition on the iron oxidation activity of *A. ferrooxidans* at 230 mmol L^{-1} , and Aston *et al.*³ observed the minimum inhibitory concentration (MIC) as 75 mmol L^{-1} Zn on *A. caldus* strain BC13. On the other hand, any significant toxic effect of Zn was not observed on *T. thiooxidans* up to 30 mmol L^{-1} ,³⁹ at 150 $\mu\text{mol L}^{-1}$ Zn on *T. ferrooxidans*,⁴⁰ and between 50 and 200 $\mu\text{mol L}^{-1}$ Zn on the growth of *Bacillus thuringiensis*.⁴¹ Thus from the studies cited above, it is seen that this *Arthrobacter* sp. JM018 can tolerate higher concentrations of Zn, compared to activated sludge, *Bacillus* sp., *Pseudomonas* sp., *Shewanella* sp., and the algae *A. doliolum* and *Chlorella* sp.

The growth curves for JM018 growing under batch conditions with 50, 100, and 150 $\mu\text{mol L}^{-1}$ Cu concentrations are given in Fig. 2. It can be seen that all Cu concentrations used in this study inhibited the growth of JM018. Higher Cu concentration caused greater inhibition of the growth rate and the extent of growth. Similar results have been reported by Cho *et al.*³⁸ on *A. ferrooxidans*; Toes *et al.*³⁴ on *S. oneidensis* MR1 and on *Shewanella* isolates MB4 and FB18; Sani *et al.*⁹ on *D. desulfuricans* G20; and Cabrero *et al.*¹⁹ and Lin *et al.*¹⁸ on activated sludge microorganisms. The NOEC, LOEC, and EC_{50} values for Cu exposure to the alga *Chlorella* sp. after 72 h was observed as 0.07 $\mu\text{mol L}^{-1}$, 0.09 $\mu\text{mol L}^{-1}$ and 0.11 $\mu\text{mol L}^{-1}$, respectively;²¹ the EC_{50} value for the alga *Cylindrotheca closterium* was shown to vary between 0.07 and 0.4 $\mu\text{mol L}^{-1}$,⁴² and the MIC value for *A. caldus* strain BC13 was observed to be 250 mmol L^{-1} Cu.³ Lin and Chen⁴³ observed EC_{50} as 90 $\mu\text{mol L}^{-1}$ for Cu to activated sludge, whereas for *Escherichia coli* they observed EC_{50} to vary from 11.7 to 180 $\mu\text{mol L}^{-1}$ with varying COD concentrations. Chen *et al.*³⁹ observed growth inhibition for *T. thiooxidans* around 2300 $\mu\text{mol L}^{-1}$ Cu. The higher toxicity of Cu compared to Zn is probably due to the unique mode of action of Cu ions, which involves loss of cell membrane integrity.²² The disruption of the cytoplasmic membrane as the major mechanism of microbial Cu toxicity has been supported by Avery *et al.*⁴⁴ and Hu *et al.*⁴⁵ Cu has been shown to induce cytotoxicity by

producing free hydroxyl radical through Fenton type reactions and promoting membrane lipid peroxidation.^{46–47} Hu *et al.*²² observed a significant loss of intracellular potassium ions (K^+), an indication of the loss of membrane integrity after the addition of Cu. The higher toxicity of Cu compared with Zn has been outlined by Nies,¹ who agreed that the latter is probably due to Cu-triggered production of hydroperoxide radicals⁴⁸ which interacts with the cell membrane,⁴⁹ resulting in many organisms being more sensitive to Cu.⁵⁰

The growth curves for JM018 growing under batch conditions with combinations of 50/50, 100/100, and 150/150 $\mu\text{mol L}^{-1}$ of Cu/Zn respectively, are given in Fig. 3. In the 50 $\mu\text{mol L}^{-1}$ Zn + 50 $\mu\text{mol L}^{-1}$ Cu combined concentrations, the growth rate of JM018 was the same as the control, while the extent of growth was slightly greater. Therefore, at these concentrations the stimulatory effect of 50 $\mu\text{mol L}^{-1}$ Zn appears to have overcome the inhibitory effect of 50 $\mu\text{mol L}^{-1}$ Cu, both for the growth rate and extent. To the knowledge of the authors, similar biostimulation effects have not been reported in the literature for any microorganism. The 100 $\mu\text{mol L}^{-1}$ Cu + 100 $\mu\text{mol L}^{-1}$ Zn and 150 $\mu\text{mol L}^{-1}$ Cu + 150 $\mu\text{mol L}^{-1}$ Zn mixed concentrations showed inhibition of both the rate and extent of growth; however, at these concentrations the growth rate and extent were higher than when JM018 was grown with 100 $\mu\text{mol L}^{-1}$ or 150 $\mu\text{mol L}^{-1}$ Cu alone. Our results indicate that the stimulatory effect of Zn overcame the inhibitory effects of Cu. This observation is in accordance with Cabrero *et al.*,¹⁹ who also reported for activated sludge that the addition of Zn in Cu solutions mitigated the toxic effects of Cu alone.

