Evaluating the enantiospecific differences of non-steroidal anti-inflammatory drugs (NSAIDs) using an ecotoxicity bioassay test battery

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Abstract: Wastewater treatment plants are a major pathway for pharmaceuticals to the aquatic environment. Many pharmaceuticals, including non-steroidal anti-inflammatory drugs (NSAIDs), are chiral chemicals and the biological activity of their enantiomers can differ. Few studies have assessed the effects of different NSAID enantiomers on non-target organisms. However, this information is important for environmental risk assessment to ensure that the effects of more potent enantiomers are not overlooked. In the current study, enantiomers of naproxen, ibuprofen, ketoprofen and flurbiprofen were evaluated in bioassays with bacteria, algae and fish cells. All enantiomers induced bacterial toxicity, with (*R*)-naproxen more toxic than (*S*)-naproxen (EC_{50} 0.75 vs 0.93 mg/L) and (*S*)-flurbiprofen more toxic than (*R*)-flurbiprofen (EC_{50} 1.22 vs 2.13 mg/L). Both (*R*)-flurbiprofen and (*S*)-flurbiprofen induced photosystem II inhibition in green algae, with (*R*)-flurbiprofen having a greater effect in the assay after 24 h (EC_{10} 6.92 vs 9.79 mg/L). Only the (*R*)-enantiomers of flurbiprofen and ketoprofen induced ethoxyresorufin-O-deethylase (EROD) activity in fish cells, while (*S*)-naproxen was 2.5 times more active than (*R*)-naproxen in the EROD assay. While enantiospecific differences were observed for all assays, the difference was less than an order of magnitude. This indicates that the risk of overlooking the effect of more potent NSAID enantiomers is minor for the studied test systems and supports the use of racemic (or single enantiomer) effect data for environmental risk assessment. However, further investigation of the (*R*)-enantiomer of commonly used NSAID ketoprofen is recommended as it was at least six times more potent in the EROD assay than the inactive (*S*)-ketoprofen.

Keywords: 2-arylpropionic acids; chiral; enantiomers; *in vitro*; pharmaceuticals

49 **1. Introduction**

50 Pharmaceuticals are present in surface waters worldwide (Hughes et al., 2013), with wastewater
51 treatment plants and combined sewer overflows acting as pathways of pharmaceuticals to the aquatic
52 environment (Kay et al., 2017). Due to the widespread presence of pharmaceuticals, there are
53 concerns about potential effects of these biologically active chemicals on aquatic organisms such as
54 bacteria, algae and fish. Around half of all available pharmaceuticals are chiral chemicals, which
55 includes classes such as β -blockers, psychotropic drugs and non-steroidal anti-inflammatory drugs
56 (NSAIDs) (McConathy and Owens, 2003). Chiral molecules, with a single chiral centre, exist in two
57 stereochemical arrangements, known as enantiomers, and have identical physicochemical properties,
58 but can differ in biological activity (Hashim et al., 2010). This is because many biological processes
59 also involve chiral chemicals (such as enzymes), which interact differentially with other chiral
60 chemicals. Some common NSAIDs, including ibuprofen and naproxen, belong to a chiral group of
61 molecules known as 2-arylpropionic acids (2-APAs). The (*S*)-enantiomer of these NSAIDs is
62 typically the biologically active enantiomer that inhibits prostaglandin synthesis in humans (Landoni
63 and Soraci, 2001). Depending on the compound, the (*R*)-enantiomer can be less potent than the (*S*)-
64 enantiomer, inactive or can even cause adverse effects (Caldwell et al., 1988). Despite this, many
65 chiral pharmaceuticals are manufactured and dispensed as racemic mixtures with both (*S*)- and (*R*)-
66 enantiomers (e.g. ibuprofen and ketoprofen). Others are dispensed as enantiomerically pure
67 formulations of an active enantiomer, such as naproxen, which is manufactured as (*S*)-naproxen.
68 However, enzymatic chiral inversion of ingested 2-APAs in humans can change the enantiomeric
69 fraction (EF) of the excreted NSAID (Wsol et al., 2004). The EF can also be altered during wastewater
70 treatment due to processes such as biodegradation (Buser et al., 1999) and, possibly, chiral inversion
71 (Hashim et al., 2011).

