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Accumulation and phytotoxicity of technical hexabromocyclododecane in maize

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A B S T R A C T

To investigate the accumulation and phytotoxicity of technical hexabromocyclododecane (HBCD) in maize, young seedlings were exposed to solutions of technical HBCD at different concentrations. The uptake kinetics showed that the HBCD concentration reached an apparent equilibrium within 96 hr, and the accumulation was much higher in roots than in shoots. HBCD accumulation in maize had a positive linear correlation with the exposure concentration. The accumulation of different diastereoisomers followed the order γ-HBCD > β-HBCD > α-HBCD. Compared with their proportions in the technical HBCD exposure solution, the diastereoisomer contribution increased for β-HBCD and decreased for γ-HBCD in both maize roots and shoots with exposure time, whereas the contribution of α-HBCD increased in roots and decreased in shoots throughout the experimental period. These results suggest the diastereomer-specific accumulation and translocation of HBCD in maize. Inhibitory effects of HBCD on the early development of maize followed the order of germination rate > root biomass ≥ root elongation > shoot biomass ≥ shoot elongation. Hydroxyl radical (·OH) and histone H2AX phosphorylation (γ-H2AX) were induced in maize by HBCD exposure, indicative of the generation of oxidative stress and DNA double-strand breaks in maize. An ·OH scavenger inhibited the expression of γ-H2AX foci in both maize roots and shoots, which suggests the involvement of ·OH generation in the HBCD-induced DNA damage. The results of this study will offer useful information for a more comprehensive assessment of the environmental behavior and toxicity of technical HBCD.
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Introduction

Hexabromocyclododecane (HBCD) is one of the most widely used brominated flame retardants and employed primarily in construction materials, upholstery textiles and electronic equipment (Marvin et al., 2011). It was reported that the current annual production of HBCD was estimated to 28,000 tons (http://www.kemi.se/en/Content/International/Conventions-and-agreements/-Stockholm-Convention-POPs/). Due to its widespread use, HBCD has been detected widely in environmental and biological matrices. HBCD was first found in fish and sediment in the Swedish River Viskan in 1998 (Sellström et al., 1998), and since then it has been detected in a wide variety of biota and abiotic samples such as rainbow trout, loach, water, sewage sludge, soils and sediments (Haukås et al., 2009; Zhang et al., 2009; Hale et al., 2006; Wang et al., 2009; Li et al., 2013).

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Very high HBCD levels have been reported in sewage sludge (36,000 ng/g dry weight) (Morris et al., 2004), soils (28,000 ng/g dry weight) (Petersen et al., 2004) and eel muscles (10,275 ng/g lipid weight) (Allchin and Morris, 2003). The concentration of HBCD has been reported to be 15.3 pg/g dry weight, with γ-HBCD as the dominant diastereoisomer, in agriculture soils in Chongming Island, the Yangtze River Delta in South China (Meng et al., 2011). A much higher concentration of HBCD was detected at a concentration of 37.5 ng/g dry weight in farmland soils near a BFR factory in North China (Zhu et al., 2014). The potential risk of HBCD in the environment is of considerable concern owing to its characteristics of long-distance transportation (Ueno et al., 2006), persistence and biomagnification (Law et al., 2008) as well as its toxicity effects on human health (Christen et al., 2010).

Commercially used HBCD (technical HBCD) is a mixture of different isomers with α-, β- and γ-HBCDs (in ranges of 10%–13%, 1%–12% and 75%–89%, respectively) as the predominant diastereoisomers (Tomy et al., 2004). Although having the same chemical formula, different configurations of HBCD diastereoisomers result in their different levels, distributions and fates in the environment. The α-HBCD concentration is usually the highest in biotic environments, whereas γ-HBCD has been reported as the dominant diastereoisomer in abiotic samples (Thomsen et al., 2007; Marvin et al., 2006). Therefore, there is a need to elucidate the uptake and contribution of each diastereoisomer when examining the toxic effect of the HBCD technical mixture.

Plant uptake of persistent organic pollutants is an important process when considering the risks associated with land contamination, the role of vegetation in global cycling, and the potential of industrial discharges to contaminate the food chain (Collins et al., 2006). Despite its ubiquitous presence in soils, there is limited knowledge on the plant uptake and phytotoxicity of HBCD. The accumulation of HBCD in cabbage and radish tissues has been determined by Li et al. (2011), with predominance of γ-HBCD in roots and α-HBCD in shoots. Nevertheless, phytotoxicity information on technical HBCD is lacking. Although we have investigated the accumulation and toxicity of each HBCD diastereoisomer in maize in our previous work (Wu et al., 2012), the contribution of each diastereoisomer in the technical mixture to HBCD accumulation in plants is still unknown.

