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Daphnid Life Cycle Responses to New Generation Flame Retardants

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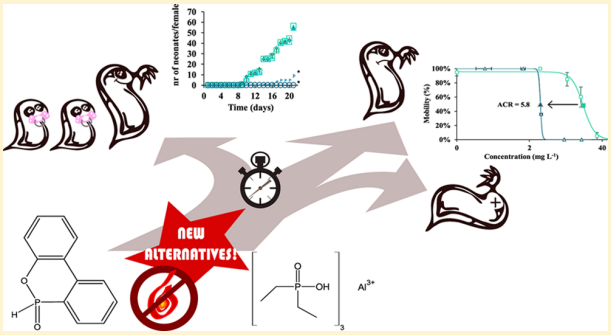
Supporting Information

ABSTRACT: Relatively hazardous brominated flame retardants (BFRs) are currently substituted with halogen-free flame retardants (HFFRs). Consequently, information on their persistence, bioaccumulation and toxicity (PBT) is urgently needed. Therefore, we investigated the chronic toxicity to the water flea *Daphnia magna* of two HFFRs, aluminum diethylphosphinate (ALPI) and 9,10-dihyro-9-oxa-10-phosphaphenanthrene-oxide (DOPO). The toxicity of ALPI increased from a 48 h LC_{50} of 18 mg L^{-1} to a 21 day LC_{50} value of 3.2 mg L^{-1}, resulting in an acute-to-chronic ratio of 5.6. This may imply a change in classification from low to moderate toxicity. ALPI also affected sublethal life cycle parameters, with an EC_{50} of 2.8 mg L^{-1} for cumulative reproductive output and of 3.4 mg L^{-1} for population growth rate, revealing a nonspecific mode of action. DOPO showed only sublethal effects with an EC_{50} value of 48 mg L^{-1} for cumulative reproductive output and an EC_{50} value of 73 mg L^{-1} for population growth rate. The toxicity of DOPO to *D. magna* was classified as low and likely occurred above environmentally relevant concentrations, but we identified specific effects on reproduction. Given the low chronic toxicity of DOPO and the moderate toxicity of ALPI, based on this study only, DOPO seems to be more suitable than ALPI for BFR replacement in polymers.



1. INTRODUCTION

To fulfill regulatory requirements on flame retardancy, chemical additives known as flame retardants (FRs) are incorporated into a wide range of polymers. Brominated flame retardants (BFRs) are frequently used because they have a low impact on the polymer's characteristics, are very effective in relatively low amounts compared to other FRs,[1] and are relatively cheap.[2] Many BFRs, however, have unintended negative effects on the environment and human health. For instance, some bioaccumulate in aquatic and terrestrial food chains,[3] and some show serious adverse effects such as endocrine disruption.[4]

Concerns about the persistence, bioaccumulation, and toxicity (PBT) of BFRs have led to a ban on the production and use of many of these compounds.[5] Hence, there is growing need to substitute BFRs with alternative halogen-free flame retardants (HFFRs), and several furniture manufacturers have already voluntarily replaced BFRs with alternative HFFRs.[6] HFFRs can be divided into several categories, the most important ones being: inorganic flame retardants and synergists (mostly used for electronics and electrical equipment), organophosphorus compounds and their salts (housings of consumer products), nitrogen-based organic flame retardants (electronics and electrical equipment) and intumescent systems (textile coatings). Because of the need for BFR substitution, many of these HFFRs are already being marketed, although their environmental behavior and toxicological properties are known to only a limited extent and their potential impact on the environment cannot yet be properly assessed. As a result, there is an urgent need for information on the PBT properties of HFFRs. Reviewing the publicly available ecotoxicity data of HFFRs, we identified large data gaps and inconsistent observations on the properties of individual compounds.[7] Therefore, we generated reliable toxicity data for a selection of HFFRs that are potential replacements for BFRs in polymers.[8] Several HFFRs exerted no acute toxicity to daphnids, making them promising substitutes, while highly toxic compounds could be discarded as alternative flame retardants. However, in order to reliably evaluate the environmental hazard of compounds showing a low acute toxicity, they should also be subjected to chronic toxicity testing, since toxicity tends to increase with increasing exposure time.[9,10] Moreover, specific effects on sublethal life history end points depending on the mode of action of a compound need time to become expressed

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and are overlooked in standard acute toxicity tests.[11,12] Therefore, the aim of the present study was to determine the life-history responses of daphnids chronically exposed to widely applied HFFRs that have been shown to elicit a low acute toxicity.

