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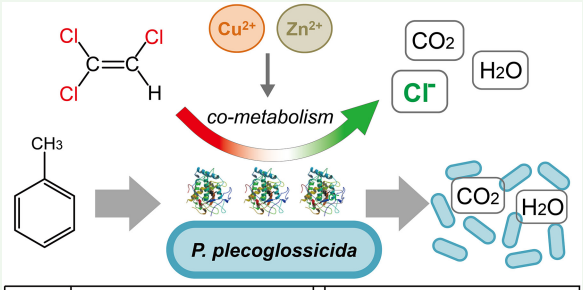
Zinc and copper supplements enhance trichloroethylene removal by *Pseudomonas plecoglossicida* in water

Lan Qiu, Keng Seng Lok, Qihong Lu, Hua Zhong, Xiaoyuan Guo and Hojae Shim

Department of Civil and Environmental Engineering, Faculty of Science and Technology, University of Macau, Macau SAR, People's Republic of China

ABSTRACT

The effects (stimulatory/inhibitory) of two representative microelements, zinc and copper, on the aerobic co-metabolic removal of trichloroethylene (10 mg/L) by the indigenous isolate *Pseudomonas plecoglossicida* were investigated. The strain was previously isolated from a petroleum-contaminated site using toluene (150 mg/L) as growth substrate. Different concentrations (1, 10 and 100 mg/L) of microelements provided with SO_4^{2-} and Cl^- as the anions were tested. The results showed the supplement of Zn^{2+} and Cu^{2+} at the low concentration (1 mg/L) significantly enhanced cell growth. The removal efficiencies for toluene and trichloroethylene were also enhanced at the low concentration (1 mg/L) of Zn^{2+} and Cu^{2+}, compared to the higher concentrations (10 and 100 mg/L). Compared to the control without zinc supplement, higher concentrations of zinc (10 and 100 mg/L) enhanced the removal efficiencies for both toluene and trichloroethylene in the first three days but showed some inhibitory effect afterward. However, the higher concentrations of Cu^{2+} (10 and 100 mg/L) always showed inhibitory to the toluene removal while showing inhibitory to the TCE removal after three days. For both Zn^{2+} and Cu^{2+}, the anions SO_4^{2-} and Cl^- did not show significant difference in their effects on the toluene removal. A possible mechanism for Zn^{2+} and Cu^{2+} to enhance the removal of toluene and trichloroethylene would be their involvement in toluene oxygenase-based transformation processes. In addition, high concentrations of Zn^{2+} and Cu^{2+} ions could be removed from the liquid by the cells accordingly. The results imply a great potential of supplementing low concentrations of zinc and copper to enhance bioremediation of the sites co-contaminated with toluene and trichloroethylene.



	Toluene Degradation			TCE Degradation		
Zn^{2+}	1mg/L	10mg/L	100mg/L	1mg/L	10mg/L	100mg/L
Day1	+ +	+	+	+	+	+
Day3	+ +	+	+	+ +	+	+ +
Day6	+	-	-	+ +	-	+
Day8	0	-	--	+ +	-	-
Cu^{2+}	1mg/L	10mg/L	100mg/L	1mg/L	10mg/L	100mg/L
Day1	+ +	---	---	+	+	0
Day3	+ +	--	--	+	+	0
Day6	+	--	---	+ +	-	--
Day8	0	--	---	+ +	-	--

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CONTACT Hojae Shim hjshim@um.edu.mo Department of Civil and Environmental Engineering, Faculty of Science and Technology, University of Macau, Macau SAR 999078, People's Republic of China

1. Introduction

Trichloroethylene (TCE) is a typical chlorinated aliphatic hydrocarbon and probably the most widely used chemical in the world [1,2]. The extensive use of TCE increases the chance of unintentional spill and disposal, resulting in its wide distribution and frequent detection in groundwater and soil [3–5]. For example, TCE was detected at 57% of the National Priorities List (NPL) sites in 2015, and 1051 out of the 1854 NPL sites in U.S.A. were contaminated with TCE [6]. TCE is of particular concern because of its classification as a human carcinogen and its detrimental effects on the nervous system [1]. Exposure to TCE is associated with detrimental effects to human health, including immunotoxicity, neurotoxicity, liver and kidney toxicity, endocrine effects and cancer [6]. Therefore, raising concerns have been aroused for developing efficient strategies for TCE remediation [2]. However, the remediation can also be challenging due to its high volatility and propensity to form a dense non-aqueous phase liquid in an aquifer. On the other hand, toluene is another frequently found soil and groundwater contaminant of great concern. A large amount of toluene is released to the environment from manufacturing use, disposal of industrial products [7], industrial wastewater discharge and gasoline leak at the gas stations [8]. The acute exposure to toluene can impact central nervous system, kidney, skin and liver, and cause loss of memory, somnolence and locomotor defects in invertebrates and mammals [9].

