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Toxicity of Plastic Additive 1-Hydroxycyclohexyl Phenyl Ketone (1-HCHPK) to Freshwater Microcrustaceans in Natural Water

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Abstract: Various potentially toxic compounds associated with plastic (e.g., plastic additives) can enter the environment during plastic fragmentation and/or weathering. 1-Hydroxycyclohexyl phenyl ketone (1-HCHPK) is a widely used photoinitiator, e.g., in UV-radiation-curable technologies such as 3D-printing, plastic coatings and construction materials. 1-HCHPK may reach aquatic ecosystems via various waste-flows, including leaching from consumer goods. However, knowledge of its potential environmental hazard is scarce. In the present study, we addressed this data gap by assessing the acute and long-term toxicity of 1-HCHPK to freshwater microcrustaceans in environmentally relevant conditions using natural waters. The results showed that the acute toxicity of 1-HCHPK ($L(E)C_{50}$) to pelagic *Thamnocephalus platyurus* and *Daphnia magna* and benthic *Heterocypris incongruens* ranged between 27 and 55 mg/L. Further, the long-term exposure of *D. magna* to low levels of 1-HCHPK (0.1 and 1.0 mg/L) did not affect ephippia hatching or organismal fitness, even in three successive daphnid generations. Thus, 1-HCHPK did not pose a hazard to the freshwater microcrustaceans at concentrations < 1 mg/L in the environmentally relevant conditions (i.e., multigenerational life cycle tests conducted in lake water at low chemical exposure concentrations). The tests employed in this study allowed for the environmentally relevant hazard assessment of emerging pollutants such as a plastic additive 1-HCHPK.

Keywords: plastic contamination; photoinitiators; hazard; crustaceans; *Daphnia magna*; toxicity; emerging contaminants; life cycle test

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1. Introduction

In recent decades, much attention has been paid to the problems of plastic pollution in the environment. Moreover, it has been shown that plastic additives play a significant role in the adverse effects of plastic on ecosystem health [1]. Importantly, plastic products differ in chemical composition due to numerous additives used to produce plastic for specific applications. The various compounds associated with plastic (i.e., plastic additives) may enter the environment not only with plastic waste during its fragmentation and/or weathering but also at all stages of their life cycle: production, transportation and application [2]. It has already been shown that plastic additives can be dangerous to living organisms and the use of some of them has been restricted [3–5]. However, there are still knowledge gaps concerning the potential hazards of various currently used plastic additives to biota due to the shortage of experimental data: (i) on the leaching of additives from different plastic products; (ii) on the ecotoxicity of plastic additives [6–8]. To date, over 10,000 plastic additives have been identified [9], which include the photopolymerization chemicals, or photoinitiators (PIs), that are increasingly important in 3D-printing and biomedical applications (e.g., dentistry and tissue engineering). Moreover, for bio-printing, biocompatible and water-soluble PIs are of high interest [10,11].

1-Hydroxycyclohexyl phenyl ketone (1-HCHPK, CAS no.: 947-19-3, IUPAC name (1-hydroxycyclohexyl)-phenylmethanone) is the most popular Norish type I PI because of

its solubility in many resins. 1-HCHPK is one of the most commonly used photoinitiators, and possesses high initiation efficiency, excellent thermal stability and yellowing resistance. The current industrial production volume of 1-HCHPK is estimated to be between 1000 to 10,000 tons [12]. The high usage of 1-HCHPK [13] in a broad range of products (e.g., ink-jet printing technology, metal coating, construction, and automotive industry) could increase risks to aquatic ecosystems as its release into the environment through leaching from discarded plastics and from food packaging (e.g., during fragmentation) [14] is a realistic scenario. The leaching of 1-HCHPK from plastics has been demonstrated through the aqueous extraction of 3D-printed plastics, where 1-HCHPK was the major leachate constituent [15]. 1-HCHPK has also been shown to migrate at high concentrations from printing inks and packaging materials into food (4260 μg/kg) [14] and from polyethylene ampoules into aqueous injection solutions (6–8 μg/mL) [16].

