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## Accepted Manuscript

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**Transcriptomic and metabolomic approaches to investigate the  
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retardant hexabromocyclododecane (HBCD)**

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## Abstract

The potential for human exposure to the brominated flame retardant, hexabromocyclododecane (HBCD) has given rise to health concerns, yet there is relatively limited knowledge about its possible toxic effects and the underlying molecular mechanisms that may mediate any impacts on health. In this study, unbiased transcriptomic and metabolomic approaches were employed to investigate the potential molecular changes that could lead to toxicity of HBCD under concentrations relevant to human exposure conditions using *in vitro* models. A concentration-dependent cytotoxic effect of HBCD to A549 and HepG2/C3A cells was observed based on MTT assays or CCK-8 assays with EC<sub>50</sub> values of 27.4  $\mu$ M and 63.0  $\mu$ M, respectively. Microarray-based transcriptomics and mass spectrometry-based metabolomics revealed few molecular changes in A549 cells or HepG2/C3A cells following a 24-hour exposure to several sub-lethal concentrations (2 to 4000 nM) of HBCD. Quantification of the level of HBCD in the HepG2/C3A exposed cells suggested that the flame retardant was present at concentrations several orders of magnitude higher than those reported to occur in human tissues. We conclude that at the concentrations known to be achievable following exposure in humans HBCD exhibits no detectable acute toxicity in A549 cells, representative of the lung, or in HepG2/C3A cells, hepatocytes showing some xenobiotic metabolic capacity.

**Key Words:** HBCD; A549 cells; HepG2/C3A cells; microarray; DIMS; cytotoxicity.

## 1. Introduction

Hexabromocyclododecane (HBCD) is a widely used brominated flame retardant (Covaci et al 2006; Marvin et al 2011). HBCD is persistent, bioaccumulative and is capable of long-range atmospheric transport (Marvin et al 2011) and has been detected in various environmental media including indoor air and dust (Roosens et al 2009). Human exposure to HBCD has been confirmed by its presence in breast milk, adipose tissue and blood (Marvin et al 2011; Rawn et al 2014). HBCD levels detected in breast milk samples in Spain were up to 188 ng/g lipid (median 27 ng/g lipid) (Eljarrat et al 2009). A recent study (Rawn et al 2014) reported concentrations of HBCD in fetal liver (median 29 ng/g lipid) and placental tissues (median 49 ng/g lipid) collected between 1998 and 2010 in Canada. HBCD has also been detected in human serum at up to 856 ng/g lipid (Thomsen et al 2007). These studies confirm that human exposure to HBCD is widespread.

Potentially adverse effects of HBCD have been reported in animal models and human cell lines (Marvin et al 2011). Disruption of thyroid function has been found in rats (van der Ven et al 2006; Ema et al 2008;), fish (Palace et al 2008; Palace et al 2010) and chicken (Crump et al 2008) following exposure to HBCD. Low micromolar concentrations of HBCD have been shown to give rise to neurotoxic effects in *in vitro* cultured neuronal cells (e.g. PC12 cells (Dingemans et al 2009) and SH-SY5Y cells (Al-Mousa and Michelangeli 2012)) by interfering with calcium ( $\text{Ca}^{2+}$ ) homeostasis and intracellular signalling pathways.

Hepatotoxicity of HBCD has been reported in a wide range of studies, which showed that liver is a major main target organ (Marvin et al 2011; Hakk et al 2012). Long term exposure to HBCD at a relatively high dose (30 mg/kg/day, 28 days) increased liver weight in female rats (van der Ven et al 2006) and induced hepatic cytochrome P450 (CYP) enzyme activities (CYP2B1 and CYP3A1) in rats (Germer et al 2006; Cantón et al 2008). In juvenile rainbow trout (Ronisz et al 2004) and Chinese rare minnow (Zhang et al 2008), HBCD inhibited

activities of cytochrome P450s (CYPs) in the liver after 28-day exposure. The mRNA expression of CYPs (*CYP2H1* and *CYP3A37*) were up-regulated in chicken (*Gallus gallus*) hepatocytes exposed to  $\geq 1\mu\text{M}$  HBCD for 24 and 36 hours (Crump et al 2008).

In several *in vitro* studies, induction of cell apoptosis by exposure to high concentrations of HBCD in HepG2 cells (Hu et al 2009; An et al 2014) and human L02 hepatocytes (An et al 2013) has been suggested to result from increased production of reactive oxygen species (ROS). However, a slight increase in ROS has also been observed after low concentration ( $10^{-7}$ – $10^{-1}$   $\mu\text{M}$ ) exposures in human L02 hepatocytes, while there was no acute toxic effect reported (An et al 2013; Zou et al 2013). More studies are necessary to better understand whether any molecular changes result from concentrations that are realistic for human exposure.

Since human exposure to HBCD can occur via inhalation as mentioned above, the lung represents one of the target organs. Therefore, in the present study an adenocarcinomic human alveolar basal epithelial cell line (A549 cells) was selected as an *in vitro* model to test the potential effects of HBCD. This cell line has been widely used in toxicology studies and shows similar metabolic properties to normal human alveolar type 2 epithelial cells in terms of xenobiotic interactions (Hukkanen et al 2000; Vulimiri et al 2009). Hepatocytes are the major site of xenobiotic metabolism and hence represent an important cellular target of xenobiotics (Deferme et al 2013). The HepG2/C3A cell line (a clonal derivative cell line of HepG2 cells that has been shown to exhibit a degree of xenobiotic metabolic capacity (Gerets et al 2012)) was employed here as a convenient alternative to primary human hepatocytes.

Toxicogenomics is a valuable and increasingly used platform for investigating the molecular changes induced by toxicants. However, there are only a few 'omics' studies of the potential toxicity of HBCD. One example showed that the hypothyroidism reported in rats following a

28-day high dose exposure (30 and 100 mg HBCD/kg/day) of HBCD (Germer et al 2006) was found to correlate well with gene expression changes in the cholesterol biosynthesis pathway in rat liver, as revealed by transcriptomic analysis (Cantón et al 2008). A proteomic study of zebrafish (*Danio rerio*) liver cells found that protein metabolism decreased after 72-hour exposure to 5  $\mu$ M HBCD (Kling and Förlin 2009). Metabolomics has been applied to a wide range of toxicological studies, including in mechanistic toxicology (Nicholson et al 2002) and ecotoxicology (Bundy et al 2009) but no metabolomics study of the effects of HBCD has yet been reported.

The aim of this study was to apply unbiased 'omics' approaches to investigate the potential molecular toxicity of HBCD to humans under as realistic experimental conditions as possible with *in vitro* cell line models, i.e. including serum within the cell culture media. A wide range of sub-lethal exposure concentrations of a commercial mixture of HBCD was investigated including levels comparable to those reported previously in human serum. Specifically, oligonucleotide microarray based transcriptomics and direct infusion mass spectrometry (DIMS) based metabolomics were applied to determine gene expression and metabolic profiles of A549 and HepG2/C3A cells exposed to HBCD, respectively. Used in combination, these 'omics' approaches can inform on both upstream regulatory processes as well as downstream functional molecular responses, providing insights into potential adverse effects of HBCD.