Oxidative stress caused by the generation of reactive oxygen species (ROS) is thought to be a major cause of physiological disorders of plants after exposure to contaminants. Overproduction of ROS can induce oxidative damage to biomolecules such as lipids, proteins and DNA, eventually leading to cell death (Mittler et al., 2004). ROS-derived DNA oxidation will result in altered bases and damaged sugar residues, leading to DNA single- and double-strand breaks (DSBs) (Roldán-Arjona and Ariza, 2009). Phosphorylation of histone H2AX (γ-H2AX) at serine 139 occurs after DNA DSBs and is probably the earliest cellular response to this lesion (Rogakou et al., 1999). The γ-H2AX foci assay showed higher sensitivity to low levels of DNA DSBs than the neutral comet assay, a classical method for detecting DNA damage (Yu et al., 2006). Because of the quantitative correspondence between the number of γ-H2AX foci and the number of DSBs, γ-H2AX has been used as a new indicator for DNA damage.

A hydroponic experiment was conducted in the present study to investigate the uptake and oxidative toxicity of technical HBCD in maize. The time- and concentration-dependent accumulations of HBCD in maize were detected. The relative diastereoisomer contributions of α-, β- and γ-HBCDs were further analyzed. The dose-effect relationship of HBCD toxicity in maize was examined by determining its toxic influence on the early growth and development of maize. ROS generation in maize exposed to different concentrations of HBCD was determined by electron paramagnetic resonance (EPR) spectroscopy. The level of γ-H2AX foci was examined to investigate genotoxicity in maize induced by HBCD. The results of the present study would offer useful information for a more comprehensive assessment of the environmental behavior of the technical HBCD mixture.

1. Materials and methods

1.1. Chemicals

A technical HBCD mixture and the native (α-, β- and γ-) HBCD standards were purchased from AccuStandard (New Haven, USA). The ^{13}C-labeled (α-, β- and γ-) HBCD standards were obtained from Cambridge Isotope Laboratories, Inc. (Andover, USA). Dimethylsulfoxide (DMSO, purity > 99.9%) and α-phenyl-N-tert-butylnitrone (PBN, purity > 98%) were obtained from Sigma-Aldrich company (USA). Anhydrous sodium sulfate (Na_2SO_4), silica gel and alumina (100–200 mesh) were washed with hexane and used after heating overnight at 150°C. Other solvents and chemicals used were of analytical or HPLC grade.

1.2. Plant cultivation and exposure

Technical HBCD was dissolved in methanol as stock solution. A series of HBCD solutions for plant exposure tests with a range of concentrations (0, 0.002, 0.005, 0.01, 0.02, and 0.05 mg/L) were prepared using the stock solution and sterile deionized water. Maize (*Zea mays* L.) was used as the test plant and the seeds were obtained from the Chinese Academy of Agricultural Sciences, Beijing, China. Prior to germination, the seeds were surface-sterilized in 3% (V/V) H_2O_2 for 10 min and rinsed thoroughly with deionized water. After germination for 4 days, five uniform seedlings were transferred to each colored vitreous pot containing 200 mL of test solution to investigate the uptake and toxicity of HBCD in maize. Pots were kept in a growth chamber with a controlled environment at a light intensity of 250 μmol/(m^2·s) provided by supplementary illumination with a photoperiod of 14 hr each day, 25/20°C day/night temperature regime, and relative humidity of 60%–70%. The seedlings were repositioned daily to minimize spatial differences in illumination and temperature, and harvested after 4 days. Unplanted and blank controls without HBCD exposure were included. Test solutions were renewed every day and all the treatments were set up in triplicate. The test solution of 0.002 mg/L was selected for the kinetic uptake assay, and maize plants were harvested at different time intervals.

Seed germination was tested on filter paper placed in Petri dishes and moistened with 60 mL of test solutions. After sterilizing, one hundred seeds were placed in each dish, covered

by a lid, and incubated at 27 ± 0.5°C in the dark. After 96 hr, the proportion of germinated seeds was counted. Seeds were considered to have germinated when both the radicle and coleoptile had achieved lengths greater than half the seed size. Controls were obtained by moistening the filter papers with 60 mL of deionized water. Test solutions were renewed every day and each treatment was set up in triplicate and repeated 3 times.

1.3. Extraction and cleanup of plant samples

Sample extraction and concentration analysis were conducted as follows. The harvested plants were separated into roots and shoots, rinsed thoroughly with deionized water and blotted with filter paper. Plant tissues were then frozen at −50°C overnight and freeze-dried for 48 hr in a lyophilizer (FD-1, China). The dried samples were chopped finely and stored in glass containers at −20°C before extraction.

