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## Effects of $\text{Cu}^{2+}$ and humic acids on degradation and fate of TBBPA in pure culture of *Pseudomonas* sp. strain CDT

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### ABSTRACT

Soil contamination with tetrabromobisphenol A (TBBPA) has caused great concerns; however, the presence of heavy metals and soil organic matter on the biodegradation of TBBPA is still unclear. We isolated *Pseudomonas* sp. strain CDT, a TBBPA-degrading bacterium, from activated sludge and incubated it with  $^{14}\text{C}$ -labeled TBBPA for 87 days in the absence and presence of  $\text{Cu}^{2+}$  and humic acids (HA). TBBPA was degraded to organic-solvent extractable ( $59.4\% \pm 2.2\%$ ) and non-extractable ( $25.1\% \pm 1.3\%$ ) metabolites, mineralized to  $\text{CO}_2$  ( $4.8\% \pm 0.8\%$ ), and assimilated into cells ( $10.6\% \pm 0.9\%$ ) at the end of incubation. When  $\text{Cu}^{2+}$  was present, the transformation of extractable metabolites into non-extractable metabolites and mineralization were inhibited, possibly due to the toxicity of  $\text{Cu}^{2+}$  to cells. HA significantly inhibited both dissipation and mineralization of TBBPA and altered the fate of TBBPA in the culture by formation of HA-bound residues that amounted to  $22.1\% \pm 3.7\%$  of the transformed TBBPA. The inhibition from HA was attributed to adsorption of TBBPA and formation of bound residues with HA via reaction of reactive metabolites with HA molecules, which decreased bioavailability of TBBPA and metabolites in the culture. When  $\text{Cu}^{2+}$  and HA were both present,  $\text{Cu}^{2+}$  significantly promoted the HA inhibition on TBBPA dissipation but not on metabolite degradation. The results provide insights into individual and interactive effects of  $\text{Cu}^{2+}$  and soil organic matter on the biotransformation of TBBPA and indicate that soil organic matter plays an essential role in determining the fate of organic pollutants in soil and mitigating heavy metal toxicity.

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### Introduction

Tetrabromobisphenol A [4,4'-isopropylidenebis (2,6-dibromophenol), TBBPA] is a brominated flame retardant widely used in electric and electronic products (He et al.,

2010). TBBPA was widely detected in the environment, such as soil, water, sewage sludge, dust, as well as human tissue (Feng et al., 2012; Gorga et al., 2013; Johnson-Restrepo et al., 2008; Tang et al., 2014; Wu et al., 2016; Xu et al., 2013). The highest level of TBBPA in soils of electronic wastes (e-wastes)

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dismantling sites detected in south China amounted to 1.94 mg/(kg-dw) (Wang et al., 2015). TBBPA is toxic to aquatic organisms and caused 100% mortality of zebrafish (*Danio rerio*) at 1.5 and 3  $\mu\text{mol/L}$  (McCormick et al., 2010). Although TBBPA showed low toxicity to mammals, cytotoxic effects of TBBPA were found in some studies (Colnot et al., 2014; Guyot et al., 2014), e.g., affecting the host immunity of mice by significantly increasing the pulmonary viral titer (Watanabe et al., 2010).

The almost ubiquitous presence of TBBPA in the environment motivated several biodegradation investigations. TBBPA can be degraded in active sludge, soil, sediments or soil-plant systems under oxic and anoxic conditions (Brenner et al., 2006; Chang et al., 2012; Gerecke et al., 2006; Li et al., 2015b; Li et al., 2014a; Sun et al., 2014). TBBPA can also be degraded in pure culture of *Ochrobactrum* sp. T or *Comamonas* sp. strain JXS-2-02 isolated from oxic and anoxic sludge, respectively (An et al., 2011; Peng et al., 2013; Zu et al., 2014).

Due to the presence of the phenolic group and its high octanol/water partition coefficient ( $\log K_{ow} = 5.9$ ), TBBPA sorbs onto organic matter and minerals in soil or sediment and forms non-extractable (or bound) residues (Li et al., 2015b; Luo et al., 2010; Tong et al., 2015). It has been reported that in surface sediments, bound residues accounted for 6.5%–48.0% of the total TBBPA detected (Luo et al., 2010). Humic acids (HA) are major component of soil organic matrix and can react with phenolic organic pollutants to form HA-bound residues of pollutants and their transformation products (Li et al., 2012a; Rieger et al., 2013; Vinken et al., 2005). Interactions of pollutants with HA can affect mobility, bioaccumulation, and biodegradation of the pollutants in the environment (Kastner and Mahro, 1996) and the effects may be compound-specific (Burgos et al., 2000; Li et al., 2007; Ortega-Calvo and Saiz-Jimenez, 1998; Vinken et al., 2004). It has been shown that biodegradation of TBBPA was responsible for the formation of bound residues of TBBPA and its metabolites in soil under both oxic and anoxic conditions (Li et al., 2015b; Liu et al., 2013). However, direct evidence for relationship between binding of TBBPA-derived residues to HA and biodegradation of TBBPA does not exist.

Coexistence of TBBPA and heavy metals has been often detected in soil (Wager et al., 2012; Wu et al., 2016; Zhou et al., 2013), because electric and electronic products contain both brominated flame-retardants and heavy metals. Copper (Cu), a common heavy metal used in electronic products (Stuhlpfarrer et al., 2016; Yang et al., 2016), can be toxic to microorganisms and enzymes (Ochoa-Herrera et al., 2011; Wang et al., 2007) and can inhibit biodegradation of organic pollutants, e.g., fluorine and biphenyl, in both pure bacterial culture and soil (Nam et al., 2015; Wannoussa et al., 2015). In addition, heavy metals can affect conformational structure and hydrophobicity of HA (Nuzzo et al., 2013; Stevenson, 1977) and therefore the interaction between HA and organic pollutants. In addition, complexation of  $\text{Cu}^{2+}$  with functional groups of HA may reduce the concentration of free  $\text{Cu}^{2+}$  (Klucakova, 2014; Li et al., 2015a) and therefore reduce Cu toxicity to microorganisms. The co-effects of HA and Cu on TBBPA biodegradation are still unknown.

In this study, we isolated a TBBPA-degrading bacterium from an oxic activated sludge and studied the dissipation, degradation, metabolism, and bound-residue formation of

TBBPA in pure culture of this bacterium in the absence and presence of  $\text{Cu}^{2+}$  and HA, in order to provide insights into the individual and combined effects of  $\text{Cu}^{2+}$  and HA on the biotransformation and fate of TBBPA in soil environment.

