Environmental Pollution 226 (2017) 174–181

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

PBDEs (polybrominated diphenyl ethers) pose a risk to captive giant pandas☆

Yi-ping Chen Dr [a, b, *], Ying-juan Zheng [a], Qiang Liu [a], Aaron M. Ellison [c], Yan Zhao [a], Qing-yi Ma [d]

[a] SKLLQG (State Key Laboratory of Loess and Quaternary Geology), Institute of Earth Environment, CAS, Xi'an 710075, China
[b] College of Life Science, Northwest Normal University, Lanzhou 730000, China
[c] Harvard University, Harvard Forest, Petersham, MA 01368, USA
[d] Shaanxi Wild Animal Research Center, Zhouzhi, Xi'an 710402, China

ARTICLE INFO

Article history:
Received 5 September 2016
Received in revised form
9 April 2017
Accepted 9 April 2017

Keywords:
PBDEs
Captive panda
Feces and blood
Food
Health risk

ABSTRACT

The Qinling subspecies of giant panda (*Ailuropoda melanoleuca qinlingensis*), is highly endangered; fewer than 350 individuals still inhabit Qinling Mountains. Previous research revealed captive pandas were exposed to bromine, so we hypothesized that captive pandas were exposed to and affected by polybrominated diphenyl ethers (PBDEs). To test this hypothesis, we tested blood and feces of captive and wild pandas, their drinking water, food (bamboo leaves) from SWARC (Shaanxi Wild Animal Research Center)and FNNR (Foping National Nature Reserve) and supplemental feedstuff given to captive panda at SWARC. We found 13 congeners of PBDEs in fecal samples, of which BDE47, BDE66, BDE71, BDE99, and BDE154 were the dominant, total PBDE concentration in feces of captive pandas was 255% higher than in wild pandas. We found nine PBDEs congeners in blood samples: BDE153 and BDE183 were the predominant congers. PBDEs in blood from captive pandas were significantly higher than in wild pandas. The total concentration of PBDEs were 5473 and 4835 (pg.g) in *Fargesia qinlingensis*, were 2192 and 1414 (pg.g) in *Bashannia fargesii* (2192, 1414 pg g), 0.066, 0.038 (pg/ml) in drinking water, and 28.8 (pg.g) in supplemental feedstuff for captive and wild pandas, which indicate that the PBDEs came from its bamboo feed, especially from *Bashannia fargesii*. Our results demonstrate that BDE99 and BDE47 could be threatening the pandas' health especially for captive panda and there are potential health risks from PBDEs for pandas. In the short term, this risk may be ameliorated by strict control of food quality. In the long term, however, reducing air, water and soil contamination so as to improve environmental quality can best reduce these risks to meet the international standard such as Stockholm Convention.

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1. Introduction

The giant panda (*Ailuropoda melanoleuca*) is one of the most and rarest endangered animals in our world. Approximately 1800 individuals remain in anthropogenically fragmented habitats (SFA, 2015), of which < 350 individuals are of the Qinling subspecies (*A. melanoleuca qinlingensis*) living in the Qinling Mountains, China (SFA, 2015). In the last several decades, there are two strategies that are now used to protect this flagship species. One strategy is *ex-situ* breeding in, for example, Beijing Zoo, Wolong Breeding Center, and Shaanxi Wild Animal Research Center. The other strategy is the establishment of natural conservation zones to preserve panda habitat. In the last several decades, 67 conservation zones, with a total area >43,600 km^2, have been established (SFA, 2015).

It is generally assumed that captive breeding centers can effectively protect giant pandas from the adverse impacts of human activities. However, the fatal virus is felling pandas at SWARC and four pandas have died rapidly within a short period of time so the news that "captive pandas succumb to killer virus" (Mara, 2015) was published, which suggested that the captive pandas were threatened and new measures are needed to protect this iconic endangered species. In addition, environmental pollution further stresses rare and endangered animals in captivity. For example, we have shown that captive pandas are exposed to heavy metals

☆ This paper has been recommended for acceptance by David Carpenter.

* Corresponding author. Institute of Earth Environment, Chinese Academy of Science, No. 97, Yan-Xiang Road, Xi'an, 710061, China.
E-mail address: chenyp@ieecas.cn (Y.-p. Chen).

http://dx.doi.org/10.1016/j.envpol.2017.04.023
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including cadmium, zinc, chromium, arsenic and lead (Chen et al., 2016). We also found that chlorine and Bromine were 690% and 330% higher in feces of captive pandas than in those of wild pandas (Chen and Ma, 2017), and we therefore hypothesized that captive pandas may be exposed to and affected by PBDEs (polybrominated diphenyl ethers), which include Br and is lipophilic.