Extraction and cleanup of plant samples for HBCD analysis were based on the method of Han et al. (2010) and Zhang et al. (2009) with some modifications. Each sample (0.1–0.5 g dry weight) was ultrasonic-assisted extracted for 2 hr using 20 mL of ethyl acetate at 60°C after spiking with ^{13}C-α-HBCD and ^{13}C-γ-HBCD. The extract was collected and evaporated to 1–2 mL. The concentrated extract was then cleaned up on a multilayer silica gel/alumina column packed with anhydrous sodium sulfate (5 g), neutral silica (2 g, 3% deactivated), acidic silica (5 g, 44% sulfuric acid), neutral silica (2 g, 3% deactivated), neutral alumina (5 g, 3% deactivated) and anhydrous sodium sulfate (5 g) from top to bottom. HBCD was eluted with 50 mL of hexane and evaporated to 1 mL. After concentrating to near-dryness under a gentle nitrogen gas, the extract was spiked with ^{13}C-β-HBCD as an internal standard and adjusted to 0.4 mL with methanol.

1.4. Determination of HBCD diastereoisomers

The extracted samples were analyzed using an ultra-high performance liquid chromatography coupled to a triple-quadrupole mass spectrometer (UPLC–MS/MS). Chromatographic separation of HBCD diastereoisomers was performed on an UPLC (Waters ACQUITY UPLC system, USA) with a column of Waters ACQUITY UPLC BEH C_{18} (50 × 2.1 mm, i.d., 1.7 μm particle size). A mobile phase of (A) 0.01 mol/L ammonium acetate and (B) methanol at a flow rate of 0.2 mL/min was applied for elution of the target compounds. The elution program started with 50% A and was ramped linearly to 15% A in 9 min, held for 4 min, followed by a linear decrease to 0% A in 1 min and held for 5 min. Then the mobile phase composition was returned to the initial conditions in 2 min and equilibrated for a further 5 min. A Waters Quattro Premier XE triple quadrupole mass spectrometer equipped with an ESI source (Waters, USA) was used. The MS system was operated in the electrospray negative ionization (ESI) and multiple reaction monitoring (MRM) mode. The MRM transitions monitored were 639.7 → 79.0 for native HBCD diastereoisomers and 651.5 → 79.0 for the ^{13}C-labeled HBCDs, respectively. The conditions for the MS system were optimized as follows: cone voltage 20 V, capillary voltage 3.5 kV, desolvation temperature 350°C, source temperature 110°C, nebulizing gas flow 400 L/hr, cone gas flow 50 L/hr and collision energy 15 eV, respectively.

Quality assurance and quality control were done by regular analyses of procedural blanks, blind duplicate samples, and random injection of solvent blanks and standards. Matrix effects on the signal intensity of UPLC-MS/MS were minimized using ^{13}C-α- and ^{13}C-γ-HBCDs as surrogate standards, and ^{13}C-β-HBCD as internal standard. The limits of detection (LOD) were 0.001 mg/kg on dry weight basis. Recovery values ranged from 79.7 to 136% with a relative standard deviation (RSD) < 10%. No HBCD was found in the blank plant samples.

1.5. PBN adduct extraction and Fenton reaction

Maize roots and shoots were analyzed for the detection of ROS generated after exposure to HBCD for 4 days. After being rinsed with ice-cold 0.1 mol/L $CaCl_2$ solution and blotted with filter paper, plant samples were weighed (0.5 g) and homogenized in a mortar with 1.0 mL freshly prepared solution of 0.1 mol/L PBN (dissolved in DMSO) in an ice bath. Then the homogenates were incubated at 37°C for 15 min, and centrifuged at 4500 r/min at 4°C for 3 min. The supernatant (30 μL) was transferred to a capillary tube with a diameter of 1.0 mm for electron paramagnetic resonance (EPR) analysis. The EPR spectra were recorded with a Bruker ESP 300 spectrometer (Bruker, Germany) at room temperature. The operation conditions were as follows: microwave power, 20 mW; microwave frequency, 9.7 GHz; center field, 347 mT; scan range, 10 mT; modulation frequency, 100 kHz; modulation amplitude, 0.25 mT; and receiver gain of 2×10^4 scans.

1.6. DNA damage measurement

Maize plants exposed to HBCD were rinsed thoroughly with deionized water, blotted with filter paper, and separated into roots and shoots for histone extraction after exposure to HBCD for 4 days. Fresh samples (1.0 g) were homogenized in 9 mL of extraction buffer (0.2 mol/L $NaH_2PO_4 \cdot Na_2HPO_4$, pH 7.4) in an ice bath. Then the homogenates were centrifuged at 4000 r/min at 4°C for 15 min and the supernatant was collected. The H2AX phosphorylation level in histone samples was analyzed with a plant γ-H2AX enzyme-linked immunosorbent assay (ELISA) kit (RB, USA).

