Uptake and biotransformation of 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) in four marine microalgae species

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Hydroxylated- and methoxylated- polybrominated diphenyl ethers (OH-PBDEs and MeO-PBDEs) are more toxic than PBDEs and occur widely in the marine environment, and yet their origins remain controversial. In this study, four species of microalgae (Isochrysis galbana, Prorocentrum minimum, Skeletonema grethae and Thalassiosira pseudonana) were exposed to BDE-47, which is synthetic and is the predominant congener of PBDEs in the environment. By chemical analysis after incubation of 2 to 6 days, the efficiency of uptake of BDE-47 and, more importantly, the potential of undergoing biotransformation to form OH-PBDEs and MeO-PBDEs by the microalgae were investigated. Growth rates of these axenic microalgae were not affected upon exposure to environmentally relevant concentrations (0.2–20 μg BDE-47 L⁻¹), and accumulation ranged from 0.772 ± 0.092 μg BDE-47 g⁻¹ lipid to 215 ± 54 μg BDE-47 g⁻¹ lipid within 2 days. Debromination of BDE-47 and formation of BDE-28 occurred in all microalgae species (0.01 to 0.87%), but biotransformation to OH-PBDEs was only found in I. galbana upon exposure to extremely high concentration. The results of this study showed that biotransformation of microalgae species is unlikely an explanation for the OH-PBDEs and MeO-PBDEs found in the marine environment.

Due to their wide use as brominated flame retardants, polybrominated diphenyl ethers (PBDEs) have emerged as ubiquitous environmental contaminants. PBDEs and their analogs, hydroxylated- and methoxylated-PBDEs (OH-PBDEs and MeO-PBDEs), have been recorded in various marine biota ranging from cyanobacteria¹, macroalgae¹,⁻², bivalves²,⁻³, fish⁴ to cetaceans⁵. They also have a wide geographic distribution¹⁻⁶,⁻¹². Despite the fact that there is no known anthropogenic source of these analogues¹³, great environmental concentrations have been reported. Total MeO-PBDEs of 3.76 μg g⁻¹ wet mass was found in the blubber of pygmy sperm whale¹⁴. In environmental samples, including the red macroalga¹, mollusks⁸, fish⁴,⁻¹¹, as well as seals and their pups⁹, OH-PBDEs generally co-exist with MeO-PBDEs. The most commonly found congeners of these two groups of PBDE analogs are 6-MeO-BDE-47 and 6-OH-BDE-47, respectively¹⁰,⁻¹²,⁻¹³. Known effects of OH-PBDEs included developmental neurotoxicity, as seen in zebrafish associated with oxidative stress and potentially disrupted cholinergic system¹⁶; genotoxicity, as shown by ROS induction in chicken DT40 cell lines¹⁷; and endocrine disruption, as demonstrated in mouse reporter gene activity assay¹⁸ and rat in vivo experiment¹⁹. In general, OH-PBDEs tend to be more potent than MeO-PBDEs and PBDEs¹⁹⁻²¹. For instance, 6-OH-BDE-47 has been found to cause dysregulation of calcium ion in cortical neurons by activation of ryanodine receptors (RyR) more readily than BDE-47²⁰. 6-OH-BDE-47 also induced a greater amount of ROS and caused more damage to DNA in human hepatoma cell line (HepG2) when compared with 6-MeO-BDE-47²². Despite the higher toxicity comparing with PBDEs and widespread occurrence in the marine environment, OH-PBDEs and MeO-PBDEs have their origins being uncertain and controversial. Some studies suggested that they could be naturally synthesized, while others suggested that they could be products of biotransformation from synthetic PBDEs²³. It has long been known that natural biogenic processes in the marine environment commonly produce a variety of organohalogen compounds²⁴. In particular, marine sponges are known to be able

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to produce various organohalogens including MeO-PBDEs, either by their own tissue or by the associated cyanobacteria within them\(^\text{25}\). The great diversity of brominated organic compounds existing in the wild tends to support natural origins of these compounds because there is a lack of relevant PBDE precursors. Industrial processes would not have produced, or led to formation of, all of the discovered brominated compounds. Natural origins of MeO-PBDEs are further supported by the identification of related radiocarbon compounds from True beak's whales\(^\text{26}\) and in sponge-cyanobacteria associations\(^\text{27}\), since radiocarbon could only be formed in nature but not in industrial processes. Disproval of biotransformation as the likely origin was also provided by the temporal trends of 6-MeO-BDE-47 and 2′-MeO-BDE-68 in pike from Swedish waters from 1967 to 2000, which were different from the trend of PBDEs production. Greatest concentrations of the MeO-PBDEs were detected in fish collected before 1970 while concentrations of PBDEs showed an increasing trend only up to the mid-1980s\(^\text{28}\).