The potential health hazards of 1-HCHPK could manifest through estrogenic activity, demonstrated in an estrogen-sensitive human breast cancer cell line in vitro at a 1-HCHPK concentration of 10^{-5} M (2 mg/L) [17]. 1-HCHPK also promoted the growth of breast tumors in mice [18] and decreased the viability of human monocytes after 24 h exposure at >125 μg/mL [16]. However, Takai et al. [19] reported that neither UV-irradiated (at ~300–400 nm) nor untreated 1-HCHPK had mutagenic effects on *Salmonella typhimurium* in the Ames assay. At the same time, the knowledge regarding 1-HCHPK's potential environmental hazards is very limited, e.g., one of the most substantial databases—EPA ECOTOX database [20]—contains neither aquatic nor terrestrial ecotoxicity data on 1-HCHPK. According to the information from the ECHA database [12], 1-HCHPK is considered a chemical of low environmental risk due to its rapid degradation in the environment. Nevertheless, the reported degradation time (80% degradation within 28 days in the activated sludge) [12] is not rapid in terms of the life cycle duration of many aquatic species, whereas experimental data on the long-term toxicity of 1-HCHPK to aquatic species are absent. In aquatic ecosystems, in the presence of continuous sources of contamination (e.g., wastewater treatment plant effluents), 1-HCHPK could accumulate in the sediments, which in turn may be considered as secondary pollution sources of 1-HCHPK, contributing to the risk of exposure of aquatic organisms. Thus, the long-term exposure of both freshwater and marine aquatic organisms to 1-HCHPK can occur, as has been reported for various low-molecular-weight plastic additives [21]. Due to the scarcity of experimental data on 1-HCHPK toxicity to aquatic species, this study addressed this crucial data gap by conducting acute and chronic aquatic toxicity testing of 1-HCHPK.

The current study aimed to evaluate the potential hazards of 1-HCHPK to freshwater micro-crustaceans—important members of the aquatic food web. We hypothesized that the adverse effects of low concentrations of 1-HCHPK on microcrustaceans may not be manifested in the first generation, but may be expressed in the second or even third generations. The potential effect of the long-term exposure of aquatic crustaceans to 1-HCHPK was evaluated in a life cycle test with *Daphnia magna*. The applied test design (i.e., long-term multi-generation test conducted in natural freshwater at low exposure concentrations of 1-HCHPK) enabled a hazard assessment of 1-HCHPK in conditions closely resembling a natural aquatic environment. Applying natural freshwater in ecotoxicity testing has been shown to improve the general understanding of the compound's potential toxicity in the environment [22]. To compare the sensitivity of *D. magna* to 1-HCHPK with the sensitivity of other aquatic micro-crustaceans, acute toxicity assays were performed with three species representing different freshwater habitats; namely, planktonic species *D. magna* and *Thamnocephalus platyurus* and benthic *Heterocypris incongruens*.

2. Materials and Methods

2.1. 1-HCHPK Solution Preparation

1-Hydroxycyclohexyl phenyl ketone (1-HCHPK) (CAS 947-19-3; BLD Pharmatech GmbH, Kaiserslautern, Germany, 99.6% purity) stock solutions (5 g/L) were prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Buchs, Switzeland, >99% purity) and stored in

glass bottles in the dark at room temperature. Exposure solutions of 1-HCHPK in respective ecotoxicity test media were prepared immediately before the toxicity tests. Nominal 1-HCHPK concentrations in the stock and test solutions were verified according to standard SIST-TS CEN/TS 16183:2012 using gas chromatography–mass spectrometry (GC-MS) in selected ion monitoring (SIM) mode using D4 IS compounds as standards. The limit of detection (LOD) was 10 µg/L and the limit of quantification (LOQ) was 30 µg/L.

2.2. Toxicity Tests

Acute toxicity tests with aquatic micro-crustaceans *Thamnocephalus platyurus*, *Daphnia magna* and *Heterocypris incongruens* were performed in two different test media: artificial freshwater (AFW) and natural freshwater (lake water) (Table 1). Life-cycle tests were performed using lake water as a test medium. Natural freshwater was collected from Lake Ülemiste (coordinates: 59°24′ N 24°46′ E, surface area −9.436 km^2) located in Northern Estonia. This is a natural unpolluted eutrophic lake, which is the potable water supply source for Tallinn (a city with a population of 450,000). Lake water was filtered through a 0.45 µm cellulose nitrate filter and stored at 4 °C in the dark before the use in the toxicity tests.

Table 1. Chemical composition of artificial freshwater (AFW) and natural (lake) water.