Bioremediation, based on the metabolic capabilities of microorganisms, is considered a cost-effective, environmentally friendly and in situ adaptive technology for treating contaminated soil and groundwater, and has been widely used in the treatment of organic contaminants including TCE and toluene [10–12]. The biotransformation of TCE can take place under both aerobic and anaerobic conditions. In anaerobic conditions, incomplete transformation of TCE can lead to the accumulation of toxic intermediates such as dichloroethylenes and vinyl chloride [13]. In comparison, aerobic degradation of TCE is more appropriate in the presence of other growth substrate and considered a faster and safer process [14]. TCE can be co-metabolically degraded by utilising toluene as the principal carbon source under the aerobic condition [15,16] and the toluene mono- or dioxygenases have been reported as the enzymes responsible for the co-metabolism of TCE in certain *Pseudomonas* spp. [17,18]. Moreover, petroleum and chlorinated aliphatic hydrocarbons have been frequently detected as co-contaminations in the environment, including landfill, subarctic groundwater and aquifer plume transect [7,19–22]. Therefore, it would be advantageous and practically significant to apply bioremediation technologies to simultaneously remove of toluene and TCE from the contaminated environment. There have been many studies focusing on TCE co-metabolism using toluene or other compound as growth substrate [4,23]. On the other hand, during the bacteria-mediated degradation process, microelements as dietary metal elements required in small quantities for the proper physiological function and growth of microorganisms have also shown effects on the co-metabolic degradation of TCE. Yang et al. investigated biological treatment of the toluene and TCE-contaminated wastewater using a sequencing batch reactor inoculated with alginate-immobilised *Pseudomonas putida* F1 and showed the presence of 1 mg/L of nickel (Ni) or 20 mg/L of iron (Fe) stimulated the degradation rates of both TCE and toluene [14]. In addition, *Dehalobacter restrictus* PER-K23 was reported to specifically use perchloroethylene (PCE) and TCE as the electron acceptor under the anaerobic condition, and Fe at a trace concentration (1.5 mg/L) was required for the dechlorination reaction [24]. Henry and Grbic-Galic reported a methanotroph, *Methylomonas* sp. MM2, exhibited significantly higher TCE oxidation rate in the culture medium containing significantly high concentrations of zinc (Zn), molybdenum (Mo), Fe and ethylene diamine tetra-acetic acid (EDTA) [25]. Gao et al. reported at 0.002 and 0.318 mg/L, Cu^{2+} increased the degradation rate of TCE in a mixed consortium SWA1 with methane as the substrate [26]. These indicate the microelements play important roles in co-metabolism of TCE, thereby improving its removal efficiency.

Zinc (Zn^{2+}), copper (Cu^{2+}) and other transition metals and nanoscale metals are considered essential and playing positive/vital roles for living organisms including plants and bacteria [27–29]. These microelements act as co-factors for various enzymes associated in the metabolism of various organic molecules such as carbohydrates, organohalides, nucleic acids, proteins and lipids [30,31]. Zn and Cu may coexist with TCE and toluene in the contaminated sites [32,33]. Soil has been considered as the largest recipient of metals entering the environment and the metals including zinc and copper are expected to increase continually given the widespread and expanding application of sewage sludge and livestock manure as well as the direct industrial discharge [34,35]. Metals combined with soil particles are subject to the movement along with the soil water and may transport through the vadose zone to groundwater [36], thereby playing potential roles in the enzyme-based co-metabolism of TCE. However, the study on the effects of such microelements on the co-

metabolism of TCE in the presence of toluene is scarce, and the knowledge about the functions of zinc and copper in this regard is not sufficient for the accurate evaluation of bioremediation technology applied for remediating the sites co-contaminated with TCE and toluene.

In this study, the main objective was to study the effects of two representative microelements (zinc and copper) on the TCE co-metabolism utilising toluene as growth substrate. The microorganism used was previously isolated from a heavily petroleum-contaminated site and identified as *Pseudomonas plecoglossicida* [37]. *Pseudomonas plecoglossicida* is a Gram-negative, aerobic and rod-shaped bacterium, which is closely related to *P. putida*, showing > 99% of similarity of the 16S rRNA gene [38]. *Pseudomonas* strains are prevalent cultivatable aerobic monoaromatic degraders across diverse environments [39], which have been demonstrated efficient in co-metabolising TCE with the presence of toluene [10,14]. Different concentrations of Zn and Cu provided in forms of sulfate and chloride salts were evaluated at the fixed concentrations of TCE and toluene. The cell growth and metal concentrations were both monitored to further characterise the TCE co-metabolism by *P. plecoglossicida*. In this study, the main objective was to assess the effects (stimulatory/inhibitory) of zinc (Zn^{2+}) and copper (Cu^{2+}) supplements on the aerobic co-metabolic removal of TCE by the isolate *Pseudomonas plecoglossicida*, utilising toluene as growth substrate. Results from this study could expand our knowledge on microelements mediated TCE co-metabolism by *P. plecoglossicida* and shed light on in situ bio-stimulation with proper zinc and copper to enhance the removal of toluene and trichloroethylene.

2. Materials and methods

2.1. Chemicals

Dimethylformamide (DMF; 99% purity) and TCE (99% purity) were purchased from Damao Chemical Co. Ltd (Tianjin, China). Toluene (99% purity) was purchased from the International Laboratory (U.S.A.). All the organic chemicals were stored according to the instructions. Other chemicals used, i.e. K_2HPO_4, KH_2PO_4, NH_4NO_3, $Fe_2(SO_4)_3$, $CaCO_3$, $MgSO_4{\cdot}7H_2O$, HNO_3, HCl, NaOH, $ZnSO_4$, $ZnCl_2$, $CuSO_4$ and $CuCl_2$, were also purchased from the International Laboratory (U.S.A.) of the highest purities available.