Alternatively, results of other studies have shown the possibility of biotransformation of PBDEs to OH-PBDEs and MeO-PBDEs. For instance, human CYP2B6 has been found to be able to transform anthropogenic BDE-47 into 6 congeners of OH-PBDEs\(^\text{29}\). Likewise, in vivo study using mice dosed with DE-71 (a commercial mixture of PBDEs) have also found OH-PBDEs as metabolites in blood plasma\(^\text{30}\), and seven congeners of MeO-PBDEs have been detected as metabolites in blood and liver of rainbow trout (Oncorhynchus mykiss) after exposure to deca-PBDEs\(^\text{31}\). It has been suggested that bacterial O-methylation of PBDEs, or the OH–PBDEs from hepatic metabolism of PBDEs, might explain the formation of MeO-PBDEs\(^\text{32}\) because such a mechanism was the major route of biodegradation of phenols in the environment\(^\text{33}\). In addition, there could be interconversion between OH-PBDEs and MeO-PBDEs. For instance, Japanese medaka has been shown to be able to transform OH-PBDEs to MeO-PBDEs and vice versa, and that demethylation of MeO-PBDEs took place, at a faster rate than hydroxylation of PBDEs, to form OH–PBDEs\(^\text{34}\). Since such biotransformation should result in lesser concentrations of OH-PBDEs and MeO-PBDEs and since biogenic production of these analogs has only been found in a few marine species, the high concentrations of OH–PBDEs and MeO-PBDEs in animals at high trophic level is difficult to explain.

It has been suggested that an unidentified source of planktonic organisms with high turn-over rates could be responsible for the high concentrations of OH-PBDEs and MeO-PBDEs found in the ocean, because the enriched \(^{14}\)C radiocarbon accumulated along the trophic level could only be acquired from surface water rather than from local hydrodynamics exposed by sessile marine sponges as suggested by many researchers\(^\text{37}\). Phytoplankton (or microalgae), which are abundant in surface waters and have a large surface to volume ratio, can be expected to accumulate PBDEs from water. They also play an important role in bioaccumulation of organic pollutants along the aquatic food chain. Despite this, uptake of PBDEs by microalgae has only been reported by a single study which investigated the efficiency of removal of PBDEs by freshwater microalgae in sewage treatment effluents\(^\text{35}\). The mechanism of passive uptake of POPs into microalgae can be described by a two-compartment model – immediate adsorption onto cell wall of the microalga followed by absorption (diffusion into the cell matrix with a mechanism similar to partitioning)\(^\text{36,37}\). However, whether microalgae could metabolize PBDEs and transform them into OH-PBDEs and MeO-PBDEs remain unknown. The significant correlations found between alkenones (a phytoplankton biomarker) and OH-PBDEs and MeO-PBDEs in surface sediment might suggest coccolithophorid species is a production source of 6-OH-BDE-47 and 6-MeO-BDE-47\(^\text{38}\).

This study was conducted to determine whether microalgae could synthesize OH-PBDEs and MeO-PBDEs by a de novo production or b) biotransformation of PBDEs acquired from the environment. Four species of marine microalgae from different taxonomic groups were selected for experiment, including the Haptophyceae Isochrysis galbana, the Dinophyceae Prorocentrum minimum, the Bacillariophyceae Skeletonema grethae and the Bacillariophyceae Thalassiosira pseudonana. The uptake efficiencies of BDE-47 in these four different microalgae were also determined. To the best of our knowledge, this is the first attempt to investigate whether microalgae, as primary producers in the aquatic environment, could be a source of OH-PBDEs and MeO-PBDEs (from de novo synthesis and/or biotransformation of anthropogenic PBDEs) in the marine environment.

