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Detection of the toxic substance dibutyl phthalate in Antarctic krill

XIANGNING HAN and DAICHENG LIU*

Key Laboratory of Animal Resistance, College of Life Science, Shandong Normal University, 88 East Wenhua Road, Jinan 250014, Peoples Republic of China
**Corresponding author: liudch@sdnu.edu.cn*

Abstract: High-performance thin layer chromatography was performed to investigate the potential presence of four phthalic acid esters, dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP) and dioctyl phthalate (DEHP), in Antarctic krill. The results revealed that in freeze-dried Antarctic krill levels of DBP (0.1043 ± 0.0005 mg g^{-1} (104.3 ± 0.05 mg kg^{-1})) were high. The structure of DBP in Antarctic krill was determined by gas chromatography-mass spectrometry. Its existence is of concern based on demonstrated harmful effects to animals and plants as Antarctic krill is a key part of the food chain in Antarctic coastal marine ecosystems. The adverse effects of DBP on Antarctic krill and the source of DBP should be explored in further research.

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Key words: DBP, HPTLC, toxicity

Introduction

Dibutyl phthalate (DBP) is a widely used plasticizer with stability, flowability, low volatility and good low-temperature resistance (Jaworek 2013). It is not chemically bound to a polymeric matrix and can migrate from the plastic (Staples *et al*. 1997). The degradation half-life of DBP is over 20 years (Bajt *et al*. 2001). The slow degradation, low volatility, high bioaccumulation and toxicity characteristics of DBP may adversely affect organisms (Guo *et al*. 2014).

The effects of DBP on aquatic algae and submerged macrophytes include growth inhibition (*Phaeodactylum tricornutum* Bohlin and *Chlorella vulgaris* Beyerinck (Beijerinck)), cell division inhibition (*Scenedesmus obliguus* (Turpin) Kützing), physiological and biochemical indices alteration (*Ceratophyllum demersum* L., *Vallisneria spiralis* L. and *Potamogeton maackianus* A.Benn.) (Kuang *et al*. 2003, Chi *et al*. 2006, Li *et al*. 2006, Yang & Duan 2010). Furthermore, DBP may lead to metabolic abnormalities in abalone (*Haliotis diversicolor supertexta* Lischke) (Zhou *et al*. 2015). The effects of DBP on arthropods include rapid maturation, reduced spawning (*Drosophila melanogaster* Meigen), increased mortality (*Folsomia fimetaria* L.) and reduced movement (*Gammarus pulex* L.) (Thurén & Woin 1991, Jensen *et al*. 2001, Atli 2010). It also poisons fish and amphibians causing organ damage (male zebrafish *Danio rerio* (F. Hamilton)) and interfering with gonad differentiation (frog *Rana rugosa* Temminck and Schlegel) (Ohtani *et al*. 2000, Xu *et al*. 2014). The effects of DBP on mammals (mainly mice and rats) include severe reproductive toxicity, reproductive abnormalities, embryo toxicity, organ damage and even neurotoxicity (Murakami *et al*. 1986, Salazar *et al*. 2004).

Though it has been classified as a priority controlled pollutant by the US Environmental Protection Agency (Xu *et al*. 2008), studies have determined that DBP remains a ubiquitous environment contaminant that can be found in food packaging materials, household items, soils, freshwater, marine water, organisms and even air (Fatoki & Ogunfowokan 1993, Staples *et al*. 1997, Lu *et al*. 2016). Unfortunately, information on DBP concentrations in Antarctic krill is still scarce.

Antarctic krill live in groups (Kokubun *et al*. 2015). This species makes up an estimated biomass of 400–1550 million tons, which may be the largest biomass of a single multicellular organism (Gigliotti *et al*. 2011). The aim of this study was to determine if there were phthalate esters in Antarctic krill and to discuss their potential sources and adverse effects.