2.2. Bacterial cultivation

The pure culture of *Pseudomonas plecoglossicida* used was previously isolated from a heavily gasoline contaminated site near a gasoline station in Xiamen, China [37], and the related enrichment and isolation procedures can be found elsewhere [40]. All the liquid media and apparatus were autoclaved at 121°C for 20 min before cultivation [10]. The constituents of mineral salts medium (MSM) used for both enrichment of *P. plecoglossicida* and bio-removal experiments included (in g/L) K_2HPO_4 1.0, KH_2PO_4 1.0, NH_4NO_3 1.0, $Fe_2(SO_4)_3$ 0.05, $CaCO_3$ 0.02 and $MgSO_4{\cdot}7H_2O$ 0.2 [41]. The pH was adjusted to 7.0 using either HNO_3 or NaOH solution. A loopful of *P. plecoglossicida* grown on the nutrient agar plate was first inoculated into 50 mL of MSM containing toluene (150 mg/L) as the sole carbon source in a 160-mL crimp-sealed serum bottle and shaken on a rotary shaker at 150 rpm and 30°C. The subculturing was performed every week using fresh toluene containing MSM with an inoculum size of 10% (v/v) until the culture was used for the experiments. Except the firstly inoculated *P. plecoglossicida* come from the solid agar plate, all the experiments were conducted in the liquid mineral salts medium.

2.3. Batch experiments

The chloride and sulfate salts of Zn^{2+} and Cu^{2+} were selected and the stock solutions of TCE, toluene, and microelements solution were prepared using the sterile MSM. The culture media with the fixed concentrations of TCE (10 mg/L) and toluene (150 mg/L) and the variable concentrations of Zn^{2+} and Cu^{2+} (1, 10 and 100 mg/L) were prepared by mixing the stock solutions and MSM at desired concentrations in serum bottles to a final volume of 47.5 mL and the bottles were then crimp sealed. These bottles were shaken on a rotary shaker at 150 rpm and 30°C for 24 h before the inoculation of *P. plecoglossicida*. Aliquot (2.5 mL) of *P. plecoglossicida* culture (inoculum size of 5%, v/v) was inoculated into each bottle using a gastight syringe, then the bio-removal experiments start up. The concentrations of TCE, toluene, and microelements (Zn^{2+} and Cu^{2+}) and the optical density (OD_{600}; measured at 600 nm) of bacterial growth were measured at each pre-determined time intervals. For the measurement of Zn^{2+} and Cu^{2+} concentrations, samples were first centrifuged at 12,000 rpm for 10 min to remove the cells. The culture medium without microelement but with inoculum was used as the positive (biotic) control, while the one without inoculum but with microelement was set as

the negative (abiotic) control. All the experiments were conducted in replicates.

2.4. Analytical methods

The headspace concentrations of toluene and TCE in serum bottles were measured by gas chromatography (GC; Agilent 6890N) and converted to their aqueous concentrations based on the corresponding headspace calibration curve. The GC was equipped with a capillary column (Agilent HP-5; 30 m × 0.53 μm × 0.88 μm) and a flame ionisation detector (FID). Aliquot (50 μL) of sample was injected using a gastight syringe. The temperatures of detector and injector were 280°C and 200°C, respectively. The initial column temperature was 80°C and incrementally increased (20°C/min) to 120°C [37]. The N_2 was used as the carry gas with a flow rate of 20 mL/min. The concentrations of microelements (Zn^{2+} and Cu^{2+}) were measured using atomic absorption spectroscopy (AAS; Thermo Scientific iCE 3000). The stock solutions (10 g/L) of Zn^{2+} and Cu^{2+} were prepared by dissolving $ZnSO_4$, $ZnCl_2$, $CuSO_4$, and $CuCl_2$ in deionised (DI) water. The calibration curves were made with the concentration range of Zn^{2+} or Cu^{2+} at 0–10 mg/L using the standard solutions prepared from diluting the stock solution. Aqueous samples without cells were then applied to the AAS. The samples with high Zn^{2+} or Cu^{2+} concentrations were diluted using DI water before the measurement. The OD_{600} of *P. plecoglossicida* culture was measured using a spectrophotometer (Shimadzu UV-1240).

2.5. Statistical analysis

For the significance of results obtained from both experimental and control groups, the independent-sample *t*-test was performed by using the Statistical Package for Social Sciences (SPSS for Windows, Version 22; SPSS Inc., Chicago) at 95% confidence level.

3. Results and discussion

3.1. Effect of Zn^{2+} on removal of TCE and toluene

Supplement of Zn^{2+} improved the removal efficiency for toluene in the first three days regardless of the Zn^{2+} concentration (Figure 1A) ($P < 0.05$). Particularly, 1 mg/L Zn^{2+} supplement greatly improved the removal efficiency for toluene by ~20% (from 65% to 85%) in the first day and about two days earlier to achieve almost 100% toluene removal, compared to the biotic control. The toluene removal was mainly achieved by the inoculant *P. plecoglossicida* while the toluene removal efficiency in the abiotic control was negligible. At 1 mg/L Zn^{2+}, higher removal efficiency for toluene was observed, compared to Zn^{2+} concentrations at 10 and 100 mg/L ($P < 0.05$) showing detectable inhibitory effects on the toluene removal from day 6. The difference in toluene removal at the same concentration of Zn^{2+} was insignificant between anions SO_4^{2-} and Cl^- (Figure 1A) ($P > 0.05$ for most cases), further indicating the anions with the minimal effect and the enhancement of toluene removal mostly resulted from the presence of Zn^{2+}.