1.7. Statistical analysis

All results were expressed as mean ± standard deviation (SD) of triplicates. Statistical analysis was performed by Microsoft Excel 2010, Origin 7.5 and SPSS 13.0 variance analysis software. One-way and Two-way ANOVA with Turkey's multiple comparison tests were used to assess differences among samples at $p < 0.05$.

2. Results and discussion

2.1. HBCD uptake by maize and diastereoisomer contribution

The initial fast uptake caused a sharp increase of HBCD concentration in maize roots and shoots, and the accumulation reached an apparent equilibrium within 96 hr (Fig. 1a). The concentration of HBCD was much higher in maize roots than in

shoots, and the apparent equilibrium concentrations were 3.71 ± 0.12 mg/kg in roots and 0.10 ± 0.01 mg/kg in shoots (on a dry weight basis) after exposure to HBCD at 0.002 mg/L, respectively. The HBCD concentration in both maize roots and shoots increased linearly with increasing concentration of HBCD in the culture solution (Fig. 1b, R^2 = 0.9842 for roots and 0.9923 for shoots, $p < 0.05$), demonstrating that plant uptake of HBCD is concentration-dependent within the tested concentration range.

Diastereomer-specific accumulation of HBCD was further investigated, and their concentrations in both maize roots and shoots were found to vary in the order γ-HBCD > β-HBCD > α-HBCD (Fig. 2). The initial exposure solution had a percentage diastereoisomer contribution of 12.87 ± 0.15, 13.12 ± 0.23 and 74.01 ± 0.35% for α-, β- and γ-HBCDs, respectively. Compared to this, the relative diastereoisomer contribution of β-HBCD increased, and that of γ-HBCD decreased with exposure time in both maize roots and shoots (Fig. 3). The relative contribution of α-HBCD increased in roots and decreased in shoots throughout the experimental period. For example, the diastereoisomer pattern in roots was 14.76, 25.69 and 58.98% for α-, β- and γ-diastereoisomers after the longest exposure time of 120 hr, respectively (Fig. 2a), corresponding to the relative increases of 14.42 ± 0.83 and 96.09 ± 8.86% for α- and β-HBCDs, respectively, and a decrease of 20.30 ± 0.87% in the relative amount of γ-HBCD in roots compared to their initial proportions in the exposure solution (Fig. 3a). In shoots, the proportions of α-, β- and γ-HBCDs were 7.89, 23.68 and 67.60% after exposure for 120 hr (Fig. 3b), which corresponded to a relative decrease of 38.87 ± 1.19 and 8.65 ± 0.67% in the proportions of α- and γ-HBCDs, and a relative increase of 80.76 ± 4.58% for β-HBCD (Fig. 3b). Therefore it can be concluded that the uptake and translocation of HBCD in maize were diastereomer-selective. Similar results were also observed in rainbow trout by Haukås et al. (2009).

The diastereoisomer contribution of HBCD was detected in many environmental samples. α-HBCD was reported to be the major diastereoisomer in the tissues of organisms such as fish, birds and marine mammals (Zegers et al., 2005; Janák et al., 2005; Morris et al., 2004), whereas γ-HBCD was found the dominant diastereoisomer in abiotic environmental samples such as soil, sediment, air and water (Marvin et al., 2006; Covaci et al., 2006). A similar result was determined in the present study, with higher γ-HBCD concentrations in both maize roots and shoots compared with α- and β-HBCDs. The time-dependent accumulation represents a balance between the uptake and elimination processes of HBCD. The highest percentage of relative accumulation for β-HBCD indicates that this diastereoisomer is more easily taken up by maize than α- and γ-HBCDs.

2.2. Effect of HBCD on maize development

The effect of technical HBCD on maize growth was determined after exposure for 4 days. The relative inhibition rates of seed