To this purpose, two water-soluble organophosphorus flame retardants (OPFRs) were selected: aluminum diethylphosphinate (ALPI), mostly used in electrical and electronic equipment, connectors, switches, and encapsulated electronic components, and 9,10-dihydro-9-oxa-10-phosphaphenanthrene (DOPO), mostly used in printed circuit boards, electronic components, and encapsulations and technical laminates.[13] With these OPFRs we performed *Daphnia magna* life cycle toxicity tests[14] and integrated the obtained data into EC_{50} values for population growth rate. In this way we aimed to assess whether or not these compounds, showing a low acute toxicity,[8] are also nonhazardous upon chronic exposure and subsequently, obtaining a first indication whether or not ALPI and DOPO are suitable candidates for BFR replacement in polymers.

2. MATERIALS AND METHODS

2.1. Test Organism and Culture Conditions. We chose the fresh water filter feeder *Daphnia magna* Straus to test for chronic toxicity of ALPI and DOPO. This water flea is frequently used as test organism in chronic ecotoxicity studies[14] due to several benefits, including its key role in the pelagic food webs of temperate regions, parthenogenetic reproduction (which excludes genetic variation) and ease of handling.[15,16] The *Daphnia magna* neonates (younger than 24 h, clone 4) used in this study were obtained from Grontmij Aquasense (Amsterdam, The Netherlands) and were cultured in Elendt M4 medium according to the OECD guideline.[14] For an extensive description of the specific culture conditions, see Waaijers et al.[8] At regular intervals (about every three months), acute toxicity tests were performed with the reference toxicant $K_2Cr_2O_7$ to check whether the sensitivity of the *D. magna* culture was within the limits (EC_{50}, 24 h = 0.6−2.1 mg L^{-1}) as set by the guideline.[17]

2.2. Test Compounds: Halogen-Free Flame Retardants. The selected HFFRs were aluminum diethyl phosphinate (CAS no. 225789−38−8, 98.5%, Clariant) and 9,10-dihydro-9-oxa-10-phosphaphenanthrene (CAS no. 35948−25−5, ≥98%, KCCS). The chemical structures of the organophosphates are shown in Figure 1.

2.3. Test Solutions. To generate homogeneous test solutions, we decided to first prepare saturated water solutions (Elendt M4 medium without additional buffer), determine the concentrations (see Section 2.5) and dilute these solutions to prepare the test concentrations. To this end we stirred an excess of compound for 7 days in Elendt medium[14] and then filtered the solution (0.1 μm, cellulose). The test concentrations for each compound were based on their acute toxicity[8] and are listed in the Supporting Information (SI), Table S1.

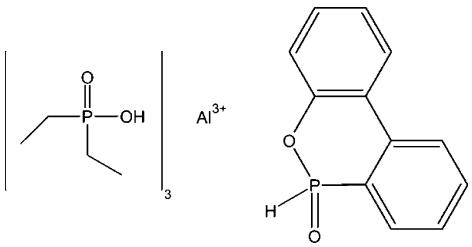

Figure 1. The molecular structures of aluminum diethyl phosphinate (ALPI, left) and 9,10-dihydro-9-oxa-10-phosphaphenanthrene (DOPO, right).

2.4. Toxicity Tests. To determine the chronic toxicity of ALPI and DOPO, *Daphnia magna* were exposed to these HFFRs in 21 day reproduction tests, following OECD guideline 211,[14] except where noted. Per test concentration (4 concentrations and a control), 15 replicates were prepared, with three additional replicates for measuring the concentration of the test compound. Each replicate consisted of a 50 mL polypropylene tube filled with 40 mL of test solution. The tubes were randomly distributed in a climate controlled fume hood (20 ± 1 °C), with a light-dark regime of 16:8 h. The experiment was started by introducing 1 neonate (younger than 24 h) into each tube using a disposable transfer pipet. Each day the number of animals not responding to gentle stimulation by tapping on the tube was scored. Juveniles and ephippia (winter eggs) were also counted and removed daily. The daphnids were fed daily with a concentrated suspension of the green algae *Scenedesmus subspicatus* (Grontmij, day 0−2: 450 μL, day 3−5: 700 μL and day 6−21: 900 μL (2850 cells/ μL)). The exposure solutions and replicates for measuring the concentrations of the compounds were refreshed two times a week using a weekly made saturated stock solution. In this way, the variation in exposure concentration during the full 21 days was sufficiently low (s.e. < 2 mg L^{-1}) for both compounds. Physical-chemical parameters (hardness, oxygen level, temperature and pH) recommended by the guideline were measured (SI Table S2). DOPO hydrolyses in water, which in turn decreases the pH of the medium significantly (pH <6, in the highest test concentrations). Therefore the DOPO test was performed with buffered medium and additional controls with buffer were included as well. Test medium was buffered to pH 7.87 ± 0.01 (s.e.) with NaOH (120 mg L^{-1}) and 3-(N-morpholino)-propanesulfonic acid (MOPS) (628 mg L^{-1}) according to De Schamphelaere et al.[18]