## 2. Materials and Methods

### 2.1 Chemicals and reagents

All chemicals, including 1,2,5,6,9,10-hexabromocyclododecane (CAS Number: 3194-55-6, purity >95%) were obtained from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

## 2.2 Cell culture

A549 cells were originally bought from European Collection of Cell Cultures (ECACC) and subcultures were kept under liquid nitrogen. A549 cells were cultured in DMEM medium (catalogue no.: D6429, Sigma-Aldrich, Dorset, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA, Cölbe, Germany), 100 U penicillin/mL and 100 µg streptomycin/mL (PAA, Cölbe, Germany), 2 µM L-glutamine (PAA, Cölbe, Germany) and incubated in 37°C with humidified air containing 5% CO<sub>2</sub>. HepG2/C3A cells were generously provided by Prof. R. Blust from the University of Antwerp, Belgium.

HepG2/C3A cells were cultured in Williams' E medium (catalogue no.:W4128, Sigma-Aldrich, Dorset, UK) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (PAA, Cölbe, Germany), 100 U penicillin/mL and 100 µg streptomycin/mL (PAA, Cölbe, Germany), 4 µM L-glutamine (PAA, Cölbe, Germany) and 0.4 µM sodium pyruvate (Sigma-Aldrich, Dorset, UK) and incubated in 37°C with humidified air containing 5% CO<sub>2</sub>. Cells were digested with 0.25% trypsin-EDTA and sub-cultured at 80% to 90% confluence.

Exponentially growing cells were used for all assays.

## 2.3 Cytotoxicity assays

The cytotoxicity of HBCD to A549 cells was assessed by the MTT assay. A549 cells were seeded in 96-well plates ( $5 \times 10^3$  cells per well) and incubated at 37°C with a humidified atmosphere containing 5% CO<sub>2</sub> for 24 hours. Then, cells were treated with HBCD at various concentrations (0.1, 1, 5, 10, 25, 50 and 100 µM; with six replicates at each concentration) for 24 hours. The percentage of viable A549 cells was calculated. Concentration-response curves were plotted and the half maximal effective concentration (EC<sub>50</sub>) values were then calculated using sigmoidal dose-response curve equation. The cytotoxicity of HBCD to HepG2/C3A cells was also evaluated by the CCK-8 assay using a commercial kit, according to the manufacturers' instructions (Dojindo Laboratories, Kumamoto, Japan). Briefly, HepG2/C3A



cells ( $2 \times 10^4$  cells per well) were cultured in a 96-well plate overnight and the cells were treated in six replicates with several concentrations (0.1, 1, 5, 10, 25, 50 and 75  $\mu\text{M}$ ) of HBCD in culture medium for 24 hours. The viability of cells in the CCK-8 assays, in terms of  $\text{EC}_{50}$  values, was calculated using the same approach as that used for the MTT assay. (Further details are available in Supplementary Material.)

## **2.4 HBCD- exposure experiments and sample preparation for metabolomics and transcriptomics**

### **2.4.1 A549 cells**

A549 cells were exposed to several HBCD concentrations: 2 nM (in the media, termed *Very low* dose group), 20 nM (termed *Low* dose group), 200 nM (termed *Medium* dose group) and 2  $\mu\text{M}$  (termed *High* dose group). In the control group, a concentration of 0.5% DMSO (v/v) was applied (used as a vehicle). Each dose group included six or eight replicates. After 24-hour exposure, cells were rapidly washed in PBS and then quenched in 60% methanol / 40% water (pre-cooled on dry ice). Cells were carefully scraped into the methanol/water solution and transferred into Eppendorf tubes for metabolite and RNA extraction. Further details of the cell harvesting can be found in Supplementary Material. To extract the metabolites, further solvents were added to the cell pellets (in 400  $\mu\text{L}$  methanol/water solution) to a final solvent ratio of methanol/chloroform/water of 1:1:0.9 (v/v/v). After vortexing and centrifuging, the mixture separated into two phases (upper polar phase and lower non-polar phase). For each sample, a 300  $\mu\text{L}$  aliquot of the polar phase was transferred into a 1.5mL Eppendorf tube and dried in a speed vac concentrator (Thermo Savant, Holbrook, NY) for 4 hours, then frozen at  $-80^\circ\text{C}$  until analysis. The non-polar aliquots were not used in this study. Further details of the extraction procedure can be found in Supplementary Material.

Total RNA of quenched cells was isolated using Qiagen's mini RNeasy Kit and QIAshredder (Qiagen, Crawley, UK) according to the manufacturer's protocol. RNA was quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA), and the integrity of RNA was verified with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

#### **2.4.2 HepG2/C3A cells**

Similar methodologies were used to culture, expose and harvest HepG2/C3A cells as above, with some subtle modifications to maximise the recovery of RNA and metabolites.

Specifically, the HepG2/C3A cells were exposed to 4  $\mu$ M HBCD or 10  $\mu$ M 7,12-dimethylbenz[*a*]anthracene (DMBA) for 24 hours; 0.5% DMSO was used as a vehicle in all groups. Each treatment group consisted of six replicates. Following the exposures, the cells were harvested and the metabolites and RNA were extracted in a similar manner to that described above. The procedure is detailed in the Supplementary Material.

### **2.5 Metabolomics analyses**

#### **2.5.1 Direct infusion mass spectrometry (DIMS) analysis and data processing**

The dried polar extracts of cells were re-suspended in 150  $\mu$ L 80:20 (v/v) methanol:water (HPLC grade) with 20 mM ammonium acetate, vortexed and centrifuged at 14000 rpm, 4°C for 10 min. Each sample was loaded into three wells of a 96-well plate (10  $\mu$ L per well) and then analysed (in triplicate) using direct infusion Fourier transform ion cyclotron resonance mass spectrometry in negative ion mode (LTQ FT Ultra, Thermo Fisher Scientific, Germany, coupled to a Triversa nanoelectrospray ion source, Advion Biosciences, Ithaca, NY, USA). Mass spectra were recorded utilising the selected ion monitoring (SIM) stitching approach from *m/z* 70 to 590 (Southam et al 2007) and then processed using custom-written Matlab scripts as previously reported (Southam et al 2007; Kirwan et al 2014). In brief, time domain ('transient') data were collected, Fourier transformed and internally mass calibrated. Only

mass spectral peaks with a signal-to-noise ratio above 3.5 were retained. Mass spectra of the three technical replicates for each sample were filtered into a single peak list (with only those peaks present in  $\geq 2$  of the 3 spectra retained). Each filtered peak list (one per sample) was then further filtered to retain only those peaks that were present in 80% of all biological samples in the entire dataset, and missing values were imputed using the k-nearest neighbours (KNN) algorithm. The resulting matrices of peak intensity data (termed “DIMS dataset”) were normalised by the probabilistic quotient (PQN) method prior to statistical analyses.

