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Triphenyltin biodegradation and intracellular material release by *Brevibacillus brevis*

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HIGHLIGHTS

- Triphenyltin, diphenyltin and monophenyltin were degraded by *Brevibacillus brevis*.
- Metabolites, ion and protein release, membrane permeability, viability were detected.
- Triphenyltin could be degraded simultaneously to diphenyltin and monophenyltin.
- Triphenyltin increased Cl^-, Na^+, Ca^{2+} and protein release, and membrane permeability.
- *B. brevis* metabolically released Cl^- and Na^+, and passively diffused Ca^{2+}.

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ABSTRACT

Triphenyltin (TPT) is an endocrine disruptor that has polluted the global environment, and thus far, information regarding the mechanisms of TPT biodegradation and intracellular material release is limited. Here, TPT biodegradation was conducted by using *Brevibacillus brevis*. Degradation affecting factors, metabolite formation, ion and protein release, membrane permeability, and cell viability after degradation were investigated to reveal the biodegradation mechanisms. The results showed that TPT could be degraded simultaneously to diphenyltin and monophenyltin, with diphenyltin further degraded to monophenyltin, and ultimately to inorganic tin. During degradation process, *B. brevis* metabolically released Cl^- and Na^+, and passively diffused Ca^{2+}. Protein release and membrane permeability were also enhanced by TPT exposure. pH ranging from 6.0 to 7.5 and relatively high biomass dosage in mineral salt medium improved TPT degradation. Biodegradation efficiency of 0.5 mg L^{-1} TPT by 0.3 g L^{-1} *B. brevis* at 25 °C for 5 d was up to 80%.

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1. Introduction

Starting from the 1940s, the usage of organotins (OTs) had increased many folds; these synthetic and multipurpose chemicals (Jadhav et al., 2011) had been extensively used as heat and light stabilizers in the manufacture of poly vinyl chloride (Adams et al., 2011), and as active ingredients of biocides, and fungicides (Antes et al., 2011) and especially antifouling paints. With the increase of marine traffic, dredging activity and construction of aquatic infrastructure, harmful levels of OTs in waters and sediments are present. Among these OTs, triphenyltin (TPT) and its degradation products are present in all compartments of the natural environment. As an endocrine disruptor highly toxic to many different non-target organisms, TPT can cause high larval mortality and imposex (Jadhav et al., 2011). Worse still, TPT bioaccumulation through food chain poses potential health threat to human beings.

Over the recent decades, TPT analytic methods, distribution and ecotoxicity were well determined (Yu et al., 2011; Zuo et al., 2012), while its degradation was less concerned. Among the physical and chemical methods (Zhao et al., 2011), OH-radical, direct photolysis (Palm et al., 2003) and thermal decomposition (Mesubi and Olatunji, 1983) have been investigated for TPT elimination. However, high cost, energy and chemical requirement in these methods limited their application. Therefore, more cost-effective and efficient methods in TPT degradation need to be developed.

Studies attempting to investigate the effects of pH, temperature and environmental factors on TPT biodegradation illustrated that some supplemental substrates and certain environmental factors seemed to have encouraged the biodegradation efficiencies

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(Stasinakis et al., 2005; Heroult et al., 2008). For example, suitable kinds and levels of surfactants, nutrients, oxygen and metals obviously enhanced TPT biodegradation (Ye et al., 2013a). As one of the trisubstituted OTs, there is an increased concern about the biodegradation of TPT organic groups. Sequential dephenylation was inferred via the calculation of mass balance in batch reactors during TPT degradation process (Stasinakis et al., 2005). After biosorption by *Brevibacillus brevis*, TPT was further transformed to diphenyltin (DPT), monophenyltin (MPT) and tin intracellularly (Ye et al., 2013a). The degradation of DPT and tributyltin (TBT) was also performed through a single successive loss of an organic group (Heroult et al., 2008). Although these reports on biodegradation process of phenyltins (PTs) are of great significance in elucidating the fate and effect of TPT in environment, there is still a paucity of information about quantitative analysis of TPT degradation products, evaluation of factors favouring the biodegradation and cellular physiological–biochemical responses during TPT degradation process, which has limited the further application of OT biodegradation in polluted environments.

