ECOTOXICOLOGICAL PROPERTIES OF KETOPROFEN AND THE S(+) ENANTIOMER (DEXKETOPROFEN): BIOASSAYS IN FRESHWATER MODEL SPECIES AND BIOMARKERS IN FISH PLHC-1 CELL-LINE

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Abstract: The increased use of non-steroidal anti-inflammatory drugs (NSAIDs) has resulted in their ubiquitous presence in the environment. The toxicological properties of these two widely prescribed NSAIDs, namely - racemic ketoprofen (rac-KP) and its enantiomer S(+)-ketoprofen (dexketoprofen, DKP) were evaluated. Firstly, by acute and chronic toxicity tests using three representative model organisms (*Vibrio fischeri*, *Pseudokirchneriella subcapitata* and *Ceriodaphnia dubia*). Secondly, by evaluating the responses of biotransformation systems and multidrug resistance associated proteins (MRP1/MRP2) using the PLHC-1 fish hepatic cell-line. Toxicity data from both acute and chronic DKP exposure indicated higher sensitivity through inhibition of bioluminescence and algal growth and through increased mortality/immobilization compared to rac-KP exposure. The growth inhibition test showed that rac-KP and DKP exhibited different values for EC\(_{50}\) (240.2 µg/L and 65.6 µg/L, respectively). Furthermore, rac-KP and DKP did not exert cytotoxic effects in PLHC-1 cells, and produced compound-, time- and concentration-specific differential effects on CYP1A and GST levels. For CYP1A, the effects of rac-KP and DKP differed at transcriptional and catalytic level. Exposure to rac-KP and DKP modulated MRP1 and MRP2 mRNA levels and these effects were also dependent on compound, exposure time and concentration of the individual drug. The present study revealed for the first time, the interactions between these NSAIDs and key detoxification systems, and different sensitivity to the racemic mixture compared to its enantiomer. This article is protected by copyright. All rights reserved

Keywords: NSAIDs, Pharmaceuticals, Bioassays, PLHC-1 cells, Biomarkers, Ecotoxicology
INTRODUCTION

The presence of pharmaceutical compounds in wastewater treatment plants (WTP) and surface water (SW) is an emerging environmental issue that poses as a potential risk for aquatic ecosystems and human health [1]. There is a large amount of data on the beneficial and adverse effects of pharmaceuticals, but there is limited information about the impact of these compounds to non-target organisms in the environment. Parent pharmaceutical compounds and their metabolites have been detected in surface, drinking and ground water, from not only hospitals and domestic sewage treatment plant (STP) effluents, but also from landfill leachates and manufacturing residues [2,3]. There is evidence that many of these substances are neither degraded in STPs nor in the environment [2,3]. Due to their widespread application, these substances can reach aquatic environments primarily via sewage effluents after human or farm animal consumption and subsequent excretion. The consumption phase represents the biggest contributor to the emissions of medicinal products into the environment [4]. Once in wastewater, STPs can partly eliminate or remove medicinal product residues, but some traces are detectable [5].

Depending on the physical and chemical properties of either the compounds or the host compartments, these pharmaceuticals can be biotransformed and the resulting metabolites may have different fates in the environment [6]. Recent studies have demonstrated the presence of measurable concentrations of medicinal drugs in ng/L-µg/L range, both in surface and sewage waters [5]. Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used pharmaceuticals, mainly due to their analgesic, antipyretic and anti-inflammatory properties [7]. Most of these drugs act as non-selective inhibitors of cyclooxygenase (COX) isoforms. COX-1 is constitutively expressed in cells under physiological conditions, while COX-2 is induced by cytokines.
mitogens and endotoxins under pathological conditions, such as inflammation. Both COX-1 and COX-2 are involved in the synthesis of different inflammatory mediators [8]. Based on estimated annual production of several kilotons, NSAIDs are the sixth top-selling class of drugs worldwide [9]. Due to the rising usage and associated pharmacokinetics (half-life, urinary and faecal excretion, metabolism and low removal efficiency STPs), several NSAIDs can reach detectable concentrations in aquatic environments [10]. Ketoprofen (KP) is one of the most widely used NSAIDs and is found as a racemic mixture (rac-KP), comprising of the two enantiomers: R(-)-KP and S(+)-KP [1]. Rac-KP has been detected at low concentrations in STPs (ranging from 0.41 - 5.7 µg/L) and at low concentrations in the environment [1].

The therapeutic effect of the rac-KP resides almost exclusively in the S(+)-enantiomer since the R(-)-enantiomer lacks such activity [11]. A useful index of the relative anti-inflammatory activity of the individual enantiomers of a chiral NSAID was obtained in vitro by comparing their effects on the COX binding site. This comparison showed that the activity resided with the enantiomer possessing the S(+) configuration, since the R (-) enantiomer produced no interaction with the COX binding site [12]. Additionally, the S(+)-KP is absorbed rapidly from the gastrointestinal tract, and is consequently characterized by a quick onset of biological action [13]. The enantiomeric distribution of NSAIDs in the aquatic environment depends on the extent of both the chiral inversion of R-enantiomers during human metabolism and the preferential biodegradation of S-enantiomer during wastewater treatment [14]. Since the efficacy is due to the S(+)-enantiomer, the marketing of the dextrorotatory enantiomer (dexketoprofen: DKP) has become a major scientific and economic interest [15]. The acute toxicity of the most common NSAIDs, including acetylsalicylic acid, diclofenac and ibuprofen, has been tested on non-target organisms belonging to different trophic levels [16]. Overall, these studies generally demonstrated that NSAIDs have low acute toxicity based on calculated short-
term 50% effect concentration (EC$_{50}$), ranging between 10 and 100 mg/L [16]. Nevertheless, considering that the effective concentrations for these tests are much higher than those currently detected in the environment, chronic effects are certainly more environmentally relevant.

An increasing number of studies have evaluated several sub-lethal effects induced by NSAIDs on non-target organisms, such as cnidarians [17], cladocerans [16], mussels [18, 19] and fish [20], highlighting that these compounds alone or in combination can produce toxic effects related to oxidative stress, reproduction, cyto-genotoxicity and survival. The use of either acute or chronic bioassays on standard test organisms represents a significant approach in the definition of ecological risk in the aquatic environment. Acute bioassays using algae, crustacean and fish are commonly used for assessing the potential impact of pharmaceuticals on the environment, however there is a lack of studies on chronic toxicity and in vitro biotransformation effects.

