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# Identification of biotransformation products of organophosphate ester from various aquatic species by suspect and non-target screening approach

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ABSTRACT

Organic pollutants that are introduced into the aquatic ecosystem can transform by various mechanisms. Biotransformation is an important process for predicting the remaining structures of pollutants in the ecosystem, and their toxicity. This study focused on triphenyl phosphate (TPHP), which is a commonly used organophosphate flame retardant and plasticizer. Since TPHP is particularly toxic to aquatic organisms, it is essential to understand its biotransformation in the aquatic environment. In the aquatic ecosystem, based on consideration of the producer-consumer-decomposer relationship, the biotransformation products of TPHP were identified, and their toxicity was predicted. Liquid chromatography-high resolution mass spectrometry was used for target, suspect, and non-target analysis. The obtained biotransformation products were estimated for toxicity based on the prediction model. As a result, 29 kinds of TPHP biotransformation products were identified in the aquatic ecosystem. Diphenyl phosphate was detected as a common biotransformation product through a hydrolysis reaction. In addition, products were identified by the biotransformation mechanisms of green algae, daphnid, fish, and microorganism. Most of the biotransformation products were observed to be less toxic than the parent compound due to detoxification except some products (hydroquinone, beta-lyase products, palmitoyl/stearyl conjugated products). Since various species exist in a close relationship with each other in an ecosystem, an integrated approach for not only single species but also various connected species is essential.



## 1. Introduction

Flame retardants are additives used in products by the manufacturing industry to inhibit combustion. Brominated flame retardants (BFRs) have been widely applied in manufacturing since the 1960s, as they are flame retardant in small amounts (Birnbaum and Staskal, 2004). However, BFRs have negative effects in the environment; they are persistent chemicals in water, and are subject to bioaccumulation (Kemmlein *et al.*, 2009). Alternative flame retardants, including organophosphate esters (OPEs), have been produced to address this issue, and made up 14% of total flame retardant production in 2004 (Brandsma *et al.*, 2013). Among the OPEs, triphenyl phosphate (TPHP) is specified by the EPA as an alternative substance, for which the total national production was 1–10 million pounds in 2015 (Epa, 2019). In addition, TPHP is widely used when manufacturing electronic products to the extent that it was investigated 2–3 times more than other OPEs in smartphones (Zhang *et al.*, 2019).

TPHP has been detected in various surface water and wastewater treatment plants (WWTPs): 11 and 165 ng/L from the River Tiber in Italy (Bacaloni *et al.*, 2007); 76–290 ng/L in the influent and up to 130 ng/L in the effluent of Swedish WWTPs (Marklund *et al.*, 2005); and an average concentration of 130 ng/L in the influent and 70 ng/L in the effluent of German WWTPs (Meyer and Bester, 2004). Since TPHP is mixed into the product without chem-

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ical bonding, it can easily flow into the water system (Li *et al.*, 2014). Therefore, research on the effects of TPHP on the aquatic ecosystem is urgently needed. The concentration of TPHP in organisms has been observed to be an average of 17.1 ng/g lipid in fish from the Great Lakes, and 76 ng/g lipid (21–180 ng/g lipid) in fish from the Swedish lakes (Guo *et al.*, 2017; Sundkvist *et al.*, 2010).

A previous study introduced that TPHP is more toxic than other halogen-free flame retardants (Waaijers *et al.*, 2013a). TPHP is particularly toxic to the aquatic ecosystem. Microalgae exposed to TPHP show inhibited cell growth and various altered metabolites. Not only membrane integrity, but also ROS (reactive oxygen species), can be reduced (Wang *et al.*, 2019). Chronic exposure to TPHP in water fleas has been shown to cause growth inhibition, decreased reproduction, and genetic modification (Yuan *et al.*, 2018). The toxicity of TPHP inhibited the expression of zebrafish heart morphogenetic nuclear receptors (Isales *et al.*, 2015).

TPHP, which exists in the aquatic environment and has a toxic effect on organisms, can be transformed in the body. Biotransformation mechanisms include phase I of oxidation–reduction, hydrolysis, and the phase II reaction, which is an enzymatic reaction (Lopardo *et al.*, 2018). When organic pollutants are introduced into an organism, the phase I reaction occurs generally, and phase II appears in different patterns depending on the chemicals or species. The substances generated at this time are called biotransformation products (BTPs). In general, the enzyme that induces oxidation in phase I is cytochrome P450 monooxygenase (CYP). CYP transforms hydrophobic organic pollutants into water-soluble compounds for detoxification and excretion from the body (Schlenk *et al.*, 2008). CYP was present in living organisms even before oxygen in the atmosphere existed, and now almost all organisms have it through evolution (Lamb *et al.*, 2009). However, enzyme reactions like sulfation and glucuronide conjugation have been identified as occurring specifically in each species (Peng *et al.*, 2014). Therefore, it is necessary to study the biotransformation of OPEs in various aqueous species.

Research has progressed to often evaluating the effect of TPHP on a single aquatic species, but comparative studies of biotransformation between species by food chain remain insufficient. Organisms increase the size by ingesting sub-trophic species in the aquatic ecosystem, and these processes could affect final predators (Steele, 2001). Hydrophobic chemicals with high $K_{ow}$ values bioaccumulate in the lipid tissues of an organism, and move along the food chain (Kim *et al.*, 2011). The log $K_{ow}$ of TPHP is 4.6 (Sids, 2002), so it is possible for it to move from a lower species to an upper species in the food chain.

Metabolism is associated with similar biotransformation pathways in different species. This is because maintaining the early evolved biotransformation pathways is the optimal result from an evolutionary biology point of view (Ebenhöh and Heinrich, 2001). On the other hand, organisms have specific biotransformation mechanisms. The metabolite profile of a particular species results from species-specific combinations of functional properties (Gargallo-Garriga *et al.*, 2020). Exposure to TPHP by aquatic species could lead to organism-specific reactions, as well as general biotransformations. Therefore, an integrated approach centered on the producer-consumer-decomposer relationship is needed.

In this study, microalgae (producer), invertebrates (primary consumer), and fish (secondary consumer) were used to confirm the biotransformation of TPHP in the aquatic ecosystem. Water from a WWTP was also obtained to identify the degradation products from microorganisms. BTPs analysis improved the accuracy, precision, and reliability, using liquid chromatography-high-resolution mass spectrometry (LC-HRMS/MS). The purpose of this study was to identify the BTPs of TPHP in the aquatic ecosystem, considering the producer-consumer-decomposer relationship, and to compare biotransformation mechanisms according to species. The prediction of toxic effects when organic pollutants are introduced into the aquatic environment is the ultimate goal of this research. The BTPs of TPHP not only lack screening data, but we also have an insufficient understanding of their toxicity. Therefore, this study estimated the effect of TPHP in the aquatic environment by predicting the toxicity of BTPs in aquatic species.

## 2. Materials and methods

### 2.1. Chemicals and reagents

For analysis of organisms, triphenyl phosphate (TPHP ≥99%), diphenyl phosphate (DPHP ≥99%), and triphenyl phosphate-d15 as an internal standard (TPHP-$d_{15}$, 98 atom % D) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and water (HPLC Grade), which were used as analytical solvents, were prepared by Thermo Fisher Scientific (St. Waltham, MA, USA). Formic acid (≥95%, Sigma-Aldrich) was added to the solvents for analysis.

### 2.2. Culture of experimental species

To elucidate the biotransformation reaction of TPHP, representative species of the aquatic food chain were selected. Producer microalgae (*Raphidocelis subcapitata*), a primary consumer invertebrate (*Daphnia magna*), and a secondary consumer fish (*Oryzias latipes*) were used as the experimental species.

*Raphidocelis subcapitata* (*R. subcapitata*), used for the biotransformation experiments, were managed according to the OECD (Oecd, 2011) and EPA guidelines (Weber, 1991). The culturing mediums were prepared with Bold's basal medium (BBM) from Bioworld (Dublin, OH, USA.): 250 mg/L $NaNO_3$, $MgSO_4 \bullet 7H_2O$ 75 mg/L, NaCl 25 mg/L, $K_2HPO_4$ 75 mg/L, $KH_2PO_4$ 175 mg/L, $CaCl_2 \bullet 2H_2O$ 25 mg/L, $FeSO_4 \bullet 2H_2O$ 8.82 mg/L, $H_2SO_4$ 1 mL/L, $Na_2EDTA \bullet 2H_2O$ 50 mg/L, KOH 31 mg/L, $H_3BO_3$ 11.42 mg/L, and trace elements ($ZnSO_4 \bullet 7H_2O$ 8.82 mg/L, $MnCl \bullet 4H_2O$ 1.44 mg/L, $MoO_3$ 0.71 mg/L, $CuSO_4 \bullet 5H_2O$ 1.57 mg/L, $Co(NO_3) \bullet 6H_2O$ 0.49 mg/L) in distilled water. *R. subcapitata* (density >2–3 × $10^7$ cell/mL) were cultured with BBM mixture in 1 L flask. The incubation was maintained 12 h/12 h as a light/dark cycle at 22±1 °C. Microalgae were incubated with aeration for at least three days for environmental adaptation. The number of cells in the microalgae solution was measured by a UV–vis spectrometer (Shimadzu, Japan) at 684 nm.

*Daphnia magna* (*D. magna*) was the experimental species representing a primary consumer in the aquatic ecosystem. The details of the experimental method have been introduced in a previous study (Choi *et al.*, 2020). Briefly, *D. magna* consumed microalgae (*R. subcapitata*) and YCT (Yeast, Cerophyl®, Trout Chow) daily. For the experiment, daphnids were collected from the new generation once they were 21-day-old adults. The obtained organisms were cultured for seven days to use in the experiment.

*Oryzias latipes* (*O. latipes*), as test organisms for the experiment, were cultured by following the EPA guidelines (Weber, 1991). The culturing medium was 0.192 g/L $NaHCO_3$, 0.12 g/L $MgSO_4$, 0.12 g/L $CaSO_4$, and 0.008 g/L KCl in Milli-Q water. Brine shrimp (*Artemia*) were fed daily to the fish, and incubation conditions were maintained as follows: 24±2 °C, 16 h/8 h light/dark cycle. Fish of at least six months of age were used for the biotransformation analysis.

### 2.3. Sample preparations

TPHP, as the target compound, was exposed to *Raphidocelis subcapitata, Daphnia magna*, and *Oryzias latipes* for the same exposure time (24 hours) and concentration (100 μg/L), to confirm the biotransformation in the study. Since the NOEC (no ob-

served effect concentration) values of microalgae, invertebrate, and fish were all over 100 μg/L (Epa, 2007; Millington *et al.*, 1988; Sitthichaikasem, 1978; Waaijers *et al.*, 2013b), the exposure concentration of 100 μg/L was judged to be suitable to identify the biotransformation products.