The removal of TCE was observed under all the conditions except the negative (abiotic) control, indicating the co-metabolism of TCE occurred in the presence of organism and toluene (Figure 1B). The TCE removal started on day 1 and proceeded during the whole stationary growth phase. Compared to the positive (biotic) control ($P < 0.05$), apparent improvement of TCE removal was achieved during the first three days in the presence of Zn^{2+}, indicating the stimulatory effect of Zn^{2+} on the initial TCE removal. The highest removal efficiency for TCE on day 8 (the end of the experiment) was observed at 1 mg/L Zn^{2+} (81% and 75% removal efficiencies for $ZnSO_4$ and $ZnCl_2$, respectively). In comparison, the Zn^{2+} concentration at 10 mg/L resulted in the lowest TCE removal efficiency (41%), compared to 1 and 100 mg/L, which was in accordance with the lowest toluene removal efficiency at 10 mg/L Zn^{2+}. This result may be somewhat contradictory to the previous report of the higher Zn^{2+} concentration causing the more severe damage on the cell [42]. The potential reasons might be (1) the bacterial strain has an extremely narrow range of the optimal zinc level before the homeostasis mechanism starting to protect cells from the zinc deficiency or zinc excess [43] and (2) the strain has only one factor expressed to mediate the zinc uptake and efflux. For example, the Zur from *Xanthomonas campestris* mediates the inverse regulation of zinc uptake and efflux system [43,44], further implying the conflicting regulation at a middle concentration (10 mg/L) compared to the low (1 mg/L) and high (100 mg/L) concentrations in current study.

Figure 1 shows a synchronised removal of toluene and TCE. Toluene was rapidly utilised in the first day with a faster removal rate than TCE, and the organism achieved the highest cell density accordingly. After that, most substrate was consumed and its removal rate was slow and the cell growth entered a stationary phase while the removal of TCE was maintained almost steady during the remaining days. Toluene was used as the sole carbon source for growth and provided the energy for the metabolic activities of *P. plecoglossicida*, and the functional enzyme involved

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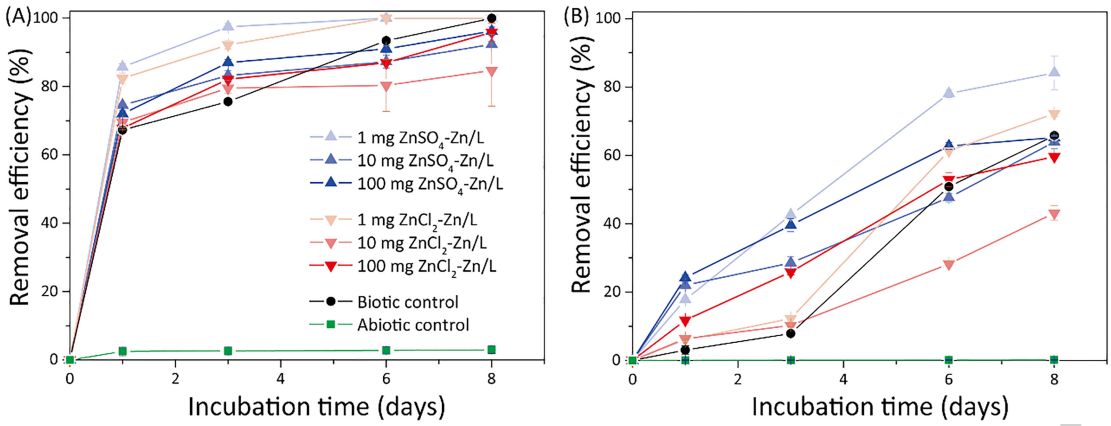

Figure 1. Removal of (A) toluene (150 mg/L) and (B) TCE (10 mg/L) by *Pseudomonas plecoglossicida* at different Zn^{2+} concentrations.

in the catabolism has been reported to be the toluene dioxygenase [45]. As shown in Figure 2, toluene is converted into 3-methylcatechol which was detected and confirmed by LC-MS and the subsequent degradation products were predicted to be 2-hydroxy-6-oxohepta-2,4-dienoic acid and acetic acid [46,47]. Simultaneously, TCE was co-metabolically removed by the isolate, and the TCE co-metabolic bacteria have been reported to primarily epoxidate the carbon–carbon double bond to chloroethene epoxides, catalysed by the toluene mono- or dioxygenase [37,48]. The chloroethene epoxides might be further hydrolysed spontaneously into glyoxylic acid which can be further mineralised to Cl^-, CO_2 and water (Figure 2) [37].

3.2. Effect of Cu^{2+} on removal of TCE and toluene

The supplemented Cu^{2+} affected the enzyme activity of *P. plecoglossicida*, thereby changing the removal efficiencies for toluene and TCE at different concentration (Figure 3). At 1 mg/L Cu^{2+} supplement, a more rapid removal of toluene occurred on day 1, resulting in a removal efficiency (~80%) higher than the positive (biotic) control (68%) ($P < 0.05$; Figure 3A), similar to the effect of Zn^{2+} at the same concentration. However, compared to the slight enhancement of toluene removal for Zn^{2+} at 10 and 100 mg/L in the first three days, the corresponding increased Cu^{2+} concentrations inhibited the toluene removal ($P < 0.05$). No significant difference was still observed between the anions SO_4^{2-} and Cl^- in their effects on the removal efficiency for toluene ($P > 0.05$ for most cases), similar to the result from Zn^{2+} supplement.

On the other hand, the TCE removal efficiency at 1 mg/L Cu^{2+} supplement, regardless of the anion associated, was higher than the positive (biotic) control ($P < 0.05$; Figure 3B), showing the stimulatory effect on TCE removal. Gao et al. also reported similar co-metabolic degradation of TCE by a mixed consortium with methane as the substrate, in which Cu^{2+} at 0.953 mg/L enhanced removal of both methane and TCE and significantly stimulated microbial growth (OD_{600}) [26]. At the higher concentrations (10 and 100 mg/L), however, Cu^{2+} showed an overall inhibitory effect on TCE removal and the inhibition was stronger at 100 mg/L. The effect of Cu^{2+} on the TCE metabolism was also observed for the anaerobic degradation of perchloroethylene (PCE). For example, Cu^{2+} concentration up to 2 mg/L enhanced the anaerobic degradation of PCE by *Dehalococcoides mccartyi* CG1 with acetate as the substrate, while the inhibitory effect was found at concentrations higher than 10 mg/L [49].