PBDEs as the brominated flame retardants that are used in electronic equipment, textiles, cabinets for television and computers and sets, and in many plastic products (WHO, 1994; Darnerud et al., 2001; Kim et al., 2012). PBDEs can be bioaccumulate in humans and other mammals' tissue via food chain, which are released slowly into environment, and are toxic to humans and other mammals (Hooper and McDonald, 2000; De Wit, 2002). Exposure of laboratory animals n the life tissue to high concentrations of PBDEs can suppress production of antibodies and proliferation of lymphocytes (Darnerud and Thuvander, 1998), decrease thymic weights (Fowles et al., 1994), cause immunomodulatory turbulence, and lead to hormonal deficits (Eriksson et al., 2001; Branchi et al., 2003). Modulating effects of PBDE exposure on wild animal endocrine systems also have been documented (Legler and Brouwer, 2003; Darnerud, 2003). The recent research had found that giant pandas were exposed to PCDDs, PCDFs, PCBs, and heavy metals in both captive breeding centers and in situ conservation areas, but concentrations of these toxins are far greater for pandas in captivity via the bamboo (*Fargesia qinlingensis* and *Bashania fargesii*), and soil of their core activity area (Chen et al., 2016). However, there is no research on the giant panda exposed PBDEs, especially PBDEs exposure in captive and wild pandas' dropping and blood.

Therefore, the objective of this paper to (1) test whether captive or wild pandas are exposed to PBDEs, blood and feces, drinking water, food (bamboo) were collected from SWARC and FNNR, and supplemental feedstuff was collected from SWARC within the Qinling Mountains (Fig. 1); (2) document and compare the concentrations of PBDEs in wild and captive pandas; and (3) identify possible sources of PBDEs contamination.

2. Materials and methods

2.1. Samples collection

The giant panda (*Ailuropoda melanoleuca*) as the most endangered animals in our world, they are protected by law in China, and capture panda is a crime behavior. Therefore using the directly giant panda sample was an impossible things, a non-invasive samples was required. Fecal samples of wild pandas which can be used as non-invasive were collected from 16 different sites of FNNR. Sampling locations were spaced 10-km apart and every four independent samples were pooled into a mixed sample. Feces of 16 captive pandas were collected from SWARC, which was established in 1987 to conserve the Qinling panda. These samples were also pooled into four samples each consisting of four independent samples.

Fresh leaves of living plants (500 g) of the two bamboo species (*Fargesia qinlingensis, Bashania fargesii*) that are the primary food for panda were collected in FNNR and around SWARC and the sampling sites were very close the droppings locations. Water samples (500 ml) were collected from streams using Pyrex borosilicate amber glass bottles, which are also near by the droppings location of at FNNR, and from the SWARC water supply. At both FNNR and SWARC, 12 samples of each bamboo species and of freshwater were collected and pooled to produce four mixed samples each consisting of three samples. In addition, four samples of mixed feedstuff, provided as a nutrient supplement for captive pandas, were also collected from SWARC.

Finally, blood samples were obtained from three similarly-aged pandas rescued from the Qinling Mountains and three captive pandas bred at SWARC because the blood sample was difficult to get in the wild, so the blood samples used in this study were