72

73 While the effects of enantiomer pairs in target systems (e.g., humans) are well known, an
74 understanding of any enantiospecific effects on non-target organisms is important for ecological risk

75 assessment. For example, the effects of chiral pesticides have been widely studied (e.g. Qu et al.,
76 2014; Xie et al., 2018), but less is known about the ecotoxicity of pharmaceutical enantiomers, with
77 most of the research to date focusing on β -blockers, such as propranolol, and the antidepressant
78 fluoxetine (Sanganyado et al., 2017). For example, Sun et al. (2014) observed no enantiospecific
79 differences in heart rate, hatching rate and mortality in zebrafish embryos after exposure to the (*R*)-
80 and (*S*)-enantiomers of propranolol and metoprolol. In contrast, Stanley et al. (2006) found that (*S*)-
81 propranolol was slightly more toxic than (*R*)-propranolol to fathead minnow larvae in a 7 d short-
82 term chronic exposure test, though no enantiospecific differences were observed after acute (48 h)
83 exposure. Fluoxetine enantiomers showed species-specific differences in effect, with (*S*)-fluoxetine
84 up to 9.4 times more toxic to fathead minnow larvae than (*R*)-fluoxetine (Stanley et al., 2007) and
85 (*R*)-fluoxetine over 27 times more toxic to protozoa *Tetrahymena thermophila* than the (*S*)-
86 enantiomer (Andrés-Costa et al., 2017). In contrast, there was no difference in effect between (*S*)-
87 fluoxetine and (*R*)-fluoxetine for zooplankton *Daphnia magna* (Andrés-Costa et al., 2017; Stanley et
88 al., 2007). The enantiospecific differences observed in the literature support the need for ecotoxicity
89 testing of other commonly detected chiral pharmaceuticals.

90
91 Chiral NSAIDs such as naproxen, ketoprofen and ibuprofen are commonly detected in treated
92 wastewater and surface water in the ng/L to μ g/L concentration range (e.g. Hashim and Khan, 2011;
93 Sanganyado et al., 2017; Tousova et al., 2017). A number of studies have reported effects of racemic
94 ketoprofen and ibuprofen and (*S*)-naproxen on algae, crustaceans and fish, with adverse effects such
95 as growth inhibition and mortality observed at mg/L concentrations (e.g. Cleuvers, 2004; Gonzalez-
96 Naranjo and Boltes, 2014; Watanabe et al., 2016). However, very few studies have considered the
97 potential ecotoxicological differences of NSAID enantiomers. In one recent study, Mennillo et al.
98 (2018) compared the effect of racemic ketoprofen to the active (*S*)-enantiomer and found that (*S*)-
99 ketoprofen was more toxic to bacteria, algae and zooplankton than racemic ketoprofen, though (*R*)-
100 ketoprofen was not tested.

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The current study aimed to evaluate the enantiospecific differences of NSAIDs naproxen, ibuprofen, ketoprofen and flurbiprofen using a battery of ecotoxicity bioassays with bacteria, algae and fish cells. Many of the previous studies evaluating enantiospecific differences have focused on effects in crustaceans and fish, which can be time consuming and expensive, so the current study used high-throughput and rapid bioassays. The studied endpoints included cytotoxicity in both bacteria and fish cells, photosystem II (PSII) inhibition in green algae and induction of cytochrome P450 1A enzymes (CYP1A) in fish cells using the ethoxyresorufin-O-deethylase (EROD) assay. Induction of CYP1A enzymes does not indicate a toxic effect per se but is an indicator of xenobiotic metabolism. The outcomes of the study can help inform ecological risk assessment of chiral NSAIDs.

2. Materials and Methods

2.1. Chemicals

All chemicals were of analytical grade and all were reported to be >96% pure. The majority of NSAIDs were purchased from Sigma Aldrich (Castle Hill, Australia), with the exception of (*R*)-ibuprofen, which was purchased from Enzo Life Sciences (Farmingdale, USA) (distributed by Sapphire Bioscience, Redfern, Australia), and (*R*)-naproxen, (*R*)-ketoprofen and (*S*)-flurbiprofen, which were supplied by Toronto Research Chemicals (Toronto, Canada) (also distributed by Sapphire Bioscience). In addition to the NSAID enantiomers, racemic ibuprofen and ketoprofen were also tested in the current study. The full list of suppliers and chemical purity is included in Table S1 of the Supplementary Material, with selected physicochemical properties shown in Table S2. Stock solutions of the test chemicals were prepared in methanol with concentrations ranging from 1.0 to 2.5 mg/mL and were stored at -20°C in the dark.