2.5. Analysis of ALPI and DOPO Concentrations in the Test Solutions. The concentrations of ALPI and DOPO in the saturated solutions were determined after a week of stirring and subsequent filtration. This was done to determine the concentration in the stock solution in order to prepare the test concentrations by dilution. During the experiment, the concentrations of the compounds were determined by taking water samples from three additional replicates containing no daphnids after 7, 14, and 21 days (end) of the toxicity tests, just before renewal of the test solutions. Of each of these replicates 15 mL water samples were taken, acidified (0.4% nitric acid and 2.38 g cesium chloride L^{-1}, both obtained from Merck) and stored until analysis at 4 °C. Samples were measured using inductively coupled plasma coupled to atomic emission spectroscopy (ICP-AES, Optima 3000XL). Phosphorus was determined to calculate the molecular concentrations for both ALPI and DOPO. These measured values were averaged over time to calculate the actual concentration per treatment during the 21 day exposure period.

2.6. Data Analysis. Concentration−response relationships and the corresponding 21-d EC_{50} values were calculated according to Haanstra et al.[19] by fitting a logistic curve (eq 1) through the data of the studied toxicity end point, that is, percentage mobility, cumulative reproductive output per

female, and population growth rate, against the HFFR concentration in the water.

$$y(x) = \frac{c}{1 + e^{b(\log_{10} x - \log_{10} a)}} \quad (1)$$

In this equation $y(x)$ is the end point at concentration x, a is the EC_{50} (mg L^{-1}), b is the slope of the curve, c is $y(0)$, which equals the average of the end point for the control, and x is the concentration of HFFR in the water (mg L^{-1}).

Cumulative reproductive output was calculated per adult (eq 2).

$$\mathrm{CRO} = \sum_{t=0}^{\Omega} m_t \quad (2)$$

In this equation CRO is the cumulative reproductive output per adult for a specific treatment (control or exposure concentration), t is the time of the experiment in days, with Ω as the last day of the experiment (21 days) and m_t is the number of neonates per adult (fecundity) at time t.

Data for CRO were first tested for normality (Shapiro-Wilks test) and homogeneity of variances (Levene's test). Then, this end point was compared with the corresponding control using one-way analysis of variance (ANOVA), followed by a Tukey post hoc test. Variances in the production of winter eggs were heterogeneous, therefore means were compared with the nonparametric Mann−Whitney U test.

Data for survival were first tested for normality (Shapiro-Wilks test) and treatments were compared with the corresponding control. The latter was calculated with the open source software program R (script developed by Arne Janssen, University of Amsterdam) using Kaplan−Meier survivorship analysis.[20] Because of multiple testing (n = 4), a Bonferroni correction was applied.

The population growth rate (r) was calculated from the integration of the age-specific data on probability of survival and fecundity by using the Lotka-Euler equation (eq 3).[21,22]

$$1 = \sum_{t=0}^{\Omega} l_t m_t e^{-r(t+1)} \quad (3)$$

In this equation t is the time of the experiment in days, with Ω as the last day of the experiment (21 days), l_t is the probability of survival at time t, m_t is the amount of living neonates per adult (fecundity) at time t and r is the population growth rate (d^{-1}). The growth rate and its standard error were calculated with the open source software program R (script developed by Arne Janssen, University of Amsterdam) using the Jackknife-method described by Meyer et al.[23]

Data analyses were performed with SPSS software (V20.0.0),[24] except for r and its standard error and for survival, which was performed with R (V2.15.1).[25]