### 2.5.2 Statistical analyses of metabolomics data

For univariate statistical analysis, one way ANOVA (or t-test) with Benjamini-Hochberg correction was conducted on the peaks in the normalised DIMS datasets to determine whether they changed intensity significantly between control and HBCD or DMBA treatment groups (at a false discovery rate (FDR)  $< 10\%$  to correct for multiple hypothesis testing). For multivariate statistical analysis, the normalized DIMS datasets were generalized log transformed and principal component analysis (PCA) performed using PLS\_Toolbox (Eigenvector Research, Wenatchee, USA) in MatLab (version 7, the Math-Works, Natick, USA). ANOVA (or t-test) with a Tukey-Kramer’s post-hoc test was conducted on the PC scores for the top few principal components from each model (with an FDR of  $< 10\%$ ) to evaluate the statistical significance of the treatments on the basis of the overall metabolic profiles.

### 2.6 Transcriptomic analysis

For RNA samples from A549 cells, six replicates (randomly selected from the total of eight replicates) were used for each of the HBCD treated groups (2 nM (*Very low*), 20 nM (*Low*), 200 nM (*Medium*) and 2  $\mu\text{M}$  (*High*)) while the solvent (DMSO) control group comprised of eight replicates. For RNA samples from HepG2/C3A cells, a total of 16 samples were subjected to microarray analyses, comprising six samples from the HBCD (4  $\mu\text{M}$ ) treated

group, six from the solvent (DMSO) control group, and the remaining four from the DMBA (10  $\mu$ M) positive control group.

The procedures for microarray analysis were performed following the manufacturer's protocols (Agilent Technologies, Santa Clara, CA). (Further details are available in Supplementary Material.) Microarray data processing and analyses were performed within GeneSpring version GX7.3.1 and GX11 (Agilent Technologies, Santa Clara, CA) and MultiExperimental Viewer v4.9 (Saeed et al 2003). The normalised microarray dataset was log<sub>2</sub> transformed and then subjected to Significance Analysis of Microarrays (SAM) (Tusher et al 2001) embedded in MultiExperimental Viewer v4.9 to identify significant differential gene expression between groups. The delta parameter was adjusted to achieve an FDR <5% and this delta value was applied to select significantly-regulated genes. Any significantly altered genes were then analysed using the Functional Annotation Clustering (FAC) tool contained in the Database for Annotation, Visualisation and Integrated Discovery (DAVID) (Huang et al 2009) to determine the most relevant pathways and processes based on the Gene Ontology annotation function, with FDR <5% selected for the significant functions. Gene set enrichment analysis (Subramanian et al 2005) was also applied to the above normalised microarray dataset to determine differentially expressed gene sets in HBCD or DMBA treated groups comparing to control. Microarray datasets are available in the ArrayExpress database under accession numbers: E-MTAB-2173 and E-MTAB-2174.

Quantitative real time-PCR (qPCR) analysis was performed to quantify the mRNA levels of a selected set of six genes in A549 or HepG2/C3A cells treated with HBCD or DMBA. (Details are available in Supplementary Material.)

## 2.7 Quantification of HBCD in A549 and HepG2/C3A cells and cell media

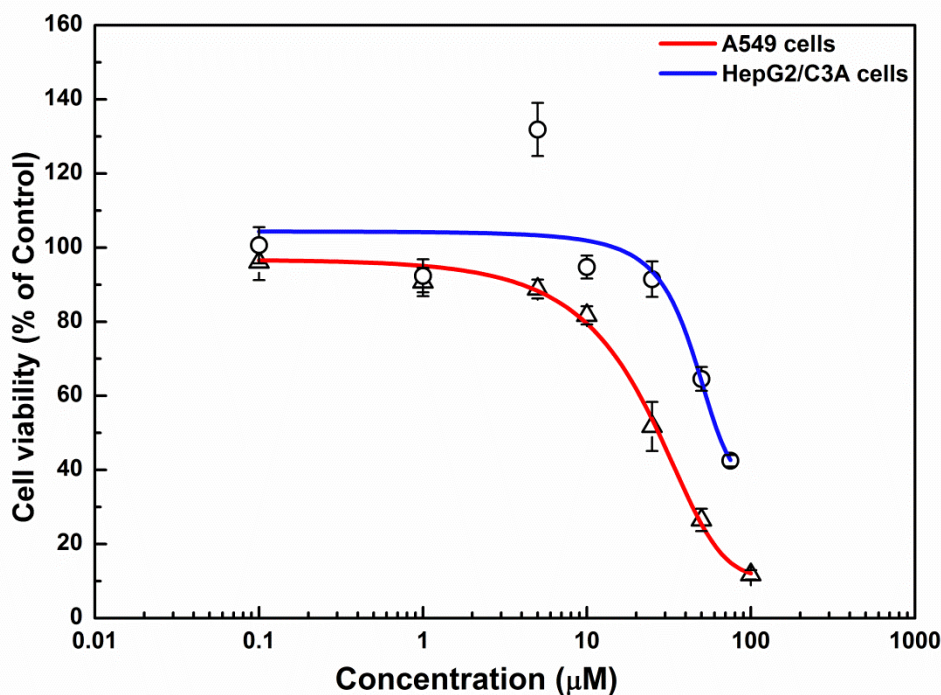
To measure the cellular uptake of HBCD after exposure, A549 and HepG2/C3A cells were exposed to 2  $\mu\text{M}$  and 4  $\mu\text{M}$  HBCD, respectively, in both FBS-containing and FBS-free media. Following 24-hour exposures, HBCD levels were quantified in both the cell pellets and media using the LC-MS/MS method (Van den Eede et al 2012). Briefly, 10 ng of each of  $^{13}\text{C}$ -labelled  $\alpha$ - and  $\beta$ -HBCDs and 25 ng  $^{13}\text{C}$ -labelled  $\gamma$ -HBCD (supplied in 10  $\mu\text{L}$  methanol) were added as internal (surrogate) standards while the samples were extracted from weighed cell pellets and cell media using liquid-liquid extraction with 3 mL of hexane:dichloromethane (DCM) (3:2) mixture in clean, stoppered, 10 mL centrifuge tubes. The clean extract was evaporated under a gentle stream of nitrogen and re-dissolved in 150  $\mu\text{L}$  of methanol containing 50  $\text{pg}/\mu\text{L}$  of  $\text{d}_{18}$ -labelled  $\alpha$ -HBCD used as a recovery determination (syringe) standard for QA/QC. Separation of the target analytes was achieved using a dual pump Shimadzu LC-20AB Prominence LC equipped with a SIL-20A autosampler, a DGU-20A3 vacuum degasser and an Agilent Pursuit XRS3  $\text{C}_{18}$  reversed phase analytical column (150 mm  $\times$  2 mm i.d., 3  $\mu\text{m}$  particle size). (Details of procedures can be found in Supplementary Material)

The concentrations of HBCD diastereomers in the samples were calculated according to the response factors of the respective  $^{13}\text{C}$ -labelled internal standard for each diastereomer. The recovery ratios were calculated from the total amount of  $\alpha$ -,  $\beta$ - and  $\gamma$ -HBCDs ( $\Sigma\text{HBCDs}$ ) in the cell pellets after exposure, divided by the nominal amount that was added into the exposure media. Average concentrations of  $\Sigma\text{HBCDs}$  detected in the cell media were also calculated where each treatment group included three biological replicates.