Continuous flow reactor experiments

In the continuous flow reactor experiments, four similar CSTRs were operated simultaneously under identical conditions, apart for heavy metal concentration in the sterile feed solution. All four reactors were operated initially without the addition of heavy metals until steady state conditions were reached and maintained for about 260 h. After 260 h of operation, 125 $\mu\text{mol L}^{-1}$ Zn plus 40 $\mu\text{mol L}^{-1}$ Cu (referred to as reactor 2), 25 $\mu\text{mol L}^{-1}$ Zn (referred to as reactor 3), and 40 $\mu\text{mol L}^{-1}$ Cu (referred to as reactor 4) were introduced in each of the three reactors, where the first reactor (referred to as reactor 1) was used as control without the addition of either Zn or Cu. The metals were introduced into the system as a simultaneous injection of Zn and/or Cu solutions into the inlet medium carboy tanks (feeding the reactors) and into the individual reactors such that the desired concentrations were instantly achieved and maintained in each reactor. The injection of metals into the individual reactors was performed to preclude any dilution effects from the feeding tanks, and thus instantly achieve the desired concentration of metals in the corresponding reactors.

The results from the continuous flow reactor system indicated that JM018 growing under continuous flow conditions was more sensitive to both Zn and Cu than when grown in batch reactors. The observed biomass and glucose concentrations with time are given in Figs 4 and 5 for the continuous flow systems. It can be seen from Figs 4 and 5 that during steady state operation (up to 260 h), both the optical density (varying between 0.13 and 0.17) and glucose concentrations (varying between 7 and 18 mg L^{-1}) remained nearly constant before metal addition. The observed variations may be attributed to slightly different behavior of CSTRs, or to sampling or measurement errors. With no metal addition, the control reactor (1) continued to remain nearly constant throughout completion of the experiment with optical

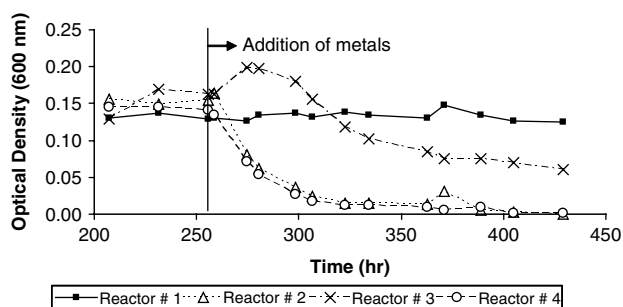


Figure 4. Optical density versus operation time for *Arthrobacter* sp. growing in a metal-free continuous flow (reactor 1), $125 \mu\text{mol L}^{-1}$ Zn and $40 \mu\text{mol L}^{-1}$ Cu (reactor 2), $125 \mu\text{mol L}^{-1}$ Zn (reactor 3), $40 \mu\text{mol L}^{-1}$ Cu (reactor 4), concentrations.

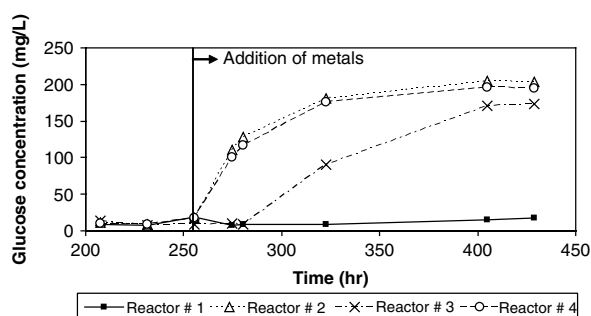


Figure 5. Glucose versus operation time graph for *Arthrobacter* sp. growing in a continuous metal-free flow reactor (reactor 1), $125 \mu\text{mol L}^{-1}$ Zn and $40 \mu\text{mol L}^{-1}$ Cu (reactor 2), $125 \mu\text{mol L}^{-1}$ Zn (reactor 3), $40 \mu\text{mol L}^{-1}$ Cu (reactor 4), concentrations.