3. RESULTS

Acute toxicity tests performed with the reference toxicant $K_2Cr_2O_7$ showed that the sensitivity of the *D. magna* culture (EC_{50}, 24 h = 1.1 mg L^{-1}, 95% CL: 0.8−1.3) was within the limits set by the guideline (EC_{50}, 24 h = 0.6−2.1 mg L^{-1}) (17). The physical-chemical parameters (hardness, oxygen level, temperature, and pH, shown in SI Table S2) were within the recommended ranges.[14] Control survival was 100%, with a mean number of neonates per female of 50 or higher, with 14% or less variation.

A clear effect of ALPI on daphnid mobility was observed during the 21 day test period (SI Figure 1). A significant (p < 0.05) drop in survival was observed for exposure to the three highest test concentrations after three days, the highest two test concentrations causing complete mortality after four d. From the mobility data at the end of the experiment (21 days) a clear concentration−response relationship was obtained (Figure 2),

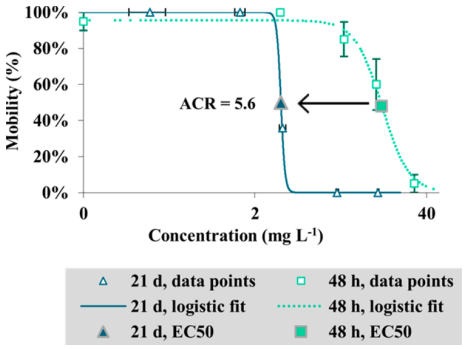

Figure 2. Average mobility (% of initial animals) of *Daphnia magna* (n = 15) exposed to a concentration range of ALPI (mg L^{-1}) in Elendt medium after 48 h (±s.e. in x and y, n = 4 × 5 individuals per concentration, published before in ref 8) and after 21 days (±s.e. in x, n = 15 individuals per concentration). The toxicity increases with increasing exposure time with an ACR of 5.8.

from which an LC_{50} value of 3.2 mg L^{-1} (21 days, 95% CL: 3.1−3.2) was derived. In Figure 2, both the chronic (21 days) and the acute concentration−response curves (acute data previously published by[8]), are plotted, as well as the corresponding LC_{50} values (LC_{50}, 2 d = 18 mg L^{-1}, 95% CL: 15−22). This comparison demonstrates that the toxicity of ALPI increased with increasing exposure time with an acute to chronic ratio (ACR) of 5.8.

In all treatments where the daphnids survived the exposure to ALPI, the cumulative reproductive output per female was significantly (p < 0.05) lower than in the controls (Figure 3, left graph). Besides a reduced reproductive output (Figure 3), the daphnids exposed to 2 and 3 mg ALPI L^{-1} also produced winter eggs (5.0 ± 1.1 s.e. and 4.8 ± 0.7 s.e., respectively), which is significantly more than those in the control did (0.2 ± 0.2 s.e.) (p < 0.001 and p < 0.05 respectively, Mann−Whitney

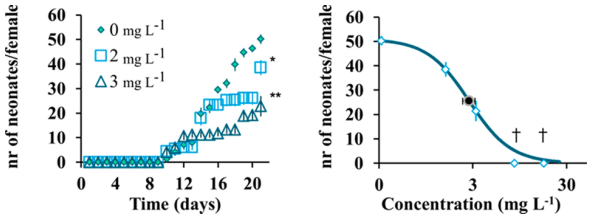

Figure 3. Average cumulative reproductive output (number of neonates per female) of *Daphnia magna* (n = 15) exposed to a concentration range of ALPI (mg L^{-1}) in Elendt medium over time (left) and after 21 days (right). In the left graph treatments where the CRO (±s.e. in y) was significantly lower than that of the controls are marked * p < 0.05 ** p < 0.001. In the right graph the average CRO (◊, female^{-1}) is shown (±s.e. in x and y, those smaller than data points are omitted). The EC50 is plotted as ● (±s.e.) and the logistic curve represents the fitted concentration−response relationship. Treatments where all daphnids were immobilized after 21 days are marked with †.