Moreover, the traditional toxicity tests are generally not designed for the specific modes of actions of each pharmaceutical and known side effects in humans are not taken into consideration [1]. Fish cell lines from different species and different organs have been used for cytotoxicity assays [21] and shown to provide the basal toxicity information, comparable to mammalian cells when correlating the EC$_{50}$ values [22]. The interactions between several pharmaceuticals on the functions and regulation of cytochrome P450 1A (CYP1A), glutathione S-transferase (GST) and efflux pumps has been used in fish cell lines to study their potential consequences in terms of cell toxicity [20,23,24]. Thus, the aims of this study were to evaluate the ecotoxicity of rac-KP and DKP in freshwater organisms using two different approaches. Firstly, we applied acute and chronic bioassays on model freshwater organisms (Vibrio fischeri, Pseudokichneriella subcapitata, Ceriodaphnia dubia). Secondly, we used in vitro fish cell model (Poeciliopsis lucida hepatocellular carcinoma, PLHC-1) to evaluate the toxicological responses on function and regulation of CYP1a, GST and efflux pumps (MRP1 and MRP2).

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MATERIALS AND METHODS

Chemicals and reagents

Racemic mixture of (RS)-ketoprofen, 2-(3-Benzoylphenyl) propionic acid (CAS No. 22071-15-4) and its enantiomer dexketoprofen, (S)-(+)ketoprofen (CAS No. 22161-81-5) were purchased from Sigma-Aldrich (Milan, Italy). Stock standard solutions of pharmaceuticals were prepared in deionized water at 10 mg/L and stored at 4°C. Amber glassware was used to prevent light degradation of pharmaceuticals. Fetal bovine serum (FBS) (heat inactivated), penicillin–streptomycin (5000 U/mL), trypsin (2.5%), Eagle’s Minimum Essential Medium (MEM), Dimethyl sulfoxide (DMSO), EDTA ethylene diamine tetra acetic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), 1-chloro-2,4-dinitrobenzene (CDNB), Ethoxyresorufin, Phenol, Potassium dichromate, Isopropyl alcohol (IPA), Hexane and Formic acid were obtained from Sigma-Aldrich.

Ecotoxicological Bioassays

An ecotoxicological test battery (bacteria, algae, daphnids) was adopted for toxicity evaluation, in order to define effect concentrations for pharmaceuticals.

Inhibition of bioluminescence (V. fischeri)

The inhibition of bioluminescence test was performed according to the ISO procedures [25]. Bacteria were obtained from Ecotox LSD (Pregnana Milanese, Italy). For identification of ecotoxicological parameters such as EC<sub>20/50</sub> a full test was performed with a dilution series (from 3.90 µg/L to 2000 µg/L, 10 dilutions). Bacteria were exposed to each dilution and their light emission was determined after 15 minutes incubation and compared to an aqueous control. The tests were performed at 15°C (pH 6–8) in triplicate. All measurements were performed using the M500 luminometer. The instrument was interfaced with a PC operating with the Microtox®
Omni 1.16 software, for acquisition, data handling and EC$_{50}$ calculation (Least Square Method). EC$_{50}$ values are expressed as means and confidence limits (95 %) of three replicate determinations. Phenol was used as the reference toxicant.

*Growth inhibition (P. subcapitata)*

The inhibition of growth was evaluated according to the protocol described in ISO procedures [26] with slight modifications. *P. subcapitata* strain CCAP 278/4 was purchased from the reference center CCAP (Culture Collection of Algae and Protozoa-Scottish Association for Marine Science/SAMS Research Services Ltd). *P. subcapitata* was cultured in 3N-BBM+V (Bold Basal Medium with 3-fold Nitrogen and Vitamins; modified). Late logarithmic phase algae were inoculated in 25 mL fresh medium to an initial concentration of 10000 cells/mL and were grown at 20±2 °C under cool white fluorescent continuous light of 7000 lux with slow shaking for 96 h of incubation. The algal growth was measured as number of cells/mL and cells were counted with a Bürker Counting Cell Chamber (HBG, Germany), using an optical microscope (Olympus CH-2). For the determination of the effective concentration causing 5%, 10%, 20% and 50% of growth inhibition (EC$_{5/10/20/50}$) and the lowest- and no effect-observed effect concentration (LOEC and NOEC), a full test was performed by serial dilutions (from 1.95 µg/L to 2000 µg/L). Experiments on rac-KP and DKP were performed in triplicate. Potassium dichromate was used as reference toxicant. The endpoint was the inhibition of growth (cells/mL) after 96 h of incubation. Assays were considered valid when the number of control cells increased by at least 16-fold over 96 h.

*Acute and chronic assays (C. dubia)*

For the acute toxicity assay, *C. dubia* (neonates < 24 h old) were exposed to high concentrations of rac-KP and DKP ranging from 1560 µg/L to 100000 µg/L in 48 h static tests, in order to assess lethal effects. Rac-KP and DKP stock solutions of 100 g/L were prepared in

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acetone and then diluted in culture medium to obtain the concentrations tested. Organisms were obtained from Ecotox LSD (Pregnanà Milanese, Italy) as dormant eggs (ephippia). Three replicates each containing ten organisms were assigned to the experimental groups (rac-KP and DKP), the control group (culture medium) and the solvent control group (acetone: 0.1% v:v). After 48 h, we evaluated the immobilization/mortality using a stereo-microscope Leica M80 (German Company). The number of dead organisms in the control group did not exceed 10%. Potassium dichromate was used as reference toxicant. The test was performed in according to US EPA 2002a [27].

For the chronic toxicity assay, third brood neonates (< 24 h old) were exposed for 7 days at different concentrations of rac-KP and DKP ranging from 1 to 1000 µg/L. The lowest concentrations were similar to concentrations detected in the environment (ranging from 0.41 to 5.7 µg/L) [1]. Ten replicates with one organism per 30 mL in a glass beaker of 100 mL were assigned to each treatment. Organisms were fed once daily with yeast and a P. subcapitata algal suspension. All test solutions were renewed daily starting from the stock solutions prepared before the start of the test. The number of neonates in each beaker was counted and recorded every day. Neonates were then discarded and the media renewed. Test solutions were characterized by alkalinity 120-138 mg/L as CaCO₃, hardness 150-184 mg/L as CaCO₃, DO 8.22-10.13, and pH 7.52-8.60 at 25°C. At least 80% of all control organisms survived, and 60% of surviving control females produced at least three broods, with an average of 15 or more neonates per surviving female. The test was performed in according to US EPA 2002b [28].