The present work aimed to effectively degrade TPT by *B. brevis*, and enhance the biodegradation by exploring the effect of initial pH, biomass dosage, degradation media, bacterium age and some potential metabolites in this process. Additionally, DPT and MPT formation, ion and protein release, cellular membrane permeability and viable count after TPT degradation were investigated to reveal the mechanisms of TPT biodegradation.

2. Materials and methods

2.1. Strain and chemicals

B. brevis was isolated from the sediment samples collected at a town called Guiyu in Guangdong Province, China, in which primitive e-waste processing and recycling activities are extensively involved (Ye et al., 2013a).

TPT was obtained from Sigma–Aldrich (St. Louis, MO, USA). Beef extract medium (BEM) contained (in g L^{-1}) 3 beef extract, 10 peptone and 5 NaCl. The concentrations of $Na_2HPO_4{\cdot}12H_2O$, KH_2PO_4, NaCl, NH_4NO_3, $MgSO_4$ and $ZnSO_4$ in mineral salt medium (MSM) were 100, 50, 40, 20, 5 and 3 mg L^{-1}, respectively.

2.2. Microbial culture

B. brevis was inoculated into BEM at 30 °C on a rotary shaker at 100 rpm for 24 h. Subsequently, the cells were separated by centrifugation at 3500*g* for 5 min, and washed three times with sterile distilled water before used in further experiments.

2.3. Biodegradation experiments

The flask with 20 mL MSM containing 0.5 mg L^{-1} TPT and 0.3 g L^{-1} *B. brevis* was inoculated in the dark at 25 °C on a rotary shaker at 100 rpm for 5 d to elucidate the best conditions and reveal the mechanism of TPT biodegradation.

The initial pH at 5.0–9.0 was adjusted by adding 0.1 M HCl or NaOH to investigate its influence on cellular membrane permeability, cell lethality and TPT degradation. The effect of 5 different media, namely MSM, distilled water, phosphate buffer solution (PBS) with pH 6.5, sterile and non-sterile water of Pearl River, on TPT biodegradation was conducted. Water quality (in mg L^{-1}) of Pearl River was as follows: DO, COD_{Mn}, COD_{Cr}, $NH_4^+ - N$, TN, TP, Zn, As, Cr(VI) and volatile phenol were 5.1, 3.1, 7.8, 0.75, 3.14, 0.157, 0.023, 0.002, 0.004 and 0.001, respectively. Growth curve of *B. brevis* cultured in BEM from 3 h to 6 d was determined by detecting the optical density of culture medium at 600 nm. Meanwhile, *B. brevis* harvested from BEM at 0.5, 1, 2, 3, 4, 5 and 6 d, respectively, was used to reveal the effect of culture time on TPT biodegradation. Based on initial concentration of TPT, ethanol, acetic acid, acetone and benzene in the range of 5–100 μM were added into MSM, respectively, to illustrate the impact of potential organic metabolites on TPT biodegradation.

To reveal metabolites of TPT biodegradation, *B. brevis* at different dosage up to 0.5 g L^{-1} degraded 0.5 mg L^{-1} TPT, DPT and MPT for 5 d, separately. Meanwhile, *B. brevis* at 0.3 g L^{-1} was inoculated in MSM with different TPT concentration up to 1.0 mg L^{-1}, respectively, to ascertain correlation between TPT biodegradation and cellular physiological–biochemical responses, including cell membrane permeability, intra- and extracellular protein concentration, and ion release. Three samples for each experiment were taken and the mean values were used in calculations.

2.4. Extraction and derivatization of PTs

After biodegradation, 2 mL HCl at 1 M and 10 mL hexane were added into 20 mL solution. PTs in the mixture were sonicated for 20 min in an ultrasonic bath and allowed to settle until phase separation. After the organic phase was removed, 10 mL hexane was added into the aqueous phase, and then the operation was repeated again. The organic part was collected, followed by concentrating using a rotary evaporator at 30 °C. The residues were dissolved by 5 mL methanol and derivatised in pH 4.5 acetate buffer with 2 mL of 2% sodium diethyl dithiocarbonate. The recoveries of TPT, DPT and MPT were 96%, 93% and 91%, separately.