U test). This is also an apparent indication of stress.[14] There was however no significant ($p > 0.05$) difference in age at first reproduction between the ALPI concentrations and the controls. A clear concentration−response relationship was observed for CRO after 21 days of exposure. From this relationship an EC_{50} value of 2.8 mg L^{-1} (21 days, 95% CL: 1.9−3.6) was derived (Figure 3, right graph). The effect on survival and fecundity of *Daphnia magna* caused by ALPI was reflected by a decrease in population growth rate with increasing ALPI concentrations in the water as shown in Figure 4. From this concentration−response relationship an EC_{50} value for population growth rate (r) of 3.4 mg L^{-1} (21 days, 95% CL: 3.4−3.4) was derived.

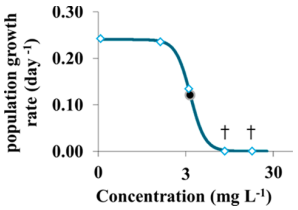

Figure 4. Population growth rate (d^{-1}) of *Daphnia magna* (n = 15) exposed to a concentration range of ALPI (mg L^{-1}) in Elendt medium after 21 days. The average population groth rate (◊) is shown (±s.e. in x and y). The EC50 is plotted as ● (s.e. smaller than data point) and the logistic curve represents the fitted concentration−response relationship. Treatments where all daphnids were immobilized after 21 days are marked with †.

No effect on mobility (>95%) was observed after 21 days of exposure to the highest tested DOPO concentration (166 mg L^{-1}). However, the daphnids exposed to this test concentration did not reproduce at all, while those exposed to the one but highest concentration (78 mg L^{-1}) started to reproduce significantly ($p < 0.001$) later and significantly ($p < 0.001$) less than the control (Figure 5, left graph). As observed with

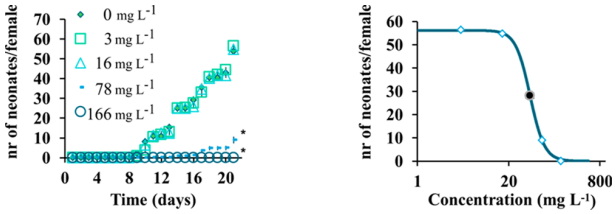

Figure 5. Average cumulative reproductive output (number of neonates per female) of *Daphnia magna* (n = 15) exposed to a concentration range of DOPO (mg L^{-1}) in Elendt medium over time (left) and after 21 days (right). In the left graph treatments where the CRO (±s.e. in y) was significantly lower than that of the controls are marked * $p < 0.001$. In the right graph the average CRO (◊, female $^{-1}$) is shown (s.e. in x and y are smaller than the data points and therefore omitted). The EC50 is plotted as ● (s.e. smaller than data point) and the logistic curve represents the fitted concentration−response relationship.

ALPI, when DOPO affected the CRO (78 mg L^{-1}), also winter eggs were produced (28 ± 2 s.e.), whereas the controls hardly produced any (0.2 ± 0.2 s.e.) ($p < 0.001$, Mann−Whitney U test). A clear concentration−response relationship was observed for CRO after 21 days, with an EC_{50} value of 48 mg L^{-1} (21 days, 95% CL: 33−62) (Figure 5, right graph). Consequently, DOPO affected the population growth rate of *Daphnia magna* as well, as shown by the concentration−response relationship in Figure 6, from which an EC_{50} value of 73 mg L^{-1} (21 d, 95% CL: 72−74) was derived.

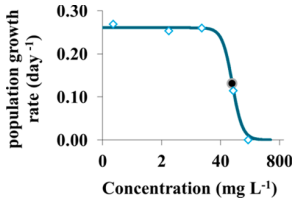

Figure 6. Population growth rate (d^{-1}) of *Daphnia magna* (n = 15) exposed to a concentration range of DOPO (mg L^{-1}) in Elendt medium after 21 days. The average population groth rate (◊) is shown (s.e. in x and y are smaller than the data points and therefore omitted). The EC50 is plotted as ● (s.e. smaller than data point) and the logistic curve represents the fitted concentration−response relationship.

Corresponding parameters and confidence limits of the concentration−response curves and EC_{50} values are reported for both compounds in SI Table S3.