**Analytical chemistry**

Concentrations of rac-KP (R(-)/S(+)-KP) and DKP (S(+)-KP) were measured in experimental media solutions for the chronic assays (algae and crustacean). Enantiomers were measured via an enantioselective HPLC-UV detection method. Samples were filtered and...
extracted with SPE (Strata™-XL 100 µm Polymeric Reversed Phase) followed by HPLC analysis with a chromatographic system that consisted of a Series 200 PerkinElmer gradient Pump coupled to a Series 200 PerkinElmer variable UV detector, which was set at 254 nm. The normal-phase column was a Lux® Amylose-1, LC Column (5 µm, 250 x 4.60 mm) (Phenomenex, CA, USA). The column was kept at room temperature. Turbochrome® software was used for data processing. A 20 µL injection was used each time. The mobile phase consisted in hexane-isopropyl alcohol with 0.1 % of formic acid. The detection limit, calculated as signal-to noise ratio 3:1, was 0.05 µg/L.

PLHC-1 cell-line

The PLHC-1 cell line, obtained from the American Type Culture Collection (ATTC CRL 2406), is derived from the hepatocellular carcinoma of the Topminnow Poeciliopsis lucida [29]. They were routinely grown at 30°C in Eagle’s Minimum Essential Medium (MEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin (5000 U/mL) in a 5% CO₂ humidified atmosphere. The cells were grown in 75 cm² culture flasks and subcultured every 3–4 days after harvesting with trypsin (0.05%) and ethylenediamine tetraacetic acid (EDTA) (0.53 mM) in phosphate-buffered saline solution (PBS) and diluted 3–4 times. Cells were used at 75% monolayer confluency.

Cell viability assay

Mitochondrial capacity to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan was used as a measure of cell viability [30]. Cells were seeded at rate of 30000 or 50000 cells per well (depending on the duration of the exposure) in 96-well plate and allowed to attach for 24 h. Cells were then exposed to rac-KP and DKP, which were dissolved in culture medium at different concentrations (0.1, 1, 10, 100 and 1000 µg/L). Plates were incubated for 48 and 72 h. The experiments were performed in triplicates.
After exposure, cells were incubated for 4 h with 0.5 mg/mL MTT dissolved in complete growth medium. The MTT solution was then replaced with 200 µL DMSO to dissolve the insoluble formazan crystals, and the plate was read in a spectrophotometric plate reader at 570 nm. Cell viability was given as relative survival (% viability) of exposed cells compared to control cells.

**Phase I and II biotransformation responses**

Ethoxyresorufin-O-deethylase (EROD) activity was used as markers for phase I biotransformation activity. Cells were seeded at the rate of 30000 or 50000 cells per well and exposed in a black fluorometric 96-well plate for 72 and 48 h respectively, to various concentrations of the rac-KP and DKP (0.1, 1, 10, 100 and 1000 µg/L). The experiments were performed in triplicates. After exposure, the medium was removed and cells were incubated in 100 µL of 0.3 mM NADPH (dissolved in PBS) for 10 minutes at 37°C. The reaction was then started by adding 1 µL of the substrate ethoxyresorufin (EROD) (180 µM in DMSO) and fluorescence was measured in a microplate reader for 20 min (ex: 535 nm; em: 590 nm) [31]. Total amount of protein was determined with the method of Bradford, using bovine serum albumin (BSA) as the standard. Quantification of the activity was made using a calibration curve with 7-hydroxyresorufin. The activities were expressed as a percentage of the control.

Glutathione-S-transferase (GST) activity was measured on cell lysates following the spectrophotometric assay described by Habig et al. (1974) [32]. Cells were seeded at the rate of 300000 or 500000 cells per well and exposed in a 6-well plate for 72 and 48 h respectively, to different concentrations of the rac-KP and DKP (0.1, 1, 10, 100 and 1000 µg/L). The experiments were performed in triplicates. After exposure, cells were harvested and resuspended in 20 mM Tris buffer pH 7.4, 100 mM NaCl, 1 mM EDTA 1x PIC (protease inhibitor cocktail). Samples were then frozen and thawed three times. Cell lysates were collected after centrifugation at 10000 x g for 30 min at 4°C. GST assay reaction contained 100 mM KH₂PO₄ pH 6.5, 1 mM GSH, 1...
mM CDNB (1-chloro-2,4-dinitrobenzene) dissolved in DMSO and 20 µL of total cell lysate. The conjugation of glutathione to CDNB was measured at 340 nm. The specific activity was calculated using the extinction coefficient of 9.6 mM$^{-1}$cm$^{-1}$.

**RNA isolation and cDNA synthesis**

Cells were seeded at the rate of 150000 or 300000 cells per well and exposed in a 24-well plate for 72 and 48 h respectively, to different concentrations of rac-KP and DKP (0.1, 1, 10, 100 and 1000 µg/L). The experiments were performed in triplicates. After exposure total RNAs from cells were isolated using Direct-zol RNA MiniPrep isolation kit (Zymo Research, Irvine, CA, USA). The RNA was quantified using NanoDrop® ND-1000 UV–vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the quality of RNA was assessed by 260/280 ratio. Total cDNA for the real-time PCR reactions were generated from 0.1 µg DNase-treated total RNA from all samples using poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad). The reverse transcription was conducted by incubating the reaction mixture in the Thermal Cycler (Bio Rad), under the following conditions: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C.

**Quantitative real-time PCR analysis (qRT-PCR)**

Quantitative (real-time) PCR was performed with gene-specific primers (cyp1a, mrp1 and mrp2, as shown in Table 1) using the Mx3000P REAL-TIME PCR SYSTEM (Stratagene, La Jolla, CA, USA). The 3 step real-time PCR program included an enzyme activation step at 95 °C (10 min) and 40 cycles of 95 °C (30 s), 60 °C depending on target gene (15 s), and 72 °C (15 s), followed by a melting analysis at 95 °C for 1 min, 65 °C (30 s) and thereafter decreasing fluorescence detection with increasing temperature between 65–95 °C. Controls lacking cDNA template was included to determine the specificity of target cDNA amplification. Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct versus

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log copy number. The criterion for using the standard curve was based on equal amplification efficiency with unknown samples and checked prior to extrapolating unknown samples to the standard curve. Standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest, following an already validated method [33]. Data obtained from triplicate runs for target cDNA amplification were expressed as percentage of the control.