Parameter	Lake Water	AFW (OECD, 2004 [1])	AFW (US EPA, 2005 [2])
pH	8.2 ± 0.1	7.8 ± 0.2	7.6 ± 0.2
Ca^{2+} (mg/L)	78.1	80.1	13.9
Mg^{2+} (mg/L)	8.43	12.1	12.0
K^{+} (mg/L)	2.79	3.0	2.1
Na^{+} (mg/L)	11.5	17.7	26.3
Cl^{-} (mg/L)	20	144.5	1.92
SO_4^{2-} (mg/L)	36	48.0	81.5
DOC (mg C/L)	10.7	0	0
P_{tot} (mg P/L)	0.062	0	0
N_{tot} (mg N/L)	2.4	0	0

Notes: DOC—dissolved organic carbon; N_{tot}—total nitrogen; P_{tot}—total phosphorus. [1] artificial freshwater used for *D. magna* acute test [23]. [2] artificial freshwater used for *T. platyurus* and *H. incongruens* tests [24].

To obtain the test organisms, dormant eggs of the micro-crustaceans were purchased from MicroBio-Tests Inc. (Gent, Belgium). Less than 24 h old animals hatched from the dormant eggs according to producer protocols [25] were used in the experiments. All the toxicity tests were performed in glass beakers. The test conditions are summarized in Table 2.

Table 2. Toxicity tests conditions.

Test Species	*T. platyurus*	*H. incongruens*	*D. magna*	*D. magna*
Feeding with microalgae [1]	no	before and during the test	2 h before the test	during the test
Exposure duration	24 h	6 days	48 h	21 days [2]
Temperature	25 ± 1 °C	25 ± 1 °C	21 ± 1 °C	21 ± 1 °C
Illumination	In dark	In dark	In dark	16 h/8 h light/dark
Total number of organisms per one test	30	30	20	15

Notes: [1] *Raphidocelis subcapitata* (previously *Pseudokirchneriella subcapitata* or *Selenastrum capricornutum*) stock culture was obtained from MicroBioTests Inc. (Gent, Belgium). [2] per generation.

In static short-term toxicity tests with *T. platyurus* [26], *D. magna* [23] and *H. incongruens* [27], the nominal concentrations of 6.25, 12.5, 25, 50 and 100 mg/L of 1-HCHPK were tested. In parallel, the solvent control (DMSO) at a concentration of 0.2%, *v*/*v*, equal to

that in the exposure medium containing the highest 1-HCHPK concentration (100 mg/L), was tested. The following endpoints were analyzed at the end of exposure: at 24 h in the *T. platyurus* assay—mortality; at 48 h in the *D. magna* assay—immobilization; and at 6 days in the *H. incongruens* assay—mortality and the size of survived organisms.

In the life-cycle test, the potential impact of 1-HCHPK on *D. magna* ephippia hatching and mortality, reproduction, body size and lipid (triacylglycerol) content of adults from three successive *D. magna* generations (each over a period of 21 days) was assessed. 1-HCHPK exposures were performed at 0.1 mg/L and 1.0 mg/L. In parallel, the solvent (DMSO) control at a concentration of 0.002%, *v*/*v*, equal to that in the 1 mg/L 1-HCHPK exposure solution, was tested. The hatching efficiency of the ephippia was calculated after incubation in 1-HCHPK solutions for 6 days under continuous illumination at 1200 lux at 21 °C. A total of 110 ephippia were hatched in each 1-HCHPK treatment and control (5 replicates, 22 ephippia per replicate). Overall, 15 neonates (<10 h old) were collected from each test solution on day 4 to use as parental organisms in the 21-day reproduction test (F0 parental generation). For the subsequent offspring generations (F1 and F2), <24 h old neonates were collected on days 19–20 of exposure of parent females. The 21-day reproduction tests were performed according to the OECD 211 guidelines [28]. Daphnids were individually exposed in glass beakers containing 50 mL test medium and fed daily with the microalga *Raphidocelis subcapitata* (~0.15 mg C/Daphnia/day). The test medium in the control and with exposure chemicals was renewed every third day. The mortality of the parent animals and number of offspring produced per live adult female were monitored and the offspring were removed daily. The lipid content in the daphnids was measured after 21 days of exposure, as described by Jemec-Kokalj et al. [29].