Results  

Growth of microalgae exposed to BDE-47.  

Compared with the respective controls, similar growth with actively dividing stage were found in all species upon exposure to BDE-47 from day 0 to day 6 (Fig. 1), except negative growth was found in I. galbana on day 3 (F\(_{5,2}\) = 18.920, \(p < 0.001\)), day 4 (F\(_{4,2}\) = 87.115, \(p < 0.001\)), and day 6 (F\(_{3,2}\) = 14.895, \(p < 0.001\)) upon exposure to extreme high concentration of BDE-47 (2000 \(\mu\text{g L}^{-1}\)).

Uptake and bioaccumulation of BDE-47 into microalgae.  

In all species tested, uptake of BDE-47 was positively related to the exposure concentrations. For example, uptake was 5\(\mu\text{g g}^{-1}\) lipid in the 2 \(\mu\text{g L}^{-1}\) treatment and 50\(\mu\text{g g}^{-1}\) lipid in the 20 \(\mu\text{g L}^{-1}\) treatment in S. grethae on day 2 (Fig. 2). It was only in I. galbana exposed to 2000 \(\mu\text{g L}^{-1}\) where uptake concentration was not as expected due to the lack of intact cells collected for measurement. Concentrations of BDE-47 in all species decreased from day 2 to day 6. As the algal cells multiplied and biomass increased from day 2 to day 6, the lipid content was likely to increase. If the total uptake amount of BDE-47 reached the maximum by day 2, increases in biomass and lipid content on subsequent days would result in reduced lipid-normalized concentration in algal cells. The background concentration of BDE-47 in filtered seawater used in this study was 0.087 ± 0.023 \(\mu\text{g L}^{-1}\), whereas targeted OH-PBDEs and MeO-PBDEs were not detected.

The percentage of BDE-47 accumulated was greatest in I. galbana, which ranged from 56 ± 13% to 76 ± 19% excluding the extreme great concentration (Table 1). Only species (F\(_{3,2}\) = 62.179, \(p < 0.001\), but not number of days of exposure (F\(_{2,32}\) = 0.741, \(p = 0.485\), had a significant effect on the uptake percentages when exposed to 2\(\mu\)g BDE-47 L\(^{-1}\) (Two-way ANOVA). The results of the Holm-Sidak test showed that besides S. grethae and T. pseudonana, other species exhibited different uptake percentages (\(p < 0.05\)).
The log of the lipid-normalized bioaccumulation factors (log BAFs) ranged from 3.87 ± 0.01, 3.40 ± 0.10, 3.35 ± 0.09, and 3.26 ± 0.13 for *I. galbana*, *P. minimum*, *S. grethae*, and *T. pseudonana*, respectively. Log BAF of *I. galbana* was significantly greater than all other algal species (one-way ANOVA, $F_{3, 8} = 33.505$, $p < 0.001$).

**Biotransformation of BDE-47 into PBDE analogs.** While all of the targeted analysts besides BDE-47 were absent in the BDE-47 stock used, BDE-28 was found in all four microalgae species after exposure to the greater treatment concentrations of BDE-47 (Fig. 3). The greatest conversion percentages to BDE-28 was found in *S. grethae* (0.58–0.87%), followed by *I. galbana* (0.22–0.36%) and *P. minimum* (0.24–0.45%). The least conversion occurred in *T. pseudonana* (0.01–0.04%) (Table 2). 2′-OH-BDE-28, 5-OH-BDE-47, and 6-OH-BDE-47 were only found in *I. galbana* exposed to 2000 μg BDE-47 L$^{-1}$. Concentrations of OH-PBDEs were small when compared

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**Figure 1.** Algal density of *I. galbana*, *P. minimum*, *S. grethae*, and *T. pseudonana* over time upon exposure to BDE-47 at concentrations of 0.2, 2, 20, 200 and 2000 μg L$^{-1}$. Microalgae were cultured using F/2 medium prepared with autoclaved filtered seawater and maintained in environmental chamber (14:10 light/dark cycle; 25°C) in culture flasks ($n = 3$). Error bars showing the standard deviation. An asterisk (*) is placed adjacent to the plot where there was significant difference in algal growth when compared to control on the same time point (Holm-Sidak test, $p < 0.05$). C: Control; SC: Solvent control (0.01% DMSO).
with BDE-28. Conversion percentages of OH-PBDEs were about 0.003%, 0.012%, and 0.005% for 2′-OH-BDE-28, 5-OH-BDE-47, and 6-OH-BDE-47 respectively (Table 3). MeO-PBDEs were not detected in any of the samples.