Materials and methods

Samples, reagents and instruments

Antarctic krill were purchased frozen whole from the Liaoning Fishery Group (Dalian, Liaoning, China). This batch (35–55 mm in body length) was caught in the first quarter of 2016 from the waters surrounding the

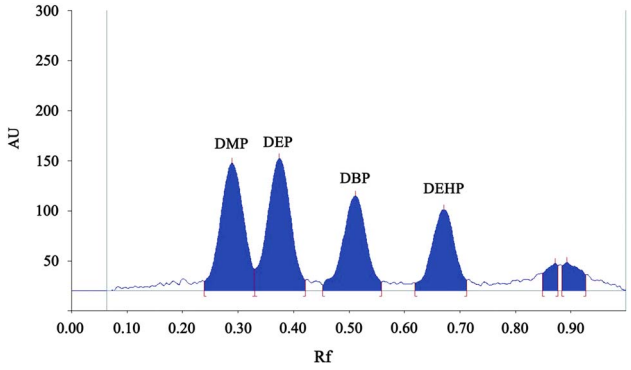

Fig. 1. The chromatogram of the standard solution showing the average peak areas for dimethyl phthalate (DMP) diethyl phthalate (DEP), dibutyl phthalate (DBP) and dioctyl phthalate (DEHP). AU = absorbance unit.

Chinese Great Wall Antarctic Station. Dimethyl phthalate (DMP), diethyl phthalate (DEP), DBP, dioctyl phthalate (DEHP) standards (≥ 99%) were obtained from Aladdin (Shanghai, China). All other reagents used in the study were of analytical reagent grade. A FUD-1200 freeze dryer (Rikakikai Tokyo, Japan), a Kun Shan Ultrasonic Instruments cleaning machine (Jiangsu, China), a Rotavapor-3 rotating evaporator (Buchi, Flawil, Switzerland) were obtained. Pre-coated silica gel GF_{254} high-performance thin layer chromatography (HPTLC) plates ($10 \times 10\,cm^2$) were obtained from Haiyang Chemical (Qingdao, China), a development chamber ($10 \times 12 \times 5\,cm^3$, consisting of a double-groove glass chamber) was obtained from Shanghai Xinyi Instrument (Shanghai, China). A Thin Layer Chromatography Scanner 3 equipped with Wincats 1.4.1 software was purchased from CAMAG (Muttenz, Switzerland). None of the experimental instruments or materials contained plastic products.

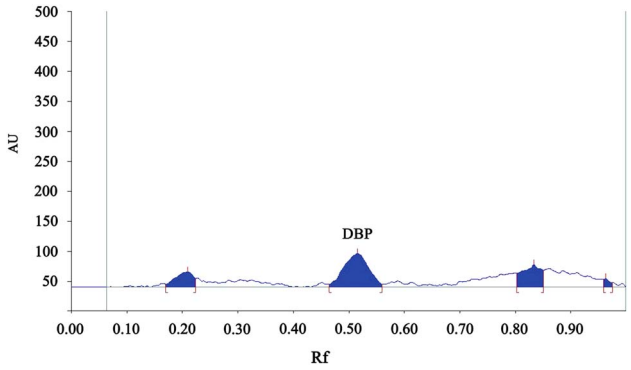

Fig. 2. The chromatogram of sample 1. AU = absorbance unit.

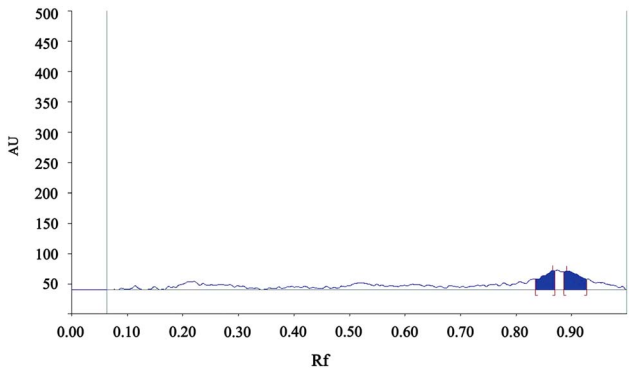

Fig. 3. The chromatogram of the blank solution. AU = absorbance unit.