*Raphidocelis subcapitata*

Incubated microalgae were prepared at a density of $5 \times 10^7$ cell/mL in 100 mL of BBM with 100 μg/L TPHP as the final concentration. *R. subcapitata* in triplicate samples were exposed for 24 h, then 100 mL of microalgae suspension was collected in a 250 mL polyethylene bottle for centrifugation (10,000 g, 10 min, 4 °C; Hanil, South Korea). Supernatants from the samples were stored separately for further treatment using solid phase extraction (SPE). Six hundred micro-liters of internal standard and TPHP-d15 injected into the cell re-suspension to make a final volume of 3 mL. The samples underwent freeze-thaw cycles three times in liquid nitrogen to extract the biotransformation products. The microalgae were homogenized two times for 2 min at 30 Hz using a TissueLyserII shaker (QIAGEN, Germany). The samples were centrifuged once again in the same conditions, and the supernatants were filtered with a 0.2 μm cellulose acetate filter (ADVANTEC®, Japan). Biota samples were kept at −80 °C before analysis.

*Daphnia magna*

For the experiment, 50 *D. magna* were collected in a 500 mL culture medium as triplicates, and individually exposed to a final concentration of 100 μg/L TPHP. After 24 hours, *D. magna* and the remaining medium were separated. Daphnids, 200 μL of internal standard, and 200 μL methanol were placed in a 2 mL microtube. The sample was homogenized twice at 15 sec, 8 Hz, using a Tissuelyser II shaker, before 600 μL of methanol was injected into the homogenization sample and it was filtered with a 0.2 μm cellulose acetate filter. It was then stored until analysis. The details have been further described in previous research (Choi *et al.*, 2020).

*Oryzias latipes*

TPHP, as an experimental compound, was prepared at 100 μg/L in 1 L of culture medium containing five individual *O. latipes* in triplicate. After exposure, the fish were rinsed in distilled water and dried on a tissue. TPHP-$d_{15}$ (internal standard) and methanol were added with the fish to give a 5 mL final volume. The samples were disrupted twice at 45 sec, 20 Hz, by a TissueLyserII shaker in a stainless steel grinding jar (10 mL) with beads (20 mm). After homogenization, the samples were added to 5 mL of methanol and filtered by a 0.2 μm cellulose acetate filter. The samples were diluted two-fold with methanol for the analysis.

### 2.4. Sample collection from the wastewater treatment plant

Samples of wastewater were obtained in April 2020 at the first wastewater treatment plant (WWTP) in Gwangju, Korea (35°09′22.7″N 126°49′51.8″E). The facility, which discharges effluent into the Youngsan River, treats 600,000 tons/day and covers an area of about 450,000 $m^2$. The samples were obtained from three stages of the treatment process in triplicate: influent, settling tank after bioreactors, and effluent. Water was collected in an amber bottle so that the final volume was more than 1 L. To minimize the biotransformation of chemicals, filtration was performed using a 125 mm diameter GF/C microfiber filter immediately after sampling.

The samples were extracted using auto solid-phase extraction (SPE; AutoTrace 280, Thermo Scientific) to identify TPHP, as well as degradation products, from the water of the WWTP. A total of 100 μL of internal standard was added, with the final volume of the wastewater samples was adjusted to 1 L, before 6 mL, 500 mg Oasis HLB cartridges (Waters, USA) were conditioned with 5 mL distilled water and the same volume of methanol. The samples were loaded at 8 mL/min, and the cartridge was dried with nitrogen gas for 40 min. Elution was obtained with methanol as a final 10 mL solution. The samples were concentrated to a final volume of 1 mL in methanol by a nitrogen concentrator (Hurricane-lite, Chongmin tech, Korea).

### 2.5. Qualitative analysis of biotransformation products

Three kinds of aquatic species (*R. subcapitata*, *D. magna*, and *O. latipes*) and wastewater samples were analyzed to identify BTPs. The samples were analyzed using an ultra-high-performance liquid chromatography-electrospray ionization-quadrupole orbitrap mass spectrometer system (UHPLC-HRMS/MS, Thermo Fisher Scientific Inc, USA). The mobile phase was water with 0.1% formic acid (A), and methanol without formic acid (B). The samples were injected on the Xbridge C18 column (2.1x50 mm, 3.5 μm; Waters, USA) for analyte separation. The UHPLC gradients are described in Supplementary Table S1. The operation conditions were as follows: 10 μL injection volume, 45 L/min sheath gas, 10 L/min auxiliary gas, and 320 °C capillary temperature. The analysis was conducted in positive and negative modes: the source voltages were 3.8 kV in the positive mode and −3 kV in the negative mode. The full scan acquisition was from 50 to 750 *m/z*, with a mass resolution of 140,000 and 10 ppm of mass accuracy.

Approaches to identifying BTPs using Orbitrap included target, suspect, and non-target screening. Target screening could be confirmed using existing reference standards. Without the standards, BTPs are verified by the suspect or non-target screening method. Suspect screening is a prediction of the structures for suspected BTPs through the mechanism of the organisms. Non-target screening is an analytical method applied to unexpected products (Schymanski *et al.*, 2015). Orbitrap was analyzed using data-dependent MS/MS fragmentation for the five most intensive ions for the parent and its BTP mass list in the full scan. The database of the mass list was constructed by target and suspect screening. For target screening, the purchased standards were used, or the mass spectral database (m/zCloud: HighChem LLC, Slovakia) was cited (https://www.mzcloud.org/). Specifically, the mass spectrum of BTPs detected in organisms and the database was compared to confirm BTPs information. Suspected structures were identified by considering the biotransformation mechanisms (phase I and phase II) in the organisms, and referring to the EAWAG-BBD pathway prediction system (http://eawag-bbd.ethz.ch/predict/). Compound Discoverer 2.0 (Thermo Scientific, USA) prediction software was used for confirmation of the BTPs. Among the workflow provided by Compound Discoverer, *Find transformation* was utilized. This workflow has the advantage of being able to identify BTPs generated through transformation pathways I and II from TPHP. BTPs predicted in the samples were selected by setting a maximum of five mechanism pathways and 10 ppm of predicted-analyzed value error. The final non-target screening compounds were determined except for cases where the intensity of the predicted compounds was less than $10^4$ and the MS/MS fragment pattern could not be interpreted.

### 2.6. Quantitative analysis of TPHP in aqueous samples

For quantification of TPHP, we used liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS/MS, Agilent Technologies, USA). The mobile phase was prepared with 0.01% formic acid in water (A) and 0.01% formic acid in methanol (B). The samples were injected at 20 μL by the Zorbax Eclipse Plus C18 column (2.1 × 150 mm, 3.5 μm: Agilent Technologies, USA)

for analyte separation. The analysis conditions were as follows: 0.3 mL/min flow rate, 30 °C column temperature, 30 V collision energy, and 200 V fragment voltage. The source flow, temperature, nebulizer pressure, and capillary potential were 6 mL/min, 320 °C, 50 psi, and 5000 V, respectively. The analysis was conducted in the positive mode by multiple reaction monitoring (MRM). Two pairs of the precursor and product ions were used for analysis: *m/z* 326.8>214.8, 151.8 (TPHP), *m/z* 251.1>251.1, 77.1 (DPHP), and *m/z* 341.9>222.9, 159.9 (TPHP-d15). The time gradient started at 50% of solvent B, increased until 100% B at 13 min, and returned to 50% B at 15 min, with 5 min remaining for the post-run interval.

### 2.7. Predicted toxicity of biotransformation products

The ecological structure-activity relationships (ECOSAR) predicted model was used to predict the toxicity of BTPs detected in organisms. The model predicts toxicity based on chemical structure similarity. ECOSAR estimates the acute and chronic toxicity for aquatic organisms (plants and animals) from data reported to USEPA . In this study, ECOSAR V 1.11, distributed by the EPA, was applied (https://www.epa.gov/tsca-screening-tools/ecological-structure-activity-relationships-ecosar-predictive-model). In the case of the identified BTPs, toxicity prediction through structure was necessary because no existing toxicity studies were available. Since the research focused on aquatic ecosystem, the toxicity values for various aquatic species were predicted using ECOSAR. For acute toxicity data, green algae (96 h), daphnids (48 h), and fish (96 h) were considered based on the LC50. Chronic toxicity data (Chronic value, ChV) used the predicted geometric mean of the lowest observed effect level (LOEC) and no observed effect level (NOEC). In the absence of LOEC, NOEC was applied alone (Epa, 2020).

## 3. Results and discussion

### 3.1. Quality assurance and quality control

Before identifying the BTPs in the aquatic organisms, QAQC (quality assurance and quality control) of the target compound TPHP and DPHP was performed. The details are shown in Supplementary Table S2. The target samples of the experiment were *R. subcapitata, D. magna, O. latipes*, and wastewater. Values were identified using LC-MS/MS analysis as quantification. For determining the accuracy of the analysis, the recovery and method detection limit (MDL) was calculated by exposure to TPHP. The average recovery was obtained at each concentration in triplicate, with 92–111% of TPHP and 90–111% of DPHP in the organisms and water. Seven replicates confirmed the method detection limit (MDL) at the same concentration. The MDLs of TPHP were as follows: 0.25 ng/$10^7$cells in microalgae, 0.02 μg/g in invertebrates, 0.15 μg/g in fish, and 0.02 μg/L in water. The MDL values of DPHP were found to be 0.57 ng/$10^7$cells in microalgae, 0.02 μg/g in invertebrates, 0.11 μg/g in fish, and 0.01 μg/L in water (Table S2). The weight of all species was expressed as wet weight.

### 3.2. Identification of biotransformation products in the aquatic ecosystem

This study confirmed the biotransformation of TPHP, an alternative flame retardant, in the aquatic ecosystem in a producer (*R. subcapitata*), consumer (*D. magna, O. latipes*), and decomposer (microorganism). Table 1 shows the BTPs of representative aquatic species exposed to TPHP. Two ion modes of analysis, positive and negative modes, were used for the analysis of BTPs. Compared to the control group, only chemicals with an intensity of $10^4$ or more were selected for the BTPs identified in the experimental group. We also checked whether all of them were present in triplicate samples. Biotransformation studies specify confidence levels to ensure the reliability of the predicted structure. Level 1 indicates accurately identified BTPs due to the presence of reference standards. Level 2 suggests they could be cited from the literature, or their structure explained following biotransformation reactions. Level 3 is classified when isotope patterns and fragment information can be defined. A prediction program lacking information to explain the precise structure is confidence level 4 (Schymanski *et al.*, 2014).