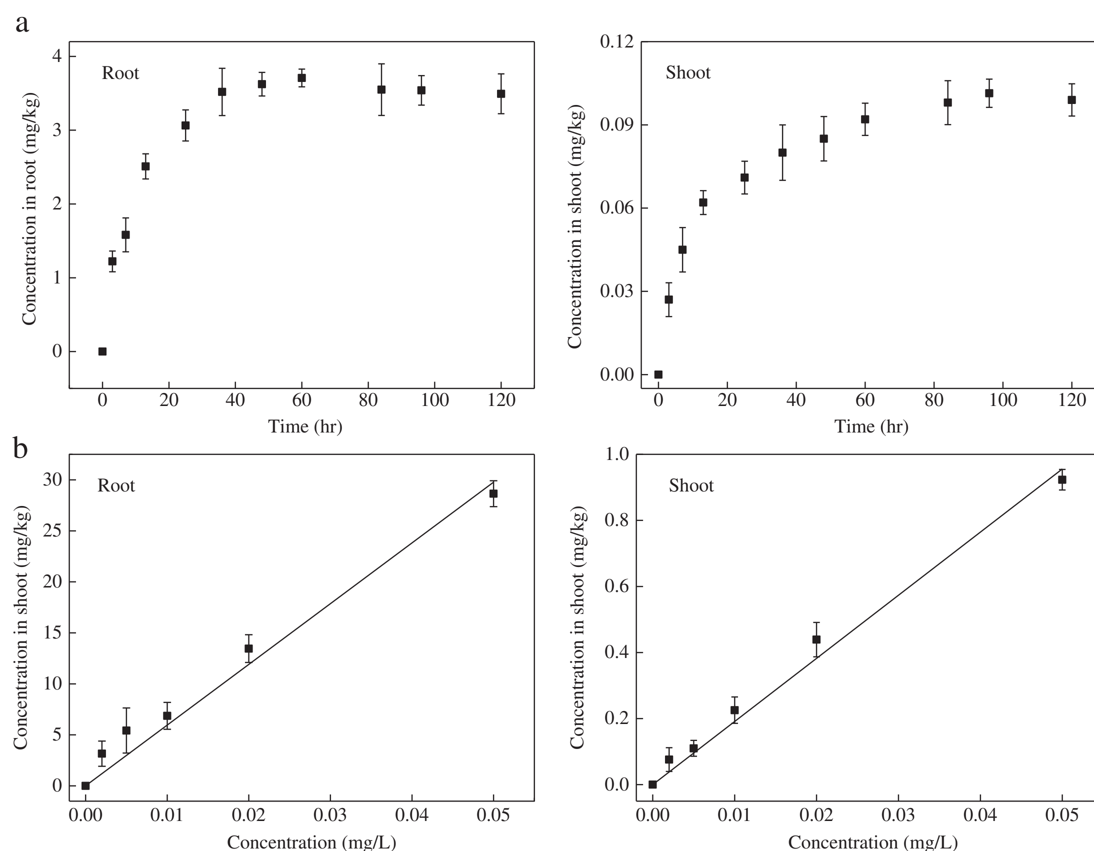

Fig. 1 – Time- and concentration-dependent accumulations (a and b) of HBCD in maize roots and shoots (on a dry weight basis). Each value is the mean ± SE of three replicate cultures.

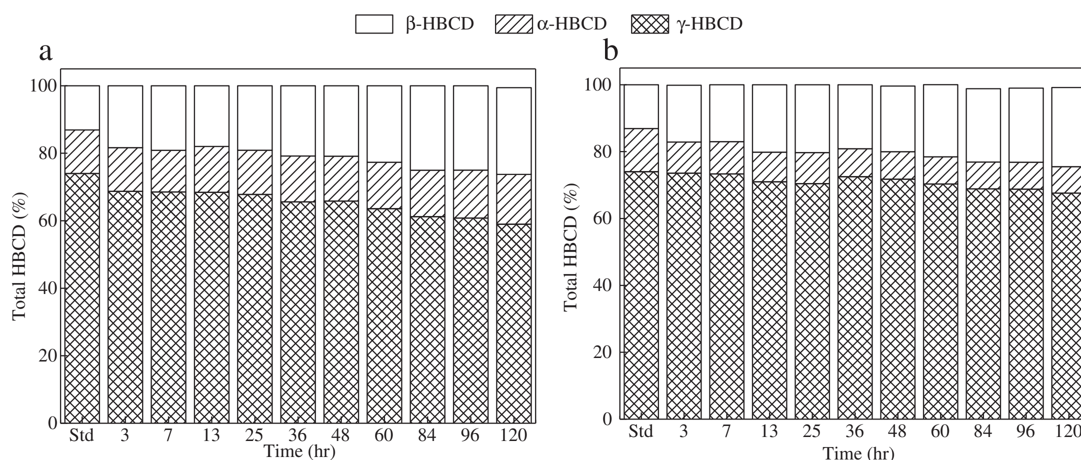

Fig. 2 – Time-dependent change of HBCD diastereomeric distribution in maize roots (a) and shoots (b). Std represents the proportion of α-, β- and γ-HBCDs in the technical standard.

germination, root and shoot biomass, and root and shoot elongation increased with increasing exposure concentration of HBCD (Table 1). The relative inhibition rates changed more noticeably at lower exposure concentrations compared with higher exposure concentrations. The toxic effects of HBCD to maize followed the order: germination rate > root biomass ≥ root elongation > shoot biomass ≥ shoot elongation. At the maximum exposure concentration of 0.05 mg/L, the inhibition rates reached 46.54, 32.71, 31.94, 14.04 and 11.97% for germination rate, root biomass, root elongation, shoot biomass and shoot elongation, respectively. Seed germination was more sensitive to HBCD than seedling growth. Seed germination relies almost exclusively on seed reserves for the supply of respiration metabolites as well as other anabolic reactions. Starch is quantitatively the most abundant storage in seeds and available evidence has indicated that seed starch is degraded during germination *via* the amylolytic pathway (Juliano and Varner, 1969). Therefore, HBCD posed a severe inhibitory effect on seed germination most probably because it damaged the starch or depressed amylase activity.