2.5. Analytical methods of PTs

TPT and its metabolites were analyzed according to previously published methods (Ye et al., 2013a) by gas chromatography–mass spectrometry (GC–MS) (QP2010, Shimadzu) equipped with a Rxi-5MS GC column (30 m × 0.25 mm × 0.25 μm). The detection limits of TPT, DPT and MPT were 250, 110 and 110 ng L^{-1}, respectively.

2.6. Cellular membrane permeability

Membrane permeability of *B. brevis* was determined by measuring the concentration of β-galactosidase released into the culture medium using *o*-nitrophenyl-β-D-galactoside (ONPG) as a substrate (Shi et al., 2013). Cells inoculated in nutrient medium for 2 d were collected, washed and suspended in 0.9% NaCl solution. The suspension was added into lactose induction medium (in g L^{-1}: KH_2PO_4 3, $Na_2HPO_4{\cdot}7H_2O$ 12.8, NaCl 0.5, NH_4Cl 1, $MgSO_4$ 0.5, $CaCl_2$ 0.01, lactose 5) and incubated at 37 °C. Subsequently, cells were collected, washed and suspended in β-galactosidase buffer (in g L^{-1}: KH_2PO_4 0.24, $Na_2HPO_4{\cdot}12H_2O$ 2.9, NaCl 8, KCl 0.2, $MgSO_4{\cdot}7H_2O$ 0.25, β-mercaptoethanol 3.9 mL). After then, cells were used to treat 0.5 mg L^{-1} TPT in the presence of 50 mg L^{-1} ONPG. The production of *o*-nitrophenol was detected at 504 nm.

2.7. Analytical methods of ions

After biodegradation, the solution was centrifuged at 3500*g* for 10 min. The resultant supernatant was filtered using a 0.22-μm polyether sulphone filter. Afterwards, the concentrations of Cl^-, Na^+ and Ca^{2+} were detected by an ICS-900 ion chromatography system (Dionex, Sunnyvale, CA, USA).

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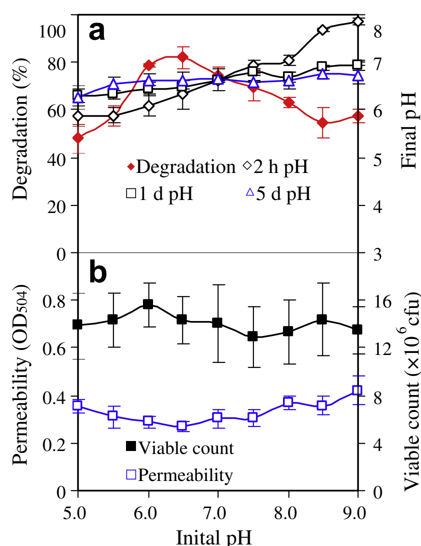

Fig. 1. Effect of pH on biodegradation of 0.5 mg L^{-1} TPT by 0.3 g L^{-1} *B. brevis* at 25 °C for 5 d. (a) Degradation efficiencies and final pH of solutions, and (b) cell membrane permeability and viable counts.

3. Results and discussions

3.1. Influence of pH on TPT biodegradation and B. brevis

As Fig. 1a shows, TPT biodegradation was apparently affected by the initial pH. With initial pH in the range of 6.0–7.5, TPT biodegradation was improved. When pH value was up to 6.0, the Sn–Cl bond in most organotin halides was early hydrolyzed (Pagliarani et al., 2013), increasing the interactions between electron donor atoms and tin, which was partially responsible for the enhanced TPT elimination in this condition. Marcic et al. (2006) also reached the conclusion that with pH over 7, about 60% of TPT in soil at 325 μg kg^{-1} was degraded. Slightly acidic and alkaline media suppressed degradation activity, since pH could impact the essential groups in active center of enzyme (Feitosa et al., 2008; Serebryakova et al., 2009) and the solubility of OTs (Graceli et al., 2013). The water solubilities of most OTs are low and dependent on pH, ionic strength and temperature. The concentration of dissolved TPT at 25 °C for 5 d in the current experiment was 121 ± 5 μg L^{-1}, while data for TBT-Cl were in the range of 5–50 mg L^{-1} (Rudel, 2003).