## 1. Materials and methods

### 1.1. Chemicals and humic material

Uniformly  $^{14}\text{C}$ -ring-labeled TBBPA ( $^{14}\text{C}$ -TBBPA) was synthesized from uniformly  $^{14}\text{C}$ -ring-labeled phenol via BPA (Liu et al., 2013).  $^{14}\text{C}$ -TBBPA had a specific activity of  $1.22 \times 10^9$  Bq/mmol and a radiochemical purity of 99%. Non-labeled TBBPA was purchased from Tokyo Chemical Industry Co., LTD with purity of 99% (Tokyo, Japan). Other chemicals of chromatographic or analytical grade were purchased from Nanjing Chemical Reagent, LTD (Nanjing, China).

HA were extracted from a pine forest soil in Aachen, Germany (Vinken et al., 2005). To prepare an HA stock solution, HA powder was added to a mineral salt medium (MSM) at pH 7.2 (see below), and then 4 mol/L NaOH was added dropwise to the medium until the HA were completely dissolved. This preparation procedure prevented oxidation of HA by air under alkaline condition.

### 1.2. MSM medium

The MSM had the following composition (per liter): 1 g of NaCl, 0.5 g of KCl, 0.1 g of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.015 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2 g of  $\text{NH}_4\text{Cl}$ , 0.2 g of  $\text{KH}_2\text{PO}_4$ , 0.15 g of  $\text{Na}_2\text{SO}_4$ , sodium phosphate buffer (pH 7.2; 20 mmol/L; autoclaved separately), and 0.5% of the trace element solution SL11 (Brune et al., 1995).

### 1.3. Isolation and identification of bacterial strains

Bacterial strains were isolated from an activated sludge sample collected from the Dachang sewage treatment plant in Nanjing, China. The sludge was washed with a phosphate buffer (20 mmol/L, pH 7.2) three times. Aliquot of the washed sludge (10 mL) with a mixed liquor suspended solids (MLSS) ( $1.33 \times 10^4$  mg/L) was added to MSM (40 mL) containing 100 mg/L TBBPA in a 150-mL flask, and incubated at 30°C on a rotary shaker (130 r/min). About 10% (V/V) of the culture was inoculated into 45 mL fresh MSM containing 100 mg/L TBBPA once every week in the first month and every other week in the next two months to enrich TBBPA-degrading bacteria. Aliquots of 100  $\mu\text{L}$  of the final enrichment were spread onto MSM-agar plates containing 100 mg/mL TBBPA. All agar plates were incubated at 30°C. Colonies with distinct morphology were picked out and streak plate method was used to get pure strains. Pure strains were inoculated to standard lysogeny broth (LB) medium, and were preserved in a mixture of glycerol (50%, in pure water) and LB medium (1:1, V/V).

The isolated strains were identified by morphology and 16S rRNA gene analysis. Cell morphologies were observed by optic microscopes (normal and phase-contrast microscope). 16S rRNA gene sequences were amplified by PCR using the forward primer 27F (AGAGTTTGATCCTGGCTCAG) and the

reverse primer 1492R (TACGGCTACCTTGTTACGACTT). The PCR reaction conditions and product purification were described by Li et al. (2014b). The obtained 16S rRNA gene sequences were annotated in GenBank database through NCBI Blast for taxonomic classification.

#### 1.4. Cell suspension

The bacterial strain was inoculated into LB medium and TBBPA degradation was induced by adding 100 mg/L TBBPA into the medium. The bacteria were cultivated in fresh LB medium at 30°C for 17.5 hr until the end of the exponential growth phase to harvest cell suspension. Cultures were then centrifuged repeatedly at 4000 r/min and washed with MSM (pH 7.2). The washing process was repeated three times and the cells were resuspended in MSM ( $OD_{600}=6.6$ ) for further experiments.

#### 1.5. Incubation of TBBPA in cell suspension

Incubation experiments were performed in 50-mL glass bottles. Bacterial cells were incubated in MSM containing 2.0 mg/L  $^{14}C$ -TBBPA (8333 Bq/bottle) at 30°C with shaking (130 rpm) in the dark. Extra yeast extract (100 mg/L) and casamino acids (100 mg/L) were added to each bottle to facilitate the bacterial growth and biodegradation as determined in pre-experiments. HA (1.0 mg/mL), Cu (10  $\mu$ mol/L  $CuCl_2$ ), or both were added to the culture to study their effects on the degradation of TBBPA. Treatments with sterilized cells (autoclaved for 20 min at 121°C) were set as controls.  $CO_2$  generated in the bottle was trapped in 1.0 mL of NaOH (1 mol/L) and radioactivity was periodically determined by liquid scintillation counter (LSC, see below). All experiments were performed in triplicate.

At 0, 8, 15, 24, 31, 41, and 87 days, individual samples were taken out and acidified with 6 mol/L HCl to adjust pH to 2 to release the dissolved  $CO_2$  from the medium. The medium (containing cells) was extracted four times with ethyl acetate (10 mL each time) and repeated sonication (1 kW, 25 kHz, 5 min), vigorous vortexing (4 min), and centrifuging (2000 r/min, 5 min). The supernatants from each step of extraction were combined and the radioactivity was determined by LSC (see below). The extracts were then evaporated to dryness on a rotary evaporator, and re-dissolved in 400  $\mu$ L methanol. The concentrated extracts were analyzed by high-performance liquid chromatography coupled to a radio flow scintillation detector (HPLC-LSC) (see below). The cultures after organic extraction were centrifuged at 5500 r/min for 10 min. The radioactivity in the cell free supernatants was determined by LSC (see below) and represented the radioactivity in hydrophilic non-extractable residues of TBBPA. The pellets were resuspended in 3 mL MSM and used for determination of radioactivity by LSC (see below).

The flasks of the treatments with HA addition, the medium was firstly extracted with ethyl acetate and then adjusted to pH 12 with 4 mol/L NaOH. The medium was centrifuged at 5500 r/min for 10 min to separate cells. The supernatant, containing HA, was acidified with 6 mol/L HCl to pH 1 and centrifuged again at 5500 r/min for 20 min. The radioactivity retained in the supernatant was determined by LSC (see

below) and represented the radioactivity in hydrophilic non-extractable residues of TBBPA. The pellet HA was re-dissolved in 3 mL NaOH (0.1 mol/L); the radioactivity was determined by LSC (see below) and represented the radioactivity of HA-bound residues derived from TBBPA.