2.3. Hydroxyl radical generation in maize

Oxidative stress, resulting from the imbalance between endogenous generation of ROS and antioxidant defense systems, is an important phenomenon in plants when exposed to contaminants (Bowler et al., 1992). Endogenous ROS produced in plants have extremely short half-lives and are present in low concentrations, thereby making detection difficult. In the present study, spin trapping followed by EPR analysis was employed to directly observe the formation of free radicals in maize exposed to HBCD. Six-line (triplet of two lines in each set) EPR spectra of ROS and PBN adducts were observed in maize tissues after exposure to HBCD (Fig. 4a, taking the concentration of 0.01 mg/L as an example). The variations of signal intensity of the PBN-radical adducts in the EPR spectra reflected the generation of hydroxyl radical (·OH), and the ·OH intensity was calculated by the signal intensity of the second couple of the triplet, according to our previous study (Wu et al., 2012). Weak ·OH signals were found in the control without HBCD exposure (Fig. 4a), indicating ·OH generation during normal cellular functions (Yin et al., 2007).

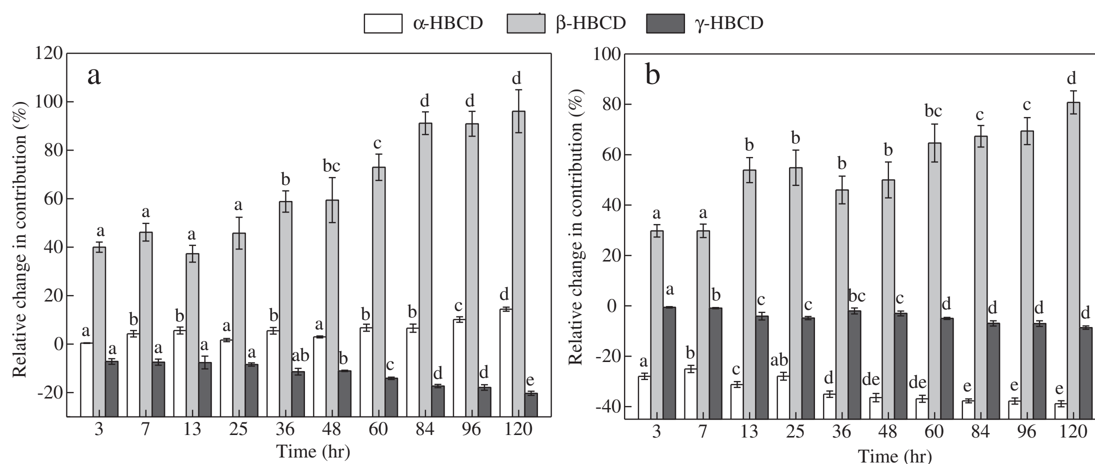

Fig. 3 – Time-dependent change (mean ± SE) of HBCD diastereoisomer contributions in maize roots (a) and shoots (b) compared with their proportions in the initial exposure solution. Different letters represent statistically significant difference between times at $p < 0.05$.

Table 1 – Relative inhibition rates (IRs) of germination (GER), root biomass (RB), shoot biomass (SB), root elongation (RE) and shoot elongation (SE) of maize exposed to technical HBCD for 4 days.

IR (%)	Concentration (mg/L)					
	0	0.002	0.005	0.01	0.02	0.05
GER	0.00 ± 3.33a	10.58 ± 4.08b	17.31 ± 3.33c	21.15 ± 3.33c	36.54 ± 5.77d	46.54 ± 7.31e
RB	0.00 ± 1.72a	6.27 ± 0.69b	9.76 ± 3.58b	18.42 ± 3.35c	24.36 ± 2.86d	32.71 ± 2.99e
SB	0.00 ± 0.66a	5.25 ± 0.90b	6.62 ± 1.37b	8.30 ± 1.93bc	11.95 ± 1.14 cd	14.04 ± 2.29d
RE	0.00 ± 2.83a	6.84 ± 3.13b	10.63 ± 2.14b	18.18 ± 2.24c	22.64 ± 2.64c	31.94 ± 3.90d
SE	0.00 ± 1.31a	4.22 ± 1.59ab	5.78 ± 1.16b	7.51 ± 0.58bc	10.60 ± 1.34c	11.97 ± 0.85c

Different letters represent statistically significant difference at $p < 0.05$.