density varying between 0.13 and 0.15 and glucose concentrations varying between 7 and 17 mg L^{-1} . The reactor with $125 \mu\text{mol L}^{-1}$ Zn (reactor 3) showed, surprisingly, a small increase in biomass concentration for the first 20 h after Zn addition, and then a steady decline throughout the experiment. Glucose concentrations in the reactors were below detection limit during the first 280 h of operation and then steadily increased to about 170 mg L^{-1} , with a parallel decrease in biomass concentration. The reactors with $40 \mu\text{mol L}^{-1}$ Cu (reactor 4) and combined $125 \mu\text{mol L}^{-1}$ Zn and $40 \mu\text{mol L}^{-1}$ Cu (reactor 2) showed a steep decline in biomass, achieving a biomass concentration close to zero, after about 130 h of heavy metal additions. Glucose concentrations showed a parallel steep increase and then reached steady values of around 200 mg L^{-1} (which was the feed glucose concentration), after about 140 h from heavy metal addition, indicating that microbial growth had ceased. The metal-free control reactor (1) on the other hand, showed a nearly constant optical density around 0.13 and a glucose concentration below detection throughout the experimental period. The glucose concentration in the feed tank was steady at 200 mg L^{-1} . These results, taken together, indicate that all metal concentrations used in the CSTRs were inhibitory. This is in contrast to results obtained in batch reactors.

Toxicity in batch reactors vs CSTR

The results indicate that Cu is more toxic than Zn to JM018 under all conditions tested, which is in accordance with previous findings,^{21,19,18} working with different microbial systems. In the batch reactors, all Zn concentrations tested ($50\text{--}150 \mu\text{mol L}^{-1}$) showed a stimulatory effect on the extent of JM018 growth.

However, in the continuous system, exposure to $125 \mu\text{mol L}^{-1}$ Zn showed significant inhibition, unlike the batch system. In the case of mixed Zn and Cu exposures in the batch reactor system, the presence of Zn reduced the severity of Cu inhibition, with a net impact of reduced growth in all cases, whereas in the continuous system the microbial growth and substrate utilization rates decreased sharply and almost ceased. Results showed that the batch reactor tests significantly underestimated heavy metal inhibition, as was observed in the continuous flow reactors. The latter is in accordance with the observations by Hu *et al.*²² and Tripathi *et al.*²⁵ The reasons for this greater sensitivity of microorganisms in continuous flow systems compared with batch reactors may be the slow kinetics of heavy metal internalization and an exacerbation effect by the continual metal exposure in the continuous system^{22,45} Alternatively, cells in the CSTR may accumulate higher heavy metal concentrations because (a) the cells in this system may remain more physiologically active than in batch systems,^{25,51} or (b) maintenance of high concentrations of heavy metals with regular dilution of metal-supplemented medium into the continuous systems.²⁵ Reduced uptake of metals from the batch system may also have occurred due to the changing physiological state of the cells in the batch reactor.²⁵ Tripathi *et al.*²⁵ reported a decline in metal content of the cells in a batch system, compared with a more or less constant level with time in a semicontinuous system, resulting in a greater toxic effect. This variability was explained by probable differences in the physiological status of the cells in batch and semicontinuous systems.

CONCLUSIONS

Batch and continuous reactors are compared to evaluate the individual and combined effects of two heavy metals on the growth patterns of *Arthrobacter* sp. JM018. In the continuous flow system, four reactors were initially run at metal free conditions until steady state was achieved. Then $125 \mu\text{mol L}^{-1}$ Zn and $40 \mu\text{mol L}^{-1}$ Cu in reactor 2, $125 \mu\text{mol L}^{-1}$ Zn in reactor 3, and $40 \mu\text{mol L}^{-1}$ Cu in reactor 4, was introduced, with the first reactor used as control (no Zn or Cu addition). Batch growth tests were carried out at 50, 100 and $150 \mu\text{mol L}^{-1}$ Zn, Cu, and 1 : 1 mol/mol mixtures of Zn and Cu. Cu was observed to be more toxic than Zn under all conditions. The batch test results showed that all Zn concentrations showed a stimulatory effect on JM018 growth and the stimulation was increased with Zn concentration, whereas in the continuous system, the $125 \mu\text{mol L}^{-1}$ Zn exposure caused inhibition. All the Cu concentrations inhibited the growth of JM018 and the higher the Cu concentration used, the greater was the inhibition. In the case of mixed Zn and Cu exposures in the batch system, the presence of Zn reduced the severity of Cu inhibition, with a net impact of slightly reduced growth in all cases, whereas in the continuous system, the microbial growth and substrate utilization rates decreased sharply and ceased. Thus, growth in batch reactors significantly underestimated the heavy metal inhibition, as was observed in the continuous system. Therefore, the results of batch reactor tests should be used with caution when heavy metal inhibition is to be interpreted for continuous flow natural environmental systems, such as rivers, wetlands, and the design and operation of biological reactors.

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