2.3. Data Analysis

The half-effective concentrations (EC_{50}) were calculated using REGTOX macro for MS Excel [30]. One-way analysis of variance (ANOVA) followed by a Tukey post-hoc test was used to determine statistically significant differences among treatments ($p < 0.05$). Data analyses were performed using Microsoft Excel v2010.

3. Results

3.1. Stability of 1-HCHPK in the Experiments

The stability of 1-HCHPK was analyzed in both the stock and the exposure solutions. The 5 g/L stock solution in DMSO was stable over the period of 2 months when stored at room temperature in the dark; beyond this time-point, an up to 10% degradation of the 1-HCHPK was observed. Consequently, fresh stock solutions were prepared monthly. In all of the acute tests performed in the dark, no statistically significant change in the concentrations of 1-HCHPK was observed at the end of the toxicity test (24–48 h or 6 days, Table 2). The exposure concentrations of the 1-HCHPK (0.1 and 1.0 mg/L) were also analyzed in the 21-day reproduction (life cycle) tests when the test medium was renewed. The GC-MS analysis showed no statistically significant differences between the 1-HCHPK concentrations in fresh medium and after 3-day exposure at 21 ± 1 °C and illumination of 1300–1400 lux with 16 h/8 h light/dark photoperiod. Therefore, nominal concentrations were used in the analysis and interpretation of the toxicity assay results.

3.2. Acute Toxicity of 1-HCHPK to Freshwater Microcrustaceans

The results of the short-term tests with two planktonic (*T. platyurus* and *D. magna*) and one benthic (*H. incongruens*) microcrustaceans showed that the acute toxicity of 1-HCHPK depended on the test organism, with EC_{50} ranging between 25.9–54.6 mg/L (Table 3). In the ostracod *H. incongruens* test, a statistically significant decrease in the organisms' body length was induced at exposure concentrations of >10 mg/L; i.e., after 6 days of exposure to 1-HCHPK at 25 mg/L, the organism size was 1.3–1.8-fold smaller than in the control. The chemical composition of the test medium did not affect the toxicity of 1-HCHPK to

aquatic microcrustaceans: there were no statistically significant differences in the acute toxicity values in artificial and natural freshwater (Tables 1 and 3).

Table 3. Acute toxicity of 1-HCHPK to three species of aquatic micro-crustaceans in two test media. Toxicity values (mg/L) are presented as mean (minimum-maximum) values.

Toxicity Test	L(E)C_{50} [1], mg/L	
	Artificial Freshwater	Lake Water
Daphnia magna (48 h immobilization)	50.4 (49.1–51.2)	54.6 (53.7–55.6)
Thamnocephalus platyurus (24 h mortality)	27.8 (22.3–33.4)	27.5 (25.3–29.8)
Heterocypris incongruens (6-day mortality)	27.5 (25.0–29.9)	26.8 (25.4–28.3)

Note: [1] L(E)C_{50}—median lethal (effective) concentration.

3.3. Chronic Toxicity of 1-HCHPK to Daphnia Magna

The life-cycle test with *D. magna* did not show any multigenerational negative effects at either of the tested 1-HCHPK concentrations: 0.1 and 1 mg/L (Figure 1). No mortality or any differences in the time to the first brood were observed. Also, the reproduction (number of neonates per female) and body size of the adults on day 21 of exposure were not statistically different from the control in any of the tests. However, an increase (hormesis) was recorded for *D. magna* ephippia hatching (31% increase at 0.1 and 18% increase at 1 mg/L of 1-HCHPK) and reproduction in F0 (13% increase at 1 mg/L). In addition, the lipid content of the parent organisms was elevated in F0 by 144% upon exposure to 0.1 mg/L of 1-HCHPK and by 98% in the 1 mg/L treatment group. Similar effects to the parental lipid content (~50% increase) were observed in F2 at the end of the 21-day tests at both 1-HCHPK exposure concentrations (Figure 1). Importantly, the solvent control (i.e., DMSO at 0.002%, *v*/*v*, corresponding to DMSO content of 1.0 mg/L 1-HCHPK) had a stimulating effect on the ephippia hatching and parental lipid content (Figure 1).