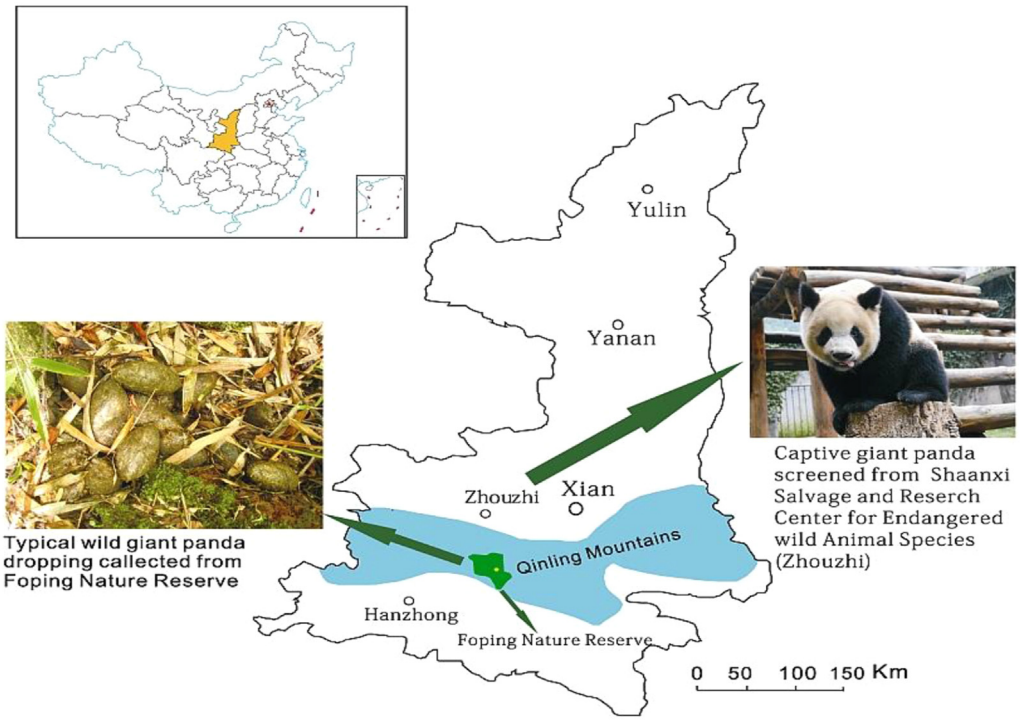

Fig. 1. Sample Collection Sites. The Shannxi Wild Animal Research Center (SWARC) is located at 34° 04′ N, 108°19′ E in Zhouzhi County, Shaanxi province. The Foping National Nature Reserve (green shaded area) is located in the area bounded by 33° 33′–33° 44′ N, 107° 40′–107° 55′ E within Qinling Mountains (blue shaded area). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

residuals from physical examinations of the individual pandas. Prior to examination, the pandas were anesthetized with 25% ketamine (dosage = 8 mg/kg). After collection, the blood was placed in EDTA tubes and frozen at −80 °C for analysis of PBDEs.

2.2. Sample preparation and extraction

PBDE congeners were analyzed using U.S. EPA method 1614 with minor modifications (Li et al., 2008). After freeze-dried, bamboo, feces, and feedstuff samples were used a stainless steel (0.5 mm) sieve to homogenize. Each 3 g was spiked with a ^{13}C-labeled surrogate standard (BDE-LCS) before accelerated solvent extraction (ASE) with dichloromethane (150 ml) and hexane (150 ml). After ASE, acidic silica (15 g, 30% w/w) was added into the sample to remove lipids. Then, 5 g of anhydrous sodium sulfate was added in the extract. The extract sample was rotary-evaporated to 2 ml and then passed through the multi-layered silica gel column that was pre-cleaned by hexane (100 ml), after sample was loaded, the congeners of PBDE were eluted with hexane followed by dichloromethane (70 ml) and hexane (70 ml). The eluant was then concentrated to 2 ml on the rotary evaporator. Its volume was further reduced with a gentle nitrogen flow and the solvent was changed to 20 μl nonane in a minivial.

PBDEs water were extracted using U.S. EPA method 1614. Prior to extraction, 1 L filtered liquid samples were spiked with a ^{13}C-labeled BDE-LCS standard, concentrated with the nitrogen stream, and through a multilayer silica gel column packed with glass wool to clean up. PBDEs in these samples were eluted with n-hexane and decreased to 200 μL.

2.3. Instrumental analysis

BDEs 17, 28, 47, 66, 71, 85, 99, 100, 138, 153, 154, 183, and 190 were analyzed by gas chromatography–mass spectrometry (Agilent 6890, USA) coupled with a high-resolution mass spectrometer (HRMS). The HRMS operated in SIM mode with resolution >10,000. Exactly 1 μL sample was injected with a CTC PAL autosampler in splitless mode into an HB-5 (30 × 250 μm i.d. × 0.1 μm film thickness) capillary column for separation. The flow rate of carrier gas was 1.2 ml/min. The program was as follows: 80 °Cheld for 1 min, increased to 200 °C at 10 °C/min, held at 200°Cfor 1 min, increased to 300 °C at 20 °C/min, and held at 300 °Cfor 5 min.

2.4. Quality assurance and quality control

All solvents were pesticide residue grade and were purchased from Fisher (Hampton, NH, USA). Silica gel was obtained from Merck (silica gel 60, Darmstadt, Germany). ^{13}C labeled surrogate and labeled injection standards were purchased from Wellington Laboratories (Guelph, Canada).

All analytical procedures were checked by the strict quality assurance and control measures to avoid samples pollution and cross pollution. A total of 3 blank control samples were analyzed in same methods. Triplicate samples were analyzed to determine repeatability and reproducibility. To monitor analyze losses, all samples were spiked with internal standards of ^{13}C-labeled BDE47, 99, and 153. The mean recoveries of ^{13}C-labeled surrogate PBDE congeners 47, 99 and 153 were in the range of 54.2 ± 12.1%, 66.0± 10.1%, 102.2± 20.1%, which were well in the limits according to U.S. EPA Method 1614 and all the content of PBDEs in control blank samples below the detection limit and if the practical results of concentrations below the detection limit, 1/2 LOD values to calculate, and if the concentrations don't check out, the value is 0.

2.5. Data analysis

Correlation analysis (CA) and principal components analysis (PCA) were used to analyze the association between 13 PBDE congers in different samples. Paired samples were analyzed using *t*-tests. All statistical analyses were used IBM statistical package SPSS 20.0 (IBM Corp., USA).