#### 1.6. Determination of radioactivity

Radioactivity in all liquid samples was quantified on a liquid scintillation counter (LS6500; Beckman Coulter, USA). For the radioactivity of  $^{14}CO_2$ , 1 mL NaOH-trap was mixed with 3 mL scintillation cocktail (Gold Star multipurpose; Meridian Biotechnologies Ltd., UK). The radioactivity of organic extracts was determined by adding 3 mL scintillation cocktail to 1 mL extract. For determining the radioactivity of hydrophilic non-extractable fraction (10 mL out of total fraction), cells and HA (3 mL), the volume of scintillation cocktail was 10 mL.

#### 1.7. HPLC-LSC analysis

HPLC-LSC analysis was performed with an Eclipse XDB-C18 column (250 mm  $\times$  4.6 mm, 5  $\mu$ m; Agilent Technology, USA) at 30°C on an Agilent HPLC 1200 series device equipped with an online radio flow detector (FlowStar LB 513; Berthold Technologies, Germany). The mobile phase contained water (22%) and methanol (78%), running isocratically at 1 mL/min for 20 min. The flow rate of the scintillation cocktail (Gold Flow multipurpose; Meridian Biotechnologies Ltd., Epsom, UK) was controlled by a pump (Liquid Scintillator Pump LB 5036; Berthold Technologies, Germany) and was 1 mL/min.

#### 1.8. Data analysis

F-test and ANOVA were used to analyze individual and mutual treatment effects of Cu or HA. A statistical probability of  $p < 0.05$  was considered significant.

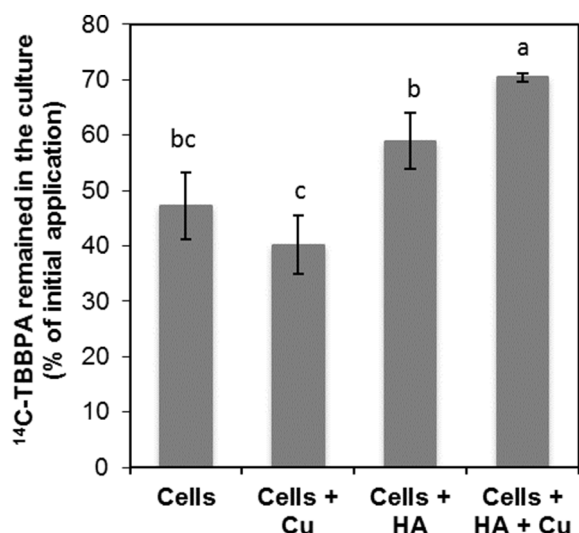
## 2. Results and discussion

### 2.1. Identification of TBBPA-degrading strain

One bacterial strain was isolated from the sludge samples. The strain was named as strain CDT and was used in further study on degradation of TBBPA. The 16S rDNA sequence analysis revealed that strain CDT was most related to *Pseudomonas pseudoalcaligenes* strain W-20 in NCBI Genbank with a similarity of 97.12%. Based on these factors, strain CDT was classified into the genus *Pseudomonas* and was named as *Pseudomonas* sp. strain CDT. Similar to previously found TBBPA-degrading microbes, like the genera *Ochrobactrum*, *Comamonas*, *Achromobacter*, and *Sphingomonas* (An et al., 2011; Li et al., 2012b; Peng et al., 2012), *Pseudomonas* sp. strain CDT also belongs to the phylum Proteobacteria and is gram-negative.

### 2.2. Dissipation of TBBPA

At the end of 87-day incubation,  $47.3\% \pm 6.0\%$  of the initial TBBPA was recovered from the culture of *Pseudomonas* sp. strain CDT alone (Fig. 1), which was similar to the results of Li



**Fig. 1** – TBBPA remaining in the culture of *Pseudomonas* sp. strain CDT at the end of incubation with active cells and in the presence of  $\text{Cu}^{2+}$  (10  $\mu\text{mol/L}$   $\text{CuCl}_2$ ), HA (1000 mg/L), as well as with both  $\text{Cu}^{2+}$  and HA (concentrations were the same as above). Data are mean values of three individual experiments. Error bars indicate standard deviation. Different letters indicate significant difference among treatments as tested with Tukey's test.

et al. (2016) who inoculated the TBBPA-degrading bacterium *Ochrobactrum* sp. T into sediments. In the presence of  $\text{Cu}^{2+}$ , the amount of recovered TBBPA was  $40.2\% \pm 5.2\%$ , which was not significantly different from treatment with active cells. In the presence of HA, the remaining TBBPA in the culture significantly increased to  $58.9\% \pm 5.0\%$ , indicating that HA inhibited the initial degradation step of TBBPA by strain CDT in the culture. This is likely attributed to the adsorption and binding of TBBPA on HA, which decreased the bioavailability of TBBPA to the bacteria. This inhibitory effect of HA contrasted with stimulation of the dissipation of nonylphenol (Li et al., 2007) and phenanthrene (Ortega-Calvo and Saiz-Jimenez, 1998) by HA in bacterial culture. Compared to TBBPA, nonylphenol and phenanthrene are more volatile, thus adsorption to HA in the culture increased the chance of pollutants to stay in the culture. Pollutants could then partially desorb from HA to become bioavailable, thus their degradation by bacteria could be increased by the presence of HA (Li et al., 2007; Ortega-Calvo and Saiz-Jimenez, 1998). On contrary, TBBPA has a much lower vapor pressure ( $1.19 \times 10^{-5}$  Pa at  $20^\circ\text{C}$ ) (Liu et al., 2016), which prevents TBBPA to volatilize from the medium, thus adsorption of TBBPA onto HA in the culture may decrease the bioavailability of TBBPA and further suppressed the initial degradation of TBBPA.

When both  $\text{Cu}^{2+}$  and HA were present in the culture, the inhibition of TBBPA dissipation was higher ( $70.4\% \pm 0.7\%$  of initial  $^{14}\text{C}$ -TBBPA recovered) than in the presence of HA alone (Fig. 1), indicating that  $\text{Cu}^{2+}$  increased the inhibition effect of HA on the dissipation of TBBPA in the culture. This may be explained by the increased adsorption of TBBPA on HA in the presence of  $\text{Cu}^{2+}$ , which decreased the concentration of free TBBPA available to bacteria. Zhao et al. (2013) found that  $\text{Cu}^{2+}$

significantly increased tetracycline adsorption on soils, because of the formation of  $[\text{tetracycline-Cu}]^+$  complexation that strongly adsorbs on soils. TBBPA has two hydroxyl groups with  $\text{pK}_{a1}$  and  $\text{pK}_{a2}$  being 7.5 and 8.5 (Kuramochi et al., 2008). At pH 7.2 of the medium, TBBPA existed mostly in neutral species, so forming  $[\text{Cu-TBBPA}]^+$  to promote the adsorption of TBBPA on HA played a less important role in our study. But  $\text{Cu}^{2+}$  as a multivalent cation can function as an ion bridge between soil organic matter and organic pollutants (MacKay and Canterbury, 2005), which also increased adsorption of TBBPA on HA.