2.2 Bioanalysis

2.2.1 Bacterial toxicity

127 Bacterial toxicity was evaluated using the Bacterial Luminescence Toxicity Screen (BLT-Screen)
 128 with *Photobacterium leiognathi* based on the protocol in van de Merwe and Leusch (2015). Briefly,
 129 the test chemicals were serially diluted in phosphate buffer (pH 4) in white 96 well plates, with each
 130 plate including positive reference compound pentachlorophenol (PCP) and solvent control methanol.
 131 The solvent concentration did not exceed 1%. Cryopreserved *P. leiognathi* stock was reconstituted in
 132 growth media and 5 µL of growth media was added per well. The samples were incubated for 30 min
 133 at room temperature, then luminescence was measured using a FLUOstar Omega Plate Reader (BMG
 134 Labtech, Ortenberg, Germany). Percent inhibition was calculated based on the relative light units
 135 (RLU) of the sample (RLU_{sample}) and the solvent control (RLU_{control}) (Equation 1). Each test chemical
 136 was run two to three times on different days.

137

$$\% \text{ Inhibition} = \left(1 - \frac{\text{RLU}_{\text{sample}}}{\text{RLU}_{\text{control}}} \right) \cdot 100\%$$

(1)

140 2.2.2 Cell viability

141 Viability of the fish liver carcinoma cell line PLHC-1 was assessed using the neutral red assay based
 142 on Repetto et al. (2008). The cells were grown in Eagle's Minimum Essential Medium (EMEM) with
 143 5% fetal bovine serum (FBS), L-glutamine and Earle's balanced salt solution and cultured every three
 144 to four days. Briefly, 200 µL of cells with a density of 4.5×10⁵ cells/mL were seeded in a clear 96
 145 well plate and incubated for 24 h at 30°C, 5% CO₂. After 24 h, the test chemicals were serially diluted
 146 in growth media and added to the cells and incubated for a further 24 h at 30°C, 5% CO₂. The media
 147 was decanted and the cells were washed once with phosphate buffered saline (PBS), then 100 µL of
 148 40 µg/mL neutral red solution in phenol red free Dulbecco's Modified Eagle's Medium (DMEM) was
 149 added and incubated for 2 h at 30°C, 5% CO₂. After 2 h, the neutral red solution was decanted and
 150 the cells were washed twice with PBS, then 150 µL of neutral red destain solution, containing 50%
 151 ethanol, 49% ultrapure water and 1% glacial acetic acid, was added to each well. The plate was shaken
 152 for 10 min at 400 rpm, then absorbance at 540 nm was measured using the FLUOstar Omega Plate

153 Reader. After subtracting the average absorbance of the cell-free wells, percent inhibition was
154 calculated based on the absorbance of the samples (Abs_{sample}) and absorbance of the control ($Abs_{control}$)
155 (Equation 2). PCP served as the positive reference compound, while methanol was used as the solvent
156 control. The maximum solvent concentration was 1.2%, with no effects observed in the solvent
157 control. Each test chemical was run twice on different days.

158

$$\% \text{ Inhibition} = \left(1 - \frac{Abs_{sample}}{Abs_{control}} \right) \cdot 100\% \quad (2)$$

161

162 2.2.3 Photosystem II (PSII) inhibition

163 PSII inhibition in green algae *Pseudokirchneriella subcapitata* was assessed based on Escher et al.
164 (2008). Briefly, the test compounds were serially diluted in Talaquil test media in a clear 96 well
165 plate, then algae with an optical density (OD_{685}) of 0.1 was added. Each plate contained positive
166 reference compound diuron and methanol as the solvent control. The solvent concentration did not
167 exceed 1.7%. PSII quantum yield ($Y(II)$) was measured after 0, 2 and 24 h using Imaging Pulse
168 Amplitude Modulation (IPAM) fluorometry (Walz GmbH, Germany). PSII inhibition was measured
169 based on the quantum yield of the sample ($Y(II)_{sample}$) and the quantum yield of the control ($Y(II)_{control}$)
170 (Equation 3). Each test chemical was run at least two times on different days.

171

$$\% \text{ PSII Inhibition} = \left(1 - \frac{Y(II)_{sample}}{Y(II)_{control}} \right) \cdot 100\% \quad (3)$$

174

175 2.2.4 Ethoxyresorufin-O-deethylase (EROD) activity

176 EROD activity in PLHC-1 cells was assessed using a method adapted from Thibaut and Porte (2008)
177 and Della Torre et al. (2011). The cells were grown as described in Section 2.2.2. Briefly, 200 μ L of