2.6. Evaluation methods

The giant panda's health risk evaluation is calculated using the equation which detailed in USEPA's Exposure Factors Handbook (USEPA, 1997). ADD, Average daily dose is analyzed as follow:

$$ADD = \frac{C \times IR_S \times EF \times ED}{BW \times AT}$$

C is PBDEs concentration (mg/kg), IR_S is ingestion rates of bamboo, the IR_S of giant panda compared with that of adult's IR_S, EF is the exposure frequency, 350 day/year, ED is the exposure duration, 10.36 years, BW is the average body weight, which is 80–130 kg (Zhang and Wei, 2006), but in our research we choose the average weight 105 kg. AT = 3781.4 which is averaging time.

Noncancer toxic risk is determined by the model hypothesis of HQ (Hazard Quotient):

$$HQ = \frac{ADD}{RfD_o}$$

RfDo is PBDEs' reference dose (USEPA, 1997). When HQ < 1, relatively safe for risk exposure was considered. When 1 < HQ < 10, considerable threaten was suggested, When HQ > 1, the high chronic risk was considered. The risk is increasing with the value of HQ increase (Hang et al., 2009).

3. Results

3.1. Concentrations of PBDEs

Total PBDEs concentrations were consistently and significantly greater of captive pandas and their food supply than in wild pandas and their food and water supply (Fig. 2). In the fecal samples, ΣPBDE of captive pandas was 2.55 times greater than in wild pandas (Fig. 2A). ΣPBDE of *Fargesia qinlingensis* was 1.13 times higher, and of *Bashania fargesii* 1.55 times higher, in leaves eaten by captive pandas (Fig. 2B and C). Water samples had low concentrations of PBDEs (Fig. 2D).

Thirteen congeners of PBDEs were found in fecal samples; BDE47, BDE66, BDE71, BDE99 and BDE154 predominated in captive pandas (Fig. 3A). Of the dozen congeners found in the two bamboo species eaten by captive pandas, BDE47 and BDE99 predominated in *Fargesia qinlingensis* and *Bashania fargesii*, respectively (Fig. 3B and C). Although captive pandas were exposed to somewhat higher concentrations of PBDEs in their water supply (Fig. 2D), the concentrations of each congener were quite low and none predominated (Fig. 3D). Ten PBDE congeners were found in the supplemental feedstuff provided for captive pandas, with BDE28 and BDE183 predominating (Fig. 3E). Finally, nine PBDE congeners were determinated in the blood samples collected from SWARC. BDE153 and BDE183 were the predominant congeners in captive panda blood samples, and occurred in significantly higher concentrations than in blood sampled from wild pandas (Fig. 3F).