Through target, suspect, and non-target screening analytical approaches, a total of 29 BTPs were identified from the aquatic ecosystem. The structures and pathways of BTPs produced from the parent compound, TPHP, in each aquatic species (microalgae, daphnids, fish, and microorganisms) were represented in Fig. 1. Six species from microalgae, nine species from daphnids, 18 species from fish, and six species from microorganisms were identified in HRMS/MS. Fig. 2 shows the major BTPs of TPHP in aquatic species. DPHP (TP_250) was commonly detected among the products, and given confidence level 1 (Fig. 2a). In this study, TP implies an abbreviation for the biotransformation product of TPHP. The number of TP indicates a neutral mass value. TP_250 was the same compound as diphenyl phosphate and was assigned to level 1 because of a reference standard. TP_250, a hydrolysis product of TPHP, is less cytotoxicity than the parent compound but affects transcriptional expressions of more genes. According to previous studies, lipid regulated genes in cells exposed to TP_250 (DPHP) are downregulated. Therefore, TP_250 might affect gene expression in the body (Su *et al.*, 2014).

*Algae*

Algae (*R. subcapitata*), the lowest level of the aquatic food chain, was used to analyze the biotransformation processes of BTPs in the study. After considering the suitability of the biotransformation and the possibility of the structure, two BTPs were identified in the positive mode and four in the negative mode of HRMS. BTPs of *R. subcapitata* exposed to TPHP were not confirmed by suspected screening. For BTPs without chemical information, a non-target screening could be employed. Meng *et al.* (2020) developed a functional group-dependent screening method through a chemical database, fragment ion pattern, and NMR analysis to discover new OPEs in indoor dust samples. In this study, the mass fragment was interpreted by applying prediction software (Compound-Discoverer) for non-target screening.

TP_594 was identified in *R. subcapitata* (Fig. 2b). It was formed by hydroxylation, palmitoyl conjugation, and desaturation from TPHP. Palmitic acid ($C_{16}H_{30}O_2$) in microalgae produces BTPs through conjugation bonds with hydroxylated TPHP. The conjugation structure of palmitic acid was predicted as palmitoleate by a reference (Nih, 2020). *M/z* 277.21698 confirmed that palmitoleate in the form of a saturated fatty acid was combined with the phenyl group of TPHP. The information of other mass fragments (especially *m/z* 223.00113) also proved that TP_594 was generated by palmitoyl conjugation of TPHP.

*Invertebrate*

TP_422 was a specific product identified in invertebrates (*D. magna*) among BTPs level 1, 2, and 3. The substances produced by sulfate conjugation in the daphnid body were TP_422 and TP_438. Fig. 2(c) shows the information of mass fragments analyzed by orbitrap for TP_422. Based on the precursor ion ($C_{18}H_{14}O_8PS$, *m/z* 421.01514), sulfo groups ($SO_3$ and $HSO_4$) were identified. It is expected that sulfate conjugation occurred after hydroxylation because hydroxylated TPHP was detected at *m/z* 341.05838. Since sulfate conjugated TPHP was commonly found in fish (TP_422, TP_438, TP_452, TP_454), sulfate conjugation can be considered an important mechanism for consumer-level species in the aquatic

**Table 1**
Information on triphenyl phosphate (TPHP) and its biotransformation products identified in algae, invertebrates, fish, and microorganisms.

| Compound[a] | Formula[b] | Mass of $[M+H]^+$or $[M-H]^-$[c] | RetentionTime (min) | FormulaChange | Biotransformations[d] | ConfidenceLevel | Detection[e] |
|---|---|---|---|---|---|---|---|
| TPHP | $C_{18}H_{15}O_4P$ | $[M+H]^+$: 327.07807 | 10.99 | | Parent compound | 1 | R, D, O, M |
| TP_110 | $C_6H_6O_2$ | $[M-H]^-$: 109.02950 | 3.58 | - $C_{12}H_9O_2P$ | Hydrolysis (2) | 1 | M |
| TP_138 | $C_7H_6O_3$ | $[M-H]^-$: 137.02440 | 2.48 | - $C_{11}H_9OP$ | Hydrolysis, Carboxylation | 1 | M |
| TP_140 | $C_7H_8O_3$ | $[M+H]^+$: 141.05460 | 2.48 | - $C_{11}H_7OP$ | Hydrolysis (2), Reduction (2), Hydroxylation, Carboxylation | 2 | M |
| TP_188 | $C_7H_8O_6$ | $[M+H]^+$: 189.03940 | 4.99 | - $C_{11}H_7P + O_2$ | Hydrolysis, Hydroxylation (2), Reduction (2), Carboxylation | 2 | M |
| TP_250 | $C_{12}H_{11}O_4P$ | $[M-H]^-$: 249.03222 | 5.73 | - $C_6H_4$ | Hydrolysis | 1 | R, D, O, M |
| TP_342 | $C_{18}H_{15}O_5P$ | $[M+H]^+$: 343.07299 | 6.78, 7.74, 9.41 | + O | Hydroxylation | 2 | D, O, M |
| TP_358a | $C_{18}H_{15}O_6P$ | $[M+H]^+$: 359.06790 | 6.16, 7.31, 8.58 | + $O_2$ | Hydroxylation (2) | 2 | O |
| TP_358b | $C_{18}H_{15}O_4PS$ | $[M-H]^-$: 357.03559 | 6.57, 8.03 | + S | GSH conjugation, Cysteine formation, Beta-lyase reaction | 4 | D, O |
| TP_372 | $C_{19}H_{17}O_6P$ | $[M+H]^+$: 373.08355 | 7.67, 9.43 | + $CH_2O_2$ | Hydroxylation (2), Methylation | 3 | O |
| TP_388 | $C_{19}H_{17}O_7P$ | $[M-H]^-$: 387.06282 | 9.2 | + $CH_2O_3$ | Hydroxylation (3), Methylation | 4 | O |
| TP_400 | $C_{21}G_{21}O_6P$ | $[M-H]^-$: 399.10029 | 2.94 | + $C_3H_6O_2$ | Reduction, Acetylation, Methylation | 4 | R |
| TP_402 | $C_{20}H_{19}O_7P$ | $[M+H]^+$: 403.09412 | 7.44, 9.51 | + $C_2H_4O_3$ | Hydroxylation (3), Methylation (2) | 3 | O |
| TP_422 | $C_{18}H_{15}O_8PS$ | $[M-H]^-$: 421.01525 | 7.76 | + $O_4S$ | Hydroxylation, Sulfate conjugation | 2 | D, O |
| TP_438 | $C_{18}H_{15}O_9PS$ | $[M-H]^-$: 437.01016 | 7.46 | + $O_5S$ | Hydroxylation (2), Sulfate conjugation | 2, 3 | D, O |
| TP_445 | $C_{21}H_{20}NO_6PS$ | $[M+H]^+$: 446.08217 | 8.03 | + $C_3H_5NO_3S$ | GSH conjugation, Cysteine formation | 2 | D, O |
| TP_452 | $C_{19}H_{17}O_9PS$ | $[M-H]^-$: 451.02472 | 7.59, 7.89 | + $CH_2O_5S$ | Hydroxylation (2), Sulfate conjugation, Methylation | 3 | O |
| TP_454 | $C_{18}H_{15}O_{10}PS$ | $[M-H]^-$: 453.00508 | 6.78 | + $C_8H_{12}N_4O_3$ | Hydroxylation (3), Sulfate conjugation | 3 | O |
| TP_463 | $C_{21}H_{22}NO_7PS$ | $[M+H]^+$: 464.09274 | 6.56 | + $C_3H_7NO_3S$ | GSH conjugation, Cysteine formation, Hydration | 3 | D, O |
| TP_502 | $C_{25}H_{27}O_9P$ | $[M+H]^+$: 503.1458 | 7.09 | + $C_7H_{12}O_5$ | Glucoside conjugation, Methylation | 4 | R |
| TP_518 | $C_{24}H_{23}O_{11}P$ | $[M+H]^+$: 519.10507 | 7.62 | + $C_6H_8O_7$ | Hydroxylation, Glucuronide conjugation | 2 | O |
| TP_534 | $C_{24}H_{23}O_{12}P$ | $[M+H]^+$: 535.0999 | 6.17, 7.31 | + $C_6H_8O_8$ | Hydroxylation (2), Glucuronide conjugation | 2 | O |
| TP_536 | $C_{24}H_{25}O_{12}P$ | $[M-H]^-$: 535.10109 | 6.97 | + $C_6H_{10}O_8$ | Hydroxylation, Glucuronide conjugation, Hydration | 4 | O |
| TP_548 | $C_{25}H_{25}O_{12}P$ | $[M+H]^+$: 549.11564 | 7.32 | + $C_7H_{10}O_8$ | Hydroxylation (2), Glucuronide conjugation, Methylation | 3 | O |
| TP_578 | $C_{26}H_{27}O_{13}P$ | $[M-H]^-$: 577.11165 | 7.41 | + $C_8H_{13}O_9$ | Hydroxylation, Glucuronide conjugation, Hydration, Acetylation | 4 | O |
| TP_594 | $C_{34}H_{43}O_7P$ | $[M-H]^-$: 593.26736 | 16.01 | + $C_{16}H_{28}O_3$ | Hydroxylation, Palmitoyl conjugation, Desaturation | 3 | R |
| TP_600 | $C_{36}H_{41}O_6P$ | $[M+H]^+$: 601.27138 | 15.88 | + $C_{18}H_{26}O_2$ | Hydroxylation, Stearyl conjugation, Desaturation | 4 | R |
| TP_617 | $C_{28}H_{32}N_3O_9PS$ | $[M+H]^+$: 618.16696 | 7.3 | + $C_{10}H_{17}N_3O_5S$ | Reduction, Dehydration, GSH conjugation | 4 | D |
| TP_628 | $C_{30}H_{33}N_2O_{11}P$ | $[M+H]^+$: 629.18948 | 12.43 | + $C_{12}H_{18}N_2O_7$ | Hydroxylation, Dehydration, Glucoside conjugation, Glutamine conjugation, Methylation | 4 | R |
| TP_669 | $C_{28}H_{36}N_3O_{12}PS$ | $[M+H]^+$: 670.18301 | 11 | + $C_{10}H_{21}N_3O_8S$ | Hydroxylation, Reduction, Hydration, GSH conjugation | 4 | D |

[a] Abbreviation: TP stands for the biotransformation product of TPHP. The following number is the neutral mass value of the product.
[b] Each structure is expressed as a neutral molecular formula.
[c] The mass values identified in the positive and negative modes are distinguished.
[d] The reaction from TPHP to biotransformation products is shown. The value in parentheses is the number of reactions.
[e] The organisms in which biotransformation products were detected are indicated by an abbreviation: *Raphidocelis subcapitata* (R), *Daphnia magna* (D), *Oryzias latipes* (O), and microorganisms (M).