Although the initial pH varied from 5.0 to 9.0, the final pH in media was approaching to neutral during the biodegradation process (Fig. 1a), which demonstrated that the excess H^+ or OH^- in solution reacted with ions or active groups on the cell to maintain the neutral condition. As a permeability barrier, cell membrane keeps organisms from being attacked by extraneous chemicals, to maintain a stable intracellular environment (Zhao et al., 2013). As shown in Fig. 1b, the lowest permeability of cell membrane and highest viable count existed in media at pH value ranging from 5.5 to 7.0. Cell membrane functions in regulating osmotic pressure, enzymatic reaction, cell recognition, material transport and electron transfer, consequently controlling overall cell metabolism (Ortiz et al., 2005; Żyłka et al., 2009). OTs are tended to disturb cellular membrane structure as a result of their interactions with the lipids and proteins, especially membrane-bound enzymes, resulting in a membrane breakdown (Bonarska-Kujawa et al., 2012; Pagliarani et al., 2013). Moreover, TPT and its derivatives are known as membrane poisons (Ortiz et al., 2005). Thereby, the exposure of *B. brevis* to TPT in the presence of excess H^+ or OH^- posed some detrimental effect on cell membrane and increased its permeability. Low membrane permeability at pH 6.0–7.5 was suitable to maintain cellular activity, enhancing TPT degradation accordingly.

3.2. TPT biodegradation in different media

The degradation efficiency of 0.5 mg L^{-1} TPT in different media reached 44–80% (Fig. 2). Particularly, in MSM *B. brevis* possessed much better performance in TPT removal, thus MSM was selected in the further studies. The degradation of TBT at 5 mg L^{-1} by the mixture of *Cunninghamella elegans* and *Cochliobolus lunatus* was also effective in synthetic medium (Bernat et al., 2013). The suitable effectiveness of *B. brevis* in TPT elimination was partially due to good adaptability of cells to TPT stress since LC_{50} values of TPT in MSM at 24, 48 and 96 h were up to 54, 12 and 1 mg L^{-1}, respectively (Fig. SM-1 in Supplemental Material (SM)).

In distilled water, even with no nutrients, the degradation efficiency of TPT was also up to 44%, which might result from the metabolism of intracellular biomacromolecules. Moreover, the removal efficiencies in non-sterile and sterile river water were about 55% and 48%, separately, which exhibited the potentiality of *B. brevis* in restoring TPT contaminated environment. While the 7% difference of removal efficiency between these two systems indicated that the indigenous microorganisms contributed little to TPT degradation, which is partially responsible for the persistence of TPT in natural environment.

3.3. Influence of culture time on TPT biodegradation

According to different growth phases reflected from OD_{600nm} values shown in Fig. 3, *B. brevis* passed adaptive phase within 3 h, and experienced logarithmic and apoptotic phases at 3–12 h and 3–6 d, respectively. The ability of *B. brevis* harvested at different culture time to degrade 0.5 mg L^{-1} TPT showed a good agreement with its biomass produced at the same culture phase. The significant positive correlation between OD_{600nm} and TPT degradation further indicated that the ability of *B. brevis* in degrading TPT was closely related to its cellular activity.

At logarithmic and stable phases, more carbon sources were utilized to maintain vigorous growth of *B. brevis*, resulting in high TPT degradation efficiency. In contrary to cells at these phases, those at apoptosis phase showed low activity, which led to the decrease of TPT-tolerant capacity, inducing the decline of degradation efficiency. Given degradation effect, incubation time and biomass, *B. brevis* cultured for 1 d was chosen for further studies.

3.4. Effect of potential organic metabolites on TPT biodegradation

Based on TPT molecular structure, ethanol, acetic acid, acetone and benzene were selected to estimate the effect of potential intermediates, which might be produced from the benzene ring-cleavage reactions, on TPT biodegradation. Although Fig. 4 revealed that all of them exerted suppression on TPT degradation to some degree, the analysis of variance test (Table SM-1) determined that only benzene significantly posed detrimental effect on TPT degradation, which inferred that TPT biodegradation relied on catabo-

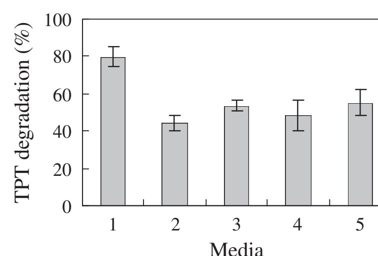

Fig. 2. Effect of degradation media on biodegradation of 0.5 mg L^{-1} TPT by 0.3 g L^{-1} *B. brevis* at 25 °C for 5 d.