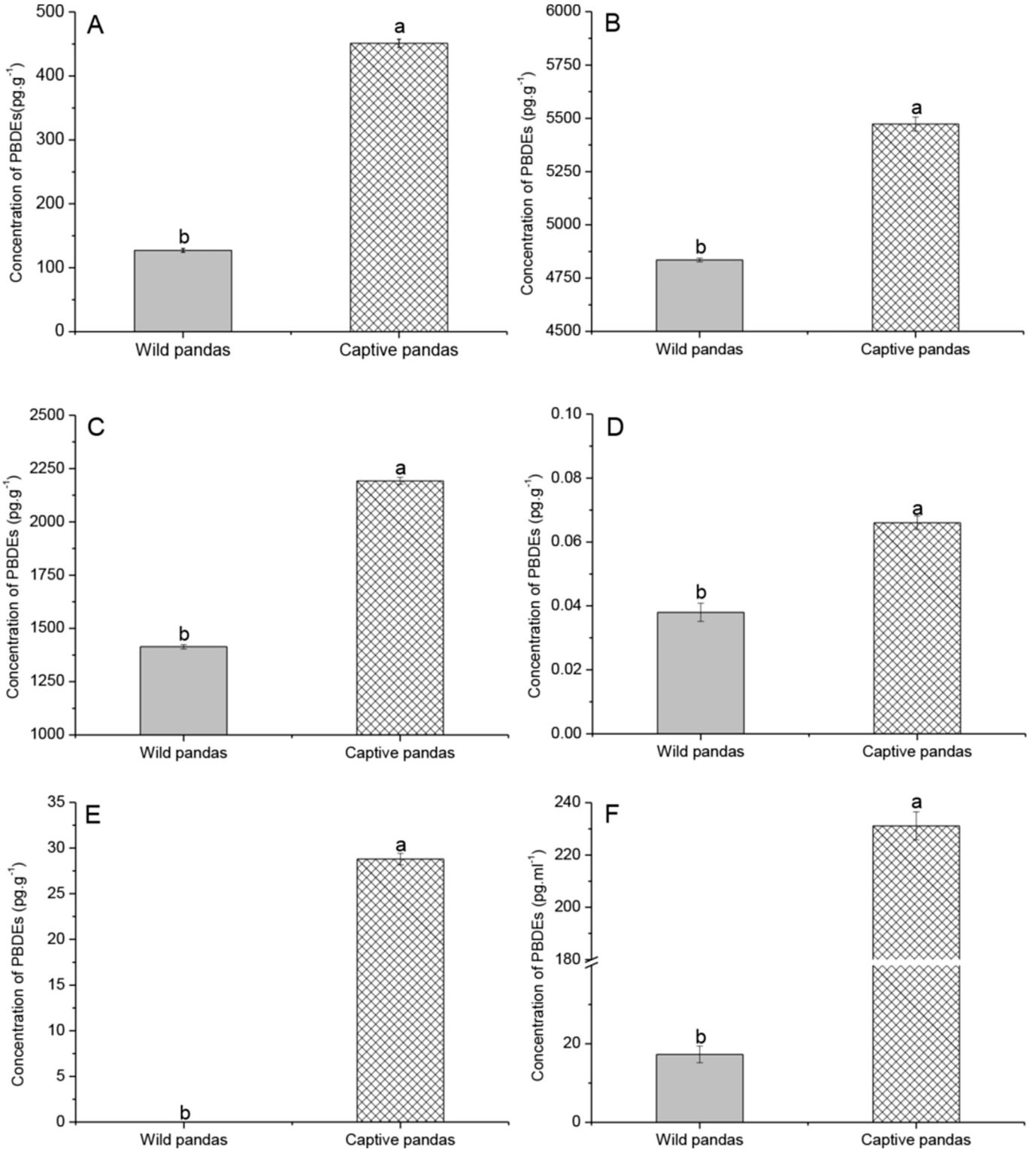

Fig. 2. Total concentrations of PBDEs in (A) fecal samples; (B) leaves of *Fargesia qinlingensis*; (C) leaves of *Bashania fargesii;* (D) drinking water; (E) supplemental feedstuff; and (F) blood sample of wild (gray bars) and captive (cross-hatched bars) giant pandas. In (F), the wild panda was three 17-year old individual rescued from Qingling and the captive panda was 8–9-years old. Bars (means ± 1 SE of the mean from n = 4 independent replicates comprising three or four pooled samples) with different letters between the wild and captive pandas (a or b), Different letters indicate significant differences identified using Tukey HSD test (all $P < 0.01$). pg.glw^{-1} = nanograms per gram lipid weights.

3.2. Statistical analysis

To analyze the association between fecal samples and other samples including the *Fargesia qinglingensis*, *Bashania fargesii*, drinking water, supplemental feedstuff and dropping samples in the captive pandas, CA and PCA were used. Before multivariate statistical analysis, all data was checked and standardized and all data met the certain requirements.