The improved biodegradation rates of organic or inorganic compounds by low Cu^{2+} concentration have also been observed in previous works. It was reported that Cu^{2+} at 0.064–0.953 mg/L significantly enhanced the expression of *lmpH* gene for the phenol hydroxylase synthesis and stimulated transformation of TCE by this enzyme in the presence of a mixed consortium SWA1 [26]. Increasing the concentration of Cu^{2+} from 0.05 to 25 μM increased the biodegradation rate of CH_4 in the methanotrophic cultures [50]. Moreover, the biogas production by methanogens has been reported an increase of 45% with the addition of copper ion at 30–100 mg/L or 250 mg/L nZVI nanoparticles, while the production was inhibited at 500 mg/L Cu^{2+} [51]. The addition of bimetallic NZVI/Cu^0 and NZVI/Fe^0 also improved phosphorus removal and bacterial growth, respectively, in mixed cultures [52–55]. When exposed to 40 mg/L copper, the anaerobic-anoxic-aerobic (A^2O) process also showed a decrease in the chemical oxygen demand and ammonia nitrogen removal, compared to 10 mg/L [50,56]. High concentration of Cu^{2+} might put pressure on the microbial community and support

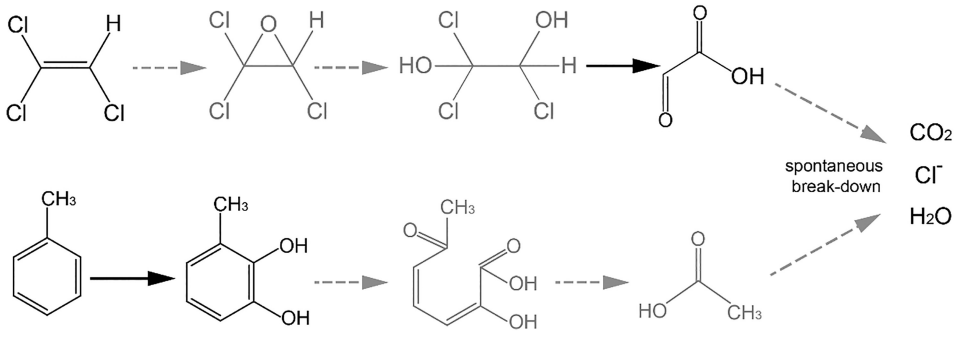

Figure 2. The aerobic degradation pathway of toluene and co-metabolic degradation pathway of TCE by *Pseudomonas plecoglossicida*. The solid arrows indicate the confirmed intermediates by LCMS and the dotted arrows indicate the predicted intermediates.

lower diversity as the more adapted bacteria could be promoted and those sensitive bacteria were inhibited in the presence of high Cu^{2+} concentration.

3.3. Cell growth in Zn^{2+} and Cu^{2+} supplemented culture and effect of anion

As shown in Figure 4, a significant cell growth was observed on the first day, in accordance with the fast consumption of toluene. After that, the cell growth entered the stationary phase. Compared to the biotic control without trace elements added, the supplement of both metal ions improved the cell density regardless of their supplemented concentrations, further indicating both metals acting important in the bacterial growth. For the same type of anions supplemented (Cl^- or SO_4^{2-}), the stimulatory effect of Zn^{2+} or Cu^{2+} on cell growth decreased with the increased metal concentrations, and almost comparable stimulatory effect was seen for both metals, except when Zn^{2+} supplemented as $ZnSO_4$ where 1 mg/L Zn^{2+} supplement showed a significantly higher stimulatory effect compared to higher concentrations (10 and 100 mg/L Zn^{2+} supplements) (Figure 4A). The stimulatory effects on cell growth were consistent with the removal efficiencies for toluene (Figure 1A). In case of Cu^{2+}, however, even though the supplemented copper stimulated the cell growth regardless of the concentrations, at 10 and 100 mg/L Cu^{2+} supplemented, it showed inhibitory to both toluene removal and TCE co-metabolism (Figure 3). In contrast, the inhibitory effect of Zn^{2+} supplemented at higher concentrations was not that severe (Figures 1 and 4A). This may further imply the toluene oxidising enzymes would have different toxicity or sensitivity to the high concentrations of Zn^{2+} and Cu^{2+}. Although copper and zinc as essential microelements can stimulate most of the biological reactions at low concentrations, they are also heavy metals which are highly toxic at high concentrations [57,58]. Previous studies have also reported different toxicities of different heavy metals toward biological reactions [57,59]. Copper was reported more toxic than Pb(II), Zn(II) and Ni(II), and posed severe inhibition on the biomass of heterotrophic bacterium in the activated sludge. Lin and Chen reported the relative toxicities of metals followed the order of Cu(II) > Cr(VI) > Cd(II) = Zn(II) > Ni(II) >> Pb(II) in terms of the CH_4 production in a methanogenic up-flow anaerobic sludge blanket