Statistical Analysis

EC$_{5/10/20/50}$ values for algal inhibition of growth and their 95% confidence limits were calculated by the linear interpolation method. Statistical analyses were performed using GraphPad Prism, version 6.01 (GraphPad Software Inc. 2012). Data were tested for normality and variance homogeneity. One-way ANOVA was performed for the chronic experiment with crustaceans to evaluate statistical differences ($p < 0.05$) between control and exposure groups using the Dunnett’s Multiple Comparison Test. Two-way ANOVA was performed to investigate possible time-effect and dose-effect relationships for the biomarker endpoints using time exposure (48 h and 72 h) and rac-KP and DKP concentrations as variables. The Two-way ANOVA was followed by Tukey post-hoc test to evaluate significant differences ($p < 0.05$) between treated and related time control, as well as among time at the same concentration. The Pearson’s correlation test was performed on all the measured variables to investigate dose-dependency.

RESULTS

Inhibition of bioluminescence on V. fischeri

In our preliminary analysis with rac-KP and DKP, we observed that bioluminescence, expressed as RBU (relative bioluminescence units at 490 nm), was not detectable in the absence of bacteria (data not shown). The assay performed with the reference toxicant (phenol) confirmed

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sensitivity within the limit of acceptability (13-26 mg/L) with an EC₅₀ value of 18.4 mg/L. In the presence of *V. fischeri*, we did not observe acute toxicity after 15 min incubation at rac-KP concentration between 3.9 - 2000 µg/L (Table 2). However, a hormetric effect (I% = 17.9) was detected at the highest rac-KP concentration. On the contrary, DKP concentration of 500 µg/L and above, inhibited bioluminescence and therefore the EC₅₀ value of DKP was calculated to be 1046 µg/L (95 % confidence limit (C.L.): 373 - 2935 µg/L, Table 2).

*Growth inhibition on P. subcapitata*

The algal strain was tested with the reference toxicant (potassium dichromate) showing an EC₅₀ value of 0.4 mg/L within the limit of acceptability (0.18-1.8 mg/L). The NOEC, LOEC and EC₅/₁₀/₂₀/₀/₅₀ values are summarized in Table 3. DKP exhibited an EC₅₀ value of 3.7-fold lower than rac-KP. The reported inhibition (%) of growth in *P. subcapitata* by plotting rac-KP, DKP and the S(+) fraction of rac-KP is shown in Figure 1. Measured concentrations of individual pharmaceutical compounds in algal media are presented in Table 4. The mean recovery percentages (%) for each exposure condition were 99.1 ± 4.6 % (Supplemental data, Table S1 and S2).

*Acute and chronic toxicity effects on C. dubia*

The crustaceans were tested with the reference toxicant (potassium dichromate) showing an EC₅₀ value of 1.1 mg/L within the limit of acceptability (0.18-1.8 mg/L). The effect of 48 h acute toxicity (mortality/immobilization) on *C. dubia* exposed to serial dilutions of rac-KP and DKP is shown in Table 5. The experimental control data is shown to be within the acceptability range of the assay. However, the obtained data were unsuitable for the regression model due to the absence of a dose-response trend. Thus, no EC values have been calculated in the acute exposure. The maximum mortality percentage observed was 40- and 60 % for rac-KP and DKP, respectively. The effect of 7-day chronic toxicity on *C. dubia* exposed to environmental relevant
concentrations of rac-KP and DKP is shown in Table 6 and 7, respectively. Control values are within the acceptability range of the assay. After 7-day exposure time period, the cumulative mortality rate was 20% for rac-KP at the highest tested concentration of 1000 µg/L, while a 20- and 40% mortality rate was observed for DKP at 100 and 1000 µg/L, respectively. For both rac-KP and DKP, the most dramatic effect was observed on fecundity, showing a significant decrease in the number of offspring/female of *C. dubia* exposed to 1000 µg/L (rac-KP) and at 100- and 1000 µg/L for DKP. The percentage of reproduction inhibition relative to the control is reported in Table 6 and 7. For the chronic toxicity assay, the measured concentrations of individual pharmaceutical compound in crustacean media are presented in Table 8. The mean recovery percentages for each exposure condition were 98.6 ± 10.6 % (Supplemental data, Table S3 and S4).

**Effects on PLHC-1 cell line**

MTT cell viability assay showed that cells exposed to serial dilutions of rac-KP and DKP for 48 h produced high survival rates at all the tested concentrations, except at 1000 µg/L, where ~65% of viability was observed. Cells exposed for 72 h to rac-KP and DKP exhibited ~70% cell viability at 10, 100 and 1000 µg/L.

**Effects on biotransformation responses in PLHC-1**

The phase I biotransformation response was measured at transcript (mRNA) and functional (EROD activity) levels (Fig. 2). The cyp1a transcript showed a rac-KP concentration-dependent increase at 48 h (*Pearson r* =0.93; *R*^2^=0.86; *p*<0.05). Exposure of cells to rac-KP for 48 h at 1000 µg/L, and for 72 h at 10, 100 and 1000 µg/L resulted in significantly increased cyp1a levels, compared to control cells (Fig. 2A). On the other hand, the EROD activity of rac-KP exposed cells was significantly decreased, compared to control cells after 48 h exposure to 10, 100 and 1000 µg/L rac-KP (Fig. 2B). Interestingly, no significant changes in EROD activity
were observed after 72 h exposure to rac-KP (Fig. 2B). Additionally, cells showed significantly different cyp1a mRNA levels and EROD activities between 48 and 72 h exposure to the same rac-KP concentrations (10, 100 and 1000 µg/L). For DKP, a significant decrease of cyp1a mRNA expression, below control level, was observed when cells were exposed to concentrations between 0.1-100 µg/L at both 48 and 72 h (Fig. 2C). However, at 1000 µg/L, DKP produced a significant increase of cyp1a mRNA expression, above control level, at both 48 and 72 h of exposure (Fig. 2C). Furthermore, for DKP, a concentration-dependent trend was observed for cyp1a mRNA at 48 h and 72 h (Pearson r=0.97; R²=0.95; p < 0.05 and Pearson r=0.98, R²=0.96; p < 0.05, respectively). The functional CYP1A response measured as EROD activity was significantly decreased below control level, in cells exposed to DKP for 72 h to 10, 100 and 1000 µg/L (Fig. 2D) and no significant changes in EROD activity were observed after 48 h exposure to DKP (Fig. 2D). The GST activity was used as a measure of phase II biotransformation response (Fig. 3). Exposure to rac-KP produced a significant increase in GST activity above control levels, in all tested concentrations at 48 h, while after 72 h exposure no differences in activity between control and treated groups were observed, except a significant decrease observed at 0.1 µg/L (Fig. 3A). At 48 h, the increase in GST activity was concentration-dependent (Pearson r=0.99; R²=0.98; p < 0.05). On the other hand, GST activity in DKP-treated PLHC-1 cells showed a significant increase at 48 h exposure to 100 µg/L, while the highest tested concentration of 1000 µg/L produced a significant decrease (Fig. 3B). Interestingly, 72 h exposure to DKP produced a significant decrease in GST activity at all tested concentrations (Fig. 3B). Rac-KP and DKP exposures showed significant time differences in GST activity.

