Bioaccumulation of hexachlorobutadiene in pumpkin seedlings after waterborne exposure†

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Hexachlorobutadiene (HCBD) has been listed as a persistent organic pollutant (POP) in the Stockholm Convention, and is now drawing more research interest. However, the understanding of its bioaccumulation, especially in plants, is still very limited. In this work, the behavior of HCBD in aqueous solution and pumpkin seedlings was studied through in-lab hydroponic exposure experiments. It was found that 69% of HCBD volatilized from water to the atmosphere after one day of exposure, and only 1% remained in the solution after four days. This high volatility might be the main cause of the low HCBD levels in aqueous environments. Although a great amount of HCBD volatilized into the atmosphere, only a small proportion of airborne HCBD was captured by the leaves and stems of the blank pumpkin seedling controls. The translocation of HCBD from the leaves to the bottom roots, as well as its release from the roots into the water, was detected. For the exposure groups, the pumpkin seedlings absorbed HCBD from both the hydroponic solution and the air via the roots and leaves, respectively. The concentration of HCBD in the exposed pumpkin roots linearly increased with the continuous addition of HCBD into the exposure system. Upward translocation from the roots to the leaves and downward translocation from the leaves to the roots existed simultaneously in the exposed pumpkin seedlings. However, the concentrations of HCBD in the leaves, stems and roots in the exposure group were much higher than those of the blank plant controls, suggesting little contribution from the airborne HCBD in the hydroponically exposed pumpkin seedlings. The lipid content did not show obvious effects on the bioaccumulation and biodistribution of HCBD in the pumpkin seedlings, indicating that the translocation of HCBD within the pumpkin seedlings might be an active process. This study provided new findings on the environmental behavior of HCBD, which will be helpful for understanding the exposure risks.

Environmental significance

Although the production and usage of HCBD in many countries has already been banned, its unintentional formation and emission during chemical production, waste disposal and some other processes are still continuing. The understanding of its bioaccumulation, especially in plants, is still limited. Here, in-lab hydroponic exposure experiments based on pumpkin seedlings revealed that the high volatility of HCBD greatly influenced its bioaccumulation. HCBD tended to evaporate from aqueous environments even when organic matter (pumpkin roots) was present in the water. Bioaccumulation and translocation of HCBD were observed in pumpkin seedlings upon waterborne exposure with continuous renewal of the exposure solutions. Pumpkin leaves could absorb airborne HCBD, and transfer it from the shoots to the roots. The findings of this study have provided empirical evidence of the bioaccumulation and biomagnification potential of HCBD, and are helpful in the evaluation of its environmental behavior and exposure risks.

1. Introduction

As a byproduct of chlorinated hydrocarbon manufacture, hexachlorobutadiene (HCBD) has been extensively used in various fields, for example, as an anti-fungal agent, as a solvent for elastomers, as a heat-transfer fluid in transformers, and so on.1,2 Though the production and usage of HCBD in many countries has already been banned, its unintentional formation and emission during chemical production, waste disposal and some other processes are still continuing. According to previous toxicological studies, HCBD can cause nephrotoxicity in rats,
especially impairing the renal proximal tubule.4–5 Hazardous effects were also reported in workers who regularly used HCBD in vineyards.6 Due to its persistence, bioaccumulation, long-range transport and high toxicity, HCBD was regarded as a candidate persistent organic pollutant (POP) in 2011,7,8 and was listed in Annex A of the Stockholm Convention as an emerging POP in 2015.9

Contamination and accumulation of HCBD has been extensively studied in various environmental samples, including air,10 soil, sewage sludge, and biota.11–14 For example, HCBD was not detectable in fish from the Rhone River in France,14 and less than 0.2 ng g⁻¹ was found in fish from four English rivers,13 indicating the low HCBD levels in natural water and aquatic biota. An investigation on animal (1.65–3.80 ng g⁻¹ lipid weight, lw), soil (<0.02–5.59 ng g⁻¹ dry weight, dw) and plant samples (0.03–24.6 ng g⁻¹ dw) from the ambient environment of a factory formerly producing the pesticide in eastern China showed that HCBD existed at relatively high levels.15 The bioaccumulation factors (BAFs) of HCBD in plants were in the range of 8.5 to 38.1. Nevertheless, a bioaccumulation modeling study suggested that the biomagnification factor of HCBD was less than 1 in all model organisms.16 The controversial findings on the bioaccumulation and biomagnification potential of HCBD still remain unresolved.