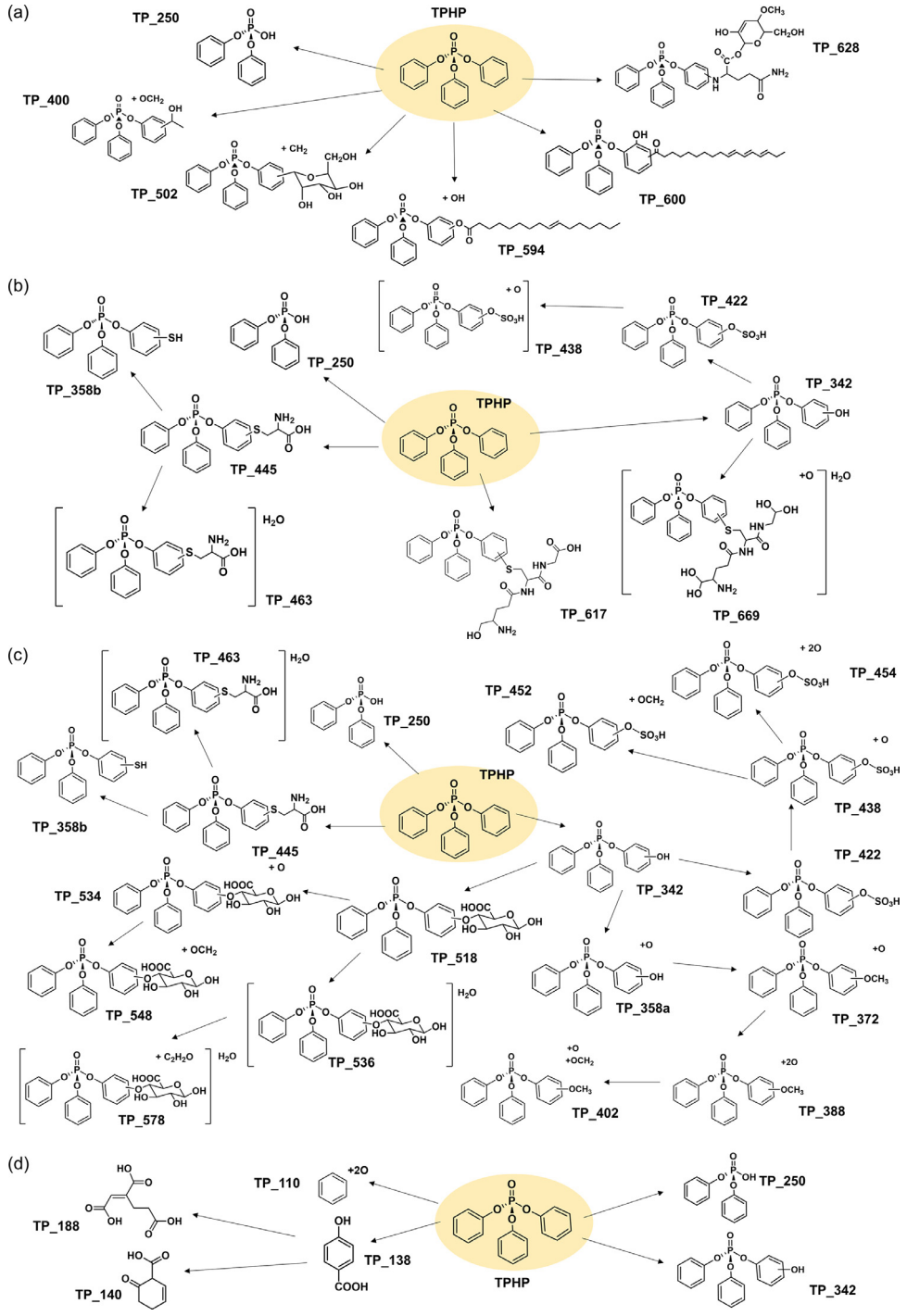


**Fig. 1.** Proposed biotransformation pathways of triphenyl phosphate (TPHP) in algae (a), invertebrate (b), fish (c), and microorganisms (d). The TP of the compounds means BTPs derived from TPHP. The number represents the neutral mass.

ecosystem. Sulfate conjugation plays an essential role in regulating and eliminating the biological activity of various chemicals or xenobiotics (Kauffman, 2004). Sulfotransferase, as an enzyme which catalyzes the sulfate conjugation of xenobiotics in the body, shows particularly high activity in the phenol group (Martin-Skilton *et al.*, 2006).

*Fish*

In fish (*O. latipes*), 18 kinds of biotransformation products of TPHP were detected in positive (nine BTPs) and negative mode (nine BTPs). Fig. 2(d) shows the MS/MS fragment patterns for TP_518 as the major product. TP_518 was generated by glucuronide conjugation (same as glucuronidation) from hydroxylated TPHP. The BTPs of fish produced through glucuronide conjugation were TP_518, TP_534, TP_536, TP_548, and TP_578. In the MS/MS fragment of TP_518, $C_5H_5O_3$ (*m/z* 113.02331) and $C_5H_7O_4$ (*m/z* 131.03401) were expected to be derived from glucuronic acid ($C_6H_{10}O_7$). As the same structure as TP_342 was observed at *m/z* 343.07266, glucuronic acid was bonded to hydroxylated TPHP to form TP_518. TP_518 was O-glucuronide conjugated from the hydroxyl group. Previous studies reported the detection of glucuronide TPHP and glucuronide hydroxyl TPHP by exposing ze-

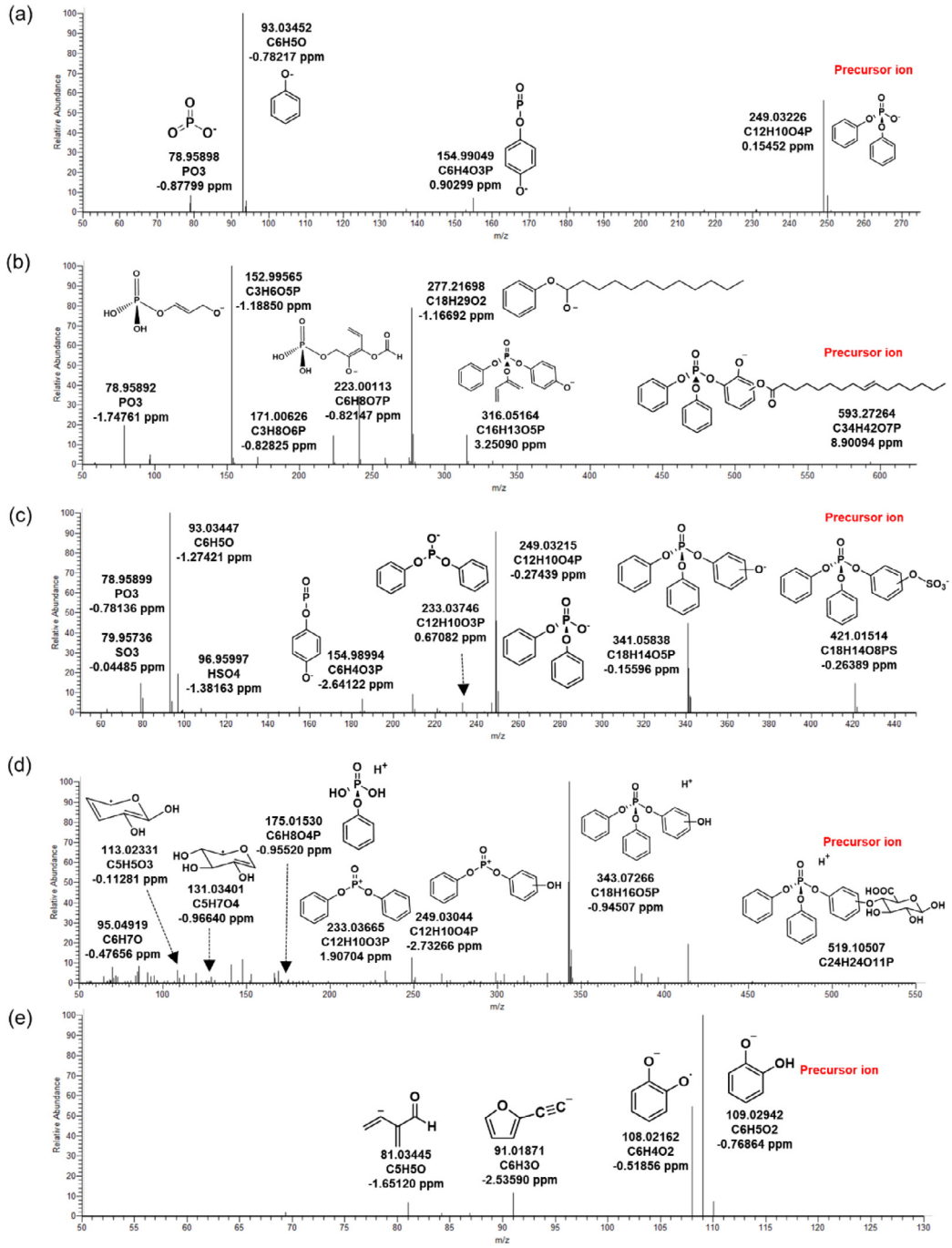


**Fig. 2.** MS/MS fragment patterns of major biotransformation products of triphenyl phosphate (TP) identified in aquatic species. (**a**) TP_250: identical biotransformation product in all species, (**b**) TP_594 from algae (*R. subcapitata*), (**c**) TP_422 from invertebrates (*D. magna*), (**d**) TP_518 from fish (*O. latipes*), (**e**) TP_110 from microorganisms.

brafish to TPHP (Su *et al.*, 2015; Su *et al.*, 2016b; Wang *et al.*, 2016). This study confirmed two BTPs (TP_518 and TP_534) in *O. latipes*, which were the same as the reference.