Preparation of sample solutions and standard solutions

The middle sections of whole frozen Antarctic krill (1000 g) were freeze-dried, leaving 200 g of dried krill. The dried krill was divided into eight equal samples (25 g per sample). Each sample was extracted separately using the following method. One sample was put into a glass beaker and dipped with three times the volume of cyclohexane (purified by distillation before use) and then extracted for 0.5 h by ultrasonic treatment at 25°C, 300 w and 40 kHz. The cyclohexane extract was filtered through a qualitative filter (after flushing with cyclohexane). The above extraction procedure was repeated three times until the extraction solution was close to colourless. All the filtrates were combined and concentrated at 60°C using a rotating evaporator to remove the cyclohexane. The residue (1.56 g) was dissolved in cyclohexane and its volume was adjusted to 5 ml (samples 1–8).

A standard solution was prepared as a mixture of DMP, DEP, DBP and DEHP dissolved in cyclohexane. The standard solution contained 1 mg ml^{-1} concentrations of DMP, DEP, DBP and DEHP standards.

High-performance thin layer chromatography

The HPTLC was performed according to the previously reported method (Chen *et al.* 2006). The standard

Table I. Average peak areas corresponding to different amounts of dibutyl phthalate (DBP).

Spot	Volume (µl)	DBP (µg)	Average peak areas (AU)
Standard 1	1.5	1.5	1430.44
Standard 2	2.0	2.0	2178.00
Standard 3	2.5	2.5	2925.44
Standard 4	3.0	3.0	3738.50
Standard 5	4.0	4.0	5444.32

AU = absorbance unit.

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Table II. The peak areas and dibutyl phthalate (DBP) concentrations for repeated sampling of freeze-dried Antarctic krill.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Mean ± SD
Peak area (AU)	1496.6	1460.9	1493.8	1476.2	1478.4	1480.8	1483.6	1466.6	1479.61 ± 12.19
DBP (μg 3 μl^{-1}) in sample	1.576	1.554	1.574	1.563	1.566	1.565	1.568	1.557	1.565 ± 0.008
DBP (mg g^{-1}) in Antarctic krill	0.1050	0.1036	0.1049	0.1042	0.1044	0.1043	0.1045	0.1038	0.1043 ± 0.0005

AU = absorbance unit, SD = standard deviation.

solution, sample 1 and a cyclohexane (blank) solution were spotted onto the same GF_{254} plate, and developed with a developing solvent that was a mixture of ethyl acetate, ethyl ether and isooctane (1:4:15 v/v/v). Double wavelength reflection absorption flying-spot scanning (λ S 275 nm, λ R 340 nm) was performed to detect the plate. Quality analysis was according to the Rf value of the spot and quantitative analysis was employed by comparison to the external standard.

Repeated trials

The eight sample solutions were detected using one GF_{254} plate (3 μl each sample). The DBP content was