Plants have been demonstrated to accumulate persistent halogenated organic pollutants, such as PBDEs and PCBs,16–19 and play roles in the environmental fates of organic compounds.20–24 Considering the limited information on HCBD bioaccumulation in plants so far, related studies to reveal the fate of HCBD in the environment and evaluate the potential health risks are urgently required. Pumpkin seedlings have been successfully applied in studies of the environmental behavior of many organic compounds.16,18,19 Their peculiarly high abilities to take up, transfer and metabolize various POPs demonstrated this species to be a very promising experimental model. In this study, in-lab hydroponic exposure experiments were performed to reveal the bioaccumulation behavior of HCBD in pumpkin seedlings, and explore its transportation within the hydroponic exposure system.

2. Materials and methods

2.1 Chemicals and reagents

HCBD (2 mg mL⁻¹, CAS no. 87-68-3) was purchased from AccuStandard (New Haven, USA).¹³C-HCBD (100 µg mL⁻¹) in isoctane (Cambridge Isotope Laboratories, Andover, MA) was used as a surrogate standard. The working solutions of HCBD for the exposure experiments (100 µg mL⁻¹) and the calibration curves (1–1000 ng mL⁻¹) were prepared in methanol and hexane, respectively.

Pesticide grade hexane and methylene dichloride and HPLC grade methanol were purchased from J. T. Baker (Philipsburg, USA). Petroleum ether (30–60 °C, AR) was obtained from Beijing Chemical Works (Beijing, China). Anhydrous sodium sulfate purchased from Sinopharm Chemical Reagent (Beijing, China) was heated at 600 °C for 6 h before use. Bio-Beads S-X Beads gel permeation chromatography (GPC) columns were supplied by BIO-RAD Company (Hercules, USA). ENVITM-carb SPE columns (Supelclean™ 0.5 g, 6 mL) were purchased from SUPELCO (Bellefonte, USA). All of the water used in this study was generated by a Milli-Q Advantage A10 system (Billerica, USA).

2.2 Hydroponic exposure

Pumpkin seeds (Cucurbita maxima × C. moschata) were purchased from Taigu Yinong Seed Co., Ltd., Shanxi province, China. The seeds were germinated in a tray covered with wet sterile gauze at a temperature of around 30 °C, and then transferred to sterile perlite beds. The seedlings were cultivated in an illumination growth chamber, which was set at 25 °C for 14 h of light time with a light intensity of 2400 lx and 22 °C for 10 h of dark time. After growing to about 4–6 cm in height, the seedlings were transferred into 50 mL glass reactors and cultivated in sterile water for two days. Then hydroponic exposure was conducted.

A schematic illustration of the exposure experiment is shown in Fig. 1. Each reactor contained 45 mL of sterile deionized water, 4500 ng of HCBD (in 45 µL of methanol) and three pumpkin seedlings. A hole plug made from polytetrafluoroethylene (PTFE) was fitted in the mouth of the reactor after the pumpkin seedlings were placed into the reactor. Then the plug hole and the reactor mouth were sealed with paraffin film immediately to avoid the loss of HCBD through volatilization. The reactor was subsequently wrapped with aluminum foil to keep the roots growing in darkness and prevent the possible photolysis of HCBD. Because of the high volatility of HCBD, the hydroponic solution was renewed daily. Namely, all of the hydroponic solutions were sampled with a syringe through the lower outlet on the reactor every 24 hours for chemical analysis. Another 45 mL of sterile deionized water and 4500 ng of HCBD were injected into the reactor through the upper outlet and the solution was gently mixed. To evaluate the HCBD loss due to its volatilization during exposure, an unplanted control was set by adding 45 mL of deionized water containing 4500 ng of HCBD in the reactor with three glass rods (diameter 3 mm) inserted inside to replace the plants. The solution in the unplanted control was not renewed during the whole experiment. The concentration variation of HCBD in the unplanted solution was monitored daily to evaluate the volatilization loss of the chemical. The blank plant control, in which three pumpkin seedlings and HCBD-free hydroponic solution were used, was set simultaneously to monitor the potential cross-contamination between different reactors caused by the volatilization of HCBD. The hydroponic solution of the blank plant control was replenished daily with sterile deionized water to 45 mL. To determine the transfer of airborne HCBD from air to the solution, a blank water control was set, in which only 45 mL of deionized water and three glass rods were added. No HCBD or pumpkin seedlings were used in the blank water control. The unplanted control, blank plant control and blank water control were all covered with paraffin film and aluminum foil in a similar way to the exposure reactors.