TP_372 was a product formed by methylation from hydroxylated TPHP (Figure S1(e)). In MS/MS fragments, *m/z* 123.0440 and 279.0412 were predicted to indicate a methoxy group (R-O-$CH_3$) attached to the phenyl group. Methylation is one of the essential phase II mechanisms in biotransformation, and is slightly different from other phase II reactions. Generally, products from phase II reactions are water-soluble, but methylation products have low solubility in water. Because methylation products are more hydrophobic than their parent compounds, methylated BTPs are not excreted differently from other BTPs (August *et al.*, 1994). TP_372 was generated by O-methylation, in which the methyl group was bonded to the oxygen of the hydroxyl functional group. Many of the methylations identified in the study were from O-methylations of hydroxyl groups (TP_402, TP_452, TP_548). S-adenosylmethionine reacts as a cofactor for methylation in organisms (Parkinson, 2001), and the main enzyme for synthesizing O-methylation is catechol O-methyltransferase (Testa, 2007). Van den Dungen *et al.* (2017) researched gene transcription from persistent organic pollutant exposure in adipocytes. Pollutants have been shown to affect adipocyte differentiation and gene expression, and interfere with DNA methylation. These results suggest that the methylated BTPs (TP_372, as well as others) identified in this study might be a potential product of disruption of gene expression.

*Microorganisms*

Water collected from a WWTP was analyzed to identify the BTPs of TPHP related to microorganisms. BTPs were detected only in samples from the effluent and biological reactors, which were compared with the sample of influent. This study considered that the BTPs of TPHP generated by microorganisms remained in the water. The BTPs in the wastewater could eventually flow into the river. Thus, the data could contribute to identifying the BTPs of TPHP in the aquatic environment. For the study of microorganisms, the existence of BTPs analyzed mainly in microbial degradation products and aquatic species (microalgae, daphnids, and fish) was investigated. As a result, a total of six BTPs were identified; three types were detected each in positive and negative mode (Table 1). Among them, the BTPs common in aquatic organisms were TP_250 and TP_342. Suspect screening using the EAWAG-BBD database in the present study revealed four types of BTPs (TP_110, TP_138, TP_140, TP_188) as decomposition products of TPHP.

The mass fragment information of the significant BTP, TP_110, is shown in Fig. 2(e). TP_110 was catechol or hydroquinone, which appeared to have been produced by microbial degradation of TPHP. Significant results were obtained by comparing the analysis data with catechol information of m/zCloud (https://www.mzcloud.org/) (Highchem, 2020b). As a result, TP_110 was ranked as confidence level 1. TP_138 was a decomposition product of TPHP (4-hydroxybenzoic acid; Figure S2a). Since the m/zCloud database was compared with the MS/MS fragments, it was described as level 1 due to the presence of a reference standard (Highchem, 2020a).

### 3.3. Comparison of biotransformation products by aquatic organisms

In the study, representative species of the food chain were selected to investigate the effects of OPEs on the aquatic ecosystem. The concentration in the body was calculated by exposing the organisms to TPHP for the same time period (24 h). The organisms were the producer (*R. subcapitata*), the primary consumer (*D. magna*), and the secondary consumer (*O. latipes*). The data were not the result of the state of equilibrium concentration, so we used the expected bioconcentration factor (BCF). The predicted BCF values were as follows: microalgae 57 L/Kg, daphnid 86 L/Kg, and fish 89 L/Kg. In previous studies, the BCF of *O. latipes* was 144 L/Kg, *Lemma minor* was 43 L/Kg, and *Typha* sp. was <1 L/Kg (Sids, 2002). BCF generally increases at higher nutritional levels, due to the increase in the lipid ratio (Hemond and Fechner, 2014). In the case of *R. subcapitata*, the BCF value was lower than that of other animal species, and similar to the BCF (*Lemma minor*, 43 L/Kg) of plants. *O. latipes* showed similar BCF values to *D. magna,* and these were lower than the reference. It was judged that the exposure time (24 h) was inadequate until the body concentration reached equilibrium.

BTPs were compared between the biological community based on the producer-consumer-degrader relationship to predict the biotransformation reaction when TPHP was introduced in the aquatic system (Fig. 3). The contribution ratio was calculated in consideration of the biotransformation steps, formed products, and intensity of BTPs. Biotransformation mechanisms showing low rates of 1% or less were expressed with 'etc.'. The biotransformations commonly seen in living organisms are hydrolysis and hydroxylation, which are representative mechanisms of phase I. Among the biotransformation of TPHP, hydrolysis is mainly used to generate TP_250 (DPHP). In this study, hydrolysis contributed to form this product at the following rates: 12% in microalgae, 2% in daphnids, 34% in fish, and 59% in microorganisms (Fig. 3). The hydrolysis reaction tends to be the general biotransformation that occurs in organisms upon the introduction of chemicals into the water. Hydrolysis is the basic process that describes the biotransformation and transport of nutrients, as well as organic pollutants, in the environment, especially biological systems (Boethling and Mackay, 2000). Each of the aquatic species used in this study has hydrolytic enzymes. In microalgae, viscozyme L and celluclast act as hydrolases (Hammed *et al.*, 2013). Carboxylic ester hydrolase and alpha/beta hydrolase, which are responsible for hydrolysis, were identified in *D. magna* (Uniprot, 2019). The major hydrolases of mammals are carboxylesterase, cholinesterase, and paraoxonase; enzymes also found in *O. latipes* (Klaassen and Amdur, 2013). Previous studies have shown that some of the OPEs in water undergo abiotic hydrolysis to generate degradation products (Su *et al.*, 2016a). DPHP-induced toxicity in developing zebrafish embryos leads to heart defects. It also causes embryonic mitochondrial dysfunction, hepatotoxicity, and renal toxicity (Mitchell *et al.*, 2019). The microbial reaction identified in this study focused on the decomposition and biotransformation of TPHP in a WWTP. Among the aquatic organisms, hydrolysis accounted for the most significant proportion of microorganisms. In addition to TP_250, hydrolysis products were generated in microorganisms by decomposition from the parent compounds to low molecular weight states. The enzyme mainly used to degrade TPHP in microorganisms is arldiakylphosphatase (Kegg, 2019a).

Hydroxylation, identified in all organisms, was a reaction that contributed to the formation of several BTPs. Hydroxylation rates of 20% in microalgae, 26% in daphnids, 45% in fish, and 24% in microorganisms were observed (Fig. 3). The hydroxylation in this study mainly reacted with the phenyl functional group of TPHP to form –OH groups. The resulting hydroxylated TPHP was transformed into new BTPs through various phase II reactions. A previous study showed that hydroxylated TPHP promotes the transcription of nuclear receptors, leading to gene modification (Kojima *et al.*, 2016). Therefore, it was predicted that CYP would also be present in the experimental species: *R. subcapitata, D. magna, O. latipes*, and microorganisms (Choi *et al.*, 2020; Kelly and Kelly, 2013; Schlenk *et al.*, 2008; Suzuki *et al.*, 2018). Because CYP plays a role in converting benzene to phenol (Parkinson, 2001), it seems that CYP in living organisms adds hydroxyl groups to the benzene of the organic pollutant TPHP.

Microalgae, positioned in the producer level of the aquatic ecosystem, exhibited different types of biotransformation reactions than other species. Desaturation (16%), palmitoyl conjugation (11%), and stearyl conjugation (5%) were identified (Fig. 3a). Desaturation is closely linked to the reactions of fatty acids in the body. In microalgae, various desaturase enzymes are targeted to different genes to increase lipid production (Vingiani *et al.*, 2019). It is estimated that the desaturase of the experimental species *R. subcapitata* in this study contributed to the biotransformation of palmitic acid and stearic acid. The major fatty acids constituting *R. subcapitata* were confirmed as palmitic acid, stearic acid, and oleic acid (Pugliese *et al.*, 2020). These fatty acids reacted with the TPHP penetrating the microalgae, to generate new types of BTPs.

The biotransformation ratios of *D. magna* and *O. latipes*, the consumers of the aquatic ecosystem, are shown in Fig. 3(b) and (c). Some general biotransformation reactions (sulfate, glutathione, cysteine conjugation) were observed in daphnids and fish, the representative animals of the aquatic environment. When organic pollutants enter the body, glutathione (GSH) and cysteine conjugated enzymes work together (in pairs). GSH and cysteine conjugation is an essential reaction in the xenobiotic detoxification pathway. Cysteine, which is a precursor of GSH, is involved in GSH synthesis and regulation. GSH and cysteine contribute to antioxidant activity, inhibition of cell damage, and neuroprotection (Jeon *et al.*, 2013). Sulfate conjugation is a phase II biotransformation mechanism in aquatic system consumers. Since most of the sulfate ions on earth exist in water, it is believed that sulfate ions contributed to the evolution of aquatic species (Ikenaka *et al.*, 2006). The biotransformation reaction characteristically observed in *O. latipes*

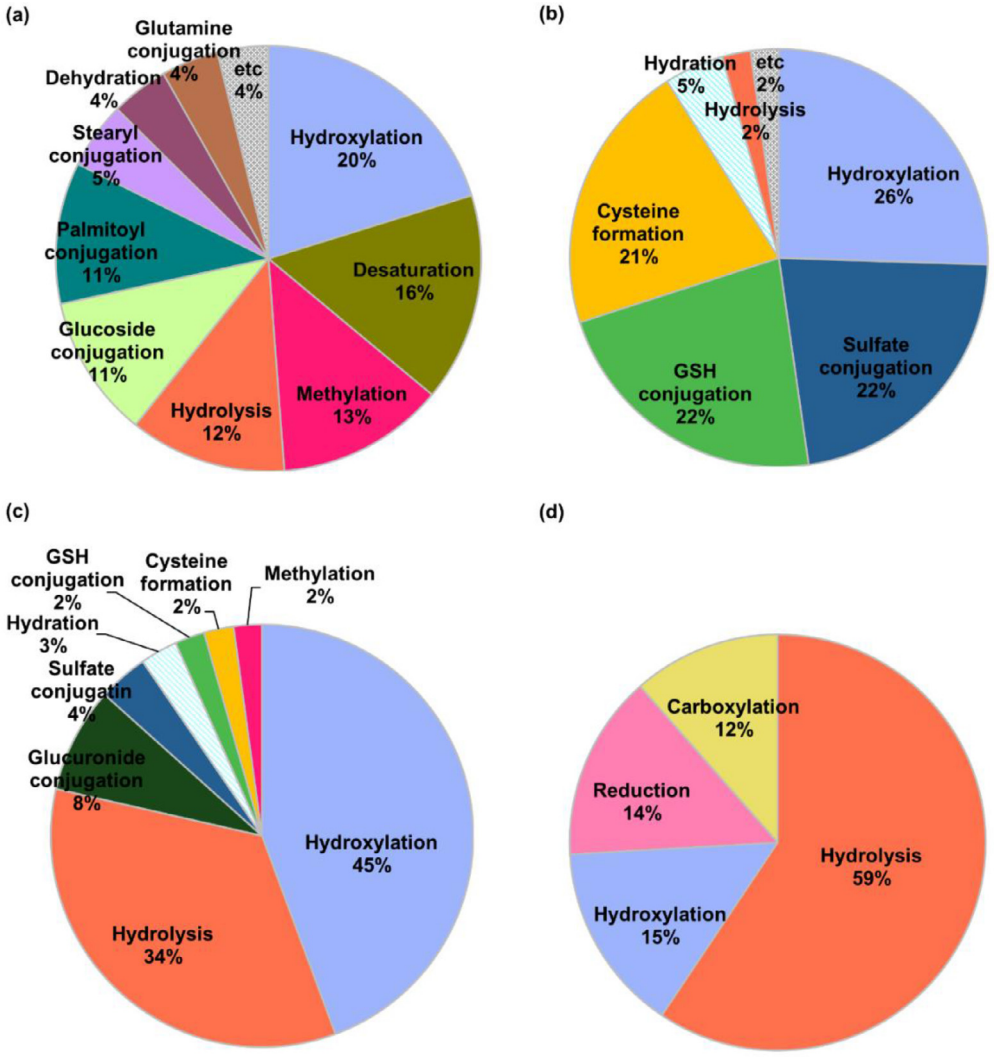


**Fig. 3.** Contribution ratio of the biotransformation of triphenyl phosphate in each organism. The graph indicates the mechanism ratios of the producer-consumer-decomposer in the aquatic ecosystem: (**a**) algae (*Raphidocelis subcapitata*), (**b**) invertebrates (*Daphnia magna*), (**c**) fish (*Oryzias latipes*), and (**d**) microorganisms.