**Effects on Multidrug resistant protein**

Changes in transcript level of the multidrug resistant protein 1 and 2 (mrp1 and mrp2) were evaluated (Fig. 4). The mRNA abundance for mrp exhibited time- and concentration-
specific changes. The levels of mrp1 were significantly increased at 48 and 72 h in cells treated with rac-KP at 10, 100 and 100 µg/L (Fig. 4A). The mrp2 mRNA expression was significantly increased after 72 h to 1000 µg rac-KP/L (Fig. 4B). On the other hand, a concentration-dependency in mrp1 and mrp2 mRNA expression was observed in PLHC-1 cells after 48 h exposure to DKP (Pearson $r=0.99$; $R^2=0.98$; $p < 0.05$ and Pearson $r=0.99$; $R^2=0.99$; $p < 0.05$, respectively). Particularly, a significant decrease was observed when cells were treated with DKP at 0.1 - 100 µg/L for 48 h (Fig. 4C and D, respectively). While at 72 h exposure, there was a significant increase in mrp1 mRNA after exposure to the highest DKP concentration of 1000 µg/L and no changes were observed in mrp2 mRNA levels (Fig. 4C and D, respectively).

**DISCUSSION**

We have evaluated the acute and chronic effects of rac-KP and its enantiomer DKP through bioassay approaches with freshwater model organisms and with fish hepatocyte model (PLHC-1). Although the racemic mixture and corresponding enantiomers of a particular compound may have the same physical properties and abiotic degradation rates, they often have different biological activity [34]. Differences in pharmacokinetics and pharmacodynamics of the two enantiomers occur in mammals and significantly vary among species [35]. Additionally, conversion of R(-)-KP to S(+)-KP occurs in many species, but the rate of conversion varies between species, and in some circumstances, between individuals within a species [35]. On the other hand, the conversion of S(+)-KP to R(-)-KP, has only been demonstrated in mice [36].

*Ecotoxicological assessment*

In order to define the ecotoxicological hazard assessment of rac-KP and DKP, we acutely or chronically exposed bacteria, algae and crustaceans to these NSAIDs. Inhibition of bioluminescence using *V. fischeri* test produced a hormetic effect at the highest tested rac-KP
concentration. Hormesis is a phenomenon that is defined as a stimulatory beneficial effect at very low concentrations of toxic chemicals [37]. However, at higher concentrations this phenomenon may have a harmful, beneficial or indifferent effect in biota [38]. On the contrary, *V. fischeri* exposed to DKP showed an inhibition of bioluminescence with an EC$_{50}$ value of 1046 µg/L.

There are only a few studies on exposure to rac-KP using *V. fischeri* and based on our knowledge, there are no available reports that have used DKP in ecotoxicological assays. However, a recent study showed an EC$_{50}$ of 118 mg/L for rac-KP [39]. The authors did not report any hormetic effect after exposure to rac-KP, which is likely due to the high concentrations used in their study. Interestingly, in our study, DKP was severely toxic with an EC$_{50}$ value 100-fold lower than the EC$_{50}$ value reported by Wieczerzak and colleagues (2016) using rac-KP [39].

We have also evaluated whether chronic exposure (96 h) to either rac-KP or DKP would produce effects on the algal growth using *P. subcapitata* as model organism, showing that algae was more sensitive to DKP (EC$_{50}$=65.6 µg/L) compared to rac-KP (EC$_{50}$=240.2 µg/L). The calculated EC$_{10}$ value for both rac-KP and DKP were comparable to the observed NOEC values for both the pharmaceuticals. While there are no ecotoxicological studies using DKP, there are few studies in the literature reporting the exposure of *P. subcapitata* to rac-KP. These studies reported different EC$_{50}$ values, with the lowest reported 4 mg/L [40] to 24.6 mg/L and the highest reported 49.3 mg/L [41,42]. Our study showed an EC$_{50}$ value in *P. subcapitata* exposed for 96 h to rac-KP that is 16.7-fold lower than the lowest value reported in literature. Specifically, our test may have revealed lower EC$_{50}$ value compared to those reported in the literature, due to the algal growth conditions, the photo-degradation of the tested compounds, test duration and different methods for evaluating the endpoint, such as optical density, electronic particle counter, chlorophyll fluorescence or number of cells/volume unit [43]. Overall, the algae test data suggest...
that these pharmaceuticals may not produce significant effects on primary producers, at least not on the algal species tested in this study.

Subsequently, acute assays using *C. dubia* (48 h) revealed the highest effect on mortality for both rac-KP and DKP at 100 mg/L. Thus, the acute risk posed by rac-KP and DKP may represent a negligible environment and biota effect, since the reported environmental concentrations are several orders of magnitude lower than the concentrations used in this study [1]. This is in accordance with previous studies showing that rac-KP, diclofenac and atenolol did not exhibit acute toxicity on organisms when tested at 1 mg/L [24]. Moreover, a study using similar cladoceran species (*D. magna*) reported a comparable EC$_{50}$ value (>100 mg/L) for rac-KP at pH 7.5 [44]. Finally, a chronic assay (7 d) using low rac-KP and DKP concentrations was also performed. *C. dubia* exposed to 1000 µg DKP/L showed a 45.4% of reproduction inhibition with mortality of 40%. A previous study in *C. dubia* tested rac-KP fecundity and found a NOEC value of 22.6 mg/L [41]. Interestingly, in our study, which tested significantly lower concentrations of rac-KP (≤1000 µg/L), we observed a 24.9 % inhibition in reproduction at the highest concentration. Since the topic of the study was to investigate environmental relevant concentrations of these compounds, we did not test concentrations of rac-KP and DKP higher than 1 mg/L. Data on bioassays showed that *V. fischeri, P. subcapitata* were more sensitive to DKP than to rac-KP. Despite that the racemic mixture rac-KP containing 50% DKP, the effects observed after exposure to rac-KP and DKP in bacteria and algae did not parallel this ratio relation. The highest sensitivity of DKP compared to rac-KP (containing 50% DKP) observed in Figure 1, suggest a possible enantioselective toxicity, partially due to the S(+) -enantiomer in the racemate.
Toxicological responses

The cytotoxicity of rac-KP and DKP was assessed in PLHC-1 cells by MTT, showing the absence of significant effects at all tested concentrations, including 1000 µg/L (~ 4 µM). Our findings are consistent with previous report by Thibaut and Porte [45].

