Environmental fate & effects of new generation flame retardants
Waaijers, S.L.

Citation for published version (APA):

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Chapter 4

Mineralization and Primary Biodegradation of Organophosphorus Flame Retardants in Activated Sludge

Sharona S. Jurgens, Rick Helmus, Susanne L. Waaijers, Dirk Uittenbogaard, Dorien Dunnebier, Melissa Vleugel, Michiel H.S. Kraak, Pim de Voogt, John R. Parsons


DOI: 10.1016/j.chemosphere.2014.04.016
Abstract

Halogen-free flame retardants (HFFRs), such as the aromatic organophosphorus flame retardants (OPFRs) triphenyl phosphate (TPP), resorcinol bis(diphenylphosphate) (RDP) and bisphenol A bis(diphenylphosphate) (BDP) have been proposed as potential replacements for brominated flame retardants in polymers and textiles. Although these OPFRs are already marketed, their environmental fate and effects are poorly characterized. The aim of this study was therefore to determine the mineralisation and primary biodegradation of these OPFRs by activated sludge. Mineralisation was monitored by measuring CO$_2$ production by means of GC analysis, whereas primary biodegradation was monitored by LC-MS/MS analysis of the OPFRs and their potential metabolites. TPP was biodegraded and mineralised most rapidly and achieved the requirement for ready biodegradability (60% of theoretical maximum mineralisation). Primary biodegradation was also rapid for RDP, but 60% mineralisation was not achieved within the time of the test, suggesting that transformation products of RDP may accumulate. Primary degradation of BDP was very slow and very low CO$_2$ production was also observed.

Keywords: organophosphorus flame retardants, biodegradation, mineralization
1 Introduction

Chemical additives known as flame retardants (FRs) are incorporated into a wide range of polymers in order to fulfil regulatory requirements on flame retardancy. Brominated flame retardants are used most frequently, due to their efficiency in low amounts, low costs, and low impairment of the polymer’s functionality (Alaee et al., 2003; Birnbaum and Staskal, 2004). Concerns about the persistence, bioaccumulation and toxicity (PBT properties) of BFRs (Law and Herzke, 2011; Darnerud, 2003; 2008) have, however, led to restrictions on the production and use of many of these compounds (Kemmlein et al., 2009).

Organophosphorous flame retardants (OPFRs), especially chlorinated OPFRs, were considered to be suitable replacements for the banned BFRs, but some of these compounds also appear to be persistent, bioaccumulative, and toxic (Reemtsma et al., 2008; van der Veen and de Boer, 2012). Interest has therefore shifted towards halogen-free flame retardants (HFFRs) as replacements for BFRs (van der Veen and de Boer, 2012; Waaijers et al., 2013b). Many of these HFFRs are already being marketed, although their environmental behaviour and toxicological properties are known to only a limited extent (Waaijers et al., 2013b). Moreover, the limited data that are available are often not accessible in the open literature and, consequently, the potential impact of these HFFRs on the environment cannot be properly assessed. Hence, there is an urgent need for information on the PBT properties of HFFRs.

The aim of the present study was therefore to determine the persistence of several aromatic organophosphorous flame retardants (OPFRs), a group of HFFRs that have been proposed as replacements for BFRs in polymers (van der Veen and de Boer, 2012; Waaijers et al., 2013b). To this purpose, three aromatic OPFRs, triphenyl phosphate (TPP), resorcinol bis diphenyl phosphate (RDP) and bisphenol-A bis diphenyl phosphate (BDP), were tested for their mineralisation and primary biodegradation in activated sludge. When mineralisation was incomplete, but degradation of the parent compound did occur, we also attempted to identify the primary degradation products.
2 Materials and Methods

2.1 Chemicals

All chemicals were obtained from commercial sources and used as received: TPP (99%, CAS: 115-86-6), triphenyl phosphate-d15 (TPP-d15, 98%, product nr: 615218), diphenyl phosphate (DPP, 99%, CAS: 838-85-3), dibenzyl phosphate (DBP, 99%, CAS: 1623-08-1), benzoic acid (BA, 99.5%, CAS: 65-85-0), benzoic acid-d5 (BA-d5, 99%, CAS: 1079-02-3), bisphenol-A (BPA, 99%, CAS: 80-05-7), bisphenol-A-d16 (BPA-d16, 99%, CAS: 96210-87-6), and ammonium acetate (99.99%) were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). RDP (≥ 80%, CAS: 125997-21-9) and BDP (≥ 80%, CAS: 5945-33-5) were only available as technical mixtures and were obtained from ICL (Tel Aviv, Israel). KH$_2$PO$_4$, K$_2$HPO$_4$, Na$_2$HPO$_4$.2H$_2$O, NH$_4$Cl, CaCl$_2$.2H$_2$O, MgSO$_4$.7H$_2$O and FeCl$_3$.6H$_2$O were obtained from Sigma Aldrich. Acetone, acetonitrile and methanol (ULC grade) were obtained from Biosolve (Valkenswaard, The Netherlands), or Merck (Darmstadt, Germany), and ultra-pure water from Biosolve. Acetic acid (100%, anhydrous) and glucose were obtained from Merck and formic acid (98-100%, reagent grade) from Biosolve.