### 2.3. Mineralization of TBBPA

At the end of incubation in active culture, only ( $2.3 \pm 0.1\%$ ) of the initial  $^{14}\text{C}$ -TBBPA was mineralized to  $^{14}\text{CO}_2$  (Fig. 2), while no  $^{14}\text{CO}_2$  was detected in the sterilized culture, indicating that the mineralization was resulted from the complete degradation of  $^{14}\text{C}$ -TBBPA by strain CDT. This much lower mineralization rate than the dissipation rate of TBBPA ( $52.7\% \pm 0.6\%$ ) (Fig. 1) indicated that compared to TBBPA parent compounds, strain CDT are less likely to degrade TBBPA metabolites. To increase the mineralization efficiency of TBBPA, different bacterial strains capable of degrading TBBPA metabolites, like 2,4-dibromophenol (2,4-DBP), 2,4,6-tribromophenol (TBP), and bisphenol A (BPA), could be added. Previous studies have found that through cooperation of many microorganisms, the degradation of complex organic compounds could be accelerated when their population sizes reached to a certain level and the function and structure of microbial consortia were stable (Cho et al., 2002; Li et al., 2016; Wongwilaiwalin et al., 2010). In the presence of  $\text{Cu}^{2+}$ , the mineralization of TBBPA at the end of the incubation became significantly lower ( $1.2\% \pm 0.2\%$ ) (Fig. 2). The inhibition of  $\text{Cu}^{2+}$  on mineralization of TBBPA was possibly due to the toxic effects of  $\text{Cu}^{2+}$  on microbial metabolism and related enzyme activity, because  $\text{Cu}^{2+}$  can suppress the activity of fermentative, aerobic glucose-degrading heterotrophic, and nitrifying bacteria (Ochoa-Herrera et al., 2011), as well as the removal efficiency of ammonia and organic constituents in bacterial culture (Chen et al., 2013, 2014; Nam et al., 2015) possibly by chelating sulfhydryl groups on proteins.

HA significantly inhibited the mineralization of TBBPA (only  $0.7\% \pm 0.1\%$   $^{14}\text{CO}_2$  generated at the end of incubation) (Fig. 2), additionally to its inhibitory effect on TBBPA dissipation in the culture (Fig. 1). However, when both  $\text{Cu}^{2+}$  and HA were present,  $0.6\% \pm 0.1\%$  of TBBPA was mineralized at the end of incubation (Fig. 2), which was almost the same as that in the presence of HA alone. This differed from the synergistic inhibitory effects of  $\text{Cu}^{2+}$  and HA on TBBPA dissipation (Fig. 1) and suggested that the interactions between  $\text{Cu}^{2+}$  and HA and between  $\text{Cu}^{2+}$  and TBBPA discussed above influenced more likely the initial degradation/dissipation of TBBPA than the mineralization (complete degradation) of TBBPA by strain CDT, possibly due to the adsorption of  $\text{Cu}^{2+}$  on HA, which decreased the effective concentration of  $\text{Cu}^{2+}$  in the medium.

### 2.4. Fate of TBBPA

During the incubation with strain CDT, TBBPA was taken up by cells, transformed to intermediates, including organic

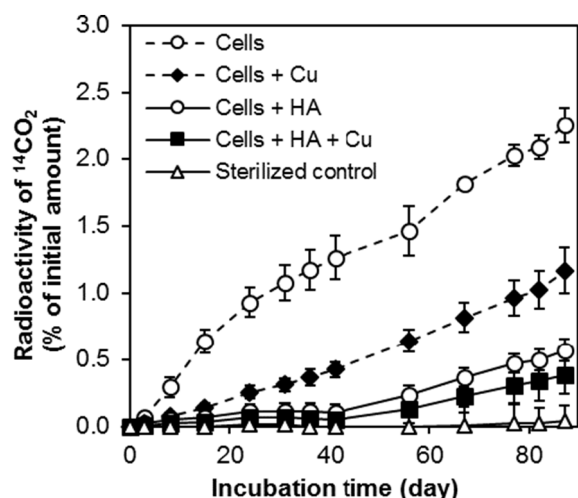


Fig. 2 – Radioactivity of  $^{14}\text{CO}_2$  during the incubation of  $^{14}\text{C}$ -TBBPA mineralized in active and sterile culture with or without the presence of  $\text{Cu}^{2+}$  (10  $\mu\text{mol/L}$   $\text{CuCl}_2$ ), HA (1 g/L) as well as with both  $\text{Cu}^{2+}$  and HA (concentrations were the same as above). Data are mean values of three individual experiments. Error bars indicate standard deviation.

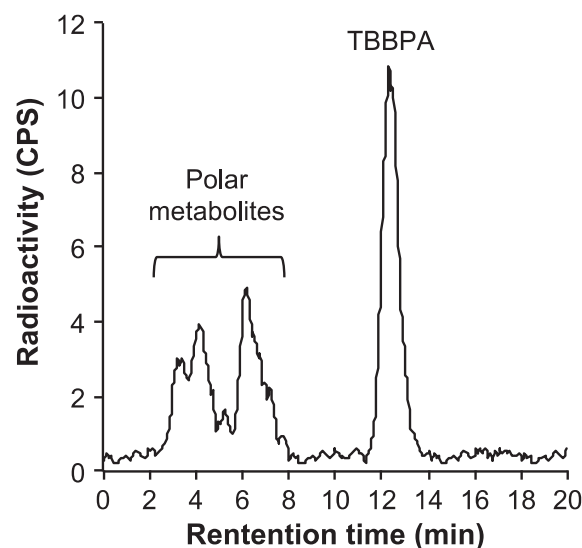


Fig. 4 – Typical radio-chromatogram of organic extracts after 87-day incubation of  $^{14}\text{C}$ -TBBPA in active culture of strain CDT. Polar metabolites (retention time: 3–8 min) of TBBPA (retention time 12.5 min) were detected.

extractable and non-extractable metabolites, and finally to a limited extent mineralized to  $\text{CO}_2$ . The major fate of TBBPA during degradation is transformation into organic extractable metabolites, accounting for 52.4%–71.2% of all transformed  $^{14}\text{C}$ -TBBPA in all treatments with active cells (Fig. 3). The metabolites detected in the organic extracts during the incubation were all more polar (retention time 3–8 min) than the parent TBBPA (Fig. 4). The structures of the metabolites were not identified, due to the low chemical concentration of metabolites generated. Previous studies on aerobic degradation of TBBPA in pure bacterial culture, nitrifying activated sludge, and soil showed that polar metabolites with retention

times shorter than TBBPA were debromination products and compounds with a single benzene ring and polar groups, such as hydroxyl and carbonyl groups, which originated from cleavage and oxidation of the alkyl chain of TBBPA (An et al., 2011; Li et al., 2014a; 2015b; Peng et al., 2013; Sun et al., 2014; Zu et al., 2014). Continuous increase of bromide concentration in the culture of strain CDT (data not shown) proved that debromination occurred in the culture, while large amounts of radioactivity remained in the aqueous phase after organic solvent extraction (Fig. 3) indicated that hydrophilic metabolites were generated during the degradation of TBBPA by strain CDT.