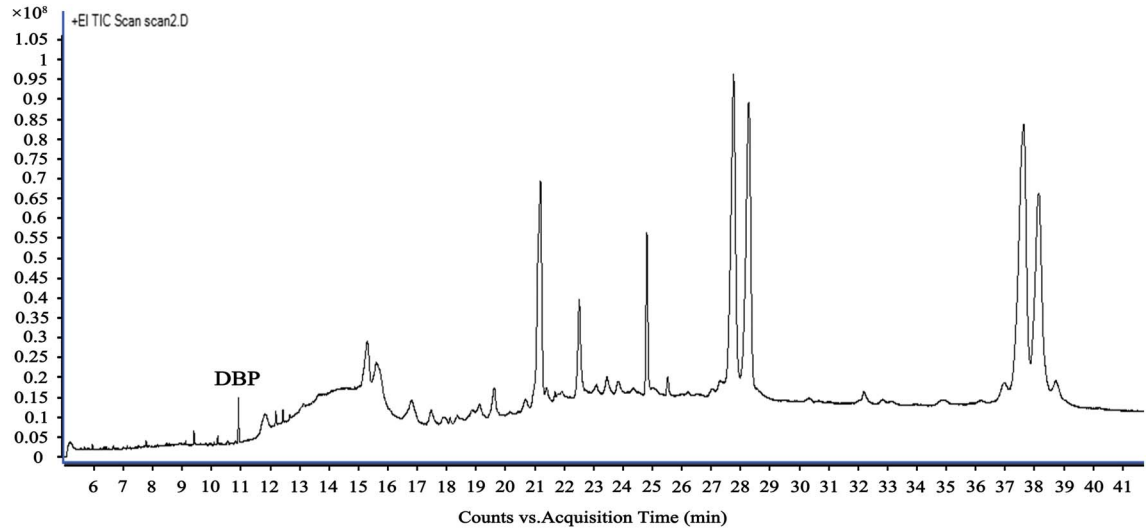

Fig. 4. The retention time of dibutyl phthalate (DBP).

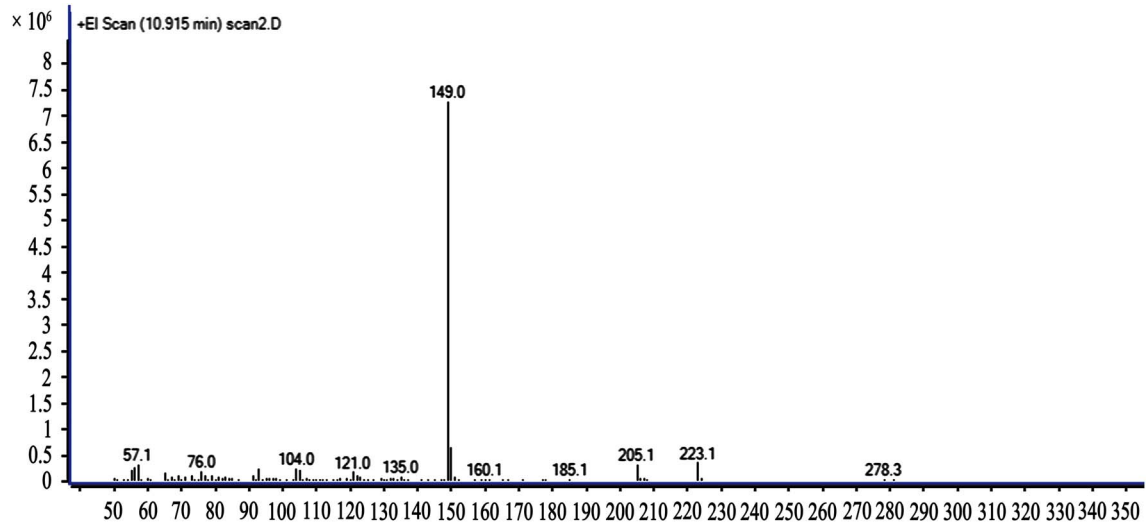

Fig. 5. The mass spectrogram of sample 1 with a retention time of 10.915 min.