is glucuronide conjugation. This reaction has been found in fish exposed to organic pollutants in a previous study (Wang *et al.*, 2016). Uridine glucuronosyltransferases present in tissue vesicles are catalysts for glucuronide conjugation. This conjugation reacts electron-rich nucleophiles (O, N, S) (Klaassen and Amdur, 2013). Glucuronide conjugation occurred in the OH functional group after TPHP hydroxylation in the organism. Although glucuronidation generally detoxifies xenobiotics, it might have toxic effects in some instances. Exposure to a phenolic drug in rats caused high cholestasis by glucuronide compounds, and finally blocked the bile ducts (Krijgsheld *et al.*, 1982; Mulder, 1990).

In order to confirm the biotransformation of TPHP by microorganisms in the WWTP, water samples were collected to analyze BTPs. As a result, it was confirmed that the decomposition process and biotransformation occurred by various microorganisms in the biological reaction tank. Microbial mechanisms showed a simpler pattern compared to the biotransformation of other aquatic organisms (Fig. 3d). The microbial community in wastewater includes a variety of taxonomic, biochemical, and physiological groups of organisms. Many microorganisms provide the functional benefits of improving water quality and removing nutrients (Numberger *et al.*, 2019). The characteristic biotransformation mechanism in microorganisms is carboxylation, which was involved in producing TP_138, TP_140, and TP_188. The reaction occurred by 4-hydroxybenzoate decarboxylase, when the carboxyl group was bound to phenols derived from TPHP (Kegg, 2019b). This enzyme is known to be involved in the biotransformation of microorganisms in various environments (Ncbi, 2020). Carboxylation catalyzes the fixation of $CO_2$ on organic molecules, and affects the carbon cycle on earth. It is also a critical reaction that contributes to several biological functions (Erb, 2011).

### *3.4. Ecotoxicological information of biotransformation products in the aquatic ecosystem*

Organophosphorus compounds form metabolites through biological biotransformation, and their toxicity is altered (Jokanović, 2001). The ECOSAR model was applied to predict the toxicity of identified BTPs in each species using a high-resolution mass spectrometer. LC50 and ChV prediction values were obtained from the chemical structures of the BTPs of TPHP. In the ECOSAR model, the chronic toxicity value is calculated as the geometric mean value of NOEC and LOEC. De Roode *et al.* (2006) confirmed that the toxicity of more than 70% of the substances in the database was accurate or overestimated by ECOSAR. In addition, the LC50 correlation between the experimental value and predicted value through the ECOSAR model showed more appropriate results for toxic chemicals than nontoxic chemicals. In previous studies, the ECOSAR pro-

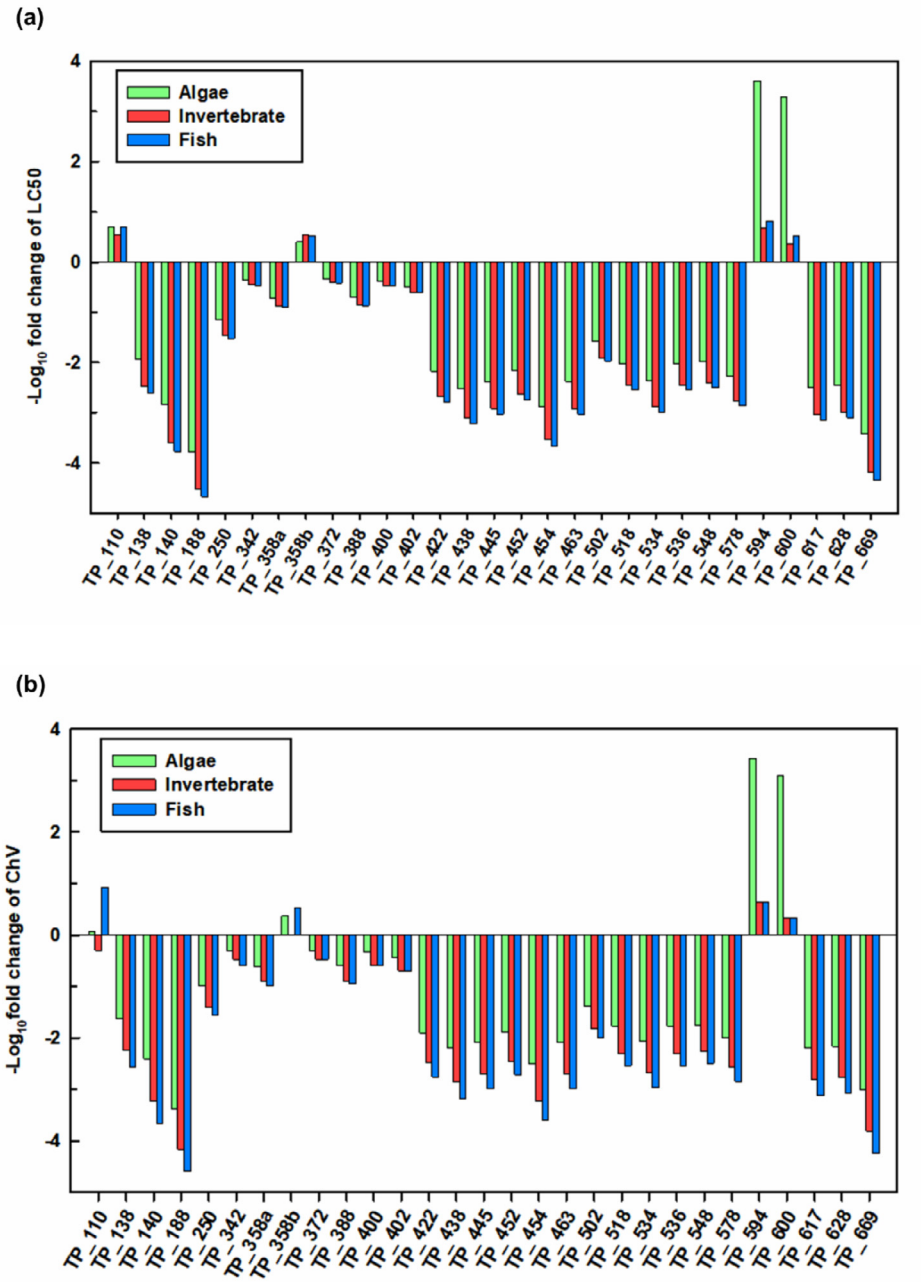


**Fig. 4.** A graph showing the toxicity values of biotransformation products of triphenyl phosphate (TP) based on a $\log_{10}$ fold change. The toxicity values of algae, invertebrate, and fish for each chemical were calculated using ECOSAR. The figure shows as follows: -$\text{Log}_{10}$ fold change of (a) 50% lethal concentration (LC50) and (b) chronic value (ChV).
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gram was used to predict the toxicity of degradation products and BTPs of various polar chemicals (Kang *et al.*, 2020; Kim *et al.*, 2018). The experimental LC50 of the parent compound TPHP was found to be 2.0, 1.3, and 1.2 mg/L in algae, invertebrates, and fish, respectively (Brooke *et al.*, 2009; Mayer *et al.*, 1981; Who, 1991). The ECOSAR model predicted values were calculated as 1.5, 0.7, and 1.1 mg/L. Since the LC50, as well as NOEC, experimental and predicted values were similar, the ECOSAR model was considered suitable for predicting BTP toxicity values of TPHP. Based on the value of the parent compound, the fold change in the toxicity value was converted into a log and expressed as a box plot for each chemical substance (Fig. 4). Most of the toxicity values of BTPs predicted by ECOSAR were decreased compared to the parent compound (-$\text{Log}_{10}$ fold change $< 0$). In particular, the toxicity of BTPs produced by the phase II enzyme reaction of aquatic organisms seemed to decrease dramatically (TP_422, 438, 445, 452, 454, 463, 518, 534, 536, 548, 578, 617, 628, and 669). The LC50 of these BTPs was predicted to be more than 100 and the ChV to be more than 10 (Table S3). Detoxification refers to a biological transformation that eliminates toxicants or prevents their production in the body. Detoxification competes with toxification and varies depending on the nature of the chemical (Klaassen and Amdur, 2013). The detoxification of organophosphorus compounds is achieved by cleavage of the phosphorus bonds or conjugation in vivo. Metabolites produced by these reactions are rapidly removed into the urine (Jokanović, 2001). It is estimated that detoxification mechanisms reduced the toxicity of most BTPs generated after exposure to the organic pollutant TPHP by aquatic organisms.