### 3. Results

#### 3.1 Cytotoxicity of HBCD to A549 cells and HepG2/C3A cells

Concentration-dependent cytotoxicity of HBCD to A549 cells and HepG2/C3A cells after 24-hour treatments were observed (Fig. 1). At relatively low concentration ranges from 0 to 10  $\mu\text{M}$ , HBCD exhibited minimal cytotoxicity with the viability of HepG2/C3A cells remaining greater than 90%, while for the A549 cells the viability remained greater than 80%. HBCD was more toxic to A549 cells than to HepG2/C3A cells with  $\text{EC}_{50}$  values of 27.4  $\mu\text{M}$  and 63.0  $\mu\text{M}$ , respectively, in the presence of serum in the exposure media.



**Fig. 1. Cytotoxic effects of HBCD to A549 cells and HepG2/C3A cells.** Cell viability of A549 cells (triangle shape) and HepG2/C3A cells (round shape) was examined using the MTT assay and CCK-8 assay, respectively, after exposure of the cells to different concentrations (0-100  $\mu\text{M}$  for A549 cells; 0-75  $\mu\text{M}$  for HepG2/C3A cells) of HBCD for 24 hours. Results are expressed as the percentage (mean  $\pm$  standard deviation,  $n=6$ ) of cell viability compared to controls treated with the appropriate vehicle (DMSO).

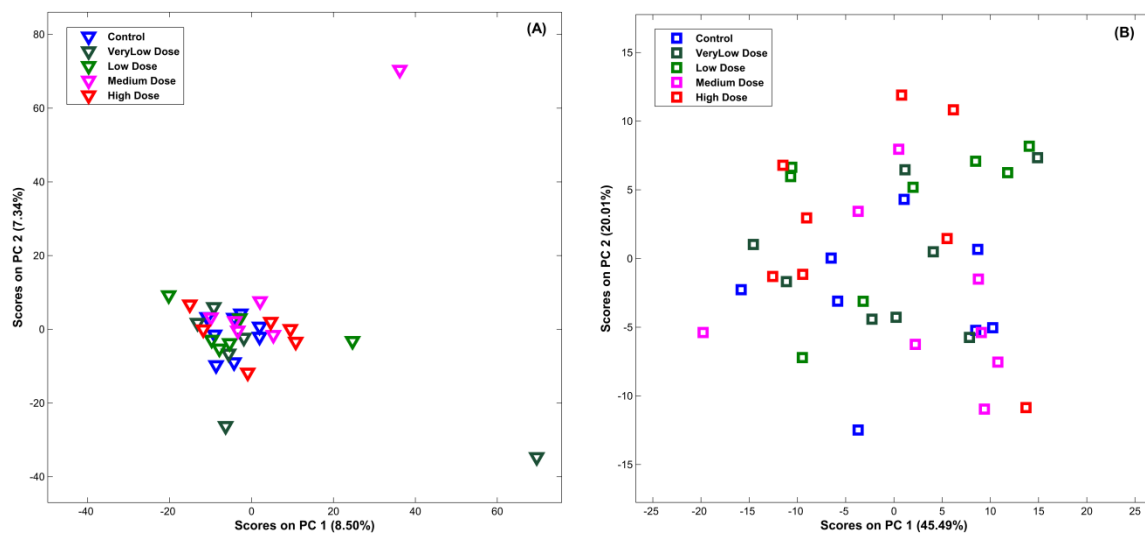
In order to explore potential molecular events in cells at sub-toxic concentrations, 2  $\mu\text{M}$  was selected as the highest concentration for exposure of A549 cells to HBCD, which was approximately 8% of  $\text{EC}_{50}$  (27.4  $\mu\text{M}$ ). Three additional treatment groups were included, each 10-fold lower in concentration. The experimental design therefore consisted of exposing A549 cells to HBCD at 2 nM, 20 nM, 200 nM and 2  $\mu\text{M}$ , with the appropriate vehicle (DMSO) as a control group, so as to maximise the relevance of our findings to likely human exposure levels. Following a similar strategy for the HepG2/C3A cells, 4  $\mu\text{M}$  was used as the exposure concentration for the 'omics' studies (which was also corresponded to approximately 8% of  $\text{EC}_{50}$ , 63.0  $\mu\text{M}$ ). DMBA at 10  $\mu\text{M}$  was employed as a reference treatment (positive control) for the gene expression study in HepG2/C3A cells. Although the 5  $\mu\text{M}$  HBCD exposure appeared to increase the HepG2/C3A cell viability (Figure 1), two further cytotoxicity assays were conducted at this concentration that showed that this increase was not significant compared to controls (data not shown).

### **3.2 Molecular responses of A549 cells exposed to HBCD**

#### **3.2.1 Gene expression profiles of A549 cells exposed to HBCD**

Oligonucleotide microarrays were employed to evaluate gene expression profiles of A549 cells after 24-hour exposures to four concentrations of HBCD, as detailed above. There was no clear separation of gene expression profiles between different treatment groups in A549 cells in PCA scores plot (Fig. 2A). The ANOVAs of the top four PC scores combined with a Tukey-Kramer's post hoc test (Table 1) also showed that there were no significant differences in the gene expression profiles between control and HBCD treated groups in A549 cells. Significance analysis of microarrays (SAM) found no significantly altered individual genes compared to controls, which is consistent with the results from the PCA of the gene