Discussion
The results from this study show that BDE-47 can be readily taken up by all four species of microalgae, either by adsorption and absorption. In just two days, uptake efficiencies of BDE-47 into microalgae had reached $76 \pm 19\%$ for the flagellates *I. galbana* and $68 \pm 6\%$ for the dinoflagellates *P. minimum*, while the diatoms *S. grethae* and *T. pseudonana* exhibited lesser uptake efficiencies (ca. 31%). This result was consistent with the finding that bioaccumulation factors (BAF) of PBDEs by *P. minimum* being greater than that of *Thalassiosira* species\(^3\). This could have been due to polysaccharides in cell walls of diatoms\(^4\), which are produced in larger amounts than by the other phytoplankton species\(^5\). The hydrophilic polysaccharides could reduce the amount of lipophilic BDE-47 adsorbed to cells, thus resulting in lesser bioaccumulation. There is only limited information on concentrations of PBDEs in marine microalgae, except that $0.023 \mu g \, BDE-47 \, g^{-1} \, lipid$ was reported in *Thalassiosira* species from the South Pole\(^6\). The concentration of BDE-47 accumulated ($4.7 \mu g \, g^{-1} \, lipid$) by *T. pseudonana* in this experiment was 100-fold greater than the reported concentration by Chiuchiolo *et al.*\(^7\). However, a more appropriate

Figure 2. Concentrations of BDE-47 ($\pm$ SD) in *I. galbana*, *P. minimum*, *S. grethae*, and *T. pseudonana* after exposure to BDE-47 at 0.2 to 2000 $\mu g \, L^{-1}$ for 2, 4 and 6 days. *N* = 3. Error bars showing the standard deviations. C: Control; SC: Solvent control (0.01% DMSO).
comparison with contaminated area is not possible since no other studies have reported concentration of PBDEs in microalgae from marine environments (also reviewed in Kosek et al.25). The log BAFs for BDE-99 obtained during a 2-day incubation for P. minimum and Thalassiosira weissflogii were approximately 8.539, which means BAFs were several orders of magnitude greater than those observed in this study for BDE-47.

Both 6-MeO-BDE-47 and 6-OH-BDE-47 were absent from the controls of all species used in this study, showing that these four species of microalgae could not produce these PBDE analogs naturally in the absence of PBDE as the precursor. Neither OH-PBDEs nor MeO-PBDE was detectable in filtered seawater used for experiments. The result of this study therefore did not support the self-production or biotransformation of 6-MeO-BDE-47 by phytoplankton. However, 2'-MeO-BDE-68 and 6-MeO-BDE-47 were detected at pg g⁻¹ dry mass concentrations in axenic cultures of Chaetoceros curvisetus, Prorocentrum donghaiense, and Emiliania huxleyi44, which suggested that biosynthesis may be possible, at least in some species. The difference in findings might be due to the differences in algal species, experimental protocol (in which seawater was only filtered through 0.45 μm, allowing passing of certain bacteria that may perform biotransformation45) and source of seawater (in which the background levels of PBDEs and their metabolites in the seawater obtained from the East China Sea were not analyzed in their study). Concentrations of PBDEs in seawater in the East China Sea has not been measured previously46, but the same MeO-PBDE congeners in the microalgae cultures in Fan et al.47 were also found at concentrations of 0.001–0.022 μg L⁻¹ in seawater in the Fanshun area. This might not directly confirm the presence of MeO-PBDEs or PBDEs in seawater because these pollutants may be transported through trophic transfer, their presence in natural seawater used for algal culture in their study cannot be ruled out. Mean concentrations of 2'-MeO-BDE-68 and 6-MeO-BDE-47 in seawater in Queensland were estimated to be 41 and 58 pg L⁻¹ (by passive samplers)48, and the presence of MeO-PBDEs in water cannot be ruled out because the concentration might be too low to be detected by solvent-extraction.