Due to their low solubility in water, individual stock solutions of TPP, RDP and BDP used for the mineralisation tests were prepared in either methanol or acetonitrile (ACN). The calibration standards for LC-MS/MS analysis were prepared in ACN.

2.2 Medium

The mineral medium was prepared according to OECD Guideline 310 (OECD 2006). The medium was made using the following stock solutions: solution A: KH$_2$PO$_4$ (8.50 g L$^{-1}$), K$_2$HPO$_4$ (21.75 g L$^{-1}$), Na$_2$HPO$_4$.2H$_2$O (33.40 g L$^{-1}$), NH$_4$Cl (0.50 g L$^{-1}$); B: CaCl$_2$.2H$_2$O (36.40 g L$^{-1}$); C: MgSO$_4$.7H$_2$O (22.50 g L$^{-1}$); D: FeCl$_3$.6H$_2$O (0.25 g L$^{-1}$) and consisted of 10 ml of stock A and 1 ml of stocks B, C and D in 1 litre of ultra-pure water. The amount of stock A prescribed by the protocol proved to be inadequate to buffer the pH of the mineralisation tests.
and was therefore increased to 50 ml. Secondary stage activated sludge from either the Amsterdam West or the Amstelveen sewage treatment plants was used as inoculum.

2.3 Mineralisation tests

The mineralisation tests were based on OECD Guideline 310 (2006) and consisted of biodegradation, abiotic and toxicity incubations in triplicate. The mineralisation incubations with TPP, RDP and BDP were prepared by adding flame retardant stock solution (to give concentrations of 2 and 20 mg L\(^{-1}\)) to 120 mL glass serum bottles and allowing the methanol to evaporate overnight, leaving the targeted compound in the vessel. Mineral medium (60 mL) and activated sludge (to give 30 mg L\(^{-1}\) total suspended solid (TSS)) were added the next day. In preliminary experiments we observed that toxicity of the OPFRs could have a negative influence on their mineralisation at high concentrations (data not shown). We therefore included toxicity tests in parallel with the mineralisation tests with OPFRs at concentrations similar to those in the mineralisation tests. Bottles used for the toxicity tests were prepared identically, but in addition contained glucose (50 mg L\(^{-1}\)). Controls to test for abiotic degradation were prepared similarly to the mineralisation incubations, but did not contain an inoculum and were sterilised with 1 mM sodium azide. The vessels were closed, the head space overpressure was set at approximately 700 mbar with synthetic air and the bottles were incubated at room temperature in the dark.

2.4 Primary biodegradation incubations

The primary biodegradation incubations consisted of biodegradation, abiotic and toxicity incubations in triplicate. For the preparation of the biodegradation incubations, 25 mL mineral medium in 100 ml-erlenmeyer flasks was inoculated with diluted secondary sludge to give a TSS of 100 mg L\(^{-1}\). Each flask was subsequently spiked with the individual stock solutions of the test compounds to give initial concentrations of 200 µg L\(^{-1}\). In addition, toxicity tests were conducted in order to monitor the vitality of the sludge microorganisms during the incubation.
These were prepared similarly to the biodegradation treatments, but additionally contained 200 mg L$^{-1}$ benzoic acid as substrate for the bacteria. Benzoic acid was chosen for this purpose as it could be analysed together with the OPFRs. The abiotic incubations were not inoculated and were sterilised with 1 mM sodium azide which was spiked weekly to maintain a sterile environment. The treatments were incubated at room temperature in the dark.