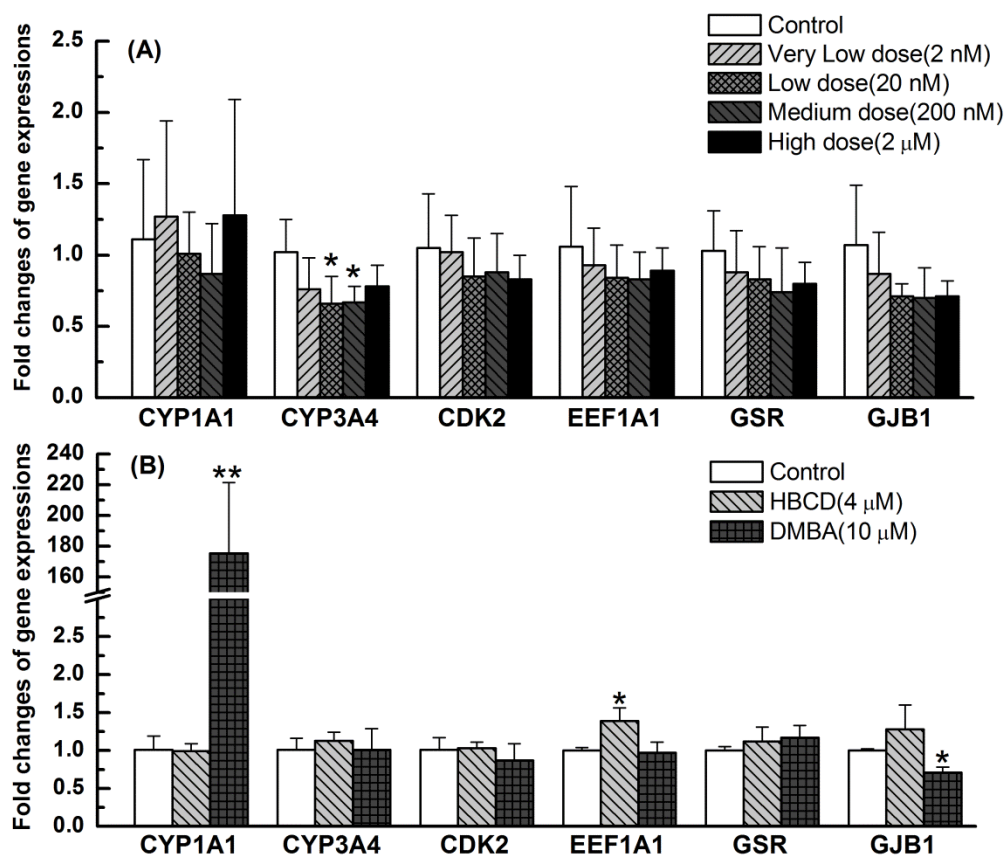
expression dataset. Gene Set Enrichment Analyses also indicated there were no significantly enriched gene sets involving KEGG pathways in HBCD treated groups (data not shown).



**Fig. 2. PCA scores plots of microarray dataset (A) and DIMS dataset (B) in A549 cells exposed to HBCD for 24 hours.** Legend: controls (blue), *Very low* dose (2 nM) group (dark green), *Low* dose (20 nM) group (green), *Medium* dose (200nM) group (pink) and *High* dose (2  $\mu$ M) group (red). Biological replication comprised n=6 (microarray) and n=8 (DIMS) in HBCD treatment groups; n=8 in the control group.

Quantitative RT-PCR analysis of six genes of interest (CYP1A1 and CYP3A4: related to xenobiotic metabolism, CDK2: cell division/growth, EEF1A1: protein biosynthesis, GSR: oxidative stress response, and GJB1: cell communication) revealed that only expression of CYP3A4 in A549 cells treated with 20 nM (*Low* dose) and 200 nM (*Medium* dose) HBCD was slightly down-regulated (Fig. 3A), while no significant changes were observed in the microarray analysis after exposure to the different concentrations of HBCD for 24 hours. The expression levels of the other five genes were not significantly altered, in agreement with the results of the microarray analysis.





**Fig. 3. qPCR analysis of mRNA expression of six selected genes in A549 cells (A) and HepG2/C3A cells (B) following HBCD or DMBA treatments for 24 hours.** Data were obtained from quadruplicate PCR reactions of four biological replicates. Differences to the solvent control were tested by one-way ANOVA or Student's t-test (\* $P < 0.05$ ). Results are presented as mean  $\pm$  standard deviation.

### 3.2.2 Metabolic responses of A549 cells exposed to HBCD

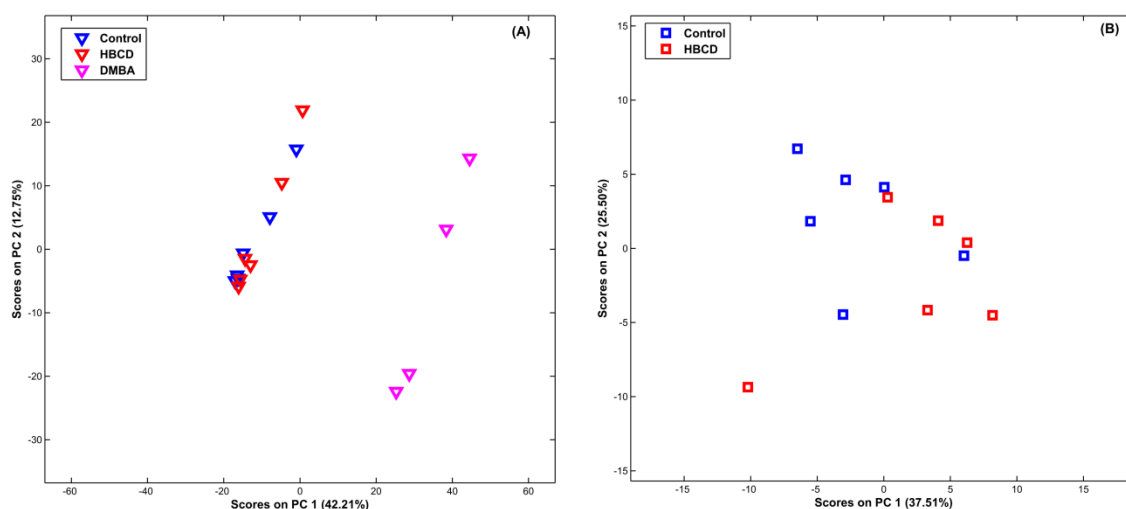
Polar metabolites extracted from HBCD-exposed A549 cells were analysed by direct infusion mass spectrometry based metabolomics. After spectral processing, the DIMS dataset comprised of a total of 1457 mass spectral peaks that were subjected to PCA to visualize the similarities and differences of the metabolic profiles between control and HBCD-treated groups. The PCA scores plot (Fig. 2B) showed no separation in the metabolic profiles measured from HBCD-treated and control groups for PC1 vs. PC2, an observation that was supported by ANOVA of the top four PC scores data and subsequent analysis using Tukey-

Kramer's post hoc tests. These results (Table 1) indicate no separation along PC1, PC2 and PC4, though HBCD-treated and control samples separated along the PC3 axis (representing 4.8% of variance in DIMS dataset) ( $p=0.005$ ). However, univariate statistical analyses of all 1457 peaks revealed that none changed significantly in response to HBCD across all four concentration groups ( $FDR < 10\%$ ).