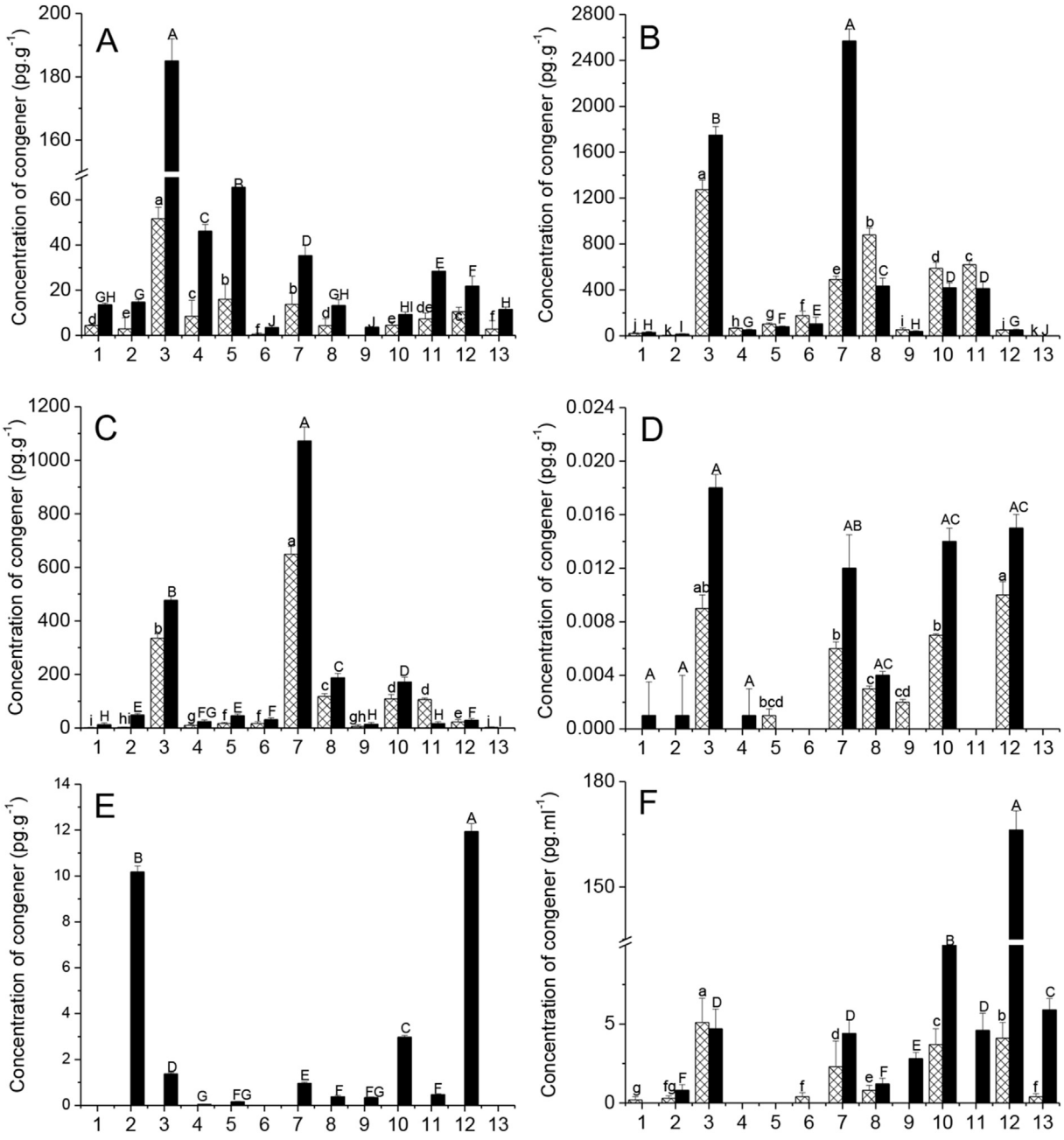

Fig. 3. The concentrations of individual PBDE congeners in (A) feces; (B) leaves of Fargesia qinlingensis; leaves of Bashania fargesii; (D) drinking water; (E) supplemental feedstuff; and (F) blood from wild (cross-hatched bars) and captive (black bars) giant pandas. Numbers on the x-axis denote different congeners: 1 = BDE17; 2 = BDE28; 3 = BDE47; 4 = BDE66; 5 = BDE71; 6 = BDE85; 7 = BDE99; 8 = BDE100; 9 = BDE138; 10 = BDE153; 11 = BDE154; 12 = BDE183; 13 = BDE190. Different letters show significant differences between pairs ($P < 0.05$).

The most significant positive correlations in ΣPBDEs were detected between feces and bamboos, blood and feedstuff ($r = 0.79–0.93$). Concentration of PBDE in water samples was not significantly correlated with any of the other samples (Table 1).

Table 1
Spearman correlation matrix for PBDEs measured in captive samples.

	Fargesia qinglingensis	*Bashania fargesii*	Blood	Water	Feces
Bashania fargesii	0.89**				
Blood	0.92**	0.92**			
Water	−0.11	−0.10	0.15		
Feces	0.90**	0.88**	0.93**	−0.09	
Feedstuff	0.73**	0.75**	0.88**	0.309	0.79**

**$P < 0.01$ level (2-tailed test).

PCA (Fig. 4) grouped the congeners into three main clusters, different samples have different main cluster. Fig. 4A was grouped the congeners into three main cluster and the cumulative variance contribution rate were 32.1%, 65.5% and 87.8%; the congeners of PBDE in leaves of Bashania fargesii and stuff were grouped into three main cluster and variance contribution rate were 16.1%, 25.6% and 20.8% (Fig. 4B), and 28.0%, 29.3% and 20.1% (Fig. 4C); that of drinking water was grouped one cluster and the rate is 19.8% (Fig. 4D). The result illustrated a clustering of congeners BDE47, BDE66, BDE71, BDE99 and BDE154 in leaves of *Bashania fargesii* that matched the predominant congeners found in fecal samples (compare Figs. 4B and 3A).

3.3. Health risk assessment

The order of Hazard Quotient of thirteen kinds of congeners of PBDEs in captive panda is BDE99 (1.60) > BDE47(1.10) > BDE100(1.07) > BDE153(0.97) > BDE154(0.55) and so on. That in wild panda is BDE47 (1.20) > BDE100(0.99) > BDE154(0.75) > BDE153(0.57) > BDE99(0.55) and so on. The HQ values > 1, showing that BDE99 and BDE47 can pose health risk to captive giant panda, whereas BDE47 is the essential congener, which can threaten the health risks of wild panda.