4. DISCUSSION

The present study demonstrated that ALPI and DOPO clearly affected the life-history of the daphnids. For ALPI only a single toxicity study was available from the literature, reporting a lower chronic toxicity (21 days, reproduction EC_{50} = 46 mg L^{-1}; immobility EC_{50} = 22 mg L^{-1})[26] than observed in the present study (2.8−3.4 mg L^{-1}). Few details on experimental conditions (such as how the concentrations were measured and the hardness) were, however, provided by the pertaining reference,[26] so it is difficult to compare this data set with our data. Daphnid clone specific differences in sensitivity potentially contribute to variation in the results of toxicity studies, such as reported by Baird et al.[27] Nevertheless, the conformity of the present toxicity data with the literature on the reference toxicant $K_2Cr_2O_7$ showed that the presently used clone met the requirements set by the guideline.[17] This, as well as the 100% control survival and EC_{50} values based on actual concentrations all substantiate the robustness of the toxicity data in the present study.

When DOPO dissolves in water, its ring P−O bond hydrolyses,[28] which opens the middle ring and creates an acidic product. This phenomenon decreased the pH of the highest test concentrations to about 4, far below the tolerance level of *D. magna*. Therefore, all treatments were actively buffered at a pH of 7.87 ± 0.01 (s.e.). There is no data available (yet) on environmental concentrations of DOPO, but we expect that the highest test concentrations used in the present study are unlikely to be environmentally relevant, unless for instance accidental spills occur. Hence, it is expected that in the environment DOPO concentrations are low enough to be naturally buffered. For DOPO only a model prediction of its chronic toxicity (23 mg L^{-1}) has been reported.[29] Although no information on the specific end point or assumed conditions were provided, the predicted effect concentration is in the same order of magnitude as our experimentally obtained effect concentrations (48−73 mg L^{-1}). To our best knowledge, apart from one study on freshwater fish (low toxicity; 48 h, EC_{50} = 370 mg L^{-1}[29]) there are no other reports on experimental ecotoxicity studies with DOPO.

The motivation to conduct this study was that these two widely applied halogen-free flame retardants are currently produced in large volumes as alternatives to brominated flame retardants, while they are poorly studied and consequently their environmental properties are not adequately characterized. For ALPI, a strong increase in toxicity was observed after three days of exposure. This shows the importance for chronic toxicity testing, since this lethal effect would be overseen in a standard acute toxicity test of 48 h.[17] This increased toxicity over time is reflected by an acute to chronic ratio (ACR) of 5.8. Based on the acute toxicity (LC_{50} 18 mg L^{-1} (8)), ALPI would be classified by the European REACH legislation as long-term chronic toxicity category 3 ($10 < LC_{50} < 100$ mg L^{-1}),[30] meaning that ALPI would be considered *harmful* to aquatic life with long lasting effects. In the present study, the chronic LC_{50} value was 2.9 mg L^{-1} ($1 < LC_{50} < 10$ mg L^{-1}),[30] whereas the chronic EC_{10} (equivalent to the NOEC) for cumulative reproductive output was 0.89 mg L^{-1} ($EC_{10} < 1$ mg L^{-1}).[30] Since ALPI has also been reported as being non rapidly degradable[7] this could lead to a change of classification to long-term chronic toxicity category 2,[30] meaning that ALPI would be considered *toxic* to aquatic life with long lasting effects. DOPO also showed an increase in toxicity with increasing exposure time, albeit the latter effect concentrations are still classified as a low toxicity.[30]

Marinkovic et al.[11] demonstrated that a chronic-to-chronic ratio can be a good predictor of the mode of action of a toxic substance. In this ratio the chronic lethal concentration (LC_{50}) is divided by a chronic sublethal concentration (EC_{50}). For ALPI the sublethal EC_{50} values are in the same order as the LC_{50} and hence, the ratio is close to 1. This implies that the compound has a nonspecific mode of action, and acts by narcosis.[11] DOPO on the other hand showed sublethal effects at concentrations considerably lower than the highest test concentrations, where still no mortality at all was observed. Therefore, the chronic LC50/EC50 would be minimally 7, suggesting a specific mode of action for DOPO acting on reproduction and thus on population growth.[11]

To conclude, the chronic experiments identified a specific effect of DOPO on daphnid reproduction and population growth, but the chronic toxicity of DOPO to *D. magna* could be classified as low and the effects occurred at concentrations unlikely to be observed in the environment. The toxicity of ALPI increased with increasing exposure time from a low toxicity upon acute exposure to a moderate toxic potential after chronic exposure. Therefore, based on this study only, DOPO seems to be a more suitable candidate for BFR replacements in polymers than ALPI.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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