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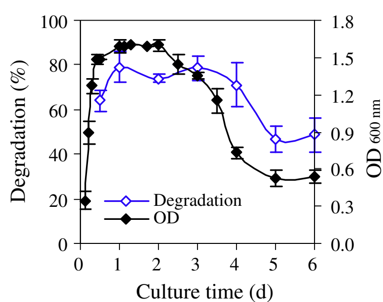

Fig. 3. Effect of culture time on biomass and biodegradation of 0.5 mg L^{-1} TPT by 0.3 g L^{-1} *B. brevis* at 25 °C for 5 d.

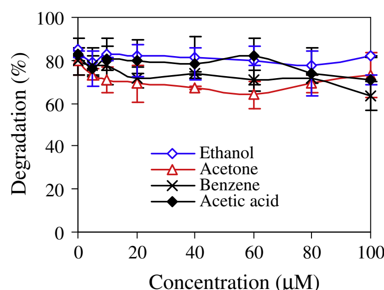

Fig. 4. Effect of potential organic metabolites on biodegradation of 0.5 mg L^{-1} TPT by 0.3 g L^{-1} *B. brevis* at 25 °C for 5 d.

lism by cells, not the co-metabolism enhanced by easily degradable carbon sources (Brandt et al., 2003). This was consistent with the conclusion that catabolic metabolism seemed to be the main mechanism of OTs biodegradation by activated sludge (Stasinakis et al., 2005). The suppressive effect of these potential metabolites might result from the inhibition of end-products. Similar to the impact of other easily degradable carbon sources, these exogenous potential metabolites were primarily metabolized by *B. brevis*, consequently posing negative effect on TPT biodegradation (Ye et al., 2013a).

3.5. Metabolites of TPT biodegradation

An apparently increasing trend of TPT degradation with ascending biomass was observed in Fig. 5a. However, when dosage was above 0.1 g L^{-1}, the uptrend gradually slowed down, which was partially attributed to the decreasing contact between unit biomass and residual TPT. This also confirmed that TPT degraded by unit biomass was even more under the condition of lower *B. brevis* dosage.

When biomass dosage increased to 0.2 g L^{-1}, viable counts after TPT degradation for 5 d changed slightly (Fig. 5a), due to high initial dosage of cells and their increasing competition for limited nutrients, which led to higher lethality. Therefore, with relatively high concentration of *B. brevis* presented, TPT degradation efficiency would not be promoted.

Fig. 5b shows that the produced DPT was quite low, while MPT concentration was found significantly higher. From MPT accumulation, it can be deduced that the cleavage of chemical bond in Sn–C, namely MPT dephenylation to form tin atom, was the efficiency controlling step in TPT degradation. This finding is consistent with previous investigations from environment monitoring that MPT is usually the predominant species of OTs (Marcic et al., 2006). Besides, since there was no benzene detected, cleavage of benzene ring may be the first step in this process. Additionally, when the biomass was up to 0.3 g L^{-1}, the descending concentration of