### **3.3 Molecular responses of HepG2/C3A cells exposed to HBCD**

#### **3.3.1 Gene expression profiles of HepG2/C3A cells exposed to HBCD**

Since no significant molecular changes had been discovered in A549 cells after exposure to the subtoxic HBCD concentration, HepG2/C3A cells, representing a system with a higher xenobiotic metabolic activity, were exposed to 4  $\mu\text{M}$  HBCD (less than 10% of  $EC_{50}$ ) and 10  $\mu\text{M}$  DMBA (as a positive control to induce gene expression). DMBA is a polycyclic aromatic hydrocarbon with carcinogenic properties and a CYP1A1 -substrate. The PCA scores plot indicated no separation of the gene expression profiles between HBCD treated group and the control group, whereas the analyses distinguished marked differences between DMBA group and the other groups (Fig. 4A). The statistical significance of the top four PC scores was verified by a t-test; no significant differences between the HBCD and control group existed, but the DMBA and control samples were significantly different along PC1 (representing 53.1% of the variance in the dataset,  $p=8.62 \times 10^{-6}$ ). In addition, SAM of individual genes showed that there was no significant modulation of gene expression in the HBCD-treated group compared to the control group, while more than 300 probe sets changed in the DMBA treated group in HepG2/C3A cells (Table S1).



**Fig. 4. PCA scores plots of microarray dataset (A) and DIMS dataset (B) in HepG2/C3A cells exposed to 4  $\mu$ M HBCD and 10  $\mu$ M DMBA for 24 hours.** Legend: controls (blue), 4  $\mu$ M HBCD group (red) and 10  $\mu$ M DMBA group (pink). Biological replication comprised n=6 (microarray) and n=6 (DIMS) in the control group and HBCD treatment groups; n=4 in 10  $\mu$ M DMBA group (as positive control in microarray analysis).

The functional annotation tool (DAVID) was employed to determine the most relevant pathway(s) or process(es) involved in these significantly dysregulated genes in the DMBA-treated group identified by SAM. The results (Table S2) suggest that p53 signalling and apoptosis are the most significantly altered pathways in DMBA-treated HepG2/C3A cells. GSEA showed similar results (Table S3) that cytokine-cytokine receptor interaction, p53 signalling pathway, apoptosis and metabolism of xenobiotics by CYPs are the top enriched gene sets in the DMBA group, while no significant enrichment of gene sets was observed in the HBCD-treated groups. In HepG2/C3A cells, the mRNA expression of six selected genes was analysed by quantitative RT-PCR. This revealed similar trends as measured by microarray; including a remarkable up-regulation of CYP1A1 (175-fold,  $p < 0.0001$ ) and slight down-regulation of GJB1 (0.7-fold,  $p < 0.0001$ ) in the DMBA group, while there was no significantly altered expression of genes in cells treated with 4  $\mu$ M HBCD, except a slightly increased expression of EEF1A1 (1.4-fold,  $p = 0.004$ ) (Fig. 3B).

### 3.3.2 Metabolomics analysis of HepG2/C3A cells exposed to HBCD

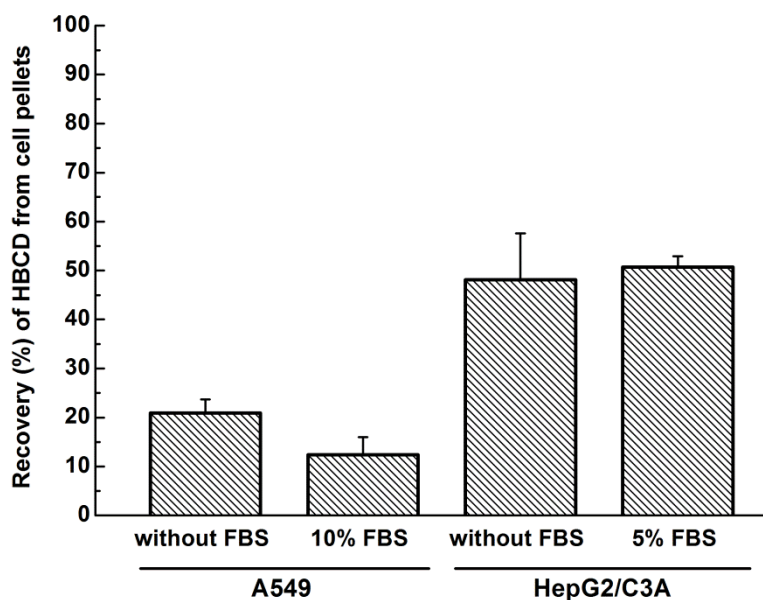
Metabolic profiles of HepG2/C3A cells exposed to 4  $\mu\text{M}$  HBCD were measured by DIMS. A total of 2128 mass spectral peaks were subjected to PCA and univariate analysis. No distinct separation was observed in the PCA scores plot (Fig. 4B) of which the top four PC scores were evaluated by t-test, confirming no significant differences (Table 1). Further t-tests on each of the 2128 individual peaks in the HBCD exposed group compared to the control group confirmed that there was no significant metabolic change in HepG2/C3A cells exposed to 4  $\mu\text{M}$  HBCD for 24 hours. However, after extended HBCD exposure to 72 hours, subtle metabolic changes were found in DIMS dataset. 85 (of total 3412) MS peaks significantly changed (Table S4) which is likely to be the results of extension of exposure time or the delayed effects of 24 hours exposure.

### 3.4 Uptake of HBCD into A549 and HepG2/C3A cells

The recovery of HBCD from cell pellets and the concentrations of HBCD in the cell media were evaluated, with the objective to facilitate comparisons between the concentrations of HBCD in the present study and those reported in human tissue and biofluids.

The results (Fig. 5) showed that when cultured in FBS-containing media, an average of 12.4% of the nominal amount of HBCD added to the exposure system was detected in A549 cell pellets, while in those cell pellets collected from the FBS-free media, approximately double the amount (20.9%) was measured in the cells. In HepG2/C3A cells, recoveries of HBCD from cell pellets exposed in FBS-containing or FBS free media were considerably higher, and 50.7% and 48.1% respectively. This suggested that the capacity for HepG2/C3A cells to uptake HBCD was higher than for A549 cells. As shown in Table 2, the average concentrations of HBCD in A549 cell pellets were 15.9 and 27.5  $\text{ng mg}^{-1}$  (wet weight) from FBS-containing exposure media and FBS-free media, respectively. However, the average concentrations of HBCD in HepG2/C3A cells from FBS-containing media and FBS-free

media were 96.2 and 99.0 ng mg<sup>-1</sup> (wet weight) respectively, which were much higher than in A549 cells.



**Fig. 5. Recovery of HBCD from cell pellets in A549 cells and HepG2/C3A cells exposed to HBCD for 24 hours.** A549 cells and HepG2/C3A cells were treated with 2  $\mu$ M and 4  $\mu$ M HBCD, respectively, in both serum (FBS)-containing media and serum-free media (without FBS). Recoveries are expressed as the percentage of detectable amount of HBCD from cell pellets after exposure, relative to the total nominal amount of HBCD added to the exposure media  $\pm$  standard deviation (n=3).