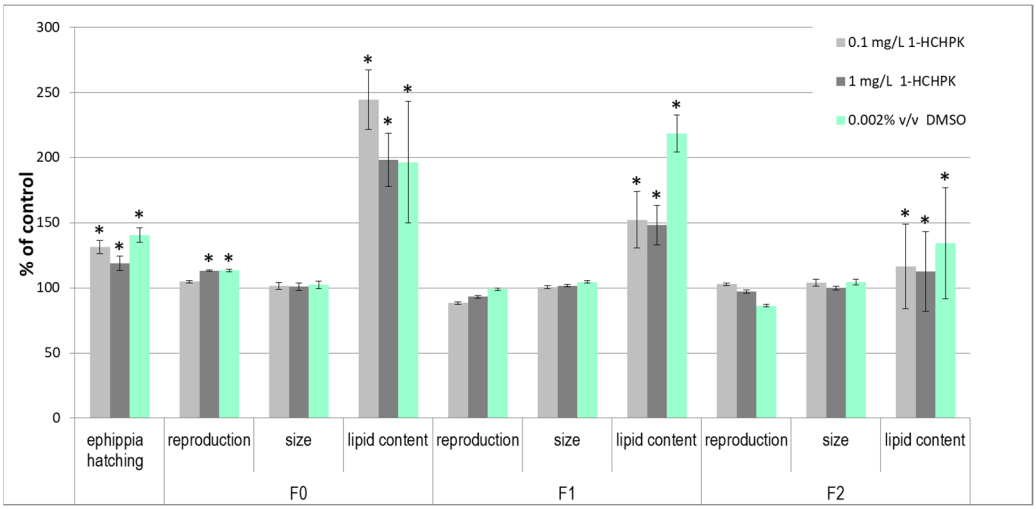


Figure 1. Results of the *Daphnia magna* life-cycle multigenerational toxicity assay upon exposure to 0.1 and 1 mg/L 1-HCHPK and 0.002% DMSO. The hatching efficiency of the ephippia was evaluated after 6 days of exposure. All endpoints for each generation (F0, F1, F2) were evaluated on day 21 of exposure. The data are presented as % of control (i.e., untreated control was considered as 100%). Asterisks represent a statistical difference from the control (lake water), $p < 0.05$.

4. Discussion

According to the OECD Guidelines for testing chemicals [23,28], the stability of the test compound under experimental conditions must be evaluated to ensure the correct interpretation of the test results. 1-HCHPK is readily biodegradable according to the OECD criteria and is considered not bioaccumulative due to the logK_{ow} of 2.81 and the low

bioconcentration factor (BCF) of ≤12. Also, the calculated half-life in the air is 129 h [12], i.e., this compound is not very stable in the environment. However, in this study, the monitoring of 1-HCHPK concentrations in the test solutions during exposure showed that the exposure concentrations were not decreased, indicating the stability of 1-HCHPK in the toxicity assays.

The 48 h EC_{50} value of 1-HCHPK for *D. magna* obtained in our study (Table 3) is very similar to the one (53.9 mg/L) listed on the ECHA website [12], confirming the quality of the test results. Also, the EC_{50} values obtained in the current study for microcrustaceans are largely comparable with the LC_{50} values for zebrafish (*D. rerio*) embryos (60 mg/L) [15] and freshwater microalgae (14 mg/L) [31]. In the acute tests, *T. platyurus* and *H. incongruens* were more sensitive ($p < 0.05$) to 1-HCHPK than *D. magna* (Table 3). However, such differences in sensitivity can be considered negligible from the environmental risk assessment perspective as all of the EC_{50} values belong to the same hazard class (harmful: EC_{50} > 10–100 mg) according to the EC Classification, Labelling and Packaging Regulation [32]. Thus, our data, as well as the available literature data, on the acute toxicity of 1-HCHPK point to the low toxicity of this compound to aquatic organisms, whereas microalgae are slightly more sensitive to the compound than micro-crustaceans and fish. The results of the acute tests also showed that the toxicity of 1-HCHPK to all of the tested species was similar in the artificial fresh water and lake water. This indicates that the results of the laboratory toxicity testing can be extrapolated to natural ecosystems with relatively low uncertainty. This is significant because the bioavailability of chemical compounds depends, to a large extent, on the chemical composition of the test media [22] and should normally be taken into account.