Since algal cells were actively dividing during incubation, the presence of BDE-28 in some of the cultures would support the assumption that BDE-47 had been available for metabolism within algal cells. The detection of BDE-28 in cells exposed to greater concentrations of BDE-47 (1 μg L⁻¹ for P. minimum and 10 μg L⁻¹ for others) demonstrated that debromination had taken place during the incubation. BDE-28 was only detected on day 4 and 6 in P. minimum while it occurred at all sampling time points for the other three species. In nature, debromination is a common process to breakdown PBDEs, and light could accelerate debromination rate49. The tetra-brominated BDE-47 itself in the environment was assumed to be formed by debromination from more brominated congeners, such as the decabrominated BDE-209, derived from flame retardants. However, in both field and laboratory situations, light must be available to support growth and metabolism of microalgae and as such the possibility of photo-debromination could not be eliminated. A recent study demonstrated that tolerant freshwater microalgae isolated from wastewater treatment plants can degrade 68–86% of tetra-brominated BDE-47 in 7 days55, suggesting that removal of BDE-47 by marine microalgae could also be possible.

The presence of OH-PBDEs at the extreme high concentration of BDE-47 (2000 μg L⁻¹) in I. galbana indicated potential capability for this species to metabolize PBDEs into OH-PBDEs by biotransformation. The metabolite 6-OH-BDE-47 has also been found in bile of crucian carp exposed to the hexa-brominated BDE-15356. Although the tetra-brominated 5-OH-BDE-47 and tri-brominated 2'-OH-BDE-28 were not congeners targeted

| Day | Concentration (μg L⁻¹) | L. galbana | P. minimum | S. grethae | T. pseudonona |
|-----|-----------------------|------------|------------|------------|--------------|
| Day 2 | 0.2 | 65 ± 7% | 50 ± 4% | 39 ± 0% | 27 ± 4% |
|     | 2   | 76 ± 19% | 36 ± 3% | 29 ± 3% |               |
|     | 20  | 71 ± 8% | 4 ± 2% |            |              |
|     | 200 | 17 ± 2% |            |              |              |
|     | 2000 | —     |            |              |              |
| Day 4 | 0.2 | 69 ± 14% | 43 ± 5% | 31 ± 4% | 31 ± 8% |
|     | 2   | 71 ± 21% | 27 ± 2% | 28 ± 12% |               |
|     | 20  | 58 ± 17% |            |              |              |
|     | 200 | 5 ± 3%  |            |              |              |
| Day 6 | 0.2 | 69 ± 9% | 49 ± 5% | 31 ± 2% | 37 ± 2% |
|     | 2   | 56 ± 13% | 27 ± 4% | 34 ± 3% |               |
|     | 20  | 64 ± 13% |            |              |              |
|     | 200 | 105 ± 8%|            |              |              |

Table 1. Uptake percentages (±SD; n = 3; corrected to the nearest 1%) of BDE-47 from nominal concentrations of 0.2, 2, 20, 200, and 2000 μg L⁻¹ in the phytoplankton species L. galbana, P. minimum, S. grethae, and T. pseudonona. Results with significant difference (Two-way ANOVA, p < 0.05) among microalgae species are represented by different alphabets.
in their study, OH-tetra-BDE and OH-tri-BDE with unknown structures were shown in their GC-MS data. Hydroxylation of PBDEs could be catalyzed by enzymes of the CYP family in mammals, and genes encoding for CYPs have also been found in single-celled diatoms. Nonetheless, further mechanistic studies such as pathway analysis are necessary to explain why biotransformation only occurred at such high concentration and when growth rate was inhibited.