The ·OH level in both maize roots and shoots was significantly increased ($p < 0.05$) at HBCD exposure concentrations lower than 0.01 mg/L and then decreased with increasing concentration (Fig. 4b). The highest ·OH intensity increase was 19.5% in roots and 25.7% in shoots at the concentration of 0.01 mg/L, compared with the levels of the controls. More ·OH was detected in maize shoots than in roots. ·OH is the most reactive species among the ROS and will immediately react with most cellular macromolecules after generation, including proteins, lipids and DNA. The production and accumulation of ROS can lead to oxidative stress. It was reported that the catalase activity significantly increased in rainbow trout after exposure to HBCD (Ronisz et al., 2004). The antioxidative enzyme catalase plays an important role in scavenging ROS generated in plants. It has been reported in a previous study that the activities of catalase, peroxidase and superoxide dismutase in wheat seedlings were enhanced significantly after HBCD exposure (Li, 2010). The elevated ·OH levels detected in this study further provided direct evidence for ROS generation and oxidative stress in maize induced by HBCD exposure. Oxidative stress is associated with numerous deleterious consequences for cells, such as lipid peroxidation and cell death (Mittler et al., 2004). Excessive production of ROS is one of the important mechanisms for plant cell damage under adverse environmental conditions (Mancini et al., 2006). Therefore, the decrease of ·OH level at higher exposure concentrations was mainly attributed to the death of plant cells, which inactivated the production and scavenging of ROS. These data are important for the risk assessment of HBCD in plants.

2.4. DNA damage responses and potential toxicity mechanism

The level of phosphorylated histone H2AX was examined to investigate the genotoxic effects of HBCD on maize. The γ-H2AX gene was identified in maize without HBCD exposure and constitutively expressed in all plant tissues (Fig. 5). The level of γ-H2AX foci was significantly elevated ($p < 0.05$) when the exposure concentration of HBCD was higher than 0.005 mg/L for roots and 0.01 mg/L for shoots, respectively. The highest γ-H2AX levels in maize roots and shoots (1.77 ± 0.54 ng/g in roots and 1.54 ± 0.29 ng/g in shoots on fresh weight basis) were observed at the exposure concentration of 0.05 mg/L, which corresponded to a relative increase of 23.10 and 16.61% in γ-H2AX level for roots and shoots compared to the controls. Several studies have already described the relevance of H2AX in the DSB responses in human and other mammalian cells (Rothkamm and Löbrich, 2003; Celeste et al., 2003); however limited analyses have so far been performed on plants. Lang et al. (2012) first characterized the expression of H2AXa and H2AXb in *Arabidopsis* and noticed the increase of γ-H2AX protein after treatment with the DSB-inducer drug bleomycin. It was demonstrated that each γ-H2AX focus was approximately equivalent to one DNA DSB, and the formation and loss of foci reflected the DSB induction and repair (Rothkamm and Löbrich, 2003). Therefore, the increase in the

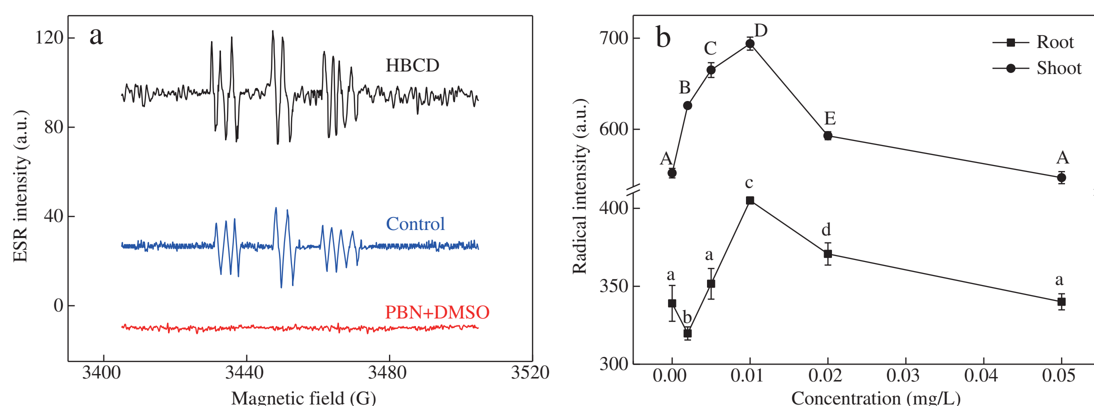

Fig. 4 – EPR spectrum (a) and radical intensity (b) of PBN-ROS-adducts determined in maize after exposure to HBCD for 4 days. Different letters represent statistically significant difference at $p < 0.05$ (the lower-case letters and the upper-case letters indicate differences in maize roots and shoots, respectively).