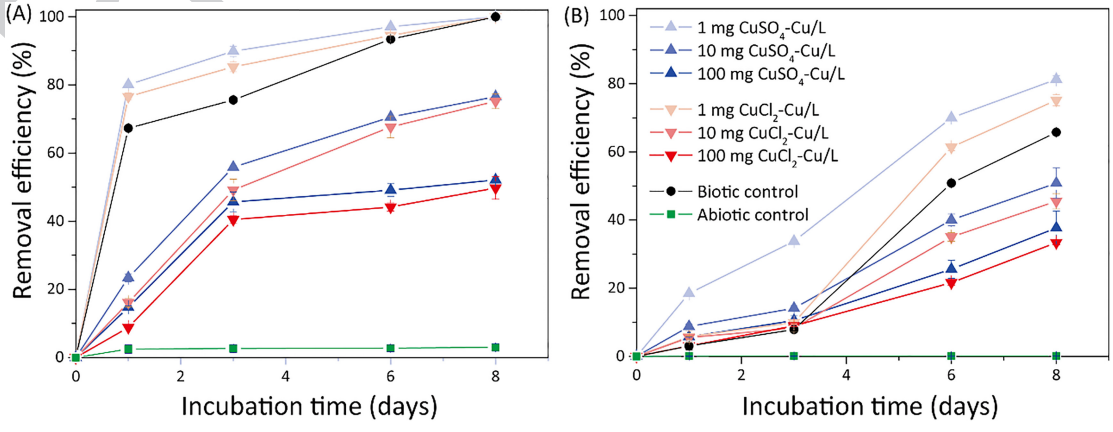

Figure 3. Removal of (A) toluene (150 mg/L) and (B) TCE (10 mg/L) by *Pseudomonas plecoglossicida* at different Cu^{2+} concentrations.

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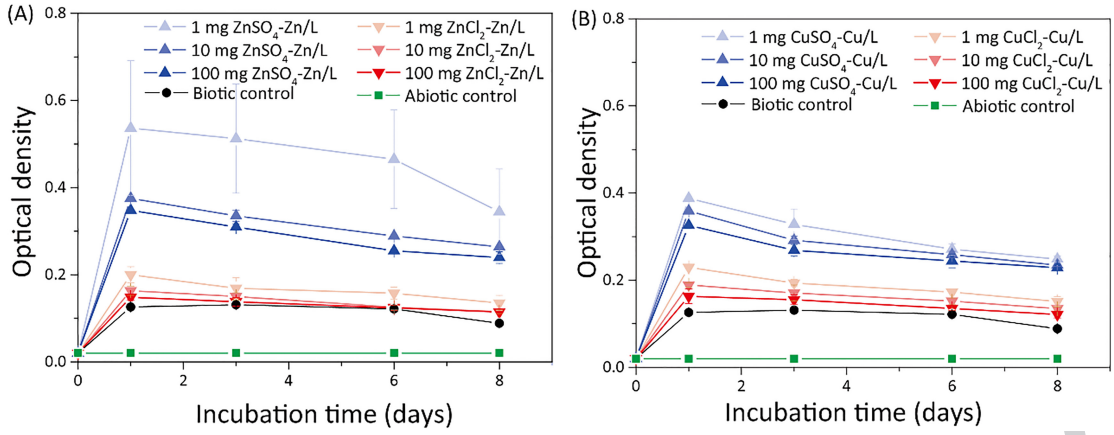

Figure 4. Cell growth (OD_{600}) at different concentrations of (A) Zn^{2+} and (B) Cu^{2+} supplemented in different forms, $ZnSO_4$/$ZnCl_2$ and $CuSO_4$/$CuCl_2$.

(UASB) reactor, and Cu was the most toxic heavy metal to the volatile fatty acid (VFA)-degrading organisms in the methanogenic UASB granule [56]. This would mean even at the high concentrations, the microbial growth kinetics and enzyme sensitivity differs from metal to metal [60,61]. Although *P. plecoglossicida* used in current study benefited from the supplemented copper, its effect on the toluene degradation enzyme differed depending on the concentrations.

As shown in Figures 3 and 5, the growth (optical density) of *P. plecoglossicida* was markedly different in the presence of different anions. The metals Zn^{2+} and Cu^{2+} with SO_4^{2-} significantly stimulated the cell growth compared to the anion Cl^- ($P < 0.05$) while the stimulatory effects were not increased with the SO_4^{2-} concentration and it was still affected by the levels of metal (cation) concentrations. Such a better cell growth in the presence of additional SO_4^{2-} was in accordance with the overall higher TCE removal efficiencies, compared to Cl^-. The results indicated SO_4^{2-} (or sulfur) is somehow important for the bacterial growth on toluene and co-metabolism of TCE. The exact mechanism behind the effects of different concentrations of metal ions warrants further study, to be elaborated by potential change of microbial morphology, metabolic products, enzymes expression and extracellular secretion of the strain [60].

3.4. Removal of Zn^{2+} and Cu^{2+}

A significant removal of Zn^{2+} and Cu^{2+} was observed on the first day and the overall removal efficiency for both microelements decreased with the increased initial concentration (Figure 5). For the Zn^{2+} added cultures, the highest removal efficiency for Zn^{2+} was obtained at 1 mg/L supplement, with the continuous removal during the whole period. This is because of the higher cell density and enzyme activity at this concentration. In comparison, at other two higher concentrations (10 and 100 mg/L), the removal efficiency for Zn^{2+} was relatively stable after day 1, and almost equal removal rate was obtained at the end of the cultivation. No significant difference was observed in the Zn^{2+} removal between SO_4^{2-} and Cl^- as anions when the initial Zn^{2+} concentration was 1 or 10 mg/L ($P > 0.05$), indicating the presence of anions did not significantly affect the removal (uptake) of Zn^{2+}.

Copper (Cu^{2+}) was more rapidly removed in the first day, regardless of the concentration supplemented, and the continuous and stable removal happened in the following days (Figure 5B) despite the bacterium in the stationary growth phase. Similar to Zn^{2+}, the Cu^{2+} removal efficiency decreased with the increased initial Cu^{2+} concentration from 1 to 100 mg/L. In addition, the culture with SO_4^{2-} supplemented showed slightly higher removal efficiencies than the Cl^- supplemented, consistent with the preference of SO_4^{2-} for the cell growth.