178 PLHC-1 cells with a density of 4.5×10^5 cells/mL were seeded in a black clear bottom 96 well plate
179 and incubated for 24 h at 30°C, 5% CO₂. Solvent concentrations up to 0.5% resulted in EROD
180 induction, so all test chemicals were blown down to dryness in glass vials and resolubilised in growth
181 media, then serially diluted in a 96 well plate. The maximum tested concentration of each chemical
182 was selected based on the neutral red assay results and did not reduce cell viability. After 24 h, the
183 growth media was removed and replaced with the serially diluted test chemicals and the cells were
184 incubated for a further 6 h at 30°C, 5% CO₂. Six hours was selected as the optimal exposure time for
185 pharmaceuticals based on Thibaut and Porte (2008). After 6 h, the test media was decanted, and the
186 cells were washed once with PBS, then 100 µL of 2 µM 7-ethoxyresorufin in 50 mM phosphate buffer
187 (pH 8) was added to each well and incubated in the dark for 20 min. Fluorescence was measured at
188 544 nm (excitation) and 590 nm (emission). After background subtraction of the cell-free wells, an
189 induction ratio (IR) was calculated using the relative fluorescence units (RFU) of the sample
190 (RFU_{sample}) and the RFU of the control (RFU_{control}) (Equation 4). Benzo(a)pyrene served as the
191 positive reference compound, while methanol was the solvent control. All samples were run two to
192 four times on different days.

193

$$\text{IR} = \frac{\text{RFU}_{\text{sample}}}{\text{RFU}_{\text{control}}}$$

(4)

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197 2.3 Data evaluation

198 Effect concentrations (EC) were derived for all assays from concentration-effect curves. Log-
199 sigmoidal concentration-effect curves were applied for the BLT-Screen and the neutral red assay,
200 with the concentration causing 50% effect (EC₅₀) determined using Equation 5. The data were
201 evaluated using GraphPad Prism 7, with 95% confidence intervals (95% CI) determined.

202

$$\% \text{ effect} = \frac{100\%}{1 + 10^{\text{slope}(\log \text{EC}_{50} - \log \text{concentration})}}$$

(5)

Log-sigmoidal concentration-effect curves are linear up to 30% effect or an IR of 4, with linear concentration-effect curves suitable for weakly potent samples (e.g. <50% effect). Consequently, linear concentration-effect curves were applied to determine the effect concentration causing 10% effect (EC₁₀) for the PSII inhibition assay (Equation 6) and the effect concentration causing an IR of 1.5 (EC_{IR1.5}) for the EROD assay (Equation 7). The standard error (SE) of the EC₁₀ and EC_{IR1.5} values were calculated using Equations 8 and 9, respectively. Further information about linear concentration-effect curves can be found in Escher et al. (2018).

$$\text{EC}_{10} = \frac{10\%}{\text{Slope}}$$

(6)

$$\text{EC}_{\text{IR1.5}} = \frac{0.5}{\text{Slope}}$$

(7)

$$\text{SE}(\text{EC}_{10}) = \frac{10\%}{\text{Slope}^2} \cdot \text{SE}(\text{Slope})$$

(8)

$$\text{SE}(\text{EC}_{\text{IR1.5}}) = \frac{0.5}{\text{Slope}^2} \cdot \text{SE}(\text{Slope})$$

(9)

225

226 The (*S*)- to (*R*)- EC ratio was calculated using Equation 10. An enantiomer pair with a (*S*)- to (*R*)- EC
227 ratio >0.1 to <10 indicates there was little difference in effect between the enantiomers (e.g. less than
228 one order of magnitude). In contrast, a (*S*)- to (*R*)- EC ratio of <0.1 or >10 demonstrates that the effect
229 of one enantiomer varies by over an order of magnitude compared to the other, warranting further
230 investigation. The (*S*)- to (*R*)- EC ratio is based on the toxic ratio (TR) concept, where chemicals with
231 a TR > 10 are considered to have a specific effect in an assay, while chemicals with a TR <10 are
232 considered to be baseline toxicants (Verhaar et al., 1992).

233

234

$$\text{(*S*)- to (*R*)- EC ratio} = \frac{\text{EC}_{(\textit{S})-}}{\text{EC}_{(\textit{R})-}}$$

(10)

237

238 3. Results and Discussion

239 3.1 Cytotoxicity

240 All of the studied enantiomers inhibited bacterial bioluminescence in a dose-dependent manner, with
241 log-sigmoidal concentration-effect curves for the four chiral NSAIDs shown in Figure 1. The EC₅₀
242 values are provided in Table 1. Of the four NSAIDs, the naproxen enantiomers were the most toxic,
243 with EC₅₀ values of 0.93 (95% CI 0.87 - 1.00) and 0.75 (95% CI 0.67 - 0.84) mg/L for the (*S*)- and
244 (*R*)-enantiomer, respectively. The (*R*)-enantiomers of ibuprofen and ketoprofen were also more toxic
245 than their corresponding (*S*)-enantiomers, but the 95% confidence intervals overlapped for the (*R*)-
246 and (*S*)-enantiomers, suggesting that the difference between the ibuprofen and ketoprofen enantiomer
247 pairs was not significant. (*S*)-flurbiprofen was more toxic than its corresponding (*R*)-enantiomer, with
248 EC₅₀ values of 1.22 (95% CI 1.07-1.38) and 2.13 (95% CI 1.94 – 2.35) mg/L for (*S*)-flurbiprofen and
249 (*R*)-flurbiprofen, respectively. For both naproxen and flurbiprofen, the difference between the (*R*)-