To avoid biodegradation caused by exogenous microorganisms, all the glass vials, rods, plugs, and hydroponic solutions...
were autoclaved before exposure. There were three parallel reactors for each group (exposure group, unplanted controls, blank plant controls and blank water controls) at each sampling time. All the reactors were placed together in a big illumination exposure chamber, which controlled the same growth conditions as the cultivation chamber. The exposure experiment lasted for 15 days.

2.3 Sampling
The pumpkin of an exposure group (3 parallel reactors) and a blank plant control group (3 parallel reactors) were sampled for HCBD analysis on day 1, 2, 3, 4, 5, 7, 11, and 15. The sampling was carried out before the solutions were refreshed to avoid the contamination of the pumpkin tissues with the newly prepared HCBD solution. The roots, stems and leaves of the pumpkin seedlings (Fig. 1) were sampled. Root samples were washed with pure water and gently dried with Kimwipes (Kimberly-Clark, Roswell, GA, USA). The root, stem and leaf samples were stored at −20 °C for 24 h, then freeze-dried to constant weight, and finally stored at −20 °C before extraction. The exposure solutions were sampled and extracted immediately for chemical determination.

2.4 Lipid content
The lipid contents of the pumpkin tissues, including the roots, stems and leaves, were determined. Pumpkin tissue samples (0.6–1.0 g, dw) were mixed with 6 g of diatomaceous earth, and then extracted with petroleum ether (30–60 °C) (flush volume 60%) at 100 °C and 1500 psi with three static extraction cycles (10 min) by ASE. The extract was evaporated to dryness by rotary evaporation and nitrogen gas stream. The residue of the extract was weighed. The lipid content was the ratio of the weight of the extracted residue to that of the pumpkin tissue.

2.5 Sample pretreatment for the analysis of HCBD and its potential metabolites
The samples were processed via extraction and purification for the analysis of HCBD and its potential dechlorinated metabolites. In brief, the water solutions were extracted under vigorous shaking with 15 mL of methylene dichloride three times after 13C-HCBD was added as a surrogate standard. The combined organic phase was dried with 6 g of anhydrous sodium sulfate. After the addition of 15 mL of hexane, the extracts were concentrated to 1 mL for the exposure groups and unplanted controls, and to 100 μL for the blank controls, and the final samples were subjected to instrumental analysis.

The freeze-dried plant samples were cut into pieces (<2 mm in length) by scissors, spiked with surrogate standard, and ultrasonically extracted with 15 mL of a mixture of hexane and dichloromethane (1 : 1 v/v) three times (15 min, each time). The crude extracts were combined and concentrated to 1–2 mL for further purification.

The extracts of the plant samples were loaded on a GPC column. The column was eluted using a mixture of hexane and methylene dichloride (2 : 1, v/v). The first 70 mL of the elutes containing impurities was discarded. The subsequent 100 mL of effluent containing the target compound was collected and concentrated to 1–2 mL. The GPC column was refreshed with 50 mL of hexane and methylene dichloride (2 : 1, v/v) between samples. SPE was chosen for further clean-up. The ENVI™-carb SPE columns were preconditioned with 10 mL of methylene dichloride and 10 mL of hexane in sequence. After loading the concentrated extracts, HCBD was eluted with 15 mL of hexane.
The organic phase was concentrated and submitted to GC-MS analysis.

2.6 Instrumental analysis

An Agilent 6890 gas chromatograph (GC) coupled with a 5973 N mass spectrometer (MS) detector (Agilent Technologies, Palo Alto, CA) was used for the analysis of HCBD and its possible dechlorinated products. Samples were injected (2 μL) by a 7683 Series Injector into a DB-5MS column (30 m × 0.25 mm × 0.25 μm J&W Scientific, Folsom, CA) in splitless mode (250 °C). Helium was the carrier gas at a constant flow of 1.0 mL min⁻¹. The oven program started at 50 °C (held for 1 min), and increased to 100 °C at 10 °C min⁻¹, and then to 120 °C at 5 °C min⁻¹. The post run was set at 300 °C and held for 2 min. Quantitative determination by GC-MS (electron ionization, EI) was performed in the optimized selected ion monitoring (SIM) mode. The selected ions for the target compounds (m/z), including HCBD and its potential dechlorinated metabolites, were shown in Table SI-1.†

2.7 Quality assurance/quality control

A calibration curve was established daily. One of the standards in the calibration curve was injected after the analysis of every three samples to control the stability of the instrument and correct the calibration curve. Hexane was injected after the measurement of every five samples, and the results ensured no memory effect of the instrument. The method detection limit (MDL) was defined as the lowest concentration of HCBD with a signal-to-noise ratio (S/N) of 3. The MDLs for HCBD in different pumpkin tissues were in the range of 0.12–0.14 ng g⁻¹. The recoveries of 13C-HCBD for solutions, leaves, stems and roots were in the ranges of 52–74%, 63–112%, 68–113% and 90–124%, respectively. All the data were corrected according to the recoveries of 13C-HCBD.