The removal of Zn^{2+} and Cu^{2+} in the culture is attributed to the biological balance for the cell uptake and efflux of these essential elements as well as the physiochemical sorption of the cells [62,63]. In bacteria, such regulation is achieved by the action of metal-responsive transcriptional regulators. For the stimulated removal of organic pollutants, the low concentrations of Zn^{2+} and Cu^{2+} were uptaken by the cells and acted as important co-factors for many enzymatic processes of the organic compounds transformation [64]. In case of zinc, bacteria regulate the zinc homeostasis via its import and export, intracellular zinc binding, and zinc-sensing and this regulation exists in most Gram-negative and many Gram-positive bacteria [43,65]. The bacterial cells are

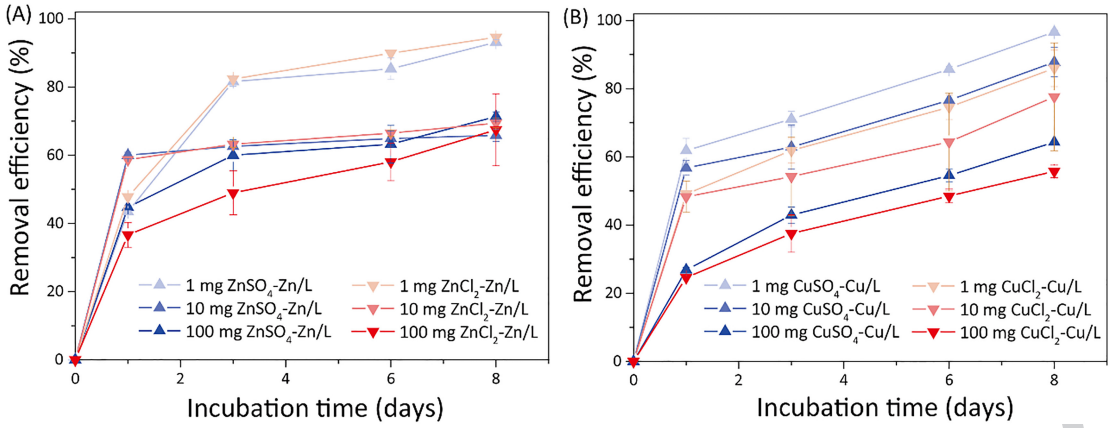

Figure 5. Removal of (A) Zn^{2+} and (B) Cu^{2+} at different concentrations supplemented in different forms, $ZnSO_4$/$ZnCl_2$ and $CuSO_4$/$CuCl_2$.

reported capable of concentrating zinc by several thousand-folds even in the zinc-poor environment [66], further explaining the zinc removal efficiency even at the high concentrations. By governing the expression of zinc-supplying and zinc-requiring proteins, bacteria regulate the intracellular total and free Zn^{2+} concentrations [43,44,67], further protecting them against both high and low external Zn^{2+} concentrations. Copper (Cu^{2+}) also plays key roles in the bacterial enzyme activities. The cytoplasmic or soluble methane monooxygenase (sMMO) is reported expressed only at low copper concentrations and the membrane-bound or particulate methane monooxygenase (pMMO) is reported constitutively expressed with respect to copper, and such expression increases with the increasing copper concentrations [68]. The copper uptake is mediated by a modified polypeptide or chalkophore, termed methanobactin, with a very high affinity [69,70]. Consequently, copper is taken by the bacteria cells, as important parts of these proteins and enzymes. On the other hand, biosorption caused by the ionic attraction might also have contributed greatly to the Zn^{2+} uptake and *Pseudomonas* sp. has previously shown the biosorption of 26.9 mg Zn^{2+}/g [63].

Table 1. Significance levels of the effects of microelements supplemented at different concentrations on the bio-removal of toluene and TCE, compared to the positive control without microelement supplement.

	Toluene removal			TCE removal		
Concentration of Zn^{2+} and Cu^{2+} (mg/L)	1	10	100	1	10	100
Zn^{2+}						
Day 1	++[a]	+	0	+	+	+
Day 3	++	+	+	+	+	++
Day 6	+	-	0	+	-	+
Day 8	0	–	0	+	-	0
Cu^{2+}						
Day 1	+	—	—	+	0	0
Day 3	+	–	—	+	0	0
Day 6	0	–	—	+	-	–
Day 8	0	–	—	+	–	—

[a] +, stimulatory; -, inhibitory; 0, not significant. + (-), ++ (–) and +++ (—) indicate the differences of removal efficiency between experimental samples and positive control at 5–15%, 15–30% and > 30%, respectively.

3.5. Significance of microelement supplement in simultaneous bio-removal of toluene and TCE

The significance of Zn^{2+} and Cu^{2+} supplements during the bio-removal of toluene and TCE is summarised in Table 1. Both microelements at the low concentration (1 mg/L) enhanced the removal of both toluene and TCE, and such enhancement was especially strong for TCE. Zinc supplemented at 10 and 100 mg/L showed a slightly stimulatory effect on the toluene and TCE removal during the early stage and a slightly inhibitory effect afterward at 10 mg/L Zn^{2+}. In comparison, copper supplemented at the same concentrations (10 and 100 mg/L) showed a strong inhibitory effect on the toluene removal during the whole 8-day period while with a medium inhibitory effect on the TCE removal after day 6.