250 and (*S*)-enantiomers was less than a factor of 2, so any enantiospecific differences in bacterial toxicity
251 can be considered minor.

252

253 In addition to the enantiomers, racemic ibuprofen and ketoprofen were tested and found to have
254 slightly lower EC₅₀ values than their corresponding enantiomers in the BLT-Screen. This difference
255 is expected to be within the range of experimental variability as the 95% confidence intervals of the
256 racemic mixtures and (*R*)-enantiomers overlapped. However, Alvarez et al. (1999) previously found
257 differences in the solubility of racemic ketoprofen and its enantiomers, with racemic ketoprofen less
258 soluble than (*R*)- and (*S*)-ketoprofen. The tested concentration ranges of the both racemic ketoprofen
259 and its enantiomers were below the solubility limit, but the differences in solubility and consequently
260 uptake over the 30 min exposure period may have potentially contributed to the slightly lower EC₅₀
261 value for racemic ketoprofen.

262

263 Racemic ketoprofen and ibuprofen, well as (*S*)-naproxen, were previously tested in another bacterial
264 bioluminescence assay, the Microtox assay with *Aliivibrio fischeri*, but the EC₅₀ values were 46 to
265 160 times higher (e.g. less toxic) than the current study (Escher et al., 2017). This can be attributed
266 to the different test conditions, specifically the pH, with the Microtox assay run at pH 7 and the BLT-
267 Screen run at pH 4, resulting in a greater fraction of the test chemical in a neutral form in the BLT-
268 Screen. In contrast, a similar EC₅₀ value was determined for (*S*)-naproxen in the BLT-Screen
269 previously (Neale et al., 2017).

270

271 In contrast to bacterial toxicity, none of the chemicals reduced cell viability in the neutral red assay
272 with PLHC-1 cells up to the maximum tested concentration (Figure S1). This was not surprising as
273 EC₅₀ values of 172 mg/L for racemic ibuprofen and 343 mg/L for (*S*)-naproxen were reported in the
274 neutral red assay with PLHC-1 cells by Caminada et al. (2006). The difference between the BLT-
275 Screen and the neutral red assay was not unexpected as the BLT-Screen is highly sensitive to organic

276 compounds (van de Merwe and Leusch, 2015) and previous studies have shown that bacterial
277 bioluminescence inhibition assays are more sensitive to contaminants and environmental extracts
278 than vertebrate cells with the neutral red assay (Neale et al., 2012; Repetto et al., 2001; Zurita et al.,
279 2007). Despite this, it was still important to test the chemicals in the neutral red assay to ensure that
280 cytotoxic concentrations were not run in the EROD assay.

281

282 3.2 PSII Inhibition

283 Only three of the tested compounds, (*R*)-naproxen, (*S*)-flurbiprofen and (*R*)-flurbiprofen, induced
284 more than 10% PSII inhibition in green algae at 2 and 24 h (Table 1), with the EC₁₀ values much
285 higher than the bacterial toxicity EC₅₀ values. All concentration-effect curves are shown in Figures
286 S2 and S3. The effect of (*S*)-naproxen at the highest tested concentration was just below the 10%
287 effect cut-off after 24 h, so the EC₁₀ value was extrapolated from the linear concentration-effect curve.
288 PSII inhibition was measured at both 2 and 24 h. PSII inhibitors, such as the herbicide diuron, inhibit
289 the quantum yield rapidly, thus the 2 h measurement is used as an indicator of specific toxicity, while
290 delayed phytotoxicity is indicated by the 24 h reading, which integrates specific and non-specific
291 effects (Escher et al., 2008). The studied chemicals are not PSII inhibitors and PSII inhibition
292 increased from 2 to 24 h (Figure S4), resulting in lower EC₁₀ values at 24 h. The (*R*)-enantiomer was
293 more toxic than the (*S*)-enantiomer for all active chemicals, but the difference was less than a factor
294 of 2. This is contrary to the bacterial toxicity results for flurbiprofen, where (*S*)-flurbiprofen was more
295 toxic, suggesting species-specific differences in enantiomer potency for flurbiprofen.