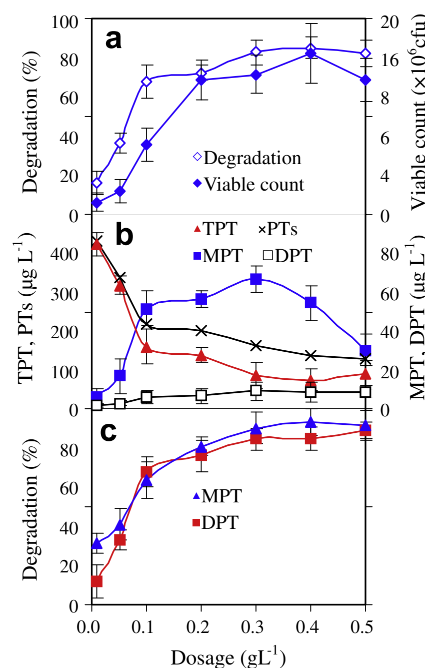

Fig. 5. Effect of biomass dosage on biodegradation of 0.5 mg L^{-1} TPT, DPT and MPT by 0.01–0.5 g L^{-1} *B. brevis* at 25 °C for 5 d. (a) Viable counts and degradation efficiencies of 0.5 mg L^{-1} TPT, (b) concentrations of residual TPT and PTs, and produced MPT and DPT after TPT biodegradation, and (c) degradation efficiencies of 0.5 mg L^{-1} DPT and MPT.

MPT exhibited that more *B. brevis* was beneficial to MPT transformation to inorganic compounds. The decreasing tendency of residual TPT and total PTs with rising biomass further determined that high biomass was in favor of TPT transformation.

While in Fig. 5c, degradation efficiency of MPT was slightly higher than that of DPT, which dismissed the inference of controlling step above. Thereby, TPT can be degraded to DPT and MPT simultaneously, with DPT further degraded to MPT, resulting in high concentration of MPT in Fig. 5b. Since no DPT was detected in soils during the 90-d experiment, TPT seemed to have also rapidly transformed to MPT in natural samples of freshwater and soils (Heroult et al., 2008). Moreover, in investigation of PT distribution in selected sites, TPT constitutes only 15.7% of total PTs in seawater, while percentage of DPT and MPT are 31.4% and 52.9%, respectively (Yi et al., 2012). It can be further inferred that direct degradation from TPT to MPT also exists in natural environments.

3.6. TPT biodegradation and cellular physiological–biochemical responses

With the initial TPT concentration ascending, TPT degradation efficiency decreased (Fig. 6a), while degradation amount increased according to the calculation results of degradation efficiency and initial TPT concentration. This was partially owing to the higher collision between TPT and *B. brevis*, resulted from the enhanced driving force induced by higher concentration of TPT to overcome mass transfer resistance between solution and *B. brevis*, since TPT bio-removal by *B. brevis* included biosorption, transport and biodegradation (Ye et al., 2013a). The rising level of MPT and DPT further revealed that TPT could be degraded to MPT and DPT simultaneously since the level of accumulated MPT was significantly higher than that of DPT. Given that in the structure of TPT, three benzene rings separately linked to Sn with symmetrical structure and the same physicochemical properties, ring-cleavage of these benzenes can occur respectively and synchronously.

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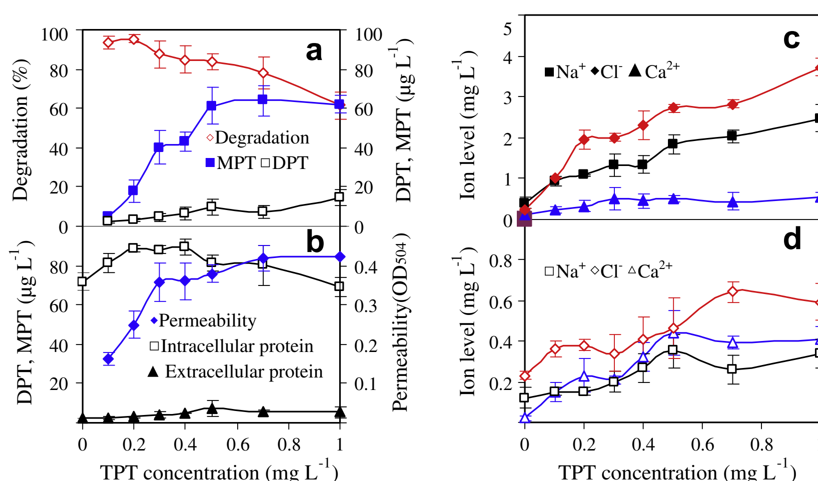

Fig. 6. Biodegradation of 0.1–1.0 mg L^{-1} TPT by 0.3 g L^{-1} *B. brevis* at 25 °C for 5 d. (a) TPT degradation efficiencies, and concentrations of produced MPT and DPT, (b) cellular membrane permeability, and concentration change of intra- and extracellular protein during TPT removal, (c) ion released by viable cells into solution, and (d) ion released by dead cells inactivated by 2.5% glutaraldehyde for 24 h.