In addition to affecting uptake in A549 cells, the presence of FBS in the media also affected the post-exposure concentrations of HBCD in the cell media itself. In A549 cells, following the 24-hour exposures, the concentration of HBCD in 10% FBS-containing media was 1133.7 ng/mL (Table 2), which is more than 20 times higher than HBCD measured in the FBS-free media. There was a similar difference in HepG2/C3A cell media (Table 2) where the concentration of HBCD in 5% FBS-containing media were considerably higher than in FBS-free media (939.7 ng/mL versus 409.9 ng/mL, respectively).

#### 4. Discussion

The widespread use of HBCD has given rise to increasing health concerns following human exposure via various routes. Inhalation has been considered as one (albeit minor) pathway of human exposure to HBCD (Marvin et al 2011). There is therefore a particular interest in investigating effects in lung cells as this organ has relatively high exposure and in liver cells that play a critical role in the detoxification (or activation) of xenobiotic chemicals and in which protective compensatory changes are often seen. A549 lung epithelial cells and HepG2/C3A liver cells were thus selected as *in vitro* models to investigate the potential toxic effects of HBCD in this study.

The EC<sub>50</sub> value of cytotoxicity of HBCD to HepG2/C3A cells in this study (63.0 µM) was similar to the value observed in HepG2 cells exposed to HBCD (ca. 50 µM) (Hu et al 2009) ), with both studies conducted containing serum in the exposure media. Furthermore, in the immortalised human hepatocyte L02 cell line, the EC<sub>50</sub> for a 24-hour exposure to HBCD was reported as ca. 60 µM (An et al 2013). Hence, the EC<sub>50</sub> value for this hepatic cell line treated with HBCD is consistent with previously published values, for the case of exposures conducted in the presence of serum which more closely reflects the *in vivo* situation.

However, in the absence of FBS in the exposure media, the EC<sub>50</sub> values of HBCD were reported as being as low as ca. 3 µM in neuronal cell lines (Reistad et al 2006; Al-Mousa and Michelangeli 2012). The discrepancy between these reported EC<sub>50</sub> values might be explained by differences in the cell type or different levels of serum in the exposure media. The higher serum protein levels could result in a reduction in the free concentration of xenobiotic such as phenanthrene, a hydrophobic polycyclic aromatic hydrocarbon (PAH), accompanied by an increase in the EC<sub>50</sub> (Kramer et al 2012). Free concentrations of compounds are believed to be appropriate to characterise the toxic potency of a chemical *in vitro* which may be used for

*in vivo* extrapolation (Gülden et al 2013). In the present study, the concentrations of HBCD in cell pellets and cell media after exposure were quantified by LC-MS/MS. The lowest concentration of HBCD detected in A549 cell pellets after exposure was ca. 15 ng/mg cell pellet (wet weight) (Table 2), which is equal to 750,000 ng/g lipid (assuming 2% lipids in cell pellets which is similar to the lipid content in human liver (Rawn et al 2014)). This is several hundred times higher than the highest concentrations reported in human fetal liver (4,500 ng/g lipid, median value: 29 ng/g lipid) and placental tissue (5,600 ng/g lipid, median value: 49 ng/g lipid)(Rawn et al 2014). Thus, taking account of the EC<sub>50</sub> values of HBCD in cells (63 µM in HepG2/C3A cells in this study), the concentrations achieved in cells that cause cytotoxicity are vastly higher than those reported in human tissues. In addition, mitochondrial reductive activity (MTT assay) and the integrity of the cell membrane (AK assay) in proliferating cells were disrupted by 60 µM HBCD to a greater extent than in confluent cells (Fig. S1 a and b). However, cell protein content was not significantly changed in the group exposed to 60 µM HBCD for 24 hours compared to the control group in either proliferating cells or confluent cells (Fig. S1 c). This suggests that 60 µM HBCD has no significant effects on cell proliferation of HepG2/C3A cells but that proliferating cells were more susceptible to HBCD toxicity.

Molecular responses in terms of transcriptomic and metabolic levels were also assessed. Particular attention was given to cytochrome P450 monooxygenases (CYPs) that play critical roles in biotransformation activities and the induction of which is a common response to organic toxicants (Danielson 2002). Forty probe sets (representing 35 mRNAs of CYPs) were detected in the A549 microarray dataset (Table S5), whereas 43 probe sets (representing 33 mRNAs of CYPs) were detected in the HepG2/C3A microarray dataset (Table S6). CYP1A1 and CYP3A4 are both particularly important in phase I metabolism of aromatic hydrocarbons. The induction of CYP1A1 mRNA expression has been used as an indicator of

exposure to PAH (Castorena-Torres et al 2008). In the present study, HBCD did not induce mRNA expression for either of these CYP genes in A549 cells or HepG2/C3A cells after a 24-hour exposure in contrast to the marked induction of CYP1A1 (175-fold) observed in response to DMBA treatment in HepG2/C3A cells.

Several studies have reported that CYP3A4 is not expressed in A549 cells (Hukkanen et al 2000; Courcot et al 2012). However, in a recent study, the expression of CYP3A4 was induced by 5  $\mu$ M of the PAH benzo[*a*]pyrene (B[*a*]P) in A549 cells exposed for 14 hours, but not in HepG2 cells (Genies et al 2013). Fery et al. (2009) also reported that HBCD failed to induce CYP3A enzyme activity in HepG2 cells, but a significant induction was observed in primary cultured rat hepatocytes. In this study, mRNA expression of CYP3A4 was detected in both A549 and HepG2/C3A cells, and this was slightly down-regulated after a 24-hour exposure to 20 nM and 200 nM HBCD in A549 cells while there were no significant changes in HepG2/C3A cells. These observations show a noticeable contrast between HBCD and DMBA in the induction of CYP expression.

The effects observed with DMBA are markedly different from those following HBCD exposure despite the latter showing intracellular accumulation. The extensive modification of the transcriptome as seen with DMBA includes evidence of a significant stress response at sub-toxic concentrations. These responses include the changes in CYP expression as mentioned above plus a range of other responses in the expression of genes involved in (e.g. apoptosis, regulation of cell proliferation, etc.). Neither two cell types showed this stress response to HBCD. This might be interpreted as a failure to react in a compensatory mode for protective purposes since no such response is seen right up to the concentrations that exhibit a toxic effect in the MTT assay or CCK-8 assay.