Predicted no-effect concentration (PNEC) is a key value in the environmental risk assessment of chemicals. The currently available PNEC value (0.014 mg/L) for 1-HCHPK for freshwater biota is calculated from the results of acute short-term toxicity assays (microalgae) using an assessment factor of 1000 [12]. However, such a large assessment factor may result in an overestimation of the potential environmental hazard of contaminants, as has been shown in the literature [33–35]. The uncertainties in environmental risk assessments depend on the quality and reliability of the experimental data. It is known that the longer the duration of the test, the smaller the assessment factor that can be applied to calculate the PNEC value [36]. As suggested before [34], our experimental results obtained in the multigenerational life cycle tests with *D. magna* support the proposition of applying a factor of 10 for the PNEC calculation. In the current study, no negative effects were observed, even in the third generation of *D. magna* exposed to 1 mg/L of 1-HCHPK. When applying factor 10, the PNEC for 1-HCHPK could be estimated as 0.1 mg/L, which is about 10-fold higher than previously established (i.e., 0.014 mg/L) [12].

Despite the fact that the reported 1-HCHPK solubility in water (442 mg/L at 23 °C, pH—7.0) [12] is higher than the maximum concentration (100 mg/L) tested in the current study, DMSO was used as the solvent in the toxicity testing because the preliminary experiment revealed that, in both test media (AFW and lake water), this compound was not completely dissolved, even after 2 h at 25 °C in solution with a nominal 1-HCHPK concentration of 100 mg/L. When a solvent is used in toxicity testing as a vehicle for poorly water solubilizing chemicals, its potential influence on the test results must be considered. Here, DMSO was used because it has been recommended as the first choice for an organic solvent due to its low toxicity to *D. magna* (48 h immobilization LC_{50} = 1.2% [37]; 25-day reproduction NOEC = 0.1% [38]) compared to other widely used organic solvents [39]. In the toxicity tests where *D. magna* was exposed to 0.002% (*v*/*v*) DMSO, we observed a stimulation of ephippia hatching, reproduction at F0 and in the lipid content of adults. Others have observed the increased hatchability of *T. platyurus* ephippia upon exposure to 0.04% DMSO, which was attributed to DMSO-enhanced Ca^{2+} inflow into embryonic cells, stimulating the hatching [40]. Regarding the observed increase in the parental lipid content, we assume that the nutrition of *Daphnia* was improved due to the stimulation of the algal growth at 0.001–0.1% DMSO [41] and increased thoracic limb activity [42]—a

potential proxy of increased filtering. However, the increased parental lipid content was accompanied by increased reproduction only in F0 when exposed to 1 mg 1-HCHPK/L and DMSO (Figure 1). Further, in the life-cycle analysis, the DMSO concentrations were 5-fold lower than recommended for toxicity testing [43] OECD, 2019), and were demonstrated to be safe for *D. magna* and its food source, *R. subcapitata* ($\leq$0.01%, v/v) [37]. We hypothesize that the stimulatory effects on the ephippia hatching and parental lipid content we observed in the life-cycle experiment (Figure 1) are (at least partially) attributable to the DMSO.

In summary, the results of the current study fill some data gaps on the toxicity of 1-HCHPK in aquatic ecosystems. The applied test design increases the data relevance for the realistic environmental hazard assessment of emerging pollutants, such as plastic additives—in this case, 1-HCHPK.

5. Conclusions

The main contributions of the study to the ecotoxicological characterization of 1-HCHPK are as follows:

(1) We showed that the results of laboratory toxicity testing can be extrapolated to natural ecosystems with relatively low uncertainty.
(2) DMSO was necessary to ensure the complete solubility of 1-HCHPK in aqueous test solutions, but care should be taken when interpreting the test results as the observed effects (hormesis in the current case) could be attributable to the DMSO rather than the tested toxicant.
(3) The results of this study provide additional information for a more reasonable estimation of the predicted no-effect concentration (PNEC) of 1-HCHPK.

This study presents new data on the long-term toxicity of photoinitiator 1-HCHPK to crustacean *D. magna*, evaluated in a multigeneration test. Until now, the potential hazards of 1-HCHPK to aquatic species have only been evaluated based on acute toxicity tests. Our results indicated that, according to the long-term life cycle experiments, the photoinitiator 1-HCHPK does not pose a hazard to freshwater micro-crustaceans at concentrations of up to 1 mg/L. Considering the current industrial volumes of 1-HCHPK, it is unlikely that emissions of this compound lead to surface water contamination at lethal or even sub-lethal levels. However, to confirm the safety of this concentration of 1-HCHPK to other aquatic species, further research is needed.

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