Biotransformation enzymes play integral roles in the activation and deactivation of xenobiotics and drugs and their alterations markedly affects their potential risks and benefits, which is consequently important from a toxicological and pharmacological standpoint [46]. A better understanding of our observed effects of rac-KP and DKP must consider the full range of possible toxic and regulatory mechanisms (transcriptional, translational and post-translational modifications) involving the P450 function or AhR signalling pathways. For toxicological responses, rac-KP and DKP produced differential effects on the CYP1A at transcriptional and functional levels. For example, while a concentration- and time-dependent increase was observed at the mRNA levels, the effects on EROD activity levels were either decreased or indifferent. This could suggest a possible mechanism that normalize the CYP-dependent function, indicating a return to the normal level. Interestingly, cells exposed to DKP did not exhibit a similar response. There is no available information on non-target organisms about the mechanisms of CYP1A regulation by NSAIDs. However, the ability of rac-KP to decrease 3-methylcolanthrene (3-MC) induced EROD activity has been reported in rat liver [47]. Elsewhere, no significant effect of NSAIDs at 1 µM on EROD activity in carp liver subcellular fractions was observed [48]. Due to the high solubility of both rac-KP and DKP, it could be reasonably inferred that these compounds may not play significant roles in the phase I biotransformation pathway. With this in mind, given that the CYP biotransformation system metabolizes both endogenous and exogenous substances (including drugs), interactions between foreign chemicals and physiological processes are possible [49]. Thus, the relationships between induction of
biotransformation enzymes and possible alteration of drug efficacy deserve more attention. The rac-KP and DKP produced reduction in CYP1A-mediated EROD activity may represent an adaptive control of drug bioavailability for optimal effects, since functional CYP1A may metabolize rac-KP and DKP, leading to their subsequent excretion. Ultimately, further investigations are needed to better understand the possible involvement of the CYP system in the metabolism and excretion of rac-KP and DKP.

GST is an important phase II enzyme that uses the products of phase I reactions to form large endogenous molecules. The production of large endogenous molecules by GST is of major importance in the conjugation and subsequent elimination of drugs and endogenous compounds [50]. Herein, we measured GST activity levels after exposure to either rac-KP or DKP, showing that rac-KP produced elevated GST activity in cells exposed for 48 h, suggesting a possible conjugation of rac-KP or rac-KP metabolites with GSH. On the contrary, DKP significantly decreased GST activity, except at 48 h with 100 μg/L, suggesting possible time-specific effects. Although phase II enzymes are crucial in eliminating NSAIDs, the interaction between GST and our tested pharmaceuticals is poorly understood. In mammals, it has been shown that rac-KP can be eliminated as either free rac-KP, conjugated (probably as a glucuronide), or as hydroxyl (OH) metabolite with or without conjugation [51]. In this regard, the high degree of ionization and solubility affects rapid excretion of the glucuronide [51]. This transformation is also known to modify sulphate and glutathione conjugates and is mediated by ATP-dependent protein transport systems - namely P-glycoprotein or MRPs [52, 53].

Protein transporters have a fundamental role in eliminating toxic compounds from the body [54]. In the present study, exposure to rac-KP resulted in a significant increase of MRP1, while cells exposed to DKP showed different mrp1 and mrp2 mRNAs expression levels,
compared to rac-KP. The effects of rac-KP on mrp1 and mrp2 expression levels could suggest a shorter cellular retention of rac-KP, compared to DKP. Thus, it is possible that rac-KP is excreted as unconjugated, conjugated by GST enzyme, or as glucuronide, prior to delivery for the phase III biotransformation process. On the other hand, DKP could remain within the cell for a longer period of time and could only be eliminated at the highest concentration. Although MRP expression has been demonstrated in several fish species [55], only limited reports are available on the regulation of these proteins, which are normally regulated at the protein level [56]. Thus, to the best of our knowledge, this is the first study in fish showing an increase of MRP mRNA by rac-KP and DKP, and as such these findings require further studies, in order to understand the pharmacological processes behind these effects.

CONCLUSIONS

This present study has demonstrated the importance of combining acute and chronic bioassays with studies of key detoxification systems at transcriptional and functional levels. This approach provides a better overview and understanding on the toxicological properties of pharmaceuticals that are commonly detected in the environment. Bacteria and algae exposed to DKP showed higher toxicity than those exposed to rac-KP. These NSAIDs did not exert cytotoxic effects in PLHC-1 cells. Nevertheless, rac-KP and DKP were observed to possibly act through different mechanism within the cells. Both NSAIDs interacted differently with CYP1A. In addition, the phase II biotransformation pathway showed different responses with significant increase and decrease in GST activity in cells treated with rac-KP and DKP, respectively. Lastly, rac-KP interacted with the efflux pump by increasing the expression levels, while DKP reduced it, except at the highest tested concentration. These results suggest that rac-KP and DKP have different toxicity effects on model organisms and different toxicological response mechanism in fish cell model.
Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

Acknowledgment—We would like to thank R. Røsbak for her technical assistance during exposure, sampling and analyses and Â. Krøkje for PHLC-1 cell line.

Data Availability—Data are available on request to E. Mennillo (elvira.mennillo@gmail.com).
REFERENCES


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Figure 1. Inhibition of growth in *Pseudokirchneriella subcapitata* (96 h incubation) exposed to different concentrations of rac-KP and DKP (expressed as Log concentration). Rac-KP, DKP and the calculated S(+) fraction of rac-KP are plotted as percentage of inhibition of control. Data are

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presented as mean % of inhibition (symbols; n=3) and regression curves for each condition are represented as lines.