2.5 Analyses

Mineralisation was monitored by analysing the CO$_2$ production every 3-4 days by injecting 2.5 ml headspace samples in a Thermo Trace Ultra GC equipped with a Hayesep Q column (80-110, 2 m) and a flame ionisation detector. Blank CO$_2$ production by the inoculum (activated sludge without any addition) was subtracted from the CO$_2$ production in the treatments. Headspace concentrations were converted to total yields using equation 1 to take account of equilibrium between gas phase and dissolved CO$_2$:

$$N(\text{liquid}) = \left( N(\text{gas}) \times a \times \left( \frac{V_l}{V_g} \right) \right) \times (1 + 10^{pH-pKa})$$

(1)

Where $N =$ amount of CO$_2$ moles in the liquid or gas phase, $a =$ solubility constant of CO$_2$ (0.88 at 20°C), $V_l/V_g =$ ratio of liquid volume to gas volume in vessel, pH = actual pH within vessel, $pK_a =$ acid dissociation constant of CO$_2$ (6.38)

Concentrations of OPFRs in the biodegradation incubations were determined following centrifugation and filtration (0.45 μm glass fibre, Ø25, GD/X, Whatman) to remove particulates. LC-MS/MS analysis was performed using an HPLC system (LC20, Shimadzu, Kyoto, Japan) coupled to a tandem mass spectrometer (QTRAP 4000, Applied Biosystems, Toronto, Canada). Chromatographic separation was carried out on a C18 stationary phase (Luna C18(2), 3 μm, 100 Å, 150 x 3.0 mm ID, Phenomenex, Torrance, CA, USA) with an injection volume of 10 μL, a flow rate of 0.25 mL min$^{-1}$ and a column temperature of 45°C. Eluent A consisted of MeOH:H$_2$O (50:50) and eluent B of pure MeOH, both containing 5 mM ammonium acetate and 3 mM acetic acid. The gradient was as follows: 0 min (0% B), 12
Electrospray ionisation (ESI), operating in positive mode, and selected reaction monitoring (SRM) were used for quantification with TPP-d15 as internal standard. ESI in negative mode was used for the analysis of BA, DPP and BPA. The transitions monitored are TPP: 327>215, 327>183; TPP-d15: 342>243, 342>162; RDP: 575>419, 575>418; BDP: 693>367, 693>327; DPP: 249>93, 249>155; BPA: 227>212, 227>133; BPA-d16: 241>223, 241>142; BA: 121>77; BA-d5: 126>82. The method is reported in more detail in Chapter 3.

3 Results

3.1 Mineralisation

Mineralisation of the OPFRs by activated sludge was studied at 2 and 20 mg L$^{-1}$. The CO$_2$ yields obtained at 2 mg L$^{-1}$ could not be distinguished from those observed in blank incubations which did not contain OPFRs, probably due to high levels of degradable organic matter in the inoculum. We therefore only discuss the results obtained for the 20 mg L$^{-1}$ OPFRs treatments. In addition, the toxicity of the OPFRs towards activated sludge microorganisms was studied by determining the effects of the OPFRs (at 20 mg L$^{-1}$) on the mineralisation of glucose.

Mineralisation of glucose in the presence of TPP was $142 \pm 14.6\%$ of what would be expected if all the glucose were mineralised (Figure 1), indicating no toxicity of TPP at this concentration and that TPP was most likely acting as an additional carbon source. Indeed, the mineralisation of 20 mg L$^{-1}$ TPP to CO$_2$ observed after 28 days was $99.0 \pm 24.3\%$, indicating that this compound achieved the ready biodegradability requirements (CO$_2$ production >60% of theoretical maximum in 28d, (OECD 2006)). This mineralisation of TPP thus explains the more than 100% yield of CO$_2$ observed in the toxicity test. In the presence of 20 mg L$^{-1}$ RDP, glucose mineralisation was $61.6 \pm 2.0\%$ compared to $64.4 \pm 1.2\%$ in the reference, indicating that RDP was not toxic at this concentration (Figure 1). Despite its lack of toxicity at this concentration, only $18.0 \pm 10.5\%$ mineralisation of RDP was reached after 28 days, indicating that this compound is not readily biodegradable. BDP at 20 mg L$^{-1}$ was slightly toxic to the activated sludge, reducing glucose mineralisation from $78.3 \pm 1.1\%$ of the maximum.
theoretically possible in the reference to 63.6 ± 1.2% in the presence of BDP (Figure 1), a reduction of about 19%. The maximum mineralisation of BDP that was observed was 11.2 ± 0.05%. This low extent of mineralisation is unlikely to be caused by its low toxicity and indicates that this compound is also not readily biodegradable.

Much lower mineralisation of all three OPFRs was observed in the sterile abiotic treatments (Figure 1), confirming that mineralisation of these compounds observed in the non-sterile experiments was the result of microbial activity only.