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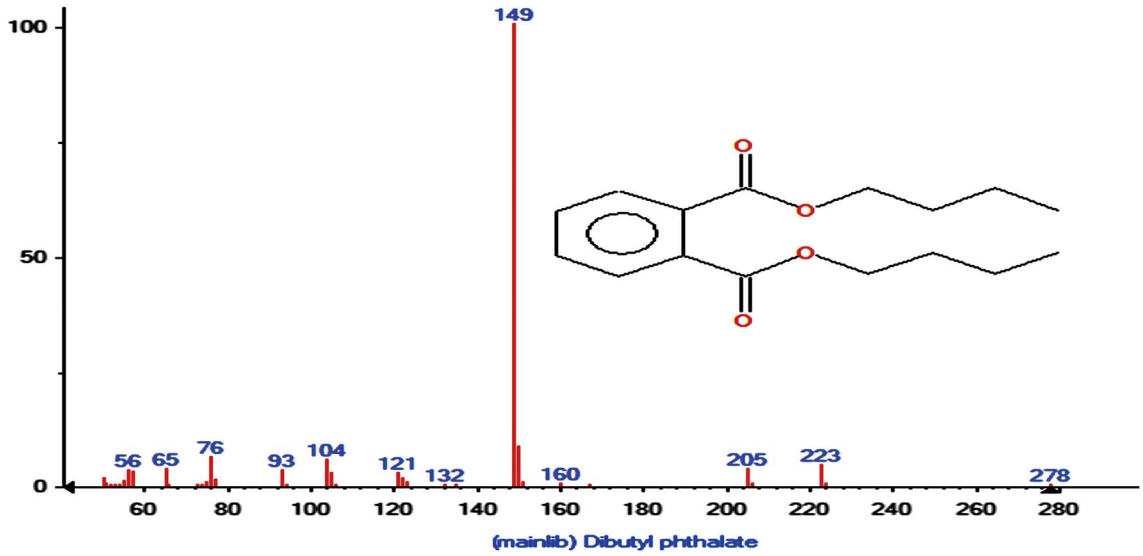

Fig. 6. The mass spectrogram of the dibutyl phthalate (DBP) standard solution.

quantified by HPTLC. Peak areas were recorded and the DBP content in Antarctic krill was calculated.

Gas chromatography-mass spectrometry

The conditions of gas chromatography-mass spectrometry (GC-MS) were those of Lahimer *et al.* (2013).

Results

High-performance thin layer chromatography

The HPTLC clearly separated the four phthalate esters, and the results of double wavelength reflection absorption flying-spot scanning of the standard solution (λ S 275 nm, λ R 340 nm) are shown in Fig. 1. The results of the scan of sample 1 are shown in Fig. 2. Only DBP was found to be

Table III. Harmful effects of dibutyl phthalate (DBP) on organisms and their related concentrations.

Species	Concentration	Harmful effects on organisms	Reference
Algae			
Phaeodactylum tricornutum	> 5 mg l^{-1}	Hardly grow for the first 2 days, decreases chl *a*, *c* and carotenoids	Yang & Duan 2010
Chlorella vulgaris	4.8 mg l^{-1}	Strongly inhibits algae growth	Chi *et al.* 2006
Scenedesmus obliguus	> 10 mg l^{-1}	Inhibits cell division, decreases chl *a*	Kuang *et al.* 2003
Submerged macrophytes			
Vallisneria spiralis	> 0.03 mg l^{-1}	Decreases glutathione and protein content, chl changes irregularly, decreases soluble carbohydrate	Li *et al.* 2006
Potamogeton maackianus	> 0.03 mg l^{-1}	Decreases protein and glutathione	Li *et al.* 2006
Ceratophyllum demersum	> 0.03 mg l^{-1}	Decreases protein and glutathione	Li *et al.* 2006
Marine mollusks			
Haliotis diversicolor supertexta	> 2 μg l^{-1}	Oxidative stress, lipid metabolism dysfunction, energy metabolism disturbance, osmoregulation imbalance	Zhou *et al.* 2015
Arthropods			
Drosophila melanogaster	> 0.25 ml l^{-1}	Accelerates mean population and mean maturation time, decreases mean fecundity	Atli 2010
Folsomia fimetaria	> 25 mg kg^{-1}	Increases juvenile mortality	Jensen *et al.* 2001
	> 14 mg kg^{-1}	Affects adult reproduction	
Gammarus pulex	500 μg l^{-1}	Significant decreases in activity	Thurén & Woin 1991
Fish			
Danio rerio	100 μg l^{-1}, 500 μg l^{-1}	Histopathological changes in the liver and gill, spawning and fertilization success significantly impaired	Xu *et al.* 2014
Amphibian			
Rana rugosa	> 0.01 μM	Interferes with gonad differentiation	Ohtani *et al.* 2000
Mammals			
Long Evans rats	12 mg kg^{-1}	Decreases pup survival	Salazar *et al.* 2004
	50 mg kg^{-1}	Marked decrease in percent of pregnancies	
	12 and 50 mg kg^{-1}	Pup weight reduced, decreased weight of thymus and testis, delays vaginal opening and occurrence of first oestrous	
Rats	100 μM	Liver enlargement, growth depression, testicular atrophy	Murakami *et al.* 1986
Immature rats	500 mg kg^{-1}	Induces neurotoxicity	Li *et al.* 2014

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present. The scan of the blank solution indicated that there was no phthalate ester present in the cyclohexane solution (Fig. 3).