4. Discussion

The first objective of our research was to test this hypothesis that giant pandas were exposed to PBDEs. Our previous research had found that Br in the captive pandas' fecal samples were 3.3 times higher than that in wild pandas (Chen and Ma, 2017), and data reported here supported the resulting hypothesis that captive pandas were exposed to PBDEs (Figs. 2A and 3A). In captive pandas, BDE47, BDE66, BDE71, BDE99, BDE154 were the major PBDE congeners in fecal samples, which matched the profile of PBDEs in leaves of the bamboo *Bashania fargesii* (Fig. 4B). This result shows that the diet supply is main source of PBDE exposure for pandas. Plants can accumulate high concentrations toxic pollutants in tissues and can bioaccumulate and biomagnify in animals' body (Burreau et al., 2006; Voorspoels et al., 2007; McKinney et al., 2011; Krieger et al., 2016). Once into the body, the PBDEs can lead deficiency in neural responses, the thyroid hormone disorders and carcinogenicity (McDonald, 2002; Staskal et al., 2005; Lee et al., 2014).

Many studies have shown that PBDEs bioaccumulate in aquatic ecosystems (Law et al., 2003), and lots of reports about terrestrial species (Huwe et al., 2002; Jaspers et al., 2005; Pirard and DePauw, 2007; Voorspoels et al., 2007; Chen et al., 2012, 2013; Crosse et al., 2012; Andersen et al., 2015). However, we are aware of so little study that sampled PBDEs in fecal samples (Zheng et al., 2015), which is an appropriately non-invasive method to sample PBDEs in a rare species like the panda.

The second objective of our research was to compare PBDE exposure of wild and captive pandas. Not surprisingly, given the anthropogenic origin of PBDEs, the ΣPBDE was significantly higher in samples taken from captive pandas. Plants often reflect the content of organic pollutants in the environment (Collins and Finnegan, 2010) because the soil-air-plant pathway (pollutants volatilized from soils into the atmosphere are deposited onto plants) describes uptake of organic pollutants from contaminated soils (e.g., Paterson et al., 1991; Trapp and Matthies., 1997; Harrad et al., 2006; Collins and Finnegan., 2010; Ding et al., 2014). We found that the ΣPBDE of in *Fargesia qinlingensis* and *Bashania*

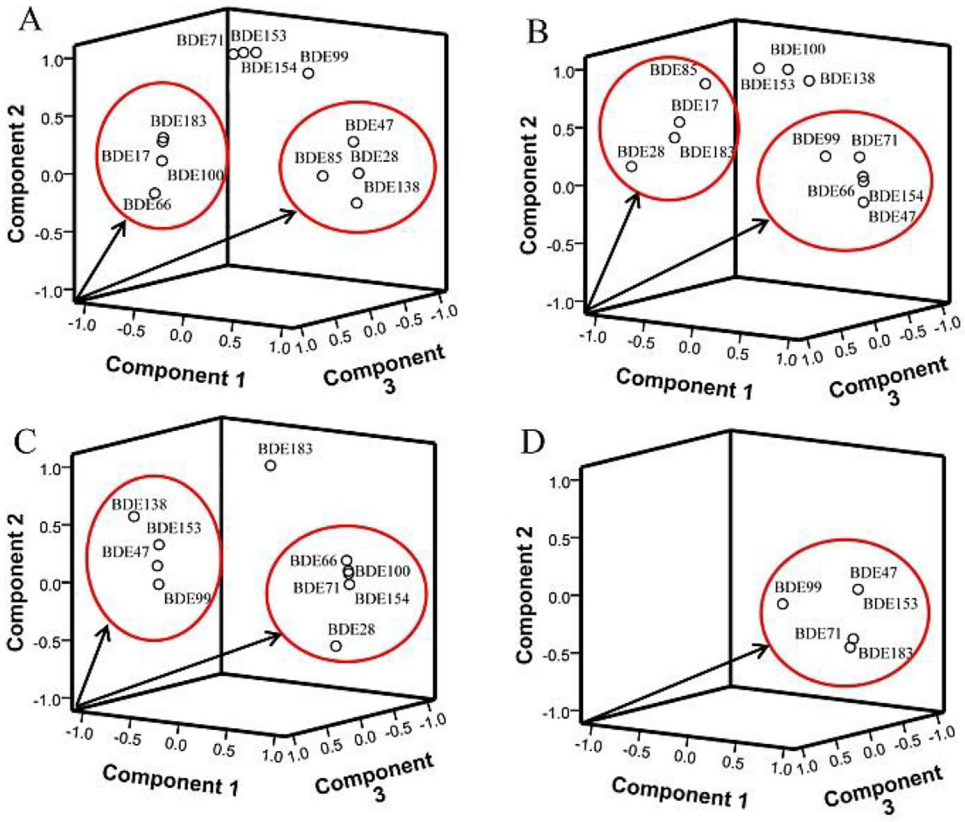

Fig. 4. Principal component triplot (principal axes 1–3) for 13 PBDE congeners in (A) leaves of Fargesia qinglingensis; (B) leaves of Bashania fargesii; (C) supplemental feedstuff; and (D) drinking water of samples taken at SWARC.

fargesii growing around the captive breeding center was significantly higher than that growing in the nature reserve (Fig. 2B and C). The panda captive breeding center at SWARC, which location is close to some city and is near to several potential sources of PBDE contamination including waste incinerators, electronic-waste processing facilities, and industrial discharges (He et al., 2014; Kosior et al., 2015; Wang et al., 2015).