2.8 Statistical analysis

Microsoft Excel and OriginPro 8.5.1 were used for data analysis. The concentration variations of HCBD over time were evaluated using linear and exponential regression models. The confidence level was 95%. An ANOVA test was performed for the regression models. Determination coefficients (R²) and p values were used to evaluate the regression models.

3. Results and discussion

3.1 The volatility of HCBD influenced its bioaccumulation

In our preliminary experiment, the exposure group were spiked with HCBD at the beginning and the solutions were not renewed during the exposure. After 10 days, the mass distributions of HCBD in the exposure systems were as shown in Table SI-2.† Only 1.2% of the initial amount of HCBD was recovered in the end. Though pumpkin roots had affinity for organic compounds in the solution, there was a big gap between the initial mass added in the exposure system and the final mass recovered. This difference could be contributed by the high volatility of HCBD.

In order to accurately evaluate the volatilization of HCBD, unplanted controls were set, and the temporal variation of the HCBD concentration in the water phase was monitored. The results showed that 3111 ± 233 ng of HCBD volatilized from the water phase to the air after 1 day, which was 69% of the initial amount added to the solution. The amount of aquatic HCBD continued to decrease sharply to a very low level, and only 1.1% of the initial amount of HCBD remained in the water phase on day 4 (Fig. 2).

According to the physicochemical properties of HCBD, it has a high volatility, a relatively high Henry’s law constant (1044 Pa m³ mol⁻¹) and a high log Kow (4.78). Therefore, most of the HCBD added to the aqueous system evaporated from the hydroponic solution to the air phase, which complicated the bioaccumulation studies based on waterborne exposure of this chemical. The exposure strategy was accordingly adjusted, and the exposure solutions were renewed daily in the following 15 day exposure experiments.

The findings on the low levels of HCBD detected in the water and pumpkin roots were consistent with what has been observed in natural rivers and some aquatic organisms. The high volatility may explain the fact that HCBD is not bioaccumulative, which has been concluded by field and modeling studies.

3.2 HCBD translocation in pumpkin seedlings due to airborne exposure

Blank plant and blank water controls were set to evaluate the possible airborne HCBD exposure which could be contributed by the other exposure group. No detectable HCBD was found in the solution of the blank water controls, indicating that the direct transfer of airborne HCBD from the air to the water was negligible. However, HCBD was detected in all samples from the blank plant groups, and the concentrations in the leaves, stems,
roots and solutions were in the ranges of 3.0–6.6, 5.1–15, and 7.0–15 ng g\(^{-1}\) dw, and 0.026–0.055 ng mL\(^{-1}\), respectively (Fig. 3 and Table SI-3\(^{†}\)). The existence of HCBD in the blank plant controls showed that the pumpkin seedlings absorbed airborne HCBD through the leaves and stems, and transferred the chemical downwards to the roots and even released it from the roots to the solutions. Compared with the total amounts of HCBD volatilized into the air phase, only a very small proportion of HCBD was absorbed by the pumpkin tissues (0.05% on day 15). This result was consistent with previous findings that a minor contribution to pumpkin contamination was detected for airborne polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/PCDF).\(^{19}\)

The time courses for the absorption of airborne HCBD by the leaves and stems and its translocation from the leaves to the roots was determined. The regression equations indicated that the adsorption/absorption of HCBD by the stems and leaves sharply increased on the first day of exposure and finally reached a plateau (Fig. 3a and b). This result indicated that HCBD was adsorbed by the leaf and stem surfaces, and diffused through the cuticle into the pumpkin seedlings very quickly. Meanwhile, the desorption\(^{18,29}\) and the translocation of HCBD to the roots caused the subsequent balance in its concentration in the leaves and stems. The HCBD concentration in the roots (Fig. 3c) increased rapidly on the first day, and exhibited a slowly increasing trend thereafter, illustrating the rapid and sustained downwards translocation of HCBD in the pumpkin seedlings. The concentration variation of HCBD in hydroponic solution showed a similar trend to those in the pumpkin tissues (Fig. 3d). Due to the fact that no measurable HCBD was found to directly transfer from air to water, the chemical detected in the solution of the blank plant control originated from root release. The continuous release of HCBD from the roots to the solution and the subsequent volatilization from the solution to the air finally led to the stable concentration of HCBD in the solution.