Figure 2. Changes in cyp1a transcript and EROD activity levels in PLHC-1 cell-line exposed for 48 and 72 h to rac-KP (A and B, respectively) and DKP (C and D, respectively). Data are presented as mean (n=3) percentage of control and standard deviation. Significant differences (two-way ANOVA, Tukey post-hoc test) were analyzed by comparing treated cells to the corresponding controls within the same exposure time (\(^p \leq 0.05\); \(^* p \leq 0.01\) and \(^** p \leq 0.001\)) and by comparing the same concentration at different exposure times (\(^# p \leq 0.05\); \(^## p \leq 0.01\) and \(^### p \leq 0.001\)).

Figure 3. GST activity in PLHC-1 cell-line exposed to rac-KP (A) and DKP (B) for 48 and 72 h. Data are presented as mean (n=3) percentage of control and standard deviation. Significant differences (two-way ANOVA, Tukey post-hoc test) were analyzed by comparing treated cells to the corresponding controls within the same exposure time (\(^p \leq 0.05\); \(^* p \leq 0.01\) and \(^** p \leq 0.001\)) and by comparing the same concentration at different exposure times (\(^# p \leq 0.05\); \(^## p \leq 0.01\) and \(^### p \leq 0.001\)).

Figure 4. Changes in mrp1 and mrp2 transcript levels in PLHC-1 cell-line exposed for 48 and 72 h to rac-KP (A and B, respectively) and DKP (C and D, respectively). Data are presented as mean (n=3) percentage of control and standard deviation. Significant differences (two-way ANOVA, Tukey post-hoc test) were analyzed by comparing treated cells to the corresponding controls within the same exposure time (\(^p \leq 0.05\); \(^* p \leq 0.01\) and \(^** p \leq 0.001\)) and by comparing the same concentration at different exposure times (\(^# p \leq 0.05\); \(^## p \leq 0.01\) and \(^### p \leq 0.001\)).
Table 1. Primer pair sequences, amplicon size, annealing temperature and GenBank ID for genes amplified by real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pairs sequences (5’-3’)</th>
<th>Amplicon size (bp)</th>
<th>Annealing T (°C)</th>
<th>GenBank ID</th>
</tr>
</thead>
</table>
| cyp1a| Forward: GCATTTGGCGTGCTCGAAGAAA  
Reverse: TTGCAGATGTGCTCCTCCAACA | 109                | 60              | JX270831.1  |
| mrp1 | Forward: GCCGTTCTCCTATCTACAC    
Reverse: GAGAGTGACCAGCGAGTA       | 107                | 60              | HM102361.1  |
| mrp2 | Forward: TGCTGCTGGTGGCTTCTTCTAC 
Reverse: GCAAGAGGTCAAACAGTGGGAGAAA | 159                | 60              | HM102360.1  |
Table 2. Inhibition of bioluminescence in *Vibrio fischeri* (expressed as I%) exposed to different concentrations of rac-KP and DKP for 15 min.

<table>
<thead>
<tr>
<th>Rac-KP (µg/L)</th>
<th>I% 15 min</th>
<th>DKP (µg/L)</th>
<th>I% 15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9</td>
<td>3.3</td>
<td>3.9</td>
<td>-4.0</td>
</tr>
<tr>
<td>7.8</td>
<td>3.8</td>
<td>7.8</td>
<td>2.6</td>
</tr>
<tr>
<td>15.6</td>
<td>-0.5</td>
<td>15.6</td>
<td>-5.6</td>
</tr>
<tr>
<td>31.3</td>
<td>0.7</td>
<td>31.3</td>
<td>4.0</td>
</tr>
<tr>
<td>62.5</td>
<td>0.6</td>
<td>62.5</td>
<td>-0.9</td>
</tr>
<tr>
<td>125</td>
<td>0.9</td>
<td>125</td>
<td>4.9</td>
</tr>
<tr>
<td>250</td>
<td>-2.8</td>
<td>250</td>
<td>-0.3</td>
</tr>
<tr>
<td>500</td>
<td>-2.9</td>
<td>500</td>
<td>18.5</td>
</tr>
<tr>
<td>1000</td>
<td>-8.4</td>
<td>1000</td>
<td>28.7</td>
</tr>
<tr>
<td>2000</td>
<td>-17.9</td>
<td>2000</td>
<td>72.9</td>
</tr>
</tbody>
</table>
Table 3. Calculated NOEC, LOEC and EC_{5,10,20,50} values with confident limits (CL: in parentheses) in *Pseudokirchneriella subcapitata* exposed to different concentrations of rac-KP and DKP for 96 h.

<table>
<thead>
<tr>
<th>NSAIDs</th>
<th>NOEC µg/L</th>
<th>LOEC µg/L</th>
<th>EC_{5} µg/L</th>
<th>EC_{10} µg/L</th>
<th>EC_{20} µg/L</th>
<th>EC_{50} µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rac-KP</td>
<td>7.8</td>
<td>15.6</td>
<td>3.1 (1.1 - 10.5)</td>
<td>9.0 (1.4 - 12.9)</td>
<td>13.9 (4.8 - 32.2)</td>
<td>240.2 (201.8 - 280.7)</td>
</tr>
<tr>
<td>DKP</td>
<td>3.9</td>
<td>7.8</td>
<td>2.9 (0.7 - 4.7)</td>
<td>4.5 (1.6 - 6.6)</td>
<td>7.4 (5.4 - 9.2)</td>
<td>65.6 (40.6 - 72.3)</td>
</tr>
</tbody>
</table>
Table 4. Nominal and measured concentration of rac-KP (R(-)/S(+) KP) and DKP (S(+))KP in algal test solutions. Data are expressed as mean ± standard deviation (n=3).

<table>
<thead>
<tr>
<th>Nominal concentration (µg/L)</th>
<th>Measured concentration of rac-KP* (µg/L)</th>
<th>Measured concentration of DKP (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>96 h</td>
</tr>
<tr>
<td></td>
<td>R (-)</td>
<td>S (+)</td>
</tr>
<tr>
<td>1.95</td>
<td>0.99 ± 0.05</td>
<td>1.11 ± 0.11</td>
</tr>
<tr>
<td>3.90</td>
<td>2.03 ± 0.06</td>
<td>1.96 ± 0.11</td>
</tr>
<tr>
<td>7.81</td>
<td>4.03 ± 0.15</td>
<td>3.96 ± 0.06</td>
</tr>
<tr>
<td>15.63</td>
<td>8.16 ± 0.29</td>
<td>7.99 ± 0.11</td>
</tr>
<tr>
<td>31.25</td>
<td>16.1 ± 0.60</td>
<td>15.93 ± 0.12</td>
</tr>
<tr>
<td>62.5</td>
<td>32.10 ± 0.36</td>
<td>32.00 ± 1.05</td>
</tr>
<tr>
<td>125</td>
<td>64.77 ± 0.40</td>
<td>64.00 ± 2.65</td>
</tr>
<tr>
<td>250</td>
<td>124.70 ± 5.03</td>
<td>112.30 ± 6.66</td>
</tr>
</tbody>
</table>