The final objective of our research was to identify possible sources of exposure for PBDE pollution by pandas. This study is the first time to measure PBDEs concentrations in bamboo, so the concentration could only be compared with unrelated plant species. The ΣPBDEs of *Fargesia qinlingensis* and *Bashania fargesii* growing at FNNR and SWARC in our study were higher than that of *Pleurozium schreberi* in uncontaminated area (the value is 755.6 pg/g dry mass) and urban areas (the value is 3062.9 pg/g dry mass). Pandas eat little besides bamboo. They can consume >20 kg/day (Tuanmu et al., 2013) and bamboo accounts for > 99% of their diet (Hu, 1991, 2000), yet only 25% of the nutrients in bamboo can be assimilated (Zhou et al., 2008). The similarities in congener profiles of panda fecal samples (Fig. 3A) and the clustering in the PCA (Fig. 4B), suggest that bamboo is the primary source of PDBE exposure for pandas.

The very low concentrations of PDBEs in water (Figs. 2D, 3D and 4D) are unlikely to be an important source of PDBEs for pandas. Likewise, the feedstuff appears an unlikely route of exposure. In captivity, pandas are fed a steamed bread supplement ("feedstuff") that includes additional ingredients, including milk powder, apple, carrot, steamed bran, rice flour, maize flour, bean flour, fishmeal, bone meal, and mineral additives that provide supplemental nutrients essential for successful breeding programs (Chen and Ma, 2017). The ΣPBDE in feedstuff (28.8 pg g^{-1}; Fig. 2E) exceeded PBDE concentrations in some farmland grains (13.7 pg g^{-1}: Luo et al., 2009) but not others (30–440 pg g^{-1}: Zheng et al., 2015). This might due to the feedstuff used in our research was purchased from local market instead of from locally-grown ingredients.

To our knowledge, this is the first investigation of exposure of pandas to PDBEs, and one of only a very few studies of PDBE exposure and bioaccumulation in a terrestrial species (Hoshi et al., 1998; Christensen et al., 2005) and there no some values be compared in our study, so we according to the HQ model to access the exposure risk and the results showed that HQ(BDE99), HQ(BDE47) and BDE(153) >1 could be threatening the health of giant panda especially BDE99 and BDE47 for captive panda.

Pandas in captive breeding centers are generally thought to be better protected from human activities than are wild pandas in nature conservation zones, primarily because these zones have become more fragmented and less suitable for supporting this species over time (Chen et al., 2016). However, our data provide direct evidence that giant pandas are exposed to PCDEs in both captive breeding centers and in situ conservation areas thought test the feces, blood and so on, but concentrations of these toxins are significant greater in captivity than that in wild.

PBDEs most likely through the bamboo they eat. Mean 30 kg bamboo shoots and leaves every day can be consumed by giant panda (Tuanmu et al., 2013). Therefore, even relatively low concentrations of PBDEs in bamboo tissue can still lead to a higher dietary exposure, which can threaten giant pandas' health. For mammals, PDBEs can be transferred to nursing offspring via mother's milk (Travis and Hattermer-Frey, 1991; Beineke et al., 2005, 2007). Because PBDEs can also be immunosuppressants (Arkoosh et al., 2010; Frouin et al., 2010; Lv et al., 2015), they could make pandas more vulnerable to bacterial and viral infections. Therefore, it should be taken seriously for other animals and human in captivity.

5. Conclusions and recommendations

Our data suggest that pandas are exposed to high levels of PDBEs in captive breeding centers, and may represent a significant health risk for pandas in captivity. We recommend that managers of these centers and captive breeding programs, including the Chinese State Forestry Administration (SFA) seek strategies to minimize PDBE exposure by pandas lest decades of successful *ex situ* conservation efforts become compromised by the increasing pollution associated with Chinese economic development. A short-term solution to addressing this issue is to reduce the supply of contaminated *Bashania fargesii* and to grow uncontaminated bamboo strictly for captive pandas. In the long term, however, sustaining a successful captive breeding program for pandas will require reduction of air, water, and soil pollution that will lead to improvements in the environmental quality of the giant panda's natural habitat.

Acknowledgements

This research was supported by a project from SKLLQQG (State Key Laboratory of Loess and Quaternary Geology) and IEECAS (Institute of Earth Environment, Chinese Academy of Sciences (ZZBS1303)).

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