296

297 Mennillo et al. (2018) assessed the effect of racemic ketoprofen and (*S*)-ketoprofen on *P. subcapitata*
298 growth after 72 h exposure, with EC₁₀ values of 9.0 and 4.5 µg/L (EC₅₀ 240 and 65.6 µg/L) reported
299 for ketoprofen and (*S*)-ketoprofen, respectively. In another study by Watanabe et al. (2016) the
300 concentration of racemic ketoprofen found to cause 50% growth inhibition of *P. subcapitata* after 72
301 h was over 100 times higher (EC₅₀ 24.6 mg/L) than Mennillo et al. (2018). No effect was observed

302 for racemic ketoprofen or either of the ketoprofen enantiomers in the current study up to the maximum
303 tested concentrations. The differences between studies can potentially be attributed to different
304 exposure times (24 h in the current study vs 72 h in the literature), test systems and experimental
305 protocols. Further, Gonzalez-Naranjo and Boltes (2014) reported racemic ibuprofen caused growth
306 inhibition in *P. subcapitata* over 72 to 96 h (EC₁₀ 11.9 mg/L), though no effect was observed in the
307 current study up to a concentration of 23.7 mg/L.

308

309 3.3 Effect on EROD activity

310 The EC_{IR1.5} values for the EROD assay are presented in Table 1, with the linear concentration-effect
311 curves shown in Figure S5. PLHC-1 cells express CYP1A, which is an important enzyme for
312 xenobiotic metabolism, with the EROD assay measuring CYP1A induction (Fent, 2001). While the
313 EROD assay was developed for dioxin-like chemicals, it has also been applied to pharmaceuticals
314 and personal care products, but typically with shorter exposure durations (e.g. 6 h vs 48 to 72 h)
315 (Della Torre et al., 2011; Thibaut and Porte, 2008). This is because pharmaceutical EROD activity is
316 often not observed after longer exposure times due to metabolism of the test compound. Therefore,
317 induction of EROD activity by 2-APAs does not necessarily translate into an adverse effect in fish,
318 but instead can provide information about chemical bioactivity. Both the naproxen enantiomers
319 induced EROD activity, with (*S*)-naproxen 2.5 times more potent than (*R*)-naproxen (EC_{IR1.5} 5.10 ±
320 0.7 mg/L for (*S*)-naproxen compared to EC_{IR1.5} 12.6 ± 1.5 mg/L for (*R*)-naproxen). In contrast, only
321 the (*R*)-enantiomers of ketoprofen and flurbiprofen were active in the EROD assay, while both
322 ibuprofen enantiomers were inactive. Based on the maximum tested concentrations, (*R*)-ketoprofen
323 is at least 6.3 times more potent than (*S*)-ketoprofen, while (*R*)-flurbiprofen is at least 1.5 times more
324 active than (*S*)-flurbiprofen. Thibaut and Porte (2008) also found that (*S*)-naproxen was a moderate
325 EROD activity inducer in PLHC-1 cells at 10 µM (2.3 mg/L), while racemic ibuprofen and ketoprofen
326 were reported as weak inducers, respectively.

327

3.4 Ecological risk of 2-APA enantiomers

While the (*S*)-enantiomer of 2-APAs is considered the bioactive enantiomer responsible for prostaglandin synthesis inhibition in humans, the (*R*)-enantiomer was often more biologically active in the bacterial toxicity, PSII inhibition and EROD assays. The exceptions were (*S*)-flurbiprofen in the bacterial toxicity assay and (*S*)-naproxen in the EROD assay. The (*S*)- to (*R*)- EC ratio ranged between 0.40 to 1.66 for all assays (Table 2), indicating less than an order of magnitude difference in effect between the enantiomer pairs. This suggests that the risk of overlooking the effect of more potent NSAID enantiomers is minor for the studied bioassays and that using racemic (or single enantiomer) effect data is sufficient for risk assessment purposes. However, it should be noted that for the EROD assay two compounds with active (*R*)-enantiomers had inactive (*S*)-enantiomers, with a (*S*)- to (*R*)- EC ratio of at least 6.3 estimated for ketoprofen. Therefore, further investigation of ketoprofen enantiomers, including additional testing in other bioassays indicative of environmentally relevant endpoints, may be warranted.