The increasing membrane permeability exhibited in Fig. 6b during TPT removal process was induced by high level TPT with high hydrophobicity and lipid solubility. Triorganotins of high concentration exerted toxic effects through their interactions with membrane lipids of microorganisms (Gadd, 2000). However, in addition to the detrimental effect, the ascended permeability also promoted TPT removal because TPT degradation took place intracellularly (Ye et al., 2013a).

The intra- and extracellular protein content, and ion release were investigated since TPT biodegradation depended on metabolism, and TPT induced the increase of cellular membrane permeability. It was revealed from the increased protein amounts in cells treated by TPT ranging from 0.1 to 0.7 mg L^{-1} (Fig. 6b) that the exposure to TPT at certain level induced the expression of proteins in cells that would be responsible for the detoxification and tolerance of TPT. However, TPT at 1.0 mg L^{-1} inhibited the expression.

The clear increase in the levels of extracellular protein, Cl^-, Na^+ and Ca^{2+} in the presence of TPT was exhibited in Fig. 6b and c, which verified the release of intracellular materials induced by TPT exposure. As for Cl^- and Na^+, their release is usually considered to be an objective verification of membrane permeability changes because of their physiological function and abundance in cells (Buckel and Thauer, 2013). When subjected to osmosis in MSM as well as exposure to TPT, cells responded by opening membrane channels, causing the efflux of these cytosolic ions (Monti et al., 2013).

Except for the efflux induced by TPT stress, some reports on biosorption proved that cells could release some light metals to balance heavy metal uptake. For example, *Stenotrophomonas maltophilia* metabolically independently exchanged Ca^{2+} for Cu (II) bioaccumulation (Ye et al., 2013b), and ion exchange was calculated to contribute 77% to Cu biosorption by *Fucus vesiculosus* (Cochrane et al., 2006). It has been confirmed that TPT biosorption by *B. brevis* was mainly owing to the physicochemical interactions independent on metabolism. Therefore, the binding of TPT in the current experiment was partially responsible for Ca^{2+} release.

To determine the contribution of metabolism to ion release, Cl^-, Na^+ and Ca^{2+} were detected after TPT treatment by dead cells inactivated by 2.5% glutaraldehyde for 24 h. Fig. 6d showed that the amounts of Cl^- and Na^+ efflux by dead cells were obviously lower than those released by viable cells. This finding inferred that Cl^- and Na^+ release coupling with TPT biodegradation was mainly regulated by metabolic activity. The final level of Ca^{2+} raised from 0.1 to 0.5 mg L^{-1} (Fig. 6c), and 0 to 0.4 mg L^{-1} (Fig. 6d) with the increase of TPT concentration, which implied that the majority of Ca^{2+} was passively diffused to solutions via exchange behavior with TPT. Without TPT in solutions, no Ca^{2+} was released by viable and dead cells further confirmed this inference.

The correlation between TPT concentration and permeability was significant (Table SM-2). Moreover, the correlation between permeability and all other items, excluding intracellular protein, was also significant at a p value of 0.05 or 0.01, further verifying that high lipid solubility of TPT ensured cell penetration and induced efflux of intracellular materials. However, the increased penetration was directly related to the enhanced biodegradation of TPT, producing more metabolites (Fig. 6a).

4. Conclusions

B. brevis could effectively degrade TPT, simultaneously transform it to DPT, MPT and inorganic tin. During this degradation process, *B. brevis* metabolically released Cl^- and Na^+, and passively diffused Ca^{2+}. Protein release and membrane permeability were also enhanced by TPT stress. With 0.3 g L^{-1} *B. brevis* separately degrading 0.5 mg L^{-1} TPT, DPT and MPT for 5 d, the degradation efficiencies were up to 80%, 84% and 90%, respectively. Degradation media, pH, potential organic metabolites, dosage and TPT concentration were of great importance in affecting the degradation process. Neutral pH, MSM and relatively high dosage benefited TPT biodegradation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2013.12.039.

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