On the other hand, BTPs with increased Log LC50 and ChV predicted values compared to the parent compound TPHP included TP_110, 358b, 594, and 600 (Fig. 4). TP_110 was detected in microorganisms, TP_358b in invertebrates and fish, and TP_596, 600 in green algae. TP_110 is hydroquinone, produced from TPHP through microbial hydrolysis, and is a major benzene metabolite. The LC50 of hydroquinone in aquatic organisms according to this experiment was found to be 0.1–0.6 mg/L, and the NOEC was found to be 0.1–5 mg/L (Echa, 2020a). Phenol compounds in ppm concentrations are highly toxic to aquatic organisms, and most of them cause changes in organisms at the ppb levels (Guerra, 2001). Hydroquinone is a substance that has carcinogenic effects, as well as causing blood toxicity (Enguita and Leitão, 2013). Long term exposure to hydroquinone by aquatic organisms could cause severe toxicity, and it therefore requires continuous observation (Echa, 2020b). TP_358b is a TP in which thiol group is generated from the cysteine conjugated compound, and has been identified in invertebrates and fish. This product was predicted to have higher toxicity than TPHP in algae, daphnids, and fish. Thiol induces the formation of free radicals in organisms, causing chemical toxicity. In particular, apoptosis occurs due to the biotransformation of red blood cells through oxidative damage to the cell membrane (Munday, 1989). The thiol functional group in TP_358b, produced from the biotransformation process of TPHP, is also changed into free radicals, and may cause toxicity to aquatic organisms.

TP_594 and TP_600 were generated from TPHP by palmitoyl/stearyl conjugation, hydroxylation, and desaturation. The main functional groups were transformed palmitic acid and stearic acid, respectively. The toxicity was higher than the parent compound according to the prediction of the ECOSAR model, especially in green algae. The toxicity values of TP_594 and TP_600 were predicted as follows: 0.7–3.6 and 0.4–3.3 (LC50), 0.6–3.4 and 0.3–3.1 (ChV), by negative log scale (Table S3). Fatty acids are a major component of cell membranes and are involved in cell signaling (De Carvalho and Caramujo, 2018). However, they may cause a cytotoxic reaction if they accumulate in large amounts, resulting in hepatic disorders, cardiac disorders, and diabetes (Park *et al.*, 2014). Palmitic acid, identified in TP_594, induces apoptosis and stress of the endoplasmic reticulum in hepatoma cells (Zhang *et al.*, 2012). Stearic acid, present in TP_600, could cause cardiac toxicity by affecting heart cell death and lipid accumulation in the body (Rabkin and Lodha, 2009).

## 4. Conclusion

This study predicted biotransformation of TPHP in the aquatic ecosystem using a producer (*R. subcapitata*), a primary consumer (*D. magna*), a secondary consumer (*O.latipes*), and a decomposer (microorganisms). As a result, 29 kinds of BTPs were identified and generated by specific mechanisms in each organism, except for hydroxylation and hydrolysis in common to all species. The form of decreasing toxicity showed a pattern of decreasing regardless of species, and the same was true when toxicity was increased. Most of the detected BTPs were less toxic than the parent compound TPHP, but some were predicted to be highly toxic (TP_594, TP_600). Therefore, continuous observation of BTPs, as well as TPHP itself, should be conducted to protect the aquatic environment. Since various species exist in a close relationship with each other in an ecosystem, an integrated approach for not only single species but also various connected species is essential. The integrated perspective of BTPs conducted in this study revealed how organic pollutants transform and accumulate in the aquatic ecosystem. Furthermore, the total toxicity effect on the aquatic ecosystem could be estimated through the toxicity values of BTPs, as well as the parent compound. The representative significance of this study lies in the fact that when BTPs of organic pollutants in the aquatic environment are detected, the path of origin (organisms such as algae, invertebrate, fish, and so forth, or WWTP) could be predicted based on the results of this study.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117201.

## References

August, J.T., Murad, F., Anders, M., Dekant, W., 1994. Conjugation-dependent Carcinogenicity and Toxicity of Foreign Compounds. Academic Press.
Bacaloni, A., Cavaliere, C., Foglia, P., Nazzari, M., Samperi, R., Laganà, A., 2007. In: Liquid Chromatography/Tandem Mass Spectrometry Determination of Organophosphorus Flame Retardants and Plasticizers in Drinking and Surface waters. Rapid communications in Mass Spectrometry, 21, pp. 1123–1130.
Birnbaum, L.S., Staskal, D.F., 2004. Brominated flame retardants: cause for concern? Environmental Health Perspectives 112 (1), 9–17.
Boethling, R.S., Mackay, D., 2000. Handbook of Property Estimation Methods for Chemicals: Environmental Health Sciences. CRC press.
Brandsma, S.H., de Boer, J., Cofino, W.P., Covaci, A., Leonards, P.E.G., 2013. Organophosphorus flame-retardant and plasticizer analysis, including recommendations from the first worldwide interlaboratory study. Trac-Trend Anal Chem 43, 217–228.
Brooke, D., Crookes, M., Quarterman, P., Burns, J., 2009. Environmental Risk Evaluation Report: Triphenyl Phosphate (CAS no. 115-86-6). Environment Agency, Bristol, UK, p. 140.
Choi, Y., Jeon, J., Choi, Y., Kim, S.D., 2020. Characterizing biotransformation products and pathways of the flame retardant triphenyl phosphate in Daphnia magna using non-target screening. Science of The Total Environment 708, 135106.
De Carvalho, C.C., Caramujo, M.J., 2018. The various roles of fatty acids. Molecules 23 (10), 2583.
de Roode, D., Hoekzema, C., de Vries-Buitenweg, S., van de Waart, B., van der Hoeven, J., 2006. QSARs in ecotoxicological risk assessment. Regulatory Toxicology and Pharmacology 45 (1), 24–35.
Ebenhöh, O., Heinrich, R., 2001. Evolutionary optimization of metabolic pathways. Theoretical reconstruction of the stoichiometry of ATP and NADH producing systems. Bulletin of Mathematical Biology 63 (1), 21–55.
ECHA, 2020a. Hydroquinone. European Chemicals Agency https://echa.europa.eu/registration-dossier/-/registered-dossier/14417/6/1.
ECHA, 2020b. Hydroquinone. European Chemicals Agency https://echa.europa.eu/substance-information/-/substanceinfo/100.004.199.
Enguita, F.J., Leitão, A.L., 2013. Hydroquinone: Environmental pollution, toxicity, and Microbial Answers. BioMed research international 2013.
EPA, 2019. Proposed Designation of Triphenyl Phosphate (CASRN 115-86-6) As a High-Priority Substance For Risk Evaluation.
EPA, 2020. Sustainable Futures /P2 Framework Manual. United States Environmental Protection Agency (EPA) https://www.epa.gov/sustainable-futures/sustainable-futures-p2-framework-manual.
EPA, I., 2007. Report On Alternatives to the Flame Retardant DecaBDE: Evaluation of Toxicity, Availability, Affordability, and Fire Safety Issues. Illionis Environmental Protection Agency A report to the Governor and the General Assembly. A Report to the Governor and the General Assembly.
Erb, T.J., 2011. Carboxylases in natural and synthetic microbial pathways. Applied and Environmental Microbiology 77 (24), 8466–8477.
Gargallo-Garriga, A., Sardans, J., Granda, V., Llusià, J., Peguero, G., Asensio, D., Ogaya, R., Urbina, I., Van Langenhove, L., Verryckt, L.T., 2020. Different “metabolomic niches” of the highly diverse tree species of the French Guiana rainforests. Scientific Reports 10 (1), 1–10.
Guerra, R., 2001. Ecotoxicological and chemical evaluation of phenolic compounds in industrial effluents. Chemosphere 44 (8), 1737–1747.
Guo, J., Venier, M., Salamova, A., Hites, R.A., 2017. Bioaccumulation of Dechloranes, organophosphate esters, and other flame retardants in Great Lakes fish. Science of The Total Environment 583, 1–9.