The finding of OH-PBDEs in this study must be interpreted with caution due to very low concentrations of metabolite found. In contrast to this study, it was found that common sole probably produced 4′-OH-BDE-49 and 4′-OH-BDE-101 as metabolite products after PBDE exposure, while 6-OH-BDE-47 in the fish was mainly of biogenic nature. Nonetheless, the fact that no OH-PBDE was found in both controls and treatments at lower concentrations showed that OH-PBDEs were not produced by biogenesis in *I. galbana*. The majority of OH-PBDEs found in biota were believed to be transformed by demethylation from MeO-PBDEs within the organisms. Direct biotransformation (hydroxylation) from BDE-47 to OH-BDE-47 with debromination before or after the transformation might therefore be the possible mechanism. Hydroxylation is generally the first step of xenobiotic detoxification, and it was suggested to serve as a mechanism for PBDE removal in tolerant microalgae. Yet, one cannot rule out the possibility that MeO-PBDEs, as intermediate metabolite, became too low to be detected since the biotransformation from MeO-PBDEs to OH-PBDEs was very efficient. The extreme high BDE-47 concentration used in this study led to the highest transformation of MeO-PBDEs to OH-PBDEs.
cent tubes and 22 W white light lamps (intensity of approximately 2700 LUX). Algal growth rate was estimated by and maintained in environmental chamber (14:10 light/dark cycle; 25 °C). Light was provided by white fluores-

at 500 °C for 7 hours to remove any organic contaminant. The four marine microalgae at the Cape d’Aguilar Marine Reserve of Hong Kong Island. All glassware was rinsed with acetone before ashing

P. minimum
and
S. grethae
in the laboratory for over a year. The microalgae were cultured using F/2 medium by Guillard 54 prepared with

Prof. KMY Leung of School of Biological Sciences, University of Hong Kong. All microalgae have been cultured (CCMP 1335) was obtained from the axenic culture from the City University of Hong Kong and the laboratory of

I. galbana, P. minimum, S. grethae, and T. pseudonana after BDE-47 treatment for 2, 4 and 6 days. ND: not detectable; —: not available.

| Metabolites          | Day 2    | Day 4    | Day 6    |
|----------------------|----------|----------|----------|
| 2′-OH-BDE-28         | 0.003 ± 0.00% | 0.003 ± 0.00% | 0.000 ± 0.000% |
| 5-OH-BDE-47          | 0.007 ± 0.001% | 0.012 ± 0.002% | 0.001 ± 0.0000% |
| 6-OH-BDE-47          | 0.004 ± 0.001% | 0.005 ± 0.001% | 0.000 ± 0.0000% |

Table 3. Conversion percentages (±SD; n = 3; corrected to the nearest 0.001%) for the OH-PBDE metabolites from *I. galbana* exposed to 2000 μg BDE-47 L^{-1}. The same congeners were not detected in *P. minimum*, *S. grethae*, and *T. pseudonana* in the same experiment.

eperiment shed light on the biological response at unrealistically great concentration. Notably, strong growth inhibition was found at the 2000 μg L^{-1} treatment within 2 days. The great BDE-47 concentration might have triggered certain atypical cell mechanism to produce OH-PBDEs, and thereby exerted toxic effects and inhibited cell growth. It has been shown that ortho-tetra-BDE radical was formed readily by photolysis of BDE-47, and that the formation of hydroxyl radical (•OH) was essential for the photo-formation of OH-PBDEs from PBDEs. If the microalgae had formed hydroxyl radical during the exposure, this might as well be the cause of the observed growth inhibition.

In conclusion, 0.2–20 μg BDE-47 L^{-1} did not inhibit growth of the four microalgae species studied within 6 days, but biotransformation of BDE-47 to BDE-28 was clearly evident in all species. Only under extreme high BDE-47 concentration that small amount of OH-PBDEs metabolites (i.e. 2′-OH-BDE-28, 5-OH-BDE-47, and 6-OH-BDE-47) were produced in *I. galbana* associated with growth inhibition. Besides, there was no natural production of the targeted OH-PBDEs nor MeO-PBDEs found in the microalgae species tested here. It should be noted that these findings were limited to the exposure conditions and analytical methods used in this study. However, with the exposure to environmentally-relevant concentration of BDE-47, this study has found that it is unlikely for the four microalgae species to produce the high concentrations of OH-PBDEs and MeO-PBDEs observed in the marine environment by either *de novo* production or biotransformation.