Linearity and repeated trials

The DBP content was calculated using the external standard method. The linear equation of DBP and the linear range (Table I) was determined as the following:

$$Y = -1033.9674 + 1606.6510\,X\,(R^2 = 0.999), \quad (1)$$

with a standard deviation of 1.94%. The results for the eight sample solutions according to the linear equation are shown in Table II. The mean content of DBP in freeze-dried Antarctic krill was 0.1043 ± 0.0005 mg g^{-1} (104.3 ± 0.05 mg kg^{-1}).

Gas chromatography-mass spectrometry

The result of the GC-MS for sample 1 is shown in Fig. 4. The retention time of DBP was 10.915 min.

The mass spectrograms of sample 1 and the standard solution are shown in Figs 5 & 6. This mass spectrogram confirms the presence of DBP in Antarctic krill.

Discussion

The levels of four phthalate esters were measured in Antarctic krill using HPTLC. Only DBP was observed. This is the first report of DBP in Antarctic krill. The structure of DBP determined by GC-MS confirmed its presence. The DBP content was 0.1043 ± 0.0005 mg g^{-1} (104.3 ± 0.05 mg kg^{-1}).

The concentrations of DBP that are toxic to organisms and the effects of DBP toxicity are summarized in Table III. Exposure to DBP has marked effects on organisms, with significant effects on reproduction. The concentrations associated with DBP toxicity range from 0.2 µg l^{-1} to 500 mg kg^{-1}. Therefore, the 104.3 ± 0.05 mg kg^{-1} level found in Antarctic krill (20.86 ± 0.05 mg kg^{-1} in fresh Antarctic krill accounting for 80% water content) is high. Although the effects of DBP on Antarctic krill have not been determined, based on the effects on other species this level of DBP may pose a serious risk to the Antarctic krill. Antarctic krill is a key part of the marine ecosystem, and the accumulation of DBP could lead to poisoning of other animals in the food chain; thereby, seriously affecting the Antarctic ecosystem.

There may be multiple pathways by which DBP may accumulate in Antarctic krill. One source of DBP could be their food (which could produce DBP and absorb DBP from marine water) (Chi *et al.* 2006, Namikoshi *et al.* 2006). Chitin adsorption may also be a possible source of DBP (McKay *et al.* 1982). Ge *et al.* (2012) reported hat fishes could absorb DBP from water; therefore, Antarctic krill may also directly absorb DBP from marine water. The source of DBP in krill requires further research.

Information on the sources and concentrations of DBP in Antarctic biota is still absent. It may reach Antarctica through long-range atmospheric and hydrospheric transport. Plastics discarded by human activities may also be a source of DBP as human activities have increased in recent years (Stark *et al.* 2006). Migratory species (such as seabirds) may introduce DBP into the local food web and may bio-transport DBP into the Antarctic (Mwangi *et al.* 2016). However, the true source of DBP in the Antarctic needs further investigation.

Fresh Antarctic krill, Antarctic krill powder and Antarctic krill oil (the main krill products used by humans) are exported and widely consumed all over the world. The maximum allowable daily level of DBP for humans is 8.7 µg day^{-1} (Kokubun *et al.* 2015). The observed concentration of 104.3 ± 0.05 mg kg^{-1} DBP in the freeze-dried Antarctic krill is high and is a serious concern for human consumption.

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Author contribution

DL designed the research. XH performed the experiments and analysed the data. XH and DL contributed to writing the manuscript.

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