Hammed, A.M., Jaswir, I., Amid, A., Alam, Z., Asiyanbi-H, T.T., Ramli, N., 2013. Enzymatic hydrolysis of plants and algae for extraction of bioactive compounds. Food Reviews International 29 (4), 352–370.
Hemond, H.F., Fechner, E.J., 2014. Chemical Fate and Transport in the Environment. Elsevier.
HighChem, 2020a. 4-Hydroxybenzoic Acid https://www.mzcloud.org/compound/reference/148.
HighChem, 2020b. Catechol https://www.mzcloud.org/compound/reference/2991.
Ikenaka, Y., Eun, H., Ishizaka, M., Miyabara, Y., 2006. Metabolism of pyrene by aquatic crustacean, Daphnia magna. Aquatic Toxicology 80 (2), 158–165.
Isales, G.M., Hipszer, R.A., Raftery, T.D., Chen, A., Stapleton, H.M., Volz, D.C., 2015. Triphenyl phosphate-induced developmental toxicity in zebrafish: Potential role of the retinoic acid receptor. Aquatic Toxicology 161, 221–230.
Jeon, J., Kurth, D., Hollender, J., 2013. Biotransformation pathways of biocides and pharmaceuticals in freshwater crustaceans based on structure elucidation of metabolites using high resolution mass spectrometry. Chemical Research in Toxicology 26 (3), 313–324.
Jokanović, M., 2001. Biotransformation of organophosphorus compounds. Toxicology 166 (3), 139–160.
Kang, D., Doudrick, K., Park, N., Choi, Y., Kim, K., Jeon, J., 2020. Identification of transformation products to characterize the ability of a natural wetland to degrade synthetic organic pollutants. Water Research 187, 116425.
Kauffman, F.C., 2004. Sulfonation in pharmacology and toxicology. Drug Metabolism Reviews 36 (3-4), 823–843.
KEGG, 2019a. ENZYME 3.1.8.1 https://www.genome.jp/dbget-bin/www_bget?ec:3.1.8.1 .
KEGG, 2019b. ENZYME 4.1.1.61 https://www.genome.jp/dbget-bin/www_bget?ec:4.1.1.61 .
Kelly, S.L., Kelly, D.E., 2013. Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us? Philosophical Transactions of the Royal Society B: Biological Sciences 368 (1612), 20120476.
Kemmlein, S., Herzke, D., Law, R.J., 2009. Brominated flame retardants in the European chemicals policy of REACH—Regulation and determination in materials. Journal of Chromatography A 1216 (3), 320–333.
Kim, J.-W., Isobe, T., Chang, K.-H., Amano, A., Maneja, R.H., Zamora, P.B., Siringan, F.P., Tanabe, S., 2011. Levels and distribution of organophosphorus flame retardants and plasticizers in fishes from Manila Bay, the Philippines. Environmental Pollution 159 (12), 3653–3659.
Kim, T.-H., Yu, S., Choi, Y., Jeong, T.-Y., Kim, S.D., 2018. Profiling the decomposition products of perfluorooctane sulfonate (PFOS) irradiated using an electron beam. Science of the Total Environment 631, 1295–1303.
Klaassen, C.D., Amdur, M.O., 2013. Casarett and Doull's toxicology: the Basic Science of Poisons. McGraw-Hill, New York.
Kojima, H., Takeuchi, S., Van den Eede, N., Covaci, A., 2016. Effects of primary metabolites of organophosphate flame retardants on transcriptional activity via human nuclear receptors. Toxicology Letters 245, 31–39.
Krijgsheld, K., Koster, H.J., Scholtens, E., Mulder, G.J., 1982. Cholestatic effect of harmol glucuronide in the rat. Prevention of harmol-induced cholestasis by increased formation of harmol sulfate. Journal of Pharmacology and Experimental Therapeutics 221 (3), 731–734.
Lamb, D.C., Lei, L., Warrilow, A.G., Lepesheva, G.I., Mullins, J.G., Waterman, M.R., Kelly, S.L., 2009. The first virally encoded cytochrome p450. Journal of Virology 83 (16), 8266–8269.
Li, J., Yu, N., Zhang, B., Jin, L., Li, M., Hu, M., Zhang, X., Wei, S., Yu, H., 2014. Occurrence of organophosphate flame retardants in drinking water from China. Water Research 54, 53–61.
Lopardo, L., Adams, D., Cummins, A., Kasprzyk-Hordern, B., 2018. Verifying community-wide exposure to endocrine disruptors in personal care products – In quest for metabolic biomarkers of exposure via in vitro studies and wastewater-based epidemiology. Water Research 143, 117–126.
Marklund, A., Andersson, B., Haglund, P., 2005. Organophosphorus flame retardants and plasticizers in Swedish sewage treatment plants. Environmental Science & Technology 39 (19), 7423–7429.
Martin-Skilton, R., Coughtrie, M.W., Porte, C., 2006. Sulfotransferase activities towards xenobiotics and estradiol in two marine fish species (Mullus barbatus and Lepidorhombus boscii): characterization and inhibition by endocrine disrupters. Aquatic Toxicology 79 (1), 24–30.
Mayer, F., Adams, W., Finley, M., Michael, P., Mehrle, P., Saeger, V., 1981. Aquatic Toxicology and Hazard Assessment. ASTM International.
Meng, W., Li, J., Shen, J., Deng, Y., Letcher, R.J., Su, G., 2020. Functional Group-Dependent Screening of Organophosphate Esters (OPEs) and Discovery of an Abundant OPE Bis-(2-ethylhexyl)-phenyl Phosphate in Indoor Dust. Environmental Science & Technology 54 (7), 4455–4464.
Meyer, J., Bester, K., 2004. Organophosphate flame retardants and plasticisers in wastewater treatment plants. Journal of Environmental Monitoring 6 (7), 599–605.
Millington, L., Goulding, K., Adams, N., 1988. The influence of growth medium composition on the toxicity of chemicals to algae. Water Research 22 (12), 1593–1597.
Mitchell, C.A., Reddam, A., Dasgupta, S., Zhang, S., Stapleton, H.M., Volz, D.C., 2019. Diphenyl Phosphate-Induced Toxicity During Embryonic Development. Environmental Science & Technology 53 (7), 3908–3916.
Mulder, G.J., 1990. Conjugation Reactions in Drug metabolism: an Integrated Approach. CRC Press.
Munday, R., 1989. Toxicity of thiols and disulphides: involvement of free-radical species. Free Radical Biology and Medicine 7 (6), 659–673.
NCBI 2020 4-hydroxybenzoate decarboxylase. https://www.ncbi.nlm.nih.gov/nuccore, 08.20.2020.
NIH, 2020. Palmitoleate. National Library of Medicine https://pubchem.ncbi.nlm.nih.gov/compound/Palmitoleate.
Numberger, D., Ganzert, L., Zoccarato, L., Mühldorfer, K., Sauer, S., Grossart, H.-P., Greenwood, A.D., 2019. Characterization of bacterial communities in wastewater with enhanced taxonomic resolution by full-length 16S rRNA sequencing. Scientific Reports 9 (1), 1–14.
OECD, 2011. Test No. 201: Freshwater alga and cyanobacteria, Growth Inhibition Test. Organisation for Economic Co-operation Development.
Park, E.-J., Lee, A.Y., Park, S., Kim, J.-H., Cho, M.-H., 2014. Multiple pathways are involved in palmitic acid-induced toxicity. Food and Chemical Toxicology 67, 26–34.
Parkinson, A., 2001. Biotransformation of Xenobiotics. McGraw-Hill, New York.
Peng, F.Q., Ying, G.G., Yang, B., Liu, Y.S., Lai, H.J., Zhou, G.J., Chen, J., Zhao, J.L., 2014. Biotransformation of the flame retardant tetrabromobisphenol-A (TBBPA) by freshwater microalgae. Environmental Toxicology and Chemistry 33 (8), 1705–1711.
Pugliese, A., Biondi, L., Bartocci, P., Fantozzi, F., 2020. Selenastrum Capricornutum a New Strain of Algae for Biodiesel Production. Fermentation 6 (2), 46.
Rabkin, S.W., Lodha, P., 2009. Stearic acid-induced cardiac lipotoxicity is independent of cellular lipid and is mitigated by the fatty acids oleic and capric acid but not by the PPAR agonist troglitazone. Experimental Physiology 94 (8), 877–887.
Schlenk, D., Celander, M., Gallagher, E.P., George, S., James, M., Kullman, S.W., van den Hurk, P., Willett, K., 2008. Biotransformation in Fishes. The toxicology of fishes, pp. 153–234.
Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. Environmental Science & Technology 48 (4), 2097–2098.
Schymanski, E.L., Singer, H.P., Slobodnik, J., Ipolyi, I.M., Oswald, P., Krauss, M., Schulze, T., Haglund, P., Letzel, T., Grosse, S., 2015. Non-target screening with high-resolution mass spectrometry: critical review using a collaborative trial on water analysis. Analytical and bioanalytical chemistry 407 (21), 6237–6255.
Sids, U.O. 2002 Triphenyl Phosphate (CAS No. 115-86-6). United Nations Environment Programme Publications (ed): United Nations Environment Programme, Organisation for Economic Co-operation and Development (OECD) Screening Information DataSets (SIDS).
Sitthichaikasem, S., 1978. Some Toxicological Effects of Phosphate Esters On Rainbow Trout and Bluegill.
Steele, J.H. 2001 Encyclopedia of Ocean Sciences (Second Edition). Steele, J.H. (Ed.), pp. 19–24, Academic Press, Oxford.
Su, G., Crump, D., Letcher, R.J., Kennedy, S.W., 2014. Rapid in vitro metabolism of the flame retardant triphenyl phosphate and effects on cytotoxicity and mRNA expression in chicken embryonic hepatocytes. Environmental Science & Technology 48 (22), 13511–13519.
Su, G., Letcher, R.J., Crump, D., Gooden, D.M., Stapleton, H.M., 2015. In Vitro Metabolism of the Flame Retardant Triphenyl Phosphate in Chicken Embryonic Hepatocytes and the Importance of the Hydroxylation Pathway. Environmental Science & Technology Letters 2 (4), 100–104.
Su, G., Letcher, R.J., Yu, H., 2016a. Organophosphate flame retardants and plasticizers in aqueous solution: pH-dependent hydrolysis, kinetics, and pathways. Environmental Science & Technology 50 (15), 8103–8111.
Su, G., Letcher, R.J., Yu, H., Gooden, D.M., Stapleton, H.M., 2016b. Determination of glucuronide conjugates of hydroxyl triphenyl phosphate (OH-TPHP) metabolites in human urine and its use as a biomarker of TPHP exposure. Chemosphere 149, 314–319.
Sundkvist, A.M., Olofsson, U., Haglund, P., 2010. Organophosphorus flame retardants and plasticizers in marine and fresh water biota and in human milk. Journal of Environmental Monitoring 12 (4), 943–951.
Suzuki, S., Yamaguchi, H., Nakajima, N., Kawachi, M., 2018. Raphidocelis subcapitata (= Pseudokirchneriella subcapitata) provides an insight into genome evolution and environmental adaptations in the Sphaeropleales. Scientific reports 8 (1), 1–13.
Testa, B., 2007. Principles of Drug Metabolism 2: Hydrolysis and Conjugation Reactions.
UniProt 2019 https://www.uniprot.org/. Universal Protein Resource.
van den Dungen, M.W., Murk, A.J., Kok, D.E., Steegenga, W.T., 2017. Persistent organic pollutants alter DNA methylation during human adipocyte differentiation. Toxicology in Vitro 40, 79–87.
Vingiani, G.M., De Luca, P., Ianora, A., Dobson, A.D., Lauritano, C., 2019. Microalgal enzymes with biotechnological applications. Marine Drugs 17 (8), 459.
Waaijers, S.L., Hartmann, J., Soeter, A.M., Helmus, R., Kools, S.A.E., de Voogt, P., Admiraal, W., Parsons, J.R., Kraak, M.H.S., 2013a. Toxicity of new generation flame retardants to Daphnia magna. Science of The Total Environment 463-464, 1042–1048.
Waaijers, S.L., Kong, D., Hendriks, H.S., de Wit, C.A., Cousins, I.T., Westerink, R.H., Leonards, P.E., Kraak, M.H., Admiraal, W., de Voogt, P., 2013b. Reviews of Environmental Contamination and Toxicology. Springer, pp. 1–71.
Wang, G., Du, Z., Chen, H., Su, Y., Gao, S., Mao, L., 2016. Tissue-Specific Accumulation, Depuration, and Transformation of Triphenyl Phosphate (TPHP) in Adult Zebrafish (Danio rerio). Environmental Science & Technology 50 (24), 13555–13564.

Wang, L., Huang, X., Lim, D.J., Laserna, A.K.C., Li, S.F.Y., 2019. Uptake and toxic effects of triphenyl phosphate on freshwater microalgae Chlorella vulgaris and Scenedesmus obliquus: Insights from untargeted metabolomics. Science of The Total Environment 650, 1239–1249.

Weber, C.I., 1991. Methods For Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms.

WHO, 1991. Environ Health Criteria 111: Triphenyl Phosphate http://www.inchem.org/documents/ehc/ehc/ehc111.htm.

Yuan, S., Li, H., Dang, Y., Liu, C., 2018. Effects of triphenyl phosphate on growth, reproduction and transcription of genes of Daphnia magna. Aquatic Toxicology 195, 58–66.

Zhang, Y., Su, H., Ya, M., Li, J., Ho, S.-H., Zhao, L., Jian, K., Letcher, R.J., Su, G., 2019. Distribution of flame retardants in smartphones and identification of current-use organic chemicals including three novel aryl organophosphate esters. Science of The Total Environment 693, 133654.

Zhang, Y., Xue, R., Zhang, Z., Yang, X., Shi, H., 2012. Palmitic and linoleic acids induce ER stress and apoptosis in hepatoma cells. Lipids in health and disease 11 (1), 1.