To put the results into an environmental context, the fraction of each NSAID enantiomer present in wastewater effluent was considered. The EF is typically expressed as the concentration of the (*S*)-enantiomer divided by the sum concentration of the (*S*)- and (*R*)-enantiomers (Harner et al., 2000; Hashim et al., 2010). The EF of the studied NSAIDs in treated wastewater reported in the literature range from 0.49 to 0.76 for ibuprofen (Hashim and Khan, 2011; Khan et al., 2014; Matamoros et al., 2009), 0.66 to 0.90 for naproxen (Hashim and Khan, 2011; Khan et al., 2014; Matamoros et al., 2009), 0.54 to 0.66 for ketoprofen (Hashim and Khan, 2011) and 0.52 for flurbiprofen (Yuan et al., 2018). The EF from the literature were calculated using either the concentration (Hashim and Khan, 2011; Khan et al., 2014; Matamoros et al., 2009) or the peak area (Yuan et al., 2018) of the enantiomers. Using the EC values of each enantiomer pair and the literature EF, the combined EC ($EC_{combined}$) value in wastewater for each NSAID was calculated using Equation 11. The minimum and maximum EF was used for the calculation, giving a range of combined EC values for each NSAID. Equation 11

354 is based on the mixture toxicity model of concentration addition, which assumes that all chemicals in
355 a mixture act by the same mode of action. Enantiomers may indeed have different modes of action,
356 so the mixture toxicity model of independent action, which assumes chemicals have different modes
357 of action, may be more suitable. However, concentration addition is widely considered to be the most
358 conservative mixture toxicity model and is recommended as a first tier assessment (Backhaus and
359 Faust, 2012), which is why it was applied in the current study.

360

$$361 \quad EC_{\text{combined}} = (EC_{(S)} \cdot EF) + (EC_{(R)} \cdot (1 - EF))$$

362 (11)

363

364 Based on the availability of effect data for both enantiomer pairs, combined EC values were calculated
365 for all NSAIDs for bacterial toxicity, though a combined EC value could only be calculated for
366 flurbiprofen for PSII inhibition and naproxen for EROD activity. For all other NSAIDs, a combined
367 EC value could not be calculated as one or both of the enantiomers were inactive.

368

369 In most cases, the combined EC value fell between the EC values of each enantiomer pair (Figure 2).
370 The exception was naproxen, where the combined EC value was close to the EC value of the (*S*)-
371 enantiomer. While naproxen is manufactured as (*S*)-naproxen, (*R*)-naproxen is detected in treated
372 wastewater (Khan et al., 2014), most likely due to chiral inversion during biological treatment
373 (Hashim et al., 2011). The (*R*)-enantiomer had a lower EC value than the (*S*)-enantiomer in the BLT-
374 Screen, meaning the combined EC value based on the wastewater EF from the literature was up to
375 7% lower (more toxic) than the (*S*)-enantiomer. This was within the 95% confidence intervals of the
376 EC₅₀ value for the (*S*)-enantiomer. The opposite was observed for EROD activity, with the combined
377 EC value slightly higher (less toxic) than the (*S*)-naproxen EC value. However, the difference in effect
378 was small, with the combined EC value between 15 to 50% higher than (*S*)-naproxen. This further

379 suggests that the enantiospecific differences of NSAIDs are minor in the studied assays, supporting
380 the use of racemic effect data for environmental risk assessment.

381

382 It should be noted that any changes in the enantiomeric fraction were not assessed after exposure in
383 the bioassays. Mennillo et al. (2018) previously measured the concentration of (*S*)-ketoprofen in algal
384 test media and found no major changes in concentration after 72 h. However, NSAID enantiomers
385 can undergo chiral inversion in *in vitro* and *in vivo* systems (Wsol et al., 2004), so chiral inversion
386 cannot be ruled out in the current study, particularly for the metabolically active PLHC-1 cells. If the
387 studied enantiomers do undergo chiral inversion in the test systems, then this can be considered as
388 part of the cell's response to the enantiomer, which is still a relevant measure of its biological activity.

389

390 **4. Conclusions**

391 The widespread use and subsequent environmental presence of chiral pharmaceuticals has
392 highlighted the need to understand potential enantiospecific effects on non-target organisms. In this
393 study the effect of NSAID enantiomers, including commonly detected naproxen, ibuprofen and
394 ketoprofen, were assessed in a battery of ecotoxicity bioassays. Differences in effect for enantiomer
395 pairs were observed for bacterial toxicity, PSII inhibition and EROD activity, with the (*R*)-enantiomer
396 often more active. However, the differences in all assays were less than an order of magnitude,
397 suggesting that using enantiospecific effect data will not have a major impact on ecological risk
398 assessment of NSAIDs based on the applied test systems. Based on EROD activity, (*R*)-ketoprofen
399 may warrant further investigation as it was at least 6.3 times more potent than (*S*)-ketoprofen, which
400 was inactive in the assay.