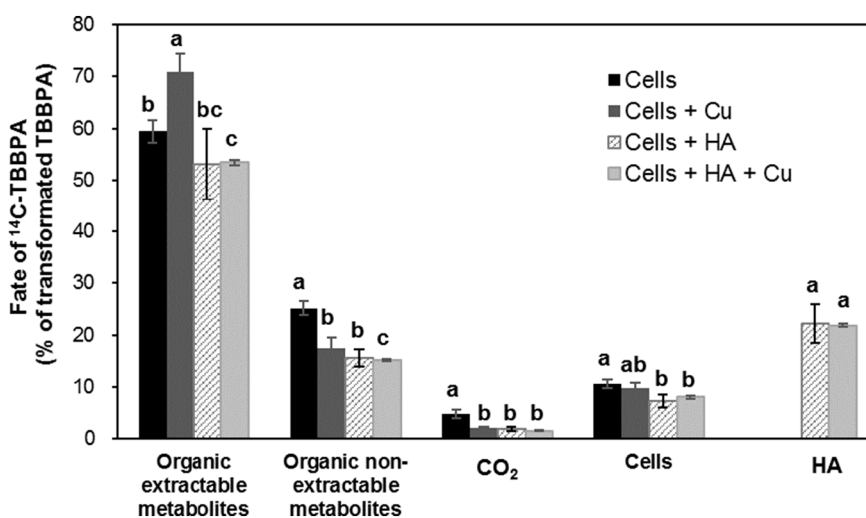
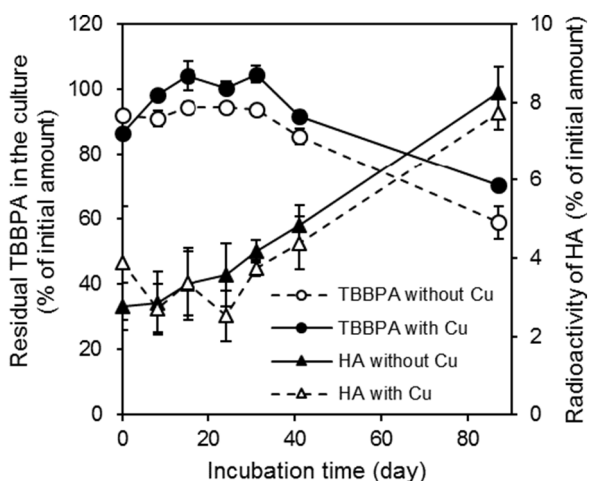


Fig. 3 – Relative radioactivity amounts of bio-transformed  $^{14}\text{C}$ -TBBPA recovered in the form of organic solvent-extractable metabolites, non-extractable metabolites (in water fraction),  $^{14}\text{CO}_2$ , cells, and associated to HA at the end of incubation of  $^{14}\text{C}$ -TBBPA in the culture of strain CDT. Data are mean values of three individual experiments. Error bars indicate standard deviation. Bars with the different letters indicate significant differences among treatments (cells, cells +  $\text{Cu}^{2+}$ , cells + HA).



**Fig. 5** – Amount of TBBPA in the culture (left y-axis) and radioactivity associated to HA (right y-axis) during incubation of  $^{14}\text{C}$ -TBBPA in active culture of strain CDT with or without  $\text{Cu}^{2+}$  ( $10\ \mu\text{mol/L}\ \text{CuCl}_2$ ). Data are mean values of three individual experiments. Error bars indicate standard deviation.

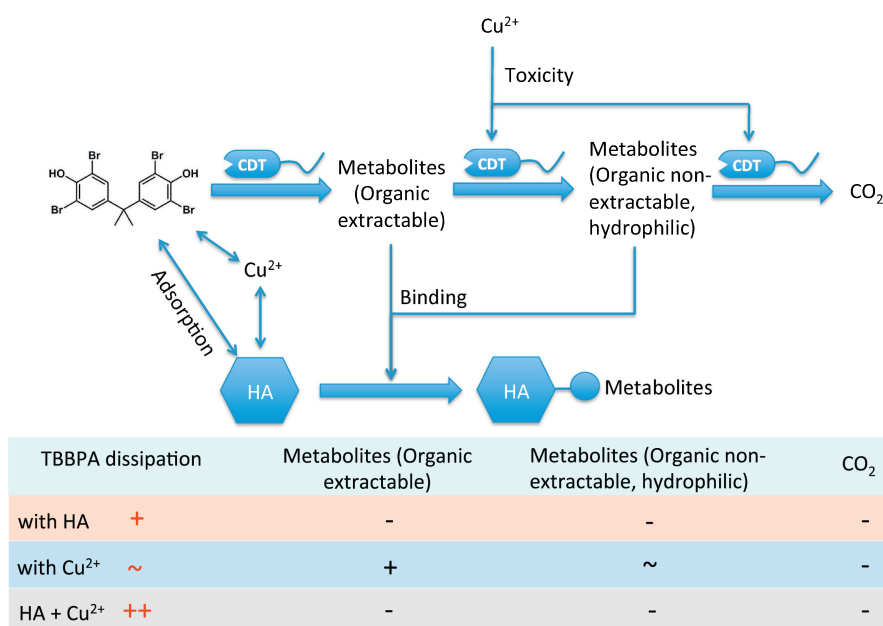
In the presence of  $\text{Cu}^{2+}$ , the amount of organic solvent-extractable metabolites significantly increased, while the amount of non-extractable, viz. hydrophilic metabolites significantly decreased (Fig. 3). Considering that  $\text{Cu}^{2+}$  did not significantly affect TBBPA dissipation (Fig. 1), this indicated that  $\text{Cu}^{2+}$  probably inhibited the transformation of organic extractable metabolites to hydrophilic metabolites and finally to  $\text{CO}_2$ .