**Methods**

**Culture and spiking of BDE-47.** Seawater used was collected from the Swire Institute of Marine Science at the Cape d’Aguilar Marine Reserve of Hong Kong Island. All glassware was rinsed with acetone beforeashing at 500 °C for 7 hours to remove any organic contaminant. The four marine microalgae *I. galbana* (clone T-ISO), *P. minimum* (CCMP 2780), *S. grethae* (CCAP 1077/3, formerly listed as Skeletonema costatum) and *T. pseudonana* (CCMP 1335) was obtained from the axenic culture from the City University of Hong Kong and the laboratory of Prof. KMY Leung of School of Biological Sciences, University of Hong Kong. All microalgae have been cultured in the laboratory for over a year. The microalgae were cultured using F/2 medium by Guillard prepared with autoclaved filtered seawater (FSW). Sodium metasilicate was added to *S. grethae* and *T. pseudonana* to achieve concentration at 1.06 × 10^{-4} M. The glass bottles carrying the microalgae were aerated with 0.22 μm filtered air and maintained in environmental chamber (14:10 light/dark cycle; 25 °C). Light was provided by white fluorescent tubes and 22 W white light lamps (intensity of approximately 2700 LUX). Algal growth rate was estimated by algal density determination with a haemocytometer.

Stock solutions of BDE-47 (Chem Service Inc.) at 2 × 10^6, 2 × 10^5, 2 × 10^4, 2 × 10^3 μg L^{-1} BDE-47 stocks were prepared with DMSO by serial dilution from a stock of 10^7 μg L^{-1}. Triplicates of BDE-47 treatments with microalgae in Erlenmeyer flasks were set up. During treatment, microalgae culture which has reached log-phase was
sub-cultured into new F/2 medium to reach 1 × 10⁶ cells mL⁻¹ (for *P. minimum*) and 1 × 10⁷ cells mL⁻¹ for all the other three species. BDE-47 stock solutions were added to the culture flasks to produce exposure concentrations of 2000, 200, 20 or 0.2 μg L⁻¹ in 300 mL of microalgae culture. In these culture media, 30 μL of BDE-47 stock solutions (or DMSO for the solvent control) was used to provide the same solvent concentrations (0.01%, v/v) in all treatments. The culture flasks were swirled twice per day during exposure. On day 2, 4 and 6 after inoculation, 50 mL of microalgae were collected from each flask and subject to centrifugation (3000 rpm, 5 min., 4 °C). The algal cells were re-suspended with FSW and then centrifuged with 15 mL glass tubes. The samples were then freeze-dried, weighed for dry weights, and stored at 20 °C before chemical analysis.

The growth rate (doubling time) and the tolerance to BDE-47 and DMSO for each species found from the literature and preliminary tests under laboratory conditions are summarized in Table 4. Result of a preliminary test for response under extreme high concentrations.

| Chemical analysis | Isopropanol 70% | Acetone | Chloroform/methanol 1:1 | Methylene chloride | Hexane/acetone 1:1 |
|-------------------|-----------------|---------|-------------------------|-------------------|-------------------|
| Instrumentation.  | Two PBE congeners (BDE-47 and BDE-28), three MeO-PBDEs congeners (2′-MeO-BDE-28, 5-MeO-BDE-47 and 6-MeO-BDE-47) and three OH-PBDEs (2′-OH-BDE-28, 5-OH-BDE-47 | | | | |

Table 4. Growth rates, resistance to the solvent DMSO and known resistance to BDE-47 of the four species of microalgae in the experiment. NOEC: No observable effect concentration; LOEC: Lowest observed effect concentration; IC₅₀: Half maximal inhibitory concentration; EC₅₀: Median effect concentration. Information with no reference was obtained from preliminary experiments in laboratory for range finding.
and 6-OH-BDE-47 (99–100%; AccuStandard Inc., New Haven, CT, USA) were the target analytes for investigation. 5-MeO-BDE-47 and 6-MeO-BDE-47 and their hydroxylated analogs were chosen because they are the most abundant congeners of their kinds in environmental samples, while the tri-brominated 2′-MeO-BDE-28 and 2′-OH-BDE-28 were targeted since they are possible metabolites either from debromination of their tetra-brominated MeO-PBDEs and OH-PBDEs analogs or from methoxylation or hydroxylation of BDE-28. They were quantified by gas chromatography interfaced to a mass spectrometry equipped with VF-5MS (Bruker 450 GC/320 MS) or DB-5MS (Agilent Technologies 7890A GC/5975 MSD) capillary column (30 m length, 0.25 mm ID, 0.25 μm film) with helium as the carrier gas for chromatographic separation for all target analytes. Detail of instrumentation with GC-MS was same as in Ho et al.\textsuperscript{57} using Electron Ionization (EI) mode with Selective Ion-Monitoring (SIM). The injector temperature was held at 280 °C for 41 min of run time. The column oven programme was as follow: from 60 °C (2 min) increased at a rate of 15 °C min\textsuperscript{−1} to 250 °C, then increased at a rate of 5 °C min\textsuperscript{−1} to 280 °C which was held for 10 min, and lastly increased at a rate of 30 °C min\textsuperscript{−1} to 290 °C and held for 10 min. The standard curves were obtained by injecting at 1 to 500 ng mL\textsuperscript{−1} for PBDEs and MeO-PBDEs, or 0.5 to 500 ng mL\textsuperscript{−1} for OH-PBDEs.