Few detectable transcriptomic and metabolomic responses after 24 hrs exposure might be due to missing the time window of some defensive or protective events. It has been reported that levels of CYP1A1 mRNA in A549 and HepG2 cells were modulated by B[a]P at an early stage of exposure (less than 4 or 6 hours) but not beyond (Genies et al 2013). Likewise, molecular responses after a relatively longer exposure may reflect chronic toxicity. van der Ven et al.(2006) reported that 28 days exposure to HBCD at a relatively high dose (30 mg/kg/day) increased liver weight in female rats and induced hepatic CYP enzyme activities (CYP2B1 and CYP3A1) in rats (Germer et al 2006; Cantón et al 2008). However, a 72-hour exposure at relative low concentration (2 to 200 nM) did not significantly change the survival rate and the malformation rate in zebrafish embryos (Wu et al 2013). In the present study, additional DIMS analysis suggested possible metabolic changes (e.g. decrease of taurine) after 72hrs exposure in HepG2/C3A cells exposed to 4  $\mu$ M HBCD, which might be responsive to stress after longer exposure or the delayed effects of 24 hours exposure.

## 5. Conclusions

'Omics' approaches were used to determine the potential molecular changes in two cell lines after exposure to HBCD over a wide range of concentrations in the presence of serum. Few molecular changes were observed in the 'omics' data despite employing cellular concentrations of HBCD up to several hundred times higher than those reported in human tissues and concentrations that approached cytotoxic levels. In contrast to the findings for the PAH, there was little evidence of a compensatory stress response to HBCD such as elevation of CYP or antioxidant systems. This lack of compensatory response does not equate to a lack of protection since cytotoxicity (mitochondrial dysfunction and loss of cell integrity) was only observed in HepG2/C3A cells exposed to a high concentration of HBCD (60  $\mu$ M), at which the cellular concentrations achieved exceeded, by several orders of magnitude, the tissue concentrations observed in environmentally exposed organisms.

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## FIGURE LEGENDS

**Fig. 1. Cytotoxic effects of HBCD to A549 cells and HepG2/C3A cells.** Cell viability of A549 cells (triangle shape) and HepG2/C3A cells (round shape) was examined using the MTT assay and CCK-8 assay, respectively, after exposure of the cells to different concentrations (0-100  $\mu$ M for A549 cells; 0-75  $\mu$ M for HepG2/C3A cells) of HBCD for 24 hours. Results are expressed as the percentage (mean  $\pm$  standard deviation, n=6) of cell viability compared to controls treated with the appropriate vehicle (DMSO).

**Fig. 2. PCA scores plots of microarray dataset (A) and DIMS dataset (B) in A549 cells exposed to HBCD for 24 hours.** Legend: controls (blue), *Very low* dose (2 nM) group (dark green), *Low* dose (20 nM) group (green), *Medium* dose (200nM) group (pink) and *High* dose (2  $\mu$ M) group (red). Biological replication comprised n=6 (microarray) and n=8 (DIMS) in HBCD treatment groups; n=8 in the control group.

**Fig. 3. qPCR analysis of mRNA expression of six selected genes in A549 cells (A) and HepG2/C3A cells (B) following HBCD or DMBA treatments for 24 hours.** Data were obtained from quadruplicate PCR reactions of four biological replicates. Differences to the solvent control were tested by one-way ANOVA or Student's t-test (\*P <0.05). Results are presented as mean  $\pm$  standard deviation.

**Fig. 4. PCA scores plots of microarray dataset (A) and DIMS dataset (B) in HepG2/C3A cells exposed to 4  $\mu$ M HBCD and 10  $\mu$ M DMBA for 24 hours.** Legend: controls (blue), 4  $\mu$ M HBCD group (red) and 10  $\mu$ M DMBA group (pink). Biological replication comprised n=6 (microarray) and n=6 (DIMS) in the control group and HBCD treatment groups; n=4 in 10  $\mu$ M DMBA group (as positive control in microarray analysis).

**Fig. 5. Recovery of HBCD from cell pellets in A549 cells and HepG2/C3A cells exposed to HBCD for 24 hours.** A549 cells and HepG2/C3A cells were treated with 2  $\mu$ M and 4  $\mu$ M

HBCD, respectively, in both serum (FBS)-containing media and serum-free media (without FBS). Recoveries are expressed as the percentage of detectable amount of HBCD from cell pellets after exposure, relative to the total nominal amount of HBCD added to the exposure media  $\pm$  standard deviation (n=3).

**Table 1. Summary of the principal component (PC) analyses of both microarray and DIMS datasets of A549 cells and HepG2/C3A cells following exposure to HBCD.**

Dataset		PC1	PC2	PC3	PC4
A549_Microarray_24h	variance	8.50%	7.34%	6.17%	5.66%
	p value	0.777	0.114	0.924	0.817
A549_DIMS_24h	variance	45.49%	20.01%	4.87%	4.11%
	p value	0.943	0.089	<b>0.005</b>	0.7
HepG2/C3A_Microarray_24h	variance	42.21%	12.75%	9.15%	6.36%
	p value	<b>2.90E-07</b>	0.502	0.272	0.962
HepG2/C3A_DIMS_24h	variance	37.51%	25.50%	9.95%	6.20%
	p value	0.251	0.14	0.78	0.873

(Groups in A549 cells: Control, *Very Low* dose, *Low* dose, *Medium* dose and *High* dose; groups in HepG2/C3A cells: Control, HBCD and DMBA). The variance explained (%) by PCs 1-4 are listed together with p values from ANOVAs (or t-tests) of these PC scores across all treatments (emboldened values indicate significance at a false discovery rate (FDR) <10%).

**Table 2. Concentration of  $\Sigma$ HBCDs in cell pellets and cell media of A549 and HepG2/C3A cells after 24-hour exposure**

Cell types	Types of Exposure Media	Nominal concentration of $\Sigma$ HBCDs in exposure media	Average wet weight of cell pellets (mg)	Concentration of $\Sigma$ HBCDs in cell pellets (ng/mg)	Concentration of $\Sigma$ HBCDs in cell media after exposure (ng/mL)
<b>A549</b>	No FBS	2 $\mu$ M (1283 ng/mL)	19.8 $\pm$ 3.3	27.5 $\pm$ 5.0	46.9 $\pm$ 7.4
	10% FBS	2 $\mu$ M (1283 ng/mL)	19.8 $\pm$ 4.0	15.9 $\pm$ 1.7	1133.7 $\pm$ 26.8
<b>HepG2/C3A</b>	No FBS	4 $\mu$ M (2566 ng/mL)	25.4 $\pm$ 3.1	99.0 $\pm$ 26.0	409.9 $\pm$ 77.8
	5% FBS	4 $\mu$ M (2566 ng/mL)	27.2 $\pm$ 2.8	96.2 $\pm$ 10.5	939.7 $\pm$ 124.3

### Highlights

1. Human cells lines were exposed to HBCD at sub-lethal concentrations for 24 hrs.
2. Molecular response was studied by unbiased transcriptomic and metabolomic approaches.
3. Few significant molecular changes were detected after 24 hrs exposure.
4. The levels of HBCD in cells were quantified using LC-MS/MS method.