The aerobic co-metabolic degradation of TCE is known as an enzymatic process with its degradation rate depending on mono- or dioxygenases, typically methane and toluene oxygenase [18,71]. Both metal ions are essential elements for some typical oxygenases that can transform TCE. Copper is required for the synthesis of particulate methane monooxygenases (pMMOs) in methanotrophs, which contain mono- and dinuclear Cu centers [72,73] and oxidise TCE through an epoxidation pathway [26]. Zinc as the second most abundant transition metal present in the majority of

organisms has both catalytic and structural functions in proteins [65]. Zinc has been detected in the metal-binding site in some pMMOs and the Zn-binding proteins account for about 4–8% of all proteins produced by prokaryotes [73]. There have been various kinds of enzymes reported to co-metabolise TCE, including toluene-2,3-dioxygenase, toluene monooxygenase, alkene monooxygenase, phenol hydroxylase, catechol 1,2-dioxygenase, methane monooxygenase and phenol hydroxylase [74,75], with the presence of respective substrates. In addition, five different pathways for the aerobic transformation of toluene and four different toluene oxygenases, i.e. toluene-2-monooxygenase, toluene-4-monooxygenase, methyl monooxygenase and toluene-2,3-dioxygenase, can be involved in the transformation process [17,18,76]. TCE can also be co-metabolised by the resting cells carrying oxygenase without growth substrate. For instance, Aziz et al. reported TCE could be removed by the resting cells of methanotrophic *Methylosinus trichosporium* OB3b PP358, with the constitutively excreted soluble methane monooxygenase (sMMO) [77]. Therefore, microelements, particularly zinc and copper, are essential for the enzymatic transformation processes and thus enhance the transformation of toluene and TCE.

In current study, high concentrations (10 and 100 mg/L) of Cu^{2+} showed a strong inhibitory effect on the removal of toluene as well as TCE during 8-day incubation, while Zn^{2+} showed a slightly inhibitory effect. One reason for their inhibitory effect at high concentration is their toxicity as heavy metals, inhibiting the co-metabolic activities. For example, Cu^{2+} can participate in the reactions that produce such reactive oxygen species as H_2O_2, $OH\cdot$ and O_2^-, which are transient and highly reactive compounds that can damage the biological macromolecules [78]. The presence of Cu^{2+} at approximately 0.1 mg/L was reported strongly inhibiting the aerobic transformation of TCE by *Methylomonas* sp. MM2, a methanotroph isolated from groundwater aquifer [25]. However, the addition of Cu^{2+} did not decrease the methane oxidation rate and the growth yield of *Methylomonas* sp. MM2, showing the increased Cu^{2+} concentration not that toxic to the methanotrophs. In some cases, zinc also showed strong toxicity to microbes for the biodegradation of organic compounds. Zinc inhibited biodegradation of 2,4-DME (dichloro-phenoxyacetic acid methyl ester) in the lake water samples inoculated with sediment and the minimum inhibitory concentration was as low as 0.006 mg total zinc/L [79]. Both copper and zinc have also been reported to decrease the microbial biomass and inhibit the soil enzyme activities in the heavy metal polluted soils [80–83]. In current study, the accumulation of intermediates from co-metabolism of TCE (e.g. glyoxylic acid) might have contributed to the stimulatory or inhibitory effect of the copper or zinc ion to some extent, further affecting the final mineralisation. The exact mechanism behind the effect caused by different concentrations of metal ions might be further elaborated by the potential change of microbial morphology, metabolic products and enzymes expression and extracellular secretion of the strain [60], which would provide further experimental evidence. The minimum inhibitory concentrations for the aerobic degradation of three chlorinated compounds, 3-chlorobenzoate, 4-chlorophenol and 2,4-dichloro-phenoxyacetic acid, were reported 29.5–736.0 mg/L for Zn^{2+} and 14.3–71.6 mg/L for Cu^{2+}, respectively [84], in accordance with the results from current study. Therefore, the exact concentration range of supplemental microelements for cell growth and enzyme activity should be considered for the optimal removal of objective pollutants.

The concentrations of Zn and Cu in groundwater are usually low, 10–40 µg/L for Zn [85,86] and ~5 µg/L for Cu [1]. In comparison, they are frequently detected in the contaminated soil sites at its soil solution concentration of as high as 1 mg/L [32,33,87,88]. The co-metabolic removal of TCE can be more favorable at the sites where a proper concentration of Zn or Cu in the aqueous phase is available. The sites in short of these microelements can be supplemented at the low aqueous concentrations (~1 mg/L) to enhance the remediation efficiency.

4. Conclusions

This study provided new insight into the effects of microelements (zinc and copper) supplement for the TCE co-metabolisation by the indigenous isolate *Pseudomonas plecoglossicida* using toluene as growth substrate. The enhanced (stimulatory) effects of Zn^{2+} and Cu^{2+} supplement on TCE co-metabolism, toluene metabolism as well as cell growth were achieved at their low concentration (1 mg/L) compared to high concentrations (10 and 100 mg/L). The higher concentrations of Zn^{2+} (10 and 100 mg/L) enhanced the removal efficiencies for toluene and TCE in the first three days but showed some inhibitory effect afterward. In comparison, the higher concentrations of Cu^{2+} (10 and 100 mg/L) always showed inhibitory to the toluene removal while showing inhibitory to the TCE removal after three days. The results may help to better understand the TCE bioremediation at the sites concurrently contaminated with TCE and toluene while increasing the removal efficiency for TCE. Further work is needed to understand how bacteria uptake zinc and

copper and the joint structure and function of these metals in the functional toluene oxidising enzymes.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All the data generated or analysed during this study are included in this published article.

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