Quality control. Procedural blanks which consisted of the three surrogate standards were used for background reduction for every 12 to 15 samples. The average recovery percentages as determined by the mass of surrogate standards in each sample was 89% (69–113%) for 13C-BDE-47, 77% (70–95%) for 13C-6-MeO-BDE-100 and 86% (71–110%) for 13C-6-OH-BDE-100. To confirm the purities of the BDE-47 stock used for treatment, the stock at 2 × 10\textsuperscript{5} μg L\textsuperscript{−1} was analyzed by the same method and no BDE-28, MeO-PBDEs nor OH-PBDEs could be detected.

Calculations and statistical analysis. For uptake percentage of BDE-47, calculations were done by dividing the mass of BDE-47 measured by the nominal BDE-47 mass for the volume collected per sample:

\[
\text{Uptake percentage} = \frac{\text{Mass of BDE-47 measured}}{\text{Mass of nominal BDE-47 added to the culture}} \times 100\% 
\]

Bioaccumulation is defined as the accumulation of chemical as a result from uptake from all sources (including surrounding water), and bioaccumulation factor (BAF, L kg\textsuperscript{−1}) is defined as the ratio of analyte in the biota and water\textsuperscript{58}. For this study, its calculation is essentially the same as the bioconcentration factor:

\[
\text{Bioaccumulation factor (L kg\textsuperscript{−1})} = \frac{[\text{BDE-47}] \text{ in microalgae}}{[\text{BDE-47}] \text{ in water (nominal)}} 
\]

For percentage of conversion to particular metabolite, it was calculated by finding the ratio of the metabolite to the BDE-47 measured:

![Figure 4. Procedures of chemical pre-treatment for analysis of PBDEs and their derivatives prior to GC-MS analysis.](image-url)
Conversion percentage = \frac{[\text{Metabolite}] \times 100}{[\text{Metabolite}] + [\text{BDE-47 measured}]}

Statistical analysis was performed using SigmaPlot 11.0. One-way ANOVA was used to find difference in algal density in different treatments at the same time point. Two-way ANOVA was used to find difference in the uptake percentage (arc sin square root transformed) of BDE-47 by number of days of exposure and by species. Only the concentration which had been tested in all species (2 \mu g L^{-1}) was subjected to test for difference among species. Data passed normality test (Shapiro-Wilk) and had equal variability (both p > 0.05). Holm-Sidak test was performed and each treatment was compared to the control if significant difference was found by ANOVA (p < 0.05).

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Acknowledgements
We would like to thank Edwina Yeung and Alan Cheng for their technical assistance. B.H.K.P was supported by PhD scholarship of The University of Hong Kong.

Author Contributions
M.H.W.L., J.P.G. and J.M.Y.C. conceived and designed the study. B.H.K.P performed the experiments. K.-L.H. carried out part of the chemical analyses. B.H.K.P. and J.M.Y.C. analysed the data and prepared the manuscript.

Additional Information
Competing Interests: The authors declare no competing financial interests.

How to cite this article: Po, B. H. K. et al. Uptake and biotransformation of 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) in four marine microalgae species. Sci. Rep. 7, 44263; doi: 10.1038/srep44263 (2017).

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