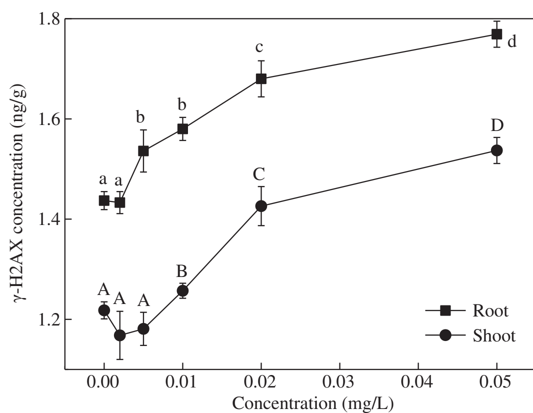

Fig. 5 – γ-H2AX induction in maize after exposure to HBCD (on a fresh weight basis). Different letters represent statistically significant difference at $p < 0.05$ (the lower-case letters and the upper-case letters indicate differences in maize roots and shoots, respectively).

γ-H2AX level in the present study suggested that DNA damage became more serious with increasing exposure concentration of HBCD.

ROS is thought to be a major cause of DNA damage (Vanderauwera et al., 2011). To further confirm whether the DNA damage in maize was induced by ·OH generation, sodium benzoate, an ·OH scavenger (Chang et al., 2005), was added to the exposure solution. Sodium benzoate significantly reduced the ·OH generation and inhibited the expression of γ-H2AX foci ($p < 0.05$) in both roots and shoots of maize exposed to 0.02 mg/L HBCD (Table 2), indicative of the involvement of ·OH generation in the HBCD-induced DNA damage. It has been reported that the highly reactive ·OH can react with DNA and cause genetic mutations (Cooke et al., 2003). Mancini et al. (2006) detected DNA damage in leaf cells of *Nicotiana tabacum* with the increase of DNA migration value and hedgehog percentage induced by hydrogen peroxide. The results of the present study indicated that ·OH generation could induce the formation of γ-H2AX foci during the DNA DSB responses in maize after exposure to HBCD. However, the decrease of γ-H2AX foci did not result in its return to the control level. By comparing the results shown in Figs. 4b and 5, we can also notice the co-occurrence of the reduction of ·OH intensity and the continual increase in the γ-H2AX level when the exposure concentration of HBCD is higher than 0.01 mg/L. Therefore, it is expected that besides ·OH generation there should be other mechanisms responsible for DNA damage in maize after exposure to HBCD.

3. Conclusions

This study demonstrates the uptake and accumulation of HBCD in maize roots and shoots, which generates phytotoxic effects on maize. HBCD accumulation in maize roots and shoots is concentration-dependent and diastereomer-specific, with γ-HBCD being more likely to be bioaccumulated than α- and β-HBCDs. Exposure to HBCD influences the early development of maize, with germination being the most sensitive. The induction of ·OH and γ-H2AX with HBCD exposure indicates the generation of oxidative stress and damage from DNA double-strand breaks in maize. The ·OH scavenger benzoate inhibits the expression of γ-H2AX foci in both maize roots and shoots, indicative of the involvement of ·OH generation in the HBCD-induced DNA damage. Given the present indications of plant accumulation and phytotoxicity for the technical HBCD mixture combined with the diastereomer-specific contribution, there is a need to elucidate the environmental fate and behavior of the diastereoisomers in technical HBCD.

Table 2 – Relative inhibition levels of ·OH and γ-H2AX compared to the controls at the exposure concentration of 0.02 mg/L after the addition of ROS scavengers.

Treatment	·OH relative intensity		γ-H2AX relative concentration	
	Roots	Shoots	Roots	Shoots
Control	1.00 ± 0.01a	1.00 ± 0.03a	1.00 ± 0.02a	1.00 ± 0.01a
Control + sb	0.99 ± 0.03a	0.98 ± 0.04a	0.98 ± 0.02a	0.97 ± 0.02a
HBCD	1.12 ± 0.03a	1.09 ± 0.02a	1.18 ± 0.03a	1.10 ± 0.03a
HBCD + sb	1.04 ± 0.04b	0.97 ± 0.05b	1.09 ± 0.03b	1.05 ± 0.01b

Different letters represent statistically significant difference between HBCD treatment and HBCD + sodium benzoate (sb) treatment at $p < 0.05$.

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