401

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405

406 **Conflict of Interest**

407 The authors declare that they have no conflict of interest.

408

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532

533 Table 1: Effect concentrations for the studied chemicals in units of mg/L.

Chemical Name	Bacterial Toxicity	Cell Viability	PSII Inhibition		EROD Activity
	<i>EC</i> ₅₀ (95% CI)	<i>EC</i> ₅₀ (95% CI)	<i>EC</i> ₁₀ ± SE (2 h)	<i>EC</i> ₁₀ ± SE (24 h)	<i>EC</i> _{IR1.5} ± SE
(<i>S</i>)-Ibuprofen	3.13 (2.83 – 3.45)	BDL (>20.7)	BDL (>29.5)	BDL (>29.5)	BDL (>19.0)
(<i>R</i>)-Ibuprofen	2.84 (2.65 – 3.06)	BDL (>13.0)	BDL (>18.5)	BDL (>18.5)	BDL (>11.9)
Ibuprofen (racemic mixture)	2.47 (2.14 – 2.82)	BDL (>16.6)	BDL (>23.7)	BDL (>23.7)	BDL (>16.6)
(<i>S</i>)-Naproxen	0.93 (0.87 – 1.00)	BDL (>13.2)	BDL (>18.8)	23.8*	5.10 ± 0.7
(<i>R</i>)-Naproxen	0.75 (0.67 – 0.84)	BDL (>18.8)	20.1 ± 1.4	19.8 ± 0.7	12.6 ± 1.5
(<i>S</i>)-Ketoprofen	4.56 (4.14 – 5.01)	BDL (>29.6)	BDL (>42.3)	BDL (>42.3)	BDL (>27.2)
(<i>R</i>)-Ketoprofen	4.23 (3.80 – 4.70)	BDL (>11.7)	BDL (>16.7)	BDL (>16.7)	4.34 ± 0.3
Ketoprofen (racemic mixture)	3.42 (3.04 – 3.86)	BDL (>13.8)	BDL (>19.7)	BDL (>19.7)	BDL (>12.6)
(<i>S</i>)-Flurbiprofen	1.22 (1.07 – 1.38)	BDL (>13.7)	9.79 ± 0.7	9.07 ± 0.6	BDL (>12.5)
(<i>R</i>)-Flurbiprofen	2.13 (1.94 – 2.35)	BDL (>13.8)	6.92 ± 0.5	5.47 ± 1.3	8.39 ± 1.2

534 BDL: below detection limit; 95% CI: 95% confidence interval; SE: standard error

535 *The maximum effect in (*S*)-naproxen was just below the 10% effect cut-off, so the EC₁₀ value was extrapolated from the linear concentration-effect curve

536 Table 2: EC_(S)/EC_(R)- for the studied NSAIDs.

Chemical Name	Bacterial Toxicity	PSII Inhibition (2 h)	PSII Inhibition (24 h)	EROD Activity
Ibuprofen	1.10	<i>not active</i>	<i>not active</i>	<i>not active</i>
Naproxen	1.24	>0.94*	1.20†	0.40
Ketoprofen	1.08	<i>not active</i>	<i>not active</i>	>6.3*
Flurbiprofen	0.57	1.42	1.66	>1.5*

537 *EC_(S)/EC_(R)- was estimated as only the (*R*)-enantiomer had an effect for naproxen, ketoprofen and flurbiprofen

538 †EC₁₀ for (*S*)-naproxen extrapolated

539 **Figure Captions**

540

541 Figure 1: Log-sigmoidal concentration-effect curves for the enantiomer pairs of ibuprofen, naproxen,
542 ketoprofen and flurbiprofen in the BLT-Screen, with the (*S*)-enantiomer shown in blue closed
543 symbols and the (*R*)-enantiomer shown in red open symbols. The racemic mixtures for ibuprofen and
544 ketoprofen are shown in green closed symbols.

545

546 Figure 2: Combined EC values ($EC_{combined}$, solid bar) for the studied NSAIDs in wastewater effluent
547 for assays indicative of bacterial toxicity, PSII inhibition (24 h) and EROD activity based on the range
548 of enantiomeric fractions provided in the literature. The EC value for the (*S*)-enantiomer is indicated
549 by the *S* symbol, while the (*R*)-enantiomer is indicated by the *R* symbol. Note the different y-axis
550 scale for each graph. Error bars indicate 95% confidence intervals for bacterial toxicity and standard
551 error for PSII inhibition and EROD activity.

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