When HAs were present in the culture, the amount of TBBPA derived hydrophilic metabolites,  $\text{CO}_2$ , and incorporated into cells

significantly decreased, and large amounts of TBBPA-derived residues bound to HA (Fig. 3), indicating an alteration of the fate of TBBPA in the culture. At the end of incubation period, the radioactivity was distributed as follows: organic solvent-extractable metabolites ( $59.4\% \pm 2.2\%$ ), non-extractable hydrophilic metabolites ( $25.1\% \pm 1.3\%$ ),  $\text{CO}_2$  ( $4.8\% \pm 0.8\%$ ), and bacterial cells ( $10.6\% \pm 0.9\%$ ) (Fig. 3). The formation of HA-bound residues was owing to the reaction of reactive metabolites of TBBPA with HA macromolecules (Tong et al., 2015) and therefore increased continually during the incubation of TBBPA (Fig. 5). As bound residues per se have lower bioavailability (Gevao et al., 2000), formation of HA-bound residues significantly inhibited the further transformation of the metabolites to  $\text{CO}_2$  (Li et al., 2015; Liu et al., 2013). The combined effects of  $\text{Cu}^{2+}$  and HA on the occurrence of metabolites in the culture were similar to that of HA (Fig. 3), suggesting that  $\text{Cu}^{2+}$  had minor effects on the transformation of TBBPA when HA were present in the culture.

## 2.5. Conclusions

In summary, TBBPA was degraded by strain CDT to organic-solvent extractable and non-extractable metabolites, and finally mineralized to a lesser extent to  $\text{CO}_2$  (Fig. 6).  $\text{Cu}^{2+}$  significantly inhibited the mineralization and transformation from organic extractable metabolites into non-extractable metabolites, but had no significant effects on initial degradation (dissipation) of TBBPA. HA markedly altered the fate of TBBPA by reacting with reactive metabolites, forming large amounts of HA-bound residues, whereas the dissipation, metabolite formation, and mineralization of TBBPA were significantly inhibited. Coexistence of  $\text{Cu}^{2+}$  and HA significantly promoted the inhibitory effects of HA on initial



**Fig. 6** – Conceptual model showing the fate of TBBPA in active culture of *Pseudomonas* sp. strain CDT with the influences of  $\text{Cu}^{2+}$  and humic acids (HA). During the incubation, TBBPA was taken up by cells, transformed to intermediates, including organic extractable and non-extractable metabolites, and finally mineralized to  $\text{CO}_2$ . Symbol “+”, “-”, and “~” indicate an increase, decrease, and no significant change in absolute (TBBPA parent compounds) or relative amounts (transformation products) of the compounds to the total transformation in the culture at the end of incubation.

degradation of TBBPA but had little or no effects on further transformation of TBBPA, owing to the complexation of  $\text{Cu}^{2+}$  and TBBPA, ion bridging function of  $\text{Cu}^{2+}$ , and neutralization of negative charge of HA by  $\text{Cu}^{2+}$ , which increased adsorption of TBBPA on HA thus decreased the bioavailability of TBBPA to strain CDT. In contrast, the presence of HA mitigated the effects of  $\text{Cu}^{2+}$  on transformation of TBBPA by strain CDT, owing to decrease of free  $\text{Cu}^{2+}$  concentration in the medium by complexation.

The results provide insights into individual and interactive effects of  $\text{Cu}^{2+}$  and HA on the biotransformation of TBBPA in pure culture systems and indicate the complexity of the interactive effects of heavy metals and soil organic matter on fate of organic pollutants in the environment. Pure culture provides a simple system for studying the effects of environmental factors on fate and biotransformation of organic pollutants and the underlying mechanisms, especially formation of bound residues in the environment, which is essential for risk assessment of organic pollutants in environments co-contaminated with heavy metals.