* rac-KP = racemic Kallol, DKP = Dextrorotatory Kallol
<table>
<thead>
<tr>
<th></th>
<th>120.70 ± 2.08</th>
<th>111.7 ± 7.64</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>245.00 ± 5.00</td>
<td>252.30 ± 6.81</td>
</tr>
<tr>
<td>1000</td>
<td>501.01 ± 11.03</td>
<td>488.70 ± 10.50</td>
</tr>
<tr>
<td>2000</td>
<td>1004.00 ± 16.92</td>
<td>992.30 ± 16.26</td>
</tr>
</tbody>
</table>

*Sum of 50% R and S enantiomers.*
Table 5. Percentage mortality/immobilization in *Ceriodaphnia dubia* after acute 48 h exposure to different concentrations of rac-KP and DKP.

<table>
<thead>
<tr>
<th>Rac-KP (µg/L)</th>
<th>Mortality/Immobilization (%)</th>
<th>DKP (µg/L)</th>
<th>Mortality/Immobilization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>Control</td>
<td>0.0</td>
</tr>
<tr>
<td>Solvent control</td>
<td>3.3</td>
<td>Solvent control</td>
<td>3.3</td>
</tr>
<tr>
<td>1560</td>
<td>13.3</td>
<td>1560</td>
<td>10.0</td>
</tr>
<tr>
<td>3130</td>
<td>20.0</td>
<td>3130</td>
<td>10.0</td>
</tr>
<tr>
<td>6250</td>
<td>23.3</td>
<td>6250</td>
<td>23.3</td>
</tr>
<tr>
<td>12500</td>
<td>23.3</td>
<td>12500</td>
<td>23.3</td>
</tr>
<tr>
<td>25000</td>
<td>23.3</td>
<td>25000</td>
<td>26.7</td>
</tr>
<tr>
<td>50000</td>
<td>26.7</td>
<td>50000</td>
<td>30.0</td>
</tr>
<tr>
<td>100000</td>
<td>40.0</td>
<td>100000</td>
<td>60.0</td>
</tr>
</tbody>
</table>
Table 6. Reproduction and survival of *Ceriodaphnia dubia* following chronic 7-day exposure to different concentrations of rac-KP.

<table>
<thead>
<tr>
<th>Rac-KP (µg/L)</th>
<th>Control</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of first reproduction (days) ± SD</td>
<td>4 ± 0.0</td>
<td>4 ± 0.0</td>
<td>4 ± 0.0</td>
<td>4 ± 0.0</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>Offspring/female ± SD</td>
<td>23.3 ± 1.9</td>
<td>18.7 ± 7.4</td>
<td>21.2 ± 2.5</td>
<td>18.2 ± 1.5</td>
<td>17.5 ± 4.4*</td>
</tr>
<tr>
<td>Total reproduction</td>
<td>233</td>
<td>187</td>
<td>212</td>
<td>182</td>
<td>105</td>
</tr>
<tr>
<td>Inhibited reproduction (%)</td>
<td>-</td>
<td>19.7</td>
<td>9.1</td>
<td>21.9</td>
<td>24.9</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

*p < 0.05 (one-way ANOVA, Dunnett’s test)
Table 7. Reproduction and survival of *Ceriodaphnia dubia* following chronic 7-day exposure to different concentrations of DKP.

<table>
<thead>
<tr>
<th>DKP (µg/L)</th>
<th>Control</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of first reproduction (days) ± SD</td>
<td>4 ± 0.0</td>
<td>4 ± 0.0</td>
<td>4 ± 0.0</td>
<td>4.4 ± 0.5</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>Offspring/female ± SD</td>
<td>20.5 ± 1.7</td>
<td>18.2 ± 2.2</td>
<td>17.4 ± 1.8</td>
<td>14.6 ± 2.6*</td>
<td>11.2 ± 1.6*</td>
</tr>
<tr>
<td>Total reproduction</td>
<td>205</td>
<td>182</td>
<td>174</td>
<td>117</td>
<td>67</td>
</tr>
<tr>
<td>Inhibited reproduction (%)</td>
<td>-</td>
<td>11.2</td>
<td>15.1</td>
<td>28.8</td>
<td>45.4</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>40</td>
</tr>
</tbody>
</table>

*p < 0.05 (one-way ANOVA, Dunnett’s test)*
Table 8. Nominal and measured concentration of rac-KP (R(-)/S(+) KP) and DKP (S(+)KP) in crustacean test solutions. Data are expressed as mean ± standard deviation (n=3).

<table>
<thead>
<tr>
<th>Nominal concentration (µg/L)</th>
<th>Measured concentration of rac-KP* (µg/L)</th>
<th>Measured concentration of DKP (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 d</td>
<td>7 d</td>
</tr>
<tr>
<td></td>
<td>R (-)</td>
<td>S (+)</td>
</tr>
<tr>
<td></td>
<td>R (-)</td>
<td>S (+)</td>
</tr>
<tr>
<td></td>
<td>S (+)</td>
<td>S (+)</td>
</tr>
<tr>
<td>1 0.43 ± 0.06</td>
<td>0.60 ± 0.10</td>
<td>0.63 ± 0.20</td>
</tr>
<tr>
<td>10 4.50 ± 0.50</td>
<td>4.53 ± 0.50</td>
<td>5.00 ± 0.10</td>
</tr>
<tr>
<td>100 47.33 ± 3.79</td>
<td>49.00 ± 6.25</td>
<td>45.67 ± 3.06</td>
</tr>
<tr>
<td>1000 489.70 ± 10.02</td>
<td>486.00 ± 6.93</td>
<td>453.30 ± 15.28</td>
</tr>
<tr>
<td></td>
<td>1.10 ± 0.10</td>
<td>0.97 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>9.62 ± 1.22</td>
<td>95.67 ± 5.86</td>
</tr>
<tr>
<td></td>
<td>993.32 ± 25.11</td>
<td></td>
</tr>
</tbody>
</table>

*Sum of 50% R and S enantiomers.
Figure 1
Figure 2
Figure 4