![Structures of the organophosphorus flame retardants used in this study.](image)

**3.2 Primary biodegradation**

### 3.2.1 TPP

In line with the mineralisation tests, rapid removal of TPP was observed in living sludge (Figure 2) with complete removal occurring within 7 days (DT$_{50}$: 2.8 days). As was observed for mineralisation, TPP removal was significantly slower in sterilised sludge (complete removal after 28 days and DT$_{50}$: 8 days). These DT$_{50}$ values correspond to significantly different first
order degradation rate constants (0.18 ± 0.04 days⁻¹ and 0.07 ± 0.01 days⁻¹ in living and sterile sludge, respectively) (paired t-test, p < 0.01). Diphenyl phosphate (DPP) was identified in the samples from these incubations by comparison of its retention time and fragmentation with those of the standard. The DPP released during biotic degradation of TPP was completely removed during the experiment, whereas it accumulated in the sterilised abiotic incubations (Figure 3), showing that in the sterile system the removal of TPP was due to hydrolysis of TPP to DPP. Both abiotic hydrolysis and microbial degradation could contribute to the conversion of TPP to DPP in the biotic incubations, but in this case DPP is degraded further by the biota present in the sludge. Other transformation products of TPP were not identified, probably due to other potential transformation products, such as monophenylphosphate, being too polar for the LC-MS system employed.

3.2.2 RDP

RDP was completely removed in the biodegradation incubations within 4 days (biodegradation rate constant 0.80 ± 0.08 days⁻¹, DT₅₀ < 1 day), while abiotic degradation was significantly slower (rate constant 0.03 ± 0.01 days⁻¹, DT₅₀ = 18 days) (p < 0.001) (Figure 4). The rapid removal of RDP is in apparent contrast to the slow mineralisation observed in the mineralisation tests and suggests that poorly degradable transformation products may have accumulated in the biodegradation incubations. As was observed for TPP, DPP was formed as RDP was degraded and reached its maximum concentration as the RDP concentration fell below the LOQ. In contrast to what was observed in the experiments with TPP, DPP was not completely removed by biodegradation, but persisted at low levels (ca. 40 nM). No other degradation products could be identified in these incubations by LC-MS analysis but as stated above, this could be due to limitations of the instrumentation used. As was observed for TPP, the DPP that is formed from RDP in sterilised sludge seems to accumulate as end product.
Figure 2: Yields of CO2 (% of theoretical) produced after 28 days mineralisation of 20 mg L⁻¹ TPP, RDP and BDP in activated sludge (biotic) and sterilised activated sludge (abiotic). Also shown is the CO2 yield from glucose in the presence of 20 mg L⁻¹ OPFRs.

Figure 3: Primary degradation of TPP (nM, line with black square data points) in biotic activated sludge (left) and the formation and degradation of the breakdown product TPP (nM, line with white triangle data points) and sterilised (abiotic) activated sludge (right).
Figure 4: Primary degradation of RDP (nM, line with black square data points) in biotic activated sludge (left) and the formation and degradation of the breakdown product TPP (nM, line with white triangle data points) and sterilised (abiotic) activated sludge (right).

Figure 5: Primary degradation of BDP (nM, line with black square data points) in biotic activated sludge (left) and sterilised (abiotic) activated sludge (right).

3.2.3 BDP

Although an initial decrease was observed in the concentrations of BDP in both the biotic and abiotic incubations (Figure 5), the results obtained after 56 days suggest that biodegradation of BDP was very slow. This is supported by the fact that the potential degradation products BPA or DPP were not detected in these incubations. The very slow biodegradation observed for BDP is consistent with the low rate of mineralisation of BDP observed in the mineralisation
tests (Figure 2). This finding and the lack of degradation products detected suggest that the rate of primary biodegradation is controlling the mineralisation of this chemical.