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## REFERENCES

- An, T.C., Zu, L., Li, G.Y., Wan, S.G., Mai, B.X., Wong, P.K., 2011. One-step process for debromination and aerobic mineralization of tetrabromobisphenol-A by a novel *Ochrobactrum* sp. T isolated from an e-waste recycling site. *Bioresour. Technol.* 102, 9148–9154.
- Brenner, A., Mukmenev, I., Abeliovich, A., Kushmaro, A., 2006. Biodegradability of tetrabromobisphenol A and tribromophenol by activated sludge. *Ecotoxicology* 15, 399–402.
- Brune, A., Miambi, E., Breznak, J.A., 1995. Roles of oxygen and the intestinal microflora in the metabolism of lignin-derived phenylpropanoids and other monoaromatic compounds by termites. *Appl. Environ. Microbiol.* 61, 2688–2695.
- Burgos, W.D., Pisutpaisal, N., Tuntolavest, M., Chorover, J., Unz, R.F., 2000. Biodegradation of 1-naphthol in the presence of humic acid. *Environ. Eng. Sci.* 17, 343–351.
- Chang, B.V., Yuan, S.Y., Ren, Y.L., 2012. Aerobic degradation of tetrabromobisphenol-A by microbes in river sediment. *Chemosphere* 87, 535–541.
- Chen, S.N., Yin, H., Ye, J.S., Peng, H., Zhang, N., He, B.Y., 2013. Effect of copper(II) on biodegradation of benzo(a)pyrene by *Stenotrophomonas maltophilia*. *Chemosphere* 90, 1811–1820.
- Chen, M.X., Wang, W.C., Feng, Y., Zhu, X.H., Zhou, H.Z., Tan, Z.L., Li, X.D., 2014. Impact resistance of different factors on ammonia removal by heterotrophic nitrification-aerobic denitrification bacterium *Aeromonas* sp HN-02. *Bioresour. Technol.* 167, 456–461.
- Cho, Y.C., Ostrofsky, E.B., Sokol, R.C., Frohnhoefer, R.C., Rhee, G.Y., 2002. Enhancement of microbial PCB dechlorination by chlorobenzoates, chlorophenols and chlorobenzenes. *FEMS Microbiol. Ecol.* 42, 51–58.
- Colnot, T., Kacew, S., Dekant, W., 2014. Mammalian toxicology and human exposures to the flame retardant 2,2',6,6'-tetrabromo-4,4'-isopropylidenediphenol (TBBPA): implications for risk assessment. *Arch. Toxicol.* 88, 553–573.
- Feng, A.H., Chen, S.J., Chen, M.Y., He, M.J., Luo, X.J., Mai, B.X., 2012. Hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) in riverine and estuarine sediments of the Pearl River Delta in southern China, with emphasis on spatial variability in diastereoisomer- and enantiomer-specific distribution of HBCD. *Mar. Pollut. Bull.* 64, 919–925.
- Gerecke, A.C., Giger, W., Hartmann, P.C., Heeb, N.V., Kohler, H.P.E., Schmid, P., Zennegg, M., Kohler, M., 2006. Anaerobic degradation of brominated flame retardants in sewage sludge. *Chemosphere* 64, 311–317.
- Gevao, B., Semple, K.T., Jones, K.C., 2000. Bound pesticide residues in soils: a review. *Environ. Pollut.* 108, 3–14.
- Gorga, M., Martinez, E., Ginebreda, A., Eljarrat, E., Barcelo, D., 2013. Determination of PBDEs, HBB, PBEB, DBDPE, HBCD, TBBPA and related compounds in sewage sludge from Catalonia (Spain). *Sci. Total Environ.* 444, 51–59.
- Guyot, R., Chatonnet, F., Gillet, B., Hughes, S., Flamant, F., 2014. Toxicogenomic analysis of the ability of brominated flame retardants TBBPA and BDE-209 to disrupt thyroid hormone signaling in neural cells. *Toxicology* 325, 125–132.
- He, M.J., Luo, X.J., Yu, L.H., Liu, J.A., Zhang, X.L., Chen, S.J., Mai, B.X., 2010. Tetrabromobisphenol-a and hexabromocyclododecane in birds from an e-waste region in South China: influence of diet on diastereoisomer- and enantiomer-specific distribution and trophodynamics. *Environ. Sci. Technol.* 44, 8357.
- Johnson-Restrepo, B., Adams, D.H., Kannan, K., 2008. Tetrabromobisphenol A (TBBPA) and hexabromocyclododecanes (HBCDs) in tissues of humans, dolphins, and sharks from the United States. *Chemosphere* 70, 1935–1944.
- Kastner, M., Mahro, B., 1996. Microbial degradation of polycyclic aromatic hydrocarbons in soils affected by the organic matrix of compost. *Appl. Microbiol. Biotechnol.* 44, 668–675.
- Klucakova, M., 2014. Complexation of metal ions with solid humic acids, humic colloidal solutions, and humic hydrogel. *Environ. Eng. Sci.* 31, 612–620.
- Kuramochi, H., Kawamoto, K., Miyazaki, K., Nagahama, K., Maeda, K., Li, X.W., Shibata, E., Nakamura, T., Sakai, S.I., 2008. Determination of physicochemical properties of tetrabromobisphenol A. *Environ. Toxicol. Chem.* 27, 2413–2418.
- Li, C.L., Ji, R., Vinken, R., Honinies, G., Bertmer, M., Schaeffer, A., Corvini, P.F.X., 2007. Role of dissolved humic acids in the biodegradation of a single isomer of nonylphenol by *Sphingomonas* sp. *Chemosphere* 68, 2172–2180.
- Li, C.L., Zhang, B., Ertunc, T., Schaeffer, A., Ji, R., 2012a. Birnessite-induced binding of phenolic monomers to soil humic substances and nature of the bound residues. *Environ. Sci. Technol.* 46, 8843–8850.
- Li, G.Y., Zu, L., Wong, P.-K., Hui, X.P., Lu, Y., Xiong, J.K., An, T.C., 2012b. Biodegradation and detoxification of bisphenol A with one newly isolated strain *Bacillus* sp. GZB: kinetics, mechanism and estrogenic transition. *Bioresour. Technol.* 114, 224–230.
- Li, X.Z., Rui, J.P., Mao, Y.J., Yannarell, A., Mackie, R., 2014a. Dynamics of the bacterial community structure in the rhizosphere of a maize cultivar. *Soil Biol. Biochem.* 68, 392–401.
- Li, F.J., Wang, J.J., Nastold, P., Jiang, B.Q., Sun, F.F., Zenker, A., Kolvenbach, B.A., Ji, R., Corvini, P.F.X., 2014b. Fate and metabolism of tetrabromobisphenol A in soil slurries without and with the amendment with the alkylphenol degrading bacterium *Sphingomonas* sp strain TTNP3. *Environ. Pollut.* 193, 181–188.
- Li, C.L., Ji, F., Wang, S., Zhang, J.J., Gao, Q., Wu, J.G., Zhao, L.P., Wang, L.C., Zheng, L.R., 2015a. Adsorption of Cu(II) on humic acids derived from different organic materials. *J. Integr. Agric.* 14, 168–177.