3.3 Uptake from water and upward translocation

Fig. 4 and Table SI-4\(^{†}\) showed the HCBD concentrations in the tissues of pumpkin seedlings collected from exposure groups at different time points. The HCBD content in the roots increased from 10 453 ± 431 ng g\(^{-1}\) dw (day 1) to 116 287 ± 17 416 ng g\(^{-1}\) dw (day 15) and was in accordance with a linear regression (Fig. 4a). The increasing trend in root HCBD concentration continued when the experiment was terminated. This was mainly caused by the continuous input of HCBD during daily solution refreshing. The root accumulation factor (RAF, \(C_{\text{root}}/C_{\text{solution}}\)) was measured to evaluate the accumulation of HCBD by the plant roots under the disequilibrium condition

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**Fig. 3** HCBD concentration (ng g\(^{-1}\), ng mL\(^{-1}\)) variations in the leaves (a), stems (b), roots (c) and solutions (d) of the blank plant controls during the exposure. The dotted lines and the formulas show the exponential regressions for the HCBD concentration trends. X stands for the time (days) and Y stands for the concentration (ng g\(^{-1}\), ng mL\(^{-1}\)); \(R^2\) is the determination coefficients.
between the roots and solution. In this work, RAFs were the ratios of HCBD concentration associated with the roots to those in the solution when sampling. log RAF (on dw basis) was increased from 3.2 to 3.7 during the whole exposure period.

The experiment conditions were similar to some extent to actual environment with continuous input of HCBD, for example, the area around chemical plants. The high log RAF observed herein showed that HCBD had high bioaccumulation potential in plant roots. Though the RAF in real soil-plant environments would be lower than that detected in this work due to the lower bioavailability of HCBD in soil than in a hydroponic system, the phenomenon was consistent with the previous finding that plants, like carrots, lettuces, rice and pumpkin, can accumulate relatively high concentrations of HCBD even when its concentration in the soil was low.11

After the pumpkin roots absorbed the organic compound from water, the compound could be translocated to the above-ground parts.16,18,19 Fig. 4b and c show the variations of the HCBD concentration in the stems and leaves. The concentration of HCBD in the stems increased from 329 ± 398 ng g⁻¹ dw to 1364 ± 461 ng g⁻¹ dw and was in accordance with linear regression during exposure, which was similar to that in the roots. The linear increase of HCBD concentration in the stems indicated that the process of translocation from the roots to the stems was more predominant than the translocation process from the stems to the leaves. The concentrations in the leaves ranged from 20 ± 3 to 59 ± 9 ng g⁻¹ dw, which fit with exponential regression during exposure. Compared to those in the blank leaf samples, the HCBD concentrations in the leaves of the exposure group were significantly higher (p < 0.05). The concentrations of HCBD in the leaves, stems and roots in the exposure group were 3.0–18, 27–254, 1483–8684 times higher than that of the blank controls, respectively. This suggested that the absorption of airborne HCBD made little contribution to the distribution of HCBD in the exposed pumpkin. Thus, HCBD translocated from the roots was the main source of the chemical in the leaves of the exposure group. Its translocation from the stems and the elimination process (possible metabolism) all contributed to the distribution and variation of HCBD in the pumpkin leaves, resulting in the chemical levelling off in the leaves. A study on the possible metabolism of HCBD in pumpkin was necessary.

### 3.4 Mass balance and possible metabolism

The mass distribution of HCBD in different compartments of the exposure systems on day 15 is shown in Table SI-5.† The loss of HCBD volatilized from the exposure reactors was calculated based on the HCBD left in the unplanted control solutions after one day of exposure. The volatilized HCBD accounted for 69% of the total amount of the chemical added into the exposure systems. Only 5% of HCBD was accumulated in the pumpkin plants. The total recovery of HCBD during the whole exposure period was 93 ± 3%. About 7% of HCBD was unrecovered. The results of the HCBD mass balance further suggested the possibility of its bio-transformation in plants.

To understand the behavior of HCBD in pumpkin, its potential metabolism was evaluated. Dehalogenation is a common reaction for halogenated organic compounds occurring in various organisms. Pumpkin and poplar plants were found to be responsible for the dehalogenation of PCBs, PBDEs and SCCPs.24–30 The dechlorination products of HCBD were also detected in both environmental media and laboratory
compared with other processes. Otherwise, the in...osempower to be translocated from the leaves to the roots...spond to several reasons. (a) There was no dechlorination metabolism after HCBD entered...e the leaves down to the roots could both occur within the pumpkin. The HCBD concentrations associated with...nvironmental fate and risk assessments.

Conflicts of interest
There are no conflicts to declare.

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