4 Discussion

In this study we determined the mineralisation and primary biodegradation of aromatic OPFRs that have been proposed as replacements for BFRs. Although some data is available on the biodegradability and persistence of these chemicals (reviewed in (Waaijers et al., 2013b)), almost none of these data are available in the open scientific literature and many of these data are contradictory. The available data show that TPP is readily biodegradable in sewage treatment plant sludge with a reported DT_{50} (time required to remove 50% of the initial concentration) of < 28 days (UNEP OECD SIDS 2002, US EPA 2005). Our data show that TPP is mineralised rapidly in sludge and that more than 60% conversion to CO₂ is achieved within 28 days. In addition, we observed rapid removal of TPP in both the primary biodegradation and the sterile abiotic incubations, with a very short DT_{50} for primary degradation in sludge (2.8 days) and a little longer value (8 days) under sterile conditions. In both cases, initial degradation consists of the conversion of TPP to DPP by hydrolysis of a phosphate ester linkage. This DPP accumulates under sterile conditions, whereas it is degraded further in non-sterilised sludge. This shows that both biotic and abiotic processes can contribute to the removal of TPP but that mineralisation depends on microbial activity. Further work employing LC columns giving longer retention times and high-resolution mass spectrometry is required to identify other transformation products and to characterise the pathway responsible for the biodegradation of DPP by these microorganisms. Nevertheless, the present study is the first to report accurate and publicly available DT_{50} values for TPP in activated sludge and to show that TPP can be considered to be readily biodegradable.

Previously reported persistency data for RDP are inconsistent, with slow primary biodegradation being reported in sludge (DT_{50} = 28 days (U.K. Environment Agency et al., 2009)) and two reports indicating either 60% or 37% mineralisation in 28-days tests with activated sludge (U.K. Environment Agency et al., 2009; ICL Industrial Products, 2011). Our results show that the persistency of RDP is much lower than previously reported, with a DT_{50}
for primary biodegradation of < 1 day. Despite the rapid primary biodegradation, the 18% mineralisation observed after 28 days in our study is lower than previously reported and indicates that this compound is not readily biodegradable. The reasons for these widely different rates of mineralisation observed for RDP remain unclear. The rapid primary biodegradation but slow mineralisation of RDP implies that significant accumulation of transformation products (or intermediates) must be taking place. We observed the formation and degradation of DPP, but were unable to identify other expected transformation products, such as a hydroxylated TPP derivative. Identification and quantification of these potentially accumulating transformation products would contribute to the assessment of environment risks of RDP. Due to its lack of mineralisation, it is concluded that RDP should not be considered ready biodegradable, in spite of its rapid primary biodegradation.

Our results for the mineralisation of BDP show that this chemical is mineralised slowly (reaching only 11.2% of the theoretical maximum yield of CO₂ after 28 days) and that this compound is therefore not readily biodegradable. This is consistent with the single previously reported study of the mineralisation of BDP in sludge, which reports that this compound is not readily biodegradable (6% mineralisation in sludge, (Australian Government Regulator of Industrial Chemicals, 2000)). Previously reported data on BDP also show that its persistence is high, with DT₅₀ values for sludge and water ranging from months to years (European Chemicals Bureau et al., 2007; EPA, 2011). Our results confirm that primary biodegradation of BDP is very slow under the conditions used in our experiments. Nevertheless, our observation of slow mineralisation of BDP shows that this chemical can be degraded by sludge and can therefore be classified as inherently biodegradable. More research is required to identify the reasons for the much slower biodegradation of BDP compared to that of the structurally similar RDP and to identify any transformation products that may accumulate.

Mineralisation (conversion to CO₂) is the basis of standardised biodegradability testing, using for example OECD protocols (e.g. OECD, 2006). This approach has a number of advantages such as the environmental desirability of mineralisation as the result of biodegradation and analytical simplicity. Nevertheless, there are some potential drawbacks to this approach, such as possible toxicity issues arising from the need to use relatively high test concentrations to achieve CO₂ production above background levels. Furthermore, such high test concentrations
may exceed the aqueous solubility of poorly soluble compounds, thereby restricting their biodegradation rates. Factors responsible for the slow biodegradation observed could include toxicity at the concentrations used in this study or the low solubility of BDP. We found no evidence for toxicity of the OPFRs significant enough to explain the mineralisation and biodegradation rates observed in this study. There is a very large distribution on the reported solubilities of RDP and BDP (Waaijers et al., 2013b) with most values in the ng L$^{-1}$ to μg L$^{-1}$ range. Whether these can account for the rapid primary biodegradation of RDP but slow primary biodegradation of BDP or that this is due to other factors is unclear.

In conclusion, the mineralisation tests show that TPP is readily biodegradable, but that this is not the case for RDP and BDP in our experimental system. Furthermore, primary biodegradation in activated sludge showed complete removal of TPP and RDP within a few days, but high persistence of the structurally similar BDP. The rapid primary biodegradation but slow mineralisation of RDP suggests that transformation products accumulate during biodegradation of this compound.

**Acknowledgements** This research is part of the EU project ENFIRO (KP7-226563) and the financial support of the European Union is gratefully acknowledged, as well as the critical input and feedback of the ENFIRO partners.