- Li, F.J., Wang, J.J., Jiang, B.Q., Yang, X., Nastold, P., Kolvenbach, B., Wang, L., Ma, Y.N., Corvini, P.F.-X., Ji, R., 2015b. Fate of tetrabromobisphenol A (TBBPA) and formation of ester- and ether-linked bound residues in an oxic sandy soil. *Environ. Sci. Technol.* 49, 12758–12765.
- Li, G.Y., Xiong, J.K., Wong, P.K., An, T.C., 2016. Enhancing tetrabromobisphenol A biodegradation in river sediment microcosms and understanding the corresponding microbial community. *Environ. Pollut.* 208, 796–802.
- Liu, J., Wang, Y.F., Jiang, B.Q., Wang, L.H., Chen, J.Q., Guo, H.Y., Ji, R., 2013. Degradation, metabolism, and bound-residue formation and release of tetrabromobisphenol A in soil during sequential anoxic-oxic incubation. *Environ. Sci. Technol.* 47, 8348–8354.
- Liu, K., Li, J., Yan, S.J., Zhang, W., Li, Y.J., Han, D., 2016. A review of status of tetrabromobisphenol A (TBBPA) in China. *Chemosphere* 148, 8–20.
- Luo, X.-J., Zhang, X.-L., Chen, S.-J., Mai, B.-X., 2010. Free and bound polybrominated diphenyl ethers and tetrabromobisphenol A in freshwater sediments. *Mar. Pollut. Bull.* 60, 718–724.
- MacKay, A.A., Canterbury, B., 2005. Oxytetracycline sorption to organic matter by metal-bridging. *J. Environ. Qual.* 34, 1964–1971.
- McCormick, J.M., Paiva, M.S., Haeggblom, M.M., Cooper, K.R., White, L.A., 2010. Embryonic exposure to tetrabromobisphenol A and its metabolites, bisphenol A and tetrabromobisphenol A dimethyl ether disrupts normal zebrafish (*Danio rerio*) development and matrix metalloproteinase expression. *Aquat. Toxicol.* 100, 255–262.
- Nam, I.H., Kim, Y., Cho, D., Kim, J.G., Song, H., Chon, C.M., 2015. Effects of heavy metals on biodegradation of fluorene by a *Sphingobacterium* sp strain (KM-02) isolated from polycyclic aromatic hydrocarbon-contaminated mine soil. *Environ. Eng. Sci.* 32, 891–898.
- Nuzzo, A., Sánchez, A., Fontaine, B., Piccolo, A., 2013. Conformational changes of dissolved humic and fulvic superstructures with progressive iron complexation. *J. Geochem. Explor.* 129, 1–5.
- Ochoa-Herrera, V., Leon, G., Banihani, Q., Field, J.A., Sierra-Alvarez, R., 2011. Toxicity of copper(II) ions to microorganisms in biological wastewater treatment systems. *Sci. Total Environ.* 412, 380–385.
- Ortega-Calvo, J.J., Saiz-Jimenez, C., 1998. Effect of humic fractions and clay on biodegradation of phenanthrene by a *Pseudomonas fluorescens* strain isolated from soil. *Appl. Environ. Microbiol.* 64, 3123–3126.
- Peng, X., Zhang, Z., Zhao, Z., Jia, X., 2012. 16S ribosomal DNA clone libraries to reveal bacterial diversity in anaerobic reactor-degraded tetrabromobisphenol A. *Bioresour. Technol.* 112, 75–82.
- Peng, X., Zhang, Z., Luo, W., Jia, X., 2013. Biodegradation of tetrabromobisphenol A by a novel *Comamonas* sp strain, JXS-2-02, isolated from anaerobic sludge. *Bioresour. Technol.* 128, 173–179.
- Riefer, P., Klausmeyer, T., Adams, A., Schmidt, B., Schaeffer, A., Schwarzbauer, J., 2013. Incorporation mechanisms of a branched nonylphenol isomer in soil-derived organo-clay complexes during a 180-day experiment. *Environ. Sci. Technol.* 47, 7155–7162.
- Stevenson, F.J., 1977. Nature of divalent transition metal complexes of humic acids as revealed by a modified potentiometric titration method. *Soil Sci.* 123, 10–17.
- Stuhlfarrar, P., Luidold, S., Antrekowitsch, H., 2016. Recycling of waste printed circuit boards with simultaneous enrichment of special metals by using alkaline melts: a green and strategically advantageous solution. *J. Hazard. Mater.* 307, 17–25.
- Sun, F.F., Kolvenbach, B.A., Nastold, P., Jiang, B.Q., Ji, R., Corvini, P.F.X., 2014. Degradation and metabolism of tetrabromobisphenol A (TBBPA) in submerged soil and soil-plant systems. *Environ. Sci. Technol.* 48, 14291–14299.
- Tang, J.F., Feng, J.Y., Lia, X.H., Li, G., 2014. Levels of flame retardants HBCD, TBBPA and TBC in surface soils from an industrialized region of East China. *Environ. Sci. Processes Impacts* 16, 1015–1021.
- Tong, F., Gu, X.Y., Gu, C., Ji, R., Tan, Y.Y., Xie, J.Y., 2015. Insights into tetrabromobisphenol A adsorption onto soils: effects of soil components and environmental factors. *Sci. Total Environ.* 536, 582–588.
- Vinken, R., Hollrigl-Rosta, A., Schmidt, B., Schaffer, A., Corvini, P.F.X., 2004. Bioavailability of a nonylphenol in dependence on the association to dissolved humic substances. *Water Sci. Technol.* 50, 277–283.
- Vinken, R., Schaffer, A., Ji, R., 2005. Abiotic association of soil-borne monomeric phenols with humic acids. *Org. Geochem.* 36, 583–593.
- Wager, P.A., Schluep, M., Mueller, E., Gloor, R., 2012. RoHS regulated substances in mixed plastics from waste electrical and electronic equipment. *Environ. Sci. Technol.* 46, 628–635.
- Wang, Y., Shi, J., Wang, H., Lin, Q., Chen, X., Chen, Y., 2007. The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. *Ecotoxicol. Environ. Saf.* 67, 75–81.
- Wang, J.X., Liu, L.L., Wang, J.F., Pan, B.S., Fu, X.X., Zhang, G., et al., 2015. Distribution of metals and brominated flame retardants (BFRs) in sediments, soils and plants from an informal e-waste dismantling site, South China. *Environ. Sci. Pollut. Res.* 22, 1020–1033.
- Wannoussa, W., Hilgsmann, S., Tasseroul, L., Masy, T., Lambert, S.D., Heinrichs, B., et al., 2015. Effect of metal ions and metal nanoparticles encapsulated in porous silica on biphenyl biodegradation by *Rhodococcus erythropolis* T902.1. *J. Sol-Gel Sci. Technol.* 75, 235–245.
- Watanabe, W., Shimizu, T., Sawamura, R., Hino, A., Konno, K., Hirose, A., Kurokawa, M., 2010. Effects of tetrabromobisphenol A, a brominated flame retardant, on the immune response to respiratory syncytial virus infection in mice. *Immunopharmacol.* 10, 393–397.
- Wongwilaiwalin, S., Rattanachomsri, U., Laothanachareon, T., Eurwilaichitr, L., Igarashi, Y., Champreda, V., 2010. Analysis of a thermophilic lignocellulose degrading microbial consortium and multi-species lignocellulolytic enzyme system. *Enzym. Microb. Technol.* 47, 283–290.
- Wu, Y.Y., Li, Y.Y., Kang, D., Wang, J.J., Zhang, Y.F., Du, D.L., et al., 2016. Tetrabromobisphenol A and heavy metal exposure via dust ingestion in an e-waste recycling region in Southeast China. *Sci. Total Environ.* 541, 356–364.
- Xu, J., Zhang, Y., Guo, C.S., He, Y., Li, L., Meng, W., 2013. Levels and distribution of tetrabromobisphenol A and hexabromocyclododecane in Taihu Lake, China. *Environ. Toxicol. Chem.* 32, 2249–2255.
- Yang, J.-G., Lei, J., Peng, S.-Y., Lv, Y.-l., Shi, W.-Q., 2016. A new membrane electro-deposition based process for tin recovery from waste printed circuit boards. *J. Hazard. Mater.* 304, 409–416.
- Zhao, Y.P., Tan, Y.Y., Guo, Y., Gu, X.Y., Wang, X.R., Zhang, Y., 2013. Interactions of tetracycline with Cd (II), Cu (II) and Pb (II) and their cosorption behavior in soils. *Environ. Pollut.* 180, 206–213.
- Zhou, X.Y., Guo, J., Lin, K.F., Huang, K., Deng, J.J., 2013. Leaching characteristics of heavy metals and brominated flame retardants from waste printed circuit boards. *J. Hazard. Mater.* 246, 96–102.
- Zu, L., Xiong, J.K., Li, G.Y., Fang, Y.J., An, T.C., 2014. Concurrent degradation of tetrabromobisphenol A by *Ochrobactrum* sp. T under aerobic condition and estrogenic transition during these processes. *Ecotoxicol. Environ. Saf.* 104, 220–225.